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The Breeding Pattern and Population Genetic Structure of *Coptotermes gestroi* (Blattodea: Rhinotermitidae) Population in Natural Woodland Habitats

Naveeta Vellupillai[1], Abdul Hafiz Ab Majid[1,2]

1 - Household & Structural Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, 11800, Minden, Penang

2 - Centre for Insect Systematics, Department of Biological Science and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor 43600, Malaysia

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Corresponding author
Abdul Hafiz Ab Majid
Household & Structural Urban Entomology Laboratory
Vector Control Research Unit
School of Biological Sciences
Universiti Sains Malaysia, 11800
Minden, Penang.
E-Mail: abdhafiz@usm.my

Abstract

Microsatellite markers are suitable tools for assessing the population structure of eusocial species, especially those with a dynamic breeding system, such as the Asian subterranean termite *Coptotermes gestroi* (Wasmann) (Blattodea: Rhinotermitidae). Therefore, this study applied seven microsatellite markers to infer the breeding pattern and population genetic structure of *C. gestroi* found in natural woodland habitats at Universiti Sains Malaysia, Penang, Malaysia. The natural woodland habitat *C. gestroi* colonies show significant deviation from HWE (all $p < 0.05$). The uncovered genetic pattern suggested that the *C. gestroi* colonies presented a combined breeding pattern of mixed- and extended-family colonies with moderate genetic differentiation and elevated inbreeding. In particular, the breeding pattern of *C. gestroi* colonies was inferred to vary depending on the demographic variation and the age of the colony. Nevertheless, the results revealed comprehensive information on the *C. gestroi* population structure, habitat-specific to natural woodlands. Furthermore, future studies with exclusive datasets on the population structure of *C. gestroi* on marginal demography are necessary to enhance the management strategies of this pest species.

Introduction

Microsatellite markers or simple sequence repeats (SSR) are widely used to evaluate the genetic divergence within and among populations. Microsatellite markers have several features that make them convenient and practical for measuring the genetic structure and gene flow among eusocial insect species, including high reproducibility, compatibility among the same and related species, and a high degree of polymorphism (Vieira et al., 2016). The eusocial system is an advanced colonial existence found in some social insects that have three main characteristics: adult members originate from overlapping generations present in one group (*i.e.*, colony), collaborative care of juveniles, and a reproductive division of labor (Batley, 2016; Bradshaw & McMahon, 2008). Therefore, from a genetic perspective, microsatellite markers are suitable tools for assessing the population structure of eusocial species (de Pletincx & Aron, 2020; Kozyra et al., 2015; Smith et al., 2011; Zima et al., 2016).

Termites are eusocial organisms that exhibit varying morphologies and extensive breeding systems and structures (Vargo, 2019). The caste system facilitates divisions in roles depending on the morphological structure of castes, behavior, and function. Mainly in lower termites, the deeper-branching families, the fate of the colony is not dependent on individual reproductives but also through succession by neotenics that have the potential to replace the primary reproductives or act as supplementary reproductives (Chouvenc, 2022; Chouvenc

& Su, 2017; Myles, 1999). Over the last decade, through field census and genetic analyses, the breeding system of subterranean species has been categorized as simple families, extended families, and mixed families (Thorne & Traniello, 2003). In brief, simple families are headed by a single pair of reproductives. Suppose there are multiple secondary reproductives from the primary pair. In that case, the colony becomes an extended family, and mixed families are formed due to multiple founding pairs or when two or more colonies merge to form one colony (Vargo, 2019).

Coptertemes gestroi, the Asian subterranean termite, is recognized as an abundant invasive pest in Peninsular Malaysia and is known to cause damage to both structural and agricultural properties (Evans et al., 2012; Bakaruddin et al., 2018). According to a study by Yeap et al. (2011), *C. gestroi* population structure analysis suggested that three cities (Penang Island, Kuala Lumpur, and Singapore) had high gene flow with no significant isolation by distance considerably connecting the populations through human-mediated transportation of infested materials. In contrast, a study by Vellupillai et al. (2023) suggested *C. gestroi* populations from urban structures in Penang Island were inbred and moderately genetically differentiated due to physical barriers caused by geographic district boundaries. Therefore, exploring the genetic diversity of *C. gestroi* species in multiple settings is necessary to understand population plasticity, survivability, and adaptation to changing environments.

Previous studies have worked on the genetic pattern distribution of *C. gestroi* across different ecologies, but most of them pivot on samples from urban regions (Yeap et al., 2011; Zhang & Evans, 2017). Limited studies have been published to analyze the population structure of *Coptotermes* species in natural woodland habitats despite its high incidence rate. This study addresses the breeding pattern and population genetic structure of *C. gestroi* found in natural woodland habitats at Universiti Sains Malaysia, Penang, Malaysia.

Materials and Methods

Termite sample collection

Termite soldier samples were collected from natural woodland environments at Universiti Sains Malaysia, Penang, between June 2021 and January 2022. Each site was recognized based on the termite infestation on live trees, and the samples were collected from underground monitoring stations established at each site prior to sample collection. The infestation in the underground monitoring stations at each site was determined to be shared by the same colony based on the marked-recapture method and G-based differentiation test, respectively (Crosland & Su, 2006; Perdereau et al., 2019). Each site was identified to be relatively occupied by the same colony as marked individuals were found in all underground monitoring stations located near the initial station released with marked individuals, and non-significant genotypic differences were obtained ($P < 0.05$, G-test) for termite soldiers collected from different underground monitoring stations located in the same site. The sample sites were separated by a linear distance ranging from 500 m to 1 km within the campus. The inter-colony interaction in the present study is supported by previous findings by Husseneder and Grace (2001), in which a distance up to 39 km was determined between nonaggressive *Coptotermes* sp. colonies and a study on preliminary fusion testing for *Coptotermes gestroi* colonies by Guaraldo and Costa-Leonardo (2009) demonstrated low agonism of the caste and high tolerance to foreign reproductives. Table 1, Figures 1 and 2 show the location coordinates for the sampling sites and the depiction of the underground monitoring stations installed at each site within the natural woodland habitat. The termite samples were morphologically identified based on Tho (1974) and were preserved in vials containing 70% ethanol at -20 ºC (Marquina et al., 2020).

Termite foraging territory

Before sampling, oven-dried survey stakes (2 cm diameter × 8 cm height) were installed surrounding infested trees at each site. The survey stakes were planted approximately 2.5 cm beneath the ground and were checked weekly for infestation. The infested survey stakes were replaced with artificial Underground Monitoring Stations (UMS) (20 cm diameter × 19 cm height) filled with nine survey stakes (18 × 1.5 × 1.5 cm). Once termite activity was noticed in all the stations, the stations were labeled as active, and the colony territory was established (Wan et al., 2020).

Table 1. Details of *C. gestroi* population sampling sites in Universiti Sains Malaysia, Penang, Malaysia.

Abbreviation	Sample Code	Collection Site	Latitude	Longitude
BD	BD1b	BumbleDees Café, USM, Penang	5°21’44.9”N	100°18’27.5”E
SJ	SJ4b	Desasiswa Restu M02, USM, Penang	5°21’25.1”N	100°17’20.3”E
FR	FR1b	School of Pharmaceutical Sciences, USM, Penang	5°21’23.5”N	100°17’52.5”E
PK	PK1b	Padang Kawad, USM, Penang	5°21’19.2”N	100°17’36.9”E
CG	CG1b	Desasiswa Cahaya Gemilang, USM, Penang	5°21’35.4”N	100°18’12.9”E
MD	MD1b	Kopa Arena USM, Minden, Penang	5°21’34.6”N	100°18’31.7”E

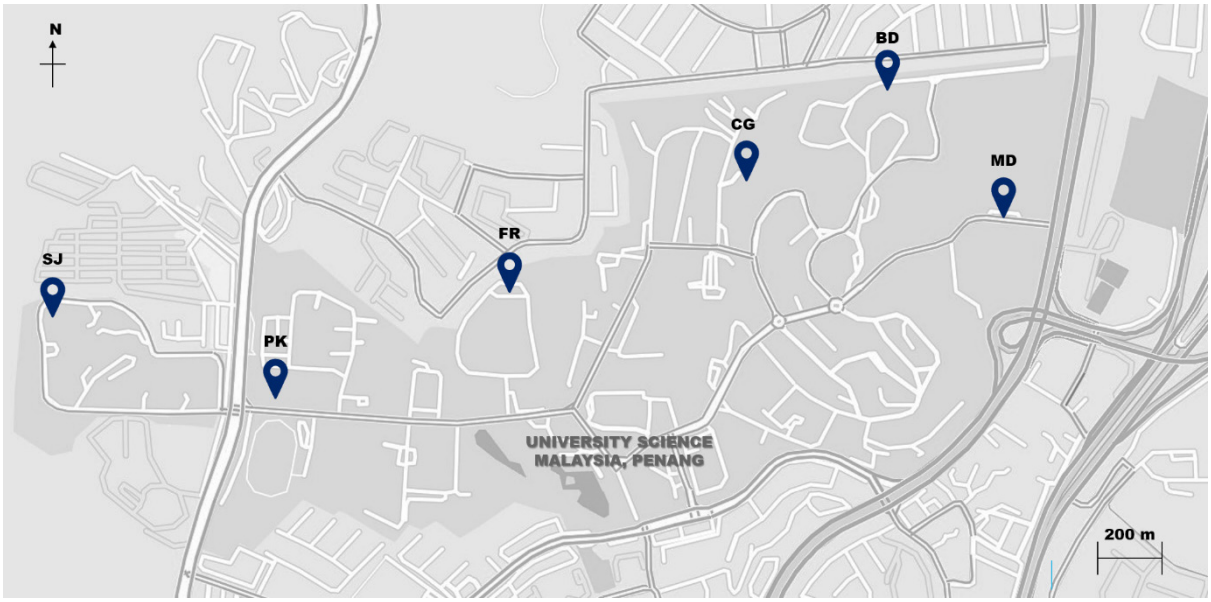

Fig 1. *C. gestroi* population sample collection sites in Universiti Sains Malaysia (USM), Penang, Malaysia.

DNA extraction and Microsatellite genotyping

The genomic DNA was exclusively isolated from the head of *Coptotermes gestroi* soldiers individually to prevent contamination by the gut microbiome. A total of 10 individuals were used for DNA extraction from each site. HiYield Plus Genomic DNA Mini Kit (Blood/Tissue/Cultured Cells) (Real Biotech Corp. Taipei Taiwan) was utilized based on the manufacturer's protocol with minimal modification to the steps (Seri Masran & Ab Majid, 2019). The elution step was modified to maximize the yield of genomic DNA. All purified DNA extractions were then quantified and validated using a spectrophotometer NanoDrop 2000c (Themoscientific, MA).

Each termite soldier was genotyped based on seven species-specific microsatellite markers: (Tm-Di08, Tm-Tr06, Tm-Tr08, Tm-Te-07, Tm-Te08, Tm-Te09, and Tm-Pe9) (Table 2) (Lim et al., 2021). The basis for selecting the seven microsatellite markers was the applicability of the markers to produce highly polymorphic analysis in a previous study for the *C. gestroi* population structure (Vellupillai et al., 2023). The sequences are accessible in Sequence Read Archive (SRA) databases under Bio Project accession number SRR13105492. The total size of the PCR reaction mixture was 12.5 µL master mix (Qiagen Valencia, CA), 5.5 µL of distilled water, 1 µL of each primer (0.4 µM), and 5 µL of gDNA. The PCR amplification was then subjected according to the following setting: initial denaturation at 94 ºC for 10 minutes followed by 35 cycles of denaturation phase at 94 ºC for 30 seconds, annealing phase at 61 ºC for 30 seconds, extension phase at 72 ºC for 1 minute and final extension phase at 72 ºC for 10 minutes. The PCR ended with being held at 4 ºC.

Table 2. Fragment analysis of seven microsatellite markers.

Loci	Primer	Type of repeat motif
TmDi08	F: GTTACACCCGATGACACTCAG R: GGCTGTTGTTTCGTCCAGAG	Dinucleotide
TmTr06	F: AGACACGTGGCAAGTATAACG R: TCGTCACTATTCCTGCTGCC	Trinucleotide
TmTr08	F: TGACACCAACAAATGCACCC R: GCATAAGTTGACGGACCCTG	Trinucleotide
TmTe07	F: TGCCCTTCACGAACGAAC R: CGACTGCGTTGCTTTACAC	Tetranucleotide
TmTe08	F: AGAGCCATGTGACTTCGTG R: AACCACGCAGATAACGAGTG	Tetranucleotide
TmTe09	F: TCTGTGGAGTTAGTTAGTTGGC R: TGCCTATCCATCCACCTGTC	Tetranucleotide
TmPe09	F: TTAGGAGTGGCAAGTGAACC R: TTGGGTTGGGTTGGTTGGTC	Pentanucleotide

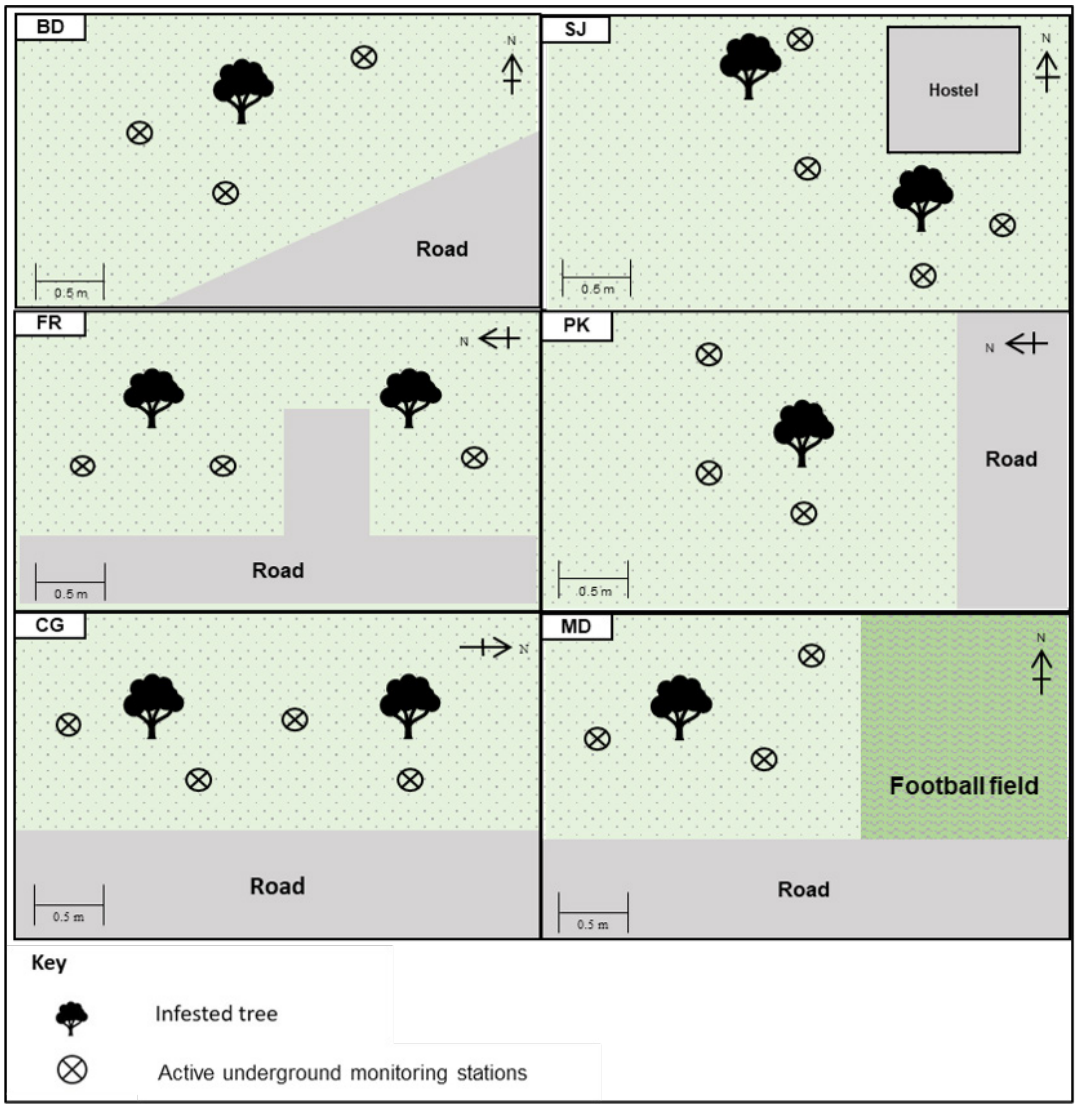

Fig 2. Overview of the locations of the study sites in Universiti Sains Malaysia where soldier samples were obtained from (BD, SJ, FR, PK, CG, and MD. Individual sites were depicted with the location of the infested trees and underground monitoring stations actively infested by *C. gestroi*. Light green areas indicate the natural woodland habitat.

The measurement of fragment sizes for all PCR products after electrophoresis visualization was performed using the Fragment Analyzer Automated CE system (Agilent Technologies, CA). Prosize version 5.0 software package was used to score the microsatellite allele data (Agilent Technologies, CA). Micro-Checker v2.2.0.3 software was used to detect any errors in the fragment analysis results due to failure in amplification, stuttering, and large allele dropout (Kim & Sappington, 2013; Van Oosterhout et al., 2004).

Population genetic diversity

The test for Hardy-Weinberg equilibrium (HWE) and Linkage disequilibrium (LD) across the loci and population was determined through the molecular biology software GENEPOP v4.7 (Rousset, 2017). Cervus v3.0.7 software was applied to process allele frequency analysis to delineate the breeding pattern of termite colony and Polymorphic Information Content (PIC) of each locus (Kalinowski et al., 2007). The FSTAT program was used to measure Wright's F-statistic (F_{IT}, F_{ST}, and F_{IS}) and relatedness coefficient of the population. The observed heterozygosity (H_O) and expected heterozygosity (H_E) in the population were further assessed through F-statistic output (Goudet, 2005; Weir & Hill, 2002). Analysis of Molecular Variance (AMOVA) was performed using a cross-platform tool, GenAIEx v6.5 to determine population differentiation among the different natural woodland habitats of *C. gestroi* population (Peakall & Smouse, 2012).

Results

Allelic diversity

A total of 60 *C. gestroi* individual soldiers were successfully genotyped using the seven appointed microsatellite markers. Within the six natural woodland habitat colonies, allelic diversity was 2 to 8 alleles per locus, with a mean of 4.22 alleles. The allelic polymorphic information content (PIC) varied from 0.674 to 0.912, averaging 0.842.

Table 3. Variability of seven polymorphic microsatellite loci for *C. gestroi* population from natural woodland habitats of Universiti Sains Malaysia, Penang.

Locus	Number of alleles							PIC	H_O	H_E	HWE	LD	F
	BD	SJ	FR	PK	CG	MD	k				(Y/N)	(Y/N)	
Tm-Di08	3	4	3	2	3	3	18	0.674	0.000	0.728	Y	N	1.0000
Tm-Tr06	4	2	2	3	4	3	18	0.811	0.000	0.840	Y	N	1.0000
Tm-Tr08	8	5	7	5	6	5	36	0.912	0.500	0.926	Y	N	0.2975
Tm-Te07	6	3	2	8	2	3	24	0.862	0.167	0.882	Y	N	0.6825
Tm-Te08	6	6	6	6	4	5	33	0.908	1.000	0.992	Y	N	0.0456
Tm-Te09	6	6	6	6	5	4	33	0.905	1.000	0.919	Y	N	0.0475
Tm-Pe09	3	3	2	2	3	2	15	0.820	0.000	0.846	Y	N	1.0000

k (number of alleles at each locus); PIC (polymorphic information content); observed heterozygosity (H_O); expected heterozygosity (H_E); Hardy-Weinberg Equilibrium (HWE); Linkage Disequilibrium (LD); F (null allele). Abbreviations refer to Table 1.

The observed heterozygosity (H_O) is 0.381, lower than the expected heterozygosity of (H_E) 0.876. TmTr08 showed the highest observed alleles per locus, while TmPe09 showed the lowest. That suggests sufficient genetic variability exists in the *C. gestroi* population assessed by the seven microsatellite markers. Table 3 shows seven loci evaluation for natural woodland habitat population varies between 15 to 36 alleles per locus.

Hardy-Weinberg Equilibrium (HWE) analyses

The HWE findings across loci and colonies are presented in Table 3 and Table 4. All seven markers show significant deviation for HWE ($p < 0.05$) with a high proportion of homozygotes, as the mean observed heterozygosity was 0.4914, and the expected heterozygosity was 0.876. However, no Linkage Disequilibrium was detected between the locus pairs after applying Bonferroni Correction ($\alpha = 0.05$). Hence, the results suggest that the loci were independent and individually segregated in the *C. gestroi* population (Du et al., 2016; Zima et al., 2016). Furthermore, Fisher's statistical definite test across the six colonies yielded highly significant p-values ($p < 0.05$) for multi-locus deviation from HWE.

Table 4. Hardy-Weinberg Equilibrium (HWE) exact test across colonies and observed heterozygosity (H_O) and expected heterozygosity (H_E) along with the inbreeding coefficient (F_{IS}) with 95% confidence intervals (CI) obtained for each *C. gestroi* colony.

Colony	HWE		H_O	H_E	F_{IS} (95% CI)
	P- value	S.E.			
BD	0.0000	0.0000	0.429	0.774	0.460
SJ	0.0005	0.0000	0.286	0.693	0.601
FR	0.0002	0.0002	0.429	0.639	0.341
PK	0.0007	0.0001	0.429	0.705	0.405
CG	0.0006	0.0001	0.429	0.703	0.403
MD	0.0014	0.0001	0.286	0.662	0.584

Markov chain parameters for all tests: Demorization: 1000; Batches: 100; Iterations per batch: 1000. S.E (standard error). Abbreviations in Table 1.

Breeding pattern and population genetic diversity

The colony breeding structure was derived according to Vargo and Husseneder (2009). *C. gestroi* colonies from six natural woodland habitats were classified as mixed-family colonies. As shown in Table 3, all the *C. gestroi* colonies exhibit complex genotypes that are not possibly produced by a single reproductive pair with five alleles or more at more than one locus. The mixed family breeding pattern is typical in mature colonies, suggesting the collaboration of several primary individuals during colony foundation responsible for production and persisted throughout the colony growth (Eyer et al., 2023). Analysis of the breeding pattern was also investigated using the F-statistic test through the FSTAT computer program. Table 4 shows observed heterozygosity and expected heterozygosity along with the inbreeding coefficient obtained for each colony, and Table 5 exhibits the summarized F-values for all the natural woodland habitat colonies. Among the colonies, the mean expected heterozygosity was 0.866 higher than the mean observed heterozygosity of 0.381. The Wright's inbreeding coefficient (F_{IS}) for the *C. gestroi* population was positive, ranging from 0.341 to 0.601 across loci, which was consistent with the excessive homozygosity exhibited within the population. The natural woodland habitat population showed an overall inbreeding (F_{IT}) of 0.573 (95% CI: lower 0.226, upper 0.896), and the average relatedness value population is 0.261 (95% CI: lower 0.200, upper 0.311). Overall, F_{ST} was estimated as 0.205 (95% CI: lower 0.142, upper 0.274), evincing moderate genetic differentiation within the population. An F_{ST} value between 0.15-0.25 was classified as moderate genetic differentiation (Curnow & Wright, 1979; Low et al., 2014). The positive F_{ST} value was more significant than zero, and through the permutation test ($P < 0.005$), significant genetic differentiation was detected among the *C. gestroi* population. The results of the *C. gestroi* breeding structure were further supported by the percentage of AMOVA. The findings suggested a significant genetic differentiation of the *C. gestroi*

population, with 78% molecular variance found within the colonies and 22% among the colonies.

Table 5. Summary of Wright's F-statistic (F_{IT}, F_{ST}, and F_{IS}) and relatedness coefficient with 95% confidence intervals (CI) for *C. gestroi* population from natural woodland habitats.

F_{IT} (95% CI)	F_{ST} (95% CI)	F_{IS} (95% CI)	Relatedness coefficient (95% CI)
0.573 (0.226-0.896)	0.205 (0.142-0.274)	0.456 (0.084-0.857)	0.261 (0.200-0.311)

Discussion

In this study, microsatellites revealed the breeding pattern and population genetic structure of the *C. gestroi* colonies found in natural woodland habitats of Universiti Sains Malaysia, Penang, Malaysia. The results suggest moderate genetic differentiation between the natural woodland habitat colonies (F_{ST} = 0.205). The positive inbreeding coefficient values F_{IS} and F_{IT} suggest substantial inbreeding within the *C. gestroi* colonies (Bankhead-Dronnet et al., 2015; Garnier-Géré & Chikhi, 2013). The seven microsatellite markers were reliable in detecting allele diversity among natural woodland habitat *C. gestroi* population, ranging from three to eight alleles per locus with high PIC value (PIC > 0.5). The colonies also exhibited a high number of alleles, with a maximum of eight alleles per locus. Similar allele frequency results were reported in a previous study by Yeap et al. (2011) performed on four large *C. gestroi* population groups found across Asia: Penang Island, Kuala Lumpur, Singapore, and Taiwan. Based on AMOVA analysis, the genetic differentiation within the colonies was more extensive than among the colonies, suggesting the natural woodland habitat *C. gestroi* colonies likely originated from a similar population source.

Besides, in a population, HWE remains constant between generations if there is no shift in the allele and genotype frequencies. This shift may occur due to generation overlap, mating systems, inbreeding, migration or selection, and gene flow in the population (Lachance, 2016). Table 4 shows that all the natural woodland habitat *C. gestroi* colonies show significant deviation from HWE (all $p < 0.05$). The likely assumption for the deviation is the occurrence of inbreeding in the natural woodland habitat colonies. Low observed heterozygosity and excessive homozygote genotype frequency found in the *C. gestroi* population further substantiate that the termite population comprised inbred individuals (Khizam & Ab Majid, 2021; Darvill et al., 2006; Jain et al., 2000). A similar result was reported for *Coptotermes lacteus* population, where deviation from HWE resulted from breeding preference for non-sibling relatives over completely unrelated mates, and confirmed deviation from HWE in *Reticulitermes chinensis* Snyder population due to inbreeding along with recent genetic bottleneck detected in the population (Huang et al., 2013; Thompson et al., 2007).

Within the natural woodland habitats of USM, Penang, Malaysia the termite colonies in all sites were recognized as a combination of mixed- and extended families. The *C. gestroi* colonies reveal complex genotypes with more than four alleles in multiple loci, suggesting mixed-family colonies. However, the possibility of extended family colonies was also considered due to the relatively high inbreeding within the colonies. Similarly, an earlier study by Zhang and Evans (2017) exhibited a combined breeding pattern of simple and extended families by *C. gestroi* in urban settings. Subterranean termite species are accustomed to both breeding patterns as mixed family colonies develop through the presence of multiple reproductives associating during the original nest foundation, and extended-family colonies appeared to have descended from the production of neotenics which may or may not be present along with primary reproductives (Aguero et al., 2020; Deheer & Vargo, 2004; Perdereau et al., 2015; Vargo, 2019). However, due to the cryptic lifestyle of *C. gestroi* and the timeline used in the study, the determination of individual colonies in natural woodland habitats was familiar to a particular breeding pattern.

According to a study by Chouvenc et al. (2022), each *Coptotermes* colony demographic trajectory is distinctive. The status of the colony may vary among the sites on the given sampling date depending on the demographic variation during that time. The breeding pattern is heavily influenced by the age of the colony, colony-colony interaction, population substructure, environmental factors, and spatial food resources that are widely available in the region (Aluko & Husseneder, 2007; Bulmer & Traniello, 2002; Majid et al., 2013). In particular, the *C. gestroi* colonies in natural woodland habitats progressively mature. Therefore, the soldiers sampled in the current study may be from mixed-generation aging and young cohorts imposing a cycle of inbreeding. This information is essential as *C. gestroi* population found in high-dynamic regions such as urban landscapes with fluctuating environmental conditions and demographic shifts may exhibit elevated genetic patterns to facilitate their survival and sustenance (Evans, 2021; Foll & Gaggiotti, 2006; Johnson et al., 2023; Vargo & Carlson, 2006).

Conclusion

To conclude, the current study focuses on extensive research with the application of microsatellite markers on the population genetic structure of the *C. gestroi* colonies found in natural woodland habitats. The *C. gestroi* colonies in natural woodland habitats of Universiti Sains Malaysia, Penang, were determined to have a combined breeding pattern of mixed- and extended-family colonies with substantial inbreeding and moderate genetic differentiation among the colonies.

However, the analysis is based on the breeding system and genetic structure of colonies from a single sampling, which needs demographic context and age of termite colony. Nevertheless, the study result represents a fraction of comprehensive information on the *C. gestroi* population structure, which is habitat-specific to natural woodlands. Hence, future studies with an exclusive dataset on the population structure of *C. gestroi* on marginal demography are necessary to enhance the management strategies of this pest species.

Authors' Contribution

NV: methodology, investigation, writing - review & editing. AHAM: conceptualization, methodology, supervision, project administration, resources, funding acquisition, writing - review & editing.

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