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# PCR diagnostic methods for *Ascosphaera* infections in bees

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**Abstract**

Fungi in the genus *Ascosphaera* are the causative agents of chalkbrood, a major disease affecting bee larval viability. Identification of individual *Ascosphaera* species based on morphological features has been difficult due to a lack of distinguishing characteristics. Most identifications are based on the size and shape of the ascomata, spore balls and conidia. Unfortunately, much overlap occurs in the size of these structures, and some *Ascosphaera* species will not produce sexual structures in vitro. We report a quick and reliable diagnostic method for identifying *Ascosphaera* infections in *Megachile* bees (leafcutting bees) using PCR markers that employ genus-specific primers for *Ascosphaera*, and species-specific primers for species known to be associated with *Megachile* spp. Using these methods, species identifications can be performed directly on bees, including asymptomatic individuals. Furthermore, the PCR markers can detect co-infections of multiple *Ascosphaera* species in a single host. We also identified a marker for *Ascosphaera apis*, the predominant cause of chalkbrood in *Apis mellifera*, the honey bee. Our diagnostic methods eliminate the need for culturing samples, and could be used to process a large number of field collected bee larvae.


*Keywords:* *Apis mellifera*; *Ascosphaera*; Bees; Chalkbrood; Disease diagnosis; *Megachile rotundata*; PCR

## 1. Introduction

The alfalfa leafcutting bee, *Megachile rotundata* (Hymenoptera: Megachilidae), is managed for the pollination of alfalfa seed crops. These bees nest in holes drilled or molded into wood or polystyrene blocks. *M. rotundata* is highly susceptible to chalkbrood, a disease caused by fungi in the genus *Ascosphaera* (Ascomycota: Plectomycetes: Ascosphaearales), and this disease is prevalent enough in the U.S. that most alfalfa seed growers rely on purchasing new bees each year to meet their pollination needs.

To date, 22 species of *Ascosphaera* have been identified (Bissett et al., 1996; Youssef and McManus, 2001), and all of these are found only in association with bees, either as a pathogen or as a saprophyte on the pollen stores in nests. The *Ascosphaera* are placed in the Plectomyces based on morphological characteristics, and this placement is substantiated by sequence analysis of the ribosomal gene region (Berbee and Taylor, 1992). Anderson et al. (1998) proposed a phylogeny of 20 *Ascosphaera* spp. based on the DNA sequence of this same region.

The *Ascosphaera* have proved to be a difficult group to identify due to a lack of distinguishing characteristics, and some species are difficult to culture, requiring specialized growth media or conditions. Most identifications are based on the appearance of the ascomata in vivo, and the size and shape of the spore balls and conidia. Unfortunately, much overlap occurs between species in the size of these structures. Molecular methods for identifying some species have been previously developed, including isozyme analysis (Maghrabi and Kish, 1985, 1987, 2001) and RAPD-PCR (Lu et al., 1996). However, both of these techniques require either pure cultures of spores, or nearly pure cultures with little or no bee tissue. The pathogens must be isolated and grown in culture, or the infected bees must be symptomatic cadavers that consist primarily of fungal tissues. Thus, it

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would be difficult to use this method to identify infections in bees that are still alive and asymptomatic. In addition, the results from such analyses are confounded if co-infections occur in a single host, a condition that is likely to arise (Johnson et al., 2005).

Polymerase chain reaction (PCR) techniques utilize highly specific primers and amplify only target DNA sequences in samples contaminated with other DNA. PCR markers could provide a quick and reliable diagnostic method for *Ascosphaera* infections. Primers developed for the different species of *Ascosphaera*, could also be used to identify co-infections. We report here on PCR diagnostic methods to identify the *Ascosphaera* species that occur in association with *Megachile* bees, and for *A. apis*, the predominant cause of chalkbrood in *Apis mellifera*, the honey bee.

## 2. Methods

### 2.1. Source of fungal cultures

We obtained pure cultures for eight species of *Ascosphaera* (Table 1), but were unable to obtain cultures of *A. columbrina*, *A. variagata*, and *A. asterophora*, which are also recorded from *Megachile* hosts. These 11 species comprise all the recorded species to occur in *Megachile* spp. or *Apis mellifera*. In addition, we obtained *Eremascus albus* and *Chrysosporium farinicola*, due to their close relationship to the *Ascosphaera*. For both of these, the strains obtained were the type specimen. *C. farinicola* is the anamorph of *Bettsia alvei*, a fungus that grows saprophytically on bee pollen provisions. *Eurotium*, *Monascus*, and *Sordaria* cultures (Table 1) were also obtained to verify that our specific primers would not amplify DNA from other common, unrelated fungal species.

Table 1
Fungal isolates used for testing genus- and species-specific primers

| Species | Strain | Source |
|---|---|---|
| *Ascosphaera aggregata* | Wild1 | Isolated from *Megachile rotundata*, Logan, UT |
| *Ascosphaera aggregata* | 690 | ARSEF[a] |
| *Ascosphaera acerosa* | 201316 | ATCC[b] |
| *Ascosphaera apis* | 13785 | ATCC |
| *Ascosphaera apis* | 13786 | ATCC |
| *Ascosphaera atra* | 693 | ARSEF |
| *Ascosphaera atra* | 5147 | ARSEF |
| *Ascosphaera flava* | 5144 | ARSEF |
| *Ascosphaera larvis* | 262708 | ATCC |
| *Ascosphaera proliperda* | 696 | ARSEF |
| *Ascosphaera proliperda* | 28358 | ATCC |
| *Ascosphaera pollenicola* | 62712 | ATCC |
| *Eurotium cheraleri* | | B. Kropp, Utah State University |
| *Chrysosporium farinicola* | 18053 | ATCC |
| *Eremascus albus* | 11665 | ATCC |
| *Monascus perperaseens* | | B. Kropp, Utah State University |
| *Sordaria* spp. | | B. Kropp, Utah State University |

[a] USDA Agricultural Research Service Entomopathogenic Fungi Collection, Ithaca, NY.
[b] American Type Culture Collection, Manassas, VA.

All fungal species were cultured on Saboraud dextrose agar and potato dextrose agar, except *Ascosphaera aggregata*, which was cultured on V-8 agar (James and Buckner, 2004). V-8 Agar consists of 50 ml low sodium V-8 Juice (Campbell Soup, Camden, NJ), 5.3 g maltose, 1.6 g yeast extract, 0.11 g $MgSO_4$, 0.04 g thiamine, 1.1 mg biotin, 7.5 ml Graces Medium (Invitrogen, Grand Island, NY), 0.5 ml canola oil, 0.5 ml 0.1% Triton X-100, and 1.5 g agar, and enough deionized water to bring the volume to 100 ml. The pH was adjusted to 6.0 before autoclaving.

### 2.2. DNA extraction and PCR conditions

DNA was extracted from pure *Ascosphaera* cultures, uninfected bee larvae, and larvae infected with *A. aggregata* using Ultra Clean plant DNA isolation kits (Mo Bio Laboratories, Solana Beach, CA). PCR analyses were conducted in 25 μl reactions consisting of 19.9 μl reverse osmosis purified water, 2.5 μl of 10× buffer, 0.5 μl of 10 mM dNTP mix, 0.5 μl of each primer (at 20 μM each), 1 μl DNA, and 0.1 μl Taq DNA polymerase (5 U/μl, Eppendorf HotMaster Taq, Brinkmann Instruments, Westbury, NY). Primer sequences are listed in Table 2. Thermocycle conditions were: an initial denaturing for 10 min at 94 °C; 30 cycles of 45 s denaturing at 94 °C, 45 s annealing at 62 °C, and 1 min extension at 72 °C; followed by a final extension for 5 min at 72 °C. PCR products were electrophoretically separated on standard agarose gels.

### 2.3. Primer design

The primers used here were all derived from conserved regions of the DNA sequences reported by Anderson et al. (1998) for the 5.8S ribosomal DNA and the internal transcribed spacers (ITS 1 and ITS 2) using the program GeneRunner (Fig. 1).

### 2.4. Testing primers on pure fungal cultures

To test the specificity of the primer pairs that we wanted to use as diagnostic markers, PCR reactions were conducted for each primer using DNA extracted from every one of our fungal cultures.

To test the sensitivity of the PCR reactions, DNA was extracted from *A. aggregata* (wild1) and *A. acerosa* (ATTC 201316), then diluted to 116 ng/ml. The DNA concentration was determined using a fluorometer (Picofluor Handheld, Turner Designs, Sunnyvale, CA) and PicoGreen (Molecular Probes, Eugene, OR), a fluorescent dye with specificity to double stranded DNA. A series of fivefold dilutions were prepared in 0.1% TE buffer to obtain solutions of 23.2, 4.64, 0.93, 0.185, and 0.037 ng DNA/ml. PCR analyses were conducted using 1 μl of each dilution as template DNA to assess the minimum concentration that would amplify. The sensitivity analyses were repeated three times.

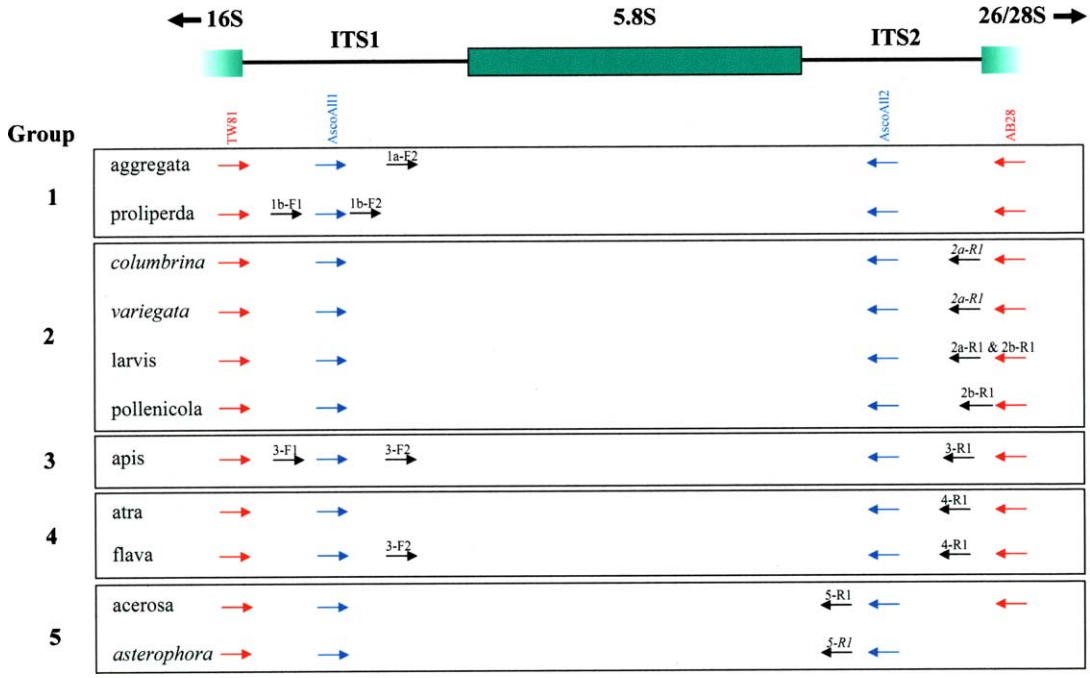


Fig. 1. Relative location of each primer on the ITS1–5.8S–ITS2 genomic region, and the *Ascosphaera* species that each primer will amplify. All species are amplified by the TW81/AB28 and AscoAll1/AscoAll2 primer pairs. Species in italics were not tested, but amplification is predicted based on the DNA sequences.

Table 2
Primers used as markers for *Ascosphaera* spp. detection

| Identifier | Forward or reverse | Primer sequence (5′–3′) |
|---|---|---|
| TW81[a] | F | GTT TCC GTA GGT GAA CCT GC |
| AB28[a] | R | ATA TGC TTA AGT TCA GCG GGT |
| AscoAll1 | F | GCA CTC CCA CCC TTG TCT A |
| AscoAll2 | R | GAW CAC GAC GCC GTC ACT |
| 1a-F2 | F | GGA AAA GAC CCT CGA CGA G |
| 1b-F1 | F | GTG CTT TCC CGG TAC TCC |
| 1b-F2 | F | ACC TGC GGG CCT TCG TG |
| 2a-R1 | R | CCA CAG AAA AAA ATA ATG GTT GGT TC |
| 2b-R1 | R | CCA CAG AAA AAA TAA TAG TTG GTT CG |
| 3-F1 | F | TGT CTG TGC GGC TAG GTG |
| 3-F2 | F | GGG TTC TCG CGA GCC TG |
| 3-R1 | R | CCA CTA GAA GTA AAT GAT GGT TAG A |
| 4-F1 | F | TGT GTT TGT GCG GCC TTC AC |
| 4-R1 | R | TAG TAG ATG GTT GGA CCG ACG |
| 5-R1 | R | TTT TCA GGT GCG TCC TTC CA |

All are on either the ITS1 or ITS2 internal transcriber regions of the 5.8S ribosomal DNA.

[a] From Curran et al. (1994).

### 2.5. Testing PCR on field collected Megachile rotundata larvae

Nests of *M. rotundata* were collected in September 2004, from an alfalfa seed grower in Warden, WA. The nests were stored at 5 °C until tested in February 2005. *A. aggregata* infections were confirmed by the formation of elongate ascomata under the cuticle of the dead insect, as described by Bissett et al. (1996). *Ascosphaera* infections should be symptomatic by the prepupal stage, and so asymptomatic prepupae were considered to be free of infection.

Fifty *A. aggregata*-infected and 50 healthy prepupae were removed from the stored boards, individually surface cleaned by submerging in a 1% chlorine solution (sodium dichloro-*s*-triazinetrione dihydrate, Quantum Biochemical, Alpharetta, GA) for 3–7 min, followed by three rinses in sterile, distilled water. New aliquots of chlorine solution were used for each insect to avoid cross contamination. After surface cleaning, DNA was extracted as described above.

To validate the surface cleaning technique, we brushed thirty healthy prepupae with *A. aggregata* spores. Ten were washed using the chlorine rinse procedure, 10 were rinsed in water four times, and the last 10 were not washed at all. DNA was extracted from all the larvae, and PCR was conducted using the *Ascosphaera* genus-specific primer pair.

## 3. Results

### 3.1. Testing primers on pure fungal cultures

*Specificity of the primers.* We identified a genus-specific primer pair (AscoAll1 and AscoAll2) (Table 2) from regions of ITS1 and ITS2 that were conserved among all the *Ascosphaera* in GenBank and Anderson et al. (1998), but distinct from *Eremascus albus*, the most closely related non-*Ascosphaera* species. In addition, we were able to develop species-specific primer pairs for *A. aggregata*,

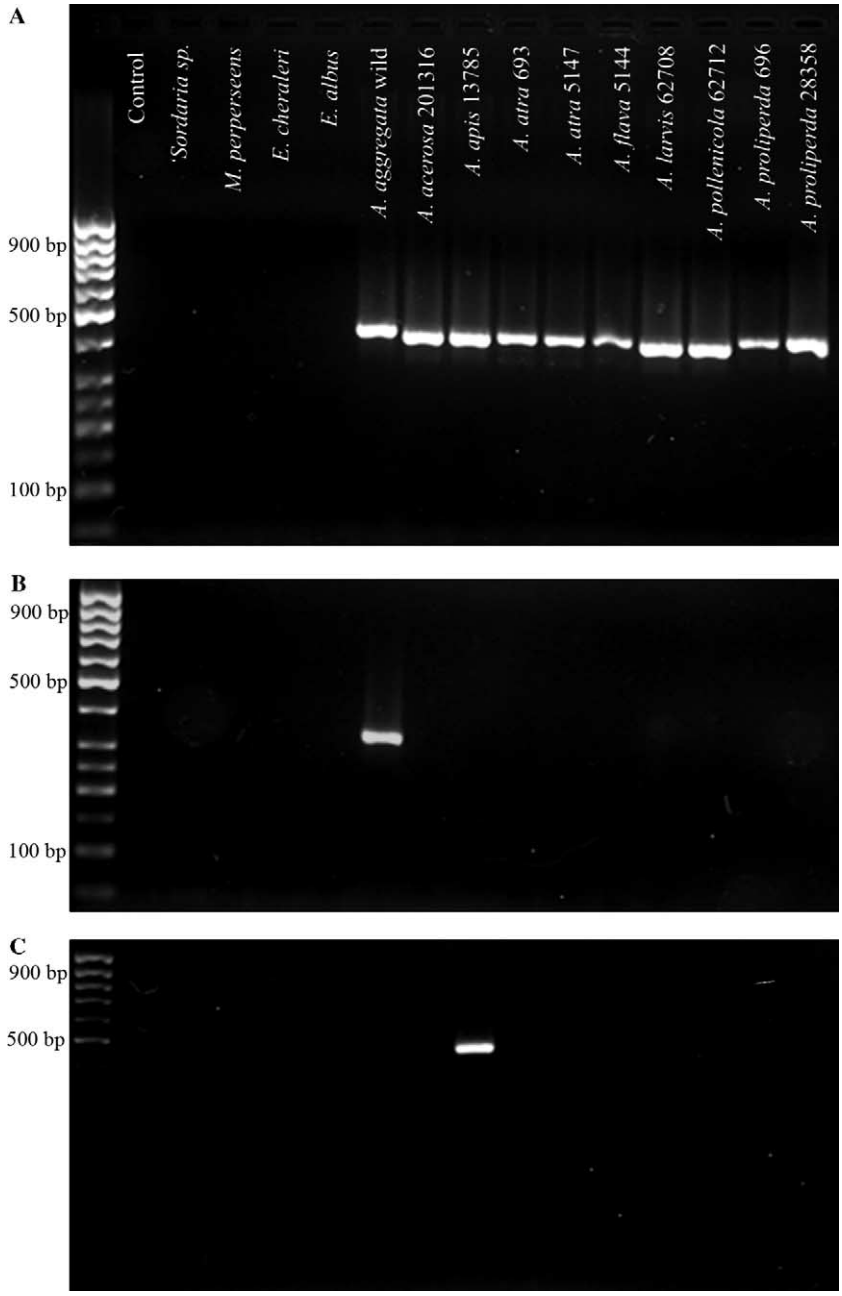


Fig. 2. Gel electrophoresis images of the PCR products when template DNA was extracted from different fungal species, and using the genus-specific primer pair AscoAll1/AscoAll2 (A), the *Ascosphaera aggregata* species-specific primer pair 1a-F2/AscoAll2 (B), and the *A. apis* species-specific primer pair 3-F1/AscoAll2 (C). The control was a PCR reaction with no template DNA.

*A. proliperda*, and *A. apis* (Table 2). Anderson et al. (1998) found *A. variegata*, *A. columbrina*, *A. larvis*, and *A. pollenicola* to be a closely related group, with only point mutations present between the various species. We called these species Group 2, and were able to design primer pairs specific for the group, or subsets of the group, but not for the individual species. A similar case existed for *A. atra* and *A. flava* (our Group 4), and *A. acerosa* and *A. asterophora* (our Group 5) (Table 2, Fig. 1).

The AscoAll1/AscoAll2 primer pair successfully primed all the *Ascosphaera* species that we tested (Figs. 1 and 2A), and the product size was approximately 550 bp. *A. aggregata* was the only species that could be distinguished on a gel based on the product size, which was slightly larger than the others (Fig. 2A). All of the primers in Fig. 1 were tested against all of the fungal species in Table 1, but amplification occurred only for those species associated with it in Fig. 1 (as shown for 1a-F2/AscoAll2 (Fig. 2B) and 3-F1/AscoAll2 (Fig. 2C)). The primer 2a-R1 is likely to amplify a specific product from *A. columbrina* and *A. variegata* based on the reported DNA sequences. Similarly, *A. asterophora* is likely to be amplified by the primer 5-R1 based on the reported sequence. However, we were unable to obtain cultures of these fungi to verify that

these primers actually yield the respective predicted products.

To readily distinguish *A. pollenicola* from *A. larvis*, one would have to run two reactions, one with AscoAll1/2a-R1 and a second with AscoAll1/2b-R1. If both reactions were positive, the species is *A. larvis*, if only the second reaction is positive, the species is *A. pollenicola* (Fig. 1). Based on the DNA sequence, we would not expect 2b-R1 to amplify the other species in Group 2. Similarly, two reactions are needed to identify *A. atra*, yet separate it from *A. flava* (AscoAlla/4-R1 and 3-F2/AscoAll2, Fig. 1).

*Sensitivity of the fungus-specific and Ascosphaera-specific primers.* Using *A. aggregata* DNA and the TW81/AB28 primers, the lowest concentration that produced a band was 0.185, 0.185, and 0.93 ng/ml (respectively, for each replicate run), yielding an average of 0.43 ng/ml, approximately equal to 0.22 mg of fresh hyphae. For the AscoAll1/AscoAll2 primer pair, the lowest concentrations that yielded detectable levels of DNA were 0.037, 0.037, and 0.185 ng/ml, with an average of 0.086 ng/ml, approximately equal to 0.044 mg hyphae. The detection limits for *A. acerosa* DNA did not differ with the primer pair. For both AscoAll1/AscoAll2 and TW81/AB28, the detection limits were 0.93, 0.185, and 0.037 ng/ml for the three replicates, with a mean of 0.38 ng/ml, approximately equal to 0.20 mg hyphae.

### 3.2. Testing PCR on field collected Megachile rotundata larvae

For the 50 field collected larvae that appeared healthy, all tested negative for *Ascosphaera* and *A. aggregata.* For the 50 field collected *A. aggregata*-infected cadavers, all showed positive for both *Ascosphaera* and *A. aggregata.* Thus, we obtained neither false positives nor false negatives when analyzing field samples. We note, however, that when we tried higher concentrations of chlorine, fewer rinses, or a shorter period of washing in chlorine, we were prone to about a 10% error rate. If the bees were not soaked long enough in the chlorine, we would occasionally obtain false positives. If the bees were rinsed in water only twice, follow-

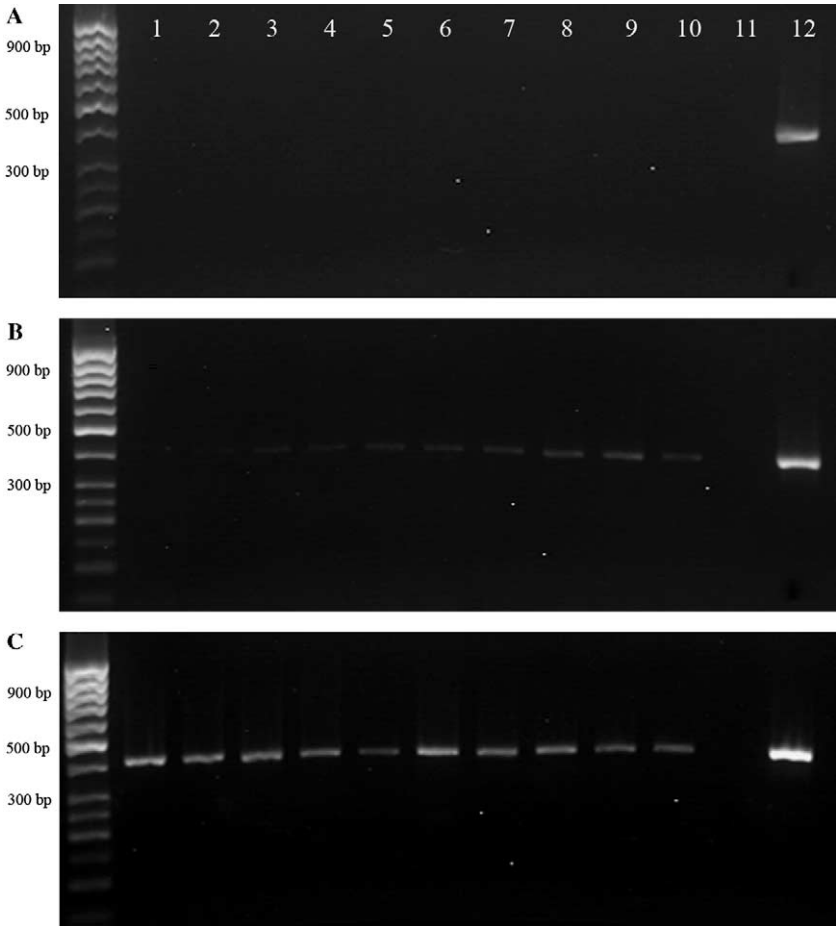


Fig. 3. Gel electrophoresis images for DNA amplified with the AscoAll1/AscoAll2 primer pair when testing adequacy of cleaning procedures for field collected larvae. First 10 lanes are *M. rotundata* larvae, 11th lane is a DNA-blank control, and the 12th lane is an *Ascosphaera aggregata*-DNA positive control. Healthy larvae were brushed with *A. aggregata* spores, then were either soaked in 1% chlorine for 3–7 min, followed by three rinses in distilled water (A), or soaked in distilled water for 3–7 min, followed by three rinses in more distilled water (B), or left untreated before DNA was extracted (C).

ing the chlorine wash, instead of three times, we would occasionally get false negatives (presumably due to residual chlorine contamination inhibiting the PCR reaction). The wash conditions with chlorine that we used adequately removed contaminating, non-infecting spores from healthy larvae, thus reducing the chances of getting a false positive (Fig. 3).

## 4. Discussion

We demonstrate that *Ascosphaera* infections can be diagnosed using PCR markers to the ITS region of ribosomal DNA, and the specific primer sets designed from species-variable segments of this region, can differentiate between *Ascosphaera* species. This diagnostic technique is very sensitive, and we could detect fungal DNA that was added to healthy larvae, an indication that we should also be able to identify disease in asymptomatic, young larvae. Our PCR marker methodology for diagnosing *Ascosphaera* infections in bee larvae should allow field samples to be quickly and accurately processed, thus large numbers of larvae could be sampled for infections throughout the nesting season. In addition, our primers could be used to determine whether bees with symptoms atypical of chalkbrood were actually infected with an *Ascosphaera*, leading to better identification of diseases in these bees, or even the discovery of new *Ascosphaera* species.

As stated earlier, the taxonomy of the *Ascosphaera* is based on spore and ascomata characteristics. This poses two problems in diagnosing disease. First, not all species sporulate readily in vitro, yet previous disease diagnosis depended on either having a fully sporulating cadaver, or getting spore production in culture. Second, the spores could not be reliably identified from environmental samples (such as honey, air samples, or scrapings from nesting materials). Our PCR markers overcome these limitations, and additionally, they could be used to detect co-infections of two or more *Ascosphaera* spp. in a single host. When identifications require culturing and co-infections are present, slower growing species are frequently overlooked. Chen et al. (2004) used a similar method for detecting virus co-infections in honey bees. However, unlike the virus PCR products, all of our products are of a similar size and so we cannot utilize a nested reaction.

Reynaldi et al. (2003) developed a PCR method for detecting *A. apis* in honey. Their technique was useful for differentiating *A. apis* strains, but it required the time consuming process of isolating the fungus and culturing it in vitro, and then sequencing the DNA to identify specific repetitive regions. Lauro et al. (2003) and Piccini et al. (2002) developed PCR markers for detecting *Paenibacillus larvae* (the causative agent for American foulbrood) in honey. Our PCR marker could probably be used for detecting *Ascosphaera* spores in honey, as well, or in other environmental samples, such as the air samples (as described by Williams et al., 2001), without the need for culturing the contaminating spores.

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