GENETIC EVIDENCE FOR TWO INTRODUCTIONS OF THE FORMOSAN SUBTERRANEAN TERMITE, *COPTOTERMES FORMOSANUS* (ISOPTERA: RHINOTERMITIDAE), TO THE UNITED STATES

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ABSTRACT

Exotic introductions of Formosan Subterranean Termite (FST) to the United States from Asia have had significant economic consequences. Multiple introductions through marine transport have been proposed, but identification of these routes has yet to reveal more than one lineage in the continental U.S. DNA sequencing of a 640-bp cytochrome oxidase II (COII) mitochondrial DNA (mtDNA) marker to 60 disjunct populations, revealed two independent lineages spanning the continental U.S., Hawaii, Japan, and China. Limited genetic variation was observed with this marker. Group I constitutes a largely Asian clade, while Group II is comprised of both Asian and southern U.S. populations. This is the first study which has documented 2 distinct lineages to continental United States and Hawaii.

Key Words: invasive species**,** DNA sequence, genetic variation, molecular diagnostics, termite

RESUMEN

Las introducciones exóticas de la termita subterránea de Formosa (TSF) de Asia a los Estados Unidos han tenido consecuencias económicas significativas. Introducciones multiples por medio del transporte marino han sido propuestas, pero la identificación de estas rutas todavia no ha revelada mas que un linaje en los Estados Unidos continental. La secuenciación de un marcador de 640-bp del citocromo-c-oxidasa II de ADN mitochondrial (mtADN) a 60 poblaciones separadas, revelo dos linajes independientes atravesando los Estados Unidos continental, Hawaii, Japan y China. El marcador mostró una variación genética limitada. El grupo I constituye un clado principalmente asiático, mientras el grupo II consiste de poblaciones asiáticas y del sur de los Estados Unidos. Este es el primer estudio que documenta los dos linajes distintas en los Estados Unidos y Hawaii.

Formosan subterranean termite (FST) *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), has long been suspected to have originated from Formosa (the Island of Taiwan), but endemic to mainland China due to the identification of a termitophile from there (Kistner 1985). FST has been reported from 14 southern provinces in China with a northern limit of 33°28' N and a western limit of 104°35'E (Gao et al. 1982; He & Chen 1981; Lin 1986) (Fig. 1). Introductions of this exotic pest have been documented around the world following closely with trade routes extending to the United States and beyond (Chhotani 1985). Historical shipping trade between the east and west over the past 450 years (Welsh 1996; Lim 1997), and the likely introduction(s) of FST to the continental U.S. after World War II (La Fage 1987), have made tracking introduction points difficult. Trading centers in Guangdong Province (e.g., Macau, Guangzhou, Shenzhen, and Hong Kong), Fujian Province (e.g., Puyuan) and Shanghai Province, China, and Taiwan have provided likely ports of origin for FST (See Province Map, Fig 1). Gay (1967) suggests that introductions of FST into Guam, Midway Island, the Marshall Islands, and the Hawaiian islands are most likely due to shipping trade.

FST is believed to have been introduced to Japan almost 300 years ago (Mori 1987; Su & Tamashiro 1987; Wang & Grace 1999; Vargo et al. 2003), and has been hypothesized to have been introduced to Hawaii almost 100 years ago (Su & Tamishiro 1987). The history of FST introductions to the continental United States is more am-

Fig. 1. Provincial Map of China based on Wang et al. (2002). Shaded provinces reflect areas with known *Coptotermes formosanus* infestations.

biguous because of likely misidentifications. For example, early samples of *Coptotermes* in Houston, Texas, during the 1950s were identified as *C. crassus* Snyder, but were later positively identified as *C. formosanus*.

Presently, FST is distributed across the southeast United States (Spink 1967; Howell et al. 1987; La Fage 1987; Su & Tamashiro 1987; Appel & Sponsler 1989; Chambers et al. 1998; Su & Scheffrahn 1998a; Cabrera et al. 2000; Hawthorne et al. 2000; Howell et al. 2000; Su & Scheffrahn 2000; Hu et al. 2001; Scheffrahn et al. 2001; Jenkins et al. 2002), and disjunct populations in southern California (Atkinson et al. 1993; Haagsma et al. 1995) are thought to have originated from Hawaii. Without doubt, their continued presence and growing distribution(s) have been exacerbated by commerce and trade practices within the United States (Cabrera 2000; Jenkins et al. 2002; Glenn et al. 2003), and by the general lack of education and research funding directed towards this problem until recently (Operation Full Stop, a FST interdiction research unit located in New Orleans, Louisiana was initiated by the United States Department of Agriculture, Agricultural Research Service in 1998).

Several studies applying genetic or biochemical interpretations of FST populations have attempted to identify introduction routes of FST. However, while multiple entry points appear likely, the lack of genetic variation in this invasive species has made identification of these routes difficult to achieve. Studies applying cuticular hydrocarbons (Haverty et al. 1990), allozymes (Korman & Pashley 1991; Strong & Grace 1993; Broughton & Grace 1994; Wang & Grace 2000), mitochondrial DNA (mtDNA) (Jenkins et al. 2002), and microsatellite DNA (Vargo & Henderson 2000; Husseneder & Grace 2000; 2001a, b; Husseneder et al. 2002) have been reported, but current literature has not conclusively established the origins of alternative routes to the United States. These studies have implicated that more than one introduction route existed, but they have not corroborated their suppositions with the inclusion of additional FST populations which might elucidate this observation.

Presumably, this could be attributed to the overall lack of genetic diversity of FST globally. In introduced populations, the lack of clear colony boundaries and the potential for considerable mixing of individuals among colonies may lead to the formation of colonies which could extend over large areas making colonial identity difficult, an observation observed in unicolonial ant species (Argentine ant *Linepithema humile*) (Tsutsui et al. 2000, 2001). Alternatively, it may be that the natural dispersal of FST alates is more significant than previous recorded distances (Messenger & Mullins 2005), an explanation proposed for the low mitochondrial DNA (mtDNA) divergence among sites spanning across states such as Georgia (Jenkins et al. 2002). However, human-aided dispersal of FST would be equally plausible as a contribution to low mtDNA divergence. Some argue that the lack of genetic diversity in FST could be due to genetic bottlenecks (Strong & Grace 1993; Broughton & Grace 1994) with limited founder effect. Others suggest the possibility of significant inbreeding due to neotenic involvement (Wang & Grace 1995). For this to be acceptable, one must assume that there would be some inbreeding depression or fixation.

Herein, we report that while multiple introductions of FST (to the United States) are presumed, limited genetic variation in this species restricts the clarification of exactly where these exotic introductions originated from when using some molecular markers. We provide evidence of 2 distinct lineages, occurring in the continental United States and in the Hawaiian Islands, with identical lineages from China.

MATERIALS AND METHODS

Coptotermes formosanus were collected from all known continental United States where FST has been reported, the Hawaiian Islands, Japan, Hong Kong, and China (Table 1). Morphological identification of specimens used in this study were performed by applying the keys of Scheffrahn et al. (1994), and verified with a FST molecular diagnostic method (Szalanski et al. 2004). Voucher specimens, preserved in 100% ethanol, are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR, the University of Florida-Ft. Lauderdale Research and Education Center, Ft. Lauderdale, FL, and the Center for Urban and Structural Entomology, Department of Entomology, Texas A&M University, College Station, TX.

Alcohol preserved specimens were allowed to dry on filter paper, and DNA was extracted from individual worker, or soldier heads by using the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 µL of Tris:EDTA and stored at -20°C. Polymerase chain reaction (PCR) was conducted with the primers TL2-J-3037 (5-ATGGCAGATTAGTGCAATGG-3) designed by Liu and Beckenbach (1992) and described by Simon et al. (1994) and Miura et al. (1998), and primer TK-N-3785 (5-GTTTAAGAGACCAGTACTTG-3) from Simon et al. (1994). These primers amplify a 3' portion of the mtDNA COI gene, tRNA-Leu, and a 5' section of the COII gene. PCR reactions were conducted with 1 µL 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 by using 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). To facilitate genetic comparison with existing GenBank DNA sequences, 113 bp from the 5' end of the sequence was removed, and the remaining 667 bp was used. GenBank accession numbers for the FST haplotypes found in this study are AY453588 and DQ386170. DNA sequences were aligned with BioEdit version 5.09 (Hall 1999) and Clustal W (Thompson et al. 1994). The distance matrix option of PAUP* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura 1980) of sequence evolution.

RESULTS AND DISCUSSION

Introduction of exotic termites to the United States is an ongoing problem that is invariably sustained by modern trade and limited or non-existent quarantine regulations.

Native populations (in China) of FST should possess greater genetic diversity. For this reason, focusing on the nature of genetic variation in populations from China and neighboring Asian countries (Vargo et al. 2003) is a logical starting point when evaluating the nature of introduced populations to the United States (Husseneder et al. 2002) and its territories. In the present study we evaluated native populations of FST from Guangdong, Shanghai, and Fujian provinces (Hong Kong, Puyuan, Guangzhou, and Xhinhui) in China. However, only two distinct COII haplotypes were observed.

Applying *C. acinaciformis* (Froggatt), *C. lacteus* (Froggatt), and *Heterotermes cardini* (Snyder) as outgroups, Haplotype group I contains locations from Hong Kong, Japan AB109529, Hsin-Hui (presently known as Xhinhui), China (from Jenkins et al. 2002), Puyuan and Guangzhou, China, Oahu, HI, Nagasaki, Japan, and Ft. Worth, TX [presumably this sample was collected from

TABLE 1. *COPTOTERMES FORMOSANUS* COLLECTION DATA.

Location	Country	N	Hap	Source
Hong Kong	China	3	1	This study
	Asia	1	1	AB109529
Ft. Worth, TX	USA	1	E(1)	Jenkins et al. 2002
Oahu, Hawaii	USA	1	1	This study
Hong Kong	China	2	2	This study
HI	USA	1	2	AY536406
Maui, HI	USA	2	2	This study
GA	USA	2	2	AY536405, AY027489
Cairo, GA	USA	2	2	AY683220
Lawrenceville, GA	USA	1	2	AY683213
Tucker, GA	USA	1	2	AY683214
Dallas, GA	USA	1	2	AY683214-15
Savannah, GA	USA	2	2	AY683217-219
GA	USA	1	2	This study
Spindale, NC	USA	1	2	This study
Forest City, NC	USA	1	2	This study
Rutherfordton, NC	USA	1	2	This study
Marco Island, FL	USA	1	2	This study
Trinity, FL	USA	1	2	This study
Niceville, FL	USA	1	2	This study
Florida City, FL	USA	1	2	This study
Temple Terrace, FL	USA	1	2	This study
Palm Beach, FL	USA	1	2	This study
Pompano Beach, FL	USA	1	2	This study
Galveston, TX	USA	2	2	This study
San Antonio, TX	USA	1	2	This study
Garland, TX	USA	1	2	This study
Rockwall, TX	USA	1	2	This study
Stennis Sp Ctr, MS	USA	4	2	This study
New Orleans, LA	USA	2	2	AY536407,AY683217
Lake Charles, LA	USA	3	2	This study
New Orleans, LA	USA	3	2	This study
St. Rose, LA	USA	1	2	This study
New Orleans, LA	USA	1	B(2)	Jenkins et al. 2002
SC	USA	1	C(2)	Jenkins et al. 2002
Nagasaki	Japan	2	2	This study
Puyuan	China	1	3	AY536403
Guangzhou	China	1	4	AY536404
Mobile, AL	USA	1	D	Jenkins et al. 2002
GA	USA	1	A	Jenkins et al. 2002
Hsin-hui (Xhinhui)	China	1	G	Jenkins et al. 2002
Hsin-hui (Xhinhui)	China	1	H	Jenkins et al. 2002
Oahu, HI	USA	1	F	Jenkins et al. 2002

Grapevine, TX, because the only known occurrences of FST in Tarrant County, TX, occur in the Northeast portion of this county (pers. Comm. Mike Merchant)]. Group II contains several FST populations from disjunct locations: Hong Kong, North Carolina, South Carolina (Jenkins et al. 2002), Georgia, Florida, Alabama (Jenkins et al. 2002), Mississippi, Louisiana, Texas, Oahu and Maui, HI (Figs. 2 and 3). Representative taxa from group I were slightly more divergent based on Maximum likelihood analysis (Fig. 3). Inclusion of FST sequence data from Jenkins et al. (2002), designated by their respective haplotype descriptions (A through H), also fall within the two groups presented herein (Table 2, Figs. 2 and 4).

Fei and Henderson (2003) noted that incipient colony establishment was somewhat more restrictive for outbred primary reproductives, owing discrepancies to environmental adaptive resource differences from two disjunct populations from Louisiana. Furthermore, Coaton & Sheasby (1976), and Lenz & Barrett (1982) suggest that dominant use of neotenics for colony growth in *C. formosanus* may be a successful strategy to in-

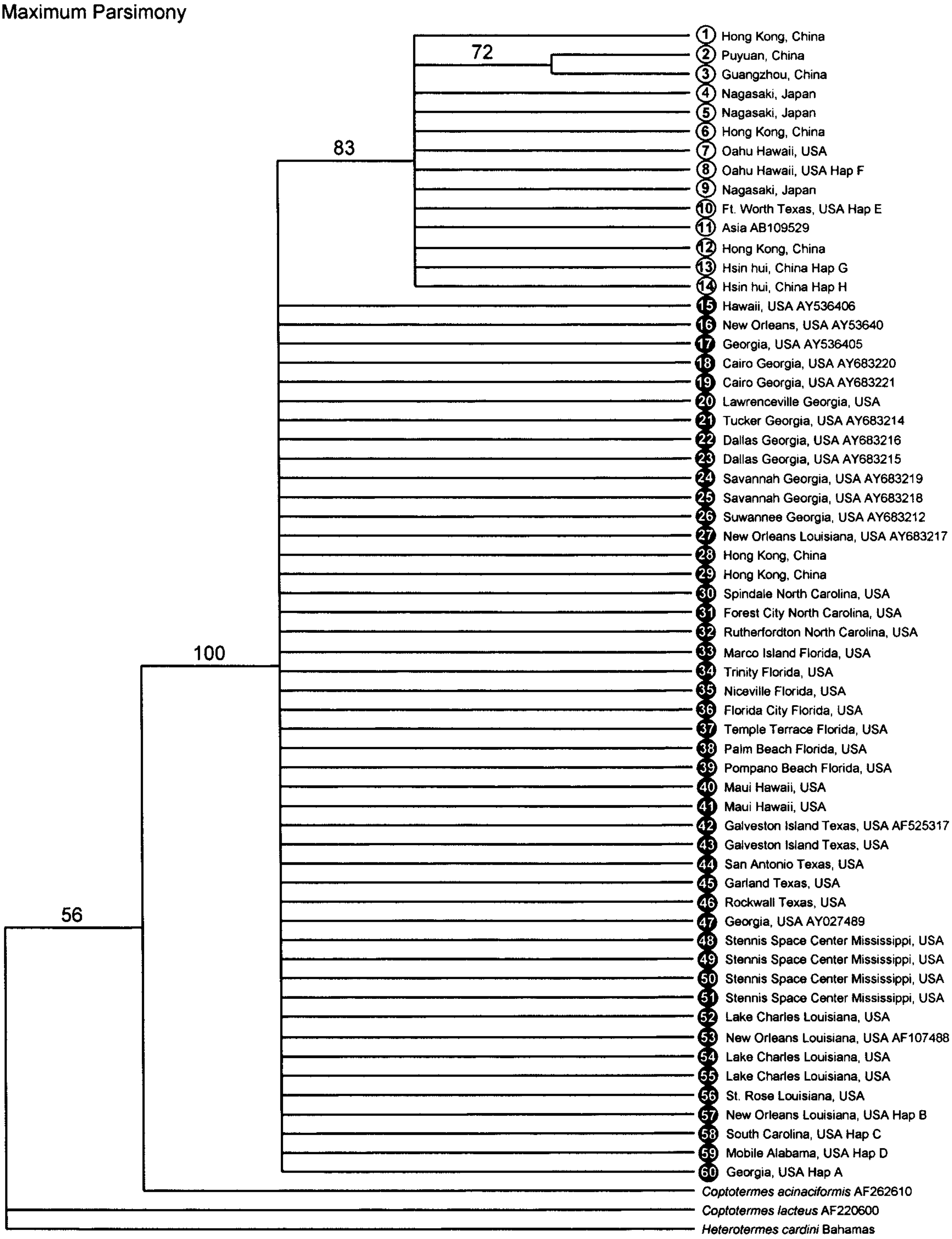

Fig. 2. Maximum Parsimony Analysis of *Coptotermes formosanus* lineages in North America. For consistency, open and closed circles reflect the different mtDNA COII lineages of *C. formosanus*, while the numbers are used for comparison and clarification of geographic location in Figures 3 and 4.

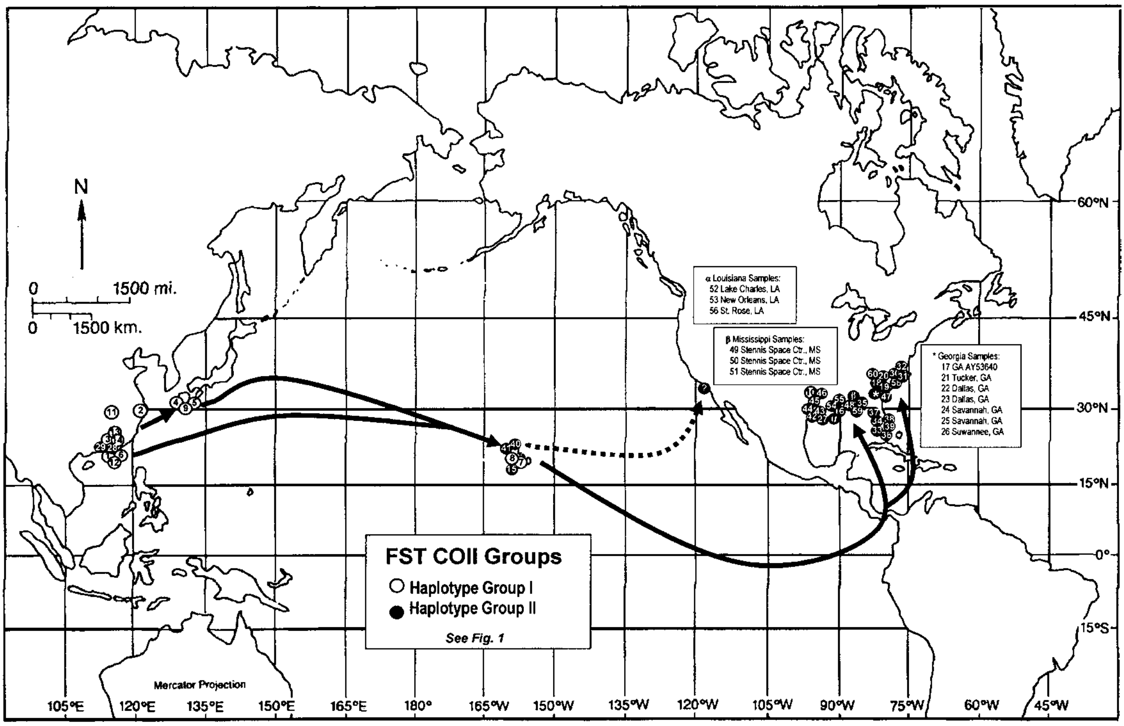

Fig. 3. Introduction routes of *Coptotermes formosanus* from Asia to North America. Dashed arrow pointing towards Southern California suggests the introduction from Hawaii based on anecdotal information that has not been corroborated in genetic studies to date.

vade new environments. If this adaptive strategy is true for *C. formosanus*, reduced genetic variation may be the result and would account for some of the limited population viscosity observed to date. Habitat fragmentation and anthropogenic disturbances significantly reduce population viscosity. More comprehensive studies of FST may not reveal significant genetic diversity. For FST, reduced genetic variation does not necessarily mean reduced fitness or vigor, but may simply imply that there is greater reproductive plasticity. For example, Hyashi et al. (2004) demonstrated that *Reticulitermes speratus* (in Japan) can utilize facultative parthenogenic reproduction. This would be a significant establishment capability for termites like FST when introduced to non-endemic locations such as the United States.

There have been numerous emigrations of people to Hong Kong throughout history. Major migrations of Chinese settlers from mainland China to Hong Kong have been recorded as early as the Song Dynasty (960-1279) (Welsh 1996). After the end of World War II and the communist takeover of mainland China in 1949, hundreds of thousands of people emigrated from China to Hong Kong (Welsh 1996). In fact, locations such as Xhinhui, a treaty port in 1904, was an important outlet for Chinese emigrants to the United States (Anonymous 2004). The introduction of FST to the U.S. likely occurred several times, perhaps more than ten different occasions (RHS, personal communication). Given this fact, it is remarkable that the established link between the U.S. and China has never been substantiated for more than one FST lineage.

Populations of FST from Japan appear only in one of the presented clades (Group I, Fig. 2), and further sampling from more locations (in Japan) may provide additional information on whether Japan could have contributed more significantly to FST introductions to Hawaii or the continental United States. Group I (Fig. 2) is largely comprised of samples from Asian/Pacific locations but has one sample (Ft. Worth, TX) that was collected in the continental U.S. (Fig. 3). This is significant because it implicates a second introduction route to the continental U.S. that has never been identified in previous studies. Group II, is comprised of FST samples from nearly all known southeastern states (Alabama, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina), Texas, Hawaii, and several FST from China. Both clades are well-supported by strong bootstrap support (>80%) by both parsimony and Liklihood analyses (Figs. 1 and 3).

Although FST distributions have been more recently updated (Wang et al. 2002), the lack of a

Table 2. Haplotype variation at 15 nucleotide sites for nine *Coptotermes formosanus* haplotypes.

Hap	8	11	19	32	33	46	176	211	222	297	333	427	643[a]	653[a]	662[a]
1	C	G	A	A	T	A	A	A	A	T	A	G	A	T	A
2	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
4	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.
A[a]	.	.	G	.	.	.	.	.	G	A	.	.	.	.	C
D[a]	.	.	.	.	.	.	.	.	G	A	C	.	.	.	.
F[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
G[a]	G	T	.	.	.	.	T	.	.	.	.	.	.	A	.
H[a]	.	.	.	T	A	T	T	T	.	.	.	.	.	.	.

[a] Jenkins et al. (2002).

geographic explanation for a second lineage introduced to the United States remains unclear (Wang & Grace 2000). Sequence data obtained from GenBank, from Jenkins et al. (2002), provides a second haplotype match in the continental United States (haplotype E from Ft. Worth, TX) that represents the first documented case corroborating multiple lineages from presumably multiple introductions (at least two in the present study). These two distinct haplotypes share one commonality—both groups have representatives with identical haplotypes (lineages) from Hong Kong, Japan, Hawaii, and the continental United States (Fig 3).

There were numerous FST samples where repeated attempts to amplify sufficient DNA for sequencing of the mtDNA COII gene were not successful (e.g., FST from San Diego, California and Tai Chuong, Taiwan). These results were not surprising, as we have routinely observed ~60% efficiency when using the COII marker with FST. However, amplification of the 16S rRNA for these samples was successful. We routinely observe >90% efficiency for this marker with FST. While the utility of the 16S marker is excellent for phylogenetic studies of the genus *Coptotermes* (JWA, unpublished), for molecular diagnostic methods (Szalanski et al. 2004), or other rhinotermitids (Szalanski et al. 2004; Austin 2004a; 2004b), it does not provide the degree of genetic variation suitable to discern the two distinct FST haplotypes observed in this study. The slightly larger COII amplicon (640 bp versus 428 bp of 16S rRNA) provides only a small increase in resolution between FST populations, even though it works well for other Rhinotermitidae (Austin et al. 2002, 2004c). Our laboratory experience with FST suggests that in general, it is more difficult to extract high quality DNA from Coptotermes for genetic studies when compared to other rhinotermitids, a problem that may be more common than reported. Additional problems may include the presence of unknown inhibitors, method of sample preservation (some preservation methods are known to provide poorer quality DNA for genetic studies (Post et al. 1993; Reiss et al. 1995; Dillon et al. 1996) or the age of samples provided.

While the idea that multiple introductions to the United States have been proposed, alternate introduction routes have never been substantiated in literature. This study provides a glimpse of some of the difficulties encountered working with FST. Most notably, it would appear that the low genetic variation detected with our COII marker in this species does not equate to reduced fitness or establishment capability.

Populations of nearly all species, social or otherwise, exhibit at least some degree of genetic differentiation among geographic locales (Ehrlich & Raven 1969). Herein, we present two distinct COII haplotypes of FST in the continental United States (one based on our own samples evaluated, and a second from Jenkins et al. (2002)). However, our results appear to contradict the degree of variation described by Jenkins et al. (2002). They describe 8 different COII haplotypes (maternal lineages) from 14 geographic locations across the southeast United States, Hawaii, and China. Applying the COII marker to 60 geographic locations (Table 1) we only identified 2 haplotypes—one in Japan, two in Hawaii, the continental United States, and China, respectively. Noting that many of the variable sites in Jenkins et al. (2002) occur at positions 651 through 685 of their slightly larger COII amplicon (total size of the amplicon was 685), it is unclear where the discrepancies occurred. One possibility may be due to sequence error that could only be detected by comparison with greater taxon sampling. Other possibilities may be due from improper sequence alignment or mispriming of template DNA during PCR. We elected to include all taxa from Jenkins et al. (2002) into our sequence dataset (COII lineages A through H), which may have provided an advantage due to our larger number of locations sampled. As with animal populations, additional genetic structure normally is to be expected over increasing spatial scales, where populations can show additional differenti-

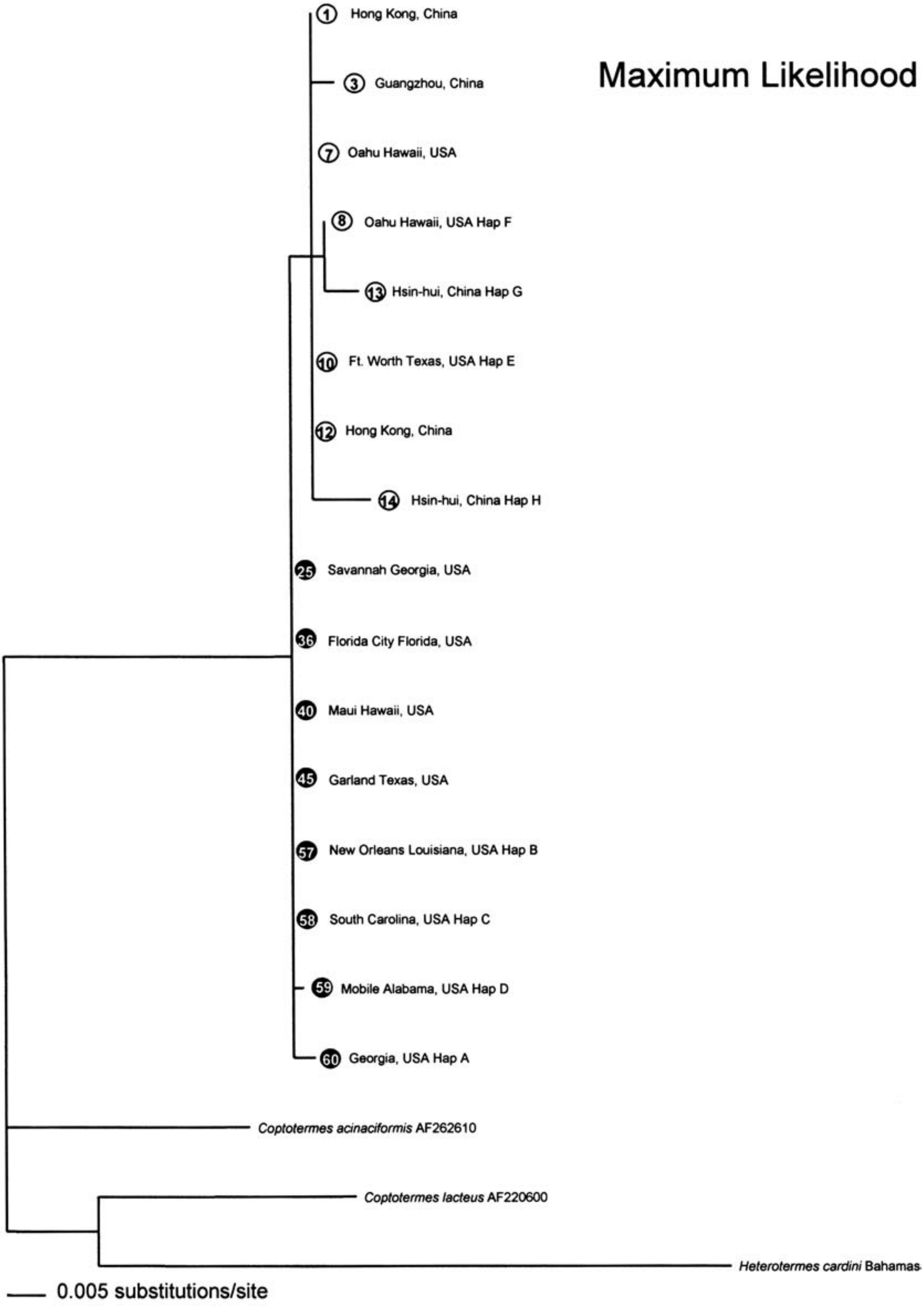

Fig. 4. Maximum Likelihood analysis *Coptotermes formosanus* lineages in North America.

ation due to spatial habitat structure and isolation by distance (Avise 2004). However, our results seem to refute this generalization for FST, a fact probably attributed to its establishment ability in fragmented urban ecosystems and their indirect interactions with humans.

The preponderance of FST research appears to support our findings. Haverty et al. (1990) found no differences in qualitative cuticular hydrocarbon profiles among four FST populations in the U.S. Korman & Pashley (1991) concluded that populations from Florida and New Orleans are in the same group and are very closely related to each other, a finding also corroborated within the present study (Fig. 3). Strong & Grace (1993) concluded that low genetic and phenotypic variability in introduced FST populations to Hawaii could have been from a single event. Broughton & Grace (1994) observed that only 9 of 16 different restriction enzymes cut mtDNA zero or once. Vargo et al. (2003) was unable to detect significant isolation by distance among colonies at the spatial scale studied (0.7-70 km) from 2 disjunct populations of FST in Japan, nor from populations in New Orleans, LA and Oahu, HI. This suggests a general lack of strong population viscosity in introduced populations of FST. The finding also seems to be contrary to Jenkins et al. (2002), whose FST samples ranged in distance from 6-37 km in Atlanta, GA. Wang & Grace (2000), applying enzymatic polymorphisms, concluded that at least two introductions to the United States have occurred, but the second clade in their study lacked sufficient samples from China to determine the origin of a second route.

More recently, the utility of mtDNA markers for identifying where exotically introduced *Heterotermes* (Szalanski et al. 2004), *Nasutitermes* (Scheffrahn et al. 2004) and *Cryptotermes*/*Procryptotermes* (RHS, unpublished) to the United States is being investigated. The principal caveat with studies of this nature is that significant representation of taxa is essential, particularly when dealing with species of limited genetic variation like FST. A secondary caveat is that tremendous skill in identifying termites morphologically is essential to ensure the validity of a genetic study based on known, identified samples. Because FST was likely misidentified when it was first observed in the continental United States, little attention was given, and subsequent populations have developed over the years. This was one of the reasons behind developing molecular diagnostics for this species (Szalanski et al. 2004), and a need to genetically review some species to corroborate their original identifications (Scheffrahn et al. 2004). As population-level studies for FST from various locations across the world continue to accumulate (see Vargo et al. 2003), perhaps a better understanding of local factors which contribute to the low genetic diversity observed in FST will become more apparent. Given the 300 years of known occurrence in Japan (Vargo 2003) and the lack of genetic variation in China, it is unlikely we will observe significant variation in this species within the U.S. Random genetic drift is unlikely to occur at a rate that we will detect anytime soon. Perhaps more intuitively, we should not assert our scientific prejudices about the nature of reduced genetic variation in FST (causing some reduction in fitness), or Isoptera in general, until we more exhaustively investigate their basic biology and reproductive systems.

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