TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2013) 37: 778-783

doi:10.3906/bot-1206-40

The phylogeny of *Heterocapsa* sp. (Dinophyceae) isolated from the south coast of Iran during a *Cochlodinium polykrikoides* bloom

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Received: 01.07.2012 • **Accepted:** 21.02.2013 • **Published Online:** 02.07.2013 • **Printed:** 02.08.2013

Abstract: The genus *Heterocapsa* Stein is a relatively small armoured dinoflagellate. Because it has few differences in morphological characteristics among its species, species identification is difficult. In this study, small *Heterocapsa* sp. cells associated with a dense bloom of *Cochlodinium polykrikoides* Margalef were collected from the south-east coast of Iran. While *Heterocapsa* sp. was isolated, *C. polykrikoides* could not be isolated by single cell or serial dilution methods. Thus, a unialgal strain of *Heterocapsa* sp. was used for molecular analysis and species identification. In order to carry out phylogenetic analysis, rDNA was extracted and large subunit domains of D1-D3 were sequenced. Similar sequences of other geographical strains were selected from GenBank and compared with the Iranian species. The Iranian strain was aligned with *Heterocapsa* spp. Phylogenetic analysis of maximum likelihood and neighbor-joining demonstrated that *Heterocapsa* sp. does not form a monophyletic group. Morphological characteristics confirmed molecular results. This is the first record of *Heterocapsa* isolation from Iranian coastal waters.

Key words: *Cochlodinium*, dinoflagellate, *Heterocapsa*, phylogeny, Oman Sea

1. Introduction

Among the most important marine and freshwater organisms are phytoplankton (Solak et al., 2012; Kükrer & Büyükışık, 2013), and dinoflagellates are one of the most important constituents of marine phytoplankton (Polat, 2007). The genus *Heterocapsa* Stein is a small armoured dinoflagellate with a Po, cp, 5′, 3a, 7′′, 6c, 5s, 5′′′, 2′′′′ thecal plate and has body scales on the cell surface (Horiguchi, 1995). There are only a few differences in morphology in the fine structure of the scales and the structure of the pyrenoid matrix among different species of the genus *Heterocapsa* (Horiguchi, 1995), which makes species identification difficult. Different species of the genus *Heterocapsa* have been studied from the aspect of genome (Waller et al., 2006). Ribosomal DNA can provide valuable phylogenetic affiliation for classifying the species. Small 5.8S and large subunit ribosomal genes (rDNA) have been widely used to evaluate phylogenetic relationships and molecular systematics of dinoflagellates (e.g., Scholin et al., 1995; Adachi et al., 1996; Saunders et al., 1997; Saldarriaga et al., 2001; Zhang et al., 2007). In some dinoflagellates, including *Heterocapsa* spp., small subunit ribosomal DNA (SSU-rDNA) genes cannot explain major evolutionary relationships and splits among taxa (Saldarriaga et al., 2004; Shalchian-Tabrizi et al., 2006). Cytochrome c oxidase I (CoxI) has potential for distinguishing closely related species based on DNA barcoding (Lin et al., 2009; Stern et al., 2010); however, dinoflagellates still need further examination within a broad variety of taxa (Zhang et al., 2007). Sequencing of internal transcribed spacer (ITS) regions, including 5.8S rDNA and phylogenetic analyses, was also performed for *Heterocapsa* species (Yoshida et al., 2003). The genus *Heterocapsa* was introduced as a gymnodinoid-shaped species for the first time by Stein (1883) in order to combine the species *Peridinium triquetrum* (Ehrenberg) Lebour and *Glenodinium triquetrum* Stein. Many species of phytoplankton can cause red tide (Feyzioğlu & Öğüt, 2006). Several blooms of the genus *Heterocapsa* are reported around the world every year (Nagasaki et al., 2004). Some species of this genus, such as *H. circularisquama* Horiguchi, have lethal effects on shellfish and have been demonstrated to kill pearl oysters and other shellfish in both cultures and field samples by Yoshida et al. (2003) and Nagasaki et al. (2004). However, some species in this genus have health benefits for humans. Peridinin is an unusual carotenoid uniquely present in some dinoflagellates including *H. triquetra*. The peridinin found in *Heterocapsa* species has a special structure with an antiproliferative effect on human colon cancer cells (Sugawara et al., 2007). A dense bloom of *Cochlodinium*

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polykrikoides began in June 2009 and lasted for 2 months along the south coast of Iran (Attaran-Fariman, 2010). *Heterocapsa* species were also associated with this bloom. In this study we describe small *Heterocapsa* cells that were isolated during the *C. polykrikoides* bloom and determine the phylogenetic relationships among the Iranian strain, different species of the genus *Heterocapsa*, and some other closely related dinoflagellate species.

2. Materials and methods

2.1. Sample collection and cultivation

The sampling was performed from 4 stations along the south-east coast of Iran, Chabahar Bay, in July 2009. Samples were taken from the surface to a maximum depth of 50 cm by Niskin bottles and were carried to the phycolab without adding fixatives. Single *Heterocapsa* sp. cells were isolated by microtube and serial dilution methods. The isolation process was continued until a unialgal strain was obtained. *Heterocapsa* sp. cells were cultured in 50-cm^3 petri dishes with 30 cm^3 of F/2 medium (Guillard, 1975). They were incubated under 12-h dark and 12-h light conditions at 25 °C and 2000 lx light. The purified samples were kept in the phycolab for further examinations.

2.2. DNA extraction, amplifications, and sequencing

DNA was extracted from the Iranian species of *Heterocapsa* sp. using the phenol:chloroform:isoamyl method (Ausubel et al., 1994; Attaran-Fariman et al., 2007). Extracted DNA quality and quantity was detected by 1.5% agarose gel electrophoresis using Tris-borate-EDTA as the buffer. Ethidium bromide was utilised for gel staining. Extracted DNA was used as a template to amplify and sequence about 950 bp of the D1–D3 regions of the large subunit ribosomal DNA (LSU-rDNA) gene, based on the method presented by Attaran-Fariman et al. (2007). While D1R (5′-ACC CGC TGA ATT TAA GCA TA-3′) was used as a forward primer (Scholin et al., 1994), 28-1483R (5′-GCT ACT ACC ACC AAG ATC TGC-3′) (Daugbjerg et al., 2000) was used as the reverse primer for polymerase chain reaction (PCR) amplification. PCR reactions were performed in 50-µL volumes in PCR tubes and contained Bioline NH_4 PCR buffer, 3 mM $MgCl_2$, 200 mM dNTPs, 10 pM of each primer, 1 U of BioTaq DNA polymerase (Bioline, UK), and 10 ng of DNA template. The thermocycling program consisted of a primary denaturation at 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, annealing (1 min, 60 °C), and elongation (72 °C, 2 min) followed by a final extension (6 min, 72 °C). PCR products were prepared for sequencing by purification through montage PCR clean-up columns (Millipore, USA), according to manufacturer protocols. PCR reaction and thermocycler profiles were performed according to the method of Attaran-Fariman et al. (2007). The Big Dye Terminator Sequencing Kit (Beckman-Coulter, USA) was used for nucleotide sequences, following manufacturer protocols. The Iranian strain's nucleotide sequence and different related species from GenBank were used for phylogenetic analysis (Table). P-distance was applied in the construction of neighbor

Table. List of species and LSU-rDNA sequences included in phylogenetic analysis.

Taxa	Strain	GenBank no.
Heterocapsa sp.	_	AF260399
Heterocapsa sp.	FIU11	EU165273
*Heterocapsa triquetr*a	GSW0206-2	EF613355
Heterocapsa triquetra	CCMP448	EU165307
Heterocapsa triquetra	_	AF206401
*Heterocapsa rotundat*a	_	AF260400
Heterocapsa niei	CS89	JN020158
Heterocapsa sp.	CCMP424	AY371082
Heterocapsa sp.	FIU12R	EU165274
Heterocapsa pygmaea	UTEX242	EU165306
Heterocapsa rotundata	CCMP173	EU165312
Heterocapsaceae sp.	CCMP2770	EU165271
Heterocapsa sp. Iranian strain	HCBC88	JN119844
Heterocapsaceae sp.	FIU10	EU165272
Gloeodinium montanum	_	EF205003
*Peridinium umbonatu*m	FACHB 329	GU001636
Scrippsiella sp.	_	AF260392
Scrippsiella trochoidea var. *aciculifera*	_	AF260393

joining (NJ) trees (Saitou & Nei, 1987), and maximum parsimony (MP) analysis (max–min branch-and-bound) were conducted for phylogenetic analysis. Sequences of similar species were compared with each other. Support for clusters in trees was estimated by bootstrap analysis (Felsenstein, 1985) using 1000 replicates of the full heuristic algorithm.

Scrippsiella trochoidea var. *aciculifera* and *Scrippsiella* sp. were used as out-groups for the purpose of rooting the analysis. Geneious 4.8.5 and ClustalX version 1.83 (Jeanmougin et al., 1998) and BioEdit (Hall, 1999) were used for the molecular analyses and phylogenetic tree tracing.

3. Results

Small dinoflagellates, observed during a *Cochlodinium polykrikoides* bloom along the south-east coast of Iran, were identified as *Heterocapsa* sp. as a result of morphological analysis. The purified Iranian *Heterocapsa* strain was named HCBC88, and it has been recorded in GenBank under this name. The cell colour is golden-brown. The cell length and width are 11–16 µm and 5–7 µm, with averages (n = 30) of 13 µm and 5 µm. The cell shape is ellipsoidal, with nearly equatorial cingulum and a small downward shift at the distal end; the epitheca and hypotheca are divided into almost equal size. The Iranian strain possesses a conical epitheca and rounded hypotheca (Figure 1). The nucleus is positioned in the hypotheca, and the pyrenoid is located above the nucleus of the epitheca (Figure 1).

The results of molecular and phylogenetic analyses by MP and NJ trees showed the same tree topology with similar branches; therefore, the MP tree is documented here. The sequence for *Heterocapsa* spp. LSU rDNA was limited in GenBank; however, both the MP and NJ trees obtained with these sequences demonstrated 3 clades (HC1, HC2, and HC3). Clade HC1 comprised *Heterocapsa* sp. and *H. triquetra* with 84% bootstrap support. In this clade all *H. triquetra* strains are in a subclade with 100% bootstrap support. Clade HC2 is a sister group of the HC3 clade, with 73% bootstrap support. This clade includes *H. niei* Morrill & Loeblich and *Heterocapsa* sp., with high bootstrap support (100%). Clade HC3 comprised *H. rotundata* (Lohmann) Hansen, *H. pygmaea* Lobelich III, and 2 *Heterocapsa* sp. species. The Iranian strain is also in this clade, with 95% bootstrap support. Its closest relatives are *Heterocapsa* sp. (strain FIU10) and *H. pygmaea* (Lobelich et al., 1981) (Figure 2). The last clade showed the relationship of *Gloeodinium montanum* and *Peridinium umbonatum* with all *Heterocapsa*; however, affinity was uncertain.

4. Discussion

Species of the genus *Heterocapsa* are small in size and have almost the same morphological characters, such as thecal plate pattern and reticulated peripheral chloroplast, which makes their identification by light microscopy difficult (Iwataki, 2008). Although tabulation does not vary much among the species of *Heterocapsa* (Uysal et al., 2003), thecal plate arrangement was noted as an important feature in the identification of most dinoflagellates (Fensome et al., 1993). In addition, many researchers noted that the fine structure of body scales, also present in the genus *Heterocapsa*, is a more reliable feature for species identification than other morphological characters (Iwataki et al., 2002; Tamura et al., 2005; Iwataki, 2008). Cell shape, cell size, and position of the nucleus and pyrenoid vary among some *Heterocapsa* species and are also useful for species identification (Iwataki, 2008); however, molecular data are necessary to address the systematic level of the species (Daugbjerg et al., 2000). The size range in most of the species overlaps. For this reason, it is not easy to recognise taxa based on cell size (Hansen,

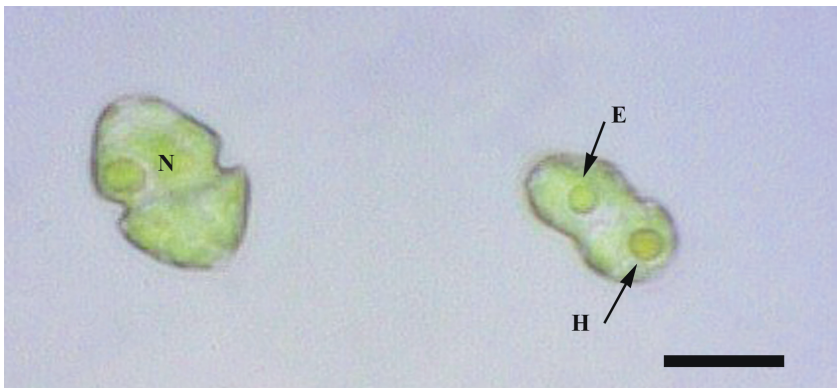

Figure 1. *Heterocapsa* sp. (strain HCBC88) isolated for the first time from south-east coast of Iran during massive *Cochlodinium polykrikoides* bloom. N = nucleus, E = epitheca, H = hypotheca, ave = average. Scale bar = 5 µm.

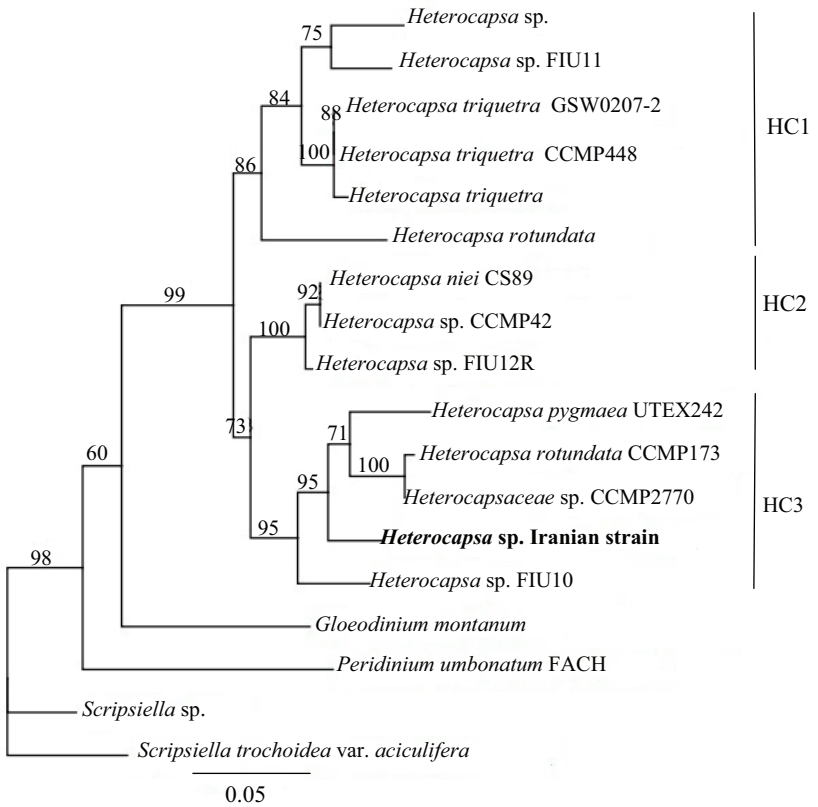

Figure 2. Molecular phylogenetic tree of *Heterocapsa* species inferred from LSU-rDNA domains D1–D3. Bootstrap consensus tree obtained by maximum parsimony analysis from 1000 replicates using Geneious 4.8.5 branch-and-bound search. *Scrippsiella* sp. and *Scrippsiella trochoidea* var. *aciculifera* are the out-groups.

1995; Horiguchi, 1997; Iwataki, 2008); however, size could be a significant factor in their identification and taxonomy. The cell length of *Heterocapsa* sp. (Iranian strain) ranged from 11 to 16 μm. *H. niei*, *H. pygmaea*, and the Iranian strain are all ellipsoidal with equally sized epitheca and hypotheca, whereas in *H. rotundata*, *H. niei*, and *H. circularisquama* the epitheca is larger than the hypotheca (Iwataki et al., 2002; Tamura et al., 2005). The pyrenoid in *H. triquetra* is in the hypotheca, while the nucleus is in the epitheca. In the Iranian strain (Figure 1) the nucleus is located in the hypotheca and the pyrenoid above the nucleus, which is similar to *H. niei* and *H. pygmaea* (Uysal et al., 2003; Iwataki, 2008). In *H. horiguchii* Iwataki, the nucleus is in the epitheca (Loeblich et al., 1981; Morrill & Loeblich, 1984; Iwataki et al., 2002; Tamura et al., 2005). In 1981, Loeblich et al. (1981) established a new species of *Heterocapsa*, *Heterocapsa pygmaea*. They reported the presence of an apical pore plate and canal plate; the eighth precingular plate was similar to the anterior sulcal plate, and this explanation has been accepted until now.

In the past decade, phylogenetic analyses of dinoflagellates based on LSU-rDNA have frequently been used to find the relationship among taxa (Daugbjerg et al., 2000; Attaran-Fariman et al., 2007). The large subunit of the gene inhabits both highly variable and conservative regions and can be useful for the study of phylogeny and evolution at different systematic levels (Hillis & Dixon, 1991). Saldarriaga et al. (2004) demonstrated that all molecular analyses agree with the placement of ciliates and apicomplexans (=Sporozoa) with dinoflagellates in a well-supported clade, the alveolates. They explained that Peridiniales is a paraphyletic group from which other dinoflagellate orders such as Dinophysiales, Prorocentrales, most Gymnodiniales, and perhaps Gonyaulacales originated. In some LSU-based trees Prorocentrales is a monophyletic group (Saldarriaga et al., 2004). In an investigation by Daugbjerg et al. (2000) based on LSU-rDNA, *H. triquetra* and *H. rotundata* were in the same clade with 100% bootstrap support; however, their relationship is not resolved, and *H. rotundata* forms a sister group with *H. triquetra* and *Heterocapsa* sp. In the study of Zhang et al. (2007), *H. triquetra* and *H. rotundata* were supported by 100% bootstrapping. Although these 2 species together with *Scrippsiella* sp. are from Peridiniales,

they were put in 2 different clades. *H. pygmaea*, *H. niei*, and *H. rotundata* with *H. triquetra* have been put in a clade with 100% bootstrap support (Zhang et al., 2007).

In this study, all *Heterocapsa* species with gene sequences similar to the Iranian strain were compared. *H. rotundata* is the first branch in clade HC1, which includes all *H. triquetra*-like species and *Heterocapsa* sp. This molecular analysis agrees with previous molecular works (e.g., Daugbjerg, 2000; Zhang et al., 2007). In all previous studies based on LSU and mitochondrial cytochrome b and c and ITS, 2 species formed a monophyletic group in comparison with other dinoflagellate species. However, in this study, which focuses mainly on gene sequences of *Heterocapsa* species and a few other species, it seems that molecular divergence has supported morphological differences, as *H. rotundata* is the out-group in clade HC1 for *H. triquetra* and *Heterocapsa* sp. (Figure 2). *H. triquetra* has a typical horn, its size is larger than *H. rotundata*, and its epi-hypotheca sizes are equal; however, *H. rotundata* has a large epitheca and small rounded hypotheca (Iwataki et al., 2002). The second clade (HC2) comprised *Heterocapsa* sp. and *H. niei* with 100% bootstrap support. The Iranian strain is in clade HC3, and its closest relatives are *Heterocapsaceae* sp. (strain FIU10) and the sister group *H. pygmaea*. The latter species has the most morphological similarity to the Iranian *Heterocapsa* species. Both species have the same epitheca and hypotheca size with rounded hypotheca, and they both have a nucleus in the hypotheca and the pyrenoid above it. The Iranian species is larger than *H. pygmaea*.

We have conclusively documented the presence of *Heterocapsa* sp. (Iranian strain) in the Iranian waters of the Oman Sea, the site of a massive *Cochlodinium polykrikoides* bloom, for the first time. Our phylogenetic analysis showed interrelationship among different strains of *Heterocapsa* in agreement with previous studies. *Gloeodinium montanum*, *Peridinium umbonatum*, *Scrippsiella* sp., and *Scrippsiella trochoidea* var. *aciculifera* lie in a monophyletic group. *Heterocapsa triquetra* and *Heterocapsa rotundata* generally form a clade in almost all phylogenetic trees with strong (more than 95%) bootstrap protection.

Acknowledgements
The authors would like to thank Fatemeh Nasseri, Seyed Ali Mousavi, and Esmat Behrouzi for their significant help with cultivation and molecular laboratory work.

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