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RESEARCH

Origins and ingredients of honey from a *Salix* community in a Janghang Wetland in Han River estuary, Korea

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Background: Janghang Wetland is a well-preserved area located in a natural estuary and brackish water zone. There exist a large community of *Salix triandra* subsp. *nipponica–S. koreensis*, with *S. triandra* subsp. *nipponica* being the dominant species in the tidal forest. The metabolite composition of honey is diverse and influenced by the floral source and environmental factors. The aim of this study is to identify the plant origins of collected honey and examine changes in metabolite composition over time within the willow community in Janghang Wetland.
Results: The study found that *S. triandra* subsp. *nipponica* was the most prominent component in the honey (50.7%), followed by *Prunus padus* (21.8%). In terms of pollen, *P. padus* was the most frequently detected (44.9%), followed by *S. triandra* subsp. *nipponica* (32.7%). The honey collected from Janghang Wetland was differentiated based on the collection time (March vs. April). Honeys collected in March exhibited a higher sucrose content than those gathered in April, while honeys collected in April demonstrated a higher mannose content compared to those obtained in March. The honey collected in Janghang Wetland had higher levels of sucrose and mannose content compared to commercial honey. In contrast, honey from an apiculture company had higher levels of lactitol and melibose. When comparing honey samples, it was found that Janghang Wetland honey showed lower levels of total phenolic content and total flavonoid content compared to commercial honeys.
Conclusions: The metabolites in honey were found to be affected by both the collection time and geographical origin, and the results of metabarcoding in honey was influenced by the floral origin. These findings can assist in identifying the origin of honey and contribute to a better understanding of metabolite diversity in honey.

Keywords: floral origin of honey, Janghang Wetland, metabarcoding, metabolite, *Prunus*, *Salix*

Introduction

Janghang Wetland is a well-preserved area situated in a natural estuary and brackish water zone. The average annual temperature ranges from 11.0°C to 12.5°C, reaching its highest temperature recorded at 29.5°C in August and dropping to its lowest at –6.1°C in January (Ramsar Sites Information Service 2021). The flora in Janghang Wetland has recently been reported to include 51 orders, 87 families, and 559 species. The Janghang Wetland was designated as a wetland protection area in 2006 and a Ramsar site in 2021. It was subsequently designated as a military facility protection area, and access to the area was restricted. This has had the effect of preserving the wetland in an optimal condition. And there exist a large community of *Salix triandra* subsp. *nipponica–S. koreensis*, with *S. triandra* subsp. *nipponica* being the dominant species in the tidal forest (Han et al. 2022). So, it is an appropriate site from which to collect honey produced by the *Salix* plant in the early spring.

Various methods have employed to determine the sources of honey such as pollen and polyphenol analyses (Adamchuk et al. 2020; Anklam 1998; Gašić et al. 2017). Among these, pollen analysis, known as melissopalynology, offers a direct means of identifying the botanical origins of honey. While melissopalynology is a valuable tool for deter-

mining uniflora honey (Balkanska et al. 2020), it comes with several drawbacks, such as being tedious and time-consuming, the challenge of identifying plants based on pollen phenotypes (Khansari et al. 2012), and the substantial influence of researcher subjectivity (Balkanska et al. 2020). Metabarcoding can be serves as an alternative tool for identifying the constituent organisms within a complex object comprising various organisms such as pollens in honey (de Sousa et al. 2019; Kim et al. 2021; Mohamadzade Namin et al. 2022b), but it also has some limitations. Regions utilized for honey metabarcoding encompass internal transcribed spacer 2 (ITS2), *rbc*L, *trn*H-*psb*A and others (de Vere et al. 2017; Özkök et al. 2023; Richardson et al. 2015). The ITS region, known for its rapid evolution and considerable variation within populations, is particularly well-suited for analysis at a low taxonomic level (Baldwin 1992; White et al. 1990). Subsequent studies have discovered several markers suitable as plant DNA barcodes, and comparisons of various regions have shown that ITS2 is an effective marker (Chen et al. 2010). This method has been reported as a valuable tool in detecting the botanical origin of honey (Hawkins et al. 2015) and recently, many studies have been conducted around the world (Milla et al. 2021; Özkök et al. 2023; Mohamadzade Namin et al. 2022a).

Nectar, produced by flowers, contains various plant metabolites produced responding to the surrounding environment. However, there is limited studies on the effects of abiotic factors on honey metabolites and existing ones focus mainly on major components such as sugars (Canto et al. 2011; Chalcoff et al. 2017). Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) can be used to analyze untargeted metabolites. In entomological studies, Wang and colleagues (2019, 2022) demonstrated the capability to differentiate honey based on honeybee species using untargeted GC-MS and LC-MS data, and several markers could be discovered through targeted approach. From a plant origin perspective, geographical and floral origins can be assessed through untargeted LC-MS analysis (Koulis et al. 2021; Li et al. 2017). The metabolite composition of honey may vary extensively, encompassing not only sugars but also other secondary metabolites, contingent upon abiotic factors. and GC-MS and LC-MS can be serve as valuable tools for metabolite analysis to discover the origin of honey.

Salidroside is predominantly extracted from the flowers of the *Rhodiola rosea* plant and has been the subject of numerous studies which have demonstrated its antioxidant properties (Choe et al. 2012; Kosakowska et al. 2018; Sun et al. 2020). Within the genus *Salix*, salidroside has been known to be produced exclusively in *S. triandra* subsp. *nipponica* (Thieme 1965). If salidroside is present in honey produced in the Janghang Wetland, where *S. triandra* subsp. *nipponica* are dominant, it can be used as a basis for determining that the honey is of *S. triandra* subsp. *nipponica* origin. Furthermore, Janghang Wetland may be a suitable location to produce high-quality honey containing salidroside.

In this study, we conducted a comparative analysis of honey collected over two months from Janghang Wetland in early spring 2023 with commercial honey brands. We employed DNA metabarcoding and metabolite analysis with the following objectives: 1) to identify the plant origins of honey collected near the extensive willow community in Janghang Wetland and 2) to comprehend the compositional changes in metabolites based on the collection time. Furthermore, it would like to examine the potential suitability of Janghang Wetland as a site for honey production.

Materials and Methods

Sample collection

There bee colonies were set in Janghang Wetland in March and April 2023, each containing 2 to 4 brood frames. The bee colonies were surrounded by steel cages to defend against disruptors such as wild animals. Five honey subsamples and five pollen subsamples were collected randomly from each brood frame. Each honey subsample was collected from an area of 2 cm × 2 cm. The pollen subsamples were collected from five cells from each brood frame, except for the C2 brood frame cell, which had no pollen present. A single sample representing each brood frame was created by mixing these subsamples. Commercial honeys were purchased from two different sources: Apiculture A, an online market, and Apiculture B, a local apiculture company near Janghang Wetland.

Preprocess for next generation sequencing from honey and pollen samples

DNA metabarcoding was conducted on beehive samples collected in March only. DNA was extracted from honey and pollen samples using the Exgene plant SV Kit (GeneAll Biotechnology, Seoul, Korea) with a modified protocol based on the kit manual. The first PCR was conducted to amplify the ITS2 region. The primers used for amplification included overhang sequences for constructing the library and distinguishing between honey and pollen samples (Table 1). PCR amplifications were performed using AmpONE Taq DNA Polymerase (GeneAll Biotechnology). The amplification conditions consisted of an initial denaturation step of 95°C for 5 minutes, followed by 45 amplification cycles of denaturation (40 sec at 95°C), annealing (60 sec at 48°C) and elongation (30 sec at 72°C) and a final extension step of 5 minutes at 72°C. The amplified PCR products were purified by using an AccuPrep® PCR Purification Kit (Bioneer, Daejeon, Korea). The honey and pollen PCR products were purified and mixed to the same con-

Table 1 The primer includes an overhang sequence designed for Illumina platform use in metabarcoding analysis

Region	Primer name	Primer sequence	Reference
ITS2	tagF_ITS2-AGC	5′ – tcgtcggcagcgtcagatgtgtataagagacagAGCatgcgatacttggtgtgaat – 3′	Chen et al. 2010
	tagR_ITS2-CTA	5′ – gtctcgtgggctcggagatgtgtataagagacagCTAtcctccgcttattgatatgc – 3′	White et al. 1990
	tagF_ITS2-ACT	5′ – tcgtcggcagcgtcagatgtgtataagagacagACTatgcgatacttggtgtgaat – 3′	Chen et al. 2010
	tagR_ITS2-CCA	5′ – gtctcgtgggctcggagatgtgtataagagacagCCAtcctccgcttattgatatgc – 3′	White et al. 1990

Capital letters within the primer sequence serve as indices for distinguishing between two samples in a mixture.
ITS2: internal transcribed spacer 2.

centration before being sent for Illumina Miseq sequencing at the Macrogen (Seoul, Korea). The paired-end sequencing method was used to obtain the sequences.

Metabarcoding analysis

The sequences were demultiplexed based on the index attached to honey and pollen. The demultiplexed reads were then paired with forward and backward reads using PEAR (version 0.9.6) (Zhang et al. 2012). The resulting reads were transformed to FASTA format and trimmed of primer sequences. Finally, entirely identical reads were merged using fastx-toolkit (version 0.0.14 http://hannonlab.cshl.edu/fastx_toolkit/).

BLAST (version 2.12.0) was used to identify the reads (Altschul et al. 1990). The database for identification was constructed using ITS2 sequences of plants reported to live in Janghang Wetland by report of Han et al. (2022) (Table S1) and plants expected to inhabit and be used by honeybees in Janghang Wetland, uploaded in genebank (https://www.ncbi.nlm.nih.gov/genbank/). The identification threshold was set at 97% or higher for the percentage of identity. Finally, the statistical analysis was performed using the amplicon sequence variant (ASV).

Preprocess for metabolite analysis of honey

A modified method based on Lisec et al. (2006) was used to preprocess metabolites in honey for GC-MS and LC-MS analysis. Honey samples were dissolved with 1,400 μL of MeOH in a 2.0 mL screw tube after being measured to 60 mg. To the polar phase, 60 μL of D-sorbitol-1-^{13}C (1 mg/mL of water; cat. no. 132144-93-5; Sigma-Aldrich, St. Louis, MO, USA) was added as an internal standard. The samples were then extracted using a thermomixer (Thermomixer comfort; Eppendorf, Hamburg, Germany) at 70°C, 950 rpm for 10 minutes. The extract was filtered using a PVDF syringe filter. For LC-MS analysis, we used 200 μL of the supernatant. To prepare for GC-MS analysis, 1 mL of the supernatant was placed in an 8 mL glass vial. Then, 750 μL of chloroform (cat. no. 67-66-3; Sigma-Aldrich) and 1.5 mL of water were added to the same vial. After vortexing and centrifugation at 2,200 g for 15 minutes, the supernatant (150 μL) of the polar solvent was transferred into a 2 mL tube and concentrated in a vacuum concentrator (CVE-2000, Eyela, Tokyo, Japan) for 12 hours. During the derivatization process, 40 μL of methoxyamination reagent (methoxamine hydrochloride [cat. no.61-16-5; Sigma-Aldrich] dissolved at 10 mg/mL in pyridine [cat. no.110-86-1; Sigma-Aldrich]) was added into a sample and then shaken at 37°C by thermomixer for 2 hours. After methoxyamination, 70 μL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, cat. no. 24589-78-4; Sigma-Aldrich) was added and the sample was shaken at 37°C for 30 minutes. The extracted samples were transferred to amber vials for GC-MS analysis.

LC-MS and GC-MS analysis of honey samples

Honey samples in amber vials (10 μL) were analyzed by LC-MS (2695 LC with micromass ZQ MSD, Waters) equipped with an Eclipse XBD-C18 column (4.6 mm × 250 mm, 5 μm; Agilent, Santa Clara, CA, USA) at a flow rate of 1 mL/min. Separation was collected over a 60 minutes period using a linear gradient of 0.01% formic acid (Sigma-Aldrich) in water (solvent A) and 0.01% formic acid in acetonitrile (solvent B). The gradient for fractions was 90:10 (A/B) to 10:90 (0–50 min), remained constant at 10:90 (50–55 min), shifted to 10:90 to 90:10 (55–58 min) and was restored to the initial condition of 90:10 proportion (58–60 min). Mass spectrometry was used for ion detection with N_2 as both nebulization and desolvation gas. Data were acquired continuously within a mass scan range of 101–1,000 atomic mass units (amu) in positive electrospray mode at 2,900 V spray and 30 V cone voltages. MS-DIAL ver. 4.80 (Tsugawa et al. 2015) was used for peak detection and alignment. Minimum peak intensity was set 100,000 and all other parameters were kept at their default value.

Honey samples in amber vials (1 μL) were analyzed using GC-MS (6890N GC with 5973 inert MSD; Agilent) equipped with an Rxi-5sil MS fused silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; Restek, Bellefonte, PA, USA). Helium was used as the carrier gas. The temperature program was set to isothermal for 5 minutes at 70°C, followed by a ramp of 10°C per minutes to 320°C, and held at this temperature for 20 minutes. M/z values were acquired by an Agilent mass spectrometer operating in the EI mode and scanning mode (mass range m/z 50–600). Chromatograms and spectra were recorded using the ChemStation software. Analyzed data for GC-MS were deconvoluted, identified and aligned by MS-DIAL ver. 4.80. (Tsugawa et al. 2015). Minimum peak intensity was set

2,500 and all other parameters were kept at their default value in MS-DIAL. All records with Kovats RI library offered MS-DIAL website (http://prime.psc.riken.jp/compms/msdial/main.html) was used to identify metabolites in honey samples. The metabolites were standardized to the internal standard (D-sorbitol-1-^{13}C). The MS-DIAL results were used for additional statistical analysis.

Total flavonoids contents and total phenolic contents analysis

To extract total flavonoids contents (TFCs), honey samples were dissolved in EtOH at 300 mg/mL. Each aliquot (100 μL) of the extracts and quercetin (Sigma-Aldrich) (0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL), was mixed with 30 μL of 95% EtOH, 20 μL 10% $Al(NO_3)_3 \cdot 9H_2O$, 20 μL of 1 M CH_3COOK (Sigma-Aldrich) and 560 μL of DW in a 2 mL tube and then vortexed. After incubation for 60 minutes at room temperature, the absorbance was determined at 415 nm with a microplate reader (INNO; LTEK Co., Ltd., Seongnam, Korea). All samples were analyzed in three times.

To extract total phenolic contents (TPCs), honey samples were dissolved in DW at 300 mg/mL. Each aliquot (200 μL) of the extracts and gallic acid (Sigma-Aldrich) (0.05, 0.1, 0.15, 0.2, and 0.3 mg/mL), was mixed with 200 μL of Folin-Ciocalteu reagent (Sigma-Aldrich) and 200 μL of 10% Na_2CO_3 (Sigma-Aldrich) in a 2 mL tube and then vortexed. After incubation for 60 minutes at room temperature, the absorbance was measured at 760 nm using a microplate reader (INNO). All samples were analyzed in three times.

Statistical analysis

Statistical analysis was performed using data matrix obtained from the results of GC-MS and LC-MS in R v3.6.1. Non-metric multidimensional scaling (NMDS) and partial least squares-discriminant analysis (PLS-DA) were conducted using the vegan package and pls package (R Package Version 2.8-1), respectively. After conducting PLS-DA, orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed using an in-house code. All plots were created using SigmaPlot 10.0 (SigmaPlot10.0; SPSS Inc., Chicago, IL, USA).

Results

Metabarcoding analysis of pollen and honey samples

As honey B4 was not amplified, metabarcoding analysis was conducted on 16 samples (honey: 8, pollen: 8) (Table 2). The sequencing yielded an average of 449,285 reads across 8 samples. After the demultiplexing process, each set of paired-end reads assembled an average of 110,501 reads. The identical reads were collapsed, resulting in an average of 32,423 ASVs per sample. Of these, 63.7% of the collapsed sequences were identified by BLAST (Table 2).

A total of 24 genera were identified in the database, including 16 genera for honey and 17 genera for pollen (Table S2, Fig. 1A). *Salix* and *Prunus* together accounted for up to 99% of the identified genera. *Salix* was more prevalent than *Prunus* in honey samples, while *Prunus* was more prevalent than *Salix* in pollen samples (Table S2, Fig. 1).

At the species level, 30 species were identified among the

Table 2 Status of data processed by type and sample at each stage

Type	Index	Sample	Raw data	Demultiplexing	Assembled	Collapsed	Identified
			Read counts	Read counts	Read counts	ASVs	ASVs (read counts)
Honey	AGC/CTA	A1	505,747	217,776	116,940	37,821	22,520 (42,744)
		A2	457,693	167,721	98,503	16,216	6,620 (17,623)
		B1	546,578	241,271	177,565	46,116	26,015 (95,088)
		B2	476,392	192,918	99,556	33,397	21,651 (39,275)
		B3	464,596	231,758	125,350	48,733	33,205 (87,487)
		C1	385,586	152,427	81,681	24,878	16,241 (31,837)
		C2	451,494	189,057	104,354	24,596	14,490 (69,190)
		C3	306,191	104,646	80,058	27,629	24,440 (65,761)
		Average	449,285	187,197	110,501	32,423	20,648 (56,126)
Pollen	ACT/CCA	A1	505,747	189,739	104,590	37,225	22,142 (60,427)
		A2	457,693	201,701	103,856	35,611	18,949 (76,581)
		B1	546,578	199,715	84,344	13,735	7,934 (20,312)
		B2	476,392	192,942	101,989	44,090	25,188 (72,878)
		B3	464,596	142,881	52,057	10,103	5,547 (12,107)
		B4	451,494	176,088	64,834	20,471	11,950 (47,941)
		C1	385,586	159,416	54,200	27,040	16,034 (36,872)
		C3	306,191	143,419	125,657	43,914	41,548 (122,423)
		Average	449,285	175,735	86,441	29,024	18,662 (56,193)

The number of reads per sample and the number of amplicon sequence variants (ASVs) are also presented. The same sample names, as well as the remaining samples (B4 and C2), were respectively pooled and analyzed together on MiSeq.

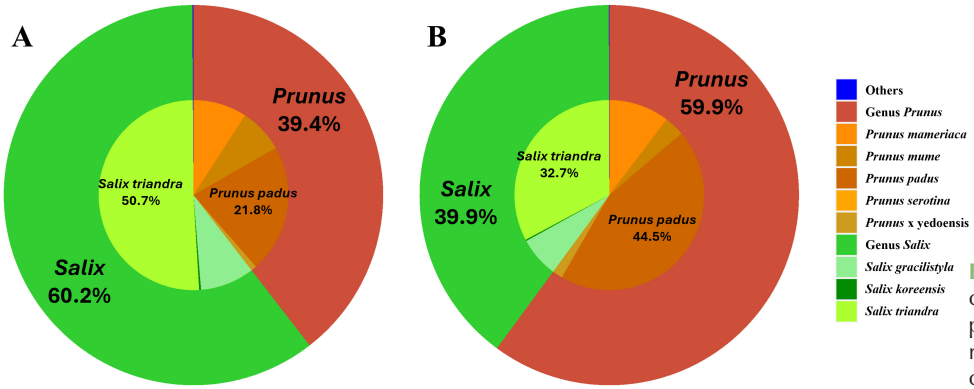

Fig. 1 Pie chart using metabarcoding data from (A) honey and (B) pollen samples. The larger circles represent genus level and smaller circles represent species level.

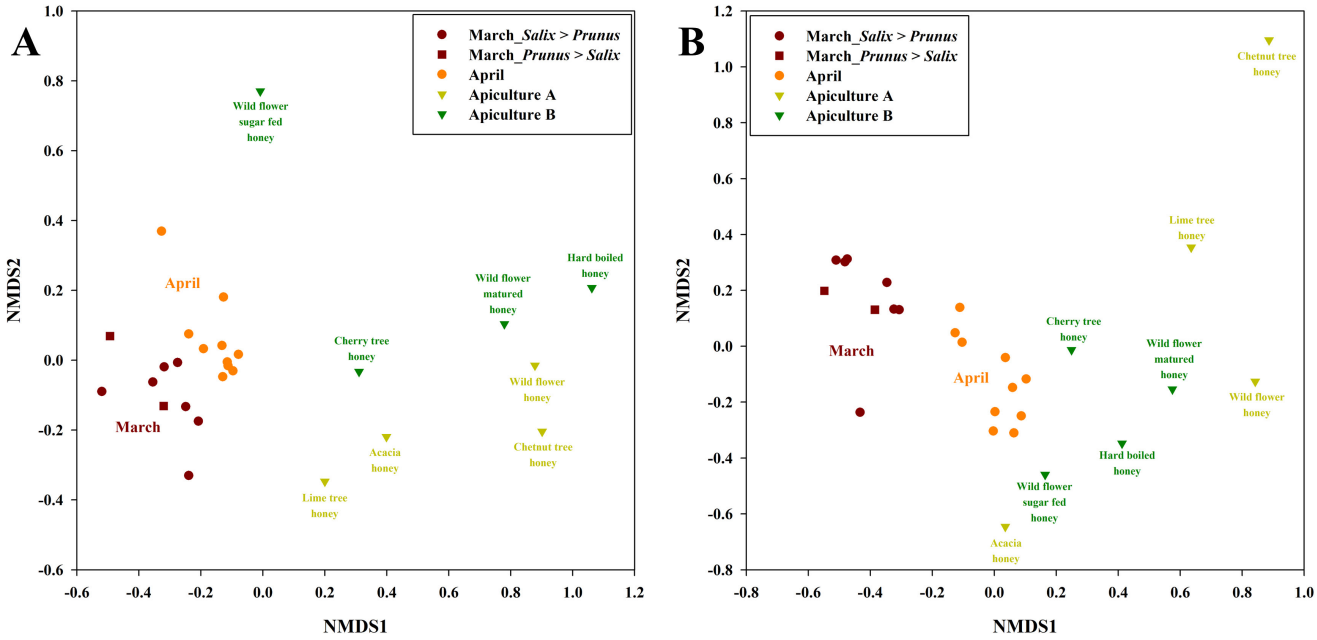

Fig. 2 Non-metric multidimensional scaling (NMDS) plot using metabolites extracted from honey samples based on (A) gas chromatography-mass spectrometry and (B) liquid chromatography-mass spectrometry analysis. The dark red circle and the dark red square in March samples are separated by the dominant genus in honey from the metabarcoding results.

249 species in the database (Table S3), with 22 species for honey and 23 for pollen. *Salix triandra* subsp. *nipponica* was the most frequently detected species in honey, accounting for 50.7%, followed by *P. padus* at 21.8% (Table S3, Fig. 1A). In contrast, *P. padus* was the most frequently found species in pollen, accounting for 44.9%, followed by *S. triandra* subsp. *nipponica* at 32.7% (Table S3, Fig. 1B). Over 90% of the total contents are made up of honey and pollen samples from 4 species: *S. triandra* subsp. *nipponica*, *S. gracilisyla*, *P. padus*, and *P. armeniaca*.

Comparison of metabolites between honey collected Janghang Wetland in March and April and commercial honey

A total of 995 peaks were detected via GC-MS analysis. Out of these, 100 metabolites were identified using the library. The NMDS result shows that the samples were well-clustered by sources and months of samples (Fig. 2A). Honey collected from Janghang Wetland was distinguished from honey produced by two beekeeping companies. Additionally, the honey collected from Janghang Wetland was differentiated based on the collection periods in March and April.

A total of 13,886 peaks were detected via LC-MS analysis. The honey collected from Janghang Wetland was categorized based on the collection time, similar to the results of the GC-MS analysis. Janghang Wetland honey was distinguishable from commercial honey, although no distinction was observed among apiculture companies (Fig. 2B). Mixed wildflower honey (wildflower sugar fed honey, hard boiled honey, wildflower matured honey, wildflower honey) from different companies tended to cluster together, while single-source honey was scattered more widely.

When comparing honey collected from Janghang Wetland in March and April, OPLS-DA analysis using GC-MS data revealed that the sucrose content was higher in March

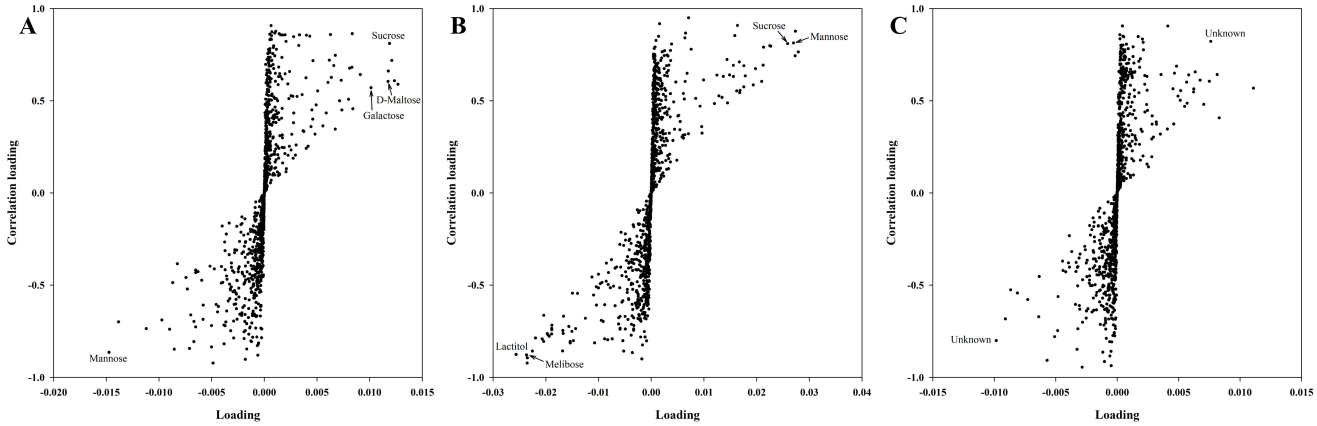

Fig. 3 S-plot based on orthogonal partial least squares-discriminant analysis using gas chromatography-mass spectrometry data (A) for comparative analysis of honeys collected from Janghang Wetland in March (positive direction) and April (negative direction), (B) for comparative analysis between honeys collected from Janghang Wetland (positive direction) and apiculture honeys (negative direction) and (C) for comparative analysis between higher *Prunus* and *Salix* ratio (*Prunus* > *Salix*: positive direction; *Prunus* < *Salix*: negative direction) among honeys collected from Janghang Wetland. Only identified metabolites were given names among the metabolites considered meaningful. Metabolites deemed meaningful were not identified in (C).

than in April, while the mannose content was higher in April than in March (Fig. 3A). The correlation is somewhat weak, but notable metabolites such as maltose and galactose were detected in the honey collected in March (Fig. 3A). When using LC-MS data in OPLS-DA, several significant metabolites were discovered that differentiate between the honey collected in March and April. Metabolites with m/z values of approximately 247 and 287 were detected in the 20-minute range for March honey, while metabolites with m/z values around 274 were predominantly detected in the 26-minute range in April honey (Fig. S1C, D).

A comparison was made between honey collected from Janghang Wetland and commercial honey. The OPLS-DA analysis, using GC-MS data, revealed that the honeys collected in Janghang Wetland had a higher sucrose and mannose content compared to those collected in commercial honey. On the other hand, honey produced by the apiculture company had a higher lactitol and melibose content (Fig. 3B). The OPLS-DA analysis using LC-MS data (Fig. S2C, D) suggests that there are no significant metabolites in S-plot, respectively, due to the low values on the y-axis and x-axis.

To compare honey samples based on their *Prunus* and *Salix* content, we grouped them according to metabarcoding results, regardless of the collection time. Although meaningful metabolites were detected in OPLS-DA using GC-MS data (Fig. 3C), they could not be identified. In the results of LC-MS data, no significant metabolites were detected, suggesting that multiple metabolites are interacting in a complex manner (Fig. S3C, D).

Measurement of total phenolic contents and total flavonoids contents

In March, the TPC was measured to be an average of 450.79 ± 46.28 mg GA/100 mg, while in April it was 331.61 ± 33.62 mg GA/100 mg. The total honey collected from Janghang Wetland had a TPC of 388.06 ± 71.77 mg GA/100 mg (Table S4). The TFC was measured to be 2.85 ± 1.18 mg QE/100 mg in March, 3.58 ± 0.67 mg QE/100 mg in April, and 3.23 ± 1.01 mg QE/100 mg in the total honey collected from Janghang Wetland in total (Table S4). Janghang Wetland honey had lower TPC and TFC levels compared to the other honey samples (Table S4, Fig. 4).

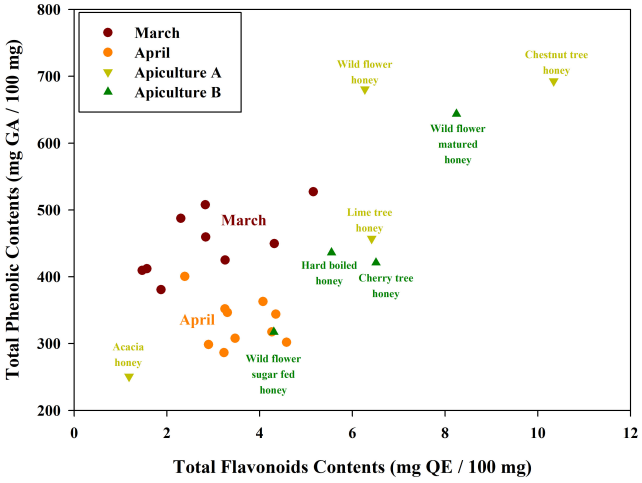

Fig. 4 Scatter plot using total phenolic contents and total flavonoids contents of honey samples.

Discussion

Origin of honey from a willow community in Janghang Wetland

The use of databases is crucial in metabarcoding analysis for species identification, but there are several challenges associated with their use (Keck et al. 2023). GenBank, as

the largest existing sequence database (Benson et al. 2013), is highly valuable, but its use requires careful consideration for appropriate use. In metabarcoding analysis, universal primers are commonly used to identify as many taxonomic groups as possible. However, it is important to note that due to the limitations of accurately classifying numerous taxonomic groups using short reads of only a few hundred base pairs obtained with universal primers, multiple taxonomic groups may have identical scores (Keck et al. 2023). Consequently, it is possible to identify a classification group even if it is not native to the area where the sample was collected. When conducting a BLAST analysis using all ITS2 sequences uploaded in the GenBank database, the plants reported as growing in Janghang Wetland were not identified at a high rank. To supplement this, longer barcode sequences could be used, but this would require cloning or long-read sequencing. However, the utilization of these techniques in metabarcoding may present additional limitations. The GeneBank database was used to identify several plants, including *S. hastata*, *S. magnifica*, *P. pseudocerasus* and *P. serrulate*. These species had not been previously reported in Korea. To address this limitation, a database created from the local flora list of the area where honey (Table S1) was collected was used for identification, resulting in practical results.

Salidroside has been isolated from various plant organs such as barks, leaves, branches, etc. and has also been found in flower buds (Julkunen-Tiitto 1989). Qualitative and quantitative analysis of salidroside can be performed by comparison with a standard material using GC-MS and LC-MS. As a result, salidroside was not detected in any of the samples including honeys, where *S. triandra* subsp. *nipponica* had been identified by metabarcoding analysis. It is thought that the concentration of salidroside did not reach the detection limit due to the relatively high sugar content in honey, or that it was produced in the flowers of *S. triandra* subsp. *nipponica*, but not transferred to the honey.

In early spring, honeybees were reported to primarily use plants from the Genus *Salix* and *Prunus* (Coffey and Breen 1997; Wood et al. 2018). Genus *Salix* and *Prunus* together accounted for over 99.9% of both honey and pollen (Fig. 1). *Salix triandra* subsp. *nipponica* dominates the community in Janghang Wetland while there have been no reports of *P. padus*. *P. padus* may be present in the Janghang Wetlands, but it may not have been recorded due to its lack of ecological or taxonomic significance. Furthermore, the average honeybee's possible foraging range has been estimated to be approximately 5.5 km (Beekman and Ratnieks 2000). The 5.5 km radius of Janghang Wetland encompasses Goyang City, Gimpo City, and western Seoul City. If honey had been collected primarily from outside the wetland, it is likely that a greater diversity of plants would have been identified in metabarcoding results. The metabarcoding analysis revealed mainly the presence of two species. It is believed that these were primarily collected in Janghang Wetland, as there was an abundance of honey plants in the vicinity of the beehive.

Honey is typically collected by honeybees from the dominant species in the area (Coffey and Breen 1997; Percival 1947). In the case of Janghang Wetland, the main species is *S. triandra* subsp. *nipponica*. However, the relatively high detection of *P. padus* in the honey and pollen suggests that honeybees may also prefer the nectar of these trees. According to Khan et al. (2021) and Ghosh et al. (2020), honeybees show a preference for pollen with a high concentration of proteins. Wood et al. (2022) demonstrated that *P. cerasus*, a species belonging to the same genus as *P. padus*, has the highest protein content when compared to *S. caprea* and *S. fragilis*, which belong to the same genus as *S. triandra* subsp. *nipponica*. These findings may explain the high contribution of *P. padus* to honey and pollen. From the perspective of plant distribution, *S. triandra* subsp. *nipponica* is a unisexual flower, while *P. padus* is a bisexual flower. the collection of pollen from *S. triandra* subsp. *nipponica* is only possible in male flowers. As in the previous discussion, if honeybee visits were concentrated near the beehive in Janghang Wetland, and the quantity of honey and pollen supposes balancing as *P. padus* is a bisexual flower, it is anticipated that the area surrounding the beehive will contain a greater proportion of female trees than male trees. This will be able to be validated by an examination of the precise plant distribution in the Janghang Wetland.

The results of the metabolite analysis comparing honey collected from Janghang Wetland and commercial honey using GC-MS and LC-MS indicate that honey collected in April from Janghang Wetland closely resembles cherry tree honey (Fig. 2). Considering *Prunus* plants flower in mainly April, the results of DNA metabarcoding and metabolites analysis show similar trends. The origin of honey can be inferred for comparison with the metabolomes in the target and reference honey. Additionally, metabolite analysis can complement findings that were not resolved through DNA metabarcoding.

The NMDS plots (Fig. 2B) based on metabolite analysis results from LC-MS indicate that commercial wild-flower honeys produced in different environments are more influenced by the composition of nectar plants than by the collected environment. However, the results from GC-MS analysis show that honeys were separated by apiculture company (Fig. 2A). GC-MS is primarily used to detect for primary metabolites, while LC-MS is employed for the detection of secondary metabolites (Lee et al. 2013; Zhang et al. 2012). Secondary metabolites may be more suitable for reflecting the origin of the nectar tree, while primary metabolites are thought to reflect the environment where the honey was collected.

Ingredient of honey from a willow community in Janghang Wetland

Variations in the composition of honey metabolites were observed even in the same collecting location (Fig. 2). Additionally, differences in sugar compositions were found between honey collected in March and April (Fig. 3A). These differences seem to be the results of changes in the composition of the nectar-producing plants, as suggested by Coffey and Breen (1997) and Wood et al. (2022), and in the type of honey plants, which depend on the time of collection, as indicated by Peters et al. (2018) and Ma et al. (2019).

When comparing honeys collected from Janghang Wetland to commercial honeys, it is apparent that honeys from Janghang Wetland contains higher levels of sucrose and mannose (Fig. 3B). These metabolites also varied with months. Figure 3A shows that honey collected from Janghang Wetland in March has a higher sucrose content, while honey collected in April has higher mannose content. The results show differences in some of the disaccharide components of Janghang Weland honey and commercial honey, which may be related to temperature or storage period. Honey contains invertase, and enzyme that hydrolyzes sucrose into fructose and glucose (Nelson and Cohn 1924; Sahin et al. 2020). Invertase is mixed with the secretion of honeybees (Nelson and Cohn 1924) and there is a significant correlation between invertase and sucrose contents (Lichtenberg-Kraag 2014). The activity of invertase increases with higher temperatures and longer storage times (Lichtenberg-Kraag 2012). It is possible that honeys collected from Janghang Wetland, which have high sugar contents, did not undergo sucrose inversion into monosaccharides due to their lower level of invertase activity compared to commercial honey.

Many significant metabolites were detected, but not identified, when comparing *Prunus* - dominant and *Salix* - dominant honey collected in March (Fig. 3C). Based on the results presented in Figure 2, it is difficult to differentiate between *Prunus* - dominant and *Salix* - dominant honey. Therefore, the unidentified metabolite shown in Figure 3C seems to have little effect on the honey's composition.

TPC and TFC in honey were reported to significantly related with antioxidant activity (Dong et al. 2013; Iurlina et al. 2009). Some studies have suggested potential correlations between the presence of flavonoids and phenolic compounds in honey and their floral and geographical origins (Dong et al. 2013; Iurlina et al. 2009; Küçük et al. 2007; Suleiman et al. 2020). Both TPC and TFC of Janghang Wetland honey were lower compared to other honeys, with the exceptions of *Robinia* honey and wildflower sugar fed honey (Fig. 4). Likewise, metabolite profile analysis, TPC and TFC in Janghang Wetland honey would have been influenced by the honey's origin and geographical source. Although the specific factors determining TPC and TFC cannot be identified, it is expected that the antioxidant capacity of honey from Janghang Wetland in spring will be lower than that of various other honey types.

Conclusions

In honey and pollen collected from Janghang Wetland in March, *Salix* and *Prunus* constituted over 99% of the total content. Specifically, *S. triandra* subsp. *nipponica* was the dominant species in honey, while *P. padus* was predominant in the pollen. This was supported by LC-MS analysis, which revealed a close grouping with cherry tree honey in contrast to commercial honey. Based on the results of GC-MS analysis, it is suggested that the secondary metabolites detected through LC-MS are more helpful in understanding the origin of honey plants compared to the primary metabolites identified by GC-MS. In comparison to commercial nectar derived from multiple plant species, the metabolite diversity of nectar collected from Janghang Wetland is comparable to that of cherry nectar. The variation in metabolite diversity suggests that it may provide a potential explanation for the geographic and environmental origins of honey considering the timing of collected honey from Janghang Wetland. These findings can contribute to identifying the geographical origin of honey and enhancing our understanding of the diversity of metabolites in honey.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.5141/jee.24.026.

Table S1. The sequence database for BLAST identification comprises a list of plant species. **Table S2.** ASVs identified at the genus level in each sample. **Table S3.** ASVs identified at the species level in each sample. **Table S4.** Total phenolic contents and total flavonoids contents of each sample. **Fig. S1.** Orthogonal partial least squares-discriminant analysis and S-plot for comparative analysis of honeys collected from Janghang Wetland in March and April. **Fig. S2.** Orthogonal partial least squares-discriminant analysis and S-plot for comparative analysis between honeys collected from Janghang Wetland and apiculture honeys. **Fig. S3.** Orthogonal partial least squares-discriminant analysis and S-plot for comparative analysis between higher Prunus and Salix ratio among honeys collected from Janghang Wetland.

Abbreviations

ITS2: Internal transcribed spacer 2

GC-MS: Gas chromatography-mass spectrometry

LC-MS: Liquid chromatography-mass spectrometry

ASV: Amplicon sequence variant
TPC: Total phenolic contents
TFC: Total flavonoids contents
NMDS: Non-metric multidimensional scaling
OPLS-DA: Orthogonal partial least squares-discriminant analysis

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Authors' contributions
YR formal analysis, and writing-original draft. DH conceptualized the study and conducted field study. IKL conceptualized the study and received a research grant. SP conceptualized the study, reviewing and editing the draft, writing-review and editing, and supervision. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Not applicable.

Consent for publication
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Competing interests
Corresponding author Sangkyu Park has been Editor-in-Chief of *Journal of Ecology and Environment* since 2019. He was not involved in the review process of this article. Otherwise, the authors declare that they have no competing interests.

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