Journal of Animal & Plant Sciences, 33(5): 2023, Page: 1184-1192
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2023.5.0711

MELISSOPALYNOLOGICAL STUDIES OF AUTUMN HONEY SAMPLES FROM KHYBER PAKHTUNKHWA, PAKISTAN

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ABSTRACT

The current study was carried out to investigate the geographical origin and botanical sources of 13 autumn honey samples collected from different areas of Khyber Pakhtunkhwa. For palynological analysis, samples were centrifuged and prepared according to the standard techniques of acetolysis. The study revealed the existence of diverse pollen in honey samples from 34 taxa, 31 genera and 18 different families. Honey was weighed, mixed with distilled water, and put in a centrifuge for analysis. Using light microscopy, pollen morphology and pollen counts were investigated. Identifications of pollen were examined from species to family level. Pollen grains of *Saccharum spontaneum* existed in every sample, revealing that these plants are distributed consistently all over the research area. In the analyzed samples, 04 were classified as dominant pollen, 08 accessory pollen and 01 isolated pollen. Unifloral and multifloral samples were recorded from the research area after analysis in the present study. *Ziziphus jujuba* and *Saccharum spontaneum* were abundant species within frequency classes. This study provides information about the flowering periods of plants, the conservation of bee flora, the importance of the autumn season regarding honey production, bee floral diversity, floral and the geographical occurrence of plants and honeybees. The diversity of bee-visiting plants led to promoting the annual yields of honey and providing opportunities for a business of beekeeping.

Keywords: Honey. Pollen. Khyber Pakhtunkhwa. Autumn season. Microscopy.

Published first online June 20, 2023 Published final September 30, 2023

INTRODUCTION

Melissa is a Greek word meaning honeybee and palynology is derived from the word palynin, which means scatter as the pollen is scattered by the source of different pollinating agents like air, water, insects and animals etc. Melissopalynology deals with the pollen collected by honeybees found in honey (Ponnuchamy *et al*., 2014). While collecting nectars, honeybees also collect pollen from flowers used as food for their larvae. Analysis of honey shows flora's botanical origin and honeybees' geographical origin (Aronne *et al*., 2010). Nectar or Pollen from flowers collected by honeybees used in honey formations are known as melliferous plants. Pollen analysis of honey determines the melliferous plants of a particular region. From the view point of melissopalynological analysis of honey samples, limited work has been done from Khyber Pakhtunkhwa.

Honeybees visit honey-foraged plants, gathering nectars and pollen for use in the production of honey (Ahmad *et al*., 2022). Pollen analysis of the melliferous flora allows the botanical species that constitute the honey to be identified. The purity of honey, floral regions, blooming duration of honeybee flora, and their significance as pollen and nectar suppliers can be determined by comparing the palynological features of botanical sources and honey content (Diatta *et al*., 2017; Cencetti *et al*., 2019; Majeed *et al*., 2023; Khan *et al*., 2022).

Honey consists of carbohydrates, proteins, fatty acids, minerals, water, vitamins and more than 180 other substances were also observed in it (Gebremedhin *et al*., 2013). Nectar collected by honeybees is used as food for themselves and their offspring. A honeybee visits approximately 500 flowers for pollen and nectar collection. While honeybees regularly visit plants, very little pollen was found during the investigation, indicating that honey specifically gathers nectar from flowers. Honeybees visit these wildflowers because their pollen grains are abundant in nutritionally essential food ingredients. This technique aids in evaluating whether honey is pure or has been contaminated with something else. Melissopalynology can be used to identify honeybees' geographical origin and plant species' botanical origin (Ahmad *et al*., 2022).

Furthermore, the study aids in determining the purity and impurity of honey. Pollen has several different characteristics that assist in identifying a species, including size, shape, sculpture, diameter, number of colpi, and pores. Honey analysis can be used to look at the food that honeybees collect. Pollen is composed of food that insects conserve for feeding their young. It is simple to research the medicinal applications of melliferous plants in a specific location. The most typical pollen in honey aids in classifying the various varieties of honey. Beekeepers are completely dependent on an area's flowering seasons (Ahmad *et al*., 2020). There are connections between the research authors on melissopalynology and other disciplines like evolution, plant ecology, genetics, molecular biology, biotechnology, biochemistry, and environmental biology. It has never been documented before to research the polliniferous flora of Khyber Pakhtunkhwa. The current study's objectives were to identify prospective floral biological sources for the honey-producing plant species in the region and assess their floral potential to understand their honey-producing potential in the region further. Identifying unifloral, bifloral, and multifloral honey and the location of the honey's origin will be made possible by the morphometric study of polinic floral species of honey (Ahmad *et al*., 2023)..

MATERIALS AND METHODS

Samples sites and their collection: Honey samples were collected from September-November 2018 from different localities in districts Lakki Marwat, Bannu, Lachi, Mardan, Swabi, Dera Ismail Khan, Karak and Kohat in Khyber Pakhtunkhwa. Maximum rainfall occurred in Khyber Pakhtunkhwa during July and August. Honeybees in the study area forage different flowers based on their structure and shape, producing different types of honey. The flora of KPK is very distinct, containing different plants, including trees, shrubs, subshrubs, herbs, climbers, and weeds. Honey samples were obtained based on a study near apiaries where beekeepers had established their businesses, and they informed us that honey production in the research area was mainly from these selected melliferous taxa. Samples were collected from beekeepers and local people in the study area. Beekeepers also took interviews about bee forage plants, honey quantity, quality, and annual production. The current honey samples were harvested from hives produced by *A. florea* and *A. mellifera*. Autumn was the most suitable season for honey formation in the research area. Our study of honeybee forage plants in nine districts of KPK is extensive, and we gathered honey samples from selected plants that were highly visited by honeybees for nectars and pollen around apiaries. The collected samples were then brought into the Plant Systematics and Biodiversity laboratory Quaid-i-Azam University Islamabad, Pakistan, to investigate its pollen analysis.

Microscopic Study: Honey samples were weighed using a digital balance, put in a cup and shaken through a metal rod. It was then kept in a centrifuge mixed with 07 ml distilled water heated up to 52°C. The sample was centrifuged at 4000 rpm for 5 minutes and decanted. Again 07 ml of distilled water was added and centrifuged to 2500 rpm for 15 minutes. Samples were put on the slides and kept drying for a few minutes following the acetolysis method (Erdtman, 1969). Two drops of glycerin jelly were added for staining purposes.

The qualitative and quantitative characteristics of pollen grain were analyzed using light microscopy. Pollen identifications were performed by comparing prepared slides with 50 reference pollen slides, pollen atlas, and research publications from this study area (Fig. 3). Pollen was counted and examined in each sample. The name of the species, genus, family and unidentified pollen were all recorded. Microphotographs of pollen were then captured using a Leica Microscope. Using light microscopy, qualitative and quantitative characteristics of pollen grains were studied. Identifications of pollen were carried out by comparing prepared slides with collected 50 reference pollen slides from selected areas, pollen atlas and research articles of study areas (Fig. 3). Pollen was examined and counted in each sample. The species name, genus, family, and unidentified pollen were noted. With Leica Microscope's help, pollen microphotographs were then taken (Pound et al., 2018; (Ahmad *et al.,* 2022).).

Data Analysis: Pollen frequency was determined using the method of (Louveaux et al., 1978). The pollen numbers on one slide were categorized into three frequency classes: "Isolated pollen" (3-15 %), "Accessory pollen" (16-45 %), and "Dominant pollen" (greater than 45 %). The percentage of pollen grain examined aids in characterizing the distinctive aspects of honey (Atrouse et al., 2004).

RESULTS

A total of 13 honey samples were examined using light microscopy in this study (Figure 3). Thirty-five floral species belonging to nine genera and 18 distinct families were identified. Pollen was identified from the species to the family level. Some plants, however, were not identified. The identified pollen varies with each other in shape, size and all other morphological characters.

In contrast, Poaceae, Apocynaceae, Cucurbitaceae, Rhamnaceae, Fabaceae, Asteraceae, Solanaceae and Convolvulaceae species were comprised as the dominant and mostly visited species by honeybees (Fig. 2). Color of honey noted was yellowish, dark-

brown, light amber and yellow-red after dilution. The sample dated Sep 2018 (2) had a maximum pollen number of 554, and Oct 2018 (5) had a minimum of 55. Details of pollen counts and frequency classes of each sample are shown in Table 1.

Overall melissopalynological data shows the dominance of *Ziziphus jujuba* Mill, *Saccharum spontaneum* L. and *Prosopis cineraria* (L.) Druce in honey samples. During field surveys and interviews with beekeepers, it was declared that in Autumn, honeybees collect nectars and pollen mostly from *Ziziphus jujuba* Mill, *Ziziphus mauritiana* Lam, *Periploca aphylla* Decne, *Citrus limon* (L.) Osbeck, *Cestrum nocturnum* L, *Ocimum basilicum* L, *Helianthus annus* L, *Prosopis juliflora* (Sw.) DC, *Saccharum spontanum* L. and *Cenhrus biflorus* Roxb. Autumn and spring are significant seasons in Pakistan for honey production and bee foraging. Rhamnaceae, Fabaceae, Apocynaceae, Cucurbitaceae and Asteraceae have mostly visited families of the study area during the Autumn season (Figure 2). While in Spring, honeybees visit *Brassica campestris* L, *Eruca sativa* Mill, *Asphodelus tenuifolius* L, *Astragalus hamosu*s L, *Brassica oleracea* L, *Raphanus sativus* L and *Zea mays* L (Ahmad *et al*. 2019).

Honey Extraction from Samples: Pollen was extracted from honey samples at the start of September and October. *Ziziphus jujuba* was estimated as the dominant pollen type, with counting 46%. No dominant and accessory pollen were recorded but contained all isolated pollen (Table I). *Launaea procumbens* have maximum pollen content in this sample. No accessory pollen was noted, but it contains isolated pollen of *Saccharum spontaneum*, *Cynodon dactylon*, *Cenhrus biflorus*, *Cymbopogon jwarancusa*, *Zea mays*, *Astragalus*, *Prosopis juliflora*, *Prosopis cineraria*, *Convolvulus arvensis*, *Duranta erecta*, *Luffa*, *Daucus carota* and 06 unidentified species.

At the end of September, honey was taken from honeycombs. *Ziziphus jujuba*, which produced 84.45% of the pollen, was regarded as the dominating species. *Cynodon dactylon*, *Cenhrus ciliaris*, *Cymbopogon jwarancusa*, *Zea mays*, *Pennisetum typhoides*, *Sorghum halepense*, *Eragrosts pilosa*, *Prosopis cineraria*, *Ziziphus jujuba*, *Convolvulus*, *Solanum*, *Cestrum nocturnum*, *Calotropis procera* and *Cucumis melo* were categorized as isolated pollen. Extracted honey during the start of October elaborates *Luffa aegyptica* and *Chenopodium murale* were classified as accessory pollen. *Cenhrus biflorus* and *Cenhrus ciliaris* were categorized as accessory pollen (Table I). *Saccharum spontaneum*, *Zea mays*, *Sorghum halepense*, *Pennisetum typhoides*, *Prosopis juliflora*, *Launaea procumbens*, *Ziziphus mauritiana*, *Convolvulus arvensis*, *Convolvulus prostratus*, *Tribulus terrestris*, *Capparis decidua*, *Cestrum nocturnum*, *Farsetia* and *Luffa cylindrica* were classified as isolated pollen.

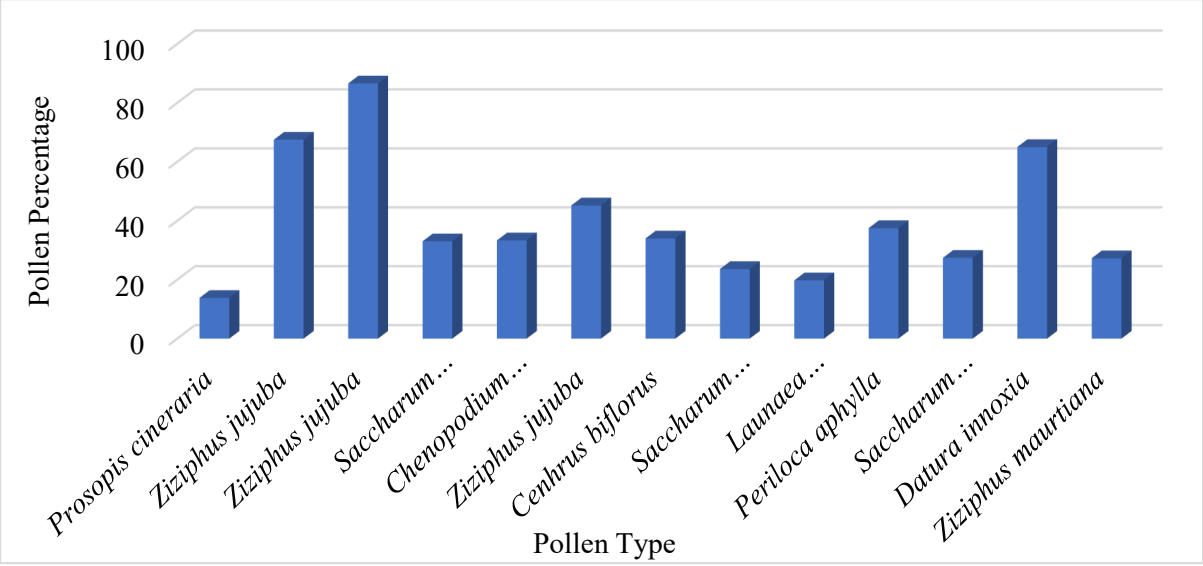


Figure 1. Relative abundance (%) of pollen types collected by honeybees used in honey formations in one slide

Prosopis juliflora and *Ziziphus mauritiana* were categorized as accessory pollen in September 2018 (Table I). *Saccharum spontaneum, Cynodon dactylon, Cenhrus biflorus, Sorghum halepense, Prosopis*, *Launaea procumbens, Ziziphus jujuba, Tribulus terrestris, Farsetia stylosa, Periploca aphylla, Phoenix dactylifera, Luffa cylindrica, Asphodelus tenuifolius, Chenopodium murale* and two unidentified species were classified as isolated pollen. In October 2018, in honey samples, no dominant pollen was recorded in this sample. *Cenhrus ciliaris* and *Prosopis cineraria* were classified as accessory pollen (Table 1). *Saccharum spontaneum,*

Cynodon dactylon, Sorghum halepense, Pennisetum typhoides, Eragrosts pilosa, Prosopis juliflora, Ziziphus jujuba, Convolvulus arvensis, Farsetia stylosa, Duranta erecta, Phoenix dactylifera, Euphorbia prostrata and *Cyperus compressus* were comprised as isolated pollen.

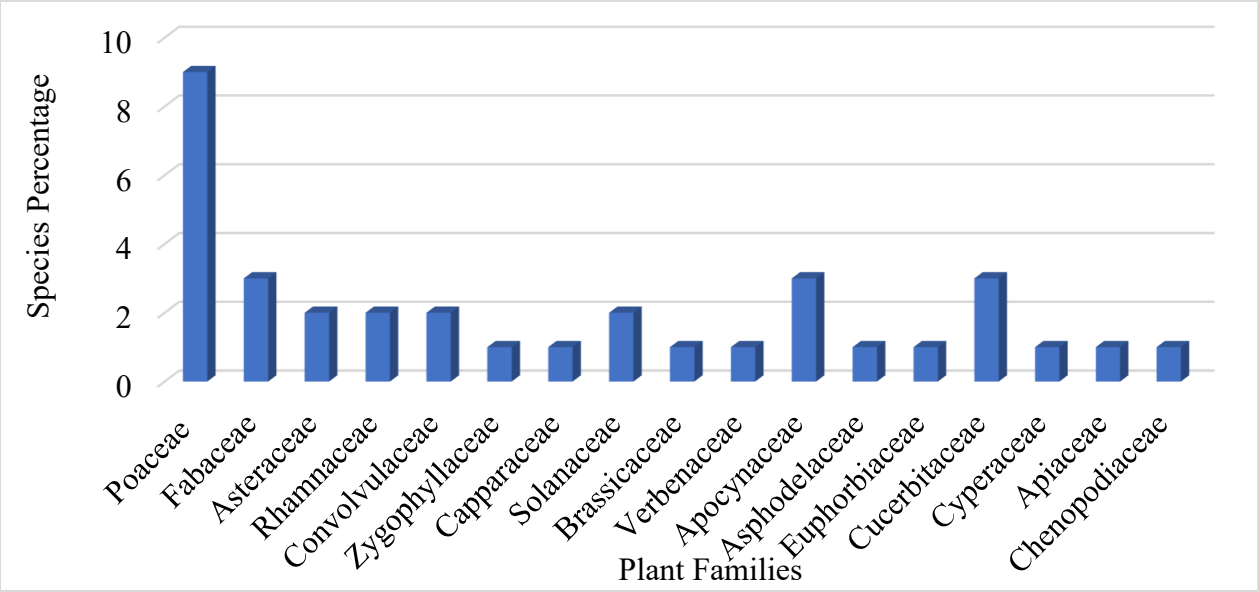
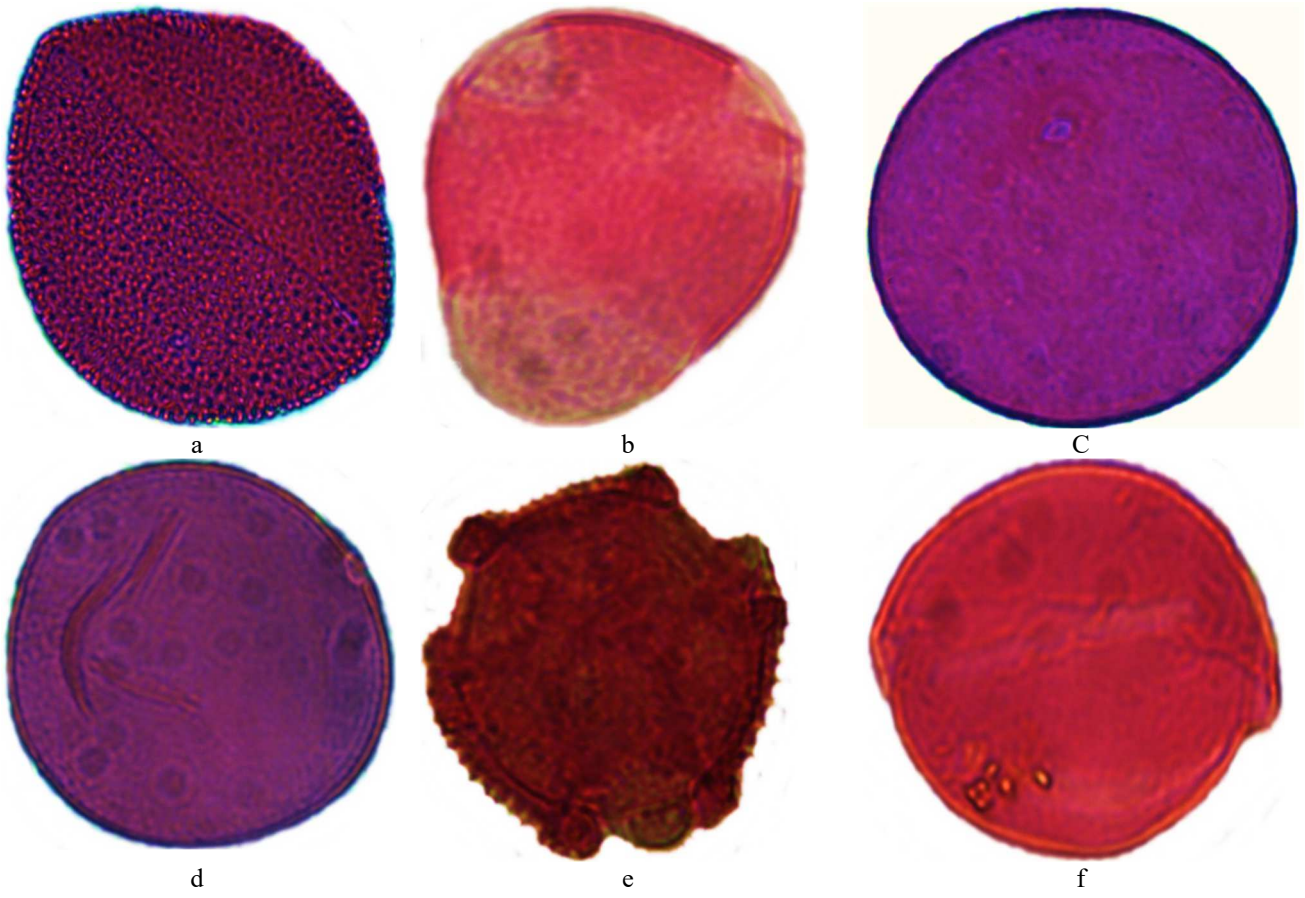


Figure 2. Distribution of investigated families in honey analysis



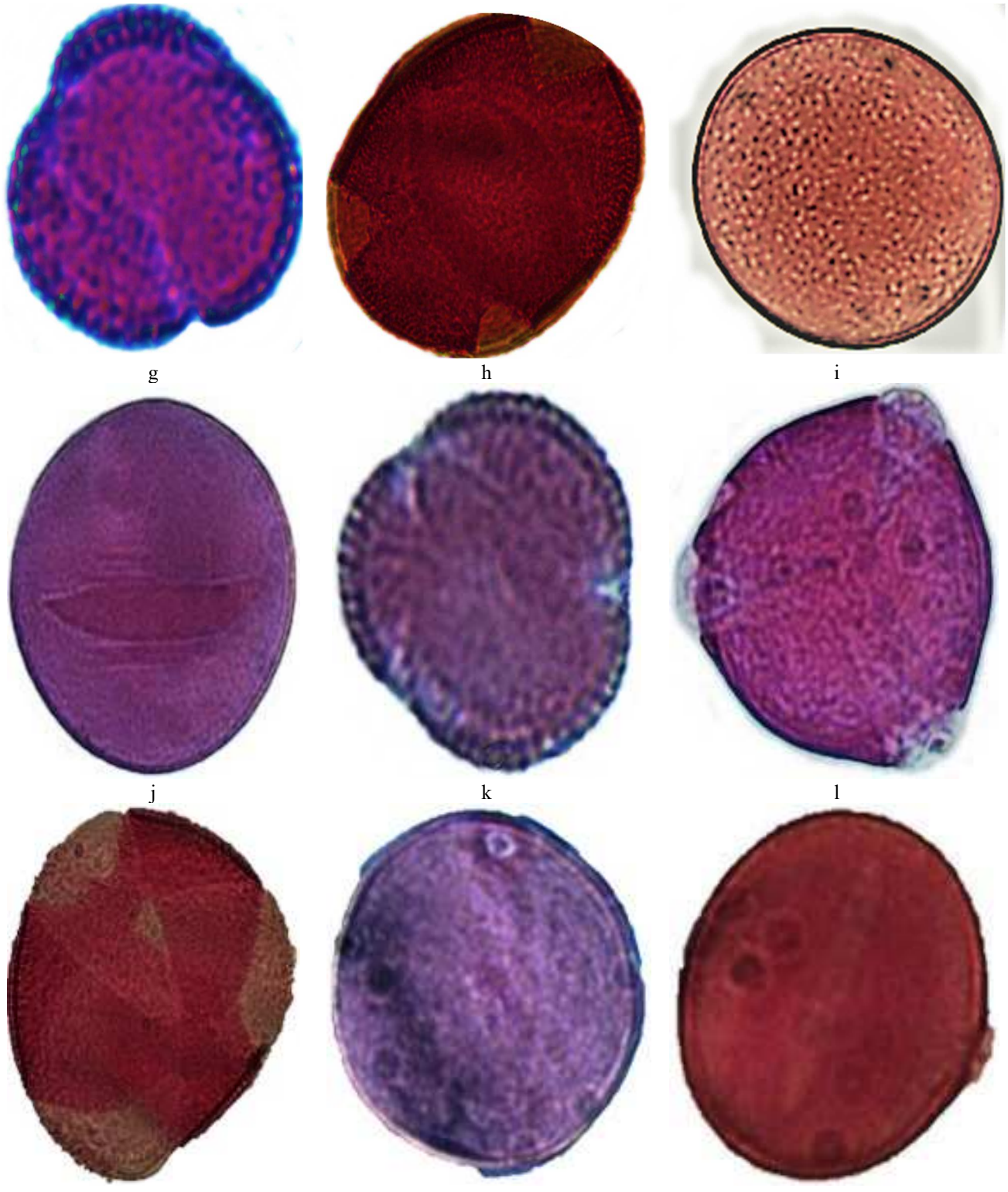


Figure 3. Light microscopy pollen micrographs- (a) *Asphodelus tenuifolius* (b) *Convolvulus arvensis* (c) *Cenchrus biflorus* (d) *Zea mays* (e) *Launaea procumbens* (f) *Prosopis cineraria* (g) *Brassica campestris* (h) *Convolvulus* species (i) *Saccharum spontaneum* (j) *Cymbopogon jwrancusa* (k) *Farsetia* species (l) *Prosopis* species (m) *Prosopis juliflora* (n) *Zea mays* (n) *Tribulus terrestris* (o) *Pennisetum typhoides*

Table 1. Pollen counts (Count), percentages (%) and frequency classifications (Frequency) for the Khyber Pakhtunkhwa honey samples.

S.No	Pollen Type	Sep 2018 (1)	Sep 2018 (2)	Sep 2018(3)	Sep 2018 (4)	Oct 2018 (1)	Oct 2018 (2)	Oct 2018 (3)	Oct 2018 (4)	Oct 2018 (5)	Oct 2018 (6)	Oct 2018 (7)	Nov 2018 (1)	Nov 2018 (2)
1	Poaceae													
	Saccharum spontaneum	10	98	1	35	1	27	14	34	1	1	2	1	1
	Cynodon dactylon	2	2	-	4	1	-	-	16	-	1	9	1	-
	Cenhrus species	3	-	1	-	1	-	-	31	-	-	-		-
	Cenhrus biflorus	4	3	-	4	-	-	33	-	1	2	6	1	1
	Cenhrus ciliaris	-	-	-	21	-	1	26	-	1	-	17	4	-
	Sorghum halepense	1	-	-	1	-	1	-	1	2	4	4	-	-
	Cymbopogon jwarancusa	-	11	1	5	-	-	-	5	-	-	-	-	5
	Zea mays	-	1	-	1	-	-	7	-	-	-	-	14	-
	Pennisetum typhoides	-	-	-	-	-	1	-	-	1	-	7	54	1
	Eragrosts pilosa	-	-	-	3	-	-	-	1	1	1	1	-	1
2	Fabaceae													
	Astragalus species	11	12	-	-	-	-	-	-	-	-	-	-	-
	Prosopis species	-	-	1	4	-	-	-	-	8	11	-	-	-
	Prosopis juliflora	3	25	-	-	3	8	1	-	-	23	4	-	8
	Prosopis cineraria	12	10	-	5	-	4	-	1	9	-	31	-	1
3	Asteraceae													
	Launaea procumbens	17	-	-	-	4	1	5	1	16	4	-	-	-
	Parthenium hysterophorus	-	-	-	-	-	-	-	3	1	-	-	-	-
4	Rhamnaceae													
	Ziziphus species	5	-	-	-	-	-	-	-	-	-	-	-	-
	Ziziphus jujuba	1	374	239	1	-	66	-	-	1	1	13	-	-
	Ziziphus mauritiana	-		1	-	-	2	4	-	-	42	-	-	4
5	Convolvulaceae													
	Convolvulus species	6	1	-	3	1	-	-	-	-	-	-	-	-
	Convolvulus arvensis	-	1	1	-	-	-	3	-	-	-	6	-	12
	Convolvulus prostratus	-	-	-	-	1	-	2	-	-	-	-	-	-
6	Zygophyllaceae													
	Tribulus terrestris	10	-	-	-	9	-	1	6	1	1	-	-	-
7	Capparaceae													
	Capparis decidua	12	-	7	-	-	-	1	1	-	-	-	-	-
8	Solanaceae													
	Solanum species	-	-	-	5	1	1	-	-	2	-	-	-	-
	Cestrum nocturnum	-	-	1	7	5	-	2	-	-	-	-	1	2

	Datura innoxia	-	-	-	-	-	-	-	15	6	-	-	2	2
9	Brassicaceae													
	Brassica species	-	-	-	-	-	-	-	-	-	-	-	-	-
	Farsetia species	4	-	1	-	-	-	4	3	-	-	-	-	-
	Farsetia stylosa	-	-	-	-	-	-	-	7	-	1	6	-	-
10	Verbenaceae													
	Duranta erecta	-	8	-	-	1	-	-	13	-	-	1	-	-
11	Apocynaceae													
	Calotropis procera	4	-	-	6	-	9	-	-	-	-	-	-	-
	Periploca aphylla	-	-	-	-	-	4	-	-	-	4	-	-	-
	Nerium oleander	-	-	-	-	-	-	-	-	-	-	-	1	1
12	Arecaceae													
	Phoenix dactylifera	-	-	14	-	-	1	-	-	-	1	04	-	-
13	Euphorbiaceae	-	-	-	-	-	-	-	-	-	-	-	-	-
	Euphorbia prostrata	-	-	-	-	-	2	-	1	-	-	1	1	-
14	Cucerbitaceae													
	*Luff*a species	12	1	-	-	-	-	-	-	-	2	-	-	-
	Luffa cylindrica	-	-	-	-	-	-	1	-	-	8	-	-	-
	Luffa aegyptica	3	-	3	-	23	1	-	-	-	-	-	1	-
	Cucumis melo	-	-	-	1	-	-	-	2	-	-	-	-	-
15	Cyperaceae	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cyperus compressus	-	-	-	-	-	1	-	-	-	-	1	1	-
16	Apiaceae	-	-		-	-	-	-	-		-	-	-	-
	Daucus carota	-	1	1	-	-	-	-	-	4	-	-	-	-
17	Asphodelaceae													
	Asphodelus tenuifolius L.	-	-	3			8	-	-		2	-	-	5
18	Chenopodiaceae													
	Chenopodium murale	3	-	-	-	18	-	-	-	-	2	-	1	-
19	Unidentified species	3	6	8	-	1	8	-	3	-	1	-	-	-

DISCUSSION

The types of pollen that LM examined were diversified. According to the findings, honey's pollen makeup was both unifloral and multifloral. In the investigated samples, species belonging to the family Poaceae, Asteraceae, Fabaceae and Convolvulaceae were frequently found, showing that it plays an essential role in honey formations. Poaceae is the only family having the maximum number of species used in honey formations in the study area. On the other hand, *Daucus carota*, *Euphorbia prostrate, Luffa cylindrica, Cyperus compressus* and *Capparis decidua* were rarely visited by honeybees. The study revealed that further cultivation of plants from such families causes an increase in the production of honey (Holzschuh *et al*., 2007). The present work is the sample collection of melissopalynological sampling of one year (De Sá-Otero *et al*., 2009).

The presence of specific honeybees and flower species is revealed by honey production in a zone (Rosdi *et al.*, 2016). Honey analysis reveals vegetation covering an area based on local bee floral resources (Lau *et al*., 2019). Research in the field of melissopalynology focuses on the local flora and the botanical and geographic origins of honeybees. Louveaux *et al.* (1978) showed that the pollen content of honey could provide information about a region's topography. The flowering periods of species that honeybees use as food sources were revealed by the pollen spectra of a region. The ability of a region's flowering cycles to develop apiary industries is essential. Beekeepers can set up their business in different parts of the province depending on the season and flowering times of the region. The study also examines honey varieties that are either pure or adulterated with deadly pollen (De Moraes *et al*., 2019).

In Pakistan, Khyber Pakhtunkhwa has a rich flora threatened by various factors. The honey analysis is essential for describing the types of nectar and pollen used in honey production. Melissopalynological insights first reported in Khyber Pakhtunkhwa help in determining qualitative and quantitative features of the pollen spectrum of honey samples. Three species of honeybees *Apis florea*, *Apis dorsata* and *Apis mellifera,* were observed in the study area. *A. florea* and *A. dorsata* are wild bees that dwell in both hilly and plain areas of the province, playing an essential source of pollination, but *A. mellifera* were kept in boxes for honey production and introduced a few years ago in the study area. Honey produced by *A. florea* is rare and high in quality. Various species of honeybees and honey-producing plants suggest that study areas are suitable for beekeeping. The analyzed samples contain both unifloral and multifloral honey. Of 13 samples, two were classified as unifloral honey of *Ziziphus jujuba* and the remaining multifloral. In the case of frequency classes, three samples were classified as isolated pollen, eight accessory pollen and four dominant pollen (Pound *et al*., 2018). The high percentage of pollen in honey revealed the significance of pollen for honeybees and honey production in the study area. No significant variations were observed in the analyzed samples collected from different regions of the province.

Conclusion: The current pollen analysis study indicated the presence of various pollen in honey samples. The presence of multiple pollens in honey demonstrates the potential for honey production in the research area's indigenous plants during autumn. *Ziziphus jujuba* and *Saccharum spontaneum* were considered the dominant and most visited species by honeybees. Purity, pollen counts, and different characterization of honey can be determined through honey analysis. Identifications of pollen spectrum of plants visited by honeybees to produce honey in the zone. A greater number of pollen in honey provides proof of the availability of pure honey in the province. This study also provides detailed knowledge for cultivating and conserving bee flora belonging to the family Rhamnaceae, Poaceae, Fabaceae, and Apocynaceae to increase honey production further and promote the business of beekeeping practices in Khyber Pakhtunkhwa.

Acknowledgments: The authors are very thankful to the Higher Education Commission of Pakistan (HEC) for financial support under project no. HEC/R&D/NRPU/2017/8420. The authors also presented special thanks to the Herbarium of Pakistan (ISL) Quaid-i-Azam University Islamabad.

Conflict of interest: The authors of this manuscript have no conflicts of interest to declare it.

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