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TECHNICAL NOTE

PATHOLOGY/BIOLOGY; TOXICOLOGY

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Tracking Plant, Fungal, and Bacterial DNA in Honey Specimens*

ABSTRACT: Consuming honey can result in adverse effects owing to poisoning by bacterial (botulism) or plant toxins. We have devised a method to extract polymerase chain reaction (PCR) amplifiable DNA of up to *c.* 400 bp in length based on dialysis of a 15-mL honey sample for 18 h against deionized water followed by sequential extraction using phenol, phenol/chloroform/isoamyl alcohol, chloroform/isoamyl alcohol, and ether. Sequence analysis of PCR products obtained using "universal" plant, fungal, and bacterial primers targeted to the ribosomal RNA genes has allowed us to identify six different orders of plants (Apiales, Fabales, Asterales, Solanales, Brassicales, and Sapindales), two orders of fungi (Entylomatales and Saccharomycetales), and six orders of bacteria (Sphingomonadales, Burkholderiales, Pseudomonadales, Enterobacteriales, Actinomycetales, and Bifidobacteriales) in a single honey specimen.

KEYWORDS: forensic science, honey DNA, bee symbionts, plants, bacteria, fungi

Since ancient times, honey has been used for its nutrient and therapeutic effects and it remains a popular product today. It has been reported that honey has beneficial effects on gastroenteritis (1,2), on gastric ulcers (3), on healing of wounds and burns (4,5), and on the diabetic patients (6,7). In addition, it is effective as a bacteriostatic and bactericidal agent (8).

Despite these benefits, sometimes honey is a potential risk to human health.

Several authors report cases of honey poisoning observed in the Black Sea region of Turkey and in various other parts of the world as well (9,10).

Honey intoxication is caused by consuming honey produced from the nectar of rhododendron species (11,12). The toxin responsible for this condition is andromedotoxin (grayanotoxin). Generally, patients complain of vomiting, salivation, weakness, and dizziness. Other symptoms are hypotension and bradycardia. Normally, complete recovery occurs in 24 h following administration of fluids and atropine treatment. In the future, there may be an increase in honey intoxication because of a widespread preference for natural products and a greater consumption of imported, unprocessed honey (9).

In other cases, hepatotoxic pyrrolizidine alkaloids (PAs) have been identified in floral honeys produced by bees visiting PA-producing plants. PA-containing plants have worldwide distribution and they belong to the families of Boraginaceae (all genera), Compositae (tribes *Senecionae* and *Eupatoriae*) and Leguminosae (genus *Crotalaria*) (13).

The International Programme on Chemical Safety has determined that contaminations of PAs in foods are a threat to human health and safety (14).

Honey consumption has also been identified as a significant risk factor for infant botulism (15).

Classic adult botulism is an intoxication caused by the ingestion of food that has been contaminated with the toxin of *Clostridium botulinum*. Infants too young to eat foods that may contain toxins generally have been thought to be safe from this disease, nevertheless clinical cases of infant botulism have been described resulting from the ingestion of spores of *C. botulinum* germinating in the intestinal tract. The infant gut harbors an immature bacterial flora and lacks the clostridium-inhibiting bile acids found in the normal adult intestinal tract, and consequently the infant gut is more susceptible to colonization by toxin-producing *C. botulinum*. Most of cases occur before the age of 1 year. Microbiological survey of honey products has reported the presence of clostridial spores in up to 25% of the products. For these reasons, honey should not be fed to children during the first year of life.

Despite the warning, two cases of infant botulism associated with consumption of honey have been reported in the United Kingdom very recently (16). In one of the cases, *C. botulinum* was found to be present in the honey fed to the infant before the onset of illness, the toxin type being the same as that isolated from the affected infant. Moreover, infant botulism is the most frequently reported form of botulism recorded each year to the Centers for Diseases Control and Prevention in the United States (annual incidence of reported cases is *c.* 1.9/100,000 live births).

Given the potential risks to human health, a sensitive method to analyze all the biological components in honey (bacteria, plants, fungi and yeasts) is crucial to establish the safety and quality of commercial honey samples. Methods currently available for honey control make use of microscopical analysis of pollen (melissopalynology), standard bacteriological examinations, or

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combination of classic microbiological techniques with molecular approaches (17). Unfortunately, these methods require professional skilled operators, previous knowledge of pollen morphology, and selective medium for the cultivation of the microorganism.

In recent years, the expectation or need for higher food safety has raised the interest in the application of DNA analyses in food. These analyses allow the detection of low amounts of nucleic acids and the screening of several components simultaneously.

The present study explores the feasibility of using polymerase chain reaction (PCR)-amplification techniques to accurately identify honey's microbial (bacteria, fungi, and yeasts) and plant components thus protecting consumers from health risks and illegal practices (substitution or addition of one component fraudulently). Because of the high carbohydrate concentration, honey is a very complex matrix and the isolation of DNA is not an easy task. In addition, sugars co-purified with target DNA could inhibit the polymerase used in the PCR amplification.

Materials and Methods

Samples

The study was carried out on three commercial (packed) honey samples produced in Italy. One sample was from Marches (National Park of Sibillini) and corresponded to multifloral honey (sample 1). The second and third samples, corresponding to unifloral (eucalyptus and lemon) honeys, were from Sicily and Sardinia islands, respectively (sample 2 and 3). The samples were directly purchased at the market.

DNA Extraction

For the DNA extraction, honeys were heated to 45°C to permit easier handling and to decrease viscosity for more uniform distribution of cellular particulates that may have been present in the sample. Fifteen milliliters of each honey sample were placed in 50 mL falcon and diluted with 2 volumes of sterile distilled water, heating at 45°C until a homogeneous mixture was obtained. The diluted samples were transferred into dialysis tubes (12–14 KDa) and dialyzed 18 h against deionized water with three water changes (18).

Following dialysis, the content of each tube was filtered (SARTORIUS SN 163 07) through a 0.45-μm-pore-size sterile membrane (Millipore®, Billerica, MA). The membranes were minced and transferred into the sterile Eppendorf tube with 500 μL buffer of the following composition: 50 mM Na_2EDTA, 50 mM Tris–HCl pH 8.0, 1% (w/v) SDS, and 6% (v/v) water-saturated phenol. Then the samples were left overnight at 4°C.

On the next day, the samples were extracted sequentially using equal volumes of phenol, phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1), and ether. The DNA fraction was precipitated from the final supernatant by centrifugation at 13,500 × *g* for 5 min after the addition of 1/10 volume of 2 M sodium acetate and 2.5 volumes cold (−20°C) ethanol. The DNA precipitates were resuspended in 20 μL sterile distilled water and stored at −20°C until use.

The extraction products were checked by electrophoresis on 1% (w/v) agarose. Amount and purity of extracted DNA was quantitated by spectrophotometry in GeneQuantpro RNA/DNA Calculator (Amersham Pharmacia Biotech, Ltd, Cambridge, U.K.).

PCR Amplification and Sequencing

PCR amplifications were performed by using the primers listed in Table 1.

All amplifications were performed in 50 μL volumes with 2.5 mM $MgCl_2$, 200 mM each dNTP, 300 ng each primer, 2 μL of DNA preparation (1:100 and 1:150 dilutions), and 2.5 units of Taq polymerase (HotStarTaq DNA Polymerase; Qiagen, Hilden, Germany) using buffer supplied by the manufacturer.

The reaction mixture was pretreated with DNAse (two enzyme units for 30 min at room temperature) to eliminate contaminant DNA. The DNAse was subsequently inactivated at 95°C for 15 min.

The thermal profile (35 cycles) was set as follows: 1 min at 94°C, 30 sec at relevant annealing temperature (Table 1), and 1 min at 72°C with a final extension of 10 min at 72°C.

PCR negative controls (no template DNA) were included in each amplification attempt, in addition to the amplifications from extraction controls.

The amplification products were checked by electrophoresis on 1.8% (w/v) agarose. The PCR products of sample 1 were purified using High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Mannheim, Germany) and cloned using the pGEM-T Easy Vector System (Promega Corporation, Madison, WI). Recombinant plasmids were isolated using Miniprep kit (Applied Biosystems, Foster City, CA) and insert size, and DNA concentration assessed by gel electrophoresis. DNA sequences were obtained using an ABI-Prism 310 automated DNA sequencer and the BigDye Terminator Cycle Sequencing v 2.0 Ready reaction kit (Applied Biosystems). Cycle sequencing products were purified by Centri-sep spin columns (Princeton Separations, Adelphia, NJ).

Sequence Data Analysis

Results were compared with the reference sequences in GenBank using the National Center for Biotechnology Information BLAST search (19). Consensus nucleotide sequences were obtained using BioEdit v.6 (20).

Results and Discussion

There is a critical factor that needs scrupulous attention when a PCR-based method is applied to the analysis of honey samples: honey consists of at least 80% sugar, and this may act as an inhibitory factor for the PCR. As a consequence, the DNA extraction

TABLE 1—*Primers used in this study.*

Primer Name	Sequence (5′ → 3′)	Target (Gene; Species)	Length of Product (bp)	Annealing Temperature (°C)
338f	AAACTGAGACACGGTCCAGAC	16S rDNA; bacteria	230	52
531r	ACGCTTGCACCCTCCGTATT			
Angio1f	TGCAGTTAAAAAGCTCGTAG	18S rDNA; plants	159	50
Angio 2r	GCACTCTAATTTCTTCAAA			
NS5	AACTTAAAGGAATTGACGGAA	18S rDNA; fungi	310	52
NS6	GCATCACAGACCTGTTATTGCCTC			

protocol has to be optimized to ensure a sufficient amount of DNA free of PCR-inhibiting substances. We found that a preliminary extensive dialysis of the samples was very important to minimize the effect of high concentration of polysaccharides. Subsequently, the samples were concentrated by filtration on a 0.45-μm-pore diameter membrane (Millipore Corporation, Bedford, MA), and the cellular elements collected on the membranes were placed in an Eppendorf tube for the extraction procedure.

As shown in Fig. 1 the method provided positive results in all the analyzed samples. However, only low molecular weight DNA can be obtained. Fragments showed a maximum length of *c.* 400 bp, thus limiting the length of the targets that can be amplified. Being a pilot study, we determined amount and purity of DNA extracted in the sole case of the sample ("Marches"), using spectrophotometry (GeneQuantpro RNA/DNA Calculator; Amersham Pharmacia Biotech, Ltd). DNA concentration was about 73.9 ng/mL with a purity coefficient A_{260}/A_{280} of 1.35.

Subsequently, the DNA isolated form the "Marches" specimen was analyzed by sequencing. All the sequencing results shown in this paper, therefore, refer to this specimen.

Bacterial DNA

With the exception of research on pathogenic microbes to bees, there are few data about the detection of microbes in honey, and most of the scientific literature is focused on the recovery of *C. botulinum*. Because of the high carbohydrate concentration and its antimicrobial activities, honey is a product with minimal types and levels of microbes. In their review on microorganisms in honey, Snowdon and Cliver (21) point out that microbes found in comb honey are principally bacteria or yeasts and come from the bees, the raw material (nectar), or from external sources. In other words, organisms found in the environment around honey (i.e., bees, hives, pollen, flowers, soil, etc.) are likely to occur in honey.

To assess the presence of bacterial DNA in the honey samples, the DNA extracts were amplified using the 338f/531r oligonucleotide primer pair (22). The PCR primers were designed to bind to a 230 bp long fragment of the nuclear 16S ribosomal RNA gene of bacteria (16S rDNA). In all cases, PCR was positive, showing the band of expected size (Fig. 2). PCR products from the selected specimen ("Marches") were then cloned and sequenced until the same groups of related sequences were repeatedly found. These sequences were grouped into clusters and the consensus sequences compared with the sequences deposited in GenBank using BLAST search. The results are summarized in Table 2, in which the 20 clones from the 16S rDNA library have been grouped into 11 clusters (HoB4cons, HoB7cons, HoB8cons, HoB25, HoB2, HoB1, HoB15, HoB6, HoB7, HoB16, and HoB5).

It appears that the most representative fraction of the bacteria belongs to the *Pseudomonadales* order. Within the *Pseudomonadales* order, *Acinetobacter* is the prevailing genus (HoB4cons), followed by *Pseudomonas* (HoB1, HoB6, HoB7, and HoB15). The order *Enterobacteriales*, on the other hand, is represented by three clones (HoB7cons). All *Enterobacteriales* belong to the *Shigella/Escherichia* genera.

Data on the gut microflora in bees have shown that intestines contain: 1% yeast-shaped microbes; 20% Gram-positive bacteria including *Bacillus*, *Streptococcus,* and *Clostridium* species, and 70% Gram-negative or Gram-variable bacteria, including *Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia, Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus,* and *Pseudomonas* (21).

In addition, species belonging to the family of the *Enterobacteriaceae* have also been detected in a microbiological monitoring performed in 14 honey houses to verify their hygienic conditions. The worst hygienic conditions were registered in the tanks and in

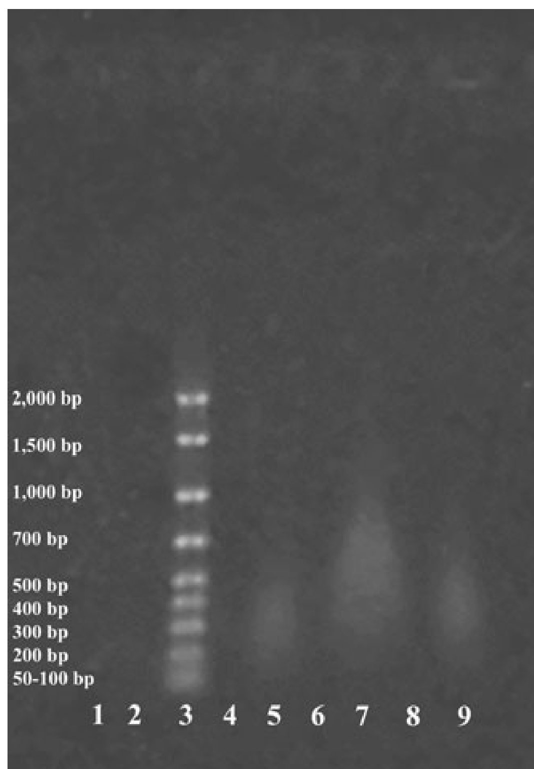

FIG. 1—*Agarose gel electrophoresis of genomic DNA extracted from three honey samples (1% w/v Certified™ Molecular Biology Agarose; Bio-Rad, Hercules, CA). Lanes 1, 4, 6, 8, no sample; lane 2, extraction blank; lane 3, Bio-Rad's 50–2000 bp ladder; lane 5, honey from Marches; lane 7, honey from Sardinia; lane 9, honey from Sicily.*

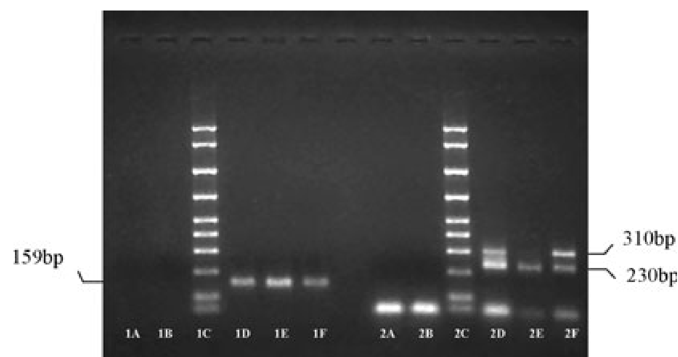

FIG. 2—*Agarose gel electrophoresis of PCR products obtained from the honey samples. Electrophoresis was performed on 1.8% w/v agarose (Certified™ Molecular Biology Agarose; Bio-Rad, Hercules, CA). Lanes 1A–1F amplifications were performed by using Angio1f/Angio2r primers; lane 1A, negative control (blank test of DNA extraction); lane 1B, negative PCR control; lane 1C, Bio-Rad's 50–2000 bp ladder; lane 1D, honey from Marches; lane 1E, honey from Sicily; lane 1F, honey from Sardinia. Lanes 2A–2F amplifications were generated by multiplex-PCR assay based on two pairs of primers 338f/531r and NS5/NS6; lane 2A, negative control (blank test of DNA extraction) control extract; lane 2B, negative PCR control; lane 2C, Bio-Rad's 50–2000 bp ladder; lane 2D, honey from Marches; lane 2E, honey from Sicily; lane 2F, honey from Sardinia. The size of products are indicated on the sides.*

TABLE 2—*Taxonomic identification of the consensus sequences for the 16S rRNA gene clones.*

Consensus	No. of Clones	Taxonomic Identification					Base Similarity
		Class	Order	Family	Genus	Species	
HoB25	1	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	–	166/167
HoB2	1	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Ralstonia*	–	191/192
HoB1	1	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*flectens*	187/192
HoB15	1	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*flectens*	187/192
HoB4cons	6	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	–	193/193
HoB6	1	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*migulae*	192/192
HoB7	1	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	–	125/125
HoB7cons	3	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Shigella/Escherichia*	–	192/192
HoB8cons	3	Gammaproteobacteria	–	–	–	–	191/192*
HoB16	1	Actinobacteria	Actinomycetales	Corynebacteriaceae	*Corynebacterium*	–	173/180
HoB5	1	Actinobacteria	Bifidobacteriales	–	*Turicella*	*otitidis*	185/186[†]

*Uncultered gammaproteobacterium from honeybee intestines identified on the basis of the DNA sequence.
[†]The family classification of the genus *Turicella* is uncertain.

the taps of the tanks that showed the greatest microbial plate count (23).

Despite the presence of a few *Shigella/Escherichia* sequences, the results of the present analysis seem basically fit with the model of Snowdon and Cliver (21), that is, we find bacteria likely to occur in the natural environment around honey, all the more so because three 16S rDNA sequences (HoB8, HoB9, and HoB21) show strong similarity (99%) to an uncultured gammaproteobacterium detected in the intestine of honeybees (24).

Finally, it seems of interest to point out that, out of the 20 clones sequenced, none belong to a clostridium.

Plant DNA

Honeys rarely come from a single plant species even if they are attributed to a single species. Taking into account the health risk from plants producing toxins, it is crucial to assess correctly the identity of the plants from which pollen comes. Traditionally, the determination of the floral composition of honey has been achieved by the melissopalynology method, which is based on the identification of pollen by light microscopy (25). However, it requires highly specialized personnel who may not always be available in the control laboratories. For this reason, there is the need for an alternative and sensitive method. From this point of view, the application of molecular methods to the floral analysis in honey offers the possibility to detect a much greater range of vegetal species in honey, overcoming the limitations of the morphological identification of plant pollen and spores.

To analyze the flora composition of honey samples, we amplified a 159-bp fragment of the nuclear 18S rRNA gene using the primer pair Angio1f/Angio2r. These primers were designed in our laboratory matching the conserved sequences of the 18S rDNA of 12 monocotiledonous and dicotiledonous plants (26). Actually, they were shown capable of binding not only to angiosperms' DNA but also to the DNA of gymnosperms, pteridophytes, and fungi. We also obtained plant sequences amplifying the DNA extracts with primers NS5/NS6 useful for amplification and sequencing of nuclear rDNA from most major groups of fungi (27).

Amplfication products were obtained from all the sample extracts by using the primer set Angio1f/Angio2r. The NS5/NS6 primers resulted in successful amplification in two of the three extracts tested (Fig. 2). Sequencing analysis of the 18S rDNA clones of Angio1f/Angio2r and NS5/NS6 libraries from the "Marches" specimen showed a significant heterogeneity in the plant composition (Table 3). The sequences correspond to six orders of flowering plants. The prevailing order is the *Apiales* followed by the *Fabales*.

It is noteworthy that *Fabaceae*, *Asteraceae*, and *Convolvulaceae* families are important in the bee foraging because they include many nectariferous and polliniferous species. Likely the identification of these families reflects the abundance of the flora surrounding the apiary.

One could remark that an identification at the taxonomic level of family may not be sufficient to identify toxic plants. In fact, we can see that, in our honey specimen, several clones belong to the family Fabaceae. The Fabaceae (synonym Leguminosae) are known to

TABLE 3—*Taxonomic identification of the consensus sequences for the 18S rRNA gene clones (plants).*

Consensus	No. of Clones	Taxonomic Identification					Base Similarity
		Class	Order	Family	Genus	Species	
HoA2	1	Angiospermae	Apiales	Apiaceae/Pittosporaceae	–	–	112/117
HoN21	1	Angiospermae	Apiales	Araliaceae	–	–	264/264
HoA7	1	Angiospermae	Apiales	Apiaceae	–	–	115/117
HoA4	1	Angiospermae	Apiales	Apiaceae	–	–	116/117
HoA16	1	Angiospermae	Fabales	Fabaceae	*Astragalus*	–	115/116
HoN1	1	Angiospermae	Fabales	Fabaceae	–	–	263/264
HoA24	1	Angiospermae	Asterales	Asteraceae	–	–	114/117
HoN14	1	Angiospermae	Solanales	Convolvulaceae	*Ipomoea*	*hederacea*	263/264
HoN17	1	Angiospermae	Brassicales	Brassicaceae	–	–	264/264
HoN4	1	Angiospermae	Sapindales	Rutaceae/Meliaceae/Simaroubaceae	–	–	259/265
HoN2	1	Angiospermae	–	–	–	–	259/265
HoA13	1	Angiospermae	Fabales/Rosales	Fabaceae/Rosaceae	–	–	112/114

TABLE 4—*Taxonomic identification of the consensus sequences for the 18S rRNA gene clones (fungi).*

Consensus	No. of Clones	Taxonomic Identification					Base Similarity
		Class	Order	Family	Genus	Species	
HoA6cons	5	Ustilaginomycetes	Entylomatales	Entylomataceae	*Tilletiopsis*	–	108/119
HoN7cons	13	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Zygosaccharomyces*	–	263/263
HoN8	1	Saccharomycetes	Saccharomycetales		*Candida*	–	248/260

include one toxic genus (*Crotalaria*). However, at the present state of development of the DNA identification tests, we cannot tell whether the Fabaceae found in our specimen belongs or not to the genus *Crotalaria*. The same consideration can be made for the family Asteraceae (synonym Compositae). The family includes two tribes (Senecionae and Eupatoriae) that produce hepatotoxic alkaloids. In this case, we are unable to tell whether the Asteraceae present in our specimen belong or not to any of the two toxic tribes.

Fungal DNA

Microorganisms other than bacteria could be present in honey.

It has been reported that molds may survive but do not grow in honey; on the contrary, yeasts can easily grow because they are not inhibited by the low level of water and the high carbohydrate concentration available in honey (21). The yeasts most commonly found in honey are *Saccharomyces spp*. (28), but other genera have been reported such as: *Nematospora*, *Schizosaccharomyces*, *Torula*, and *Zygosaccharomyces* (29).

To characterize the fungal composition of the "Marches" honey specimen, we have analyzed 19 sequences of 18S rDNA clones amplified with both primer set NS1/NS2 and Angio1f/Angio2r. Related sequences were grouped into clusters and the consensus sequences were compared with the sequences deposited in GenBank using BLAST search. As shown in Table 4, the 19 clones could be grouped into three clusters (HoA6cons, HoN7cons, and HoN8) that were identified at the genus level. The highest number of clones was assigned to the *Zygosaccharomyces* genus. This corresponds with report from Schneider et al. (30), who tested four fermented honey samples from different geographical and botanical origins and isolated 20 yeast strains. By using morphological and physiological criteria, all strains were identified as *Zygosaccharomyces rouxii* or its imperfect form *Candida mogii*.

Five other clones (HoA6cons) were grouped within the genus *Tilletiopsis*, which comprises typical phylloplane-inhabiting fungi. *Tilletiopsis* species were reported as part of the apple phylloplane during later stages of the growth season and aerobiological studies demonstrated that *Tilletiopsis* spp. occur abundantly in air (31). In addition to *Tilletiopsis* spp. and *Zygosaccharomyces* genera, one clone displaying high similarity to *Candida* DNA was found. This result agrees with previous studies describing the isolation of yeast species related to *Candida* from bees, honey comb, and honey (32–34).

It seems important to remark that yeasts may ferment honey producing the loss of the tasteful qualities yet they are not toxin-producing organisms.

Conclusions

The outcome of this study demonstrates the feasibility of using DNA analysis to detect a wide range of natural components in honey. Despite the technical difficulties because of the high carbohydrates concentration, our results indicate that: (i) the experimental protocol to extract DNA from honey, developed in our laboratory, provides enough DNA suitable for PCR amplification; (ii) the method allows the detection of DNA from minority constituents such as symbiotic bacteria of the intestinal tract of honeybees; and (iii) the PCR analysis enables the identification of a wide range of plant species.

On the other hand, the results show that a better discriminating power is advisable, particularly in the case of plant DNA identification. In principle, this can be achieved by raising the length of the PCR systems. We know that, because of the state of fragmentation of the DNA from honey, it is possible to raise the length of the PCR systems up to a maximum of *c.* 400 bp. This length should be sufficient to raise the discriminating power of the test to the tribe (i.e., subfamily)—genus level in the case of plants and to the species level in the case of bacteria.

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