

Genetic Analysis of *Periplaneta americana* (Blattodea: Blattidae) in Central Texas Using the ITS1 Region¹

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ABSTRACT American cockroaches, *Periplaneta americana* (Blattodea: Blattidae) (L.), are common pests of urban environments. Analyzing spatial distribution of *P. americana* populations in an artificial, outdoor environment provided insight of gene flow among populations collected in central Texas. This information provides a better understanding of how and if populations were segregated, or if there was a single unified population. Populations can be genetically differentiated through determining variation of specific gene regions within populations. This study revealed a ubiquitous distribution of cockroach populations, and their ability to indiscriminately inhabit areas within an urban environment. Overall, cockroaches were identified from a large interbreeding population with no discernable relationship between genetic variation of *P. americana* and spatial distribution.

KEY WORDS *Periplaneta americana*, ITS1 region, central Texas

Cockroaches can passively and actively disperse to new locales (Jobet et al. 2000). Gene flow may be caused by long range passive travel, such as cockroaches traveling in properties of people moving from one location to another. Active movement appears to be confined to temperate climate zones when alternative, ideal habitats are within close proximity (Cloarec et al. 1999). Schoof and Siverly (1954) indicated a lack of dispersal among American cockroach, *Periplaneta americana* (L.) (Blattodea: Blattidae), populations through sewer systems in Phoenix, Arizona, USA. This inability to disperse may have resulted from the ideal habitat a sewer system provided, including ample amounts of water, food, and harborage. It appeared that when requisites for life were fulfilled the necessity to actively disperse was reduced.

Genes usually occur in repeating, tandem units and have NTS regions between repeating segments of RNA, while ITS regions separate genes within each strand. Despite looking at the lesser of the two variable spacer regions, ITS regions still can provide an ample amount of variation to reveal a relatively moderate level of gene flow amongst the given cockroach population in central Texas (Mukha et al. 2007).

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Defining a population depends on several factors such as spatial distribution, structures from which collections were made, ecological niches occupied by a population, or the general bias of the collector(s) may contribute to the definition of a "population." Differences in allelic frequencies may also be used to distinguish populations. Hypothetically, genetic variability decreases in populations secluded from other populations (Cloarec et al. 1999). In regards to cockroaches, isolated populations may have limited gene fluctuation because of minimal migration from outside populations contributing to the non-diverse gene pool (Mukha et al. 2007).

Only a few cockroaches are needed to establish a new population in a given area. Mukha et al. (2007) identified three *Blattella germanica* (Linnæus) (Blattodea: Blattellidae) populations with substantial genetic differentiation, hence, isolated populations separated between 15 and 115 km. In contrast, Cloarec et al. (1999) analyzed isoenzymatic genetic markers from *B. germanica* populations from two French cities (Rennes and Sète) approximately 900 km apart and demonstrated limited genetic variation. Consequently, due to contrasting results in previous studies it is inconclusive as to whether or not populations analyzed over distances are homologous.

Genetic variation among dispersing populations may result from various genetic events. Genetic drift, founder effects, natural selection, migration, and gene flow are some factors that might contribute to genetic variation (Jobet et al. 2000). Founder effects occur more frequently in cockroach populations because only a limited number of individuals are required to establish new populations (Cloarec et al. 1999). Cloarec et al. (1999) suggested that populations within a defined geographical area (i.e., a city) were more homologous than populations compared between greater distances (i.e., city to city). Populations separated by variable distances retaining similar allelic frequencies indicated a homologous correlation between populations, hence, gene flow (Cloarec et al. 1999).

The objective of this study was to determine gene flow among populations collected in central Texas. This information may allow for a better understanding of how and if populations were segregated, or if there was a single unified population.

Materials and Methods

Sampling technique for cockroaches. *Periplaneta americana* (L.) were collected within 50 m of neighboring urban structures in College Station, TX and investigated for potential gene flow by phylogenetic analysis among the collected population(s). Collecting sites on campus were selected from locations with the highest cockroach populations based on preliminary trapping. Once locations were established, three collecting containers were placed within a 1.83 m² square at each trapping location. Coordinates of each site were determined with a Gormin eTrex® Vista Cx GPS unit (Garmin Ltd., Olathe, KS, USA). Additional samples from the following cities in Texas were obtained from the Texas A&M University Insects in Human Society (ENTO 322) Student Insect Collection including: Pleasanton, Del Rio, Bryan, and Hempstead, Texas. The cockroaches from the Texas A&M University Insects in Human Society Student Insect Collection were preserved by pinning and stored in boxes turned in by the

students. Data points for all cockroaches collected were uploaded to Google Earth.

Containers used for collection at College Station, TX were glass mason jars (430 ml) coated with Elmer's Acid Free Craft Bond (Elmer's Products, Inc., Columbus, Ohio, USA) and rolled in Quickrete® Playsand (Quickrete® International, Inc., Atlanta, GA, USA), according to Granovsky (1983). The top 2 cm of the jar opening was lined with H-E-B brand petroleum jelly (H-E-B, San Antonio, TX, USA), and baited with approximately 51.76 ml beer (Miller Brewing Co., Milwaukee, WI, USA) and 7.04 g of H-E-B brand white bread (H-E-B, San Antonio, TX, USA) for specimen collections (Barcay 2004). Baited containers were placed in the field immediately after adding the beer/bread mixture. Jars were set out prior to dusk and collected from the field after 8–12 h.

Cockroaches were collected from each jar and stored in individual plastic bags (16.5 × 14.9 cm) with up to three plastic bags containing cockroaches from each site. Collected specimens were stored in a freezer at –20°C until further analyses were conducted.

Molecular analysis. Molecular probes were used to identify different haplotypes within each cockroach sample. The hind femur from each specimen was used for genetic analysis. The specific region providing the greatest amount of information about the genetic flow is the ITS1 region located between the 18S and 5.8S gene. The success of fragments of both the 18S and 5.8S genes, and the entire ITS1 region, to make up the probe in identification of individuals and their genetic composition from the provided specimens has been demonstrated in recent studies (Mukha et al. 2007).

A 562-bp section of the nuclear 3' portion of 18S rDNA, all of ITS1 region, and the 5' portion of 5.8S were amplified with the primers rDNA2 (5'-TTGAT-TACGTCCCTGCCCTTT-3') and rDNA 1.58S (5'-GCCACCTAGTGAGCC-GAGCA-3') with a thermal cycler profile consisting of 40 cycles of 94°C for 45 s, 53°C for 1 min and 72°C for 1 min as described by Szalanski and Owens (2003) (Vrain et al. 1992, Cherry et al. 1997). Amplified DNA from individual cockroaches was purified and concentrated with minicolumns according to the manufacturer's instructions (Wizard PCRpreps, Promega). Samples were sent to the University of Arkansas Medical School DNA Sequencing Facility (Little Rock, AR, USA) for direct sequencing in both directions. Consensus sequences were derived from both of DNA sequences from an individual with Bioedit 5.09 to verify nucleotide polymorphisms (Hall 1999).

DNA sequences were aligned by CLUSTAL W (Thompson et al. 1994). The distance matrix option of PAUP* 4.0b10 was used to calculate genetic distances according to the Kimura 2-parameter model of sequence evolution (Kimura 1980, Swofford 2001). 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, will be determined). These parameters were used to carry out a heuristic search by PAUP* with a neighbor joining tree as the starting tree. Gene flow was

Table 1. Sample sites and haplotypes frequencies from each collection site within Texas counties.

City (County)	N	Haplotype (frequency)
Pleasanton (Atascosa)	1	17(1)
Bryan (Brazos)	2	1(1), 5(1)
College Station (Brazos)	48	1(10), 2(1), 3(14), 4(1), 6(1), 7(1), 8(1), 9(1), 10(4), 11(1), 12(1), 13(1), 14(3), 15(1), 16(2), 18(1), 19(1), 20(1), 21(1), 22(1)
Hempstead (Waller)	1	1(1)

evaluated applying Mitochondrial DNA haplotypes aligned by MacClade v4 (Sinauer Associates, Sunderland, MA). Haplotype distribution between populations, number of haplotypes, number of unique haplotypes, haplotype diversity (h), and nucleotide diversity (π) was calculated with DNAsp v3.51 and Genealogical relationships among haplotypes were constructed using TCS, with the method described by Templeton et al. (1992) (Rozas & Rozas 1999, Clement et al. 2000).

Results

DNA sequencing of the ITS1 region from 52 sampled cockroaches resulted in an average size of 560 bp. There were 22 haplotypes observed from four Texas counties with the 3 haplotype being the most common (Table 1). There were 25 unique haplotypes. Del Rio, Texas is approximately 462 km from College Station; Pleasanton, Texas has a distance of approximately 274 km from College Station, Texas; Hempstead, Texas is approximately 62 km away from College Station, Texas; Bryan, Texas is a sister city to College Station, Texas separated by approximately 8 km.

There were 41 polymorphic sites (Table 2). The average number of pairwise nucleotide differences was 3.992. Out of the 22 haplotypes there were 25 singletons or unique sequences. Nucleotide diversity, π , was 0.007, and the mean number of pairwise nucleotide differences between haplotypes, k , was 3.992. Tajima's D test of neutrality of mutations against excess of recent mutations were not significant (Table 3).

Applying PAUP* version 4.0b10 software, both Neighbor-Joining (NJ) and Maximum Parsimony (MP) analyses were conducted. Results of the NJ tree using uncorrected "P" distances are presented as an unrooted cladogram (Figure 1). For MP analysis, parametric bootstrapping (50% majority-rule) with a full heuristic search was employed for 1000 pseudoreplicates with a starting seed = 632095753. A total of 560 characters were evaluated with all characters equally weighted; 513 characters remained constant and 20 characters were parsimony informative. Gaps in nucleic sequences were treated as "missing" with the starting tree(s) obtained via stepwise addition. The Branch-swapping algorithm: tree-bisection-reconnection (TBR) was employed. The sum of minimum possible

lengths = 48; the sum of maximum possible lengths = 140. A single tree (Figure 1) was produced with length = 113, CI = 0.425 and RI = 0.293. Uncorrected ("P") distances were used to construct the NJ tree.

Phylogenetic trees were also obtained using a Bayesian analysis with the GTR+G model by applying Bayesian Evolutionary Analysis Sampling Trees (BEAST) version 1.4.7 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 (Figure 2); MP branch support are presented above the major branches with posterior bootstrapping probabilities presented behind each node (Figure 2).

TCS spanning tree analysis revealed that haplotype 3 had the highest outgroup possibility for all of the 22 haplotypes (Figure 3)

Discussion

The purpose of this study was to analyze the spatial distribution of *P. americana* populations in an outdoor, urban environment and to determine the extent of gene flow among the populations. This study attempted to determine genetic variability among *P. americana* collected on Texas A&M University in College Station, TX.

Genetic differentiation occurs between populations in diverse locations for all organisms (Austin et al. 2004). Inward et al. (2007) suggested that the orders Isoptera and Blattodea are related, thus their genes would coalesce to a single common ancestor. It can be assumed that the individual lineages would comprise similar genetic material, thus specific gene regions would be applicable for amplification purposes in both orders. Phylogenetic studies and population genetics performed on termites commonly used the 16S region of the gene for amplification. The 16S region of the gene was initially chosen as the amplification site in this study to determine variability among cockroach populations collected on campus. During this study, the 16S gene region amplification protocol commonly used in termite studies failed to amplify cockroach DNA. Differing genetic compositions of the 16S gene region selected may have resulted from evolution of separate ordinal lineages over time. The universal primers that annealed for termite DNA simply would not work for cockroach DNA and/or the annealing temperature may have been too low thus inhibiting annealing or too high which would damage the primers or DNA. Consequently, the ITS1 region was chosen for amplification because of the availability of comparable sequences available on Genbank (National Center for Biotechnology Information).

The ITS1 region functions in primary rRNA processing and has a higher rate of differentiation than the 18S gene region of rRNA (James et al. 1996). Mukha et al. (2007) reported rRNA genes as being the most conserved among populations, while non-transcribed spacer regions have the most variation, and transcribed spacer regions between the two extremes. There are conflicting results when analyzing the ITS1 region for genetic variability in insect populations. Szalanzski et al. (2008) determined a lack of diversity in the nuclear gene region (ITS1 region) with high levels of differentiation when examining the mitochondrial DNA region (16S gene) in *Cimex lectularius* (L.) (Hemiptera: Cimicidae). The ITS1 region may have indicated low levels of diversity in this species at these specific loci (Szalanzski et al. 2008). When the ITS1 region was used, it failed to

Table 2. Base pair differences between *P. americana* haplotypes from Texas.

Haplotype	Nucleotide site															
	N	27	33	52	55	58	67	69	82	85	92	136	137	179	186	198
1	12	T	T	C	C	A	C	A	C	C	A	C	G	C	C	T
2	1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3	14	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
4	1	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*
5	1	*	*	*	*	G	G	G	*	*	*	*	*	*	*	C
6	1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
7	1	*	*	*	*	*	*	*	*	*	C	*	*	A	*	*
8	1	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*
9	1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
10	4	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
11	1	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*
12	1	*	*	T	T	*	G	*	T	T	C	T	*	A	T	*
13	1	*	*	T	*	*	*	*	T	T	*	T	*	*	*	*
14	3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
15	1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
16	2	*	*	*	*	*	G	*	T	*	*	*	*	*	*	*
17	1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
18	1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
19	1	*	*	*	*	*	G	G	*	*	*	T	T	*	*	*
20	1	*	*	T	T	*	*	*	T	T	*	T	*	A	T	*
21	1	*	*	T	T	*	*	*	T	T	*	T	*	A	T	*
22	1	*	*	T	*	*	*	*	T	T	C	T	*	*	*	*

Haplotype	Nucleotide site														
	N	199	225	239	264	272	303	314	355	366	437	463	488	514	515
1	12	T	A	G	G	G	G	A	A	G	C	A	C	A	A
2	1	*	*	*	*	*	*	T	T	*	T	*	*	C	*
3	14	*	T	*	*	*	*	*	*	*	*	*	*	*	*
4	1	*	T	*	*	*	*	*	*	*	*	*	*	*	*
5	1	*	T	*	*	*	*	*	*	*	*	*	*	*	*
6	1	*	T	*	*	*	*	*	*	*	*	*	*	*	T
7	1	*	*	G	*	*	*	*	*	*	*	*	*	*	*
8	1	*	*	*	C	*	*	*	*	*	*	*	*	*	*
9	1	A	*	G	*	*	*	*	*	*	*	*	*	*	*
10	4	A	T	*	*	*	*	*	*	*	*	*	*	*	*
11	1	*	*	G	*	*	*	*	*	*	*	*	*	*	*
12	1	A	*	G	*	*	*	*	*	*	*	*	*	*	*
13	1	A	*	G	*	*	*	*	*	*	*	*	*	*	*
14	3	*	*	G	*	*	*	*	*	*	*	*	*	*	*
15	1	A	*	*	*	*	*	*	*	*	*	*	*	*	*
16	2	*	*	*	*	*	*	*	*	*	*	*	*	*	*
17	1	*	*	G	*	G	C	*	*	C	*	C	*	*	*

Table 2. Continued.

Haplotype	N	Nucleotide site														
		199	225	239	264	272	303	314	355	366	437	463	488	514	515	
18	1	*	*	*	*	*	*	*	*	C	*	*	G	*	*	
19	1	*	*	G	*	*	*	*	*	*	*	*	*	*	*	
20	1	*	*	G	*	*	*	*	*	*	*	*	*	*	T	
21	1	*	*	G	*	*	*	*	*	*	*	*	*	*	*	
22	1	*	*	G	*	*	*	*	*	*	*	*	*	*	*	

determine phylogenetic relationships between *Reticulitermes* termites (Tripodi et al. 2006). On the other hand, there was sufficient variability in the ITS1 region used to identify diversity among *Diabrotica* (Coleoptera: Chrysomelidae) species (Szalanski & Owens 2003). Additionally, Szalanski et al. (2000) demonstrated differentiation between *Nicrophorus americanus* (Olivier) (Coleoptera: Silphidae) based on results from the ITS1 region. The current study may have demonstrated biotic homogenization within populations of *P. americana* based on data from the ITS1 region (McKinney & Lockwood 1999).

Haplotypes are defined by at least a single nucleotide difference within the same gene region between sequences thus identifying unique genes. Haplotype diversity is the number of haplotypes compared to their relative frequency and determined the probability of two sequences chosen from a population being different (Austin et al. 2004). Tajima’s D is a statistical determination of the neutral mutation hypothesis in natural populations (Tajima 1989). Positive values of D indicate population bottlenecks while negative values of D suggest expansion of a population (Tajima 1989). Nucleotide diversity (Pi) in populations assumed neutrality based on the infinite alleles model (Austin et al. 2004).

Among the 52 cockroaches sampled there were 22 haplotypes indicating a high amount of variation in the population. TCS spanning tree analysis defined lineages from nuclear markers which implied populations moderate levels of gene flow. The lack of isolated populations was reconfirmed by maximum likelihood and Bayesian phylogenetic analyses.

Periplaneta americana samples from Bryan, College Station, Hempstead, and Pleasanton, TX were in a single clade, including *P. americana* sequence obtained from Genbank (AF321248). Sequence comparisons reconfirmed speciation and

Table 3. Summary of statistics for rDNA genetic variation.^a

Sample	<i>n</i>	<i>h</i>	<i>s</i>	Hd	π (<i>k</i>)	θ_s	θ_g	D ^{***}	F ^{**}	D [*]
Texas	52	28	41	0.918 ± 0.025	0.007 (3.992)	0.017	9.29	-3.39	-3.41	-1.94

*P < 0.05; **P < 0.02.

^a*n* is the number of sequences, *h* is the number of haplotypes, *s* is then number of polymorphic sites, Hd is haplotype diversity ± SD, π is nucleotide diversity, *k* is mean number of pairwise nucleotide differences, θ_s is the theta per site, θ_g is theta per gene, D⁺ and F⁺ are statistics per Fu and Li, and F^{**} is Tajima D statistic.

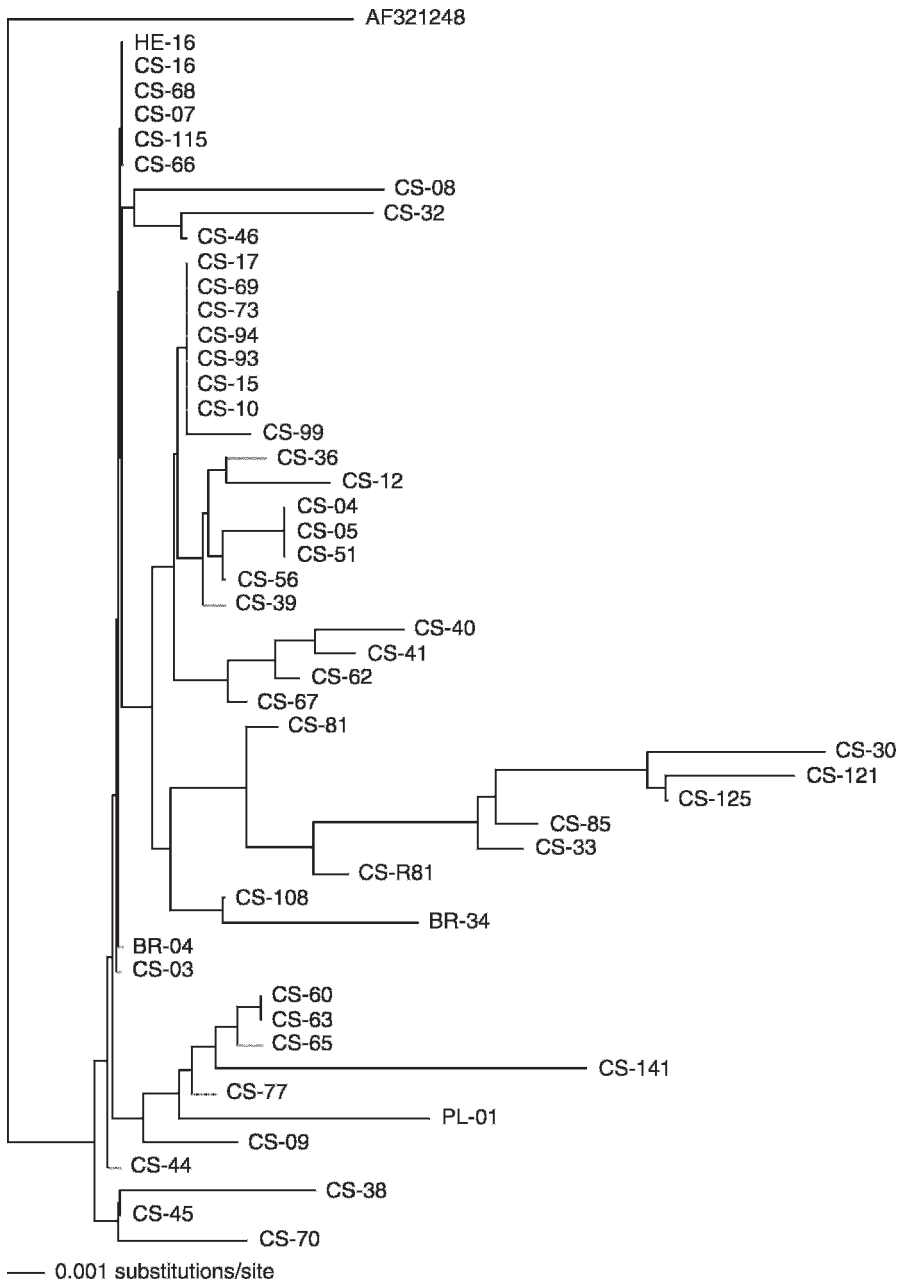


Fig. 1. Phylogenetic relationship of *P. americana* rDNA ITS1 region. Neighbor-joining tree with a length = 113, CI = 0.425, and RI = 0.293 resulting from samples collected from quadrants on the Texas A&M University campus College Station, Texas, and from Bryan, Hempstead, and Pleasanton, Texas.

revealed moderate interbreeding between *P. americana*. The Smokey Brown cockroach (*Periplaneta fuliginosa*) (Serville) and Brown cockroach (*Periplaneta brunnea*) (Burmeister) were chosen as outliers because their sequences were available on Genbank, AF321250 and AF321249, respectively, and are members of the same genera as are American cockroaches. Comparing various species allowed a broader analysis of *P. americana* to varying genetic sequences as a result of speciation within the same genera. Comparing the 52 sequences amplified to 22 haplotypes suggested a moderate amount of variation in the population based on nuclear markers. The lack of isolation indicated interbreeding populations on campus. Differentiation of genetic variation based on spatial distribution of *P. americana* populations indicated the success and ability of breeding with independence among various populations.

Migration of individuals to new locations provided opportunities for new genetic material to be introduced into a population thus increasing some haplotypic diversity. Szalanski and Owens (2003) suggested lack of variation among southern corn rootworm resulted from motility or population expansion. Diversity among populations collected on campus most likely resulted from the ability of cockroaches to travel successfully in urban environments and breed effectively with cockroaches from other areas thus contributing to a constant influx of genetic material into various populations. It remains unknown what degree of genetic variability is observed among other cockroach species.

Genetic variability in populations can be achieved through genetic drift, genetic flow, natural selection, and founder effects (Slatkin 1987). Genetic drift can affect nuclear genes though the fixation of loci in various locations, but gene flow can impede the permanent fixation of the alleles (Slatkin 1987). Lenormand (2002) determined gene flow limited adaptation of genes to specific locations because new genes from outside sources prevent loci from becoming fixed in the environment. Gene flow can prevent speciation because introduced genetic material can be adapted for survival in a particular environment differing from the population in which it emigrated (Slatkin 1987). Gene flow is an indirect method of determining movement within a population. Bossart and Prowell (1998) indicated that the only method that definitively determined gene flow among a population was through the use of genetic tags used to track movement which had been successful in marine organisms. Cloarec et al. (1999) described gene flow (in *B. germanica*) from the movement of cockroaches over long distances by passive transportation, thus increasing the rate of homogenization among the genetic material between populations. Results found in the current study were similar to those found in Cloarec et al. (1999) when they determined that the German cockroach populations were not isolated in two French cities 900 km apart. Mukha et al. (2007) determined three *B. germanica* populations found in farms separated by 10–100 km and had three populations differentiated by rDNA markers, but they were still not completely isolated. Species, including highly mobile organisms such as cockroaches, disperse through an environment until geographical impediments such as oceans, deserts, and mountains limit expansion (Slatkin 1987).

To date, this study is the first using rDNA markers to identify spatial relationships and gene flow among *P. americana* populations in the United States. Future studies may analyze a broader range of genes including mitochondrial DNA to determine if there are lineages formed by maternal

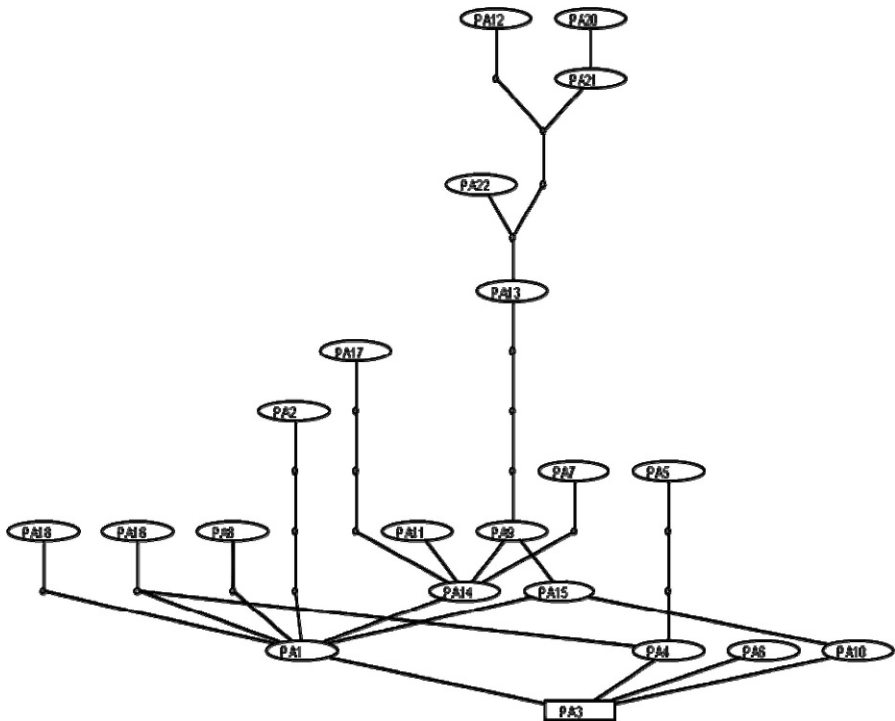


Fig. 3. Genealogical relationship among haplotypes of *P. americana* estimated by TCS. The square is the most Bayesian haplotype among the collected populations in Texas. Ovals are haplotypes not observed and each branch represents a single mutation.

genetic material. Also, analyzing gene flow at several differing sequences within DNA may determine a more comprehensive evolutionary lineage of divergences in cockroach populations.

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Fig. 2. Phylogenetic trees using a Bayesian analysis with MP branch support are presented above the major branches with posterior bootstrapping probabilities presented behind each node for samples collected from quadrants on the Texas A&M University campus College Station, Texas, and from Bryan, Hempstead, and Pleasanton, Texas.

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