

Molecular Phylogeography of the Subterranean Termite *Reticulitermes tibialis* (Isoptera: Rhinotermitidae)¹

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ABSTRACT A molecular genetics study involving DNA sequencing of a portion of the mitochondrial DNA 16S gene was undertaken to determine the extent of genetic variation within the subterranean termite, *Reticulitermes tibialis*, in the United States. A total of 106 samples were analyzed from 18 states. Thirty-two nucleotide sites were variable in the 428 bp 16S rDNA sequence, and 41 distinct haplotypes were observed. Twenty-three haplotypes (55%) occurred only once, while the most common haplotype, T2, occurred in 24% of the samples. Genetic diversity among haplotypes ranged from 0.2 to 2.3%. Bayesian phylogenetic and TCS spanning tree analysis revealed several distinct clades that appeared to be geographically isolated; however, regression and Mantel tests did not support any populational structure. The role of glaciation and sky islands on haplotype variation of *R. tibialis* is supported by a molecular clock and may have contributed to the large amount of genetic variation observed within this species.

KEY WORDS mitochondrial DNA, DNA sequence, genetic variation, *Reticulitermes*, termite

The majority of pestiferous subterranean termites in North America belong to the endemic genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Reticulitermes* species are found in every state in the continental United States except Alaska, but are most common in the warm and humid southeastern region (Su et al. 2001). There are presently six described species of *Reticulitermes* in North America, four of which occur in western United States: the eastern subterranean termite, *Reticulitermes flavipes* Kollar, the light southern subterranean termite *R. hageni* Banks, the western subterranean termite, *R. hesperus* Banks, and the arid subterranean termite, *R. tibialis* Banks (Banks 1920, Snyder 1954, Weesner 1965, Messenger 2003). There is also genetic evidence for a new species of *Reticulitermes* in western United States and British Columbia, Canada (Szalanski et al. 2006).

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The arid subterranean termite, *R. tibialis*, is found from California to eastern Texas, and as far north as Iowa to Idaho (Banks & Snyder 1920, Snyder 1954, Weesner 1965, Nutting 1990). An isolated population has also become established in sand dunes located in Indiana (Park 1929, Ye et al. 2004).

This species is an economic pest in western United States. In its natural habitat, it can cause a small amount of damage to roots and stems of plants. It can also cause economic damage to man made structures. This termite is capable of building shelter tubes up to wooden structures; however, it is much less persistent in building tubes than other *Reticulitermes* species. When it does attack structures, it is more likely to attack moist and decaying wood than dry lumber (Pickens 1934), and this may be due to its preference for wood with high humidity (Williams 1934).

Genetic variation for a species is best determined if sampling from populations is distributed within the species range (Mayr & Ashlock 1991). Given the known distribution of *R. tibialis* in North America, the objective of this study was to document the extent of its genetic variation in North America. Avise (1994) suggests that smaller scale investigations may not provide a discernable population structure which might be more easily observed with larger scale studies. To our knowledge, this is the first comprehensive attempt to determine the extent of genetic variation for *R. tibialis* in North America, and the first study which encompasses most of the known geographic distribution of the species.

Materials and Methods

Termites were collected from various locations in the United States from our own collecting efforts, from samples provided by collaborators, and from the 2002 National Termite Survey (Fig. 1). With the exception of the samples from Utah and Texas, only one to three samples were analyzed from the remaining states. All samples collected from the field were preserved in 90–100% ethanol. *Reticulitermes* were morphologically identified to species when alates were available using the keys of Krishna & Weesner (1969), and from labral measurements of soldiers (Hostettler et al. 1995, Heintchel et al. 2006) and other morphometrics outlined by Light (1927) and compiled by Castle (1930). For the remaining samples, species identification was conducted using mtDNA 16S sequences (Szalanski et al. 2003). Voucher specimens preserved in 100% ethanol are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR.

Alcohol-preserved specimens were dried 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 μ l of Tris:EDTA and stored at -20°C . Polymerase chain reaction was conducted using 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 1 μ l 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, Madison, WI)



Fig. 1. Genealogical relationship of *R. tibialis* mtDNA haplotypes. Each circle/node represents a single nucleotide substitution. Haplotypes T1 and T4 were collected from type locality specimens in Beeville, TX 2005, and haplotypes 43–46 from Missouri are not presented.

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. Additional *R. tibialis* mtDNA 16S sequences were obtained from previous studies of *Reticulitermes* from Texas (Austin et al. 2004a), California (Tripani et al. 2006) and Indiana (Ye et al. 2004). GenBank accession numbers were FJ610446 to FJ610472 for the new *R. tibialis* haplotypes found in this study. Consensus sequences for each sample were obtained using Bioedit 5.09 (Hall 1999). Mitochondrial DNA haplotypes were aligned using MacClade v4 (Sinauer Associates, Sunderland, MA). Genealogical relationship of *R. tibialis* haplotypes was constructed using TCS (Clement et al. 2000).

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. okanaganensis*', *R. hesperus* Banks, *R. mallei* Clément, *R. hageni* Banks, *R. flavipes* (Kollar) and *R. virginicus* (Banks) (Szalanski et al. 2003, Austin et al. 2004a,b,c, Szalanski et al. 2006, Austin et al. 2007) were added to the *R. tibialis*

dataset along with DNA sequences from the Formosan termite, *Coptotermes formosanus* Shiraki (GenBank AY558910), and *Heterotermes aureus* (Snyder) (GenBank AY280399), which were added to act as outgroup taxa. DNA sequences were aligned using CLUSTAL W (Thompson et al. 1994). Maximum likelihood (ML) and maximum parsimony (MP) analysis on the alignments was conducted using PAUP* 4.0b10 (Swofford 2001). Gaps were treated as a fifth character state for the maximum parsimony analysis, and a Ti/Tv ratio of 2.77 was used for the ML analysis. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1000 resamplings using the Branch and Bound algorithm of PAUP* (Fig. 3).

For Bayesian analysis, the best-fitting nucleotide substitution model was chosen according to the general time reversible + gamma (GTR+G) model among 64 different models using the ModelTest v 3.7 (Posada & Crandall 1998) and PAUP* 4.0b10 (Swofford 2001) programs. Phylogenetic trees were obtained using Bayesian inference with the GTR+G model using Bayesian Evolutionary Analysis Sampling Trees (BEAST) v1.4.2 software (Drummond & Rambaut 2007). For Bayesian inference, four Markov chains run for 10^6 generations with a burn-in of 2×10^4 were used to reconstruct the consensus tree.

To test for correlation between genetic similarity and geographical distances (Km) we applied a linear regression using JMP version 5.1 software (SAS Institute, Cary, NC). A Mantel test (Sokal 1979, Manly 1985, 1997, Epperson 2003) was conducted to ensure interdependence was not involved via PopTools Version 3.0.5 (Hood 2000) using a matrices correlation (Pearson r).

Results and Discussion

The phylogeographic patterns of *Reticulitermes* have been reported for over 80 yr now, but given the high degree of confusion for species identification and the inability to recover all diagnostic castes, updated records which utilize more objective identification tools such as genetics are needed. This approach has been effective for interpreting the distribution and evolutionary history of this morphologically ambiguous genus. *Reticulitermes tibialis* from 18 U.S. states were subjected to DNA sequencing analysis (Fig. 1, Table 1). DNA sequencing of the 16S rRNA amplicon revealed an average amplicon size of 428 bp. The average base frequencies were A = 0.41, C = 0.23, G = 0.13, and T = 0.23. Among the 106 *R. tibialis* mtDNA 16S DNA sequences, a total of 32 nucleotide sites were variable (Table 2). Forty one distinct haplotypes (lineages) were observed (Tables 1 and 2), and intraspecific genetic divergence among these haplotypes ranged from 0.2% to 2.3%. Twenty-three haplotypes occurred only once, while the most common haplotype, T2, occurred in 23% of the samples (Tables 1 and 2).

Some haplotypes appeared to have some geographical isolation significance as evident from the TCS spanning tree analysis and the Bayesian phylogenetic analysis. We conducted a TCS spanning tree analysis on all 42 *R. tibialis* haplotypes (Fig. 2). Regression of genetic distances and spatial distances (Km) can be observed in Figure 4. Although TCS results appear to indicate the possibility of structure among *R. tibialis* populations, this was not statistically supported. There was a poor relationship between genetic and geographic distances ($r^2 = 0.291$, $P = 0.008$, from 83 (of 106 total) distinct populations >2 km distances apart each) as only 29% of the variance of genetic *p*-distances can be

attributed to changes in geographic distance and the linear relationship between them. In addition, in order to account for any possible interdependence associated with input variables assigned to matrices, a permutation test (Mantel Test) was performed. The Mantel test was conducted by applying the Mantel Test option of PopTools version 3.0.5, released 30 May 2008 that employed 10,000 permutations. The simple matrix correlation (Pearson r) between genetic and geographic distance was 0.061 ($P < 0.001$ with 10,000 permutations); consequently, the overall poor relationship of geographic structure as evidenced by more distant populations was even less supported than the linear regression value ($r^2 = 0.291$) (Fig. 4).

The basal haplotype, T23, was recovered from Salt Lake Co., Utah. Reports of problems with *R. tibialis* attacking both ornamental plantings and structures have been known for decades (Rees & Gaufin 1939) in Utah. Many distinct haplotype lineages were observed; however, most of these lineages were only a single nucleotide in divergence from other observed haplotypes. The exceptions were haplotypes T25 from Washington Co., Utah and T41 from Ada Co., Idaho (Fig. 2). Two haplotypes were recovered from the type locality, Beeville, TX. Haplotype T4 was also found from New Braunfels, TX, while haplotype T1 was recovered from three other locations in Texas.

Both northern and eastern populations of *R. tibialis* pose interesting questions into the possibilities of biotic radiation of the species; in particular, the possibility that some populations have followed glacial refugia patterns (Comes & Kardereit 1998) as has been proposed in other studies of the genus from both nearctic (Austin et al. 2006, McKern et al. 2007) and palearctic (Uva et al. 2004, Luchetti & Mantovani 2005) regions. Although some have proposed migration or accidental introductions of *Reticulitermes* into Canada and north central states (Myles 2004), it is clear that many have continuously occupied these areas (Park 1929, Emerson 1936) and that termite occurrences, due to synanthropic associations with man, may be more directly related to the incidence of urban sprawl and development of these areas.

Of the 438 characters used for the phylogenetic analysis, a total of 71 characters (16.2%) were parsimony informative. The maximum parsimony analysis resulted in a tree of a length of 271 and a confidence index value of 0.531. The maximum likelihood analysis resulted in a single tree with a $-\ln L$ value of 1492.37750. As expected, the MP, ML, and Bayesian phylogenetic analysis revealed that *R. tibialis* formed a sister clade with *R. hesperus*, which was a sister group to another western *Reticulitermes* termite, '*R. okanaganensis*' (Szalanski et al. 2006) (Fig. 3).

Among western populations, Castle (1930) applied morphological comparisons of several samples of *R. tibialis* to distinguish it from other congeners such as *R. hesperus*. Furthermore, Castle (1930) attempted to distinguish *R. tibialis* from *R. humilis* (not to be confused with *R. humilis* var. *hoferi* which is now known to be *Heterotermes aureus*) and *R. tumiceps*, both of which have been synonymized with *R. tibialis* by Snyder (1954). Recent genetic investigations confirm the presence of an undescribed nearctic *Reticulitermes* that occupies a range consistent with *R. tibialis* occurring on the eastern range of the Sierra Nevada and Rocky mountains (Szalanski et al. 2006) but is not as broadly distributed. This is not surprising given that other cryptic *Reticulitermes* species are being identified (Austin et al. 2007). Castle (1930) evaluated *R. tibialis* workers and

Table 1. *Reticulitermes tibialis* collection locations, haplotypes (frequency), and number of samples per U.S. state (n).

State (n)	County/Parish, haplotype(s) (n)	Lat/Long	
AZ (9)	Cochise T19	315555N	1094432W
	Coconino T11	360500N	1120803W
	Mohave T10, T12, T24(2)	360500N	1134603W
	Santa Cruz T19(2)	313500N	1103502W
	Yavapai T10	343030N	1122233W
CA (6)	Los Angeles T9	340143N	1174837W
	Riverside T9, T10	335503N	1164714W
	San Bernadino T3(2), T10	342535N	1171803W
CO (6)	Larimer T27	403507N	1050504W
	Mesa T17, T18(3), T26	390350N	1083302W
ID (2)	Ada T30, T41	434144N	1162114W
IN (2)	Porter T33	413947N	0870213W
	Tippicanoe T2	402500N	0865231W
KS (5)	Butler T7	374700N	0965001W
	Harvey T33(2)	380005N	0973031W
	Reno T33	380356N	0975452W
	Russell T32	385317N	0985114W
LA (1)	St. Mary T8	295003N	0913448W
MO (4)*	Eureka	383009N	0903740W
	Green City	401607N	0925712W
	Springfield	371255N	0931754W
	St. Louis	383741N	0901131W
MT (1)	Yellowstone T37	454700N	1083002W
ND** (1)	Golden Valley	471727N	1020353W
NE (1)	Cheyenne T2	410804N	1025807W
NM (7)	Bernalillo T11	350913N	1064042W
	Dona Ana T3, T13	321844N	1064642W
	San Juan T12, T14	364341N	1081307W
	Sandoval T12, T15	351419N	1064002W
NV (1)	Clark T31	360223N	1145855W
OK (7)	Delaware T7	363537N	0944609W
	Greer T2	345219N	0993015W
	Payne T5	360656N	0970330W
	Texas T8	363543N	1013812W
	Tulsa T8(2)	361021N	0955627W
SD (1)	Lyman T27	435435N	1000331W
TX (40)	Aransas T2(5)	280246N	0970215W
	Bee T1(2), T4	282403N	0974454W
	Brewster T42	302131N	1033940W
	Carson T36	352044N	1012250W
	Collin T1	331200N	0963401W
	Comal T4	294211N	0980728W
	Culberson T14	310223N	1044951W
	Dallas T6	323523N	0965125W
	Denton T1	330052N	0970549W
	El Paso T3, T9(2), T34	314531N	1062913W
	Goliad T8, T35, T39	284006N	0972318W

Table 1. Continued.

State (n)	County/Parish, haplotype(s) (n)	Lat/Long	
	Henderson T7(2)	321218N	0955120W
	Hill T35	320101N	0970801W
	Hudspeth T6	315607N	1051201W
	Kinney T2	291838N	1002504W
	Leon T6	312750N	0960329W
	Llano T5, T35, T38	305301N	0982821W
	Midland T2	315950N	1020440W
	Palo Pinto T2	324502N	0981801W
	Swisher T5	344437N	1015117W
	Tarrant T1	324331N	0971915W
	Travis T2	302944N	0975524W
	Ward T2	313100N	1030402W
	Wilson T2, T40(2)	290801N	0980922W
UT (16)	Duchesns T10	401758N	1095920W
	Grand T2, T3, T10, T12, T16	383424N	1093259W
	Salt Lake T11, T12(2), T19, T20, T21(2), T22, T23	404539N	1115328W
	Washington T25	371502N	1131505W
WY (1)	Park T10	443135N	1090324W

*Pinzon-Florian (2007).

**Sample morphologically identified but not sequenced.

paratype samples (alates and soldiers) provided by T. E. Snyder (USNM collection) and from other collected samples from California, Oregon, Washington, Arizona, and Mexico. Although Castle (1930) suggests there was little variation in morphological measurements, we find significant genetic variation across its range. Given the breadth of the collection at his disposal, it is unclear why Castle (1930) evaluated *R. tibialis* specimens from Tracy, CA instead of type material from Beeville, TX. One of the key elements described in Castle's thesis suggests gular measurements were significantly different, and might be supported by more recent morphological studies of the same type (Brown et al. 2005). However, these results can vary considerably as has been viewed with type locality material of *R. tibialis* recently evaluated (Heintschel et al. 2006), and depending on what characters are applied.

Recent investigations into the dispersal capabilities of Rhinotermitids such as *Coptotermes formosanus* (Messenger et al. 2005) imply that many studies underestimate just how far termites can migrate through natural dispersal events. Tethered experiments with *R. flavipes* suggest a flight distance of almost 500 m is possible (Shelton et al. 2006), and earlier observations of *R. virginicus* have demonstrated that alates can be collected at significant altitudes ranging from 6 to 1000 m (Glick 1939). Wind speed at or below 1.0 m/s has been shown to be one of the most important microenvironmental factors involved in dispersal flight activity (Leong et al. 1983). Species such as *R. virginicus* have been observed making dispersal flights from the tops of trees as opposed to *R. flavipes* near the ground (DeHeer & Vargo 2006). This type of ecological separation and establishment may dramatically influence dispersal capabilities of *R. tibialis*.

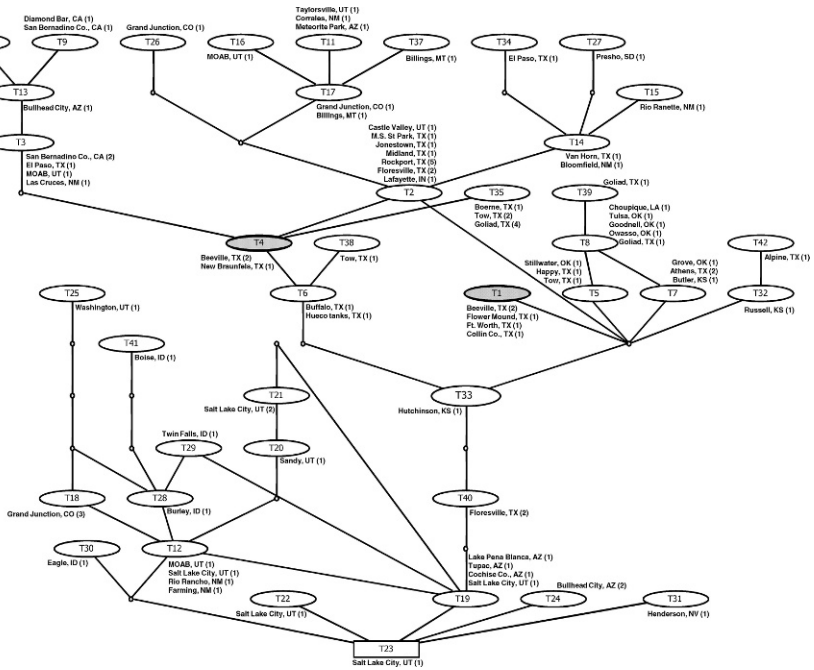


Fig. 2. Distribution of *Reticulitermes tibialis* haplotypes from the United States. Size of shaded circles is proportional to sample size, white circles are populations which were not used for genetic analysis.

Furthermore, the intense intraspecific competition of newly founded reproductives in close proximity to mature nests is also a consideration (Pickens 1934, Kitade et al. 2004, Green et al. 2006) which likely influences successful range expansion for this species, particularly since they are well established in more northerly locations, such as western Montana (Castle 1944), Idaho, Colorado, North Dakota (Austin et al. 2006), and likely extend well into Canada.

This is the first comprehensive genetic analysis of *R. tibialis* from the United States as a whole, and the phylogenetic relationships of this species provide potentially important information on the history of evolutionary change and range expansion of the species.

Because mitochondrial DNA is a single circular molecule which is apparently inherited only through females and does not undergo recombination, lacks complicating features such as introns and transposable elements, and evolves as much as ten to twenty times faster than most nuclear genes (Brown et al. 1979, Futuyma 1998), it is a highly efficient means to understanding the rate of mutation and provides valuable insight into historical radiation of *R. tibialis* in the Nearctic. Furthermore, the substitution rate commonly estimated for insects mtDNA, 2.3% genetic divergence / Myr (Brower 1994), was considered. Applying a molecular clock to all western congeners, we find 1.1% intraspecific genetic variation within *R. tibialis* with an estimated time of divergence approximating 478 kya. The interspecific genetic variation and estimated divergence of *R.*

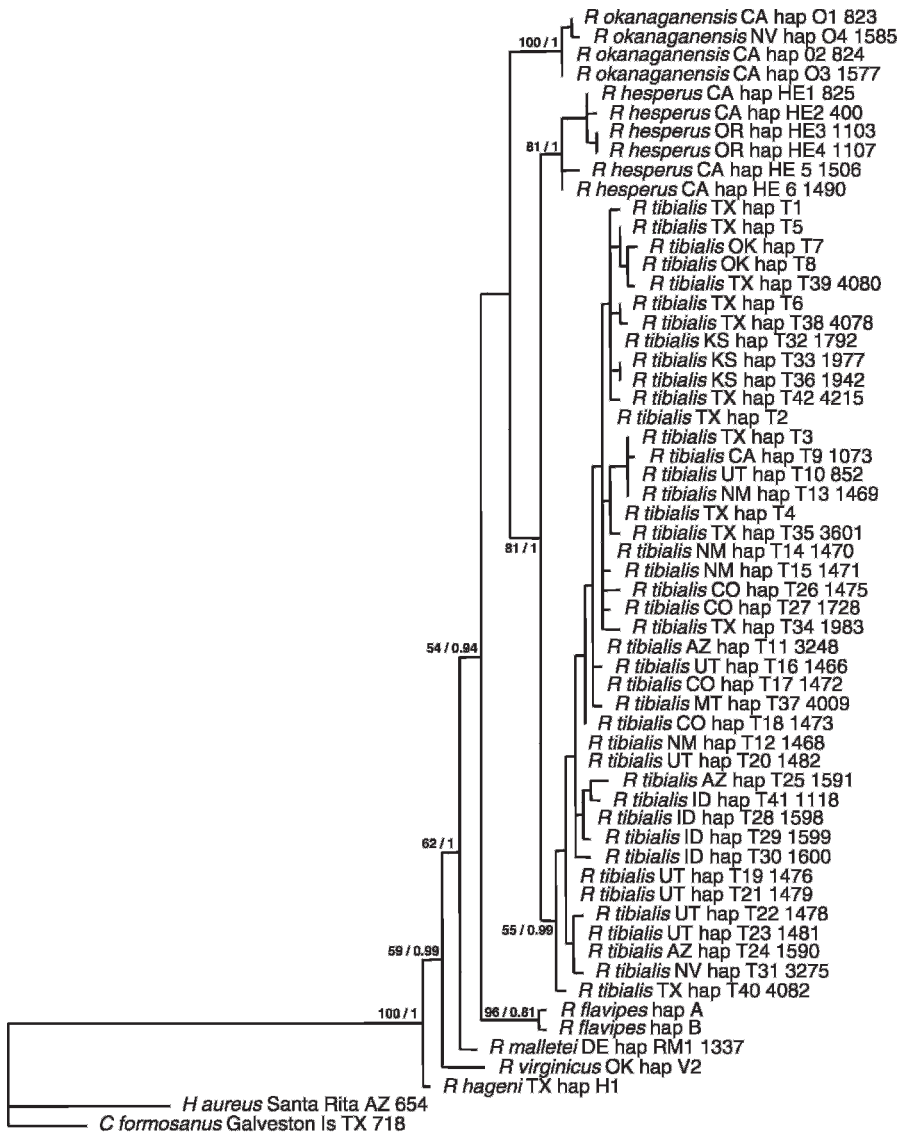


Fig. 3. Maximum likelihood phylogenetic tree of *Reticulitermes tibialis* haplotypes relative to other Rhinotermitidae termites. Maximum parsimony bootstrap values are provided and Bayesian posterior bootstrap values are listed nearest their respective branches right to left, respectively. Haplotypes 43–46 from Missouri, Courtesy of O. Pinzon-Florian are not presented (unpublished and unavailable on GenBank).

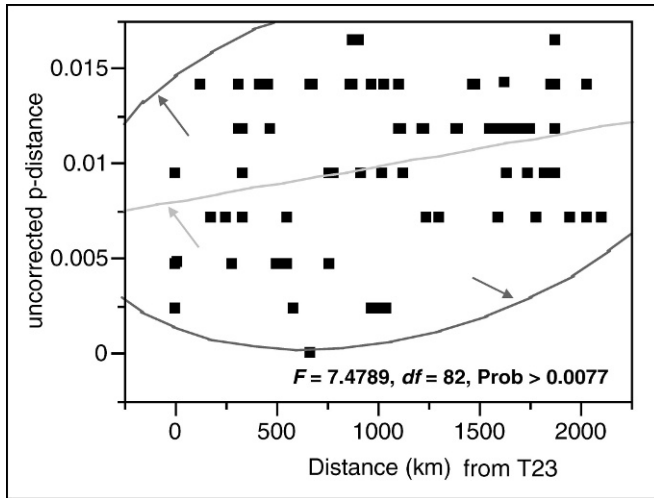


Fig. 4. Spatial relationships among *R. tibialis* in the United States applying a linear regression. Geographic distances from the most basal 16S rRNA haplotypes originating from Salt Lake City, Utah (T23) are on the X-axis; Uncorrected P-distances for all 46 *R. tibialis* haplotypes are on the y-axis. The Bivariate correlation and associated 2-tailed probability are listed just above the x-axis, with the light grey arrow pointing to the linear fitted line and red ellipse (demarcated by dark grey arrows) representing the 0.95 probability.

tibialis from other congeners was 2.1% (913 kya) for *R. hesperus*, and 2.8% (1.217 mya) for *R. okanaganensis*, respectively. Spatially and temporally distinct zones where ice sheets have receded and subsequently encroached over the last ice age likely play significant roles (Comes & Kardereit 1998) in the fragmentation, isolation and genetic divergence of this genus in the Nearctic, as have been proposed for populations of *R. hesperus* in British Columbia (Spencer 1937, 1945), the isolation of *R. okanaganensis* from western populations of *R. hesperus* (McKern et al. 2007, Szalanski et al. 2006), and the likely eastern radiation of *R. tibialis* across the American Great Plains following glacial events (Austin et al. 2006).

Genetic analysis revealed a large amount of genetic variation within *R. tibialis*, second only to *R. flavipes* (Austin et al. 2006). Variation in genes, as well as in environmental conditions, is translated by processes of development into variation in phenotypic characters, such as morphological features (Futuyma 1998), which provides some ideas for the morphological variation described in literature for this species.

While one goal of applying molecular markers such as 16S is to identify morphologically ambiguous samples (Szalanski et al. 2003), another might be to identify the source of introductions to non-native areas (Szalanski et al. 2004). By comprehensive sampling across broad geographic zones, as presented in this study, it affords others the opportunity to develop molecular diagnostics for future invasive termite species to the United States. This study is just a small step in a long journey towards this goal, but an important one as it represents the most current view of

this species known to date. This study also demonstrates that *R. tibialis* has a large amount of genetic diversity in western United States; a fact not surprising given the climatic and geographic conditions where this species exists.

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