ABSTRACT

NINO, BERNARDO D. Colony Genetic Structure and Effects of Inbreeding on Body Size in Three Populations of *Reticulitermes flavipes* in the Southeastern U.S. (Under the direction of Edward Vargo.)

In this study, I expanded the knowledge base of the eastern subterranean termite *R. flavipes* by investigating colony and population genetic structure in previously unstudied areas in Florida and Mississippi. I used microsatellite markers to infer colony breeding structure and population genetic structure and genotyped 20 workers in each of 20-30 colonies per population at eight microsatellite loci. I conducted pedigree analysis on the worker genotypes to determine the proportions of colonies that were simple families, extended families or mixed families. I also estimated the coefficient of relatedness and *F*-statistics and compared these values to those based on computer simulations of different breeding systems to infer levels of inbreeding and numbers of reproductives within colonies.

An unexpected finding was the presence of two distinct populations in one collection site in Mississippi (MS). These two populations, MS1 and MS2, differed in the predominant family type. MS1 consisted mainly of simple family colonies whereas MS2 was composed primarily of extended family colonies. The breeding structure in the extended family colonies in both populations was consistent with simulations for colonies with a low number of effective reproductives (2-6) which have been interbreeding for few generations. In Florida I found a high proportion of extended family colonies (~ 63%); the breeding structure in these colonies was consistent with the presence of a higher number of effective reproductives (> 6) which had been interbreeding for many generations. Mixed family colonies were collected in all three populations and composed about 10% of all colonies in each population.

In addition, I investigated possible effects of inbreeding and colony family type on worker and soldier body size. Worker and soldier head widths were measured and correlated to colony inbreeding coefficient (F_{IC}). This statistic is highly sensitive to the number of effective reproductives heading colonies. A negative correlation was discovered between worker and soldier body size and the effective number of reproductives heading colonies in two populations. I found a similar trend in the third population (MS1) but it was not significant, most likely due to small sample size. In population MS2 I found a significant effect of colony family type on body size; workers and soldiers in extended family colonies were smaller than individuals in simple family colonies. These finding may indicate a previously little appreciated consequence of inbreeding in termites.

Colony Genetic Structure and Effects of Inbreeding on Body Size in Three Populations of *Reticulitermes flavipes* in the Southeastern U.S.

by
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DEDICATION

I dedicate this work to my parents Miguel Niño and Susan Gallego. Mom and dad thank you so much for all your love and support. You both have always inspired me to do my best.

BIOGRAPHY

Bernardo Nino was born on July 11th 1980 to Miguel Niño and Susan Gallego. He grew up in Austin, TX and love it so much he stayed to complete is BS in Biology at Saint Edward's University. He worked under the direction of Dr. Allan Hook who sparked his interest in the field of entomology. After taking a year off of school to work for the Bureau of Land management, Bernardo moved to pursue his Master's degree in Entomology under supervision of Dr. Edward L. Vargo

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Chapter 1- Literature Review

The investigation of breeding systems in subterranean termites had been particularly challenging, however the development of sophisticated molecular techniques has allowed scientists to gain a more in depth understanding of their cryptic lifestyles. With this new information, scientists are now able to compare empirical data to previous theoretical models, providing new insights into mechanisms and pressures governing termite breeding systems. In reviewing the primary literature it was clear that subterranean termite populations vary greatly in levels of inbreeding, effective numbers of reproductives heading colonies, relatedness of primary reproductives, and the predominance of colony family types. The aim of this review is to highlight the variation reported in subterranean termite breeding systems and the possible effects that inbreeding can have on termite societies.

A species' breeding system is determined by a suite of factors ranging from large scale events including dispersal tactics and patterns, to fine scale events like mate choice and relatedness between individuals. Some major components of breeding structure include which individuals reproduce, number of individuals reproducing, reproductive skew, ratio of male to female reproductives, and the genetic relatedness of the individuals reproducing (Ross 2001). Traditionally, most studies of breeding systems have focused on vertebrates and plants (Clutton-Brock 1989, Hamrick and Godt 1996, Ligon 1999). Insect researchers have focused on the order Hymenoptera when studying breeding systems and kin structure among insects (Ross 2001). The order Isoptera, another eusocial group, remains underrepresented when addressing these

questions. Therefore, a better understanding termite breeding systems may uncover possible mechanisms which drive the evolution of eusociality in insects.

Understanding the subterranean termite life cycle is important when addressing questions pertaining to their breeding structure. Colony formation usually begins with two winged primary reproductives known as alates. Termite colonies generally produce a large number of alates once per year (Nutting et al. 1969). Mating flights or "swarms" are often linked to seasonality and associated with high temperatures and abundant rainfall (Banks and Snyder 1920, Nutting et al. 1969). The alates disperse and begin to search for a potential mate. After the alates land they shed their wings, and the female assumes a "calling" pose with her abdomen raised in the air awaiting a male (Nutting et al. 1969). Once a male locates the calling female, they form a tandem running pair and settle into wood or soil (Nutting et al. 1969). After they find a suitable location, they tunnel down into the substrate and construct a nuptial chamber, where the pair mates and the female lays the first clutch of eggs (Nutting et al. 1969). The female reproductive is responsible for rearing the brood, until a sufficient number of workers have developed and can assume the role of caretakers (Nutting et al. 1969, Lainé and Wright 2003). At this stage of colony development, the queen is then free to lay eggs exclusively and adopts a physogastric state (Lainé and Wright 2003). In addition, the number of functional ovarioles increases, leading to greater egg production and colony growth (Nutting et al. 1969).

Once the colony has matured the production of new alates is initiated. This process is very costly for a colony to undergo and therefore a colony must reach a critical size in order to

produce alates (Nutting et al. 1969). However, over time the primary reproductives of the colony die off and new secondary reproductives assume the role of reproduction within the colony (Snyder 1935, Thorne et al 1999). These neotenics remain in the colony and allow the colony to persist.

Breeding Systems in Subterranean Termites

Termites, like other eusocial insects, evolved a highly cooperative, morphologically specialized caste system which generally consists of workers, soldiers, and reproductives. Termites vary greatly in their breeding structures, primarily in the effective number of reproductives heading each colony, and the degree of relatedness between these reproductives. All termite colonies can be classified into one of three family types: simple, extended, or mixed. Simple family colonies are headed by a monogamous pair of reproductives, usually a pair of outbred primary reproductives known as alates (Lepage and Darlington 2000, Myles 1999, Thorne et al. 1999, Shellman-Reeve 1997). Extended family colonies arise from simple family colonies and are headed by multiple inbred secondary reproductives called neotenics and their offspring (Thorne et al. 1999, Vargo 2003). Mixed family colonies are headed by multiple unrelated same sex reproductives and their offspring. These colonies may form from such events as colony fusion, exchange of workers, and pleometrosis (Atkinson and Adams 1997, Bulmer et al. 2001, Goodisman and Crozier 2002, Hacker et al. 2005, DeHeer and Vargo 2004, 2008).

Studies of termite breeding structure have increased exponentially since the advent of molecular markers (Ross 2001). Researchers are now able to infer such parameters as relatedness of individuals within colonies, relatedness of colony founders, numbers of reproductives heading each colony, colony boundaries, and levels of inbreeding, both at the population and colony level. One of the most significant findings is that termite breeding structure is highly variable across taxa, as well as within species. In particular, there is considerable variation in the proportions of colony family types and levels of inbreeding within colonies. In most subterranean termite species, simple family colonies are founded by two unrelated primary reproductives; this is the case for example in *Reticulitermes flavipes* (Bulmer et al. 2001, Vargo 2003, DeHeer and Vargo 2004, 2006; Vargo and Carlson 2006, Vargo et al. 2006 a, b), *R. virginicus* (DeHeer and Vargo 2006; Vargo and Carlson 2006, Vargo et al. 2006a, b), and *Coptotermes formosanus* (Husseneder et al. 2005, Vargo et al 2006a). In contrast, simple family colonies in *R. grassei* (DeHeer et al. 2005) and *R. hageni* (Vargo and Carlson 2006, Vargo et al. 2006b) are often founded by related primary reproductives.

Among extended family colonies, the effective number of reproductives heading colonies is the main source of variation. Studies of *R. flavipes* in Massachusetts and France revealed extended family colonies that were highly inbred and headed by numerous secondary reproductives (Bulmer et al. 2001, Dronnet 2004). The extended family colony type was the only one found in France (Dronnet 2004), while in Massachusetts extended family colonies made up the majority of the colonies collected (Bulmer et al. 2001). Colonies analyzed in Tennessee were found to have levels of inbreeding consistent with highly inbred extended family colonies

(Reilly 1987). However, due to the small number of workers analyzed per colony, Reilly (1987) was unable to conduct pedigree analysis and was therefore unable to determine specific colony family types. In contrast, extended family colonies in North and South Carolina were found to be slightly inbred and headed by relatively few reproductives (Vargo 2003, DeHeer and Vargo 2004, Vargo and Carlson 2006; Vargo et al 2006b, Parman and Vargo 2008). In addition, simple families were the predominant family type in these areas. Extended family colonies of *C. formosanus* studied in southeastern United States, where this species was introduced, have been found to be headed by few reproductives (<9) and are typically less common than simple family colonies (Husseneder et al. 2005, Vargo et al. 2006b). However, different populations of *C. formosanus* exhibit varying levels of inbreeding most likely due to differing levels of relatedness between reproductives heading individual colonies (Vargo et al. 2006b). Native populations of this species were found to be composed of exclusively extended family colonies, while an introduced population in Hawaii was found to have a higher proportion of extended family colonies (Husseneder et al. 2008). Extended family colonies in these populations were consistent with colonies headed by a relatively few reproductives (Husseneder et al. 2008).

Studies of the European species *R. grassei* by Clément and co-workers as summarized in Clément et al (2001), documented a high percentage of simple family colonies (in some cases 77%) over much of its range. Yet, Clement et al (2001) also asserted that during the summer months a drop in aggression between workers occurred and may have led to a single super colony which spanned form southern France to northern Spain. DeHeer et al. (2005) examined the breeding structure of *R. grassei* in three populations in France and reported a high prevalence

of extended family colonies (56-100%). Furthermore, the extended family colonies had high levels of inbreeding and were likely headed by a high effective number of reproductives. Some simple family colonies found in this study were headed by related primary reproductives and others were headed by unrelated primary reproductives.

In the genus *Reticulitermes,* mixed family colonies have been documented in only two species: *R. flavipes* (Bulmer et al. 2001, DeHeer and Vargo 2004, 2008; Vargo and Carlson 2006, Vargo et al 2006a, Parman and Vargo 2008) and *R. grassei* (Nobre et al. 2008). The behaviors between the reproductives heading mixed family colonies are not well understood. For example, the extent to which reproductives may be interbreeding, displaying aggression, or avoidance is unknown. In addition, it is unclear whether they are even in the same vicinity as one another. Our poor knowledge of reproductives within mixed family colonies is due in part to the low frequency at which these colonies are encountered (DeHeer and Vargo 2008). In a 2004 study DeHeer and Vargo documented a mixed family colony which formed via colony fusion. In most cases workers collected from these mixed family colonies could be sorted into distinct family groups, suggesting a lack of interbreeding among the reproductives (DeHeer and Vargo 2004, Vargo and Carlson 2006, Vargo et al. 2006a). The lack of interbreeding between family groups in these mixed family colonies may be attributed to spatial separation between reproductive centers. In a few cases, workers in mixed family colonies could not be separated into distinct family groups (DeHeer and Vargo 2008). This may indicate interbreeding among reproductives or cooperative colony foundation. However, there is little evidence for cooperative colony foundation in subterranean termites (Matsuura et al. 2002). It is currently

thought that colony fusion is the most common event leading to the formation of mixed family colonies (DeHeer and Vargo 2008).

Role of Inbreeding on Termite Eusociality

Inbreeding in termites has been thought to be instrumental to their social organization and largely responsible for their success. Bartz (1979) hypothesized that cyclical outbreeding and inbreeding was the mechanism driving the evolution of eusociality in termites. Inbreeding occurs when the secondary reproductives remain in their natal colony and interbreed. According to this hypothesis, after multiple cycles of interbreeding between related neotenics, alate offspring are produced that are assumed to be highly inbred and predominately homozygous. During the outbreeding cycle, highly inbred alates disperse and form pairs with other unrelated highly inbred alates. These reproductive pairs will found a colony in which the progeny of the colony founders are more closely related to one another than they are to their potential offspring, thus creating an incentive for cooperative brood care. Subsequent studies reexamined Bartz's (1979, 1982) conclusions and outlined the limited role of cyclical inbreeding. Pamilo (1984), and a more recent paper (Myles and Nutting 1988) dissected Bartz's assumptions and highlighted some flaws in his argumentation. These latter authors acknowledged that inbreeding may have played a role in the evolution of eusociality, but downplayed its importance in the evolution of eusociality. Thorne (1997) hypothesized that termites' life history traits along with their ecological role may have predisposed them to the eusocial system. In addition, Husseneder et al. (1998) tested the role of genetics in the development of eusociality in termites by

examining the with-in colony relatedness of the subterranean termite *Schedorhinotermes lamanianus*. These authors found that the colonies studied were not highly inbred and secondary reproductives, while present, were likely to have been inbreeding for a single generation. The authors concluded that the high level of relatedness between individuals within a colony alone may be insufficient to maintain a eusocial system. Again ecological mechanisms were cited as the primary cause for the maintenance of eusociality in termites. Furthermore, Roisin (1999) proposed that the intrinsic benefits of cooperation with in a colony specifically during the early stages of colony formation may be a possible mechanism behind termite social evolution. The exact mechanisms behind the evolution of eusociality in termites remain unclear. More studies regarding breeding systems over a wider range of taxa may help elucidate these mechanisms.

A study of the basal termite, *Mastotermes darwinensis*, utilized microsatellites to determine the relatedness of individuals (Goodisman and Crozier 2002). In contrast to low levels of relatedness in *Schedorhinotermes lamanianus* (Husseneder et al. 1998), this study found workers to be highly related and colonies to be headed by multiple related neotenics and highly inbred. This could be evidence for the importance of inbreeding in the evolution of eusociality in termites (Goodisman and Crozier 2002). However, recent studies have shown low levels of inbreeding in subterranean termites (Husseneder et al. 1998, Vargo 2003a, b; DeHeer and Vargo 2004, 2006; Vargo and Carlson 2006; Vargo et al. 2006a) and possible consequences to inbreeding (DeHeer and Vargo 2006, Husseneder et al. 2008). This empirical evidence shows that inbreeding may not be as widespread and as important to the evolution of eusociality in termites as previously believed.

Inbreeding occurs in nearly all studied taxa (Jimenez et al. 1994, Madsen et al. 1996, Brown and Brown 1998, Keller 1998, Saccheri et al. 1998, Keller and Waller 2002). Ordinarily, inbreeding is seen as a threat to populations primarily due to allelic reduction or fixation, and the possible accumulation of deleterious mutations; this is known as inbreeding depression (ID) (Keller and Waller 2002). ID can be a serious problem for endangered species, because populations of endangered species generally tend to be small and genetically isolated. These two factors can accelerate negative effects of inbreeding. Generally ID is more pronounced in natural populations as opposed to the laboratory setting. This can be explained by the mild conditions prevailing in the latter which may alleviate the burden of deleterious alleles (Keller and Waller 2002). Some previous studies have outlined inbreeding avoidance mechanisms in wild populations (Pusey and Wolf 1996). In addition, others have proposed that inbreeding depression can be alleviated, because inbreeding can expose deleterious recessive mutations to selection, thus purging them from the population (Lande and Schemske 1985). Clearly inbreeding can affect populations in a variety of ways.

While most consequences of inbreeding are seen in immune responses or reproductive success (reviewed in Keller and Waller 2002), an examination of morphological consequences of inbreeding is important in discerning the impact of inbreeding on a species. In addition, arthropods are an ideal group to test this relationship due to the short generation time. A study of the sub-social spider, *Stegodyphus lineatus* (Latreille), showed a significant negative correlation between the levels of inbreeding with respect to development and adult body size (Bilde et al. 2005). However, other studies showed no effect of inbreeding levels on individual size both in

crickets (Roff and Derose 2001) and bumble bees (Gerloff et al. 2003). Two studies of the invasive subterranean termite *Coptotermes formosanus* reported a significant negative correlation between worker body size and inbreeding level within of colonies (F_{IC}) (Husseneder et al. 2005, 2008), meaning colonies with high effect numbers of reproductives are composed of workers with smaller body size.

In general, smaller size is considered to be detrimental rather than advantageous (Keller and Waller 2002). Nevertheless, there has been some speculation that in *C. formosanus* smaller worker size may be beneficial, because smaller workers may be more vigorous and therefore more active foragers (Grace et al. 1995). Grace et al. (1995) noted that a decrease in colony population size correlated with an increase in worker body size in aging colonies, suggesting that large workers in this species may be an indication of an older colony close to collapse. In contrast, Campora and Grace (2001) reported that larger workers are able to construct larger tunnels which may increase foraging rates and thereby be advantageous. Husseneder and Simms (2008) found that smaller primary reproductives of *C. formosanus* were less likely to successfully pair with other primaries during tandem pair formation, resulting in lower fitness.

In conclusion, recent studies of subterranean termites have provided great insight into their breeding systems. Extended family colonies can range from highly inbred colonies headed by numerous reproductives to colonies with low levels of inbreeding and headed by a relatively low effective number of reproductives. The importance of inbreeding has been challenged as an essential mechanism for the evolution of eusociality in termites. Recent empirical data point to other factors as playing important roles in the evolution of eusociality in termites. While

morphological consequences to inbreeding may not be as profound as other possible manifestations, it is still an important aspect to examine.

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Chapter 2

Colony genetic structure and effects of inbreeding on body size in three populations of *Reticulitermes flavipes* in the southeastern U.S.

Introduction

An in depth understanding of the genetic structure of populations can allow for a detailed view into a variety of evolutionary and ecological mechanisms (Neigel 1997, Bossart and Prowell 1998). In social systems, genetic structure is particularly important when examining the evolution of sociality, as well as investigating the occurrence and persistence of multiple-queen societies, reproductive skew, sex ratio conflict, and worker reproduction (Mehdiabadi et al. 2003, Pamilo et al. 1997, Ross 2001, Sundström and Boomsma 2001). Since the order Hymenoptera served as a basis for both original theory on kin selection (Hamilton 1964) and work on sex ratio conflict (Trivers and Hare 1975), expectedly this group has received the majority of the attention regarding experimental genetic studies devoted to eusocial insect societies (Crozier and Pamilo 1996). Despite the fact that termites (Isoptera) are a globally distributed group of eusocial insects which are important both ecologically and economically their basic colony breeding structure and population genetics have not been well characterized.

Termites, like other eusocial insects, evolved a highly cooperative caste system. Isopteran societies possess non-reproductive worker and soldier castes and morphologically specialized reproductive castes. Within this basic colony organization the termites vary greatly in their breeding structures, both in the number of reproductives and the degree of relatedness between

these reproductives. The variation largely depends on the colony's family type. All termite colonies form one of three colony family types: simple, extended, or mixed. Simple family colonies are headed by a monogamous pair of reproductives, usually a pair of primary reproductives which disperse via mating flights (Shellman-Reeve 1997, Myles 1999, Thorne et al. 1999, Lepage and Darlington 2000). Extended family colonies arise from simple family colonies and are headed by multiple inbred secondary reproductives called neotenics and their offspring. The production of neotenics varies widely across taxa. Multiple neotenics are commonly found in Mastotermitidae, Termopsidae, and Rhinotermitidae (Lenz 1985), but supernumerary neotenics are eliminated by fighting in Kalotermitidae, leaving a single pair (Ruppli 1969, Myles and Chang 1984, Lenz 1985).

The production of neotenics may be tied to an ancestral trait linked to the founding of temporary nest sites near low quality food sources (Shellman-Reeve 1997, Myles 1999, Thorne et al. 1999). Neotenics remain within their natal colony and interbreed with other nestmates, thus producing inbred offspring. Mixed family colonies are headed by multiple unrelated reproductives and their offspring. These colonies may arise through such events as colony fusion, exchange of workers, and pleometrosis (Atkinson and Adams 1997, Bulmer et al. 2001, Goodisman and Crozier 2002, DeHeer and Vargo 2004, Hacker et al. 2005, DeHeer and Vargo 2008).

Subterranean termites are common in the eastern United States (Austin et al. 2004a, b, c), and due to their highly variable colony breeding structure this group of termites serves as an excellent model system for studies examining the causes and consequences of social insect

breeding systems. However, because of their cryptic nesting and foraging behavior many questions regarding their breeding structure remain unanswered. Nevertheless, with advances in molecular biology techniques, specifically molecular markers, recent studies have begun to reveal new details about colony breeding structure and to tease apart the underlying factors influencing it (Clement et al. 1981, Clement 1984, Bulmer et al. 2001, Clement et al. 2001, Bulmer and Traniello 2002a, b; Vargo 2003a, b; DeHeer and Vargo 2004, Dronnet et al. 2004, DeHeer et al. 2005, Dronnet et al. 2005, Vargo and Carlson 2006). *Reticulitermes* remains the most extensively studied genus with respect to breeding systems in termites. Many of these studies have examined one species in particular, the eastern subterranean termite *R. flavipes* (Kollar). Over the geographic range of this species recent studies have documented variation in colony breeding structure. Using five polymorphic allozymes and double-strand conformation polymorphism (DSCP) analysis, Bulmer et al. (2001) reported that six of 22 colonies collected in Massachusetts were simple family colonies. Of the remaining colonies, 13 were extended family colonies, and three colonies were mixed families. This ratio of simple to extended family colonies is far different from findings in recent studies in North Carolina which used highly variable microsatellites for determining the breeding structure of *R. flavipes*. In these studies 79% of 318 colonies studied were found to be simple family colonies, while 24% fit the extended family colony model (Vargo 2003, DeHeer and Vargo 2004, 2006; Vargo and Carlson 2006, Parman and Vargo in press). In addition, a recent study in South Carolina reported similar findings regarding the predominance of simple family colonies (Vargo et al. 2006a).

Within extended family colonies the effective number of reproductives can range from one male and one female to well over 100 (Thorne et al. 1999). In addition, the degree of relatedness between the inbreeding neotenics can vary as can the number of generations of inbreeding a colony has undergone. Consequently, the degree of inbreeding can vary dramatically among colonies within a population as well as among populations across the geographic range. Typically, Wright's *F*-statistics, specifically the F_{IC}-value, is used to determine the specific breeding structure within extended family colonies. F_{IC} is defined as the level of inbreeding within individuals relative to their respective colonies. The F_{IC}-value is highly sensitive to the effective number of reproductives heading a colony. When F_{IC} is highly negative (-0.35 to -0.10) the effective number of reproductives is expected to be low; as F_{IC} approaches zero the effective number of reproductives is expected to be high. A positive F_{IC}-value indicates non-random mating by spatially separate reproductive centers or the presence of mixed family colonies (Thorne et al. 1999, Bulmer et al. 2001).

Extended family colonies studied in Tennessee and Massachusetts (Reilly 1987, Thorne et al. 1999, Bulmer et al. 2001) had higher F_{IC}-values ($F_{IC} > 0.05$) than extended family colonies in North and South Carolina ($F_{IC} < -0.14$), suggesting that extended family colonies in Tennessee and Massachusetts were headed by more secondary reproductives; in addition these reproductives may have formed spatially separated centers, and the effective number of reproductives was on the order of tens to hundreds. Based on F_{IC} values, these colonies likely underwent more generations of inbreeding than the colonies studied in North and South Carolina, where relatively few secondary reproductives appeared to be heading colonies, on average fewer

than 10 (Vargo 2003a, b; DeHeer and Vargo 2004, 2006; Vargo and Carlson 2006, Vargo et al. 2006a).

Although inbreeding in termites has been thought to be instrumental to their social organization and responsible to a large extent for their success (Hamilton 1964, Husseneder et al. 1999), it is largely accepted that inbreeding generally has negative consequences on individuals as well as populations (Keller and Waller 2002). However only recently have researchers begun to examine the effects in detail in termites. One study, conducted by DeHeer and Vargo (2006), examined possible consequences of inbreeding in subterranean termite colonies. The authors reported an indirect effect of inbreeding depression in *R. flavipes*. When colony founding occurs via two primary reproductives, the relatedness between the founding pair ranges from zero, in the case of an unrelated pair, to 0.5, in the case of a pair of full siblings. The authors used microsatellite markers to calculate the degree of relatedness between collected tandem running pairs, which form after the primary reproductives dealate following their mating flight, but prior to colony foundation. These individuals were compared to reproductives that had successfully founded mature colonies. The degree of relatedness of tandem partners was significantly greater than the level of relatedness of reproductives heading established mature colonies, indicating a reduction in colony survivorship among founding pairs comprised of closely related individuals.

There is contradictory evidence for the effects of inbreeding in the dampwood termite *Zootermopsis angusticollis* (Hagen). One study reported that grouped inbred termites had significantly greater mortality when exposed to a relatively high concentration of fungal pathogens when compared to groups of outbred termites, indicating a negative effect of

inbreeding (Calleri et al. 2006). However, an earlier study (Rosengaus and Traniello 1993) found a reduction in survivorship in outbred colony founders, yet reported no difference in the growth of incipient colonies founded by these outbred primary reproductives when compared to related founding pairs. Clearly, additional detailed studies regarding potential effects of inbreeding on termite societies are necessary in order to elucidate the costs or benefits of inbreeding.

Results vary in regards to morphological consequences of increased levels of inbreeding across taxa. A study on the sub-social spider, *Stegodyphus lineatus* (Latreille), showed a significant negative correlation between the levels of inbreeding with respect to development and adult body size (Bilde et al. 2005). However, other studies showed no effect of inbreeding levels on individual size both in crickets (Roff and DeRose 2001) and bumble bees (Gerloff et al. 2003). In termites, Husseneder et al. (2005) reported that the invasive species *Coptotermes formosanus* (Shiraki) had a significant negative correlation of worker weight to the inbreeding levels of the colony (F_{IC}) which translates into colonies with high effective numbers of reproductives being composed of workers with smaller body size.

The aim of the present study was to extend our knowledge of the population and breeding structure of *R. flavipes* by analyzing populations located in previously unstudied parts of its range in Mississippi and Florida and to determine if there was an effect of inbreeding on size of colony members or on fluctuating asymmetry. In order to accomplish this I used microsatellite genotypes of workers to infer the colony social organization of the study populations. In addition, I utilized both worker and soldier morphological characteristics to investigate the

effects of colony family type, as well as effects of colony inbreeding levels, on worker and soldier body size. This study is the first to investigate morphological consequences of elevated inbreeding levels in *R. flavipes*.

Materials and Methods

Collection

Samples of *Reticulitermes* spp. were collected from natural wooded sites in DeSoto National Forest in Harrison County, Mississippi (N 30˚ 37.429' W 89˚ 03.332') on June 30, 2005, and Ocala National Forest in Putnam County, Florida (N29˚ 25.806' W81˚ 44.238') on October 10, 2005 (coordinates correspond to the first collection point at each location). *Reticulitermes* spp. were the only subterranean termites found, and all samples were collected regardless of species. The DeSoto National Forest site consisted primarily of longleaf (*Pinus palustris*), slash (*P. elliotii*) and loblolly (*P. taeda*) pines (USFS http://www.fs.fed.us/). This site was an even-growth forest, planted in rows with trees approximately 2 m apart. The Ocala National Forest site contained comparable flora (USFS http://www.fs.fed.us/). However, the latter forest was mixed-growth, and trees did not appear to be evenly spaced.

Samples were collected from a total of 106 and 66 collection points, in DeSoto and Ocala National Forests respectively (Fig 2 and Fig 3 respectively). Each collection point consisted of naturally occurring wood debris, e.g., a tree stump or fallen branch. In the case of a large log or tree stump, individuals were collected from a localized area not exceeding 1 m in diameter. At each collection point termites were manually aspirated and placed in individually labeled 1.5 ml

Nalgene collection tubes containing 95% ethanol. Collection points were spaced at least 15 m apart in order to increase the likelihood of sampling distinct colonies (DeHeer and Vargo 2004). Compass bearings and the distances between neighboring collection points were taken to construct a map of the collection area. Global Positioning System (GPS) data were also recorded in triplicate for each collection point. A minimum of 30 workers were collected from each point to ensure sufficient individuals for genotyping (n = 20; see Methods below under Genotyping) and morphometrics (n = 5; see Methods below under Morphometrics). Soldiers, when observed, also were collected for morphometric analysis. Once in the laboratory, dirt and debris were removed from the vials, and the ethanol was replaced with a fresh solution (95%). The samples were stored at −20°C prior to DNA extraction.

DNA Isolation and Species Identification

DNA was isolated from whole termite bodies (n = 1115) following the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) protocol with some slight modifications. After the final ethanol wash, the remaining DNA pellet was air-dried overnight and resuspended in 250-300 μl of 1× TE buffer (Sigma-Aldrich, St. Louis, MO, USA).

Species identification proved to be challenging and involved several steps. In the eastern United States there are four recognized species: *R. flavipes*, *R. virginicus*, *R. malletei* and *R. hageni* (Scheffrahn and Su 1994; Austin et al. 2007). Initially, I attempted to use morphological characteristics of soldiers for identification following the keys of Scheffrahn and Su (1994) and Hostettler et al. (1995). According to these keys, *R. flavipes* soldiers have a pronotal width

measuring greater than 0.85 mm and the point of the left mandible curving inward at 70˚-90˚. *R. virginicus* pronotum measures 0.71-0.80 mm wide with the point of the left mandible curving inward at 70˚-90˚. In *R. hageni*, the soldier pronotum is less than 0.70 mm wide with the point of the left mandible curving inward at a 45˚ angle. This method alone was insufficient for identifying all samples to species because soldiers were not present in some samples (approximately 80% contained soldiers). Moreover, according to Szalanski et al. (2003) these morphological characteristics are unreliable for accurate species identification due to considerable overlap in soldier pronotal widths among species (see also DeHeer and Vargo 2004, Vargo and Carlson 2006). However, the morphological characteristics offered preliminary data on the species composition for each collection site.

The second method I used was the molecular approach of Szalanski et al. (2003) where species were identified based on restriction digest patterns of a portion of the mitochondrial *Cytochrome oxidase* II (COII) gene. These authors reported that PCR-restriction fragment length polymorphism (RFLP) analysis with the restriction enzyme *Taq*I for the COII gene was a reliable method for determining species identity for the genus *Reticulitermes* in the eastern United States. I applied this method to two individuals per collection point. DNA was amplified in 40 µl reaction volumes each containing 4 ng genomic DNA, 1X PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.06 U *Taq* polymerase (Bioline Randolph, MA), and 1.5 pmol each of the primers designed by Szalanski et al. (2003): tercoII-i*df* (TCTTCTTCCACGAYCAYACAYTAATAA) and tercoII-idr (TTTATGGGTAGTACYATTCGYTT). PCR amplification was carried out in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) using the following conditions: an

initial denaturation step at 94 °C (2 min), followed by 35 cycles at 94 °C (1 min), 50 °C (1 min) and 70 °C (1 min), with a final extension step at 70 °C (5 min) and then held indefinitely at 4 °C.

Following amplification, 4 µl of the PCR product of each sample was combined with 1 µl of Bromophenol blue loading buffer (25% glycerol, 20% 0.5M EDTA, 0.25% Bromophenol blue) and subsequently loaded on a 1% 0.8X TBE agarose gel stained with ethidium bromide (1% solution, Fisher Biotech Subiaco, Australia) to verify expected amplicon size. DNA standards (Hyperladder II, Bioline Randolph, MA) were loaded in the first and last lanes of the agarose gel to determine fragment size. The gel was run at 80V on a Maxicell Primo (EC Apparatus, Holbrook, NY) electrophoretic gel system for approximately 1 h. Gels were then visualized by UV trans-illumination using the Kodak Digital Science (Rochester, NY) 1D gel analysis software system.

Samples containing the amplified COII region were subsequently digested with the restriction enzyme *Taq*I (New England Biolabs, Ipswich, MA). The reactions were carried out in 20 µl volumes containing 10 µl PCR reaction mixture, 2.0 µl Buffer 3 (New England Biolabs, Ipswich, MA), and 4 U of *Taq*I (New England Biolabs, Ipswich, MA) enzyme. Samples were digested according to the manufacturer's instructions: 60°C for 3 h followed by 80°C for 20 min. Following digestion, 4 µl of the product was combined with 1 µl Bromophenol blue loading buffer and then loaded on a 2.5% 0.8X TBE agarose gel containing ethidium bromide. DNA standards (Hyperladder II, Bioline Randolph, MA, USA) were again loaded in the first and last lanes of the gel. Gels were run at 80V on a Maxicell Primo electrophoretic gel system for approximately 1 h. DNA fragment patterns were then visualized as described above to determine

the presence or absence of diagnostic restriction sites. Species identity was tentatively assigned based on the fragment length size of the individuals from each collection point (Szalanski et al. 2003). *R. flavipes* has fragment sizes of 292-bp and 87-bp, whereas *R. virginicus* and *R. hageni* do not have a restriction site for *Taq*I and therefore have a fragment length of 379 bp. Figure 1 shows representative results using this method.

In addition, microsatellite genotyping of five individuals from each collecting point (n = 860 individuals) at locus *Rf* 24-2 (protocol given in Genotyping below) was employed for further clarification based on consistent differences in allele sizes among species (Vargo, unpublished). Samples with allele sizes greater than 125 bp at this locus were considered to be *R. flavipes*, whereas those with allele sizes less than 125 bp were considered to be either *R. virginicus* or *R. hageni*. Some of these data were contradictory to the species assignments based on the PCR-RFLP method (see Results below). To reconcile these differences, I utilized the genetic analysis program STRUCTURE v2.0 (Pritchard et. al. 2000) which used the genotypic data from all eight of the loci investigated (see Genotyping below) to infer group structure and assign individuals to putative clusters (see Population Definitions under Methods below). This program was also implemented to analyze the genetic relationships of colonies within and among sites (see Population Designation below), and further clarify species identity. Ordinarily, the PCR-RFLP method described by Szalanski et al. (2003) is sufficient for species identification of *Reticulitermes*; however, in this study the presence of possible undescribed species confounded the results (see Results below). Therefore, I based identification of *R. flavipes* samples on microsatellite allele sizes with support from the STRUCTURE results.

Microsatellite Genotyping

Once I had identified the *R. flavipes* samples, I isolated the DNA from an additional 15 workers per collection point (195 individuals), bringing to 20 the total number of workers analyzed per collection point. These were genotyped at eight polymorphic trinucleotide microsatellites loci: *Rf* 24-2, *Rf* 21-1, *Rf* 1-3, *Rf* 5-10, *Rf* 6-1, *Rf* 15-2, (Vargo 2000) *Rs 10*, and *Rs* 15 (Dronnet et al. 2004). PCR reactions were carried out in 5 µl volumes, each containing 10× NH_4 buffer (160 mM $(NH4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20), 2 mM $MgCl_2$, 0.2 µg/µl Bovine Serum Albumin (BSA), 0.2 mM dNTPs, 4 ng of DNA template, and 0.033 U *Taq* polymerase (Bioline Randolph, MA, USA). The forward primer of each set contained a fluorescent IRD label (Li-Cor Biosciences, Lincoln, NE, USA). Concentrations of the labeled and unlabeled primers used varied according to locus (Table 1). Primers were multiplexed in the following combinations: *Rf* 1-3 with *Rf* 5-10, *Rf* 6-1 with *Rf* 15-2, and *Rs* 10 with *Rs* 15. *Rf* 24-2 and *Rf* 21-1 were amplified and screened separately due to their large ranges in allele sizes. PCR cycling profiles varied according to locus as shown in Table 1. The specific conditions are as follows. Touch1: Initial denaturation step at 94 °C for 30 s, followed by 6 cycles of 94 °C for 30 s, annealing step starting at 60 °C for 30 s, dropping 1°C, per cycle and an extension step at 72 °C for 30 s. This was then followed by 30 cycles at 94 °C (30 s), 54 °C (30 s) and 72 °C (30 s), with a final extension step at 72 °C (5 min) and held indefinitely at 4°C. MICRO57: Initial denaturation step at 95 °C (5 min), followed by 35 cycles of 95 °C (30 s), 57 °C (30 s), 72 °C (30 s) and held indefinitely at 4°C. MICRO-F1: Initial denaturation step at 94 °C (5 min), followed by 30 cycles at 94 °C (1 min), 55 °C (1 min) and 72 °C (15 s), with a final extension step at

72 °C (5 min) and held indefinitely at 4°C. All PCR programs were carried out using PTC-100 thermal cyclers (MJ research, Watertown, MA, USA).

Amplified PCR products were then combined with 5 µl of loading buffer (95% formamide, 10mM EDTA, 10mM NaOH and 0.01% Pararosaniline). Reactions were subsequently denatured at 90 °C for 4 min and approximately 0.2 µl of each sample was loaded onto 25 cm 6.5% 1× TBE polyacrylamide gels. IRD labeled size standards spanning a range of 50-350bp (IRDye700/IRDye800, Li-Cor Biosciences, Lincoln, NE) were loaded every 20 samples to enable accurate sizing of allelic fragments. Gels were run on a Li-Cor 4300 automated DNA sequencer (Li-Cor Biosciences, Lincoln, NE) for 120 min at 1500V, 38mA at 40W and 50 °C. GENEPROFILER (v4.5) software (Scanalytics, Inc Rockville, MD, USA) was used to genotype individuals. All data were subsequently compiled into an Excel (Microsoft) spreadsheet prior to statistical analysis.

Basic Genetic Analysis

Descriptive statistics for the microsatellite loci (allelic richness, frequency of the most common allele, observed versus expected heterozygosity, and mean number of alleles per locus) were obtained from the program Genetic Data Analysis (GDA) v1.1 (Lewis and Zaykin 2000). The tests were based on a single individual per colony. Tests of Hardy-Weinberg equilibrium were performed for each population (n =3) (see Defining a Population under methods). The program GENEPOP ON THE WEB (Raymond and Rousset 1998) was used to perform exact tests of Hardy-Weinberg equilibrium, in which each locus in the population was examined by a

probability test (Option 1, Sub-option 3, Markov chain parameters were the following default values: dememorization number 1000; number of batches 100; and number of iterations per batch 1000). Enumeration of alleles was not done. In addition, GENEPOP ON THE WEB was used to test for linkage disequilibrium by testing each pair of loci in each population (Option 2, Sub-option 1). I used the default Markov chain parameters (dememorization number 1000, number of batches 100, and number of iterations per batch 1000).

Determination of Population Identity

To determine if further population substructure existed within each of the sites I used the program STRUCTURE v2.0 (Pritchard et al. 2000). This program uses Bayesian analysis to estimate the minimum number of putative clusters to which the data match and then assigns each individual probabilistically to one of the clusters independent of prior assignment to specific groups. Once the number of putative clusters is identified, analysis can then be rerun implementing geographic information (USEPOPINFO = 1) to provide a more robust estimate of population structure (Pritchard et al. 2000). For the initial analysis, a single individual per colony was used under the following parameters: USEPOPINFO = 0, which uses only the genotypic data to form groups rather than predesignation based on geographic location. I used a range of one to four for the number of putative clusters (populations) to which the data may match. One was the minimum number of clusters possible, i.e., if all colonies belonged to the same population. The upper limit was calculated by multiplying the suspected number of clusters (populations) by two. In the case of the Mississippi collection site I suspected 2 separate

populations based on data generated by examining genetic isolation by distance, hence the upper limit was 4 possible clusters. In order to determine the most likely number of clusters (populations), the posterior probability of *K* (P (K|X)) was estimated for each of the possible values of *K* (1-4), and P (K|X) will approximate one for the most likely number of clusters as described in Pritchard et al. (2000). All runs were based on 100,000 iterations, following a burn-in period of 50,000 iterations and three repetitions. Analysis was further replicated two additional times, each time selecting a different individual from each colony. Finally, the analysis was rerun using population geographic information (USEPOPINFO = 1; *K* = 1 - 4).

To further investigate population structure, I conducted exact tests of genotypic differentiation. For this test I examined individuals (n = 20) from each colony from each of the three populations identified by STRUCTURE. One group consisted of all *R. flavipes* colonies found in Florida, whereas the *R. flavipes* colonies from Mississippi were split into two groups, MS1 and MS2 (see Results). Analysis was carried out under the following default parameters: dememorization number: 1000, number of batches: 100, number of iterations per batch: 1000. Groups were considered separate if the P-values were less than or equal to 0.05.

Colony Affiliations

Colony affiliations for the individuals from all collection points were determined from the microsatellite genotypes at all of the eight loci examined. The program GENEPOP ON THE WEB was used to determine whether each collection point was a distinct colony or whether multiple collection points belonged to the same colony. Exact tests of genotypic differentiation

were conducted for all pairs of populations (Option 3, Sub-option 4). Markov chain parameters were the following: dememorization number: 1000, number of batches: 100, number of iterations per batch: 1000. Collection points were considered separate colonies if the *P*-values were less than 0.05 (DeHeer et al. 2005, Vargo and Carlson 2006).

Colony Classification

Consistent with terminology used by Vargo (2003) and DeHeer and Vargo (2004), colonies were classified into three different family types: simple, extended, and mixed. Simple family colonies are colonies headed by a single pair of monogamously breeding reproductives. Worker genotypes from this family type were consistent with those from a single pair of parents, and the frequencies of the genotypes did not differ significantly from expected. Deviations from the expected genotypic frequencies were calculated by a *G*-test (goodness of fit test). *G* -values at each locus were calculated and summed to obtain an overall G-value. Colonies were considered to be simple families if $P > 0.05$ for the overall *G* -value.

Extended family colonies arise from simple family colonies and are headed by multiple inbred reproductives (neotenics). Worker genotypes from extended family colonies are expected to consist of a maximum of four alleles at any given locus, but may have more than four genotypic classes at a locus, more than two homozygous classes at a locus, or genotypic frequencies that deviate significantly from expected Mendelian frequencies ($P < 0.05$).

Mixed family colonies are headed by multiple unrelated same-sex reproductives. Worker genotypes in this group are characterized by having more than four alleles at one or more loci which could only arise through the presence of two or more unrelated kings or queens.

Population and Colony Breeding Structure

The genetic structure of both the colonies and populations were examined at multiple levels. Colony as well as site-level structure was assessed concurrently by estimating *F*-statistics using the methods of Weir and Cockerham (1984) in the program *F*STAT (Goudet 2001). I used terminology developed by Thorne et al. (1999) and Bulmer et al. (2001) in this study. Distinct colonies are viewed as individual populations, and genetic variation is divided among the individual (I), colony (C), and total (T) components. Under this notation, F_{IT} is a measure of the genetic differentiation between an individual and the total population, and is also the measure of the standard inbreeding coefficient (F_{IS}). F_{CT} is a measure of the genetic differentiation between colonies within a population, and it is analogous to F_{ST} in solitary systems. F_{IC} is referred to as the colony inbreeding coefficient, and is exclusive to social systems. F_{IC} is highly influenced by the number of effective reproductives heading a colony, as well as the number of generations of inbreeding a colony has undergone. F_{IC} is especially descriptive of colony breeding (Thorne et al. 1999). In particular, F_{IC}-values should be highly negative (F_{IC} = -0.33) in simple family colonies (Thorne et al. 1999). The F_{IC}-value increases as the number of effective reproductives heading a colony increase. In addition, F_{IC} will increase as the number of generations of inbreeding increase. Highly inbred colonies headed by numerous effective reproductive have an

F_{IC}-value which approaches zero. Non-random mating and mixing of individuals from different colonies may drive F_{IC} to a positive value (Thorne et al. 1999). Standard errors were constructed by bootstrapping over loci with 1000 replications. *F*-statistics at each site were estimated for all colonies within a given population, and subsequently estimated for each family type separately (simple, extended and mixed). Values obtained were compared to values previously generated from computer simulations of possible breeding systems of *Reticulitermes* spp. by Thorne et al. (1999). Above the level of the colony, genetic structure among populations was quantified by determining F_{ST} values among the different populations in this study. I utilized the program KINSHIP 1.3.1 (K.F. Goodnight; http://www.gsoftnet.us/GSoft.html) in order to identify the likelihood that a pair of reproductives heading a simple family colony was full siblings. Genotypes for the reproductive pairs were inferred from the worker genotypes of each simple family colony. For these calculations I used a relatedness coefficient of zero for the null hypothesis and the observed relatedness coefficient for within colony relatedness in each respective population as the alternative hypothesis. I used 10,000 permutations to asses the statistical significance. In addition, each population was examined separately.

Morphometrics

To investigate potential trends in body size and symmetry in relation to population and colony breeding structure, morphometric analysis was performed on a number of body measurements taken from both workers and soldiers (when the latter were collected) from each of the colonies sampled. Measurements taken for workers were body weight, head width and

lengths of right and left hind tibiae. For soldiers, I measured pronotal width, head width and head length. These measurements were used as an indication of termite body size (Matsuura 2001, Campora and Grace 2004), with the exception of the hind tibiae which were used to detect possible fluctuating asymmetry.

Worker Morphometrics

Mass of 10 individual workers was taken for each colony (MS1: n = 8; MS2: n = 15; FL: n = 7). Worker termites were removed from 95% ethanol and allowed to air dry for approximately 15 min which allowed the termites to reach a constant dry weight before being placed individually on an analytical scale (AB 104, Mettler-Toledo Columbus, OH). Each termite was weighed to the nearest 0.1 mg. Head widths of five workers were measured from all colonies. Measurements were taken by decapitating the worker and orienting the head on a Petri dish filled with clay. The dorsal side of the head was facing upward and the posterior side was distal to the front of the microscope. Measurements were then taken at the widest point of the head capsule using a dissecting microscope (Nikon SMZ-2T SM-instruments, Atlanta, GA) fitted with an ocular micrometer which was calibrated with a stage micrometer. Different castes were measured at different levels of precision. Worker head widths and tibiae lengths were measured to the nearest 0.015 mm, and soldier pronotal width, head width and head length were measured to the nearest 0.022 mm. To determine the degree of fluctuating asymmetry, hind tibia lengths were measured by removing both hind legs of an individual (n = 5 per colony) and lying them flat to ensure accuracy of all measurements. Each measurement of head width and tibia length was duplicated by removing and reorienting the respective body part, and repeating the

measurement after all other individuals from the colony were measured. In all cases, second measurements did not differ from initial measurements.

Soldier Morphometrics

Variable numbers of soldiers were collected from different colonies, and often no soldiers were encountered or collected. Therefore not all colonies could be included in the analysis of soldier morphometry. Head width measurements were taken in a manner similar to that described above for worker head widths. Decapitated heads were then turned 90 degrees and head length at the longest point was taken. Pronotal width measurements were taken at the widest point of the dorsal surface of the pronotum. All soldier measurements were again duplicated by the same method used for the workers (see above).

Analysis

All morphometric data (worker body weight, head width and lengths of right and left hind tibiae, soldier pronotal width, head width and head length), as well as the relationship between the morphometric measurements (Correlations, Tukey's comparison of means, and ANOVA), were analyzed using the computer program JMP v 6.0 (SAS Institute Inc., Cary, NC).

Results

Species Identification

Soldiers were obtained from 88 of the 106 collection points sampled in Mississippi. These specimens were used for species identification based on morphological characteristics. The mean pronotal width of soldiers was 0.86 mm and ranged from 0.83-0.92 mm. Soldiers from

37 of the 88 collection points containing soldiers had morphological features characteristic of *R. flavipes* (pronotal width > 0.90 mm and angle of $70^{o} – 90^{o}$ curved inward for the left mandible), as described by Scheffrahn and Su (1994) and Hostettler et al. (1995). In Florida, 57 of the 66 collection points contained soldiers. Of these, specimens from 28 collection points had morphological characteristics consistent with *R. flavipes* with a mean pronotal width of 0.97 mm and a range of 0.84-1.18 mm. Some samples had conflicting morphological characteristics for soldiers. Samples that had mandible curvature consistent with *R. flavipes* but slightly smaller than expected head widths were tentatively classified as *R. flavipes* pending results from the molecular data.

PCR-RFLP analysis indicated that samples from 27 of 106 collection points from the Mississippi site had *cytochrome oxidase* II fragments containing the *Taq*I restriction site, resulting in the 287-bp subfragment diagnostic of *R. flavipes* (Szalanski et al. 2003). Only 19 of these samples were a part of the 37 collection points classified as *R. flavipes* based on morphology alone. Of the 66 collection points sampled in Florida, only 23 contained samples with the *Taq*I restriction site. Species specific allelic size range for *R. flavipes* (111–216 bp) at the microsatellite locus *Rf* 24-2 was used to help clarify the classifications described above. All 27 collection sites from Mississippi possessing the *Taq*I restriction site also had the *R. flavipes* characteristics *Rf* 24-2 allele sizes; these were therefore classified as *R. flavipes*. In the FL population 23 collection points contained the *Taq*I restriction site. Only 13 of these samples were part of the 28 collection points classified as *R. flavipes* based on morphology alone. However, only 15 of the 23 (65%) collection points containing the *Taq*I restriction site had *Rf* 24-2 allele

sizes consistent with *R. flavipes*. The remaining eight collection points containing the *Taq*I restriction site were not considered *R. flavipes*, because these samples had allele sizes ranging from 75 to 90 bp, which were below the range expected for *R. flavipes*. In addition, soldier morphology for these collection points was inconsistent with morphological characteristics of *R. flavipes* (see Appendix for details).

The Bayesian assignment program, STRUCTURE v 2.0 (Pritchard et al. 2000), resolved the contradictions between the PCR-RFLP and microsatellite methods regarding species identification within the FL sample. This program estimates the most probable number of population clusters (K) into which the total sample can be arranged with no prior knowledge of structure, based on allele associations and conformance of designated clusters to Hardy Weinberg Equilibrium (HWE). Individuals are then given an assignment value for each putative cluster. For the collection points within the FL population which contained the *Taq*I restriction site (n = 23), the most likely number of putative clusters describing the allelic data was two prior to applying population flags (i.e., known structure). Individual assignment values to these clusters ranged from 68.3% to 89.3%. These values increased to 95.4% and 98.6% once population flags were applied. Examination of these two putative clusters resulted in one consisting exclusively of collection points containing both the PCR-RFLP patterns and microsatellite genotypes expected for *R. flavipes*. Thus, this group was considered to be *R. flavipes*. The other group was comprised of collection points consistent with the PCR-RFLP designation as *R. flavipes*; however, these displayed microsatellite allelic sizes smaller than expected for this species. This second group was considered to belong to another species or

subspecies, yet to be determined. Based on a combination of morphological and molecular characteristics, I concluded that *R. flavipes* was present in 29 of the 106 collection points from Mississippi and 14 of 66 collection points in Florida. However, it is important to note that whenever morphological data contradicted molecular data I used the molecular data to determine species identity.

Colony Designation

Once collection points were assigned to populations (FL, MS1 and MS2 see Population Differentiation below), colony affiliations were determined. Fisher's exact test of the genotypic differentiation for all pairwise combinations of collection points in the FL population varied from non significant ($P > 0.78$) to highly significant ($P < 0.001$). Based on these values, I determined that the FL population contained two colonies comprised of multiple collection points, and seven single collection point colonies (Fig. 3). One colony consisted of five collection points spanning over 110 linear meters. In addition, another colony consisted of two separate collection points covering 35 linear meters. The mean F_{CT} for collection points that were a part of the larger colony was -0.012, this value was not significantly different from zero (two sample *t*-test; $P < 0.05$; $df = 14$). The mean F_{CT}-value between all other collection points was 0.322. In contrast, both Mississippi populations consisted exclusively of single collection site colonies (all $P < 0.0001$, for all pairwise tests), with a mean F_{CT} value of 0.355 for all pairs of collection points. Thus, population MS1 consisted of nine colonies and population MS2 contained 18 colonies (Fig. 2).Overall, genetic differentiation among colonies in the FL

population was significantly lower than in MS2 (two sample *t*-test; $P = 0.006$, $df = 14$) but not MS1 (two sample *t*-test; $P = 0.07$, $df = 14$).

Population Differentiation

While comparing pairwise F_{CT}s, I noticed that some pairs of colonies in the Mississippi population that were separated by larger distances were more strongly differentiated than other pairs located closer together. This was not the case for colonies found in the Florida collection site. Once mapped out the colonies from Mississippi appeared to form two spatially separated clusters, suggesting the presence of two distinct groups.

In order to test whether these colonies were part of a single population or a number of distinct populations, I utilized the genetic analysis program STRUCTURE v 2.0. All these tests were based on the genotypes present among the workers in the populations; the posterior probability of *K* (P (K|X)) approximates one for the most likely number of clusters (K). Based on prior assignment analysis described above, the *R. flavipes* present at the Florida site were considered a single population because the most likely number of putative clusters was $K = 1$. Individual assignment values ranged from 87.3% to 95.1%. In contrast, the most likely number of putative clusters into which the *R. flavipes* present at the Mississippi site could be grouped was $K = 2$ (Fig. 3). Individual assignment values ranged from 72.3% to 82.3%.

To further confirm the results given by STRUCTURE, I performed exact tests of genotypic differentiation using GENEPOP ON THE WEB (Raymond and Rousset 1998). These tests revealed significant genotypic differentiation ($P < 0.0001$) between the two Mississippi

populations identified in STRUCTURE (MS1 and MS2). The F_{ST} between these two populations was 0.163 (SE = 0.040). Surprisingly, the pairwise F_{ST}-value between FL and MS2 (F_{ST} = 0.101; SE = 0.026) was significantly lower than the value between MS1 and MS2 (two-sample *t*-test; $P < 0.05$, $df = 14$; Table 2), indicating that the MS2 population was genetically more similar to the FL population than to the MS1 population with which it was sympatric.

General Genetic Data

A total of 1100 workers of *R. flavipes* were genotyped at eight microsatellite loci. Basic summary statistics are given in Table 3. Number of alleles per locus ranged from two to 29. Allelic richness ranged from 12.4 to 24.4 across populations. Allelic richness in population MS2 was significantly greater than the other two populations studied (two-sample *t*-test; $P < 0.05$, $df = 14$). Levels of observed and expected heterozygosity ranged from 0.27 to 0.80 and 0.66 to 0.95, respectively. Observed heterozygosity in MS2 was significantly greater than both MS1 and FL (two-sample *t*-test; $P < 0.05$, $df = 14$). No population deviated significantly from Hardy-Weinberg Equilibrium. None of the populations were found to have a significant excess or deficit in heterozygotes at any of the loci.

Colony Breeding Structure

All three populations were slightly inbred (Table 4). The average colony inbreeding levels (F_{IC}) in both Mississippi populations were significantly lower than the inbreeding levels in FL (two sample *t*-test; $P < 0.05$, $df = 14$). In addition, nestmates were closely related to one

another in both MS1 and MS2 ($r > 0.5$ in both cases), and significantly more so than nestmates in the FL population ($r < 0.4$) (two-sample *t*-test; $P < 0.05$, $df = 14$). Estimates of relatedness and *F*-statistics are shown in Table 4, as well as computer simulations for different potential breeding systems generated by Thorne et al. (1999) and Bulmer et al. (2001) for comparison. The proportions of family types for all populations are shown in Table 5. Population MS1, with mostly simple family colonies, differed significantly from the MS2 and FL populations (Fisher's exact test; $P < 0.05$), both of which had predominantly extended families. Populations MS2 and FL did not differ significantly from each other in the proportion of simple and extended family colonies (Fisher's exact test; $P > 0.05$). Most extended family colonies in all three populations had genotypes consistent with simple family colonies, but the frequencies of the genotypes deviated significantly from the expected Mendelian ratios (Table 6). Mixed family colonies for all populations were identified by the presence of more than four alleles at at least one locus, with the number of alleles ranging from five to 12 at the most polymorphic locus.

In populations MS1 and FL simple family colonies did not differ significantly from any of the computer generated values for colonies founded by pairs of outbred monogamous reproductives (*t*-test; $P > 0.05$, $df = 7$; Table 4 case A). Results for the MS2 population were different. Workers in the simple family colonies in this population were more closely related than expected (*t*-test; $P < 0.05$, $df = 7$), and colonies were more genetically distinct from each other than expected (*t*-test; $P < 0.05$, $df = 7$), In addition, output from KINSHIP revealed that in the MS1 population no colonies were headed by a significantly related pair of reproductives. Both population MS2 and FL contained a single colony in which the reproductives were closely

related (MS2 = 17%; FL = 50%). These finding support the idea that the majority of simple family colonies in the populations were not headed by full siblings.

Extended families in MS1 and MS2 had significantly lower F_{IC} values than the FL population (two-sample *t*-test; $P < 0.05$, $df = 14$). F_{IT}, F_{IC}, and *r* values for populations MS1 and MS2 did not differ significantly from values expected for a small number of reproductives with relatively few generations of inbreeding (Table 4 case B2). Based on these results, I can exclude colony breeding structures with greater than six reproductives on average actively reproducing (*t*-test; $P > 0.05$, $df = 7$) for the extended family colonies in these populations. Values from the FL population did not differ significantly from values expected for a larger number of reproductives and few generations of inbreeding (Table 4 case B4) (*t*-test; $P > 0.05$, $df = 7$). Thus, breeding structures with fewer than six and greater than 200 reproductives actively reproducing within colonies can be excluded for extended families in this population.

I was unable to sort workers in any of the mixed family colonies into obvious separate family groups as has been done in previous studies (DeHeer and Vargo 2004, Vargo and Carlson 2006, Vargo et al. 2006a). F_{IC} values from all mixed family colonies ranged from -0.341 to 0.082. The mixed family colonies in MS2 were inconsistent with simulated values generated by computer models (Thorne et al. 1999, Bulmer et al. 2001) with the exception of colonies potentially founded via pleometrosis, which is not known to be common in *Reticulitermes* (Bulmer et al. 2001, DeHeer and Vargo 2004, Vargo and Carlson 2006, Vargo et al. 2006a). From the number of alleles found among the workers' genotypes in these mixed family colonies, I can infer that there must have been three or more reproductives in one of the colonies (five

alleles present at one locus), four or more in another (seven alleles present at one locus), and no fewer than six reproductives in the third colony (11 alleles at one locus). The mixed family colony in FL must have been headed by a minimum of four reproductives based on the presence of seven alleles at one locus among the workers' genotypes. In population MS1 the mixed family colony was likely headed by a minimum of three reproductives based on six alleles present at one locus.

Morphometric Analysis

General Correlations

I did not detect a significant relationship between worker mass and any other morphological measure using a pairwise correlation analysis (all $r < 0.34$; $P > 0.05$). Worker head widths and tibia lengths were significantly positively correlated in all populations (all $r > 0.945$; $P < 0.05$). For soldiers, a strong positive pairwise correlation was detected between all morphological measurements: head width, head length, and pronotal width (all $r < 0.85$; $P < 0.05$).

Morphometric Variation across Populations

Overall there was no significant difference among populations in average worker head width (ANOVA; $P > 0.05$, $F = 1.067$, $df = 144$). In contrast, there was a significant difference in the average soldier pronotal width across populations (ANOVA; $P < 0.05$, $F = 9.295$, $df = 58$).

Morphometric Variation across Colonies within Populations

I found significant variation in soldier pronotal width among colonies in all populations and significant variation in worker head width among colonies in MS2 (ANOVA; $P < 0.05$ $df = 14$) and FL (ANOVA; $P < 0.05$ $df = 6$), but not in MS1 (ANOVA; $P > 0.05$ $df = 6$).

Morphometric Correlations to Family Type

Analyses of the effect of colony family structure on worker/soldier body size were limited to simple and extended families. This was because mixed family colonies may be a combination of multiple family types, therefore confounding any possible effects of colony family type. In population MS2 there was a significant effect of colony family type; the average sizes of individuals within simple family colonies were greater than of those within extended family colonies (two-way ANOVA; workers: $P < 0.035$, $F = 6.72$, $df = 14$; soldiers: $P < 0.045$, $F = 4.93$, $df = 9$). In contrast, populations MS1 and FL had no significant effect of colony family type on either measure of worker or soldier size (two-way ANOVA; workers: $P > 0.05$, $df = 6,6$, MS1 and FL respectively; soldiers: $P > 0.05$ $df = 3,5$, MS1 and FL respectively), although the results were in the same direction as in the MS2 population. No doubt small sample sizes in these populations prevented a strong test of this relationship.

Effect of Inbreeding on Workers and Soldiers Size

I examined possible negative effects of inbreeding on worker and soldier body size. I examined the relationship between body size and colony F_{IC}-value. The size of workers and soldiers within MS2 and FL was significantly negatively correlated to colony F_{IC} values (Fig. 4c, d, e, f). In population MS1 no significant correlations were found between any measure of body

size and colony inbreeding level (Fig. 4a, b), but results were in the same direction and there were only four colonies where soldiers were collected, again preventing a robust analysis in this population.

Discussion

Colony Breeding Structure

The results of this study, in which three different populations were analyzed in previously unstudied parts of this species' range, help extend the knowledge of the colony breeding structure of *R. flavipes.* These results, when combined with those from previous studies, allow us to examine the variation in breeding structure across much of the East Coast of the U.S. The three populations investigated showed similarities and differences between them, as well as to previously studied populations (Jenkins et al. 1999, Bulmer et al. 2001, Bulmer and Traniello 2002, DeHeer and Vargo 2004, Vargo and Carlson 2006, Vargo et al. 2006a, Parman and Vargo in press). Based on the previous studies, my expectation was to find a high percentage of simple family colonies (~80%) headed by outbred primary reproductive and a low percentage of extended family colonies (~ 15%) with low levels of inbreeding headed by a low effective number of reproductives. These assumptions were based on the positive correlation between inbreeding levels and latitude documented for much of the East Coast (Vargo unpublished data). In this study I confirmed the high level of geographic variation in regards to colony breeding structure. The populations I studied did have relatively low levels of inbreeding, but populations

MS2 and FL had a much higher percentage of extended family colonies than expected given the putative latitudinal trend.

The predominance of colony family type can vary greatly across the range of *R. flavipes*. Simple family colonies of *R. flavipes* comprised 79% of 318 colonies in North Carolina (Vargo 2003, DeHeer and Vargo 2004, Parman and Vargo in press) and 72% of 18 colonies in South Carolina (Vargo et al. 2006a). The high proportion of simple family colonies is consistent with one of the populations in the present study. In population MS1 66% of colonies examined were found to be simple family colonies. However, I found that the other two populations, MS2 and FL, had a much lower occurrence of simple family colonies, no more than 30%. Yet, these populations had a very small sample size of *R. flavipes* colonies; therefore, I cannot exclude the possibility of sampling error in these estimates and that the actual proportion of simple families in these populations may be higher. Nonetheless, it is interesting to note that these latter two populations were comparable to a study completed in Massachusetts, at the far northern part of the range for this species, in which only 36% of the colonies were simple family colonies (Bulmer et al. 2001).

The estimated *F*-values and the results from KINSHIP for the simple family colonies in all populations in the current study indicated these colonies are likely headed by outbred primary reproductives. These findings are consistent with several previous studies of *R. flavipes* (Bulmer et al. 2001, Vargo 2003, DeHeer and Vargo 2004, 2006; Vargo and Carlson 2006, Vargo et al. 2006a). In these studies a low percentage (5%) of simple family colonies were likely headed by

a pair of sibling reproductives (DeHeer and Vargo 2004, 2006; Vargo and Carlson 2006, Vargo et al. 2006a).

The colony inbreeding coefficient (F_{IC}) is particularly sensitive to the effective number of reproductives heading colonies For this reason I utilized this statistic to infer details about the breeding structure in extended family colonies. Highly negative F_{IC}-values for extended family colonies are indicative of colonies headed by a relatively low effective number of reproductives that have undergone few generations of inbreeding. The F_{IC}-values for the extended family colonies observed in the Mississippi populations were found to be consistent with the values associated with colonies headed by no more than six reproductives (F_{IC} = -0.212 to -0.276), and are similar to values found for extended family colonies in NC and SC (Vargo 2003, DeHeer and Vargo 2004, Vargo and Carlson 2006, Vargo et al. 2006a, Parman and Vargo in press). In contrast, the F_{IC}-value for the extended family colonies in the FL population was high (F_{IC} = -0.054), indicating a high effective number of reproductives, estimated to be no less than fourteen but no more than 100. Results from this population are more consistent with the study completed in Massachusetts (mean F_{IC} = 0.08 for extended family colonies at two study sites) (Bulmer et al. 2001). It is important to note that while many other secondary reproductives may be present in the colony, the effective number of reproductives may be lower than the actual number present due to unequal levels of reproduction. Such reproductive skew is common among social insects (Reeve and Keller 2001).

In most cases, workers in mixed family colonies can be sorted into family groups based on their genotypes (DeHeer and Vargo 2004, 2008; Vargo and Carlson 2006, Vargo et al 2006a).

These family groups can then be classified into either extended or simple family colonies from their genotypes and genotypic frequencies. The ability to separate these family groups into specific colony family types would also indicate a mixture of workers from different sets of reproductives. Another possibility is that workers from separate colonies were foraging in the same food source upon collection. These results are in sharp contrast to the present study. I could not separate workers into distinct family groups in any of the mixed colonies analyzed. In a similar study, Parman and Vargo (in press) documented three mixed families where workers could not be separated into distinct family groups as well. The inability to assign workers to distinct family groups within mixed family colonies could be explained by the interbreeding of separate groups of unrelated reproductives, each heading independent reproductive centers. An alternative explanation is that the mixed family colonies were cooperatively founded by multiple unrelated primary reproductives. However, there has been little documentation that colonies are successfully founded in this manner (Matsuura et al. 2002). In addition, I cannot exclude the possibility that workers in the present study colonies belonged to distinct family groups, but that the numbers of alleles observed in the twenty individuals screened were too low to assign them to specific families**.** More definitive conclusions regarding family assignments of the workers within mixed family colonies in the study populations may require screening more individuals and/or using more loci.

Possible interactions between reproductives heading mixed family colonies were elucidated in a detailed study which examined colony foraging over time and space (DeHeer and Vargo 2004). In that study, the authors documented the formation of a mixed family colony via

colony fusion of two distinct colonies. In addition, the newly produced larvae present 1 yr after the fusion event belonged solely to one set of reproductives which headed one of the original colonies. In a laboratory study, microsatellite analysis was performed on the nymphs and eggs of two fused colonies (Fisher et al. 2004). The authors reported that only one set of reproductives from one of the colonies which fused had produced all the progeny. This result suggests that reproductives from the two original colonies did not interbreed (Fisher et al. 2004). Furthermore, the genotypic patterns seen in the offspring are highly indicative of reproductives from one of the colonies monopolizing the reproduction. Therefore, all the progeny will belong to that set. These results suggest that in many cases mixed family colonies may be an ephemeral state with all of the workers eventually being produced by reproductives of only one of the original colonies that merged together. Yet, documentation of interbreeding between unrelated sets of reproductives, as has been shown in this and previous studies also indicates that mixed family colonies may also persist for prolonged periods.

Species Abundance

Relative species abundance plays an integral role in understanding community structure and can help answer many ecological questions (Hairston 1959). Social insect populations are a special case because a colony is the basic ecological unit, rather than an individual (Wilson 1971, Thorne et al. 1999, Lepage and Darlington 2002). It is therefore vital to determine the number of distinct colonies of a species present in a given location in order to accurately assess relative species abundance in termites.

Another component to an accurate assessment of species abundance requires proper species identification and the assignment of foragers to distinct colonies. According to Szalanski et al. (2003) determining species identity can be a challenge when examining solely the morphological characteristics of soldiers and/or alates. As a result, the dichotomous keys commonly used for the identification of *Reticulitermes* (Scheffrahn and Su 1994, Hostettler et al. 1995) may be problematic when used as the only method of identifying specimens to species. The use of these two castes for species identification provides further challenges as alates are present only for a short period during the year, and soldiers generally make up only a small fraction of the total population (1% to 8%) (Lainé and Wright 2003).

Throughout much of the eastern United States there are four recognized species of *Reticulitermes*: *R. flavipes*, *R. virginicus*, *R. hageni*, and *R. malletei* (Austin et al. 2007, Scheffrahn and Su 1994). While there have been several recent studies examining the distribution of these species (Austin et al. 2004a, 2004b, 2004c, 2005, 2007), there have been few detailed studies investigating their relative abundance (Vargo et al. 2006a).

In order to ensure proper species identification I used a combination of methods: morphological characteristics (Scheffrahn and Su 1994), restriction fragment length polymorphisms (RFLP) (Szalanski et al. 2003), and species specific allelic size variation revealed by the highly variable microsatellite locus *Rf* 24-2 (Vargo unpublished data). Given the disparity in results between morphology and the molecular methods, the latter methods were considered to provide the greatest accuracy for species identification. Once species were properly identified I determined the colony affiliations for samples of workers. From these data

it was determined that the relative abundance of *R. flavipes* was lower than anticipated in both the Florida and Mississippi sites studied (18% and 25% respectively). In addition, there appeared to be an undescribed cryptic species in Florida (Vargo unpublished data). This conclusion was supported by results from the genetic analysis program STRUCTURE.

My results indicate a lower percentage of *R. flavipes* colonies than other studies have documented. In the previous studies, *R. flavipes* was found to be the most commonly collected species over much of the southern part of this species' range, often comprising nearly 75% of the species collected (Scheffrahn et al. 1988, Messenger et al. 2002, Austin et al. 2004). Several of these studies (Scheffrahn et al. 1988, Messenger et al. 2002, Austin et al. 2004a, 2004b, 2004c, 2005) were based primarily on residential properties treated by Pest Management Professionals (PMP) and thus carry caveats that must be taken into consideration when evaluating the strength of the data. For example, these studies do not take into account the possible ability of *R. flavipes* to exploit structures and/or disturbed habitats more readily than other species within the genus. Therefore, urban sites may not be indicative of the true species abundance in a particular geographic area. Additionally, colony identity of the foraging workers was not confirmed in these studies. Thus, the results of these studies on relative species abundance should be viewed with caution.

As in the present study, other recent studies utilized molecular techniques, to identify species and assign workers to their particular colonies. In the Coastal Plain of North Carolina, Vargo (unpublished data) reported 75% of subterranean termite colonies were *R. hageni*, whereas only 21% of the colonies were identified as *R. flavipes*. A study completed in

Charleston, South Carolina, also in the Coastal Plain, reported a slightly higher abundance of *R. hageni* (~ 43%) compared to *R. flavipes* (~37%) (Vargo et al. 2006a). In contrast, DeHeer and Vargo (2004) reported that *R. flavipes* colonies accounted for 91% of 33 colonies sampled in two 484 m^2 forest plots in the Piedmont of central North Carolina over a 3-yr period. In a study of infested residential properties in NC, Parman and Vargo (in press) found nearly 90% of 188 colonies were identified as *R. flavipes*. The remaining colonies were identified as *R. hageni* and *R. virginicus*. These studies along with the results from the current study confirm the geographical differences in relative species abundance found in *Reticulitermes*.

There was significant variation found between the current study and a previous study by Wang et al. (2003) conducted in Harrison Co. Mississippi only 5 miles from the site investigated in the present study. These authors reported that *R. flavipes* was the most abundant species collected, comprising 49% of the total species sampled. This is nearly twice as common as I found in the present study. There are two possible reasons for the discrepancy between the present results and those of Wang et al. (2003). First, Wang et al. (2003) relied solely on morphological characteristics described by Scheffrahn and Su (1994) for species identification, possibly resulting in inaccuracies in their species assignments. In the current study only 51% of the *R. flavipes* samples from the Mississippi collection site were identified correctly based on morphology alone. If I had relied exclusively on morphology, my results would have been very similar to those of Wang et al. (2003). Second, no attempt was made in the earlier study, or indeed possible given the methodology used, to identify distinct colonies. Because the spatial scale used by Wang et al. (2003) would likely have resulted in multiple collections of the same

colony for all species, their estimate of species abundance may be inaccurate. Species in which colonies have large foraging areas may be collected more frequently; therefore the level of abundance of such species may be inflated compared to species with a more limited foraging range. The methodology used in the present study alleviates this problem by properly assigning foragers to distinct colonies. Thus, the results of the present study are more likely to reflect the true species abundance of *R. flavipes* in this area of Mississippi.

There is very little known regarding the factors that determine the relative success of a particular species of subterranean termites in a given area. A minimum of three species within the genus *Reticulitermes* (*R. flavipes, R. virginicus,* and *R. hageni*) occur sympatrically over the majority of their ranges (Snyder 1954). These species play virtually identical ecological roles and occupy apparently similar niches, suggesting some level of resource partitioning may exist among them. Variation in soil moisture along with temperature may be key factors influencing the relative distribution and activity of subterranean termites in a given locale (Howard et al. 1982, Houseman et al. 2001, Green et al. 2005). This hypothesis is supported by studies in both Texas (Houseman et al. 2001) and Mississippi (Howard et al. 1982). Houseman et al. (2001) noted that *R. flavipes* increased foraging activity during the cool and moist part of the season, whereas *R. hageni* increased foraging activity during the warmer more arid part of the season. Howard et al. (1982) documented *R. virginicus* occupying wetter areas than *R. flavipes*. In the current study collections in MS were made in the warmest time of year (June 30). In addition, recent rainfall created very moist soil conditions. In FL collections were made during a cooler period with less rainfall (October 10). Despite the different environmental conditions, a low

relative abundance of *R. flavipes* was documented in both sites. This may indicate that other factors besides soil moisture and temperature were affecting the relative species abundance in these collection sites. Further research investigating the underlying factors promoting the dominance of a given species of *Reticulitermes* in a given locality is required. Subsequent studies should implement molecular methods to determine colony affiliation and utilize appropriate techniques to guarantee accurate species identification. The use of these methods in concert is crucial to the proper assessment of species abundance in the genus *Reticulitermes*.

Higher Genetic Structure

Across a large part of its range, previous studies of *R. flavipes* have shown little or no genetic differentiation ($F_{ST} < 0.10$, most values were close to zero) between populations at spatial scales of 0.5 to 100 km (Reilly 1987, Bulmer et al. 2001, Vargo 2003, DeHeer and Vargo 2004, Vargo and Carlson 2006). In my study, the presence of such strong population structure between MS1 and MS2 ($F_{ST} = 0.1891$) was unexpected given that they were completely sympatric. The only species within this genus documented with strong population structure at spatial scales of 50 km is *R. hageni* (Vargo and Carlson 2006). Vargo and Carlson (2006) proposed that the inability to disperse over long distances leads to frequent pairing with siblings during colony foundation and this promotes strong population structure in *R. hageni*. This colony founding behavior creates smaller, more genetically differentiated sub-groups (DeHeer and Vargo 2004, Vargo and Carlson 2006, Vargo et al. 2006a). This does not appear to be the case in *R. flavipes* since alates can disperse fairly long distances up to 400 m (Shelton et al.

2006) and successful colonies are rarely founded by related reproductives (Thorne et al. 1999, Bulmer et al. 2001, Vargo 2003, DeHeer and Vargo 2004, Vargo and Carlson 2006, DeHeer and Vargo 2006).

The exact mechanisms maintaining the genetic separation between populations MS1 and MS2 is unclear. A temporal separation is possible due to the fact I collected alates from a single colony in MS1 and none from MS2 in late June, which is few months after the expected swarming date (Nutting 1969). Typically *R. flavipes* swarms in a simultaneous event to ensure maximum pairing of alates (Thorne et al. 1999). The swarming of alates from MS1 and MS2 at different intervals may be sufficient to maintain separation of these two populations since the reproductives are never exposed to each other. However, the collection of alates at a single colony does not offer overwhelming support of this theory. The existence of high levels of genetic differentiation found in this study is uncommon for the majority of taxa. Ideally, a subsequent collection and repeated analysis of the Mississippi site would be able to clarify whether these two populations are homogenizing or maintaining their genetic differentiation.

In order to gain a better understanding of possible origins of these populations, a closer inspection of the genetics of the workers within the populations was completed. These results revealed that allelic richness in population MS1 was significantly lower than in MS2. Furthermore, alleles present in population MS1 were not simply a subset from population MS2. Furthermore, this may indicate that population MS1 was a recently introduced population, and the high percentage (66%) of simple family colonies in MS1 may be another indicator that population MS1 was recently established. Extended family colonies arise from simple family

colonies, therefore a population with a higher frequency of simple family colonies may be a "younger" population, because the colonies have not been established long enough for neotenics to have differentiated (Thorne 1997, Thorne et al. 1999). Given the high level of genetic differentiation between MS1 and MS2 it is unlikely that MS1 arose from MS2 or vice versa. Although I am extremely confident in the proper identification of the species in this study, I cannot exclude the possibility that population MS1 represents an unrecognized subspecies.

Colony Spatial Structure

Generally, colonies of *R. flavipes* in their native range span less than 30 linear meters (Bulmer et al. 2001, Vargo 2003, DeHeer and Vargo 2004, Vargo and Carlson 2006, Vargo et al. 2006, Parman and Vargo in press). In addition, there has been no evidence that colony family type has any bearing on the expansiveness of colonies (Bulmer and Traniello 2002, Vargo et al. 2006, Parman and Vargo in press). However, there is evidence of a positive correlation between the F_{IC}-value and foraging range in extended family colonies (Bulmer and Traniello 2002, DeHeer and Vargo 2004). In the present study I found that population FL was unique because two colonies spanned multiple collection points, whereas all collection points in populations MS1 and MS2 were found to be distinct colonies. One of the mixed family colonies in the FL population spanned 110 linear meters and five separate collection points. This distance surpasses the previous record of 83 linear meters in the native range (Vargo and Carlson 2006). The other FL multi-collection point colony spanned 54 linear meters and two separate collection points, and was an extended family colony.

Mixed family colonies can form in a number of ways and one that has been documented in both the field and the laboratory is colony fusion (DeHeer and Vargo 2004, Fisher et al. 2004). Several studies have verified the possibility for a break down in colony borders in *R. flavipes* (Grace 1996, Bulmer and Traniello 2002, Fisher et al. 2004, DeHeer and Vargo 2004, Vargo and Carlson 2006, DeHeer and Vargo 2008, Parman and Vargo in press). Nonetheless, field studies have revealed that *R. flavipes* may be more territorial than previously thought (DeHeer and Vargo 2004). A recent study by DeHeer and Vargo (2008) compiled a database of 354 colonies of *R. flavipes* collected from the field and found only a small percentage (2.3%) of colonies to be mixed family colonies. Presently, I collected mixed family colonies in each of the three populations, where the proportion of such colonies was slightly higher than reported for other populations in the southern part of the range (11%-17%), but more consistent with studies in Massachusetts and Nebraska (Bulmer et al. 2001, DeHeer and Kamble 2008). The percentage of mixed family colonies found in the present study may be inflated somewhat due to sampling error given the few *R. flavipes* colonies analyzed in two of the study populations.

Evidence of colony border breakdown has been documented in a European species of *Reticulitermes* as well. Studies of *R. grassei* in Portugal reported sharing of haplotypes among the different locales (Nobre et al. 2006, 2008). The authors cite anthropogenic effects as well as lack of aggression and mixing of workers as a possible explanation for the presence of mixed family colonies. In France, Clément et al. (1981, 1986, 2001) conducted a number of studies examining colony boundaries, nestmate recognition, and genetic variations across populations of *R. grassei*. He found that colonies in the southwest region of France displayed lower aggression

in the summer months which lead to an exchange of workers between colonies. In addition he indicated that these colonies should be considered one large supercolony. These studies were based on genetic data from two allozyme loci, cuticular hydrocarbons, and behavioral assays. However, a more recent study by DeHeer et al. (2005) utilized highly polymorphic microsatellites at eight loci to extrapolate genetic information. In this study the authors examined two populations within the region previously studied by Clément and coworkers, and found distinct differences in the population structure. DeHeer et al. (2005) reported a low prevalence of colony border breakdown, and the majority of colonies studied were localized around a single collection point. These latter authors cite the inability for two allozyme loci to accurately describe the spatial structure in some populations to explain the conclusions regarding colony breeding structure in *R. grassei* reached by Clément et al. (1981, 1986, 2001). The use of highly variable microsatellite loci with numerous individuals per colony (> 20) was required to achieve the level of sensitivity necessary to properly reveal the spatial structure of *R. grassei* colonies (DeHeer et al. 2005).

Colony fusion appears to readily induced in laboratory settings. For example, a laboratory study which examined colony fragments of *R. flavipes* reported colony fusion in 55% of pairings (Fisher et al. 2004). Fisher et al. (2004) cited lack of aggression as the means by which these colonies fused. Another laboratory study examining a related species, *R. speratus,* reported (Matsuura and Nishida 2001) that different termite colonies could fuse and create mixed family colonies. The authors of this study, Matsuura and Nishida (2001), cited the nymph ratio (number of nymphs: number of workers) of each colony as an important factor determining the

likelihood of colony fusion. Colony members with a lower nymph ratio than the host colonies were accepted into the host colony, whereas colonies with a higher nymph ratio than the host colony were attacked and killed. Matsuura and Nishida (2001) concluded that under some conditions the costs of combat among neighboring colonies may be less attractive than cooperation. The authors also asserted that colony fusion may be an adaptive strategy in social insects. Nonetheless, this condition may not present itself commonly in their natural habitats.

The prevalence of mixed family colonies varies across taxa in termites. Such colonies have been previously reported in low percentages in the most basal group of termites, *Mastotermes darwiniensis* (Mastotermitidae) (Goodisman and Crozier 2002), and the neotropical arboreal termite, *Nasutitermes corniger* (Termitidae) (Atkinson and Adams 1997), while in the mound building termite, *Macrotermes michaelseni* (Termitidae), mixed family colonies occur in high proportions (Hacker et al. 2005). This study offers more evidence that mixed family colonies in termites are generally the least frequently encountered family type.

Although it is clear from a number of studies, *Reticulitermes* has the capacity for a breakdown in colony borders, and while there may be variation among species, it seems that factors present in the field and not in the laboratory must be maintaining these borders. More studies are required to identify these factors and to discover the mechanisms for creating and maintaining relatively strict colony borders as well as the events leading to the breakdown in these borders within the termite's natural environment. In addition, it is important to examine the possible trade offs between maintaining tight colony borders and fusing with neighboring colonies in termites.

Effects of Inbreeding

There is little consensus on the effect inbreeding has on termite colonies and populations. In the present study I found a significant negative correlation between the body size of both soldiers and workers and colony inbreeding coefficient (F_{IC}) in all populations except MS1. It should be noted that the sample size was too small in MS1 to provide a robust test. In addition, I found a colony family type effect in population MS2, the population for which I had the largest sample size. In this population, the extended family colonies, which were more inbred than simple family colonies, were composed of significantly smaller workers and soldiers than the simple family colonies. The other populations followed the same trend, but the relationship was not significant, possibly due to the limited sample sizes (N = 8 and 9 for FL and MS1, respectively). No evidence of fluctuating asymmetry (FA) was detected in any of the studied populations. These findings are unsurprising since FA has been rarely shown to directly affect fitness unless under stressful ecological conditions (Nosil and Reimchen 2001). It may be more informative to examine the reproductives or newly produced alates for possible fluctuating asymmetry since they are more susceptible to fitness costs.

In earlier studies inbreeding was proposed as an essential mechanism for the establishment of social organization, evolution, and maintenance of eusociality in termites (Hamilton, 1972, 1978; Syren and Luykx, 1977, Bartz 1979). Nevertheless, recent empirical studies have found conflicting evidence regarding the direct and indirect effects of inbreeding in termites. DeHeer and Vargo (2006) examined the pairing of primary reproductives in *R. flavipes*. The authors screened 65 primary reproductive pairs during colony founding at 12

microsatellite loci. More than one-quarter of all *R. flavipes* tandem running pairs (26.1%) were likely siblings. However, the average relatedness of pairs heading established colonies as inferred from the genotypes of their worker offspring, was much lower (5%) when compared to the proportion of full-sibling tandem running pairs. Thus, during colony foundation, selective pressures appear to operate that do not favor the foundation of colonies by pairs comprised of full siblings. The authors suggested inbreeding depression as the probable cause of mortality in sibling tandem pairs.

Two studies of the invasive termite *C. formosanus* documented an effect of the colony inbreeding coefficient (F_{IC}) on average worker size, as measured by weight and head width in three geographically separate locations (HI, China, and LA) (Husseneder et al. 2005, 2008). These studies showed that colonies with high F_{IC}-values, associated with high numbers of reproductives, consisted of workers with a lower body weight and smaller head width than workers in colonies with low F_{IC}-values, indicative of low numbers of reproductives. These studies suggest a morphological influence of having higher numbers of effective reproductives in termites. The authors propose that inbreeding may be detrimental to the development of workers arresting their growth. In the current study I used different measures of body size; however, the trend of smaller body size in relation to high levels of within colony inbreeding was in the same direction as that reported by Husseneder et al. (2005, 2008). In addition, Husseneder and Simms (2008) discovered that larger males and females were more likely to form mating pairs in *C. formosanus*. This seems to indicate that smaller size in the alate caste could be a hindrance to obtaining a mate. The negative correlation between worker and soldier body size and F_{IC} in my

study provides preliminary evidence of the potential detrimental effects of inbreeding at the colonial level in an additional species within the Rhinotermitidae. However, in other studies of *C. formosanus* (Grace et al. 1995) there has been some speculation that smaller worker size may be beneficial. Grace et al. (1995) assert that smaller workers may be more vigorous and therefore more active foragers, yet there is no direct evidence either way concerning the advantages or disadvantages of worker size in this species. Therefore, it seems clear that inbreeding can affect worker size in subterranean termites, but whether this effect is detrimental remains to be determined.

A study completed by Rosengaus and Traniello (1993) of *Zootermopsis angusticollis* reported fitness costs for non-sibling reproductive pairs by showing a higher mortality rate than for sibling pairs. Susceptibility to disease, especially fungal pathogens, was proposed as a mechanism driving the inbreeding cycle in termites (Rosengaus and Traniello 1993). Inbreeding may aid in the retention of alleles beneficial to the defense of pathogens (Rosengaus and Traniello 1993). However, a study examining the effect of inbreeding on immunocompetence and heterozygosity in *Z. angusticollis* reports a possible negative consequence to termite colony health (Calleri et al. 2006). Calleri et al. (2006) found that when inbred termites were grouped and exposed to relatively high concentrations of fungal conidia, they had a significantly greater mortality than outbred grouped termites. In addition, inbred termites also had significantly higher cuticular microbial loads, which the authors interpreted as resulting from a reduction in grooming by nestmates, compared to workers in outbred colonies. Clearly these contrasting results highlight the ambiguity of positive or negative effects of inbreeding on the health and

mortality of termites. Furthermore, this question should be addressed in *R. flavipes* given the wealth of information regarding this species' breeding structure.

Mixed results exist relating to the morphological consequences of increased levels of inbreeding in arthropods. A recent study reported that the sub-social spider, *Stegodyphus lineatus*, showed a significantly negative correlation between the levels of inbreeding, with respect to development, and adult body size (Bilde 2005). In contrast, other studies on a cricket, *Gryllus firmus* (Roff and Derose 2001) and a bumble bee, *Bombus terrestris* (Gerloff et al. 2003), showed no significant effect of inbreeding levels on individual size. Typically, the major manifestation of inbreeding depression is not found in morphological characteristics, but rather in immunological or genetic defects (Keller and Waller 2002). Therefore, future studies of possible effects of inbreeding on fitness in termites should concentrate on reproductives.

Overall, effects of inbreeding on wild populations vary across taxa, populations, and environments (Keller and Waller 2002). However, rarely does it effect both individual and population performance (Keller and Waller 2002). The effect of inbreeding in termites, whether positive or negative, remains unclear. Future studies of termite populations should provide valuable insights into both, the mechanisms that drive inbreeding and its possible consequences on fitness.

In summary, this work provides us with a more complete picture of the highly variable breeding structure of *R. flavipes* in the southeastern U.S. and raises some important questions regarding subterranean termite biology. Given the geographical trend in colony breeding structure previously proposed (Vargo, unpublished data), I expected to find a high proportion of

simple family colonies in all populations and very low levels of inbreeding among the extended family colonies. However, this was not the case. Mixed family colonies were documented in all populations, and workers could not be assigned to specific sets of reproductives which may indicate interbreeding among reproductives from different source colonies or cooperative colony foundation. Closer examination of the mixed family colonies and the mechanisms which lead to their formation need further scrutiny. I also documented a negative correlation between the inferred number of reproductives heading a colony and worker size. These results suggest that reduction in worker size may be a possible negative consequence of inbreeding in *R. flavipes*. *R. flavipes* was less abundant than other *R.* spp. in all collection sites and in previous studies completed in MS. It was unexpected to discover two genetically distinct populations occupying the same location. The mechanism which maintains the separation between these two populations is unclear, but provides an interesting question for future research. Future studies should continue to examine possible consequences of inbreeding, as well as obtain additional basic breeding structure information in this and other closely related species.

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TABLES

Table 1. List of primer concentrations and corresponding PCR programs used for genetic analysis.

Locus	PCR Program	Primer Concentrations	
		Unlabeled Strand Conc. [pmol/µl]	Labeled Strand Conc. [pmol/µl]
Rf 24-2	MICRO57	0.2	0.04
Rf 21-1	MICRO57	0.2	0.05
Rf 1-3	MICRO-F1	0.2	0.05
Rf 5-1	MICRO-F1	0.2	0.05
Rf 6-1	MICRO57	0.2	0.06
Rf 15-2	MICRO57	0.15	0.03
Rs 10	TOUCH1	0.2	0.05
Rs 15	TOUCH1	0.2	0.07

Table 2. FST values between the three study populations: Ms1 and MS2 from Mississippi and FL from Florida.

	MS2	FL
MS1	0.1894	0.2302
MS2	*	0.1045

Table 3. Basic genetic data for the three study populations: Ms1 and MS2 from Mississippi and FL from Florida.

Sample		Microsatellite Locus								
		Rf 24.2	Rf 21.1	Rs 10	Rs 15	Rf 1.3	Rf 5.10	Rf 6.1	Rf 15.2	AVG
	N	9	9	9	9	9	9	9	9	9
	na	5	8	7	4	3	6	5	2	5.00
MS1	He	0.72	0.89	0.78	0.58	0.62	0.84	0.68	0.47	0.70
	Ho	0.78	0.89	0.78	0.44	0.44	0.44	0.22	0.44	0.56
	N	18	18	18	18	18	18	18	18	18
	na	20	14	6	11	7	5	7	3	9.13
MS2	He	0.95	0.91	0.80	0.87	0.79	0.70	0.82	0.56	0.80
	Ho	1.00	0.83	0.61	0.66	0.77	0.39	0.83	0.50	0.70
	N	8	8	8	8	8	8	8	8	8
	na	5	6	3	8	5	3	5	3	4.75
FL	He	0.60	0.77	0.54	0.88	0.71	0.58	0.80	0.49	0.67
	Ho	0.38	0.63	0.50	0.63	0.63	0.25	0.63	0.50	0.52

Table 3 Continued

Sample		Microsatellite Locus								
		Rf 24.2	Rf 21.1	Rs 10	Rs 15	Rf 1.3	Rf 5.10	Rf 6.1	Rf 15.2	AVG
	N	35	35	35	35	35	35	35	35	35
	na	24	28	7	14	8	8	11	3	12.88
TOTAL	He	0.91	0.92	0.80	0.82	0.83	0.78	0.87	0.53	0.81
	Ho	0.77	0.80	0.54	0.57	0.63	0.43	0.60	0.43	0.60

N: Number of colonies, Na: Number of alleles, He: Expected Heterozygosity, Ho: Observed heterozygosity

Table 4. Mean observed and expected colony level *F*-statistics and nestmate relatedness ® broken down by population and by family structure for the three study populations.

	F_{IT}	F_{CT}	F_{IC}	*r*
Empirical Values				
MS 1				
All colonies n = 9	0.175	0.355	-0.28	0.61
SE	0.104	0.072	0.04	0.075
Simple colonies n = 6	0.089	0.344	-0.389	0.638
SE	0.11	0.073	0.066	0.084
Extended colonies n = 2	0.328	0.493	-0.212	0.741
SE	0.219	0.182	0.087	0.178
MS 2				
All colonies n = 18	0.133	0.318	-0.271	0.562
SE	0.022	0.02	0.019	0.026
Simple colonies n =6	0.062	0.292	-0.326	0.551
SE	0.036	0.02	0.028	0.025

Table 4 Continued

	F_{IT}	F_{CT}	F_{IC}	r
Extended colonies n = 9	0.17	0.349	-0.276	0.598
SE	0.042	0.026	0.032	0.028
Mixed colonies n = 3	0.071	0.22	-0.191	0.412
SE	0.039	0.03	0.058	0.054
FL				
All colonies n = 8	0.187	0.229	-0.054	0.386
SE	0.038	0.021	0.058	0.038
Simple colonies n = 2	0.012	0.255	-0.322	0.504
SE	0.074	0.056	0.1	0.099
Extended colonies n = 5	0.254	0.296	-0.057	0.472
SE	0.056	0.047	0.078	0.068
Simulated Breeding system				
(A) Simple family colonies headed by outbred reproductives	0	0.25	-0.33	0.5

Table 4 Continued

	F_{IT}	F_{CT}	F_{IC}	r
(B) Extended Family colonies headed by inbred neotenics				
(1) $N_f = N_m$ X = 1	0.33	0.42	-0.14	0.62
(2) $N_f = 2$ $N_m = 1$ X = 1	0.26	0.35	-0.14	0.55
(3) $N_f = 2$ $N_m = 1$ X = 3	0.52	0.59	-0.17	0.78
(4) $N_f = 5$ $N_m = 1$ X = 1	0.27	0.34	-0.11	0.53
(5) $N_f = N_m = 10$ X = 1	0.33	0.34	-0.01	0.51
(6) $N_f = N_m = 10$ X = 3	0.37	0.38	-0.02	0.56
(7) $N_f = 200$ $N_m = 100$ X = 3	0.34	0.34	0	0.71
(C) mix of colonies headed by primary reproductives (35%) and neotenics				
(1) $N_f = N_m = 10$ X = 1	0.28	0.35	-0.12	0.55
(D) Pleometrosis				
(1) Colonies headed by two queens and one king	0	0.19	-0.23	0.38

For simulated breeding systems: N: Number of pairs of reproductives, X: Number of generations of inbreeding among replacement reproductives, f: Females neotenics, m: Male neotenics,

Table 5. Percentage of colony family type for the three study populations.

Population	no. of Simple Family Colonies	no. of Extended Family Colonies	no. of Mixed Family Colonies	Total no. of Colonies
MS1	6 (66.6%)	2 (22.2%)	1 (11.1%)	9
MS2	6 (29.4%)	9 (53.0%)	3 (17.6%)	18
FL	2 (25.0%)	5 (62.5%)	1 (12.5%)	8

Table 6. Classification of extended family colonies for the three study populations.

Population	Colony ID	Significantly Deviated from Mendelian Ratios	Number of Genotypic Classes Greater than Four
MS1	LA-57	$P = 0.00453$	*
	LA-62	$P = 0.0288$	*
MS2	LA-8	*	5 @ RF-24-2
	LA-27	*	5 @ RF-21-1
	LA-29	*	5 @ RF-24-2
	LA-30	$P = 0.0007$	*
	LA-31	*	5 @ RF-24-2
	LA-35	*	5 @ RF-24-2
	LA-40	$P = 0.00453$	*
	LA-61	*	5 @ RF-24-2
	LA-18	$P = 0.014$	*
FL	FL-23	*	5 @ RF-24-2; 9 @ 21-1; 5 @ 15; 6 @ 6-1
	FL-35	$P = 0.00049$	*
	FL-37	*	7 @ 15; 6 @ 6-1

Table 6 Continued

FL-38	*P* = 0.001	*
FL-64	*	7 @ RF-24-2; 6 @ 21-1; 5 @ 15; 6 @ 6-1

FIGURE LEGENDS

Figure 1. Representative image of COII region cut with *Taq*1; white arrows indicate *R. flavipes*; other bands represent other *R. spp*

Figure 2. Map of Collection points in the site located in DeSoto National Forest, Mississippi. Blue dots are undetermined *R.* spp.; black diamonds are *R. flavipes* colonies in population MS2, green squares are *R. flavipes* colonies in population MS1

Figure 3. Map of collection points in the Florida site located in Ocala National Forest; Blue dots are undetermined *R.* spp.; black triangles are *R. flavipes* colonies; encircled symbols denote a single colony consisting of multiple collection points

Figure 4. Relationship between F_{IC} and body size of 3 studied populations; one from Florida (FL) and two from Mississippi (MS1) and (MS2)

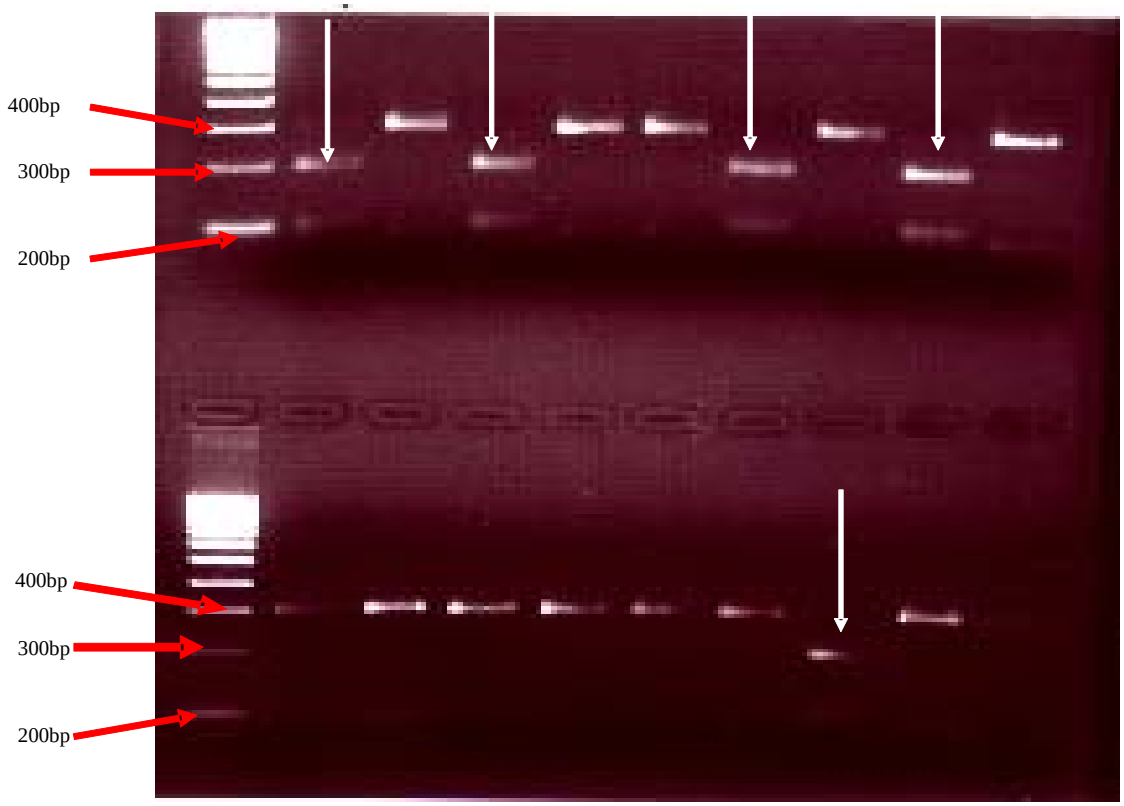

Fig. 1. Representative image of COII region cut with *Taq*1; white arrows indicate *R. flavipes*; other bands represent other *R. spp*

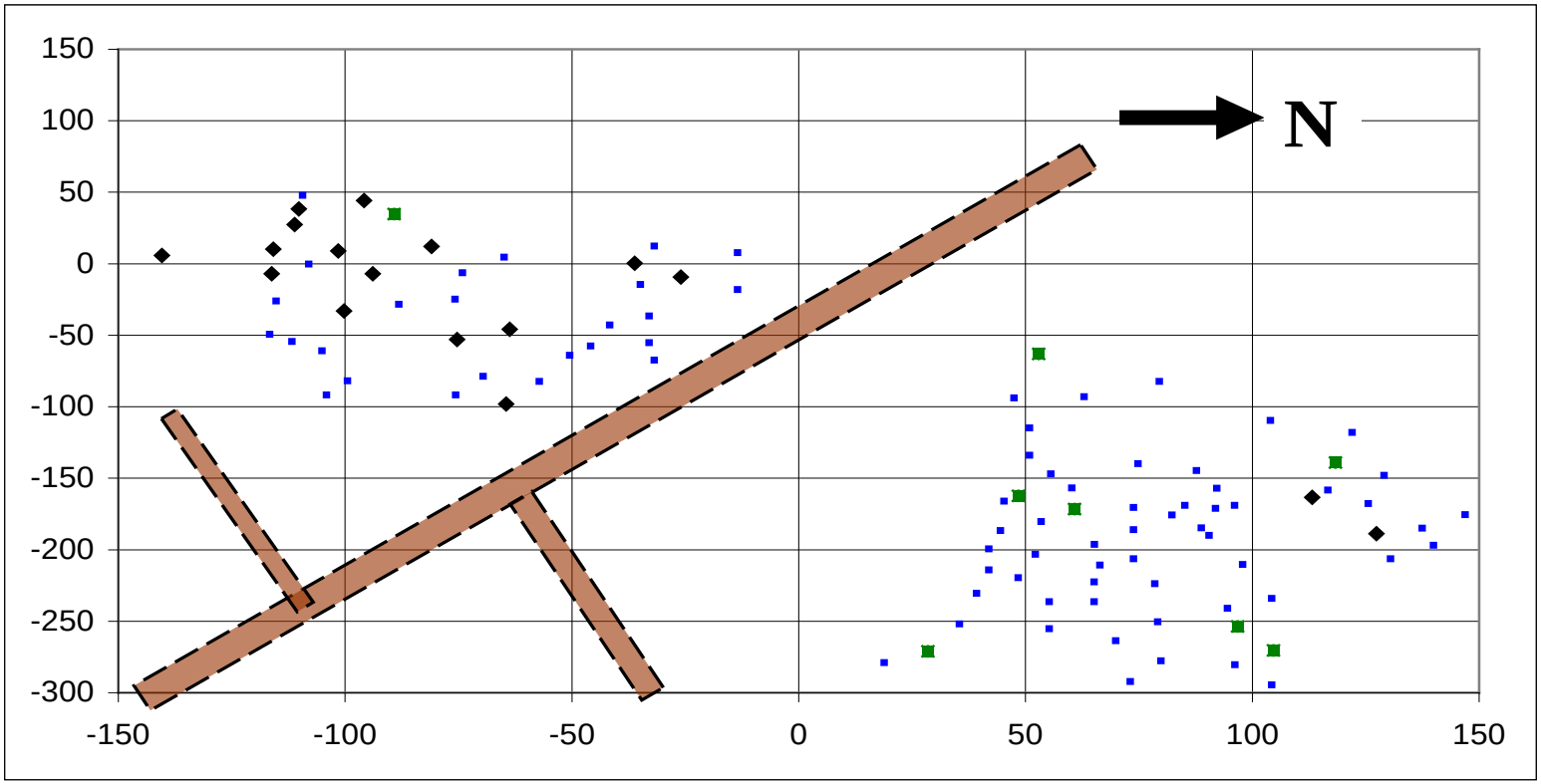

Fig. 2. Map of Collection points in the site located in DeSoto National Forest, Mississippi. Blue dots are undetermined *R.* spp.; black diamonds are *R. flavipes* colonies in population MS2, green squares are *R. flavipes* colonies in population MS1

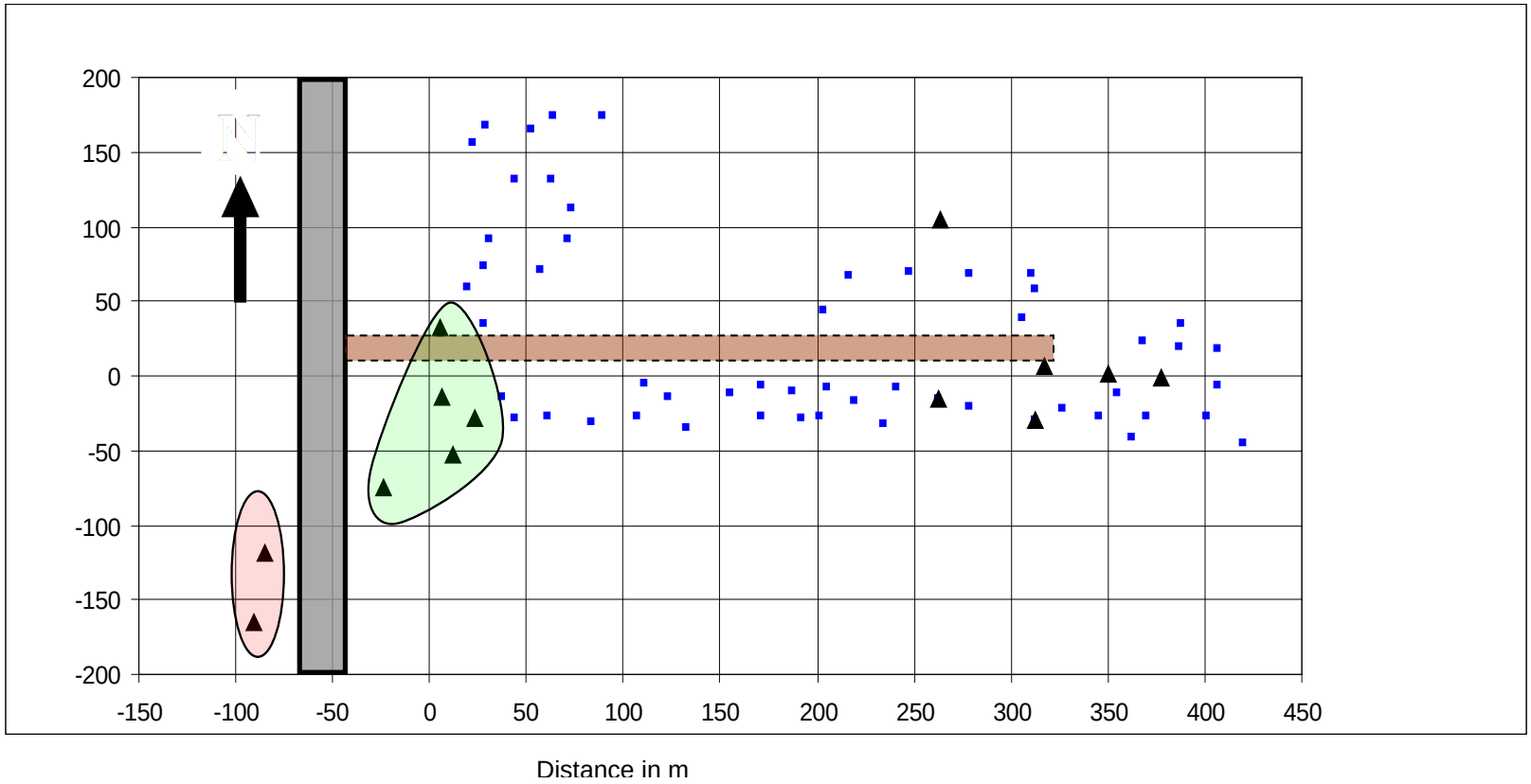

Fig. 3. Map of collection points in the Florida site located in Ocala National Forest; Blue dots are undetermined *R.* spp.; black triangles are *R. flavipes* colonies; encircled symbols denote a single colony consisting of multiple collection points

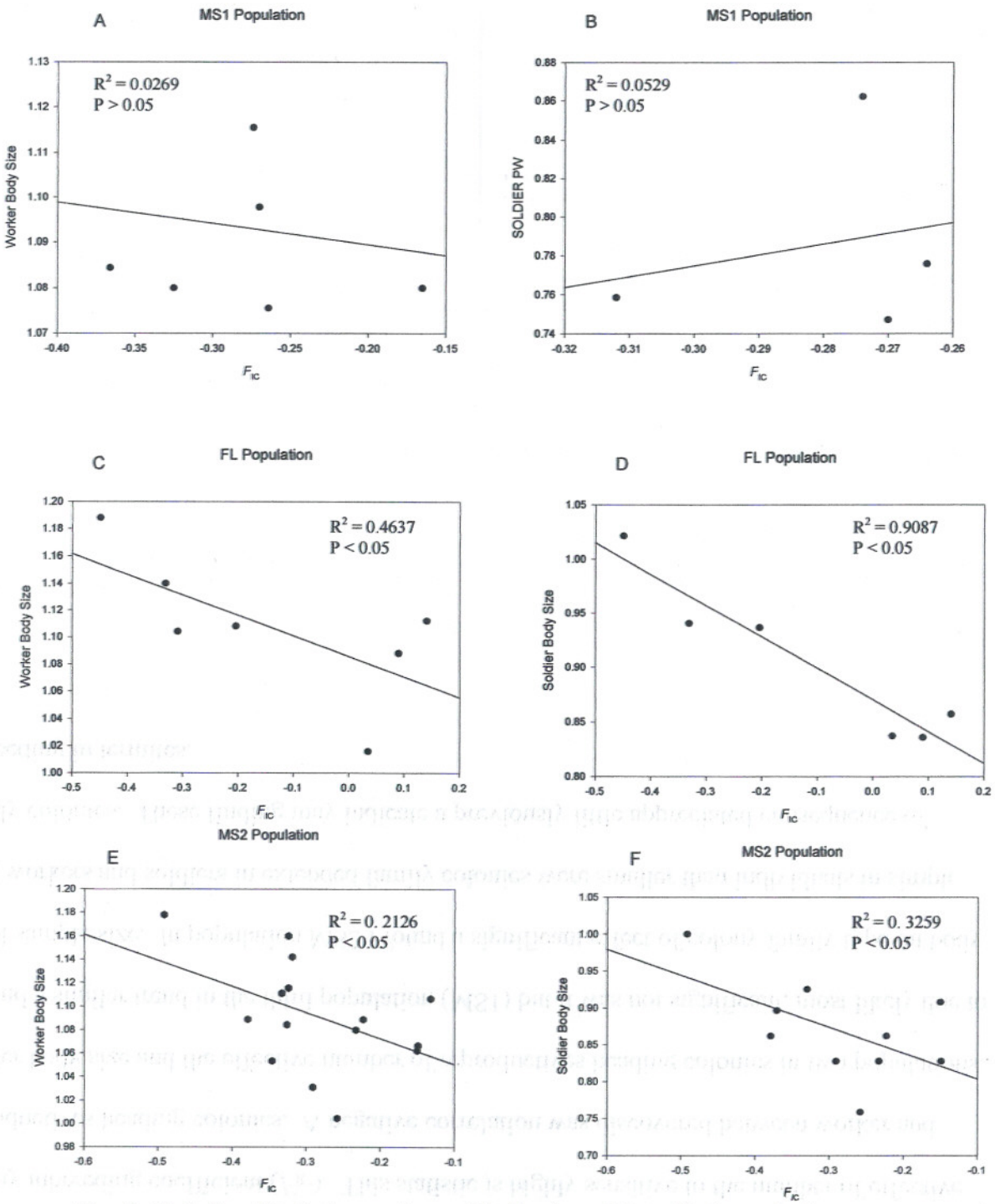

Fig. 4. Relationship between F_{IC} and body size of 3 studied populations; one from Florida (FL) and two from Mississippi (MS1) and (MS2)

APPENDIX

An asterisk indicates positive species ID for *R. flavipes* for each method used in identification; morphological species identity verified by key from Scheffrahn and Su (1994); PCR-RFLP species identity verified by fragment lengths when cut with *Taq*1 (Szalanski et al 2003); microsatellite species identity verified by alleles sizes at locus *Rf* 24-2 (Vargo unpublished).

Collection site MS			
Colony ID	Morphology	PCR-RFLP	Microsatellite
MS-4			
MS-5	*		
MS-6	*	*	*
MS-7	*	*	
MS-8	*	*	*
MS-9			
MS-11			
MS-12	*		
MS-13	*		
MS-14	*	*	*
MS-15	*		
MS-16			
MS-17	*		
MS-18	*	*	*
MS-19			
MS-20			
MS-21	*		
MS-22			
MS-23			
MS-24	*		
MS-25			
MS-26	*	*	*
MS-27	*	*	*
MS-28			
MS-29	*	*	*

Appendix Continued

MS-30		*	*
MS-31	*	*	*
MS-32	*	*	*
MS-33			
MS-34	*	*	*
MS-35		*	*
MS-36	*		
MS-37			
MS-38	*	*	*
MS-39	*		
MS-40	*	*	*
MS-41	*		
MS-42			
MS-43			
MS-44			
MS-45	*	*	*
MS-46	*	*	*
MS-47			
MS-48			
MS-49	*		
MS-50			
MS-51			
MS-52	*		
MS-53			
MS-54			
MS-55			
MS-56			
MS-57	*	*	*
MS-58			

Appendix Continued

MS-59			
MS-60			
MS-61	*	*	*
MS-62	*	*	*
MS-63			
MS-64			
MS-65			
MS-66	*		
MS-67			
MS-68			
MS-69			
MS-70			
MS-71	*		
MS-72			
MS-73			
MS-74	*	*	*
MS-75			
MS-76			
MS-77			
MS-78	*		
MS-79			
MS-80			
MS-81			
MS-82			
MS-83			
MS-84			
MS-85			
MS-86			
MS-87			

Appendix Continued

MS-88				
MS-89				
MS-90				
MS-91				
MS-92				
MS-93				
MS-94				
MS-95				
MS-96				
MS-97				
MS-98				
MS-99				
MS-100				
MS-101				*
MS-102	*	*	*	
MS-103				
MS-104				
MS-105	*	*	*	
MS-106				
MS-107				
MS-108				
MS-109				
MS-110				

Appendix Continued

Collection site FL			
Colony ID	Morphology	PCR-RFLP	Microsatellite
FL-1		*	*
FL-2	*	*	*
FL-3	*	*	*
FL-4	*	*	*
FL-5	*	*	*
FL-6			
FL-7			
FL-8	*	*	
FL-9	*	*	
FL-10		*	
FL-11	*		
FL-12		*	
FL-13	*		
FL-14	*		
FL-15			
FL-16			
FL-17			
FL-18			
FL-19			
FL-20			
FL-21		*	*
FL-22	*		
FL-23	*		
FL-24			
FL-25	*		
FL-26			
FL-27	*		
FL-28	*		
FL-29			

Appendix Continued

FL-30	*	*	*
FL-31		*	*
FL-32	*		
FL-33			
FL-34	*		
FL-35	*	*	*
FL-36			
FL-37	*	*	*
FL-38	*	*	*
FL-39			
FL-40			
FL-41	*		
FL-42	*		
FL-43			
FL-44			
FL-45		*	*
FL-46			
FL-47			
FL-48		*	
FL-49			
FL-50			
FL-51		*	
FL-52			
FL-53	*		
FL-54		*	
FL-55			
FL-56		*	
FL-57	*		
FL-58	*		

Appendix Continued

FL-59			
FL-60			
FL-61			
FL-62			
FL-63			
FL-64	*	*	*
FL-65	*	*	*
FL-66	*	*	*