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## MOLECULAR DIAGNOSTICS OF THE FORMOSAN SUBTERRANEAN TERMITE (ISOPTERA: RHINOTERMITIDAE)

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## ABSTRACT

Formosan subterranean termite, *Coptotermes formosanus* Shriaki, is a serious pest of structures in portions of United States. A 467-bp region of the mtDNA 16S rRNA gene was subjected to DNA sequencing from 12 *Coptotermes* species, including 64 populations of *C. formosanus*. Genetic diversity among species ranged from 1.8% to 7.0%, with *C. formosanus* at least 3.0% divergent to the other *Coptotermes* taxa. No genetic variation was detected among the *C. formosanus* populations for this marker making it ideal for diagnostics. Comparison of nucleotide sequence of mitochondrial rRNA 16S was used to design polymerase chain reaction (PCR) primers specific for *C. formosanus*. The diagnostic assay consists of two independent PCR runs of the 16S primer pair along with the *C. formosanus* primer set. PCR product from samples that are not *C. formosanus* can be subjected to DNA sequencing and compared with the database of termite 16S sequences on GenBank for identification. This technique provides a non-morphological method to identify field collected termites and may facilitate future quarantine programs for *C. formosanus*.

Key Words: *Coptotermes formosanus*, invasive species, PCR, genetic variation, molecular diagnostics, termite.

## RESUMEN

La termita subterráneo de Formosa, *Coptotermes formosanus* Shriaki, es una plaga seria de las estructuras en algunas partes de los Estados Unidos. Una región de 467 bp del gen 16S rARN de la ADN mitocondrial fue sujeta de la secuenciación de ADN de 12 especies de *Coptotermes*, incluyendo 64 poblaciones de *C. formosanus*. La diversidad genética entre las especies fue de 1.8% hasta 7.0%, con la *C. formosanus* por lo menos 3.0% divergentes de los otros especies de *Coptotermes*. Ningún variación genética fue detectada entre las poblaciones de *C. formosanus* para este marcador haciéndole ideal para un diagnóstico. Una comparación de la secuencia del nucleótido del gen 16S rARN de la ADN mitocondrial fue usada para diseñar unos cebadores (primers) específicos de la reacción en cadena por la polimerasa (PCR) para *C. formosanus*. El ensayo diagnóstico consiste de dos pruebas independientes de PCR del par 16S del cebador junto con el grupo de cebadores de *C. formosanus*. El producto de PCR de las muestras que no son *C. formosanus* puede ser sujeta de la secuenciación de ADN y comparados con el base de datos de las secuencias de 16S de termitas en el GenBank para la identificación. Esta técnica provee un método no morfológico para identificar las termitas recolectadas en el campo y puede facilitar los futuros programas de cuarentena para *C. formosanus*.

The Formosan subterranean termite (FST) *Coptotermes formosanus* Shriaki (Isoptera: Rhinotermitidae), is a major economic pest worldwide and has become a serious pest to the United States and its territories. Native to China, FST has been introduced into Japan, Guam, Sri Lanka, South Africa, Hawaii and the continental United States (Mori 1987; Su & Tamashiro 1987; Wang & Grace 1999). *Coptotermes formosanus* was first recorded in continental United States at Charleston, SC in 1957 (Chambers et al. 1988). Numerous well-established colonies were discov-

ered in Florida in 1980, 1982, and 1984 (Oi et al. 1992) with many additional finds since (Su & Scheffrahn 2000). Introductions to San Diego, CA (Atkinson et al. 1993; Haagsma et al. 1995), the Gulf Coast states, and southeastern US also have been documented (Spink 1967; LaFage 1987; Su & Tamashiro 1987; Howell et al. 1987; Appel & Sponsler 1989; Oi et al. 1992; Su & Scheffrahn 1998; Hawthorne et al. 2000; Howell et al. 2000; Scheffrahn et al. 2001; Hu et al. 2001; Jenkins et al. 2002). Since 2002, *C. formosanus* has been considered a quarantine pest in Mississippi (Missis-

issippi Department of Agriculture and Commerce, Rule 40). In the city of New Orleans, the control and repair costs due to FST are estimated at \$300 million annually (Lax & Osbrink 2003) and annual damage to the entire United States is estimated to exceed \$1 billion. It is considered the single most economically important insect pest in the state of Hawaii (Su & Tamashiro 1987).

The inability to quickly discriminate what *Coptotermes* species one is dealing with could lead to difficulties in evaluating the source of the infestation. While introductions of FST have gained recent notoriety, less is reported or known about other potentially damaging *Coptotermes* species. Exotic introductions of *Coptotermes havilandi* to Florida (Su et al. 1997; Su et al. 2000; Scheffrahn & Su 2000) have been detected. Although not established, *C. havilandi* Holmgren, which recently has been synonymized as *C. gestroi* (Wasmann) by Kirton & Brown (2003), has been recovered in shipping crates in Tennessee imported from East Asia (RHS, unpublished data). *Coptotermes* from South America, Central America, and the Caribbean also pose potential problems for the US. There are three described endemic species of *Coptotermes* in the Americas including *C. crassus* Snyder, *C. testaceus* L., and *C. niger* Snyder, and possibly others which have not been identified. More recent genetic surveys have uncovered old world *Coptotermes* species, *C. sojesti*, introduced to the West Indies (Scheffrahn et al. in press).

Mistakes have been made in both the naming and correct identification of some *Coptotermes*. For example, the inconsistencies in the pest status of *C. havilandi* in different regions of its geographic range have been due to misidentification and taxonomic confusion between *C. travians* (Haviland), *C. havilandi*, and *C. gestroi* (Kirton & Brown 2003). An examination of the type series of *C. travians* indicates the species has been misidentified in Peninsular Malaysian literature (Tho 1992) as *C. havilandi* and also has been referred to as *C. borneensis* Oshima (Kirton & Brown 2003).

Correct identification is critical for pest insects. Identification of termite workers is possible at the generic level only, and finding an alate, which can be identified, in a collection is seasonal and can be rare. We have developed a molecular diagnostic method capable of differentiating FST from other *Coptotermes* species regardless of the caste encountered or locality obtained. It has been demonstrated that both nucleotide sequencing and restriction enzyme digestion of polymerase chain reaction (PCR) amplicons can be used to differentiate between various termite species (Austin et al. 2002; Austin et al. 2004; Szalanski et al. 2003; Clement et al. 2001; Jenkins et al. 2002; Uva et al. 2004). DNA sequence differences reported between *Coptotermes* species from numerous disjunctive populations from around the world in a small portion of the mtDNA were

exploited to design species-specific PCR primers and to develop a DNA-based assay that can discriminate FST from other *Coptotermes* species.

#### MATERIALS AND METHODS

*Coptotermes* termites were collected from various locations in North America, South America, the Caribbean, Australia, Africa, and Asia (Table 1). Morphological identification of specimens used in this study was achieved by using Snyder (1922), Emerson (1925, 1928), Hill (1942), Scheffrahn et al. (1990), Scheffrahn & Su (1994), Tho (1992), and Su et al. (1997). Voucher specimens, preserved in 100% ethanol, are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR, USA.

Alcohol preserved specimens were allowed to dry on filter paper, and DNA was extracted from individual worker or soldier heads with the Pure-gene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50  $\mu$ l of Tris:EDTA and stored at -20°C. Polymerase chain reaction was conducted with the primers LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati & 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. PCR reactions were conducted with 1  $\mu$ l of the extracted DNA per Szalanski et al. (2000), and a profile consisting of 35 cycles of 94°C for 45 s, 46°C for 45 s and 72°C for 45 s. Amplified DNA from individual termites was purified and concentrated by Microcon-PCR Filter Units (Millipore, Bedford, MA). Samples were sent to The University of Arkansas Medical School DNA Sequencing Facility (Little Rock, AR) for direct sequencing in both directions with an ABI Prism 377 DNA sequencer (Foster City, CA). GenBank accession numbers for the *Coptotermes* termites subjected to DNA sequencing in this study are AY558898 to AY558914. DNA sequences were aligned by the PILEUP command of GCG (Accelrys, San Diego, CA), and the distance matrix option of PAUP\* 4.0b10 (Swofford 2001) was used to calculate genetic diversity according to the Kimura 2-parameter model (Kimura 1980) of sequence evolution.

#### RESULTS AND DISCUSSION

The 428-bp region of the mtDNA 16S rRNA gene was subjected to DNA sequencing from 12 described species of *Coptotermes*, including 64 populations of *C. formosanus* (Table 1). Within the genus, genetic diversity ranged from 1.8% between *C. testaceus* and *C. crassus* to 7.0% between *C. acinaciformis* and *C. vastator*. No genetic variation was observed between the two *C. testaceus* samples, and up to 0.7% variation was observed among the *C. gestroi* samples. No genetic varia-

TABLE 1. *COPTOTERMES* COLLECTION DATA.

Species	Location	Country	N <sup>a</sup>
<i>C. acinaciformis</i>		Australia	1
<i>C. carvinatus</i>		Malaysia	1
<i>C. crassus</i>	Belize City	Belize	1
<i>C. formosanus</i>	San Diego, CA	USA	1
	Jacksonville, FL	USA	1
	Wilton Manors, FL	USA	1
	Stone Mt., GA	USA	1
	Oahu, HI	USA	2
	Maui, HI	USA	2
	Lake Charles, LA	USA	8
	New Orleans, LA	USA	1
	Baton Rouge, LA	USA	1
	St. Rose, LA	USA	1
	Stennis Space Ctr, MS	USA	7
	Spindale, NC	USA	1
	Forest City, NC	USA	1
	Rutherfordton, NC	USA	1
	Rockport, TX	USA	1
	Rockwall, TX	USA	1
	Galveston, TX	USA	8
	Garland, TX	USA	7
	Grapevine, TX	USA	2
	Lewisville, TX	USA	1
	San Antonio, TX	USA	1
	Beaumont, TX	USA	1
	La Porte, TX	USA	1
	Hong Kong	China	8
	Guangzhou	China	1
		Taiwan	1
	Nagasaki Prefecture	Japan	2
<i>C. heimi</i>		India	1
<i>C. gestroi</i>		Grand Turk	1
	Monroe, FL	USA	1
	Miami, FL	USA	1
		Singapore	1
		Taiwan	1
	Hong Kong	China	1
<i>C. lacteus</i>	Berrburrum	Australia	2
<i>C. intermedius</i>	Togo	Africa	1
<i>C. michaelsoni</i>	Perth	Australia	4
<i>C. sjostedti</i>	Nongo	Guinea	1
<i>C. testaceus</i>		Tobago	1
		Grenada	1
<i>C. vasator</i>	Oahu, HI	USA	1

<sup>a</sup>Number sequenced.

tion was observed in *C. formosanus*, and *C. formosanus* was most similar to the *C. intermedius* sample from Togo Africa, with 3.0% DNA sequence divergence. Phylogenetically, *C. formosanus* forms a distinct clade among non-Australian *Coptotermes* (*C. vasator*, *C. testaceus*, *C. crassus*, *C. sjostedti*, *C. intermedius*, *C. gestroi*, *C. heimi*, and *C. carvinatus*) (Scheffrahn et al. in press).

Formosan subterranean termite 16S DNA sequences along with sequences from other *Copto-*

*termes*, *Reticulitermes* (Szalanski et al. 2003) and *Heterotermes* (Szalanski et al. 2004) were aligned and examined for mismatches that reflected either substitutions or deletions. The mismatches were exploited to design primers that were unique to FST (Table 2). Two primers, one from each strand FST-F (5'-TAAAACAACAAACAA-CAAACAAC-3') and FST-R (5'-ATGGCTTGAC-GAGGCACAA-3') were designed. Based on the sequence, the expected sizes of the amplicon is

TABLE 2. DNA SEQUENCE ALIGNMENT OF 12 *Coptotermes* SP., AND *C. formosanus* SPECIFIC PCR PRIMERS.

FST-F	TAAACAAAC	AAACAACAAA	CAAAC			
	10	20	30	40	50	60
<i>C. formosanus</i>	TAAACAAAC	AAACAACAAA	CAAACAAATA	AACCCAAA-TG	TTAAACTCTA	TAGGGTCTTC
<i>C. vastator</i>	-AAACAAAAC	AAACAACATA	AAA-TAAATA	GGCCAAA-TG	TTAAACTCTA	TAGGGTCTTC
<i>C. testaceus</i>	-AAACAAAAC	AAACAACATA	AA--TAAATA	AGCCAAA-TG	TCAAACCTCTA	TAGGGTCTTC
Tobago						
<i>C. crassus</i>	-AAACAAAAC	AAACAACATA	AA--TAAATA	AGCCAAA-TG	TTAAACTCTA	TAGGGTCTTC
<i>C. sjostedti</i>	-AAACAAAAC	AAACAACAAA	GAAATAAACA	AACCCAAA-GG	TAAACTCTA	TAGGGTCTTC
<i>C. intermedius</i>	-AAACAAAAC	AAACAACAAA	AAAATAAATA	AACCCAAA-TG	TTAAACTCTA	TAGGGTCTTC
<i>C. gestroi</i>	--AATAAAC	AAACAACAAA	CAAGTAAATA	AACCCAAA-TG	TCAAACCTCTA	TAGGGTCTTC
<i>C. heimi</i>	--AAACAAAAC	AAACAACAAA	TAAGCAAATA	AACCCAAA-TG	TCAAACCTCTA	TAGGGTCTTC
<i>C. carvinatus</i>	CAAAACAAAAC	AAACAACCAA	ACAGGAAATA	AACCCAAA-TG	TCAAACCTCTA	TAGGGTCTTC
<i>C. lacteus</i>	TAAATAAAC	AAACAAC-AA	CAAATAAGTA	AGCCAAAATG	TCAAACCTCTA	TAGGGTCTTC
<i>C. acinaciformis</i>	CAAAATAAAC	AAACAACCAA	CAAATGAATA	AACCCAAA-TG	TTAAACTCTA	TAGGGTCTTC
<i>C. michaelsoni</i>	TAAATAAAC	AAACAACCAA	TAAATGAATA	AACTAATATG	TCAAACCTCTA	TAGGGTCTTC
	70	80	90	100	110	120
<i>C. formosanus</i>	TCGTCCCACA	AAAACATCTA	AGAATTTTAA	CTCAAAAACC	AAATTCAATA	AAA-CATTCA
<i>C. vastator</i>	TCGTCCCATA	AAAACATCTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-CATTCA
<i>C. testaceus</i>	TCGTCCCATA	AAAACATCTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-CATCCA
Tobago						
<i>C. crassus</i>	TCGTCCCATA	AAAACATCTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-CATTCA
<i>C. sjostedti</i>	TCGTCCCACA	AAAACATTTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-CATTCA
<i>C. intermedius</i>	TCGTCCCACA	AAAACATCTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-TATTCA
<i>C. gestroi</i>	TCGTCCCACA	AAAACATCTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-CATTCA
<i>C. heimi</i>	TCGTCCCACA	AAAACATCTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-CATTCA
<i>C. carvinatus</i>	TCGTCCCACA	AAAACATTTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-CATTCA
<i>C. lacteus</i>	TCGTCCCATA	AAAACATTTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-TATCCA
<i>C. acinaciformis</i>	TCGTCCCATA	AAAACATTTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAAATATTCA
<i>C. michaelsoni</i>	TCGTCCCATA	AAAACATCTA	AGAATTTTAA	CTCAAAGACC	AAATTCAATA	AAA-TATTCA
		ATGGCTT	GACGAGGCAC	AA FST-R		
	130	140	150			
<i>C. formosanus</i>	ACATTAAGAC	AGCTTGTGCC	TCGTCAAGCC	AT		
<i>C. vastator</i>	TCACTAAGAC	AGCCAGTGCC	TCGTCAAGCC	AT		
<i>C. testaceus</i>	TCACTAAGAC	AGCCAGTGCC	TCGTCAAGCC	AT		
Tobago						
<i>C. crassus</i>	TCACTAAGAC	AGCCAGTGCC	TCGTCAAGCC	AT		
<i>C. sjostedti</i>	TCACTAAGAC	AGCCGGTGCC	TCGTCAAGCC	AT		
<i>C. intermedius</i>	TCACTAAGAC	AGCCCCTGCC	TCGTCAAGCC	AT		
<i>C. gestroi</i>	ACACTAAGAC	AGCCCCTGCC	TCGTCAAGCC	AT		
<i>C. heimi</i>	ACACTAAGAC	AGCCCCTGCC	TCGTCAAGCC	AT		
<i>C. carvinatus</i>	ACACTAAGAC	AGCCCCTGCC	TCGTCAAGCC	AT		
<i>C. lacteus</i>	ACACTAAGAC	AGCTCATGTC	TCGTCAAGCC	AT		
<i>C. acinaciformis</i>	ACACTAAGAC	AGCTCATGTC	TCGTCAAGCC	AT		
<i>C. michaelsoni</i>	ACACTAAGAC	AGCTCGTGCC	TCGTCAAGCC	AT		

151 bp. Proper sized PCR products were obtained with conspecific DNA, whereas no product was obtained with template from the other species, i.e., no false positives were observed with known DNA. Each FST primer paired with a common primer will only amplify Formosan subterranean termite DNA.

The FST species-specific primers were tested for optimal annealing performance in a 47°-59°C temperature gradient with 2°C intervals. The optimal annealing temperature for the FST specific

primers was 57°C. PCR reactions were the same as the 16S conditions with the exception of the PCR profile which consists of 30 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 45 s. This annealing temperature, however, is too high for the 16S universal primers, preventing multiplex PCR with both primer pairs. To resolve this, both PCR reactions are conducted individually and 10 µl of each PCR reaction are loaded onto a single well of a 2% agarose gel (Fig. 1). The FST specific primer set successfully amplified for 52 individual FST



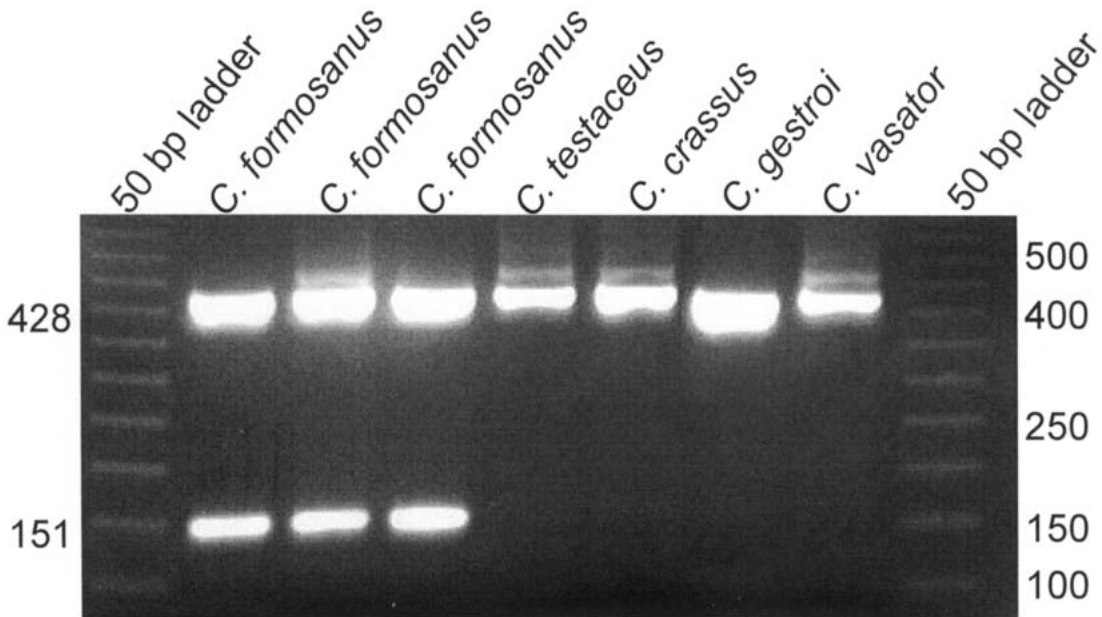


Fig. 1. Two percent agarose gel of 428-bp mtDNA 16S amplicon and 151-bp *C. formosanus* diagnostic amplicon for 4 *Coptotermes* spp.

from all 23 FST populations, and did not yield a PCR product for the other *Coptotermes*, *Reticulitermes* and *Heterotermes*. PCR product from samples that are not *C. formosanus* can be subjected to DNA sequencing and compared with the database of termite 16S sequences on GenBank for identification by a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>). This technique provides a method to identify field collected termites and facilitates the screening of the monitoring for this species and for the introduction of other invasive *Coptotermes* termites.

In the context of determining the species for a large number of samples collected in connection with distribution or competition studies, simplifying the identification of the worker caste is advantageous. FST has no genetic polymorphism across its geographic distribution for the 16S marker, whereas genetic variation has been observed in the mtDNA COII gene (ALS unpublished data, Jenkins et al. 2002). This lack of mtDNA 16S intraspecific variation makes this marker ideal for molecular diagnostics.

These primers provide a convenient way to identify individual termites without resorting to more time consuming restriction fragment-length polymorphism analysis, or extensive morphological data which may result in overlap due to clinal variations in size as observed in many insects including termites. The approach is equally applicable to other castes, such as soldiers and alates, but given their obvious taxonomic importance should be constrained to either morphologically

ambiguous samples, or when the more diagnostic castes are unavailable. This should be an important new tool for substantiating the identity of FST before the onset of regulatory procedures (i.e., quarantine).

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