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HOST SPECIES AND FOREST HABITAT SHAPE THE EXTERNAL BACTERIAL MICROBIOMES OF FOREST BLOWFLIES (DIPTERA: CALLIPHORIDAE) IN MALAYSIA

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ABSTRACT

Necrophagous flies harbour diverse bacterial communities that reflect their interactions with decomposing substrates and the surrounding environment. This study characterised the external bacterial microbiomes of two forest-associated blowflies, *Chrysomya villeneuvi* Patton and *Phumosia promittens* Walker, across two urban forest fragments (Ayer Hitam Forest Reserve and Bukit Rupa UKM Forest Reserve) and one continuous rainforest (Kuala Keniam Rainforest, Pahang National Park). Sampling was conducted using modified hanging traps baited with 72-hour decomposed mackerel fish. In total, 74 *Ch. villeneuvi* and 158 *P. promittens* individuals were captured, and external microbiomes from five samples per species at each location were aseptically isolated and characterised using 16S rRNA gene amplicon sequencing. Principal coordinates analysis demonstrated clear separation between the microbiomes of *Ch. villeneuvi* and *P. promittens* in Kuala Keniam, whereas samples from Ayer

Hitam and UKM exhibited greater overlap. PERMANOVA revealed a significant effect of host species identity on bacterial community composition across rainforest habitats ($P<0.05$). Core microbiome analysis indicated that *Ch. villeneuvi* uniquely contained *Klebsiella*, while *P. promittens* possessed several unique taxa, including *Orbus*, *Streptococcus*, *Kaistella*, *Ligilactobacillus*, *Staphylococcus*, and *Orbus sasakiae*. Microbiome composition also varied among forest habitat contexts, with *Ch. villeneuvi* from Kuala Keniam forming more distinct clusters than populations from fragmented forests, while *P. promittens* showed greater overlap among locations. PERMANOVA further indicated a significant effect of forest type on bacterial community composition in both species ($P<0.05$). Collectively, these findings provide evidence that host species identity is a key factor shaping the external bacterial microbiomes of forest-associated blowflies. Additionally, habitat context, particularly differences between continuous and fragmented rainforest ecosystems, may contribute to microbiome variation. These patterns are considered preliminary and require further investigation using larger sample sizes and synchronised sampling designs to minimise potential temporal effects. The findings provide baseline information for future studies exploring blowfly-associated microbiomes as potential indicators of tropical rainforest ecosystem health.

Keywords: Necrophagous flies; Ayer Hitam Forest Reserve; Metagenomic analysis; Kuala Keniam; 16S rRNA sequencing

ABSTRAK

Lalat nekrofagus membawa komuniti bakteria yang pelbagai yang boleh menggambarkan interaksinya dengan bahan yang mereput serta persekitaran sekeliling. Dalam kajian ini, mikrobiom bakteria luaran pada dua spesies lalat iaitu *Chrysomya villeneuvi* Patton dan *Phumosia promittens* Walker, yang disampel di dua hutan terpisah, iaitu Hutan Simpan Ayer Hitam dan Hutan Simpan Bukit Rupa UKM, serta satu hutan hujan tidak terpisah, iaitu Hutan Hujan Kuala Keniam, Taman Negara Pahang telah dicirikan. Persampelan dijalankan menggunakan perangkap gantung yang diubahsuai menggunakan umpan ikan kembung yang berusia 72 jam pereputan. Sebanyak 74 *Ch. villeneuvi* dan 158 *P. promittens* telah ditangkap, dan mikrobiom pada badan daripada lima sampel bagi setiap spesies di setiap lokasi telah disample secara aseptik dan dicirikan menggunakan penjujukan amplikon gen 16S rRNA. Analisis koordinat menunjukkan pemisahan yang jelas antara mikrobiom *Ch. villeneuvi* dan *P. promittens* di Kuala Keniam, manakala bagi sampel dari Ayer Hitam dan UKM, pertindihan yang lebih besar telah dilihat. PERMANOVA menunjukkan spesies lalat memberi pengaruh yang bererti bagi komposisi komuniti bakteria bagi setiap habitat hutan hujan ($P<0.05$). Analisis mikrobiom teras menunjukkan bahawa *Ch. villeneuvi* mengandungi *Klebsiella*, manakala *P. promittens* mempunyai beberapa taksa unik, termasuk *Orbus*, *Streptococcus*, *Kaistella*, *Ligilactobacillus*, *Staphylococcus* dan *Orbus sasakiae*. Komposisi mikrobiom juga didapati berbeza antara habitat hutan, dengan *Ch. villeneuvi* dari Kuala Keniam membentuk kelompok yang lebih tersendiri berbanding populasi dari hutan terpisah, manakala *P. promittens* menunjukkan pertindihan yang lebih besar di antara lokasi. PERMANOVA menunjukkan kesan jenis hutan yang bererti terhadap komposisi komuniti bakteria bagi kedua-dua spesies ($P<0.05$). Secara keseluruhannya, penemuan ini menunjukkan bahawa spesies lalat merupakan faktor penting yang membentuk mikrobiom bakteria luaran manakala dalam konteks habitat, perbezaan antara ekosistem hutan hujan berterusan dan terasing, juga menyumbang kepada variasi mikrobiom. Walau bagaimanapun, corak ini ditafsirkan sebagai penemuan awal dan memerlukan kajian lanjut dengan menggunakan saiz sampel yang lebih besar dan masa persampelan yang diseragamkan bagi meminimumkan kesan temporal.

Penemuan ini menyediakan maklumat asas untuk kajian masa hadapan dalam penerokaan mikrobiom lalat yang berpotensi sebagai penunjuk status ekosistem hutan hujan tropika.

Kata kunci: Lalat nekrofagus; Hutan Simpan Ayer Hitam; analisis Metagenomik; Kuala Keniam; jujukan 16S rRNA

INTRODUCTION

Necrophagous flies are characterised by their ability to feed on dead animal tissue. They are regarded as one of the most important insect groups involved in decomposition, nutrient recycling, and forensic investigations. A major proportion of these necrophagous flies is represented by blowflies (Diptera: Calliphoridae) (Banerjee & Hore 2017), which are a prominent group inhabiting both synanthropic and natural environments. Blowflies have a wide geographical distribution, occurring across many regions with moderate climates. They occupy diverse habitats, including forests, grasslands, and mountainous regions, and their distribution and abundance may be influenced by anthropogenic activities such as urbanisation (Hwang & Turner 2005; Kavazos & Wallman 2012; Laprise et al. 2024; Oh et al. 2024; Smith et al. 2023). As they are found in various habitats and interact significantly with humans (Nasser et al. 2021), they have become useful indicators for assessing environmental changes, whether caused by human activities or natural events that affect ecosystems (Moophayak et al. 2024). In addition, they have been used as model organisms to investigate population dynamics and are often studied in decomposition due to their close association with animal decay (Abdul Rahim et al. 2024; Boudreau et al. 2021; Laprise et al. 2024). Apart from this, it has been reported as a potential vector of pathogenic microorganisms, although its association with specific human diseases remains poorly documented (Junqueira et al. 2017).

The ecological roles of blowflies in decomposition and their potential involvement in pathogen transmission are closely linked to the diverse microbial communities on their bodies, particularly on their external surfaces and in their digestive tract. By carrying and dispersing bacteria through their digestive tracts and on their external surfaces, these flies can alter surrounding microbial communities and influence ecological interactions at multiple biological levels (Deel et al. 2022; Jiménez et al. 2020; Othman et al. 2022). Differences in the environment, for instance between disturbed and undisturbed habitats, can affect microbial communities present in those ecosystems (Nguyen et al. 2021). Industrialisation, pollution and land degradation usually introduce contaminants such as heavy metals and hydrocarbons, which disrupt microbial communities and impair soil health. Hence, shifts in blowflies' microbial composition after interacting with this affected area may reflect current ecosystem conditions, making them potential bioindicators of environmental disturbance. Flies specialised morphological features facilitate recognition of microbial shifts in such an example. For instance, bacteria can easily stick to their adhesive leg pads, fine body hairs, electrostatically charged exoskeleton and sponged-like mouthparts (Wiktorczyk-Kapischke et al. 2022). Therefore, identifying microbial communities associated with blow flies helps to reveal the core microbiome of species of interest and potential indicators of emerging health threats and pollution.

Malaysian tropical rainforests are extensive ecosystems that support high biodiversity and habitats for decomposer insects, primarily necrophagous blowflies. However, these ecosystems may vary in their degree of anthropogenic disturbance, ranging from relatively undisturbed continuous rainforests to disturbed forest fragments surrounded by human-modified landscapes. A continuous rainforest is described as a large, relatively undisturbed

tract of natural habitat. In contrast, fragmented rainforests are typically remnant patches embedded within highly urbanised landscapes, such as residential, commercial, and transportation areas (Tripathi et al. 2016). Unlike continuous forests, fragmented forests may experience varying degrees of anthropogenic disturbance, including edge effects, altered microclimates, modified vegetation structure, and increased exposure to human-associated environmental inputs (Kovacs et al. 2025). These structural changes may influence environmental microbial reservoirs, including those found in soil, and can contribute to the homogenization or convergence of microbial communities (Kovacs et al. 2025). As adult blowflies interact closely with their habitat and may acquire microbes from their immediate surroundings, changes in these environmental microbial pools may influence the bacterial communities that assemble on their external surfaces (Junqueira et al. 2017). Therefore, blowflies collected from continuous rainforests exhibit distinct, highly species-specific external microbiomes due to the environment's diversity and undisturbed nature (Mikaelyan et al. 2025). In contrast, blowflies in fragmented forests exhibit considerable microbial overlap among species, directly reflecting the homogenised microbial exposure conditions and convergent microbial reservoirs characteristic of such forests (Kovacs et al. 2025; Mikaelyan et al. 2025).

Forest-associated blowflies could be a suitable subject for microbiome-based rainforest assessment because they interact with decomposing organic matter, soil, vegetation, and other microbial reservoirs. Their external bacterial communities may therefore reflect local environmental conditions and provide preliminary insights into differences among rainforest ecosystems. For example, *Chrysomya villeneuvi* and *Phumosia promittens* could be potential candidates for rainforest monitoring as they were known as forest-associated blowflies (Kurahashi et al. 1997). This species has been reported from decomposed human remains in a high mountainous forest in northern Thailand, indicating its occurrence in forest-associated and non-urban habitats (Monum et al. 2017)—the adult behaviour of *Ch. Villeneuvi* remains unclear; however, it has not been observed in indoor or synanthropic environments (Sukontason et al. 2003). Meanwhile, *P. promittens* remains poorly studied, with available information largely limited to taxonomic identification and collection records. This species is characterised by a testaceous-yellow body and four postsutural dorsocentral bristles on the thorax. It has been reported from rainforest habitats and is attracted to decaying animal material and fruits (Kurahashi et al. 1997).

Kuala Keniam Rainforest, located within Pahang National Park, is an example of a continuous rainforest ecosystem in Peninsular Malaysia. Pahang National Park encompasses approximately 2,477 km² of largely protected primary tropical rainforest, with Kuala Keniam situated at elevations of approximately 120–200 m above sea level. However, information on blowflies and their microbiome in this protected lowland rainforest remains unexplored. On the other hand, rainforests in Malaysia are increasingly fragmented due to intense urbanisation. Ayer Hitam Forest Reserve and Bukit Rupa UKM Forest Reserve are examples of fragmented forest habitats embedded within human-modified landscapes shaped by residential, institutional, and transportation infrastructure. As such, these reserves may function as isolated forest patches within an increasingly urbanised matrix. Comparing the two rainforest ecosystems may provide preliminary insight into the extent to which habitat context influences external bacterial community composition and whether forest fragmentation is associated with detectable shifts in microbial profiles.

This study aimed to characterise and compare the external bacterial microbiomes of *Ch. villeneuvi* and *P. promittens* collected from three rainforest locations in Malaysia: Kuala

Keniam Forest, Ayer Hitam Forest Reserve and Bukit Rupa UKM Forest Reserve. By examining microbiome composition across host species and habitat contexts, this study provides preliminary insights into host-associated and location-associated variation in bacterial communities of forest-associated blowflies. The findings contribute to baseline knowledge on tropical rainforest insect–microbe associations and may support future ecological comparisons, microbial biodiversity assessments, and conservation-related research in tropical forest ecosystems.

MATERIALS AND METHODS

Study Locations

The sampling was carried out across three rainforests; Ayer Hitam Forest Reserve (AHFR) (3.014722°N, 101.647500°E) and Bukit Rupa UKM Forest Reserve (UKM) (2.919517° N, 101.766731° E) which categorised as urban forest fragments and Kuala Keniam Rainforest (KKR) (4.517611° N, 102.474397° E) which is a continuous rainforest (Figure 1). The first two locations represent remnant lowland tropical forest patches embedded within highly urbanised landscapes. These locations are surrounded by residential, institutional, commercial, and transportation infrastructure, resulting in varying degrees of habitat fragmentation and anthropogenic influence. The third location is a large contiguous tract of protected tropical rainforest with no urban development, located within Pahang National Park. This location is a dipterocarp forest situated at elevations ranging from 58 to 86 metres above sea level (asl).

For all study locations, three plots were set at distances of 300-500 metres apart. Environmental data of each study site were gathered, including ambient temperature (°C), relative humidity (RH) (Digital Hygro-Thermo Meter (DHT-1), Daeyoon Scale Industrial Co., Ltd., Seoul, South Korea), and coordinates (Garmin™ eTrex Handheld GPS, Garmin China Co., Ltd., Chaoyang, China). Sampling at Ayer Hitam Forest Reserve (AHFR) was carried out on 18 February 2025. Sampling at the Bukit Rupa UKM Forest Reserve (UKM) was conducted on 20 February 2025. Sampling at Kuala Keniam Rainforest was carried out on 9 March 2024.

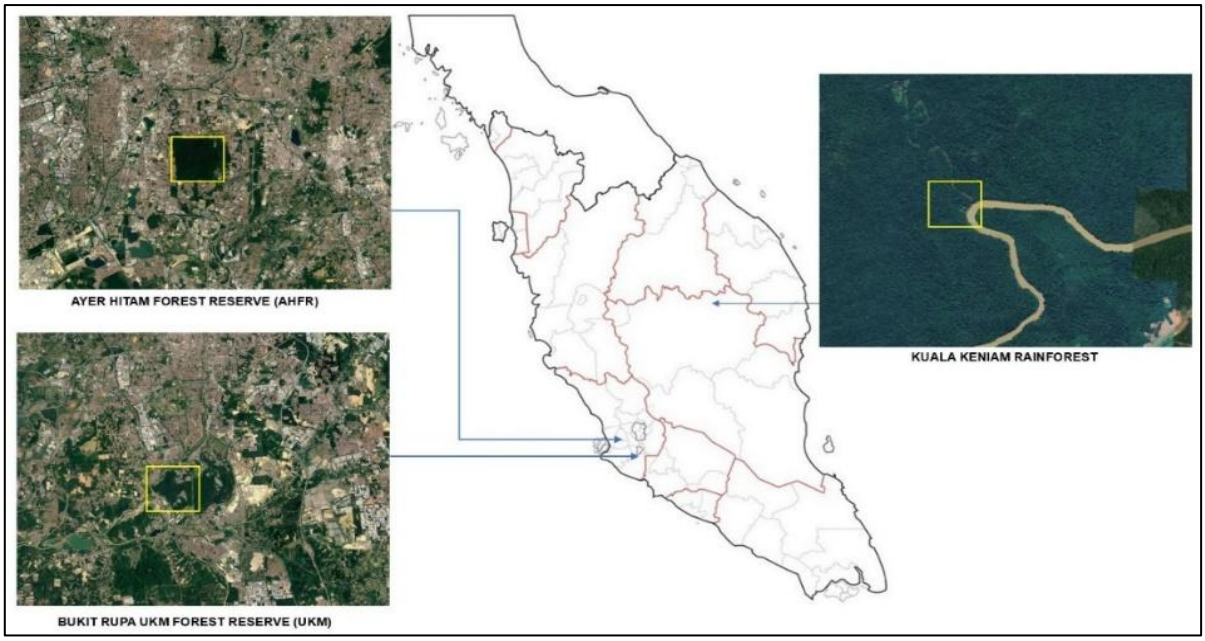

Figure 1. Location of the three rainforest study sites in Peninsular Malaysia: Ayer Hitam Forest Reserve (AHFR), Bukit Rupa UKM Forest Reserve (UKM), and Kuala Keniam Rainforest, Pahang National Park

Sample Collection

A modified hanging fly trap (IP No: CRL Y2024W04670) was utilised for fly collection (Figure 2). The trap was developed by enhancing a commercially available hanging fly trap with additional components to increase its efficiency and durability. Mackerel fish (*Scomber scombrus*) aged 72 hours were used as bait, with one fish per plot placed in the plastic bait container. The containers were designed to prevent flies from landing directly on the decomposing fish, thereby avoiding the deposition of eggs or the development of larvae within the bait. For the 72-hour-old fish preparation, fresh mackerel were obtained from a fish market and immediately placed in a polystyrene box. The box cover was removed and replaced with plastic netting to ensure that decomposition occurred naturally. The box was kept at room temperature, allowing for natural decomposition while preventing access by flies and ants. This preparation was carried out 72 hours before trap deployment. During field setup, traps were securely hung (2m height) in open areas away from tree branches to minimise disturbance or interference by animals, such as iguanas. Traps were set up in the morning between 9 am and 10 am, using bait aged for 72 h. Captured flies were collected after 24 h of trap exposure. Therefore, the flies collected in the trap represented individuals attracted to the bait and aged between 72 and 96 h.

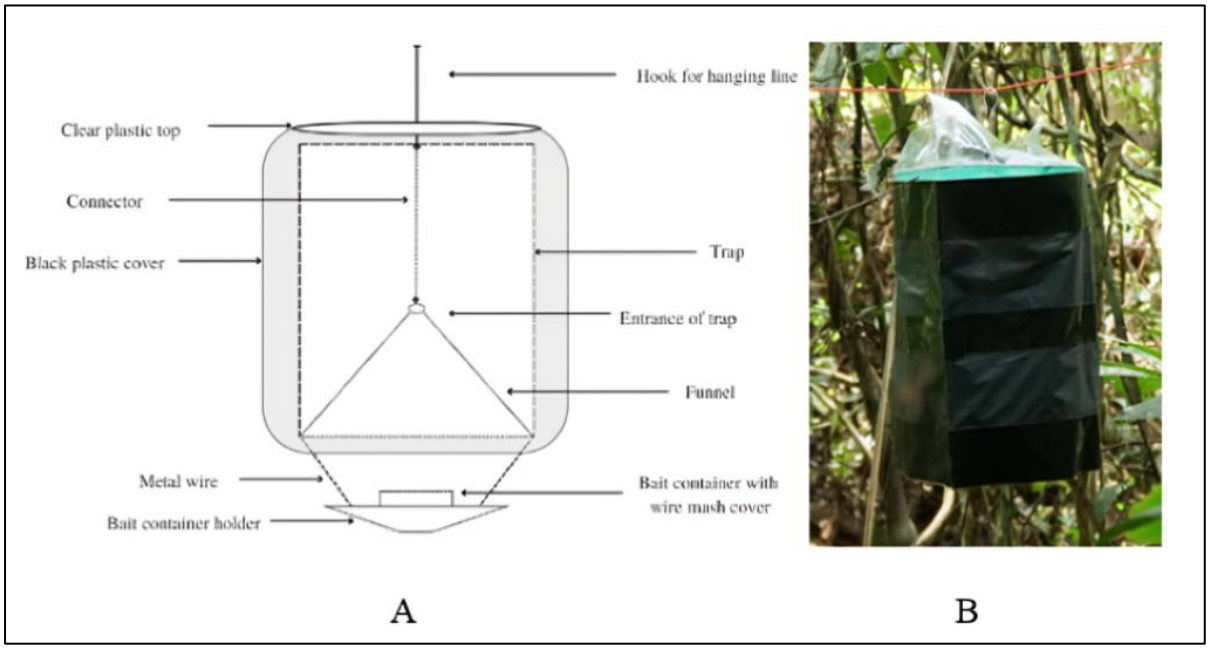

Figure 2. A modified funnel trap. (A) Schematic drawing of the modified trap. (B) A trap was set by hanging it in one plot of the study location

Immobilisation of Blowflies

The fly trap bodies were removed from the location and placed in a ziplock bag. The samples were kept in a zipped plastic bag containing a small cotton ball dipped in ethyl acetate, taped inside (top) of the zip bag to confine the vapour and immobilise the captured flies. The plastic bags were placed in an ice box and sealed to facilitate transport and maintain sample sterility. The ice was brought back to the nearby research station as soon as the 3rd trap was completed, to ensure the samples were processed promptly and minimise sample assimilation.

Sorting, Identification and DNA Preservation of *Chrysomya villeneuvi* and *Phumosia promittens*

Upon arrival at the research station, 15 suspected *Ch. villeneuvi* and 15 *P. promittens* individual blowflies were collected from traps using sterile forceps and placed into sterile Falcon tubes, for a total of 30 specimens. Species identification was then conducted by observing external morphological characters through the wall of the Falcon tube under a stereomicroscope, thereby avoiding direct contact with the specimens. Identification was made by referring to the common features of both blowflies, and for more detailed features, they were referred to the taxonomic key of Kurahashi et al. (1997). To avoid contamination from surrounding microbes, disposable gloves were worn throughout the process, and forceps were disinfected with 70% ethanol between specimens. After identification, five confirmed individuals out of 15 individual *Ch. villeneuvi* and *P. promittens* from Falcon tubes were selected for 16S rRNA gene sequencing. These same procedures were repeated in all locations, resulting in a total of 30 specimens. The selected samples were then transferred into Falcon tubes containing DNA/RNA Shield (Zymo Research) to preserve DNA. The preserved samples were labelled and sent to a sequencing service company for 16S rRNA gene sequencing and metagenomic analysis. The remaining blowfly samples were sorted and, if possible, identified to species level. After species sorting, the number of individuals collected was counted and recorded by plot number. The samples were then stored in plastic containers and brought to the laboratory for preservation.

Genomic DNA Extraction

Thirty selected samples were submitted to a commercial biotechnology service provider for DNA extraction, library preparation, and sequencing. Upon receipt, samples preserved in DNA/RNA Shield™ were centrifuged to separate particulate material from the preservation medium. The resulting supernatant was used for genomic DNA extraction. DNA was purified using a magnetic bead-based protocol in which the supernatant was mixed with an equal volume of SPRI beads and incubated at room temperature to facilitate DNA binding. The bead-bound DNA was subsequently washed with 70% ethanol to remove impurities and eluted in 100 µL of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). No whole-body homogenisation or crushing of fly specimens was performed before DNA extraction. The extracted DNA was subsequently used to amplify the bacterial 16S rRNA gene (V3–V4 region). This region offers a practical advantage due to its lower sequence complexity, which simplifies bioinformatic analysis and reduces computational demands (Alfonsi et al. 2023; Fadeev et al. 2021).

Library Preparation and Sequencing

The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′). To facilitate inline barcoding, a 5-bp barcode and partial Illumina adapter sequence were incorporated at the 5′ end of each primer. Each sample was amplified using a unique combination of barcoded forward and reverse primers. PCR amplification was performed using Rediant II PCR Master Mix (Apical Scientific, Malaysia) under the following cycling conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 30 s. PCR products were visualised by agarose gel electrophoresis and purified using 0.8× SPRI beads. Purified amplicons were subjected to an 8-cycle indexing PCR to attach full-length Illumina adapter sequences and dual-index barcodes. Final libraries were size-selected using 0.8× SPRI beads, pooled into a single library, and quantified using a DeNovix high-sensitivity assay. Sequencing was performed on an Illumina NovaSeq 6000 platform using 2 × 250 bp paired-end chemistry.

Bioinformatic and Statistical Analyses

Raw paired-end sequences were demultiplexed and primer-trimmed using Cutadapt v1.18. Trimmed reads were quality-filtered (Q>20) and merged using fastp v0.21. The processed reads were imported into QIIME2 v2023.9, where denoising and amplicon sequence variant (ASV) inference were conducted using DADA2 v1.26.0. Taxonomic assignments were generated using the q2-feature-classifier plugin trained on the GreenGenes2 reference database. ASVs that could not be classified at least to the phylum level were excluded from subsequent analyses. The resulting ASV feature table and taxonomy file were exported as tab-delimited text files. In contrast, the metadata file was prepared separately to include the corresponding sample information, including host species, sampling location, and forest type. To standardise sequencing depth across samples, all libraries were rarefied to 10,000 reads per sample.

MicrobiomeAnalyst 3.0 was used for the statistical analysis, visualisation, and interpretation of the microbiome data. The input files consisted of the produced ASV feature table, a taxonomy file, and a metadata file containing the corresponding sample information. Bacterial community composition was characterised across multiple taxonomic ranks, from phylum to species level. The 10 most abundant ASVs were identified to determine the dominant bacterial taxa associated with the samples. Alpha diversity was assessed using observed features and Chao1 as richness estimators, whereas Shannon and Simpson diversity indices were used to evaluate community diversity. For beta diversity analysis, principal coordinates analysis (PCoA) was performed to visualise differences in bacterial community composition across fly species and forest types. Permutational multivariate analysis of variance (PERMANOVA) was then conducted to statistically test the effects of fly species and forest type on bacterial community structure. Core microbiome analysis was performed to identify shared and unique bacterial taxa among fly species and forest types.

Fly Sample Preservation

Once arrived at the laboratory, selected samples from each species were pinned to preserve their structure. An entomological pin size #2 or #3 was inserted through the thorax at 90˚, with the wings, legs and antennae positioned and spread for clear display of diagnostic traits. Each specimen was labelled with the following information: collection site, elevation, date of collection, collector's name and species. If the species is unidentified, it was labelled with an ID number and regarded as a morphospecies. To ensure proper preservation for long-term storage, the pinned samples were dried at 70-80°C for 72 hours (Junqueira et al. 2017). These specimens were referred to taxonomic experts for the identification and confirmation of unknown species.

RESULTS

Weather Data

Microclimatic conditions varied slightly among the three sampling locations. At Ayer Hitam Forest Reserve, temperature showed minimal variation among plots, ranging from 29.1°C to 29.9°C, with a mean relative humidity of 86.6%. Plot 2 recorded the highest temperature at 29.9°C, followed by Plot 1 at 29.3°C and Plot 3 at 29.1°C. At Bangi Forest Reserve, the plots differed slightly in elevation and temperature. Plot 1 was located at 92.29 m above sea level and recorded the highest temperature at 29.4°C. Plot 2, located at the highest elevation of 110.67 m, recorded a lower temperature of 28.8°C, while Plot 3, at an intermediate elevation of 102.37 m, recorded the lowest temperature of 28.5°C. The mean relative humidity at this location was 75.6%. At Kuala Keniam, temperatures ranged from 28.9°C to 29.3°C. Plot 1 recorded the highest temperature at 29.3°C, followed by Plot 3 at 29.1°C and Plot 2 at 28.9°C.

The mean relative humidity was 77.1%. Overall, microclimatic conditions during sampling were broadly comparable among the three locations, with small differences in temperature and relative humidity.

Bacterial Community Composition and Relative Abundance

Table 1 summarises the number of *Ch. villeneuvi* and *P. promittens* collected from three traps at Ayer Hitam Forest Reserve (AHFR), Bukit Rupa UKM Forest Reserve (UKM), and Kuala Keniam Rainforest (KKR). A total of 232 blowfly individuals were captured across the three sampling locations, with the highest abundance recorded in Kuala Keniam (n = 111), followed by Ayer Hitam (n = 65) and UKM (n = 56). Overall, 74 individuals of *Ch. villeneuvi* and 158 individuals of *P. promittens* were collected. The highest trap-level abundance was recorded for *P. promittens* in Trap 1 at Kuala Keniam (n = 65), whereas no *P. promittens* individuals were collected from Trap 3 at this location. For microbiome analysis, a minimum of one and a maximum of three individuals from each trap were randomly selected for 16S rRNA gene sequencing. However, no *P. promittens* sample from Trap 3 at Kuala Keniam was available for sequencing because no individuals of this species were captured there.

Table 1. The frequency of *Ch. villeneuvi* and *P. promittens* captured in the study. Values in parentheses indicate the number of samples taken for 16S rRNA sequencing.

Trap Number	**Ayer Hitam Forest Reserve (AHFR)**		**Bukit Rupa UKM Forest Reserve (UKM).**		**Kuala Keniam Rainforest (KKR)**	
	Ch. villeneuvi	*P. promittens*	*Ch. villeneuvi*	*P. promittens*	*Ch. villeneuvi*	*P. promittens*
1	5(1)	24(1)	2(1)	11(1)	8(2)	65(3)
2	2(2)	3(2)	6(2)	29(2)	8(1)	7(2)
3	14(2)	17(2)	6(2)	2(2)	23(2)	0(0)
Total	21(5)	44(5)	14(5)	42(5)	39(5)	72(5)

Relative abundance of bacterial phyla associated with blowfly specimens for each sampling location is shown in Figure 3(A). Proteobacteria consistently dominated the bacterial communities across all samples. This was followed by the Firmicutes and Bacteroidota phyla, although their relative abundances varied among individuals and locations. Samples from Kuala Keniam exhibited greater variability, particularly in two samples (CV28 and CV29), which showed elevated proportions of Firmicutes and reduced Proteobacteria.

In contrast, most samples from Ayer Hitam and UKM displayed a more uniform community composition dominated by Proteobacteria. Other phyla, including Actinobacteriota, Firmicutes_A, Firmicutes_C, Desulfobacterota, and Cyanobacteria, were also found but at relatively low abundances and not present in all samples. Overall, the bacterial communities across both blowfly species and locations were characterised by the dominance of Proteobacteria, with inter-individual variation mainly driven by fluctuations in Firmicutes_D and Bacteroidota.

The genus-level relative abundance is shown in Figure 3(B). Bacteria genera exhibited substantial variability among specimens and sampling locations, although several dominant genera were consistently detected across all populations. The microbiomes were primarily composed of *Dysgonomonas*, *Ignatzschineria*, *Vagococcus*, *Wohlfahrtiimonas*, *Orbus*, *Providencia*, members of *Enterobacteriaceae*, and *Acinetobacter*, while a considerable

proportion of unclassified sequences (Others). Across the three study sites, *P. promittens* generally harboured higher relative abundances of *Orbus* and *Wohlfahrtiimonas*, particularly in several individuals from UKM and Kuala Keniam, whereas *C. villeneuvi* showed greater representation of *Ignatzschineria*, *Vagococcus*, and *Enterobacteriaceae* in many specimens.

Notably, marked inter-individual variation was observed even within the same species and locality, with some flies being dominated by a single bacterial genus. In contrast, others possessed a more even community structure. Samples from Kuala Keniam exhibit higher proportions of *Dysgonomonas* and *Enterobacteriaceae*, whereas individuals collected from UKM displayed increased abundances of *Ignatzschineria* and *Orbus*. Despite these location-specific trends, no single genus was exclusively associated with a particular site, suggesting the existence of a shared core microbiota among forest-associated blowflies. The pronounced heterogeneity in bacterial composition among individual flies indicates that host species identity, local environmental conditions, and individual ecological histories may collectively influence microbiome assembly and contribute to the observed variation in bacterial community structure.

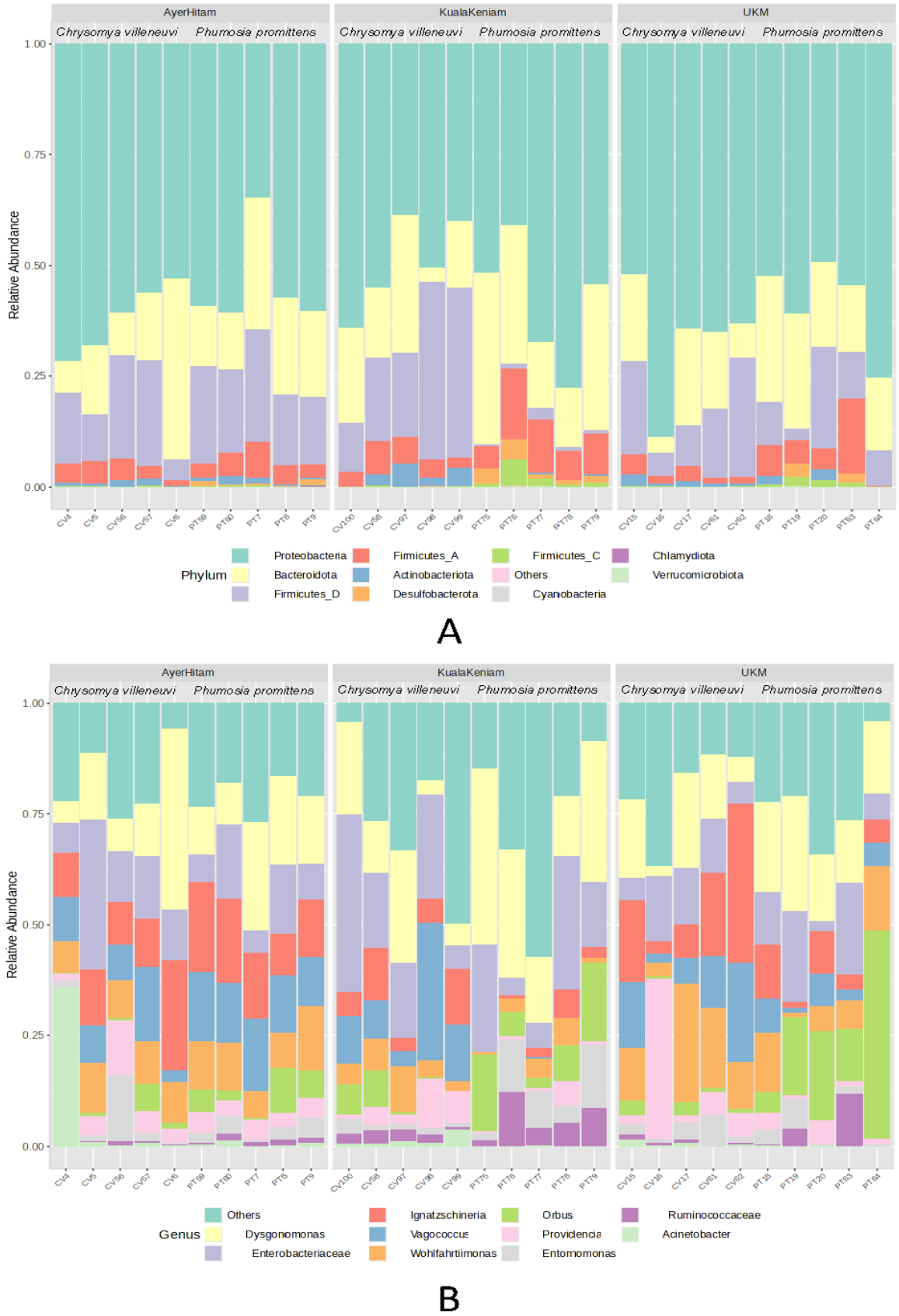

Figure 3. Relative abundance of the top ten associated with individual *Ch. villeneuvi* and *P. promittens* specimens collected from three sampling locations. A: Top ten bacterial phyla; B: Top ten bacterial genera

Host Species Variation in the External Bacterial Microbiomes of *Chrysomya villeneuvi* and *Phumosia promittens*

The effect of host species on bacterial microbiome composition was evaluated using Alpha diversity, Principal Coordinates Analysis (PCoA) and Permutational Multivariate Analysis of Variance (PERMANOVA). Figure 4 shows bacterial richness and diversity assessed using observed ASVs, Chao, Shannon, and Simpson indices for pooled samples (n=30). Observed richness was slightly higher in *Ch. villeneuvi*, but the difference was not statistically significant ($t = 1.1445$, $P = 0.26247$). Similarly, Chao1 richness did not differ significantly between the two species ($t = 0.71213$, $P = 0.4823$). Shannon diversity was comparable between species ($t = -0.4805$, $P = 0.63471$), indicating similar bacterial richness and evenness, while Simpson diversity also showed no significant difference ($t = -1.3802$, $P = 0.17925$). Overall, these results suggest that both blowfly species harboured comparable levels of bacterial alpha diversity.

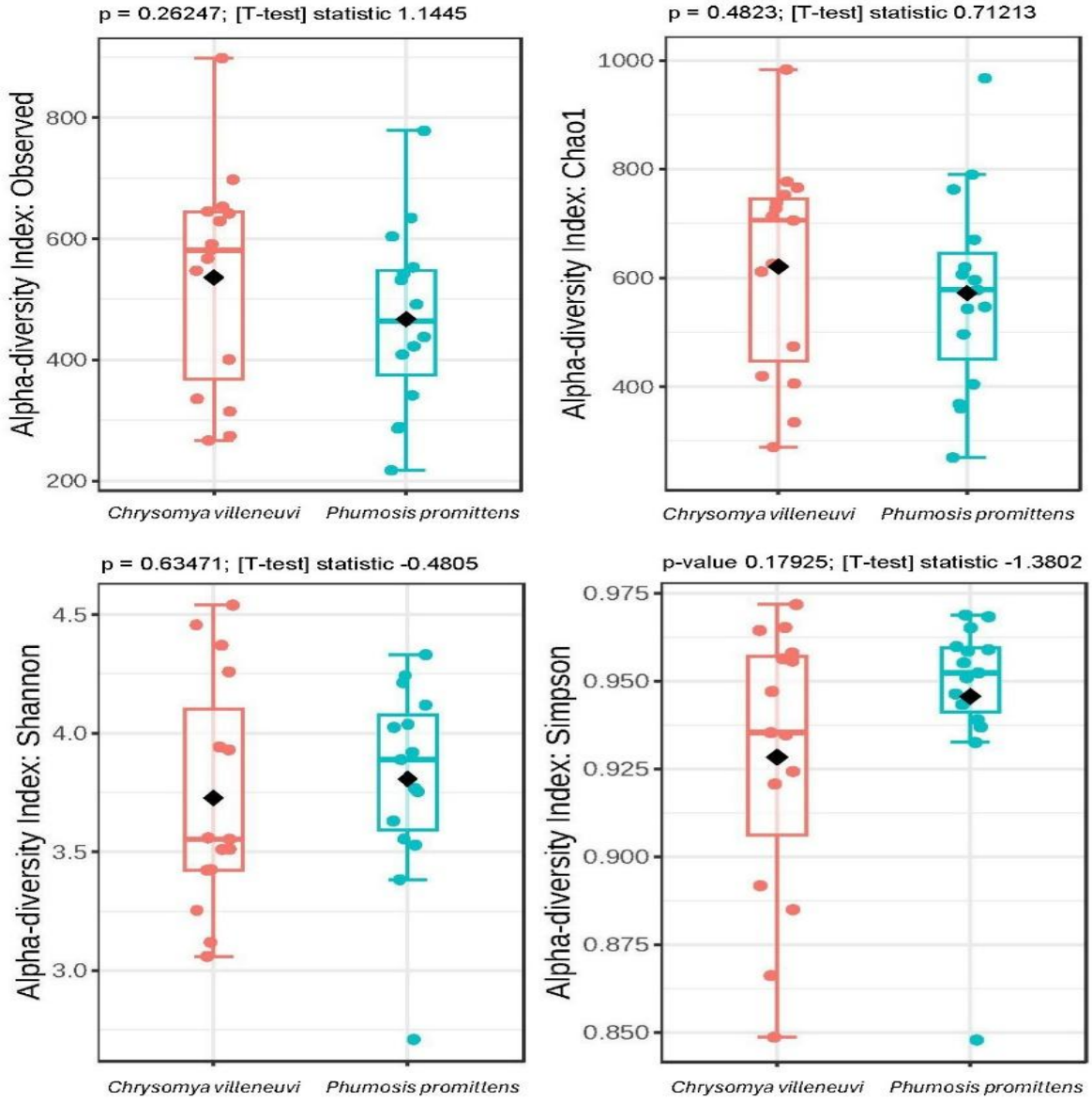

Figure 4. Alpha diversity of bacterial communities associated with *Chrysomya villeneuvi* and *Phumosia promittens.* The results are pooled data from Ayer Hitam, Kuala Keniam, and UKM.

For beta diversity, PCoA based on Bray–Curtis dissimilarity was used to visualise differences in bacterial community composition between *Ch. villeneuvi* and *P. promittens* using both the pooled dataset (n = 30) and separate datasets for each of the three sampling locations (n = 10) (Figure 5). In the pooled dataset, separation of bacterial communities between the two blowfly species was evident. The two groups show some separation, mainly along the first PCoA axis, with one species forming a broader cluster toward the positive side of the axis. At the same time, the other is more concentrated toward the centre-left. However, the confidence ellipses overlap, indicating that the bacterial communities are not completely distinct and that some individuals from both species share similar bacterial profiles. Statistical analysis confirmed that the bacterial communities differed between *Ch. villeneuvi* and *P. promittens* in pooled samples (PERMANOVA: F = 3.8883, R^2 = 0.12194, *P<0.001*). Host species factor explained approximately 12.2% of the variation in bacterial community composition, indicating a significant but moderate host-associated effect.

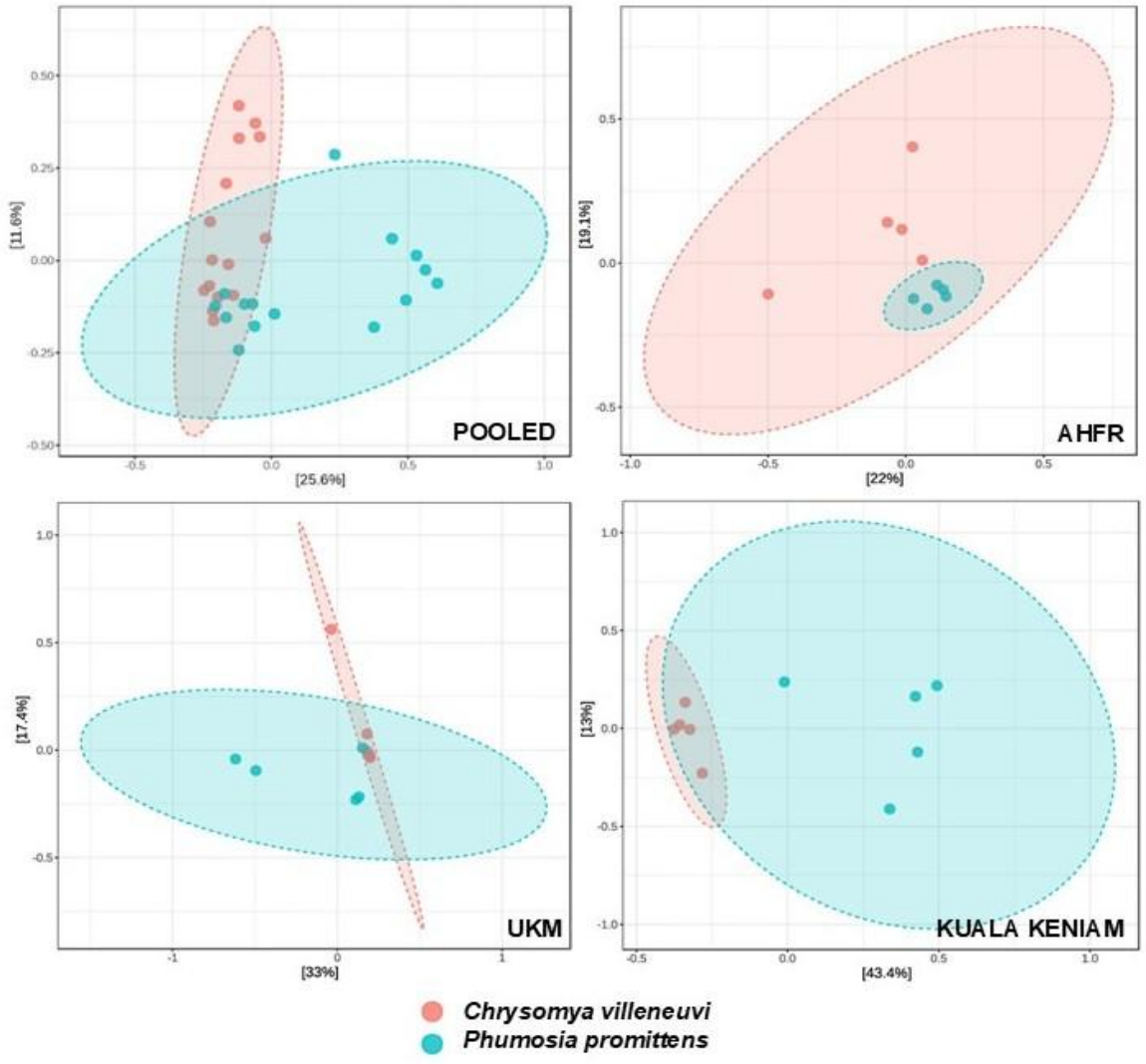

Figure 5. Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarity showing bacterial community variation between *Chrysomya villeneuvi* and *Phumosia promittens* across three forest locations. Ellipses represent the 95% confidence intervals around each species group. Percentages on the axes indicate the proportion of variation explained by the principal coordinates.

Similarly, location-wise analysis showed partial overlap between the two species in Ayer Hitam and UKM, indicating relatively similar bacterial community structures between species within these fragmented forests. In contrast, samples from Kuala Keniam formed more distinct, clearly separated clusters, reflecting greater differences in microbiome composition among species at this location (Figure 5). PERMANOVA was used to compare the magnitude of differences in host-species-associated bacterial communities across rainforest locations. In Ayer Hitam, bacterial communities differed significantly between *Ch. villeneuvi* and *P. promittens* ($F = 1.7262$, $R^2 = 0.17748$, $P<0.05$), with host species explaining approximately 17.7% of the variation. In UKM, the species effect was weaker and not statistically significant ($F = 1.6576$, $R^2 = 0.17163$, $P = 0.071$), although the R^2 value suggests a possible trend towards host-associated differentiation. The strongest species-associated separation was observed in Kuala Keniam, where host species explained approximately 38.8% of the variation in bacterial community composition ($F = 5.076$, $R^2 = 0.38819$, $P<0.05$).

Core microbiome comparison between *Ch. villeneuvi* and *Ph. promittens* at a 20% prevalence threshold revealed both shared and species-specific bacterial taxa across the three sampling locations (Figure 6). In Ayer Hitam, both species commonly harboured *Dysgonomonas*, *Ignatzschineria*, *Providencia*, *Vagococcus*, *Wohlfahrtiimonas*, *Ignatzschineria indica*, *I. ureiclastica*, and *Wohlfahrtiimonas chitiniclastica*. However, *Ch. villeneuvi* contained only *Klebsiella*, whereas *P. promittens* contained unique taxa, including *Orbus*, *Streptococcus*, *Kaistella*, *Ligilactobacillus*, *Staphylococcus*, and *Orbus sasakiae*.

In UKM, both blowfly species shared dominant bacterial taxa, including *Dysgonomonas*, *Ignatzschineria*, *Vagococcus*, *Wohlfahrtiimonas*, *Entomomonas moraniae*, and *Ignatzschineria ureiclastica*. Nevertheless, *Providencia* was more associated with *Ch. villeneuvi*, while *P. promittens* showed unique occurrence of *Saezia* and *Orbus sasakiae*. In Kuala Keniam, fewer bacterial taxa were shared between the two species, indicating greater microbiome differentiation within the protected forest. Shared taxa included *Dysgonomonas*, *Ignatzschineria*, *Providencia*, *Wohlfahrtiimonas*, and *Ruminococcaceae*. *Ch. villeneuvi* uniquely harboured *Mammaliicoccus fleurettii*, *Acinetobacter*, *Corynebacterium*, and *Rosenbergiella*, whereas *P. promittens* uniquely contained *Neisseriaceae*, *Weeksellaceae*, *Saezia*, *Christensenellales*, and *Orbus*. Overall, the disturbed forests (Ayer Hitam and UKM) exhibited greater overlap in core bacterial taxa between the two blowfly species. In contrast, Kuala Keniam showed higher species-specific bacterial composition and reduced microbiome overlap.

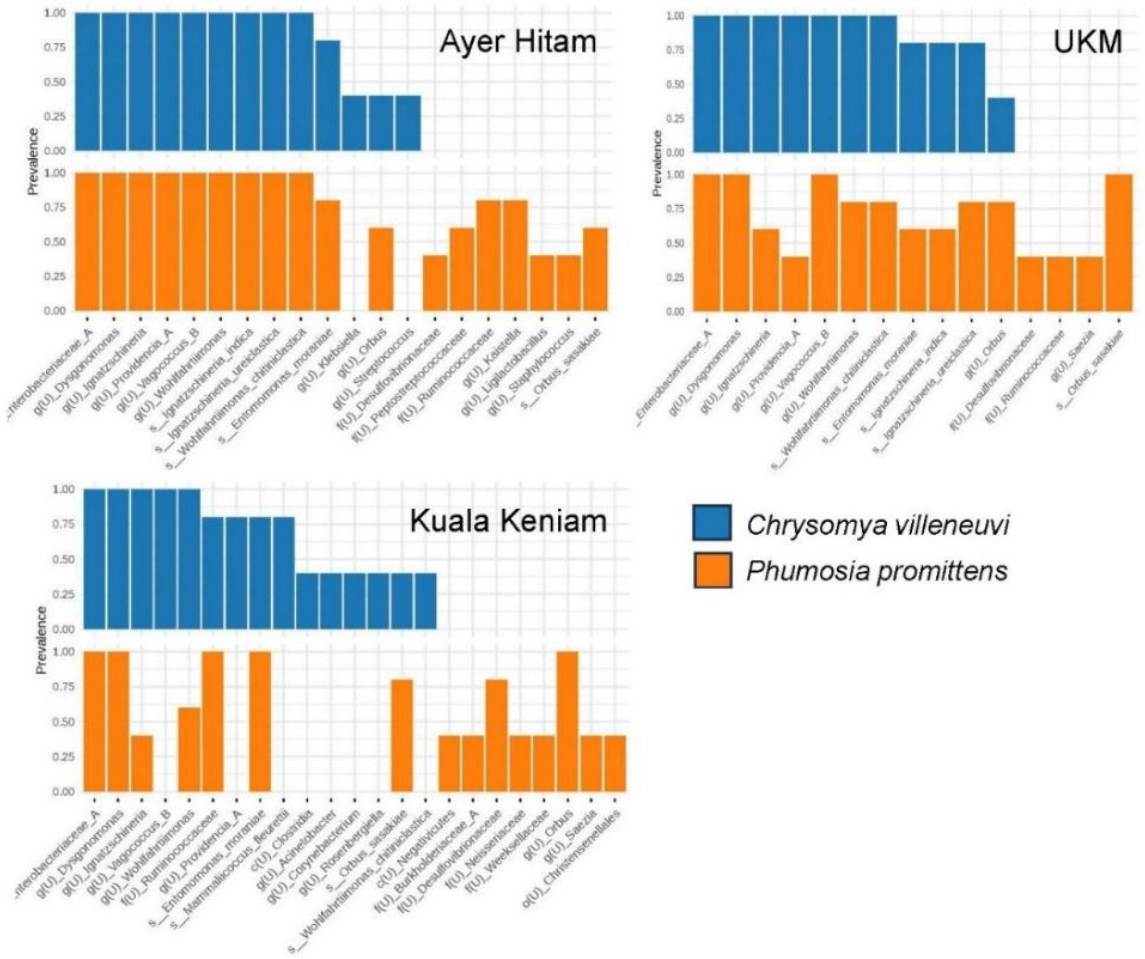

Figure 6. Core microbiome of *Chrysomya villeneuvi* and *Phumosia promittens* collected from Ayer Hitam, Kuala Keniam, and UKM forest reserves

Forest-Associated Variation in the External Bacterial Microbiomes of *Chrysomya villeneuvi* and *Phumosia promittens*

Figure 7 shows bacterial richness and diversity assessed using observed ASVs, Chao, Shannon, and Simpson indices. Alpha diversity analysis showed no significant differences in bacterial richness or diversity among Ayer Hitam, Kuala Keniam, and UKM. Observed richness ($F = 2.63$, $P=0.090$) and Chao richness ($F = 2.45$, $P=0.105$) exhibited a non-significant trend towards lower richness in UKM samples compared with the forest sites. Similarly, Shannon diversity ($F = 0.53$, $P = 0.593$) and Simpson diversity ($F = 0.13$, $P = 0.875$) did not differ significantly among locations, indicating comparable bacterial diversity and community evenness between fragmented and continuous rainforests.

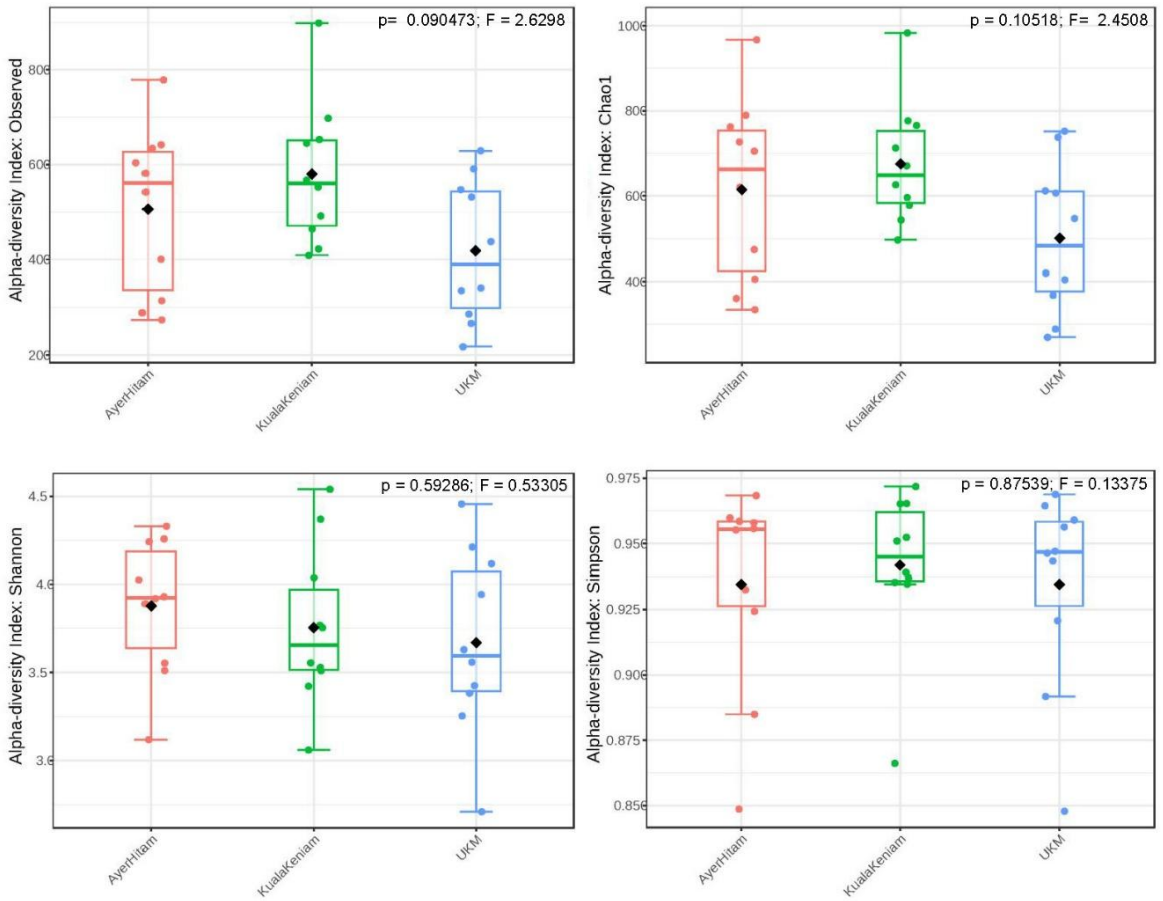

Figure 7. Alpha diversity of bacterial communities associated with flies collected from Ayer Hitam, Kuala Keniam, and UKM. The results are pooled data of *Chrysomya villeneuvi* and *Phumosia promittens*

In contrast to alpha diversity, beta diversity, as revealed by PCoA ordinations, revealed spatial variation in the composition of bacterial communities associated with the external surfaces of the sampled flies across the three locations (Figure 8). In the *Ch. villeneuvi* plot, the first and second principal coordinates explained 21.4% and 14.0% of the total variation, respectively, accounting for 35.4% of the overall community variation. Samples from continuous rainforest (Kuala Keniam) formed a relatively distinct cluster along the positive axis of PCoA1. In contrast, samples from the fragmented forests (UKM and Ayer Hitam) showed partial overlap, indicating greater similarity in bacterial community composition between the two locations. However, the overlapping confidence ellipses suggest that the separation among locations was not complete, reflecting considerable within-location variation.

In the *P. promittens* plot, the first two axes explained 35.4% and 11.9% of the total variation, respectively, accounting for 47.3% of the variation in bacterial community composition. Samples from UKM showed a tight cluster, indicating lower variability in bacterial communities among individuals. In contrast, Kuala Keniam and Ayer Hitam exhibited broader dispersion and greater overlap, indicating greater heterogeneity in their external microbiota.

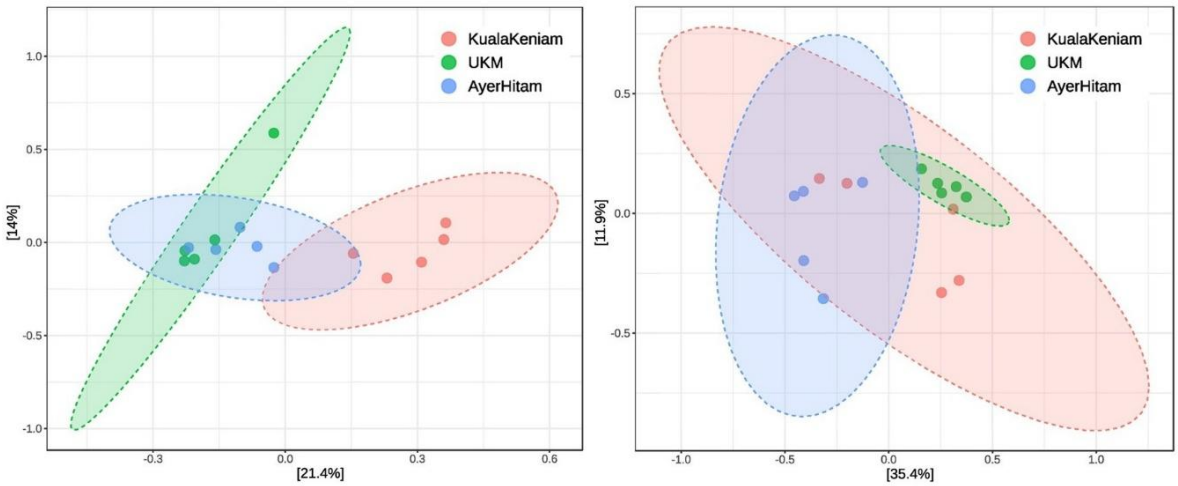

Figure 8. Principal Coordinates Analysis (PCoA) of bacterial community composition associated with (A) *Chrysomya villeneuvi* and (B) *Phumosia promittens* across three forest locations based on Bray–Curtis dissimilarity.

Further PERMANOVA analysis demonstrated that sampling location significantly influenced the composition of external-surface bacterial communities in both *Ch. villeneuvi* and *P. promittens*. For *Ch. villeneuvi*, location explained 26.1% of the variation in bacterial community composition ($F = 2.1209$, $R^2 = 0.2612$, $P = 0.002$). Pairwise comparisons revealed significant differences between Kuala Keniam and UKM ($F = 2.8418$, $R^2 = 0.2621$, adjusted P =0 .009) and between Kuala Keniam and Ayer Hitam ($F = 2.4841$, $R^2 = 0.2369$, adjusted $P = 0.009$). In contrast, no significant difference was observed between UKM and Ayer Hitam ($F = 1.0511$, $R^2 = 0.1161$, adjusted $P = 0.414$). This concludes that there are differences in bacterial communities between fragmented and continuous rainforests.

In contrast, the bacterial communities associated with *P. promittens* exhibited a stronger location effect, with sampling location accounting for 34.3% of the total variation ($F = 3.1373$, $R^2 = 0.3434$, $P = 0.001$). Pairwise comparisons showed significant differences among all locations, including UKM versus Ayer Hitam ($F = 1.9613$, $R^2 = 0.1969$, adjusted P=0.026), UKM versus Kuala Keniam ($F = 2.3608$, $R^2 = 0.2279$, adjusted P=0.026), and Ayer Hitam versus Kuala Keniam ($F = 5.6284$, $R^2 = 0.4130$, adjusted P=0.026), with the greatest compositional dissimilarity observed between Ayer Hitam and Kuala Keniam. Collectively, these results indicate that geographical location significantly shaped the external bacterial communities of both blowfly species, although the effect was more pronounced in *P. promittens*.

Core microbiome analysis performed at the species level (detection threshold = 0.01%; prevalence threshold = 20%) revealed differences in the prevalence of bacterial taxa among the three forest locations (Figure 9). Across all locations, Enterobacteriaceae and *Dysgonomonas* were detected in all samples (100% prevalence). *Ignatzschineria* also exhibited high prevalence, occurring in all samples from Ayer Hitam and UKM and in 80% of samples from Kuala Keniam.

Ayer Hitam exhibited the highest number of highly prevalent taxa, with *Providencia*, *Vagococcus*, *Wohlfahrtiimonas*, *Ignatzschineria indica*, *Ignatzschineria ureiclastica*, *Wohlfahrtiimonas chitiniclastica*, and *Entomomonas moraniae* detected in all samples.

Additionally, *Ruminococcaceae* was present in 80% of samples, whereas *Kalsiella*, *Peptostreptococcaceae*, *Klebsiella*, and *Orbus sasakiae* exhibited lower prevalence, ranging from 30% to 50%. In Kuala Keniam, only Enterobacteriaceae and *Dysgonomonas* reached 100% prevalence. *Ignatzschineria*, *Wohlfahrtiimonas*, *Ruminococcaceae*, and *Kalsiella* occurred in 80–90% of samples, while *Providencia*, *Vagococcus*, *Orbus*, *Orbus sasakiae*, *Desulfovibrionaceae*, *Rosenbergiella*, *Christensenellales*, *Ignatzschineria larvae*, and *Mammaliicoccus fleurettii* were detected in 30–60% of samples.

An intermediate prevalence pattern was observed at the UKM forest reserve. Enterobacteriaceae, *Dysgonomonas*, and *Vagococcus* were detected in all samples, whereas *Wohlfahrtiimonas*, *Wohlfahrtiimonas chitiniclastica*, and *Entomomonas moraniae* occurred in 90% of samples. *Ignatzschineria*, *Ignatzschineria indica*, and *Ignatzschineria ureiclastica* showed prevalence values ranging from 70% to 80%. *Orbus* and *Orbus sasakiae* were present in approximately 60% of samples, while *Ruminococcaceae* occurred in 70% of samples.

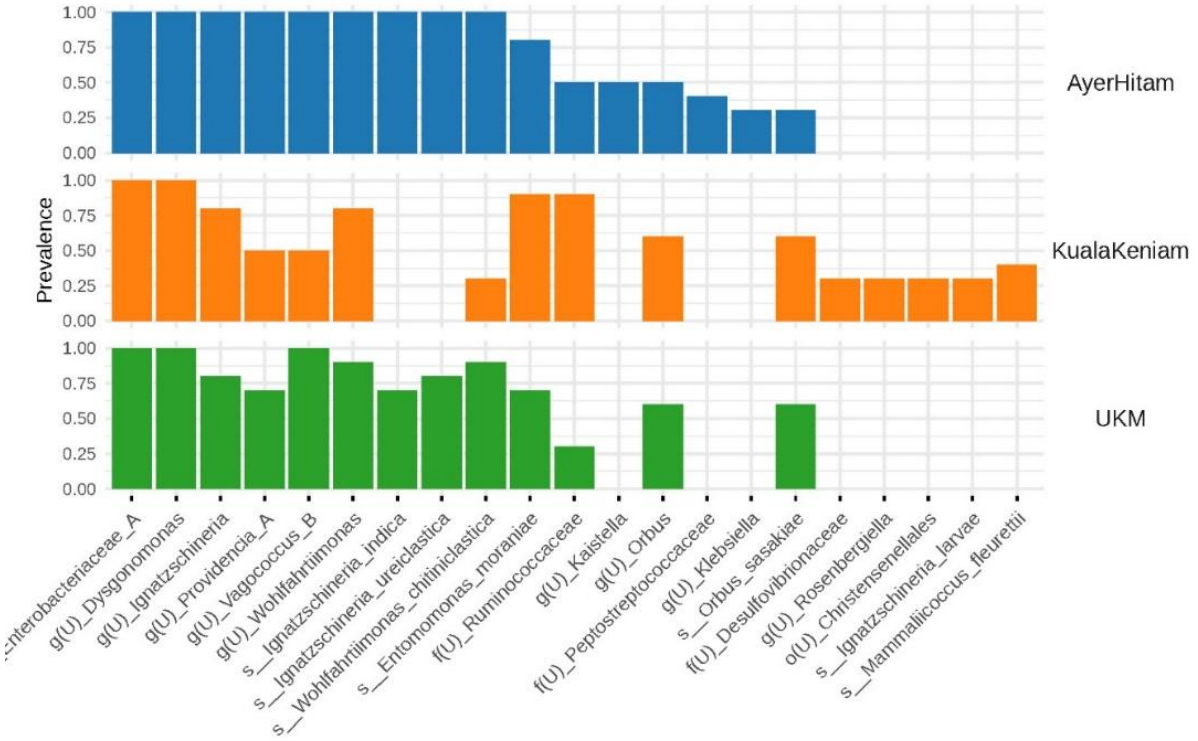

Figure 9. Comparisons of core microbiome at species level at Ayer Hitam, Kuala Keniam, and UKM forest reserves. Bar plots represent the prevalence of bacterial taxa detected at a minimum relative abundance threshold of 0.01% and a prevalence threshold of 20% across samples from each location.

DISCUSSION

This study demonstrates that species identity significantly influences the external bacterial microbiomes of forest-associated blowflies, suggesting that host-specific selection plays an important role in the microbial assembly of *Ch. villeneuvi* and *Ph. promittens.* Although both species consistently interact with similar transient nutritional sources in the study locations, their distinct microbial profiles were preserved. This partitioning indicates that microbiome assembly in these species aligns with the "ecosystem on a leash" concept, in which the local

environmental species pool supplies accessible bacteria. At the same time, host-specific features act as selective filters (Foster et al. 2017). Host-specific filtering has increasingly been recognised as a principal determinant of microbiome composition among sympatric fly species; for example, wild populations of the blowflies *Lucilia sericata* and *Phormia regina* exhibit highly species-specific microbiomes that go beyond mere stochastic environmental acquisition (Mikaelyan et al. 2025). Similarly, comparative studies of blowflies and houseflies have shown that fly-associated bacterial communities contain both shared core taxa and host-associated bacterial signatures, further supporting the role of host identity in shaping microbiome structure (Junqueira et al. 2017). By focusing specifically on external bacterial communities, the present study extends this evidence to non-synanthropic forest blowflies. It suggests that host identity may influence the acquisition and retention of bacteria on fly external surfaces.

Principal Coordinates Analysis revealed that blowfly microbiomes from Kuala Keniam Rainforest formed discrete clusters, distinct from those of the urban-fragmented Ayer Hitam and UKM forest reserves. Moreover, whereas the microbiome profiles of *Ch. villeneuvi* and *P. promittens* were clearly distinct in the relatively undisturbed Kuala Keniam habitat, they showed significant overlap in the disturbed Ayer Hitam and UKM forests. The greater microbiome overlap among fly samples from fragmented forests may reflect more similar microbial exposure conditions in these fragments. Fragmented forests often share disturbance-related characteristics, including edge effects, altered microclimates, modified vegetation structure, and greater exposure to human-associated environmental inputs, which may contribute to convergence in environmental microbial reservoirs (Kovacs et al. 2025).

This phenomenon is consistent across multiple taxa; for instance, anthropogenic habitat disturbances have been shown to alter microbial diversity in surrounding soil and water, thereby reshaping the commensal microbiota of local amphibian hosts (Chen et al. 2023). Similarly, land-use changes in tropical regions directly influence underlying soil properties, driving significant shifts in the structure of soil microbial communities (Tripathi et al. 2016). As blowflies consistently acquire bacteria from their surroundings, shared environmental conditions in fragmented forests may have contributed to the overlapping bacterial profiles observed between the two fly species. This pattern indicates that human-induced habitat disturbances and microclimatic variations between fragmented and primary forests may affect the environmental microbial reservoir and, consequently, alter microbial assemblages in local fly populations (Jiménez et al. 2020). However, the interpretation of the forest-type effect has been approached cautiously because the locations were not sampled concurrently. Therefore, the observed clustering patterns may reflect a combination of habitat context and temporal differences in microbial exposure. Synchronised and repeated sampling across rainforest types would further clarify how forest conditions and temporal dynamics influence blowfly-associated bacterial communities.

Although the two factors discussed above may contribute to variation in microbial community composition, the relatively small sample size is acknowledged as a limitation of this study. Microbiome data frequently exhibit considerable variability among individual samples, attributable in part to environmental changes and disparities in microbial exposure (Nguyen et al. 2021), which may have reduced the statistical power to identify small variations in bacterial richness, evenness, and low-abundance taxa (Kers & Saccenti 2022). Despite this limitation, the PERMANOVA test revealed significant differences in bacterial communities by host species and sample location, thereby demonstrating that the effects remain detectable within the current dataset. Moreover, multiple diversity metrics were applied in this study, including the Bray-Curtis metric, which is generally considered the most sensitive measure for

detecting differences between groups with smaller sample sizes (Kers & Saccenti 2022). Considering these arguments, these findings have been regarded as preliminary evidence of microbiome variation with species and forests as the main factors. Additionally, because environmental sources such as bait, soil, vegetation, and trap surfaces were not sequenced, the specific origins of the detected bacterial taxa cannot be determined, and future studies should include environmental controls to clarify bacterial transmission pathways (Junqueira et al. 2017). Hence, additional research employing bigger sample sizes, repeated temporal sampling and soil samples is recommended to enhance the reliability and generalisability of these patterns. Besides that, expanded geographic sampling may yield a better understanding of ambient microbial dynamics and the influence of local habitats on blowfly-associated bacterial communities (Park et al. 2019).

Besides the host species and forest variability, the elevated prevalence of specific genera, such as *Dysgonomonas, Ignatzschineria, Providencia, Vagococcus,* and *Wohlfahrtiimonas,* corresponds with the literature characterising the microbiomes of necrophagous and saprophagous flies. The widespread presence of *Dysgonomonas* and *Enterobacteriaceae suggests* a conserved functional role for these bacteria. *Dysgonomonas* is commonly present in significant quantities in detritivorous insects and is associated with the enzymatic breakdown of complex polysaccharides and resistant organic materials (Koch et al. 2026). Meanwhile, *Ignatzschineria* and *Wohlfahrtiimonas* are characteristic taxa of carrion-associated flies. Both genera were initially characterised from the larvae of the myiasis-inducing meat fly *Wohlfahrtia magnifica* (Toth et al. 2008). Their persistence across various forest ecosystems in both *Ch. villeneuvi* and *P. promittens* indicates a shared ecological niche and suggests overlapping nutritional needs associated with carrion consumption and decomposition.

Our findings indicated notable taxonomic biases between the two host species. *P. promittens* often exhibited higher relative abundances of *Orbus* and *Wohlfahrtiimonas*, whereas *Ch. villeneuvi* showed a greater prevalence of *Ignatzschineria* and *Vagococcus.* These specific taxonomic biases mirror patterns observed in other sympatric blowflies. For example, previous study identified *Ignatzschineria* and *Vagococcus* as the primary predictive genera that consistently differentiate wild populations of *Phormia regina* and *Lucilia sericata* respectively (Mikaelyan et al. 2025). Meanwhile, members of the genus *Orbus* are identified as specialised gut bacteria in insects, commonly associated with wild-caught dipteran species, such as *Drosophila* (Phillips et al. 2024). The species *Orbus sasakiae* was isolated from the gut of the butterfly *Sasakia charonda*, showing that this genus is not restricted to blowflies but is also associated with other insects (Kim et al. 2013). The differential prevalence of these key taxa between *Ch. villeneuv*i and *P. promittens* underscores the notion that species-specific physiological or behavioural factors govern microbial colonisation, even in cohabiting flies within the same habitat (Mikaelyan et al. 2025; Muñoz-Hernández et al. 2026).

Identification of genera such as *Providencia, Acinetobacter, Ignatzschineria,* and *Wohlfahrtiimonas* on *Ch. villeneuvi* and *P. promittens* warrants substantial consideration. Many of these groups are reported to contain opportunistic pathogens with clinical significance. For instance, *Wohlfahrtiimonas chitiniclastica* has been reported as an emerging opportunistic pathogen associated with bacteraemia and myiasis, while *Ignatzschineria ureiclastica* and related *Ignatzschineria* species have also been linked to wound-associated and myiasis-related infections in humans and animals (Campisi et al. 2015; Reed et al. 2021). Furthermore, a previous study reported that *Providencia* was detected alongside antibiotic resistance genes in houseflies from human-associated environments, highlighting the capacity of flies to acquire

and carry clinically relevant bacteria from their surroundings (Monyama et al. 2023). Although antimicrobial resistance was not assessed in the present study, the presence of these genera on the external surfaces of forest-associated blowflies suggests that their potential ecological and public health significance warrants further investigation.

CONCLUSION

This study provided preliminary evidence of host-species-associated variation in the external bacterial microbiomes of *Ch. villeneuvi* and *P. promittens*. Although both species shared several consistently detected bacterial taxa, their overall community structures differed, suggesting that host species identity is an important factor shaping the composition of the external bacterial microbiome. Whole-genome metagenomics, metatranscriptomics, and culture-based isolation should be considered to determine whether host-species-associated bacteria play functional roles in decomposition, environmental adaptation, or pathogen carriage. Meanwhile, rainforest types were also found to shape the microbial community. However, the evidence is limited because temporal factors may have acted as confounding factors that could also contribute to microbial changes. Therefore, the potential use of forest-associated blowfly microbiomes as indicators of rainforest condition remains exploratory and requires further study. Future studies are recommended to include larger sample sizes, synchronised and repeated temporal sampling, environmental microbial controls, and broader geographical coverage to distinguish better host, habitat, spatial, and temporal effects.

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AUTHORS DECLARATIONS

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Conflict of Interest

The authors have declared no conflicts of interest.

Ethics Declarations

Ethics declarations are not applicable for this research.

Data Availability Statement

The datasets generated during and/or analysed during the present study are available from the corresponding author.

Authors' Contributions

Ahmad Azani Othman designed the methodology, conducted the formal analysis, administered the project, secured funding, collected data, prepared the original draft, and reviewed and edited the manuscript. Atikah Insyirah Azizi contributed to research sampling, investigation, formal and software analyses, data collection, and manuscript review and editing. Zolkapli Eshak and Mohammad Adam Adman contributed to formal and software analyses, funding acquisition, project administration, data collection, and manuscript review and editing. Zulkiflee Abd Latif contributed to funding acquisition, software analysis and manuscript review and editing. Muhamad Azizi Mustapa contributed to logistics and data collection. Mohamad Faiz Foong Abdullah contributed to formal analysis, supervision and manuscript review and editing. Azwandi Ahmad designed the methodology, supervised the research, conducted the formal analysis, administered the project, secured funding, conducted data collection, prepared the original draft, and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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