Honey sample collection methods influence pollen composition in determining true nectar-foraging bee plants

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ABSTRACT

This study investigated the suitability of honey sample collection methods for determining the botanical origin of honey through palynological analysis. We used three methods to collect honey samples in three different modes viz. extracted honey using a honey extractor, squeezed honey and pipetted honey (collected by micropipette/dropper from honey cells only) during 2017 to 2019 in West Bengal, India. We considered two native honey bee species (*Apis dorsata* and *Apis florea*) and one introduced bee species (*Apis mellifera*). Pollen composition differed significantly, both quantitatively and qualitatively, among the honey samples of the different methods. The number of pollen grains in extracted honey and squeezed honey was significantly higher than that of pipetted honey. Furthermore, some pollen types of nectar deficient, but polleniferous plants (*viz. Capparis zeylanica*, *Echinochloa frumentacea*, *Papaver somniferum*, Poaceae type, *Nelumbo nucifera*, *Solanum melongena*, and *Solanum sisymbriifolium*), were also present in extracted and squeezed honeys. We concluded that some pollen grains present in extracted and squeezed honey samples came from stored pollen loads or bee bread in the hive. Hence, the pollen spectrum for pipetted honey samples was more accurate in depicting the bees foraging on nectariferous plants.

Keywords: extracted honey, nectariferous plant, pipetted honey, pollen spectrum, squeezed honey

Introduction

Most eusocial bees including honey bees depend on floral resources for survival, because their main food sources are nectar and pollen (Haydak 1970; Michener 2007; Wright *et al.* 2018). Nectar is the principal source of carbohydrates from which honey bees obtain their energy (Freitas 1991; Ramalho *et al.* 1991; Winston 1991; Nicolson 2011). After collecting nectar, worker bees process the nectar in their honey stomach and then store it in honey cells in the hive, where it forms ripened honey. The nectariferous plants that honey bees forage on therefore play a pivotal role as a source of honey and support bee colony health in a particular locality. The identity of local nectariferous plants is also important for the establishment of beekeeping within a biozone. Honey composition depends on its botanical origin, the climatic conditions where it was formed, and the insect itself (Oroian *et al.* 2014; Attanzio *et al.* 2016). The taste, smell, color and consistency of honey differ according to its botanical origin (Truchado *et al.* 2008; Kaškonienė & Venskutonis 2010; Dobre *et al.* 2013).

The presence of floral pollen grains within honey enables the identification of the nectariferous plants that a honey sample was sourced from. Pollen analyses of honey (*i.e.,* melissopalynology) have been used to accurately determine the botanical and geographic origin of honey samples (Louveaux *et al.* 1978; Ohe *et al.* 2004; Ponnuchamy *et al.*

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2014) and also help us to verify honey authenticity. For these purposes, the proper sampling of honey is important for identifying the plants providing nectar. The open-nesting honey bee species *Apis dorsata* and *Apis florea* build a single comb, containing both honey and pollen. Honey samples obtained carelessly or by squeezing the comb may therefore be contaminated with pollen coming from pollen loads stored in pollen cells. We have also seen that most of the migratory beekeepers who are beekeeping with *Apis mellifera* in the Kanchanpur of Bankura, Lokhata and Jhakra of Paschim Medinipur, and Bagula of Nadia districts in West Bengal do not use a honey super because honey supers are difficult to transport. Instead, they use frames containing honey, pollen, and bee bread during honey extraction. Such extracted honey samples also have greater possibilities of pollen contamination from nectar-deficient plants. Researchers unfortunately use such traditionally extracted honey samples for palynological analysis to determine honey's botanical origin, but the presence of non-nectariferous pollen types may provide an ambiguous result.

Here, we performed palynological analyses of honey samples collected via three different sampling methods (using a micropipette/dropper to obtain honey from honey cells, using a honey extractor, and by squeezing the comb cells) to determine the effect of sampling methods on the pollen composition of honey. We hypothesized that sampling methods have a significant effect on the pollen composition of honey. Specifically, we expected melissopalynological analysis of pipetted honey to be more reliable for the accurate identification of nectariferous plants than analyses of squeezed or extracted honey.

Materials and methods

Sampling area

We collected honey samples from seven districts (Bankura, Birbhum, Hooghly, Paschim Burdwan, Paschim Medinipur, Purba Burdwan, and Purba Medinipur) in southern West Bengal, India. The state lies between 21º38'-27º10' N and 85º50'-89º50' E. Six seasons (summer, monsoon, autumn, late autumn, winter, and spring) are clearly discernible in the study areas. Summer (April to mid-June) is the warmest season, with daytime high temperatures ranging from 38 °C to 45 °C. The monsoon brings rain to the whole state from mid-June to August; of the average annual rainfall of 175 cm, about 125 cm occurs during this period. Autumn (September to mid-October) is characterized by sporadic rainfall with patches of white clouds in the sky. After that, the temperature gradually decreases and during late autumn (mid-October to November) the mean day temperature remains near 23 °C. During winter (December to mid-February), the temperature falls sharply to 7 ºC, and the daytime humidity level is very low [relative humidity (RH) 42-65 %]. Spring is a transitional season between winter and summer, with a moderate temperature (average 26 °C) and humidity (average RH 66 %). Agriculture is the primary economic activity in southern West Bengal, and depends on rainfall as well as irrigation. Some regions within the study areas are covered by natural dipterocarp (*Shorea robusta* Roth) forest and artificially planted forests of *Acacia auriculiformis* A. Cunn. ex Benth. and *Eucalyptus* spp.

Collection of honey samples

We collected honey samples (25 ml for each sample) from 2017 to 2019 using three different methods. Namely, we pipetted honey only from honey cells using a micropipette/dropper, we extracted honey using a honey extractor, and we squeezed honey from combs. We evaluated honey from two native honey bee species (*A. dorsata* F. and *A. florea* F.) and one introduced bee species (*A. mellifera* L.). Bees were first either partially or fully removed from the comb using a smoker. We then collected one pipetted honey sample. For *A. dorsata* and *A. florea*, we collected another squeezed honey sample, and for *A. mellifera*, we collected an extracted honey sample from a single comb. A total of 68 honey samples (36 pipetted, 22 squeezed, and 10 extracted) were collected.

Palynological analyses of honey samples

Honey samples were processed using the methodology of Louveaux *et al.* (1978) with the modification recommended by Jones & Bryant Jr. (2004). In brief, 10 g of honey was dissolved in 10 ml of warm water (not above 40 °C), to which 50 ml of 95 % ethanol was added. The solution was centrifuged for 10 min at 2500 rpm (1036 g) and the supernatant was discarded. The pollen sediment was prepared for microscopy using the acetolysis method described by Erdtman (1960) and then mounted on permanent slides with glycerine jelly. Pollen types were identified using reference slides prepared from the local flora as well as with the help of published articles (Pal & Karmakar 2013; Layek & Karmakar 2016; 2018; Layek *et al.* 2020). Microscopy was performed using a Leica DM 1000 Ergo trinocular microscope and a Nikon Eclipse LV100 POL polarising microscope, and pollen types were micro-photographed at suitable magnifications. Pollen was classified according to the pollen type system (Joosten & Klerk 2002; Klerk & Joosten 2007) which was based on morphological features.

To determine the frequency of each pollen type, we counted the number of pollen grains of each type that could be observed in a microscope field. Due to fewer pollen grains within a microscope field for pipetted samples relative to the other sample types, we counted a lower number of pollen grains for pipetted samples (~100 pollen grains per sample) compared to extracted and squeezed honeys (~300 pollen grains per sample). Pollen types were then classified into one of the following frequency classes (Louveaux *et al.* 1978):

predominant (>45 %), secondary (16-45 %), important minor (3-15 %), and minor (<3 %).

To determine the pollen concentration of honey samples, we followed the methodology of Pérez *et al*. (1994), with a slight modification. Acid water (3.5 ml of concentrated H_2SO_4 in 1 L of distilled water) was added to 10 g of honey to create a 20 ml suspension. The suspension was homogenized with a stirrer and then loaded into a haemocytometer using a pipette. Pollen grains were counted under an optical microscope and counts were normalized to the number of pollen grains per 20 ml of suspension (*i.e.,* per 10 g of honey). Based on the number of pollen grains per 10 g honey, we classified the honey sample according to Maurizio's (1939) representative pollen classes: class I (<20000), class II (20000-100000), class III (100000-500000), class IV (500000-1000000) and class V (>1000000).

Statistical analysis

Statistical analyses of honey samples were conducted to obtain the arithmetic mean and standard deviation. A one-way ANOVA followed by Duncan's multiple range test (DMRT) was used to analyze data and $p \leq 0.05$ was considered statistically significant.

Results

A total of 67 pollen types belonging to 36 plant families were identified (Tab. 1, Tab. S1 in supplementary material). The predominant pollen types were *Borassus flabellifer*, *Brassica nigra*, *Coriandrum sativum*, *Eucalyptus* type, and *Sesamum indicum* (Fig. 1). Important pollen types (predominant and secondary) of nectariferous plants were common among all the honey bee species and among all the honey sampling methods. However, squeezed honeys revealed a greater diversity of pollen types (67 types from 22 samples) than pipetted honeys (50 types from 36 samples) and extracted honeys (25 types from 10 samples). A few pollen types (namely *Mimusops elengii*, *Semecarpus anacardium*, and *Xanthium strumarium*) were absent from pipetted honeys, even though these are known nectariferous plants for the bee species we studied. Pollen types like *Capparis zeylanica, Echinochloa frumentacea*, *Papaver somniferum*, *Nelumbo nucifera*, Poaceae type, *Solanum melongena*, and *Solanum*

Table 1. Pollen composition of honey samples (Sample: e-extracted, p-pipetted, s-squeezed; Pollen type: P- predominant, S-secondary; In bold: pollen type of non-nectariferous plant).

Season	Honey bee	Comb	Sample	Pollen/10 g honey	Pollen type
Summer	A. florea	Af-1	p	2487	*Aegle marmelos, Azadirachta indica, Borassus flabellifer* (S)*, Trema orientalis*
		Af-1	s	601342	*Aegle marmelos, Azadirachta indica, Borassus flabellifer* (S), **Capparis zeylanica**, *Syzygium reticulatum, Trema orientalis*
		Af-2	p	3208	*Alangium salviifolium, Borassus flabellifer* (P), *Syzygium reticulatum, Tridax procumbens*
		Af-2	s	132714	*Alangium salviifolium, Borassus flabellifer* (P), **Capparis zeylanica**, **Solanum melongena**, *Syzygium reticulatum* (S), *Tridax procumbens*
		Af-3	p	5852	*Momordica charantia, Peltophorum pterocarpum, Sesamum indicum, Terminalia arjuna* (S)
		Af-3	s	625137	*Croton bonplandianum, Momordica charantia, Peltophorum pterocarpum, Sesamum indicum,* **Solanum sisymbriifolium**, *Terminalia arjuna* (S)
	A. dorsata	Ad-1	p	3415	*Borassus flabellifer* (S)*, Delonix regia, Momordica charantia, Peltophorum pterocarpum, Sesamum indicum, Terminalia arjuna*
		Ad-1	s	125342	*Albizia lebbeck, Borassus flabellifer* (S)*, Delonix regia, Momordica charantia,* **Nelumbo nucifera**, *Peltophorum pterocarpum, Sesamum indicum* (S), *Syzygium reticulatum, Terminalia arjuna*
		Ad-2	p	2316	*Borassus flabellifer, Millettia pinnata, Sesamum indicum*(P)*, Tamarindus indica, Terminalia arjuna*
		Ad-2	s	664248	*Borassus flabellifer* (S)*, Hygrophila auriculata, Millettia pinnata, Sesamum indicum* (S), **Solanum melongena**, *Tamarindus indica, Terminalia arjuna*
	A. mellifera	Am-1	p	1921	*Millettia pinnata, Sesamum indicum*(P)*, Tamarindus indica*
		Am-1	e	82724	*Croton bonplandianum, Delonix regia, Millettia pinnata,* **Nelumbo nucifera**, *Sesamum indicum* (S), *Tamarindus indica, Trema orientalis* (S)
		Am-2	p	1136	*Borassus flabellifer, Sesamum indicum* (P), *Terminalia arjuna*
		Am-2	e	23045	*Azadirachta indica, Borassus flabellifer,* **Capparis zeylanica**, *Croton bonplandianum, Sesamum indicum* (P), *Terminalia arjuna*

Table 1. Cont.

Season	Honey bee	Comb	Sample	Pollen/10 g honey	Pollen type
Summer	*A. mellifera*	Am-3	p	1927	*Alangium salviifolium* (S), *Albizia lebbeck*, *Mangifera indica*, *Millettia pinnata*
		Am-3	e	168705	*Aegle marmelos, Alangium salviifolium* (S), *Albizia lebbeck, Mangifera indica, Millettia pinnata, Syzygium reticulatum*
Monsoon	*A. florea*	Af-4	p	16282	*Citrus × aurantiifolia, Cocos nucifera* (S), *Momordica charantia, Tridax procumbens*
		Af-4	s	271475	*Citrus × aurantiifolia* (S), *Cocos nucifera* (S), *Momordica charantia,* **Poaceae type**, *Semecarpusanacardium, Tridax procumbens*
		Af-5	p	13806	*Citrus × aurantiifolia* (S), *Cocos nucifera, Peltophorum pterocarpum, Phyla nodiflora*
		Af-5	s	182723	*Citrus × aurantiifolia* (S), *Cocos nucifera,* **Echinochloa frumentacea**, *Peltophorum pterocarpum, Phyla nodiflora, Ziziphus mauritiana*
	A. dorsata	Ad-3	p	11940	*Cucurbita maxima, Lippia alba, Peltophorum pterocarpum* (S), *Tridax procumbens* (S), *Vitex negundo*
		Ad-3	s	234619	*Cleome viscosa, Cucurbita maxima,* **Echinochloa frumentacea**, *Lippia alba, Murraya paniculata, Peltophorum pterocarpum,* **Solanum sisymbriifolium**, *Tridax procumbens* (S), *Vitex negundo*
		Ad-4	p	14070	*Acacia nilotica, Leucaena leucocephala, Neolamarckia cadamba* (S), *Peltophorum pterocarpum*
		Ad-4	s	308621	*Acacia nilotica, Citrus × aurantiifolia, Leucaena leucocephala, Mimusops elengi, Neolamarckia cadamba* (S), *Peltophorum pterocarpum*
	A. mellifera	Am-4	p	6284	*Citrus × aurantiifolia, Cocos nucifera* (S), *Cucurbita maxima, Peltophorum pterocarpum, Tridax procumbens*
		Am-5	p	4702	*Cleome viscosa, Momordica charantia, Murraya paniculata, Peltophorum pterocarpum* (S)
Autumn	*A. florea*	Af-6	p	12835	*Acacia auriculiformis, Bridelia retusa* (S), *Cocos nucifera, Ziziphus mauritiana*
		Af-6	s	183547	*Acacia auriculiformis, Bridelia retusa* (S), *Cocos nucifera, Tephrosia purpurea, Ziziphus mauritiana*
	A. dorsata	Ad-5	p	17219	*Acacia auriculiformis, Bridelia retusa* (S), *Haldina cordifolia, Luffa aegyptiaca, Ziziphus mauritiana*
		Ad-5	s	408926	*Acacia auriculiformis* (S), *Bridelia retusa, Haldina cordifolia, Luffa aegyptiaca,* **Poaceae type**, *Trema orientalis, Xanthium strumarium, Ziziphus mauritiana*
	A. mellifera	Am-6	p	17293	*Acacia auriculiformis* (S), *Cocos nucifera, Luffa aegyptiaca, Ziziphus mauritiana*
		Am-7	p	14764	*Acacia auriculiformis* (S), *Cocos nucifera, Tridax procumbens, Ziziphus mauritiana*
Late autumn	*A. florea*	Af-7	p	18072	*Acacia auriculiformis* (S), *Cocos nucifera, Eucalyptus* type (P), *Ziziphus mauritiana*
		Af-7	s	1023858	*Acacia auriculiformis* (S), *Cocos nucifera, Eucalyptus* type (P), *Mikania scandens, Ziziphus mauritiana*
		Af-8	p	15329	*Acacia auriculiformis* (S), *Cyanotis axillaris, Eucalyptus* type (P), *Ziziphus mauritiana*
		Af-8	s	731046	*Acacia auriculiformis* (S), *Cyanotis axillaris, Eucalyptus* type (P), *Ricinus communis, Ziziphus mauritiana*
	A. dorsata	Ad-6	p	15207	*Acacia auriculiformis, Antigonon leptopus, Eucalyptus* type (P), *Hygrophila auriculata, Ricinus communis, Ziziphus mauritiana*
		Ad-6	s	362853	*Acacia auriculiformis* (S), *Antigonon leptopus, Cyanotis axillaris, Eucalyptus* type (P), *Hygrophila auriculata, Ricinus communis, Ziziphus mauritiana*
		Ad-7	p	10894	*Acacia auriculiformis* (S), *Cocos nucifera, Eucalyptus* type (P), *Mikania scandens, Ziziphus mauritiana*
		Ad-7	s	75362	*Acacia auriculiformis* (S), *Cocos nucifera, Eucalyptus* type (P), *Grewia asiatica, Mikania scandens, Ziziphus mauritiana*
	A. mellifera	Am-8	p	6314	*Acacia auriculiformis, Eucalyptus* type (P), *Phoenix sylvestris, Ziziphus mauritiana*

Table 1. Cont.

Season	Honey bee	Comb	Sample	Pollen/10 g honey	Pollen type
Late autumn	*A. mellifera*	Am-8	e	140827	*Acacia auriculiformis, Cocos nucifera, Eucalyptus* type (S), *Phoenix sylvestris, Ziziphus mauritiana*
		Am-9	p	2507	*Eucalyptus* type (P)
		Am-9	e	32483	*Acacia auriculiformis, Eucalyptus* type (P), *Mikania scandens*
Winter	*A. florea*	Af-9	p	8236	*Acacia auriculiformis, Cocos nucifera, Eucalyptus* type (P), *Phoenix sylvestris, Ziziphus mauritiana*
		Af-9	s	31752	*Acacia auriculiformis, Cocos nucifera, Eucalyptus* type (S), *Mikania scandens, Phoenix sylvestris* (S), *Ziziphus mauritiana*
		Af-10	p	2473	*Brassica nigra* (P), *Coriandrum sativum, Eucalyptus* type, *Moringa oleifera, Phoenix sylvestris*
		Af-10	s	23845	*Acmella radicans, Brassica nigra, Coriandrum sativum* (S), *Eucalyptus* type (S), *Moringa oleifera, Phoenix sylvestris*
	A. dorsata	Ad-8	p	6104	*Acacia auriculiformis, Cocos nucifera, Eucalyptus* type (P), *Phoenix sylvestris, Ziziphus mauritiana*
		Ad-8	s	36183	*Acacia auriculiformis, Brassica nigra, Cocos nucifera, Coriandrum sativum* (S), *Eucalyptus* (S), *Phoenix sylvestris, Ziziphus mauritiana*
	A. mellifera	Am-10	p	3258	*Brassica nigra* (P), *Coriandrum sativum, Eucalyptus* type
		Am-10	e	18206	*Brassica nigra* (P), *Coriandrum sativum* (S), *Eucalyptus* type, *Mikania scandens, Ricinus communis*
		Am-11	p	10724	*Coriandrum sativum* (P), *Eucalyptus* type, *Mangifera indica*
		Am-11	e	26952	*Cocos nucifera, Coriandrum sativum* (P), *Eucalyptus* type (S), *Mangifera indica, Phoenix sylvestris*
Spring	*A. florea*	Af-11	p	4617	*Alangium salviifolium, Butea monosperma* (S), *Ceiba pentandra, Coriandrum sativum, Moringa oleifera, Lannea coromandelica*
		Af-11	s	147386	*Alangium salviifolium, Butea monosperma, Ceiba pentandra, Coriandrum sativum* (S), *Moringa oleifera, Lannea coromandelica,* **Solanum melongena**
		Af-12	p	17403	*Bombax ceiba, Borassus flabellifer* (P), *Coriandrum sativum, Nigella sativa, Syzygium cumini*
		Af-12	s	265848	*Bombax ceiba, Borassus flabellifer* (P), *Coriandrum sativum, Holoptelia integrifolia* (S), *Nigella sativa, Syzygium cumini*
	A. dorsata	Ad-9	p	4049	*Alangium salviifolium, Borassus flabellifer* (S), *Lannea coromandelica, Gmelina arborea, Madhuca longifolia, Mangifera indica, Shorea robusta*
		Ad-9	s	208329	*Alangium salviifolium, Borassus flabellifer,* **Capparis zeylanica**, *Dalbergia sissoo, Gmelina arborea, Lannea coromandelica* (S)*, Madhuca longifolia, Mangifera indica,* **Papaver somniferum**, *Shorea robusta*
		Ad-10	p	11572	*Alangium salviifolium, Bombax ceiba, Butea monosperma* (S), *Coriandrum sativum, Lannea coromandelica*
		Ad-10	s	209381	*Ailanthus excelsa, Alangium salviifolium, Bombax ceiba, Butea monosperma, Coriandrum sativum, Helianthus annuus, Lannea coromandelica* (S), **Solanum melongena**
	A. mellifera	Am-12	p	2805	*Alangium salviifolium, Borassus flabellifer* (P), *Butea monosperma, Coriandrum sativum*
		Am-12	e	25294	*Alangium salviifolium, Borassus flabellifer* (P), *Butea monosperma,* **Capparis zeylanica**, *Coriandrum sativum, Shorea robusta*
		Am-13	p	17807	*Alangium salviifolium, Bombax ceiba, Borassus flabellifer* (P)
		Am-13	e	58432	*Alangium salviifolium, Bombax ceiba, Borassus flabellifer* (S), *Coriandrum sativum* (S), **Solanum melongena**
		Am-14	p	1869	*Butea monosperma, Ceiba pentandra* (S), *Coriandrum sativum, Dalbergia sissoo, Syzygium cumini*
		Am-14	e	607192	*Butea monosperma, Ceiba pentandra, Coriandrum sativum* (S), *Dalbergia sissoo, Nigella sativa, Syzygium cumini*

sisymbriifolium were found in squeezed and extracted honeys but were absent from pipetted honeys (Fig. 2). These plants are polleniferous rather than nectariferous, a fact that was additionally confirmed by field observations. Among these pollen types of nectar-deficient plants, *Capparis zeylanica* and *Solanum melongena* frequently occurred in honey samples.

The number of pollen types of nectar-deficient plants and their frequency of occurrence were higher during summer (four types, 43.75 % of occurrence), spring (three types, 28.57 % of occurrence) and the monsoon season (three types, 30 % of occurrence) compared to autumn, late autumn and winter. In some cases (combs no. Ad-2, Ad-8, Af-9, Af-10, Am-1, and Am-13), honey types that were predicted to be unifloral based on observations of pipetted samples wrongly appeared as multifloral when we sampled via extraction or squeezing. Furthermore, the frequency class of some nectariferous plant pollen types changed from "minor" and "important minor" in pipetted honeys to "secondary" in squeezed or extracted honeys samples collected from same comb.

The number of pollen types per honey sample also differed significantly among pipetted, extracted and squeezed honeys ($F_{2, 65}$ = 26.94, P = 3.01E-09). Squeezed honeys contained the most pollen types per sample (6.63 ± 1.36) and pipetted honeys contained the fewest (4.33 ± 1.10) (Tab. 2).
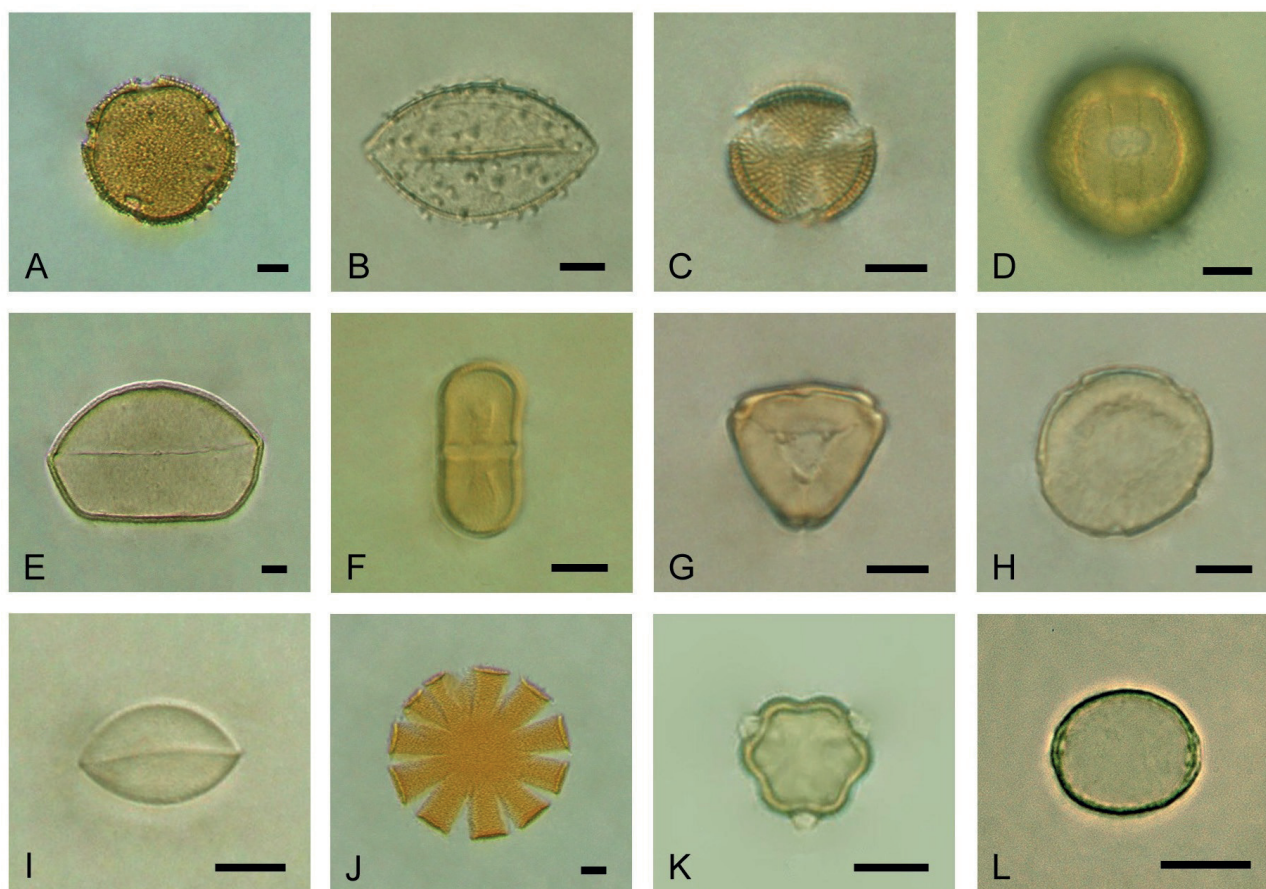

Figure 1. Some important (predominant and secondary) pollen types found in pipetted honeys. **A**. *Alangium salviifolium*, **B**. *Borassus flabellifer*, **C**. *Brassica nigra*, **D**. *Butea monosperma*, **E**. *Cocos nucifera*, **F**. *Coriandrum sativum*, **G**. *Eucalyptus* type, **H**. *Holoptelea integrifolia*, **I**. *Phoenix sylvestris*, **J**. *Sesamum indicum*, **K**. *Terminalia arjuna*, **L**. *Trema orientalis*. Scale bar- 10 µm.

Table 2. Number of pollen types per sample and quantitative pollen content of different sampling types.

Sampling types	Pollen types/sample		Number of pollen grains/ 10 g honey	
	Range	Mean ± SD	Range	Mean ± SD
Pipetted honey	1-7	4.33[c] ± 1.10	1136-18072	8630.47[c] ± 5924.51
Extracted honey	3-7	5.40[b] ± 1.07	18206-607192	118386[b] ± 179618.34
Squeezed honey	5-10	6.68[a] ± 1.36	23845-1023858	311569.86[a] ± 262964. 78

Means in the column followed by same alphabets do not differ significantly by DMRT at 5%, SD-standard deviation.
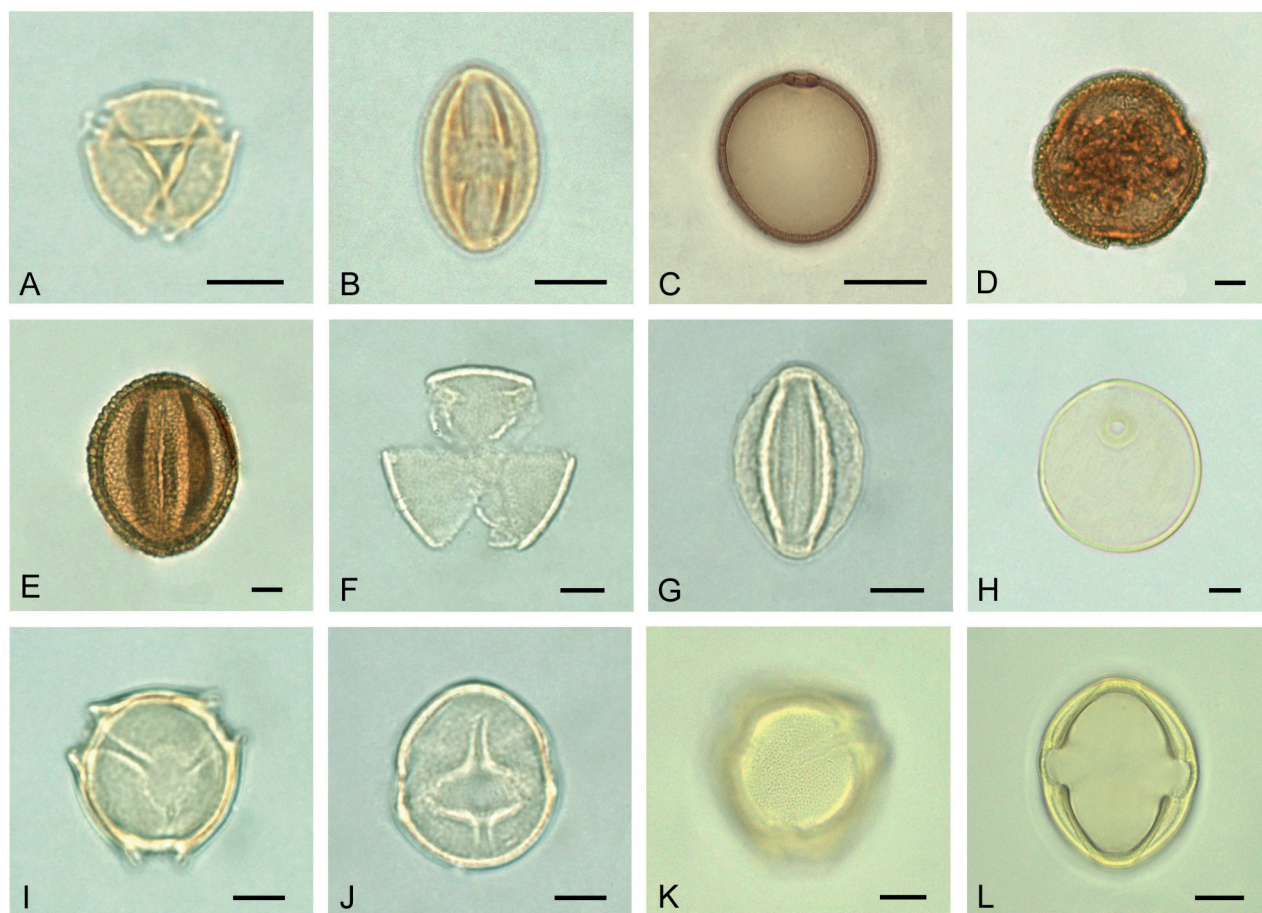
Figure 2. Some non-nectariferous pollen types found in extracted and squeezed honeys. **A-B**. *Capparis zeylanica*, **C**. *Echinochloa frumentacea*, **D-E**. *Nelumbo nucifera*, **F-G**. *Papaver somniferum*, **H**. Poaceae type, **I-J**. *Solanum melongena*, **K-L**. *Solanum sisymbriifolium*. Scale bar- 10 μm.

In addition, the concentration of pollen grains per 10 g of honey varied significantly among pipetted, extracted and squeezed honeys ($F_{2, 65}$ = 23.36, P = 2.27E-08). The average number of pollen grains was the lowest (8630.47 ± 5924.51 grains/10 g honey) in pipetted honeys and the highest (311569.86 ± 262964.78 pollen grains/10 g honey) in squeezed honeys (Tab. 2). According to Maurizio's classification, all pipetted honeys belonged to class I, whereas the majority of extracted honeys (60 %) and squeezed honeys (59.09 %) belonged to class II and class III, respectively (Fig. 3). However, the number of pollen grains per 10 g of pipetted honey did not vary among honey bee species ($F_{2, 33}$ = 1.29, P = 0.29).

Discussion

Qualitative analysis showed that diverse pollen types (67 types) were present in the different honey samples; however, the few predominant pollen types were common to all the studied honey bee species. Most pollen types observed in this study were previously documented in West Bengal by Layek & Karmakar (2016; 2018). The primary focus of the present study was to determine the suitability of different honey collection methods, and we showed that pipetted samples contained only pollen types that corresponded to nectariferous plants, whereas extracted and squeezed
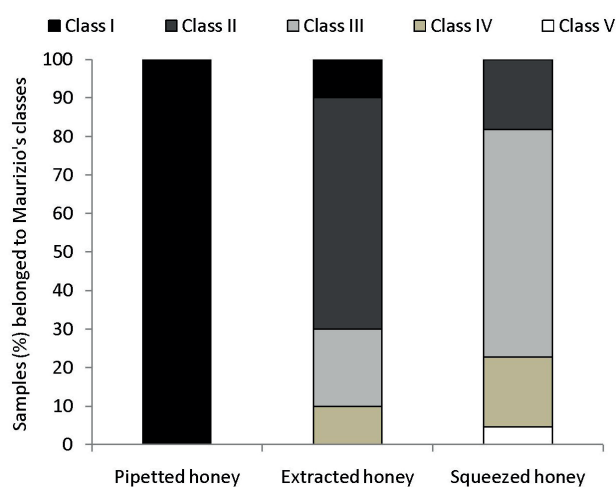

Figure 3. Distribution of different honey samples in Maurizio's classes.

samples contained pollen types of some nectar-deficient plants in addition to nectariferous taxa. The occurrence of pollen grains from nectar-deficient plant species has also been documented for apiary honeys, presumably sampled via extraction, from various countries including Australia (Warakomska & Wojtacki 1988), Morocco (Terrab *et al*. 2005), New Zealand (Moar 1985), Norway (Maurizio 1979) and Poland (Wróblewska *et al*. 2006; Stawiarz 2009). In addition, Upadhyay *et al*. (2014) documented the presence of pollen grains from nectar-deficient plants in squeezed honeys from Orissa, India. However, our study is the first documentation of the presence of pollen grains from nectar-deficient plants (including both anemophilous and entomophilous plants) in extracted and squeezed honeys from West Bengal. The nectar-deficient plants identified in this study serve only as pollen sources for the honey bees and are an important part of bee flora in West Bengal (Layek *et al*. 2015; 2016; 2020). There are many potential reasons why pollen types of nectar-deficient plants could occur in honey samples, including (i) incidental contamination with airborne pollen of anemophilous plants, (ii) adherence of pollen grains from polleniferous plants to honey bees during forage, and (iii) contamination caused by pollen types of nectar-deficient plants entering the honeys during sample collection, either from stored pollen in pollen cells when comb cells were squeezed or from bee bread when samples were extracted. Among these reasons, the last one is more agreeable due to: (i) the absence of pollen types of nectar deficient plants in pipetted honeys, (ii) greater number of pollen types per sample in squeezed and extracted honeys compared to pipetted honeys, and (iii) the higher absolute pollen count in squeezed and extracted honeys compared to pipetted honeys. The number of identified pollen types of nectar-deficient plants and their frequency of occurrence were much higher in honeys collected during spring, summer and the monsoon season compared to honeys from autumn to winter. From the foregoing discussion, we can infer that this greater seasonal occurrence of pollen types of nectar-deficient plants is associated with the availability of large numbers of non-nectariferous, polleniferous taxa around the bee colonies.

Quantitative analysis revealed that all pipetted honey samples had low pollen content and belonged to Maurizio's class I. This result corroborates the previous work of Layek & Karmakar (2018), who reported that the majority of *A. dorsata* honeys belong to class I. However, the pollen content of extracted and squeezed honeys was much higher than the pollen content of pipetted honeys and mostly belonged to class II and class III, respectively. Among extracted honeys, the dominance of class II honeys has been recognized by several authors (Fagúndez & Caccavari 2006; Ramos & Ferreras 2006; Boi *et al*. 2013). Some authors (Ramos *et al*. 2002; Sá-Otero *et al*. 2006) also reported an abundance of class III in extracted honeys. Upadhyay *et al*. (2014) analyzed the absolute pollen count of squeezed honeys of

A. dorsata and *A. florea* and documented the abundance of class II honeys. The pollen count Upadhyay *et al*. (2014) recorded for squeezed honeys was slightly lower than the counts observed in this study, but the quantitative pollen content of a squeezed honey sample can vary depending on the availability of stored pollen and the distribution of pollen cells within the comb.

In summary, our study provides information on how honey sampling methods can influence the pollen composition of honey. Pollen composition significantly differed among the studied honey samples, both qualitatively and quantitatively. Pipetted honeys were less rich in pollen content and contained only pollen types of nectariferous plants, whereas squeezed and extracted honeys were rich in pollen content and contained pollen types of both nectariferous and nectar-deficient (non-nectariferous) plants. This excess pollen presumably came from stored pollen loads or bee bread in the comb cells. Squeezed and extracted honey samples can therefore be utilized to determine the bee flora (rather than only the nectariferous plants) in a region. However, the pollen spectrum from pipetted honey samples depicts the nectariferous plants more accurately.

Acknowledgements

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