

**USE OF SOLDIER PRONOTAL WIDTH AND  
MITOCHONDRIAL DNA SEQUENCING TO  
DISTINGUISH THE SUBTERRANEAN TERMITES,  
*RETICULITERMES FLAVIPES* (KOLLAR) AND  
*R. VIRGINICUS* (BANKS) (ISOPTERA:  
RHINOTERMITIDAE), ON THE DELMARVA  
PENINSULA: DELAWARE, MARYLAND,  
AND VIRGINIA, U.S.A.<sup>1</sup>**

Susan Whitney King,<sup>2</sup> James W. Austin,<sup>2</sup> and Allen L. Szalanski<sup>3</sup>

**ABSTRACT:** Termite alates and accompanying soldiers were collected during a 5-year period from diverse habitats on the Delmarva Peninsula, including inland hardwood sites (Newark, Delaware, U.S.A.; Galena, Maryland, U.S.A.), and a pine scrub beach (Lewes, Delaware, U.S.A.). Alates from 34 colonies were identified to species based on taxonomic keys. Pronotal width was measured for 1,447 accompanying soldiers from 33 of the colonies and compared to similar studies in Florida, U.S.A. Mitochondrial DNA 16S sequencing was conducted on soldiers from 31 of the colonies. *Reticulitermes flavipes* and *R. virginicus* were identified from pine scrub, whereas only *R. flavipes* was collected from the hardwood sites. DNA sequences showed three lineages of *R. virginicus*, two of which matched that from specimens previously reported to be a new species (*R. mallei nomen nudum*). Soldier pronotal width ranges at the 95% confidence level were: *R. flavipes*, 0.84-1.04 mm; *R. virginicus*, 0.63-0.83 mm. Statistical analyses indicated that species identification could be based on a sample of 5 soldiers with a confidence level of >95%. The observed pronotal width range for *R. virginicus* overlapped with that reported for *R. hageni* Banks in Florida, U.S.A.

**KEY WORDS:** Identification, taxonomy, sample size, subterranean termites, Isoptera, Rhinotermitidae, soldier, pronotal width, mitochondrial DNA, *Reticulitermes flavipes*, *R. virginicus*, Delmarva Peninsula, Delaware, Maryland, Virginia, U.S.A.

It is difficult to distinguish species of *Reticulitermes* in a study site when alates are not present. Worker termites have no morphological characteristics that can be used to separate species. Soldiers' pronotal measurements have been used to distinguish species; however, there is overlap between *R. flavipes* and *R. virginicus* (Hostettler et al., 1995). This study was initiated to (1) determine how many subterranean termite species are present in Lewes, Delaware, and inland sites on the Delmarva Peninsula and (2) genetically and morphometrically characterize soldiers of each species.

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<sup>2</sup> Department of Entomology and Wildlife Ecology, University of Delaware, Newark, DE 19716-2160, U.S.A., swhitney@udel.edu.

<sup>3</sup> Center for Urban and Structural Entomology, Department of Entomology, Texas A&M University, College Station, TX 77843, U.S.A., jwaustin@ag.tamu.edu.

<sup>3</sup> Department of Entomology, University of Arkansas, Fayetteville, AR 72701, U.S.A., aszalan@uark.edu.

## METHODS

Six study sites were established during 1995 on a 15-acre pine scrub beach at Cape Henlopen on the Delaware Bay in Lewes, Delaware, U.S.A. Soils in each site were sand. Three inland hardwood study sites, approximately one acre each, also were established: one on the University of Delaware (UD) farm in Newark, Delaware, in 1994 and two on a private farm near Galena, Maryland, U.S.A. in 1997. Soils in the hardwood sites were silt loam (Newark) and clay loam (Galena). From 1997 through 2001, subterranean termite alates and soldiers were collected from the nine sites.

Monitoring stakes (*Picea* sp., 2.0 cm x 3.5 cm x 45 cm) were vertically driven into the ground to a depth of approximately 30 cm at each site. Stakes were placed randomly around vegetation at approximately one meter intervals. Approximately 100 stakes were used at each inland site and approximately 1,500 stakes were used in Lewes. Stakes were examined weekly from March of each year through November for signs of termite infestation.

Stakes that became termite-infested were subsequently driven deeper into the ground leaving 3-4 cm exposed. A one-gallon plastic bucket (19.0 cm x 17.5 cm diameter) with the bottom cut off was placed around each infested stake and sunk 10 cm into the soil. Soil was then removed from the bucket, a pine block "sandwich" was placed inside on the ground next to the exposed stake, and the bucket was capped. Each sandwich was constructed of six Southern Yellow Pine, *Pinus taeda* L., boards (9.0 cm x 2.0 cm x 12.5 cm). Two boards served as "bookends" that held the other four boards in the center perpendicular to the bookends. Six dowels (3.0 mm diameter x 11.0 cm) were placed between the inner four boards and the sandwich was nailed together. The dowels created crevices between the boards within which workers constructed their tunnels.

Buckets were monitored weekly for termite activity from March through November each year from 1997 through 2001. When alates were observed, they were collected and the wood block sandwich was dismantled and examined for soldiers. Alates and soldiers were collected and preserved in 80% ethanol.

Previous mark-release-recapture studies (King, unpublished data) showed that marked worker termites moved less than one meter in Lewes and less than 30 meters at inland sites. Buckets that yielded alates for this study were more than one meter from each other in Lewes and those in inland sites were more than 30 meters from each other; thus, each bucket with alates was designated a separate colony.

Termites were examined at 62.5x using a dissecting microscope equipped with an ocular micrometer. Species identification on alates from 34 colonies was made using taxonomic keys (Scheffrahn and Su 1994). The distance of the ocellus from the compound eye was determined for alates. Furthermore, alate body color was compared with *R. hageni* alates collected on 4/28/2001 from Cumberland Island, Georgia, U.S.A. Pronotal width was measured on 1,447 soldiers. The mean pronotal width for *R. flavipes* in both pine scrub and hardwood was calcu-

lated as was the mean pronotal width for *R. virginicus*. Confidence intervals (95, 99, and 99.9%) for these means and their margins of error were calculated. The sample size needed to separate these species was determined. Voucher specimens were deposited in the University of Delaware Insect Reference Collection in Newark, Delaware, U.S.A.

Alcohol-preserved soldiers from each of 31 colonies were sent to the University of Arkansas for species identification using mtDNA 16S sequences (Szalanski et al., 2003). Specimens were dried on filter paper, and DNA was extracted according to Liu and Beckenbach (1992) and Jenkins et al. (1999) on individual whole termites with the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, Minnesota, U.S.A.). Extracted DNA was resuspended in 50 µl of Tris:EDTA and stored at -20°C. Polymerase chain reaction was conducted using the primers LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati and Smith 1995) and LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3') (Simon et al. 1994). These PCR primers amplify an approximately 428 bp region of the mtDNA 16S rRNA gene. The PCR reactions were conducted with 1 µl of the extracted DNA (Szalanski et al., 2000), having a profile consisting of 35 cycles of 94°C for 45 s, 46°C for 45 s and 72°C for 60 s. Amplified DNA from individual termites was purified and concentrated with minicolumns (Wizard PCRpreps, Promega) according to the manufacturer's instructions. Samples were sent to The University of Arkansas Medical Center DNA Sequencing Facility (Little Rock, Arkansas, U.S.A.) for direct sequencing in both directions. GenBank accession numbers were DQ422137 and DQ422138 for the two new haplotypes corresponding to *R. malletei* found in this study. DNA sequences were aligned using CLUSTAL W (Thompson et al., 1994). Mitochondrial DNA haplotypes were aligned by MacClade v4 (Sinauer Associates, Sunderland, Massachusetts, U.S.A.).

Voucher specimens preserved in 100% ethanol were deposited at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, Arkansas.

## RESULTS AND DISCUSSION

Alates were collected from 26 colonies in Lewes (Table 1). These were morphologically identified as either *R. flavipes* (7 colonies) or *R. virginicus* (19 colonies) applying the taxonomic keys of Scheffrahn and Su (1994). Alates were collected from eight colonies in the hardwood sites. These were all identified as *R. flavipes*. Only *R. flavipes* and *R. virginicus* were present on the Delmarva Peninsula sites; *R. hageni* was not found.

**Table 1.** Pronotal width of *Reticulitermes* soldiers

Species <sup>a</sup>	Haplotype <sup>b</sup>	# of Colonies	Site	# of Soldiers	Observed Range	Pronotal Width (mm) mean $\pm$ SD
<i>R. flavipes</i>	Z	7	Pine scrub <sup>c</sup>	586	0.76 - 1.09	0.94 $\pm$ 0.05
<i>R. flavipes</i>	Z, KK, SS, TT	8	Hardwood <sup>d</sup>	267	0.80 - 1.09	0.94 $\pm$ 0.05
<i>R. virginicus</i>	RM1, RM2	18	Pine scrub <sup>c</sup>	594	0.62 - 0.91 <sup>e</sup>	0.73 $\pm$ 0.04
<i>R. virginicus</i>	V6	1	Pine scrub <sup>c</sup>	72	0.73 - 0.87 <sup>f</sup>	

<sup>a</sup>Determination based on taxonomic characteristics of accompanying alates.

<sup>b</sup>Determination based on DNA analysis. RM1, RM2 refers to cryptic species *R. mallei* shown genetically distinct (J. W. Austin, unpublished) and behaviorally and chemotaxonomically distinct (Clément et al. 1986).

<sup>c</sup>Six beach sites in Lewes, Delaware, U.S.A.

<sup>d</sup>Inland sites on the Delmarva Peninsula, including one site in Newark (Delaware), and two sites near Galena, Maryland.

<sup>e</sup>This range overlaps with that of Florida *R. virginicus* (0.71-0.87 mm and 0.70-0.84 mm) and that of Florida *R. hageni* (0.55-0.71 mm and 0.65-0.71 mm) (Hostettler et al., 1995).

<sup>f</sup>Haplotype V6 omitted from morphometric study.

Samples from 31 of the 34 colonies were subsequently subjected to DNA sequencing; three *R. flavipes* colonies were not included in the DNA analysis. Seven distinct haplotypes (lineages) were obtained: Z, KK, SS, TT, RM1, RM2, and V6 (Table 1). The most abundant haplotype was Z (*R. flavipes*, GenBank DQ001953) from nine samples. This is a common haplotype observed from the northeast United States (Austin et al., 2005). Three additional haplotypes of *R. flavipes* were obtained: KK (GenBank DQ001963), SS (GenBank DQ001971), and TT (GenBank DQ001972) (one sample each). Eighteen of the samples were identified as haplotypes RM1 (GenBank DQ422137) and RM2 (GenBank DQ422138). These lineages belong to a cryptic *Reticulitermes* species that has previously been reported to be a new species, *R. malletei* (Clément et al., 1986), but has not been described according to the International Code of Zoological Nomenclature (ICZN). The original description of *R. malletei* has been designated *nomen nudum* (Scheffrahn et al., 2001), but subsequent evaluation has determined that indeed it appears to bear all the necessary requirements as a discrete species (Austin, unpublished) and as such is being prepared for publication. One sample of *R. virginicus* was identified as haplotype V6 (GenBank AY257243). This haplotype is found in several other states (Austin et al., 2004a, b, c).

Pronotal width measurements for *R. flavipes* and *R. virginicus* soldiers are provided in Table 1 and Figure 1. Because only one colony of *R. virginicus* (haplotype V6) was recovered, this sample was not included in the morphometric analysis. For a sample size of five soldiers, the following margins of error were calculated for various confidence levels: 0.0403 (95%); 0.0530 (99%); 0.0677 (99.9%). The difference between the average pronotal widths for *R. flavipes* versus *R. virginicus* (haplotypes RM1, RM2) was 0.21; thus, a sample size of five should reliably separate these two species in Delaware.

Reported values for *R. virginicus* pronota (0.71-0.87 and 0.70-0.84mm) (Hostettler et al., 1995) appear to be supported by our measurements for this species too, with the pronota of 72 soldiers from the single colony (haplotype V6) in Lewes ranging 0.73-0.87mm in width. Pronotal width ranges at the 95% confidence level for *R. flavipes* were 0.84-1.04 mm. According to the taxonomic keys of Scheffrahn and Su (1994) and Hostettler et al. (1995), *R. flavipes* generally has a soldier pronotal width usually greater than 0.90 mm. Our morphological observations, supported with mtDNA sequence data, affirm these measurements. Both Scheffrahn and Su (1994) and Hostettler et al. (1995) describe measurements of *R. hageni* as generally  $\leq 0.70$  mm. At the 95% confidence level, the pronotal width range for *R. virginicus* (haplotypes RM1, RM2) soldiers in Lewes, Delaware (0.63-0.83 mm) overlaps with that of *R. hageni* in Florida (Hostettler et al., 1995). This could lead to termite misidentification if keys based on Florida specimens are used to identify termite soldiers from Delaware.

**Figure 1. Pronotal Width of *Reticulitermes* spp. Soldiers**

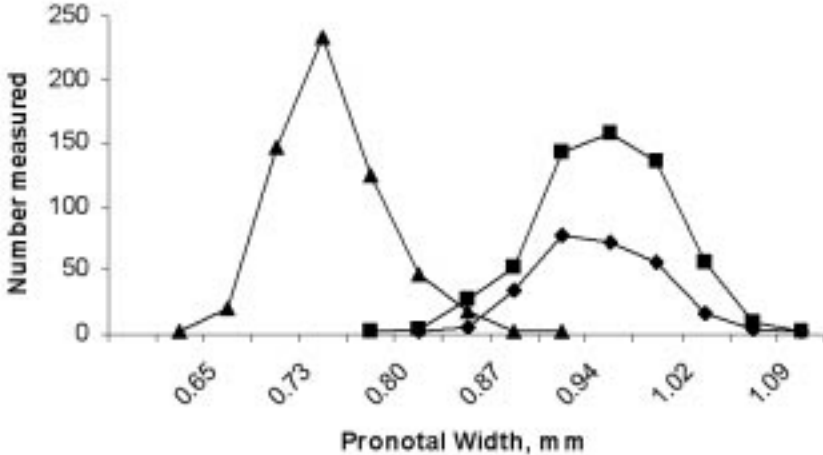


Figure 1. Pronotal width of *Reticulitermes* spp. soldiers. ◆— *R. flavipes*, Hardwood, Haplotypes Z, KK, SS, TT; ■— *R. flavipes*, Pine Scrub, Haplotype Z; ▲— *R. virginicus*, Pine scrub, Haplotypes RM1, RM2.

There are no published accounts of *R. hageni* in Delaware, which prompted a genetic inquiry for species confirmation. Evaluation of comparative sequence data and phylogenetic interpretation through Maximum Parsimony and Maximum Likelihood analyses (see Austin et al. 2005) suggest our samples identified as *R. virginicus* (haplotypes RM1, RM2) from morphological keys is likely incorrect and constitute a discrete species. This same observation has been recently discussed, where Vargo and Carlson (2006) determined that two residing populations of *R. hageni* constituted two distinct taxa, referring to one population as *R. hageni sensu stricto* and the other as *Reticulitermes* n. sp., since the two had not been formally split. We in like kind have resolved to describe these as a single species, *R. virginicus sensu stricto* until the completed description of *R. malletei* is available (Austin, unpublished) and because there are no known occurrences of *R. hageni* in Delaware. However, to clarify this important relationship, it should be understood that the specimens in Vargo and Carlson (2006) taken from Duke Forest, NC, share the same 16S rRNA haplotype (RM1) as found in Lewes, DE (Austin and Vargo, unpublished).

This research demonstrates the discrepancy between taxonomic keys which apply metrics from populations which may vary in size and shape due to the variable nature of the habitats which *Reticulitermes* occupy. Transitions in topography and environment can have significant influences on *Reticulitermes* phenolo-

gy, distribution, and genetic composition. Recent studies demonstrate that underlying *Reticulitermes* phenology is a genetic component which influences both chemotaxonomy (Jenkins et al., 2000) and morphology (Heintschel et al., 2006), when evaluated with mtDNA sequence data. In essence, genes drive phenotypes, and reliance of morphology alone can be misleading when attempting species identification. From these results, there are likely 3 species of *Reticulitermes* which occupy the Delmarva Peninsula, and future investigations which clarify the abundance of these species should be considered.

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