

Phylogeography illuminates maternal origins of exotic *Coptotermes gestroi* (Isoptera: Rhinotermitidae)

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Abstract

Coptotermes gestroi, the Asian subterranean termite (AST), is an economically important structural and agricultural pest that has become established in many areas of the world. For the first time, phylogeography was used to illuminate the origins of new found *C. gestroi* in the US Commonwealth of Puerto Rico; Ohio, USA; Florida, USA; and Brisbane, Australia. Phylogenetic relationships of *C. gestroi* collected in indigenous locations within Malaysia, Thailand, and Singapore as well as from the four areas of introduction were investigated using three genes (16S rRNA, COII, and ITS) under three optimality criteria encompassing phenetic and cladistic assumptions (maximum parsimony, maximum likelihood, and neighbor-joining). All three genes showed consistent support for a close genetic relationship between *C. gestroi* samples from Singapore and Ohio, whereas termite samples from Australia, Puerto Rico, and Key West, FL were more closely related to those from Malaysia. Shipping records further substantiated that Singapore and Malaysia were the likely origin of the Ohio and Australia *C. gestroi*, respectively. These data provide support for using phylogeography to understand the dispersal history of exotic termites. Serendipitously, we also gained insights into concerted evolution in an ITS cluster from rhinotermitid species in two genera.

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1. Introduction

The genus *Coptotermes* is widespread in the tropical and subtropical regions of the world. All species of *Coptotermes* consume wood, and 28 species are economically important structural pests, the largest number for any termite genus (reviewed by Su and Scheffrahn, 2000). *Coptotermes* species are readily transported by human commerce in part due to

their habit of constructing carton material for nests and for filling aboveground excavations and voids. Carton is comprised of soil and cellulose admixed with fecal and salivary secretions, and it readily retains moisture, likely enhancing the survival of these termites when inadvertently transported in infested materials (Jenkins et al., 2002).

The genus *Coptotermes* also readily produces neotenic reproductives (reviewed by Myles, 1999; Lenz and Barrett, 1982), further enhancing the termites' establishment potential. Neotenic reproductives are capable of replacing or supplementing primary reproductives. Neotenic enable

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the social organization to be maintained following death of primary reproductives—they provide an alternative to dispersal by alate flight; and they regulate population size over time (Lenz et al., 1986; Myles, 1999).

Coptotermes gestroi (Wasmann) (= *Coptotermes havilandi* Holmgren per Kirton and Brown (2000)), the Asian subterranean termite (AST), is the primary pest species of *Coptotermes* originating from the Indo-Malayan Region; its native distribution is from Assam through Burma and Thailand to Malaysia and the Indonesian archipelago (Kirton and Brown, 2000). It is one of the most important and widespread urban pests in Southeast Asia (Tho, 1992; Sor-nnuwat et al., 1996a,b; Lee, 2002; Klangkaew et al., 2002).

Coptotermes gestroi has been introduced into other zoogeographic regions of the world, including the Caribbean (Tucker, 1939; Snyder, 1956; Scheffrahn et al., 1990, 2003); the Americas—Brazil (da Costa Lima, 1936; Araujo, 1958; Goncalves and Silva, 1962; Ferraz and Canello, 2001; Constantino, 1998, 2002; Costa-Leonardo and Barsotti, 1998) and Florida (Su et al., 1997; Cabrera et al., 2005); the Marquesas Islands (French Polynesia) in the Pacific Ocean (Light, 1932); and Mauritius Island (Moutia, 1936), Reunion Island (Paulian, 1957), and Madagascar (Edwards and Mill, 1986) in the southern Indian Ocean.

Coptotermes gestroi is often intercepted outside its native range, typically in cargo onboard ships and in wooden components of sailing vessels (Gay, 1969; Costa-Leonardo et al., 1999; Kirton and Brown, 2000). Populations of *C. gestroi* have been scattered across a wide and ecologically varied geography (Baker, 1986; Shigesada and Kawasaki, 1997, p. 24), and such populations have so successfully established themselves in new habitats that it can be difficult to determine their country of origin (Gay, 1969). Because *C. gestroi* has been found infesting a number of Caribbean-based yachts that docked in southern Florida, Scheffrahn and Su (1999) suggested that land-borne infestations of *C. gestroi* in Miami “likely originated from the West Indies.”

Phylogeography is a method that combines phylogenetic analyses with geographic distributions to map the spatial and temporal history of intraspecific mtDNA lineages (Avice, 2000). The overall purpose of the present study was to use phylogeography to examine the maternal dispersal history of *C. gestroi* that we recently collected in Ohio, USA, Florida, USA, the US Commonwealth of Puerto Rico, and Brisbane, Australia (Table 1). Phylogenetic relationships of *C. gestroi* collected in indigenous locations within Malaysia, Thailand, and Singapore as well as from the four areas of introduction were investigated using three genes: 16S ribosomal RNA (16S rRNA), cytochrome oxidase subunit II (COII), and the internal transcribed spacer array (ITS) that includes both ITS1 and ITS2. Until this study, neither the origin nor the dispersal history of exotic AST infestations has been investigated using molecular markers.

The genes, 16S rRNA, COII, and ITS, are useful for investigating phylogenetic relationships. 16S rRNA and

COII gene trees are expected to be concordant since mitochondrial DNA is a haploid, non-recombining molecule that shares the genetic history of common descent (Wilson et al., 1985), which has been shown to be good at inferring phylogenetic relationships among closely related extant populations (Hillis et al., 1996; Simon et al., 1994). ITS regions have also been shown to be suitable for intraspecific phylogenetic studies in insects (Rokas et al., 2002). The 16S rRNA gene, although more conserved than the COII gene, has been successfully used in arthropod molecular taxonomy and population genetic studies (Whitfield and Cameron, 1998; Simon et al., 1994), including subterranean termites generally (Dopman et al., 2002; Miura et al., 2000; Kambhampati, 1995) and *Coptotermes* specifically (Scheffrahn et al., 2004; Szalanski et al., 2004). The availability of *C. gestroi* sequences in GenBank, including 16S rRNA (Scheffrahn et al., 2004) and COII (Tsai and Chen, 2003) sequences, allowed us to expand our molecular dataset to include AST haplotypes from additional exotic locales.

The current study employed molecular markers to explore the relationships among *C. gestroi* from indigenous and exotic locations. We used a molecular dataset composed of 16S rRNA, COII, and ITS genes to accomplish three objectives: (1) to estimate phylogenetic relationships among AST individuals using multiple algorithms, (2) to apply principles of phylogeography to determine AST dispersal scenarios and geographic origins(s), and (3) to relate phylogeography data to anecdotal introduction histories in order to understand mode and tempo of dispersal patterns of the AST.

2. Materials and methods

2.1. Termites

Samples of *C. gestroi* were collected in Puerto Rico; Ohio, USA; Florida, USA; Brisbane, Australia; Malaysia; Thailand; and Singapore (Table 1). Termites were preserved in 95–100% ethanol, except the Ohio collection consisted solely of desiccated specimens. Termites were initially identified to species using morphometric characters (Tho, 1992) and then verified using molecular techniques and phylogenetic analyses.

2.2. DNA extraction, amplification, and sequencing

The joined head and thorax was excised from worker termites for molecular analyses. DNA was extracted from an individual termite using the E.Z.N.A. Mollusc DNA kit (Omega Bio-Tek, Inc., Doraville, GA). DNA was extracted from three termites per collection locality (Table 1), except four individuals were analyzed from site 2 in Bangkok, Thailand.

Two mitochondrial genes were amplified with polymerase chain reactions (PCRs) and sequenced. The 16S rRNA gene (~428-bp) fragment was amplified and sequenced with primers LR-J-13007 (5'-TTACGCTGTTATCCCT

Table 1
Coptotermes gestroi collection data

Sample code ^a	Collection locality	Date ^b	Source	GenBank No. ^c	
				16SrRNA	COII
CgA2	Hamilton, Queensland, Australia	28-IX-2004 ^d	C. Harvey	DQ004487	
CgB1	Royal Forest Department, Bangkok, Thailand	01-III-2005	C.-Y. Lee	DQ004488	EF092290
CgB2	Royal Forest Department, Bangkok, Thailand	01-III-2005	C.-Y. Lee	DQ004494	
CgM1	Penang National Park, Malaysia	20-VII-2004	C.-Y. Lee	DQ004484	
CgM2	Penang National Park, Malaysia	30-VII-1999	C.-Y. Lee	DQ004481	
CgM3	USM, ^e Penang, Malaysia	21-VI-2004	C.-Y. Lee	DQ004482	DQ923416
CgM4	USM, ^e Penang, Malaysia	25-VIII-2004	C.-Y. Lee	DQ004483	DQ923417
CgP1	Las Mareas, Puerto Rico	17-VII-2004	S.C. Jones	DQ004485	DQ923418
CgO1	Cleveland, Ohio, USA	22-XI-2002 ^d	S.C. Jones	DQ004495	DQ923420
CgF2	Key West, Florida, USA	17-IX-2005	S.C. Jones	EF156760	EF092291
CgS1	Tampines, Singapore	16-IX-2003	C.-Y. Lee	DQ004477	DQ923419
CgS2	Sommerville Wak, Singapore	17-IX-2003	C.-Y. Lee	DQ004476	
CgS3	Sime Ave., Singapore	18-IX-2003	C.-Y. Lee	DQ004478	
CgS4	Jalan Piala, Singapore	03-IX-2003	C.-Y. Lee	DQ004479	
CgS5	Pandan Loop, Singapore	01-IX-2003	C.-Y. Lee	DQ004480	
sCgS6	Kim Keat Rd., Singapore	22-X-2003	C.-Y. Lee	DQ915942	
CgS7	Tisifun Ave., Singapore	20-XI-2003	C.-Y. Lee	EF092285	
CgS8	20A Jalan Piala, Singapore	03-IX-2003	C.-Y. Lee	EF092287	
GA1	Atlanta, Georgia, USA	05-XI-2003	D. Suiter	DQ007344	AY683220
Nsp	Penang National Park, Malaysia	24-IV-2003	C.-Y. Lee	EF092286	AY940140

^a Designates the country where the *C. gestroi* (Cg) sample was collected and the assigned number for an individual collection, e.g., CgA2 is *C. gestroi* from Australia, collection 2. The outgroups are designated GA1, *Coptotermes formosanus*, and Nsp, *Nasutitermes* sp. Three worker termites were analyzed from each collection, except four were analyzed for CgB2.

^b Collection day, month, year as per entomological convention.

^c ITS consensus sequence has GenBank No. EF092288.

^d When the sample was received for identification.

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AA-3') (Kambhampati and Smith, 1995) and LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3') (Simon et al., 1994). The COII gene fragment (619-bp) was amplified and sequenced from a subsample of *C. gestroi* collections (CgM3, CgM4, CgB1, CgS1, CgO1, CgP1, CgF2) (Table 1) with primers TL2J3037 (Alias AtLeu) (5'-ATGGCA-GATTAGTGCAATGG-3') and TKN3785 (Alias BtLys) (5'-GTTTAAGAGACCAGTACTTG-3') (Liu and Beckenbach, 1992; Simon et al., 1994).

Overlapping contiguous regions of the nuclear ribosomal DNA (rDNA) gene cluster also were amplified with PCR and sequenced. The entire rDNA array or cluster includes (in order): 18S rDNA, internal transcribed spacer 1 (ITS1), 5.8S rDNA, internal transcribed spacer 2 (ITS2), and 28S rDNA. The ITS1 and ITS2 regions have been shown to be functionally independent (Musters et al., 1990). In addition the ITS2 region is generally more conserved than the ITS1 region (Schlötterer et al., 1994). Therefore, we amplified the entire ITS1, 5.8S, and ITS2 regions (935-bp) of the cluster for all *C. gestroi* samples, analyzing a single individual from each. We used *Drosophila* primers: CS249 (5'-TC GTAACAAGGTTTCCG-3') anchored in the 18S rRNA gene, and CS250 (5'-GTT(A/G)GTTTCTTTTCCTC-3') anchored in the 28S rRNA gene (Schlötterer et al., 1994).

All PCR was performed in a standard 25- μ l reaction with 5–20 ng of total genomic DNA. The reaction for the 16S rRNA fragment had 1 pmol of each primer, 2.5 mM MgCl₂, 1 \times buffer, 0.64 mM dNTP, and 0.05 U/ μ l *Taq* DNA polymerase. Amplification was done in a Perkin-El-

mer Gene Amp PCR system 9600 or 9700 (Applied Biosystems, Foster City, CA). The procedure included a pre-cycle denaturation at 94 °C for 2 min, a post-cycle extension at 72 °C for 5 min, and 35 cycles of a standard three-step PCR (94 °C for 45 s, 46 °C for 45 s, and 72 °C for 45 s). Reactions for the COII fragment were according to Jenkins et al. (1999). The ITS fragment was amplified according to Schlötterer et al. (1994) and included a pre-cycle denaturation at 94 °C for 2 min, a post-cycle extension at 72 °C for 2 min, and 35 cycles of a standard three-step PCR (94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min). All PCR fragments were treated with exonuclease I (10 U/ μ l) and shrimp alkaline phosphatase (1 U/ μ l) (Jenkins et al., 1999, 2001) to remove single stranded DNA (ssDNA) fragments and cleave phosphate groups, respectively. PCR products were further purified according to protocol using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). All PCR samples from individual termites were then sent to the Sequencing and Synthesis Facility (SSF) at Integrated Biotechnology Laboratories (Athens, GA) for direct sequencing in both directions.

Individual electropherograms were first analyzed and contigs were formed using Sequencher 3.1.1 software (Gene Codes Corp., Ann Arbor, MI). All sequences were then aligned with CLUSTALW 1.83 (<http://align.genome.jp>) (Thompson et al., 1994; Higgins et al., 1996) and formatted for PHYLIP 3.65 (<http://align.genome.jp>) (Felsenstein, 1993). Rooted trees (Fig. 1) were generated in TREEVIEW v. 3.2 (Page, 1996).

Sequences were entered in GenBank, with accession numbers listed in Table 1. The sample code (Table 1) typically represents a consensus sequence for the three individuals sampled from each collection locality, e.g., CgM2. The consensus sequence designated CgB1 (Table 1), however, represents three individuals from Bangkok site 1 and three individuals from Bangkok site 2, whereas CgB2, also from Bangkok site 2, represents a single sample with a different 16S rRNA sequence (Table 1).

2.3. Phylogenetic algorithms and analyses

The accurate estimate of phylogeny, within the parameters of our dataset, was essential if the demographic history of AST dispersal was to be constructed (Leaché and Ree-

der, 2002). Thus, heuristic searches for the best tree were done using three phylogenetic algorithms in PHYLIP 3.65 (Felsenstein, 1993) under either cladistic or phenetic assumptions (Avise, 1994, p. 124; Jenkins et al., 2001; Uva et al., 2004). Conspecific gene flow was estimated with maximum likelihood (ML) using DNAML, unweighted maximum parsimony (MP) using DNAPARS, and neighbor-joining (NJ) (Saitou and Nei, 1987) using NEIGHBOR. The DNADIST program of PHYLIP (Felsenstein, 1993) was used to calculate genetic distances according to the Kimura 2-parameter model of sequence evolution. Consensus trees were determined in CONSENSE using the majority rule (extended) model.

Maximum likelihood, the method least affected by sampling error, can accurately reconstruct relationships

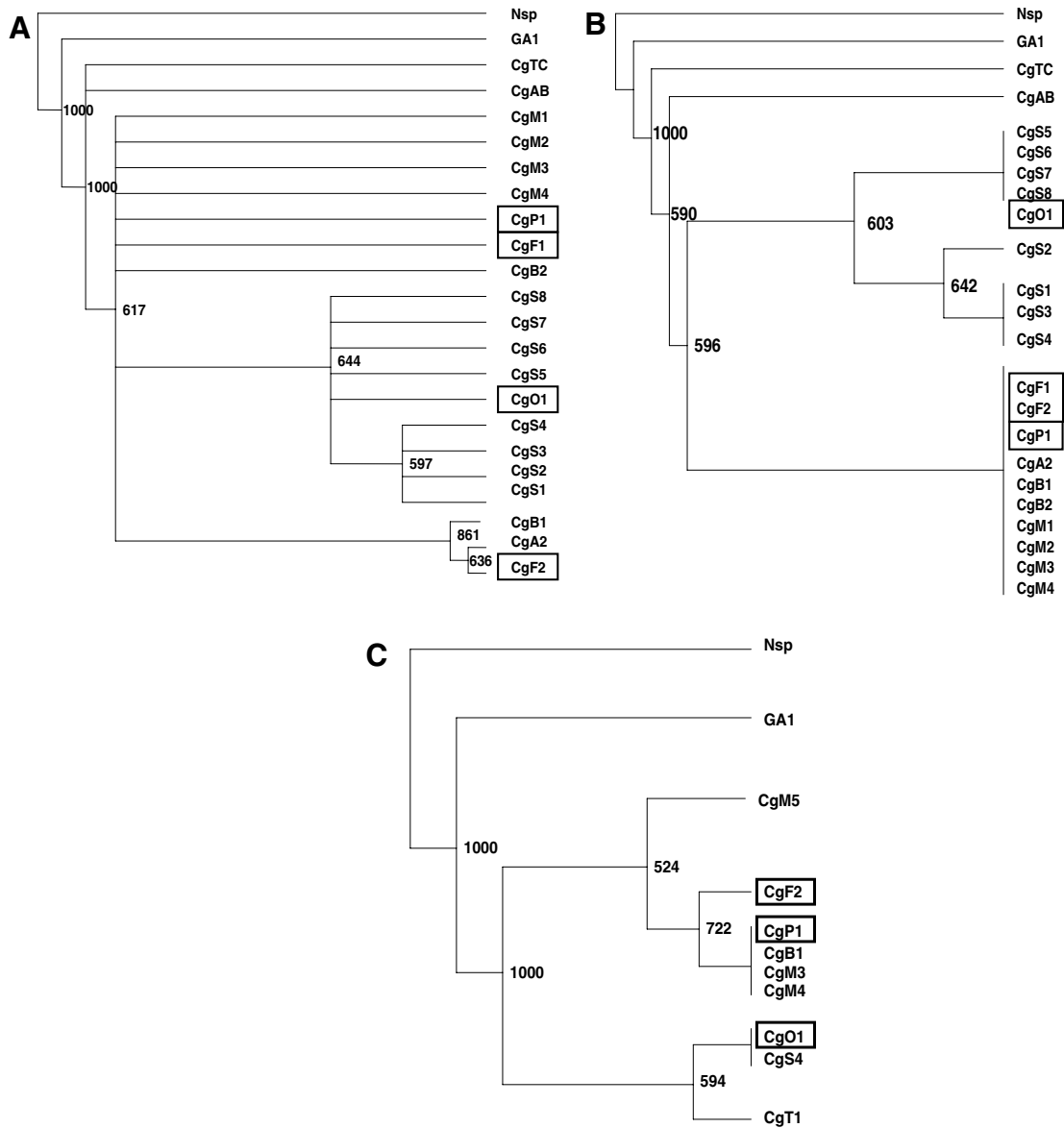


Fig. 1. Gene tree topologies for *C. gestroi* collections from Singapore, Malaysia, Taiwan, Australia, the US mainland, and several Caribbean Islands (see Table 1): (A) 16S rRNA MP tree; (B) 16S rRNA NJ tree; (C) COII NJ tree. Rectangles designate AST samples from Florida, USA (Key West [CgF2] and Miami [CgF1]); Ohio, USA (CgO1); and Puerto Rico (CgP1). All trees were rooted with *Nasutitermes* sp. (Nsp) and *Coptotermes formosanus* (GA1). Bootstrap values are indicated at the nodes and represent 1000 pseudoreplicates; all nodes are >50%.

between long-diverged or rapidly evolving DNA sequences and has been shown to be consistent when parsimony is inconsistent (Holder and Lewis, 2003; Dopman et al., 2002; Leaché and Reeder, 2002; Swofford, 1996; Kuhner and Felsenstein, 1981). Parsimony tree construction is based on searching all alternative trees for minimum total length. It performs best when tree branch lengths do not vary substantially (Holder and Lewis, 2003). Neighbor-joining (Saitou and Nei, 1987) is a distance-matrix methodology that is best employed on extant populations when sequence divergence is low (Holder and Lewis, 2003; Kuhner and Felsenstein, 1994).

Character state polarities (Avice, 1994, p. 125) and a test for minimal ingroup monophyly (Smith, 1994, p. 26) were accomplished by rooting 16S rRNA and COII trees with two other species: *Coptotermes formosanus* (Isoptera: Rhinotermitidae), a sister group to *C. gestroi*, and *Nasutitermes* sp. (Isoptera: Termitidae). Specimens of *C. formosanus* and *Nasutitermes* sp. were collected from Atlanta, GA and Penang National Park, Malaysia, respectively (Table 1).

Three 16S rRNA sequences of *C. gestroi*, AY558905, AY55906, and AY558907, were downloaded from GenBank to expand the phylogeographic analyses and to test Scheffrahn and Su's (1999) hypothesis that *C. gestroi* from Florida, USA, likely originated in the Caribbean. These *C. gestroi* had been collected from Antigua and Barbuda Islands (CgAB), Turks and Caicos Islands (CgTC), and Miami, FL (CgF1), respectively (Scheffrahn et al., 2004). To also further expand the phylogeographic analyses of the COII sequence data, two COII sequences of *C. gestroi* were downloaded from GenBank: AY536408 is a sample from Penang, Malaysia (CgM5) and AY295078 is from Taiwan (CgT1) (Tsai and Chen, 2003).

Node support for 16S rRNA and COII consensus dendrograms (CONSENSE) was assessed by 1000 non-parametric pseudoreplicates (Hillis et al., 1996, p. 523) generated in SEQBOOT. Bootstrap values are considered superior to other estimates (Sanderson, 1989), even though they are not so much a measure of support as they are a predictor of whether the results, especially clade designations (Hillis and Bull, 1993), would hold with more data (Holder and Lewis, 2003). Thus, all tree branches with nodal support <50% were collapsed (Hillis and Bull, 1993). Jackknife pseudoreplicates (1000) were also generated (not shown).

2.4. Population structure

An analysis of molecular variance (AMOVA) (Weir and Cockerham, 1984; Excoffier et al., 1992; Weir, 1996) with 10,000 permutations was conducted to determine the partitioning of the genetic variation of the conserved 16S rRNA and the less conserved COII sequences (Simon et al., 1994) within and among collection sites, particularly the Malaysia and Singapore sites, using Arlequin v. 3.0 (Excoffier et al., 2005; [http://lgb.unige.ch/arlequin]). Because AMOVA uses a hierarchically partitioned matrix of

squared genetic distances to determine by permutation the significance of variance components associated with each level of genetic partitioning, it is analogous to a nested analysis of molecular variance (Yannarell et al., 2006).

3. Results

3.1. Phylogeny analysis

The 16S rRNA and COII gene trees (Fig. 1A–C), based on heuristic searches for best trees, partitioned the *C. gestroi* samples into two major clusters: a Malaysia cluster and a Singapore cluster. The 16S rRNA maximum parsimony tree (Fig. 1A) and the neighbor-joining gene tree (Fig. 1B) showed that CgP1, CgF1, CgF2, CgA2, CgB1, CgB2, CgM1, CgM2, CgM3, and CgM4 all cluster into the Malaysia group and CgS1, CgS2, CgS3, CgS4, CgS5, CgS6, CgS7, CgS8, and CgO1 all cluster into the Singapore group with node support 62% and 60%, respectively. The maximum likelihood tree (not shown) likewise separated the Malaysia and Singapore samples into two distinct clades with node support of 62%; CgP1 was placed into the Malaysia group and CgO1 into the Singapore group with strong node support (62%). In contrast, these three phylogenetic algorithms showed that the termite sample from the Caribbean Islands of Antigua and Barbuda (CgAB [AY55905]) and that from the Turks and Caicos (CgTC [AY55906]) did not cluster with either the Malaysia or Singapore groups or with each other (node support 100%) (Fig. 1A and B). Jackknife analyses (not shown) produced similar results.

The COII NJ gene tree was composed of a subset of the samples (CgP1, CgF2, CgB1, CgO1, CgM3, CgM4, and CgS4) together with a representative sample from both Taiwan (CgT1 [AY295078]) and Penang, Malaysia (CgM5 [AY536408]). The NJ tree topology (Fig. 1C) clustered the collection from Florida (CgF2) and from Puerto Rico (CgP1) with the Malaysia samples, and it clustered the Ohio collection (CgO1) with the Singapore sample. The sample from Taiwan (CgT1) clustered in the Singapore group and the sample from Penang (CgM5) clustered in the Malaysian group (node support 100%). The maximum parsimony and maximum likelihood COII gene trees (not shown) had the same tree topology as the NJ tree (Fig. 1C).

3.2. Internal transcribed spacer sequence

All ITS sequence (935-bp) from each *C. gestroi* collection (Table 1) was the same, meaning that the sequence was homogenized across all samples. The homogenized sequence was 62% GC, with individual base frequencies of A = 18%, T = 20%, C = 30% and G = 32%. For comparison, the ITS sequence from six samples each of *C. formosanus* from New Orleans, LA and *Heterotermes convexinotatus* from Puerto Rico was also amplified (unpublished data). The ITS sequence was intraspecifically homogenized for each of these three species. Phylogenetic

analysis partitioned these consensus ITS sequences into species groups with strong node support (>70%).

3.3. Genetic structure

Population differentiation was measured directly from the DNA data using AMOVA to test the hypothesis that *C. gestroi* samples in the Malaysia cluster differed from the Singapore cluster for the 16S rRNA gene sequences (Fig. 1A and B) and the COII subsample of sequences (Fig. 1C). The 16S rRNA gene sequences had only five polymorphic sites and two that separated Singapore from Malaysia. There were 428 usable loci with a 0.05 allowed level of missing data. The null distribution was computed using 10,000 permutations. Malaysia and Singapore samples were significantly differentiated from each other ($F_{st} = 0.592$, $P = 0.0090 \pm 0.0091$). When all AST samples were included, Malaysia and Singapore clusters were again significantly differentiated ($F_{st} = 0.61058$, $P = 0.0000$). For the COII gene, population differentiation between the Malaysia and Singapore clusters with 619 usable loci was significant ($F_{st} = 0.68000$, $P = 0.0050 \pm 0.0007$).

4. Discussion

4.1. Phylogeography and insights into AST origins

Phylogeographic analysis of 16S rRNA, COII, and ITS DNA sequences illuminated the maternal dispersal history of *C. gestroi* recently collected in North America. Three phylogenetic algorithms encompassing phenetic and cladistic assumptions (MP, NJ, and ML) consistently showed that *C. gestroi* samples from two locations in Florida (Key West [CgF2] and Miami [CgF1]) as well as from Puerto Rico (CgP1) clustered with Malaysia samples. An Australia collection (CgA2) also clustered with the Malaysia collections, whereas the AST sample collected in Cleveland, OH (CgO1) clustered with the Singapore samples (Table 1; Fig. 1A–C).

The 16S rRNA and COII phylogenetic tree topologies (Fig. 1A–C) confirmed species identification based on morphological taxonomy and showed that all AST samples formed a monophyletic group consistent with genealogical concordance (Avice et al., 1987). *C. gestroi* origins of dispersal and intraspecific phylogeography were illuminated by comparing the 16S rRNA and COII phylogenetic tree topologies to geographic sampling sites that included exotic locales as well as indigenous locations within Malaysia, Thailand, and Singapore. The two major clades, Malaysia and Singapore, provided insight into New World introductions of the AST: the termites transported to Ohio, USA, appear to have originated in Singapore, whereas infestations in Puerto Rico; Florida, USA; and Australia appear to have originated in Malaysia. Based on our phylogeographic analyses, we suggest scenarios that would explain these phenomena.

Interstate and international commerce have been documented in the passive transport of exotic subterranean termites (Constantino, 2002; Costa-Leonardo and Arab, 2004; Jenkins et al., 2002; Scheffrahn and Su, 1999). In Brazil, the AST was likely introduced from Asia through the ports of Rio de Janeiro and Santos (Araujo, 1958) where, facilitated by its social structure and reproductive strategy, it rapidly spread throughout the country (Costa-Leonardo and Arab, 2004). Numerous ports in Southeast Asia handle cargo bound for countries throughout the world, and we propose that maritime trade associated with the Indo-Malayan Region is a likely route for the importation of *C. gestroi* into exotic locales. We further propose that *C. gestroi* were off-loaded concealed in trade goods at ports in New York (S. Kmetz, personnel communication) and Miami, FL, USA; Puerto Rico; and, Brisbane, Australia.

Thailand, Malaysia, and the island city-state of Singapore share a well-developed transportation infrastructure, including world-renowned port systems and a railway network and modern highway system that accommodate intra- and inter-country travel and commerce. Furthermore, a partnership between railway systems in Thailand and Malaysia allows minimum inspection of sealed railway containers (http://travelvideo.tv/news/more/php?id=A3674_0_1_0_M). The numerous modes of transportation, many characterized by minimal inspection of cargo, may explain the haplotype homogenization and/or close relationship between *C. gestroi* samples collected from Thailand and Malaysia (Fig. 1A–C).

Our analyses of the AST recently discovered in Taiwan (Tsai and Chen, 2003) indicate that the sample is more closely related to the Singapore collections than Malaysia collections (Fig. 1C). We hypothesize that AST were introduced into Taiwan from Singapore via maritime trade along the 3,500,000 km² South China Sea, which supports extensive trade between these two countries and others.

Considering the phylogeography data, the fact that *C. gestroi* is indigenous to the Indo-Malayan Region (Kirton and Brown, 2000) and that maritime sea lanes readily support transport of *C. gestroi*, it is likely that AST samples from Australia, Puerto Rico, and Miami and Key West, FL originated in Malaysia (Fig. 1A–C), although their interim history is unknown. Likewise, the origin of the AST sample from Ohio is most probably Singapore.

We propose that AST populations in Florida and Puerto Rico originated via passive transport of goods from ship to shore, although Su et al. (1997) suggest that infestations in Florida likely resulted from AST alates that swarmed onboard then serendipitously reached land. However, a number of biological factors affect the plausibility of the latter scenario. For example, the mortality rate of dispersing termite alates is very high (Behnke, 1977). Furthermore, if individual colonies have male- or female-biased sex ratios, such as have been reported for *C. formosanus* (Jones et al., 1988), the likelihood of successful pairing is further decreased. In addition, growth rates of incipient colonies of *C. gestroi* are initially quite low (Costa-

Leonardo and Barsotti, 1998; Ferraz and Canello, 2004), which is inconsistent with this species' high rate of dispersal in exotic locations (Fontes and Milano, 2002; Constantino, 2002). In Brazil, the AST has dispersed at a rate of 250–300 m/year (Fontes and Milano, 2002). Its high dispersal rate led Constantino (2002) to predict that the AST may eventually invade most of tropical South America.

Studies by Fontes and Milano (2002) suggest a one-to-one correlation between urban growth and termite population expansion and dispersion. *C. gestroi* infestations in urban structures and construction favor further dispersion, while urban trees, lawns, and other flora can serve as reservoirs for this exotic insect (Fontes and Milano, 2002). With continuing population growth and urbanization in Thailand, Malaysia, and Singapore, further AST range expansion is expected.

4.2. Anecdotal introduction histories

Anecdotal historical data confirmed the molecular phylogenetic analyses and geographic coalescent histories for the *C. gestroi* in Ohio and also in Australia. These records were obtained after our phylogenetic analyses had been completed.

Shipping documents substantiated that the cargo sent to Cleveland, OH, had been warehoused in Singapore. The cardboard boxes, which were filled with medical latex gloves, were stored on the dirt floor of a warehouse in Singapore, where they apparently were infested by *C. gestroi*. This is consistent with observations that many cardboard boxes had wet stains, particularly near their base (S. Kmetz, personnel communication). The cardboard boxes apparently dried out during the ~40-day period that they were containerized, and no live termites were found when the boxes were opened in Cleveland.

Australian quarantine and inspection records revealed that the infested yacht in Brisbane was imported as break bulk cargo from China, but the load port was Port Klang, Malaysia. This is one of the largest international ports in the region. Approximately 11,000 ships arrive annually in Port Klang, which has a capacity of 80 million tons of cargo (www.pemsea.org). Houses in this region of Malaysia were built on ex-rubber and oil palm plantation lands containing large quantities of buried tree roots that sustain large *C. gestroi* populations (Lee, 2002). Thus termite infestation could have been part and parcel of the bulk cargo and ultimately the infestation of the yacht.

4.3. Internal transcribed spacers: ITS1, ITS2

ITS1 and ITS2 have been popular for population genetics studies (Ji et al., 2003). Although the ITS2 region has been shown to be functionally independent of the ITS1 region (Schlötterer et al., 1994), the ribosomal gene cluster, 18S rDNA, ITS1, 5.8S rDNA, ITS2, 28S rDNA, is initially transcribed into RNA as a single unit. This means that ITS1 and ITS2 can be treated as a single locus that is not

under selection pressure to be conserved (Cruickshank, 2002). Thus, we hypothesized that sequences of individual amplicons that encompassed both internal transcribed spacer regions would be intraspecifically variable. However, this hypothesis was rejected in favor of subterranean termite concerted evolution due to the discovery that the ITS sequence was homogenized across all *C. gestroi* individuals from each collection in Table 1, as well as from several other rhinotermitids: *C. formosanus* and *H. convexinotatus*.

The ITS2 region has been shown to be intraspecifically variable and phylogenetically informative in invertebrates generally and insects specifically (Ji et al., 2003), including Diptera (Beebe et al., 1999; Xu and Qu, 1997) and Hymenoptera (Alvarez and Hoy, 2002). In contrast, in several rhinotermitids, the ITS2 region has been homogenized (Jenkins et al., 2001), and this current study indicated that the more variable ITS1 region also has been homogenized. Thus since the ITS cluster was shown to be a multiple homogenous copy sequence that has partitioned interspecific subterranean termite variation, it can be effectively used in phylogenetic studies designed to corroborate or illuminate interspecific taxonomic relationships among the Rhinotermitidae.

The process of production and maintenance of homogeneity within repeated gene families, like the ribosomal gene cluster, is likely due to unequal crossing over and biased gene conversion (Hillis et al., 1991). This interlocus homogenization process, or concerted evolution, results in homogenization of rDNA internal transcribed spacers across all nucleolar organizer regions regardless of initial genome dosage (Hillis et al., 1991). The ITS1 and ITS2 spacers could be “evolving along separate chromosomal lineages” (Schlötterer et al., 1994). But, although confined to a single chromosomal lineage, the concerted evolution pattern may be the result of selection acting to homogenize both internal transcribed spacers (Schlötterer et al., 1994), which suggest that the spacers may have a molecular function. The homogenization of the ITS1 and ITS2 sequence in subterranean termites (Rhinotermitidae) could, therefore, be due to the interaction of genomic and natural selection processes. Further studies of the ITS region in subterranean termites could provide an opportunity to determine the interaction of natural selection with genomic processes (Dover et al., 1993) to illuminate the process of concerted evolution in termites.

4.4. Concluding remarks

The extensive transportation infrastructure of Southeast Asia, coupled with the termite's biology, appear to be important factors that have made it possible for the AST to invade throughout the world. Identifying where exotic termites originate is a crucial step for developing possible interdiction policies to curtail future introductions of destructive species. Our findings support the use of phylogeographic analysis using the 16S rRNA and COII gene

fragments to determine the origin of termite infestations. We show that *C. gestroi* in Key West and Miami, FL, USA, the US Commonwealth of Puerto Rico as well as Brisbane, Australia, most likely were ultimately dispersed from Malaysia, whereas the *C. gestroi* found in Cleveland, OH, most likely originated in Singapore. Given the destructive nature of the AST and its propensity to infest cargo onboard ships and wooden components of sailing vessels, interdiction efforts likely should center on ports in Malaysia and Singapore.

We serendipitously discovered that, although not a good molecular marker for intraspecific subterranean termite studies, the ITS sequence marker can be used to validate interspecific taxonomy or phylogeny of subterranean termites. We also suggest that the ITS cluster could be used in genome studies to elucidate selection pressures on DNA sequences.

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