한국어병학회지 제 17 권 제 2 호 (2004)
J. Fish Pathol., 17(2) : 145 ~ 149 (2003)

- Brief Communication -

# Differentiation of Three Scuticociliatosis Causing Species in Olive flounder (*Paralichthys olivaceus*) by Multiplex PCR

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The definitive identification of ciliate species by morphological characteristics relies on time-consuming and laborious staining techniques. Therefore, in this study, we discriminated 3 scuticociliatosis causing species - *Pseudocohnilembus persalinus, Uronema marinum* and *Philasterides dicentrarchi* - in cultured olive flounder by multiplex PCR. The multiplex PCR based on the species-specific amplification of small subunit ribosomal RNA (SS rRNA) gene sequence enabled us to distinguish the 3 scuticociliate species in a simple and rapid manner, even in the sample containing the three species simultaneously.jThese data suggest that the multiplex PCR strategy would make it possible to avoid the cumbersome and time-consuming procedures of morphological analysis for the definitive identification of scuticociliates.

*Key words*: *Pseudocohnilembus persalinus*, *Uronema marinum*, *Philasterides dicentrarchi*, Scuticociliatosis, Olive flounder, SS rRNA gene, Multiplex PCR

Scuticociliates belonging to the genera *Uronema, Miamiensis* and *Philasterides*, free-living protozoans which are ubiquitous in the marine environment, have been associated with focal or systemic infections in marine fish (Thompson and Moewus, 1964; Cheung *et al.*, 1980; Dragesco *et al.*, 1995; Munday *et al.*, 1997; Iglesias *et al.*, 2001; Jee *et al.*, 2001). In Korea, *Uronema marinum* was reported as a causing species of scuticociliatosis in olive flounder, *Paralichthys olivaceus* (Jee *et al.*, 2001). Recently, we identified 2 other scuticociliatosis causing species - *Pseudochonilembus persalinus* and *Philasterides dicentrarchi* - from cultured olive flounder by morphological characteristics and small subunit ribosomal RNA (SS rRNA) gene full sequences (Kim *et al.*, 2004 a,b; submitted).

Although several attempts have been done to treat scuticociliatosis by various chemotherapeutics (Iglesias *et al.*, 2002; Quintela *et al.*, 2003), there is at present no effective *in vivo* chemotherapeutics especially for internal infections. Therefore, development of effective vaccines may be the best way to control scuticociliatosis. Recently, Iglesias *et al.* (2003) reported that *Philasterides dicentrarchi* expressed immobilization antigens (i-antigens) on its surface which was recognized by the turbot immune system and which might be useful for vaccination. They pointed out, however, the existence of different serotypes of the ciliate and outbreaks of the disease in turbot by another scuticociliate, *U. marinum*, which very probably expresses i-antigens of its own. Thus identification and differentiation of ciliate species responsible for scuticociliatosis in a host species would be the first step for development of effective vaccines.

The definitive identification of ciliate species by morphological characteristics relies on time-consuming and laborious staining techniques. There-

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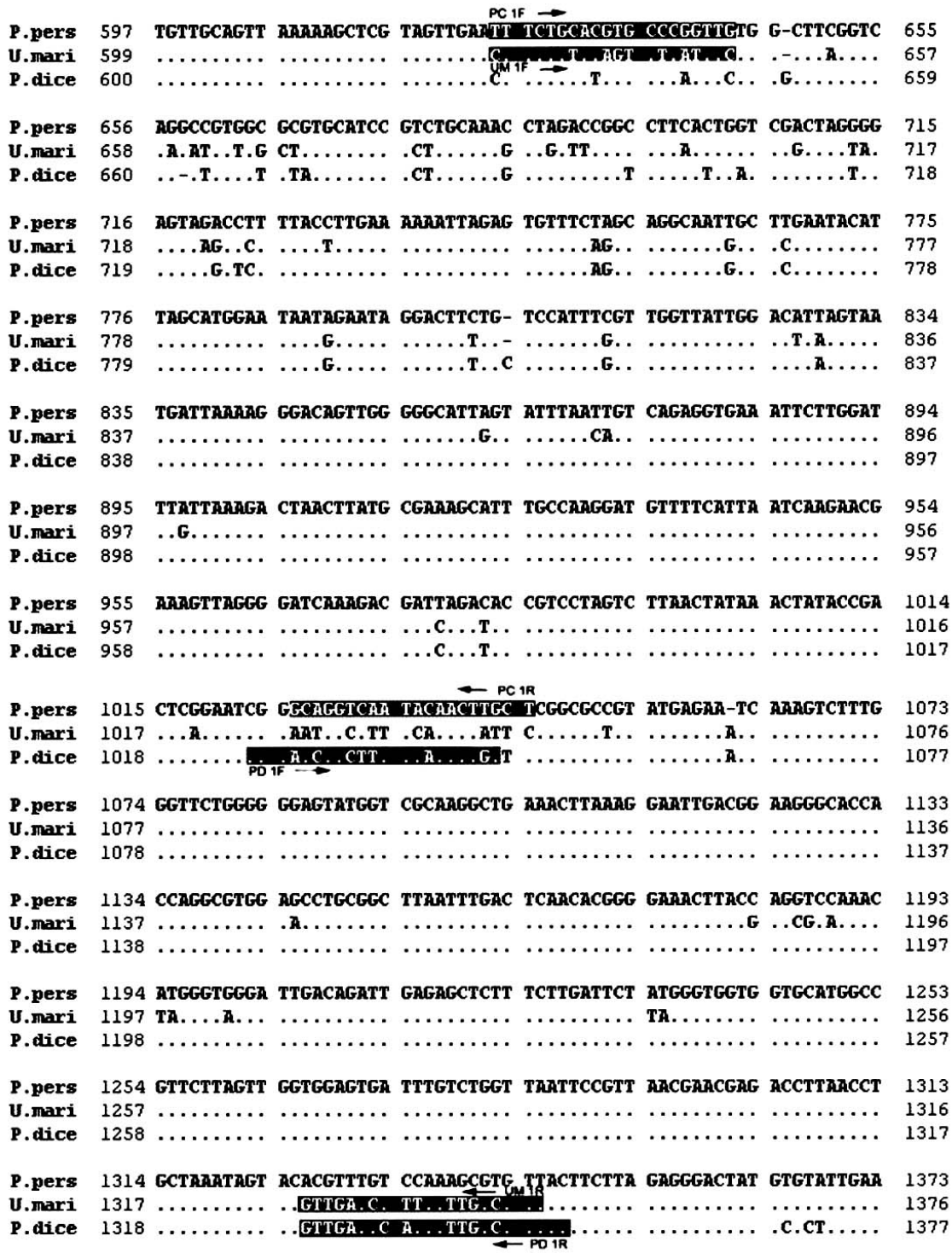


Fig. 1. Sequence alignment of SS rRNA genes from three scuticociliate species: *Pseudocohnilembus persalinus* (P. pers; GenBank AY551906), *Uronema marinum* (U. mari; GenBank AY551905) and *Philasterides dicentrarchi* (P. dice; GenBank AY642280). The six oligonucleotides primers (PC 1F and PC 1R for *P. persalinus*; UM 1F and UM 1R for *U. marinum*; PD 1F and PD 1R for *P. dicentrarchi*) used for multiplex PCR were shown in black boxes.

fore, in this study, we discriminated those 3 scuticociliatosis causing species in cultured olive flounder by multiplex PCR based on the specific primers of SS rRNA genes.

Ciliates were isolated from brain, gill or ulcerated skin of olive flounders *Paralichthys olivaceus* collected from several local fish farms in Korea, and were identified by morphological characteristics and SS rRNA gene sequences. Each species of ciliates were cultured separately in filtered seawater supplemented with autoclaved yeast extracts at 20℃.

Based on the comparison of SS rRNA gene sequences of *P. persalinus* (Gene Bank accession number AY551906), *U. marinum* (AY551905) and *P. dicentrarchi* (AY642280), hyper-variable regions were selected to design PCR primers for the species-specific amplification of SS rRNA gene segments using multiplex PCR. The sequence and location of each forward and reverse primer were shown in Fig. 1. Genomic DNA of each ciliate species was extracted using Accuprep® Genomic DNA Extraction Kit (Bioneer Co., Daejeon, Korea). To perform the multiplex PCR, equal amount of six oligonucleotides (10 pmoles each) was included in a PCR reaction. The reaction (20 $\mu\ell$) was 30 cycled at 95℃ for 30 sec, 50℃ for 30 sec and 72℃ for 30 sec. Five $\mu\ell$ of amplified PCR product was separated onto a 1% agarose gel and visualized using ethidium bromide staining. The expected sizes of PCR products are 421 bp for *P. persalinus*, 722 bp for *U. marinum*, and 326 bp for *P. dicentrarchi*.

DNA fragments of the expected size were obtained using the PCR procedure to amplify SS rRNA genes from DNA extracted from the ciliates, *Pseudochonilembus persalinus* (421 bp), Uronema marinum (722 bp) and *Philasterides dicentrarchi* (326 bp) (Fig. 2). DNA from the sample containing 2 species association - *P. persalinus-U. marinum, P. persalinus-P. dicentrarchi* and *U. marinum-P. dicentrarchi* - generated amplicons corresponding each species. respectively. All three amplicons were obtained when DNAs extracted from the 3 ciliate species were present simultaneously.

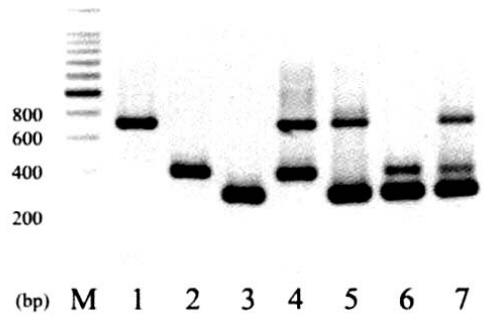


Fig. 2. Ethidium bromide-stained 1.0% agarose gel showing the multiplex PCR products amplified with genomic DNAs from the three scuticocilate species using the multiplex PCR primer mix.
Lane (M) Molecular weight size marker, (1) *Uronema marinum* (2) *Pseudocohnilembus persalinus* (3) *Philasterides dicentrarchi* (4) *U. marinum* and *P. persalinus* (5) *U. marinum* and *P. dicentrarchi* (6) *P. persalinus* and *P. dicentrarchi* (7) *U. marinum, P. persalinus* and *P. dicentrarchi*.

In the present study, the multiplex PCR based on the species-specific amplification of SS rRNA gene sequence enabled us to distinguish 3 scuticociliate species - *Pseudocohnilembus persalinus, Uronema marinum* and *Philasterides dicentrarchi* - in a simple and rapid manner, even in the sample containing the 3 species simultaneously. These data suggest that the multiplex PCR strategy would make it possible to avoid the cumbersome and time-consuming procedures of morphological analysis for the definitive identification of ciliates.

Considering no known chemotherapeutics against internal infection of scuticociliates, development of vaccine for scuticociliatosis would be the most effective control measure. Although immobilization antigen (i-antigen) on the surface of patho-

genic ciliates has been intensively studied as a target for inducing protective antibody (Clark *et al.*, 1995; Iglesias *et al.*, 2003), there are markedly higher diversity and variability of i-antigen in ciliates even in a same species at different conditions (Lin *et al.*, 2002; Wang *et al.*, 2002). Occurrences of scuticociliatosisin olive flounder by various ciliate species suggest that common and more stable target antigens should be explored to develop effective vaccines.

We here describe a genetic method which discriminates between different scuticociliate species. Such approaches to that described here may contribute to a better understanding of the epizootiology of scuticociliatosis together with more rational approaches to the control of this disease. Although, *P. persalinus, U. marinum* and *P. dicentrarchi* were reported as causing species of scuticociliatosis, it can not be excluded that other scuticociliate species may be involved in scuticociliatosis in olive flounder, and more extended studies are needed.

## Acknowledgements

This study was supported by a grant from the Ministry of Maritime Affairs and Fisheries, Republic of Korea.

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Manuscript Received : January 22, 2004
Revision Accepted : April 26, 2004
Responsible Editorial Member : Sung-Ju Jung (Yosu Univ.)