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

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Abstract

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

Subterranean termites are both important ecological players and major economic pests. In the United States, there are 12 recognized species of subterranean termites, two of which are invasive (Krishna et al. 2013). In the eastern United States, there are six recognized species of *Reticulitermes: R. flavipes* (Kollar), *R. hageni* Banks, *R. malletei* Howard and Clement, *R. nelsonae* Lim and Forschler, *R. tibialis* (Banks), and *R. virginicus* (Banks) (Fig. 1). Three of these species, *R. flavipes*, *R. tibialis*, and *R. virginicus*, along with the western subterranean termite, *R. hesperus* (Banks), and the invasive Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Blattodea: Rhinotermitidae) account for 90% of termite control business in the United States (Forschler and Lewis 1997). Americans spend approximately $11 billion USD annually for the prevention, treatment, and repair of termite damage (Su 2002).

Identification is the first step to successfully control termites. While some genera are relatively simple to distinguish from each other, species within *Reticulitermes* and *Coptotermes* are difficult to identify morphologically. Alates are the most morphologically distinct caste but are only abundant seasonally when they swarm for several days. Soldier morphology can be used to distinguish species (Scheffrahn and Su 1994), but they are a small fraction of the colony (1–10%) for most species (Haverty 1977). Workers do not have readily discernable species-specific characteristics, and are only genera-specific within the Rhinotermitidae from dentation (Ahmad 1950), and thus are not generally used for morphological species identification.

Subterranean termite identification currently relies on morphological keys, cuticular hydrocarbon (CHC) profiling, and genetic methods. Initially, dichotomous keys were developed for soldiers and alates (Banks and Snyder 1920, Snyder 1954, Weesner 1965, Scheffrahn and Su 1994). Additionally, an online lucid key was

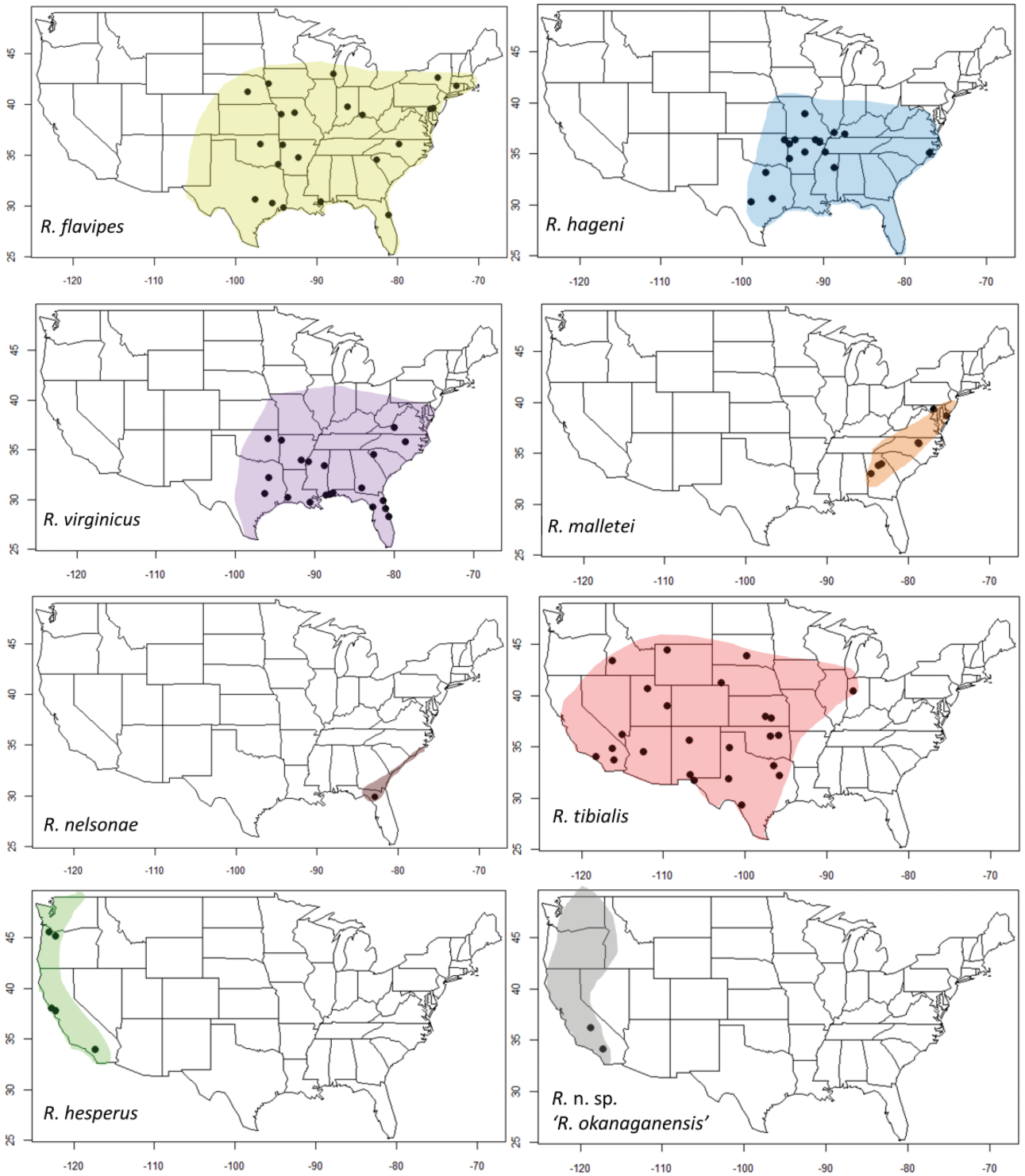

Fig. 1. Range map of *Reticulitermes* species based on in-house databases (A. L. Szalanski, unpublished data; E. L. Vargo, unpublished data) and literature records (Clément et al. 1986, Ye et al. 2004, Austin et al. 2007, Pinzon and Houseman 2009, Lim and Forschler 2012, Arango 2015, Arango et al. 2015, Hyseni and Garrick 2019). Dots indicate sampling for this study.

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

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

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

Table 2. Unique fragments used for species identification with IS01

	Diagnostic species-specific fragment (bp)					
Species	138	143	162	190	342	345
Reticulitermes flavipes				X		O
Reticulitermes hageni				O		X
Reticulitermes malletei		O	O	O		O
Reticulitermes tibialis	X					
Reticulitermes virginicus					X	

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

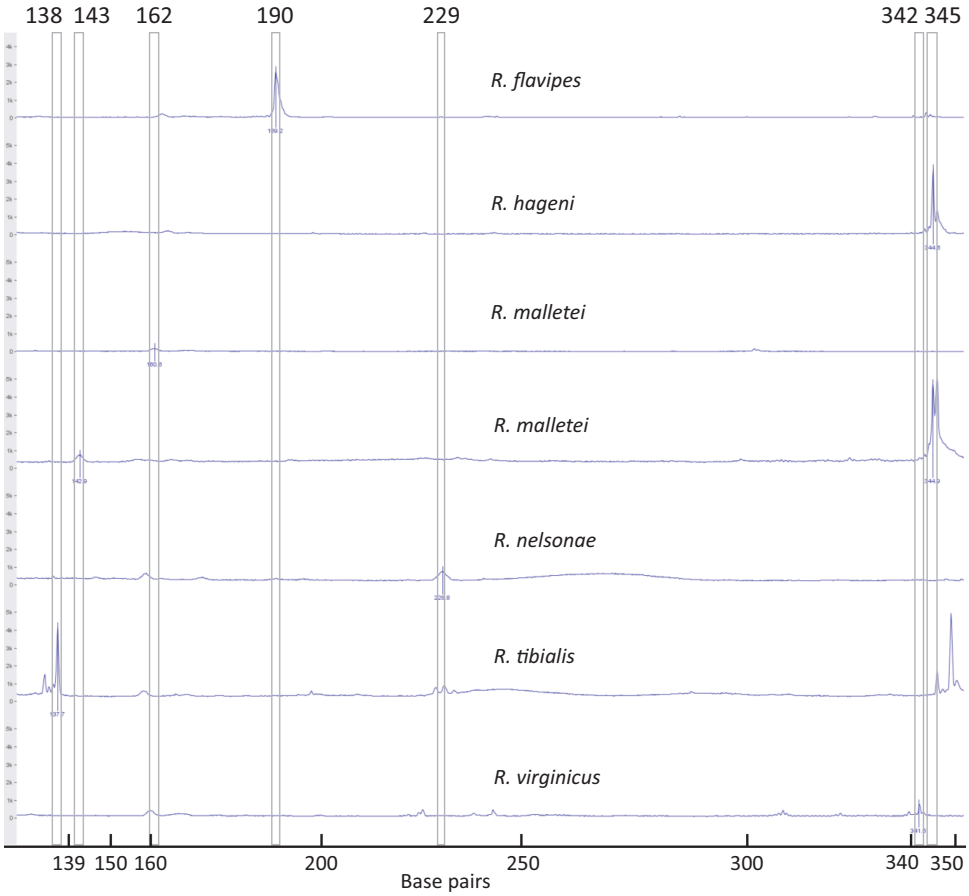

Fig. 2. Representative ISSR results for each species (rows). Bins are drawn at diagnostic species-specific locations.

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. malletei* 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

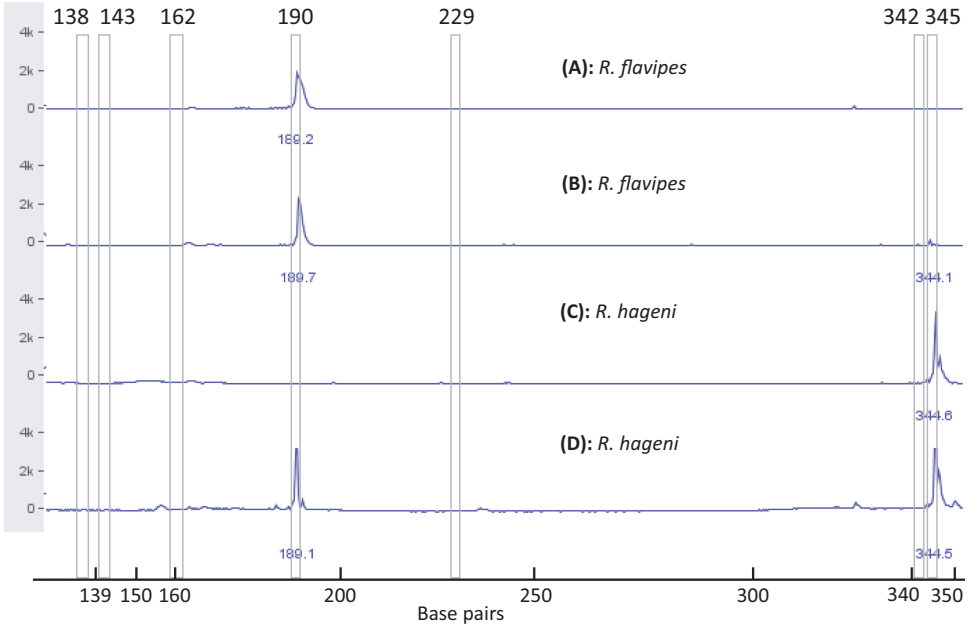

Fig. 3. Example of ISSR profiles of *R. flavipes* and *R. hageni* with only species-specific fragments (A and C) and with both species' fragments (B and D). When both fragments were present, the stronger sized fragment corresponded to the correct species.

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: Simuliidae) (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. hageni*, *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′-CGCCTGTTTATCAAAAA 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 5× Promega (contains $MgCl_2$ 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 aureus* (Snyder), *Prorhinotermes simplex* (Hagen), *R. nelsonae*, *R. hesperus*, 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 *Amitermes 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 ISO1 fragments

Species	No. of localities (no. of samples)	Diagnostic species-specific fragment (bp)						Notes
		138	143	162	190	342	345	
		R. tibialis	*R. malletei*	*R. malletei*	*R. flavipes*	*R. virginicus*	*R. hageni*	
A. wheeleri	2 (5)							No conserved fragments
C. formosanus	7 (11)		O		O		O	Conserved fragments not species-specific
C. gestroi	2 (7)				O		O	Unique fragment at 515 bp
H. aureus	3 (7)				O	O		Unique fragments at 565 and 627 bp
P. simplex	2 (2)							No conserved fragments
R. nelsonae	1 (2)							Unique fragment at 229 bp
R. hesperus	6 (6)				O		O	Conserved fragments not species-specific
R. n. sp. '*R. okanaganensis*'	2 (3)		O		O			No conserved fragments
R. banyulensis	6 (16)			X	O	O		Conserved fragment shared with *R. malletei* (162 bp)
R. grassei	1 (8)				O		X	Unique fragment at 135 bp
R. lucifugus	4 (4)				O		X	Conserved fragments not species-specific
R. speratus	3 (3)							Conserved fragments not species-specific

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

of AC with a T as an attached base (Long et al. 2009). We examined the complete trace which included fragments ranging from 70 to 650 bp. Each species had between 6.6 and 8.7 alleles per sample, on average, from the potential of up to 48 different alleles (Table 1).

There was sufficient variability between 130 and 350 bp to distinguish species. In this size range, all five species had a diagnostic, species-specific fragment besides *R. malletei* (Table 2; Fig. 2). Samples of *R. malletei* had diagnostic alleles of both *R. flavipes* or *R. hageni*, but they additionally had one of two fragments, 143 or 162 bp, that were specific to *R. malletei*. Some samples of *R. flavipes* and *R. hageni* had diagnostic fragments for both species; however, in those cases, the fragments occurred with different intensities and the stronger sized fragment always corresponded to the correct species (Fig. 3).

When testing other species, many had individuals with fragments in the *R. flavipes* and *R. hageni* diagnostic areas (Table 3). Every sample of *R. banyulensis* contained a 162-bp fragment overlapping with the second *R. malletei*-specific fragment (Table 3). Both *R. grassei* and *R. lucifugus* had the *R. hageni*-specific fragment in all samples (Table 3). From the samples tested, *R. nelsonae*, *C. gestroi*, *H. aureus*, and *R. grassei* had species-specific diagnostic fragments sized 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 identity (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.

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