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RESEARCH NOTE

NEW COMBINATION OF PRIMER PAIRS FOR PCR-DGGE DETECTION OF SOIL CILIATES

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Ciliates (Protozoa: Ciliophora) are motile unicellular eukaryotes which can be found ubiquitously almost in all environments (Lynn, 2008; Vaerewijck *et al*., 2008). They are important components in soil food webs as they prey on microorganisms mainly bacteria and in turn, are consumed by other protozoa and metazoa (Foissner, 1999; Vaerewijck *et al*., 2008). They participate directly and indirectly in enhancing energy and nutrient flows in soil (Foissner, 1999; Li *et al*., 2008; Shimano *et al*., 2012). In addition, their unique physiological features such as delicate external membranes, short life cycle and fast reproduction have suited them as potential bioindicators of ecosystem changes (Foissner, 1999; Chen *et al*., 2008; Lara & Acosta-Mercado, 2012).

In recent years, denaturing gradient gel electrophoresis (DGGE) has become a popular and powerful molecular tool in studying soil ciliate community structures (Jousset *et al*., 2010; Shimano *et al*., 2012; Zhao *et al*., 2013). It is a rapid method which detects both culturable and non-culturable microorganisms (Muyzer, 1999; Marschner, 2007; Li *et al*., 2012) and aids non-taxonomists in identifying the species (Wu *et al*., 2009; Lara & Acosta-Mercado, 2012). As DGGE is a PCR-based method, the use of group-specific primers with high sensitivity in differentiating the species (Junier *et al*., 2008) is needed for an accurate study of ciliate communities (Dopheide *et al*., 2008; Jousset *et al*., 2010; Zhao *et al*., 2013). Several specific primers which have been designed and applied successfully in screening 18S rDNA of soil ciliates by DGGE were: (1) CilF, CilR I, CilR II and CilR III (Lara *et al*., 2007), (2) CilDGGE-r (Jousset *et al*., 2010) and (3) CS322F (Shimano *et al*., 2012). In this study, we presented new combinations of ciliate 18S rDNA primer pairs to provide alternative primers for PCR-DGGE detection of soil ciliates.

A total of 24 soil ciliates 18S rDNA sequences from various taxa of ciliate were retrieved from the National Centre for Biotechnology Information (NCBI) GenBank database. These DNA sequences were aligned with BioEdit software (Hall, 1999) using program ClustalW. The potential primer identified through this process was 5'-AAC CTG GTT GAT CCT GCC AGT-3'. The specificity of this primer was examined by conducting nucleotide BLAST searches in the GenBank database (http://www.ncbi.nlm.nih.gov/blast/). The BLAST results showed that it matched 100% with the deposited ciliate sequences in GenBank. However, it also matched a wide range of non-ciliate organisms. Furthermore, our finding matched exactly with a eukaryote-specific forward primer, EukA which was designed by Medlin *et al*. (1988).

Then we tested a new combination of primers to amplify the 18S rDNA of soil ciliates. Primer EukA was used with CilDGGE-r (a ciliate-specific reverse primer) to increase the specificity of PCR amplification. The specificity of this primer pair was tested in PCR with DNA templates extracted from a pure culture of fungi (*Shanorella spirotricha*), yeast (*Saccharomyces cerevisiae*), algae (*Trentepohlia annulata*) and two soil samples. The PCR mixture (25 µl) consisted of 1x *Taq* buffer, 0.2 mM dNTP mixture, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.75 U *Taq* DNA polymerase (Thermo Scientific, Lithuania) and 0.5 µl template DNA. The PCR program applied was: 95°C for 5 min, 30 cycles of

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94°C for 45 sec, 52°C for 1 min and 72°C for 1 min, and followed by a final extension at 72°C for 10 min. The result showed that this primer pair had low specificity for soil ciliates because the DNA of non-targeted organisms (fungi, algae and yeast) was amplified (Fig. 1).

Previous studies showed that the nested PCR is needed to increase the sensitivity and specificity of the detection of soil ciliates (Puitika *et al*., 2007; Martin *et al*., 2008; Jousset *et al*., 2010). Thus, a semi-nested PCR was performed and primers EukA/CilDGGE-r were used as the external primer pair. This external primer pair EukA/CilDGGE-r is located at the 18S rDNA of *Tetrahymena australis* at positions 1 and 940 respectively (Fig. 2). The expected PCR product is ~1,000 bp. Meanwhile, ciliate-specific primers CilF-GC/CilDGGE-r were used as the internal primer pair. This internal primer pair CilF-GC/CilDGGE-r is located at the 18S rDNA of *T. australis* at positions 315 and 940 respectively. The expected PCR product is ~600 bp.

The specificity of these primer pairs was tested in a semi-nested PCR-DGGE with DNA templates extracted from a pure culture of fungi (*Shanorella spirotricha*), yeast (*Saccharomyces cerevisiae*), algae (*Trentepohlia annulata*) and three soil samples. The first amplification was carried out as described in the previous section. The resulting PCR product was diluted and re-amplified using the internal primer pair CilF-GC/CilDGGE-r. A 40-bp GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to the 5' end of the forward primer. In the second amplification, the PCR mixture was prepared as in the first amplification while the PCR program was set according to Jousset *et al*. (2010). PCR products were examined on 1% agarose gel.

The result showed that the DNA of non-targeted organisms (fungi, algae and yeast) was not amplified in the second amplification (Fig. 3A). PCR products of three soil samples were further analyzed in DGGE to confirm the specificity of these primers on soil ciliates. The DGGE was performed using the D-Code System (Bio-Rad, USA) with a slight optimization of the parameters described by Jousset *et al*. (2010). Approximately 180 ng of PCR products were loaded on the 6% polyacrylamide gels with denaturing gradient ranging from 30-40% (100% denaturant contained 7 M urea and 40% formamide). Gel was stained with ethidium bromide for 30 min and destained with 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 5 min prior to UV transillumination, FluorChem 5500 (ProteinSimple, USA). Predominant DNA bands were excised and

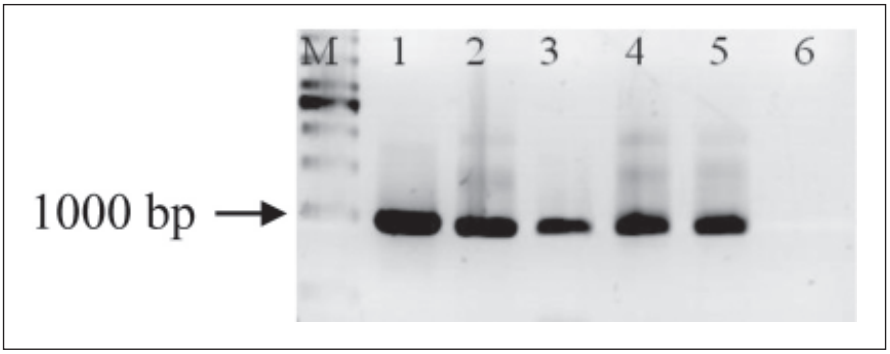

Fig. 1. PCR products amplified by primer pair, EukA/CilDGGE-r were analyzed on 1% agarose gel. The size of amplicons was ~1000 bp. Lane M, 1kb DNA ladder; lane 1, *S. spirotricha*; lane 2, *T. annulata*; lane 3, *S. cerevisiae*; lane 4, soil sample I; lane 5, soil sample II; lane 6, negative control.

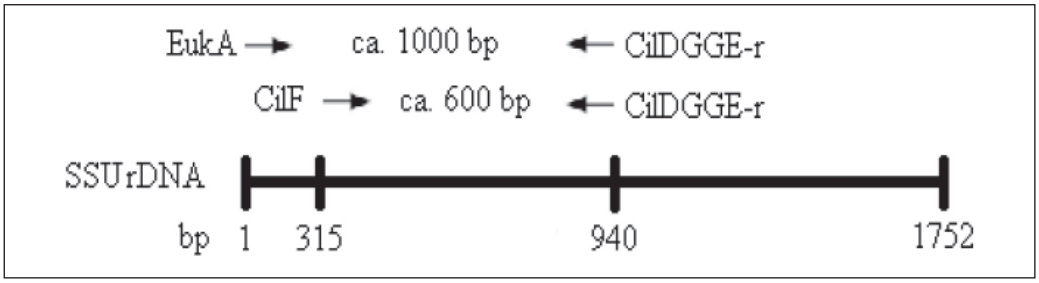

Fig. 2. Schematic diagram of the rDNA region targeted by the primers in reference to the SSU rDNA gene of *T. australis* (GenBank accession no. X56167).

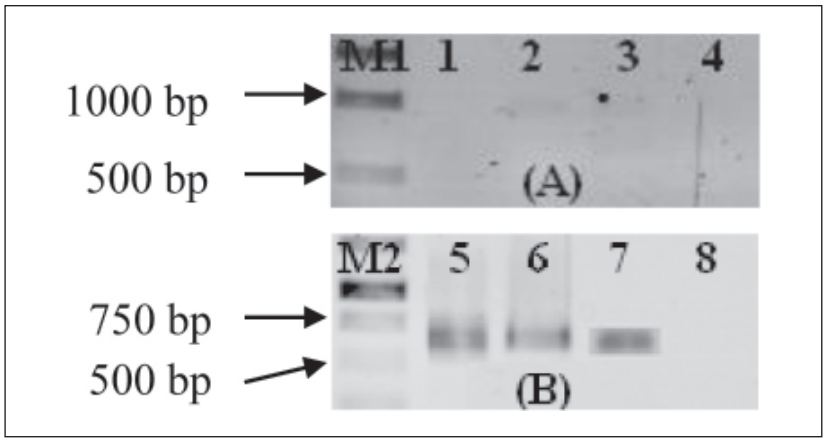

Fig. 3. Semi-nested PCR amplification using primer pairs EukA/CilDGGE-r and CilF-GC/CilDGGE-r. (A) Lane M1, 1kb DNA ladder; lane 1, negative control; lane 2, *S. spirotricha*; lane 3, *T. annulata*; lane 4, *S. cerevisiae*. (B) Lane M2, GeneRuler 1 kb DNA Ladder; lane 5, soil sample I; lane 6, soil sample II; lane 7, soil sample III; lane 8, negative control.

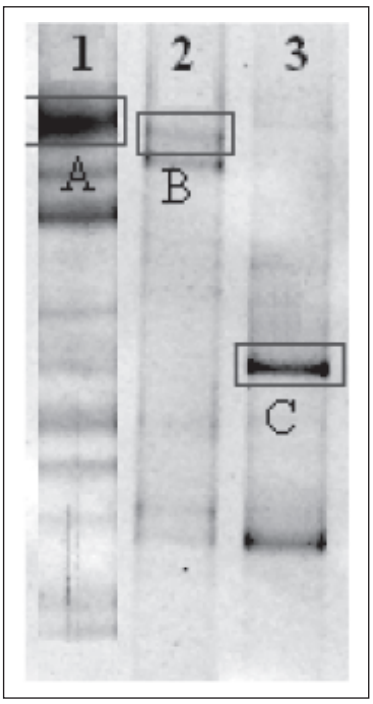

Fig. 4. DGGE profiles of 18S rDNA amplicons from soil samples. Lane 1, soil sample I; lane 2, soil sample II; lane 3, soil sample III. Three selected DGGE bands (A, B and C) were excised and sequenced.

suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C overnight. Then, 2 µl of the eluted DNA was used as a template in the re-amplification. The re-amplified PCR products were purified using GeneJET PCR Purification Kit (Thermo Scientific, Lithuania) and subjected to DNA sequencing using an ABI BigDye® Terminator v3.1 cycle sequencing kit and an ABI 3730XL Genetic Analyzer (Applied Biosystems, USA).

Multiple bands generated in DGGE gel (Fig. 4) showed a good resolution of DGGE profiles was obtained using these primer pairs. Three predominant DGGE bands were excised, sequenced and compared to the known ciliate 18S rDNA sequences in GenBank using the BlastN program (http://www.ncbi.nlm.nih.gov/blast/). Sequence analysis of the excised bands confirmed that these primers produced only amplicons from ciliates: (A) *Bistichella variabilis* (GenBank accession no. HQ699895.1)/ Oxytrichidae environmental sample clone Elev_18S_1189 (EF024702.1), (B) uncultured ciliate clone WIM26 (AM114813.1) and (C) Oxytrichidae environmental sample clone Elev_18S_1438 (EF024903.1). Identical matches between the excised sequences and known ciliate 18S rDNA sequences in GenBank were ranged from 94-98%. This showed that the specificity of the internal ciliate-specific primer set is high and the newly combined primer pairs are suitable and efficient in amplifying ciliate 18S rDNA from soil samples.

We further applied these primer pairs and semi-nested PCR-DGGE to study ciliate diversity in both agriculture and forest soils from the year 2010 to 2012. There was a total of 73 species of soil ciliates (including the unknown species) affiliated to 7 classes and 17 orders detected (data not shown). When compared with the previous studies on soil ciliates using PCR-DGGE approach with different primer pairs (Table 1), a wider range of ciliates (7 classes) was detected in our study. Besides, the newly combined primer pairs involved only three primers which reduce the cost, time and complexity in PCR. In addition, the size of our PCR products was larger (~600 bp) than the PCR products (~260 bp) obtained in Shimano *et al*. (2012). A larger

Table 1. Comparison of three primer sets for PCR-DGGE detection of soil ciliates

Method	Primer pair	Sequence (5' - 3')	Specificity	Position	Product size (bp)	Ciliate classes discovered in the study	Reference
Semi-nested PCR-DGGE	1. Forward: EukA	AACCTGGTTGATCCTGCCAGT	Eukaryotes	1[a]	600	Armophorea, Colpodea, Heterotrichea, Litostomatea, Nassophorea, Oligohymenophorea, Spirotrichea	This study
	Reverse: CilDGGE-r	TGAAAACATCCTTGGCAAATG		940[a]			
	2. Forward: CilF-GC	[GC clamp] TGGTAGTGTATTGGACWACCA	Ciliates	315[a]			
	Reverse: CilDGGE-r	TGAAAACATCCTTGGCAAATG		940[a]			
Semi-nested PCR-DGGE	1. Forward: CilF	TGGTAGTGTATTGGACWACCA	Ciliates	315[a]	600	Colpodea, Litostomatea, Nassophorea, Oligohymenophorea, Spirotrichea	Jousset *et al*., 2010; Zhao *et al*., 2012
	Reverse: CilR I	TCTGATCGTCTTTGATCCCTTA		959[a]			
	Reverse: CilR II	TCTRATCGTCTTTGATCCCCTA		959[a]			
	Reverse: CilR III	TCTGATTGTCTTTGATCCCCTA		959[a]			
	2. Forward: CilF-GC	[GC clamp] TGGTAGTGTATTGGACWACCA	Ciliates	315[a]			
	Reverse: CilDGGE-r	TGAAAACATCCTTGGCAAATG		940[a]			
Nested PCR-DGGE	1. Forward: EU60F	GAAACTGCGAATGGCTCATT	Eukaryotes	79[b]	260	Colpodea, Heterotrichea, Litostomatea, Oligohymenophorea, Phyllopharyngea, Spirotrichea	Shimano *et al*., 2012
	Reverse: EU929R	TTGGCAAATGCTTTCGC		913[b]			
	2. Forward: CS322F	GATGGTAGTGTATTGGAC	Ciliates	313[b]			
	Reverse: EU581RGC	[GC clamp] ATTACCGCGGCTGCTGGC		557[b]			

[a] Corresponding position in the SSU rDNA in *Tetrahymena australis* (GenBank accession no. X56167).
[b] Corresponding position in the SSU rDNA in *Tetrahymena corlissi* (GenBank accession no. U17356).

size of the PCR product provides more taxonomic information and produces a higher resolution of DGGE profiles (Berglund *et al*., 2005).

In conclusion, the results herein validated the use of this newly combined primer pairs in the semi-nested PCR-DGGE for the detection of a wide range of soil ciliates. We also believe that these primers will give valuable insight into the biodiversity of soil ciliates. Thus, we recommend these primer pairs for studying soil ciliate communities. Slight optimization of these primers and semi-nested PCR-DGGE might be needed for screening ciliates from different types of soil.

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