Journal of Microbiological Methods xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi

Bart Lievens [a,b,*], Stefan van Kerckhove [a,d], Annelies Justé [a,b], Bruno P.A. Cammue [c], Olivier Honnay [d], Hans Jacquemyn [d]

[a] Scientia Terrae Research Institute, 2860 Sint-Katelijne-Waver, Belgium
[b] Research Group Process Microbial Ecology and Management, Lessius Hogeschool, Campus De Nayer, Department of Microbial and Molecular Systems, KULeuven Association, 2860 Sint-Katelijne-Waver, Belgium
[c] Centre of Microbial and Plant Genetics (CMPG), Department of Microbial and Molecular Systems, KULeuven, 3001 Leuven, Belgium
[d] Laboratory of Plant Ecology, Department of Biology, KULeuven, 3001 Leuven, Belgium

ARTICLE INFO

Article history:
Received 15 September 2009
Received in revised form 6 November 2009
Accepted 7 November 2009
Available online xxxx

Keywords:
Mycorrhizal association
Oligonucleotide
Orchis
Sequencing
Tulasnellaceae

ABSTRACT

A DNA array was developed from extensive clone library sequence data sets for the assessment of dominant members of mycorrhizal fungi that associate with terrestrial orchid species. As a-proof-of-concept, the array was developed for the basidiomycetous mycorrhizal partners from three closely related perennial *Orchis* species, including *Orchis anthropophora*, *O. militaris* and *O. purpurea*. Based on internal transcribed spacer regions, oligonucleotides were developed for seven operational taxonomic units (OTUs; defined as groups of sequences sharing at least 97% sequence similarity), corresponding to members of the *Tulasnellaceae* family. In order to cover a broader spectrum of tulasnelloid fungi, oligonucleotides were as well developed for two subsets of closely related OTUs. The array was evaluated using multiple primer pairs. In addition, hybridization results were validated by recovery and sequencing of the hybridized amplicons as well as by hybridizing reference DNA samples. Considering the unlimited expansion possibilities of DNA arrays to include specific detector oligonucleotides for other and more microorganisms, the method described here has the major advantage that it provides a powerful, rapid and cost-effective way for the simultaneous detection and identification of a wide range of orchid mycorrhizae. The design, development and advantages of the array are discussed in relation to its potential for future research in mycorrhizal ecology.

1. Introduction

Microbial ecology has undergone a profound change in the last 20 years with regard to methods for the analysis of microbial communities. In general, recent methods to assess microbial communities rely on PCR amplification of target nucleic acid sequences and subsequent analysis by, for example, terminal restriction fragment length polymorphism (T-RFLP) (Marsh et al., 2000; Edel-Hermann et al., 2004), denaturing gradient gel electrophoresis (DGGE) (van Elsas et al., 2000; Joynt et al., 2006) or temperature gradient gel electrophoresis (TGGE) (Beier et al., 2008; Kubartová et al., 2009). These fingerprinting techniques have demonstrated their potential to assess, for example, fungal diversity and facilitate ecological studies, but do not provide direct identification of individual community members or populations. Species association may be accomplished through sequencing, but this requires additional labour to physically isolate each amplified sequence of interest. More efficient identification methods include sequence analysis of clone libraries of PCR amplified genes (Schadt et al., 2003; Timke et al., 2005), or sequencing clones from metagenomic DNA libraries (Rondon et al., 2000). Because the accurate description of a microbial community requires at least a few hundred sequences per sample (Dunbar et al., 2002), this is however a costly and labour-intensive procedure. In addition, the production of aberrant recombinants or sequence inaccuracies due to cloning-related artefacts (Speksnijder et al., 2001) may hamper clone library analysis. These drawbacks may be circumvented by, for example, high-throughput pyrosequencing (Roesch et al., 2007; Cardenas and Tiedje, 2008), enabling to generate thousands of sequences in a single run without a cloning step. Although this technology offers many advantages over the traditional cloning-Sanger sequencing methodology, it is currently still relatively cost-demanding, hampering its widespread use in ecological studies. Moreover, before this technology can be routinely used in studying the microbial diversity in natural ecosystems, additional advances in data mining and bioinformatics are necessary, improving the way in which the huge datasets of sequences that are generated can be handled and analyzed (Pop and Salzberg, 2008).

In recent years, several techniques have been developed that use distinctive short DNA sequences (also called oligonucleotides) for

* Corresponding author. Scientia Terrae Research Institute, 2860 Sint-Katelijne-Waver, Belgium. Tel.: +32 15 305590; fax: +32 15 305599.
E-mail address: bli@scientiaterrae.org (B. Lievens).

0167-7012/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2009.11.004

microbial identification. Among these is DNA array technology, originally developed for gene expression analysis (Schena et al., 1996), currently the most suitable technique for simultaneous detection and identification of several microbial strains, species, genera or higher taxa (Lievens et al., 2005b). With this technology, specific surface-bound detector oligonucleotides hybridize with homologous, labeled target amplicons, which then can be detected. This strategy has proven to be successful for routine detection and identification of pathogenic and environmental microorganisms from various sources, including soil, plants, food and animals (Lievens et al., 2003; Warsen et al., 2004; Kostrzynska and Bachand, 2006), with a discrimination level down to a single nucleotide polymorphism (SNP) (Lievens et al., 2006). In comparison with the above mentioned methods, DNA arrays have the potential to overcome some limitations associated with the restricted phylogenetic resolution of many fingerprinting methods, the requirement of large numbers of sequencing reactions per sample when analyzing clone libraries, and the high costs associated with pyrosequencing (Mazzola, 2004). In addition, DNA array-based microbial detection enables straight forward data interpretation.

Nowadays, DNA arrays have been generally developed based on the huge body of DNA sequences from phylogenetic studies of known microbial species (Lievens et al., 2003; Tambong et al., 2006). Likewise, based on an in-depth sequence analysis of (previously unknown) microbial communities it should be possible to develop DNA arrays for the efficient detection of selected targets or indicator populations from the community that are not yet extensively characterized (Justé et al., 2008). However, so far no studies have been reported that draw together these strategies to ultimately develop a comprehensive DNA array. To test this idea, the overall goal of this study was to evaluate and apply this approach for its use in ecological studies. More specifically, the objective of this study was to develop a DNA array from clone library sequence data sets enabling cost-effective, high-throughput screening of different orchid mycorrhizal fungi by which the mycorrhizal community composition on orchid roots can be efficiently characterized. Orchid species are known to associate with a wide range of fungi in what is believed to be a mutualistic relation (Rasmussen and Rasmussen, 2009). Nevertheless, the diversity and specificity of mycorrhizal fungi that associate with orchid species is poorly known. This is largely due to the fact that mycorrhizal fungi are extremely difficult to culture in the absence of a suitable host and are difficult to identify based on morphological criteria alone. The few studies that used more advanced molecular tools suggest that individual orchid species are fairly selective in their association with mycorrhizal fungi, with different orchid species associated with distinct phylogenetic clades of fungal partners (Shefferson et al., 2005, 2007; McCormick et al., 2006; Bonnardeaux et al., 2007; Irwin et al., 2007). However, as most of these studies were based on fungal isolations or on the sequencing of a limited number of cloned fungal genes from roots, the available information on the host range of orchids is likely biased or incomplete. The approach used in this study, however, should allow for more detailed information on the mycorrhizal community composition on orchid roots. As a-proof-of-concept, in this study we focussed on three closely related perennial *Orchis* species, including *Orchis anthropophora*, *O. militaris* and *O. purpurea*, and their associated basidiomycetous mycorrhizal partners, representing the vast majority of the mycorrhizae found on orchid species (Rasmussen, 1995; Taylor and McCormick, 2008).

2. Materials and methods

2.1. Study samples and DNA extractions

Root samples were collected from *O. anthropophora*, *O. militaris* and *O. purpurea*. Two individuals per plant species were sampled from one population in a calcareous grassland with shrub encroachment (Eben-Emael, Belgium). In addition, one individual of each species was sampled from another calcareous grassland or forest on limestone (Table 1). The numbers of individuals sampled per species were kept low for conservation concerns. Roots were surface sterilized (30 s submergence in 1% sodium hypochlorite, followed by three 30-s rinse steps in sterile distilled water) and microscopically checked for mycorrhizal colonization. Subsequently, DNA was extracted from 0.5 g mycorrhizally colonized root pieces using the UltraClean Plant DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc., Solana Beach, CA, USA), and 10 times diluted afterwards.

Table 1
Samples used in this study.

Name	*Orchis* species	Origin[a]	Habitat
OanA/A148	*O. anthropophora*	Eben-Emael (B)	Shrub
OanA/A177	*O. anthropophora*	Eben-Emael (B)	Shrub
OanAC05	*O. anthropophora*	Wonck (B)	Calcareous grassland
OmiA05	*O. militaris*	Eben-Emael (B)	Shrub
OmiA/P120	*O. militaris*	Eben-Emael (B)	Shrub
OmiAB03	*O. militaris*	Wijlre (NL)	Calcareous grassland
OpuA05	*O. purpurea*	Eben-Emael (B)	Shrub
OpuA06	*O. purpurea*	Eben-Emael (B)	Shrub
OpuE02	*O. purpurea*	Sint-Pieters Voeren (B)	Forest on limestone

[a] B = Belgium, NL = the Netherlands.

2.2. ITS clone libraries

In order to describe the basidiomycetous mycorrhizal fungal community accurately, multiple primer pairs were used in parallel to amplify the ribosomal RNA internal transcribed spacer (ITS) regions.

Table 2
Detector oligonucleotides used in this study.

Name	Intended specificity	Sequence (5′–3′)	Origin
OTU1-1	OTU 1	AACCCCTAAGGGATGCGGTT	ITS I
OTU1-2	OTU 1	CTTAGGGGTTGTGCGTTCAAC	ITS I
OTU1-3	OTU 1	GGGGTTGTGCGTTCAACCTTT	ITS I
OTU1-4	OTU 1	GGGGTTGTGCGTTCAACCTT	ITS I
OTU2-1	OTU 2	GACTCGTCCTGCCGGCCT	ITS II
OTU2-2	OTU 2	GACTCGTCCTGCCGGCCTA	ITS II
OTU2-3	OTU 2	TCTGTTGGCCTTCCCAGGT	ITS II
OTU2-4	OTU 2	CTCTGTTGGCCTTCCCAGG	ITS II
OTU3-1	OTU 3	TCGTCCCGCCAGTCTAACG	ITS II
OTU3-2	OTU 3	TGACGACTCGTCCCGCCAG	ITS II
OTU3-3	OTU 3	TCTGTCGGCCTTCTCAGGT	ITS II
OTU3-4	OTU 3	CTCTGTCGGCCTTCTCAGG	ITS II
OTU1/2/3-1	OTU 1, OTU 2, OTU 3	ATCAGCGATGACGTCCCTTG	ITS II
OTU1/2/3-2	OTU 1, OTU 2, OTU 3	CGTCCCTTGGGTCCATCTC	ITS II
OTU1/2/3-3	OTU 1, OTU 2, OTU 3	TAAGTTGATCGTCGCAAAGG	ITS II
OTU1/2/3-4	OTU 1, OTU 2, OTU 3	GATAAGTTGATCGTCGCAAAG	ITS II
OTU4-1	OTU 4	CATCTTTTGTAACGAAGCGAA	ITS I
OTU4-2	OTU 4	TCTTTTGTAACGAAGCGAATGA	ITS I
OTU4-3	OTU 4	GTCGAGGACATTTCTTAGGAG	ITS II
OTU4-4	OTU 4	GTCGAGGACATTTCTTAGGA	ITS II
OTU5-1	OTU 5	CTCGTCTTTCGACGGGGG	ITS I
OTU5-2	OTU 5	TGAAATTGATTTAACAATGTAC	ITS I
OTU5-3	OTU 5	AATGAAATTGATTTAACAATGTAC	ITS I
OTU5-4	OTU 5	GCGAATGAAATTGATTTAACAATG	ITS I
OTU4/5-1	OTU 4, OTU 5	CTTGCGCCCTCTGGTATTC	ITS II
OTU4/5-2	OTU 4, OTU 5	TTGCGCCCTCTGGTATTCC	ITS II
OTU4/5-3	OTU 4, OTU 5	TGGGTT(T/C)GGTGTGATGCCG	ITS II
OTU4/5-4	OTU 4, OTU 5	GGCATCACACC(G/A)AACCCAT	ITS II
OTU6-1	OTU 6	GGCGCGTTCTCCCATCCA	ITS I
OTU6-2	OTU 6	TCTGTGCATCGTGACGCGT	ITS I
OTU6-3	OTU 6	ACACCTCAATCCAACGGTCT	ITS II
OTU6-4	OTU 6	AAAGGCCCAAGCCCGACG	ITS II
OTU7-1	OTU 7	TTTGTAATCGAGCGAACGTCT	ITS I
OTU7-2	OTU 7	AATCGAGCGAACGTCTTGTA	ITS I
OTU7-3	OTU 7	GCCGAAAGCGCGCGTTTGA	ITS II
OTU7-4	OTU 7	CTTTCGAGGTGGCCGAAAG	ITS II
Uni1[a]	Universal	TCCTCCGCTTATTGATATGC	28S rRNA
Dig1[a,b]	None	GTCCAGACAGGATCAGGATTG	–

[a] Lievens et al. (2003).
[b] 5′-end thiol and 3′-end digoxigenin-labeled reference oligonucleotide.

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004

Table 3
Hybridization results[a] of digoxigenin-labeled PCR amplicons from selected fungal and environmental samples.

Species	Isolate	Host (origin)	Oligonucleotides													
					OTU 3				OTUs 1, 2 and 3				OTU 7			
			Dig1	Uni1	OTU3-1	OTU3-2	OTU3-3	OTU3-4	OTU1/2/3-1	OTU1/2/3-2	OTU1/2/3-3	OTU1/2/3-4	OTU7-1	OTU7-2	OTU7-3	OTU7-4
Ceratobasidium cornigerum	CBS 570.83	*Sarcochilus dilatatus*	●	●												
Ceratobasidium obscurum	UAMH 5443	*Orchis rotundifolia*	●	●												
Epulorhiza calendulina	UAMH 7782	*Amerorchis rotundifolia*	●	●							●	●				
Epulorhiza calendulina	UAMH 7783	*Amerorchis rotundifolia*	●	●							●	●				
Epulorhiza calendulina	UAMH 7784	*Amerorchis rotundifolia*	●	●							●	●				
Epulorhiza sp.	CBS 118625	*Platanthera holochila*	●	●												
Epulorhiza sp.	CBS 487.93	*Dactylorhiza incarnata*	●	●												
Epulorhiza sp.	CBS 606.93	*Orchis militaris*	●	●												
Epulorhiza repens	UAMH 6095	*Dactylorhiza maculata*	●	●												
Epulorhiza repens	UAMH 6096	*Dactylorhiza maculata*	●	●												
Epulorhiza repens	UAMH 6097	*Orchis morio*	●	●												
Moniolopsis solani	UAMH 6098	*Orchis morio*	●	●												
Mortierella isabellina	UAMH 5163	*Orchis rotundifolia*	●	●												
Phialocephala fortinii	UAMH 5425	*Orchis rotundifolia*	●	●												
Rhizoctonia sp.	CBS 298.32	*Orchis morio*	●	●												
Rhizoctonia sp.	CBS 486.93	*Dactylorhiza majalis*	●	●												
Rhizoctonia sp.	CBS 496.93	*Gymnadenia conopsea*	●	●												
Rhizoctonia sp.	CBS 499.93	*Platanthera* sp.	●	●												
Rhizoctonia sp.	CBS 501.93	*Orchis militaris*	●	●												
Rhizoctonia sp.	CBS 505.93	*Dactylorhiza iberica*	●	●												
Rhizoctonia sp.	CBS 508.93	*Orchis militaris*	●	●												
Rhizoctonia sp.	CBS 602.93	*Orchis mascula*	●	●												
Rhizoctonia sp.	CBS 604.93	*Orchis ustulata*	●	●												
Rhizoctonia sp.	CBS 605.93	*Dactylorhiza sambucina*	●	●												
Rhizoctonia sp.	CBS 610.93	*Orchis pauciflora*	●	●												
Rhizoctonia solani	CBS 339.36	*Vanda tricolor*	●	●												
Uncultured *Tulasnellaceae* isolate[b]	P48	*Cypripedium montanum*	●	●									●	●	●	●
Uncultured *Tulasnellaceae* isolate[b]	612	*Cypripedium guttatum*	●	●												
Uncultured *Tulasnellaceae* isolate[b]	836	*Cypripedium reginae*	●	●												
Uncultured *Tulasnellaceae* isolate[b]	972	*Cypripedium macranthon* var. *speciosum*	●	●			●	●	●	●	●	●				

[a] Results are limited to those oligonucleotides that generated hybridization signals in this experiment. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and classified into three categories: blank = no signal (rIOD ≤ 2); ○ = weak signal (rIOD > 2 and ≤ 15); ● = moderate or strong signal (rIOD > 15).
[b] Environmental DNA extract from orchid roots (kindly provided by Dr. R. Shefferson, University of Georgia, USA).

Table 4
Phylogenetic affiliation of operational taxonomic units (OTUs) in the phylum of Basidiomycota.

OTU	Sequence length (bp)	Phylogenetic relationship			No. of clones	Abundance[a] (%)
		Family	Closest match in GenBank (Accession no.)	Sequence identity (%)		
OTU 1	660	*Tulasnellaceae*	*Epulorhiza* sp. SC 034 (AB369932)	95	45	7.5
OTU 2	659	*Tulasnellaceae*	*Epulorhiza* sp. Ep/Sst/07 (EU418851)	97	5	0.8
OTU 3	656	*Tulasnellaceae*	Uncultured *Tulasnellaceae* isolate 968 (DQ925661)	98	18	3.0
OTU 4	684	*Tulasnellaceae*	*Epulorhiza* sp. RO 02 (AB369933)	92	132	22.0
OTU 5	687	*Tulasnellaceae*	*Epulorhiza* sp. RO 02 (AB369933)	98	59	9.8
OTU 6	699	*Tulasnellaceae*	Uncultured *Tulasnellaceae* isolate 451 (EU195344)	97	89	14.8
OTU 7	677	*Tulasnellaceae*	Uncultured *Tulasnellaceae* isolate A1.14 (EU583697)	98	240	39.9
OTU 8	667	*Thelephoraceae*	Uncultured ectomycorrhiza (*Tomentella*) isolate UBCOCS640F (EF218835)	93	3	0.5
OTU 9	702	*Cortinariaceae*	*Hebeloma senescens* (AY312987)	99	10	1.7

[a] Relative to the total number of sequences comprising the nine OTUs.

These primer sets included ITS1 (White et al., 1990) and ITS4-OF (Taylor and McCormick, 2008), ITS1-OF (Taylor and McCormick, 2008) and ITS4 (White et al., 1990), and ITS1-OF and ITS4-OF. Whereas ITS1 and ITS4 are universal eukaryotic primers, ITS1-OF and ITS4-OF are broad-spectrum basidiomycete-specific primers that are increasingly used to characterize fungal symbionts of orchids (Taylor and McCormick, 2008; Shefferson et al., 2007, 2008). PCR amplification was performed in a reaction volume of 20 µl, containing 0.15 mM of each deoxynucleoside triphosphate, 0.5 µM of each primer, 1× Titanium *Taq* DNA polymerase, 1× Titanium *Taq* PCR buffer (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. Amplification conditions consisted of 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C, with a final elongation step at 72 °C for 10 min. PCR products were cloned into *Escherichia coli* plasmids using the pCR2.1 vector and the Topo-TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Ninety-six white colonies were randomly picked from each library and sequenced using the M13 forward primer by the Genome Center of the Washington University. DNA sequences were aligned using MEGA4 (Tamura et al., 2007; http://www.megasoftware.net) followed by manual editing. Conserved sequence motifs were identified in the regions flanking each ITS sequence and the sequences were shortened to these motifs to ensure all sequences had the same start and end point. As a provisional surrogate for "species", operational taxonomic units (OTUs) were defined as a group of sequences sharing at least 97% pairwise similarity, which is within the range of intraspecific ITS sequence similarity (O'Brien et al., 2005). Representative sequences for each OTU were queried against GenBank using BLAST to identify the sequence and gather closely related sequences to be included in our analysis. All sequences for which the ITS I and/or ITS II regions matched totally different sequences were excluded from the analysis. In addition, sequences with high similarity to non-basidiomycete fungi were excluded from the analysis. Subsequently, a global phylogenetic analysis was performed. For ease of visualization of the resulting trees, highly similar sequences were pruned from each OTU to two sequences per sample. Phylogenetic trees were calculated by the neighbour-joining method (Saitou and Nei, 1987) implemented in Clustal X and displayed by TreeView (v. 1.6.6; Page, 1996). Representative sequences for each OTU were deposited in GenBank (Accession numbers GQ907249–GQ907285).

Table 5
Clone distribution within the different operational taxonomic units (OTUs).

Sample	Primer pair	No. (abundance)[a] of clones within								
		OTU 1	OTU 2	OTU 3	OTU 4	OTU 5	OTU 6	OTU 7	OTU 8	OTU 9
OanA/A148	ITS1-OF/ITS4-OF	3 (23)						10 (77)		
	ITS1/ITS4-OF	8 (17)						38 (83)		
	ITS1-OF/ITS4	1 (12)						7 (88)		
OanA/A177	ITS1-OF/ITS4-OF		1 (100)							
	ITS1/ITS4-OF		1 (100)							
	ITS1-OF/ITS4				9 (100)					
OanAC05	ITS1-OF/ITS4-OF							35 (94)	1 (3)	1 (3)
	ITS1/ITS4-OF							39 (78)	2 (4)	9 (18)
	ITS1-OF/ITS4				8 (100)					
OmiA05	ITS1-OF/ITS4-OF							22 (100)		
	ITS1/ITS4-OF			13 (20)				52 (80)		
	ITS1-OF/ITS4			5 (12)				37 (88)		
OmiA/P120	ITS1-OF/ITS4-OF						24 (100)			
	ITS1/ITS4-OF	4 (12)					29 (88)			
	ITS1-OF/ITS4	6 (14)					36 (86)			
OmiAB03	ITS1-OF/ITS4-OF	1 (8)				12 (92)				
	ITS1/ITS4-OF	22 (71)				9 (29)				
	ITS1-OF/ITS4		3 (60)			2 (40)				
OpuA05	ITS1-OF/ITS4-OF				8 (100)					
	ITS1/ITS4-OF				37 (100)					
	ITS1-OF/ITS4				7 (100)					
OpuA06	ITS1-OF/ITS4-OF				5 (100)					
	ITS1/ITS4-OF				54 (100)					
	ITS1-OF/ITS4				4 (100)					
OpuE02	ITS1-OF/ITS4-OF					7 (100)				
	ITS1/ITS4-OF					28 (100)				
	ITS1-OF/ITS4					1 (100)				

[a] Relative to the total number of basidiomycete sequences generated per clone library.

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004

2.3. DNA array analysis

2.3.1. DNA array design

For each OTU, a set of four detector oligonucleotides was designed as described previously (Lievens et al., 2003, 2006) (Table 2). Oligonucleotides were designed in such a way that they perfectly matched all sequences of a given OTU, but were dissimilar from sequences outside the OTU. In addition, two sets of four oligonucleotides were developed that matched a group of closely related OTUs (Table 2). In addition to the OTU-specific oligonucleotides, a non-specific oligonucleotide (Uni1) and a digoxigenin-labeled control oligonucleotide (Dig1) (Lievens et al., 2003, 2006; Table 2) were used as a check for hybridization and control for detection and calibration, respectively. The selected oligonucleotides were synthesized with a 5′ NH_2 group followed by a C6 linker and spotted in duplicate (8.0 fmol per spot) on nylon membranes as reported previously (Lievens et al., 2003, 2006). Subsequently, membranes were air dried, blocked for 30 min at room temperature, again air dried, vacuum-packaged and stored at room temperature until use.

2.3.2. PCR amplification, labeling and DNA array hybridization

Target ITS regions were amplified and simultaneously labeled with alkaline-labile digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany). In order to check potential differences between different primers pairs, amplification was performed using seven primer combinations. These included the universal primer pair ITS1 and ITS4, the fungal-specific primer pair ITS1-F (Gardes and Bruns, 1993) and ITS4, the basidiomycete-specific primer pairs ITS1 and ITS4-B (Gardes and Bruns, 1993) and ITS1-OF and ITS4-B, and the three primer pairs used for clone library construction. DNA samples were amplified as described above with the exception that 5% dTTP was replaced by digoxigenin-11-dUTP (Dig-dUTP; Roche Diagnostics, Mannheim, Germany). Hybridization (20 µl of labeled amplicons in 12 ml hybridization buffer), washing and detection were performed as described previously (Lievens et al., 2003, 2006). Hybridization signals were quantified and analyzed using Labworks 4.0 Image Acquisition and Analysis Software. Hybridization signal strength was reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). All hybridizations were performed twice using amplicons from a single PCR reaction.

2.3.3. DNA array validation

To validate DNA array hybridization with the expected target sequences as well as to explain unexpected hybridization signals, DNA sequences were recovered from a selection of representative spots, re-amplified and sequenced (CA Lévesque, Agriculture and Agri-Food Canada, Canada, pers. commun.). For each selected oligonucleotide, small-scale membranes were produced containing three replicate spots of the oligonucleotide (24 fmol per spot; surface of nearly 7 mm^2). Following overnight hybridization with unlabeled amplicons, two washes in stringency buffer (6× SSC and 0.1% SDS) and immersion in washing solution (0.1 M maleic acid and 0.15 M sodium chloride; pH 7.5), the area containing the spots was excised and added to a 0.5 ml PCR tube with 37.75 µl sterile water and 5 µl of 10× Titanium *Taq* PCR buffer. Following heating of the mixture for 10 min at 95 °C, 30 µl of the mixture was transferred into a 0.2 ml PCR tube containing 2.625 µl of a 2.0 mM dNTP mix, 0.875 µl of 20 µM forward, and reverse primer and 0.7 µl of 50× Titanium *Taq* DNA polymerase. Samples were amplified with the same thermal cycling profile as described above, with the exception of an annealing temperature of 53 °C instead of 58 °C. As no amplicons could be generated using the same primers that were used for the first amplification, reamplification was done with primers annealing within the generated region (i.e. initial amplification with ITS1-OF and ITS4-OF; reamplification with ITS1 and PH-R1 (5′- TTAAGTTCAGCGGGTAGTCC-3′)). Re-amplified products were cloned as described above. Subsequently, five to eight white colonies were selected for sequencing using the M13 forward primer. Sequences were compared to the target OTU sequences and oligonucleotides.

In addition, specificity of the array was evaluated using a collection of reference samples. As we were not successful in culturing the mycorrhizal fungi from the investigated samples *in vitro*, and no isolates representative for our target fungi could be obtained from international culture collections, diagnostic accuracy was tested using DNA samples from another study in which similar and related sequences were found (Shefferson et al., 2007). In addition, diagnostic exclusivity was tested using 26 mycorrhizal fungi, originally isolated from orchid species and phylogenetically related to our target fungi (Table 3). PCR amplification and DNA array hybridization were performed as described above. However, hybridization using amplicons

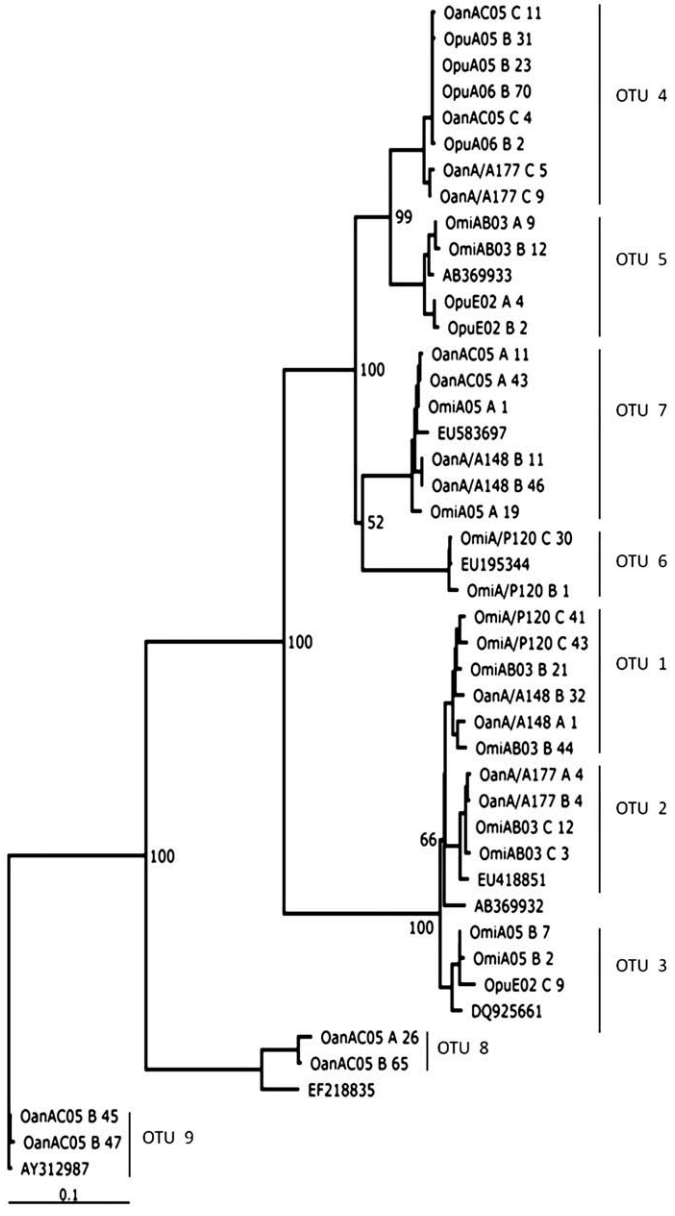


Fig. 1. Neighbour-joining phylogram showing genetic relationships between the different sequences obtained in this study. For each sample, two representative sequences of each operational taxonomic unit (OTU) found were, together with their closest related sequence found in GenBank, used to construct the phylogenetic tree. Sequences are annotated by the sample name, the primer pair with which they were generated (A = ITS1-OF/ITS4-OF; B = ITS1/ITS4-OF; C = ITS1-OF/ITS4), and the number of the clone. Bootstrap percentages based on 1000 replications are shown at the major nodes.

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004

Table 6
Hybridization results[a] of digoxigenin-labeled PCR amplicons obtained with different primer pairs.[b]

Sample	Primer pair	Oligonucleotides targeting the different OTUs:															
		OTU 1				OTU 2				OTU 3				OTUs 1,2 and 3			
		OTU1-1	OTU1-2	OTU1-3	OTU1-4	OTU2-1	OTU2-2	OTU2-3	OTU2-4	OTU3-1	OTU3-2	OTU3-3	OTU3-4	OTU1/2/3-1	OTU1/2/3-2	OTU1/2/3-3	OTU1/2/3-4
OanA/A148	ITS1-OF/ITS4-OF	●	●	●	○									●	●	●	●
	ITS1/ITS4-OF	●	●	●	○									●	○	●	●
	ITS1-OF/ITS4	●	●	●	●									●	●	●	●
	ITS1/ITS4	●	●	●	○									●	●	●	●
	ITS1-F/ITS4	●	●	●	●									●	●	●	●
OanA/A177	ITS1-OF/ITS4-OF[c]					●	●	●	●					●	●	●	●
	ITS1/ITS4-OF					●	●	●	●					●	●	●	●
	ITS1-OF/ITS4					●	●	●	●					●	●	●	●
	ITS1/ITS4					●	●	●	●					●	●	●	●
	ITS1-F/ITS4					●	●	●	●					●	●	●	●
OanAC05	ITS1-OF/ITS4-OF																
	ITS1/ITS4-OF																
	ITS1-OF/ITS4																
	ITS1/ITS4																
	ITS1-F/ITS4																
OmiA05	ITS1-OF/ITS4-OF									●	●	○	○	●	●	●	●
	ITS1/ITS4-OF									●	●	○	○	●	●	●	●
	ITS1-OF/ITS4									●	●	○	○	●	●	●	●
	ITS1/ITS4									●	●	○	○	●	○	●	●
	ITS1-F/ITS4									●	●	○	○	●	●	●	●
OmiA/P120	ITS1-OF/ITS4-OF[c]	●	●	●	○									●	●	●	●
	ITS1/ITS4-OF	●	●	●	○									●	○	●	●
	ITS1-OF/ITS4	●	●	●	●									●	●	●	●
	ITS1/ITS4	●	●	●	○									●	●	●	●
	ITS1-F/ITS4	●	●	●	○									●	●	●	●
OmiAB03	ITS1-OF/ITS4-OF	●	●	●	●									●	●	●	●
	ITS1/ITS4-OF	●	●	●	●									●	●	●	●
	ITS1-OF/ITS4	●	●	●	●									●	●	●	●
	ITS1/ITS4	●	●	●	●									●	○	●	●
	ITS1-F/ITS4	●	●	●	●	○	○	○	○			○	○	●	●	●	●
OpuA05	ITS1-OF/ITS4-OF[c]																
	ITS1/ITS4-OF																
	ITS1-OF/ITS4																
	ITS1/ITS4																
	ITS1-F/ITS4																
OpuA06	ITS1-OF/ITS4-OF																
	ITS1/ITS4-OF																
	ITS1-OF/ITS4																
	ITS1/ITS4																
	ITS1-F/ITS4																
OpuE02	ITS1-OF/ITS4-OF																
	ITS1/ITS4-OF																
	ITS1-OF/ITS4																
	ITS1/ITS4																
	ITS1-F/ITS4																

[a] Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and classified into three categories: blank = no signal (rIOD ≤ 2); ○ = weak signal (rIOD > 2 and ≤ 15); ● = moderate or strong signal (rIOD > 15).
[b] When the reverse primer ITS4-B was used in combination with either ITS1 or ITS1-OF no PCR products or hybridization signals were obtained.
[c] Illustrated with the corresponding blot in Fig. 2.

from pure fungal strains was performed using 4 µl of labeled amplicons in 12 ml hybridization buffer (Lievens et al., 2006).

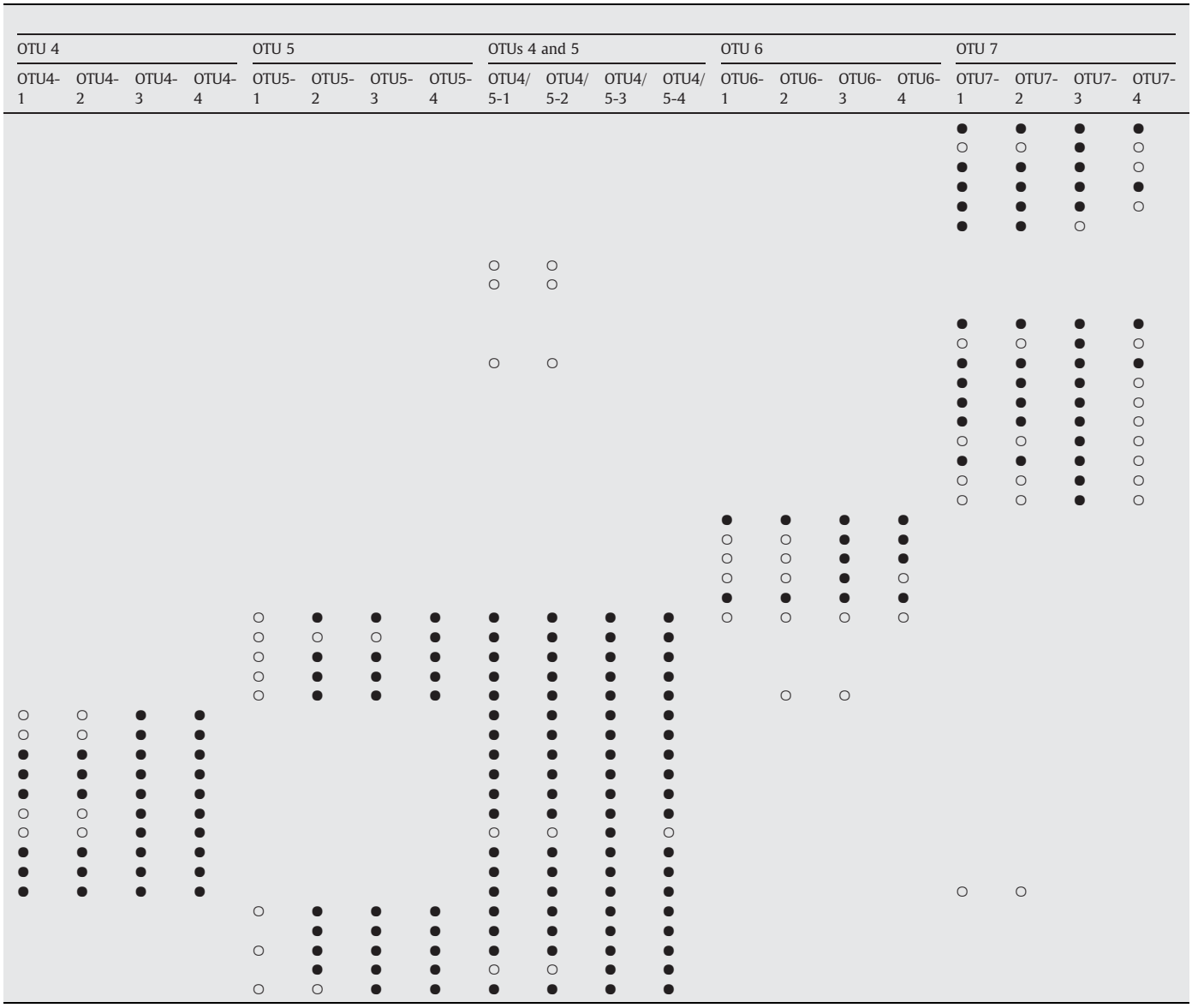
3. Results and discussion

3.1. Clone library analysis

In order to assess the mycorrhizal fungal diversity and community composition on the roots of the selected orchids, twenty seven clone libraries representing dominant members of the root-associated fungal community were generated using three basidiomycete-specific primer pairs, including ITS1/ITS4-OF, ITS1-OF/ITS4, and ITS1-OF/ITS4-OF. In total, over 1100 high-quality sequences (with sequence lengths of over 600 Q20 quality bases) were obtained of which the majority corresponded to basidiomycete sequences. On the basis of at least 97% DNA sequence similarity, the basidiomycete sequences could be grouped into nine OTUs, ranging in abundance from 3 (OTU 8) to 240 sequences (OTU 7) (Table 4). Some OTUs were found to exhibit relatively low levels of sequence similarity between each other (e.g. 50% between OTU 1 and OTU 9). On the other hand, others were found to share a high degree of sequence similarity: OTUs 1, 2 and 3, for example, share over 95% sequence similarity.

rDNA genes are known to occur in multiple copies in fungal and other genomes. In order to eliminate that different ITS sequences from a single fungal isolate (Pannecoucque and Höfte, 2009) would be assigned to different OTUs, ITS sequences of eight clones of five basidiomycete isolates originally isolated from orchids were analyzed. These isolates included three *Rhizoctonia* species isolated from different *Orchis* species (CBS 501.93, CBS 602.93 and CBS 604.93) and two *Epulorhiza* species isolated from *Platanthera holochila* (CBS 118625) and *Amerorchis rotundifolia* (UAMH 7782). For each isolate, all sequences were similar (>99% DNA sequence identity), justifying that the different OTUs at least correspond to different isolates.

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004



Upon BLAST analysis, seven OTUs (OTU 1–OTU 7) were affiliated with members of the *Tulasnellaceae* family. For five out of these OTUs (OTUs 2, 3, 5, 6 and 7) more than 97% DNA sequence identity was found with a GenBank sequence (Table 4). Sequences that fell within OTU 1 and OTU 4 displayed 95% and 92% DNA sequence identity to the most related GenBank sequence, respectively (Table 4). In all cases, the most related GenBank sequence represented a mycorrhizal fungus from orchid species, including species of *Cypripedium* (OTUs 1, 3, 4, 5 and 6) and *Orchis* (OTU 7). Additionally, two OTUs, including OTU 8 and OTU 9, were related to the family *Thelephoraceae* and *Cortinariaceae*, respectively (Table 4). More specifically, OTU 9 sequences almost perfectly matched (>99% sequence similarity) isolates of the ectomycorrhizal basidiomycete species *Hebeloma senescens*. Remarkably, although the use of primer ITS1-OF resulted in many self-ligated cloning vectors or short sequences corresponding to one or multiple copies of the used primers, for some samples (i.e. for the samples OanA/A177, OanAC05 and OmiAB03) certain sequence types were only obtained when this primer was used (Table 5). This could potentially be explained by a different preferential template amplification by the different primer pairs, in combination with the number of sequences obtained for each clone library. For each sample two representative sequences of each OTU found were selected and, together with their closest related sequences found in GenBank, used to construct a phylogenetic tree. The resulting phylogram is shown in Fig. 1 which displays the different OTUs as well-supported clades (bootstrap support between 52 and 100%).

3.2. DNA array analysis

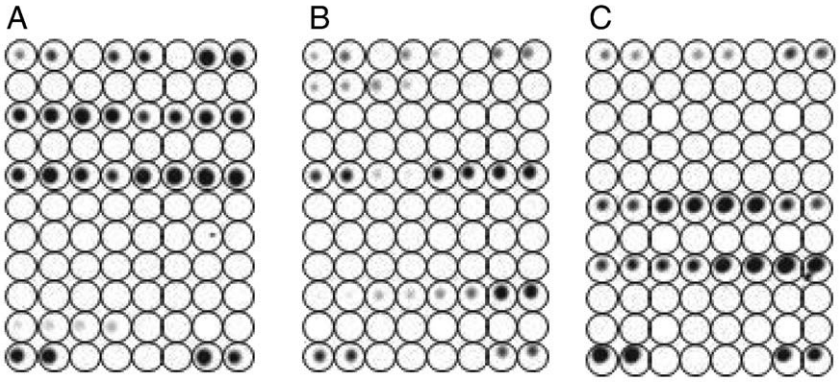
Based on the sequences obtained by cloning, a comprehensive DNA array was developed for rapid and effective detection and identification of the dominant mycorrhizal fungi that occur on the studied orchid species. More in particular, OTU-specific oligonucleotides were developed for the different OTUs that were assigned to the

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004

Tulasnellaceae family (OTU 1–OTU 7) (Table 2). In addition, in order to cover a broader spectrum of tulasnelloid fungi, group-specific oligonucleotides were developed for those OTUs sharing over 90% sequence similarity: one set of oligonucleotides was developed targeting members of OTUs 1, 2 and 3 (more than 95% sequence similarity) and another set of detector oligonucleotides was developed for OTUs 4 and 5 (92% sequence identity) identification (Table 2). First, hybridization experiments were performed using labeled amplicons generated with the same primers that were used to construct the clone libraries. In contrast to the clone library analysis (Table 5), in general no difference in species richness (number of detected OTUs) was observed between the three primer pairs (Table 6). Nevertheless, minor differences in hybridization patterns were observed for a few reactions, i.e. when the oligonucleotides produced weak hybridization signals (Table 6). However, such variation experienced at the detection limit of the assay is also observed in between different hybridization runs using amplicons from different PCR reactions from a single sample (Robideau et al., 2008). One explanation for the discrepancy observed between the clone library analysis and the DNA array analysis includes the higher sensitivity of the array by which targets can be easily detected down to 0.1% of the microbial community from which the template DNA is amplified by the same primer pair (Lievens et al., 2005a), demonstrating a major advantage of this technique above other techniques. Similar hybridization patterns were obtained for the universal primer pair ITS1/ITS4 and the fungal-specific primer pair ITS1-F/ITS4 (Table 6). However, when the basidiomycete-specific primer ITS4-B was used in combination with either ITS1 or ITS1-OF no PCR products or hybridization signals were obtained, which may be explained by the fact that DNA from *Tulasnellaceae* amplifies poorly with this primer (Taylor et al., 2003; Taylor and McCormick, 2008). Remarkably, different OTUs were found to associate with individuals from the same plant population (e.g. samples OanA/A148 *vs* OanA/A177 and OmiA05 *vs* OmiA/P120), while single OTUs (e.g. OTUs 1, 5 and 7) were also found to associate with plants belonging to different plant species (Table 6), suggesting that these orchids are fairly unselective with respect to the mycorrhizae with which they associate. A selection of representative DNA array analyses from the studied samples is shown in Fig. 2.

3.3. Confirmation of additional OTUs detected by the DNA array

In order to check correct DNA array hybridization, and whether the additional OTUs detected by the DNA array were not the result of unforeseen cross-hybridizations, three hybridization reactions (for OanA/A177, OmiA05 and OpuA05) were selected for further investigation. Sequences were recovered from the oligonucleotides OTU2-1, OTU3-1, OTU4-3 and OTU7-2 (Table 2), re-amplified and sequenced. Irrespective some OTUs were not represented in certain libraries, sequencing in all cases revealed similarity to the oligonucleotide-corresponding OTU sequences (data not shown). However, among the different clones analyzed, few additional sequences were obtained corresponding to other OTUs that were also found in the investigated sample. Due to the high nucleotide diversity between these sequences and the oligonucleotide, this discrepancy is probably not caused by cross-reaction (Kawasaki and Chehab, 1994; Lievens et al., 2006). However, possibly, it may be explained by the fact that genomic DNA or non-target amplicons got stuck to the membrane during hybridization, leading to these non-target amplicons. Alternatively, this may be explained by an aspecific binding between two closely related



D

Dig1		Uni1		Uni1
OTU1-1	OTU1-2		OTU1-3	OTU1-4
OTU2-1	OTU2-2		OTU2-3	OTU2-4
OTU3-1	OTU3-2		OTU3-3	OTU3-4
OTU1/2/3-1	OTU1/2/3-2		OTU1/2/3-3	OTU1/2/3-4
OTU4-1	OTU4-2		OTU4-3	OTU4-4
OTU5-1	OTU5-2		OTU5-3	OTU5-4
OTU4/5-1	OTU4/5-2		OTU4/5-3	OTU4/5-4
OTU6-1	OTU6-2		OTU6-3	OTU6-4
OTU7-1	OTU7-2		OTU7-3	OT1U7-4
Uni1				Dig1

Fig. 2. Simultaneous detection and identification of several mycorrhizal fungi on (A) *Orchis anthropophora* (OanA/A177), (B) *O. militaris* (OmiA/P120) and (C) *O. purpurea* (OpuA05). Amplicons were generated using primer pair ITS1-OF and ITS4-OF. A scheme for the localization of the detector oligonucleotides on the DNA array is shown in panel D. The oligonucleotides were horizontally spotted in duplicate.

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004

amplicon strands of which one was also correctly bound to the oligonucleotide. Specificity of the array was further evaluated using a collection of 26 mycorrhizal fungi originally isolated from orchid species, and which are related to our target fungi (Table 3). In general, for most tested isolates no hybridization signals were obtained. The three tested *Epulorhiza calendulina* isolates (UAMH 7782, UAMH 7783 and UAMH 7784), however, produced strong hybridization signals for the oligonucleotides OTU1/2/3–3 and OTU1/2/3–4, targeting isolates belonging to OTU 1, 2 or 3 (Table 3). Sequencing of the ITS region revealed these isolates have sequences identical to these oligonucleotides, explaining the observed hybridization signals. In addition, the array was validated using environmental DNA from mycorrhizal colonized *Cypripedium* orchid roots. Of these samples, samples P48 and 972 represented an isolate with over 97% ITS sequence identity with OTU 7 and OTU 3 sequences, respectively. Samples 612 and 836 represented less closely related *Tulasnellaceae* isolates. For each sample, DNA array hybridization resulted in the expected hybridization pattern (Table 3). Altogether, these experiments show the robustness and accuracy of the assay.

4. Conclusions

In this manuscript, we have demonstrated the usefulness of an integrated molecular ecological approach to characterize and monitor microbial communities, starting with a sequence analysis from unknown microbial communities leading to the development of a comprehensive DNA array for the efficient screening of the targets of interest. Whereas previously DNA arrays were generally developed from phylogenetic datasets, in this study DNA arrays were developed from an in-depth sequence analysis of an environmental sample and subsequently validated by sequence recovery and sequencing. In addition, this manuscript illustrates the use and power of DNA arrays for the assessment of the dominant members of mycorrhizal fungi that associate with terrestrial orchid species. The importance of mycorrhizae for the persistence of orchid populations is widely recognized (e.g. Jacquemyn et al., 2007), yet very little is known about the nature and specificity of orchid-mycorrhizae associations. The DNA array-based method described here has the major advantage that it allows for the simultaneous detection and identification of a wide range of orchid mycorrhizae and thus provides a powerful way of incorporating multiple associations in the study of mycorrhizal associations in natural orchid populations. Moreover, given the unlimited expansion possibilities of DNA arrays to include oligonucleotides, the array can be easily enlarged for the identification of other mycorrhizal fungi. Ultimately, this may lead to a complete orchid mycorrhizal fungi assessment assay for providing a better understanding of orchid-mycorrhizae associations.

Compared to other methods currently used, such as sequencing clones from DNA libraries, neither cloning, which is generally considered as a major bottleneck, nor sequencing is necessary for DNA array-based analysis. Importantly, the high sensitivity of the technique (Lievens et al., 2003, 2005a) increases the likelihood of detecting species which occur at low densities and which would remain undetected with the relatively limited number of screened clones (Table 5 *vs* Table 6). As sensitivity is a very important feature in microbial ecology, the high sensitivity obtained using this technology includes a major advantage of the technique. In addition, the technology offers a cost-effective and user-friendly way for the high-throughput screening of several selected target fungi in a single assay. However, as the detection with DNA arrays is typically limited to the organisms for which oligonucleotides are developed, complementing fingerprinting and/or sequencing-based techniques are recommended for initial characterization of unknown ecosystems.

We believe that the presented method will be useful in at least three ways. First, a method for rapid detection of orchid mycorrhizae will enhance our understanding of orchid-mycorrhizae associations and we expect this new approach to prove especially useful in the analysis of the evolutionary history of mycorrhizal specificity. Moreover, because our and previous data (e.g. Shefferson et al., 2007; Roy et al., 2009) suggest multiple orchid fungal partners, simultaneous detection of several fungi allows investigating host specificity in an unprecedented way. Second, the use of DNA arrays provides a new tool for quickly evaluating how local environmental characteristics affect mycorrhizal associations and how they may generate spatial variation in orchid-mycorrhiza associations. Subsequently, one may examine whether and how variation in spatial distribution of mycorrhizal partners affects germination rates in the field. Third, the DNA array-based method can be used to analyze the accumulation of fungal partners during successive stages in the life cycle of orchid species (e.g. protocorm, tuber, seedling, and juveniles to adults). In such a case, also the possibility of switching from mycorrhizal partner (McCormick et al., 2006) can be investigated in further detail.

Although in general no differences were observed between the different primer pairs used for the DNA array analysis (with the exception of ITS4-B), we recommend the use of ITS1-OF and ITS4-OF as these primers were shown to be specifically effective against a wide range of Basidiomycota and to minimize amplification of plant sequences (Taylor and McCormick, 2008). Consequently, the use of this primer pair should enable a less biased view of orchid associations, which may be not the case when more universal primers like ITS1, ITS1-F and ITS4 are used.

Acknowledgements

The authors thank the Fund for Scientific Research-Flanders for financial support of this study (project G.0592.08). In addition, we are grateful to Dr. C. A. Lévesque (Agriculture and Agri-Food Canada, Canada) for providing the DNA array validation protocol, to Dr. R. Shefferson (University of Georgia, USA) for providing reference DNA samples, and to Dr. H. Elifantz (the Volcani Center, Israel) for help in analyzing the sequence data sets.

References

Beier, S., Witzel, K.-P., Marxsen, J., 2008. Bacterial community composition in central European running waters examined by temperature gradient gel electrophoresis and sequence analysis of 16S rRNA genes. Appl. Environ. Microbiol. 74, 188–199.

Bonnardeaux, Y., Brundrett, M., Batty, A., Dixon, K., Koch, J., Sivasithamparam, K., 2007. Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. Mycol. Res. 111, 51–61.

Cardenas, E., Tiedje, J.M., 2008. New tools for discovering and characterizing microbial diversity. Curr. Opin. Biotechnol. 19, 544–549.

Dunbar, J., Barns, S.M., Ticknor, L.O., Kuske, C.R., 2002. Empirical and theoretical bacterial diversity in four Arizona soils. Appl. Environ. Microbiol. 68, 3035–3045.

Edel-Hermann, V., Dreumont, C., Pérez-Piqueres, A., Steinberg, C., 2004. Terminal restriction fragment length polymorphism analysis of ribosomal RNA genes to assess changes in fungal community structure in soils. FEMS Microbiol. Ecol. 47, 397–404.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes—Application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.

Irwin, M.J., Bougoure, J.J., Dearnaley, J.D.W., 2007. *Pterostylis nutans* (Orchidaceae) has a specific association with two *Ceratobasidium* root associated fungi across its range in Eastern Australia. Mycoscience 48, 231–239.

Jacquemyn, H., Brys, R., Vandepitte, K., Honnay, O., Roldan-Ruiz, I., Wiegand, T., 2007. A spatially-explicit analysis of seedling recruitment in the terrestrial orchid *Orchis purpurea*. New Phytol. 176, 448–459.

Joynt, J., Bischoff, M., Turco, R., Konopka, A., Nakatsu, C.H., 2006. Microbial community analysis of soils contaminated with lead, chromium and petroleum hydrocarbons. Microb. Ecol. 51, 209–219.

Justé, A., Thomma, B.P.H.J., Lievens, B., 2008. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiol. 25, 745–761.

Kawasaki, E.S., Chehab, F.F., 1994. Analysis of gene sequences by hybridization of PCR-amplified DNA to covalently bound oligonucleotide probes. In: Isaac, P.G. (Ed.), Protocols for nucleic acid analysis by non-radioactive probes. : Methods in Molecular Biology, vol.28. Humana Press Inc, Totowa, NJ, pp. 225–242.

Kostrzynska, M., Bachand, A., 2006. Application of DNA microarray technology for detection, identification, and characterization of food-borne pathogens. Can. J. Microbiol. 52, 1–8.

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004

Kubartová, A., Ranger, J., Berthelin, J., Beguiristain, T., 2009. Diversity and decomposing ability of saprophytic fungi from temperate forest litter. Microb. Ecol. 58, 98–107.
Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Lévesque, C.A., Cammue, B.P.A., Thomma, B.P.H.J., 2003. Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. FEMS Microbiol. Lett. 223, 113–122.
Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Lévesque, C.A., Cammue, B.P.A., Thomma, B.P.H.J., 2005a. Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. Environ. Microbiol. 7, 1698–1710.
Lievens, B., Grauwet, T.J.M.A., Cammue, B.P.A., Thomma, B.P.H.J., 2005b. Recent developments in diagnostics of plant pathogens: a review. Recent Res. Dev. Microbiol. 9, 57–79.
Lievens, B., Claes, L., Vanachter, A.C.R.C., Cammue, B.P.A., Thomma, B.P.H.J., 2006. Detecting single nucleotide polymorphisms using DNA arrays for plant pathology. FEMS Microbiol. Lett. 255, 129–139.
Marsh, T.L., Saxman, P., Cole, J., Tiedje, J., 2000. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl. Environ. Microbiol. 66, 3616–3620.
Mazzola, M., 2004. Assessment and management of soil microbial community structure for disease suppression. Annu. Rev. Phytopathol. 42, 35–59.
McCormick, M.K., Whigham, D.F., Sloan, D., O'Malley, K., Hodkinson, B., 2006. Orchid-fungus fidelity: a marriage meant to last? Ecology 87, 903–911.
O'Brien, H.E., Parrent, J.L., Jackson, J.A., Moncalvo, J.-M., Vilgalys, R., 2005. Fungal community analysis by large-scale sequencing of environmental samples. Appl. Environ. Microbiol. 71, 5544–5550.
Page, R.D.M., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.
Pannecoucque, J., Höfte, M., 2009. Detection of rDNA ITS polymorphism in *Rhizoctonia solani* AG 2-1 isolates. Mycologia 101, 26–33.
Pop, M., Salzberg, S.L., 2008. Bioinformatics challenges of new sequencing technology. Trends Genet. 24, 142–149.
Rasmussen, H.N., 1995. Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press, New York, USA. 444 pp.
Rasmussen, H.N., Rasmussen, F.N., 2009. Orchid mycorrhiza: implications of a mycophagous life cycle. Oikos 118, 334–345.
Robideau, G.P., Caruso, F.L., Oudemans, P.V., McManus, P.S., Renaud, M.A., Auclair, M.E., Bilodeau, G.J., Yee, D., Désaulniers, N.L., DeVerna, J.W., Lévesque, C.A., 2008. Detection of cranberry fruit rot fungi using DNA array hybridization. Can J. Plant Pathol. 30, 226–240.
Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub, S.H., Camargo, F.A.O., Farmerie, W.G., Triplett, E.W., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J. 1, 283–290.
Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Liles, M.R., Loiacono, K.A., Lynch, B.A., MacNeil, I.A., Minor, C., Tiong, C.L., Gilman, M., Osburne, M.S., Clardy, J., Handelsman, J., Goodman, R.M., 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. 66, 2541–2547.
Roy, M., Watthana, S., Stier, A., Richard, F., Vessabutr, S., Selosse, M.-A., 2009. Two mycoheterotrophic orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of ectomycorrhizal fungi. BMC Biol. 7, 51.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Schadt, C.W., Martin, A.P., Lipson, D.A., Schmidt, S.K., 2003. Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science 301, 1359–1361.
Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P.O., Davis, R.W., 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc. Nat. Acad. Sci. U. S. A. 93, 10614–10619.
Shefferson, R.P., Weiß, M., Kull, T., Taylor, D.L., 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. Mol. Ecol. 14, 613–626.
Shefferson, R.P., Taylor, D.L., Weiß, M., Garnica, S., McCormick, M.K., Adams, S., Gray, H.M., McFarland, J.W., Kull, T., Tali, K., Yukawa, T., Kawahara, T., Miyoshi, K., Lee, Y.-I., 2007. The evolutionary history of mycorrhizal specificity among lady' s slipper orchids. Evolution 61, 1380–1390.
Shefferson, R.P., Weiß, M., Kull, T., Taylor, D.L., 2008. Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. Am. J. Bot. 95, 156–164.
Speksnijder, A.G.C.L., Kowalchuck, G.A., De Jong, S., Kline, E., Stephen, J.R., Laanbroek, H.J., 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16 S rRNA gene sequences. Appl. Environ. Microbiol. 67, 469–472.
Tambong, J.T., de Cock, A.W.A.M., Tinker, N.A., Lévesque, C.A., 2006. Oligonucleotide array for identification and detection of *Pythium* species. Appl. Environ. Microbiol. 72, 2691–2706.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Taylor, D.L., McCormick, M.K., 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. New Phytol. 177, 1020–1033.
Taylor, D.L., Bruns, T.D., Szaro, T.M., Hodges, S.A., 2003. Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. Am. J. Bot. 90, 1168–1179.
Timke, M., Wang-Lieu, N.Q., Altendorf, K., Lipski, A., 2005. Community structure and diversity of biofilms from a beer bottling plant as revealed using 16S rRNA gene clone libraries. Appl. Environ. Microbiol. 71, 6446–6452.
van Elsas, J.D., Duarte, G.F., Keijzer-Wolters, A., Smit, E., 2000. Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. J. Microbiol. Methods 43, 133–151.
Warsen, A.E., Krug, M.J., LaFrentz, S., Stanek, D.R., Loge, F.J., Call, D.R., 2004. Simultaneous discrimination between 15 fish pathogens by using 16S ribosomal DNA PCR and DNA microarrays. Appl. Environ. Microbiol. 70, 4216–4221.
White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.S., White, T.J. (Eds.), PCR protocols: a guide to methods and applications. Academic Press, New York, USA, pp. 315–322.

Please cite this article as: Lievens, B., et al., From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi, J. Microbiol. Methods (2009), doi:10.1016/j.mimet.2009.11.004