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Multi-omics and palynology of selected Philippine forest honey

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The Sierra Madre Mountains, which happen to be the longest mountain range in the Philippines, is home to lush floral and faunal species as well as forest-based indigenous communities actively involved in preserving local biodiversity. With active reforestation efforts ongoing for decades, the locals are further encouraged to continue their long-standing practice of honey gathering as a form of cultural manifestation and as an important source of livelihood. To further inspire ongoing conservation efforts, we aim to show that the small molecule diversity in Sierra Madre forest honey reflects the local floral composition and is reflective of the positive impact of previous reforestation initiatives. In order to do this, liquid chromatography—mass spectrometry (LC–MS) based metabolomics was used to profile and compare metabolite diversity in honey produced by *Apis cerana*, *Apis breviligula* Maa. and *Tetragonula biroi* (Friese) honey from Palaui Island and Laiban in Northern and Southern Sierra Madre, respectively. Surprisingly, the Philippine National Tree and unfortunately endangered *Pterocarpus indicus* Willd (loc. Narra) proved to be important, especially in Palaui Island where honey from *A. cerana* is close to being monofloral. Aside from *P. indicus* and its small molecule marker hypaphorine, caffeine was detected in Palaui honey beautifully reflecting the way of life of native Agtas who manage a small coffee plantation. The abundance of caffeine, however, is higher in stingless honey samples from Tanay, Rizal where *Coffea* trees have been extensively included in restoration activities over the past few decades. Our results imply the possibility of using honey as an ecological monitoring tool while generating baseline chemical information that reflects the state of Philippine forests. Furthermore, the identification of unique chemical components in forest honey can be further used in programs that assist indigenous communities in safeguarding the ownership and origin of forest honey sources.

Keywords Philippine forest honey, Metabolomics, DNA barcoding, Palynology, Chemical ecology, Forest conservation

The Philippine archipelago, consisting of over 7641 islands, is considered a treasure trove of tropical biodiversity[1,2]. Within its lush rainforests are about 10,000–13,000 species of plants and about 1200 wildlife species, half of which are endemic[1,3]. A major concern, however, is the rapid decline of forest cover (70% in the last 100 years) from deforestation caused by logging and agricultural and urban development[4,5]. The largest remaining contiguous forest area is in the Sierra Madre Mountain Range, spanning across ten provinces and covering 1.4 million hectares of land[6]. Also called the "backbone of Luzon Island"[6], the Sierra Madre mountains provide a natural barrier against tropical storms[7], supply fresh water to the nation's biggest watersheds[8], provide habitat for diverse flora and fauna[9], and serve as the ancestral domain for several indigenous groups[10]. The Sierra Madre is usually

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divided into the Northern Sierra Madre (NSM) and the Southern Sierra Madre (SSM), with a biogeographical divide in Aurora Province[11].

At the very northern tip of the NSM lies Palaui Island (Fig. 1A, C), a 7,415 ha protected area which is also the ancestral domain of an indigenous Agta community. Forest honey gathering here is an important ritual and livelihood activity for the Agta. Forest honey is collected by "honey hunters" during March–May, by cutting part of the honeycomb after using smoke to deter stinging bees. The bees return and rebuild the comb after harvesting, and thus this manner of honey collection is deemed sustainable. Similarly, honey gathering is being carried out by the Laiban community in the Municipality of Tanay in Rizal Province (Figs. 1B & 1D). Located at the southern region of the Sierra Madre Mountain Range, a 180-hectare region has been the focus of forest conservation and livelihood programs aided by partnerships forged with academic and conservation organizations[12]. Furthermore, public awareness about the local biodiversity and cultural rights of indigenous inhabitants has defended much of the area from a proposed dam construction project[13] that will destroy the habitat of endemic and endangered species such as the Philippine Eagle (Philippine Brown Dear, Philippine Wart Pig[14], and the pollinating *Apis breviligula*, a subspecies of giant honey bee that can only be found in the Philippines[15].

Because of its environmental and socio-economic importance, comprehensive biochemical characterization of Philippine forest honey is important to associate its dependence to local flora as well as establish its uniqueness and authenticity. A recent report reveals that 80% of commercially available honey in the Philippines is adulterated[16]. Even though this study didn't cover forest honey extensively, the mentioned research certainly elevates public distrust and skepticism toward the consumption of forest honey products. Furthermore, classifications such as "wild" and "forest-sourced" often associated with honey are highly misused in the supply chain. Outside radioisotope-based methods, there is certainly a need for alternative authentication, quality control, and traceability frameworks[17]. Furthermore, spectroscopic methods could highlight beneficial small molecules, such as the potent antimicrobial isorhamnetin from Philippine stingless bee honey[18]. Determination of these important metabolites could serve as the basis for the value-added development of forest honey as an important functional food[19]. Aside from the socio-economic impact, small molecules in honey represent the ecological footprint of

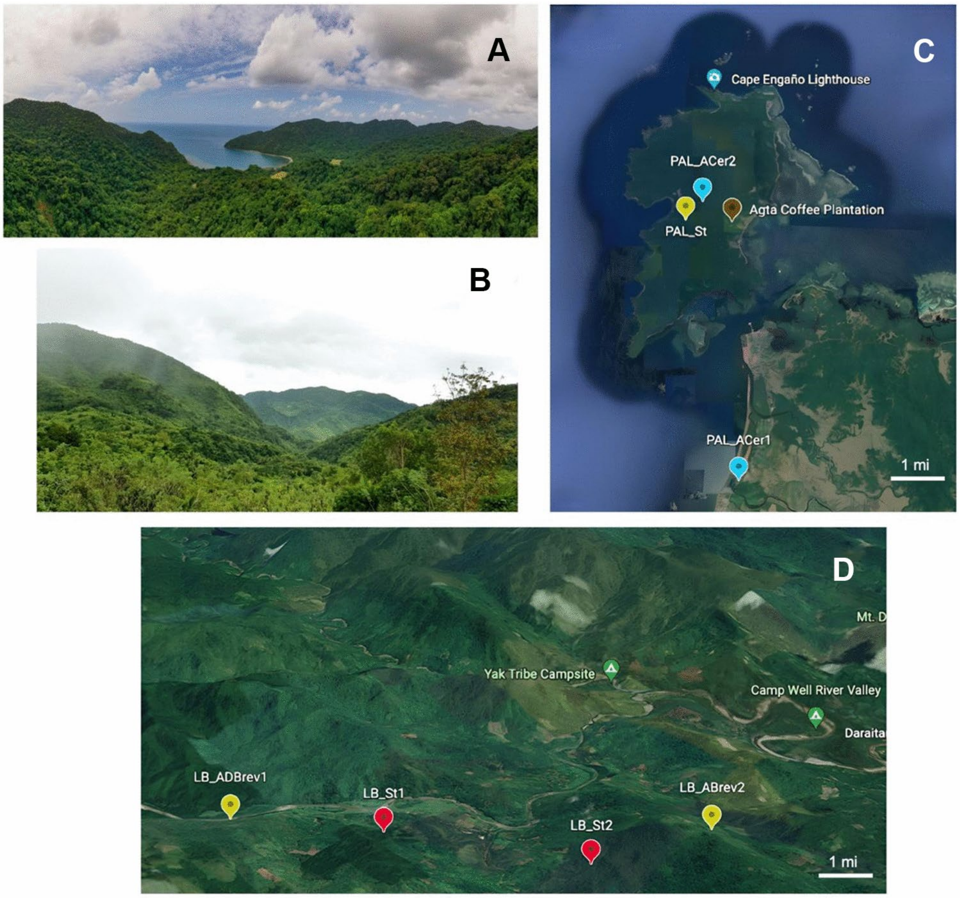

Fig. 1. Focal regions of the study are biodiversity hotspots in Palaui Island (PAL) and the Laiban Community in Tanay, Rizal (LB) situated in the Northern and Southern Sierra Madre Mountains, respectively. Unique terrain in Palaui Island and Laiban are shown in (**A**) and (**B**), respectively. Geographic coordinates of samples are shown in (**C**) for Palaui, and (**D**) for Tanay. The bee species are indicated in the location labels and abbreviated as St: stingless bee, ACer: *Apis cerana*, and ABrev, *Apis breviligula*. Sampling map generated using Google Maps (maps.google.com).

native bee species that are significantly influenced by the surrounding environment[20]. Guided by the traditional knowledge of forest communities, we seek to explore and establish unique ecological relationships involving bees by performing comparative metabolomics and conducting genomics- and palynology-based verification studies.

Focused on the detection, characterization, and comparative analysis of small molecules present in complex biological mixtures, metabolomics is often used to verify ecological hypotheses and can also unravel novel insights. The application of metabolomics in honey research has been well demonstrated in the literature[21] in terms of quantitative determination of biomarker compounds[22], molecular forensics linking propolis and resin sources[23], and even biotechnology-driven product development of honey[24]. In this research, we seek to identify unreported metabolites in *Apis cerana* Fabr. and stingless *Tetragonula biroi* (Friese) from Palaui Island and *A. breviligula* Maa[15] as well as *T. biroi* honey from the Laiban community in Rizal. Information generated can be used for value-added product development and quality assessment, as well as possible health monitoring of bee colonies. Furthermore, baseline information generated in this study can also guide future environmental monitoring in forest landscapes.

Results

Untargeted metabolomics explored the untapped chemical biodiversity of honey sourced within the protected regions of the Sierra Madre Mountain Ranges. Representative base peak chromatograms (BPI) in Fig. 2 comparing honey from Northern (Palaui, **A1-3**) and Southern (**B1-4**) Sierra Madre showed similarities but with evident differences as well. There is significant complexity of Laiban compared with Palaui samples based on the semipolar and nonpolar compounds ($t_R = 3.00–7.00$ min). Moreover, many divergent features emerged related to geographic locations, and can be attributed to the different bee species that produce the honey. To further account for this uniqueness, the focus shifted towards identifying the small molecules using their characteristic mass spectral fingerprints.

Without extensive purification, data-dependent acquisition enabled tandem MS of highly intense ions in the crude mixtures. Utilizing the high selectivity and resolution of the qToF mass analyzer, specific fragmentation patterns were obtained for each compound, which was then matched to public reference files in the community-curated Global Natural Products Social Molecular Networking (GNPS) platform[25]. Complete lists of annotated metabolites are listed in Table S1 with amino acids ($t_R = 0.30–0.70$ min), sugars and catabolites ($t_R = 0.50–1.10$ min), alkaloids ($t_R = 1.50–3.00$ min), flavonoids ($t_R = 3.00–5.50$ min), phenolamides ($t_R = 4.75–5.50$ min), and lipids ($t_R = 7.00–11.00$ min) included. Structural relatedness of the molecular scaffolds of these compound families are shown in Fig. 3A. Congruent search of other natural products databases, as well as manual fragmentation analysis, further increased the coverage of annotated compounds. For small molecules annotated using GNPS, confidence in the putative identification is based on the cosine (similarity) score calculated from the alignment of experimental (black) and reference (green) mass spectra (Fig. 3A–D). Reported spectral hits are only those with cosine values that exceeded the 0.70 threshold set[25]. Furthermore, a high-resolution qTOF instrument is capable of measuring mass-to-charge ratio with high mass accuracy having lower than 5.0 ppm error[26].

Examples of tail-to-tail alignments are shown for important metabolites such as niacin (Vitamin B3, Fig. 3A), and the immunomodulatory naringenin (Fig. 3B)[27]. In addition, essential for quality monitoring is the detection of Maillard (browning) reaction products such as 1-(1-deoxy-1-fructosyl) phenylalanine (Fig. 3C)[28]. Ecologically important phenolamides such as dicoumaroylspermidine (Fig. 3D) will be noted as fundamental for environmental monitoring[29].

As expected, the presence and varied abundances of small molecules affected the multigroup comparison of samples using XCMS Online[30]. Principal component analysis (PCA) plot in Fig. 4A shows distinct clustering of Palaui samples apart from Laiban and Isugod honey from Palawan island. Loadings plot (Fig. 4B) accounted for this distribution affected by the metabolite hypaphorine and many lipid types enriched in Palaui honey, while phenolic compounds were significantly present in Laiban samples.

To further contrast Palaui samples that exhibited significant overlap in chemical profiles, statistical analysis was focused on the three groups of samples collected in this region. Figure 4C highlights the separation of stingless bee honey compared to *A. ceran*a honey, with higher levels of flavonoids (Fig. 4D) on the former contributing significantly to 58.9% of variance projected in Fig. 4C. Similarly, kaempferol, isorhamnetin, and quercetin were abundant in stingless bee honey from the Laiban collection site, compared to *A. cerana* honey collected from the remote Mt. Toyang (Fig. 4E, PC1 = 44.4%). Within species variation between the *T. biroi* and *A. breviligula* samples was also described for Laiban honey across PC2 (22.5%). which was associated with the levels of primary metabolites (sucrose, tryptophan), lipids, and Maillard reaction products (Fig. 4F) that could vary depending on the maturation of the hive[24].

Aside from global profile comparison, individual metabolites were also compared throughout the samples. Biologically active flavonoids such as isorhamnetin (Fig. 5A) were more abundant in stingless bee honey from Palaui and Laiban (p-value < 0.001, Fig. 5B). On the other hand, plant-derived abscisic acid (Fig. 5C) appears to be independent of bee species as it was detected in all *A. cerana* and *T. biroi* samples from Palaui as well as *A. breviligula* honey from Laiban (p-value < 0.001, Fig. 5D). Surprisingly, the levels of the *Coffea* sp. related alkaloid caffeine (Fig. 5E) were significantly rich in stingless bee honey from Laiban (p-value < 0.001, Fig. 5F), and marginally present in a Palaui sample (PAL_St, Fig. 5G).

During the collection period, *P. indicus* trees found throughout Palaui island were profusely blooming. Metabolomics analysis revealed that the tryptophan metabolite hypaphorine synonymously called lenticin (Fig. 6A), is significantly present in all three Palaui samples (Fig. 6B). Matching the extracted chromatograms for this m/z 247.1422 feature verified that it is also significantly contained in *P. indicus* nectar and pollen (Fig. 6C). This discovery prompted verification studies through DNA barcoding and palynological analysis.

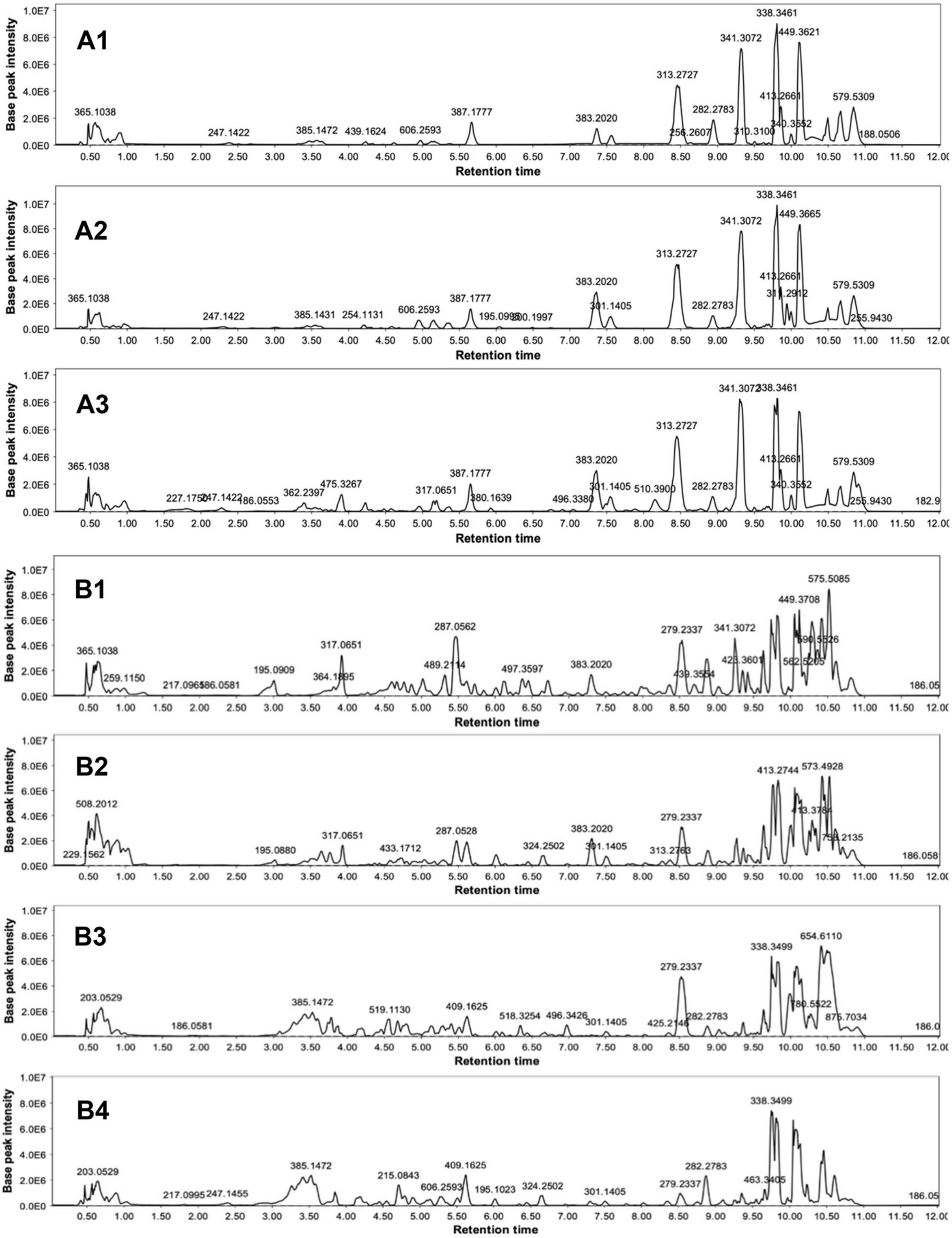

Fig. 2. Base peak ion (BPI) chromatograms of representative honey samples. A1 corresponds to PAL_ACer1, A2-PAL_ACer2, A3-PAL_St. Meanwhile B1-4 represents the metabolite profile of LB_St1, LB_St2, LB_ABrev1, and LB_ABrev2, respectively.

As can be seen in Fig. 6E, Palaui honey labeled as PAL_ACer2 sample contains *P. indicus* pollen with trace amounts of *Coffea* sp. pollen. Meanwhile, the PAL_ACer1 sample contains more *Coffea* sp. pollen (Fig. S1). Unfortunately, limited pollen was noticed in the PAL_St sample which could be due to poor sample quality during the time of palynological examination. To further obtain supporting evidence, Sanger-based DNA barcoding was used to verify the occurrence of *Pterocarpus* and *Coffea* pollen. To determine the plant species match from the honey samples, DNA sequences were subjected to BLAST (Table 1). Top BLAST results for SIW415 using

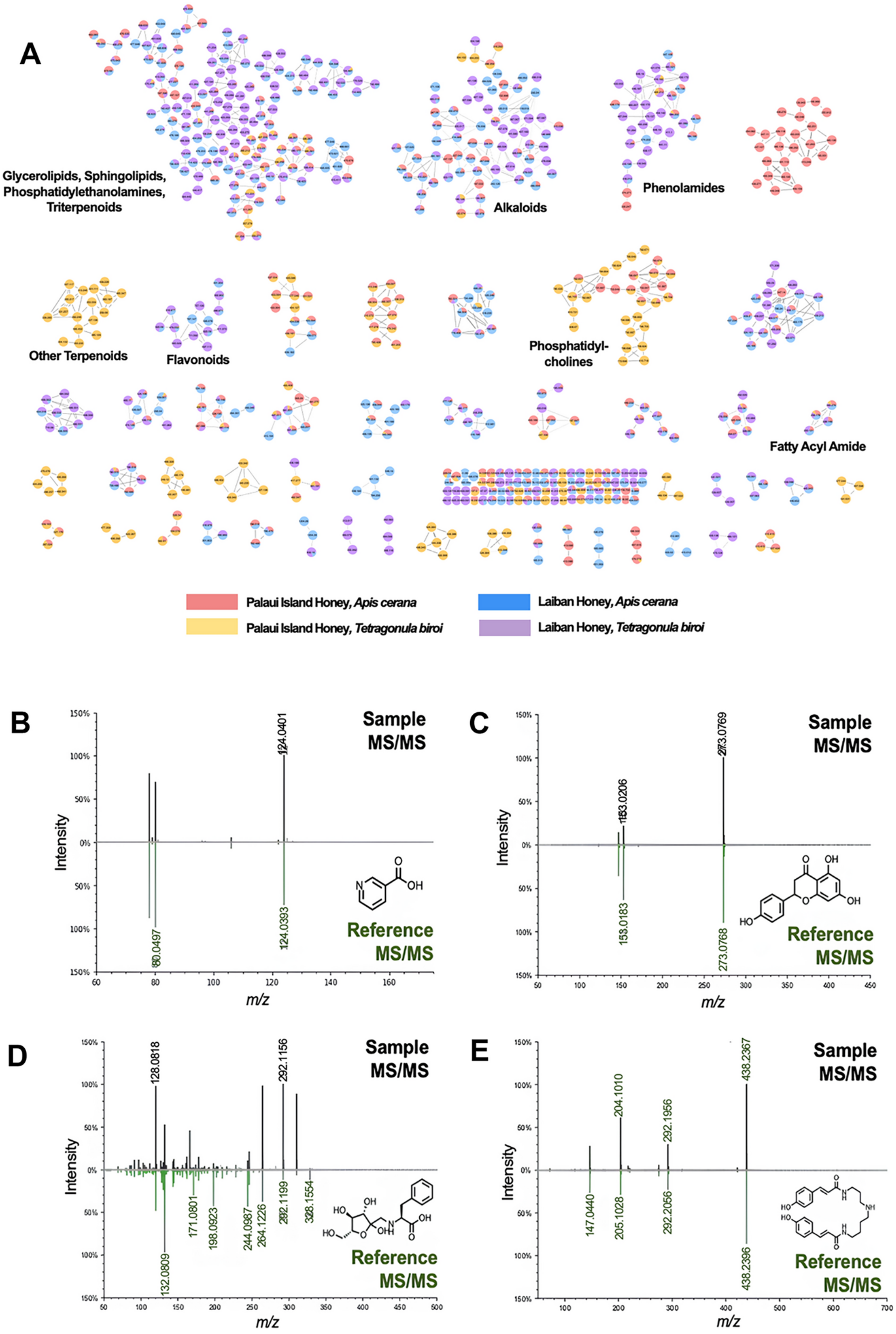

Fig. 3. Metabolite characterization and structural comparison using the GNPS platform. Molecular Network in (**A**) shows structural relatedness of honey-derived small molecules clustered according to compound classes. In addition, tail-to-tail matches are shown for important compounds such as niacin [(**B**), cosine: 0.95, 2.42 ppm], naringenin [(**C**), cosine: 0.95, 0.55 ppm], N-(1-deoxy-1-fructosyl)phenylalanine [(**D**), cosine: 0.91, 4.02 ppm], and dicoumaroyl spermidine [(**E**), cosine: 0.90, 4.63 ppm].

the rbcL primers were various *Pterocarpus* species. Using ITS1(A) and ITS1(B) primers narrow the species down to *P.indicus*. BLAST results for STA. ANA when using rbcL indicates the presence of another *Pterocarpus* species, *P. santalinoides*. BLAST results for SIW420 using rbcL also reveal the *Coffea* species as a possible source. Commonly cultivated in the Philippines is *Coffea. arabica* L., which originated from the hybridization of *C. canephora* and *C. eugenioides*. This might explain why *C. eugenioides* was included in the BLAST results. On the other hand, *C. liberica* var. *dewevrei* (De Wild. & T.Durand) Lebrun, also known as Excelsa coffee, was detected

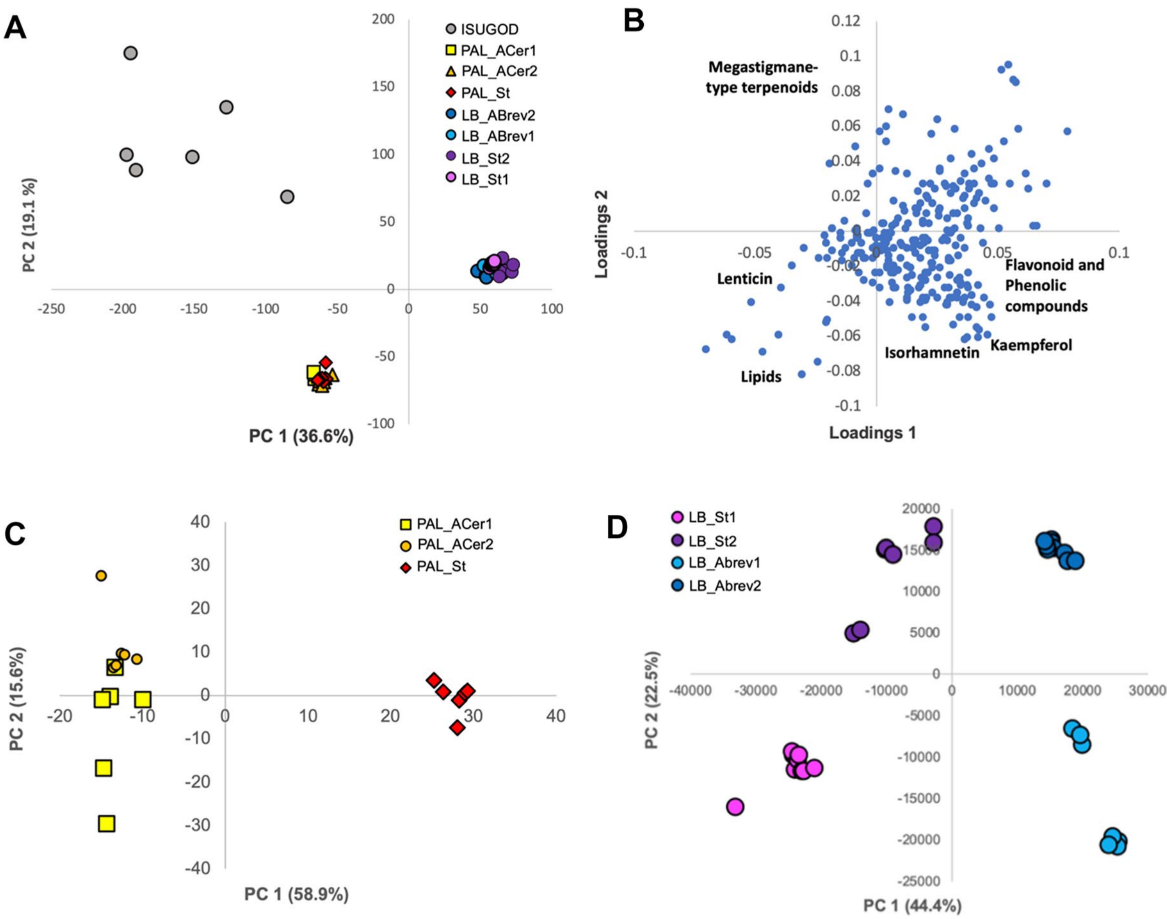

Fig. 4. Multivariate statistical comparison of Palaui and Laiban honey. Log-transformed scores plot in (**A**) shows distinct grouping of Palaui and Laiban honey against honey from Palawan island. Particular clustering is accounted for by higher levels of phenolic compounds in multifloral Laiban honey compared to unifloral Palaui island honey, enriched with lenticin and various lipids. (**B**) Aside from inter-location comparison, within-group variations were further explored across the Palaui (**C**) and Laiban (**E**) samples.

using the ITS1(A) primers. Excelsa coffee is another *Coffea* species widely cultivated in the Philippines, and is also under the same species as the locally known "Kapeng Barako" or *C. liberica* W. Bull ex Hiern *var. liberica*[31]. Interestingly, the BLAST results for ITS1(B) reveal the presence of a mangrove species, *Rhizophora apiculata* DC.

Discussion

Multi-omics investigation of understudied Palaui and Laiban forest honey verified existing hypotheses and unraveled fascinating insights on the chemical diversity of Philippine forest honey, and its important association with local flora. Initial component of the research work is LC–MS based metabolomics that enabled rapid survey of compounds present in crude honey blends. Analyses were first carried out in full-scan (MS^1) mode to obtain comprehensive information on the detectable mass-to-charge ratio and signal intensities of small molecules (Fig. 2). Prior to these, preliminary separation by way of ultraperformance liquid chromatography (UPLC®) resolves highly polar (such as sugars and amino acids), semi-polar (e.g. flavonoids and terpenoids), and non-polar metabolites (e.g. lipids). Thousands of features were detected in the samples and served as the basis for multivariate statistical comparison. Variations across these metabolites were examined and the model generated clustering of samples sourced from the same geographic location (Fig. 3A). Honey from Northern Sierra Madre (Cagayan) was undoubtedly discriminated from those coming from Southern Sierra Madre (Laiban) and the southern Luzon Island of Palawan (Isugod) which is not part of the Sierra Madre Mountain Range. Important for the data clustering is the floral source in the specified area. Palynological analysis (Fig. 7) revealed that Palaui honey is highly likely unifloral in terms of *Pterocarpus indicus* pollen while Laiban and Isugod honey are extensively multifloral. This explained the less convoluted chromatograms of Palaui honey (Fig. 2 A1-3) compared to that of Laiban (Fig. 2 B1-4).

To further examine smaller differences within locations, multigroup comparisons were carried out separately for the Palaui and Laiban samples. Both PCA scores plots were able to differentiate *Apis* and stingless bee honey across the first principal axis, explaining 58.9% (Fig. 4C) and 44.4% (Fig. 4E) of observed variation among Palaui and Laiban samples, respectively. Meanwhile, within-species variation was accounted for by differentially distributed sugars, amino acids, amidori products, and lipids, the relative abundance of which can vary depending

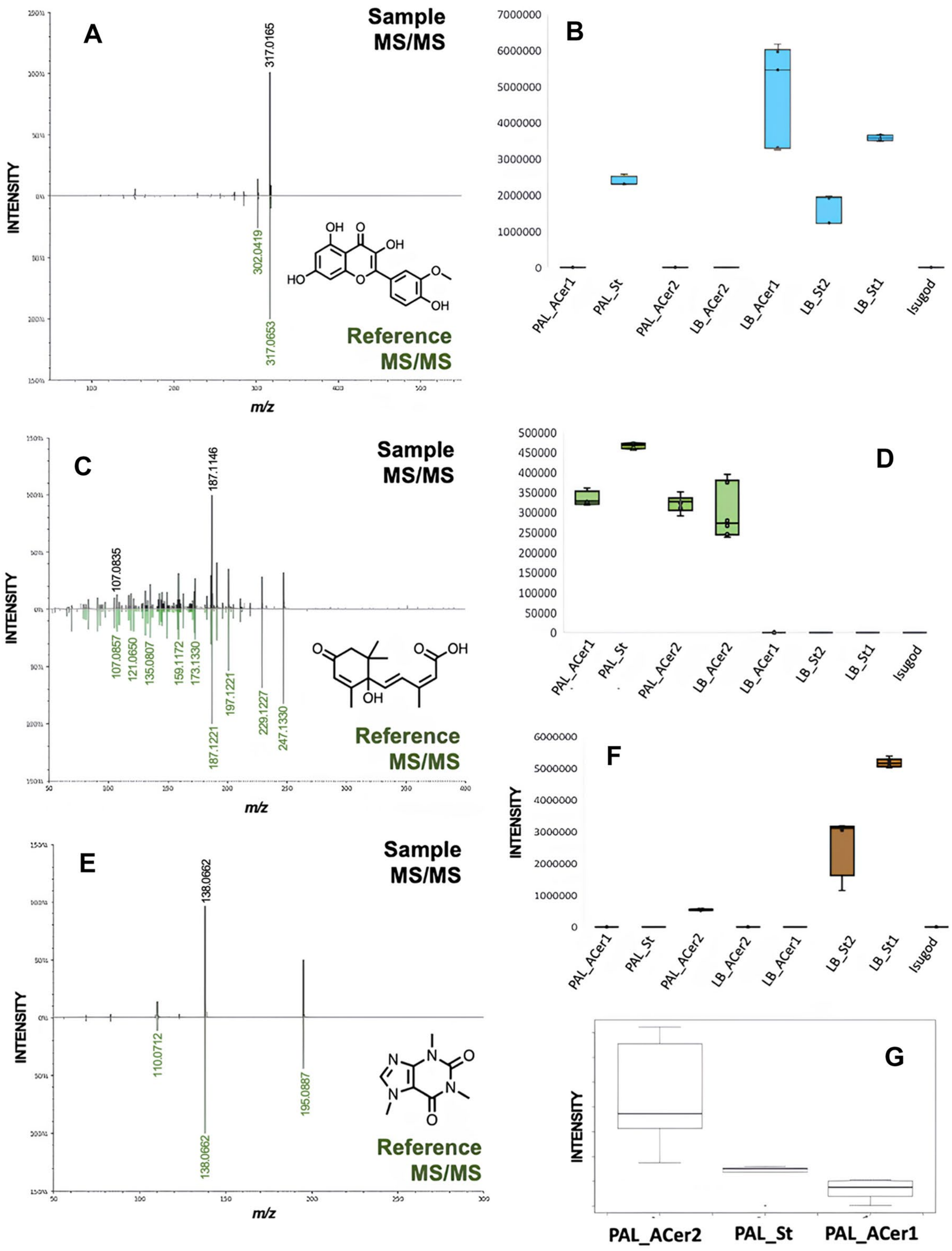

Fig. 5. Statistical comparison highlights significance of specific metabolites in Laiban and Palaui honey. Tail-to-tail matches and box plot signal intensity comparison for isorhamnetin, abscisic acid, and caffeine, are shown in (**A**,**B**), (**C**,**D**), and (**E**–**G**), respectively. p-values from one-way ANOVA analysis of the three compounds are all < 0.001.

on several factors such as temperature, moisture content, as well as in-hive fermentation of honey prior to the collection period[32]. Interestingly, dipeptides such as Ser-Leu, Ile-Val, Val-Pro, and Ile-Pro, were detected in *A. breviligula* honey from Rizal Province (Fig. 4F, Table S1). Mentioned compound class has been reported in yeast

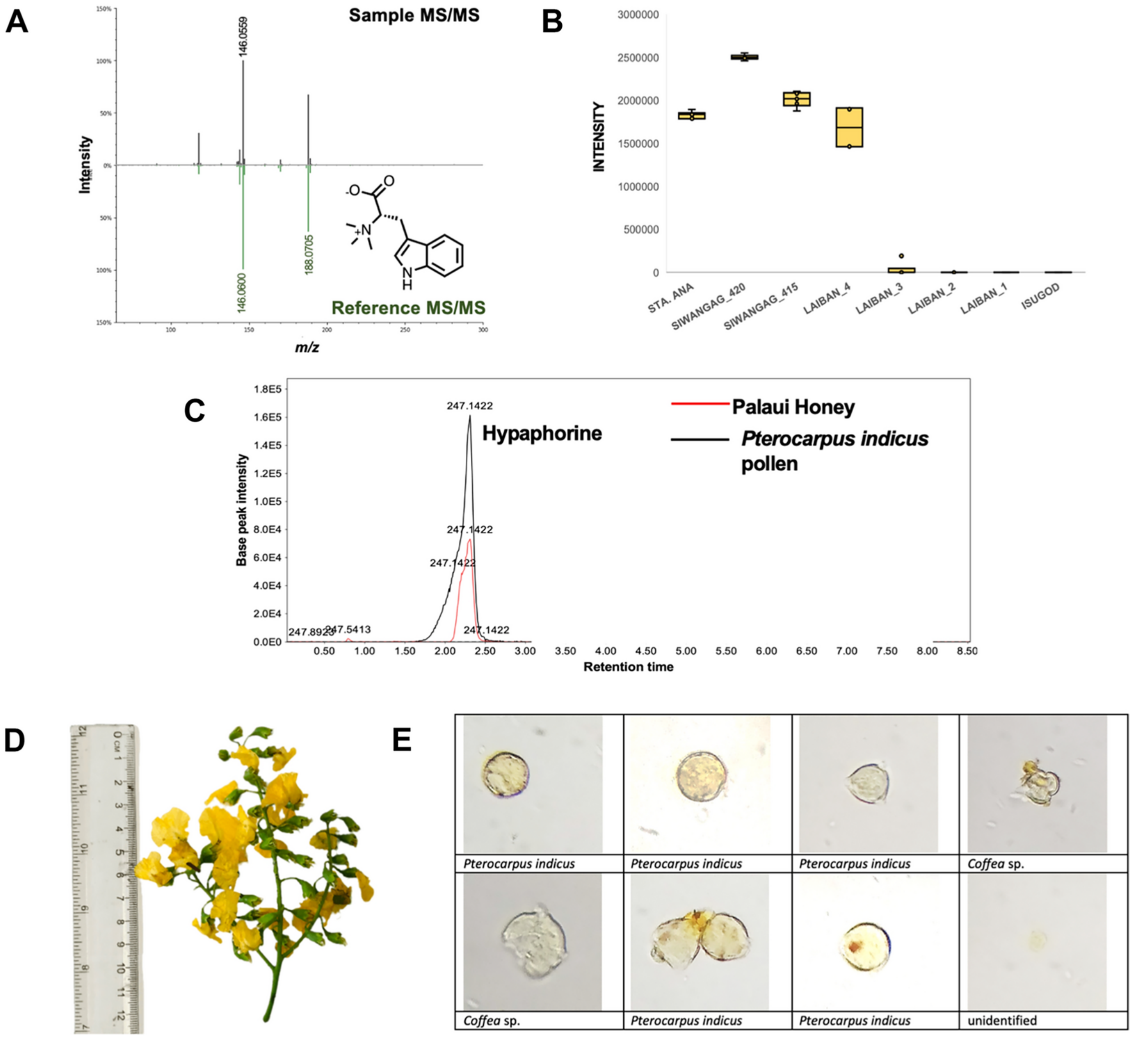

Fig. 6. Striking detection of hypaphorine in the honey samples and *Pterocarpus indicus* Willd. nectar and pollen. GNPS identification for the tryptophan catabolite is shown in (**A**). Prevalence of this compound in Palaui honey and one sample from Mt. Toyang, in Rizal Province is highlighted in [(**B**), $p < 0.001$], while alignment of the m/z 247.1422 feature between Palaui honey and *Pterocarpus indicus* floral alcoholic extract is shown in (**C**). Photo taken of *P. indicus* inflorescence with dimensions scaled in centimeters (**D**). Occurrence of *P. indicus* pollen in the honey samples is further verified by microscopy analysis in (**E**).

Sample	Bee species	Target gene amplified	Top blast results	Percent identity
PAL_Acer2	*Apis cerana*	rcbL	*Pterocarpus spp.*	100% (across multiple species)
		ITS1(A)	*Pterocarpus indicus*	99.29%
		ITS1(B)	*Pterocarpus indicus*	99.72%
PAL_St	*Tetragonula biroi*	rbcL	*Coffea arabica*	100%
			Coffea eugenioides	100%
		ITS1(B)	*Rhizophora apiculate*	99.23%
PAL_Acer1	*Apis cerana*	rbcL	*Pterocarpus santalinoides*	98.11%
		ITS1(A)	*Coffea liberica var. dewevrei*	98.83%

Table 1. Top BLAST results from DNA barcoding of honey samples highlight the significance of *Pterocarpus* and *Coffea sp.* in Palaui honey.

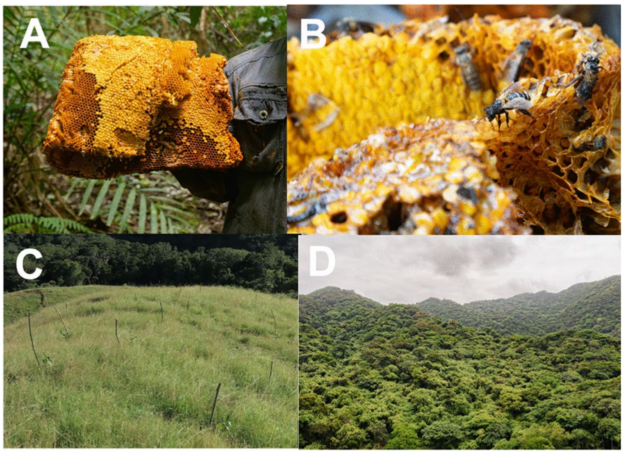

Fig. 7. Deeper understanding of the ecological relationship between vital forest bee species and local flora further inspired the community to protect the environment. Photos from honey gathering during the month of April are shown on (**A**,**B**). (**C**) is from the tree planting of Narra tree in August of the same year. After a few years, the planted seedlings are expected to further fortify the Palaui rainforest (**D**), safeguarding the home of native bee species and other pollinators as well as exotic fauna.

(*Saccharomyces cerevisiae*) and parasitic microbes[32,33]. Possible sources could also very well be the endogenous digestion in the bee gut of ingested proteins to dipeptides that can be more readily absorbed by the bee's intestinal system[34].

Aside from chemometrics-based authentication and geographic provenance and bee source identification, the characterization of amadori products such as N-(1-deoxy-1-fructosyl) phenylalanine and N-fructosylisoleucine (Fig. 3D) will be important for future quality monitoring of Palaui and Laiban honey[35]. Also known as Maillard reaction products, these compounds formed from the condensation of monosaccharide sugars to amino acids[35]. Contributing to the observed "browning" of honey, the detection of these polar metabolites could aid in the standardization of fresh and fermented honey throughout its shelf-life. In addition to this, the prospect of further developing Philippine forest honey as a functional food[36] was shown in terms of the detection of high-value and bioactive small molecules such as the potent antimicrobial isorhamnetin[18] found in all stingless honey samples analyzed, immunomodulatory naringenin[27] enriched in LB_St2 sample, lipid-lowering vitamin B3 (niacin) in LB_ABrev1[37], as well as 5-methoxy-L-tryptophan (LB_St1-2a and LB_ABrev2) with well-documented anti-inflammatory and tumor-suppressing benefits[38,39]. Furthermore, beta-carboline alkaloids harman and tetrahydroharman-3-carboxylic acid (Table S1), found in Laiban and Palaui honey, respectively, have been studied for their anti-cancer effects[40]. In addition, they are considered potent monoamine oxidase inhibitors that induce anti-depressant benefits by upregulating the biosynthesis of serotonin, dopamine, and norepinephrine[41]. Aside from these alkaloids, wide-range of phenolic flavonoids (quercetin, apigenin, isorhamnetin glycosides, C-glycosylated flavonoids, Fig. 3A) were also determined to impart antioxidant activity, especially for LB_St1 & LB_St2 (Fig S1).

Interestingly, specific phytochemical classes exist in the samples, which is not only beneficial because of their bioactivities but also for their potential role in assessing bee and colony health. The first one is pyrrolizidine alkaloids, found in both Palaui and Laiban honey. The compound lycoperodine-1 (Table S1) is considered an agonist of Ca^{2+} sensing receptors (CaSR) implicated in many diseases[42]. Equally important is the reversible transformation of pyrrolizidine alkaloids to the more hydrophobic N-oxide form[43]. Sequestering the highly-soluble quaternary aminated form from plants, insects have been shown to use N-oxidases to make the alkaloid more hydrophobic and non-toxic[43]. Meanwhile, hydrolysis in the gut of parasitic organisms renders these honey-associated molecules a lethal weapon for chemical defense[43]. The same effect is exerted by phenolamides (Fig. 5E), derived from the condensation of phenolic acids (such as coumaric acid, caffeic acid, and ferulic acid) to polyamines (putrescine, spermidine, spermine)[44]. Known to exhibit potent antioxidant, antimicrobial, and probiotic effects[45], phenolamides have been described to act as poison in the control group, but then act as a potion and suddenly became actively sought by bumblebees (*Bombus terrestris*) infested with *Chritidia bombi* parasite[29]. Whether or not phenolamides play a similar role in Apis and stingless bees remain to be explored in future work.

Another interesting ecological marker is the plant hormone abscisic acid (ABA, Fig. 5C)[46]. Known to regulate the flowering of plants[46], ABA has been shown to enhance cold tolerance and the survival of bee larvae[47,48]. Interestingly, the levels of ABA are significantly high in high-altitude regions such as Palaui Island (PAL_ACer2, PAL_St), Mainland Cagayan (PAL_ACer1), and Mt. Toyang in Rizal (LB_ABrev2). Meanwhile, the levels of ABA are very low in stingless honey sourced in lowland regions in Tanay, Rizal (Fig. 1D). It is important to highlight that abscisic acid is not exclusive to *Apis* species since a high prevalence of ABA was recorded in *T. biroi* honey from Palaui (Fig. 5D). The influence of flora and altitude in the abscisic acid content could further be established by doing metabolomics and palynological comparison of samples from other regions in the Sierra Madre

Mountain Range. Succeeding research could also investigate if other megastigmane-type terpenoids detected in the honey sample exert similar or cooperative effects to abscisic acid.

Perhaps the most remarkable finding from the study is the detection of the tryptophan catabolite hypaphorine in PAL and LB_ABrev2 honey (Fig. 6A). Identification of probable floral sources was fostered through strong communication with the local Agta community with a rich understanding of the forest landscapes including important pollinators to native bee species. An important source highlighted was *Pterocarpus indicus*, locally known as narra. Metabolite profiling of *P. indicus* nectar and pollen confirmed that the flower indeed contains hypaphorine that was detected with the same mass m/z 247.1422 and retention time ($t_R = 1.78$ min) as that of the honey samples (Fig. 6C). To provide further supporting evidence, DNA barcoding analysis and pollen morphological examination of Palaui honey were carried out. The level of evidence provided by Sanger-based DNA barcoding is on the existence of *Pterocarpus* sp. and *Coffea* sp. genetic material in the specimen (Table 1). Complementing this is the result of microscopic examination of pollen that describes Palaui as highly monofloral in terms of *P. indicus* pollen. It has been reported that *P. indicus* is one of major plant sources of *A. cerana*[49]. In fact, bees were observed to be the pollinators of Narra within its one to two day floral longevity[50] as the plant provides profuse rewards of nectar and pollen to them. In addition to this, a small amount of *Coffea* sp. pollen was observed especially in *the* PAL_ACer2 sample. This correlates with the measured intensity.

On the contrary, levels of caffeine are significantly high in PAL_ACer2 and PAL_St. Tracing probable origin, field mapping (Fig. 1B) illustrates that the PAL_ACer2 sampling location is most proximal to a small-scale coffee plantation by the Siwangag Agta community. This is further corroborated by community records that have shown coffee trees blooming during the honey harvesting period in April. Meanwhile, historical tracing revealed that planting *Coffea* trees has been widely prioritized over the years in Laiban throughout many reforestation programs. This clearly showcases the intimate association and responsiveness of forest bee species to indigenous way of living as well as external conservation efforts. At the center of this ecological interaction are native flora such as *P. indicus* which is under endangered status and need urgent protection. Moving forward the results of the research give a sense of heightened pride and inspiration to forest communities, supporting conservation organizations, and the local governing and regulatory bodies. Everyone is further motivated to continue collaborative partnership towards sustainable honey gathering, indigenous cultural manifestation, as well as broader public education through effective scientific communication.

Conclusion

For the first time, untargeted metabolomics was utilized to profile small molecule diversity in forest honey sourced from protected regions in the Sierra Madre Ranges in the Philippines. Specifically, honey sourced from Palaui Island, Cagayan in the Northern Sierra Madre region was shown to be distinct from Laiban in the Southern Sierra Madre region, and as well as Isugod in Palawan. Primary discriminators were determined to be hypaphorine and lipids found in Palaui samples, and phenolic compounds enriched in Laiban and Isugod honey. The identification of hypaphorine in Palaui Island samples is highlighted as it is associated with *P. indicus*, corroborated by morphological and pollen DNA analysis. This implies the preference of bees to the endangered *P. indicus* and thus emphasizes the importance of preserving its population in Sierra Madre.

The study can further be expanded by looking at more regions of Sierra Madre to further correlate surrounding flora with honey metabolites. The effect of sampling periods, especially during the wet and dry season, can also be studied to associate metadata with metabolite profiles to monitor honey quality. Lastly, the methods developed in this study can be applied to gather data on other honey-producing regions to establish baseline information for validation and authentication purposes.

Methodology

Honey collection and specimen identification

All the methods were carried out in accordance with relevant Institutional guidelines and regulations. Collection and transportation of bee specimens and *Pterocarpus indicus* pollen were through a gratuitous permit provided by the Department of Environment and Natural Resources CENRO Aparri Office. Honey gathering was carried out between the months of April to June 2021 in specific locations in Palaui Island, Cagayan Province and Tanay, Rizal (Table S2). Initiated by the local Agta communities, sampling was assisted by the Mabuwaya Foundation (Palaui Island) and Miriam College—Environmental Science Institute (Laiban). Over two visits in the community separated by seven days, three hives (two *A. cerana*, one *T. biroi*) were collected in Palaui while four bee colonies (two *A. breviligula*, two *T. biroi*) were collected in Laiban. Additional hives were not collected as the amount harvested was deemed by the community sufficient for the month in terms of what they needed to put in the market and keep for their personal use.

Following the traditional process, smoke from underneath the tree was used to deter the bees, after which, only a small portion of the hive was taken such that the bees can rebuild their colony after. Immediately after, the honeycomb, immersed in honey, is transported in the laboratory for small molecule extraction and pollen analysis. At least three bottles of honey were collected from each hive and subjected to analysis.

Alongside, taxonomic determination of the bee specimens was provided by the UPLB Museum of Natural History. Meanwhile, *Pterocarpus indicus* flowers collected was verified by Mr. Jayson Mansibang of the Philippine Taxonomic Initiative. Voucher specimens are deposited in an in-house collection in our laboratory under (Accession Code: 2022-015).

Metabolomics analysis

Small molecule extraction and solution preparation

Extraction of semi-polar, aromatic, and hydrophobic small molecules in honey were done through solvent-based partitioning. Appropriate amounts of lyophilized samples were resuspended in 100% LC–MS grade water (Merck, LiChrosolv, Darmstadt, Germany) to a ratio of 100 mg/mL. Small volumes of solution (approximately 5 mL) were then subjected to liquid–liquid partitioning with the same volume of isobutanol. This process was repeated four times pooling the organic layer together which were then dried using a rotary evaporator (Stuart XE 3500). Prior to LC–MS analysis, samples were reconstituted in absolute ethanol to a final concentration of 10 mg/mL.

UPLC-QTOF analysis

Separation, detection, and characterization of small molecules in the crude extracts were then carried out using a Waters Acquity UPLC® System hyphenated to a XeVo G2-XS quadrupole time-of-flight mass spectrometer. Samples were introduced with a 5 µL volume in a Waters Acquity BEH® Fluoro-Phenyl column (1.7 µm, 50 mm × 2.1 mm i.d.) kept at 30 °C. The elution method includes a binary mobile phase system of water (H_2O) and acetonitrile (ACN) both with 0.1% formic acid. Percentage composition of the organic phase is 5% at 0.0–0.5 min, 20% (2.0 min), 40% (4.0 min), 50% (6.5–7.0 min), 100% (9.5 min), and back again to 5% (11.5–13.0 min).

Simultaneous to chromatography is the detection of ions from the electrospray ionization of small molecules. The capillary, cone, and offset voltages were set at 2.20 eV, 40 eV, and 80 eV, respectively while the source and desolvation temperatures were at 150 °C and 500 °C, respectively. Full-scan acquisition in the positive mode was at a rate of 0.50 s per scan within the range of m/z 50–1500. Similar scan time and mass range were specified in the data-dependent acquisition of tandem-MS spectra, with additional selection criteria for the top eight precursor ions that exceed 3×10^5 ion counts. Improved database matching was also obtained by running programmed DDA analysis in 15 eV, 15–30 eV, and 30–45 eV collision energies.

GNPS data analysis

Proprietary waters .RAW files were converted to open-source .mzXML format using the MSConvert tool of Proteowizard. Files were then uploaded in the Center for Computational Mass Spectrometry (CCMS) repository of the University of California—San Diego (UCSD). Mentioned platform enabled convenient analysis of metabolomics data in the cloud based Global Natural Products Social Molecular Networking (GNPS, http://gnps.ucsd.edu) platform. The library search workflow was mainly used to match experimental results against the public spectral libraries of GNPS. Parameters for precursor and fragment ion mass tolerances are m/z 0.02 and m/z 0.05, respectively. Spectral hits considered are those with at least 7 matched peaks, less than 5 ppm precursor mass error, and cosine (similarity) score above 0.70.

Multivariate statistical analysis

Unsupervised principal component analysis was carried out using the XCMS Online platform (https://xcmsonline.scripps.edu). Specifically, multigroup analysis was carried out specifying data pre-processing paramaters which begin with peak detection guided by 10 ppm mass error tolerance, minimum and maximum peak widths of 2 s and 15 s, respectively. Further elimination of background noise was ensured by specifying a pre-filter and noise filter of 1×10^5 and 1×10^3, respectively as well as limiting features to those with signal-to-noise ratio of 10. The rest of the parameters were based on the recommended setting by XCMS for the high-resolution Waters instrument used. Furthermore, univariate comparison of metabolites across samples was through non-parametric testing (Kruskal–Wallis) with $p = 0.01$ and $p = 0.001$ as minimum thresholds for significant and highly significant features, respectively. Scores and loading plot coordinates were exported and plotted using Microsoft Excel.

DNA barcoding analysis

Sample preparation and DNA extraction

The honey sample preparation used in the study was based on the method of Wirta et al. (2021). Subsamples of 0.5 g of honey were each diluted with 1.5 ml of nuclease-free water in 2 ml microcentrifuge tubes. Nine subsamples were used for the PAL_ACer2 and PAL_ACer1 samples, and six subsamples were used for PAL_St. The subsamples were allowed to dissolve in a 60 °C dry bath for 1 h. To recover the tissue material and reduce water content, the sub-samples were centrifuged at 8000*g* for 1 h. The supernatant was discarded and the pellet from all the subsamples were combined into one tube. Samples were then centrifuged at 11,000*g* for 5 min, and the supernatant was discarded. DNA from the sample pellets were extracted using the DNeasy Plant Pro Kit (QIAGEN) following the manufacturer's protocol. DNA concentration and purity were determined using the Epoch Microplate Spectrophotometer (Bio Tek).

PCR amplification and sequencing

The PCR protocols and primers (Table S3) used in this study were adapted from Chen et al. (2010) and Rashmi et al. (2016). PCR amplification was done in 50 µL reaction mixtures containing: 10 µL 5× MyTaq Reaction Buffer (Bioline), 30 µL nuclease-free water, 1 µL MyTaq DNA Polymerase (Bioline), 2 µL 10 µM primer, and 5 µL DNA. PCR products were run through agarose gels, which were then excised and purified using GF-1 Gel DNA recovery kit (Vivantis). Purified products were sent to Macrogen (S. Korea) for bidirectional sequencing.

Molecular identification of plant species from honey samples
The forward and reverse sequences of each sample were aligned using Mega X software (Stecher et al., 2020). The consensus sequence was subjected to the Basic Local Alignment Search Tool (BLAST) against the NCBI database to determine the species composition of the samples (Altschul et al., 1990).

Palynological analysis

Samples were acetolyzed using a modification of the mellisopalynological procedures suggested by Erdtman approved by the International Commission for Bee Botany (ICBB). Due to the limited amount of samples, 0.2 mL of honey was dropped onto a slide. Honey from the sides of the container were utilized as these contain the most pollen grains. Around 0.2 mL of acetolysis solution (nine parts acetic anhydride; one part sulfuric acid) was dropped directly onto the sample and mixed thoroughly. This removes the exine and clarifies the pollen grains. The solution was allowed to dry for 2 min. Afterwhich, a drop of glycerine was added and mixed to the processed sample to prevent dessication. A coverslip was then placed and nail polish was used for permanent mounting. The slides were scanned for the presence of pollen grains and were set aside for identification. Photomicrographs of the distinguishable pollen grains were taken for identification by comparing with reference pollen slides and available flora and taxonomic characterization.

Data availability

All metabolomics data acquired using LC–MS and processed under the GNPS pipeline is stored as a public dataset under MassIVE (massive.ucsd.edu, MSV000093476). Meanwhile, Genomic results are stored in the Genbank Repository (https://www.ncbi.nlm.nih.gov/genbank/) with accession codes described correspondingly for PAL_ACer2-ITS1a (OR768480), PAL_ACer1-ITS1a (OR768481), PAL_ACer2-ITS1b (OR768482), PAL_St-ITS1b (OR768483), PAL_ACer2-rbcL (OR778587), PAL_St-rbcL (OR778588), and PAL_ACer1-rbcL (OR778589).

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Author contributions

R.J.E.M., K.F.R., V.T., H.J., M.V.W., L. Parunga, and L.P. were involved in the conceptualization of the research work. M.V.W. and L.P. coordinated the sampling in Palaui Island and Tanay, Rizal, respectively. R.J.E.M., K.F.R., V.T performed the metabolomics experiments and data analysis. M.M. & D.M.S. carried out DNA barcoding while A.M.F. provided pollen morphometrics data.

Competing interests

The authors declare no competing interests.

Additional information

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