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Contributed Paper

Dynamics of Fungal Communities in Corbicular Pollen and Bee Bread

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ABSTRACT

Mimosa pudica L., a sensitive plant, is a major pollen source for honey bees in Thailand. In this study, fungal communities in honey bee pollen collected over different periods of time (corbicular pollen (0) and bee bread stored in comb cells for 1, 2, 3, 4 and 6 weeks) in an apiary surrounded by *M. pudica* L. were investigated using Polymerase Chain Reaction denaturing gradient gel electrophoresis techniques (DGGE) and culture methods. Fresh pollen collected from corbicular pollen showed the highest fungal abundance ($3.96\pm0.20 \times 10^4$ $CFUg^{-1}$ pollen). The fungal population and diversity decreased after storage in hives when the pH and moisture content decreased over time. The variation of DGGE band patterns indicated a high diversity of the population of filamentous fungi and yeasts. At the beginning of bee pollen storage, the fungal community patterns were more diverse and showed a significant decline over time. Dominant fungal species found in bee bread using both culture and molecular methods were yeast in the genus *Zygosaccharomyces*, and filamentous fungi in the genera *Cladosporium* and *Aspergillus*. This is the first report of the fungal community in bee pollen and bee bread during storage in managed European honey bee hives.

Keywords: DGGE, honey bee pollen, micro flora, *Mimosa*

1. INTRODUCTION

The European honey bee *(Apis mellifera)* is a highly social insect that typically maintains intra-colony food reserves. Pollen from plants represent the most important source of proteins (25-30%) for honey bees; however, they also serve as a source of carbohydrates (30-55%), lipids such as fatty acids and sterols (1-20%), vitamins, as well as minerals [1]. During foraging activities, honey bees pack collected pollen grains onto specialized hind leg structures called corbicular pollen baskets; glandular secretions, including associated microbes, are used to moisten pollen to facilitate packing [2]. Inside the hive, pollen is

stored by foragers in wax cells surrounding the bee larvae [3]. During storage, biochemical changes caused by microbial action [4] help to preserve the pollen, now called bee bread, for long-term storage [5].

Recently, microorganisms associated with honey bees and their food have been identified [2, 6]. Most predominant were fungi from the genera *Penicillium*, *Mucor* and *Aspergillus*, as well as bacteria from the genus *Bacillus* [2, 4]. It is believed that microbial communities may differ according to floral pollen source [4]; however, this has not been fully explored. In northern Thailand, *Mimosa pudica* L., a sensitive plant ("Mai Ya Rab" in Thai) in family Fabaceae is a major pollen source for honey bees [7], and is commonly employed by Thai beekeepers as a bee food supplement due to its ubiquitous. Microbes associated with *M. pudica* L. pollen collected by honey bees have not been reported. Moreover, previous studies only investigated the microbial diversity in bee pollen by using the culture dependent method that is limited both by strict cultivation conditions as well as number of tested microbes. In this study, we aimed to examine fungal communities in corbicular pollen and bee bread collected from bee hives using both cultural and molecular approaches.

2. MATERIALS AND METHODS

2.1 Sample Collection

Three colonies of *A. mellifera* used for this experiment were maintained at the Bee Protection Laboratory (BeeP), Faculty of Science, Chiang Mai University, Thailand. The apiary was found to be surrounded by *M. pudica* L., and colonies were equipped with entrance mounted pollen. Corbicular pollen was collected using traps placed in front of the hives (Week 0) and bee bread stored in cells for 1, 2, 3, 4 and 6 weeks were collected from newly drawn combs of colonies of bees during winter (October-December, 2010).

2.2 Identification and Physical Characterizaton of Honey Bee Pollen

To confirm the pollen source, pollen samples (corbicular pollen and bee bread) were mounted in glycerin jelly. Pollen measurement and morphological observation were carried out under a compound microscope (Olympus CX31). The pH values of pollen samples were measured by a digital pH meter (713 pH Meter Metrohm Herisau, Switzerland). The moisture content determination was made through gravimetry until constant weight, using a vacuum oven at 70°C (2 g of sample) [3]. Normality and homogeneity of data variances were checked using SPSS version 17.0 (SPSS, Inc.) Data were analyzed using one - way ANOVA.

2.3 Fungal Isolation and Identification from Honey Bee Corbicular Pollen and Bee Bread

Samples (0.1 g) were each suspended in 900 µL sterile water. From the first suspension, a series of decimal dilutions were performed and used to inoculate Petri dishes containing 25 mL Dichloran Rose Bengal Chloramphenicol agar (DRBC). Plates were incubated for 3-5 days at 25°C. Fungal enumeration ($CFUg^{-1}$ sample) was examined [8]. All assays were performed in triplicates to allow mean and standard deviation calculation. All isolated fungi were grouped and maintained in cryovials containing 20% glycerol at -80°C, and deposited at Bee Protection Center, Faculty of Science, Chiang Mai University, Thailand. The isolated fungi were identified using conventional morphological characters [9-13]. Moreover, molecular methods were used to confirm the morphological identification of isolated fungi. For this, genomic DNA of each fungal

isolate was extracted by a SDS-CTAB (sodium dodecyl sulfate-cetyltrimethyl-ammonium bromide) method [14]. Fresh fungal mycelia were scraped from the surface of the agar plate and transferred in to a 1.5 microcentrifuge tube. The freeze-dried mycelium was mixed with sterile white quartz sand and 600 µL of preheated (60°C) CTAB buffer (2% (v / w) CTAB,100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0). The sample was then ground with a pestle for 5µ10 min. SDS was added to a final concentration of 2% (w/v), vortexed and incubated at 60°C for 60 min with occasional swirling every 15 min. Chloroform:isoamyl alcohol (24:1, v/v) (600 µL) was added into each tube and mixed. The mixture was centrifuged at 13,000 rpm for 30 min, and the aqueous extraction layer was transferred into a new 1.5-mL centrifuge tube. Phenol:chloroform (1:1, v/v) extraction was repeated twice or until no interface was visible. Two volumes of cold absolute ethanol were added into each tube, and the tube was inverted gently. The tube was stored overnight at -20°C to precipitate DNA and centrifuged at 14,000 rpm for 15 min at 4°C, and the DNA pellet was washed twice with 70% cold ethanol and dried at room temperature (27±2°C). The dried pellet was resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and incubated at 37°C for 1h [15]. DNA samples were checked for purity by electrophoresis in 1% agarose gel stained with ethidium bromide under UV light. The suspended DNA was stored at -20°C pending use for PCR amplification. The internal transcribed spacer (ITS) regions of nuclear rRNA gene were amplified by polymerase chain reaction (PCR) with ITS1 and ITS4 primers [15]. The PCR amplification was performed in total volume of 25 µL containing 2 µL DNA extract, 12.5 pM of each primer, 0.2 mM of each dNTP, 0.2 mM $MgCl_2$, 1X reaction buffer and 2.5 unit of *Taq* DNA polymerase (Invitrogen) under the following thermal conditions: 95°C for 2 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and 72°C for 10 min. Amplicons were checked on 1% agarose gels stained with ethidium bromide under UV light. PCR products were purified using PCR clean up Gel Extraction NucleoSpin® Extract II Purification Kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced. Sequencing reactions were performed, and the sequences were automatically determined in a genetic analyzer (1st Base, Selangor, Malaysia) using PCR primers mentioned above. Sequences were used to query GenBank via the BLAST program (http://blast.ddbj.nig.ac.jp/top-e. html). All covering ITS1, 5.8S gene, and ITS2 region sequences obtained in this study can be accessed as NCBI GenBank entries (http://www.ncbi.nlm.nih.gov;fungal accession number: **KF527993**-**KF528006**).

2.4 DNA Extraction from Honey Bee Corbicular Pollen and Bee Bread

Genomic community DNA was extracted from honey bee corbicular pollen and bee bread samples (approximately 0.25 g) using the ZR Soil Microbe DNA Kit™ (ZYMO RESEARCH, New York, USA.). DNA extraction was performed following the manufacturer's protocols.

2.5 Polymerase Chain Reaction for Denaturing Gradient Gel Electrophoresis (DGGE)

PCR was employed to amplify a region of fungal 18S rRNA gene display in honey bee pollen collected at different time periods. A partial sequence of 18S rRNA gene (about 350 bp) was amplified using forward primer: NS1 (5'-GTAGTCATATGCTTG TCTC-3')

[16] and reverse primer: GCFung (5'-CGCC CGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCATTCCCCGTTACCCGT TG-3') [17]. In addition, the GCFung primer includes a 40 base GC clamp (underlined) required for optimal denaturing gradient gel electrophoresis (DGGE) analysis [18]. The PCR amplification was performed in a total volume of 25 µL containing 2 µL DNA extract, 12.5 pM of each primer, 0.2 mM of each dNTP, 0.2 mM $MgCl_2$, 1X reaction buffer and 2.5 unit of *Taq* DNA polymerase (Invitrogen). Amplification was performed in a thermal cycler using an initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. The reaction was completed by a final elongation step at 72°C for 10 min.

2.6 Denaturing Gradient Gel Electrophoresis

DGGE analysis of the PCR products was performed using by Dcode™ Universal mutation Detection System (Bio-Rad) with 7% (w/v) polyacrylamide (acrylamide: bis-acrylamide (37.5:1, w/w) gel in running buffer (1X TAE: 20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gel was made with denaturing gradients ranging from 25 to 40 % (where 100% denaturant defined as 7 M urea and 40% formamide). The electrophoresis was run at a constant voltage of 50 V and temperature of 60°C for 20 h. After electrophoresis, the gels were incubated for 30 min in ethidium bromide and photographed UV transillumination.

2.7 Sequencing of DGGE Bands and Phylogenetic Analysis

Separated DNA bands from DGGE gels were removed and eluted in 25 µL of TE buffer at 4°C overnight. The eluted DNA was re-amplified by PCR performed primers NS1 and Fung (without GC) using conditions described above. The PCR products were separated on a 1.5% agarose gel, excised, and purified using Nucleic Acid and Protein Purification (NucleoSpin Extract II, Macherey-Nagel, Germany) prior to sequencing according to the manufacturer's protocols. DNA sequencing was carried out by 1st BASE DNA Sequencing (Malaysia). Database searches with determined sequences were conducted by using the BLAST program in the GenBank. The sequences were aligned using ClustalW, version 7.0, and the alignments were refined by visual inspection. All 18S rRNA gene sequences obtained in this study can be accessed as NCBI Genbank entries (http://www.ncbi. nlm.nih.gov; accession number: **JN817288**-**JN817297**).

For the phylogenetic analysis, closely related 18S rRNA gene sequences were retrieved from the GenBank database by BLAST-N Searches (http://blast.ncbi.nlm.nih.gov/). Multiple alignments of sequences determined in this study and reference sequences obtained from databases were taken together in the calculations of levels of sequence similarity using CLUSTALW 1.74, with arithmetic averages tree-making algorithms taken from the MEGA package version 5.0 [19]. The topologies of the neighbor-joining phylogenetic trees were evaluated based on bootstrap analyses of 1,000 replicates.

3. RESULTS

3.1 Identification and Physical Characterization of Honey Bee Pollen

Pollen measurement and morphological observations analyzed using light microscopy found that *M. pudica* L. was predominant plant pollen (more than 45%) found in our bee pollen samples. The pantocolpate

pollen grain occur in tetrads with spheroidal shape. The pollen size ranges from 9-10 µm (Figure 1). During storage of pollen, the pH value gradually decreased from 4.93±0.03 to 4.55±0.02. The differences in pH between corbicular pollen (4.93±0.03), stored pollen (4.87±0.04) and 2-6 weeks after storage in comb cells was significant elevated (ANOVA, $P < 0.000$) (Table 1). The moisture content peaked one week after storage (35.22%) (ANOVA, $P < 0.000$); however, it decreased after six weeks of storage in the comb (6.53%).

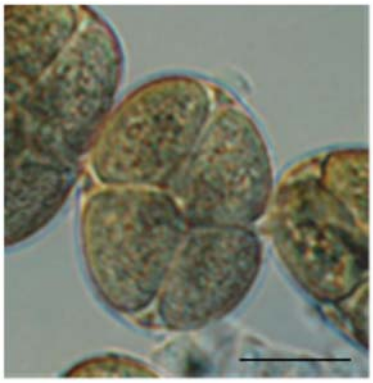

Figure 1. Pollen of *Mimosa pudica* L. observed under a light compound microscope.

Table 1. Fungal population and physical characteristics of bee corbicular pollen and bee bread.

Source of pollen	$CFUg^{-1}$ pollen	pH of pollen	Moisture content (%)
Corbicular pollen	$3.96±0.20 \times 10^4$(d)	*4.93±0.03(b)	7.54±1.41(a)
Comb cells after 1 week	$2.50±0.10 \times 10^4$(c)	*4.87±0.04(b)	*35.22±3.45(b)
Comb cells after 2 weeks	$2.20±0.17 \times 10^4$(c)	4.57±0.01(a)	13.88±1.87(a)
Comb cells after 3 weeks	$0.76±0.11 \times 10^4$(b)	4.55±0.02(a)	8.95±2.62(a)
Comb cells after 4 weeks	$0.33±0.11 \times 10^4$(a)	4.56±0.02(a)	6.09±3.00(a)
Comb cells after 6 weeks	$0.20±0.10 \times 10^4$(a)	4.56±0.01(a)	6.53±3.93(a)

Asterisks and label alphabets (a, b, c, and d) indicate statistically significant differences (Anova $P = 0.05$). Normality and homogeneity of variances of data was checked using SPSS version 17.0 (SPSS, Inc.).

3.2 Fungal Isolation and Identification from Honey Bee Corbicular Pollen and Bee Bread

After fungal isolation of samples on DRBC agar, incubated aerobically for 3-5 days at 25°C, fresh corbicular pollen collected in pollen trap showed the highest numbers in fungal population ($3.96±0.20 \times 10^4$ $CFUg^{-1}$ pollen) (38 isolates in 8 fungal genera) (Table 1 and 2). The data also suggest that the number of fungal isolates decreased as storage period increased. In this study, ninety-nine fungal isolates were grouped by morphological characters and ten fungal genera were recovered, including *Aspergillus*, *Cladosporium, Curvularia*, *Eupenicillium, Fusarium, Gibberella, Mucor*, *Penicillium, Pestalotiopsis* and *Rhizopus*. The majority of fungi were from the genus *Cladosporium* (31.31 %), followed by the genus *Aspergillus* (21.21 %) and the genus *Penicillium* (17.17 %). Based on morphology fungal isolates CS-P/F05 and CS-P/F16 were identified to be *Curvularia lunata* and *Rhizopus nigricans*, respectively. This was confirmed by molecular analyses of ITS1-5.8S-ITS2 sequences (Table 3). Both morphological and molecular identification tools identified 16 species of culturable fungi. The ITS1-5.8S-ITS2 sequences of mycelia sterilia CS-P/F17 showed 99% of sequence similarity with *Pestalotiopsis heterocornis* (JN943626). Fungus genus *Mucor* and *Pestalotiopsis* were only found in corbicular pollen samples. After six weeks of storage in comb cells, only members of genera *Aspergillus* and *Cladosporium* were presented. The phylogenetic tree based on

DNA sequences of ITS 1, ITS 2 and 5.8 ribosomal RNA gene of fungal isolates was constructed (Figure 2) and this found that the fungal species found were in accordance with the morphological identification (Table 3).

Table 2. Incidence of culturable fungal species isolated from honey bee corbicular pollen and bee bread samples.

Fungal Taxa	Isolate	Pollen sample collected in different time (week)					
		0	1	2	3	4	6
Aspergillus sp. 1	CS-P/F02	2	4	4	-	-	-
Aspergillus sp. 2	CS-P/F03	3	-	1	-	-	1
Aspergillus sp. 3	CS-P/F04	1	1	2	1	-	1
Curvularia lunata	CS-P/F05	2	-	2	-	-	-
Cladosporium sp. 1	CS-P/F06	7	3	1	2	2	1
Cladosporium sp. 2	CS-P/F07	9	3	2	-	1	-
Eupenicillium sp. 1	CS-P/F08	1	-	-	-	-	-
Eupenicillium sp. 2	CS-P/F10	1	3	-	-	-	-
Eupenicillium sp. 3	CS-P/F11	-	-	1	-	-	-
Fusarium sp. 1	CS-P/F12	2	2	-	-	-	-
Fusarium sp. 2.	CS-P/F13	-	-	-	1	-	-
Gibberella sp.	CS-P/F09	-	-	2	1	-	-
Mucor sp.	CS-P/F01	2	-	-	-	-	-
Penicillium sp.	CS-P/F14	4	6	4	2	1	-
Pestalotiopsis sp.	CS-P/F15	1	-	-	-	-	-
Rhizopus nigricans	CS-P/F16	-	4	2	-	-	-
Mycelia Sterilia	CS-P/F17	3	-	-	-	-	-
Total		38	26	21	7	4	3

Table 3. The PCR product size (bp) and GenBank sequence accession numbers of ITS1-5.8S-ITS2 sequences of isolated fungi in this study.

Fungal isolate	size (bp)	Database match (accession no)	% similarity	Accession number.
Mucor sp. CS-P/F01	621	*Mucor hiemalis* (JN206248)	100	KF527993
Aspergillus sp. CS-P/F02	563	*Aspergillus niger* (JF838357)	99	KF527994
Aspergillus sp. CS-P/F03	564	*Aspergillus. aff. dimorphicus* (JN246074)	99	KF527995
Aspergillus sp. CS-P/F04	548	*Aspergillus. wentii* (GU934502)	99	KF527996
Cladosporium sp. CS-P/F06	512	*Cladosporium cladosporioides* (JN084017)	99	KF527997
Cladosporium sp. CS-P/F07	524	*Cladosporium oxysporum* (KC137278)	99	KF527998
Eupenicillium sp. CS-P/F08	516	*Eupenicillium shearii* (AJ004893)	98	KF527999
Eupenicillium sp. CS-P/F11	550	*Eupenicillium* sp. (HQ649853)	99	KF528001
Fusarium sp. CS-P/F12	523	*Fusarium subglutinans* (HQ995669)	99	KF528002
Fusarium sp. CS-P/F13	517	*Fusarium verticillioides* (KP132243)	99	KF528003
Gibberella sp. CS-P/F09	540	*Gibberella moniliformis* (JF499676)	99	KF528000

Table 3. Continued.

Fungal isolate	size (bp)	Database match (accession no)	% similarity	Accession number.
Penicillium sp. CS-P/F14	545	*Penicillium sumatrense* (AY213677)	99	KF528004
Pestalotiopsis sp. CS-P/F15	570	*Pestalotiopsis uvicola* (JF923829)	99	KF528005
Mycelia 1 CS-P/F17	557	*Pestalotiopsis heterocornis* (JN943626)	99	KF528006

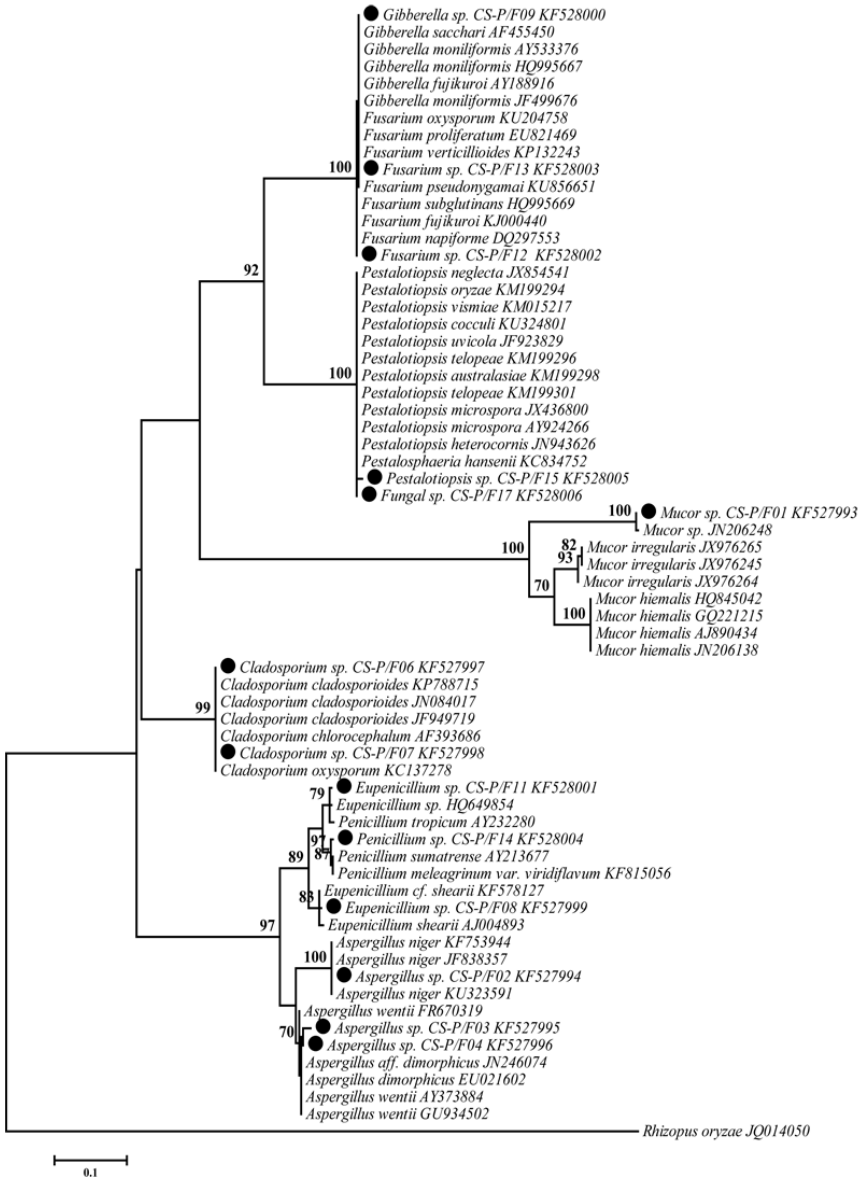

Figure 2. The Maximum Likelihood tree based on the partial internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 sequence alignments of 66 isolates. *Rhizopus oryzae* was used to root the tree. Bar represents 0.1 showed substitutions per nucleotide position

3.3 Denaturing Gradient Gel Electrophoresis

The comparison of fungal community in the pollen at different storage times was performed by the PCR-DGGE analyses with universal fungus-specific primers. PCR product size after amplification using the NS1 and GCFung primers was about 350 bp and the DGGE profile is shown in Figure 3. The results indicated that DGGE profiles from samples revealed that the fungal communities of each comb cell at the same duration of storage (0, 1, 2, and 3 weeks) were quite similar. After 2-3 weeks of storage in bee hive, the fungal community shifted to OTU F and H (*Pseudozyma parantarctica* and *Cladosporium cladosporioides*). Additionally, OTU F could be found in all samples with different intensity. Ten PCR product bands from the DGGE profile were selected, purified and sequenced. The BLAST result of each band is shown in Table 4. The results showed that A and F bands were closely related to the yeasts *Zygosaccharomyces mellis* and *Pseudozyma parantarctica*. Six bands such as B, D, E, G, H and J were closely related to the filamentous fungi, *Cladosporium bruhnei, Trimmatostroma abietis*, *Tilletia goloskokovii*, *Paraconiothyrium sporulosum*, *Cladosporium cladosporioides*, and *Helicascus kanaloanus*. In addition, one amplified DNA band was homologous with uncultured fungus clone (K; uncultured fungus clone (DQ412127)), and band C had only low similarity with an insect environmental sample (AB385856) with the similarity of 94 %. (Table 4). All DGGE bands from the same age of pollen showed similar pattern based on clustering analysis (Figure 4). These results were in accordance with the cultivation method in which members of *Cladosporium* were found predominant. The phylogenetic tree constructed by the sequences of 18S rRNA gene (Figure 5) and this found that the fungal species found were in accordance with the cultivation methods.

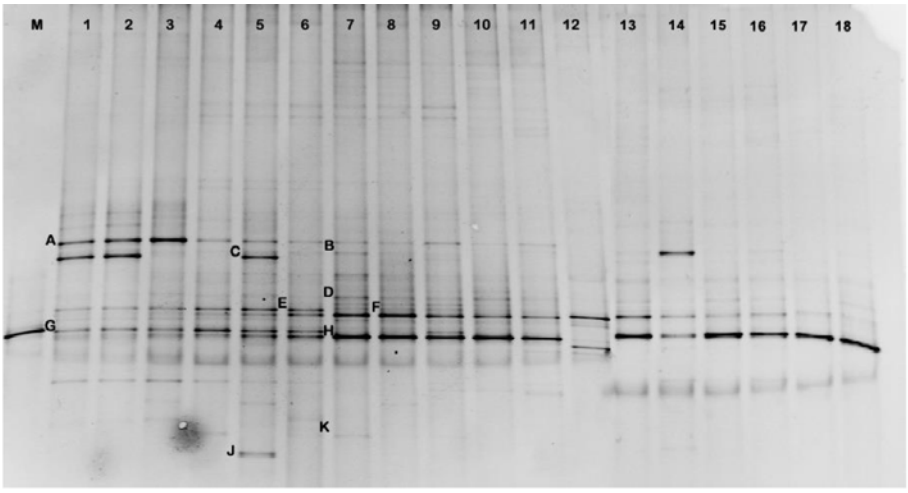

Figure 3. 18S rRNA gene PCR-Denaturing gradient gel electrophoresis gel of fungi from the honey bee pollens collected at different period of time (bases 20-38 to 351-368 relative to *Saccharomyces cerevisiae*). Gel gradient ranging from 20-45% denaturant. Lane M, Marker (Ascomyces); Lane 1, 2, 3 The corbicular pollen at Week 0; Lane 4, 5, 6 bee bread at Week 1; Lane 7, 8, 9 bee bread at Week 2; Lane 10, 11, 12 bee bread at Week 3; Lane 13, 14, 15 bee bread at Week 4; Lane 16, 17, 18 bee bread at Week 6.

Table 4. Identification of selected DGGE bands.

Band position/ OTUs	Data base match (accession number)	% similarity	Accession number[b]
1. P CS-F/A	*Zygosaccharomyces mellis* AF339891[b]	99	JN817288
2. P CS-F/B	*Cladosporium bruhnei* strain CPC 5101 AY251096[b]	99	JN817289
3. P CS-F/C	*Insect environmental* AB385856[b]	94	JN817290
4. P CS-F/D	*Trimmatostroma abietis* strain TRN127 AY559396[b]	99	JN817291
5. P CS-F/E	*Tilletia goloskokovii* DQ832247[b]	98	JN817292
6. P CS-F/F	*Pseudozyma parantarctica* strain JCM 11752 JN940457[b]	99	JN817293
7. P CS-F/G	*Paraconiothyrium sporulosum* AB303549[b]	99	JN817297
8. P CS-F/H	*Cladosporium cladosporioides* DQ678004[b]	99	JN817294
9. P CS-F/J	*Helicascus kanaloanus* AF053729[b]	98	JN817296
10. P CS-F/K	Uncultured fungus clone L20 DQ412131[b]	97	JN817295

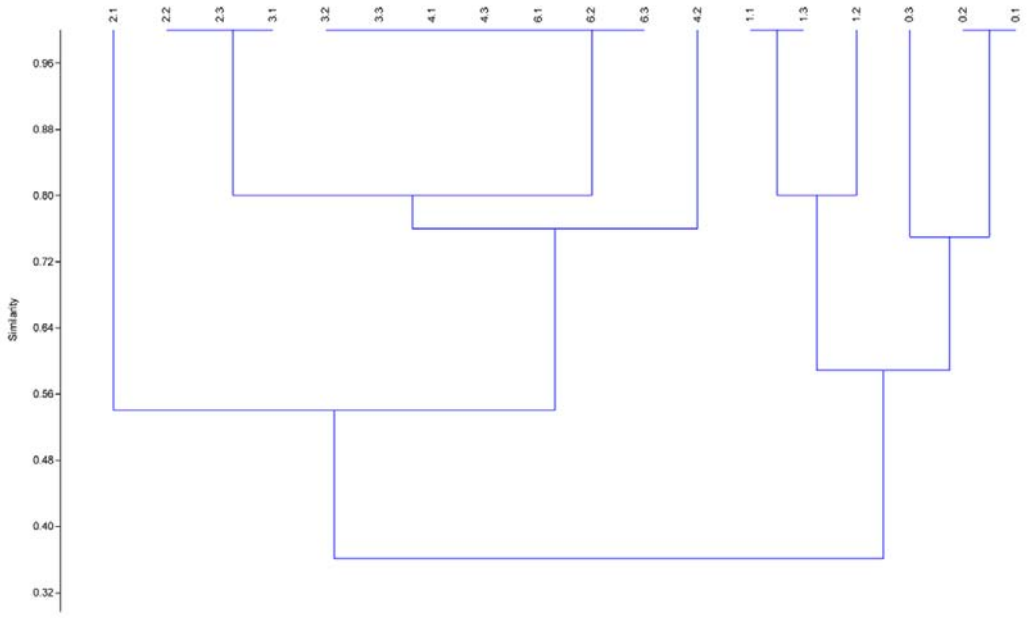

Figure 4. Clustering analysis of the DGGE pattern of the honey bee pollens collected at different period of time (0.1, 0.2, 0.3 represents corbicular pollen: 1.1, 1.2, 1.3 represents the bee bread at Week 1: 2.1, 2.2, 2.3 represents bee bread at Week 2: 3.1, 3.2, 3.3 represents bee bread at Week 3: 4.1, 4.2, 4.3 represents bee bread at Week 4: 6.1, 6.2, 6.3 represents bee bread at Week 6).

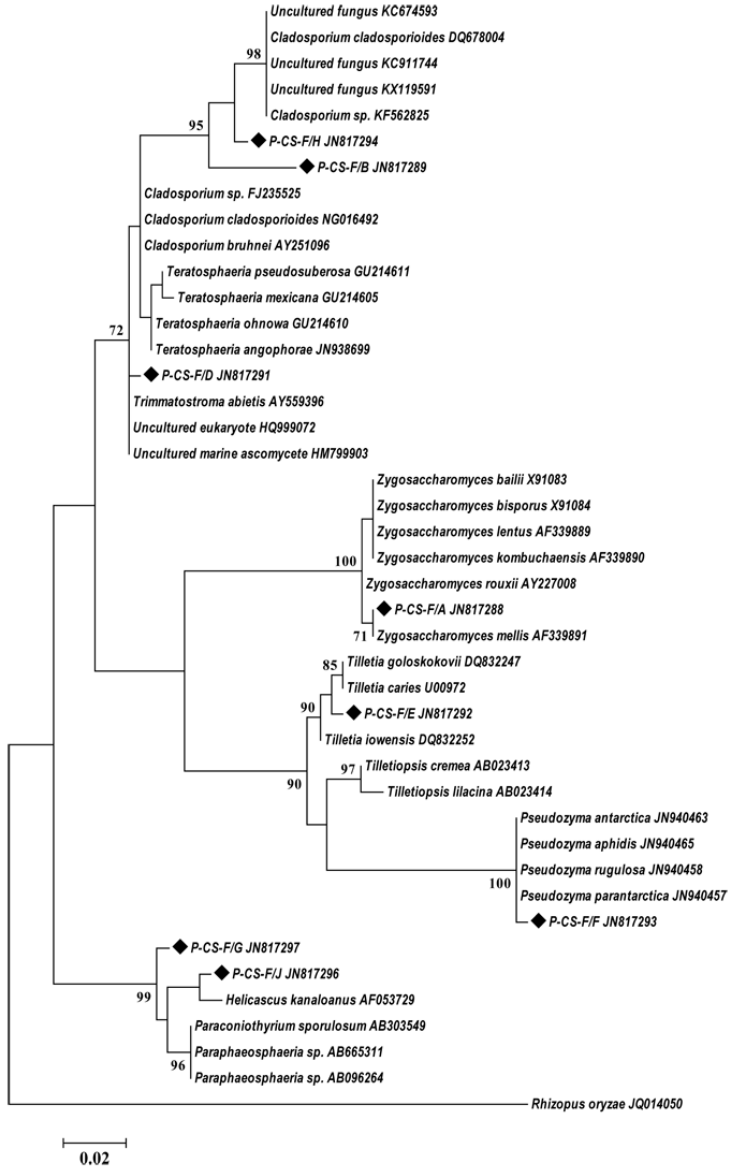

Figure 5. The Maximum Likelihood tree based on 18S ribosomal RNA gene sequence alignments of 43 isolates. *Rhizopus oryzae* was used to root the tree. Bar represents 0.02 showed substitutions per nucleotide.

4. DISCUSSION

According to the study observations analyzed using light microscopy, pollen grains of the genus *M. pudica* L., were consistent in size as a previous *M. pudica* pollen study in Brazil [20].

Our examination of fungal communities in honey bee corbicular pollen and bee bread demonstrated that Mimosaceae (*Mimosa pudica* L.) was commonly collected by colonies and predominant pollen during this study, increasing time caused changes of color and texture of collected pollen. Collected bee pollen became darker in color, thinner wall and pH of samples were becoming more acidic.

Foraging worker bees pack a thin layer of honey, which enhance pH and has high osmotic pressure, on collected pollen to prevent spoilage [3]. As a result, stored pollen displays chemical changes. The differences in pH between *M. pudica* L. corbicular pollen (4.93±0.03) and stored bee bread (4.55±0.02) after 7 and 42 days of storage in comb cells suggest that both the bee activity, by adding honey on top of stored pollen, as well as microflora activity, might promote acid to lower the pH of corbicular pollen as it is converted to bee bread [2].

Moisture content of the pollen peaked one week after storage (35.2±3.45%), and after the addition of honey in the pollen cell by the worker bees; however, it decreased after 6 week of storage in comb (6.5±3.93%). Such low pH values and humidity condition could limit or reduce the microbial growth. In our study, fungi from the genera *Cladosporim, Aspergillus* and *Penicillium* were found in corbicular pollen, with *Cladosporium* sp. most prevalent in both corbicular pollens and bee bread. *Cladosporium cladosporioides* is a very common, cosmopolitan and saprobic species [21]. Kelly et al (2014) also displayed that fungi in genus *Cladosporium* and *Aspergillus* were predominant in bee bread [22]. Molds especially Aspergilli, Mucorales and Penicillia, as well as yeast such as *Cladosporium* sp., *Peyronelia* sp. [5, 23] were also previously reported as predominant microorganisms in pollen and bee bread. Moreover, Gilliam et al. [4] reported new records of molds (*Alternaria* sp., *Rhizopus nigricans*). Magan and Lacey [24] reported the most frequently isolated fungi were *Penicillium* spp., whereas *Aspergillus* spp. and *Fusarium* spp. were found less often. Furthermore, our study is the first to report of *Curvularia* sp. isolated from *M. pudica* L. pollen.

The conversion of pollen to bee bread, and accompanying biochemical changes, have been suggested to result from microbial action such as lactic acid fermentation caused by bacteria and yeasts [25]. Some fungi may be unable to grow in highly acidic conditions because fungal spores are not resistant to environmental conditions like bacterial endospores [3, 7]; pollen grains in flowers can also secret substances that inhibit microbial spore germination [26]. In addition, some fungi found in collected pollen may produce secondary metabolites like antibiotics, organic acids, and enzymes that are effective in reducing the number of pathogens and other microbes [4]. The comparison of the fungal communities in the pollen at different storage times was performed by the PCR-DGGE analyses with universal fungus-specific primers. Variation of DGGE band patterns indicates the diversity of the population of fungi and yeasts (Figure 3). At the beginning of storage, the strongest band (OTU A) had only 99 % homogeneity with *Zygosaccharomyces mellis* (AF339891). *Zygosaccharomyces* is a yeast often concerned with food spoilage [27]. *Z. mellis* also possesses extreme osmotolerant characteristics and is a spoiling agent of high sugar foods and honey. Some fungi, *Zygosaccharomyces* yeasts in particular, could be contributing to pollen chemical dynamics due to their ability to ferment sugar, as well as their osmotolerance, resistance to preservatives, formation of heat-resistant ascospores, and fructophily [27]. Later in the storage period, the fungal community shifted to OTU F and H (*Pseudozyma parantarctica* and *Cladosporium cladosporioides*). Pseudozyma fungi are yeast-like Basidiomycetes that are mostly epiphytic or saprophytic and are not pathogenic to plants. Several Pseudozyma species have been reported to exhibit biological activity against powdery mildews [28]. According to Magan and Lacey [24]; Gillium [5, 23], members from the genus *Cladosporium* are some of the most abundant

airborne fungi worldwide [29]. Our results were also in accordance with the cultivation method in which members of *Cladosporium* were found to be predominant. Band E, which had similarity of 98 % with *Tilletia goloskokovii* (DQ832247), was found in week 0 (corbicular pollen) and week 1 (bee bread). Tilletiales have been poorly represented in previous phylogenetic analyses of smut fungi [30].

5. CONCLUSIONS

Our results show fungal communities in corbicular pollen and stored pollen (bee bread), as well as highlighting the diversity of fungi using both cultural and molecular approaches. In this study it can be concluded that there are specific groups of fungi that have adapted to reside in honey bee corbicular pollen and stored bee bread; it is possible that honey bee activities could affect the fungal population. The fungi might be derived from flowers or outside environment however, the condition of bee pollen when preserved by honey bees restricted the fungal diversity and shape of the fungal communities of stored bee pollens in honey bee hives.

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