Morphology And Molecular Characterization Of *Alexandrium Tamutum* In Sarawak Water

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DECLARATION OF AUTHORSHIP

I, Ellianna Ellsa Harry declare that the final year project in this report entitled:

Morphology and Molecular characterization of *Alexandrium Tamutum* in Sarawak Water

and the work presented in the report are both my own and have been generated by me as the result of my original research. I confirm that:

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- I have acknowledged all main sources of help;
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Signed:

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ABSTRACT

Alexandrium tamutum is a solitary, small single cell that is known to not produce paralytic shellfish poisoning toxins. Despite the increased frequency of algal bloom episodes observed in Malaysia, which have been linked to negative environmental and health repercussions, there is a lack of research on *Alexandrium tamutum* that can help with species identification and future plankton monitoring. A study was done to characterize the *A. tamutum* species based on their morphology and molecular characterization in Sarawak waters. Four strains of *Alexandrium* were collected and established from Santubong Beach, Kuching was maintained in L1 medium under light intensity 12:12 hour light-dark photoperiod. The detailed morphological observation was carried out by using a compound and scanning electron microscope. The morphotype such as the the anterior sulcal plate (Sa), ventral pore (vp), first antapical plate (1'), sixth precingular plate (6''), apical pore complex (APC), and the posterior sulcal plate (Sp) was similar to *Alexandrium tamuntum.* A Bayesian Inference (BI) and Maximum Likelihood (ML) tree were constructed by DNA amplification and sequencing of Large Subunit (LSU) of length 580bp. Based on the BI and ML phylogeny tree, *Alexandrium* in this study were cladded together with *A.tamutum* with a supported bootstrap value of (0.95/100%).

Keywords : *Alexandrium* species, *Alexandrium tamutum*, LSU rDNA, morphology, phylogeny

ABSTRAK

Alexandrium tamutum merupakan sel tunggal kecil yang tidak menghasilkan toksin. Di Malaysia, terdapat peningkatan kes ledakan alga yang boleh dikaitkan dengan kesan negatif terhadap alam sekitar dan kesihatan.Walau bagaimanapun, terdapat jurang penyelidikan tentang spesis Alexandrium tamutum yang boleh membantu dengan mengenal pasti spesis dan pemantauan plankton pada masa hadapan.Kajian telah dilakukan untuk mencirikan spesis A. tamutum berdasarkan morfologi dan filogeni molekular di perairan Sarawak. Empat strain A. tamutum yang diperolehi dari Pantai Santubong, Kuching dikekalkan dalam medium L1 dibawah intensiti cahaya 12:12 jam cahaya gelap. Pemerhatian morfologi secara terperinci telah dijalankan dengan menggunakan pemerhatian mikroskopik. Susur galur Inferens Bayesian (BI) dan Kemungkinan Maksima (ML) telah dibina melalui amplifikasi DNA dan penujukan sub-unit besar (LSU). Dapatan daripada kajian ini telah menunjukkan bahawa keputusan morfometrik daripada Alexandrium yang dikaji adalah padanan dengan A.tamutum dengan nilai bootstrap yang disokong (0.95/100%).

Kata Kunci : Spesis *Alexandrium*, *Alexandarium tamutum*, LSU rDNA, morfologi, filogeni

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List of Abbreviations

HABs	Harmful algal blooms
LSU	Large subunit
ITS	Internal transcribed spacer
rDNA	Ribosomal ribonucleic acid
BLAST	Basic Local Alignment Search Tool
NCBI	National Centre of Biotechnology Institute
PCR	Polymerase Chain Reaction
STXs	Saxitoxins
PSP	Paralytic shellfish poisoning
rpm	Rotation per minute
MP	Maximum Parsimony
ML	Maximum Likelihood
BI	Bayesian Inference
KNO_3	Potassium nitrate
KH_2PO_4	Potassium dihydrogen phosphate
$MgCL_2$	Magnesium chloride
ddH_2O	Double distilled water
dNTP	Deoxyribonucleotide triphosphat

1.0 Introduction

HABs (harmful algae blooms) are naturally occurring phenomena that occur when a significant number of microalgal species congregate in marine and freshwater habitats. The occurrence of HAB has been shown to have a negative impact on both the environment and humans. This occurrence has been increasingly documented throughout the country, not only because of the frequency and severity of the incidents but also because of the diversity of species involved, as indicated by Lim *et al*., (2012). HABs come in a variety of forms, as they can be produced by a range of algae groups that produce different types of toxins, one of which being the *Alexandrium* species from the *Goniodomataceae* family (Leung *et al*., 2018). *Alexandrium* can be found in a variety of maritime settings, primarily along the shore. According to AlgaeBase, 33 species are presently accepted taxonomically as of 2021. *A.catenella*, *A.minitum*, *A.ostenfeldii*, *A.tamarense*, and *A.monilatum* are among the 33 species known to generate paralytic shellfish toxins (PSTs) (Brown *et al*., 2010; Gu *et al*., 2013). Saxitoxin (STXs), a neurotoxin that causes Paralytic Shellfish Poisoning (PSP), is the most well-known toxin. Humans can develop serious gastrointestinal and neurological problems after consuming infected mussels. (Arnich & Thebault, 2018). Although not all members of the *Alexandrium* genus are known to produce toxins, an overpopulation of algae can result in a lack of oxygen, which can pose a threat to the marine environment (Heisler *et al*., 2008).

Alexandrium tamutum are oval or round in shape that measures approximately 24-35 µm long and 26-32 µm wide with a thin and smooth thecal plate. The cell is not heavily pigmented with a centrally positioned ventral pore (Montresor *et al*., 2004).*Alexandrium tamutum* in essence is very similar morphologically to a toxic species *Alexandrium minitum* as they both share similarities in both shape and size. They are mainly distinguished by detailed microscopic observation on their thecal plate arrangement (Kon *et al*., 2013).

The morphological aspects used when distinguishing the *Alexandrium* species as mentioned by Balech, (1995) includes the shape of the pore plate (Po), the anterior (Sa) and posterior sulcal plates (Sp), the first apical plate (1'), the sixth precingular plate (6''), the presence of ventral pore, the connection between the Po and 1' plate, and the ability to form chains. As more species have surfaced, it has become exponentially harder for species to be distinguished solely based on their morphological characteristics. Having said that, molecular phylogeny analysis can be done by using the gene markers such as large subunit (LSU) and internal transcribed spacers (ITS) with the help from programs such as Bioedit and ClustalX.

In Malaysia, the first-ever reported case of HAB was in 1970 in Sabah, in which almost 396 people were affected (Syakira *et al*., 2019). The first fish kills were reported in 2002 in Kota Kinabalu, Sabah (Usup *et al*, 2002). This has propelled researchers to conduct more studies and research on the PSP-toxin producing species which includes the species from the genus *Alexandrium*. Kon *et al*., (2013) was the first report of *A.tamutum* in Malaysian waters from Sabah in 2013.

The study of the *Alexandrium* species is scattered in Malaysian waters and the *A.tamutum* species in comparison to other blooming species is not well studied on. Thus, this study is conducted mainly to study the morphology and molecular phylogeny of *A.tamutum* and to provide a clearer differentiation between the related species. Furthermore, the distribution of *A.tamutum* was documented.

The objectives of this study are :

- To analyze the morphological characteristics of *A. tamutum*.
- To study the phylogeny relationship of *A. tamutum* based on LSU phylogeny.
- To document presence of *A. tamutum* in Sarawak waters.

2.0 Literature Review

2.1 Genus of *Alexandrium*

The genus *Alexandrium* was originally classified into a different genus known as *Gonyaulax* in 1911 by Kofoid by using the Kofoid system that enables the identification of dinoflagellate by size as well as their plate morphology (Taylor,1999). According to AlgaeBase (2021), there are currently 33 species that are accepted taxonomically. Many of the *Alexandrium* species are known to be toxic as they produce paralytic shellfish poisoning (PSP) toxins. Some of the species that are known to be toxic are *A.ostenfeldii, A.minitum*, *A.catenella* and *A.monilatum* (Kremp *et al*., 2009 ; Delgado *et al*., 1990; Mackenzie and Lincoln, 2014; Ray and Aldrich 1967). The high concentration of *Alexandrium* can lead to the formation of harmful algae bloom or also known as the red tide which has an adverse effect on humans, fish, and shellfish.

As mentioned by Amandine *et al*., (2018), not all the *Alexandrium* species produce the same toxin as toxin profile varies between species and strains which can also be influenced by abiotic conditions such as temperature, salinity, and CO_2 concentration which is apparent in *A.catenella*. This marine dinoflagellate is globally distributed and can be found mainly in coastal waters. Additionally, they can survive unfavorable living conditions as cysts which can act as a seed for bloom initiation which results in the formation of blooms that occur seasonally (Joyce & Pitcher, 2006).

2.2 Morphological characterization of *Alexandrium tamutum*

Alexandrium tamutum is a small species that resembles *Alexandrium minitum* which is a toxin-producing dinoflagellate. They both share similarities in the size, shape, and morphology of their cyst. Hence, the difference between the two species can be made by

detailed observation on the thecal plate using a Scanning Electron Microscope (SEM). (Montrsor *et al*., 2004)

Species identification of the *Alexandrium* species by morphological observation is difficult as the differences are subtle which includes the shape of the pore plate (Po), the anterior (Sa) and posterior sulcal plates (Sp), the first apical plate (1'), the sixth precingular plate (6''), presence or absence of a ventral pore, the connection between the Po and 1' plates, and the ability to form chains (Balech, 1995). However as debated by Anderson *et al*., (1994); Kim *et al*., (2002); Hansen *et al*., (2003) the existence of ventral pore is arguably not a suitable key characteristic to be used for species identification.

The cells of *A.tamutum* are generally known to be round in shape and small in size similar to *A.minutum*. The shape is described to be oval or round. The epitheca is dome-shaped meanwhile the hypotheca is hemispherical and is slightly tilted. The thecal plates are thin, smooth with small pores (Gu *et al*., 2013). The posterior sulcal plate (Sp) on the hypotheca is short and wide and is roughly rectangular in shape. The nucleus is located in the middle of the cell and resembles a kidney (Kon *et al*., 2013). The ventral pore is present and is located toward the anterior of the first apical plate. The sixth precingular plate is wider than long and is adjacent to the first plate. The presence of brown chlorophyll pigment indicates the photosynthetic nature of the cell. The plate formula (Figure 1) is Po, 4', 0a, 6", 6C, 9S, 5"', 2"" (Montresor *et al*., 2004).

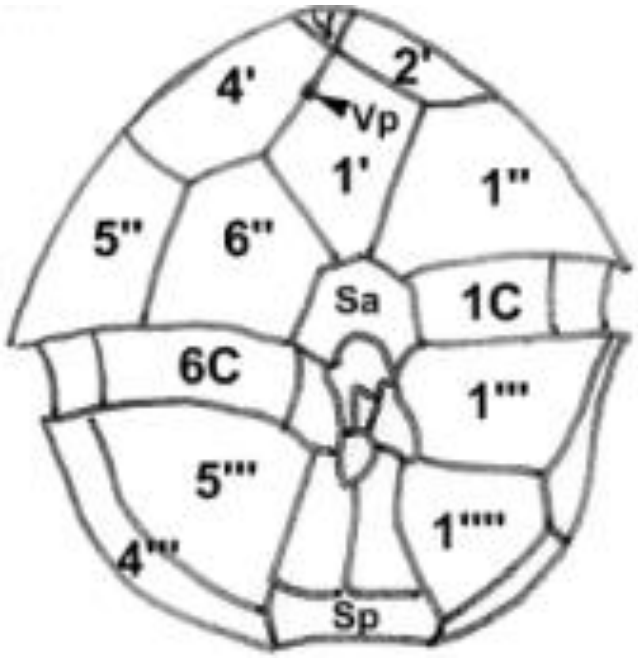

Figure 1 : *Alexandrium tamutum*.Ventral view of thecal plate patterns (Montresor *et al*., 2004)

2.3 Molecular characterization of *Alexandrium tamutum*

Based on previous phylogenetic studies on the *Alexandrium* genus using 18S rDNA (Scholin 1993), the D1/D2 region of 28S rDNA (Scholin *et al*. 1994; Medlin *et al.* 1998; Higman *et al*., 2001) and ITS sequences (Adachi *et al*., 1996) it is easy to amplify by PCR despite the low quantity of DNA and has a high degree of variation even between closely related species. The study done by Baggesen *et al.,* (2012) shows Bayesian Inference (BI) being used to establish the phylogenetic relationship on the genus *Alexandrium*. That being said, BI is beneficial when analyzing a relatively large quantity of data in a short period of time, uses a likelihood function and an efficient search strategy, and can be accounted for uncertainty in parameter estimates by marginalizing over parameters that allow for a more stable phylogenetic analysis (Soltis and Soltis, 2003).

In a study where the SSU, ITS region and LSU rDNA sequences were analyzed, the genus *Alexandrium* is identified to be not monophyletic (Li *et al*., 2019) . *A.tamutum* is closely related to *A. affine, A. andersoni A. catenella, A. minutum, A. ostenfeldii, A.*

peruvianum A. tamarense, and A. tamiyavanichii. Although there are major morphological similarities between *A.tamutum* and *A. minitum* results from the phylogenetic inferences states that both form a different clade and are a sister taxon. (Kon *et al*., 2013).

2.4 Distribution of *Alexandrium* in Southeast Asia

In Southeast Asia, there has been a high diversity of the genus *Alexandrium* identified throughout the years due to the overgrowing cases of harmful algae blooms (HAB) relating to the *Alexandrium* genus that has sparked an interest for researches (Yñiguez *et al*., 2020). In Malaysia as reported by Lim *et al*., (2005); Kon *et al*., (2013) and Usup *et al*., (2002) there are 8 species recorded including *A.taylori, A.peruvianum, A.tamutum* and *A.minutum* which has caused the red tide in Sungai Geting, Kelantan recently (TheStar, 2021). *A.tamutum* was first recorded in Malaysia in the coastal waters of Sarawak which has aided in providing further information for monitoring purposes in our country (Hii *et al*., 2012).

In Korea, after the Paralytic Shellfish Poisoning (PSP) outbreak in Jinhae-Masan Bay 4 species of *Alexandrium* have been identified to be responsible which are *A.tamarense, A.catenella, A.inseutum, A.fundyense* (Kim *et al*., 2017). There have been a few species that have been associated to the Philippines and this includes *A. minutum, A. affine, A. leei, and A.minutum* which was reported in Manila Bay and Pangasinan.(Azanza and Benico, 2013).

2.5 Harmful Algal Bloom (HAB) reports in Malaysia

Malaysia is no exception when it comes to the countries that are affected by harmful algal blooms (HAB) event which is associated with shellfish toxicity. HABs events have seen an increase in frequency in our country with more species being associated with it (Lim *et al*., 2012). This reason for this escalation could be from the increased human activities in coastal areas that led to eutrophication. The expansion of our industrial sector due to urbanization

added on with commercial farming has ushered the release of nutrients into our water systems that ensuingly promotes algal bloom

HABs events can lead to huge losses to the aquaculture industry which is apparent in Penang where the total loss was estimated to be RM 20 million (Sin Chew Daily 2005). Humans were not an exception as reported by Usup *et al*. 2012 there were hundreds of poisoning cases related to HABs. *Alexandrium minutum* was reported in Tumpat, Kelantan in 2001 which resulted in 1 casualty and 6 hospitalizations from PSP (Lim *et al*. 2004). *Alexandrium tamiyavanichii* bloom in Sebatu, Malacca in 1991 have caused a countrywide ban on the consumption of mussels (Usup *et al*. 2002). Recently, the same species has also caused the hospitalization of 10 individuals in Kuantan Port, Pahang in 2017. *Noctiluca scintillans* blooms reported from 2014-2015 in Tanjung Kupang, Johor has caused fish kills that have affected the fishing sector. (Lim *et al*. 2014; Teng *et al*. 2016). In Kota Kinabalu, Sabah red tides were reported after the bloom of *Pyrodinium bahamense*

3.0 Materials and Methods

3.1 Study site

Sampling was done in Telaga Air ,Sarawak, Malaysia (Figure 2). Plankton samples was collected with a 20µm mesh phytoplankton net. A Van Dorn water sample was used to collect surface water samples. A refractometer (Agato, Japan) was used to measure *in-situ* water characteristics such as salinity.. Meanwhile, a portable pH metre (Hanna Instrument, USA). was used to record temperature and pH values

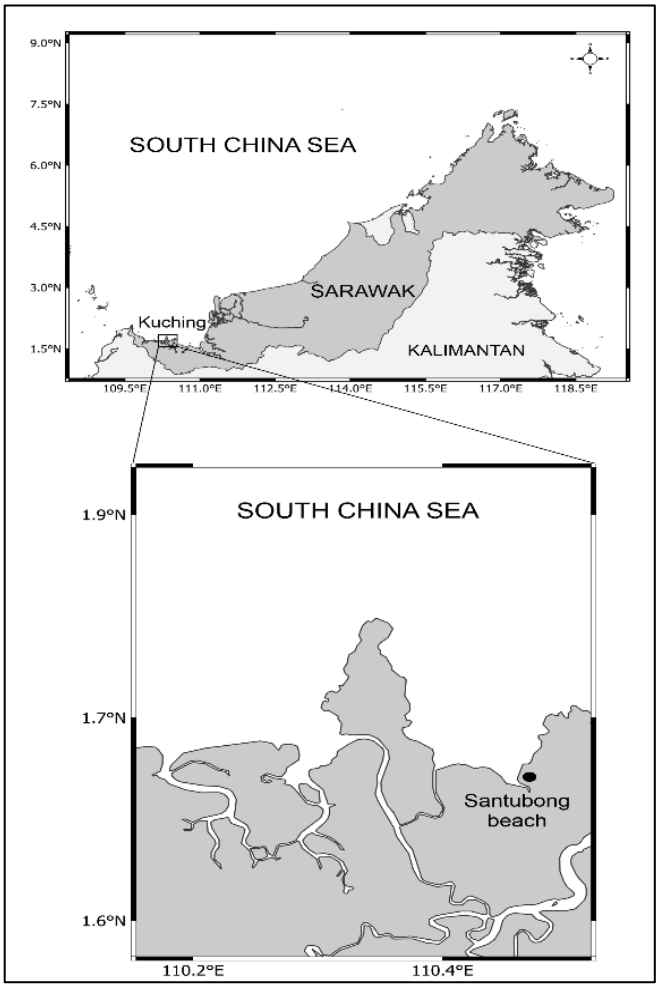

Figure 2: The map of Sarawak showing Santubong Beach, Sarawak

3.2 Algal culture

The cell was isolated by micro pipetting under an inverted light microscope to obtain clonal cultures. The samples was cultured and maintained in a L1 medium (Table 1) which is

silicate-free and prepared with natural seawater. The culture was placed in an incubator chamber and was provided with a light intensity of 50 µmol photons m^{-2} s^{-1} provided under a 12:12 h light-dark cycle.

Table 1 : L1 medium recipe (Guillard and Hargraves, 1993)

Elements	Volume to be aliquoted (mL)
$NaNO_3$	1.0
NaH_2PO_4	1.0
Li Trace Metal	1.0
f/2 vitamin solution	1.0
NH_4Cl	1.0

3.3 Microscopic analysis

20 µl of dense culture was added to the slide with seawater used as the medium base. The cultures was observed by using a compound light microscope, (Olympus, BX 51) and a Scanning Electron Microscope (SEM). The morphological characteristics such as cell diameter (width and length), cell shape, and theca plate tabulation were measured and tabulated.

3.4 Molecular Analysis

3.4.1 DNA extraction

The clonal cultures was harvested for DNA extraction during the mid-exponential growth phase. DNeasy Plant Mini Kit (Qiagen) was used for genomic DNA extraction. The 1ml sample was transferred into a 1.5ml microcentrifuge tube and centrifuged at 3,500 rpm for 20 minutes using centrifuge 5415R (Eppendorf). 400 µl buffer API and 4 µl RNase A was

added then vortexed using vortexer (vx 100, Labnet) and later, incubated at 65 °C for 10 min in a water bath (WiseBathR). 130 µl buffer P3 was added and mixed and later incubated in a mini cooler box with ice for 5 min. The lysate was centrifuged at 13, 200 rpm for 5 min then pipetted into a QIA shredder spin column placed in a 2 ml collection 10 tube and later be centrifuged at 13, 200 rpm for 2 min. The flow-through was transferred into a new microcentrifuge and added 1.5 volumes of buffer AW1 and mixed by pipetting. The 650 mL of mixture is transferred to a Dneasy mini spin column, which is placed in a 2 ml collection tube, and centrifuged at 8, 000 rpm for 1 minute, after which the flow-through is discarded, and the procedure is repeated with the remaining sample. The spin column was transferred to a fresh 2 ml collection tube, and 500 mL of AW2 buffer was added before centrifuging for 1 minute at 8,000 rpm.The flow-through was discarded and added with another 500 µl buffer AW2 and centrifuged at 13, 200 rpm for 2 min. The spin-column was transferred into a new 1.5 ml microcentrifuge and the DNA pellet was dissolved in 20 µl distilled water for elution and incubated at room temperature for 5 min and later centrifuged at 8, 000 rpm for 1 min. Lastly, the DNA pellet was stored overnight at 4 °C and at -20 °C for long-term storage.

3.4.2 PCR amplification

The D1-D3 region of the LSU rDNA was amplified using the D1R (5'-ACC CGC TGA ATT TAA GCA TA-3') and D3Ca (5'- ACGAAC GAT TTG CAC GTC AG-3') as forward and reverse primers (Scholin *et al*., 1994).

The amplification process was followed by PCR with the PCR mixture that was prepared according to Table 3. The PCR was perfomed by using the Mastercycler Eppendorf gradient thermocycler. The total cycle in the PCR reaction was 35 cycles and the thermal cycling steps that was applied in this study are specified in Table 2. The annealing

temperature was set to 55°C. Next, 1% agarose gels that are stained with ethidium bromide was used in the process of electrophoresis to check the amplification products under ultraviolet light (Lee *et al*., 2012). A DNA purification kit was used to purify the successful reactions. Lastly, the purified DNA products was sent to a sequencing service laboratory to proceed with sequencing both forward and reverse strands.

Table 2 : Thermal cycling steps

Steps	Temperature (°C)	Duration
Initial denaturation	94	5 mins
Denaturation	94	30s
Annealing	55	30s
Elongation	72	1 min
Final elongation	72	7 mins

Table 3 : Preparation of PCR mix

Reagents	Stock Concentration	Final Concentration	Volume (μL)
PCR buffer	10x	5x	5.0
$MgCL_2$	25mM	1.5mM	1.5
Forward primer	25 μM	1.5μM	1.0
Reverse primer	25μM	1.5μM	1.0
dNTPs	25μM	0.2mM	0.2
ddH_2O	-	-	15.1
DNA template	-	1-100 ng	1.0
Taq polymerase	5U μL-1	1U μL-1	0.2
Total volume			25.0

3.4.3 Sequence analysis, taxon sampling, and phylogeny analyses

The obtained raw nucleotide sequence data was analyzed and the reverse strands was reversed complemented and edited using Bioedit sequence Alignment Editor v7.2.6 6 (Hall, 1999). This was then be followed by aligning both forward and reverse sequence through CLUSTAL-X v2.1 (Thompson *et al*., 1997) and aligned manually.

The cleaned nucleotide sequence was analyzed through BLAST in conjunction with the National Center of Biotechnology Institute (NCBI) to acquire related sequences. All the LSU rDNA of *Alexandrium* that was obtained from NCBI Genbank undergo multiple-aligned through Multiple Sequence Comparison by Log-Expection, MUSCLE (Edgar, 2004). to enable the construction of a phylogeny tree. During the construction of the phylogeny tree, an outgroup was chosen for LSU rDNA.

Mr. Bayes v3.0 (Ronquist & Huelsenbeck, 2001) was used to perform Bayesian Inference (BI) with the best fitting model (GTR+R). PAUP *4b10 (Swofford, 2011) was used for the Maximum Likelihood (ML) algorithms to create an estimation of phylogeny.

4.0 Result

4.1 Algal Culture

In this study, the established clonal culture of *Alexandrium tamutum* (Strain AUSBT01) isolated from Santubong, Sarawak was donated by UNIMAS Phytoplankton Culture Collection (Laboratory of Aquatic Botany and IBEC Molecular). Cultures were sub-cultured upon reaching the mid-exponential growth phase within two weeks of the incubation period and maintained in a sterilized test tube containing an L1 medium.

4.2 Morphological Analysis

Cell culture from one strain (AUSBT01) was examined in detail for morphological characteristics. The main plate shapes observed in this study were the anterior sulcal plate (Sa), ventral pore (vp), first antapical plate (1’), sixth precingular plate (6’’), apical pore complex (APC), and the posterior sulcal plate (Sp). AUSBT01 consisted of the typical plate formula of Alexandrium which is Po, 4’ , 0a, 6’’, 6C, 9S, 5’’’, 2’’’’.

***Alexandrium tamutum* strain AUSBT01**

The shape is round and elliptical (Figure 3A). A horseshoe-shaped nucleus is positioned in the center of the cell and comprises of multiple brownish chloroplasts (Figure 3, A-C).Cells appear to be clustered in some observations (Figure 3D). The anterior sulcal plate (Sa) is twice as long as it is wide and contacts plate 1' on the backside (Figure 4A). Sa has a straight left margin that is in contact with plate 1C, while the right margin is in contact with the 6" (Figure 4A). The apical pore plate (P0) is directly related to the first apical plate (1'), which is irregularly rhomboidal (Figure 4D). A tiny ventral pore is present on the right border of the first apical plate, toward the anterior (Figure 4, A, D, and G).The APC or foramen is connected to the 1’ and is bordered by margins the marginal pores are present on the pore plate (Figure 4I). The sulcal area has Sa, sulcal anterior plate; Sma, sulcal median anterior plate; Smp, sulcal median posterior plate; Sda, sulcal right anterior plate; Ssa, sulcal left anterior plate; Sdp, sulcal right posterior plate; Ssp, sulcal left posterior plate; Sp, sulcal posterior plate (Figure 4B). The posterior sulcal plate (Sp) is essentially rectangular in shape, with a longer width than a longer length. (Figure 4E).The thecal plates appears to be smooth with the presence of small pores . Plate 2’’’’ is large and has a pentagonal shape (Figure 4F)

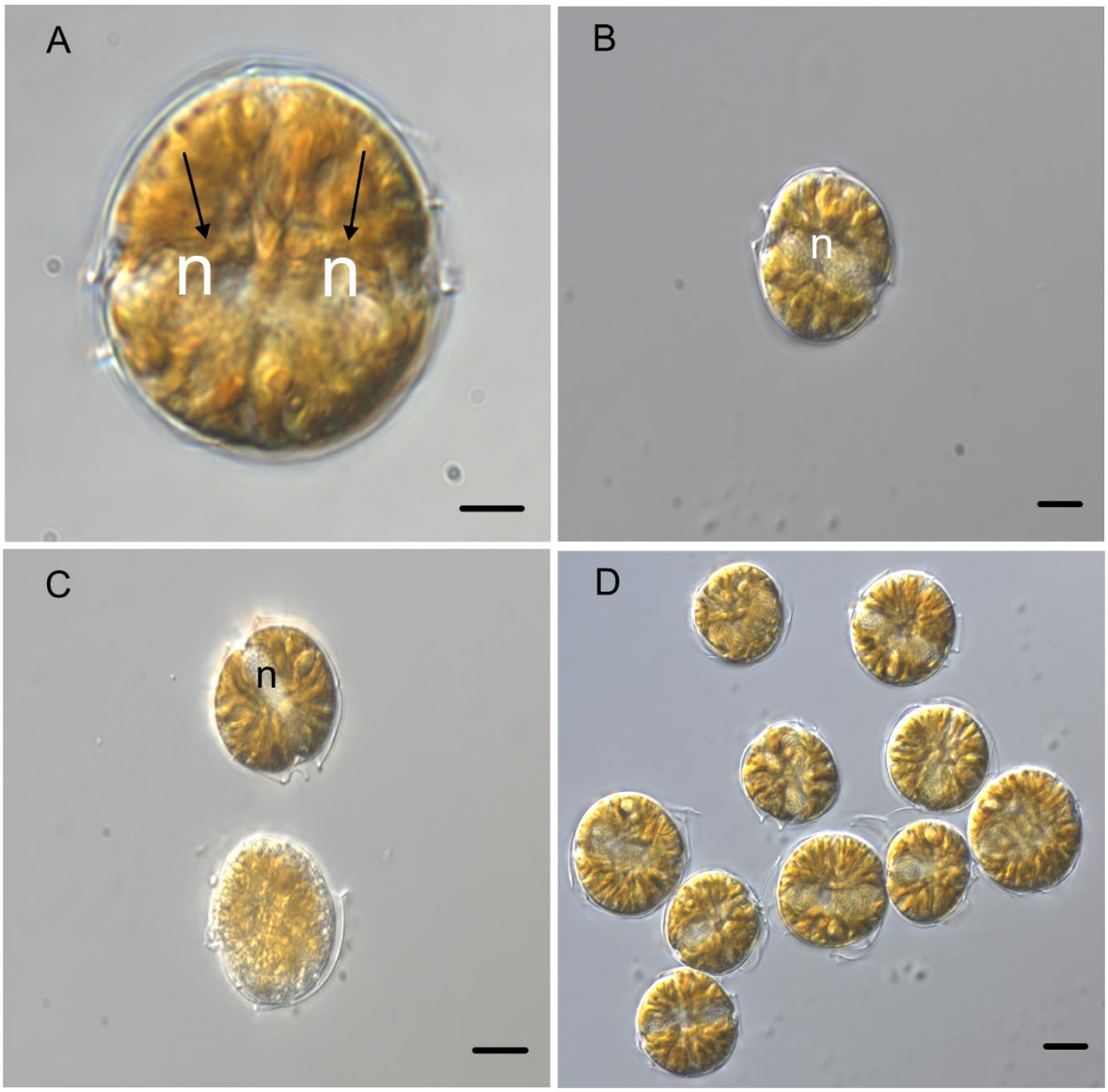

Figure 3. *Alexandrium tamutum* sp. Light micrographs of vegetative cells. (A) Micrograph of a cell in ventral view (strain AUSBT01) the two ventral arms of the horseshoe-shaped nucleus (*n*) are shown by an arrow. (B-C) Dorsal view showing the path of the nuclues (*n*). (D)Shows the cell in a cluster.Scale bars, 10μm.