



# The Genus *Alexandrium* (Dinophyceae, Dinophyta) in Brazilian Coastal Waters

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A review of the dinoflagellate genus *Alexandrium* occurring in Brazilian coastal waters is presented based on both published information and new data. Seven *Alexandrium* species have been recorded from Brazil so far: *Alexandrium catenella*, *Alexandrium fraterculus*, *Alexandrium gaardnerae*, *Alexandrium kutnerae*, *Alexandrium tamiyavanichi*, *Alexandrium tamutum*, and *Alexandrium* sp. While *A. gaardnerae* and *A. kutnerae* were identified based only on morphological characteristics, phylogenetic analysis (ITS and LSU rDNA) were performed for the remaining species based on cultures and/or field populations. Monoclonal cultures of the analyzed species were isolated from field samples obtained from Bahia (*A. tamiyavanichi*, two strains), Rio de Janeiro (*A. tamutum*, three strains; *Alexandrium* sp., two strains), Santa Catarina (*A. fraterculus*, one strain), and Rio Grande do Sul (*Alexandrium tamarense*, three strains). This is the first record of *A. tamutum* for the South Atlantic. In addition, molecular data for Brazilian strains of *A. fraterculus* are presented for the first time, as well as sequences from the ITS region for *A. catenella* (previously reported as *A. tamarense*) from Brazilian coastal waters. The morphological characters of the three species corresponded to those typically recorded in the literature and their identification was confirmed by molecular analysis. Based on the LSU rDNA and ITS regions, the three strains of *A. catenella* showed a high degree of similarity with strains from Southern Chile and North America. The implications and limitations of these findings for the monitoring protocols within the global and regional context are discussed.

**Keywords:** *Alexandrium catenella* (group I), *Alexandrium tamutum*, phenotypic plasticity, taxonomy, phylogeny, paralytic shellfish poisoning

## INTRODUCTION

The dinoflagellate genus *Alexandrium* Halim currently encompasses more than 30 species (Anderson et al., 2012), some of them known worldwide as the causative agents of blooms and/or production of neurotoxins associated to the Paralytic Shellfish Poisoning (PSP) syndrome (Wang, 2008; Etheridge, 2010). Recognized as the most geographically widespread algal-related shellfish poisoning syndrome, PSP constitutes a serious human illness caused by the ingestion of seafood contaminated with saxitoxin (STXs) and its derivatives (Deeds et al., 2008; Anderson et al., 2012). PSP symptoms include spreading numbness and tingling sensations, headache and nausea, and in more severe cases may cause paralysis of the muscles of the chest and abdomen leading to death

(Etheridge, 2010). Besides the evident relevance to human health, accumulation of PSP toxins in filter-feeding bivalves constitutes an additional issue for the shellfish industry and fisheries activities (Etheridge, 2010; Rhodes and Munday, 2016).

Many factors have been suggested as the cause of the observed increase in the worldwide extension and frequency of *Alexandrium* blooms and other Harmful Algal Bloom (HAB) species, such as ocean currents, climate change, pollution, ballast water dispersal, and colonization of newly generated niches (Nagai et al., 2007; Matsuyama et al., 2010; Anderson et al., 2012, 2015; Anderson, 2017; Gobler et al., 2017). It has also been suggested that the higher number of blooms reported nowadays is a direct result of increased monitoring activities and the implementation of more sensitive tools to detect and prevent the negative effects of HABs worldwide, particularly in aquaculture areas (Anderson, 2007; Anderson et al., 2015). Both toxic and non-toxic blooms of *Alexandrium* species have been reported from distinct geographic regions, e.g., Japan (Kodama, 2010), Mediterranean Sea (Karydis and Kitsiou, 2012), China (Lu et al., 2014), Argentina Sea (Fabro et al., 2017), Australia (Ajani et al., 2017), and the Bering Sea (Natsuike et al., 2017).

In Brazil, records of HABs related to human intoxications in coastal areas increased in the last 20 years, becoming a national issue with socio-economic repercussions (Silva et al., 2013). Particularly, reports of *Alexandrium* species in the Brazilian coast have been intensifying (Figure 1). To date, five species were previously identified based on light microscopy: *Alexandrium gardnerae* Nguyen-Ngoc & Larsen [= *Alexandrium concavum* (Gaarder) Balech], *Alexandrium fraterculus* (Balech) Balech, *Alexandrium kutnerae* (Balech) Balech, *Alexandrium catenella* (Whedon & Kofoid) Balech [= *Alexandrium tamarensis* (Lebour) Balech], and *Alexandrium tamiyavanichi* Balech (Balech, 1995; Persich et al., 2006; Menezes et al., 2010). STX production has been confirmed for isolates of *A. catenella* (Persich et al., 2006) and *A. tamiyavanichi* (Menezes et al., 2010). A toxic bloom of *Alexandrium* sp. (previously identified as *Alexandrium minutum* Halim by Menezes et al., 2007) and a non-toxic bloom of *Alexandrium fraterculus* were recorded, respectively, in beaches at Rio de Janeiro (Menezes et al., 2007) and Santa Catarina (Omachi et al., 2007). Cysts of *A. catenella* and *A. cf. minutum* were additionally detected in sediments from Patos lagoon and Sepetiba bay, respectively (Persich and Garcia, 2003; Juliano and Garcia, 2006). Among these studies, only Persich et al. (2006) and Menezes et al. (2010) applied molecular techniques to study the phylogeny and biogeography of the identified species.

Here, we present a review of the dinoflagellate genus *Alexandrium* in Brazilian coastal waters, based both on available published information and new morphological and molecular data (28S rDNA e ITS) for *A. fraterculus*, *A. catenella*, *Alexandrium* sp., and *Alexandrium tamutum* Montresor, Beran & John (the latter being the first record for the South Atlantic).

## MATERIALS AND METHODS

This review was based on previously published information for *A. gardnerae* (Balech, 1995), *A. kutnerae* (Balech, 1995), *A.*



*tamiyavanichi* (Menezes et al., 2010), and *A. catenella* (formerly identified as *A. tamarensis* by Persich et al., 2006). New morphological and molecular data were obtained from the analysis of monoclonal strains of *A. tamutum* and *Alexandrium* sp. (same species identified as *A. minutum* by Menezes et al., 2007) isolated from Rio de Janeiro, and *A. fraterculus* isolated from Santa Catarina (Figure 1). Cells were grown using in K medium at 16 and 24 PSU (*A. tamutum*), K medium at 30 PSU (*Alexandrium* sp.), and F/2 medium at 34 PSU (*A. fraterculus*). Cultures were maintained at 20–23°C with photon flux density of 70–136  $\mu\text{E m}^{-2}\cdot\text{s}^{-1}$  and a 12:12-h light:dark photoperiod. We also performed the morphological characterization and ITS rDNA sequencing for three of the *A. catenella* strains (ATBR1A7, ATBR2E, ATBR3D) used by Persich et al. (2006), and obtained from the Woods Hole Oceanographic Institution. Finally, the morphology of *Alexandrium* sp. was further re-analyzed by scanning electron microscopy (SEM) using environmental Lugol fixed samples obtained from the bloom recorded in 2007 from Rio de Janeiro by Menezes et al. (2007). The precedence of all strains/samples used here is informed in Table 1.

## Morphological Analysis

The tabulation of the species was characterized by the addition of calcofluor MR2 (10 ng ml<sup>-1</sup>) under an Olympus BX51 epifluorescence microscope (Fritz and Triemer, 1985). For SEM, cells were harvested by filtration on 0.45  $\mu\text{m}$  pore acetate membranes (Millipore). Filters were immediately fixed for 2 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH

**TABLE 1** | Records of *Alexandrium* species in Brazilian coast.

Species	Years	Location	Coordinates	Source	Strains	References
<i>Alexandrium catenella</i>	1997 1999	Patos lagoon estuary mouth (Rio Grande do Sul)	32°81'00'' to 32°82'20''S; 51°85'10'' to 51°85'50''W	Cysts in sediment samples	ATBRA ATBR2B ATBR2C ATBR2D <b>ATBR2E</b> ATBR2F ATBR2G ATBR3A ATBR3C <b>ATBR3D</b> ATBR1A7 <b>ATBR1A10</b> ATBRC6	Persich et al., 2006
<i>Alexandrium catenella</i>		Cassino beach (Rio Grande do Sul)	32°14'56''S 52°9'36''W	Bloom sample ( $2 \times 10^5$ cells L <sup>-1</sup> )		Odebrecht et al., 1997
<i>Alexandrium fraterculus</i>	2010	Alegre beach (Santa Catarina)	26°46'21''S, 48°39'31''W	Plankton samples	<b>AF#53</b>	This study
<i>Alexandrium fraterculus</i>	2004	Itajaí (Santa Catarina)	26°57'03''S, 48°45'44''W	Bloom sample ( $8.8 \times 10^4$ cells L <sup>-1</sup> )		Omachi et al., 2007
<i>Alexandrium gaarderae</i>	Unknown	Rio Grande do Sul	Unknown	Plankton sample		Balech, 1995
<i>Alexandrium kutnerae</i>	Unknown	Rio Grande do Sul	Unknown	Plankton sample		Balech, 1995
<i>Alexandrium tamiyavanichi</i>	2004 2006	Porto Seguro inner coastal platform	16°36'S, 39°05'W	Plankton samples	A1PSA B2PSA PSII	Menezes et al., 2010
<i>Alexandrium tamutum</i>	2013 2014	Guanabara bay, (Rio de Janeiro)	22°81'16''S 43°15'45''W	Plankton samples ( $1 \times 10^6$ cells L <sup>-1</sup> )	<b>UFRJ-MN03</b> <b>UFRJ-MN04</b> <b>RJ_BG</b>	This study
<i>Alexandrium</i> sp.	2007	Leblon beach (Rio de Janeiro)	22°54'14''S 43°13'16''W	Bloom samples ( $4.3 \times 10^5$ L <sup>-1</sup> )		Menezes et al., 2007
<i>Alexandrium</i> sp.	2014	Guanabara bay (Rio de Janeiro)	22°81'16''S 43°15'45''W	Plankton samples ( $6.7 \times 10^5$ cells L <sup>-1</sup> )	<b>UFRJ-MN01</b> <b>UFRJ-MN02</b>	This study

Strains analyzed in this study are depicted in bold.

7.2). After that, they were washed three times (10 min) using the same buffer and postfixed in 1% osmium tetroxide in sodium cacodylate buffer (pH 7.2). Filters were finally dehydrated in an ascending ethanol series of 10 min each 35, 50, 70, 90, and 100%, followed by two washes for 10 min each in 100% ethanol, critical-point dried (CPD 020 Balzers Union). Dried filters were mounted on stubs, coated with gold (Balzers/Union FL-9496), and viewed with a JEOL 5310 scanning electron microscope (Akishima, Tokyo, Japan). The metric data that represented outliers for minimal and maximal cell dimensions were shown in parentheses.

## PCR Amplification and DNA Sequencing

DNA from *A. tamutum* and *Alexandrium* sp. was extracted from exponentially growing cultures using NucleoSpin® Plant II extraction kit (Macherey-Nagel GmbH & Co., KG, Germany) following the manufacturer's protocol. The ITS (including 5.8S rDNA gene) and LSU (D1–D3) regions of rDNA were amplified using primers ITSA/ITSB (Sato et al., 2011) and D3F/D1R (Scholin et al., 1994; Litaker et al., 2003), respectively. The PCR mix (25 µl final volume) contained 1 U GoTaq® DNA

polymerase (Promega, Madison, WI, USA), 1X GoTaq® Flexi buffer (Promega), 1.25 mM MgCl<sub>2</sub> solution (Promega), 0.16 mM dNTPs (Thermo Scientific Inc., USA), 8 pmol of each primer, 0.2 µg of Bovine Serum Albumin (BSA) (New England Biolabs Inc.), and ~15 ng genomic DNA. The PCR protocol was as follows: initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 50 and 55°C (ITS and LSU rDNA, respectively), and 1 min extension at 72°C, plus a final extension of 5 min at 72°C. The PCR products were purified and sequenced by Macrogen (Seoul, Korea) in both directions using the PCR primers. The sequences obtained were deposited in GenBank (**Supplementary Tables S1, S2**).

DNA extraction from *A. fraterculus* strain was performed using a modified CTAB protocol (Lebret et al., 2012), whereas DNA extracts from fixed samples of *A. catenella* strains were obtained using a modified guanidinium isothiocyanate protocol (Alves-de-Souza et al., 2011). Both ITS and LSU (D1–D3) regions of rDNA were obtained for *A. fraterculus* whereas only ITS rDNA sequences were obtained for *A. catenella* using the same primer mentioned above. For both species, the PCR mix (15 mL final volume) contained 1–6 mL of the

DNA extract, 330 mM of each deoxynucleoside triphosphate (dNTP), 2.5 mM of MgCl<sub>2</sub>, 1.25 U of GoTaq1 DNA polymerase (Promega Corporation), 0.17 mM of both primers, 1X reaction buffer (Promega Corporation). PCR protocol included an initial denaturing step at 95°C for 5 min, 35 cycles at 95°C for 1 min, annealing at 55°C for 45 s, extension at 72°C for 1 min 15 s, with a final extension step at 72°C for 7 min. PCR products were purified using the ExoSAP-IT kit (USB) following the manufacturer's recommendations and directly sequenced on an ABI Prism 3100 automatic sequencer (Applied Biosystems).

Phylogenetic analyses were performed for each rDNA region (ITS and LSU) individually. *Alexandrium* sequences obtained from this study and from GenBank (Supplementary Tables S1, S2) were aligned using the online package MAFFT version 7 (<https://mafft.cbrc.jp/alignment/server/>) followed by manual editing. The Maximum Likelihood (ML) analyses were conducted using the software MEGA 7.0 (Kumar et al., 2016). The evolutionary models used were the Hasegawa-Kishino-Yano model (HKY) for ITS rDNA and the Tamura-Nei model (TN93) for LSU rDNA, both with gamma distribution. These models were the best available for the tree of the rDNA region data (ITS and LSU). All positions containing gaps and missing data were eliminated. The robustness of the inferred topology was tested by bootstrap resampling (1,000 replicates). Bayesian inferences (BI) were obtained with the software MrBayes 3.2 (Ronquist et al., 2012). Rather than selecting a nucleotide substitution model by *a priori* model selection, we used the "Iset nst = mixed" Markov Chain Monte Carlo procedure, consisting of two independent trials with four chains. The chains were run for 5,000,000 generations and sampled every 100th cycle. Posterior probability (PP) values for the resulting 50% majority rule consensus tree were estimated after discarding the first 25% of the trees as burn-in. All trees were rooted using *Alexandrium lee* and *Alexandrium diversaporum* sequences as outgroup.

## RESULTS

Based on both published information and the new data, seven *Alexandrium* species have been identified from Brazilian coastal waters so far (Figure 1, Table 1). Except for *A. catenella* and *A. fraterculus*, which were reported for different locations in Rio Grande do Sul and Santa Catarina (southern Brazil), the other five species were recorded only once for the northeast (*A. tamiyavanichi*), southeast (*Alexandrium* sp., *A. tamutum*), and south (*Alexandrium gaardnerae* and *A. kutnerae*) parts of the country. This is the first report of *A. tamutum* for the South Atlantic.

### Morphology

#### *Alexandrium catenella* (Figures 2A,B)

Cells isodiametric (40–50 μm long, 30–50 μm wide). The first apical plate (1') was irregularly rhomboidal connected direct or indirectly to the apical pore (Po). The third apical plate (3') was asymmetrical and the ventral pore (v.p.) was always located along the margin between plate 1' and plate 4'. The posterior sulcal plate (s.p.) was always pentagonal without a connecting pore.

The morphology of the three isolates agreed with the description reported by Balech (1995) for the morphospecies *A. tamarensis*. No morphological variability regarding presence/absence of the both v.p. and s.p. connecting pore. Although previously identified as *A. tamarensis* (Persich et al., 2006), these three isolates along with others from South and North America (*A. tamarensis* complex group I, sensu John et al., 2014) should be currently named as *A. catenella* (Prud'homme van Reine, 2017). Besides the three isolates analyzed in this study, Persich et al. (2006) analyzed the toxin content of ten more strains isolated from the same locality (Patos Lagoon estuary) (Table 1). These isolates showed toxicity from 7.07 to 65.92 pg STX eq cell<sup>-1</sup> and total toxin content ranged from 42 to 199 fmol cell<sup>-1</sup>. Toxin C1,2 was predominant in most of the strains, comprising up to 78.4% of the total toxins (Persich et al., 2006). More recently, high concentrations of PSP toxins (601.13 to 3,199.6 μg STX eq.kg<sup>-1</sup>) were detected in bivalves (*Perna perna* and *Crassostera gigas*) on October 2017 from several coastal locations in Santa Catarina (<http://www.cidasc.sc.gov.br/defesasaniaanimal/>). These PSP levels were associated with the presence of *A. tamarensis* morphospecies complex in the plankton (7,350–11,750 cells L<sup>-1</sup>) and lead to the closure of all mariculture farms in the area for ~2 months. Although several toxic *Alexandrium* species have been previously reported in Brazil, this is the first record of high PSP toxins in shellfish associated to a species of this genus.

#### *Alexandrium fraterculus* (Figures 2C–E)

Chains formed by of 2–8 isodiametric, pentagonal cells (30–40 μm long, 30–40 μm wide). The first apical plate (1') was directly attached to the Po. The ventral pore (v.p.) was located in the middle of the right margin, in the suture with the fourth apical plate (4'). The posterior sulcal plate (s.p.) was isodiametric, rhomboidal with a large connecting pore located in the center of the plate. The anterior sulcal plate (s.a.) was slightly longer than it was wide, with a deep posterior sinus.

No toxicity was found in the analyzed strain (L.A.O. Proença, personal communication).

#### *Alexandrium gaardnerae*

This species was reported by Balech (1995) based on environmental samples from the Brazilian coast as *A. concavum* (locality not informed), along with a description but no illustrations. Cells were 50–81 μm long, 45–64.5 μm wide. The first apical plate (1') was rhomboidal, connected indirectly or directly to the apical pore (Po). A ventral pore (v.p.) occurred enclosed within the right margin of 1'. The posterior sulcal plate (s.p.) was longer than wide, symmetrical.

#### *Alexandrium kutnerae* (Figure 2F)

This species was described by Balech (1995) as having rounded cells, 51–65 long, 41–57 wide. The first apical plate (1') is connected directly or indirectly to the apical pore (Po). A ventral pore (v.p.) occurs inside of the first apical plate (1'). The anterior sulcal plate (s.a.) had a quadrangular or hexagonal precingular part (p.pr.). The posterior sulcal plate (s.p.) was longer than wide, symmetrical, without an attachment pore.

### *Alexandrium tamiyavanichi* (Figures 2G–K)

This species was characterized by Menezes et al. (2010) as having small and rounded cells, sometimes slightly wider than long, 31–41  $\mu\text{m}$  long and 26–35  $\mu\text{m}$  width. A small ventral pore (v.p.) occurred within the right margin on the first apical plate (1'). The anterior sulcal plate (s.a.) showed a triangular precingular part (p.pr). The posterior sulcal plate (s.p.) was longer than wide, symmetrical, with an attachment pore.

Six PSP toxins have been identified for one *A. tamiyavanichi* strain (A1PSA): STX, Neo-STX, GTX4, GTX3, dcGTX2, and dcGTX3. STX was the main toxin (16.85 fmol. cell<sup>-1</sup>), accounting for 67.06% of the overall cell toxin content (Menezes et al., 2010).

### *Alexandrium tamutum* (Figures 3A–J)

Cells were ovate to spherical, 20.1–35.0  $\mu\text{m}$  long, and 19.2–33.6  $\mu\text{m}$  wide. The theca surface was smooth and very thin. The first apical plate (1') was rhomboidal with a direct connection to the APC and posterior margin straight. A ventral pore was present on the anterior right margin of the 1' plate. The sixth precingular (6'') plate was pentagonal, longer than wide or wider than long. Apical pore complex (APC) with comma-shaped. The anterior sulcal plate (s.a.) was longer than wide with anterior margin straight and posterior margin concave, often extending to the epitheca. The posterior sulcal (s.p.) plate was wider than long, asymmetrical, and without a posterior attachment pore.

The three analyzed strains presented cell morphology matching with the original description of the species (Montresor et al., 2004).

### *Alexandrium* sp. (Figure 2L)

Cells were usually solitary, sometimes forming short chains of up to four ovate to irregularly elliptical cells (17.1)19–31.2(32.3)  $\mu\text{m}$  long, (15.2)17.1–31.2(32.3)  $\mu\text{m}$  wide. The sixth precingular (6'') plate was generally pentagonal, longer than wide, sometimes as long as wide. The first apical plate (1') showed a clear ventral pore and connected directly or through a channel with the Po plate. Epitheca surface was smooth whereas hypotheca surface showed strong reticulation.

This species was at first identified as *A. minutum* by Menezes et al. (2007) based on samples obtained from a bloom in 2007 from the Rio de Janeiro coast. However, DNA sequencing (see Phylogenetic Analysis section) of strains recently isolated from Guanabara bay depicting the same unusual theca ornamentation pattern (epitheca smooth and hypotheca strongly reticulated) indicated that both populations belong to a new *Alexandrium* species, currently under description. Four analogous of STX have been found in the cells from the 2007's bloom: Neo-STX, STX, GTX4, and GTX2 (Menezes et al., 2007). Neo-STX was the dominant toxin, accounting to 83.47% of STX equivalents (Menezes et al., 2007).

## Phylogenetic Analyses

Maximum-likelihood (ML) and BI analyses based on ITS and LSU rDNA resulted in phylogenetic trees with similar topologies (Figures 4, 5). All phylogenies exhibited two main clades: (i) a clade composed of the *A. tamarensis* complex (e.g., *A. catenella*, *Alexandrium fundyense*, *Alexandrium*

*mediterraneum*, *A. tamarensis*, *Alexandrium australiense*, *Alexandrium pacificum*), *A. tamiyavanichi*, *A. fraterculus*, and *Alexandrium affine* sequences (ML = 86% and BI = 1.0 for ITS rDNA; ML = 100% and BI = 1.0 for LSU rDNA), and (ii) a clade composed of *Alexandrium insuetum*, *A. minutum*, *A. ostenfeldii*, *A. tamutum*, and *Alexandrium* sp. sequences (ML = 72% and BI = 0.98 for ITS rDNA; ML and BI < 0.5 for LSU rDNA).

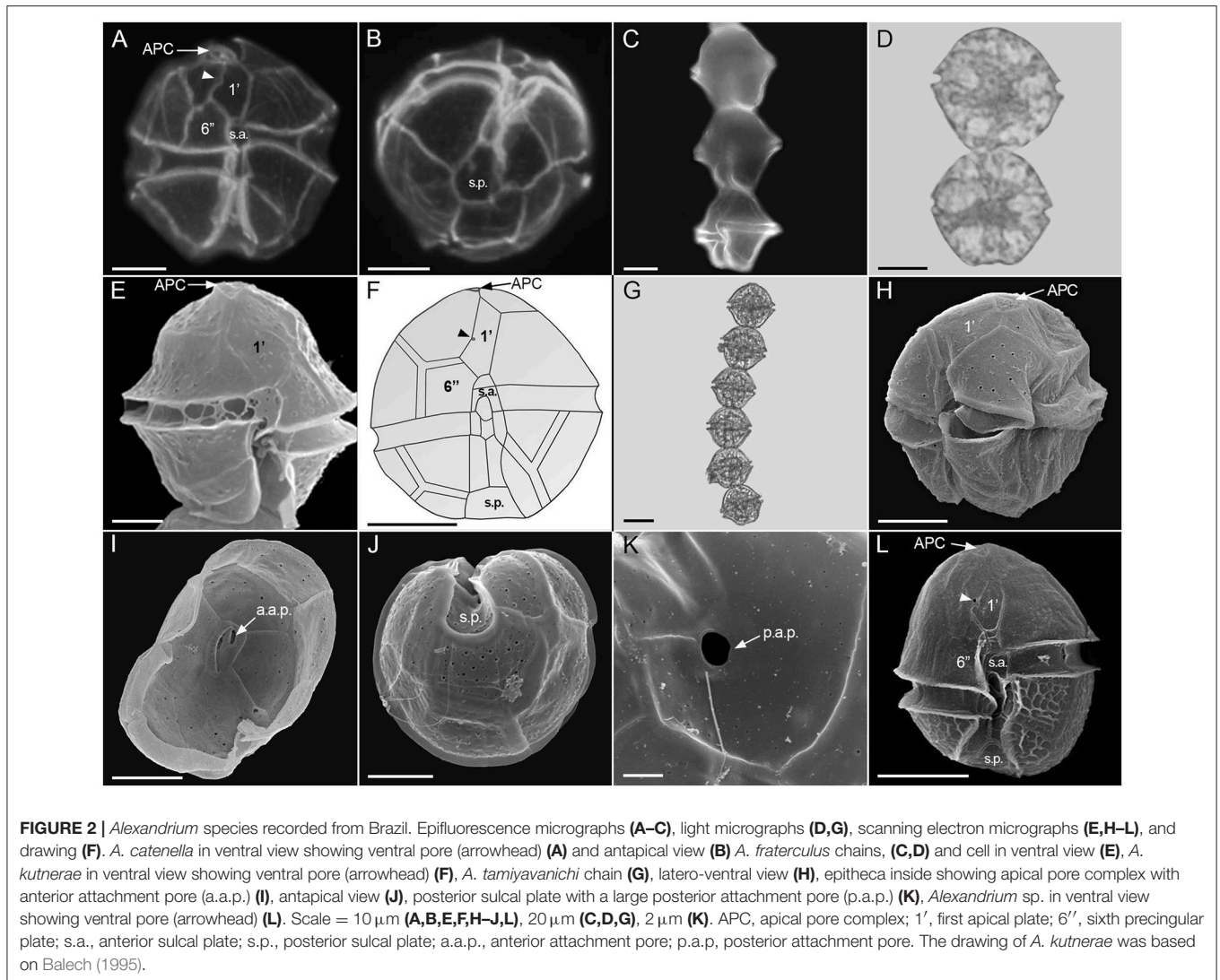
The *A. tamarensis* complex formed a large monophyletic group made up by sequences of *A. catenella*, *A. mediterraneum*, *A. tamarensis*, *A. australiense*, and *A. pacificum* (ML = 99% and BI = 1.0 for ITS; ML = 100% and BI = 1.0 for LSU rDNA). *Alexandrium catenella* sequences from Brazil grouped with sequences of this species from USA and Chile, North America (USA and Canada), and Japan (ML = 99% and BI = 1.0 for ITS rDNA; ML = 100% and BI = 1.0 for LSU rDNA). Two subclades were formed inside the *A. catenella* clade in trees based on ITS rDNA: a clade composed only by sequences from Chile (ML = 99% and BI = 1.0) and a clade composed with sequences from Canada, USA and Norway (ML = 98% and BI = 1.0). The latter subclade was also recognized inside the *A. catenella* clade formed in the trees based on LSU rDNA (ML = 100% and BI = 1.0).

*Alexandrium tamiyavanichi* formed a monophyletic clade consisting of two different subclades. The first subclade was formed only by sequences from Brazil (ML = 92% and BI = 0.96 for ITS rDNA; ML = 96% and BI = 0.89 for LSU rDNA) whereas the second subclade included sequences from Japan and Malaysia (ML = 99% and BI = 1.0 for ITS rDNA; ML = 96% and BI = 1.0 for LSU rDNA). *Alexandrium tropicale* formed a sister group of *A. tamiyavanichi* in the trees based on LSU rDNA (ML = 100% and BI = 1.0). The *A. fraterculus* clade grouped with the *A. tamiyavanichi* clade (ML = 75% and BI = 1.0 ITS rDNA trees; ML = 98% and BI = 1.0 for LSU rDNA) and included sequences from Brazil, New Zealand, Yellow Sea, Japan, and Korea (ML = 100% and BI = 1.0 for ITS and LSU rDNA).

*Alexandrium tamutum* sequences formed a monophyletic group (ML = 80% and BI = 0.84 for ITS; ML = 74% and BI = 0.9 for LSU). A subclade with a small branch composed of sequences from Italy was observed inside the *A. tamutum* clade in trees based on ITS rDNA (ML = 97% and BI = 1.0) whereas Brazilian strains grouped with sequences from Italy, Greenland, and United Kingdom (ML = 99% and BI = 0.83). *Alexandrium* sp. sequences formed a monophyletic group (ML = 99% and BI = 1.0 for ITS; ML = 100% and BI = 1.0 for LSU) clearly separated from other *Alexandrium* clades.

## DISCUSSION

*Alexandrium* species reported from Brazilian coastal waters so far have been identified based mostly on traditional morphological features, such as plate patterns, cell size and shape, presence and localization of the ventral pore, presence of a sulcal pore, and

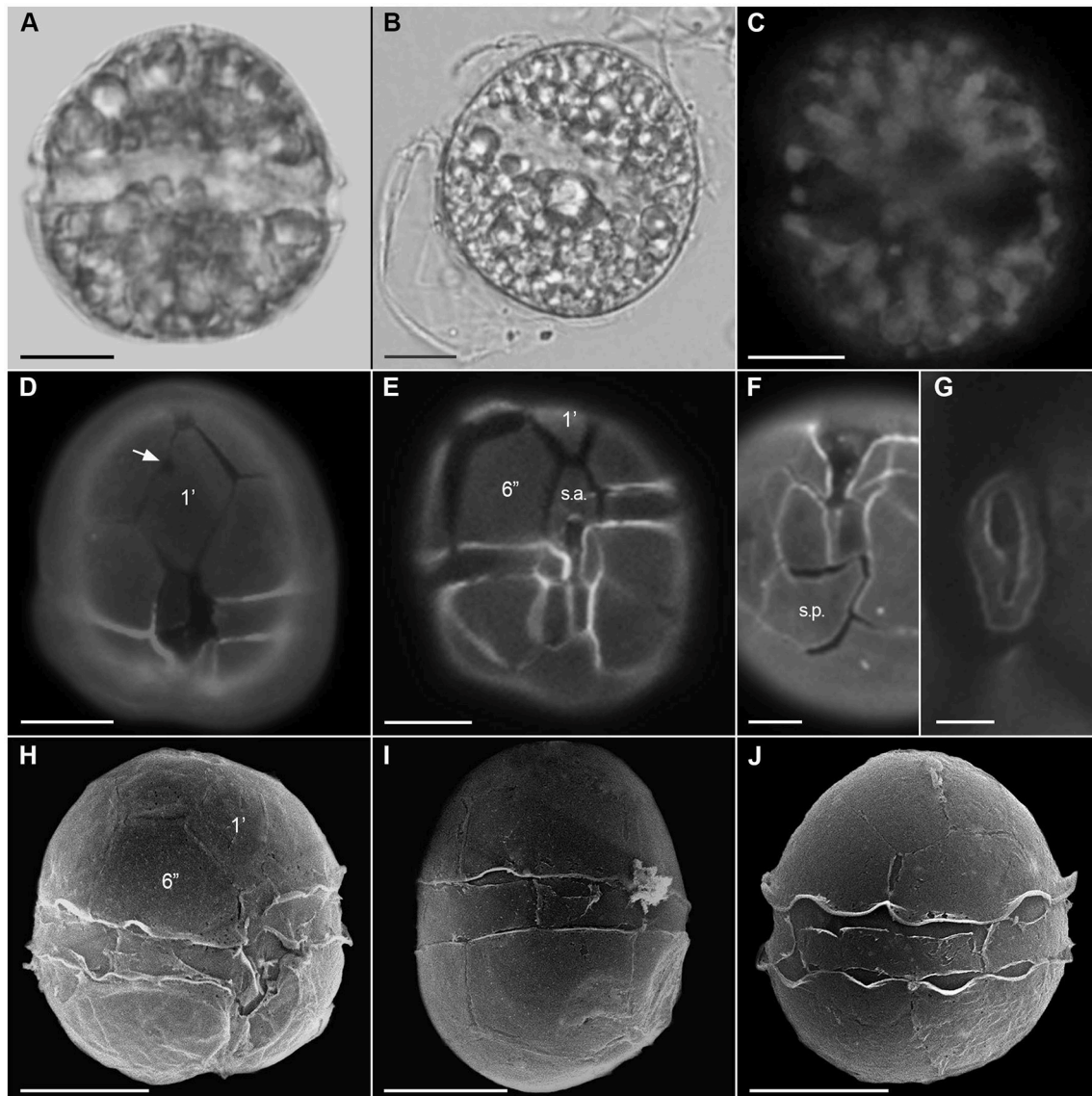


chain formation. These morphological traits, however, are widely variable often with overlapping or transitional forms between species and therefore are shown to be less robust for species discrimination (e.g. Lilly et al., 2005; Anderson et al., 2012).

*Alexandrium gaarderae* and *A. kutnerae* have been reported only once from Brazilian coastal waters (Balech, 1995). In addition to Brazil, *A. gaarderae* has been recorded from Argentina, Gulf of Mexico, the Caribbean Sea, and Vietnam (Balech, 1995; Larsen and Nguyen-Ngoc, 2004), whereas *A. kutnerae* has records for Argentina and Western Mediterranean (Balech, 1995; Bravo et al., 2006). While *A. gaarderae* has a distinctive cell shape characterized by a conical epitheca (Larsen and Nguyen-Ngoc, 2004), the general cell appearance of *A. kutnerae* (Figure 2F) reminds as observed in the *A. tamarensis* complex. The presence of a pre-cingular part in the sulcal plate (s.p.) in *A. kutnerae* is the main morphological characteristic separating this species from those of the *A. tamarensis* complex. However, it is not clear if this feature is discriminative enough to keep the former apart from representatives of the *A. tamarensis*

complex. As a matter of fact, phylogenetic analysis including ITS sequences of an isolate of *A. kutnerae* from the Catalan Sea (Spain) indicated this species belongs to the *A. pacificum* clade (Group IV, sensu John et al., 2014), with a high support value (Gu, 2011).

Taxonomic and phylogenetic reviews of the genus *Alexandrium* revealed several cryptic species in this genus, not only invalidating some of the described species in the literature but also precluding the application of a morphospecies concept for the species circumscriptions (Lilly et al., 2007; Miranda et al., 2012; John et al., 2014; Wang et al., 2014). An example of that was observed in the *A. tamarensis* complex (formed by the morphospecies *A. tamarensis*, *A. catenella*, and *A. fundyense*), where species were segregated into five genetic distinct clades, each one corresponding to a different cryptic species: *A. catenella* (Group I), *A. mediterraneum* (Group II), *A. tamarensis* (Group III), *A. pacificum* (Group IV), and *A. australiense* (Group V) (Lilly et al., 2007; Miranda et al., 2012; John et al., 2014; Wang et al., 2014).

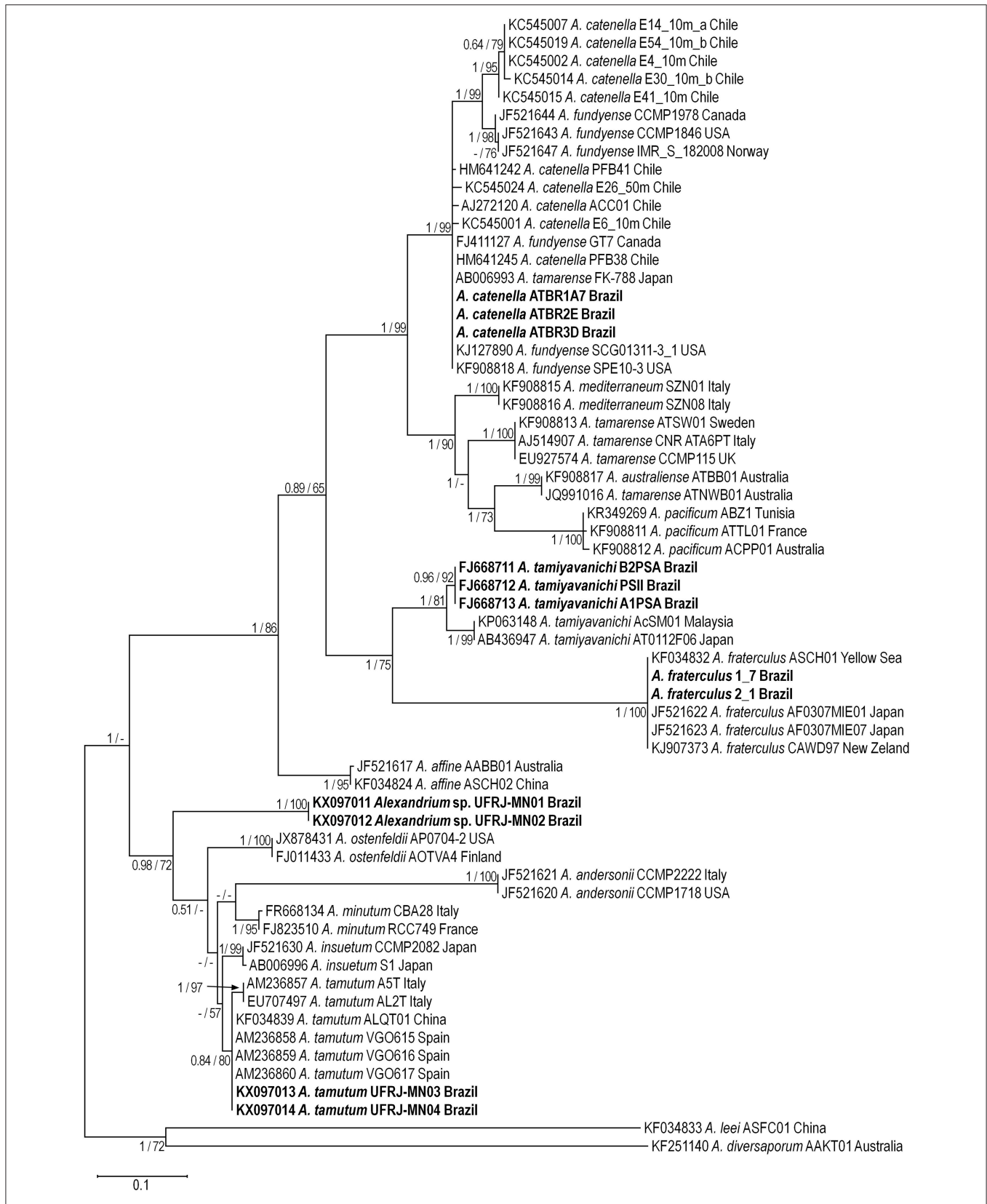


**FIGURE 3 |** *Alexandrium tamutum*. Light micrographs (A,B), epifluorescence micrographs (C–G), and scanning electron micrographs (H–J). Live cell (A), cyst (B), chloroplast autofluorescence (C), ventral view (D,E), showing the first apical plate (1') with ventral pore (arrow), sixth precingular plate (6''), and anterior sulcal plate (s.a.), antipical view showing posterior sulcal plate (s.p.) (F), apical pore complex (G), ventral view showing the first apical plate (1') and large sixth precingular plate (6''); lateral view (I), and dorsal view (J). Scale = 10  $\mu\text{m}$  (A–E,H–J), 5  $\mu\text{m}$  (F), 2  $\mu\text{m}$  (G).

In our morphological analysis, strains of *A. catenella* (Group I sensu John et al., 2014; formerly identified as the morphospecies *A. tamarensis*) presented cells always with a ventral pore, which is consistent with the *A. tamarensis* morphospecies description provided by Balech (1995). According to this author, the presence of a ventral pore in the morphospecies *A. tamarensis* constitutes a diacritic character separating this morphospecies from *A. catenella* and *A. fundyense* (the latter two morphospecies lacking this feature). Stability in the presence of a ventral pore has been observed in strains of the morphospecies *A. tamarensis* from Australia (MacKenzie et al., 2004), Europe (Thau Lagoon) (Genovesi et al., 2011), and from western Greenland (Tillmann

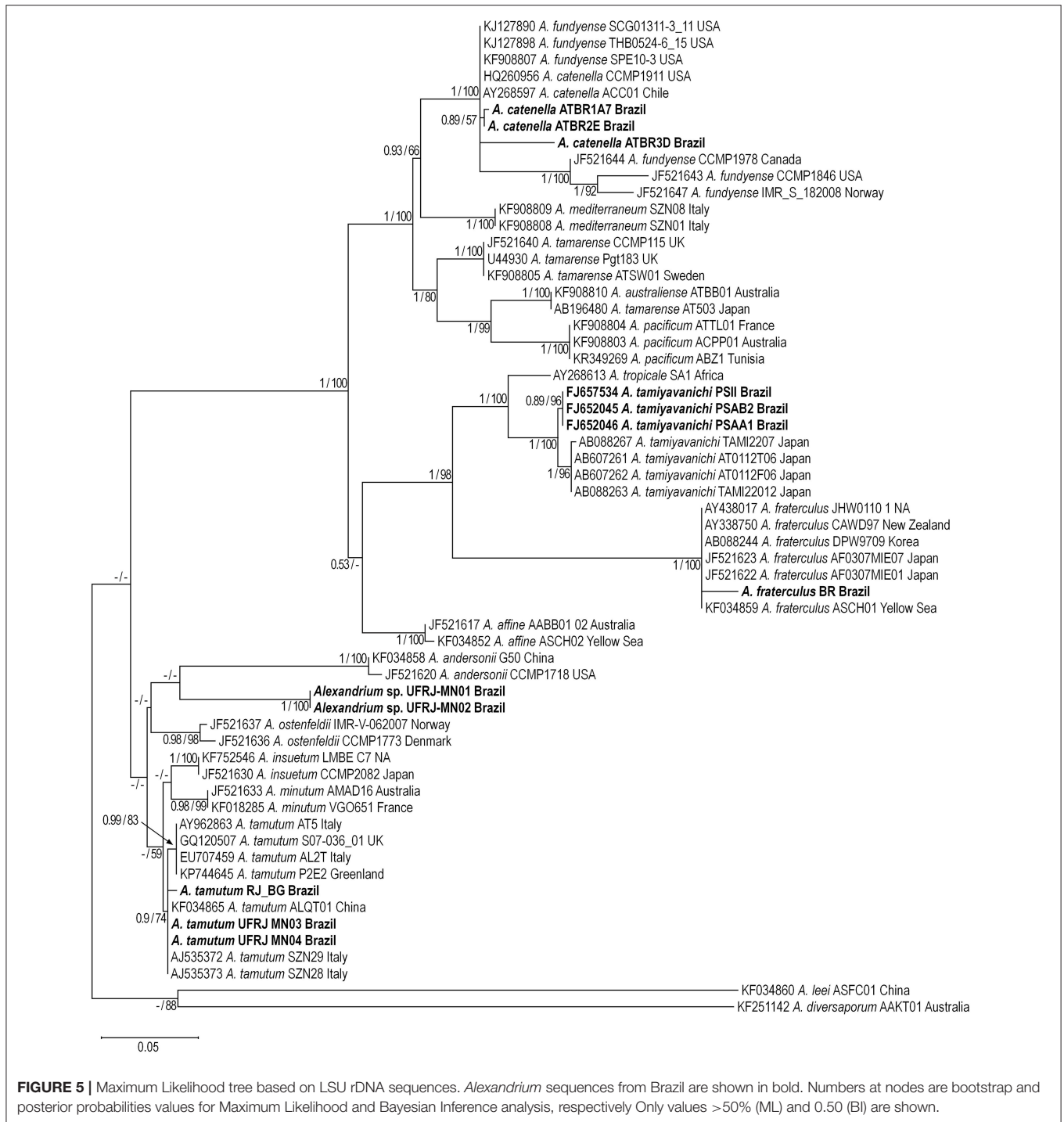
et al., 2016), the former one corresponding to Group IV (now *A. pacificum*) and the latter two to Group III (now *A. tamarensis*). In contrast, many studies based on wild populations and cultures have demonstrated intermediate morphology between *A. catenella*, *A. fundyense*, and *A. tamarensis* morphospecies, regarding the presence/absence of a ventral pore and/or chain formation (for more details see Orlova et al., 2007; Wang et al., 2008, 2014; John et al., 2014).

Phenotypic plasticity and genetic variability has been reported for *A. catenella* strains (Group I) from Chile, Mediterranean Sea, and Japan (Penna et al., 2005; Cruzat et al., 2018). Alpermann et al. (2009) reported phenotypic and genetic variability in *A.*



**FIGURE 4 |** Maximum Likelihood tree based on ITS rDNA sequences. *Alexandrium* sequences from Brazil are shown in bold. Numbers at nodes are bootstrap and posterior probabilities values for Maximum Likelihood and Bayesian Inference analysis, respectively. Only values >50% (ML) and 0.50 (BI) are shown.





*tamarensis* isolates (Group III) from the North Sea coast of Scotland that were interpreted, respectively, as having strong potential for adaptability of the population and evidence for a lack of strong selective pressure on respective phenotypic traits at the time the population was sampled. Logares et al. (2007, 2009) reported almost identical DNA sequences for the dinoflagellate morphospecies *Peridinium aciculiferum* and *Scropsiella hangoei*,

which are well-differentiated based on morphology (Logares et al., 2007, 2009). Similar results were reported for *P. aciculiferum*, *Peridinium euryceps*, and *Peridinium baicalense* by Annenkova et al. (2015). These authors hypothesized that identical sequences do not necessarily mean the same microbial species, and these taxa are not a case of phenotypic plasticity but originated via recent rapid diversification (radiation) into several

species followed by dispersion to environments with different ecological conditions (Logares et al., 2007, 2009; Annenkova et al., 2015).

Phenotypic plasticity is a single genotype's ability to produce variable phenotypes in response to environmental conditions thereby enabling it to buy additional time for adaptation to occur (Sunday et al., 2014) and provide a mechanism for adaptation to occur rapidly (Pigliucci et al., 2006; Ghalambor et al., 2015). Although it is recognized that phenotypic plasticity can increase organism survival under specific conditions, there is no consensus on its effects on the organisms: whether they drive the evolution of novel traits and promote taxonomic diversity, or whether they act more on acceleration or evolutionary changes (Pfennig and Pfennig, 2010). Little is known about the extent of the phenotypic plasticity in *Alexandrium*. Studies on the phenotypic plasticity on this genus should be addressed aiming to respond to questions such as: Could this phenotypic plasticity be a pre-adaptation or genetic shift after an invasive process of this genus to different localities? Could the acclimatization process cause definitive change in morphology?

The phylogenetic analysis of ITS and LSU rDNA sequences corroborated that the isolates of *A. catenella* from southern Brazil belong to the clade formed by *A. catenella* (Group I, sensu John et al., 2014), along with other isolates from Chile, Japan, Norway and North America, as already pointed out by Persich et al. (2006). The phylogeny resulted from ITS rDNA sequences also confirmed the existence of genetic diversity within this clade (Cruzat et al., 2018) with a subclade formed by some isolates from Chile and a second subclade formed by isolates from the United States, Canada and Norway. The presence of the Group I in South America has been hypothesized as being a result of ballast water introduction (invasive species) or natural dispersion (see Lilly et al., 2007).

According to John et al. (2003), the ancestral population of *Alexandrium* was globally distributed, diverging into the ancestors of Groups I and II that occurred in waters off North America and in the tropical Atlantic. Posteriorly, Group II evolved in the tropical Atlantic, becoming distinct from the Group I populations and later colonizing the Mediterranean (John et al., 2003). There is evidence that Group I populations were transported to the west coast of South America by a natural dispersal mechanism during a period in which the weather was substantially cooler than nowadays, e.g., as in one of the recent ice ages (Persich et al., 2006; Lilly et al., 2007). However, the presence of Group I on the east Atlantic coast of South America is hypothesized to have resulted recently from Chilean populations, given the similarity of the LSU rDNA sequences (D1–D2) among the isolates from both sites (Persich et al., 2006; Lilly et al., 2007), as corroborated by the our phylogeny results based on ITS rDNA. Although Persich et al. (2006) suggested the possibility that *A. catenella* (Group I) is spreading northward by current ocean circulation patterns, environmental changes due to global warming would likely explain the recent spread of *A. catenella* to the east coast of South America (Lilly et al., 2007).

*Alexandrium tamiyavanichi* from northeastern Brazilian coast was included in a distinct clade along with isolates from Japan

and other Asian locations with medium support value, with the Brazilian population diverging earlier (Menezes et al., 2010). One of the hypotheses that seems plausible to explain the presence of the species in Brazil is due to an invasion event that could not be recent or occurred several times (Menezes et al., 2010). *Alexandrium tamiyavanichi* is a tropical species but it also occurs in warm-temperate regions such as Japan, Baja California, and South Africa (Sierra-Beltrán et al., 1998; Ruiz-Sebastián et al., 2005; Nagai et al., 2011). The origin of this species in Brazil could be explain by closure of Tethys Ocean and the emergence of the Isthmus of Panama that formed a land bridge between the two American continents and its final closure when there was a final interruption of the Atlantic and Pacific oceans' connection disrupting the flow of genes of many species (Hou and Li, 2018). On the other hand, after the closure of Tethys Ocean the dispersal between the Atlantic and Indo-Pacific could have occurred via southern Africa during interglacial periods (Bowen et al., 2006). These same hypotheses could be applied to *A. fraterculus* that segregated with isolates from Japan, Korea, China, and New Zealand, forming a sister clade of *A. tamiyavanichi*.

Data on the geographical distribution of *A. tamutum* are still scarce. Previously recorded as *Alexandrium* sp. for the northwest Pacific (Yoshida, 2002), *A. tamutum* was formally described based on material isolated from the Mediterranean (Montresor et al., 2004). Since then, the species has been reported in morphological studies of populations from Russia, Malaysia, and Scotland (Selina and Morozova, 2005; Hii et al., 2012; Swan and Davidson, 2014) and in morphological and genetic studies of populations from Ireland, Shetland, China, Malaysia, Greenland (Touzet et al., 2008; Brown et al., 2010; Gu et al., 2013; Kon et al., 2013; Tillmann et al., 2016). Our results demonstrated a genetic variability within the *A. tamutum* clade, which was formed by some subclades, including the Brazilian subclade. Genetic variability between isolates from different geographical areas of *A. tamutum* based on LSU and ITS rDNA has been reported by Montresor et al. (2004), Penna et al. (2008) and Tillmann et al. (2016). However, more sequences from distinct locations are necessary to confirm the occurrence of a geographical partitioning of *A. tamutum* species. The two isolates of *Alexandrium* sp. formed a monophyletic clade, however it is necessary for more sequences to make any inference about its geographical distribution. These populations have been found in Guanabara Bay and constitute a new species for the science (currently under description), related to *A. minutum* and *A. tamutum*.

This review constitutes the first approach on the occurrence of the *Alexandrium* genus in Brazilian coastal waters. The number of species recorded was very incipient considering the 7,491 km of the Brazilian coast, with the new record of *A. tamutum* and the discovery of a new species (*Alexandrium* sp.) indicating a wide gap in knowledge on the genus diversity in the country. Brazil has a Program for the National Sanitary Control of Bivalve Molluscs (PNCMB) established in 2011 designed to monitor for the presence of toxins in seafood and the presence of harmful algae in the coastal area. Although it is a national program, until now it has been implemented

only in Santa Catarina. The presence of toxic *Alexandrium* strains in other areas on the country points out to the need of expand the current coverage of the PNCMB and improve our ability to identify causative species of HABs through training courses and international technical and research collaborations.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00421/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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