Phylogenetic Analysis of the *Noctiluca scintillans* Based on SSU rDNA from the Persian Gulf

Zahra Yarahmadi[1], Bita Archangi [1*], Ahmad Savari [1], Seyed Mohammad Bagher Nabavi [1]

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Abstract

Noctiluca scintillans is the first described dinoflagellate that has been widely scrutinized because of its potential for red tide formation and bioluminescence. Nevertheless, its phylogenetic position with members of the Noctilucales order is still unstable and challenging. In this research, the morphology of *N. scintillans* collected from the Persian Gulf was described by observation under an inverted microscope, and then the Small Subunit ribosomal DNA (SSU rDNA) gene fragment was amplified by the single-cell method. The Small subunit ribosomal RNA (SSU rRNA) gene sequence or SSU rDNA sequence was determined for the Iranian strain of *N. scintillans*. In the phylogenetic analysis of SSU rDNA in this study, the order Noctilucales appeared as a monophyletic clade of free-living species in a basal position of the tree, being an early divergent lineage within dinoflagellates branching before the core dinoflagellates. Although this position is supported moderately, the current study generally supports the hypothesis that noctilucids are placed in an ancestral origin within dinoflagellates and are regarded as an ancestral lineage for dinoflagellates.

Keywords: Dinoflagellates, Noctilucid, Morphology, Single-cell, Phylogeny

Introduction

N. scintillans is one of the most common heterotrophic and unarmored dinoflagellates that feed by phagotrophy (Kraberg et al., 2010). It is well known for the role of its populations in algal bloom formation (Miyaguchi et al., 2006) and bioluminescence (Lebour, 1925; Larink and Westheide, 2006). Because of its ability to produce high concentrations of ammonium (toxic levels) in dense populations when algal blooms occur, it depletes water oxygen levels, and harms fish gills (Horner, 2002; Escalera et al., 2007; Asefi and Attaran-Fariman, 2023), and causes the death of many marine invertebrates (Smithsonian, 2012; Asefi and Attaran-Fariman, 2023).

The process of identifying and classifying *Noctiluca* has evolved numerously. *N. scintillans* was initially identified as a jellyfish (Slabber, 1771). Hackel (1873) proposed that *Noctiluca* should be included in the group Cystoflagellata within the dinoflagellates. Later, Kofoid (1920) created a new order termed Noctilucales based on

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
Doi: 10.48308/jpr.2024.231742.1046

the morphological characteristics of the trophont (sulcus, flagellum, cingulum, etc.), which is closely related to Gymnodiniales and was accepted. However, Zingmark (1970) claimed *Noctiluca* not to be a true dinoflagellate because its trophont nucleus is eukaryotic rather than dinokaryotic (A dinokaryon is a eukaryotic nucleus present in dinoflagellates in which the chromosomes are fibrillar in appearance (i.e. with unmasked DNA fibrils) and are more or less continuously condensed. Histones are absent and nuclear envelope does not break down during mitosis, which is thus termed closed mitosis, or "dinomitosis". The mitotic spindle is extranuclear). Notwithstanding, Fensome et al. (1993) assigned *Noctiluca* to the class Noctiluciphyceae based on the dinokaryotic nature of the gamete nucleus.

In the 1990s, the phylogenetic position of *Noctiluca* was evaluated using molecular data. The first phylogenetic analysis including noctilucids using Large subunit ribosomal DNA (LSU rDNA) revealed *N. scintillans* in the ancestral position within the dinoflagellates (Lenaers et al., 1991). An analysis by Saunders et al. (1997) based on SSU rDNA also placed *N. scintillans* in an ancestral position. Although a phylogenetic analysis by Cavalier-Smith and Chao (2004) placed *Noctiluca* in a basal position, the *Oxyrrhis* was considered a specialized species from core dinoflagellates. On the other hand, Saldarriaga et al. (2004) indicated that the placement of *Noctiluca* was unstable in phylogenetic trees and its position is influenced by the number of sites in the used sequences so that this placement can change from a basal position among the dinoflagellates to a position in the order Gymnodiniales. Moreover, there are two variable viewpoints on the position of noctilucids based on morphological characteristics and phylogenetic analysis using 18S rDNA and Hsp90, and their phylogenetic position is still disputable (Fukuda and Endoh, 2008). The phylogenetic analysis by Gómez et al. (2010) using SSU rDNA indicated a basal position for Noctilucales after several parasitic lineages of alveolate. However, in a study by Hoppenrath and Leander (2010) using the Hsp90 sequence, *Noctiluca* never occupied a basal position among the core dinoflagellates, and rather they were placed deeply among other dinokaryotic lineages (close to Gymnodiniales). Phylogenetic analysis using Cytochrome c oxidase subunit III (COX3) also showed an early branching position for *Noctiluca* (Janouškovec et al., 2017). But Cooney et al. (2022) showed recently in phylogenomic analyses based on SSU rDNA that Noctilucales are sister to *Amphidinium* and not an independent clade outside the core dinoflagellates.

In this study, *N. scintillans* was identified by an inverted microscope. In addition, the first SSU rDNA gene sequence was provided for the Iranian strain of *N. scintillans* collected from the waters of Bandar Abbas in the Persian Gulf. Moreover, their position in phylogenetic trees and their evolutionary relations with other dinoflagellate groups were studied through phylogenetic analyses.

Material and methods

Sampling

In December 2017, water samples from

the Persian Gulf, Bandar Abbas coasts (59" 17′ 56° E and 10" 10′ 27° N, Fig. 1) were collected by a standard phytoplankton net (mesh size: 50µ) equipped with a flowmeter from the water surface to a depth of 3 m with three replicates using 200cc Polyethylene bottles. 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).

Light microscope observation

To examine the species morphology, fixed specimens were observed under an inverted microscope (Olympus, Tokyo, Japan) and photographed with a Dino capture lens. The specimens were identified morphologically using phytoplankton identification keys. (Norris 1969; Hasle et al., 1996; Botes 2003; Al-Kandari 2009; Okolodkov 2010, 2014).

Single-cell Isolation

The fixed specimen (0.5 cc) was mounted on a microscopic slide, supplemented with 5 µl of 1M sodium thiosulfate solution to discolor the Lugol's solution and prepare the cells for isolation. The target specimen was isolated by pipetting, and the isolated cell was rinsed with deionized water 5-8 times to ensure that the sample was void of any likely contamination. Next, the isolated cell with 2 µ of deionized water was transferred to a 0.2 ml microtube, and the successful transfer of the specimen to the microtube was confirmed under a microscope.

PCR Conditions and Amplification

The single-cell fixed with Logul's solution was included in three rounds of polymerase chain reaction (PCR). The PCR mixture containing 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 forward (EK-42F) and reverse (EK-1520R) primers (10 pmol), and 0.3 µl of Taq polymerase (5 U/µl) was poured into a microtube containing the single cell, and the solution final volume was made to

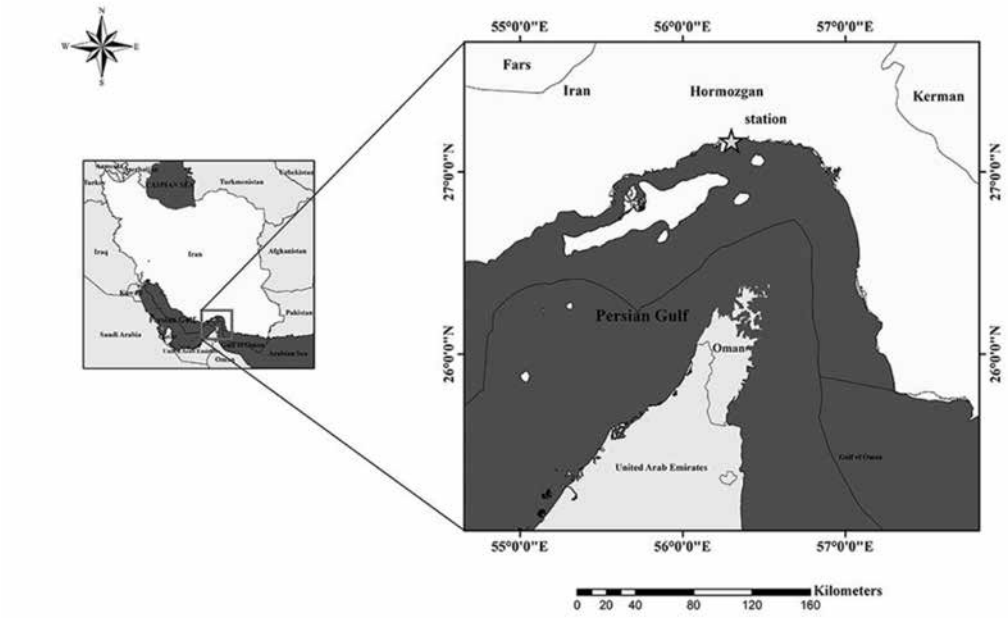

Fig. 1. Map of the Persian Gulf showing the sampling location in Bandar

30 µl per reaction by deionized water.

Nested PCR was necessary because the amount of DNA to be tested was very low, (Guillou et al., 2002). 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 Sec, and elongation at 72 °C for 2 min. This was followed by a further 20 cycles including denaturation at 94 °C for 15 S, annealing at 55 °C for 30 Sec, 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 and EK-1498R, 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 (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., 2009; 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. The specifications of the primers are shown in Table 1.

PCR products were electrophoresed on 1% agarose gel and observed by a UVITEC-Cambridge gel documentation system. Suitable 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

Table 1. primers used for the amplification and sequenceing in this study

marker	Gene	sequence (5´ - 3´)	application
EK-42F	eukaryotic-specific SSUrDNA	CTCAARGAYTAAGCCATGCA	Initial amplification
EK-1520R	eukaryotic-specific SSUrDNA	CYGCAGGTTCACCTA	Initial amplification
EK-82F	eukaryotic-specific primers	GAAACTGCGAATGGCTC	nested PCR
EK-1498R	eukaryotic-specific primers	CACCTACGGAAACCTTGTTA	nested PCR, sequencing
DIN464F	dinoflagellate specific primer	TAACAATACAGGGCATCCAT	Semi-nested PCR, sequencing

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 maximum likelihood (ML), maximum parsimony (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

Microscopic Observation

In microscopic observation, isolated cells were spherical and swollen (balloon-like), with a large (500-1000 µm) diameter (Fig. 2) and They had food vacuoles (Fig. 2-a). Since they are a type of unarmored dinoflagellates, no distinction was observed between their epicon and hypocon. The cells lacked chloroplasts, and the ventral groove was deep and broad, comprising a flagellum, a tooth, and a tentacle (Fig. 2-c).

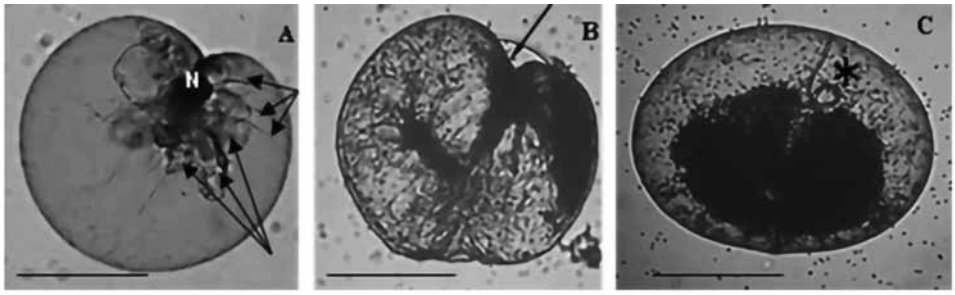

Fig 2. Inverted microscope images of *N. scintillans* of the Persian Gulf; A: the nucleus is marked with **N**, the long arrow indicates the food vacuoles and the small black arrows show the cytoplasmic filaments. B: a cell dividing asexually with binary fission; the arrow indicates the fission site. C: the asterisk indicates the tentacle. The cell is fixed with Lugol's solution. Scale line 250 µm

There is only one flagellum in this species (equivalent to a transverse flagellum in other dinoflagellates). The tooth is a particular extension of the cell wall. The transverse flagellum was transformed by the tentacle. There is a large eukaryotic nucleus near the groove from which the cytoplasmic filaments are extended to the cell edge (membrane) (Fig. 2-a). These filaments are involved in the movement, short-term deformation, and complex anthogenetic process of these organisms. Cells isolated from the Persian Gulf waters were often mature and undergoing asexual division by binary fission in some cases (Fig. 2-b). Based on the above characteristics and the comparison with valid identification keys, the isolated cells were identified as *Noctiluca scintillans* ((Macartney) Kofoid and Swezy, 1921). Unfortunately, there were no representatives of other Noctilucoid dinoflagellates in our samples to be used to enhance the results of the molecular phylogenetic analysis.

Molecular Phylogeny

In this study, the SSU rDNA sequence from the Iranian *N. scintillans* strain, identified under a microscope and isolated individually, was acquired by PCR and deposited with accession numbers OK356406, OK356475 and OK356482 in the GenBank. Until now, no registered SSU rDNA sequences for *N. scintillans* from the Persian Gulf are available in the GenBank, and the sequences obtained in this study have been reported for the first time. In addition, the SSU rDNA sequences from the Iranian *N. scintillans* strain did not wholly correspond to any of the sequences registered in the GenBank, and the extent of similarity to the earlier GenBank-deposited sequences of *N. scintillans* was about 90%. The phylogenetic analysis was performed using new sequences with the

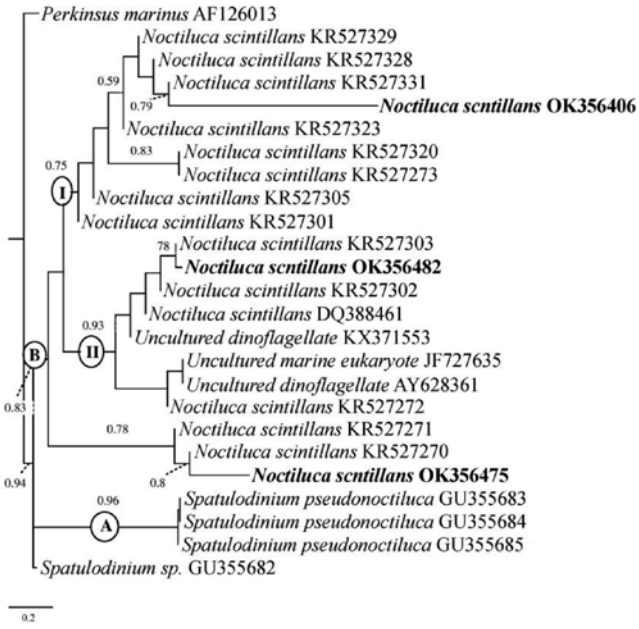

Fig. 3. Phylogenetic tree of the Bayesian analysis based on the SSU rDNA gene fragment; *N. scintillans*; values on branches represent Bayesian posterior probabilities. Probabilities less than 50% are overlooked here. The sequences of the present study are shown in bold. The accession number of the sequences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

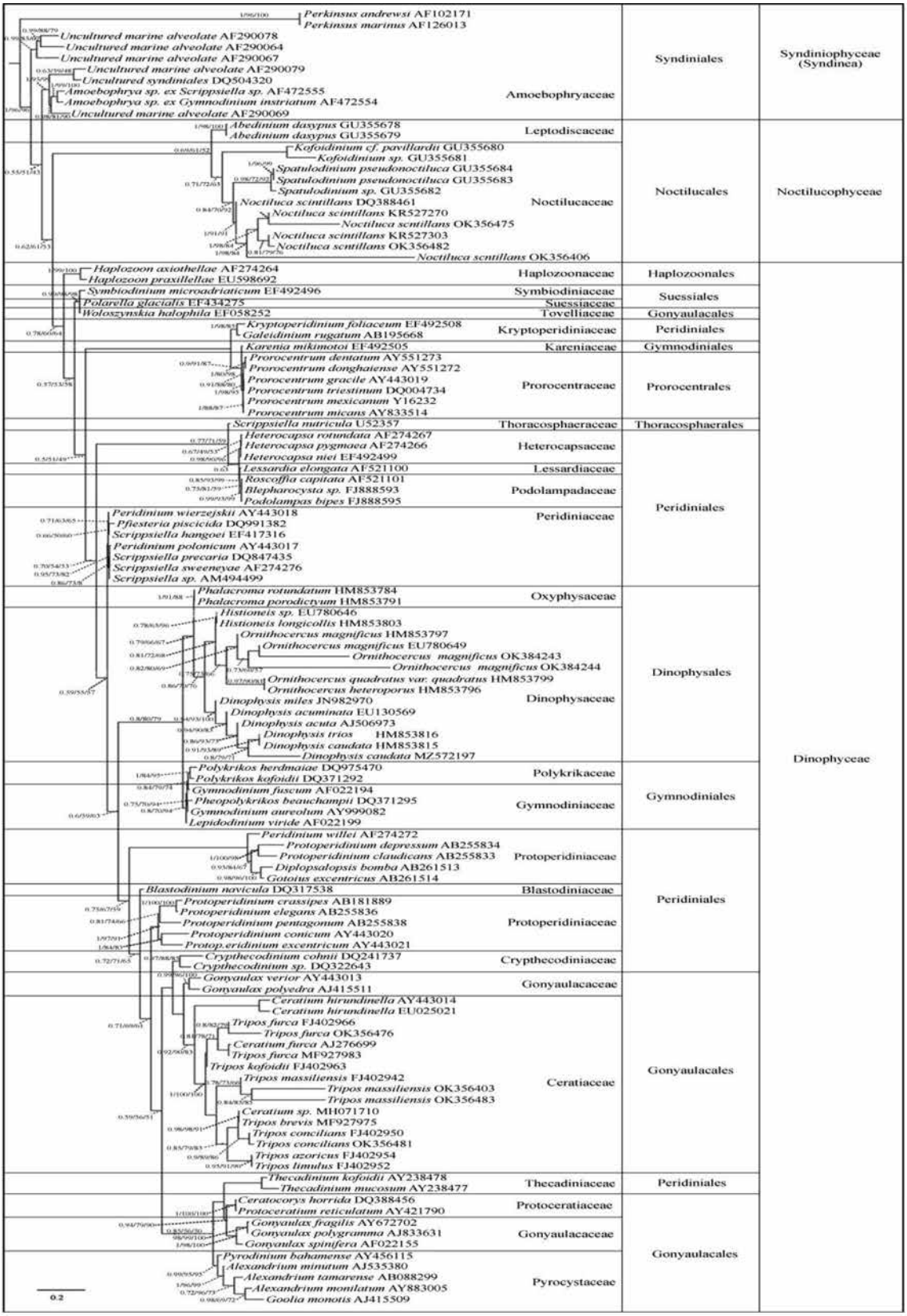

Fig. 4. Bayesian phylogenetic tree of dinoflagellate SSU rDNA sequences; numbers on branches represent Bayesian posterior probabilities/maximum likelihood values of 1000 trees/bootstrap values of maximum parsimony of 1000. Probabilities less than 50% are overlooked here. The sequences of the present study are shown in gray highlight.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

most similar sequences and those from the main orders of the dinoflagellates registered previously in the Gen Bank. Sequences from representatives of perkinsozoan and marine alveolate were used as outgroups.

Results of molecular and phylogenetic analyses revealed the identical topology of the trees. Hence, only the tree derived from the Bayesian phylogenetic analysis has been documented here. All three sequences of the Iranian strain (identified morphologically as *N. scintillans*) were placed under the clade B, the members of which are from diverse populations of *N. scintillans*. Moreover, individuals from the genus *Spatulodinium* form the sister group clade B (Fig. 3).

As shown in the Bayesian tree (Fig. 4), the sequences of the Iranian *N. scntillans* strain along with individuals from populations of the order Noctilucales, including the families Leptodiscaceae, Noctilucaceae, and Kofoidiniaceae with moderate support (PP = 0.69, ML = 61, and MP = 52), appear in a position between the Syndiniales and the core dinoflagellates. The closest relatives of the order Noctilucales are clades consisting of species from the genus *Haplozoon* (PP = 78, ML = 60, and MP = 64) (Fig. 4).

Discussion

The order Noctilucales comprises three families of aberrant dinoflagellates (Noctilucaceae, Leptodiscaceae, and Kofoidiniaceae) that, at least at some stages in their life, lack the typical characteristics of dinoflagellates, including transverse flagellum or condensed chromosomes (Gómez et al., 2010). In our phylogenetic analysis of the SSU rDNA, the order Noctilucales as a monophyletic clade of free-living species is placed in the basal position of the tree, close to a variety of parasitic chloroplast-free species of the alveolate lineage (perkinsoids, marine alveolate, and Syndiniales), and as the sister group of a larger clade consisting of the non-photosynthetic parasite of the genus *Haplozoon* (from the order Blastodiniales) and other dinokaryots (Fig. 4). In most published phylogenetic analyses, Noctilucales as an early divergent lineage is placed among dinoflagellates in the branches after *Oxyrrhis* and before the core dinoflagellates (Lenaers et al., 1991; Saunders et al., 1997; Saldarriaga et al., 2001; Cavalier-Smith and Chao, 2004; Shalchian-Tabrizi et al., 2006; Liu and Hastings 2007; Moore et al. 2008; Fukuda and Endoh 2008; Gómez et al. 2010; Zhang and Lin, 2008; ki, 2010; Gómez et al., 2011; Janouškovec et al., 2017). They all share the lack of well support and high bootstraps from Noctilucales' position in the mentioned phylogenetic trees.

The branching pattern may differ depending on the sequences and the phylogenetic approach used in the analysis (Leaw et al., 2005; Touzet et al., 2008). In phylogenetic studies, the position of the order Noctilucales is unstable and may be influenced by the number of nucleotides used in the alignment (Saldarriaga et al., 2004; Gómez et al., 2010). Phylogenomic analyses by Cooney et al. (2022) suggests that the Noctilucales are sister to Amphidinium rather than an independent branch outside the core dinoflagellates. In phylogenetic trees rooted using the sequences of *Perkinsus Levine* and Syndiniales (Saldarriaga et al.,

2004), *Noctiluca* was placed in a clade of the order Gymnodiniales. They suggested the gymnodinioid as a common ancestor for the entire group and emphasized the re-examination of a basal position for Noctilucales among dinokaryotic dinoflagellates. Interestingly, the two groups gymnodinioid and noctilucoids have similarities in some unique morphological characters. First, the presence of the special ampullae chamber in the nuclear pores in *Noctiluca* trophont (Afzelius, 1963; Soyer, 1969), *Petalodinium* of noctilucoid types (Gómez and Furuya, 2005), and in various representatives of the *Gymnodinium sensu stricto* group (Daugbjerg et al., 2000; Hansen and Moestrup, 2005; Hoppenrath and Leander, 2007), and second, the absence of transverse flagella in *Gymnodinium* species and *Noctiluca* zoospores (the common feature of other dinoflagellates) (Hansen et al., 2000).

Generally, general characters can be helpful for the diagnosis of the group, but the cell shape has little classification value. In addition, the cell morphology changes several times during the life cycle, and diverse shapes can be assigned to a species using molecular data. An excellent example is a report on *Gymnodinium lebouriae*, which was explicitly identified as a life stage of *S. pseudonoctiluca* (Gómez et al., 2011). In this study, species of the genus *Noctiluca* (with a sac-like appearance) are branched with species of the genus *Spatulodinium* (laterally flattened) (Fig. 4). Consequently, the present results do not support the division of Noctilucales into the families Noctilucaceae and Kofoidiniaceae.

As mentioned above, phylogenetic analyses have ascertained two probable hypotheses about the origin of noctilucid members among dinoflagellates. This group is either from the clade of ancestral dinoflagellates (an ancestral lineage for dinoflagellates) or has recently evolved from the order Gymnodiniales. This study supports the hypothesis that noctilucids placed in anancestral position within dinoflagellates. Regarding, the evolutionary origin of core dinoflagellates, Fukuda and Endoh (2008) offer a very interesting hypothesis based on the morphological observations of the trophont and gamete forms of *Noctiluca*. Based on this hypothesis, most dinoflagellates presumably branch from an ancestral cell with a haploid nucleus, such as the *Noctiluca* gamete. The *Noctiluca* trophont form has lost some of the common features of dinoflagellates (transverse flagellum and condensed chromosomes).

In contrast, *Noctiluca* gametes have maintained the essential and primitive characteristics of dinoflagellates, including the presence of two grooves, flagella of different lengths, paraxial bodies, and condensed chromosomes (Soyer, 1970; Soyer, 1972; Fukuda and Endoh, 2006). This feature of gametes is seemingly a reflection of the primary features of oldest dinoflagellates. Given the diploid trophont form in *Oxyrrhis* and *Noctiluca*, Fukuda and Endoh (2008) considered a diploid biflagellate as a common ancestor of dinoflagellates. This ancestral species may have undergone sexual reproduction during a period of its life cycle. After the *Oxyrrhis* branching from the ancestral genus and

the loss of two grooves in its descendants, the ancestral species has evolved into *Noctiluca* (and most of the remaining core dinoflagellates). Following these events, *Noctiluca* diverged from the main line of dinoflagellates. While the ancestors of *Noctiluca* show specialized trophonts due to the lack of typical features in dinoflagellates, the features derived from the ancient ancestor are preserved in gametes. The common ancestor of the core dinoflagellates has evolved from an ancestor with a haploid nucleus (such as the *Noctiluca* gamete) by the neoteny or juvenilization, making it possible to produce haploid trophonts (core dinoflagellates) from diploid types (*Oxyrrhis* and *Noctiluca*). With ancestral haploidization, the core dinoflagellates have evolved rapidly, which is reflected in low supportaion of their phylogenetic relationships (Fukuda and Endoh, 2008).

In summary the present research is the first attempt to establish the *N. scintillans* SSU rDNA sequence in the Persian Gulf performed by a thorough genetic study of individual cells. Evolutionarily, our phylogenetic analysis supports the hypothesis that members of noctilucids are placed ancestrally within dinoflagellates; however, this support is not associated with high bootstraps. Molecular techniques complement classical classification methods and are more important when morphological diagnostic features are inadequate or misleading. Molecular techniques seem to be the only option for solving the phylogenetic relationships of organisms. Future studies should be accompanied by broader sampling to include more representatives of the order Noctilucales. Other molecular markers should also be used to help enrich their gene bank and elucidate their phylogenetic position.

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