
New primers for detection of *Smittium* spp. (*Trichomyces*, *Zygomycota*) in insect hosts

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The distinctive thalli of trichomyces can only be isolated from the larval stage of the host. There is a general lack of knowledge on the presence of trichomyces in other developmental stages of their host. Two new primers, TR3F and TR4R, were designed based on SSU ribosomal DNA sequences from two *Harpella* species, one *Harpellomyces* species, four *Smittium* species and *Aedes aegypti* (host). These primers amplified DNA from species of the *Harpellales* but discriminated against other groups of organisms including other fungal phyla. The presence of *Smittium* species was detected in laboratory-infected pupae and adult flies of *Aedes aegypti* and in field-collected larvae of *Culex pipiens* by PCR using the new primers.

Key words: *Aedes*, *Culex*, fungi, *Harpellales*, rRNA.

Introduction

Trichomyces (*Zygomycota*) are fungi living in the digestive tracts of arthropods (Alexopoulos *et al.*, 1996). The class includes three orders: *Asellariales*, *Eccrinales* and *Harpellales* (Gottlieb and Lichtwardt, 2001). Traditionally, the *Amoebidiales* was recognised as a trichomycetous order but was found to be related to the Protozoa based on morphology and rRNA gene phylogeny (Walker, 1984; Benny and O'Donnell, 2000).

Recently, the phylogeny of a number of trichomyces has been examined (O'Donnell *et al.*, 1998; Benny and O'Donnell, 2000; Benny and White, 2001; Gottlieb and Lichtwardt, 2001). O'Donnell *et al.* (1998) investigated the inter-ordinal relationships between *Harpellales* and *Kickxellales*, while Gottlieb and Lichtwardt (2001) tested the monophyly of the genus *Smittium*. All isolates used in both studies were culturable, but overall only 16% of the known genera and approximately 13% of the species can be cultured axenically and all belong to the *Harpellales* (Misra and Lichtwardt, 2000). None of the known species of

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the *Asellariales* and *Eccrinales* grows in culture, which makes it difficult to extract and sequence their DNA for phylogenetic studies. Non-culturability of most trichomycetes may relate to specific nutritional requirements for germination (Horn, 1989).

Propagules of trichomycetes are mostly isolated from the larval stage of the insect host. Presence of the fungi in other developmental stages (pupae, adult) of the host has not been verified although it has been postulated. Fungal-like structures have been observed in those stages, but their identities cannot be validated by microscopy (A.M. Rizzo, pers. observ.). This study is aimed at examining the infection cycle of the mosquito, *Aedes aegypti*, by *Smittium culicis* (*Legeriomycetaceae*, *Harpellales*) using polymerase chain reaction. Two trichomycete-specific primers targeting the 18S rRNA gene were designed to amplify the fungal DNA from members of *Harpellales*. The specificity of these primers to amplify fungal DNA from different developmental stages (larval, pupal and adult) of the mosquito was also tested.

Materials and methods

Primer design

Two new oligo-DNA primers, TR3F (5'-GGCACTGTCAGTGGTGAA ATAC-3') and TR4R (5'-GATTTCTCTTACGGTGCCAAGCA-3'), were designed based on alignments of the SSU ribosomal RNA gene sequences from *Aedes aegypti* (U65375), *Harpella melusinae* (unpublished), *H. tica* (unpublished), *Harpellomyces eccentricus* (unpublished), *Smittium caudatum* (AF277031), *S. phytotelmatum* (AF277025), *S. simulii* (AF277015, AF277046) and *S. tipulidarum* (AF277043). The priming regions of TR3F and TR4R are shown in Fig. 1. The sequence of the primers was compared against other sequences in the GenBank using the nucleotide-nucleotide BLAST (blastn) search to determine their target organisms.

Test of primer specificity

In order to test primer specificity, genomic DNA from a range of different organisms was used as templates for PCR (Table 1). Two DNA extraction procedures were adopted. For the animal and fungal DNA, biomass (~100 mg) was washed twice with sterile distilled water, blotted dry by filter paper and immediately frozen in liquid nitrogen. Material was ground into fine powder using a mortar and pestle, and DNA extracted with the DNeasy Plant DNA Extraction Kit (Qiagen) according to the manufacturer's instructions. For

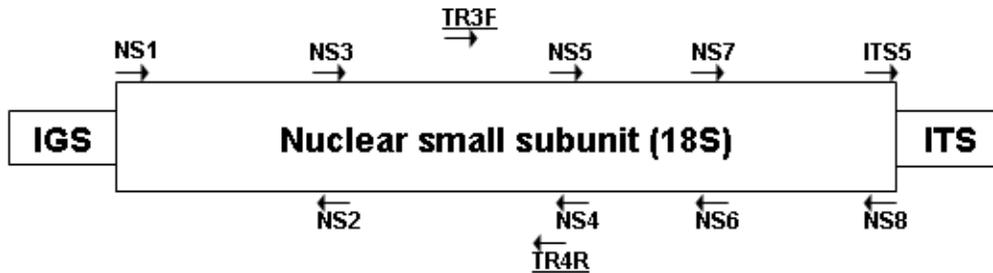


Fig. 1. The primer map of the nuclear small subunit (18S) showing the priming regions of TR3F and TR4R.

Amoeba sp., *Chlorella* sp. and *Thraustochytrium aureum*, a microwave DNA extraction method was used (Lee and Taylor, 1990). The organisms were centrifuged down at ~6,000 g for 5 minutes, resuspended in 300 μ l lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulphate, 1% β -mercaptoethanol) in a 1.5 ml Eppendorf tube, vigorously vortexed for 1 minute, microwaved three times for 10 seconds each at maximum power, and subsequently incubated at 70°C for 10 minutes. One volume of phenol:chloroform:isoamyl alcohol (25:24:1, by volume) was added and the tube inverted several times. The tube was centrifuged (~15,000 g) for 10 minutes at room temperature, and the upper aqueous layer was transferred to a new 1.5 ml Eppendorf tube. Half a volume of ammonium acetate (7.5 M) and 2.5 volume of absolute ethanol were added with the tube being left on ice for 10 minutes. The tube was centrifuged (~15,000 g) for 10 minutes at 4°C. The DNA pellet was washed with 100 μ l 70% ethanol, centrifuged for another 10 minutes, air-dried and resuspended in 20 μ l Tris-EDTA buffer.

The nuclear SSU rRNA gene was amplified from the genomic DNA with primers NS5 and NS8 (White *et al.*, 1990). These products were further amplified with the new primers TR3F and TR4R. PCR reactions were performed in 50 μ l containing about 20 ng DNA, 0.2 μ M of each primer, 0.2 mM of each dNTP, 2.5 mM MgCl₂ and 1.25 U of Taq Polymerase (Gibco). The amplification cycle consisted of an initial denaturation step of 95°C for 2 minutes, followed by 35 cycles of (i) denaturation (95°C for 1 minute), (ii) annealing (55°C for 1.5 minutes) and (iii) elongation (72°C for 1.5 minutes) and a final 10 minute elongation step at 72°C. The PCR products were separated in a 1% agarose gel supplemented with 0.2 μ g/ml ethidium bromide at 80V for an hour and visualised under a gel documentation system.

Table 1. Polymerase chain reaction on genomic DNA of different groups of organisms using primer pairs NS5-NS8 and TR3F-TR4R. (+ sign for successful amplification, - sign for unsuccessful amplification).

Group	Organisms	Primer pair	
		NS5-NS8	TR3F-TR4R
Metazoa	<i>Culex pipiens</i>	+	-
Metazoa	<i>Aedes aegypti</i>	+	-
Metazoa	<i>Simulium simulii</i>	+	-
Fungi (<i>Ascomycota</i>)	<i>Jahnula</i> sp.	+	-
Fungi (<i>Basidiomycota</i>)	<i>Poria monticola</i>	+	-
Fungi (<i>Trichomycetes</i>)	<i>Smittium culicis</i>	+	+
Fungi (<i>Trichomycetes</i>)	<i>Smittium culisetae</i>	+	+
Fungi (<i>Trichomycetes</i>)	<i>Smittium macrosporium</i>	+	+
Fungi (<i>Zygomycetes</i>)	<i>Mucor hiemalis</i>	+	-
Viridiplantae	<i>Chlorella</i> sp.	+	-
Stramenopiles	<i>Thraustochytrium aureum</i>	+	-
Lobosea	<i>Amoeba</i> sp.	+	-

Detection of Smittium species in insect hosts

Inoculum was prepared by subculturing *Smittium culicis* in a modified TG medium containing 20 g l⁻¹ tryptone, 5 g l⁻¹ glucose, 0.28 g l⁻¹ KH₂PO₄, 0.35 g l⁻¹ K₂HPO₄, 0.26 g l⁻¹ (NH₄)₂SO₄, 0.1 g l⁻¹ MgCl₂·6H₂O, 0.07 g l⁻¹ CaCl₂·2H₂O, 200 µg l⁻¹ Thiamine HCl and 50 µg l⁻¹ Biotin (Whisler, 1962). Spores were separated from mycelium by filtering 50 ml culture through sterile glass wool. Spore count was standardised to 5 × 10⁴ spores/ml. Five drops of the spore suspension were added to tubes containing 10 ml of liver extract and ten larvae of *Aedes aegypti*, which were less than 2 hours old. These infected larvae, which pupated within 14 days of hatching, were then allowed to develop into adult flies in a temperature controlled room at a constant 28°C ± 1°C with a 12-hour light and 12-hour dark photoperiodicity. Concurrently, larvae of *Culex pipiens* were collected from a site within the grounds of the University of Portsmouth, UK. Genomic DNA of laboratory-infected pupae and adult flies of *A. aegypti* and field-collected larvae of *C. pipiens* was extracted with the DNeasy Plant DNA Extraction Kit (Qiagen) according to the manufacturer's instructions. PCR was performed on the genomic DNA with primers NS5-NS8 and TR3F-TR4R using the PCR profile described above. The PCR product amplified from TR3F-TR4R of laboratory-infected pupae was purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and sequenced at the University of Dundee. Sequence identity was elucidated using the nucleotide-nucleotide BLAST (blastn) search in the GenBank.

Results

Primer Specificity

BLAST search results revealed that both TR3F and TR4R are very specific towards taxa of the *Harpellales*. Sequence matches of TR3F with 100% identity include a number of *Smittium* spp., *Furculomyces boomerangus* and *Genistelloides hibernus* (*Harpellales*). On the other hand, the sequence match with the highest score and 100% identity of TR4R is *Smittium simulii*. Sequences having 1 nucleotide mismatch with TR4R include *Smittium* spp., *Genistelloides hibernus* and *Undatella* sp. (straminopiles).

Specificity of these primers against other groups of organisms was tested using PCR (Table 1). All DNA tested was amplified by NS5-NS8, which are universal primers for eukaryotic organisms. TR3F-TR4R amplified DNA from all three *Smittium* species but not from other groups of organisms including: (1) Metazoan (*Aedes aegypti*, *Culex pipiens*, *Simulium simulii*); (2) Viridiplantae (*Chlorella* sp.); (3) stramenopiles (*Thraustochytrium aureum*); (4) Lobosea (*Amoeba* sp.); and (5) other assemblages of fungi (*Jahnula* sp., *Mucor hiemalis* and *Poria monticola*).

Detection of Smittium species in insect hosts

Genomic DNA of laboratory-infected pupae and adult flies of *A. aegypti* and field-collected larvae of *C. pipiens* was amplified by the universal eukaryotic primer pair NS5-NS8 and the new primers TR3F-TR4R (Fig. 2). The PCR product amplified from TR3F and TR4R of laboratory-infected pupae of *A. aegypti* was sequenced to determine its identity. Four sequences having the highest score from the BLAST search included two *Smittium culicis* isolates, *S. annulatum* and *S. simulatum* but the highest similarity was with *S. culicis* and *S. simulatum*. The GenBank accession number of the new sequence is AY677681.

Discussion

We developed two primers, TR3F and TR4R, that specifically detect *Smittium* species from different developmental stages of their insect hosts. These primers amplified specifically *Smittium* species discriminating not only against their insect hosts, but also against other eukaryotic organisms including other assemblages of fungi (*Ascomycota*, *Basidiomycota*, *Zygomycetes*).

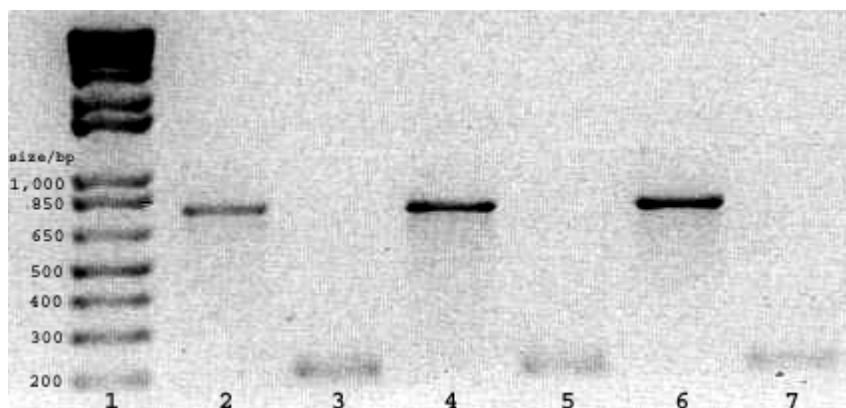


Fig. 2. Lane 1, DNA size marker; lane 2, PCR with NS5-NS8 on larval stage of *Culex pipiens* from environment; lane 3, PCR with TR3F-TR4R on larval stage of *C. pipiens* from environment; lane 4, PCR with NS5-NS8 on pupal stage of infected *Aedes aegypti* with *Smittium culicis*; lane 5, PCR with TR3F-TR4R on pupal stage of infected *A. aegypti* with *S. culicis*; lane 6, PCR with NS5-NS8 on adult stage of infected *A. aegypti* with *S. culicis*; lane 7, PCR with TR3F-TR4R on adult stage of infected *A. aegypti* with *S. culicis*.

Several applications are possible using TR3F and TR4R to study trichomycetes, including environmental detection of populations in insect larvae (e.g. López Lastra *et al.*, 2003). Although the primers were originally designed to amplify the SSU rRNA gene of *Smittium* spp., they could amplify other genera including *Capniomyces*, *Furculomyces*, *Genistelloides*, *Harpella*, *Harpellomyces* and possibly other genera in the *Harpellales* as there is only one nucleotide mismatch between sequences of these taxa and the primers. PCR amplification occurs even if there is one nucleotide mismatch between the primers and the template sequences, e.g. in *S. culicis* (Table 1). The new primers may be useful in obtaining sequences from non-culturable harpellalean taxa for phylogenetic study. Although TR4R is specific to a *Undatella* sp. (one base difference), TR3F is specific towards the *Harpellales*.

The distinctive thalli of the trichomycetes have only been observed in the larval stage of the hosts, providing morphological information of these fungi in this stage. There is a lack of knowledge on the morphological form these fungi might exist in other developmental stages, i.e. egg, pupa and adult. Moss and Descals (1986) observed a species of the *Harpellales* associated with oviposited simuliid eggs. The infection experiment in this study confirmed the presence of *S. culicis* in laboratory-infected pupae and adults of *A. aegypti* and larvae of *C. pipiens* from environment. The infestation of larvae by most species of *Smittium* is not detrimental to the host. Horn (1989) reported that the presence of these fungi in the gut may have a beneficial effect under certain

environmental conditions. The potential effect(s) of infestation in the pupae and adults is still not fully understood. However, presence of the ovarian stage in the gravid adult female can partly/wholly replace the eggs, rendering the individual biologically sterile (Moss and Descals, 1986). The detection of *S. culicis* in the pupal and adult stages of *A. aegypti* suggests that, the fungus is passed from larva to egg through the pupal and adult stages. Unfortunately, the morphological form of the trichomycetes present in those stages cannot be identified and consolidated by microscopy. Fungus-like structures have been observed from infected adults (A.M. Rizzo, pers. observ.) but cannot be confirmed as trichomycetes due to the lack of distinctive identification features. Molecular techniques combined with microscopic techniques (light or electron microscopy) may therefore lead to a more complete understanding of the infection cycle of trichomycetes.

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