Morphological and molecular characterization of some HAB-forming dinoflagellates from Philippine waters

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Various species of toxic dinoflagellates have been isolated from various sites in the Philippines. In this study, we characterized a number of these isolates using a molecular approach and Confocal Laser Scanning Microscopy (CLSM). Phylogenetic analysis using genetic markers (LSU rDNA, SSU rDNA, and cytochrome oxidase *b*) confirmed the identity of the isolates as *Pyrodinium bahamense* var. *compressum, Alexandrium affine, Gymnodinium catenatum, Prorocentrum sigmoides, P. micans* and *P. rhathymum*. Morphological differences among strains of *Pyrodinium* were very minor and could not be readily correlated with geographical variation. Intra-species polymorphism in the form of single nucleotide polymorphisms (SNPs) and deletions/insertions in the sequences were also observed. However, in general, the SNPs in these markers are too few and may not provide sufficient resolution to allow these markers to be used for population genetic studies.

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INTRODUCTION

Harmful algal blooms (HABs) are natural events but their frequencies, scale of impact and geographical distribution have recently increased (Azanza and Taylor, 2001; Hallegraeff et al. 2003). The accurate identification of species that cause HABs is crucial in the development of strategies for mitigating the adverse impacts of these events. Detection and identification methods are frequently based on traditional microscopy that requires special skills and training. It has been established that many species of dinoflagellates exist in different morphological forms and some closely related species exhibit little or no visible variation making them very difficult to differentiate (Godhe et al. 2001a, 2001b; Culverhouse et al. 2003). Hence, there is a necessity for a more reliable and accurate identification approach that does not depend on morphology alone.

The advent of molecular approaches for taxonomic identification such as polymerase chain reaction (PCR) coupled

with gene sequencing and phylogenetic analysis has made possible a more rapid, efficient and accurate classification of organisms isolated from the field (Ki et al. 2005; Ki and Han, 2007). Application of molecular tools for the identification of dinoflagellates, especially the HAB-causative organisms, has significantly increased. Various genetic markers from nuclear and mitochondrial genomes were used to assess the genetic variability of target organisms. The small subunit (SSU) rDNA gene (18S) for example has been extensively used to determine the phylogeny and diversity of phytoplankton in culture collections and environmental samples (e.g. Lin et al. 2006; Hong et al. 2008; Handy et al. 2009). Alternatively, Daugbjerg et al. (2000) used the large subunit (LSU) rDNA gene (28S) to infer phylogenetic relationships among different dinoflagellates alongside morphological characteristics. Genetic diversity and identification of new species of *Gymnodinium* from Danish waters was also accomplished by the use of the same gene (Ellegaard and Oshima, 1998). Zhang et al. (2008) used mitochondrial genes such as cytochrome oxidase b (COB) and cytochrome oxidase 1 (*co*1) to infer the phylogenetic position of *Dinophysis* in the dinoflagellate lineage. They also suggested a COB region that can be used as a universal barcode marker for dinoflagellates. In addition, molecular data can help reveal other types of important information, such as intra- and interspecies variability of algal isolates. Such information is useful in investigating population connectivity and might lead to a better understanding of the geographic scale and adaptation of harmful species (Casabianca et al. 2012). For example, 28s rDNA

Figure 1. A. Photomicrograph of chains of *Gymnodinium catenatum* with 12-cell in chain. Note that cell in longer chain are more compressed, B. A more elongated cell of *Gymnodinium catenatum* showing a more pronounced cingulum, C. CLSM image of *Gymnodinium catenatum*

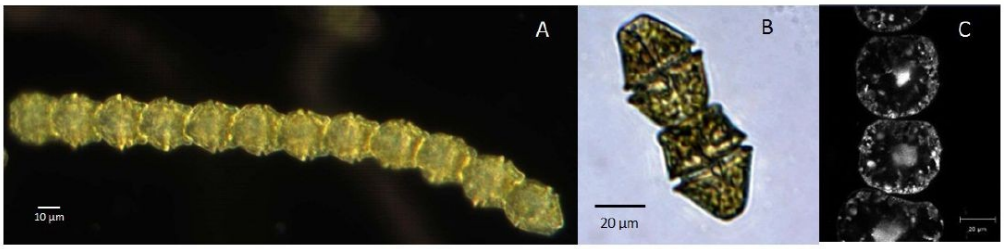


Figure 2. A. Ventral view of thecal plates of *Alexandrium affine* showing anterior part, B. Apical pore complex without connecting pore, C. Fourth apical plate, 4', D. Sixth precingular plate, 6', E. Posterior sulcal plate, s.p, F. First apical plate showing ventral pore, G. First precingular plate. H. First apical plate with ventral pore not connected to fourth apical plate, I. Posterior sulcal plate without a pore

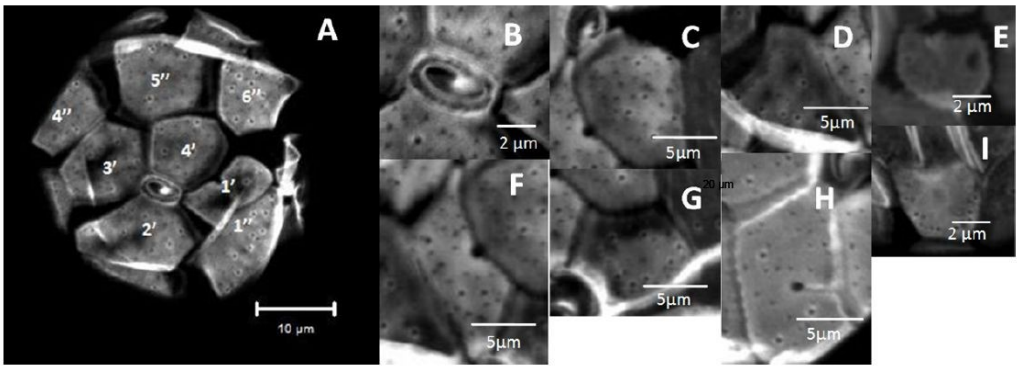
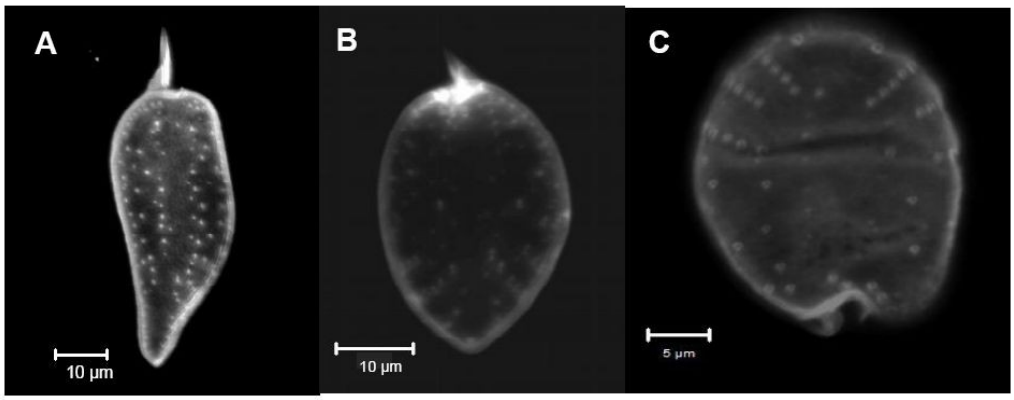


Figure 3. Laser scanning images of three species of *Prorocentrum,* A. *Prorocentrum sigmoides* showing an elongated cell with apical spines. Note the presence of pores in the surface, B. *Prorocentrum micans* showing a heart-shaped cell with shorter apical spine and pointed anterior end. Note the presence of trichocyst pores in the anterior end radially, C. *Prorocentrum rhathymum* showing an ovoid cell with a simple small thecal spine. Note the thecal surface is relatively smooth with numerous pores lying in shallow depressions radiating from the center.

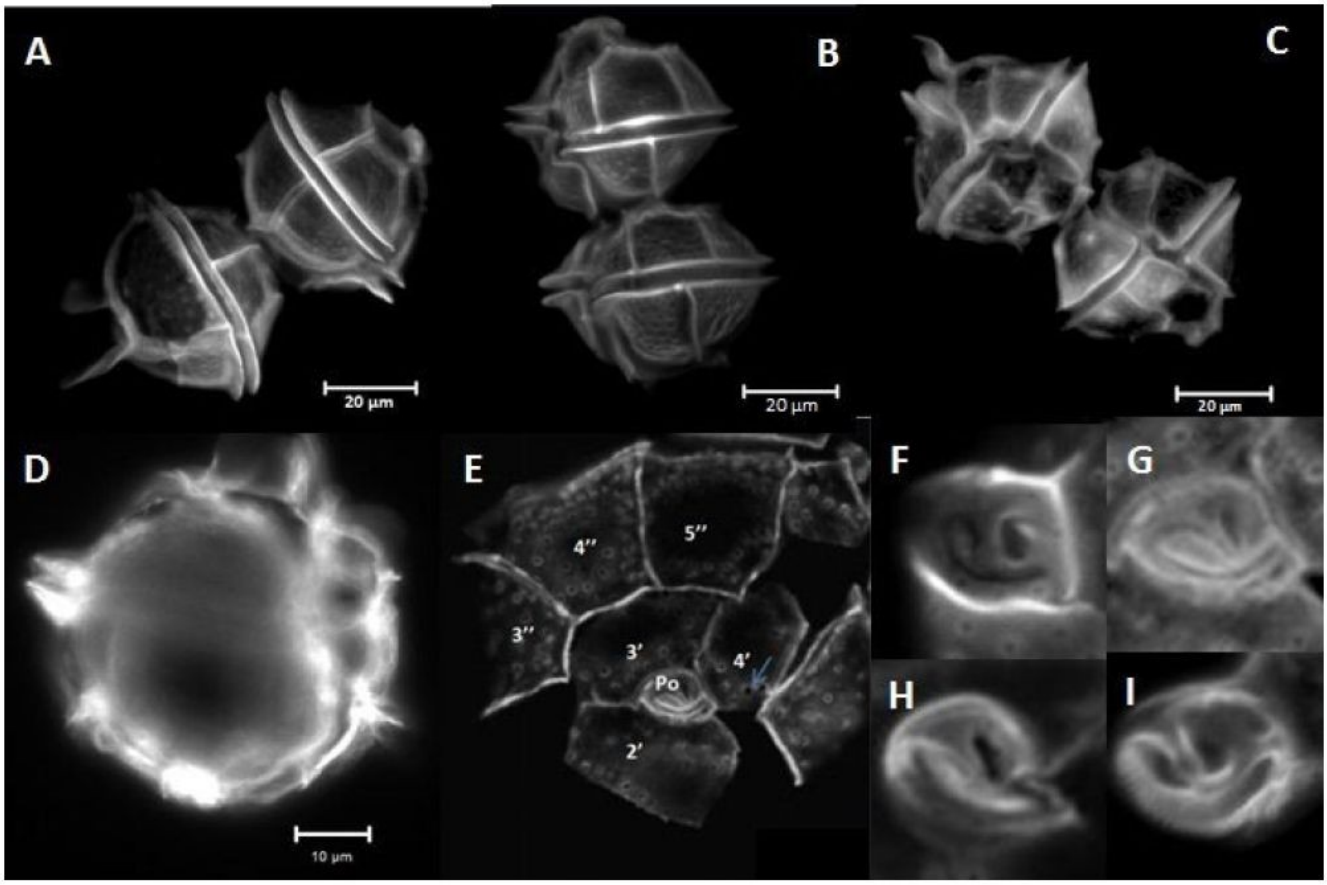


sequences and their associated single nucleotide polymorphisms (SNPs) have been utilized to study the global distribution and biogeography of the toxic *Alexandrium* "tamarense complex" populations (Scholin et al. 1994; Lilly et al. 2007) and has been beneficial in understanding the scope, width and potential mechanism of dispersal of the organism. However, some problems may also arise with the use of sequences deposited in public databases affecting analysis and comparisons due to lack of quality control and misidentification of species (Shen et al. 2013). In these cases, a multi-locus analysis approach is used alongside with morphological and biochemical properties to verify identities.

In the Philippines, a number of HAB-causing species have been reported to form blooms, particularly those belonging to the genera *Alexandrium, Gymnodinium, Amphidinium, Prorocentrum* and *Pyrodinium,* with the last species being the most dominant in terms of reported occurrence and distribution (Azanza and Taylor, 2001). Given the hazards associated with such species, there is a clear need then to improve detection and mitigation strategies by understanding their ecology and dynamics to protect public concerns. Environmental factors that could potentially account for blooms, as well as the seasonality of their occurrences have been investigated (Bajarias and Relox 1996; Azanza et al. 2004). To date however, molecular identification of the Philippine isolates, assessment of their genetic diversity, and investigation on potential population connectivity have not been carried out.

In this study, we demonstrated the application of a molecular phylogeny-based approach coupled with morphological characterization to determine the taxonomic identity down to the species level of the cultured isolates. Laboratory cultures are generally used as references and model species in characterizing certain groups; thus, accurate identification is a key step to their understanding. Furthermore, we explored the use of the same set of data to determine intraspecific variation (i.e. *Pyrodinium*) which could probably delineate different populations (i.e. represented by strains from various geographic locations). We attempted to detect SNPs and

Figure 4. Laser scanning images of *Pyrodinium bahamense* var. *compressum*, A. Masinloc, Zambales, B. Honda Bay, C. Sorsogon Bay, D. Matarinao Bay. E. Apical view of different thecal plates, Apical pore complex of F. PbcMZRVA042595, G. PbcHBRVA102905, H. PbcSB121510 and I. PbcMTB091610.



insertions/deletions in gene sequences which may be used for such purpose. The high image resolution coupled with 3D-reconstruction capability of confocal laser scanning microscopy also allowed for more detailed observations on the morphological traits of the isolates.

MATERIALS AND METHODS

Algal cultures

Algal batch cultures were obtained from the RVA Microalgal Collection of the HABs laboratory of The Marine Science Institute, maintained in 250 mL Guillard's f/2 medium (without silicic acid) and incubated at 24 ± 2ºC temperature condition with 12:12 light-dark cycle provided by cool white fluorescent lamps emitting an approximated 200 µmol photons m^{-2} s^{-2} of irradiance (Azanza 1997; Santos and Azanza, 2012). The following isolates were used: *Pyrodinium bahamense* var. *compressum* (Masinloc, Zambales: PbcMZRVA042595; Matarinao Bay, Samar: PbcMTBRVA091610, Honda Bay, Palawan: PbcHBRVA102905 and Sorsogon Bay, Sorsogon: PbcSB121510), *Alexandrium affine* (Honda Bay, Palawan: AlexHBRVA102905), *Gymnodinium catenatum* (Manila Bay: GCMBRVA011810), *Prorocentrum sigmoides* (Manila Bay: PSMBRVA111510), *P. rhathymum* (Sorsogon Bay: PRSB121510) and *P. micans* (Manila Bay: PMMBRVA091309).

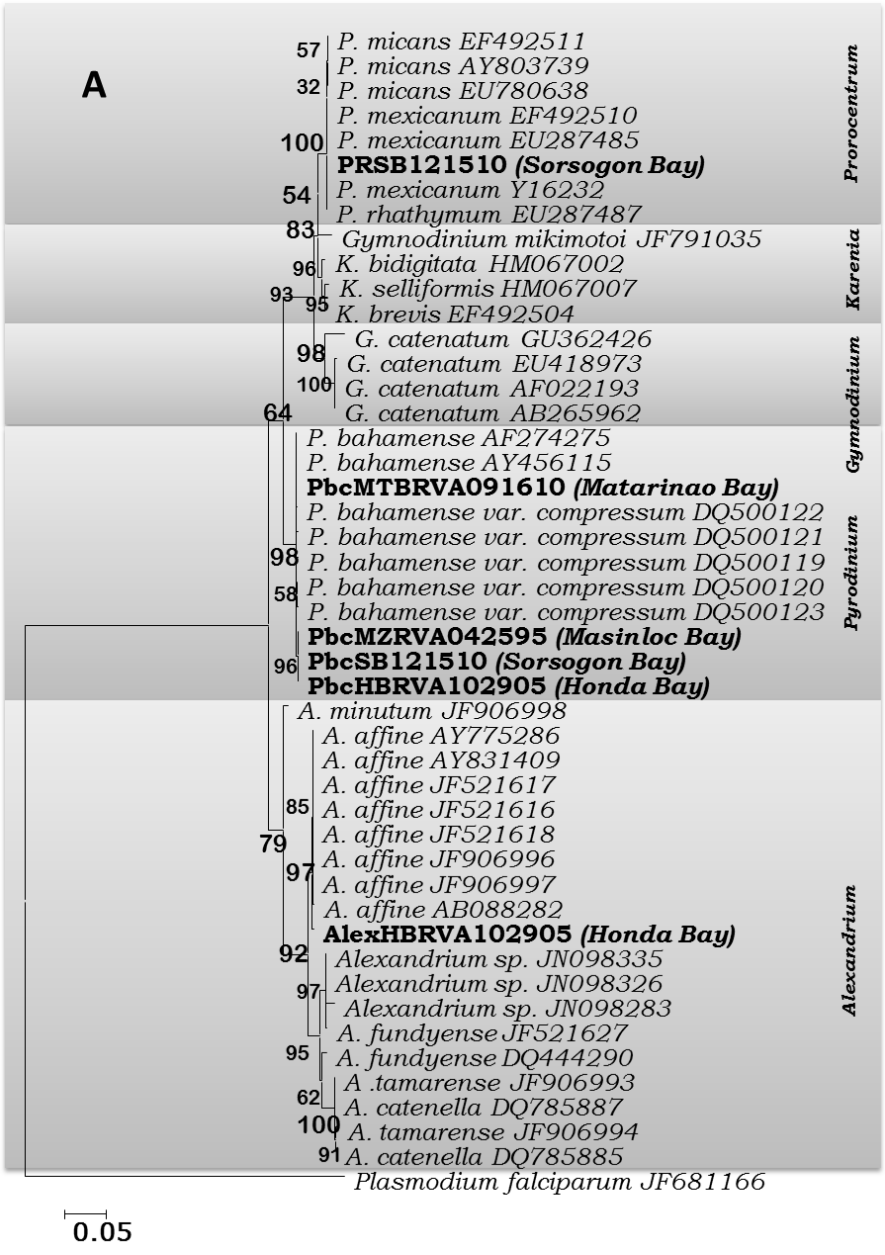
Morphological characterization

A confocal laser scanning microscope (CLSM 710, Carl Zeiss, Germany) was employed to visualize and characterize the distinguishing structures of the isolates. Briefly, 15 mL of each culture was fixed by adding glutaraldehyde (1% final concentration, [Sigma-Aldrich]), incubated at room temperature for 15 minutes and pelleted by centrifugation at 2600 x *g* for 15 minutes. The supernatant was subsequently removed and cells were resuspended in 100 µL 1x Phosphate Buffer Saline (PBS). To mount, 30 µL cells were transferred to a sterile polycarbonate membrane with a hole punched at the center and mounted on a glass slide. Calcofluor white (5 µL, 10x final concentration, [Sigma-Aldrich]) was added to the cells, which were then incubated at the same temperature in the dark for another 15 minutes before the slide was covered with a glass slip. For imaging, fluorescence was detected at 420 nm wavelength (cell wall) using the CLSM.

Algal DNA extraction

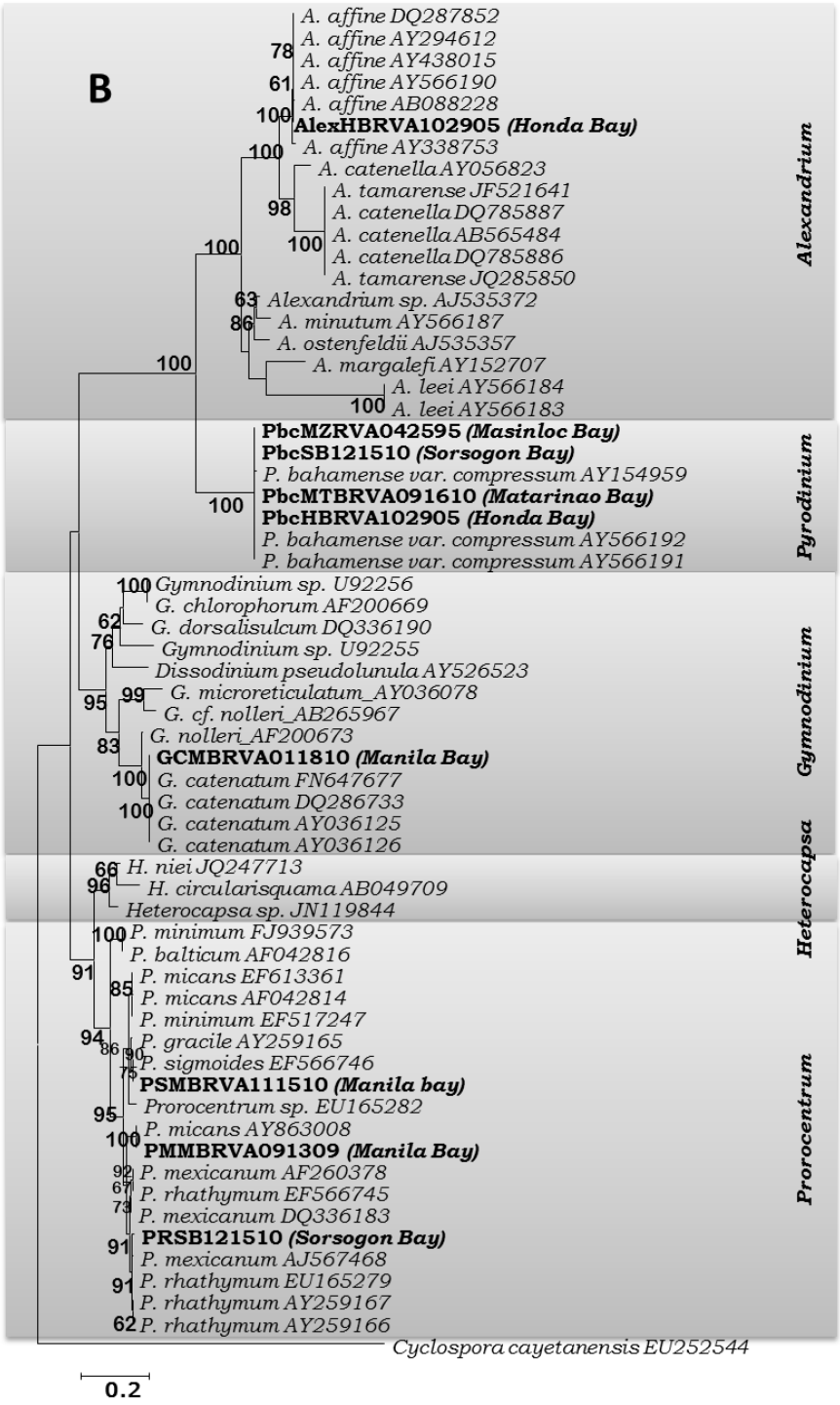
Total genomic DNA was extracted from microalgal samples

Figure 5. Phylogenetic trees inferred from the partial sequences of the nuclear genes A. small subunit rRNA and B. large subunit rRNA using the Maximum-Likelihood algorithm. Bootstrap values (in %) are shown next to the nodes (see Materials and Methods for details).



using the DNEasy™ Plant Mini-Kit (Qiagen) following the manufacturer's protocol (with some modifications). Briefly, 50 mL of samples in their logarithmic growth phase (~10-12 days) were taken from culture and then pelleted by centrifugation at 2300 × *g* for 15 minutes at room temperature. The supernatant was then removed and cells were resuspended in 1 mL sterile seawater and transferred to 1.5 mL centrifuge tubes. Cells were again pelleted at the same speed, the supernatant was then removed and 400 µL AP1 buffer was added as prescribed by the kit. Four to six sterile silica beads were added into the tube and vortexed for 5 minutes to disrupt the cells. Subsequently, 4uL of RNAse was added into the lysate and incubated at 65ºC in a heat block for 10 minutes with frequent inversion. This was then followed by the procedures prescribed by the kit's manufacturer. Eluates that contained the DNA material were stored at - 20ºC until use.

Figure 5. continued.



PCR Amplification, Sequencing and Analysis

The universal primer pairs 4616F (5'-AACCTGGTTGATCCTGCCAG-3') and 4618R (5'-GATCCTTCTGCAGGTTCACCTAC-3'), and DinFi (5'-GCATATAAG TAMGYGGWGG-3') and DinRi (5'-CCGTGTTTCAAGACGGGTC-3') were used to amplify the 18S rDNA (SSU) and 28S rDNA (LSU) genes respectively following the conditions described by Logares et al. (2007). However, 60ºC was used as the annealing temperature instead of 55ºC in amplifying the ~1.5 kb product for the SSU gene fragments. Mitochondrial cytochrome oxidase *b* (COB) was amplified using the barcoding primers Dinocob4F (5'-AGCATTTATGGGTTATGTNTTACCTTT – 3') and Dinocob3R (5'-AGCTTCTANDGMATTATCTGGATG-3') reported by Zhang et al. (2007). All PCR reactions were done in a 25 µL reaction mix with 0.26 U Titanium *Taq* DNA polymerase, 0.13 µM dNTPs, 1x *Taq* buffer (Clonetech) and at least 50 ng of template. Amplicons were purified using QIAquick Gel Purification Kit (Qiagen) and sent to 1st Base (Malaysia) for single pass sequencing via the Sanger method.

Generated sequences were used to search the NCBI database for most similar sequences using BLASTn (Altschul et al. 1990). The reference and related sequences were downloaded from GenBank and aligned together with the generated sequences using the Muscle and ClustalW alignment software in MEGA v. 5.0 (Tamura et al. 2011;

http://www.megasoftware.net/). Sequence comparison and determination of polymorphisms/insertions-deletions were done in BioEdit Sequence Alignment editor (http://www.mbio.ncsu.edu/bioedit). A model-test was completed separately for each dataset of each gene and was then used for the phylogenetic analysis using Maximum Likelihood Method. Clade support was computed by bootstrap analysis with 100 replicates.

RESULTS AND DISCUSSION

Morphological characterization

In general, most of the isolates in this study were correctly identified down to the species level based on morphology particularly for *Gymnodinium*, *Pyrodinium* and some *Prorocentrum* species (Fig. 1, 3A-B & 4). However, morphological landmarks traditionally used in taxonomy showed limitations in distinguishing species in the *Alexandrium* complex and other *Prorocentrum* isolates.

Alexandrium (Fig. 2A) from Honda Bay have cells which are 32-40 µm in trans diameter and non-chain forming with observable U-shaped nucleus. It could be identified as *A. affine* by the presence of the anterior attachment pore; however this was not found in all specimens in culture (Fig 2B). The same was true for the first apical plate which was in direct contact with the apical pore complex (APC) with a large ventral pore adjacent to the fourth apical plate (Fig 2C). In some cells, the ventral pore does not touch the fourth apical plate and is located toward the middle of the plate (Fig 2H). The posterior attachment plate is similar to *A. tamarense* (Balech 1995; Taylor 1995) with a posterior attachment pore present in some (Fig. 2E) but absent in other cells (Fig 2I).

Another case of morphological similarity was the *Prorocentrum* isolate from Sorsogon Bay which was observed to be ovoid to oblong with a simple apical spine (Fig. 3C) and could be mistaken for *P. mexicanum*. The valve surface was less rugose and had large trichocyst pores arranged radially in furrowed depressions, characteristics that are shared by both *P. rhatymum* and *P. mexicanum*. The two species had been reinstated into separate species based on slightly different structures of the apical spine, details of pyrenoid, trichocyst number and placement, and habitat [i.e., *P. rhatymum* being benthic and *P. mexicanum* as planktonic] (Cortés-Altamirano and Sierra-Beltrán 2003).

Table 1. Summary of putative identity of the isolates based on the alignment scores from the BLASTn search against the NCBI database (nr).

Isolate Code (Source)	Marker	Closest Identity Match[a] (BLASTn)	Ref Sequence NCBI Accession No.	Maximum identity[b]
PbcMZRVA042595 (Masinloc Bay)	18s rRNA	*P. bahamense* var. *compressum*	AY456115.1	99%
	LSU	*P. bahamense* var. *compressum*	AY566192.1	100%
	COB	*P. bahamense* var. *compressum*	AY456114.1	99%
PbcSB121510 (Sorsogon Bay)	18s rRNA	*P. bahamense* var. *compressum*	AY456115.1	99%
	LSU	*P. bahamense* var. *compressum*	AY566192.1	99%
	COB	*P. bahamense* var. *compressum*	AY456114.1	99%
PbcMTBRVA091610 (Matarinao Bay)	18s rRNA	*P. bahamense* var. *compressum*	AF274275.1	99%
	LSU	*P. bahamense* var. *compressum*	AY566192.1	99%
	COB	*P. bahamense* var. *compressum*	AY456114.1	99%
AlexHBRVA102905 (Honda Bay)	18s rRNA	*P. bahamense* var. *compressum*	AY456115.1	99%
	LSU	*P. bahamense* var. *compressum*	AY566192.1	100%
	COB	*P. bahamense* var. *compressum*	AY456114.1	99%
AAHBRVA100510 (Honda Bay)	18s rRNA	*Alexandrium affine*	JF906997.1	99%
	LSU	*Alexandrium affine*	U44935.1	99%
	COB	*Alexandrium catenella*	AB374243.1	99%
GCMBRVA011810 (Manila Bay)	18s rRNA	*n/a*	-	-
	LSU	*Gymnodinium catenatum*	DQ779989.2	100%
	COB	*Gymnodinium catenatum*	EU840167.1	100%
PRSB121510 (Sorsogon Bay)	18s rRNA	*Prorocentrum mexicanum*	Y16232.1	99%
	LSU	*Prorocentrum rhathymum*	EU165279.1	99%
	COB	*Prorocentrum micans*	AY585525.1	100%
PMMBRVA091309 (Manila Bay)	18s rRNA	*n/a*	-	-
	LSU	*Prorocentrum micans*	AY863008.1	99%
	COB	*Prorocentrum micans*	AY585525.1	99%
PSMBRVA111510 (Manila Bay)	18s rRNA	*n/a*	-	-
	LSU	*Prorocentrum sigmoides*	EF566746.1	99%
	COB	*Prorocentrum micans*	AY585525.1	99%

[a] Full name or description of the closest matched sequence.

[b] Maximum percentage of identical nucleotides within the noted alignment length.

In other organisms and algal species, morphological traits carry information that may distinguish one strain from another and therefore could indicate presence of distinct natural populations. In the case of the *Pyrodinium* strains examined, gross morphology and structure were the same but slight differences in overall appearance could be observed. Strains isolated from Masinloc Bay (Fig. 4A), Honda Bay (Fig. 4B), Sorsogon Bay (Fig. 4C) and Matarinao Bay (Fig. 4D) were closely related to *P. bahamense* var. *compressum* common in the Indo-Pacific region (Taylor and Pollingher, 1987) as compared to *P. bahamense* var. *bahamense* found in Tropical and Subtropical Atlantic regions (Steidinger et al. 1980). Balech (1985) argued that the separation between the two varieties is artificial and only a result of ecological factors rather than genetics. Morphological variations among the four strains were very minor and could possibly be the result of differences in the length/duration of acclimatization to the laboratory condition rather than the consequence of geographical variation. The most notable difference was the variation in the overall form and shape among the strains. The oldest strain of *P. bahamense* var. *compressum* (PbcMZRVA042595) isolated from Masinloc Bay, Zambales (Fig. 4A) on April 25, 1995 exhibited the form and shape as described in other isolates (Taylor and Fukuyo, 1989; Garate-Lizarraga and Gonzalez-Armas, 2011). The most recently isolated *Pyrodinium* strain from Matarinao Bay, Eastern Samar exhibited abnormally shaped cells characterized by thicker thecal plates. Cells were antero-posteriorly compressed, chain-forming up to eight cells in length and had a less developed apical spine. These features are typical characteristics that are enough to separate them from the *P. bahamense* var. *bahamense* at least morphologically. The APC of PbcMZRVA042595 (Fig. 4F), PbcHBRVA102905 (Fig. 4G), PbcSB121510 (Fig. 4H) and PbcMTB091610 (Fig. 4I) were almost similar with minor differences in the shape of the attachment pore.

These observations highlight the limitations and inconsistencies of gross morphological landmarks as basis for identification and classification in some species (i.e. *Alexandrium* and *Prorocentrum*). In the case of intraspecies variability, morphology is also not useful in demonstrating patterns to distinguish populations (i.e. *Pyrodinium*). Use of landmarks would also need standardization and comparison across large number of representatives from different populations which would be tedious and time-consuming. Thus, there is a need to utilize a more variable yet conclusive marker than morphology to allow distinction between species/strains such as the information encoded in genes.

Molecular identification of species

Nuclear genes (SSU and LSU rDNA) complimented the limitations of the morphological traits and confirmed the identity of most isolates down to the species level. Results showed high congruence for the 2 nuclear markers in most species including *Gymnodinium* and *Alexandrium* isolates (Table 1). This is supported by the phylogenetic tree which consistently clustered the Philippine strains with their putative related sequences (Fig. 5A and B).

Phylogenetic analysis showed the clustering of AlexHBRVA102905 with *A. affine* sequences and the "tamarense complex" cluster in the same clade, consistent with previous observations in trees generated from 18s and 28s rDNA sequences (Hansen et al. 2003 and Scholin et al. 1995). Morphologically, species under this clade and complex are not exactly similar but further studies have shown that the molecular evidence has not actually supported the separation of the members of the complex into distinct species and suggested that a re-evaluation is necessary before new species are proposed (Lilly et al. 2007). Other studies, on the other hand, suggested that the branching pattern may differ depending on the sequences and phylogenetic approach used in the analysis (Leaw et al. 2005; Touzet et al. 2008). For this study however, we identified our isolate as *A. affine* based on the combined characteristics inferred from phylogenetics and morphology as well as toxin profiles (C. Mendoza et al. unpublished observations).

A similar pattern of ambiguity was also observed for some of the *Prorocentrum* species. For example, our isolate (PRSB121510) that was morphologically identified as *P. rhathymum* clustered with other *P. rhathymum* SSU sequences (EU165279, AY259167 and AY259166) and one *P. mexicanum* (AJ567486) using different phylogenetic models. It could be argued that some of the reference sequences were just mislabelled / misidentified (Hoppenrath and Leander, 2008) because in the past, the species *P. rhathymum* was dissolved and reclassified as *P. mexicanum* after a recharacterization but was later verified as a separate species based on morphology (Cortés-Altamirano and Sierra-Beltrán, 2003). However, a more recent phylogenetic reclassification of *Prorocentrum* species confirmed the monophyly of *P. rhathymum* and *P. mexicanum*, supporting the notion that these are cryptic species (Cohen-Fernandez et al. 2010).

The phylogenetic tree generated from the COB sequences was similar to the nuclear gene trees which did not resolve the affinity of *A. affine* with other *Alexandrium* species (data not shown). This observation, including that of the *Prorocentrum* species, highlights the limitations of some genetic markers in distinguishing species. The COB region used in this study was from the report of Lin et al. (2009) which was also the proposed universal barcode marker. COB is a well-known mitochondrial genetic marker that has been extensively used for barcoding of some species and is considered to be one of the most useful markers for phylogenetic analysis. It is generally characterized by the presence of rapidly evolving codon positions which make it suitable for assessing genetic divergence (Farias et al. 2001). The faster rate of mutation of such a gene made it ideal to be used as a barcode (Barrientos et al. 2002). They also contain enough conserved regions for which primers can be designed and be used to distinguish strains of microalgae (Lin et al. 2009).

Present results suggest that even if the COB worked for some species, the proposed barcode still seems to be not informative enough to distinguish between species in some genera such as *Alexandrium*. It does, however, appear to have more potential in assessing population/community diversity than the nuclear genes. The high number of variable sites allows for more convenient sequencing of shorter regions enough to discriminate genera and, in some cases, species (Lin et al. 2009). Furthermore, aside from not providing a uniform species-discriminating distance threshold especially for dinoflagellates (Lin et al. 2009) and contrary to previous reports (Zhang et al. 2008), another limitation of the use of this gene is the lack of sequences for a number of species which could have implications for the correct identification of samples, especially those that had been collected from the field. For example, our search for the most similar sequences for the *Prorocentrum* COB sequences did not return hits for *P. sigmoides* and *P. rhathymum* in the NCBI database (Table 1). The sequences we generated could be the first few contributions to the COB references for the said species.

This study has been the first attempt to genetically characterize some HAB species in the Philippines using different markers and phylogenetic analysis. The sequences had been useful in verifying the putative identity of the isolates. Though single-gene model phylogenetic inferences have shown the possibility of resolving ambiguities, the use of multiple gene approach or concatenation has been popular in recent years though it has its advantages and disadvantages. In this study however, the results of the SSU rDNA, LSU rDNA and COB phylogenetic trees showed consistency and congruence with one another. Their utility had been most useful in resolving genus-level relationships, but species-level relationships still lack the necessary resolution for some species.

A number of variable sites were also observed in the markers. These variations, however, were not informative enough to allow discrimination of isolates (hence populations) and to reveal possible patterns of spatial distribution of the organisms. On the other hand, there were no detectable genetic variation/polymorphisms for the *G. catenatum* sequences, which are consistent with the other reports (Bolch and Reynolds, 2002; Ordas et al. 2004).

Species occurrence and distribution: implications to monitoring

Most species presented in this study are toxin producing and/or fish-killing. *Pyrodinium, Gymnodinium* and *Alexandrium* are all capable of producing toxins causing Paralytic Shellfish Poisoning (PSP). Though most cases of PSP in Manila Bay and Sorsogon Bay were widely associated with *Pyrodinium*, recent reports indicate the dominance/co-occurrence of *G. catenatum* in high density (3000-9000 cells/mL) for both bays especially during the Southwest monsoon (Benico and Azanza, 2012). In addition, preliminary studies detected STX/neo-STX as well as GTX1 and GTX2 from the *A. affine* cultures in our laboratory which also varied temporally (C. Mendoza et al. unpublished results). Interestingly, a strain/isolate of this species from California is non-toxic (Band-Schmidt et al. 2003). Due to the capacity of these species/isolates to produce toxins, it is possible that episodic poisoning in some areas was caused, at least in part, by these organisms and not only by *Pyrodinium*. Another interesting group is Prorocentrales which, though not yet dominant, may also pose possible threats. *Prorocentrum rhathymum* produces Okadaic Acid (OA) that when consumed in potent amounts, can cause severe diarrhea or Diarrheic Shellfish Poisoning (DSP) (An et al. 2010). DSP cases have not been well-monitored in the Philippines, and no data is available, but may possibly be occurring. Other related species can also cause hypoxia during their bloom termination causing fish-kills (Azanza et al. 2005).

The presence of these species and their continuously increasing occurrence in key coastal areas pose threats to public health. Therefore, species detection can greatly help in risk management and prevention. Though identification of polymorphisms did not reveal phylogeography of the isolates, they can still be further utilized to design primers/probes that may be used for detection purposes. Possible application of SNPs as a marker of toxicity may also be explored, as different strains of some species may or may not be toxic as in the case of the toxic *A. affine* from the Philippines. Also, molecular characterization may capture other types of data that may help in efforts to model a possible distribution and pattern of dispersal which will be useful in management, prevention and mitigation strategies. However, the lack of resolution at intraspecific levels in this study indicates that the use of more genes and more polymorphic markers (e.g., microsatellite markers) may be necessary to determine population structures and connectivity especially for key species such as *Pyrodinium.*

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CONFLICT OF INTERESTS

The authors expressed no conflict of interests.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

DFL Onda, G Benico, AF Sulit and PL Gaite contributed in the collection of isolates, generation of sequences, images, data analysis and preparation/finalization of the manuscript. AO Lluisma and RV Azanza contributed in the experimental design, data analysis and interpretation, and finalization of the manuscript.

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