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GENETIC DIVERSITY AND PHYLOGENETIC RELATIONSHIP OF GERMAN COCKROACHES, *Blattella germanica* (L.) (BLATTODEA: ECTOBIIDAE) FROM URBAN COMMERCIAL SPACES

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ABSTRACT

Infestations of German cockroach (*Blattella germanica*) pose a significant threat to public health and the economy. Understanding its population's genetic diversity can improve pest control strategies to tackle this resilient pest. Therefore, this study focused on the genetic analysis of several populations of *B. germanica* collected from urban commercial spaces in Penang, Malaysia. Five urban commercial spaces, comprising restaurants and shopping centres, were selected from Bukit Mertajam, Juru, Bayan Lepas, and Gelugor. A total of 30 isolates were collected, and fragments of the mitochondrial cytochrome c oxidase subunit I (COI) gene and the 16S rRNA gene were amplified and sequenced. Genetic analysis revealed the formation of two haplotypes, separating *B. germanica* populations from shopping complexes and restaurants, with slightly higher nucleotide diversity observed in populations from shopping centers. Phylogenetic analysis using the Neighbor-Joining method also supported the observed separation between populations from restaurants and shopping centers. These findings suggest that the population genetics of *B. germanica* in urban areas is influenced

by human-mediated activities, reflecting their adaptability and ongoing evolution. This indirectly underscores the significance of refining the integrated pest management (IPM) strategies for more effective pest control in high-traffic commercial areas.

Keywords: German cockroach; *Blattella germanica*; genetic; pest; *COI*, 16S rRNA

ABSTRAK

Serangan lipas German (*Blattella germanica*) memberi ancaman yang signifikan kepada kesihatan awam dan ekonomi. Pemahaman tentang kepelbagaian genetik populasinya dapat menambah baik strategi pengawalan makhluk perosak yang berdaya tahan ini. Oleh itu, kajian ini fokus kepada penganalisisan genetik *B. germanica* daripada beberapa populasi yang di kumpulkan daripada kawasan komersial di bandar Pulau Pinang, Malaysia. Lima kawasan komersial bandar yang terdiri daripada restoran dan pusat membeli belah telah dipilih di Bukit Mertajam, Juru, Bayan Lepas, dan Gelugor. Sebanyak 30 isolat telah dikumpul, dan sebahagian daripada gen mikondria sitokrom c oksidase subunit I (COI) serta gen 16S rRNA telah diamplifikasi dan dijujuk. Analisis genetik menunjukkan pembentukan dua haplotip yang memisahkan populasi *B. germanica* daripada kompleks membeli belah dan restoran. Analisis filogenetik menggunakan kaedah Neighbor-Joining juga telah menyokong perpisahan di antara populasi restoran dan kompeks membeli belah. Hasil penemuan ini mencadangkan bahawa genetik populasi *B. germanica* di bandar telah dipengaruhi oleh aktiviti-aktiviti manusia, menunjukkan keupayaan mereka dalam beradaptasi dan sentiasa berevolusi. Secara tidak langsung, ini menekankan kepentingan penambahbaikan strategi pengurusan perosak bersepadu (IPM) ke arah yang lebih berkesan untuk pengawalan perosak di kawasan komersial tumpuan ramai.

Kata kunci: Lipas German; *Blattella germanica*; genetic; perosak; *COI*;16S rNA

INTRODUCTION

The German cockroach, *Blattella germanica* (Linnaeus) (Blattodea: Ectobiidae), is a significant urban pest worldwide. It is frequently found in human structures such as houses, restaurants, grocery stores, hospitals, and sewers, as well as other areas where food, water, and shelter are available (Marín-Miret et al. 2024; Nasirian & Salehzadeh 2019; Rajab et al. 2020). *Blattella germanica* is an omnivorous species that feeds on a diverse range of materials, such as human and pet food, cardboard, dead insects, and live plants, with a particular preference for protein- and carbohydrate-rich diets (Cruz & Sfara 2018; Marín-Miret et al. 2024; McPherson et al. 2021). Due to its presence in human environments, *B. germanica* is considered a health pest, as it can transmit harmful diseases to humans and act as a vector for various pathogens, including bacteria, viruses, and parasites responsible for vector-borne illnesses (Gits et al. 2023; Mond & Pietri 2023; Nasirian 2017a; Nasirian 2019a; Oz et al. 2024; Yulianti et al. 2023).

Previous studies have indicated a global rise in cockroach infestations and asthma cases, suggesting a possible correlation between the two (Nasirian 2017b). Later, a study by Evcen et al. (2024) revealed a connection between the sensitivity and frequency of asthma patients and their increasing sensitivity to cockroaches during the COVID-19 pandemic. The global mean infestation rate of German cockroaches (55.2%) (Nasirian 2017b) and their bacterial contamination rate (62.9%) (Nasirian 2019b) highlight their significance as a persistent pest. These infestations threaten human health and result in economic losses through food

contamination and increased medical expenses due to exposure to cockroach allergens (Tisgratog et al. 2023; Yulianti et al. 2023).

Despite its status as a significant pest affecting the economy and public health, controlling *B. germanica* remains challenging because of its resistance to 42 distinct insecticide-active ingredients worldwide (Gao et al. 2023; Zhu et al. 2016). To date, researchers have been actively working to tackle this issue by conducting studies to determine the genetic factors associated with insecticide resistance (Gao et al. 2023; Hu et al. 2021; Konkala & Narra 2024; Tisgratog et al. 2023; Tseng et al. 2024). In addition, researchers are also evaluating the effectiveness of new active ingredients such as isocycloseram (Lee et al. 2024b), bioinsecticides like essential oils (Manzanares-Sierra et al. 2025), and antibiotics (Marín-Miret et al. 2024). Moreover, they are investigating the artificial sweetener sucralose as an alternative control agent (Lee et al. 2024a) and assessing the impact of different baiting strategies, such as solid versus aqueous formulations, in controlling the German cockroach population (Oz et al. 2024).

Most of these studies have incorporated multiple cockroach populations in their research, including laboratory and wild strains. This approach is essential because each population is unique, as different strains exhibit different resistance profiles toward insecticides (Gits et al. 2023; Lee et al. 2024b). Therefore, understanding this important pest's genetic structure and diversity is critical for developing effective control strategies. Advancements in molecular analysis have significantly contributed to understanding genetic diversity and evolutionary patterns within different populations of the same species. Molecular analysis of the cytochrome c oxidase I (COI) and 16S rRNA genes have been widely used for species identification, population studies, genetic diversity, and phylogenetic relationships in diverse range of insects, for example, cockroaches (Crissman et al. 2010; Hashemi-Aghdam et al. 2017; Rajab et al. 2020; Tang et al. 2024; Vargo et al. 2024), fruit flies (Doorenweerd et al. 2024), stingless bees (Sayusti et al. 2023), and honeybees (Joaty et al. 2022).

Therefore, the main goal of our study was to investigate the genetic diversity and phylogenetic relationships of *B. germanica* populations using the COI and 16S rRNA gene from different urban commercial spaces in Penang, Malaysia. This study is significant as it can provide insights into how high-human-traffic urban commercial environments may influence the genetic adaptability of *B. germanica* populations.

MATERIALS AND METHODS

German Cockroach Sample Collection

Collections of *B. germanica* were made from five urban commercial spaces located across different areas in Penang, Malaysia, designated as ABM (Aeon Bukit Mertajam), RR (Ryujin Restaurant), AQB (Aeon Queensbay Mall), TP (T Palace Dining), and G (Gelugor) (Figure 1). Based on the sampling map, G and AQB were located on Penang Island, while RR, ABM, and TP were located on the mainland. The cockroaches collected from these locations were labeled as Population 1 through Population 5, respectively (Table 1). Cockroaches were captured from restaurants and shopping centres using traps or hand vacuum and immediately stored in vials containing 95% ethanol. A total of 30 adult *B. germanica* were collected from each location.

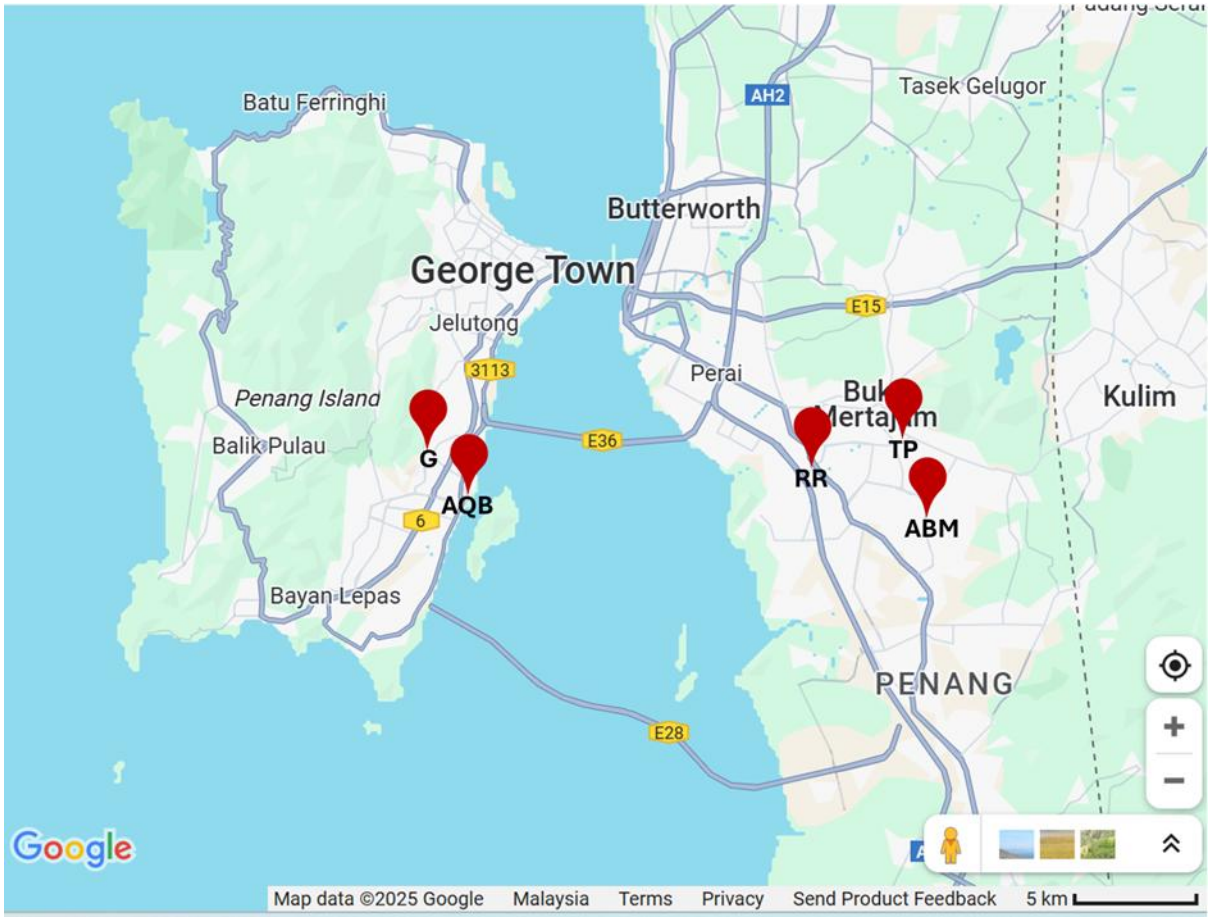

Figure 1. Map of Penang, Malaysia, showing the sites of *B. germanica* populations collected. The abbreviation details are listed in Table 1. The map was edited and retrieved from Google

Table 1. Population number, location code, location, sources, and GPS coordinates of each *B. germanica* population in this study

Population	Location Code	Location	Source	Coordinate
1	ABM	Bukit Mertajam, Penang	Shopping Centre	5° 19' 15.9835" N 100° 28' 39.0901" E
2	RR	Juru, Penang	Restaurant	5° 20' 34.432" N 100° 26' 9.3281" E
3	AQB	Bayan Lepas, Penang	Shopping Centre	5° 19' 55.443" N 100° 18' 26.5623" E
4	TP	Bukit Mertajam, Penang	Restaurant	5° 21' 18.5982" N 100° 28' 11.0304" E
5	G	Gelugor, Penang	Restaurant	5° 21' 31.3816" N 100° 17' 33.1146" E

DNA Extraction

Adult *B. germanica* specimens (one per site, in triplicate) were used for total genomic DNA extraction. The DNA extraction was performed using HiYield Plus^{TM} Genomic DNA Mini Kit (Blood/Tissue/Cultured Cells) from Real Biotech Corporation (Taiwan). The specimens were

meticulously washed three times with sterile distilled water, and then, the leg tissues were carefully dissected and homogenized in a cell lysis buffer containing Proteinase K. The homogenate was incubated for an hour at 60°C using a Mini Dry Bath to ensure efficient cell lysis. Subsequently, the lysates underwent protein precipitation and ethanol washes. Finally, the DNA was eluted twice using 50 µL elution buffer. The concentration and purity of extracted DNA were measured using an OPTIZEN™ NanoQ Lite Microvolume Spectrophotometer (KLAB, Korea) at the wavelength of 260/280 nm, and fragments of DNA were observed using 1% agarose gel electrophoresis. Then, the extracted DNA samples were subjected to PCR amplification targeting the COI and 16S rRNA genes.

COI and 16S rRNA Genes PCR Amplification

The COI gene was amplified using a pair of primers (Table 2), forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and reverse primer HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). The 16S rRNA gene was amplified using a combination of primer pair (Table 2), forward LR-J-13017 (5'-TTA CGC TGT TAT CCTAA-3), and reverse LR-N-13398 (5'-CACCTGTTTAACAAAAACAT-3') (Rajab et al. 2020). The PCR reaction mixture (50 µL) contains 25 µL of 2x TopTaq Master Mix, producing a final concentration of 1.5 mM MgCl2, 1.25 units TopTaq DNA polymerase, 1× PCR buffer, and 200 µM of each dNTP. Additionally, 1 µL (10 µM) of each primer, 5 µL (10x) CoralLoad Concentrate (as a substitute for loading dye), 10- 100 ng of bulk DNA template, and sterile distilled water were added. The PCR program comprised an initial denaturation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 45 seconds, primer annealing at 45°C for COI and 38°C for 16S rRNA for 45 seconds, the first extension step at 72°C for 60 seconds, and the final extension step at 72°C for 5 minutes. The PCR products were purified with MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea).

Sequencing and Analysis of COI and 16S rRNA Gene Sequences

The purified PCR products were sequenced using Sanger sequencing at First Base Laboratories Sdn. Bhd., Malaysia. Raw sequence data were extracted and processed using FinchTV 1.4 (Geospiza Inc.). Multiple sequence alignment was performed using T-Coffee (Notredame et al. 2000). Following alignment, the sequences were manually edited to remove low-quality bases. Finally, the edited sequences were converted to FASTA format. The FASTA files were then used for a BLASTn search against the NBCI GenBank database to identify similar COI gene sequences. Species identification was based on sequences with ≥99% similarity. The partial COI sequences were subsequently submitted to GenBank for public access.

Genetic Diversity and Phylogenetic Analysis

All sequences were automatically aligned using ClustalX v2.1 software under default settings (Larkin et al. 2007; Thompson et al. 1997). Genetic characteristics, including haplotype diversity (Hd) and nucleotide diversity (π), were calculated using DnaSP v6 (Rozas et al. 2017). Phylogenetic relationships were inferred through Neighbour-joining (NJ) trees, which were constructed based on p-distance using MEGA 7 (Kumar et al. 2016). To assess the robustness of NJ trees, bootstrap analysis with 1000 resamplings was performed.

RESULTS

Five distinct cockroach populations were sampled, with six individuals collected from each sampling site. From each cockroach population, three individuals were sequenced for the COI gene and three for the 16S rRNA gene, resulting in 30 sequences. The primer pair LCO1490

and HCO2198 (Table 2) amplified approximately 710 base pairs (bp) of the COI gene, while the primer pair of LR-J-13017 and LR-N-13398 (Table 2) amplified approximately 410 bp of the 16S rRNA gene. BLASTn analysis confirmed the cockroach species was *B. germanica*. The edited and trimmed sequences of this study were submitted to the NCBI GenBank database for an accession number. The assigned accession numbers for the COI and 16S rRNA gene sequences were listed in Tables 3 and 4, respectively. The general morphology of *B. germanica* is illustrated in Figure 2.

Table 2. List of primers and their sequences. COI primers, LCO1490 and HCO2198 (Folmer et al. 1994) and 16S primers LR-J-103017 and LR-N-13398 (Rajab et al. 2020). Dots represent identical nucleotides at specific locations compared to *Blattella germanica* in the GenBank with accession number NC_012901.1 (Xiao et al. 2012)

Primer (*COI*)	**Sequence**										**Position (Exon)**
(LCO1490)	5'-	GG	TCA	ACA	AAT	CAT	AAA	GAT	ATT	GG	-3'	
B. germanica		TT		…	…	…	…	…	…	..		1452
(HCO2198)	5'-	TGA	TTT	TTT	GGT	CAC	CCT	GAA	GTT	TA	-3'	
B. germanica		…	..C	…	..A	..T	..A	…	…	..		2162
Primer (16S rRNA)											
(LR-J-103017)	5'-	TTA	CGC	TGT	TAT	CCT	AA				-3'	
B. germanica		…	…	…	…	..C	T.					12835
(LR-N-13398)	5'-	CAC	CTG	TTT	AAC	AAA	AAC	AT			-3'	
B. germanica		GCG	GAC	AAA	TTG	TTT	TTG	TA				13246

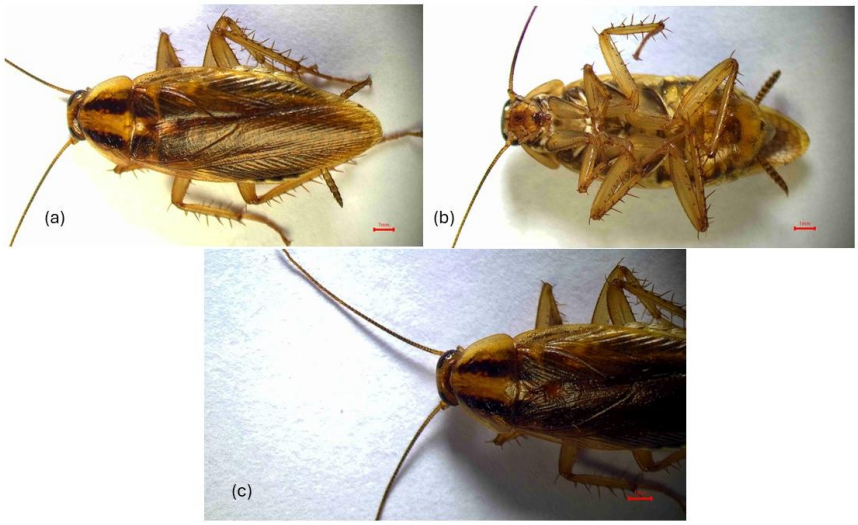

Figure 2. Morphological features of *Blattella germanica* specimens collected from urban commercial spaces. (a) The dorsal view shows a light brown colour body with two dark longitudinal stripes on the pronotum. (b) The ventral view shows the abdomen, legs, and antennae. (c) close-up of the head and antennae, highlighting the pronotum and its dark stripes

Table 3. Details of samples based on *COI* gene sequences.

Population	Location Code	Source	Sample	Haplotype	Accession Number
1	ABM	Shopping Centre	ABM01	2	PP751793
			ABM02	2	PP754525
			ABM03	2	PP751831
2	RR	Restaurant	RR01	1	PP752033
			RR02	1	PP752032
			RR03	1	PP758930
3	AQB	Shopping Centre	AQB01	2	PP752063
			AQB02	2	PP754240
			AQB03	2	PP752155
4	TP	Restaurant	TP01	1	PP754236
			TP02	1	PP754231
			TP03	1	PP752154
5	G	Restaurant	G01	1	PP752117
			G02	1	PP754233
			G03	1	PP754234

Table 4. Details of samples based on 16S rRNA gene sequences

Population	Location Code	Source	Sample	Haplotype	Accession Number
1	ABM	Shopping Centre	ABM01	1	PP751933
			ABM02	1	PP751999
			ABM03	1	PP752012
2	RR	Restaurant	RR01	1	PP752054
			RR02	1	PP752057
			RR03	1	PP757920
3	AQB	Shopping Centre	AQB01	2	PP752066
			AQB02	2	PP752067
			AQB03	2	PP752091
4	TP	Restaurant	TP01	1	PP757922
			TP02	1	PP754436
			TP03	1	PP752116
5	G	Restaurant	G01	1	PP752127
			G02	1	PP752139
			G03	1	PP752138

Genetic Diversity of COI and 16S rRNA gene

In this study, the genetic variation of *Blattella germanica* was examined across different types of urban commercial spaces on Penang Island and the mainland. From the results, both mitochondrial genes COI and 16S rRNA showed a similar haplotype distribution where *B. germanica* populations were clustered based on the type of urban commercial spaces rather than the geographic separation. For the COI gene (Table 5), haplotype 1 was associated with restaurants (RR, TP, and G), while haplotype 2 was associated with shopping centres (ABM and AQB). For the 16S rRNA gene (Table 5), haplotype 1 was associated with all populations

except AQB, and haplotype 2 was solely grouped into the shopping centre (AQB). By contrast, the COI gene shows clearer separation by commercial space type than 16S.

In terms of haplotype diversity, all *B. germanica* populations in shopping centres exhibited moderate diversity values (Hd = 0.667). In contrast, populations collected from restaurants showed no haplotype diversity with Hd values of 0.000. These values indicate that the genetic diversity of *B. germanica* was higher in shopping centres and lower in restaurants. A similar pattern was observed for nucleotide diversity (π) (Table 5), where populations in shopping centres displayed greater genetic variability. For the COI gene, population ABM had a value of $\pi = 0.338$ for nucleotide diversity, while population AQB had a slightly higher value of $\pi = 0.051$. These values indicated moderate variation at the nucleotide level. In contrast, for the 16S rRNA gene, only population AQB showed nucleotide diversity with the value $\pi = 0.003$, which indicates low genetic variation. All restaurant populations (RR, TP, G) displayed no nucleotide diversity ($\pi = 0.000$) for both gene markers.

Table 5. Haplotype (Hp), haplotype diversity (Hd), nucleotide diversity (π) of *Blattella germanica* populations from five locations

Population	Code	Hp (COI)	Hp (16S rRNA)	Hd (COI)	Hd (16S rRNA)	π (COI)	π (16S rRNA)
1	ABM	2	1	0.667		0.338	
2	RR	1	1				
3	AQB	2	2	0.667	0.667	0.351	0.003
4	TP	1	1				
5	G	1	1				

Phylogenetic Relationship Inferred from COI and 16S rRNA Genes

Two phylogenetic trees of *B. Germanica* were constructed using the Neighbor-joining (NB) method with p-distance, based on the COI (Figure 3) and 16S rRNA (Figure 4) gene sequences. For the COI sequences, the NJ tree showed that German cockroaches from commercial spaces in Penang, Malaysia, clustered into two clades, distinguishing *B. germanica* populations from restaurants and shopping centers.

Similarly, the NJ tree for 16S rRNA sequences displayed a pattern consistent with the COI sequences, forming two distinct clades. However, only population 3 (AQB) appeared in the second clade.

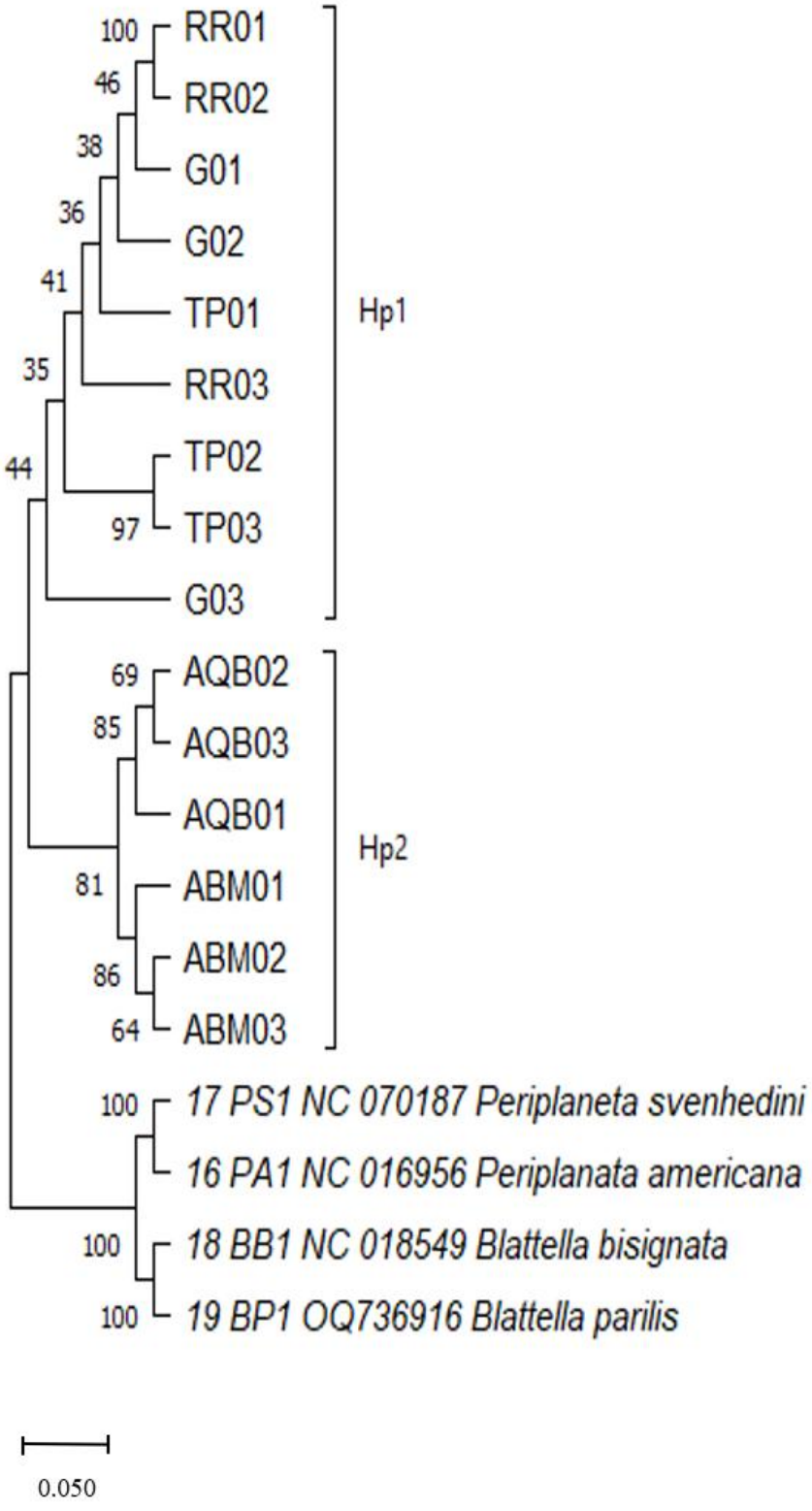

Figure 3. A Neighbor-joining tree constructed from COI gene sequences of 15 *B. germanica* specimens collected from five urban commercial spaces in Penang, Malaysia (see Table 3 for the name codes). The third clade corresponds to the outgroup species, including *Periplaneta svenhedini*, *P. americana*, *B. bisignata*, and *B. parilis*. The numbers represent bootstrap percentages (%) based on 1000 replications

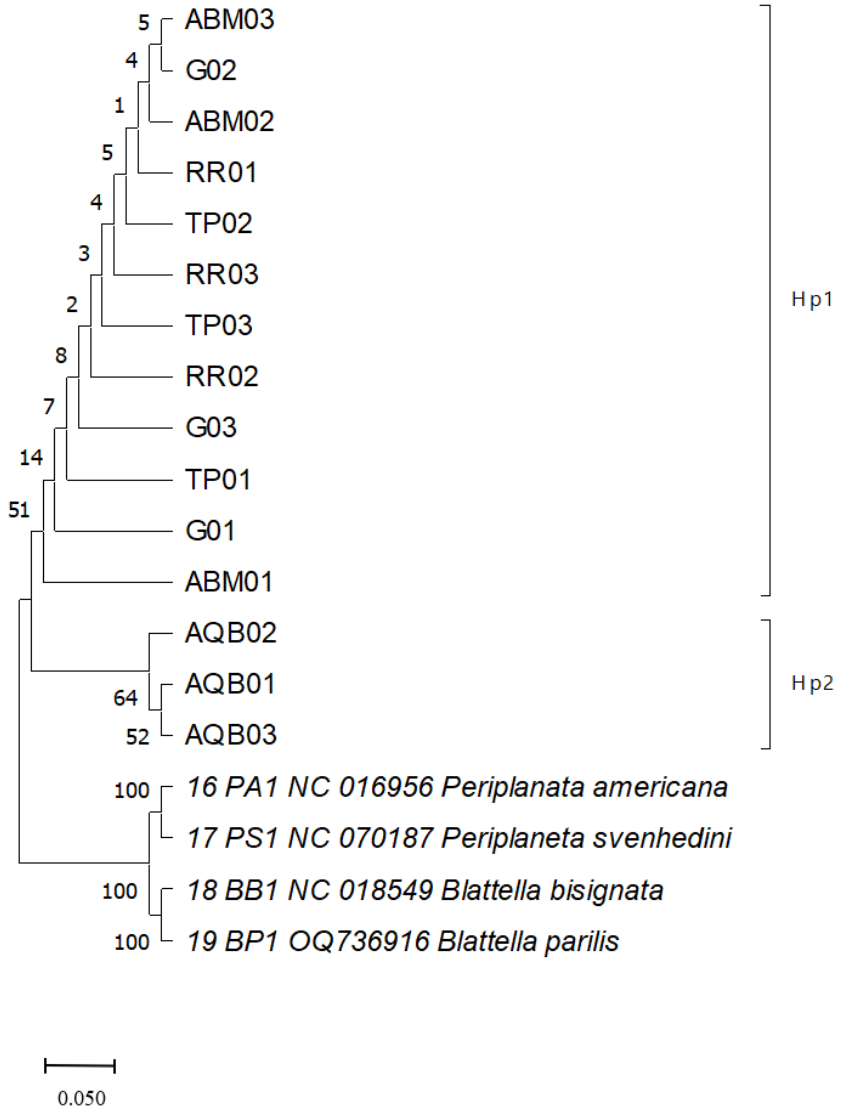

Figure 4. A Neighbor-joining tree constructed from 16S rRNA gene sequences of 15 *B. germanica* specimens collected from five urban commercial spaces in Penang, Malaysia (see Table 4 for the name codes). The third clade corresponds to the outgroup species, including *P. svenhedini*, *P. americana*, *B. bisignata*, and *B. parilis*. The numbers represent bootstrap percentages (%) based on 1000 replications

DISCUSSIONS

This study compared the genetic variation of *B. germanica* populations isolated from various locations in Penang, Malaysia, including the island (Bayan Lepas, Gelugor) and mainland (Bukit Mertajam, and Juru), using two mitochondrial gene markers: *COI* and 16S rRNA. Instead of reflecting geographic segregation (island versus mainland), both gene markers consistently showed two distinct haplotypes where populations were separated according to the type of urban commercial spaces (shopping centres versus restaurants) rather than geographic origin (Penang Island versus mainland). This pattern suggests that habitat type has a significant role in shaping the genetic structure of *B. germanica* populations than physical distance. These

findings may be explained by the natural behavior of German cockroaches, which are generally incapable of dispersing outside human dwellings and rely solely on human-mediated movement for passive dispersal (Booth et al. 2011; Vargo et al. 2014).

Following this, we observed moderate genetic diversity (Hd = 0.667, π = 0.338-0.351) in shopping centre populations (AQB and ABM) compared to restaurant populations (RR, G, TP), which showed no genetic diversity. It is well known that shopping centres have bigger spaces and higher levels of human activity compared to restaurants. Thus, the increased human movement in shopping centres might allow for greater dispersal and mixing of cockroach populations, potentially influencing their genetic structure through frequent introductions from various locations.

In contrast, despite the high level of human activity, abundant food sources, and water availability in restaurants, the genetic diversity of restaurant populations was low (Hd = 0.00, π = 0.00). Moreover, *B. germanica* was found to be the most prevalent cockroach species in restaurants compared to other species (Jeffery et al. 2012). Based on the findings from Crissman et al. (2010) and Vargo et al. (2014), we suggest that the limited movement of cockroaches within buildings may contribute to this pattern. They also proposed that every population should function as a single unit, which may explain why restaurant populations are more isolated, leading to reduced genetic mixing and lower genetic variation. However, factors such as hygiene practices, environmental conditions, and pest control measures may potentially shape the genetic makeup of *B. germanica* populations. Therefore, further investigation is needed to understand these influences better.

The genetic divergence between cockroach populations from shopping centres and restaurants is further supported by phylogenetic analysis using Neighbor-Joining, which shows two distinct clades in the resulting trees. Both phylogenetic trees (Figures 3 and 4) distinctly separated restaurant and shopping centre populations, except for the 16S rRNA tree (Figure 4), where population 3 (AQB) formed a separate clade. This suggests that while both are efficiently used for DNA barcoding and genetic differentiation, the COI gene has drastic evolutionary changes compared to other genes (Srinu 2018). The COI gene permits more specific identifications, but 16S rRNA covers broad taxa, but is low at taxonomic resolution (Magoga et al. 2022). Thus, combining these markers allows for a comprehensive understanding of genetic diversity and the evolutionary history of the populations.

Additionally, the observed genetic differentiation may have important implications for pest control strategies. Different populations may have different levels of susceptibility to insecticides. For example, a study on four German cockroach populations collected from the field showed mortality rates ranging from 0% to 58% when exposed to deltamethrin (Tseng et al. 2024). Another study reported that two out of five strains of German cockroaches collected from the field demonstrated resistance when treated using topical isocycloseram assays (Lee et al. 2024b). Future research should further investigate insecticide susceptibility between these two genetic clusters, as this could help refine targeted pest control strategies for managing urban infestations. Although the sample size in this study is relatively small, the data obtained can serve as an important baseline for future research on insecticide resistance profiles in urban commercial pests.

CONCLUSION

In conclusion, both gene markers COI and 16S rRNA showed separation of the *B. germanica* populations according to habitat type rather than geographic origin. The genetic diversity was moderately higher in populations from shopping centres compared to those from restaurants. We suggest that this differentiation may be influenced by human-mediated dispersal activity. These findings contribute to a broader understanding of German cockroach population genetics and emphasize the need for additional research into how genetic variation affects pest resilience and the development of effective management strategies in urban environments.

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AUTHORS DECLARATIONS

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Conflict of Interest
The authors declare no conflict of interest.

Ethics Declarations
The research protocol received approval from the Human Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/19120868). All procedures were executed in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Credit Authorship Contribution Statement
Nurul Akmar Hussin: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Hussein Ali Baqir**: Data curation, Formal analysis & Investigation. **Li Lim**: Data curation, Formal analysis & Investigation. **Martini Martini**: Data curation, Formal analysis & Investigation. **Retno Hestiningsih**: Data curation, Formal analysis & Investigation. **Sri Yuliawati**: Data curation, Formal analysis & Investigation. **Ameilia Zuliyanti Siregar**: Data curation, Formal analysis & Investigation. **Wan Aisyah Natasya Wan Gidi Sairee:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Abdul Hafiz Ab Majid**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing –review & editing.

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