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Application of a Multiplex PCR with Specific PCR Primers for the Detection of the Genus *Paramecium* and the *Paramecium aurelia* Complex

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Summary. The representatives of the genus *Paramecium* are well-studied ciliates and can be used in water quality assessment and the determinations of saprobic levels. For these applications, a clear and unambiguous identification of ciliate assemblages is essential, which is typically based on morphological characters requiring a sound taxonomic knowledge and experience in species determination including microscopic identification of both living and stained specimens. Therefore, we developed and applied specific PCR primers for the detection of species belonging to the genus *Paramecium* and the *Paramecium aurelia* complex. These primers were successfully tested with different *Paramecium* species including representatives of the *P. aurelia* complex as well as closely related species like *Frontonia* sp. and *Tetrahymena* sp. in both experimental and environmental samples. These primers can be used in a simultaneous approach achieving fast and reliable results with regard to determination of ciliate community and water assessment.

Key words: Multiplex PCR, *Paramecium*, saprobic level, species specific primers.

INTRODUCTION

To date, 17 morphospecies have been described in *Paramecium* (Fokin *et al*. 2004). Several of these species have been used in water quality assessment and determination of the saprobic level (Berger *et al*. 1997). For example, the presence of species of the *P. aurelia* complex indicate good quality of effluent to slightly polluted waters (Curds and Cockburn 1970). By contrast, the presence of *P. putrinum* is an indicator of heavily polluted and oxygen-deficient water (Berger *et al*. 1997).

To draw conclusions about water quality using the presence or absence of the specific *Paramecium* species, clear and unambiguous determinations are required. These determinations have traditionally been carried out by the evaluation of morphological characters in combination with different silver-staining methods. However, these exact identifications require a profound morphological knowledge (Fokin *et al*. 2004). Investigations of ciliate communities were soon ex-

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panded by the use of molecular markers after their taxonomic significance had been realized. One approach is based on the analyses of clone libraries followed by sequence comparisons with GenBank data (e.g. Epstein and López-García 2008, and references therein). This sequencing approach is time consuming and expensive.

An alternative approach to identification is the use of diagnostic species- or clade-specific primers. Such diagnostic primers have been used especially of pathogenic protists; e.g. for the detection of Legionellae (Wellinghausen *et al*. 2001), *Cryptosporidium parvum* (Monis and Saint 2001, Udeh *et al*. 2000), and *Giardia lamblia* (Ghosh *et al*. 2000). This technique has been used to a lesser extent for other protists, including ciliates. Hide *et al*. (2003) presented a set of specific primers targeting *Blepharisma japonicum.* Also, for the differentiation of the genus *Spirostomum* species-specific PCR primers are available (Schmidt *et al*. 2007).

Moreover, specific primers have been applied in the differentiation of morphologically nearly indistinguishable species complexes. Petroni *et al*. (2003) developed a set of species-specific probes targeting three closely related species in *Euplotes,* and applied them successfully by *in situ* hybridization. The sibling species *Stylonychia mytilus* and *Stylonychia lemnae* can be readily distinguished with specific FISH-probes (Schmidt *et al*. 2006) and species specific PCR primers (Haentzsch *et al*. 2006).

In our investigation we aimed to develop a hierarchical primer set for the genus *Paramecium* and therein for the *Paramecium aurelia* complex. These primers should be applicable simultaneously, allowing a fast, reliable and unambiguous determination. Subsequently, based on such amplification results, assessment of water quality should be reliable, even without taxonomic knowledge.

MATERIAL AND METHODS

Origin of samples

Genomic DNA of all *Paramecium* isolates was kindly provided by D. Barth (Leipzig), C. Zschornack (Dresden), and E. Przyboś (Kraków, Poland).

Development of PCR-primers for the Multiplex-PCR

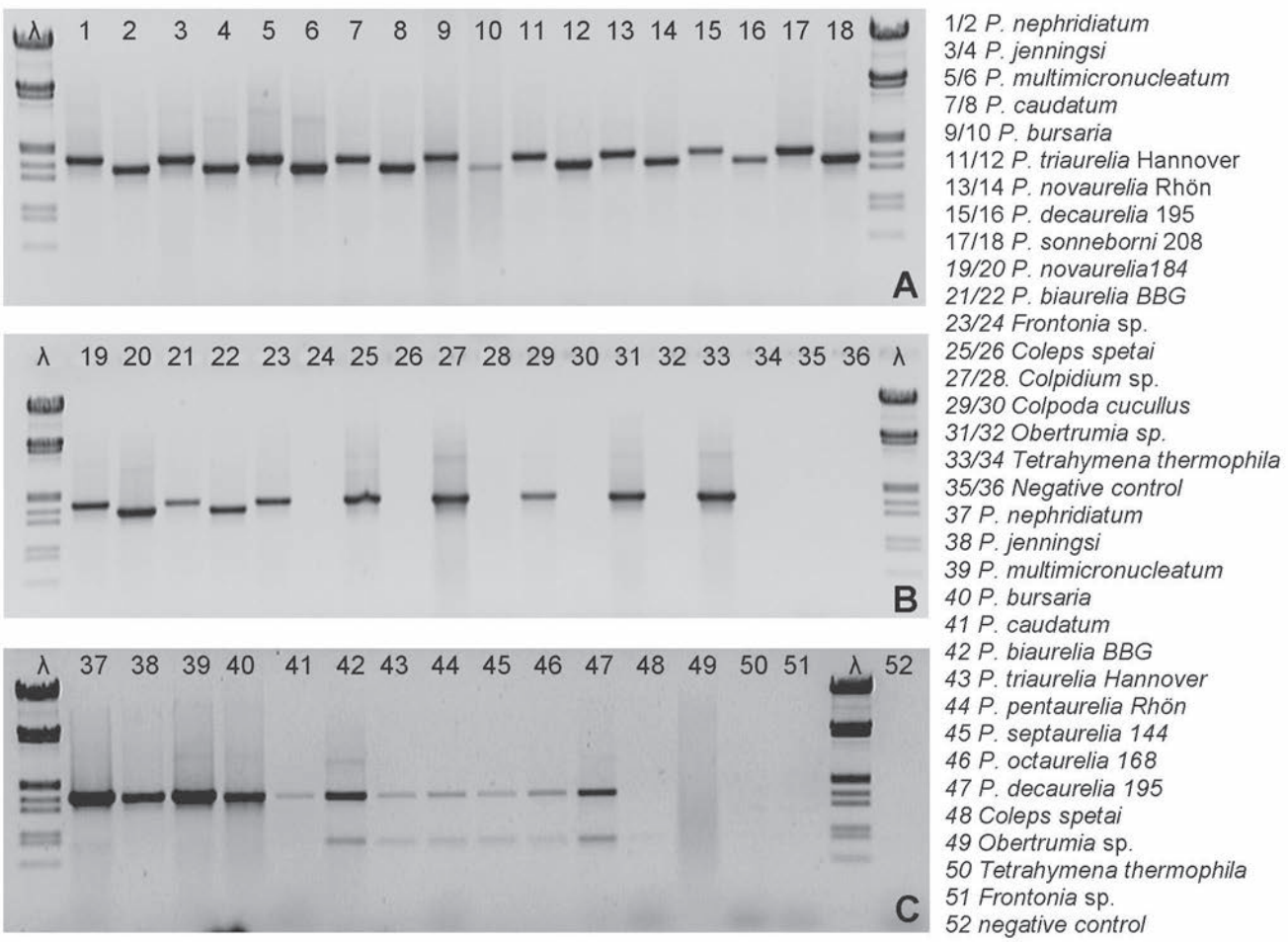
Based on an alignment using ClustalX (Thompson *et al*. 1997) of all available 18S rRNA gene sequences of *Paramecium* spp. (13 species) and 35 further ciliate species out of all ciliate classes, a genus-specific reverse primer (5'-GGCTGATGACCTRTGCTTACA-3') was developed. This primer targeting *Paramecium* was checked for cross matching with other DNA sequences using the program BLAST (Altschul *et al*. 1990), and then paired with a universal eukaryote specific SSU rDNA forward primer (Elwood *et al*. 1985) to amplify a 1564bp fragment. Additionally, for each investigated isolate a second amplification with two universal SSU rDNA primers (Elwood *et al*. 1985) was performed spanning the complete SSU rDNA. The PCR was done with 0.2 µM of each primer (18SF and *Paramecium* R), 1.5 U Taq polymerase (Fermentas), 10 × PCR buffer supplied with Taq, 200 µM dNTPs and 3–5 µl genomic DNA in a total volume of 50 µl. The reactions were carried out in a Mastercycler (Eppendorf) using the following PCR program: 5 min. denaturation at 95°C, followed by 35 cycles (45 s denaturation at 94°C, 1 min. annealing at 58°C, 1 min. elongation at 72°C), and a final elongation step at 72°C for 10 min. Amplifications were checked by gel electrophoreses.

After the first PCR that detected *Paramecium*, a second specific primer for the *P. aurelia* complex was developed and tested. To develop this *P. aurelia*-specific primer, 77 SSU rDNA sequences (Catania *et al*. 2009) from all sequenced species in this complex were added to the above alignment to find specific regions targeting the *Paramecium aurelia* complex. The specificity of this newly designed primer (5'-TCAAGTCCTAAAACCAACAA-3') was also determined using the BLAST search as above. In combination with the universal 18S forward primer this specific primer yielded an 813bp long fragment. To preclude the formation of primer dimers or multiple annealing sites, all oligonucleotides used were tested by the program Fast PCR (Kalendar *et al*. 2009). The amplification with three primers (18SF, *Paramecium* R and *P. aurelia* R) was done with 15 species out of five genera. The PCR conditions were the same as described above with the only exception of a modified annealing temperature (60°C).

RESULTS AND DISCUSSION

The detection of the genus *Paramecium* as well as the *Paramecium aurelia* complex was carried out by applications with newly developed specific primers in a Multiplex PCR. First, the *Paramecium*-specific primer was tested in combination with a universal 18S forward primer. In total, 16 different species were used for this analysis and each investigated species was tested twice. A first amplification was done as a positive control with two universal SSU rDNA primers that amplified the whole 18S rDNA gene, whereas a second amplification was performed with the new *Paramecium*-specific primer. The results of these amplifications and the following gel electrophoreses are shown in Figs 1A, B.

The lanes labeled with even numbers show the amplifications with the *Paramecium*-specific primer, and the odd numbers the positive controls (whole SSU rDNA).



Figs 1A–C. A–B – results of the amplification with universal 18S rDNA primers (odd numbers) and the specific *Paramecium* primer (even numbers) using different DNA samples of the genus *Paramecium* and other ciliated species; **C** – results of the Multiplex PCR employing three primers (universal 18SF and two *Paramecium*-specific primers) in combination with DNA derived from different ciliate species.

All tested *Paramecium* species (lanes 1 to 22) showed clear bands in both amplifications. Furthermore, the lengths of the PCR products corresponded to the expected size of 1564bp with the *Paramecium*-specific primer, and 1800bp for the whole SSU rRNA. In addition to the *Paramecium* isolates, six related species of other ciliated genera were investigated. These species showed bands (lane 23 to 34) only after amplification with the universal SSU rDNA primers, but no visible bands with the *Paramecium*-specific primer.

Only members of the genus *Paramecium*, therefore, yielded bands. All other tested species including related species generated no amplification products and we hypothesize that also other non-tested ciliate species will be discriminated.

After the application of the *Paramecium*-specific primer, a Multiplex PCR was performed using the *P. aurelia* primer (Fig. 1C).

The first five lanes show the results of representatives of the genus *Paramecium* that do not belong to the *P. aurelia* complex. As expected only the 1500bp long *Paramecium*-specific fragment from the first round PCR was visible. By contrast, amplifications using DNA from members of the *P. aurelia* complex (lane 42 to 47) clearly showed both bands; i.e. the *Paramecium*- and the *P. aurelia*-specific PCR-products. All non-*Paramecium* species as well as the negative control yielded no products. A Multiplex PCR using bulk genomic DNA isolated from a constructed wetland produced the same two bands (data not shown). This

result is in agreement with light microscopy control of the wastewater sample in which we identified members of the *Paramecium aurelia* complex (between 2 to 10 cells per ml).

In summary, the established Multiplex PCR – using *Paramecium*- and the *P. aurelia*-specific primers – produced the expected results for the tested isolates. Furthermore, the comparison with SSU rRNA data from all species out of the *Paramecium aurelia* complex (Catania *et al*. 2009) revealed no sequence differences within the primer binding site. This suggests optimal results also for other *P. aurelia* species that we did not test. Furthermore, the application of the developed primer set on related species like *Frontonia* sp. or *Tetrahymena thermophila* was discriminative. Based on this result, we assume that distantly related species will also yield no products using this primer set.

The development of these tools represents an easy, reliable, and cheap method for the determination of species in the genus *Paramecium* and the *P. aurelia* complex. In combination with already available specific primers fast insights into ciliate assemblages can be gained, especially by non-morphologists. For more detailed analyses of the different *Paramecium* species within an environmental sample, for each species specific primers have to be developed in order to increase the spectrum of diagnostics devices for the assessment of water quality and saprobic levels.

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REFERENCES

Altschul S. F., Gish W., Miller W., Myers E. W., Lipman D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215:** 403–410

Berger H., Foissner W., Kohmann F. (1997) Bestimmung und Ökologie der Mikrosaprobien nach DIN 38 410. G. Fischer, Stuttgart, Jena, Lübeck, Ulm

Catania F., Wurmser F., Potekhin A. A., Przybos E., Lynch M. (2009) Genetic diversity in the *Paramecium aurelia* species complex. *Mol. Biol. Evol.* **26:** 421–431

Curds C. R., Cockburn A. (1970) Protozoa in biological sewage-treatment processes–II. Protozoa as indicators in the activated-sludge process. *Water Res.* **4:** 237–249

Elwood H. J., Olsen G. J., Sogin M. L. (1985) The small-subunit ribosomal RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata. Mol. Biol. Evol.* **2:** 399–410

Epstein S., López-García P. (2008) "Missing" protists: a molecular prospective. *Biodivers Conserv*. **17:** 261–276

Fokin S. I., Przybos E., Chivilev S. M., Beier C. L., Horn M., Skotarczak B., Wodecka B., Fujishima M. (2004) Morphological and molecular investigations of *Paramecium schewiakoffi* sp. nov. (Ciliophora, Oligohymenophorea) and current status of distribution and taxonomy of *Paramecium* spp. *Eur. J. Protistol.* **40:** 225–243

Ghosh S., Debnath A., Sil A., De S., Chattopadhyay D. J., Das P. (2000) PCR detection of *Giardia lamblia* in stool: targeting intergenic spacer region of multicopy rRNA gene. *Mol. Cell. Probes* **14:** 181–189

Haentzsch M., Schmidt S. L., Bernhard D., Ammermann D., Berendonk T. U., Schlegel M. (2006) A PCR-based method to distinguish the sibling species *Stylonychia mytilus* and *Stylonychia lemnae* (Ciliophora, Spirotrichea) using isocitrate dehydrogenase (IDH) gene sequences. *J. Eukaryot. Microbiol.* **53:** 343–347

Hide G., Hughes J. M., McNuff R. (2003) A rapid and simple method of detection of *Blepharisma japonicum* using PCR and immobilisation on FTA paper. *BMC Ecol*. **3:** 7

Kalendar R., Lee D., Schulman A. H. (2009) FastPCR Software for PCR Primer and Probe Design and Repeat Search. *Genes, Genomes and Genomics* **3:** 1–14

Monis P. T., Saint C. P. (2001) Development of a nested-PCR assay for the detection of *Cryptosporidium parvum* in finished water. *Water Res.* **35:** 1641–1648

Petroni G., Rosati G., Vannini C., Modeo L., Dini F., Verni F. (2003) In situ identification by fluorescently labeled oligonucleotide probes of morphologically similar, closely related ciliate species. *Microb. Ecol.* **45:** 156–162

Schmidt S. L., Bernhard D., Schlegel M., Fried J. (2006) Fluorescence in situ hybridization with specific oligonucleotide rRNA probes distinguish the sibling species *Stylonychia lemnae* and *Stylonychia mytilus* (Ciliophora, Spirotrichea). *Protist* **157:** 21–30

Schmidt S. L., Treuner T., Schlegel M., Bernhard D. (2007) Multiplex PCR approach for species detection and differentiation within the genus *Spirostomum* (Ciliophora, Heterotrichea). *Protist* **158:** 139–145

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25:** 4876–4882

Udeh P., Veenstra J., Abraham A. J., John G. H. (2000) Quantitative polymerase chain (QPCR) reaction using the MIMIC approach to estimate *Cryptosporidium parvum* oocysts, an intestinal pathogen, in municipal water treatment sludge samples. *Mol. Cell. Probes* **14:** 121–126

Wellinghausen N., Frost C., Marre R. (2001) Detection of Legionellae in hospital water samples by quantitative real-time LightCycler PCR. *Appl. Environ. Microbiol*. **67:** 3985–3993

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