

## Molecular identification of *Uronema marinum* (Protozoa, Ciliophora, Scuticociliatia) in cultured turbot (*Psetta maxima*) larvae

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Article Info	Abstract
<b>Article history:</b> Received: 25 June 2019 Accepted: 08 January 2020 Available online: 15 March 2021	Scuticociliates are dangerous parasitic pathogens causing systemic tissue destruction and high mortality in marine fish worldwide. In this study, the first identification of <i>Uronema marinum</i> (Ciliophora, Scuticociliatida) from cultured turbot ( <i>Psetta maxima</i> ) larvae using mitochondrial cytochrome c oxidase 1 ( <i>cox1</i> ) gene sequence as well as species-specific primers was reported. The mean prevalence values of infected fish were calculated, and partial sequencing obtained from the mitochondrial <i>cox1</i> gene region was also compared with isolates registered in the Genbank database. The sequence comparison showed 93.00% identity to <i>U. marinum</i> , and the parasite has also been deposited in the GenBank database. This study is the first case of <i>U. marinum</i> infection in Turkish marine aquaculture, contributing to the systematics and molecular epidemiology of scuticociliate in Turkey.
<b>Keywords:</b> Cytochrome c oxidase 1 Parasite Polymerase chain reaction Turbot <i>Uronema marinum</i>	

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### Introduction

The aquatic environment may offer proper conditions for sustaining parasites' life cycles, and both wild and cultured fish species may be suitable hosts for the parasites.<sup>1</sup> Because of the numerous stress factors, parasitic infections' prevalence and adverse effects are usually more severe in cultured fish.<sup>2</sup>

The ciliates belonging to the scuticociliates class are obligate parasites causing significant economic losses in aquatic animals. These ciliates in fish tissues has been associated with various pathological changes, including anemia, ascites, hemorrhages, ulcers, muscular necrosis, and encephalitis in the brain.<sup>3,4</sup> In the early stages of infection, the ciliates are encountered in the connective tissue of skin and fins and nervous tissue. During the following stages, the whole organism can get infected.<sup>5,6</sup>

Turbot is a carnivorous flatfish living at or near the bottom of the marine environments from North Africa to the Atlantic. On the other hand, the Black Sea turbot (*Psetta maxima*) is an endemic subspecies having potential for aquaculture in the Black Sea region.<sup>7</sup> For the last two decades, the Black Sea turbot has been cultured by

Central Fisheries Research Institute (CFRI) in Turkey in partnership with the Japan International Cooperation Agency. About 20,000 larvae are produced annually in a seawater based recirculation system, and juvenile turbot is tagged and released into the Black Sea coast of Turkey.<sup>8</sup>

Ciliates present in cultured fish species in Turkey are mostly unknown. *Miamensis avidus*, a scuticociliate species, has only been isolated from reared common dentex (*Dentex dentex*).<sup>2</sup> Also, presumptive identification of *Philasterides dicentrarchi* in cultured juvenile turbot (*P. maxima*), based on a parasitological examination of the skin and fins, has been reported.<sup>9</sup> The *P. dicentrarchi* was the source of severe internal and external clinical signs in farmed turbot *Scophthalmus maximus* in Spain.<sup>4</sup>

As an alternative to or support the microscopy-based identification methods, mitochondrial cytochrome c oxidase 1 (*cox1*) gene sequencing has been recommended as a new and additional taxonomic approach scuticociliate species identification. This technique is a sensitive, rapid, and specific diagnostic tool<sup>10</sup> and has been used to identify scuticociliates named *U. marinum*, *Pseudocohnilembus persalinus*, *P. longisetus*, and *M. avidus* in various fish species, including olive flounder (*Paralichthys olivaceus*)

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and black rockfish (*Sebastes schlegelii*).<sup>11</sup> As a result, in this study, we report the first identification of *U. marinum* (scuticociliatida) from cultured turbot (*P. maxima*) larvae using mitochondrial *cox1* gene sequencing.

## Materials and Methods

**Examined fish.** Turbot is cultured in the hatchery of CFRI, Turkey. About 20,000 larvae are produced annually in a seawater-based system. All examined fish were collected from CFRI Turbot Hatchery, Trabzon, Turkey. Fish were randomly sampled. Sampling was taken place in late October 2018. The water temperature, pH, and salinity recorded at the sampling time were 15.00 °C, 7.20, and 18.00 ppt, respectively. Thirty turbot larvae (2.00 - 4.00 g) were sampled for parasitic examination after a suspected disease. All sampled fish were examined externally and internally.

**Ciliate isolation.** Scuticociliatosis parasites cause many fish mortality (20.00 - 25.00% annually) in turbot hatchery. Turbot larva is especially sensitive to the pathogen. The presumptive identification of ciliates was accomplished by microscopic observation of affected fish. Skin and fin tissues were dissected from moribund turbot larvae and examined via light microscopy (E400; Nikon, Tokyo, Japan). Morphological studies were also carried out using the wet Chatton-Lwoff silver nitrate impregnation method described by Foissner.<sup>12</sup> Live ciliates from all affected fish were cultured in seawater containing 0.18% NaCl at hatchery temperature (15.00 °C) for ten days. The ciliates were concentrated by centrifugation at 1000 *g* for 5 min, and the supernatant was removed.<sup>4</sup> In this study, the prevalence of ciliata was also calculated in sampled fish.<sup>13</sup>

**DNA extraction.** Fresh tissue samples of 13 ciliata affected fish were mixed in a tube, and DNA extraction and polymerase chain reaction (PCR) assays were conducted. According to the manufacturer's guidelines, ciliata genomic DNA was extracted by the DNA extraction kit (Qiagen, Hilden, Germany). The concentration and quality of DNA were assessed by a Nanodrop (ND 8000; Thermo Fisher Scientific, Waltham, USA), and the final DNA concentration was adjusted to 20.00 ng  $\mu\text{L}^{-1}$ .

**PCR amplification of *cox1* and sequence analysis.** The presumptive identification of ciliates was made by microscopic observation of fish. Molecular identification of ciliates was performed by amplifying the mitochondrial *cox1* gene by species-specific primers (Table 1) and sequence analysis of amplified fragments.<sup>11</sup> The DNA amplification was conducted using AmpliTaq Gold Master Mix (Thermo Fisher Scientific) in a thermal cycler (Applied Biosystems, Foster City, USA). The PCR reaction components were used according to the manufacturer's instructions. The following PCR conditions were used for all primer sets: Pre-denaturation at 95.00 °C for 10 min, 35 cycles of

95.00 °C for 30 sec, 52.00 °C for 45 sec and 72.00 °C for 30 sec and a final extension at 72.00 °C for 7 min. Analysis of amplification product was performed using 1.00% agarose gel containing SYBR Green. DNA fragment size was evaluated using the 100-bp DNA ladder (BioBasic Inc., Ontario, Canada). The expected sizes of the strong PCR products for *U. marinum*, *M. avidus*, *P. persalinus*, and *P. longisetus* were 285, 422, 229, and 341 bp, respectively (Table 1). The sequencing reaction was performed by BigDye Terminator Cycle Sequencing kit (version 3.1; Applied Biosystems), according to the manufacturer's instructions and sequences were determined in an ABI PRISM 3500 Genetic Analyzer and ABI Prism DNA Sequencing Analysis Software (version 5.1; Applied Biosystems). The nucleotide sequences were compared with previously published data in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). *Uronema marinum*, *Cyclidium glaucoma*, and *Tetrahymena* sp. nucleotide sequences were aligned by CLUSTALW Multiple Sequence Alignment Program (UCD, Dublin, Ireland), and the phylogenetic tree was constructed on MEGA software (version 10.0; Biodesign Institute, Tempe, USA).<sup>14</sup> When designing the phylogenetic tree, Tamura-Nei substitution model,<sup>15</sup> gamma-distributed with invariant sites for rates among sites, partial deletion for gap/missing data treatment was chosen. The phylogenetic tree was constructed by maximum likelihood method with 1000 replicates.<sup>16</sup>

## Results

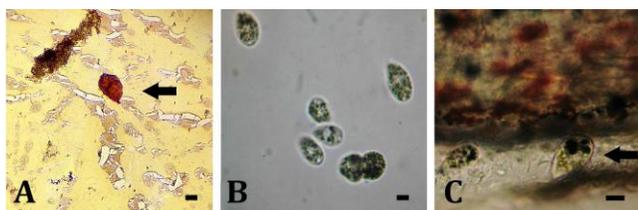
Skin lesions were observed as the most common finding in larvae with scuticociliates infections. The affected fish displayed petechial hemorrhages on various locations such as skin, operculum, and around the mouth. They also exhibited anorexia, lethargy, and darkened skin. Also, ascites in the internal organs were seen. The existence of infection caused by *U. marinum*, a pathogenic parasite, was identified in 13 out of 30 specimens. The mean prevalence of infected fish in this facility was calculated as 43.33%. The scuticociliates isolated from the naturally-infected fish larvae were identified by the traditional technique of light microscopy to evaluate morphological characters. The ciliate's morphological characteristics included its distinct size of approximately 40.00 - 50.00  $\times$  20.00 - 30.00  $\mu\text{m}$  and pear-like shape (Fig. 1).

An expected PCR fragment size of about 285-bp was obtained (not shown), and the 251 bp mitochondrial *cox1* gene sequence was aligned to the GenBank database. The sequence comparison showed 93.00% identity to the sequences of *U. marinum* (Accession no.: MG372368.1). Thus, based on the sequence identity, the ciliata isolated from the diseased turbot were confirmed as *U. marinum*.

The sequencing results obtained from the mitochondrial *cox1* gene regions were deposited in GenBank database and compared with isolates from different countries registered in the database.

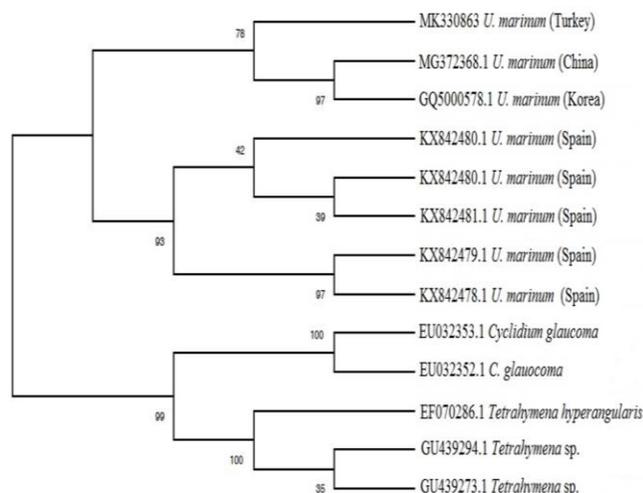
**Table 1.** Primers used for this study.

Organism	Primer	Sequence (5'-3')	Size (bp)
<i>Uronema marinum</i>	UM-F	AACATAGAGCATATAGAGAGTACTCTAA	285
	UM-R	TTCATCCAGCTGTTGTTAATGT	
<i>Miamiensis avidus</i>	MA-F	AGTAATAATAGAACATTTAACGAATTTAATAACAC	422
	MA-R	CGTCTTGTAATTAATAAATTTGTAAACGATAC	
<i>Pseudocohnilembus persalinus</i>	PP-F	TAAATCTAATCATCGTAATAATAGAGAATTGTTAG	229
	PP-R	CTTATCGATACGACTAACTGCAT	
<i>Pseudocohnilembus longisetus</i>	PL-F	AAATCAAATCATAGAAAATAATAGAGAATTTTAAATG	341
	PL-R	GCTCCAACACCAGTATATTTAATG	



**Fig. 1.** Morphological characteristics of *Uronema marinum* (arrow) from turbot. **A)** Silver nitrate stain; **B)** Ciliates observed through light microscopy; **C)** A ciliata in sub-dermal layer. (Scale bars = 10.00 µm).

The parasite has also been deposited in GenBank databases (Accession no: MK330863). The phylogenetic tree showed *U. marinum*, *C. glaucoma*, and *Tetrahymena* sp. came from a common ancestor, and then *U. marinum* separated from its relatives. The *U. marinum* strains from Spain are under the same clade, while the *U. marinum* strain from Turkey separated from *U. marinum* strains belonging to Korea and China (Fig. 2).



**Fig. 2.** Phylogenetic tree based on mitochondrial cytochrome *cox1* gene sequence comparison, obtained by the MEGAX package program based on the maximum-likelihood method with 1000 bootstraps showing the *Uronema marinum* strains from different countries.

## Discussion

*Uronema* is a genus under scuticociliatida found worldwide in both freshwater and marine environments.<sup>11,17</sup> In a

previous study, new *Uronema* species, including *Uronemella binucleata*, *U. filificum*, and *U. cymruensis* have been reported from China's marine waters.<sup>18</sup> In this study, *U. marinum* was identified as an etiological agent of the disease outbreak in cultured turbot in the CFRI, the first *Uronema* species reported in Turkey's marine waters.

*Pleuronema coronatum*, *Cohnilembus verminus*, *Philasterides armatalis*, *Porpostoma notatum*, *Cyclidium varibonneti*, *Ancistrum crissum*,<sup>19</sup> *Philasterides dicentrarchi*,<sup>4</sup> *Uronemella filificum*, *U. binucleata*, *U. cymruensis*, *Pseudocohnilembus hargisi*, *Cyclidium citrullus*,<sup>18</sup> *Miamiensis avidus*,<sup>10</sup> *Pseudocohnilembus persalinus*, *P. longisetus*, *Uronema marinum*,<sup>11</sup> *Pleuronema elegans*, *P. setigerum*, *P. grolierei* and *U. orientalis*<sup>17</sup> are important ciliata species belonging to the orders scuticociliatida. The existence of ciliata with the capacity to destroy cells and tissues of fish has been known for many years. The typical features of all ciliata are tissue damage and high mortality in aquatic animals. Similarly, the results detailed in previous reports, hemorrhage in the external and internal organs, skin color changes, abnormal swimming behavior, anorexia, and lethargy were detected in affected fish. The cumulative mortality was approximately reached 25.00%.

The mitochondrial *cox1* gene appears to be a new candidate for use as a DNA barcode. The particular regions of the *cox1* gene are highly conserved. This case allows the design of universal primers. The *cox1* gene also contains regions of the hypervariable sequence, which allows generating species-specific primers.<sup>20</sup> In our study, *U. marinum* was identified based on the mitochondrial *cox1* gene sequence in cultured turbot larvae. Phylogenetic tree displayed strain from Turkey diverged from strains belonging to Spain, and it is closer to strains from Korea and China. The divergence between strains can be caused due to different geographical conditions. This result indicates that the hypervariable sequences of the *cox1* gene may represent a useful diagnostic tool for rapid identification of scuticociliate species.

Turbot larva is a susceptible species to stress factors, including water temperature changes, parasitic infections, and handling. Thus, in the case of parasitic infection, high mortalities must be expected in larvae culture.<sup>4,8</sup> Various chemicals and chemotherapeutics have been used to treat scuticociliatosis throughout aquaculture production. However, the desired recovery could not be achieved.

Therefore, preventive measures such as environmental stress reduction may be essential to decrease the risk of infection. To our knowledge, this study represents the first *U. marinum* identification in Turkey by targeting the mitochondrial *cox1* gene. In our opinion, the results of this study will help to advance the systematics and molecular epidemiology of scuticociliate in Turkey.

### Acknowledgments

The author would like to thank Dr. Attila Karsi (Mississippi State University, USA) and Recep Parlak (CFRI, Turkey) for their help. This research was supported by CFRI, Turkey. The author followed all applicable international, national, and/or institutional guidelines for the care and use of animals. The research permission for the animal experiment (Protocol No: 42208298-040-04-05) was received from the Animal Experiments Local Ethics Committee of CFRI, Turkey.

### Conflict of interest

The author declares that there is no conflict of interest regarding the publication of this article.

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