Combined Morphological and Molecular Phylogenetic Analysis of the Genus Tripos (Dinophyceae) from the Persian Gulf

Zahra yarahmadi[1], Bita Archangi[1]*, Ahmad Savari[1], Seyyed Mohammad Bagher Nabavi[1]

Received: 2022.12.22 Revised and accepted: 2023.02.26

Abstract

The genus *Tripos* (the marine Ceratium) often comprises a major part of micro-phytoplankton in terms of abundance and diversity that plays an important role in annual primary production; however, it has received little attention in the Persian Gulf. The morphology and phylogeny of *Tripos furca*, *T. massiliense,* and *T. concilians* were investigated in the present research. Samples collected from the Persian Gulf were identified morphologically under an inverted microscope, and the *SSU* rDNA sequence from individual cells was amplified using a polymerase chain reaction. The *SSU* rDNA sequence was determined for Iranian strains of these species and registered in the GenBank. The results of the phylogenetic analysis indicated the formation of a monophyletic clade of the family *Ceratiaceae* with high support. In this clade, freshwater species of *Ceratium* were grouped in a highly supported subclade, and marine species comprised another subclade. These results well demonstrated the separation (divergence) of marine and freshwater species of *Ceratium*. Altogether, the present results agree with previous studies where a considerable evolutionary distance is suggested for marine and freshwater species and support the formation of two highly distinct monophyletic clades based on *SSU* rDNA and *LSU* rDNA fragments and the number of cingular plates.

Keywords: *Tripos*, Ceratiaceae, Single Cell, Phylogeny, SSU rDNA

Introduction

Ceratium F. Schrank, 1793 is a genus of important micro-phytoplankton in the phylum *Myzozoa* and the superclass Dinoflagellata with a cosmopolitan distribution. *Ceratium* sp. contains chloroplasts and drastically contributes to the annual primary production (Nielsen, 1991; Dodge and Marshall, 1994; Ibrahim, 2014). The presence of food vacuoles suggests their mixotrophy (Steidinger and Tangen, 1997) and rarely forms resting cysts (Gómez et al., 2010). Most species of this genus are non-toxic but their high population density causes red tide,

1-Department of Marine Biology, Faculty of Marine Sciences and Oceanography, Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran
* Corresponding author email address: bita.archangi@gmail.com

which in turn depletes nutrients and oxygen (Zingone and Enevoldsen, 2000; Kudela et al., 2017; Orellana et al., 2004; Morton et al., 2011; Pitcher and Probyn et al., 2011; Moreira et al., 2015). Some species of this genus (e.g., *Ceratium furca* (Ehrenberg) Claparède and Lachmann, 1859) produce ichthyotoxins (Mijares et al., 1985; Tan, 2016) that extensively kill fishes (Teen et al., 2012).

The widespread cosmopolitan distribution, abundance, and species richness have made this genus one of the preferred models to study different eco-physiological aspects of dinoflagellates, including bioluminescence (Sullivan and Swift, 1995; Latz et al., 2004), flagellar motility (Brokaw and Wright, 1963; Sato et al., 2004), cell division and growth rate (Elbrächter, 1973; Weiler, 1980), daily cycles (Pizay et al., 2009), trophic relationships, and mixotrophy (Smalley et al., 1999; Skovgaard et al., 2000). *Ceratium* species are sensitive to temperature in terms of biogeography (Dodge and Marshall, 1994), seasonality, and morphology (Sournia, 1968) and therefore serve as biological indicators of water masses (Raine et al., 2002; Ibrahim, 2014) and climate change (John et al., 2003; Tunin-Ley et al., 2009).

Ceratium is the first genus name used for dinoflagellates (Schrank, 1793) and is still in use. It was, however, reported 7 years before that by O.F. Müller (1786) through recording the illustrations of *Bursaria hirundinella* O.F. Müller, 1773 (freshwater) and *Cercaria tripos* O.F. Müller, 1776 (saltwater). Later, *Cercaria tripos* was transferred into *Ceratium* (Nitzsch, 1817), and *Bursaria hirundinella* was transferred into the genus Schrank by Dujardin (1841). Gómez (2013) proposed the separation of marine and freshwater species of *Ceratium* based on morphological and ecological differences; *Tripos* includes marine species and *Ceratium* remained as a genus for marine species. *Tripos* is very diverse and contains over 155 species (Guiry and Guiry, 2017). Considering the morphological variability at the intraspecific level, varieties, and different forms, there are 450 organisms classified in this genus (Gómez, 2013).

Due to their intrageneric similarities, it is sometimes not possible to identify dinoflagellate species based on morphological characters (Reguera et al., 2012; Hansen et al., 2000). The phenotypic variability of species often leads to false identification (Gaston and O'Neill, 2004; Accattatis et al., 2020) and, consequently, the identification based on morphological Characters will require high taxonomic expertise (Accattatis et al., 2020). This is particularly true for dinoflagellates because of their considerable morphological variations (Culverhouse et al., 2003) so the morphological variability in Ceratium species is referred to as "diabolical" by Sournia (1967).

Owing to extreme physical conditions (e.g. high temperature and salinity levels), the marine ecosystem of the Persian Gulf and Oman Sea contains valuable natural resources and biodiversity specific to plant and animal species (Sheppard et al., 2010; John, 2012) being one of the most fragile ecosystems

in the world (IPCC, 2014). Despite the scientific and strategic importance of this area and a considerable number of articles, there are still fundamental gaps concerning the food web dynamics, health, biodiversity, and stability of the ecosystem, particularly in Iranian waters (Dorgham, 2013). Overall, a small number of published articles are about dinoflagellates in Iranian waters (Jalilli and Rezai 2010; Rabbaniha et al., 2012; Aein Jamshid et al., 2016; Shapoori and Gholami, 2014), and only morphological identification for a rather large group of dinoflagellates at the species level has been investigated by Zarei Darki and Krakhmalnyi (2017). Some part of research in the Persian Gulf has focused on the causes of harmful algal blooms on the Iranian coasts (Rezai, 1995; Attaran-Fariman et al., 2011; Fatemi et al., 2012; Hamzehei et al., 2012). Specifically, molecular and phylogeny studies have not attracted much attention in Iranian waters of the Persian Gulf and Oman Sea, and often include blooming species, such as *Cochlodinium polykrikoides* Margalef, 1961 (Tamadoni Jahromi et al., 2011), *Scrippsiella trochoidea* (F. Stein) A.R. Loeblich III, 1976 (Attaran-Fariman et al., 2011), and *Heterocapsa* Stein, 1883 (Attaran-Fariman et al., 2013). Alemzadeh et al. (2014) also identified a wide range of phytoplankton by the molecular method.

This study aims to document the molecular identification and phylogeny of three dominant species of Tripos from the Persian Gulf (Bandar Abbas) using the combined approach of morphological and molecular data.

Materials and methods

Sampling and isolation

Sampling was carried out in December 2017 in the Persian Gulf at Bandar Abbas (59” 17′ 56° E and 10” 10′ 27° N, Figure 1).

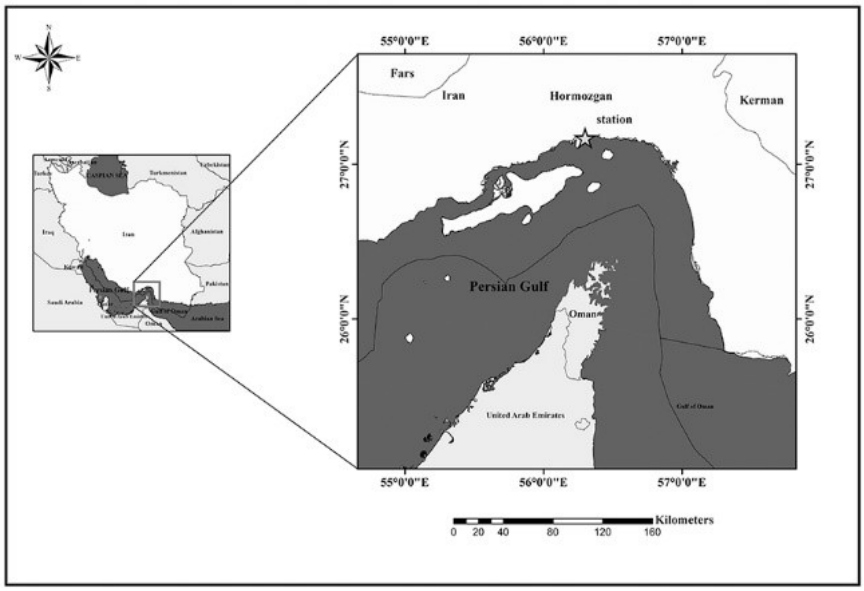

Fig. 1. Map of the Persian Gulf showing the sampling location in Bandar Abbas. The map is prepared in GIS software

Cells were collected using a phytoplankton net (mesh size: 20 µm) equipped with a flowmeter in surface waters. The water samples were fixed with 1% Lugol's solution, which has a more negligible effect on DNA than other fixatives (Galuzzi et al., 2004). The plankton samples were scanned at ×100 magnifications with an inverted microscope (Olympus, Tokyo, Japan). The fixed specimens were photographed with a Dino capture lens mounted on the microscope's eyepiece. The specimens were identified morphologically based on their shape and size using phytoplankton identification keys.

After being photographed each specimen was micropipetted individually with a fine capillary disposable on a glass slide and washed five times a Milli-Q water to assure that the sample was void of any likely contamination. Next, the isolated cell with 2 µl of Milli-Q water was transferred to a 0.2 ml microtube, and the successful transfer of the specimen to the microtube was confirmed under a microscope. Jusbeforeto isolation, 5 µl of 1M sodium thiosulfate solution was added to the samples and hand mixed until the Lugol's iodine solution was discolored.

Cell preparation, PCR amplification, and sequencing

Single cells in a PCR tube were subjected to the freeze-thaw cycles to lyse the cell, as follows: plunging sealed tube into liquid nitrogen and 1 min incubation at 95 °C with the use of a Thermoblock, PeQlab and used as template in an initial PCR reaction. Then mixed with the PCR solution consisting of 2.5 µl of PCR 10X buffer, 1.5 µl of $MgCl_2$ (mM 50), 0.5 µl of dNTPs (10 mM), one µl of each EK-42F: CTCAARGAYTAAGCCATGCA (forward) and EK-1520R: CYGCAGGTTCACCTA (reverse) primers (10 pmol), and 0.3 µl of Taq polymerase (5 U/µl). SSU rDNA sequences from the individual cells were obtained by polymerase chain reaction (PCR) using the fixed single-cell method described in Gómez et al. (2010).

The amplification was done with a Touch-down program in a Corbett CGI-96, Palm-Cycler Thermal Cycler as follows: initial denaturation at 94 °C for 2 min, 10 cycles of denaturation triple steps at 94 °C for 15 Sec, annealing at 55-65 °C (1 °C decrease per cycle) for 30 s, and elongation at 72 °C for 2 min. This was followed by further 20 cycles including denaturation at 94 °C for 15 Sec, annealing at 55 °C for 30 s, elongation at 72 °C for 2 min, and final elongation for 7 min. In the second round, the nested PCR was run using 2 µl of PCR first-round product, PCR mixture with the same composition and concentrations as the previous round, internal primers EK-82F: GAAACTGCGAATGGCTC and EK-1498R: CACCTACGGAAACCTTGTTA, a thermal program similar to the previous step. The third round of reaction was performed by semi-nested PCR using 2 µl of the second round product with a specific dinoflagellate primer DIN46F: TAACAATACAGGGCATCCAT (forward primer) and EK-1498R and a thermal program similar to the previous stages,

except that the total number of cycles increased from 30 to 35 (Gómez et al., 2010; Source: López-García et al., 2001). A DNA-free sample reaction (lysed cell or PCR product) as a negative control was used in all steps.

PCR products were electrophoresed on 1% agarose gel and observed by a UVITEC-Cambridge gel documentation system. At the next step, appropriate samples were purified based on the size and quality of formed bands, and amplicons in the expected size (~1200 bp) were sequenced by EK-1498R and DIN46F primers as paired-end read with the 3130 xl Genetic Analyzer sequencer by the Sanger method using the Sequencing Analysis v5.2 program.

Phylogenetic Analysis

At first, the Iranian strains sequence (related to forward and reverse primers) were combined to achieve a full sequence using the DNA Sequence Assembler ver. 4 software (Heracle BioSoft, 2013). The nucleotide sequence of the Iranian strain and 105 sequences extracted from GenBank were used for the phylogenetic analysis. Sequence alignment was performed using trimAl v1.2 (Capella-Gutiérrez et al., 2009) to eliminate ambiguous areas and gaps. The aligned data matrix was analyzed by ML, MP, and Bayesian methods.

According to the Akaike information criterion (AIC), the GTR + G evolution model was chosen for data using MrModeltest Ver. 2.3 software (Nylander, 2004). Maximum Likelihood analysis (ML) was performed in the raxmlGUI 1.5* (Silvestro and Michalak, 2012) with 1000 bootstrap to obtain the support values of branches. The Maximum parsimony (MP) was performed using PAUP *4.b10 software (Swofford, 2002) by the heuristic search method with 1000 replications and the branch-swapping rearrangements by the Tree Bisection and Reconnection (TBR) method. MP clade support was assessed by non-parametric bootstrapping with 1000 replicates and the same heuristic-search parameters. Bayesian analysis was conducted in the MrBayes Ver. 3.2 program (Ronquist et al., 2012) starting from random trees and four Markov chains (with one cold chain and three heated chains). Sample trees estimated the posterior probability (PP) of trees using the Markov Chain Monte Carlo (MCMC). After the analysis, the standard deviation was <0.005 (i.e., the distribution of the sampled trees converged with the target distribution). After the convergence study, 25% of the trees that did not reach the convergence stage were burned and one consensus tree was made out of every 100 trees.

Results

Morphological characterization of PG07 and PG08 samples

A species with a very long horn and a cell size of 65-100 µm. The cell body shape is robust or angular and subtriangular, and the posterior margin is almost smooth and oblique. The apical horn is high and straight, somewhat rightward in the ventral view. The hypothecal (antapical) horns are open-ended. In the ventral view, the proximal end of the

left antapical is wider and more curved than the right horn. The cell is the widest next to the antapical horns. The cingulum and sulcus are visible (Figure 2 A-B). Based on the mentioned morphological features, both PG08 and PG09 samples were identified as *Tripos massiliense* (Gourret) F. Gómez, 2013.

Morphological characterization of the PG09 sample

This is a relatively large cell with a length of 62-88 µm, which is almost smooth and sometimes with a concave ventral surface and a convex dorsal surface. The cell is wider next to the cingulum (Figure 2 C-E). The epitheca gradually changes into tapers forming a rather high antapical horn. Two epithecal antapical horns are parallel, slightly divergent, or convergent (often parallel), and one of them has a length almost twice the

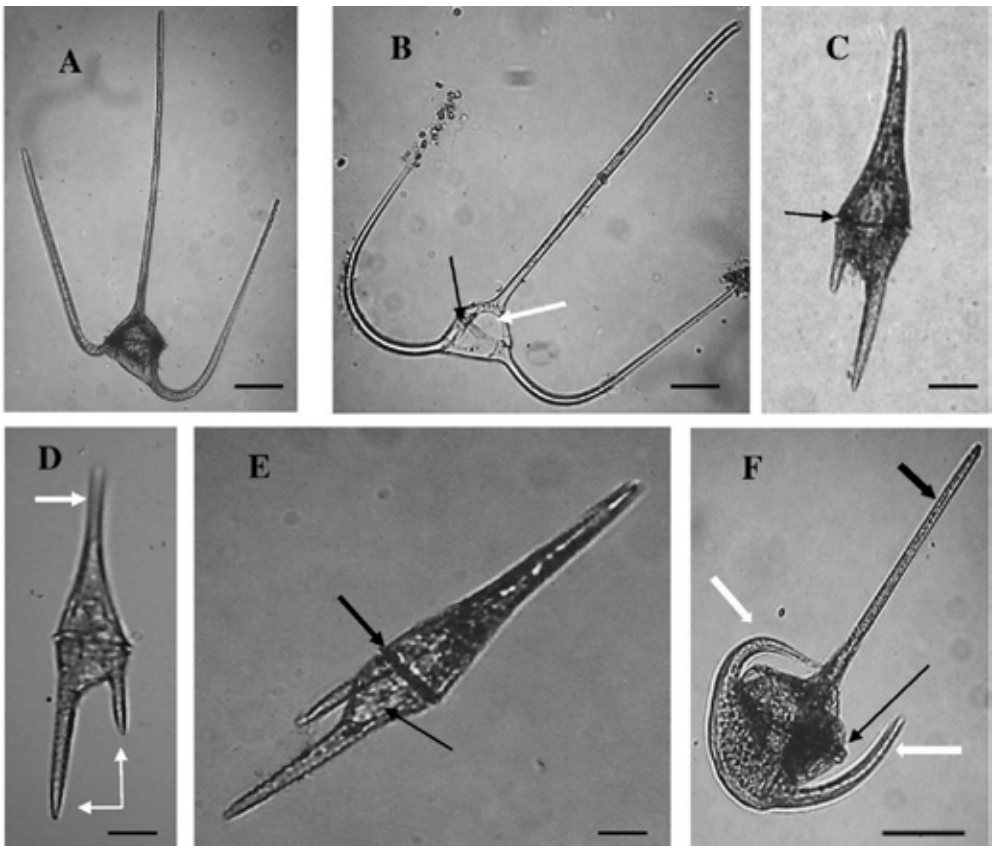

Fig. 2. Inverted microscope images of *Tripos* sp. of the Persian Gulf. A and B: *T. massiliense* (Gourret) F. Gómez, 2013; the black arrow indicates the Cingulum and the white arrow indicates the sulcus. C, D, and E: *T. furca* (Ehrenberg) F. Gómez, 2013; the bulky white arrow shows the apical horn (D), the thin white arrows show the antapical horns (D), the thick black arrow indicates the Cingulum (E) and the thin black arrow show the sulcus (E). F: *T. concilians* (Jørgensen) F. Gómez, 2013; the long black arrow indicates the Cingulum, the bulky white arrow indicates the antapical horns and the thick black arrow shows the apical horn. The cell is fixed with Lugol's solution. Scale bars: A-B, 20 µm; C-E, 10 µm; F, 50 µm

other. The horns are open-ended. The nucleus is situated in the epicone, and cells are seen as single or chains of two cells. Based on the mentioned morphological features and comparisons with valid identification keys, the PG07 sample was identified as *Tripos furca* (Ehrenberg) F. Gómez, 2013.

Morphological characterization of the PG10 sample

The cell body is not robust with no angle. The theca is smooth and without decorations. The dorsal margin of the body is more or less hunched to the left. The apical horn is high and slightly deflected to the left (in the dorsal view). The antapical horns are short. The left antapical horn (in the dorsal view) is bent toward the cell body (Figure 2 F). The PG 10 sample was identified as *Tripos concilians* (Jørgensen) F. Gómez, 2013.

The results of phylogenetic analysis of Ceratium

In this study, four new sequences of SSU rDNA from three marine species of *Tripos* identified microscopically and isolated individually, were obtained using PCR and deposited with the accession numbers OK356403 and OK356483 (*Tripos massiliensis*), OK356476 (*Tripos furca*) and OK356481 (*Tripos concilians*) in the GenBank. Up to now, there are no registered sequences of SSU rDNA for *Tripos* sp. in the Persian Gulf, and the obtained sequences are reported in this study for the first time. Moreover, the SSU rDNA sequences from the Iranian strains of this genus corresponded to none of the sequences registered in the GenBank. The sequences of PG07 and PG08 samples, which were detected based on the morphological features of *T. massiliense*, corresponded to the FJ402942 sequence (94.54% and 92.58%, respectively) from the same species in the GenBank. The results of the NCBI BLAST for the PG09 sequence shares 93.85% identity with the available sequence in databases from *T. furca* (accession numbers: FJ402966). This sample was detected according to the morphological characteristics of *T. furca*. Based on the results of the NCBI BLAST, a close relation (Identity: 94.3%) was also found between the PG10 sequence and that of *T. concilians* (FJ402950).

The phylogenetic position of *Tripos* species was analyzed using a matrix consisting of available sequences of different dinoflagellate orders (thecate and athecate) in the GenBank. The phylogenetic trees were rooted with two perkinsozoan sequences. The results of molecular and phylogenetic analyses revealed a similar topology for all MP, ML, and MrB threes. Therefore, only the tree obtained from the Bayesian phylogenetic analysis is documented here. In the phylogenetic tree of Figure 3, individuals were first separated from different populations of *T. furca* (subgenus *Biceratium*) with moderate support (PP = 0.84, ML = 71, and MP = 62). The next clade, which is separated with high support (PP = 1, ML = 97, and MP = 80), belongs to individuals from *T. fusus* (Ehrenberg) F. Gómez, 2013 (Subgenus *Amphiceratium*). The next clade is represented by species from the genus *Tripoceratium* including

T. azoricum (Cleve) F. Gómez, 2013, *T. petersii* (Steemann Nielsen) F. Gómez, 2013, *T. paradoxides* (Cleve) F. Gómez, 2013, *T. limulus* (Pouchet) F. Gómez, 2013, *T. declinatus* (G. Karsten) F. Gómez, 2013, *T. platycornis* (Daday) F. Gómez, 2013, *T. massiliensis*, *T. contrarius* (Gourret) F. Gómez, 2013, and *T. concilians*. As shown in the branching pattern of the tree, the origin of the Iranian species of *Tripos* is closer to the Mediterranean populations. In the resulting phylogenetic tree Figure 4, the Ceratiaceae Kofoid, 1907 appeared among several families from orders Gonyaulacales and Peridiniales after important orders such as Gymnodiniales, Prorocentrales, and Dinophysales. An important result of the resulting phylogenetic tree is the separation of freshwater and marine species of *Ceratium* with high support (PP = 0.92,

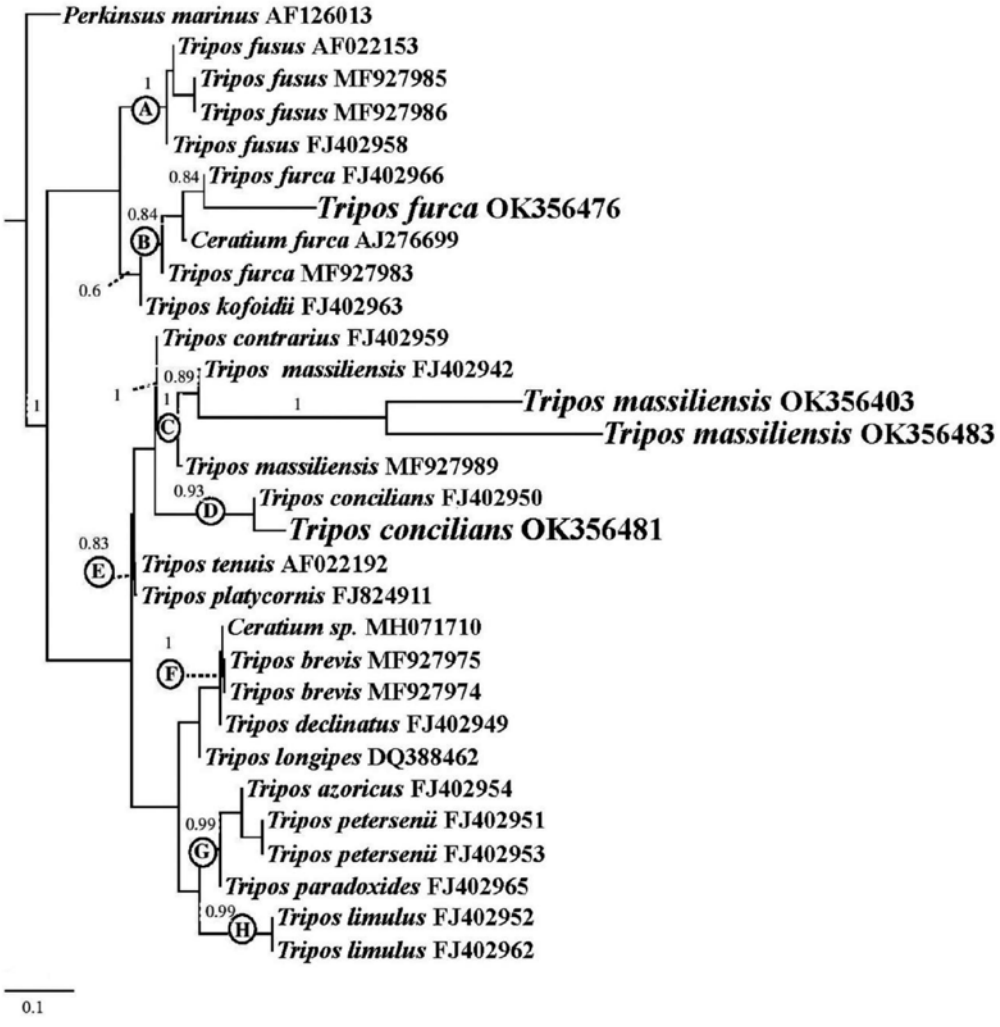

Fig. 3. Phylogenetic tree of the Bayesian analysis based on the SSU rDNA gene fragment; *Tripos* sp. Values on branches represent Bayesian posterior probabilities. Probabilities less than 50% are omitted here. The sequences obtained in the present study are shown in bold. The accession number of the sequences extracted from the GenBank is placed next to the species name. The scale bar exhibits the number of substitutions for a unit branch length

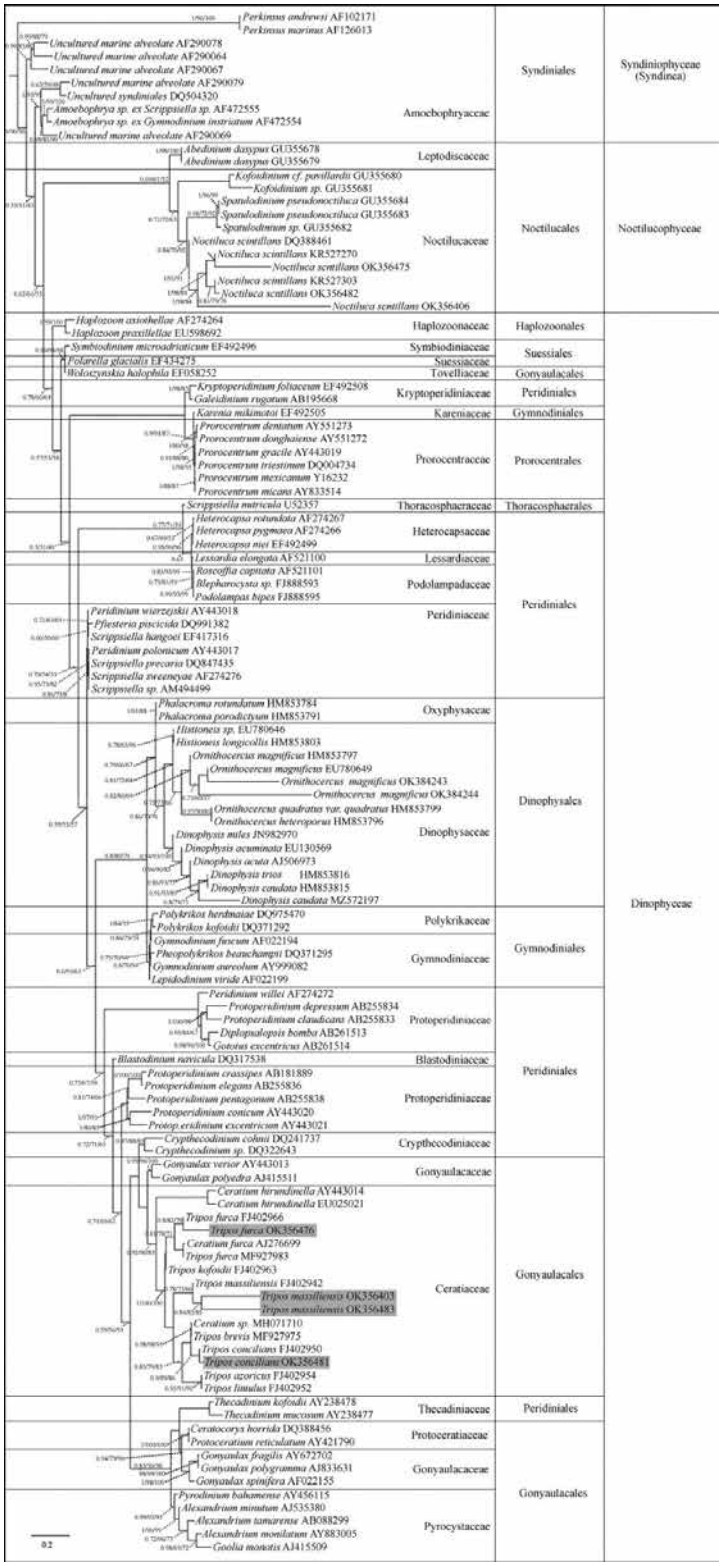

Fig. 4. Bayesian phylogenetic tree of dinoflagellate SSU rDNA sequences; Values on branches represent Bayesian posterior probabilities/maximum likelihood percentages of 1000 trees/bootstrap percentages of maximum parsimony of 1000. Probabilities less than 50% are omitted here. The sequences of the present study are shown in the gray box. The accession number of the sequences extracted from the GenBank is placed next to the speme. The scale bar exhibits the number of substitutions for a unit branch length

ML = 90, and MP = 83).

Discussion

The morphology and phylogenetic position of *T. furca*, *T. massiliense*, and *T. concilians* were investigated here. Some morphological similarities may confuse the species-level identification. morphologically, *T. concilians* may be confused with *T. gibberus* (Gourret) F. Gómez, 2021. An important difference between the two species is a somewhat reticulate theca in the *T. gibberus* (Hasle et al., 1996). Similarly, *T. massiliense* may be confused with *T. trichoceros* (Ehrenberg) Gómez, 2013 with a smaller, somewhat rounded body and almost parallel apical and antapical horns that make a U-shaped form. The antapical horns in this species are shorter than those of *T. massiliense* (Al-Kandari et al., 2009). Furthermore, *C. massiliense* can easily be confused with, *C. macroceros* (Ehrenberg) F. Gómez, 2013 but the hypothecal horn development and the right-directed apical horn clearly distinguish *C. macroceros* (Hasle et al., 1996). Sometimes *T. furca* is confused with *C. hircus* Schröder, 1909. even though *C. hircus* is smaller and has almost equal hypothecal horns and a more robust body. The right hypothecal horn of *C. hircus* is directed ventrally (Hasle et al., 1996). However, the BLAST results of sequences obtained in this study showed no close identity between these species. The classic taxonomic scheme was closely related to freshwater species (Jørgensen, 1911; Schiller, 1937; Sournia, 1968), but this is not supported by our results and indicates no close relation of freshwater species of *Ceratium* with *N. furca* (*T. furca*) as a member of the subgenus *Biceratium* (Figure 4).

In the present research, the SSU rDNA phylogenetic analyses significantly supported (MP = 83, ML = 90, and PP = 0.92) the formation of a monophyletic clade of marine and freshwater species of *Ceratium* (as the only member of the Ceratiaceae family) (Figure 4). In this clade, freshwater sequences of *Ceratium* formed a well-supported subclade, and sequences of marine *Ceratium* were well clustered into monophyletic groups (Figure 4). These results strongly support the splitting of the marine and freshwater species of *Ceratium* into two distantly related groups, suggesting that they have no close relations, Marine and freshwater species of *Ceratium* drive into two distinct genera which correspond to the findings of Saldarriaga et al. (2004), Logares et al. (2007), and Gómez et al. (2010).

In the phylogenetic analyses of Gómez et al. (2010), the marine and freshwater *Ceratium* appeared separated into two monophyletic clades with strong support based on the number of cingular plates and the significant evolutionary distance of their SSU rDNA sequences. Thus, they suggested transferring marine species into the new genus "*Neoceratium*". However, this name was not approved. Currently, the accepted genus *Tripos* includes marine species, and *Ceratium* has remained for freshwater species (Gómez, 2013). Similarly, this formerly occurred for

Peridinium sensu lato. The marine species of *Peridinium* Ehrenberg, 1830 were transferred into the genus *Protoperidinium* Bergh, 1881 (Balech, 1974), and *Peridinium* was assigned to freshwater species. Then, the genera *Peridinium* sensu stricto and *Protoperidinium* were placed into separate families (Balech, 1988).

Also based on extensive phylogenies of 18-28 SSU rDNA, Logares et al. (2007) concluded that marine and freshwater *Ceratium* are only distantly related species and appeared separated. They suggest that the marine-freshwater transitions have not occurred recently, and few marine lineages seem to have successfully colonized freshwater.

Some morphological and biological differences between marine and freshwater *Ceratium* also suggest that these lineages did not diverge recently. There are six cingular thecal plates in freshwater species while marine species possess five plates (Gómez et al., 2010). Since almost all Gonyaulacales have at least six cingular plates and *Tripos* (*Neoceratium*) exceptionally possesses five cingular plates, *Tripos* has supposedly derived from a gonyaulacean ancestor with six cingular plates where the third and fourth cingular plates have been merged into a single plate (Gómez et al., 2010). The formation of resting cysts is unknown in marine *Ceratium* species (Sournia, 1986) while it is a common feature of the freshwater species. In freshwaters, the populations of *Ceratium* are influenced by extreme environmental changes including desiccation. Thus, resting cyst formation is considered to be a vital strategy for surviving unfavorable environmental conditions; these cysts are also used for airborne dispersal. whereas, extreme environmental conditions (e.g. desiccation) rarely occur in open marine environments. In marine *Ceratium* species, mixotrophy (having chloroplasts) is a common feature (Jacobson, 1999). Accordingly, marine *Ceratium* populations may be better adapted to survive under conditions of nutrient depletion, which may be the reason for the rarely formed resting cysts in *Neoceratium*. However, the resting cyst formation cannot be considered an exclusive feature of the freshwater *Ceratium* species. For example, cyst formation was reported in senescent populations of *N. furca* (Gómez et al., 2010). The number of cingular plates seems to be a stable taxonomical character for the generic separation of thecate dinoflagellates (Balech, 1980).

During the evolution, the different physicochemical properties of marine and freshwater habitats have acted as a barrier to dinoflagellates and a small number of lineages have been able to pass through this barrier (Logares et al., 2007). This matter is confirmed by molecular data from other microbial lineages, suggesting that displacement and transitions have not occurred frequently between saltwater and freshwater environments (Methe et al., 1998; Zwart et al., 1998, 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Warnecke

et al., 2004; Katz et al., 2005; Richards et al., 2005; Figueroa and Rengefors, 2006; Scheckenbach et al., 2006; Sims et al., 2006; Alverson et al., 2007; Lefevre et al., 2007). The difference in habitat prevents the free interchange of microbes between saltwater and freshwater in a way similar to metazoans (Lee and Bell, 1999). In addition, only a few marine lineages of multicellular organisms have been successful in colonization into freshwater (Lee and Bell, 1999; Miller and Labandeira, 2002; Vermeij and Wesselingh, 2002; Lovejoy et al., 2006). The osmoregulatory mechanisms may be one of the main barriers to freshwater migration in metazoans. Unlike metazoans, microbial populations typically have very large population sizes, high reproductive rates, enormous genetic diversity (Snoke et al., 2006), and dispersal ability in long distances (Finlay, 2002). Hence, microbial strains are expected to be good candidates to acquire the necessary mutations to create a transition environment (Logares et al., 2007), but this has practically occurred contrastingly in nature. The barrier(s) preventing the constant and/ or even reversible transition of marine microbes to freshwater is not exactly known. Salinity gradient, competitive deprivations imposed by adapted residents (De Meester et al., 2002), and the high extinction rate of small limnic habitats are some factors that have been proposed to be investigated as Possible reasons and barriers to colonization in freshwater (Logares et al., 2007).

To put it briefly, we performed the first report of *T. furca*, *T. massiliense*, and *T. concilians* in the Persian Gulf combining morphological and molecular phylogenetic data of Individual cells from the natural population. Evolutionarily, present molecular phylogenetic analyses based on SSU rDNA significantly supported the formation of a monophyletic clade of the family Ceratiaceae. Our results also indicated that marine and freshwater species of *ceratium* are not closely related, split into two subclades (two genera), and are supported with good bootstraps. Subsequently, these taxonomic and phylogenetic schemes are discussed. Molecular data are used to help enrich the Genebank and improve phylogenetic resolution. Furthermore, the analysis of molecular data can greatly benefit the large number of samples that are required for a biogeographical study. We hope that in future studies, molecular data will be useful in identifying and monitoring *Tripos* species as biological indicators of water masses aiming to assess biodiversity all over the Persian Gulf.

Acknowledgment

We are grateful to Dr. Musa keshavarz of the Department of Marine Biology, Hormozgan University for his contribution to the sampling collection. We also extend our gratitude to the personnel and facilities of the Medical Biotechnology Department of the Lorestan University of Medical Sciences. We thank David Moreira of the Unit of Ecology, System, and Evolution, CNRS, and University Paris-Sud for his support and helpful guidance in phylogenetic

analysis. The authors would like to thank the reviewers for their helpful comments.

References

Aein Jamshid K, Mohsenizadeh F, Omidi S. (2016). Effects of environmental parameters and nutrients on microalgae communities around the shrimp farm complexes in Bushehr province, in the Persian Gulf. Iranian Journal of Fisheries Sciences. 5 (3): 1044-1054. https://jifro.ir/article-1-2319-en.html.

Accattatis V, Piccini C, Huber P, Metz S, Rueda E, Devercelli M.)2020(. Identifying invaders: the case of *Ceratium furcoides* (Gonyaulacales, Dinophyceae) in South America. Journal of Phycology. 56(5): 1362-1366. DOI: 10.1111/jpy.13015.

Alemzadeh E, Haddad R, Ahmadi AR. (2014). Phytoplanktons and DNA barcoding: characterization and molecular analysis of phytoplanktons on the Persian Gulf. Iranian Journal of Microbiology. 6 (4): 296-302. PMID: 25802715

Al-Kandari M, Al-Yamani F, Al-Rifaie K. (2009). Marine phytoplankton atlas of Kuwait's waters. Kuwait Institute for Scientific Research. 351.

Alverson AJ, Jansen RK, Theriot EC. (2007). Bridging the Rubicon: phylogenetic analysis reveals repeated colonizations of marine and fresh waters by thalassiosiroid diatoms. Molecular Phylogenetics and Evolution. 45 (1): 193-210. DOI: 10.1016/j.ympev.2007.03.024.

Attaran-Fariman G, Khodami S, Bolch CJS. (2011). The cyst-motile stage relationship of three *Protoperidinium* species from the southeast coast of Iran. Iranian Journal of Fisheries Sciences. 10 (1): 1-12. http://jifro.ir/article-1-120-fa.html.

Attaran-Fariman G, Javid P. (2013). The phylogeny of *Heterocapsa* sp. (Dinophyceae) isolated from the south coast of Iran during a *Cochlodinium polykrikoides* bloom. Turkish Journal of Botany. 37(4): 778-783. DOI: 10.3906/bot-1206-40.

Balech E. (1974). El genero '*Protoperidinium*'Bergh, 1881 ('*Peridinium*' Ehrenberg, 1831, partim). Rev. Museo Argentino de Ciencias Naturales Bernardino Rivadavia e Instituto Nacional de Investigación de las Ciencias Naturales. 4: 1-79.

Balech E. (1980). On the thecal morphology of dinoflagellates with special emphasis on cingular and sulcal plates. Anales del Instituto de Ciencias del Mar y Limnologia, Universidad Nacional Autonoma de Mexico. 7: 57-68.

Balech E. 1988. Los dinoflagelados del Atlántico sudoccidental. Publicaciones Especiales. Instituto Espanol de Oceanografia. 1: 1-310.

Brokaw CJ and Wright L. (1963). Bending waves of the posterior flagellum of *Ceratium*. Science. 142 (3596): 1169-1170. DOI: 1126/science.142.3596.1169.

Culverhouse PF, Williams R, Reguera B, Herry V, González-Gil S. (2003). Do experts make mistakes? A comparison of human and machine identification of dinoflagellates. Marine Ecology Progress Series. 247: 17-25. DOI: 10.3354/

MEPS247017.

De Meester L, Gómez A, Okamura B, Schwenk K. (2002). The Monopolization Hypothesis and the Dispersal–gene flow paradoxes in aquatic organisms. Acta Oecologica. 23 (3): 121-135. DOI: 10.1016/S1146-609X(02)01145-1.

Dodge JD and Marshall HG. (1994). Biogeographic analysis of the armored planktonic Dinoflagelate *Ceratium* in the North Atlantic and adjacent seas 1. Journal of Phycology. 30 (6): 905-922. DOI: 10.1111/j.0022-3646.1994.00905.x.

Dorgham MM. (2013). Plankton research in the ROPME Sea Area, Achievements and Gaps. International Journal of Environmental Research. 7: 767-778. DOI: 10.22059/IJER.2013.656.

Dujardin F. (1841). Histoire naturelle des zoophytes: Infusoires, comprenant la physiologie et la classificatin de ces animaux, et la manière de les étudier à l'aide du microscope. (Vol. 1). Roret. DOI: 10.5962/bhl.title.10127.

Elbrachter M. (1973). Population dynamics of Ceratium in coastal waters of Kiel Bay. Oikos. 15: 43-48. https://cir.nii.ac.jp/crid/1571135650390010624.

Fatemi SMR, Nabavi SMB, Vosoghi G, Fallahi M, Mohammadi M. (20120. The relation between environmental parameters of the Hormuzgan coastline in the Persian Gulf and the occurrence of the first harmful algal bloom of Cochlodinium polykrikoides (Gymnodiniaceae). Iranian Journal of Fisheries Sciences. 11 (3): 475-489. http://dorl.net/dor/20.1001.1.156229 16.2012.11.3.3.3.

Figueroa RI, Rengefors K. (2006). Life cycle and sexuality of the freshwater Raphidophyte *Gonostomum* semen (Raphidophyceae) 1. Journal of Phycology. 42 (4): 859-871. DOI: 10.1111/j.1529-8817.2006.00240.x.

Finlay BJ. (2002). Global dispersal of free-living microbial eukaryote species. Science. 296 (5570): 1061-1063. DOI: 10.1126/science.1070710

Gaston KJ, O'Neill MA. (2004). Automated species identification: why not? Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences. 359 (1444): 655-667. DOI: 10.1098/rstb.2003.1442.

Gómez F, Moreira D, López-García P. (2010). *Neoceratium* gen. nov., a new genus for all marine species currently assigned to Ceratium (Dinophyceae). Protist. 161 (1): 35-54. DOI: 10.1016/j.protis.2009.06.004.

Gómez F. (2013). Reinstatement of the dinoflagellate genus Tripos to replace Neoceratium, marine species of Ceratium (Dinophyceae, Alveolata). Cicimar Oceánides. 28 (1): 1-22. DOI: 10.37543/oceanides.v28i1.119.

Guiry MD and Guiry GM. AlgaeBase (2017). World-wide electronic publication, National University of Ireland, Galway.

Hamzehei S, Bidokhti AA, Mortazavi MS, Gheiby A, Ebrahimi M, Saraji F. (2012). Field study of the 2008-2009 red tide in the northern Strait of Hormuz. Life Science Journal. 9 (4): 4727-4733.

Hansen G, Daugbjerg N, Henriksen P.

(2000). Study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum*, comb. nov. (= *Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data. Journal of Phycology. 36 (2): 394-410. DOI: 10.1046/j.1529-8817.2000.99172.x.

Hasle GR, Syvertsen EE, Steidinger KA, Tangen K, Tomas CR. (1996). Identifying marine diatoms and dinoflagellates. Elsevier. P. 598. DOI: 10.1016/B978-0-12-693015-3.X5000-1.

Hoef-Emden K, Marin B, Melkonian M. (2002). Nuclear and nucleomorph SSU rDNA phylogeny in the Cryptophyta and the evolution of Cryptophyte diversity. Journal of Molecular Evolution. 55 (2): 161-179. DOI: 10.1007/s00239-002-2313-5.

Holzmann M, Habura A, Giles H, Bowser SS, Pawlowski JAN. (2003). Freshwater foraminiferans were revealed by analysis of environmental DNA samples. Journal of Eukaryotic Microbiology. 50 (2): 135-139. DOI: 10.1111/j.1550-7408.2003. tb00248.x.

Ibrahim AMM. (2014). Marine plankton and Genus *Ceratium* on the west coast of the Red Sea. Blue Biotechnology Journal. 3(3): 295-341.

IPCC. (2014). Climate Change: Synthesis Report. Contribution of working groups I, II, and III to the fifth assessment report of the intergovernmental panel on climate change [Core Writing Team, Pachauri RK and Meyer LA. IPCC, Geneva, Switzerland. p. 151.

Jacobson DM. (1999). A Brief History of Dinoflagellate Feeding Research. Journal of Eukaryotic Microbiology. 46 (4): 376-381. DOI: 10.1111/j.1550-7408.1999. tb04616.x.

Jalilli M and Rezai H. (2010) Composition, abundance and distribution of phytoplankton in the Strait of Hormuz, Persian Gulf. In a first national conference on the biological science of Makran Sea. 30 (2): 97.

John DM. (2012). Marine algae (seaweed) are associated with coral reefs in the Gulf. In: Riegl BM, Purkis SJ (eds) (2012) Coral Reefs of the Gulf. Dordrecht: Springer, Netherlands. 309-335.

Johns DG, Edwards M, Richardson A, Spicer JI. (2003). Increased blooms of a dinoflagellate in the NW Atlantic. Marine Ecology Progress Series. 265: 283-287. DOI: 10.3354/meps265283.

Jorgensen E. (1911). Die Ceratien. Eine kurze Monographie des Gattung *Ceratium* Schrank. Internationale Revue der gesamten Hydrobiologie und Hydrographie. 4 (2): 1-124.

Katz LA, McManus GB, Snoeyenbos-West OL, Griffin A, Pirog K, Costas B, Foissner W. (2005). Reframing the 'Everything is everywhere' debate: evidence for high gene flow and diversity in ciliate morphospecies. Aquatic Microbial Ecology. 41 (1): 55-65. DOI: 10.3354/ ame041055.

Kudela RM, Berdalet E, Enevoldsen H, Pitcher G, Raine R, Urban E. (2017). GeoHAB: The Global Ecology and

Oceanography of Harmful Algal Blooms Program; motivation, goals, and legacy. Oceanography. 30 (1): 12-21. DOI: 10.5670/oceanog.2017.106.

Latz, MI, Nauen, JC, Rohr J. (2004). Bioluminescence response of four species of dinoflagellates to fully developed pipe flow. Journal of Plankton Research. 26 (12): 1529-1546. DOI: 10.1093/plankt/fbh141.

Lee CE and Bell MA. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. Trends in Ecology and Evolution. 14 (7): 284-288. DOI: 10.1016/S0169-5347(99)01596-7.

Lefèvre E, Bardot C, Noël C, Carrias JF, Viscogliosi E, Amblard C, Sime-Ngando T. (2007). Unveiling fungal zooflagellates as members of freshwater picoeukaryotes: evidence from a molecular diversity study in a deep meromictic lake. Environmental Microbiology. 9 (1): 61-71. DOI: 10.1111/j.1462-2920.2006.01111.x.

Logares R, Shalchian-Tabrizi K, Boltovskoy A, Rengefors K. (2007). Extensive dinoflagellate phylogenies indicate infrequent marine–freshwater transitions. Molecular Phylogenetics and Evolution. 45 (3): 887-903. DOI: 10.1016/j.ympev.2007.08.005.

Lovejoy NR, Albert JS, Crampton WG. (2006). Miocene marine incursions and marine/freshwater transitions: Evidence from Neotropical fishes. Journal of South American Earth Sciences. 21(1-2): 5-13. DOI: 10.1016/j.jsames.2005.07.009

Methé BA, Hiorns WD, Zehr JP. (1998). Contrasts between marine and freshwater bacterial community composition: analyses of communities in Lake George and six other Adirondack lakes. Limnology and Oceanography. 43 (2): 368-374. DOI: 10.4319/lo.1998.43.2.0368.

Mijares AJ, Sevcik C, Barboza CA, Saavedra JA. (1985). Ichthyotoxism by a paralytic toxin produced by marine dinoflagellates of the genus *Ceratium*: relationship to fraction β isolated from the sponge *Tedania ignis*. Toxicon. 23 (2): 221-233. DOI: 10.1016/0041-0101(85)90145-X.

Miller MF and Labandeira CC. (2002). Slow crawl across the salinity divide: delayed colonization of freshwater ecosystems by invertebrates. Geological Society of America. 12: 4-10. DOI:10.1130/1052-5173(2002)012<0004:SCATSD>2.0.CO;2.

Moreira RA, Rocha O, Santos RM, Laudares-Silva R, Dias ES, Eskinazi-Sant'Anna EM. (2015). First record of *Ceratium furcoides* (Dinophyta), an invasive species, in a temporary high-altitude lake in the Iron Quadrangle (MG, Southeast Brazil). Brazilian Journal of Biology. 75. DOI: 98-103. 10.1590/1519-6984.08013.

Morton SL, Shuler A, Paternoster J, Fanolua S, Vargo D. (2011). Coastal eutrophication, land use changes, and *Ceratium furca* (Dinophyceae) blooms in Pago Pago Harbor, American Samoa 2007–2009. Chinese Journal of Oceanology and Limnology. 29 (4): 790-794. DOI: 10.1007/s00343-011-0507-7.

Nielsen TG. (1991). Contribution of

zooplankton grazing to the decline of a *Ceratium* bloom. Limnology and Oceanography. 36 (6): 1091-1106.

Nitzsch CL. (1817). Beitrag zur Infusionenkunde oder Naturbeschreibung der Zerkarien und Bazillarien. Neue Schriften der Naturforschenden Gesellschaft zu Halle. 3: 1-128.

Orellana-Cepeda E, Granados-Machuca C, Serrano-Esquer J. (2002). *Ceratium furca*: One possible cause of mass mortality of cultured bluefin tuna at Baja California, Mexico. In Proceedings of the Xth International Conference on Harmful Algae. St. Petersburg Beach, Florida, USA. pp. 514-516.

Pawlowski J, Holzmann M. (2002). Molecular phylogeny of Foraminifera a review. *European Journal of Protistology.* 38(1): 1-10. DOI: 10.1078/0932-4739-00857

Pitcher GC, Probyn TA. (2011). Anoxia in southern Benguela during the autumn of 2009 and its linkage to a bloom of the dinoflagellate Ceratium balechii. Harmful Algae. 11: 23-32. DOI: 10.1016/j. hal.2011.07.001

Pizay MD, Lemée R, Simon N, Cras AL, Laugier JP, Dolan JR. (2009). Night and day morphologies in a planktonic dinoflagellate. Protist. 160 (4): 565-575. DOI: 10.1016/j.protis.2009.04.003.

Rabbaniha M, Izadpanahi GH, Mohsenizadeh F, Owfi F. (2012). Comparing of phytoplankton assemblage in inshore and offshore waters of the Persian Gulf (Bushehr province waters). In International conference on land-sea interactions in the coastal zone, Lebanon. 10 pp.

Raine R, White M, Dodge JD. (2002). The summer distribution of net plankton dinoflagellates and their relation to water movements in the NE Atlantic Ocean, west of Ireland. Journal of Plankton Research. 24 (11): 1131-1147. DOI: 10.1093/plankt/24.11.1131.

Reguera B, Velo-Suárez L, Raine R, Park MG. (2012). Dinophysis species: A review. Harmful Algae. 14: 87-106. DOI: 10.1016/j.hal.2011.10.016.

Rezai H. (1995). Blooms of phytoplankton along the northeastern coast of the Persian Gulf. Iranian Fisheries Research and Training Organization Report. 1: 1-55.

Richards TA, Vepritskiy AA, Gouliamova DE, Nierzwicki-Bauer SA. (2005). The molecular diversity of freshwater picoeukaryotes from an oligotrophic lake reveals diverse, distinctive, and globally dispersed lineages. Environmental Microbiology. 7 (9): 1413-1425. DOI: 10.1111/j.1462-2920.2005.00828.x.

Saldarriaga JF, Cavalier-Smith T, Menden-Deuer S, Keeling PJ. (2004). Molecular data and the evolutionary history of dinoflagellates. European Journal of Protistology. 40 (1): 85-111. DOI: 10.1016/j.ejop.2003.11.003.

Sato H, Greuet C, Cachon M, Cosson J. (2004). Analysis of the contraction of an organelle using its birefringence: The R-fibre of the *Ceratium* (Dinoflagellate) *flagellum*. Cell Biology International. 28 (5): 387-396. DOI: 10.1016/j.cellbi.2004.03.007

Scheckenbach F, Wylezich C, Mylnikov AP, Weitere M, Arndt H. (2006). Molecular comparisons of freshwater and marine isolates of the same morphospecies of heterotrophic flagellates. Applied and Environmental Microbiology. 72 (10): 6638-6643. DOI: 10.1128/AEM.02547-05.

Schiller J. (1937). Dinoflagellatae (Peridinineae) in monographischer Behandlung. In: Rabenhorst L (ed) Kryptogamen-Flora von Deutschland, Österreichs und der Schweiz. Akad Verlag, Leipzig. 10 (3): 1-589.

Schrank FVP. (1793). Mikroskopische Wahrnehmungen. Der Naturforscher (Halle). 27: 26-37. https://www.algaebase.org/search/species/detail/?species_id=56083.

Sheppard C, Al-Husiani M, Al-Jamali F, Al-Yamani F, Baldwin R, Bishop J, Benzoni F, Dutrieux E, Dulvy NK, Durvasula SRV, Jones DA. (2010). The Gulf: a young sea in decline. Marine Pollution Bulletin. 60 (1): 13-38. DOI: 10.1016/j.marpolbul.2009.10.017

Sims PA, Mann DG, Medlin LK. (2006). Evolution of the diatoms: insights from fossil, biological and molecular data. Phycologia. 45(4): 361-402. DOI: 10.2216/05-22.1

Skovgaard A, Hansen PJ, Stoecker DK. (2000). Physiology of the mixotrophic dinoflagellate *Fragilidium subglobosum*. I. Effects of phototrophy and irradiance on photosynthesis and carbon content. Marine Ecology Progress Series. 201: 129-136. https://www.jstor.org/stable/24863575.

Smalley GW, Coats DW, Adam EJ. (1999). A new method using fluorescent microspheres to determine grazing on ciliates by the mixotrophic dinoflagellate Ceratium furca. Aquatic Microbial Ecology. 17(2): 167-179. DOI: 10.3354/ame017167.

Snoke MS, Berendonk TU, Barth D, Lynch M. (2006). Large global effective population sizes in Paramecium. Molecular Biology and Evolution. 23 (12): 2474-2479. DOI: 10.1093/molbev/msl128.

Sournia A. (1967). Le genre *Ceratium* (*Péridinien planctonique*) dans le canal du Mozambique. Contribution à une révision mondiale. Vie et milieu/ Laboratoire Arago, Université P. et M. *Curie,* 18: 375-440. https://www.sudoc.fr/020993137.

Steidinger KA and Tangen K. (1997). Dinoflagellates. In Tomas CR (ed) Identifying Marine Phytoplankton. Elsevier, Academic Press, SanDiego, 387-584.

Sullivan JM and Swift E (1995). Photo enhancement of bioluminescence capacity in natural and laboratory populations of the autotrophic dinoflagellate *Ceratium fusus* (Ehrenb.) Dujardin. Journal of Geophysical Research: Oceans. 100 (4): 6565-6574. DOI: 10.1029/94JC01511.

Tamadoni Jahrom S, Sofiman Othman A, Saraji F, Abdolalian E, Moezzi M, Roohani K (2011). Identification and molecular phylogeny of the dinoflagellate (*Cochlodinium polykrikoides*) from the Persian Gulf. International Journal of

Review in Life Science.

Tan TH, Leaw CP, Leong SCY, Lim LP, Chew SM, Teng ST, Lim PT. (2016). Marine micro-phytoplankton of Singapore, with a review of harmful microalgae in the region. Raffles Bulletin of Zoology. 34: 78-96. http://ir.unimas.my/id/eprint/12646.

Teen LP, GireS USUP, Pin LC. (2012). Harmful algal blooms in Malaysian waters. Sains Malaysiana. 41 (12): 1509-1515. http://ir.unimas.my/id/eprint/2272.

Tunin-Ley A, Ibañez F, Labat JP, Zingone A, Lemée R. (2009). Phytoplankton biodiversity and NW Mediterranean Sea warming: changes in the dinoflagellate genus *Ceratium* in the 20th century. Marine Ecology Progress Series. 375: 85-99. DOI: 10.3354/meps07730.

Vermeij GJ and Wesselingh FP. (2002). Neogastropod mollusks from the Miocene of western Amazonia, with comments on marine to freshwater transitions in mollusks. Journal of Paleontology. 76 (2): 265-270. DOI:10.1666/0022-3360(2002)076<0265:NMFTMO>2.0.CO;2.

Warnecke F, Amann R, Pernthaler J. (2004). Actinobacterial 16S rRNA genes from freshwater habitats cluster in four distinct lineages. Environmental Microbiology. 6 (3): 242-253. DOI: 10.1111/j.1462-2920.2004.00561.x.

Weiler CS. (1980). Population structure and in situ division rates of *Ceratium* in oligotrophic waters of the North Pacific central gyre. Limnology and Oceanography. 25 (4): 610-619. DOI: 10.4319/lo.1980.25.4.0610.

Zarei Darki B and Krakhmalnyi A. (2017). Report of armored dinoflagellates from waters surrounding Hormuz island (the Strait of Hormuz). Iranian Journal of Botany. 23 (2): 145-160. DOI: 10.22092/IJB.2017.114012.

Zingone A and Enevoldsen HO. (2000). The diversity of harmful algal blooms: a challenge for science and management. Ocean and Coastal Management. 43 (8-9): 725-748. DOI: 10.1016/S0964-5691(00)00056-9.

Zwart G, Hiorns WD, Methé BA, van Agterveld MP, Huismans R, Nold SC, Zehr JP, Laanbroek HJ. (1998). Nearly identical 16S rRNA sequences recovered from lakes in North America and Europe indicate the existence of clades of globally distributed freshwater bacteria. Systematic and Applied Microbiology. 21 (4): 546-556. DOI: 10.1016/S0723-2020(98)80067-2.

Zwart G, Crump BC, Kamst-van Agterveld MP, Hagen F, Han SK. (2002). Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquatic Microbial Ecology. 28 (2): 141-155. DOI: 10.3354/ame028141.