Identification of *Reticulitermes* Subterranean Termites (Blattodea: Rhinotermitidae) in the Eastern United States Using Inter-Simple Sequence Repeats

M. A. Janowiecki, J. W. Austin, A. L. Szalanski, and E. L. Vargo

Identifying species of *Reticulitermes* subterranean termites is important for pest management and future ecological research. In the eastern United States, there are nine species of subterranean termites in three genera: *Reticulitermes* (six species), *Coptotermes* (two species), and *Prorhinotermes* (one species). These species serve as important ecological players by decomposing cellulose material, and some are important structural pests. Many of these species are difficult to discriminate morphologically and require examining the reproductive or soldier castes, which can be difficult to collect. While some genetic tools have been developed for species identification, they are often expensive and time-consuming. To help facilitate identification, we developed a more cost-effective and rapid genetic method to identify *Reticulitermes* species by screening 10 PCR primers that amplified inter-simple sequence repeats (ISSRs) in other termite species. From these, one primer was amplified in all five focal *Reticulitermes* species and contained conserved, species-specific fragments. We further screened this identification method on samples of each species covering a diversity of mitochondrial DNA haplotypes and localities. This identification method utilizing ISSRs can be used to quickly identify five species of *Reticulitermes* subterranean termites in the eastern United States in a matter of hours, providing a useful technique for pest management as well as future ecological research.

**Key words:** molecular diagnostic, genetics, Isoptera
created for the species in the state of Georgia (Lim 2011). However, a minimum of six alates or up to 29 soldiers per colony are needed to obtain 95% confidence levels for morphological species identification (King et al. 2007, Lim and Forschler 2012). CHCs have also been used to identify species and lineages (Howard and Blomquist 1982; Clément et al. 1986; Haverty et al. 1991, 1996, 1999; Haverty and Nelson 1997; Page et al. 2002; Copren et al. 2005), but variation in CHC profiles between colonies and species can be difficult to distinguish. DNA sequencing has also been used to identify species (Austin et al. 2004, Foster et al. 2004). As with all genetic methods, this technique can be applied to any caste including workers. Szalanski et al. (2003) developed a method using restriction fragment length polymorphisms (RFLPs) to distinguish species.
polymorphism (RFLP) to distinguish four species of *Reticulitermes* (*R. flavipes*, *R. hageni*, *R. tibialis*, and *R. virginicus*) using two restriction enzymes on the 16S and COII mitochondrial DNA (mtDNA) genes. Garrick et al. (2015) updated this protocol using three restriction enzymes on a single mtDNA gene (COII) and broadened this to include *R. mallei* and *R. nelsonae*. However, *R. tibialis* was not included in this study. Multiplex polymerase chain reaction (PCR) methods have been used to distinguish the invasive Formosan subterranean termite from other native subterranean termites (Szalanski et al. 2004, Janowiecki and Szalanski 2015). Microsatellite markers

**Table 1.** Haplotypes, samples screened, and fragments amplified by IS01 for each termite species

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of 16S haplotypes</th>
<th>No. of localities (no. of samples)</th>
<th>Average number of fragments</th>
<th>Number of conserved fragments</th>
<th>Total fragments in all samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Reticulitermes flavipes</em></td>
<td>20</td>
<td>23 (45)</td>
<td>7.1</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td><em>Reticulitermes hageni</em></td>
<td>4</td>
<td>16 (29)</td>
<td>8.7</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td><em>Reticulitermes mallei</em></td>
<td>2</td>
<td>7 (20)</td>
<td>6.9</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td><em>Reticulitermes tibialis</em></td>
<td>16</td>
<td>24 (26)</td>
<td>6.7</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td><em>Reticulitermes virginicus</em></td>
<td>10</td>
<td>20 (25)</td>
<td>6.6</td>
<td>1</td>
<td>28</td>
</tr>
</tbody>
</table>

**Table 2.** Unique fragments used for species identification with IS01

<table>
<thead>
<tr>
<th>Species</th>
<th>Diagnostic species-specific fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>138 143 162 190 342 345</td>
</tr>
<tr>
<td><em>Reticulitermes flavipes</em></td>
<td>X</td>
</tr>
<tr>
<td><em>Reticulitermes hageni</em></td>
<td>O</td>
</tr>
<tr>
<td><em>Reticulitermes mallei</em></td>
<td>X</td>
</tr>
<tr>
<td><em>Reticulitermes tibialis</em></td>
<td>O</td>
</tr>
<tr>
<td><em>Reticulitermes virginicus</em></td>
<td>X</td>
</tr>
</tbody>
</table>

‘O’ indicates fragment present in some samples while ‘X’ indicates fragment conserved in all samples.

**Fig. 2.** Representative ISSR results for each species (rows). Bins are drawn at diagnostic species-specific locations.
have been used for species identification (Husen et al. 2006) to distinguish between *R. flavipes* and *R. tibialis*.

Most of the previous methods for subterranean termite identification have so far focused on regional species assemblies. Therefore, there is a critical need for a robust method to identify termite species broadly in the eastern United States. Additionally, current methods are often time- and resource-intensive, requiring that samples be sequenced for accurate, comprehensive species identification. Inter-simple sequence repeats (ISSRs) amplify regions of DNA between identical microsatellite regions using a single PCR primer complementary to the microsatellite repeat (Pradeep Reddy et al. 2002). These markers have been applied primarily for quantifying variation in plant breeding (Pradeep Reddy et al. 2002) but have also been used for population genetic studies of fungi, vertebrates, and insects (Wolfe 2005), including termites (Long et al. 2009, 2013). ISSRs have been used for species identification in seven species of black flies (Diptera: Simulidae) (Dušinský et al. 2006) and five species of necrophagous flies (Muscidae, Calliphoridae, Sarcophagidae) (He et al. 2007). Since ISSRs have been found to successfully differentiate insect species, our objective was to create a genetic method using ISSRs to quickly identify five species of *Reticulitermes* (*R. flavipes*, *R. hagenii*, *R. virginicus*, *R. tibialis*, and *R. malletei*) in the eastern United States.

**Materials and Methods**

We screened *Reticulitermes* samples representing many unique 16S mtDNA haplotypes from across the range of each species when possible (Table 1; Supp Table S1 [online only]). Samples were identified with morphological keys (Banks and Snyder 1920, Snyder 1954, Weesner 1965, Scheffrahn and Su 1994) and confirmed with 16S mtDNA sequencing. DNA was extracted from whole termites using a salting-out procedure with in-house reagents (Sambrook and Russell 2001). The 16S mtDNA fragment was amplified with the primers LR-J-13007 (5′-TTACGCTGTTATCCCTAA-3′) (Kambhampati and Smith 1995) and LR-N-13398 (5′-CGGCTGTTATTCAAAAA CAT-3′) (Simon et al. 1994). Consensus sequences were aligned with Geneious v6.1.8 (Kearse et al. 2012) and assigned haplotypes by comparing to an in-house database (A. L. Szalanski, unpublished data) (Supp Table S1 [online only]).

We screened 10 ISSR primers previously used in *R. chinensis* Snyder and *R. speratus* (Kolbe) (Long et al. 2009). PCRs were setup in 12 µl reactions with 0.8 µl primer (10 µM), 2 µl M13 tail (10 µM), 2.5 µl Buffer 5x Promega (contains MgCl₂ and dNTP), 0.1 µl Taq polymerase, 5.6 µl water, and 1 µl DNA. The thermocycler conditions, as per Long et al. (2009), consisted of an initial denature step of 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s. The final extension step was 72°C for 10 min. The ISSR-amplified products were visualized using fragment analysis on an ABI 3500 Genetic Analyzer (Applied Biosystems, Life Technologies, Grand Island, NY) with GeneScan 500 Liz dye size standard (Applied Biosystems, Life Technologies).

Additional native subterranean termites (*Heterotermes aures* (Snyder), *Proxiboltermes simplex* (Hagen), *R. nelsonae*, *R. besperus*, and a new *Reticulitermes* from Western United States and British Columbia based on genetic data referred to as *R. n. sp. ‘R. okanaganensis’* (Szalanski et al. 2006)), as well as invasive species currently established (*C. formosanus*, *C. gestroi* (Wasmann) (Blattodea: Rhinotermitidae)) and European species of *Reticulitermes* (*R. grassei* Clement, *R. lucifugus* (Rossi), *R. banyulensis* Clement) (Supp Table S1 [online only]) were screened from a limited number of samples to test potential overlap in species-specific ISSR amplicons. The higher termite *Anitermes wheeleri* (Desneux) (Blattodea: Termitidae) was also included since it lives in similar habitats in the southwestern United States (Texas to California) and roughly resembles *Reticulitermes* morphologically.

**Results and Discussion**

Of the 10 ISSR primers tested, only four amplified in our samples (IS01, IS09, IS13, IS15). We selected IS01 because it had species-specific fragments. This primer consisted of eight repeats
Table 3. Assessment of other species of termites for overlap in diagnostic IS01 fragments

<table>
<thead>
<tr>
<th>Species</th>
<th>Diagnostic species-specific fragment (bp)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Max conserved fragments reported</td>
</tr>
<tr>
<td>R. tibialis</td>
<td>No conserved fragments</td>
<td>Unique fragment at 138 bp</td>
</tr>
<tr>
<td>R. malletei</td>
<td>No conserved fragments</td>
<td>Unique fragment at 138 bp</td>
</tr>
<tr>
<td>R. flavipes</td>
<td>No conserved fragments</td>
<td>Unique fragment at 135 bp; 162 bp</td>
</tr>
<tr>
<td>R. virginicus</td>
<td>No conserved fragments</td>
<td>Unique fragment at 162 bp</td>
</tr>
<tr>
<td>C. formosanus</td>
<td>Conserved fragments not species-specific</td>
<td>Unique fragment at 515 bp</td>
</tr>
<tr>
<td>C. gestroi</td>
<td>Conserved fragments not species-specific</td>
<td>Unique fragments at 565 and 627 bp</td>
</tr>
<tr>
<td>H. aureus</td>
<td>Conserved fragments not species-specific</td>
<td>Unique fragment at 135 bp</td>
</tr>
<tr>
<td>P. simplex</td>
<td>Conserved fragments not species-specific</td>
<td>Unique fragment at 135 bp</td>
</tr>
<tr>
<td>R. nelsonae</td>
<td>Conserved fragments not species-specific</td>
<td>Unique fragment at 135 bp</td>
</tr>
<tr>
<td>R. hesperus</td>
<td>Conserved fragments not species-specific</td>
<td>Unique fragment at 135 bp</td>
</tr>
<tr>
<td>R. okanaganensis</td>
<td>Conserved fragment shared with R. malletei(162 bp)</td>
<td>Conserved fragment shared with R. malletei(162 bp)</td>
</tr>
</tbody>
</table>
| R. n. sp.                     | Conserved fragments not species-specific  | Conserved fragments at 229, 515, 565 and 627, and 135 bp respectively. All other species either had no conserved fragments or had conserved fragments that were not species-specific and were shared by other species. This ISSR marker amplifies conserved, species-specific fragments in *Reticulitermes*, expanding the utility of ISSRs to termites. This technique is advantageous over other molecular markers because it produces a large number of reproducible fragments that are distributed widely across the genome. Additionally, no prior sequence data are required for primer design, making ISSR methods relatively simple and quick to develop for new systems. For these reasons, ISSRs have been applied in many studies of plant breeding to identify lineages containing different traits (Pradeep Reddy et al. 2002) and for species identification in flies (Dušinský et al. 2006, He et al. 2007). ISSRs are inexpensive, simple molecular markers that can be applied to identify species or lineages as well as to investigate genetic diversity and evolutionary biology.

This method updates previous PCR-RFLP (Szalanski et al. 2003, Garrick et al. 2015) and microsatellite (Husen et al. 2006) methods by including a wider array of species and requiring fewer steps. DNA sequencing can be used to identify these species of *Reticulitermes* but this new ISSR method requires less time and is less expensive. This ISSR method can be conducted in approximately 11 h, whereas 14 and 34 h are needed for the RFLP and sequencing methods, respectively. In addition, because different dyes can be multiplexed and run simultaneously on a genetic analyzer, this method can be incorporated into microsatellite genotyping protocols for colony identification (Vargo 2003), thus saving additional time and resources in studies involving identification of individual colonies. While ISSR identification is successful for these five species of *Reticulitermes* in the eastern United States, it does not extend to western species or potential invasive species. Other species screened (Table 3) have similar fragments as eastern species and could be easily confused if this was applied at a wider scale.

In this study, we present a new method using ISSRs to quickly and affordably identify five eastern species of *Reticulitermes* through species-specific ISSR fragments. We robustly tested this method with termites across the species’ ranges covering numerous unique 16S mtDNA haplotypes (Fig. 1). This method should be helpful in ecological studies that require screening a large number of samples (e.g., Hyseni and Garrick 2019) and when species identification is
required in controlling this economically important genus of subterranean termites containing multiple pest species.

Supplementary Data
Supplementary data are available at Journal of Economic Entomology online.

Acknowledgments
We thank Carlos Aguero, Thomas Chouvenc, and Joe Eger for providing samples. This research was supported by the Texas A&M University Urban Entomology Endowment and Texas A&M University Dr. Roger E. Gold Endowed Graduate Scholarship at Texas A&M University.

References Cited


