

# Strains of *Uronema marinum* (Scuticociliatia) co-isolated with amoebae of the genus *Neoparamoeba*

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**ABSTRACT:** Mixed infections of histophagous ciliates and *Neoparamoeba* spp. Page, 1987 were diagnosed in gill tissue of farmed turbot *Psetta maxima* (synonym: *Scophthalmus maximus*) and Atlantic salmon *Salmo salar* during a study of amoebic gill disease. Ciliates co-isolated from lesions grossly visible on gills of both fish hosts and from 2 species of red algae *Lithophyllum racemus* and *Palmaria palmata* were characterized morphologically and by using molecular markers. Sequences of small subunit (SSU) rDNA were determined for 6 strains of ciliates isolated from hosts collected in geographically distant localities. Of these, sequences of 5 strains revealed a surprisingly high level of similarity and identified the corresponding strains with *Uronema marinum* Lynn et Small, 1997. Thus, the set of environmental sequences of *U. marinum* available in the GenBank database to date was supplemented with the first sequences of potentially histophagous strains. On the basis of SSU rDNA, the 6th strain, also isolated from affected fish gills, was identified as *Ariasterostoma* sp.

**KEY WORDS:** *Uronema marinum* · SSU rDNA · Histophagous strains

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## INTRODUCTION

Marine scuticociliates described as free-living organisms and symbionts or 'harmless' commensals of invertebrates are well known also as primary agents causing serious disease conditions, both in invertebrate and fish hosts (Cheung et al. 1980, Dyková & Figueras 1994, Dragesco et al. 1995, Cawthorn et al. 1996, Munday et al. 1997, Crosbie & Munday 1999, Alvarez-Pellitero et al. 2004, Kim et al. 2004a,b, Paramá et al. 2006, Rossteuscher et al. 2008). Etiological studies of amoebic gill disease (AGD) that focused on the isolation of the agent of AGD from gill tissues turned our attention to ciliates that were found in primary isolates together with *Neoparamoeba* spp. (Dyková et al. 1999, Dyková & Novoa 2001). Since the ciliates were found in mixed infections with trophozoites of *Neoparamoeba* spp. and also in histological sections of affected gill tissues of turbot *Psetta maxima*

(synonym: *Scophthalmus maximus*) and Atlantic salmon *Salmo salar*, exact determination of the identity of ciliates established in cultures became of great interest.

## MATERIALS AND METHODS

### Primary isolation, culture and subculture of ciliates.

Ciliates included in this study were isolated together with amoebae of the genus *Neoparamoeba* Page, 1987 in the course of extensive screening of fish gill tissues for the presence of an agent of AGD and during the search for amoebae of the genus *Paramoeba* Schaudinn, 1896 in invertebrate and environmental samples from 1996 to 2008. Generic assignment of amoeba strains was based on light and electron microscopic observation. Species determination of 3 *Neoparamoeba* strains was supplemented with molecular

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characterization (Dyková et al. 2007, 2008). Ciliates included in the present study are listed in Table 1. This set of strains contains only those co-isolated with *Neoparamoeba* strains from a same sample (gill tissue or organic material covering the surface of red algae). Non-nutrient 75% seawater agar (BD Bacto™ Agar) was routinely used for primary isolations. To establish the ciliates in culture, a clean population of each isolate of ciliates was selected with the aid of a light microscope and transferred (together with piece of agar) to MY75S (Malt & Yeast Extract–75% seawater) medium (catalogue of the UK National Culture Collection). The same medium was used for the cloning procedures carried out in cell wells at 20°C. For subculturing of ciliates, initially MY75S medium supplemented with autoclaved pieces of freshwater fish gill tissue was used. Later, the ciliates were fed fathead minnow *Pimephales promelas* (FHM) cell line cells. Culture medium used for the FHM cell line was removed from each culture flask and substituted with MY75S medium containing ciliates of the parent culture. Ciliates were subcultured weekly. Both methods of subculturing were used alternately with varying success.

**Morphological characterization of ciliates.** Gross morphological features of living ciliates were observed with light microscopy by means of Nomarski differential interference contrast. Details of ciliature, not visible in living specimens, were studied in stained preparations and with scanning electron microscopy (SEM). For purposes of SEM, ciliates were fixed in 3% cacodylate buffered glutaraldehyde, washed in 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide. Samples were dehydrated in a graded acetone series, critical-point dried with liquid CO<sub>2</sub>, sputter-coated with gold-palladium and examined with a JEOL 6700F electron microscope. The wet silver nitrate method by Chatton and Lwoff as described in Foissner (1991) and the ammoniacal silver carbonate method (Fernández-Galiano 1994) were used for morphological studies of clonal cultures of individual strains. To eliminate negative influence of salts from the culture medium, repeated washing of samples was applied in both methods after ciliates were fixed.

#### DNA isolation, amplification and sequencing.

Genomic DNA was extracted from pelleted ciliates using the JETQUICK Tissue DNA Spin Kit (Genomed) according to the manufacturer's protocol. Universal eukaryotic primers (5'-ACC TGG TTG ATC CTG CCA G-3' and 5'-CTT CCG CAG GTT CAC CTA CGG-3') reported by Barta et al. (1997) were used for amplification of the small subunit (SSU) rRNA gene. PCR was carried out in a 25 µl reaction volume using 1 µM of each primer, 200 µM of each dNTP, 2.5 µl of 10 × Taq polymerase buffer and 1 unit of TaqDNA polymerase (Top-Bio). The PCR amplification was performed with a Tpersonal Thermocycler (Biometra). The cycling conditions were as follows: initial 5 min denaturation at 95°C followed by 5 amplification cycles (each comprising 94°C for 1 min, 44°C for 1.5 min and 72°C for 2 min), and another 25 cycles (each comprising 94°C for 1 min, 48°C for 1.5 min and 72°C for 2 min). The final 10 min extension ran at 72°C. The amplified products were purified from 1% agarose gel using JETQUICK Gel Extraction Spin Kit (Genomed) and cloned into pDrive Cloning Vector using the QIAGEN PCR Cloning Kit (Qiagen GmbH). Sequencing was carried out on an automatic sequencer ABI 3130x1 using the ABI PRISM BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems).

The forward primer G-FOR 5'-GGG ATC CGT TTC CGT AGG TGA ACC TGC-3' and reverse G-REV 5'-GGG ATC CAT ATG CTT AAG TTC AGC GGG T-3' (Coleman & Vacquier 2002) were used for PCR amplification of the whole internal transcribed spacer (ITS) region (ITS1, 5.8S and ITS2). The cycling conditions included an initial 5 min denaturation at 95°C followed by 30 cycles of a 1 min denaturation at 95°C, a 2 min annealing at 46°C, a 1 min extension at 72°C and a final 10 min extension at 72°C.

**Phylogenetic analysis.** To determine phylogenetic relationships of SSU rDNA sequences representing our strains of ciliates and those available from the GenBank database, the following methods were employed. The alignment was prepared in ClustalX 2.0.6 (Larkin et al. 2007). Ambiguously aligned positions were deleted manually in BioEdit (Hall 1999). The resulting alignment

Table 1. Denomination and origin of strains of ciliates co-isolated with *Neoparamoeba* strains. Generic assignment of amoebae co-isolated with CTAS/I, CTAS2/II and CTAS4/I was based on morphology of trophozoites

Strain/clone	Host species	Geographic origin	Sample origin	Co-isolated amoeba
CESP/I	<i>Psetta maxima</i>	Atlantic Ocean, NW Spain farm	Gills	<i>N. pemaquidensis</i> AFSM2/I
CTAS/I	<i>Salmo salar</i>	Tasman Sea, floating sea cages	Gills	<i>Neoparamoeba</i> sp.
CTAS2/II	<i>Salmo salar</i>	Tasman Sea, floating sea cages	Gills	<i>Neoparamoeba</i> sp.
CTAS4/I	<i>Salmo salar</i>	Tasman Sea, floating sea cages	Gills	<i>Neoparamoeba</i> sp.
CLIT/I	<i>Lithophyllum racemus</i>	Adriatic Sea, Croatia		<i>N. pemaquidensis</i> LITHON/I
CPAL2/I	<i>Palmaria palmata</i>	Norwegian Sea, reg. Trondheim		<i>N. pemaquidensis</i> PAL2/I

contained 1648 positions. Maximum likelihood (ML) analyses were performed with the program RAxML 7.0.3 (Stamatakis 2006) with the use of GTR+ $\Gamma$  model and rapid bootstrapping (100 replicates). Program PAUP\* 4.0b10 (Swofford 2000) was used to compute maximum parsimony (MP) analyses. Heuristic searches were conducted with 10 repeated searches in which the starting tree was constructed by random taxa addition and swapped with the tree bi-section–reconnection (TBR) algorithm. The number of bootstrap replicates was 1000.

## RESULTS

During our study of AGD, a limited number of mixed infections of ciliates and amoebae of the genus *Neoparamoeba* was diagnosed histologically (Fig. 1) compared with the number of samples used for isolation attempts. Co-isolation of ciliates and free-living amoebae from samples of gill tissues occurred quite frequently. Of 40 primary isolates of *Neoparamoeba* spp., 22 (55%) were accompanied by ciliates. If present, ciliates started to multiply as soon as the decay of gill tissue samples began after being placed with pieces of algae on the surface of agar plates. Actively moving and multiplying ciliates were always restricted to liquefying parts of tissue samples (Fig. 2). As the quantity of liquid gradually decreased, trophozoites became less active and more rounded.

Five of six strains of ciliates co-isolated with *Neoparamoeba* spp. included in this study revealed a conspicuous similarity in their morphology (see Figs. 1, 3 & 4 for details). Trophozoites were ovoid to elongate-ovoid with an estimated maximum body length and width averaging 24.2 and 10.1  $\mu\text{m}$ , respectively. Somatic ciliature revealed holotrichous distribution with 14 somatic kineties (Fig. 7). The oral region was in the anterior half of the cell (Figs. 6 & 8). Oral ciliature was relatively inconspicuous. Scutica, the hook-like configuration of kinetosomal structure, was observed in specimens impregnated with the wet silver nitrate method. A globular to ellipsoid macronucleus was located midlength along the body and the micronucleus in its vicinity was visualized by the ammoniacal silver carbonate method (Figs. 10 & 11). A single contractile vacuole was located in the posterior half of the body (Fig. 5). Caudal cilium was not clearly seen in any individual observed. Based on these morphological features, clonal cultures of 5 strains (CLIT, CESP, CPAL2, CTAS and CTAS4) were assigned to the genus

*Uronema* Dujardin, 1841. Unfortunately, we did not succeed in cryopreservation of these *Uronema* strains although repeated attempts were made by means of various protocols. However, samples of DNA are stored in our collection.

The 6th strain (CTAS2) was included in this study on the basis of superficial morphological similarities observed in the living individuals; nevertheless, it differed from the other strains in stained preparations. The results obtained using the same staining procedures did not seem sufficient for the generic assignment of this strain on the basis of morphological features. Unlike the previous group of strains, cryopreservation of CTAS2 strain was successful and samples of DNA were also stored.

Sequences of SSU rDNA and internal spacers (ITS1, ITS2) that were determined for the strains studied have been deposited in the GenBank database. Accession numbers of sequences are listed and sequence lengths are summarized in Table 2.

Phylogenetic analyses based on SSU rDNA revealed a close relationship of sequences of 5 strains of ciliates morphologically assigned to the genus *Uronema* and positioned them to the well-supported clade of *Uronema marinum* (Fig. 12). Within this clade, the sequences of 4 strains (CESP, CLIT, CPAL2 and CTAS) were of identical length, consisting of 1751 bp (SSU rDNA) and 484 bp (ITS). The sequence of the CESP strain differed in only one nucleotide in the ITS2 region. In the sequences of CTAS4 there were 8 differences in the ITS1 region and 6 in ITS2, although the total length of both ITS and SSU rDNA differed by one nucleotide only (485 and 1752 bp, respectively).

The sequence of the CTAS2 strain isolated from gills of Atlantic salmon branched as a sister sequence of *Aristerostoma* sp. (EU264563). Together with the sequence of *A. marinum* (EU264562) obtained by Dunthorn et al. (2009), it formed a well-supported clade. Although we were unable to determine the CTAS2 strain morphologically, the unambiguous result of phylogenetic analysis provided a basis of assignment of the strain CTAS2 to the genus *Aristerostoma* Kahl, 1931.

Table 2. Strains of ciliates, ordered by the base pair length of their internal transcribed spacer (ITS) and small subunit (SSU) rDNA sequences

Strain/clone	ITS1	5.8S	ITS2	Total ITS	SSU	Accession nos. ITS/SSU
CESP/I	133	120	231	484	1751	GQ259754/GQ259744
CLIT/I	133	120	231	484	1751	GQ259751/GQ259745
CPAL2/I	133	120	231	484	1751	GQ259752/GQ259746
CTAS/I	133	120	231	484	1751	GQ259753/GQ259747
CTAS4/I	134	120	231	485	1752	GQ259755/GQ259749
CTAS2/II				632	1754	GQ259750/GQ259748

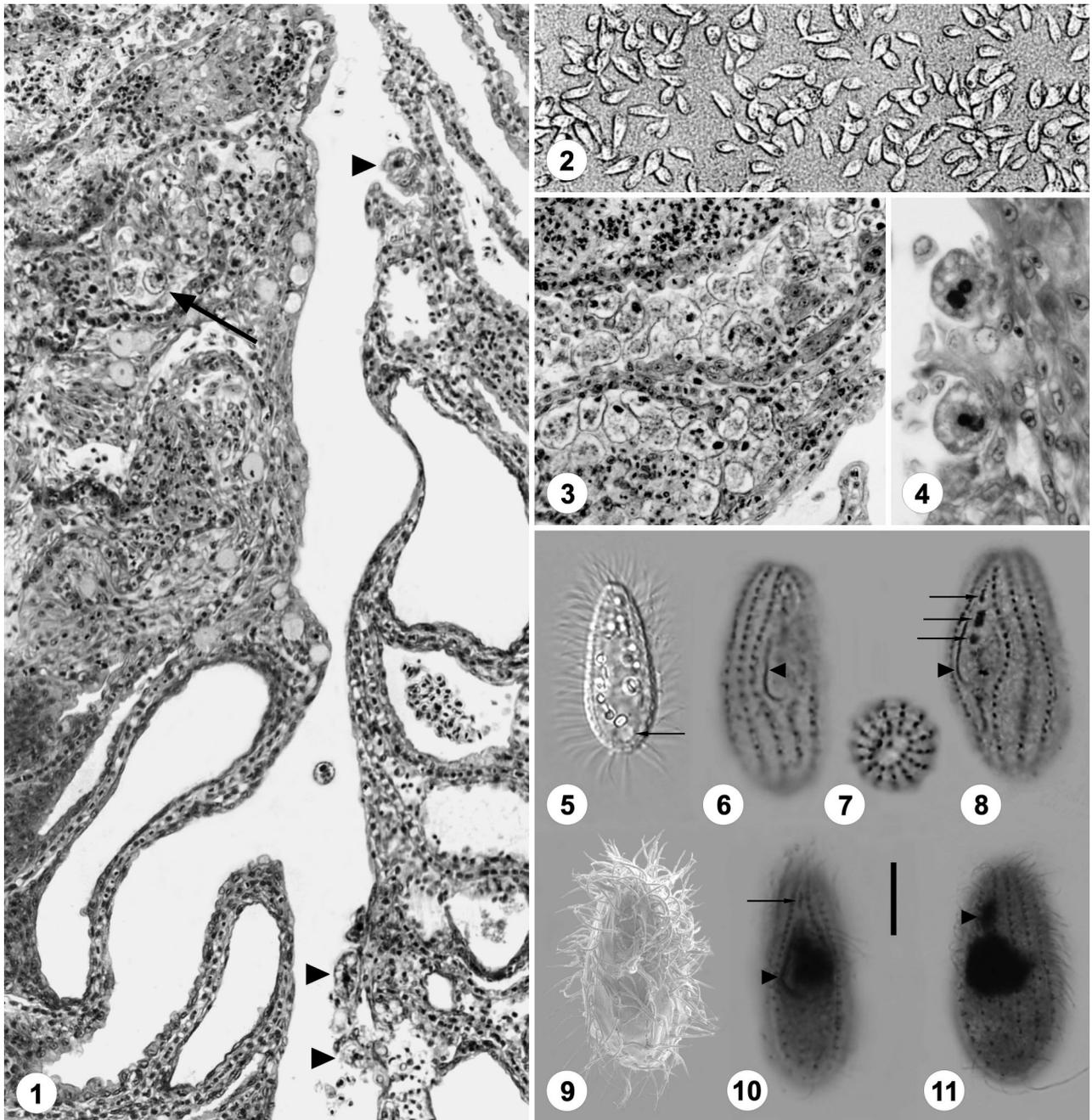


Fig. 1. *Psetta maxima*. Mixed gill infection of histophagous ciliates and *Neoparamoeba* sp. in turbot. Arrow indicates ciliates in the interlamellar epithelium; arrowheads mark attached trophozoites of *Neoparamoeba* sp. Low magnification was used to document the mixed infection in one field of view. Stained with haematoxylin and eosin (H&E). Magnification  $\times 130$ . Fig. 2. Trophozoites of histophagous ciliates co-isolated with *Neoparamoeba* sp. as seen on the surface of an agar plate. Fig. 3. Detail of ciliates localised in the interlamellar epithelium of the sample shown in Fig. 1. Stained with H&E. Magnification  $\times 220$ . Fig. 4. Trophozoites of *Neoparamoeba* sp. attached to the surface of hyperplastic epithelium of the sample shown in Fig. 1. Stained with H&E. Magnification  $\times 570$ . Figs. 5 to 11. *Uronema marinum*. Morphological features of isolated strains. Fig. 5. Live trophozoite of CTAS4 strain as observed in Nomarski DIC; arrow points at the contractile vacuole. Figs. 6 to 8. Wet silver nitrate impregnation demonstrating buccal apparatus and longitudinal kineties in lateral and apical views (CTAS4 strain). Arrowheads point at the paroral membrane. Fine arrows point at the 3 oral polykinetids in Fig. 8. Fig. 9. Trophozoite of CESP strain as seen in SEM in ventral view. Figs. 10 & 11. Silver carbonate impregnated trophozoites of the strain CTAS4. Fine arrow marks 1 oral polykinetid in Fig. 10; arrowhead marks paroral membrane in Fig. 11. Arrowhead marks micronucleus side to macronucleus. Scale bar = 10  $\mu\text{m}$  for Figs. 5 to 11

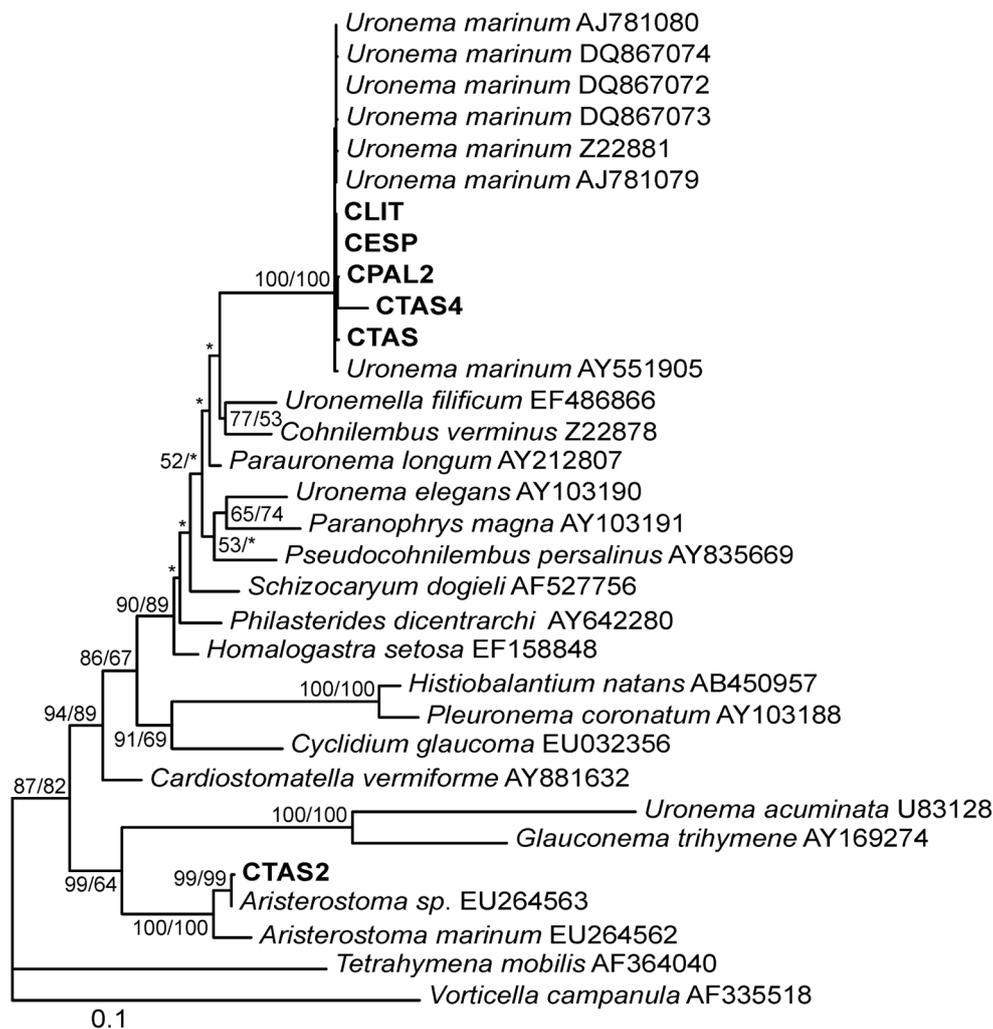


Fig. 12. Phylogenetic tree resulting from maximum likelihood (ML) analysis of a dataset comprising 30 SSU rDNA sequences of philasterid ciliates plus 2 outgroups. Six sequences determined in the present study are indicated in bold text. Numbers at the nodes are bootstrap values for ML/maximum parsimony analysis, respectively. Asterisks (\*) represents bootstrap values < 50. (Note: *Philasterides dicentrarchi* is a junior synonym of *Miamiensis avidus*)

## DISCUSSION

The role of several species of histophagous ciliates has been studied in relation to their effect on the health of ornamental fish (Cheung et al. 1980, Bassleer 1983, Rossteuscher et al. 2008) and on farmed fish species, especially seabass *Dicentrarchus labrax* (Dragesco et al. 1995), southern bluefin tuna *Thunnus maccoyii* (Munday et al. 1997), turbot (Iglesias et al. 2001, Paramá et al. 2003) and olive flounder *Paralichthys olivaceus* (Kim et al. 2004a,b, Jung et al. 2007). *Philasterides dicentrarchi*, which is considered to be a junior synonym of *Miamiensis avidus* (Jung et al. 2007), has been frequently reported as an agent of systemic infections, including those that infect gills. This species has been characterized in detail, both morphologically and at a molecular

level (Jung et al. 2005). A highly invasive and destructive ciliate similar to free-living *Uronema marinum* has been described from Atlantic and Pacific marine fishes kept in the New York Aquarium (Cheung et al. 1980). *Uronema*-like ciliates have been reported as agents of severe outbreaks of systemic infections in farmed turbot (Sterud et al. 2000). Also amoebae of the genus *Neoparamoeba* have been widely reported as a cause of serious problems in farmed turbot, but unlike scuticociliates, they were always restricted to gill lesions. The primary role of *Miamiensis avidus* in gill scuticociliatosis (Iglesias et al. 2001, Jung et al. 2005, 2007) as well as the primary role of *Neoparamoeba perurans* in aetiology of AGD (Young et al. 2007, 2008) was clearly demonstrated. Less attention has been paid to component causes of both infections and possible mixed infec-

tions by these 2 groups of free-living organisms that possess a fascinating amphizoic way of life. Histological examination of mixed infections of both organisms makes it possible to determine trophozoites of *Neoparamoeba* spp. in gill tissue owing to the presence of a kinetoplastid endosymbiont in the vicinity of the amoeba nucleus (Dyková & Novoa 2001). Ciliates can be recognized in histological sections, but the morphological features distinguishing individual genera of scuticociliates are not detectable in sectioned material (Dyková & Figueras 1994, Ramos et al. 2007) and, therefore, the isolation of ciliates facilitating their morphological and molecular characterization is important.

The interpretation of results of staining procedures might be ambiguous as, for example, in the case of stomatogenesis (Ma et al. 2004); therefore, molecular characterization is desirable to make a proper diagnosis in ciliates. Unfortunately, sequences representing many species, even those that were established in culture, are missing. This applies also to *Uronema nigricans*, which is found to cause fatal encephalitis in southern bluefin tuna (Munday et al. 1997, Crosbie & Munday 1999). The results of attempts by Soldo et al. (1981) to characterize nucleic acids of this ciliate are incomparable with current data in the GenBank database. The subclass Scuticociliatia Small, 1967 is considered basically monophyletic as determined with SSU rRNA gene sequences (Lynn & Strüder-Kypke 2005); nevertheless, a call for more sequences is urgent because classification based on morphostatic and stomatogenic oral features receives little support from SSU rRNA gene sequences (Lynn 2008).

To date, only environmental isolates and strains of *Uronema marinum* derived from them have been available for phylogenetic analysis. Based on the results of the present study, the clade of *U. marinum* also now contains sequences of strains isolated from gills of 2 fish species (turbot and Atlantic salmon) that were collected in geographically distant localities. Although there is no guarantee that the ciliate found in mixed infections with *Neoparamoeba* sp. (Table 1) is *U. marinum*, the sequences obtained can be used in designing specific probes. These, together with species-specific *Neoparamoeba* probes, can help in pathogenetic studies of mixed infections of both agents or in retrospective disease outbreak surveys and case-control studies. The search for pathogenic species of both groups of organisms continues due to their cosmopolitan distribution and possible effect on the economy of fish farms.

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