

Species Composition of Bee and Non-bee Hymenoptera as Effective and Less Effective Pollinators in the Tengku Hassanal Wildlife Reserve Forest, Pahang, Malaysia, with New Insights from DNA Barcoding

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ABSTRACT

The species composition of bee and non-bee Hymenoptera in the Tengku Hassanal Wildlife Reserve (THWR) Forest, Pahang, Malaysia, was studied to document their diversity and to explore their relatedness using DNA barcoding of the cytochrome c oxidase subunit I (*COI*) region inferred by Neighbour-Joining tree. DNA of 56 specimens were successfully extracted from the trapping between February 2023 and January 2024 by using 12 malaise traps. The collection represents 34 species from 33 genera across 11 families; eight families identified as an effective pollinators

(Apidae, Halictidae, Liopteridae, Tenthredinidae, Crabronidae, Pompilidae, Tiphiidae and Vespidae), while three families were classified as less effective pollinators (Braconidae, Evaniidae, and Ichneumonidae). Unique DNA sequences revealed limited or no matches in global databases, highlighting Malaysia's underexplored hymenopteran diversity. This study provides essential baseline data on pollinator diversity, emphasizing the roles of effective pollinators (*Apis cerana*, *Tetrigona apicalis*) and less effective pollinators (e.g., parasitoid wasps) through flower visitation in sustaining ecosystem stability. The inclusion

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of genetic tools alongside morphological approaches offers valuable insights for biodiversity conservation and highlights the global importance of preserving tropical ecosystems.

Keywords: Biodiversity conservation, DNA barcoding, Hymenoptera diversity, pollinators, tropical ecosystems

INTRODUCTION

The order Hymenoptera which includes bees, wasps, ants, and sawflies, represents one of the most diverse and ecologically significant insect groups globally (Blaimer et al., 2023). While bees are critical pollinators in natural and agricultural ecosystems, other hymenopterans such as parasitoid wasps support pest control and ecosystem stability (Khalifa et al., 2021). However, knowledge of hymenopteran biodiversity remains limited, particularly in tropical regions like Malaysia. This underscores the need for comprehensive studies on both bee and non-bee species to better understand their ecological roles and conservation needs, especially regarding their function as pollinators.

Numerous studies in Malaysia have catalogued hymenopteran pollinators species for forest trees (Idris et al., 2023; Lee et al., 2002; Nagamitsu et al., 1999; Santos et al., 2022) and from agricultural crops (Azmi et al., 2017; Fahimee et al., 2021). Most of the studies conducted in Malaysia focus on other insect groups such as moths, butterflies, beetles, flies, and also hymenopteran species, which include bees and ants as important pollinators (Momose et al., 1998; Pfeiffer et al., 2011). Some parasitoids, particularly those from the families Ichneumonidae and Braconidae, play a role as less effective pollinators. However, their function as pollinators cannot be ignored as bees and wasps have comparable pollination activities (Borchardt et al., 2024).

DNA barcoding data remain scarce for hymenopteran species, particularly in Malaysia and minimally covered in existing publications. Generally, most identifications conducted use morphological identification and field observations, which may lead to misidentification. Although morphological identification is commonly used as an initial approach, it is time-consuming compared to DNA barcoding (Chan et al., 2014; Schenk et al., 2020).

The longer approach to morphological identification persists due to the scarcity of hymenopteran taxonomists and the lack of comprehensive taxonomic keys for Malaysian species. Interestingly, molecular identification offers a rapid and precise method for taxonomic resolution (Schenk et al., 2020; Tahir et al., 2018). Additionally, it provides genetic baseline data for advanced analyses, such as diet assessment using Next-Generation Sequencing (NGS) for metabarcoding analysis of *trnL*, contributing to a better understanding of the functional role of hymenopterans as pollinators of forest trees (Bell et al., 2017; Namin et al., 2022).

Malaysia's tropical forests, including the Tengku Hassanál Wildlife Reserve (THWR) in Pahang, are home to a rich diversity of Hymenoptera species. THWR serves as a model

site for studying the natural dynamics of these pollinators and their interactions with forest trees. The underexplored hymenopteran diversity in THWR can act as a bioindicator for forest health (Catalano et al., 2024) and provide indirect insights into herbivorous wildlife populations. Documenting this diversity is crucial for understanding ecological contributions and informing conservation efforts. Therefore, this study aims to identify the species composition of hymenopteran pollinators and to barcode all species collected from THWR.

MATERIALS AND METHODS

Sampling Location

The hymenopteran samplings were conducted at the model forest, THWR, located at the east coast of Peninsular Malaysia (Figure 1). It covers approximately 60,551.608 hectares. Four locations were chosen as the sampling sites, namely Lembah Klau (LK), Bukit Rengit (BR), Kuala Lompat (KL), and Perlok (PP) (Table 1). These sites were strategically chosen to represent the northern, southern, eastern and western regions of the forest, to get the holistic view of the hymenopteran species diversity within the area.

Sample Collection and Identification

Three malaise traps were placed at each location in the THWR for hymenopteran samples collection (Table 1), making a total of 12 traps. The traps were left for 12 months, from February 2023 to January 2024. The samples were preserved in 70% ethanol for the molecular work.

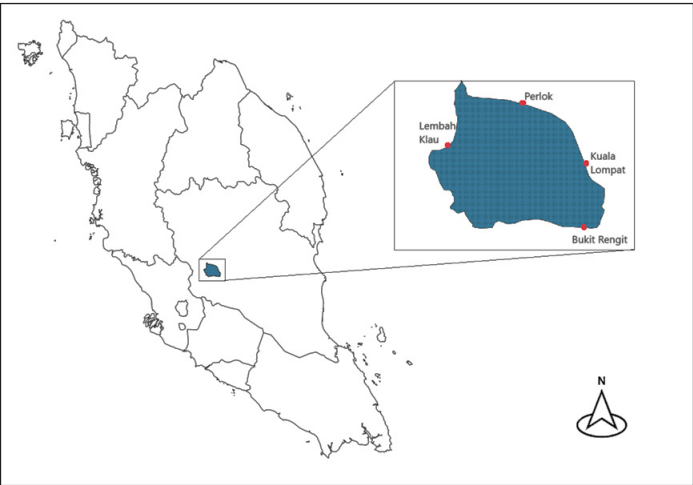


Figure 1. Map of Tengku Hassanal Wildlife Reserve (THWR), Pahang, Malaysia
Source: Author's own work

Table 1
List of locality, Global Positioning System (GPS) coordinates and the type of ecosystem for all study sites

Sample code	Location	Grid	Ecosystem
LK	Lembah Klau	03°41.354’N, 102°1.196’E	Lowland dipterocarp forest
BR	Bukit Rengit	03°35.580’N, 102°10.817’ E	Lowland dipterocarp forest
KL	Kuala Lompat	03°42.957’N, 102°17.286’E	Lowland dipterocarp forest, Riverine
PP	Perlok	03°50.544’N, 102°12.234’E	Lowland dipterocarp forest

Morphological Identification

All hymenopteran samples were identified using a stereomicroscope (StemiD4, Zeiss, Germany). Identification was done based on the morphological characteristics of the hymenopteran species following species key provided by Goulet and Huber (1993), Michener (2007) and Van Noort and Broad (2024). The morphological identification was conducted prior to DNA barcoding analysis.

Digital Photograph of Specimens

A comprehensive observation was conducted on adult samples by using a StemiD4 stereomicroscope (Carl Zeiss, Germany) and a Dino-Lite digital microscope to facilitate detailed species identification. Detailed habitus images of the samples were acquired utilising a ZEISS SteREO Discovery V20 stereomicroscope with AxioCam MR ToupTek software.

Species Record and Their Pollination Detection

All identified species, based on morphological characters were listed up to species level where applicable in a table to illustrate the diversity of the sampled hymenopteran pollinator species from THWR. Their functional role as pollinators (effective and less effective) were confirmed based only on published literatures (Klein et al., 2019; Michener, 2007; Rader et al., 2020; Stavert et al., 2016; Van Noort & Broad, 2024; Wojcik, 2021; Yokota et al., 2024).

DNA Extraction and Amplification

Identification was further confirmed through DNA barcoding analysis. The samples were extracted using NucleoSpin DNA Insect Kit (Macherey-Nagel) and DNeasy Blood and Tissue Kit (Qiagen) following manufacturer’s protocol with slight modification according to Mohammed et al. (2017) and Yaakop et al. (2013). The extraction was then amplified through Polymerase Chain Reaction (PCR) by targeting cytochrome c oxidase subunit I (COI) with universal forward primer LCO1490 (GGTCAACAAATCATAAAGATATTGG) and reverse primer HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994). The amplification was performed under the following protocol: initial denaturation of 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing

ranged from 42-55°C depending on the species for 30 s and extension at 72°C for 30 s, with final elongation at 72°C for 5 min (Fahimee et al., 2021; Idris et al., 2023).

Sequencing, Aligning and BLAST Analysis

All PCR products were sent to Apical Scientific Sdn. Bhd., Selangor, Malaysia for sequencing. The obtained sequences were edited using Sequencher 5.4.6 by Gene Codes Corporation. The resulting datasets were manually and automatically aligned using MEGA version 11 (Kumar et al., 2016). Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) was used to compare the sequences with other sequences available in the GenBank database.

Tree Reconstruction

The species separation was visualised by implementing distance-based criteria through Neighbour-Joining (NJ) analysis using PAUP version 4.0 (Swofford, 2003). The distance-based method using the Kimura 2-parameter model with 1000 replications of the bootstrap analysis was selected.

RESULTS

Species Composition and Species Richness

A total of 56 individuals of hymenopteran pollinators were collected from THWR throughout the sampling duration, comprising eleven families. 34 species under 33 genera from families of Apidae, Crabronidae, Halictidae, Pompilidae, Tiphiiidae, Vespidae, Braconidae, Evaniidae, Ichneumonidae, Liopteridae and Tenthredinidae were recorded and presented in Table 2. The largest family comprising the largest number of species belong to Ichneumonidae (22%) while Halictidae and Tenthredinidae had the lowest record, each comprising only 2% of the total. The species composition and richness are illustrated in Figure 2.

Table 2
List of species, genera and families collected from the samplings identified based on morphological characters

No.	Sample code	Family	Subfamily	Genus	Species
1	KL13	Apidae		Apis	Apis cerana
2	KL15	Apidae		Apis	Apis cerana
3	KL16	Apidae		Apis	Apis cerana
4	PP24	Apidae		Lepidotrigona	Lepidotrigona terminata
5	BR12	Apidae		Tetragonilla	Tetragonilla collina
6	BR3	Apidae		Tetrigona	Tetrigona apicalis

Table 2 (*continue*)

No.	Sample code	Family	Subfamily	Genus	Species
7	BR4	Apidae		<i>Tetrigona</i>	<i>Tetrigona apicalis</i>
8	BR5	Apidae		<i>Tetrigona</i>	<i>Tetrigona apicalis</i>
9	KL3	Crabronidae	Crabroninae	<i>Ectemnius</i>	<i>Ectemnius</i> sp.
10	BR14	Crabronidae	Crabroninae	<i>Liris</i>	<i>Liris</i> sp.
11	BR18	Crabronidae	Crabroninae	<i>Lyroda</i>	<i>Lyroda subita</i>
12	LK16	Crabronidae	Crabroninae	<i>Rhopalum</i>	<i>Rhopalum clavipes</i>
13	BR7	Halictidae	Nomiinae	<i>Nomia</i>	<i>Nomia strigata</i>
14	KL1	Pompilidae	Pepsinae	<i>Auplopus</i>	<i>Auplopus</i> sp.
15	KL2	Pompilidae	Pepsinae	<i>Auplopus</i>	<i>Auplopus</i> sp.
16	LK10	Pompilidae		<i>Leptodialepis</i>	<i>Leptodialepis ceylonica</i>
17	LK3	Tiphiidae		<i>Tiphia</i>	<i>Tiphia</i> sp. 1
18	PP10	Tiphiidae		<i>Tiphia</i>	<i>Tiphia</i> sp. 1
19	PP16	Tiphiidae		<i>Tiphia</i>	<i>Tiphia</i> sp. 2
20	PP18	Tiphiidae		<i>Tiphia</i>	<i>Tiphia</i> sp. 1
21	PP15	Vespidae	Eumeninae	<i>Euodynerus</i>	<i>Euodynerus</i> sp.
22	BR24	Vespidae	Stenogastrinae	<i>Liostenogaster</i>	<i>Liostenogaster flavolineata</i>
23	BR11	Vespidae		<i>Polistes</i>	<i>Polistes</i> sp.
24	BR15	Vespidae		<i>Polistes</i>	<i>Polistes</i> sp.
25	BR17	Vespidae		<i>Polistes</i>	<i>Polistes</i> sp.
26	BR9	Vespidae		<i>Polistes</i>	<i>Polistes</i> sp.
27	BR13	Vespidae	Polistinae	<i>Polybioides</i>	<i>Polybioides</i> sp.
28	BR21	Vespidae	Polistinae	<i>Polybioides</i>	<i>Polybioides</i> sp.
29	BR22	Vespidae	Polistinae	<i>Polybioides</i>	<i>Polybioides</i> sp.
81	KL11	Vespidae	Polistinae	<i>Polybioides</i>	<i>Polybioides</i> sp.
31	LK14	Vespidae	Polistinae	<i>Polybioides</i>	<i>Polybioides</i> sp.
32	KL12	Vespidae		<i>Stenodyneriellus</i>	<i>Stenodyneriellus similiguttulatus</i>
33	BR38	Braconidae	Rogadinae	<i>Aleiodes</i>	<i>Aleiodes procoronarius</i>
34	BR39	Braconidae	Rogadinae	<i>Aleiodes</i>	<i>Aleiodes procoronarius</i>
35	BR29	Braconidae	Microgastrinae	<i>Choeras</i>	<i>Choeras</i> sp.
36	BR30	Braconidae	Microgastrinae	<i>Choeras</i>	<i>Choeras</i> sp.
37	BR32	Braconidae	Microgastrinae	<i>Neoclarkinella</i>	<i>Neoclarkinella</i> sp.
38	BR20	Braconidae	Braconinae	<i>Syntomernus</i>	<i>Syntomernus</i> sp.
39	LK2	Evaniidae		<i>Prosevania</i>	<i>Prosevania</i> sp.
40	LK20	Evaniidae		<i>Prosevania</i>	<i>Prosevania</i> sp.
41	BR28	Evaniidae		<i>Zeuxevania</i>	<i>Zeuxevania</i> sp.
42	BR33	Ichneumonidae		<i>Acrolyta</i>	<i>Acrolyta</i> sp.
43	BR25	Ichneumonidae	Orthocentrinae	<i>Aperileptus</i>	<i>Aperileptus</i> sp.

Table 2 (continue)

No.	Sample code	Family	Subfamily	Genus	Species
44	BR19	Ichneumonidae	Campopleginae	<i>Hyposoter</i>	<i>Hyposoter</i> sp.
45	BR23	Ichneumonidae			Ichneumonidae sp.
46	BR26	Ichneumonidae			Ichneumonidae sp.
47	BR27	Ichneumonidae	Acaenitinae	<i>Jezarotes</i>	<i>Jezarotes</i> sp.
48	KL10	Ichneumonidae	Cryptinae	Cryptinae	Cryptinae sp.
49	BR36	Ichneumonidae	Orthocentrinae	<i>Orthocentrus</i>	<i>Orthocentrus</i> sp.
50	BR34	Ichneumonidae	Orthocentrinae	<i>Plectiscus</i>	<i>Plectiscus callidulus</i>
51	BR35	Ichneumonidae	Orthocentrinae	<i>Plectiscus</i>	<i>Plectiscus callidulus</i>
52	BR37	Ichneumonidae	Orthocentrinae	<i>Plectiscus</i>	<i>Plectiscus callidulus</i>
53	BR40	Ichneumonidae	Orthocentrinae	<i>Plectiscus</i>	<i>Plectiscus callidulus</i>
54	LK7	Liopteridae		<i>Paramblynotus</i>	<i>Paramblynotus</i> sp.
55	PP17	Liopteridae		<i>Paramblynotus</i>	<i>Paramblynotus</i> sp.
56	BR2	Tenthredinidae	Heterarthrinae	<i>Caliroa</i>	<i>Caliroa</i> sp.

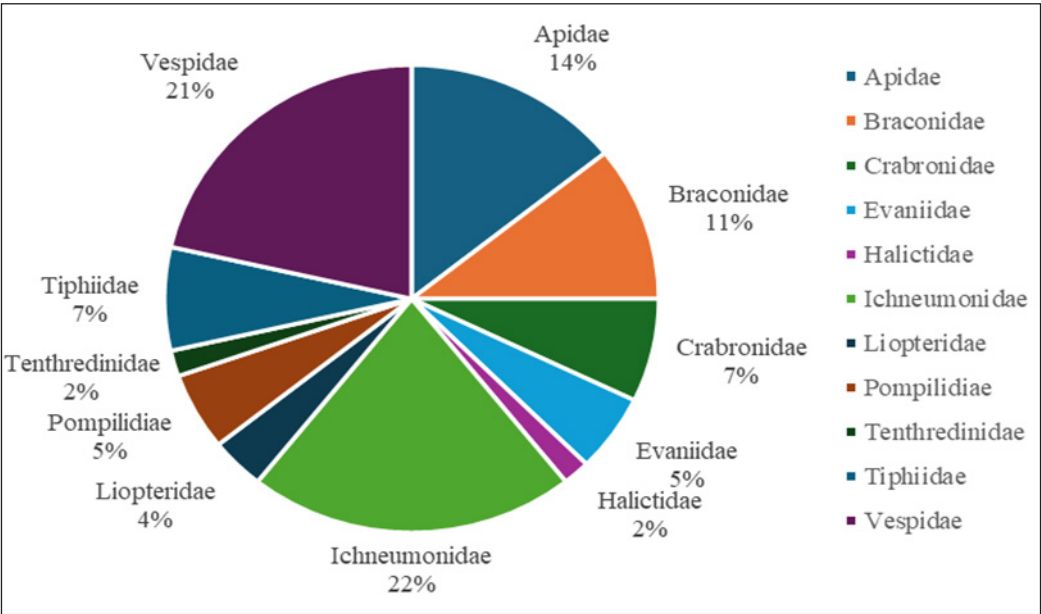


Figure 2. Family composition for the Hymenopteran pollinators collected from Tengku Hassanal Wildlife Reserve (THWR), Pahang, Malaysia

DNA Barcode Information

All 56 morphologically identified samples were barcoded using *COI* marker. The BLAST results and accession numbers obtained from GenBank are listed in Table 3.

Table 3
List of barcoded species along with their maximum score, total score, query coverage, E-value, similarity percentage, and accession number, including the additional GenBank sequences used in the phylogenetic analysis

No.	Sample code	Locality	Family	Subfamily	Maximum Score	Total Score	Query cover (%)	e-value	Similarity Percentage (%)	GenBank Accession number	Species Identified
1	KL13	Malaysia: Pahang, Kuala Lompat	Apidae		1118	1118	86	0	99.52	PQ788155	<i>Apis cerana</i>
2	KL15	Malaysia: Pahang, Kuala Lompat	Apidae		1118	1118	86	0	99.52	PQ788154	<i>Apis cerana</i>
3	KL16	Malaysia: Pahang, Kuala Lompat	Apidae		1118	1118	87	0	99.52	PQ788153	<i>Apis cerana</i>
4	PP24	Malaysia: Pahang, Perlak	Apidae		707	766	83	0	86.36	PQ815050	<i>Lepidotrigona terminata</i>
5	BR12	Malaysia: Pahang, Bukit Rengit	Apidae		789	789	72	0	93.67	PQ815047	<i>Tetragonilla collina</i>
6	BR3	Malaysia: Pahang, Bukit Rengit	Apidae		919	919	88	0	92.14	PQ788158	<i>Tetrigona apicalis</i>
7	BR4	Malaysia: Pahang, Bukit Rengit	Apidae		992	992	89	0	94.28	PQ788156	<i>Tetrigona apicalis</i>
8	BR5	Malaysia: Pahang, Bukit Rengit	Apidae		923	923	88	0	92.27	PQ788157	<i>Tetrigona apicalis</i>

Table 3 (continue)

No.	Sample code	Locality	Family	Subfamily	Maximum Score	Total Score	Query cover (%)	e-value	Similarity Percentage (%)	GenBank Accession number	Species Identified
9	KL3	Malaysia: Pahang, Kuala Lompat	Crabronidae	Crabroninae	748	748	84	0	87.36	OR782830.1	<i>Ectermius</i> sp.
10	BR14	Malaysia: Pahang, Bukit Rengit	Crabronidae	Crabroninae	1183	1183	94	0	98.67	PQ827100	<i>Liris</i> sp.
11	BR18	Malaysia: Pahang, Bukit Rengit	Crabronidae	Crabroninae	820	820	87	0	89.1	PQ827101	<i>Lyroda subita</i>
12	LK16	Malaysia: Pahang, Lembah Klau	Crabronidae	Crabroninae	893	893	91	0	90.15	PQ788160	<i>Rhopalum clavipes</i>
13	BR7	Malaysia: Pahang, Bukit Rengit	Halictidae	Nomiinae	1145	1145	95	0	96.94	PQ834802	<i>Nomia strigata</i>
14	KL1	Malaysia: Pahang, Kuala Lompat	Pompilidae	Pepsinae	612	612	91	2.00E-170	81.04	PQ827097	<i>Auplopus</i> sp.
15	KL2	Malaysia: Pahang, Kuala Lompat	Pompilidae	Pepsinae	900	900	98	0	88.65	PQ800421	<i>Auplopus</i> sp.
16	LK10	Malaysia: Pahang, Lembah Klau	Pompilidae		900	900	86	0	92.37	PQ800422	<i>Leptodialepis ceylonica</i>

Table 3 (continue)

No.	Sample code	Locality	Family	Subfamily	Maximum Score	Total Score	Query cover (%)	e-value	Similarity Percentage (%)	GenBank Accession number	Species Identified
17	LK3	Malaysia: Pahang, Lembah Klau	Tiphiidae		820	820	92	0	87.8	PQ800426	<i>Tiphia</i> sp. 1
18	PP10	Malaysia: Pahang, Perlok	Tiphiidae		810	810	90	0	87.81	PQ800425	<i>Tiphia</i> sp. 1
19	PP16	Malaysia: Pahang, Perlok	Tiphiidae		812	812	81	0	90.77	PQ800424	<i>Tiphia</i> sp. 2
20	PP18	Malaysia: Pahang, Perlok	Tiphiidae		708	708	79	0	86.94	PQ800423	<i>Tiphia</i> sp. 1
21	PP15	Malaysia: Pahang, Perlok	Vespidae	Eumeninae	1137	1137	88	0	99.69	PQ800408	<i>Euodynerus</i> sp.
22	BR24	Malaysia: Pahang, Bukit Rengit	Vespidae	Stenogastrinae	886	886	91	0	89.97	PQ800409	<i>Liostenogaster flavolineata</i>
23	BR11	Malaysia: Pahang, Bukit Rengit	Vespidae		752	752	88	0	86.49	PQ827095	<i>Polistes</i> sp.
24	BR15	Malaysia: Pahang, Bukit Rengit	Vespidae		747	747	88	0	86.33	PQ800412	<i>Polistes</i> sp.
25	BR17	Malaysia: Pahang, Bukit Rengit	Vespidae		780	780	91	0	86.34	PQ827096	<i>Polistes</i> sp.

Table 3 (continue)

No.	Sample code	Locality	Family	Subfamily	Maximum Score	Total Score	Query cover (%)	e-value	Similarity Percentage (%)	GenBank Accession number	Species Identified
26	BR9	Malaysia: Pahang, Bukit Rengit	Vespidae		719	719	92	0	84.19	PQ800413	<i>Polistes</i> sp.
27	BR13	Malaysia: Pahang, Bukit Rengit	Vespidae	Polistinae	775	775	86	0	87.26	PQ819650	<i>Polybioides</i> sp.
28	BR21	Malaysia: Pahang, Bukit Rengit	Vespidae	Polistinae	765	765	86	0	87.03	PQ819651	<i>Polybioides</i> sp.
29	BR22	Malaysia: Pahang, Bukit Rengit	Vespidae	Polistinae	784	784	85	0	87.58	PQ800411	<i>Polybioides</i> sp.
81	KL11	Malaysia: Pahang, Kuala Lompat	Vespidae	Polistinae	780	780	85	0	87.42	OR782831.1	<i>Polybioides</i> sp.
31	LK14	Malaysia: Pahang, Lembah Klau	Vespidae	Polistinae	775	775	86	0	87.26	PQ800410	<i>Polybioides</i> sp.
32	KL12	Malaysia: Pahang, Kuala Lompat	Vespidae		1110	1110	88	0	98.74	OR782829.1	<i>Stenodyneriellus similiguttulatus</i>
33	BR38	Malaysia: Pahang, Bukit Rengit	Braconidae	Rogadinae	1011	1011	81	0	98.29	PQ800415	<i>Aleiodes procoronarius</i>

Table 3 (continue)

No.	Sample code	Locality	Family	Subfamily	Maximum Score	Total Score	Query cover (%)	e-value	Similarity Percentage (%)	GenBank Accession number	Species Identified
34	BR39	Malaysia: Pahang, Bukit Rengit	Braconidae	Rogadinae	1009	1009	81	0	98.29	PQ800414	<i>Aleiodes procoronarius</i>
35	BR29	Malaysia: Pahang, Bukit Rengit	Braconidae	Microgastrinae	1187	1187	81	0	100	PQ800417	<i>Choerax</i> sp.
36	BR30	Malaysia: Pahang, Bukit Rengit	Braconidae	Microgastrinae	1010	1010	78	0	99.82	PQ800416	<i>Choerax</i> sp.
37	BR32	Malaysia: Pahang, Bukit Rengit	Braconidae	Microgastrinae	839	839	88	0	89.32	PQ800418	<i>Neoclarkinella</i> sp.
38	BR20	Malaysia: Pahang, Bukit Rengit	Braconidae	Braconinae	878	878	83	0	89.98	PQ800419	<i>Syntomermus</i> sp.
39	LK2	Malaysia: Pahang, Lembah Klau	Evanidae		680	680	97	0	81.6	PQ827103	<i>Prosevania</i> sp.
40	LK20	Malaysia: Pahang, Lembah Klau	Evanidae		575	575	96	5.00E-159	78.44	PQ845697	<i>Prosevania</i> sp.
41	BR28	Malaysia: Pahang, Bukit Rengit	Evanidae		604	604	87	1.00E-167	79.48	PQ800420	<i>Zeuxevania</i> sp.
42	BR33	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	-	860	860	91	0	87.8	PQ827102	<i>Acrolyta</i> sp.

Table 3 (continue)

No.	Sample code	Locality	Family	Subfamily	Maximum Score	Total Score	Query cover (%)	e-value	Similarity Percentage (%)	GenBank Accession number	Species Identified
43	BR25	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Orthocentrinae	786	786	90	0	86.76	PQ800429	<i>Aperileptus</i> sp.
44	BR19	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Campopleginae	784	784	87	0	87.54	PQ827104	<i>Hyposoter</i> sp.
45	BR23	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	-	781	781	90	0	86.74	PQ827106	Ichneumonidae sp.
46	BR26	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	-	989	989	80	0	98.25	PQ827105	Ichneumonidae sp.
47	BR27	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Acaenitinae	800	800	79	0	90.92	PQ800428	<i>Jezarotes</i> sp.
48	KL10	Malaysia: Pahang, Kuala Lompat	Ichneumonidae	Cryptinae	753	753	89	0	85.78	PQ834819	Cryptinae sp.
49	BR36	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Orthocentrinae	882	882	86	0	91.47	PQ800427	<i>Orthocentrus</i> sp.
50	BR34	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Orthocentrinae	958	958	92	0	92.4	PQ800433	<i>Plectiscus callidulus</i>
51	BR35	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Orthocentrinae	957	957	91	0	92.25	PQ800432	<i>Plectiscus callidulus</i>

Table 3 (continue)

No.	Sample code	Locality	Family	Subfamily	Maximum Score	Total Score	Query cover (%)	e-value	Similarity Percentage (%)	GenBank Accession number	Species Identified
52	BR37	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Orthocentrinae	957	957	91	0	92.25	PQ800431	<i>Plectiscus callidulus</i>
53	BR40	Malaysia: Pahang, Bukit Rengit	Ichneumonidae	Orthocentrinae	953	953	91	0	92.1	PQ800430	<i>Plectiscus callidulus</i>
54	LK7	Malaysia: Pahang, Lembah Klau	Liopteridae		877	877	90	0	89.67	PQ800434	<i>Paramblynotus</i> sp.
55	PP17	Malaysia: Pahang, Perlak	Liopteridae		884	884	99	0	87.71	PQ827098	<i>Paramblynotus</i> sp.
56	BR2	Malaysia: Pahang, Bukit Rengit	Tenthredinidae	Heterarthrinae	810	810	91	0	87.6	OR782832.1	<i>Caliroa</i> sp.
57	-	Malaysia	Pyrilidae	Pyrilinae	-	-	-	-		JN277359.1	<i>Viteessa splendida</i>
58	-	Thailand	Apidae		-	-	-	-		PV197633.1	<i>Lepidotrigona terminata</i>

Neighbour-Joining (NJ) Tree

All sequences obtained from this study were included in the phylogenetic analysis together with several GenBank sequences that were listed in Table 3. The NJ tree showed distinct separation between species, with each species cladded together and located at a specific lineage, supported by bootstrap values ranging between 51%–100%. Furthermore, a clear separation between ingroups and outgroups visualised, supported by 91% bootstrap value. The grouping or classification of species into families, genera and species is indicated in the tree in Figure 3, with the hymenopteran families were found to be paraphyletic. Habitus image of hymenopteran representative recorded are shown in Figures 4A and 4B.

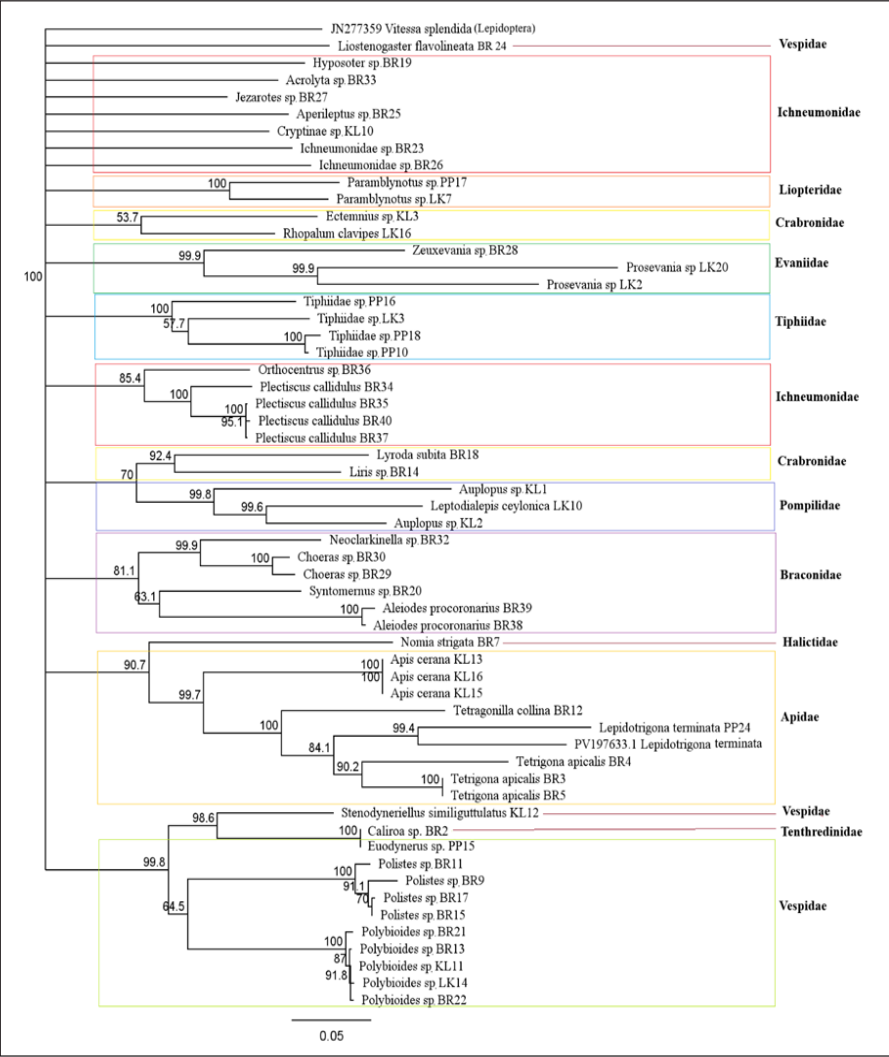


Figure 3. The NJ tree resulted from the Neighbour-Joining (NJ) analysis (distance criterion) based on *COI* sequences of hymenopteran pollinators collected from Tengku Hassan Wildlife Reserve (THWR), Pahang, Malaysia. The bootstrap values, based on 1000 replications, are indicated above the branches

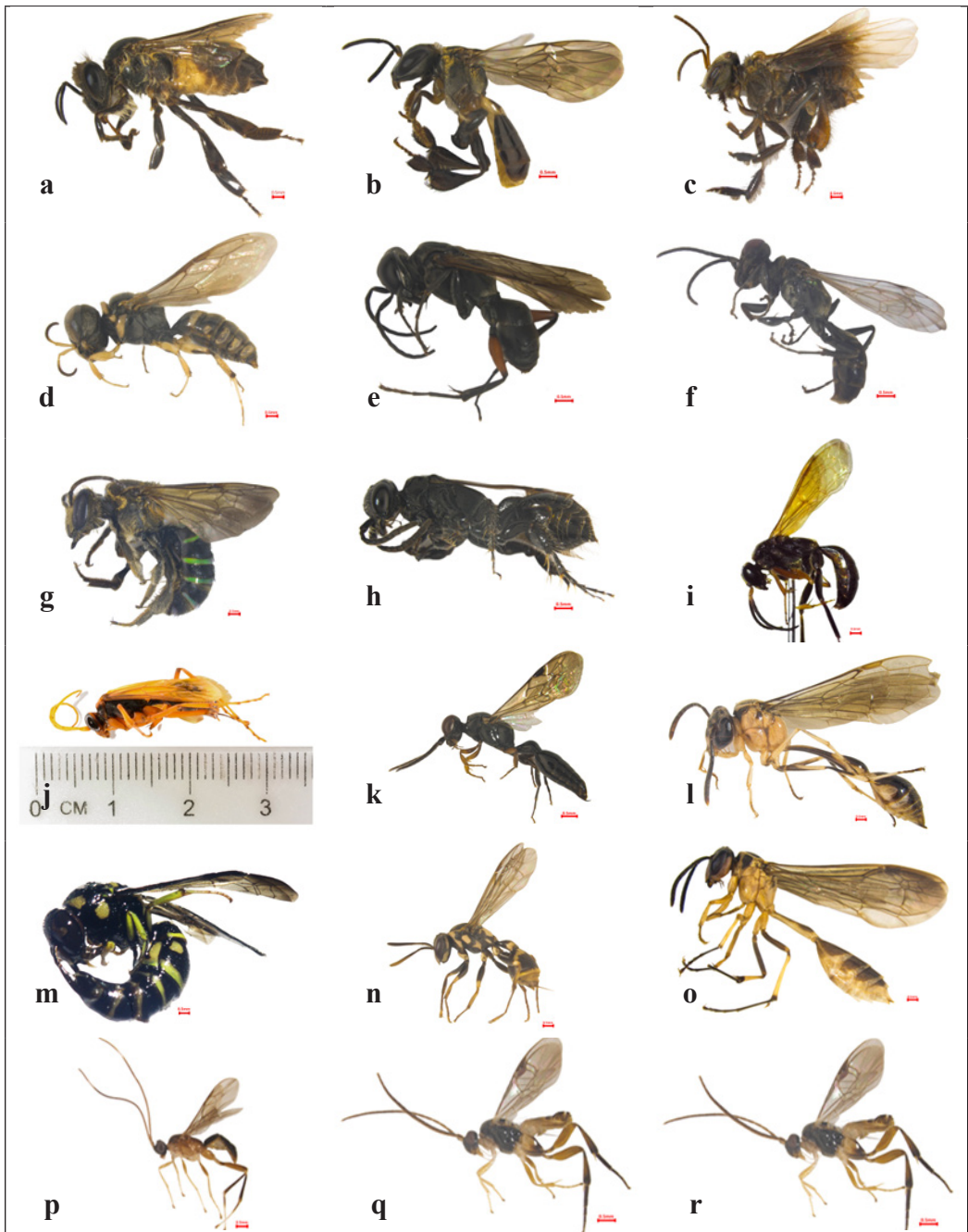


Figure 4A. a, *Apis cerana* (KL13); b, *Lepidotrigona terminata* (PP24); c, *Tetrigona apicalis* (BR4); d, *Ectemnius* sp. (KL3); e, *Liris* sp. (BR14); f, *Lyroda subita* (BR18); g, *Nomia strigata* (BR7); h, *Tiphia* sp. 1 (PP18); i, *Auplopus* sp. (KL1/KL2); j, *Leptodialepis ceylonica* (LK10); k, *Tiphia* sp. 2 (PP16); l, *Liostenogaster flavolineata* (BR24); m, *Stenodyneriellus similiguttulatus* (KL12); n, *Polistes* sp. (BR9); o, *Polybioides* sp. (LK14); p, *Aleiodes procoronarius* (BR39); q, *Choeras* sp. (BR30); r, *Neoclarkinella* sp. (BR32)

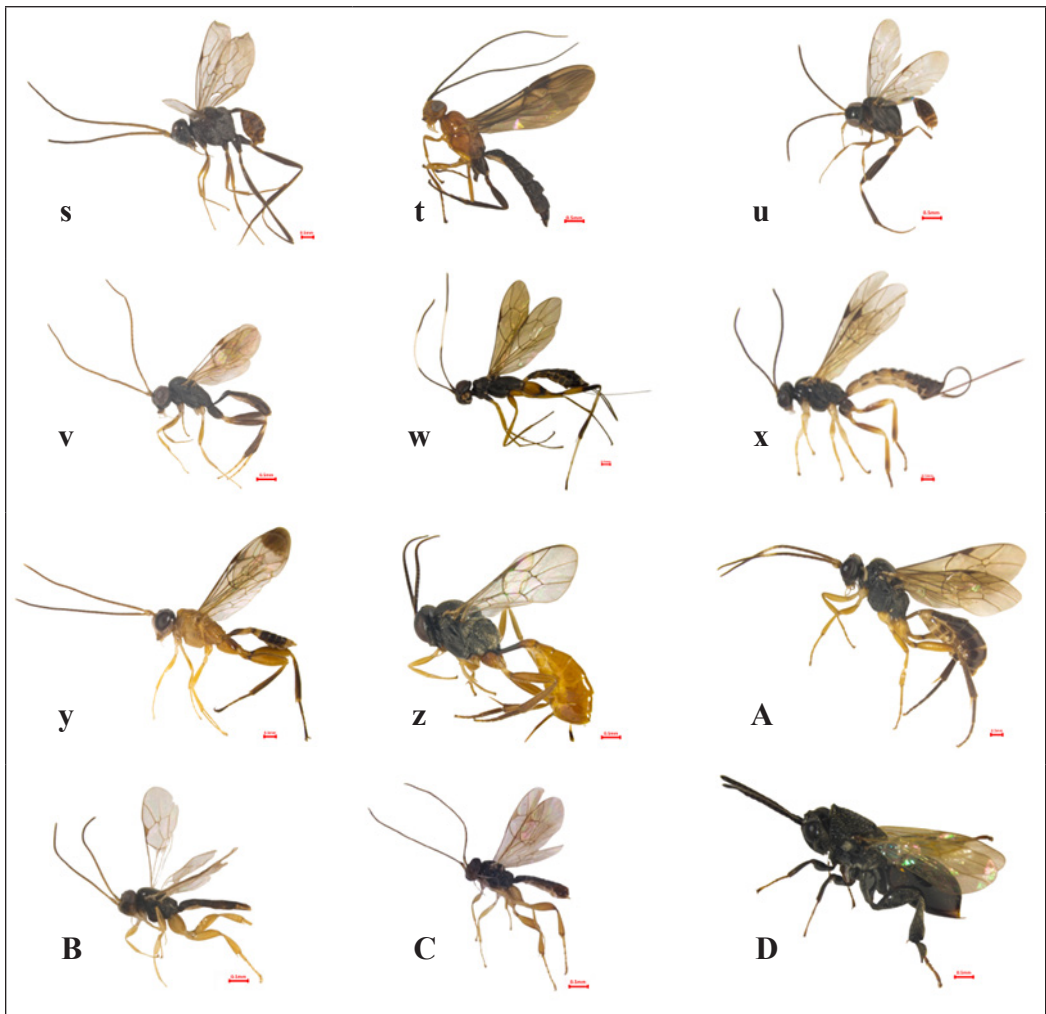


Figure 4B. s, *Zeuxevania* sp. (BR28); t, *Syntomernus* sp. (BR20); u, *Prosevania* sp. (LK20); v, *Acrolyta* sp. (BR33); w, *Aperileptus* sp. (BR25); x, *Ichneumonidae* sp. (BR26); y, *Jezarotes* sp. (BR27); z, *Hyposoter* sp. (BR19); A, *Cryptinae* sp. (KL10); B, *Orthocentrus* sp. (BR36); C, *Plectiscus callidulus* (BR34); D, *Paramblynotus* sp. (LK7)

DISCUSSION

This study focused on understanding a group of hymenopteran species composition, specifically as pollinators in THWR, Pahang, Malaysia. While many previous studies on hymenopteran species in Peninsular Malaysia have focused on species found in plantations (e.g., Aman-Zuki et al., 2019) and primarily in secondary forests (Rabibah et al., 2018), nevertheless these species rarely discussed in a wildlife reserve forest. Hymenoptera has been identified as one of the most abundant and highly diverse groups within the forest ecosystem (De Souza Amorim et al., 2022), serving both as pollinators and parasitoids

(Larsen et al., 2014). However, they have often been overlooked in previous studies due to several factors, such as the lack of comprehensive research, the unavailability of identification keys, limited resources, and high taxonomic diversity. Given their ecological roles and diversity, this study fills a critical gap by exploring hymenopteran pollinators in a protected forest using both morphological and molecular approaches.

A total of 34 species from 11 families have been successfully collected. This finding is in line with Yahaya et al. (2023) that recorded 11 hymenopteran families from Kuala Keniam National Park, Pahang. In this study, eight families were identified as an effective pollinators (Apidae, Halictidae, Liopteridae, Tenthredinidae, Crabronidae, Pompilidae, and Tiphiidae, Vespidae), while three families (Braconidae, Evaniidae, Ichneumonidae) consisted of species classified as less effective pollinators. The effective pollinators were identified based on specific criteria for effectiveness (their ability to transfer pollen efficiently, frequency of flower visitation). In contrast, the less effective pollinators are those incidentally carry pollen while foraging for nectar, without playing a direct role in pollination (Klein et al., 2019; Wojcik, 2021; Yokota et al., 2024).

In this study, bee species from Apidae and Halictidae exhibited traits associated with effective pollination such as dense body pilosity (Roquer-Beni et al., 2020). Three Apidae stingless bees collected in this study, namely *Lepidotrigona terminata*, *Tetragonilla collina*, and *Tetrigona apicalis*, are recognized as pollinators. Among them, *T. apicalis* has been particularly noted as an important pollinator in forest ecosystems (Dumesh & Sabu, 2014). In addition, collected *Apis cerana* has also been documented as a significant pollinator of native forest trees and is considered a keystone species that contributes valuable pollination and other ecosystem services (Chen et al., 2018).

In contrast, non-bee Hymenoptera, while more taxonomically diverse, varied widely in their pollination roles. Families such as Vespidae and Tenthredinidae contributed to pollination but often incidentally or opportunistic manner, while others like Braconidae and Ichneumonidae were more likely to act as parasitoids or nectar feeders with limited pollination impact (Borchardt et al., 2024). This contrast highlights the importance of bees as primary pollinators in forest ecosystems, with non-bees contributing supplementary roles in maintaining floral diversity (Katumo et al., 2022).

Two families namely Vespidae and Ichneumonidae had the highest number of species identified as observed, with nine and seven species, respectively. Vespidae are known as generalist pollinators of forest flowering plants and contribute to maintaining plant biodiversity (Rojas-Nossa et al., 2023). Although Ichneumonidae are not as efficient as bees in pollination, they do serve as pollinators. In contrast, the families Halictidae, Liopteridae, and Tenthredinidae were represented by only one or two species, belonging to a single genus, despite being recognised as very effective pollinators in forest and other ecosystem (Garibaldi et al., 2013; Michener, 2007; Müller & Kuhlmann, 2008;), except

for Evaniidae, which is not commonly associated with pollination. Several other families such as Apidae, Crabronidae, Pompilidae, Tiphidae, and Braconidae, consisting of effective and less effective pollinators, with moderate number of species and genera (Roubik, 2023) (Tables 2 and 3).

Species-level resolution is critical in pollination ecology to accurately delineate pollinator-plant interactions (Van der Niet et al., 2014). Molecular tools, particularly DNA barcoding, significantly enhance identification reliability by resolving taxa that are morphologically cryptic or exhibit high intraspecific variation. When integrated with classical morphological diagnostics, DNA barcoding improves taxonomic precision and facilitates more accurate ecological inferences. Such integration combining molecular and morphological datasets, have become necessary in contemporary systematic and biodiversity research (Schlick-Steiner et al., 2010; Simonsen et al., 2012).

The barcoding analysis revealed that some species share over 98% similarity with reference sequences previously deposited in GenBank (NCBI). However, several other specimens could only be identified up to the genus level, with similarity scores greater than 80% to the available GenBank sequences. This highlights the need for barcoding analysis in hymenopteran species, which some are minute organisms that show high morphological variation. In this case, the species or genus-level identification based on morphological characteristics alone was difficult and impossible, leading to misidentifications which required confirmation through DNA barcoding (Chac & Thinh, 2023). Only 20 out of 56 (35.7%) specimens were successfully identified up to species level based on molecular data. However, the remaining specimens could only be identified up to genus level, both morphologically and molecularly. Although BLAST analysis yielded low sequence similarity with GenBank entries, morphological identification was consistent and accurate. This discrepancy likely reflects database limitations rather than misidentification. Many GenBank records are uncurated or lack representative sequences for closely related species, particularly in under-sampled taxa. Moreover, BLAST performs local alignments and may not capture full-sequence similarity, especially when reference sequences are incomplete or geographically divergent (Spouge & Mariño-Ramírez, 2012).

This study provides new insights into the barcode data for hymenopteran species. Even though morphological identification was conducted, barcode information was very important and informative in supporting species identification (Powell et al., 2019). The molecular approach proved to be highly effective in providing accurate species identification, especially given the close morphological similarities among hymenopteran species (Jeong et al., 2010) and their significant intraspecific variation (Rasool et al., 2018). Such challenges were particularly evident in hymenopterous parasitoids (Farrokhdeh et al., 2014) and members of the Braconidae family (Sharanowski et al., 2011). The *COI* marker used in this study was shown to be suitable for the barcoding approach (Lv et al., 2014), as mitochondrial DNA (mtDNA) evolves rapidly, making it ideal for resolving

taxonomic relationships at the species or genus level (Guo et al., 2022) as seen in similar studies by Hussain et al. (2024).

The NJ tree provided valuable insights into distinguishing species and families relatedness (Figure 3). However, it did not account for the evolutionary process, as it did not incorporate mutation rates (Kalinowski, 2009). Nonetheless, clear separations were observed between the ingroups and outgroups, with outgroup species *Vitessa splendida* (Pyralidae: Lepidoptera) showing significant divergence, which helped differentiate the more primitive ancestors from the more derived Hymenoptera species. *Polybioides* sp. formed a well-supported cluster with high bootstrap values, indicating good confidence in this grouping. This species clustered closely with *Polistes* sp., reflecting their taxonomic relationship within the family Vespidae. Similarly, *Neoclarkinella* sp. and *Choeras* sp. from the Braconidae family and *Prosevania* sp. and *Zeuxevania* sp. of the Evaniidae family, also formed closely related cluster. *Tetrigona apicalis* samples form a distinct cluster with *Lepidotrigona terminata*. The high bootstrap value supports their grouping within the Meliponini tribe of stingless bees.

The exclusion of species within Crabronidae and Vespidae family to cluster together (Figure 3) may reflect long-branch attraction or limited resolution of the NJ method. Branch lengths nonetheless offer useful insights into evolutionary relationships (Kumar & Gadagkar, 2000). Short branch lengths among species within a family generally suggest closer relatedness, which should align with their taxonomic classification (Fernández et al., 2023). However, the NJ tree may not accurately reflect complex evolutionary relationships as it simplifies evolutionary history by relying on distance matrices. This can obscure complex evolutionary scenarios such as hybridisation, rate heterogeneity among lineages, and multiple substitutions at the same site (Zou et al., 2024). Moreover, the use of mtDNA alone may be inadequate for resolving deeper divergences or relationships influenced by introgression or incomplete lineage sorting (Toews & Brelsford, 2012). Additionally, it is recommended to integrate mtDNA with other nuclear markers to enhance resolution and provide a more comprehensive view of evolutionary history (Yoshida & Nei, 2016; Zou et al., 2024).

CONCLUSION

This study offers new insights into the taxonomic composition and relatedness of THWR's bee and non-bee hymenopterans, documenting 34 species across 11 families. By incorporating both morphological identification with DNA barcoding, it improves species-level resolution in a taxonomically challenging group while reducing the gap in current knowledge for Peninsular Malaysia. Identifying both effective and incidental pollinators has important conservation implications, supporting the needs to protect pollination services and maintaining the forest ecosystem. Future studies could explore pollinator-

plant interactions within seasonal and spatial variation to further strengthen conservation and ecological understanding.

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