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PRIMER NOTE

Group-specific polymerase chain reaction amplification of SSU rRNA-encoding gene fragments from 12 microbial taxa

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Abstract

Starting from an alignment of all known representatives in GenBank, we designed group specific primers targeting SSU rRNA-encoding sequences of 12 microbial taxa known to contain insect pathogens and symbionts. We tested the specificity of these primers using representative species of all 12 groups as control templates. Polymerase chain reaction amplification conditions were modified until only group-specific templates yielded a positive signal. The presented primer pairs thus allow for the amplification of SSU rRNA-encoding sequences representing specific microbial groups directly from the environment (a social insect host in our study). We discuss possible applications of the identified molecular tools.

Keywords: 16S, 18S, endosymbionts, environmental sample, ribosomal RNA, uncultivable

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Recent trends in microbial ecology include the use of molecular techniques to amplify DNA sequences directly from environmental samples (Ward *et al*. 1992; Amann *et al*. 1995). This allows the detection of uncultivable micro-organisms that remain invisible with conventional culture-based bacteriological techniques (Göbel 1995). In particular, the use of Small SubUnit ribosomal RNA (SSU rRNA)-encoding sequences has dramatically increased the understanding of microbial phylogenies and taxon diversity in a wide range of environments, ranging from plankton communities (Schmidt *et al*. 1991) through communities in sewage plants (Godon *et al*. 1997) and communities living in hot springs (Barns *et al*. 1994), to species complexes of uncultivable endosymbionts (Clark *et al*. 1992; Berchtold & König 1996; Noda & Kodama 1996; Krueger & Cavanaugh 1997).

Normally, either universal or very specific (genus level) primers are used to pick up DNA sequences from an environmental sample, but specific polymerase chain reaction (PCR) primers to amplify SSU rDNA sequences from precisely defined taxonomic groups are still scarce. As part of a study on the microbial symbionts of fungus-growing ants, we developed specific primers to amplify SSU rDNA from 12 groups of eukaryotic and eubacterial micro-organisms which are known to harbour parasites and symbionts of insects. The use of the reported primers surpasses the field of insect–microbe symbiosis, as they are also likely to be valuable tools in medical and environmental studies of free-living or pathogenic bacteria.

To design primers for group-specific amplification of SSU rRNA-encoding genes, we downloaded all known SSU rDNAs for each group from GenBank (Benson *et al*. 2000), aligned them using CLUSTALW (Thompson *et al*. 1994) (with some manual refinements of the alignment) and calculated a consensus sequence for each group. Potential primer pairs annealing to this consensus sequence were designed using PRIMER 0.5 (Lincoln *et al*. 1991). We gradually restricted primer-annealing properties by manipulating the melting temperature, the G + C content and potential secondary structures. Subsequently, potential primers annealing to group-specific conserved regions were tested against all known sequences of that group in GenBank using a BLAST search. Primers producing too many nonspecific hits were rejected or combined with a second primer not amplifying these nonspecific fragments, until complete group-specificity was achieved.

To optimize the specificity of the PCR, we obtained a representative species for each of the groups from a type culture collection (Table 1). DNA was extracted from these

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Table 1 Representative species used in the cross-amplification tests

Group (Kingdom)	Representative species	Source, accession no.
Microsporidia (EUK)	*Nosema locustae*	ATCC 30860
	Endoreticulatus sp.	ATCC 50040
Parabasalidea and Diplomonadida (EUK)	*Spironucleus barkhanus*	ATCC 50467
	Trepomonas agilis	ATCC 50286
	Hypotrichomonas acostata	ATCC 30069
Entamoebidae and Heterolobosea (EUK)	*Entamoeba invadens* Rodhain	ATCC 30016
	Naegleria lovaniensis	Local strain*
Euglenozoa and relatives (EUK)	*Euglena gracilis*	CCAP 1224/5Z
	Leptomonas seymori	ATCC 30220
	Blastocrithidia culicis	ATCC 30268
Eumycota — Fungi (EUK)	*Saccharomyces cerevisiae*	ATCC 287
	Beauveria bassiana	ATCC 9453
	Termitomyces albuminosus	ATCC 42010
Alveolata and Stramenopiles (EUK)	*Alexandrium tamarense*	CCAP 1119/1
	Ochromonas danica	ATCC 30004
	Paramecium tetraurelia	ATCC 30300
Flavobacteria (EUB)	*Flexibacter filiformis*	DSMZ 527
	Cytophaga aurantiaca	ATCC 12208
	Flavobacterium aquatile	ATCC 11947
Fibrobacter (EUB)	*Fibrobacter succicogenes*	ATCC 51216
Spirochetes and relatives (EUB)	*Spirochaeta aurantia*	DSMZ 1902
	Treponema denticola	ATCC 33520
	Brachypira innocens	ATCC 29796
Proteobacteria/Orientia (EUB)	*Pseudomonas aeruginosa*	ATCC 97
	Agrobacterium sp.	ATCC 12210
	Nitrosomonas europaea	ATCC 19718
Gram-positive hi GC (EUB)	*Streptomyces coelicolor*	DSMZ 40233
	Propioniferax innocua	ATCC 49929
	Pseudonocardia thermophila	ATCC 19285
Gram-positive low GC (EUB)	*Bacillus subtilis*	ATCC 82
	Mesoplasma lactucae	ATCC 49193
	Lactobacillus fermentum	ATCC 8289

Kingdoms of organisms are indicated with EUK (Eukaryota) or EUB (Eubacteria) for each taxon. Sources of pure cultures were: ATCC (American Type Culture Collection, Manassas, USA), CCAP (Culture Collection of Algae and Protozoa, Oban, UK), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, DE).
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GC = Guanine and Cytosine content.

type cultures by a 3-h incubation at 55 °C and 20 min boiling in 500 μL of a 10% BioRad Chelex 100 resin solution. Subsequently, the samples were centrifuged and stored at −20 °C until use. To avoid contamination, all pre-PCR procedures were carried out under a laminar flow hood. In a cross-amplification test, we tested a whole range of PCR conditions until we found conditions under which only the group-specific templates yielded a positive signal. We tested ranges for annealing temperature (45–65 °C), $MgCl_2$ concentration (0.5–2.0 mM), primer concentration (0.25–0.75 μM), number of amplification cycles (25–40) and extension time (60–90 s) in all possible combinations.

Each PCR included a negative control (double-distilled water), two or three group-specific positive controls selected to span the whole diversity of the group (Table 1) and 10 additional negative control templates (representatives from other groups, Table 1). PCR reactions were carried out in 25 μL reaction volumes, including 0.2 mM of each dNTP, 1 μL of the crude DNA extract, specific primers and $MgCl_2$ according to the tested conditions and 0.5 U of *Taq* DNA polymerase (AmpliTaq, Perkin Elmer Cetus). All PCRs were performed with an initial denaturation at 95 °C for 3 min and an additional denaturation at the beginning of each cycle at 95 °C for 30 s. The extension temperature was always 72 °C. Other cycling parameters varied with the conditions to be tested. Ten microlitres of the resulting reaction mixture was electrophoresed with a 100-bp DNA ladder size standard (GibcoBRL) on 1.5% agarose minigels. DNA bands were visualized by ethidium bromide staining.

Table 2 Primer pairs, concentrations of primer and $MgCl_2$, and conditions for group-specific PCR amplification of different groups of micro-organisms

Group (Kingdom)	Primers (5′-3′)	Bp	T_a (°C)	[$MgCl_2$] (mM)	[primer] (μM)	c	t_{ext} (s)
Microsporidia (EUK)	MicF1: GGACGAAGGCTAGAGGATCG Mic + R2: GACGGGCGGTGTGTACAA	612	58	0.75	0.375	40	60
Parabasalidea and Diplomonadida (EUK)	EukF1: AGCAGCCGCGGTAATTCC ParabaR1: GGCGGTAGTCTCGCTCGTT	814	66	1.25	0.5	35	60
Entamoebidae and Heterolobosea (EUK)	EukF1: AGCAGCCGCGGTAATTCC EntamoeR1: CAATCCTCGATCCCTGTCA	1833	65	1.5	0.5	35	60
Euglenozoa and relatives (EUK)	EugF1: TAANGCTGTTGCTGTTAAA UNIR2: GGTTACCTTGTTACGACTT	1058	45	2	0.5	40	60
Eumycota – Fungi (EUK)	EukF1: AGCAGCCGCGGTAATTCC EumycR2: ATCGCCCGATCCCYAGTC	517	62.5	1	0.5	35	60
Alveolata and Stramenopiles (EUK)	StramAlvF1: CAAGTTTCTGCCCTATCAGC UNIR5: TACGGYTACCTTGTTACGACTT	1565	45	1.5	0.5	40	90
Flavobacteria (EUB)	EubEukF1: CCTACTGGAGGCAGCAG FlavoR1: AGCCCTGGTAAGGTYCCTCG	694	64.5	1.5	0.5	35	60
Fibrobacter (EUB)	FibroF1: GAAATACCCGTGCCAACGC UNIR4: TGACGGGCGGTGTGTRCAAG	1376	63	1.5	0.5	40	90
Spirochetes and relatives (EUB)	SpirochF1: RYGCGTCTTAAGNATGCAAGTC Spiroch + R1: GACGGGCGGTGTGTACAA	1446	62	1	0.375	35	90
Proteobacteria (EUB)	EubEukF1: CCTACGGGAGGCAGCAG ProteoR1: CAAACCMAYTCCCATGGYITGAC	1105	63	1	0.375	35	90
Gram-positive high GC (EUB)	EUBF3: ACTCCTACGGGAGGCAGC GrpohiGCR2: CTGATCTGCGATTACTAGCGAC	1299	63	1.5	0.5	35	90
Gram-positive low GC (EUB)	GrpoloGCF1: ATACATAGGTGGCAAGCGTTATCCG UNIR4: TGACGGGCGGTGTGTRCAAG	1296	63	1	0.375	35	90

Bp = length of amplified fragments, T_a = annealing temperature, c = number of cycles, t_{ext} = extension time in seconds, GC = Guanine and Cytosine content.

Using the above-described procedure, we designed group-specific primers for six eukaryotic groups and six eubacterial groups known to contain insect-associated pathogens and/or symbionts (Table 2). These primers resulted in the amplification of partial SSU rRNA-encoding sequences between 517 and 1833 base pairs in length. Specific PCR amplification conditions for these primer pairs were determined using cross-amplification tests, resulting — for each primer pair — in a combination of annealing temperature, $MgCl_2$ concentration, primer concentration, extension time and number of amplification cycles for which only the group-specific template was amplified (Table 2, columns 4–8). During this optimization procedure, $MgCl_2$ concentration, primer concentration, annealing temperature, number of cycles and extension time were increased in steps of 0.25 mM, 0.125 μM, 0.5 °C, five cycles and 30 s, respectively. The optimal conditions given in Table 2 are the least stringent conditions under which amplification was still group-specific. Preliminary screenings using these primer sets indicated that representatives of at least five of these microbial groups occur inside *Acromyrmex* leaf-cutter ants (S.V.B., unpublished data).

The group-specific primers that we present here are likely to be generally useful tools to sample SSU rRNA-encoding sequences directly from any environment without prior cultivation. The amplification products yielded by these PCRs can be used as templates for targeted cloning and further sequencing studies. This will not only allow a better focus of such studies on peculiar microbial taxa of interest, but is also likely to enhance their resolution compared to alternative techniques using general PCR primers. In the latter case, a biased PCR amplification of sequences from micro-organisms that do not belong to the taxon of interest, but are very abundant in the environment, might prevent minority groups of micro-organisms from being detected. The primer pairs presented here can thus be used to amplify SSU rDNA in a wide variety of environments of biological and medical relevance.

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References

Amann RI, LudwigW, Schleifer K-H (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, 143–169.

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proceedings of the National Academy of Sciences of the USA*, **91**, 1609–1613.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA, Wheeler DA (2000) GenBank. *Nucleic Acids Research*, **28**, 15–18.

Berchtold M, König H (1996) Phylogenetic analysis and in situ identification of uncultivated Spirochetes from the hindgut of the termite *Mastotermes darwiniensis*. *Systematic and Applied Microbiology*, **19**, 66–73.

Clark MA, Baumann L, Munson MA, Baumann P, Campbell BC, Duffus JE, Osborne LS, Moran NA (1992) The eubacterial endosymbionts of whiteflies (Homoptera: aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. *Current Microbiology*, **25**, 119–123.

Göbel UB (1995) Phylogenetic amplification for the detection of uncultured bacteria and the analysis of complex microbiota. *Journal of Microbiological Methods*, **23**, 117–128.

Godon J-J, Zumstein E, Dabert P, Habouzit F, Moletta R (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Applied and Environmental Microbiology*, **63**, 2802–2813.

Krueger DM, Cavanaugh CM (1997) Phylogenetic diversity of bacterial symbionts of *Solemya* hosts based on comparative sequence analysis of 16S rRNA genes. *Applied and Environmental Microbiology*, **63**, 91–98.

Lincoln SE, Daly MJ, Lander SE (1991) PRIMER: A computer program for automatically selecting PCR primers: http://www.genome.wi.mit.edu/ftp/distribution/software/primer.0.5/.

Noda H, Kodama K (1996) Phylogenetic position of yeastlike endosymbionts of anobiid beetles. *Applied and Environmental Microbiology*, **62**, 132–167.

Schmidt TM, DeLong EF, Pace NR (1991) Analysis of a marine picoplankton community by 16S rRNA gen cloning and sequencing. *Journal of Bacteriology*, **173**, 4371–4378.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 6008–6013.

Ward DM, Bateson MM, Weller R, Ruff-Roberts AL (1992) Ribosomal RNA analysis of microorganisms as they occur in nature. In: *Advances in Microbial Ecology*, **Vol. 12** (ed. Marshall KC), pp. 219–286. Plenum Press, New York.