Biol Fertil Soils (2003) 37:70–72
DOI 10.1007/s00374-002-0556-3

SHORT COMMUNICATION

Carsten Renker · Jörn Alphei · François Buscot

Soil nematodes associated with the mammal pathogenic fungal genus *Malassezia* (Basidiomycota: Ustilaginomycetes) in Central European forests

Received: 16 May 2002 / Accepted: 27 September 2002 / Published online: 12 November 2002

Abstract Screening forest soil nematodes for associated fungi by PCR, and sequencing the internal transcribed spacer detected the human, and other mammals, pathogenic fungus *Malassezia* in association with soil nematodes for the first time in Europe. *Malassezia restricta* and *M. globosa* were associated with the nematode genus *Malenchus* sp., whereas another nematode, *Tylolaimophorus typicus* hosted only *M. restricta*.

Keywords *Malassezia* · Nematodes · Pathogenic fungi · Ustilaginomycetes

Introduction

Malassezia is a distinct fungal genus within the Ustilaginomycetes (Begerow et al. 2000), and seven species are currently known on the basis of molecular and morphological features (Guého et al. 1996; Gupta et al. 2000). The impact of nearly all the species of *Malassezia* on different skin diseases has been well documented in humans and other mammals (Aizawa et al. 2001; Ashbee and Evans 2002; Crespo et al. 2000). Besides its pathogenicity, *Malassezia* is an anthropophilic fungal genus belonging to the established physiological skin flora. It can grow in both yeast and mycelial phases. On non-affected skin, it is mainly prevalent in the yeast phase (Ashbee and Evans 2002; Schmidt 1997).

Nematodes are the most numerous metazoans in soil. Interactions between fungi and nematodes in soils may be grouped as follows: (1) nematodes as predators of fungi (Bakhtiar et al. 2001), (2) fungi as predators or parasites of nematodes (e.g. Viaene and Abawi 2000), (3) etiological relations between nematodes and fungi as partners of plant disease complexes (Zahid et al. 2002), and (4) nematodes acting as vectors for fungal spores (Mendoza de Gives et al. 1999).

In South American cattle, a correlation of nematode infestation with appearance of external otitis provoked by *Malassezia* was suggested (Duarte et al. 2001). While studying the nematode populations in the soil of a beechwood forest in Central Europe, species were screened by PCR with fungal primers to reveal their feeding abilities and the presence of parasitic or other nematode-associated fungi. This study validates the assumption that nematodes may act as vectors for species of *Malassezia*.

C. Renker · F. Buscot (✉)
Institute of Ecology, Department of Environmental Science, University of Jena, Dornburger Str. 159, 07743 Jena, Germany
e-mail: Francois.Buscot@uni-jena.de
Tel.: +49-3641-949451
Fax: +49-3641-949452

J. Alphei
Institute of Zoology and Anthropology, Ecology Group, University of Göttingen, 37073 Göttingen, Germany

Materials and methods

Nematodes were obtained from fresh samples of the Lf-layer of a moder humus beechwood soil in the Solling mountains, near Göttingen (Lower Saxony, Germany). Specimens of the three taxa *Malenchus* sp., *Tylolaimophorus typicus* and *Prionchulus* sp. were screened by PCR with the broad range fungal primers ITS5/ITS4 (White et al. 1990) to reveal their feeding abilities and the presence of associated fungi. The internal transcribed spacer region (ITS) within the nuclear ribosomal DNA was chosen as target region because of its high resolution for many fungi at the species level (Buscot et al. 2000).

Following extraction by a modified wet funnel method (Alphei 1998), nematodes were picked alive, crushed with a rounded Pasteur pipette and used directly in PCR without specific DNA extraction. In each nematode taxon investigated, 10–20 specimens were analyzed individually. Amplification of the ITS region by PCR was performed on a HYBAID Limited OmniGene TR3 CM220 Thermo Cycler (MWG-Biotech, Ebersberg, Germany) in a total volume of 50 µl containing 2 U Taq DNA polymerase (Promega, Heidelberg, Germany), 5 µl 10× Taq polymerase reaction buffer (Promega), 4 µl 25 mM $MgCl_2$, 10 nmol each dNTP (MBI-Fermentas, St. Leon-Rot, Germany), 50 pmol each of the two primers and the crushed nematode. Reactions were performed as hot-start-PCR with a 10-min initial denaturation at 94°C before adding the Taq polymerase at 80°C. The PCR program comprised 40 cycles (40 s at 94°C, 30 s at 54°C, 40 s at 72°C). For RFLP analyses PCR products were cut with *Alu*I, *Bsu*RI, *Hinf*I,

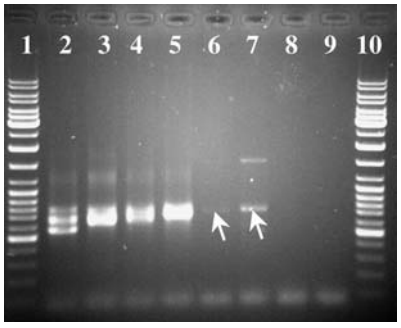

Fig. 1 PCR of the internal transcribed spacer region (ITS1, 5.8S, ITS2) with the fungi-specific primer pair ITS4/ITS5 done on three different nematodes isolated from litter of a beechwood soil. *Lanes 2–5 Malenchus* sp., *lanes 6 and 7 Tylolaimophorus typicus*, *lanes 8 and 9 Prionchulus* sp., *lanes 1 and 10* Gene Ruler DNA Ladder Mix. *Arrows* indicate PCR products corresponding to the fungal mammal pathogen *Malassezia restricta* detected in *T. typicus* and visible as a distinct band. In *Malenchus* sp. the PCR products belonging to *Malassezia* are part of the more complex banding pattern

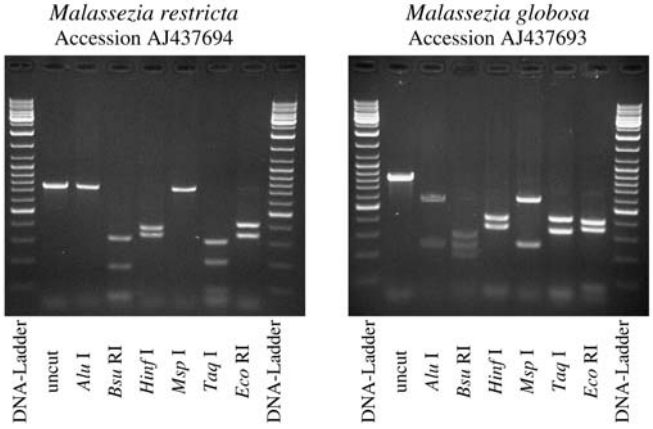

Fig. 2 RFLP patterns of the ITS region of *Malassezia restricta* and *M. globosa* obtained after cloning the PCR products amplified with the primer pair ITS4/ITS5 from nematodes of the genus *Malenchus*, isolated from litter of a beechwood soil. Restriction fragments were separated on 2% agarose gels. *Lanes 1 and 9* Gene Ruler DNA Ladder Mix

*Msp*I, *Taq*I or *Eco*RI (all restriction enzymes were from MBI Fermentas, St. Leon-Rot, Germany).

PCR products were cloned into the pCR4-Topo vector using the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into TOP10 chemically competent *E. coli*. Sequencing was done on a LI-COR DNA Sequencer Long Reader 4200 using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, England). Sequence data were compared with GenBank using the BLASTN program (http://www.ncbi.nlm.nih.gov/blast/).

Results and discussion

Sequence analysis of multibands (Fig. 1) gathered in PCR with the nematodes *Malenchus* sp. and *T. typicus* revealed the presence of the fungal species *Malassezia restricta* and *Malassezia globosa*. *M. globosa* was only associated with *Malenchus* sp., while *M. restricta* was associated with both *Malenchus* sp. and *T. typicus*. Sequencing revealed an ITS fragment length of 714 bp for *M. restricta* and 786 bp for *M. globosa*. Sequence identities of 100% with the only available ITS1 regions of *M. restricta* and *M. globosa* (AB019340 and AB019343, Makimura et al., unpublished) were found with BLASTN (Table 1). The ITS2 regions of these species are not available in GenBank. Nucleotide sequences are deposited at the European Molecular Biology Laboratory (EMBL) under the accession numbers given in Table 1. Restriction patterns of the *Malassezia* ITS regions are given in Fig. 2.

Table 1 Identity of the internal transcribed spacer region 1 (ITS1) fragments extracted from the soil nematode taxa *Malenchus* sp. and *Tylolaimophorus typicus* and amplified with fungi-specific primers ITS5/ITS4. Identification was carried out with existing ITS1 sequences of the fungal genus *Malassezia* in GenBank

Malassezia species	Sequence type 1 from *Malenchus* sp. AJ437693 (266-bp ITS1)	Sequence type 2 from *Malenchus* sp. AJ437694 (210-bp ITS1)	Sequence type 2 from *Tylolaimophorus typicus* AJ437695 (210-bp ITS1)
M. furfur AB019335 (208 bp)	82% on 202 bp + 71 bp gaps	63% on 199 bp + 20 bp gaps	63% on 199 bp + 20 bp gaps
M. globosa AB019343 (266 bp)	100% on 266 bp	73% on 210 bp + 56 bp gaps	73% on 210 bp + 56 bp gaps
M. obtusa AB019336 (213 bp)	69% on 205 bp + 71 gaps	67% on 201 bp + 22 gaps	67% on 201 bp + 22 gaps
M. pachydermatis AB019339 (193 bp)	72% on 192 bp + 75 gaps	68% on 192 bp + 19 gaps	68% on 192 bp + 19 gaps
M. restricta AB019340 (210 bp)	73% on 210 bp + 53 bp gaps	100% on 210 bp	100% on 210 bp
M. slooffiae AB019350 (196 bp)	63% on 196 bp + 70 bp gaps	62% on 195 bp + 17 bp gaps	62% on 195 bp + 17 bp gaps
M. sympodialis AB019347 (162 bp)	68% on 162 bp + 104 gaps	69% on 161 bp + 50 gaps	69% on 161 bp + 50 gaps

No fungal DNA was detected with *Prionchulus* (Fig. 1), probably due to the carnivorous status of this species.

The results clearly indicate the presence of *Malassezia* in groups other than vertebrates, confirming observations from Brazil where nematodes and even acarids seem to act as vectors for *Malassezia* (Duarte et al. 2001). *M. globosa* favors temperatures below 38°C and might thus be well adapted to the temperate climate of Europe (Duarte et al. 2001). In many studies, *M. globosa* was found to be the most common species of *Malassezia* associated with patchy flaking skin in humans (pityriasis versicolor, Ashbee and Evans 2002). Therefore the proof of potential hosts other than mammals is a significant increase in the knowledge about the biology of this human pathogenic fungus. That *Malassezia* has now been found in association with soil animals suggests that the soil mycoflora harbors this pathogen and that further studies are required to examine the character and consequences of possible animal-fungus associations.

Acknowledgements Many thanks to Prof. Ajit Varma, Dr. V. Temperton and two anonymous reviewers for critical reading and improvement of the manuscript.

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