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MELISSOPALYNOLOGICAL ANALYSIS AND POLLEN DIVERSITY OF STINGLESS BEE HONEY IN DAKSHINA KANNADA DISTRICT, KARNATAKA

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ABSTRACT: Stingless bees which belong to the family Apidae and sub-family Meliponinae, are the smallest of the honey-producing bees. They are highly social insects like honey bees living in perennial colonies and nesting in concealed places. The stingless bee genus (*Tetragonula spp.)* plays an important role as a plant pollinator. These bees are actively involved in pollination of crops and are known to prefer to select flowers for pollination. The aim of this study was to analyse the pollen load in *Tetragonula iridipennis.* We collected 20 honey samples during each season in the time span of October 2017 to February 2021 from five different districts of Dakshina Kannada. The pollen was homogenized and acetolyzed before identifying microscopically to classify them to the lowest taxonomic level. After identification, each of the pollen type was then classified basing on its overall relative abundance. Species richness and pollen diversity was also calculated and compared across states and seasons using the Shannon-Weaver diversity index. Stingless bee honey and Pollens attached to the body of bees mostly belong to 13 plant families, i.e., *Euphorbiaceae, Oxalidaceae, Asteraceae, Solanaceae, Areacaceae, Capparaceae, Anacardiaceae, Cucurbitaceae, Fabaceae, Myrtaceae, Poaceae, Sapindaceae* and *Polygonaceae*. *Cucurbitaceae* and *Solanaceae* pollen were found to be more predominant. *Cucurbitaceae* was seen to be predominantly collected during the seasons. In summer season, the load of pollen was found to be high at all regions ($p<0.05$). There was a significant difference in the total overall species diversity across all the five taluks ($H = 13.69$, $P<0.005$). Post-hoc tests also revealed a greater overall species diversity in Belthangady and lower species diversity in Mangalore compared to other taluks (Dunn's all pairs test, $P<0.05$). This study will surely aid in broadening our understanding of stingless bees ecology and nutrition in various environments and seasons. This could also help in promoting the use of plants which not only provides aesthetic value but also nutritious forage for the bees.

Keywords: Stingless bee, Dakshina Kannada district, Pollen diversity, Shannon-Weaver diversity

index.

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1.INTRODUCTION

Stingless bees (Apidae, Meliponini) are native to tropical and subtropical parts of the planet like Central and South America, Africa, Asia, and northern Australia [5]. All stingless bees build elaborate nests with structures that are often characteristic for the species or for higher taxa [17, 25]. Meliponini is the tribe, little studied, with two genera: Lisotrigona and Trigona found to occur in Asia [18]. The Indian stingless bee or dammar bee, *Tetragonula iridipennis* is predominantly found in India. Various studies have been conducted on *T. iridipennis* colonies located in Karnataka, Kerala, and Tamil Nadu states [8]. The tropical savanna climate, varying physiographic environment, higher altitudes, and luxuriant flora offers an abode for the rich and wide distribution of stingless bee (*T. iridipennis*). Like Apis honeybees, most species of eusocial stingless bees, produce honey. Stingless bee honey is very valued as a food source. Melissopalynology is an applied branch of classical technology concerned with the study of pollen grains in honey samples and its application in beekeeping. The pollen and nectar collected by bees used as food for the colony [21]. Melissopalynological studies had been widely used to determine the geographical and floral origin of honey. Honey consists of pollen grains collected by honeybees. Hence, pollen analysis of honey will assist in the identification of plant species origin. The identification of different plant species is vital as they contribute towards the composition of honey and helps to verify honey authenticity. Honey is classified into unifloral or multifloral honey. Unifloral honey consists of nectar collected from one type of floral source. Unifloral honey contains distinct flavour and color reflecting the type of flowers from which the nectar is collected [2]. Evaluation of plants for their usefulness as a food source for bees provides the information needed to assess beekeeping potential in an area [20]. Therefore, melissopalynological studies are useful in managing bees and promoting the development of beekeeping. Research on pollen analysis of honey samples in India is sketchy. Important work has been done [27]. Pollen and nectar are the main source of attraction for bees. Pollen grains have thin walls made of exine and intine [14]. Pollen contains proteins and amino acids as a source of nutrients [31]. Pollen is the main source of protein to meet the nutritional needs of bees. The bee's ability and need to collect pollen representing a symbiotic relationship between bees and flowering plants. The bee acts as pollinator that helps transfer of pollen to the

pistil. Pollen in the form of a solid ball will be brought back to the nest and placed in the existing chalk pot in the nest. Pollen placed in the pollen vial will be covered with propolis, if it is full [10]. The superiority of bees in the production of honey and propolis is one of the reasons why bees are so widely kept. Stingless bees (Hymenoptera: Apidae: Meliponinae) is a social insect are known to be plant pollinator [33, 12] and very active in visiting agricultural crops, such as peppers and tomatoes [6, 26]. There are about 100 plant species visited by stingless bees belonging to the families *Acantaceae, Bignoniaceae, Caesalpiniaceae, Cucurbitaceae, Ciperaceae, Poaceae, Malvaceae, Myrtaceae, Rutaceae, Euphorbiaceae, Leguminosae, Rubiaceae, Sapindaceae* which are food sources for these species [28]. These bees have a special structure for collecting pollen, including a pollen basket located in the tibia of the hind legs. The amount of pollen attached to the bee's body is called the pollen count. During feeding, bees also show floral consistency. The latter shows the bee's preference to visit the flowers of a particular plant during a single trip. In foraging, non-stinging bees typically visit only one flower with an average of 97% and 3% from other plant species [23]. Stingless bee, *T. sapiens* is a type of bee bread in Indonesia and known as meliponiculture. The practice of honey beekeeping can take advantage of knowledge of food sources important for the development of populations of stingless bees [16]. In addition, this information is also important to enrich the knowledge of bee-plant interactions [23] In this study, we aimed to depict the significance in pollen diversity and melissopalynogical analysis of the stingless bees within the five taluks of Dakshina Kannada at different seasons (summer and winter) from 2017 to 2021.

2. MATERIALS AND METHODS

Collection of honey samples:

In the present study, 20 honey samples (Table-1) were collected during winter and summer season in the time span of October 2017 to February 2021 from the different locations of five taluks of Dakshina Kannada district namely - Buntwal, Putturu, Belthangady, Sullia and Mangalore taluks (fig:2). A total of 120 samples were collected during the period and studied. About 20 samples were collected during the following sampling periods like October 2017 to February 2018 (Winter); March 2018 to May 2018 (Summer); October 2018 to February 2019 (Winter); March 2019 to May 2019 (Summer); November 2019 to February 2020 (Winter) and October 2020 to February 2021 (Winter). All samples were subjected to melissopalynological analyses.

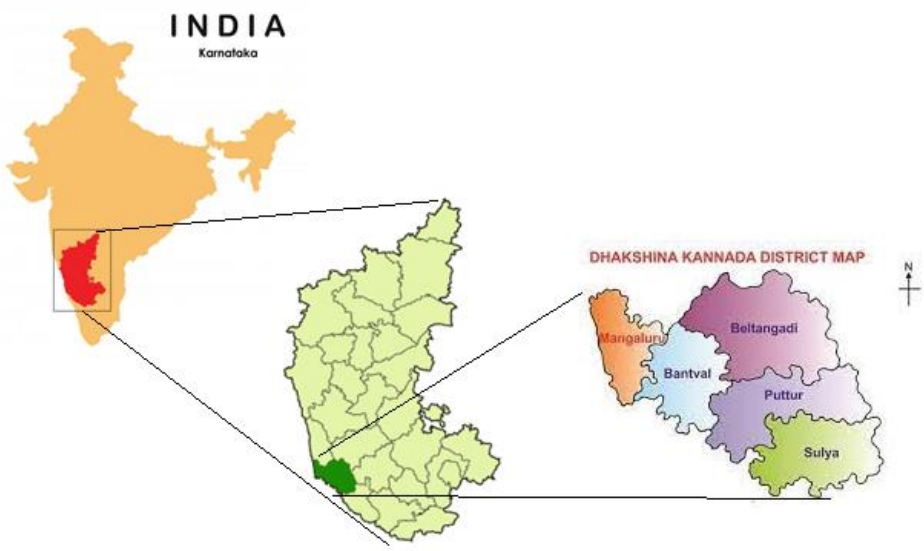

Figure 1: Map showing the study area of Dakshina Kannada district of Karnataka in India

The Dakshina Kannada district area (fig: 1) falls under the Western Ghats and is known for its rich biodiversity of flowering plants. It has a geographical area of 4,866 sq km and lies between 12.8438° N latitude, 75.2479° E longitude. All honey samples were collected from domesticated *Tetragonula iridipennis* hive bees as well as from the beekeepers. All honey samples were raw and unprocessed. A small portion of honey cells was collected by cutting with a knife from the hive and squeezed for the honey, although slight mixing with a few pollen loads could not be ruled out (20-50gm of honey was collected from each hive). The honey was filtered with a single thickness fine cloth to remove suspended particles like dirt, beeswax, and other impurities. All the honey samples were collected in sterilized polythene bottles from the place of honey extraction and it was stored at room temperature.

Table 1: Table showing the number of sampling sites per taluk and season.

Region (Taluks)	**No. of colonies**	Oct 2017- Feb 2018	March 2018 - May 2018	Oct 2018 - Feb 2019	March 2019 to May 2019	Nov 2019 - Feb 2020	Oct 2020 - Feb 2021
		Winter	**Summer**	**Winter**	**Summer**	**Winter**	**Winter**
Belthangady	20	41	35	42	38	32	24
Buntwal	20	42	34	45	34	34	28
Mangalore	19	40	31	42	40	40	38
Puttur	20	40	40	38	40	42	42
Sullia	20	38	38	42	40	40	38

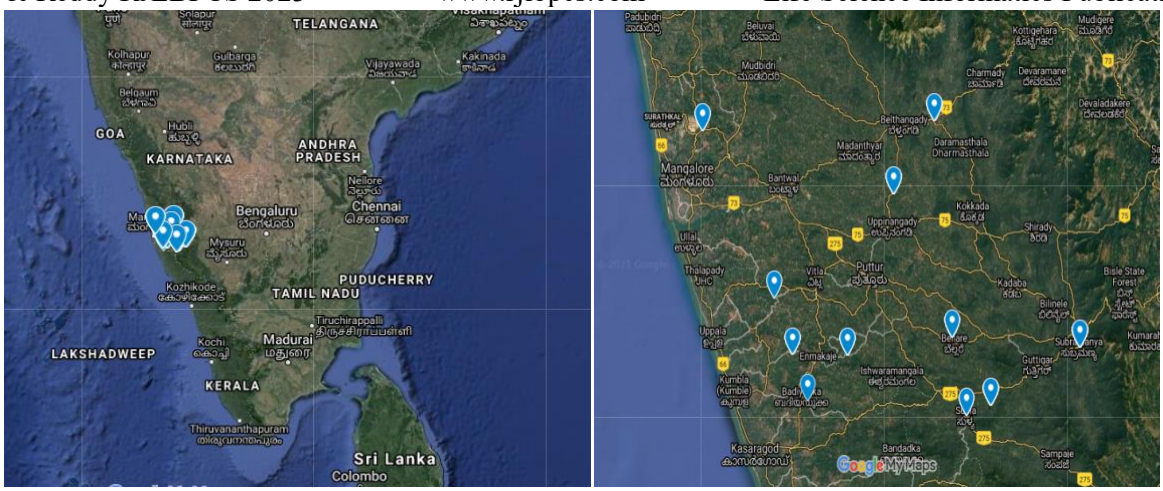

Figure 2: Map showing the location sites for honey sample collection in Dakshina Kannada district

Pollen removal from stingless bee individuals: Each worker stingless bee was collected and anesthetized using ethyl acetate and then was treated with 1.5ml micro-tube containing 0.5ml 70% ethanol: glycerol (4:1). Then, the samples were rotated at maximum speed, and the bee was removed from the micro-tube. Afterwards, the solution was centrifuged at 3000 rpm for about 10 minutes and the 0.1ml of sediment which contained pollen was retained and used for further analysis [7].

Measurements of pollen loads on Stingless bees: This sediment which contained pollens is used to measure pollen load. The number of pollens in the solution were counted using a compound microscope. The same method was replicated 4–5 times. Total pollen loads in 0.1ml was measured and counted

The absolute pollen counts (APC) (number of pollen grains per 10 g honey) was calculated using a haemocytometer [30]. The pollen was counted under a microscope at 100× magnification over a haemocytometer (counting chamber). For each sample, we counted the pollen grains of five medium squares at the center, left and right corners at the top and bottom of the chamber, which was repeated for making 100 individual observations. Average of the 100 observations per 100µl of pollen suspension was used in the study.

Pollen analysis from the honey samples: In the laboratory, samples were processed as follows. Approximately 7ml of warmed honey was taken from each sample jar, using an open-ended, disposable plastic syringe, transferred to a 50ml, pre-weighed polypropylene centrifuge tube, then weighed to two significant digits on a laboratory balance. Each sample weighed approximately 10gm. Approximately 40ml of distilled water was added to each sample. The honey samples were placed into a hot water bath at 80^0C until it was possible to homogenize the honey + water mixtures using a vortex mixer. The homogenized dilute honey samples were then centrifuged at 3500 rpm (relative centrifugal force = 2355) for 3minutes, decanted, rinsed and decanted again. After dehydrating the residues in glacial (100%) acetic acid, the samples were centrifuged and decanted,

then acetolysed using a 9:1 mixture of acetic anhydride [$(CH_3CO)_2O$] and concentrated Sulphuric acid (H_2SO_4) [9, 13, 24]. After twice centrifuging and decanting, the pollen pellets were dehydrated with 100% ethanol, then small quantities of each pellet was mounted in glycerol on permanent glass slides. Pollen grains were counted on a Zeiss Axioscope A1 compound microscope at 300×, 600× and 1500× magnification, with EC Plan-Neofluar objectives and 16× eyepieces. Pollen was identified by reference to the author's modern pollen reference collection, published manuals (Song, 2012), published studies of individual families [32]. These pollen types were classified based on the recommendations of the International Commission for Bee Botany [15, 19]. Identified pollen grains were placed into one of the following four pollen frequency classes: Predominant (more than 45%), Secondary (45-16%), Important Minor (3-16%) and minor pollen types (Less than 3%) and the data was graphically represented in colour intensity charts. Based on the frequencies of pollen grains in various honey samples, the absolute pollen count. Species richness and pollen diversity was also calculated and compared across states and seasons using the Shannon-Weaver diversity index.

Taxonomic diversity: Pollen taxonomic diversity in each sample was calculated using the Shannon-Weaver diversity index to characterize the taxonomic richness and evenness for each season in each district. The Shannon-Weaver diversity index (*H'*) was calculated using the equation:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

Where *pi* is the proportion of each pollen type (*i*) in the sample and *ln* is the natural logarithm. Greater the *H'* value greater is the taxonomic diversity. Shannon-Weaver diversity indices are calculated for each individual site for each season in every district. We used this index to compare taxonomic diversity at the seasonal level and at district scale [11].

3. RESULTS AND DISCUSSION

Pollen species diversity: We found a significant difference in the total overall species diversity across all the five taluks (H = 13.69, $P<0.005$). Post-hoc tests also revealed a greater overall species diversity in Belthangady and lower species diversity in Mangalore compared to other taluks (Dunn's all pairs test, $P<0.05$). Also, total diversity was also significantly higher in the summers across all taluks (H = 31.47, $P<0.005$) compared to winter season. Seasonal differences were also seen in species diversity within each taluk. For instance, honey bees in Belthangady collected pollen from significantly fewer (H = 18.67, $P<0.005$) plant species in the winter than in the summer season ($P<0.05$). Likewise, in Buntwal, Mangalore, Puttur and Sullia, the overall species diversity was significantly higher with index values of 11.94, 13.45, 15.92 and 11.66 respectively ($P<0.05$). Although we could trace a significant effect of seasonal species diversity in the taluks (H = 3.15, $P = 0.05$), we found no significant pairwise differences in species diversity among seasons within that particular taluk ($P>0.05$).

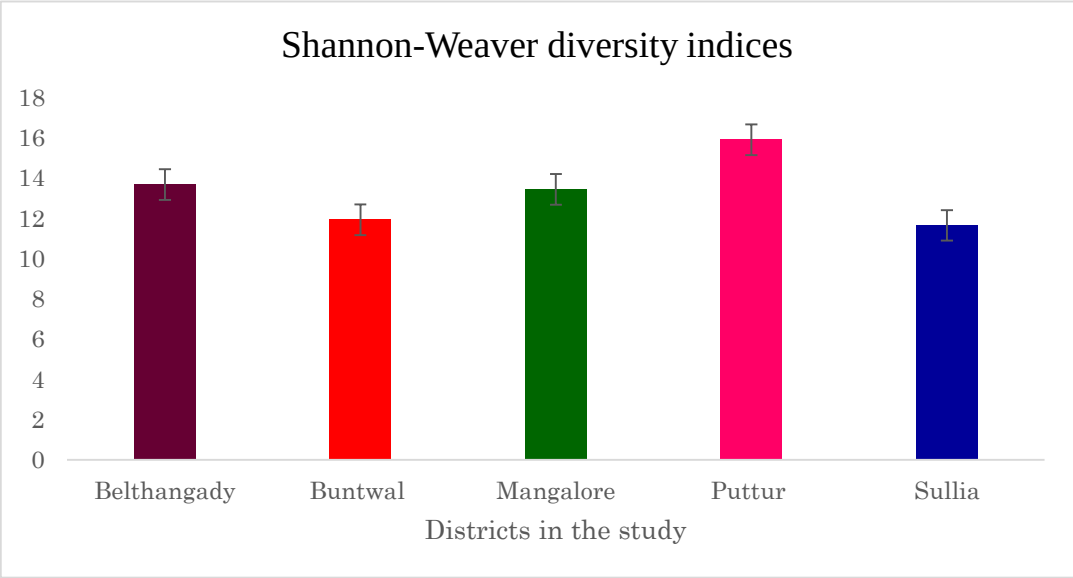

Figure 3: Graph showing the Shannon-Weaver diversity indices in each taluk. Summary of Shannon-Weaver diversity index values (mean ± S.E) for all sites in the study.

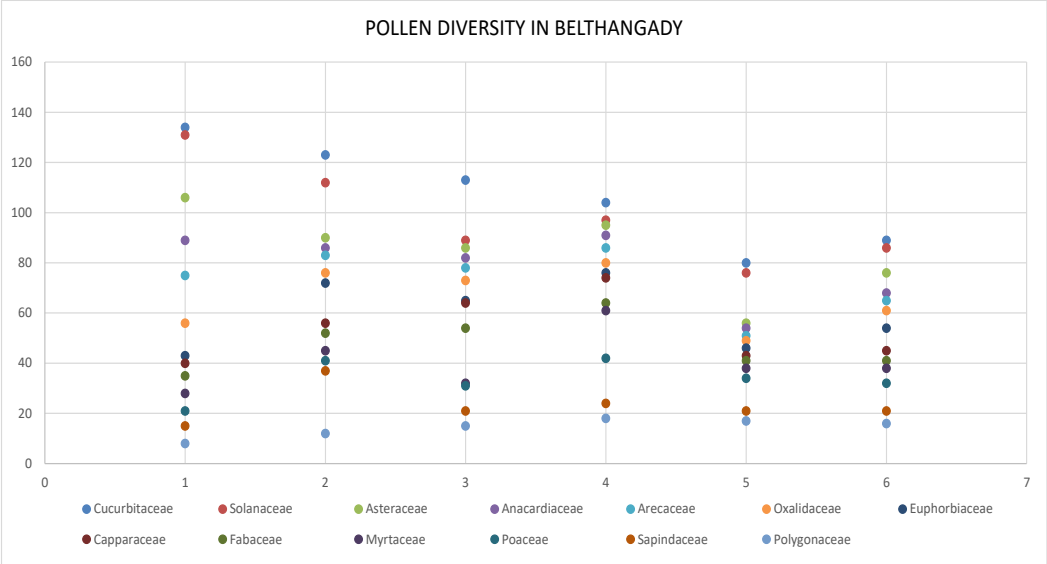

Figure 4: Graph showing the scatter chart of the pollen diversity within the Belthangady for the study period. 1: Winter; 2: Summer; 3: Winter; 4: Summer; 5: Winter; 6: Winter. All the values are average of triplicates.

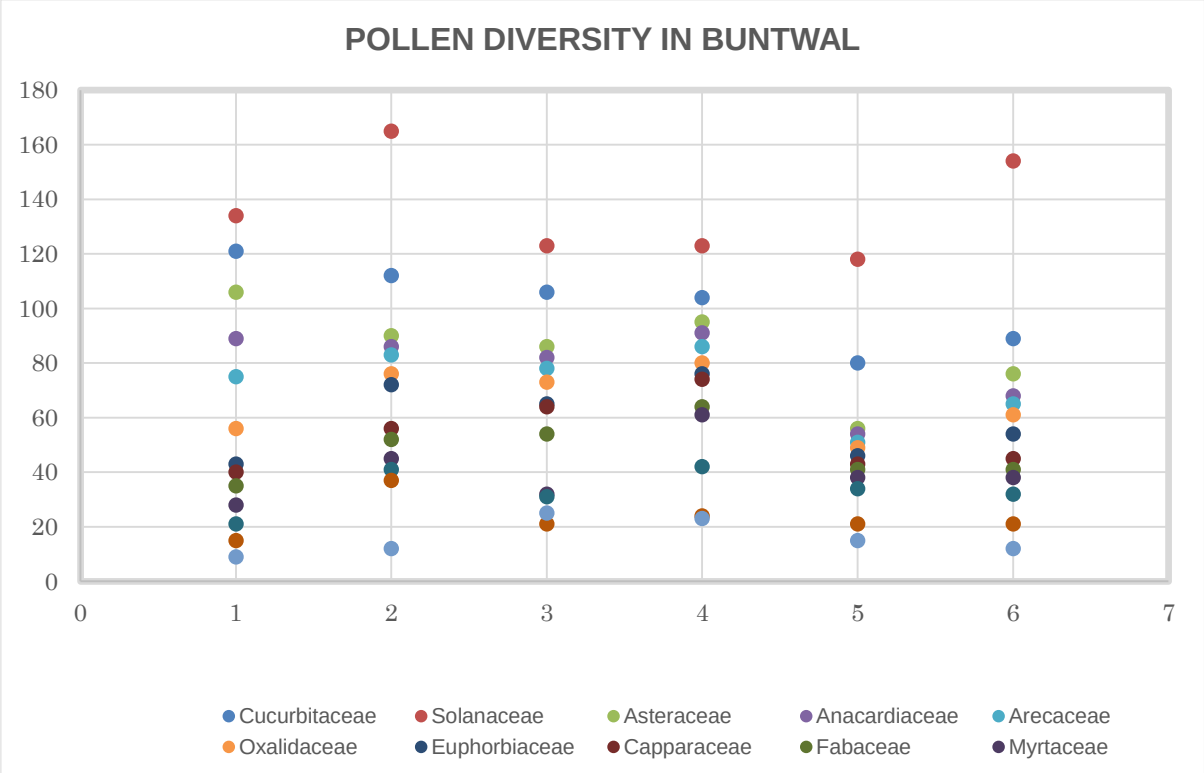

Figure 5: Graph showing the scatter chart of the pollen diversity within the Buntwal for the study period. 1: Winter; 2: Summer; 3: Winter; 4: Summer; 5: Winter; 6: Winter. All the values are average of triplicates.

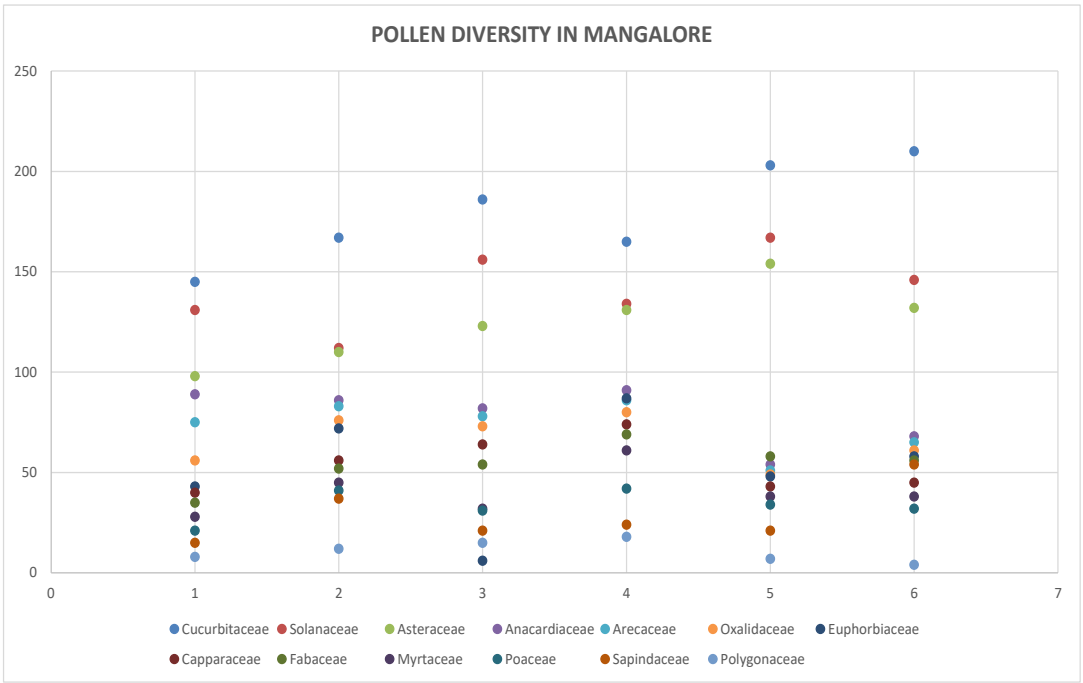

Figure 6: Graph showing the scatter chart of the pollen diversity within the Mangalore for the study period. 1: Winter; 2: Summer; 3: Winter; 4: Summer; 5: Winter; 6: Winter. All the values are average of triplicates.

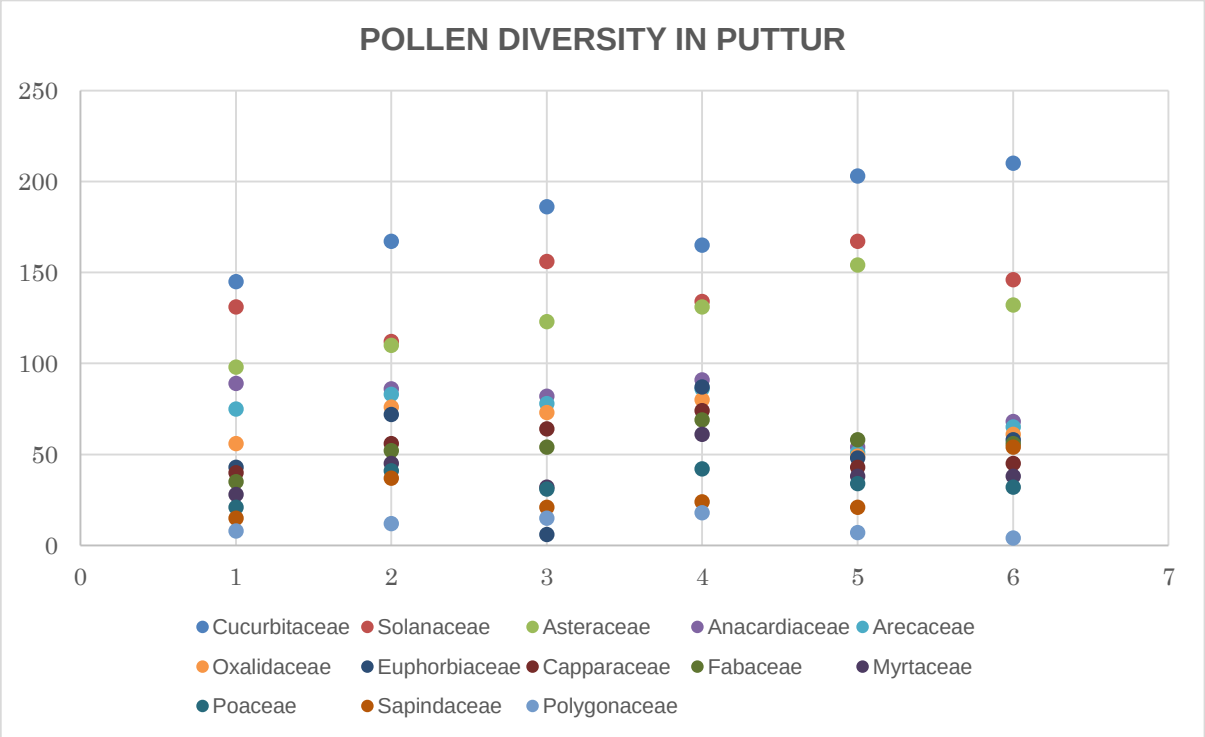

Figure 7: Graph showing the scatter chart of the pollen diversity within the Puttur for the study period. 1: Winter; 2: Summer; 3: Winter; 4: Summer; 5: Winter; 6: Winter. All the values are average of triplicates.

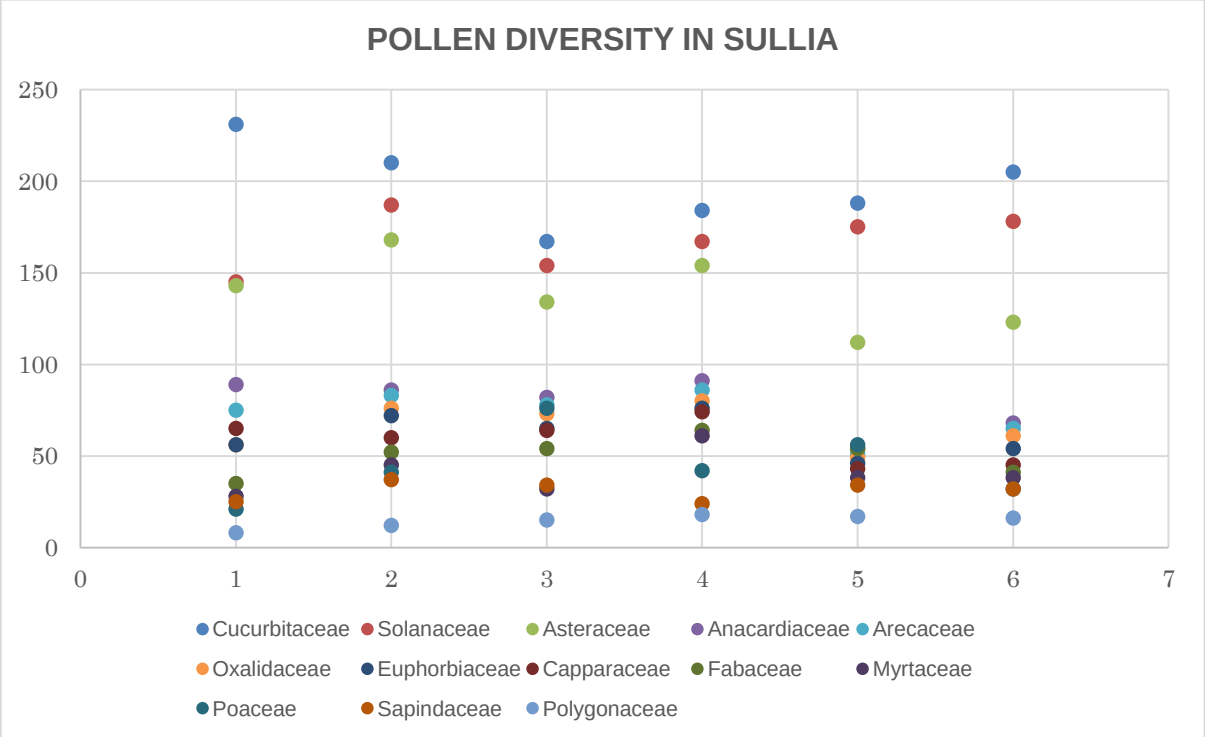

Figure 8: Graph showing the scatter chart of the pollen diversity within the Sullia for the study period. 1: Winter; 2: Summer; 3: Winter; 4: Summer; 5: Winter; 6: Winter. All the values are average of triplicates.

Amount of Pollen Collected: The pollens most collected by *T. iridipennis* are pollen from the flowers of *C. sativus* (Cucurbitaceae) and *Solanum sp.* (Solanaceae) which has yellow flowers. In addition to its attractive colour, cucurbitaceae has a flower shape with an open corolla that makes it easy for various types of insects collect pollen. In *C. sativus* flowers, *T. iridipennis* takes pollen with a technique commonly used by other insects to land on a corolla and then collect pollen easily. This is the reason why most pollen grains are collected from *C. sativus* flowers.

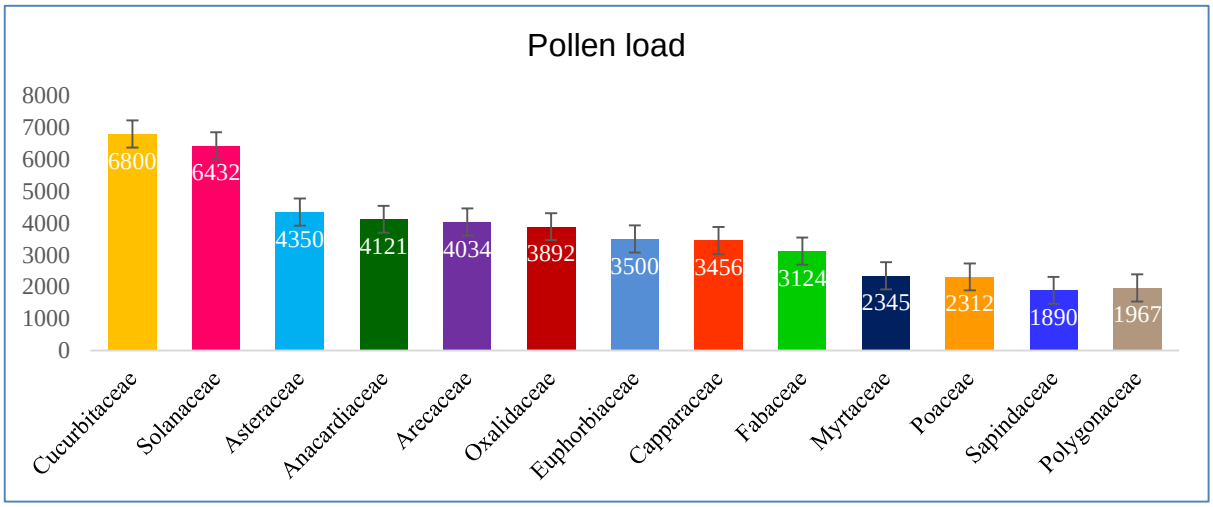

Figure 9: Graph showing the average number of pollen grains collected from the bees in the sampling sites.

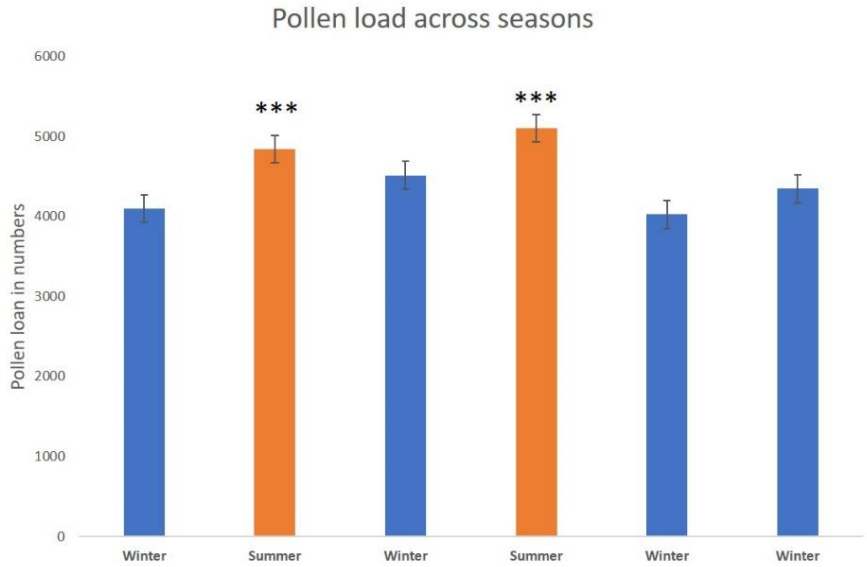

Figure 10: Graph Showing the total pollen load collected during the seasons in the study. Summer season the load was significantly higher than the winter seasons ($p<0.05$).

Pollen load was found to be significantly higher during the summer seasons than the winter seasons ($p<0.05$). This was found across the regions. The load was found to be 4839 and 5100 for the summer seasons in the study. The mean of the winter seasons was found to be 4244.

The Pollen types attached to the bees: Pollens attached to the body of bees mostly belong to 13 plant families, i.e., *Euphorbiaceae, Oxalidaceae, Asteraceae, Solanaceae, Areacaceae, Capparaceae, Anacardiaceae, Cucurbitaceae, Fabaceae, Myrtaceae, Poaceae, Sapindaceae* and *Polygonaceae*. The bees predominantly fed on *Cucurbitaceae* and *Solanaceae*.

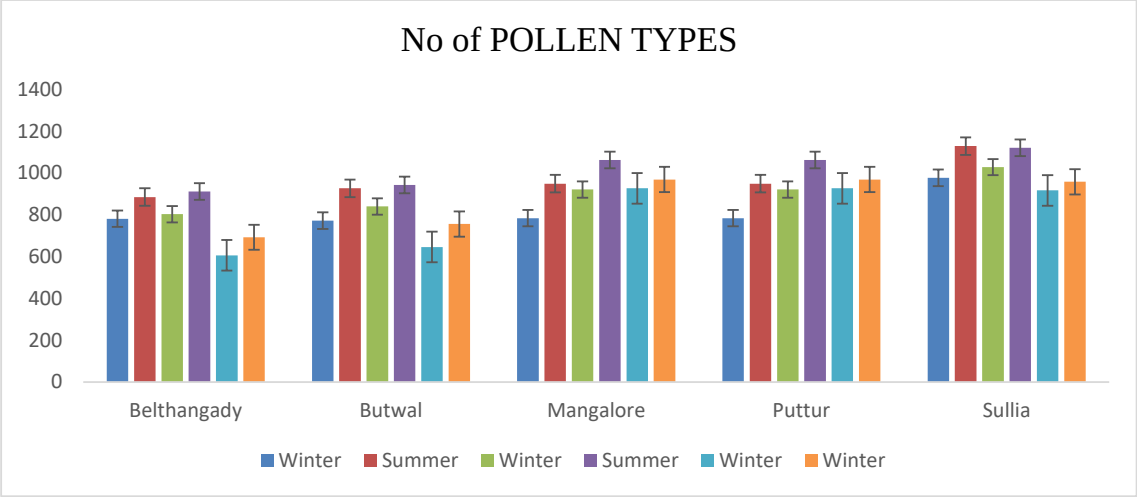

Figure 11: Graph showing the average number of pollen grains observed under the microscope following acetolysis.

Region	Study period	Season	*Cucurbitaceae*	*Solanaceae*	*Asteraceae*	*Anacardiaceae*	*Arecaceae*	*Oxalidaceae*	*Euphorbiaceae*	*Capparaceae*	*Fabaceae*	*Myrtaceae*	*Poaceae*	*Sapindaceae*	*Polygonaceae*
BELTHANGADY	Oct 2017- Feb 2018	W	1	1	1		2	2	2	2	2	3	3	4	4
	March 2018 - May 2018	S	1	1		1		2	3		3	3		4	4
	Oct 2018 - Feb 2019	W	1	1	1			2	2	2		3	3	4	4
	March 2019 to May 2019	S	1	1	2	1	2	2	3	4	3			4	4
	Nov 2019 - Feb 2020	W	1	1	1			2	2	2		3		4	4
	Oct 2020 - Feb 2021	W	1	1	1	1	1	2	2	2			3	4	4
BUNTWAL	Oct 2017- Feb 2018	W	2	1	1			2	2	2	2				
	March 2018 - May 2018	S	2	1		1		2	3	3	3	4	4	4	4
	Oct 2018 - Feb 2019	W	2	1	1		2	2	2	2	3	3	4	4	4
	March 2019 to May 2019	S	2	1		1	2	2	2	2	3	3	3	4	4
	Nov 2019 - Feb 2020	W	2	1	1	2	2		3		4	3	4	4	4
	Oct 2020 - Feb 2021	W	2	1				2	2	2			3	4	4
MANGALORE	Oct 2017- Feb 2018	W	1	1	1	1	2	2	2	3	3	3	4	4	3
	March 2018 - May 2018	S	1	1	1	2	2	2	2	2	2	3	4	4	4
	Oct 2018 - Feb 2019	W	1	1	1	1	1	2	2	3	3	4	4	4	4
	March 2019 to May 2019	S	1	1	1	2	2	2	2	2	2	3	3	4	4
	Nov 2019 - Feb 2020	W	1	1	1	1		2	2	3	3	3	3	4	4
	Oct 2020 - Feb 2021	W	1	1	1	1	2	2		2	3		3	3	4
PUTTUR	Oct 2017- Feb 2018	W	1	1	1	1	2	2	3	3	3	4	4	4	4
	March 2018 - May 2018	S	1	1	1	2	2	2	2	3	3	3	4	4	4
	Oct 2018 - Feb 2019	W	1	1	2	2	2	2	3	3	3	4	4	4	4
	March 2019 to May 2019	S	1	1	1		2	2	3	3		3	4	4	4
	Nov 2019 - Feb 2020	W	1	1	1	2	2	3	3	3	3	4	4	4	4
	Oct 2020 - Feb 2021	W	1	1	1	1	2	2	2	2	3	3	2	4	4
SULLIA	Oct 2017- Feb 2018	W	1	1	1	1	1	2	2	3	3	4	4	2	4
	March 2018 - May 2018	S	1	1	1	1	2	2	2	3	3	4	4	4	4
	Oct 2018 - Feb 2019	W	1	1	1	1	2		2	3	3	3	4	4	4
	March 2019 to May 2019	S	1	1	1	1	1	2	2	2	2	2	3	4	4
	Nov 2019 - Feb 2020	W	1	1	2		2	3	3	3		3	3	4	4
	Oct 2020 - Feb 2021	W	1	1	1	1	2	2	2	2	3	3	4	4	4

Figure 12: Colour scale graph showing the presence and absence of types of pollen in the samples collected from 5 taluks and within the seasons. 1: Predominant (>45%); 2: Secondary Pollen (16-45%); 3: Important Minor pollen (3-15%); 4: Minor pollen (<3%).

Floral diversity in the five district: Identified almost 32 plant taxa belonging to 13 families in the summer, consisting of predominantly *Cucurbitaceae and Solanaceae*. This pattern was seen across all the taluks except Buntwal where *Solanaceae* members are predominant. The average Shannon-Weaver diversity index for each taluk across seasons was not that significant, however, diversity of pollen was seen.

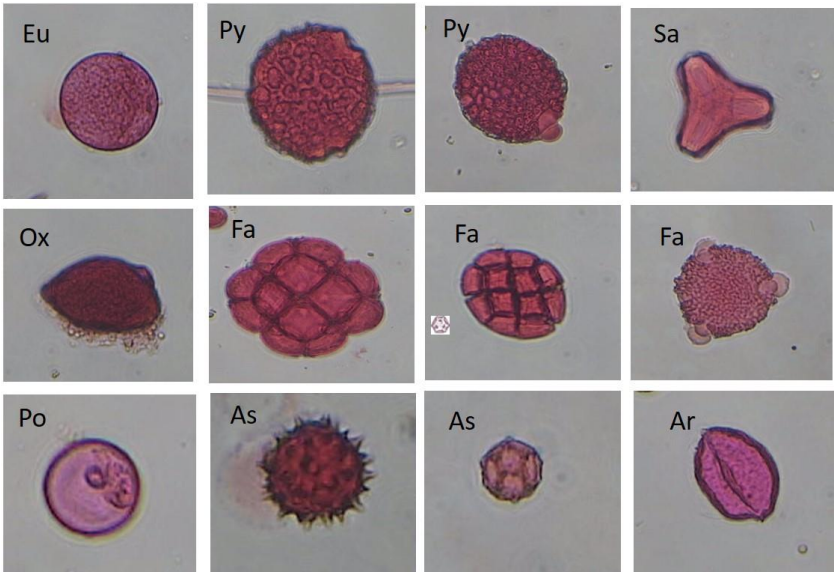

Figure 13: Photo Micrographs of Pollen types identified from samples. All the figures are magnified to X1000. Eu: *Euphorbiaceae;* Po: *Polygonaceae*; Sa: *Sapindaceae*; Ox: *Oxalidaceae*; Fa: *Fabaceae;* Po: *Poaceae;* As: *Asteraceae;* Ar: *Arecaceae.*

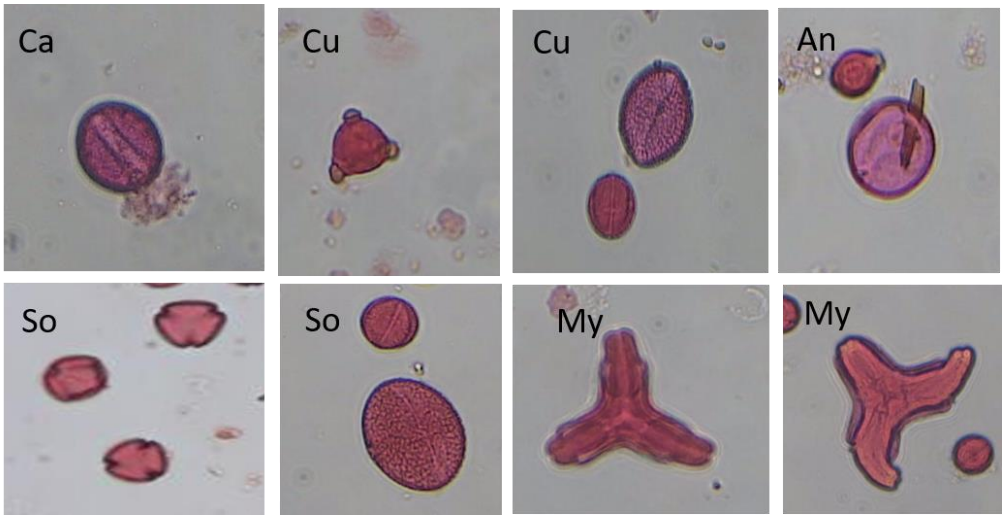

Figure 14: Photo Micrographs of Pollen types identified from samples. All the figures are magnified to X1000. Ca: *Capparaceae*; Cu: *Cucurbitaceae*; An: *Anacardiaceae*; So: *Solanaceae*; My: *Myristicaceae*.

4. CONCLUSION

Honey analysis shows a good potential for the growth of these local bee colonies. The bees used pollen to feed their parent bees, increase the strength of the colony and nectar for their carbohydrate needs. Identifying sources of pollen and nectar in honey will help beekeepers maintain their colonies [22]. The area Dakshina Kannada district selected for this study has good potential to support beekeeping business due to the diversity of nectar and pollen taxa. As pollen from *Cucurbitaceae* and *Solanaceae* are the main sources of food for honey bees, efforts should be made to increase the cultivation of them as well as of plants of the *Asteraceae, Poaceae, Euphorbiaceae* and *Fabaceae* in these areas. To improve the beekeeping industry, there is a need for a good understanding and reciprocity between bees and the plant taxon available in the region and at a particular season. The taxa identified are not only economic crops but also play an important role in the development of beekeeping in these regions. These data reflect the floral situation of where a particular honey is produced, and the geographical origin is based on the presence of a combination of pollens from that particular region [4]. Similar studies also stated that honey samples from Bhagamandala and Puttur districts belong to *Sapindaceae* [27]. Data clearly showed that majority of pollen source available was of *Cocus nucifera* throughout the year and also *Terminalia sp*. was considered as good pollen and nectar source [1]. Even the samples from Gutti, Mudigere, Kundur, Sringeri, Banur and Kumbadiall belong to *Coffea arabica* with pollen ranging almost to 62%. The total pollen load seems to be significantly higher in summer season than in winter season, which was observed across all the regions ($p<0.05$). *Cucurbitaceae* pollen was collected predominantly across the regions, suggesting of the nature of vegetation within these regions. The pollen collected was not uniform and a large variation was seen in terms of diversity and evenness which indicates foragers did not depend on the pollen evenly during a particular sampling period or season. Instead, they likely collected pollen from a few resources in each collection. Our work is consistent with previous reports stating that, even in cases when there is a massive diversity of plant resources, bees tend to focus their foraging patterns on a few species might be due to the preferred sources are more abundant, or such that they do provide selective nutrients that the colonies are in need of at a particular time. In addition, honey bees have been known to monitor their environment and share several species of plants at any given time until the resource is nearly exhausted [3].

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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