Sociobiology
An international journal on social insects

RESEARCH ARTICLE - TERMITES

Population Genetic Structure and Breeding Pattern of Higher Group Termite *Globitermes sulphureus* (Haviland) (Blattodea: Termitidae)

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Article History

Edited by
Qiuying Huang, HAU, China
Received 05 August 2020
Initial acceptance 13 February 2021
Final acceptance 11 March 2021
publication date 31 March 2021

Keywords
Globitermes sulphureus; higher group termite; population genetics; polymorphism; natural regions; metropolitan regions.

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Abstract

The subterranean termite *Globitermes sulphureus* (Blattodea: Termitidae) can be found in tropical regions. We chose seven novel species-specific microsatellite markers to infer the breeding pattern of *G. sulphureus* based on its colony and population genetic structure in eight selected populations (natural-n = 4 and metropolitan-n = 4) in Kedah and Penang, Malaysia. A strong correlation with their geographical location is shown by the acquired genetic gap for all studied populations from this study. The breeding pattern of family structure and comparisons of estimated F-statistics among *G. sulphureus* workers suggests 60% of all colonies are mixed families, whereas the remaining are simple families. Average relatedness values within simple and mixed family colonies are similar (r = 0.121). Positive fixation index F_{ST} values (F_{ST} = 0.086) indicate all eight populations (>500 m apart) have a significantly moderate genetic differentiation and low levels of inbreeding based on the low overall inbreeding coefficient F_{IT} value of 0.391. Furthermore, four populations; Palapes USM (PU), Tmn Astana (TA), Kg Teluk (KT), and Penang National Park (NP), deviate from Hardy–Weinberg equilibrium (HWE, all p = 0.000) and five studied polymorphic loci (GS1, GS10, GS15, GS27 and GS29) are possibly under selection. The findings also reveal signs of a bottleneck effect in two populations: Tikam Batu (TB) and Penang National Park (NP), indicating genetic drift.

Introduction

The subterranean termite *Globitermes sulphureus* (Haviland) (Blattodea: Termitidae) is a secondary pest species that has been introduced from its natural habitat to many parts of the metropolitan areas in Peninsular Malaysia after the elimination of primary termite pests such as *Coptotermes* sp. (Ab Majid et al., 2007**).** In this study, we found infestations of *Globitermes sulphureus* species around suburban or metropolitan environments at several locations in northern Peninsular Malaysia. The successful migration and establishment of *G. sulphureus* populations in metropolitan environments have created considerable concerns regarding biological invasions. Invasive social insects such as termites often interrupt ecological communities and trigger serious economic destruction in their newly invaded locations (Vargo & Carson, 2006**,** Ab Majid & Ahmad, 2011).

In particular, *G. sulphureus* termites are considered a unique model system for analysing and distinguishing ecological elements that play a prominent role in the evolution of the introduced population (Luchetti et al., 2013). Furthermore, most comparative studies to date, including measuring the genetic variation level, genetic structure, and breeding pattern of G. sulphureus species, have failed to reveal any meaningful information. Additionally, most termite species' population studies have shown some genetic structure levels, and these population patterns are critical points for evolutionary biology research. Thus, knowledge of the *G. sulphureus* breeding system and dispersal behavior is crucial to understand the observed patterns of the population genetic structure.

Molecular data such as microsatellite marker analysis enables researchers to infer the breeding system of the species and its dispersal behavior responsible for producing the pattern

structure and characterize the population's subdivisions (Thompson et al., 2007; Fougeyrollas et al., 2018). Khizam and Ab Majid (2019) developed highly polymorphic species-specific microsatellite markers for *G. sulphureus* to investigate the breeding pattern and population genetic structure. Microsatellite markers are suitable instruments for understanding a colony's breeding structure in social insects (Ross, 2001). Besides, there are a growing number of genetic research studies observing the social organization of termites colonies, especially among subterranean termites such as *G. sulphureus* (Goodisman & Crozier, 2002; Bulmer et al., 2001; Clement et al., 2001; Dronnet et al., 2005; DeHeer et al., 2005**).** In general, the knowledge gained from species-specific microsatellite analysis helps assess the genetic structure, genetic diversity, and population structure, which can be applied in breeding system strategies and termite management.

This study's main objectives are to analyze and understand the breeding system and organization of the population genetic structure of *G. sulphureus* between natural and metropolitan populations in Penang and Kedah, Malaysia.

Materials and methods

Termites collection

We collected termite workers of *G. sulphureus* from eight different assigned sampling sites in natural and metropolitan locations in Kedah and Pulau Pinang between September 2017 and February 2018. We collected a total of 10 worker termites from each nesting site and identified termite specimens based on Tho (1974). Figure 1 shows the sampling locations marked on the map using the PinMap web application. We recorded the addresses and geographic coordinates for the sampling sites (Table 1) using a hand-held Garmin® GPS 72H unit (Garmin Ltd, Inc. USA). After collection, all worker termites from each sampling site were preserved in vials containing 90% ethanol. The sample was kept at -20°C before DNA isolation.

Sampling site location

Geographical areas and environments determine the types of research sites, either natural or metropolitan regions. Natural sites such as forest and agricultural areas were selected if the sites are in rural areas with a low-density population. Consequently, metropolitan sites consist of many infrastructures with many human settlements such as cities, towns, urban and suburban areas.

Genomic DNA isolation

DNA was extracted from the head of 10 termite workers individually from each sampling site using the Real Biotech Corporation (RBC) DNA extraction kit with modified protocols (Seri Masran & Ab Majid, 2018). All purified genomic DNA was then quantified and tested for quality using a spectrophotometer NanoDrop 2000c (Thermoscientific, USA). The purified DNA samples were observed under 2.0% agarose gel electrophoresis.

Microsatellite genotyping

Each termite worker was genotyped based on species-specific microsatellite loci obtained from Khizam and Ab Majid (2019) (GS 1, GS 3, GS 4, GS 10, GS 15, GS 27, and GS 29) (Table 2). We deposited all sequences in the National Centre for Biotechnology Information (NCBI) and Sequence Read Archive (SRA) databases under accession number

Table 1. Detailed of eight collection points of *G. sulphureus* worker individuals.

Isolated Code	Location (GPS)	State	Sources	Nesting sites	Collection_Date
TJ	N 5°38' 51"; E 100°29'3"	KEDAH	TMN JUBLI	DEAD LOG	28 Oct 2017
PU	N 5°21. 525"; E 100°17.596"	PENANG	PALAPES USM	MOUND	31 Oct 2017
TA	N 5°35. 367"; E 100°31.134"	KEDAH	TMN ASTANA	MOUND	9 Nov 2017
AU	N 5°21. 648"; E 100°18.365"	PENANG	ARKEOLOGI USM	LIVING TREE	14 Nov 2017
*TB	N 5°35. 042"; E 100°25.915"	KEDAH	*TIKAM BATU	DEAD LOG	26 Nov 2017
*SL	N 5°39'23"; E 100°28'14"	KEDAH	*SG. LAYAR TENGAH	MOUND	13 Jan 2018
*KT	N 5°39'1"; E 100°27'44"	KEDAH	*KG. TELUK	MOUND	19 Jan 2018
*NP	N 5°27'43"; E 100°12'14"	PENANG	*PENANG NATIONAL PARK	MOUND	20 Jan 2018

Tikam Batu (TB), Sg. Layar Tengah (SL), Kg. Teluk (KT), Penang National Park (NP), Tmn. Jubli (TJ), Tmn. Astana (TA), Palapes USM (PU), Arkeologi USM (AU)

*Natural/Rural regions was labelled.

Fig 1. Eight sampling locations of *G. sulphureus* termite species between natural and metropolitan region Abbreviations refer to Table 1.

SRP132022 associated with BioProject PRJNA432461. The gDNA was amplified using a PCR thermocycler machine in volumes of 50 µL containing 25 µL master mix (Qiagen, Valencia, CA), 15 µL of double-distilled water, 2.5 µL of each primer (0.05 µM), and 5 µL gDNA. The PCR touchdown reaction was then subjected to the following settings; initial denaturation at 94 °C (30 seconds), 30 denaturation cycles at 94 °C (30 seconds), annealing at 60 °C (30 seconds), and extension at 72 °C (1 minute), followed by another 30 cycles at 94 °C for 30 seconds, 45 °C for 30 seconds, and 72 °C for 1 minute. The reaction was terminated at 72 °C for 10 minutes and held at 4 °C (Seri Masran & Ab Majid, 2018; Khizam & Ab Majid, 2019).

We measured exact fragment sizes for all PCR products after electrophoretic separation during fragment analysis. To separate the fragments according to their respective size, we used the Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Ankeny, Iowa, USA) with an internal size standard of 35-1500 bp. Microsatellite data were analyzed and hand-scored using the Prosize 2.0 software package (Advanced Analytical Technologies, Ankeny, Iowa, USA).

Microsatellites Genotyping and Tests for HW Equilibrium

To determine the definite tests of genotypic differentiation, we used GENEPOP version 4.2 (available at http://wbiomed.curtin.edu.au/genepop) (Rousset, 2008). The significant result obtained from the test exhibits different genotype frequencies among different groups of *G. sulphureus* workers, which indicates they were drawn from different colonies. Using the Hardy–Weinberg equilibrium (HWE)

Table 2. Information for seven chosen loci used in genotyping of microsatellite.

Loci	Primer3 Calculated Value of Possible Allele Size (bp)	Primer (5'-3')	Type of repeat motif
GS1	236	F: AGCGATCGGATGAGCAAGG R: ACACGTCTGTGTAAAGGCAG	Dinucleotide
GS3	186	F: GTGCCATTCCACCTTCGTG R: CGTCTCACTAGCAGCAATTATG	Dinucleotide
GS4	316	F: TGGTGTGAGATGGTGAACCC R: TCAGTTAGCAAATGGGAAGCC	Dinucleotide
GS10	303	F: TCCAGTAGGTGTCCTGTTGC R: AAGGCTAGCTTCCAGTTCAG	Dinucleotide
GS15	180	F: TGTTGCTGAAACTAAATGGCTG R: CTGCACGTAAGGAGAAGTCTG	Dinucleotide
GS27	294	F: ACAATGAAGGGCACGTTTGG R: GCAATGGAGTCTAGGTGTCG	Tetranucleotide
GS29	431	F: GGACGACTGCTTAAAGTTGC R: ACTATGCCTGGGTTTGATCC	Tetranucleotide

concept, the observation of allele frequency patterns within and between population(s) could also be carried out. Unbalanced distribution of homozygote and heterozygote allele frequencies within the population indicate a deviation from the HWE principle (Vargo & Husseneder, 2009; Perdereau et al., 2010; Ab Majid et al., 2018).

Colony Breeding Pattern

A subterranean termite's colony breeding structure is classified into three categories: simple, mixed, or extended family structure (Vargo & Husseneder, 2009; Perdereau et al., 2010; Ab Majid et al., 2018). The simple family structure consists of a single pair of primary (winged) reproductive members in the subterranean termite colony. Colonies are characterized as simple families if worker genotype patterns are consistent with a direct offspring expected from a single pair of reproductive parents. Primary pair of reproductive parents can disperse and build a new colony, later producing secondary reproductive parents or neotenic (Perdereau et al., 2010). The neotenic replaces the primary reproductive parents, fly, and initiate inbreeding. Meanwhile, multiple neotenic forms an extended family structure having fewer than four alleles at any locus (Vargo & Husseneder, 2009; Perdereau et al., 2010). On the other hand, colonies form mixed families if more than four or five alleles are observed at one or more loci and headed by more than a single pair of primary reproductive parents.

Colony Genetic Structure and relatedness coefficient

Additional understanding of the genetic structure of colonies is possible by analyzing relatedness coefficients. Firstly, we used F_{IT} to measure the homozygosity of individuals relative to their population. Secondly, F_{IS} indicates the level of inbreeding in individuals relative to the population. Thirdly, F_{ST} refers to the inbreeding coefficient of individuals relative to their colony. 95% confidence intervals (CIs) and standard errors (SEs) were obtained by jack-knifing over loci. We used the program FSTAT to compute the F-statistics (Goudet, 2001) and to calculate the expected heterozygosity (He), the observed heterozygosity (Ho), and the allelic diversity in the population. On the other hand, the F-statistic output can estimate the coefficient of relatedness (*r)* among individual workers from each population. Thus, the colony data and population genetic structure of *G. sulphureus* within populations can be successfully assessed to extrapolate the number of alleles per loci and population.

Genetic isolation by distance

GenAIEx v6.5 (Peakall & Smouse, 2012) - a cross-platform tool - was used to perform population genetic analysis, which runs within Microsoft Excel. Data were received in the form of co-dominant genotypic microsatellite data with two columns per locus. The loci scored as fragment sizes were obtained from the previous fragment analysis. We performed an Analysis of Molecular Variance (AMOVA) (Meirmans, 2012) to determine the extent of population differentiation and the distribution of genetic variation within and among the eight selected population sites of *G. sulphureus*. Estimations of pairwise F_{ST} and significance were evaluated by a probability distribution from permutation tests (N = 1000). By comparing different populations, pairwise F_{ST} values were quantified for linear correlation with gene diversity values (He). Besides, we used principal coordinates analysis (PCoA) (Peakall & Smouse, 2012) to characterize the population structure among the eight different population sites. This analysis generates the number of genetic clusters among the population sites based on each termite's coordinate along the variation axes.

Bottleneck effect test

The effect of a mutation on allele frequency in a population can be computed using two models of mutation–

drift equilibrium. We reviewed the results using the BOTTLENECK v1.2.02 program (Piry et al., 1999). The models used were the Infinite Allele Model (I.A.M.) and Stepwise Mutation Model (S.M.M.). As a newly formed population in metropolitan areas, the *G. sulphureus* termite population is expected to exhibit a recent genetic bottleneck. Thus, we tested worker genotypes for excessive heterozygosity to obtain evidence of a bottleneck effect in each population. The first test aimed to detect any existence of significantly excessive heterozygosity with a relatively larger proportion of loci for a population at mutation-drift equilibrium. Whereas the second test – known as the Wilcoxon sign-rank test – aimed to detect significant average excessive heterozygosity across loci.

Results

Allelic diversity

We detected a total of 363 alleles at seven appointed microsatellite loci assessed in 560 *G. sulphureus* genotypes. Within the eight populations, allelic diversity is between 3 to 9 alleles per locus, with an average of 2.685. The mean percentage of the PIC value is 94.9%. Observed heterozygosity (Ho) is 0.601, lower than the expected heterozygosity (He) of 0.967. From these findings, all of the studied populations in natural and metropolitan areas demonstrate a considerable variance in their genetic variability. As shown in Table 3, 7 of the 30 loci examined in natural and metropolitan populations varies between 40 to 74 alleles per locus.

Table 3. Variability of seven polymorphic species-specific microsatellite loci in Peninsular Malaysia (Kedah and Penang) for natural and metropolitan populations.

Locus	k	Number of individuals (*N*)	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	PIC	F
GS1	74	80	0.73	0.98	0.963	0.1481
GS3	40	80	0.44	0.95	0.946	0.3692
GS4	49	80	0.79	0.97	0.961	0.1004
GS10	46	80	0.45	0.96	0.959	0.3634
GS15	49	80	0.44	0.97	0.913	0.3768
GS27	43	80	0.53	0.96	0.942	0.2957
GS29	62	80	0.83	0.98	0.961	0.0837

k (number of alleles at each locus); F (null allele); PIC (polymorphic information content).

Colony Breeding Pattern

The subterranean termites collected from eight population sites were morphologically identified as *G. sulphureus* termite species according to Hussin and Ab Majid (2017) and Hussin et al. (2018). There is a diverse variation in terms of the distribution among simple and mixed family colonies presented in the eight assigned populations in Peninsular Malaysia. As shown in Table 4, most colonies are derived from mixed families (60%) in both natural and metropolitan areas. Mixed family colonies have more genotypes produce by a single pair of reproductive parents. Each natural and metropolitan region also includes simple family colonies of *G. sulphureus*.

Table 4. Breeding structure of natural and metropolitan populations of the *Globitermes sulphureus* subterranean termite.

Population sites	Number of colonies	Number of simple family colonies	Number of mixed family colonies
Natural (TB, SL, KT, NP)	10 Subtotal = 80	1 (20%)	3 (60%)
Metropolitan (TJ, TA, PU, AU)	10 Subtotal = 80	1 (20%)	3 (60%)

All population sites combined = 160. Abbreviations refer to Table 5.1.

Genetic Differentiation, Colony Genetic Structure, and Relatedness

Table 5 exhibits the F-values obtained from the computer simulations. For simple family colonies, all workers in two populations (PU and TB) are the most significantly inbred (F_{IT} = 0.483). The inbreeding (FIT) measure has positive values, which indicates a general deficit of heterozygosity compared to the expected genotypic HWE. Furthermore, both are relatively connected due to the moderate value of the relatedness coefficient (r = 0.121). Simple families show a moderate genetic differentiation (F_{ST} = 0.090) since the F_{ST} ranges between 0.05 and 0.25. F_{ST} of 0 indicates the populations are not genetically differentiated. Therefore, the populations show identical allele frequencies. Meanwhile, F_{ST} of 1 indicates populations are fixed for different alleles.

Table 5. Summary of F-statistics (F_{IT}, F_{IS}, and F_{ST}) and relatedness coefficient (*r*) for workers of *Globitermes sulphureus* subterranean termite from simple, mixed family colonies and all populations focused in the Northern part of Peninsular Malaysia.

Colonies	F_{IT} (S.E)	F_{ST} (S.E)	F_{IS} (S.E)	*r* (S.E)
Simple families (n=2)	0.483 (0.128)	0.090 (0.020)	0.432 (0.130)	0.121 (0.019)
Mixed families (n=6)	0.358(0.055)	0.082 (0.010)	0.301 (0.056)	0.121 (0.013)
All populations (n=8)	0.391(0.066)	0.086 (0.011)	0.333 (0.067)	0.124 (0.013)

Compared to simple family colonies, mixed family colonies show less substantial inbreeding (F_{IT} = 0.358) in all six populations. Average relatedness values within simple and mixed family colonies are similar (r = 0.121), as shown in Table 5.5. F_{ST} of 0.082 is recorded for mixed families, showing a moderate genetic level differentiation among colonies. F_{ST} values in the range of 0 - 0.05 indicate the populations have little genetic differentiation. F_{ST} values between 0.05 - 0.25 represent moderate genetic differentiation, while F_{ST} values higher than 0.25 manifest a high genetic differentiation level. This study found a positive F_{IS} value (0.301) for mixed family colonies, indicating an absence of excessive heterozygosity, similar to the simple family colonies (F_{IS} = 0.432) of *G. sulphureus*.

F_{ST} values of eight *G. sulphureus* populations are significantly greater than zero (positive), according to the permutation test (P < 0.005), which shows a significant genetic differentiation among colonies. Thus, all eight populations of *G. sulphureus* have a moderate genetic differentiation (F_{ST} = 0.086) and a low inbreeding level based on the low F_{IT} value of 0.391.

Table 6 summarizes the F-statistics for *G. sulphureus* workers from natural and metropolitan populations, particularly in Peninsular Malaysia. The total inbreeding (F_{IT}) level for metropolitan populations (F_{IT} = 0.415) is higher than natural population sites (F_{IT} = 0.371). These values indicate metropolitan populations have a greater inbreeding level variation among individuals of *G. sulphureus* compared to natural populations. Although both populations experience a moderate genetic differentiation, *G. sulphureus* individuals in metropolitan populations show slightly less differentiation, as evident from a relatively lower F_{ST} (0.082) than natural populations (F_{ST} = 0.097). Moreover, both natural (F_{IS} = 0.303) and metropolitan (F_{IS} = 0.363) populations experience a low level of inbreeding indicated by F_{IS} < 1. Thus, the populations have a high level of heterozygosity in all seven loci. The finding suggests the Wahlund effect may not affect the seven loci allele distribution within the eight populations.

Table 6. Summary of F-statistics (F_{IT}, F_{ST}, and F_{IS}) for workers of *Globitermes sulphureus* subterranean termite from natural and metropolitan populations focused in the Northern part of Peninsular Malaysia.

Population sites	Classification	F_{IT}	F_{ST}	F_{IS}
Natural (TB, SL, KT, NP) n=4	Forests Paddy field Oil palm trees or plantations	0.371	0.097	0.303
Metropolitan (TJ, TA, PU, AU) n=4	Street trees Housing areas Business premises	0.415	0.082	0.363

*Abbreviations refer to Table 1.

Inbreeding coefficients (F_{IS}) for simple families' reproductive and relatedness values between reproductive nestmates in the colonies are based on genotypes, as inferred from the worker offspring (Table 7). Both populations in Penang (Palapes USM) and Kedah (Tikam Batu) have a high relatedness reproduction coefficient of r = 0.087 and r = 0.154, respectively. However, neither show a significance greater than zero (both P > 0.1, t-test). In Palapes USM (Penang), simple family colonies' functional reproduction shows slightly less inbreeding than the workers in Tikam Batu (Kedah). However, the differences are insignificant based on the overlapping 95% confidence intervals.

Table 7. Relatedness coefficient (*r*) between nestmate reproductive and inbreeding (F_{IS}) reproductive in simple family colonies of *G. sulphureus*.

Population	*r*	F_{IS} (95% C*I*)
Palapes USM (PU), Penang	0.087	0.204
Tikam Batu (TB), Kedah	0.154	0.678

Table 8 shows the measured genetic diversity for *G. sulphureus* workers. Mean of alleles per loci, observed (Ho) and expected (He) heterozygosities, and estimations of F_{IS} within eight *G. sulphureus* termite populations (natural and metropolitan regions). The mean of alleles across loci is higher than 9 in most of the populations. Two populations (MU and NP) show the lowest mean of alleles per loci. Interestingly, population MU is located in a metropolitan area, while population NP is in a natural area.

Also, the heterozygosity deficit measured by F_{IS} is positive in most populations when averaged across loci, ranging from 0.015 to 0.066. The average F_{IS} across loci and populations ranging from 0.190 (population AU) to 0.640 (population MU). Among eight populations of *G. sulphureus*, pair-wise F_{ST} values show an overall genetic differentiation of 0.086 and pair-wise F_{ST} values ranging from 0.047 to 0.134. Significant (α = 0.05) genetic differentiation is found after sequential Bonferroni correction is performed to evaluate population pairs' significance.

Table 8. Gene diversity measures for workers of *Globitermes sulphureus* subterranean termite from both natural and metropolitan populations.

Population	*Q*	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	F_{IS} (IC 95%)
TJ	9.57	0.671	0.887	0.253
PU	8.29	0.314	0.844	0.640
TA	11.29	0.586	0.923	0.378
AU	10.71	0.743	0.908	0.190
*TB	9.14	0.686	0.877	0.228
*SL	9.71	0.657	0.893	0.275
*KT	9.71	0.700	0.881	0.214
*NP	7.71	0.429	0.836	0.501

Q (Mean number of alleles per loci); * indicate natural populations. Abbreviations refer to Table 5.1.
** 10000 Bootstrap over F_{IS} by population, IC 95% = confidence interval at 95%.

Genetic distance by PCoA and AMOVA analysis

Based on the PCoA analysis (Nm values), samples from four populations failed to prove different due to overlapping in a particular location site (Figure 2). Nm values are based on F_{ST} values, which indicate the genetic distance between populations. This study shows that PU and AU populations are the most genetically related populations with an F_{ST} of 0.088. The SL population is related to the TJ population with an F_{ST} of 0.045. Although PU, AU, SL, and TJ populations might be closely related to each other, PU and AU populations have the highest genetic correlation. The acquired genetic distance for *G. sulphureus* populations in this study shows a positive relation with geographical location for eight populations in Peninsular Malaysia (Figure 1). TJ, TA, KT, TB, and SL populations are located in northeast Peninsular Malaysia (near each other), while AU, PU, and NP populations are in southwest Peninsular Malaysia.

The moderate level of individual relatedness within aggregated populations suggests *G. sulphureus* individuals are moderately related. The observed moderate values are due to the moderate genetic differentiation among populations and the moderate gene flow among them. These results demonstrate individuals of *G. sulphureus* could interbreed because of active diffusion, with a percentage of AMOVA within the population of 94% and among populations of 6% (Table 9).

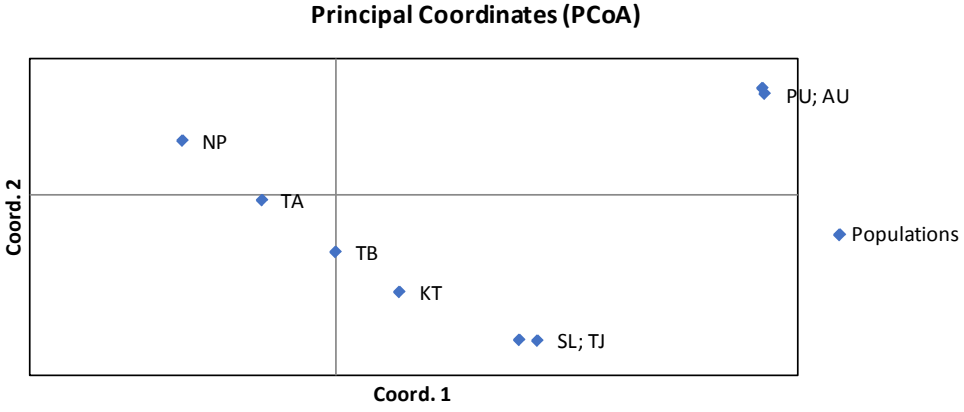

Fig 2. Principal coordinates analysis (PCoA) among the eight population sites based on *G. sulphureus* colony coordinates along axes of variation. Abbreviations refer to Table 1.

Table 9. Summary of AMOVA analysis.

Source	df	Est. Var.	%
Among Populations	7	0.219	6%
Within Populations	72	3.260	94%
Within Individual	80	0.000	0%
Total	159	3.479	100%

*Est. Var. (Estimation variance)

Hardy–Weinberg Equilibrium (HWE) tests

HWE results across loci and populations are shown in Table 10 (a and b). Following Fisher's statistical definite test for HWE, highly significant p-values ($p < 0.001$) for multi-locus departures from HWE proportions are found in most populations. Significant p-values ($p < 0.05$) are observed in TJ, AU, and TB populations. However, the single locus test across populations to access the deviation from HWE shows

no significant p-values other than for the GS4 microsatellite marker. Other loci mostly show highly significant p-values.

Regarding the linkage disequilibrium for seven microsatellite loci pairs, the definite test results show seven of eight population combinations with significant linkage disequilibrium. However, inconsistent patterns are found across loci or populations. Thus, the seven polymorphic microsatellite loci used in the analysis are independently assorted markers conforming to HWE and suitable for the colony and population genetic analysis.

Table 10 (a). Hardy-Weinberg Equilibrium (HWE) exact test in the subterranean termite *G. sulphureus* populations based on populations.

Population	P-value	S.E.
TJ	*0.0282	0.0091
PU	0.0000	0.0000
TA	0.0000	0.0000
AU	*0.0174	0.0045
TB	*0.0300	0.0045
SL	0.0016	0.0016
KT	0.0000	0.0000
NP	0.0000	0.0000

Markov chain parameters for all tests: Demorization: 1000; Batches: 100; Iterations per batch: 1000. S.E. (standard error). Abbreviations refer to Table 5.1.
*No significant departure from Hardy-Weinberg equilibrium.

Table 10 (b). Hardy-Weinberg Equilibrium (HWE) exact test in the subterranean termite *G. sulphureus* populations based on loci.

Locus	P-values	S.E.
GS1	0.0000	0.0000
GS3	0.0003	0.0002
GS4	*0.0422	0.0122
GS10	0.0000	0.0000
GS15	0.0000	0.0000
GS27	0.0000	0.0000
GS29	0.0000	0.0000

Markov chain parameters for all tests: Demorization: 1000; Batches: 100; Iterations per batch: 1000. S.E. (standard error).
* No significant departure from Hardy-Weinberg equilibrium.

Bottleneck tests

Table 11 shows evidence of a bottleneck effect in two populations (TB and NP). Both populations undergo a heterozygosity deficit with the shift-mode estimation of allele frequencies, thus suggesting the occurrence of genetic drift (Piry et al. 1999). Also, the probabilities of mutation–drift equilibrium using both I.A.M. and S.M.M. are high for the AU population at 0.46 and 1.00, respectively. KT population shows a moderate probability of mutation–drift equilibrium using I.A.M. (0.375) and S.M.M. (0.039) (Table 12).

Table 11. Bottleneck test analysis that showed P-value within eight populations of *G. sulphureus* in Peninsular Malaysia.

Population	I.A.M	T.P.M	S.M.M	Mode Shift	P(He)
TJ	0.464	0.592	0.575	Normal	0.886
PU	0.025	0.174	0.641	Normal	0.844
TA	0.020	0.034	0.652	Normal	0.923
AU	0.373	0.637	0.602	Normal	0.908
TB	0.027	0.032	0.158	Shifted	0.877
SL	0.162	0.444	0.305	Normal	0.893
KT	0.394	0.552	0.110	Normal	0.394
NP	0.403	0.384	0.419	Shifted	0.836

I.A.M (Infinite Allele Model); T.P.M (Two-Phase Model);
S.M.M (Stepwise Mutational Model)

Discussion

Microsatellite genotyping is a tool to amplify and analyze individual loci. This method provides robust markers for target alleles containing simple repetitive sequences with a high mutation rate in the genome's non-coding regions (Guichoux et al., 2011). For instance, several studies on termites have used microsatellite marker accessibility to study the wood-dwelling termite *Kalotermes flavicollis* (Luchetti et al., 2013), the subterranean termite *Reticulitermes grassei* (Dronnet et al., 2015), and the soil-feeding termites *Embiratermes neotenicus* and *Silvestritermes minutus* (Fougeyrollas et al., 2018).

Table 12. Summary of probability value for mutation-drift equilibrium from Wilcoxon test.

Population	I.A.M	S.M.M
TJ	0.375	0.812
PU	0.007	0.578
TA	0.007	0.468
AU	0.468	1.000
TB	0.007	0.015
SL	0.023	1.000
KT	0.375	0.039
NP	0.109	0.812

I.A.M (Infinite Allele Model);
S.M.M (Stepwise Mutational Model)

Additionally, a genotyping assay from microsatellite markers reveals information on ancestries and relationships of individual termites, colonies, and populations, as discussed in the Mendelian inheritance rules (Glass, 2017). Alleles with high variability in repetitive numbers and co-dominant characteristics have possible variations in length (Keller & Waller, 2002). Therefore, the proportion of termite individuals carrying different alleles at gene loci on corresponding chromosomes (heterozygotes) in *G. sulphureus* colonies and populations can be detected and analyzed using F-statistics procedures (Goodisman & Crozier, 2002). From eight colonies of *G. sulphureus* screened for variability using seven primer pairs, all loci show variation within all population sites with 3 to 9 alleles per locus. We conclude that a large amount of genetic variation is detected.

From the results shown in Table 2, observed heterozygosity (Ho) is less than expected heterozygosity (He) in seven polymorphic loci (GS1, GS3, GS4, GS10, GS15, GS27, and GS29) with significant deviation from HWE ($P < 0.05$). This might be due to the sample collection strategy or unique only to the tested populations. Genetic diversity within the population varies as all seven tested loci are observed in all eight populations of *G. sulphureus* (Table 7) due to the various populations' heterozygosity levels. We propose that these populations are genetically diverse and have excellent survival prospects. Genetically diverse populations with high heterozygosity levels have greater survival prospects during regular environmental changes. The opposite occurs with genetically homogenous populations with high homozygosity levels (Frankham, 2005).

Although Palapes USM (PU) and Penang National Park (NP) are island populations, a constant phenomenon was not observed. An island population usually experiences low genetic diversity due to high genetic drift caused by its small population size. The outcome of genetic drift is severe in small and isolated populations (Keller & Waller, 2002). However, this population did not suffer low genetic diversity (HE for PU = 0.844; HE for NP = 0.836), probably due to selection. Kaeuffer et al. (2007) reported selection is the most likely mechanism responsible for heterozygosity to increase in a small population over time. Selection may affect the changes in allele frequency. However, the genetic drift effect caused by selection is sometimes hidden. Thus, selection may reduce the impact of genetic drift on the loss of genetic diversity. Genetic drift can cause alterations in allele frequencies of a population from time to time due to variability of the reproductive success rate within a population. Some individuals produce more offspring than others. The impact of genetic drift can be seen frequently in small populations such as an island population.

However, all eight populations of *G. sulphureus* show no homozygosity (HO = 0). This finding suggests all of the studied populations have not experienced a high level of genetic drift and natural selection. Therefore, some alleles are fixed in the populations and low genetic diversity. Under natural selection, individuals tend to adapt to their local environmental conditions, leading to local adaptation (Lenormand, 2002). There are two possibilities: (i) if an environmental condition prefers an allele, the selection direction skews towards it in the population, and (ii) upon a negative effect of mutation or the allele is less preferred, the selection process removes the allele from the population (Kawecki et al., 1997), decreasing the genetic diversity level within a population.

Meanwhile, under genetic drift, alleles are randomly fixed or lost, leading to a low genetic diversity level (Lande, 2015). Genetic diversity is essential for the survival and adaptation of a population (Frankham, 2005). Reducing genetic diversity lowers alleles fitness and thereby decreases the evolutionary potential of species to adapt to a changing environment.

This study establishes two types of colonies: simple family colonies (40 %) and mixed family colonies (60 %). Previous research on the colony's breeding structure suggested most subterranean termite populations are composed of different proportions of simple and extended colonies, while mixed colonies are generally less common (Vargo & Husseneder, 2009). However, current data show mixed family colonies are predominant in six populations (SL, KT, NP, TJ, TA, and AU), followed by simple family colonies (TB and PU). Simple family colonies in Palapes USM (PU) and Tikam Batu (TB) are headed by inbred, related, monogamous reproductive pairs, which suggests that dispersal by primary reproductive parents is limited in those populations. These findings support the notion that simple family colonies are classified as being headed by the original colony-founding reproductive pairs.

On the contrary, colonies headed by more than a pair of primary reproductive parents having more than five alleles per locus are identified as mixed family colonies. Mixed colonies form when two different individuals from two different colonies fuse and breed together (Ab Majid et al., 2013). The fusion of colonies, invasion of mature colonies by other alates, or sharing of foraging galleries by neighboring colonies is other factors that may lead to the formation of a greater mixed colonies proportion (DeHeer & Vargo, 2004; Aldrich & Kambhampati, 2007). For instance, mixed family colonies result from colony fusions in natural and metropolitan populations of *G. sulphureus* termites. Other than that, this study uses a large sample size, which might contribute to the greater percentage of mixed family colony formation in *G. sulphureus* populations (Ross & Carpenter, 1991). Besides, high mixed families proportion in this study is commonly found in nature (KT, NP, and SL) as supported by studies on *Zootermopsis nevadensis* termite (Howard et al., 2013) and wood-dwelling termite *Kalotermes flavicollis* (Luchetti et al. 2013).

The breeding structure could not be resolved in colonies that did not fit the expected genotype frequencies for the progeny of a simple family containing less than four alleles at all loci. Populations of *G. sulphureus* termites focus

in northern Peninsular Malaysia reveal a substantial variation in colony breeding structures, probably due to the mixed colonies' inbreeding levels in the TB population. Proportions of termite colonies with different breeding systems vary across populations depending on the age of colony structure, dynamics of the colony–colony interactions, food quantity and quality, soil characteristics, and disturbance or treatments (Bulmer et al., 2001; Aluko & Husseneder, 2007).

It has been suggested that F_{ST} between 0.05 to 0.15 indicates a moderate genetic differentiation, while values in the range of 0.15 to 0.25 indicate a great genetic differentiation. F_{ST} above 0.25 indicates an exceptionally great genetic differentiation. Based on this rule, the F_{ST} of this study is in the range of 0.05 to 0.15 (F_{ST} = 0.082 to 0.097), indicating a moderate genetic differentiation. Furthermore, the GST range is equivalent to F_{ST} obtained between natural and metropolitan populations of G. *sulphureus* (G_{ST} = 0.069), which also show a moderate genetic differentiation. The moderate genetic differentiation between both populations might indicate incomplete isolation (Pamilo et al., 2016;).

The dispersal or migration of *G. sulphureus* termites occurs in several ways. For instance, Julio et al. (2002) claimed rafting of wood pieces containing reproductive pairs could be an effective means of dispersal for some termite species. In a tropical country such as Malaysia, the rainy season occurs several times a year, facilitating the transportation of infested wood pieces to a new infestation area via rainwater flow. Besides, blowing out alates from their nest by strong winds could be considered another possible dispersal mode. Winged termites can disperse over 800 m. Most studies on termites reveal that the emergence of alates usually occurs during the middle of the year, which corresponds to Malaysia's annual rainy season between July and November (Tong et al., 2017).

Therefore, an alate flight phenomenon might occur since the collection of *G. sulphureus* termites was performed from October 2017 to February 2018. Additionally, frequent migration among populations is necessary to achieve a moderate genetic differentiation between natural and metropolitan populations. The frequencies observed rarely occur by wood rafting due to a very stochastic process, but most probably is caused by the most frequent migration, the alates' flight. Furthermore, relatively low G_{ST} values between natural and metropolitan populations demonstrate a gene flow phenomenon between both populations.

The current study demonstrates a considerable variation in the *G. sulphureus* colony structure over a small spatial scale, including colonies headed by monogamous outbred primary reproductives and colonies containing multiple inbred neotenic reproductives. This result reflects the number of reproductives and nestmate relatedness. Polymorphic species-specific microsatellite markers are employed to determine the social organization of *G. sulphureus* colonies at two sites in Peninsular Malaysia. The level of nestmate relatedness and inbreeding coefficient within and among colonies are estimated. This information helps to infer the nature of colony founding and reproductive structure by comparing the empirical results with computer simulations for different breeding schemes. The results indicate a remarkable variation in *G. sulphureus* colony organization.

Thorne et al. (1999) stated the inbreeding phenomenon is common in subterranean termites. F_{IS} is the measure of inbreeding in populations under random mating. The positive value for all eight populations, as shown in Table 7, indicates there are more related individuals in the population under random mating (Wright, 1965). In a previous study on *Odontotermes* termite, the high level of inbreeding suggests a shorter mating flight range; thus, they are more likely to pair with relatives during colony founding (Cheng et al., 2013). Inbreeding reduces heterozygosity, which has also called the Wahlund effect. Inbreeding also contributes to a high level of genetic relatedness among workers in colonies (Dronnet et al. 2005). The Wahlund effect shows excessive homozygosity in a population is due to the existence of subdivision fragmentation. This effect may have occurred in the current study since the seven tested loci had an overlapped distribution for all eight tested populations with unknown stratification. In summary, the high and significant F_{IT} and F_{ST} close to zero indicates lots of related breeders in each colony, leading to a significant genetic differentiation among colonies and inbreeding (Painter et al., 2000).

Nm values are based on F_{ST}, which is equivalent to genetic distances between populations. In this study, several samples from four populations did not reach a clear distinction due to overlapping at a particular location site (Figure 2). This study shows PU and AU populations are the most genetically closely related populations with an F_{ST} of 0.088. In contrast, SL populations are close to TJ populations with an F_{ST} of 0.045, a coefficient possibly resulting from the short distances between PU, AU, SL, and TJ sampling sites. Comparison between island populations (NP, PU, and AU) and mainland populations (SL, TJ, KT, TB, and TL) generally shows a high genetic distance. This outcome may be due to the small sample size of island populations which yields a high value of Nei's genetic distance (Nei, 1987.

As Julio et al. (2002) described, the dispersal or migration of termites in islands such as PU and AU populations may occur in several ways. For instance, the rafting of wood pieces containing reproductive parents could be an effective means of dispersal for *G. sulphureus* species (Gathorne-Hardy et al., 2000). Strong winds hit Penang island several times a year, facilitating the transportation of infested wood pieces to the sea via inland temporal and permanent rivers, carrying them to other islands. A similar study on *Nasutitermes takasagoensis* termite in Japan showed that strong typhoon winds blow out alates, possibly establishing a mode of overseas dispersal. Nevertheless, anthropogenic dispersal is another possible means of putative dispersion for *N. takasagoensis* termites (Julio et al. 2002).

In particular, the obtained genetic distance for all eight populations in this study is positively related to the populations' geographical locations in Peninsular Malaysia (Figure 2) (Pironon et al., 2015; Schwalm et al., 2016). TJ, TA, KT, TB, and SL populations are in northeast Peninsular Malaysia (near each other), while AU, PU, and NP populations are in southwest Peninsular Malaysia.

Several factors influence the relationship between allele frequencies and genotype frequencies, e.g., mutation, population size, mating strategy, natural selection, and gene flow. In the absence of these factors, allele and genotype frequencies conform to a simple relationship known as the Hardy–Weinberg equilibrium (HWE) (Harrison et al., 2018. The concept of HWE states in non-evolving populations, allele and genotype frequencies remain constant from generation to generation. The populations are then regarded as being in HWE. Therefore, at a single locus, any deviation from HWE is eradicated after one generation of random mating (Allendorf, 2017). From Table 10, four populations (PU, TA, KT, and NP) deviate from HWE (all $p = 0.000$), indicating they are under selection, causing rapid changes in allele frequencies since many alleles are lost except for the favorable alleles (Miller et al., 2001; Williams, 2018). Five polymorphic loci (GS1, GS10, GS15, GS27, and GS29) are probably under selection. Besides, allele frequencies may also deviate from HWE if the sample size is small (Salanti et al., 2005). However, the effect of sample size is very modest (Ioannidis al., 2001). The sample size used in this study is sufficient to obtain a good HWE analysis ($n = 10$ per population). Another factor contributing to HWE deviation is non-random mating or inbreeding, which commonly occurs in isolated populations (Reddy, 2017). Since PU and NP are island populations, the effect of inbreeding may be high; thus, these populations deviate from HWE.

When a population deviates from HWE, genotype frequency shifts. For example, as inbreeding occurs, the population stops undergoing random mating, and the homozygote genotypes frequency increases with the decreasing frequency of heterozygote genotypes (Hamilton, 2011). This is due to relatives, by definition, are more likely to inherit the same ancestral alleles from a common ancestor; this is known as being identical by descent (IBD) (Powell et al., 2010). A population consisting of inbred individuals is expected to show excessive homozygosity over HWE expectations, which can be detrimental as recessive mutations continue to segregate within the population, resulting in inbreeding depression (Charlesworth & Charlesworth, 1987).

S.M.M signifies that an allele only mutates by losing or gaining single tandem repetitive alleles, possibly among alleles already present in the population. On the contrary, under I.A.M, a mutation involving any number of tandem repeats always produce alleles not commonly encountered in the population (Hardy et al., 2003; Roussel et al., 2004). Since the estimation of effective population size and mutation rates depend on the mutation model, computational assays are performed to test the adequacy of each model with the observed data. The results (Table 10) show five populations (TJ, AU, SL, KT, and NP) might have a low probability of undergoing the mutation and genetic drift for the seven tested loci using both I.A.M. and S.M.M ($p > 0.05$). In contrast, MU, TA, and TB populations have high chances of mutation and genetic drift ($p < 0.05$) for I.A.M but not for S.M.M (p-value MU = 0.641; p-value TA = 0.652; p-value TB = 0.158). The cause of these outcomes may be these populations' geographical location (Wlasiuk et al., 2003). The nesting sites of *G. sulphureus* in these populations are surrounded by sub-optimal local environments such as being placed far from any food source (Taylor & Hellberg, 2003).

In particular, the probability of mutation-drift equilibrium using both I.A.M. and S.M.M. is high for the AU population (0.46; 1.00) and moderate for the KT population (Table 5.11). The latter shows a moderate (0.37) and low (0.03) probability of mutation-drift equilibrium using I.A.M. and S.M.M., respectively. This result can be evidence that the KT population may be suffering from a mutation effect. The mutation–drift equilibrium test can also be applied to study inbreeding depression by measuring the correlation between individual fitness and inbreeding. The result shows the heterozygosity level of individuals or the population. This test also provides insights into evolutionary interpretation using the information on relative values of mutation rates compared to the migration rate or population divergence (Hardy et al. 2003).

Conclusion

This study is the first one to focus on the population, colony genetic structure, and dispersal pattern of *G. sulphureus* termites within natural and metropolitan regions. In conclusion, geographic variation and urbanization affect the genetic structure of *G. sulphureus* colonies. A suitable and flexible environmental habitat plays a vital role in driving genetic differentiation. The current study suggests *G. sulphureus* in northern Peninsular Malaysia can be characterized by moderately related and inbred individuals between populations. The high level of genetic diversity among and within the populations and the moderate genetic differentiation found out in this study show that *G. sulphureus* is most likely to mate with moderately related mates. With increasing globalization due to trade, human-mediated transportation, seasonal weather, and tourism, subterranean termites of *G. sulphureus* spread rapidly to infest new regions such as housing and business areas. A comprehensive understanding of the population dynamics of *G. sulphureus* colonies provides an improvement in response to higher group termite colonies and population management tactics, especially for higher termite group baiting.

Acknowledgments

The Universiti Sains Malaysia funded this research work (USM) Bridging Research Grant: 304/PBIOLOGI/6316010.

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