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## GENETIC EVIDENCE FOR A NEW SUBTERRANEAN TERMITE SPECIES (ISOPTERA: RHINOTERMITIDAE) FROM WESTERN UNITED STATES AND CANADA

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### ABSTRACT

Genetic evidence for a new subterranean termite species herein named *Reticulitermes okanaganensis* is provided based on DNA sequence analysis. Partial sequences of the mitochondrial DNA rRNA 16S gene were obtained from 27 samples of *R. okanaganensis* from British Columbia, Idaho, Oregon, Nevada and California. Five nucleotide sites were variable among the four observed haplotypes. One haplotype occurred only once, while the most common haplotype, O3, occurred in 37% of the samples. Molecular phylogenetic analysis of *R. okanaganensis* relative to five other North American *Reticulitermes* species has clarified its distinct position within the genus.

Key Words: 16S gene, DNA sequence, *Reticulitermes*, termite

### RESUMEN

Se provee evidencia genética para una nueva especie de termita aquí nombrada *Reticulitermes okanaganensis* basada sobre el análisis de la secuencia de ADN. Secuencias parciales del gen 16s de rARN del ADN mitocondrial fueron obtenidas de 27 muestras de *R. okanaganensis* de Columbia Británica, Idaho, Oregon, Nevada, y California. Cinco sitios de nucleótidos fueron variables entre los cuatro haplotipos observados. Un haplotipo ocurrió solamente una vez, mientras que el haplotipo más común, "O3", ocurrió en 37% de las muestras. El análisis filogenético molecular de *R. okanaganensis* en relación con las otras cinco especies de *Reticulitermes* norteamericanas ha clarificado su posición distinta dentro del género.

There is a general consensus that the genus *Reticulitermes* is in need of revision (Weesner 1970; Nutting 1990; Scheffrahn & Su 1994; Forschler & Jenkins 1999). This is an especially difficult because of the lack of discrete morphological characters which provide for accurate identification of specimens to the species level. For this reason, the application of non-morphological identification methods such as the evaluation of cuticular hydrocarbon analysis and mtDNA markers have been used.

Most recently, the application of the mitochondrial rRNA 16S gene has been applied to identify *Reticulitermes* populations from the south central United States (Austin et al. 2004a, b, c) for species across North America (Austin et al. 2005b), and for clarification of exotic introductions of nearctic *Reticulitermes* around the world (Austin et al. 2005a; Su et al. 2006). The application of this marker has tremendous potential for molecular diagnostics of *Reticulitermes*, with increased accuracy of positive species identifications (Szalanski et al. 2003), and clarifying the identities of exotic

introductions around the world (Austin et al. 2005 a) and from North America (Austin et al. 2005 b). Recently, Copren et al. (2005) found evidence for as many as seven new species of *Reticulitermes* from the western United States based upon cuticular hydrocarbon phenotypes, but resolved to designate them as putative new species after attempting to corroborate their relationship with molecular phylogenetics and reproductive flight dates. These discrepancies likely are attributed to the environmental plasticity of cuticular hydrocarbons and stresses the need for fixed character states for species identification such as mtDNA sequences.

We provide the first genetic evidence of a new species, named herein as *R. okanaganensis* after its first collection site in the Okanagan region of British Columbia, Canada, and provide a phylogenetic analysis of *Reticulitermes* by applying the 16S mtDNA gene. In addition, the geographic distribution of *Reticulitermes* species and haplotypes of species throughout the region are discussed.

## MATERIALS AND METHODS

Termites were collected from various locations in British Columbia, Idaho, Oregon, Nevada, and California, both from our own collecting efforts and from the 2002 National Termite Survey (Table 1). Samples were preserved in 100% ethanol.

Alcohol-preserved specimens were allowed to dry on filter paper, and DNA was extracted according to Liu & Beckenbach (1992) on individual whole worker termites with the Puregene 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. The PCR reactions were conducted with 2  $\mu$ l of the extracted DNA (Szalanski et al. 2000), with 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, AR) for direct sequencing in both directions. GenBank accession numbers were DQ389178-DQ389180 and DQ438936 for the *Reticulitermes okanaganensis* haplotypes found in this study. Consensus sequences for each sam-

ple were obtained with Bioedit 5.09 (Hall 1999). Mitochondrial DNA haplotypes were aligned with MacClade v4 (Sinauer Associates, Sunderland, MA).

The distance matrix option of PAUP\* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model of sequence evolution (Kimura 1980). Mitochondrial 16S sequences from *R. flavipes*, *R. hesperus*, *R. tibialis*, *R. hageni*, *R. flavipes*, and *R. virginicus* (Szalanski et al. 2003; Austin et al. 2004a,b,c) were added to the DNA sequence dataset for comparison. DNA sequences from the Formosan termite, *Coptotermes formosanus* Shiraki (GenBank AY558910), and *Heterotermes aureus* (Snyder) (GenBank AY280399), were added to act as outgroup taxa. DNA sequences were aligned by CLUSTAL W (Thompson et al. 1994). Maximum likelihood and unweighted parsimony analysis on the alignments were conducted by PAUP\* 4.0b10 (Swofford 2001). Gaps were treated as missing characters for all analysis. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1,000 resamplings with the Branch and Bound algorithm of PAUP\*. For maximum likelihood analysis, the default likelihood parameters were used (HKY85 six-parameter model of nucleotide substitution, empirical base frequencies with the exception of the transition/transversion ratio, which was set to 2.596954:1). These parameters were used to carry out a heuristic search by PAUP\* with a neighbor joining tree as the starting tree.

TABLE 1. COLLECTION DATA, AND HAPLOTYPES (HAP) FOR *RETICULITERMES OKANAGANENSIS*

State/Prov	City	County	Lat/Long	Hap	n
BC	Osoyoos		49:02:09 N 119:27:51 W	O1	2
CA	Placerville	El Dorado	38:43:47 N 120:47:51 W	O1	1
NV	Reno	Washoe	39:31:47 N 119:48:46 W	O1	3
ID	Boise	Ada	43:36:49 N 116:12:09 W	O1	1
ID	Lewiston	Nez Perce	46:25:00 N 117:01:00 W	O1	1
CA	Chino	San Bernardino	34:00:44 N 117:41:17 W	O2	1
CA	Grass Valley	Nevada	39:13:09 N 121:03:36 W	O2	1
CA	Irvine	Orange	33:40:10 N 117:49:20 W	O2	1
CA	Napa	Napa	38:17:50 N 122:17:04 W	O2	1
NV	Reno	Washoe	39:31:47 N 119:48:46 W	O2	2
NV	Carson City	Carson City	39:09:50 N 119:45:59 W	O2	1
CA	Auburn	Placer	38:53:48 N 121:04:33 W	O3	2
CA	Bakersfield	Kern	35:22:24 N 119:01:04 W	O3	1
CA	Dinuba	Tulare	36:32:36 N 119:23:10 W	O3	1
CA	Lafayette	Contra Costa	37:53:09 N 122:07:01 W	O3	1
CA	Lake Arrowhead	San Bernardino	34:15:52 N 117:11:04 W	O3	1
CA	Napa	Napa	38:17:50 N 122:17:04 W	O3	1
CA	Strathmore	Tulare	36:08:44 N 119:03:35 W	O3	1
CA	Walnut Creek	Contra Costa	37:54:23 N 122:03:50 W	O3	1
OR	Klamath Falls	Klamath	42:13:30 N 121:46:50 W	O3	1
NV	Reno	Washoe	39:31:47 N 119:48:46 W	O4	2

RESULTS

DNA sequencing of the 16S rRNA amplicon from *R. okanaganensis* revealed an average size of 428 bp. The average base frequencies were A = 0.41, C = 0.23, G = 0.13, and T = 0.23. Among the 27 *R. okanaganensis* mtDNA rRNA 16S DNA sequences, a total of 5 nucleotide sites were variable (Table 2). Four distinct haplotypes (lineages) were observed (Table 1), and genetic divergence among these haplotypes ranged from 0.2 to 0.5 percent. One haplotype, O4, occurred only once, while the most common haplotype, O3, occurred in 37% of the *R. okanaganensis* samples. While haplotype O4 was only found in Nevada, haplotype O1 was found over the largest geographical area (Table 1).

We conducted a phylogenetic analysis on *R. okanaganensis* relative to all described North American *Reticulitermes* species to clarify the phylogenetic relationships of *R. okanaganensis* within the genus. Parsimony analysis of the aligned *Reticulitermes* spp. and the outgroup taxa used 436 characters, of which 90 were variable (20%) and 59 (14%) were parsimony informative. This analysis had a single consensus tree with a length = 154, and a CI value of 0.695 and verified the distinct monophyly of '*R. okanaganensis*' in the genus (Fig. 1). Maximum likelihood analysis (-ln L = 1363.74728) also supported a distinct clade for *R. okanaganensis* and was identical to the maximum parsimony analysis. *Reticulitermes okanaganensis* formed a sister group to *R. flavipes*, while *R. hesperus* grouped with *R. tibialis*, and *R. hageni* formed a distinct clade with *R. virginicus*.

DISCUSSION

Phylogenetic analysis of North American *Reticulitermes* revealed a distinct clade for *R. okanaganensis*. There is clear genetic evidence for *R. okanaganensis* being identified as a discrete species, possessing multiple haplotypes over a broad geographical range including California, Idaho, Oregon, Nevada, and British Columbia (Fig. 2). The genetic isolation observed demonstrates that this group represents a distinct taxonomic entity, albeit cryptic due to overlapping morphological characters with other congeners from the region that merits further investigation.

The identification of new species from northern California has been reported by Haverty & Nelson (1997), Haverty et al. (1999) applying cuticular hydrocarbons, and further investigated with ethological data (Getty et al. 2000a,b). It has been proposed that extensive collecting from western states may produce as many as 6 new *Reticulitermes* species, and perhaps at least three *Reticulitermes* species in Mexico (Myles 2000). We have already identified other morphologically and genetically distinct nearctic *Reticulitermes* from this region and we are attempting to collect additional material in the near future so that subsequent investigations can be performed and the overall relationships of western nearctic *Reticulitermes* can be better understood.

*Reticulitermes okanaganensis* has become a problem in British Columbia, where there is an unprecedented number of attacks to structures, particularly around Kelowna, BC (Hugh Philip unpublished). Reports of difficulties controlling *Reticulitermes* infestations from urban structures in northern California (Kistner & Sbragia 2001) prompts us to consider whether these difficult control scenarios are due to the structures themselves, or the possibility of infestations from this newly identified species, that may have slightly different nutritional, ecological, and behavioral requirements.

The western subterranean termite, *Reticulitermes hesperus*, is the most destructive termite found in California (Lewis 2001), but it is highly probable that *R. okanaganensis* accounts for a significant portion of damage previously thought to be associated with *R. hesperus*. Snyder (1949) originally described *R. hesperus* based on material from Little Bear Lake, San Bernadino mountains, California and samples previously collected from Vancouver, British Columbia, and suggests the range to extend from British Columbia through Washington, Oregon, California, and Nevada, extending down to Baja, California (Spencer 1937). The northern limit of its range occurs in the upper Frazer valley from Lytton to Kamloops, British Columbia (Spencer 1937, 1945). Our genetic evaluation of populations from along the Pacific and throughout the western states suggest that *R. hesperus* does not generally occur away from the Pacific coast region, as is described by Weesner (1965), and is isolated west of the Sierra Nevada/Cascades mountain ranges (unpublished). *Reticulitermes tibialis* overlaps entirely with the range of *R. okanaganensis*, and extends east as far as Indiana, but is not commonly found east of Texas and Oklahoma (Banks & Snyder 1920; Austin et al. 2004a). Light and Pickens (1934) indicate collections of *R. tibialis* from the northern tip of Idaho, while the U.S. Dept. of Agriculture (1959a,b) have reports from Twin Falls and Lewiston to Lapwari, Idaho. Weesner (1970) concedes that collections from western California to as far east as Elko and

TABLE 2. BASE PAIR DIFFERENCES BETWEEN *RETICULITERMES OKANAGANENSIS* HAPLOTYPES.

haplotype	75	160	364	369	375
O1	C	G	T	—	T
O2	.	A	.	A	.
O3	.	.	.	A	.
O4	T	.	.	.	.

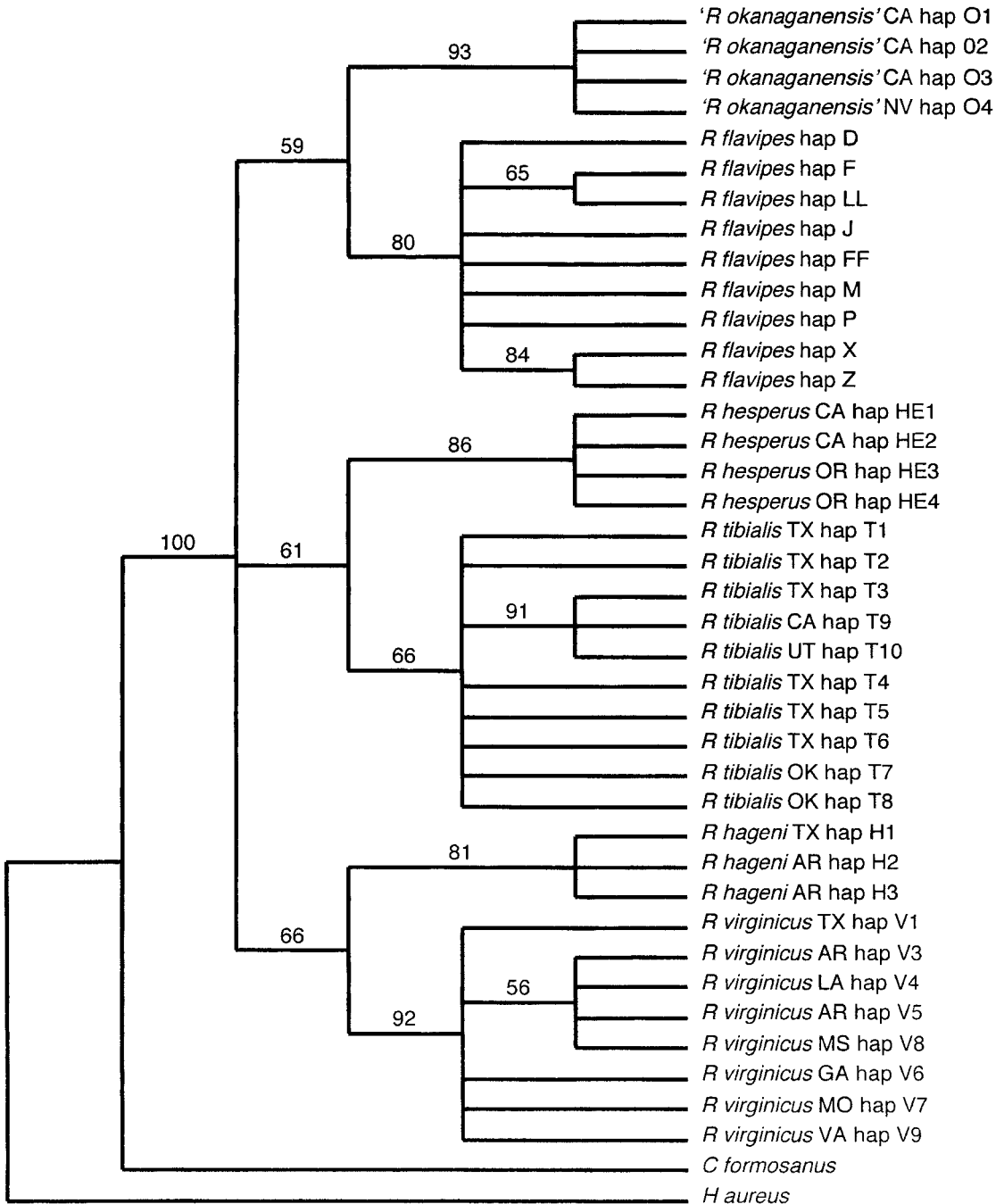


Fig. 1. Maximum parsimony cladogram of *Reticulitermes okanaganensis* and related taxa.

Reno, Nevada appear to be *R. tibialis*, but are too difficult to assign to either *R. hesperus* or *R. tibialis*.

*Reticulitermes okanaganensis* haplotype O1, which has been reported in Osoyoos, BC, Canada, is also found in El Dorado county California, Washoe county Nevada, and Ada and Nez Perce

counties in Idaho. Osoyoos, B.C. is located in the southern interior ecoprovince of Canada, a region characterized as a desert climatic zone. We are currently investigating the distribution of *Reticulitermes* species throughout the western United States. From this study and ongoing research, we

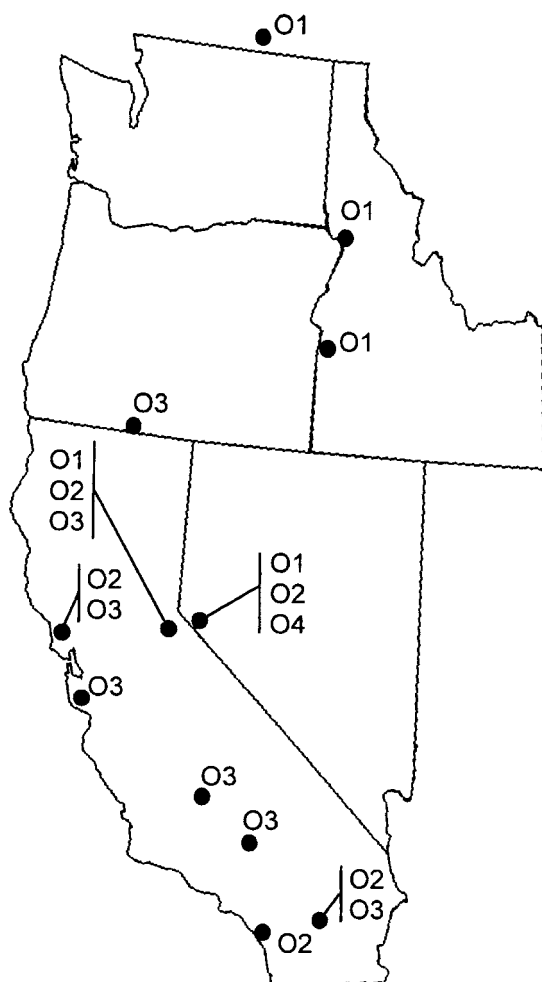


Fig. 2. Distribution of *Reticulitermes okanaganensis* haplotypes in British Columbia, Idaho, Oregon, Nevada, and California.

expect that *R. okanaganensis* will be found in the drier regions north of the Cascades and East of the Sierra Nevada Ranges. While the possible importation of this species either to the United States or to Canada should be considered, in all probability we are dealing with a cryptic endemic species. Establishment of this species in Canada may be attributed to trade between the US and Canada, and may account for the unprecedented number of structures being attacked in British Columbia. It may be equally plausible that urban sprawl and encroachment into previously undeveloped areas for housing needs has contributed to the increase frequency of attacks to structures there.

An important criterion for determining the extent of genetic variation for a species lies in the ability to sample from populations evenly distributed within the species range (Mayr & Ashlock

1991). For this reason, future studies of this unknown species, including a proper species description, are required. This study represents an important first step towards this endeavor.

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