A bloom of *Karlodinium australe* (Gymnodiniales, Dinophyceae) associated with mass mortality of cage-cultured fishes in West Johor Strait, Malaysia

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**A B S T R A C T**

A recent (February 2014) mass mortality of fishes was observed in the cage-farming region of the West Johor Strait of Malaysia, involving over four different species of cultured fishes, numbering ~50,000 fish. A field investigation at six stations along the West Johor Strait collected water samples and examined for the presence of harmful species. Dead fishes were collected for necropsy. The phytoplankton composition was dominated by a species of *Karlodinium*, at a considerably high cell concentration (0.31–2.34 × 10⁶ cells l⁻¹), and constituting 68.8–98.6% of the phytoplankton relative abundance at all stations. Detailed morphological assessment by light and scanning electron microscopy revealed that the species was *Karlodinium australe* de Salas, Bolch and Hallegraeff. This was supported by molecular evidence of the nuclear encoded large subunit ribosomal gene (LSU rDNA) and the second internal transcribed spacer (ITS2) via single-cell PCR. The sequences of LSU rDNA yielded 3.6–4.0% divergence when compared to the sister taxon, *K. armiger*; and >6.5% when compared to other *Karlodinium* species. Fish necropsy showed symptoms similar to those affected by karlotoxin ichthyotoxins. This is the first report of a mass mortality of cage-cultured and wild fishes attributed to the unarmored dinoflagellate *K. australe*.

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1. Introduction

Many coastline countries in Southeast Asia, including Malaysia, are experiencing a rapid growth in aquaculture/mariculture industries, owing to high demands from domestic and international markets, and the involvement of the government and profit-driven companies (Hishamunda et al., 2009). In Malaysia, the industry has grown by more than 10% annually between 1990 and 2002, with total production of nearly 176,000 tonnes in 2005 (FAO, 2007). While the industry has expanded, it has also suffered huge production losses due to frequent fish kills caused by water quality degradation or the proliferation of harmful marine diatoms and ichthyotoxic dinoflagellates at aquacultures sites.

Several marine microalgal bloom events, some resulting in massive fish kills, have been documented in Malaysia (reviewed in Lim et al., 2012). In 2002, a bloom of *Proorocentrum minimum* (Pavillard) Schiller (at >2 × 10⁶ cells l⁻¹) was observed along the Johor Strait, causing massive water discoloration but no fish kills, even near finfish cages (Usup et al., 2003). Subsequent fish-kill events were recorded in the Straits of Malacca, the most important finfish-farming area in the country. In 2005–2006, the most notable protracted algal bloom associated with the mass mortality of caged finfish, with an estimated loss of at least 6 million USD, was encountered in Penang, northern Peninsular Malaysia (Sin et al., 2007).
The suspected causative species was the unarmored dinoflagellate, *Cochlodinium polykrikoides* Margalef. Concurrently, fish kills in cage farms were also reported in Malaysia Borneo (Sepanggar Bay, off Kota Kinabalu, Sabah), where blooms and water discoloration were observed. It was later found that *C. polykrikoides* was responsible for the event, with a maximum cell density of 6 × 10⁶ cells l⁻¹ (Anton et al., 2008). In a recent field investigation along the Johor Strait, potentially harmful unarmored dinoflagellates, *Karlodinium veneficum* (Ballantine) Larsen and *Kareania mikimotoi* (Miyake & Kominnami ex Oda) Hansen and Moestrup, were documented (Tan et al., 2013).

The genus *Karlodinium* gained significant scientific scrutiny after the first described bloom of *Karlodinium veneficum* (syn. *K. micrum*; Bergholtz et al., 2005) in Walvis Bay (Namibia), in 1950, harmed fishes and marine invertebrates (Ballantine, 1956; Abbott and Ballantine, 1957; Braarud, 1957). In the 2000s, periodic fish-kill incidents associated with *K. veneficum* blooms were reported in the United States (Deeds et al., 2002; Kempton et al., 2002; Fensin, 2004; Goshorn et al., 2004). Fish assays using cultured *K. veneficum* cells showed toxic effects on juvenile fish and molluscan shellfish (Nielsen and Strømgren, 1991; Nielsen, 1993; Galimany et al., 2008).

After decades of fish-kill events associated with *Karlodinium veneficum*, the responsible toxic compounds were discovered in 2002, and named karlotoxins (KmTxs) (Deeds et al., 2002, 2004a; Kempton et al., 2002). These potent amphipathic ichthyotoxins are hemolytic, cytotoxic, algidinal and ichthyotoxic (Mooney et al., 2010). Karlotoxins cause pore formation in biological membranes, increasing their ionic permeability (Deeds, 2003). Their biological activity is dependent on the sterol composition (Adolf et al., 2006a,b; Deeds and Place, 2006). Organisms with a predominance of 4-desmethyl sterols, such as fishes, are susceptible to the toxins, which damage gill epithelial tissues (Deeds et al., 2006; Place et al., 2012). Toxin production varies geographically by station and eco-physiological condition (Deeds et al., 2004a; Adolf et al., 2009).

On February 11, 2014, wild as well as caged fishes were found dead in the West Johor Strait of Malaysia. A field investigation was undertaken to investigate the cause of this mass mortality. Here, we reveal the causative bloom species, based on morphological and molecular evidence.

### 2. Materials and methods

#### 2.1. Study site and sampling

The study area is located in the West Johor Strait, Tanjung Kupang, Malaysia (1.2151° N, 103.3758° E) (Fig. 1). The West Johor Strait receives freshwater inflows from the Pendas River and is subjected to tidal influence by the Straits of Malacca. Sampling was undertaken a week after the fish-kill event was reported in the press on February 20–21, 2014 (Sin Chew, The Star, 2014). Six sampling stations (Stn 1–6) along the West Johor Strait were selected to include cage-farming areas (Stn 3), as well as areas influenced by anthropogenic activities such as land reclamation (Stn 2), a jetty (Stn 4), a river mouth (Stn 5), and a marina (Puteri Harbour) (Stn 6). Station 1, a fishing kelong (a platform built predominantly with wood) served as a control site (Fig. 1).

Quantitative water samples were collected at each station using a Van Dorn water sampler. Plankton samples were collected using a 20-μm mesh-size plankton net. Samples containing live dinoflagellates were brought back to the laboratory for culture establishment and molecular characterization.

#### 2.2. Determination of phytoplankton composition and water parameters

Duplicate water samples were collected for analyses. Aliquots (1 l) were concentrated by filtration and preserved with 1% acidic Lugol’s solution for phytoplankton enumeration and composition. Cell counts were performed using a Sedgwick-Rafter counting chamber under a Leica CME light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Cell abundances of each station were plotted with Surfer 9 (Golden Software Inc., CO, USA). Phytoplankton composition was plotted using GraphPad Prism 5 (GraphPad Software Inc., CA, USA).

Temperature, salinity and pH of seawater were measured in situ. The data were plotted with Surfer 9, and Ocean Data View (http://odv.awi.de/) was used to contour the distribution of depths, sea-surface temperature, salinity and pH in the study area.

Fig. 1. Map of the western Johor Strait, showing sampling sites.
2.3. Detailed morphological characterizations

Lugol’s preserved cells were examined with an Olympus BX51 microscope (Olympus, Tokyo, Japan), using differential interference contrast (DIC) optics. For SEM examination, Lugol’s preserved field samples were fixed with 2% osmium tetroxide overnight. The samples were then washed with a decreasing salinity of filtered seawater and finally rinsed with distilled water before dehydration by a graded series of ethyl alcohol. Samples then underwent critical point drying, after intermediate substitution by acetone. The samples were coated with gold-palladium using a JEOL JFC-1600 magnetron sputter coater (JEOL Ltd., Tokyo, Japan), and observed under a JEOL JSM-6390LA analytical scanning electron microscope (JEOL).

2.4. DNA isolation, single-cell PCR and sequencing

Single cells collected from the field were isolated by micropipette under an Olympus IX51 inverted light microscope (Olympus). Each single cell was rinsed in droplets of, sterile-filtered seawater prior to transferring into a 0.2-ml PCR tube containing 1 µl of TE buffer (Tris 1 M, EDTA 0.5 M, pH 8). Genomic DNAs (gDNAs) of the single cells were extracted by Proteinase K (20 mg·ml⁻¹) at 56°C for 30 min, followed by 10 min at 95°C. The gDNAs was then used as template for gene amplification.

Gene amplification was performed using the universal primer pair of D1R (5'-ACC CGC TGA ATT TAA GCA TA-3') and D3Ca (5'-ACG AAC GAT TGC ACG TCA G-3'), targeting the large subunit ribosomal RNA gene (LSU rDNA, domain 1–3) (Scholin et al., 1993), and the primer pair ITS1F (5'-TGG TCA TAA GGT TTC TGT AGG TG-3') and ITS1R (5'-TA TGG TTA AGT TCA GCG GG-3'), targeting the internal transcribed spacer region (ITS) of rDNA (Leaw et al., 2001). Amplification was performed using an Arktik™ Thermal Cycler (Thermo Scientific, Vantaa, Finland), with the following cycling conditions: pre-denaturation at 94°C for 4 min, 35 cycles of 94°C for 35 s, 55°C for 50 s and 72°C for 35 s. A final extension was carried out at 72°C for 7 min.

The amplicons were then separated by 1% agarose gel electrophoresis, stained with SYBR Safe DNA Stain (Invitrogen, Life Technologies, Carlsbad, CA, USA), and visualized under a blue transilluminator (Invitrogen). The amplicons were further purified using the Wizard® SV Gel PCR Clean-Up System (Promega, Madison, WI, USA) prior to sequencing. The purified amplicons were directly sequenced for both strands by First Base Laboratories (Selangor, Malaysia). The nucleotide sequences obtained were deposited in NCBI GenBank (Table 1).

2.5. Sequence alignment and phylogenetic analyses

Nucleotide sequences of LSU rDNA obtained in this study, and sequences of other Karlodinium retrieved from the NCBI nucleotide database, were aligned with MUSCLE (Edgar, 2004) (Table 1). The resulting alignment obtained from MUSCLE was further edited using BioEdit Sequence Alignment Editor v7.2.5 (Hall, 1999).

The alignment comprised 23 operational taxonomic units (OTUs), with two outgroup taxa: Takayama tuberculata de Salas and T. acrotrocha (Larsen) de Salas, Bolch & Hallegaard. Outgroup selection was based on de Salas et al. (2008). Phylogenetic analyses of maximum parsimony (MP) and maximum likelihood (ML) were performed using PAUP* 4b10 (Swofford, 2011). Bayesian inference (BI) was performed using MrBayes v 3.2.1 (Huelsenbeck and Ronquist, 2001), as described in Lim et al. (2013). The estimates of evolutionary divergence between LSU rDNA sequences of Karlodinium spp. were conducted in MEGA 6.06, through pairwise distance analysis using the p-distance model (Tamura et al., 2013).

2.6. ITS2 transcript modelling

The proximal stems of 5.8S–28S rRNA interaction were determined to annotate the termini of ITS2 in Karlodinium species (Gottschling and Plötner, 2004; Keller et al., 2009). The ITS2 secondary structure of K. australis (KJ670418) was predicted using the free energy minimization in RNAstructure v. 5.02 (Reuter and Mathews, 2010). The ITS2 secondary structure terminology was further confirmed based on Müller et al. (2007). The sequence-structure modelled was used as a template for homology modelling (Wolf et al., 2005), using the IT52 Database (Koetschan et al., 2012; Merget et al., 2012). We modelled four sequences of K. australis isolated from West Johor Strait, one from Aman Island, three of K. armiger from Spain, and 35 of K. veneficum from both Pacific and Atlantic regions (Table 1).

The multiple sequence-structure alignment of Karlodinium ITS2 was generated using an ITS sequence structure-specific scoring matrix (Seibel et al., 2006) in 4SALE v. 1.7 (Seibel et al., 2006, 2008). The compensatory base changes (CBCs) and hemi-compensatory base changes (HBCs) were identified using the CBCAnalyzer option implemented in 4SALE (Wolf et al., 2005; Seibel et al., 2006, 2008). The ITS2 secondary structure was illustrated using VARN (Darby et al., 2009).

3. Results

3.1. Fish kills in Tanjung Kupang cage cultures, West Johor Strait

The cage-fish cultures are located along the coasts of Tanjung Kupang, Johor, the West Johor Strait, Malaysia. Nine cages were operated at the area. Most of the affected cages were situated at close quarters between Stn 3 and Stn 4 (Fig. 1). The affected cultured fish included estuary cod (Epinephelus cooides Hamilton), barramundi (Lates calcarifer Bloch), paddletail snapper (Lutjanus gibbus Forsskål) and fourfinger threadfin (Eleutheronema tetradactylum Shaw). Fish kills started on February 11, 2014, becoming more serious during the subsequent three days, and continued for about a week (Fig. 2A–D). The net cages were unable to be moved during the incident and no aeration system was available. About four tonnes of fish died during the incident. Other than the cage-cultured fishes, some small wild fishes in the surrounding area were also affected; their carcasses were found floating and washing away from the coastal area.

The dead fishes observed during the incident showed some symptoms similar to those of other fishes exposed to high levels of karlotoxins (see Mooney et al., 2010). These included protruding eyes and reddening of the iris, discoloured and sloughing skin, reddening at the base of fins, and damaged gills showing a brownish cast (Fig. 2E–I). Human skin irritation, swelling, rashes and itchiness were also observed (Fig. 2J).

3.2. Phytoplankton composition and its related water parameters

A total of 33 taxa were identified from the area: 26 taxa of Bacillariophyceae (19 centric and 7 pennate diatoms) and seven of Dinophyceae. Karlodinium sp., a minute unarmed dinoflagellate, dominated at most of the sampling stations. The average cell density of Karlodinium sp. was 1.25 × 10⁶ cells l⁻¹, with relative abundances of 68.8–98.6% (Fig. 3A). Cell densities increased inwardly along the West Johor Strait; the lowest cell density was observed at Stn 1 (the outermost station; 0.31 × 10⁶ cells l⁻¹), and the highest at Stn 6 (the innermost station; 2.34 × 10⁶ cells l⁻¹) (Fig. 3B). Other dinoflagellates (Ceratium, Prorocentrum, Protoperidinium, Gyrodinium, Ostreopsis) were observed in the samples, but comprised only a minority of the overall phytoplankton composition (<0.2%). Cell densities of these
other dinoflagellates were relatively low ($<4 \times 10^3$ cells$^{-1}$).
 Ostreopsis sp. was found only at Stn 2 (a land reclamation area
 with dredging activity) and Stn 6 (the marina, Puteri Harbour).
 Water depths at the stations ranged from 6–30 m (Fig. 4A). The
 distribution of two basic physical properties (sea-surface tempera-
 ture and pH) in the vicinity of the sampling stations was rather
 homogenous, with temperatures ranging from 27.2–27.5 °C
 (Fig. 4B) and pH of ~8 (Fig. 4C). However, salinity was lower in
 the inner channel of the West Johor Strait ($<30$, Stn 4–6) compared
 to the outer stations (Fig. 4D). A low water visibility ($<0.5$ m) was
 observed, compared to a normal visibility of $\geq 1$ m. The water
 appeared to be stagnant.
3.3. Species description

Samples collected in the area were comprised mainly of a single species of an unarmed dinoflagellate. Detailed morphological observations and comparisons showed that the species resembled *Karlodinium australi*, as described in de Salas et al. (2005).

3.3.1. Morphology

Cells are ovoid (Fig. 5), with a mean length of 22.5 ± 2.15 μm and a mean width of 16.16 ± 1.86 μm (n = 30); the mean length:width ratio is 1.4 ± 0.11 μm. Cells are lightly pigmented, with 7–19 (n = 10) ribbon-shape chloroplasts observed along the cell periphery, distributed in both epì- and hypocone (Fig. 5A–B). The cell nucleus is at the anterior of the cell (Fig. 5A and C). The epicone is slightly smaller and conical compared to the round hypocone (Fig. 5C).

Under SEM, cells are ovoid without a dorsal-ventral compression (Fig. 5D–G). A ventral pore is present to the left of the apical groove, and slightly above the sulcal intrusion. A tube-shaped peduncle-like structure is present, lying along the sulcus (Fig. 5D–E). An apical groove descends 1/3 the length, to the dorsal side of the epicone (Fig. 5F). The deeply excavated premedian cingulum invades into the epicone and descends to the right, and is about two cingulum widths (1/3 the total cell length). A smooth, curly transverse flagellum is observed in the cingulum (Fig. 5G). The amphisema (alveola) is polygonal (mostly pentagonal-hexagonal), with some rounded trychocysts observed (Fig. 5H).

3.3.2. Diagnosis

Cells are larger compared to *Karlodinium antarcticum*, *K. armiger*, *K. ballantinum*, *K. corrugatum* and *K. vitiligo* (Bergbott et al., 2005; de Salas et al., 2008), but are in the range of those observed in the type species, *K. australi* (de Salas et al., 2005). The cingulum displacement was 1/3 the total cell length, which resembles that of *K. corsicum* and *K. decipiens* (de Salas et al., 2008; Siano et al., 2009), and is slightly larger than the type specimens (25% of total cell length; de Salas et al., 2005). Generally, the number of chloroplasts is larger (7–19) than the type species (6–10; de Salas et al., 2005).

3.3.3. Distribution

*Karlodinium australi* is documented for the first time in Malaysian coastal waters, in samples collected along the West Johor Strait. Concurrently, *K. australi* was also found in Aman Island (Penang), the Straits of Malacca. The species was previously reported from Australia (New South Wales [Tuggerah Lake]; Victoria [Port Phillip Bay]; South Australia [Port Lincoln]; Tasmania [Grants Lagoon, Moulting Bay]) (de Salas et al., 2005) and Singapore (de Salas et al., 2008).

3.4. Molecular dataset and phylogenetic inferences

The LSU rDNA D1–D3 nucleotide sequences of *Karlodinium* species were aligned and yielded 900 characters (including gaps), of which 729 were constant and 129 were parsimony informative. The best substitution and rate heterogeneity models of TIM1 + I + G were selected for ML (parameter values set: A = 0.2423, C = 0.1957, G = 0.2851, T = 0.2769; rate matrix of A–C = 1.0000, A–G = 0.8872, A–T = 0.6048, C–G = 0.6048, C–T = 5.3201, G–T = 1.0000, with the proportion of invariable sites estimated at 0.5110 and a gamma shape parameter of 0.6290). For
BI, the best model selected with Bayesian Information Criterion (BIC) was TrN + I + G (parameter values set: $A = 0.2408$, $C = 0.1948$, $G = 0.2865$, $T = 0.2779$; rate matrix of $A$–$C = 1.0000$, $A$–$G = 1.1010$, $A$–$T = 1.0000$, $C$–$G = 1.0000$, $C$–$T = 6.6521$, $G$–$T = 1.0000$; proportion of invariable sites estimated at 0.5070 and a gamma shape parameter of 0.6110).

The LSU rDNA phylogenetic inferences based on MP, ML and BI yielded identical tree topologies. Only the ML tree is shown here, with MP, ML bootstraps supports and BI clade credibility values presented (Fig. 6). The phylogenetic trees consistently revealed a highly support group consisting of the *K. australae* isolates from the West Johor Strait, one strain of *K. australae* from Aman Island (Malacca Strait), one strain from Singapore, and two strains from Australia (including the type specimens, KDACT03) (MP/ML/BI = 100%). Its sister taxon, *K. armiger*, formed the basal of the clade of *K. australae*, with moderately strong bootstrap supports (MP/ML/BI: 75/76/99).

The LSU rDNA sequence divergence of *Karlodinium australae* and *K. armiger* strains ranged from 3.6–4.0%; and were ≥6.5% when compared to other species. The lowest sequence divergence observed was between *K. antarcticum* de Salas and *K. decipiens* de Salas and Laza-Martínez, by a sequence divergence of 0.8–0.9%. The intraspecific LSU rDNA sequence divergence of the eight *K. australae* strains ranged from 0–0.3%, comparable to that in *K. decipiens* (0.1–0.2%), while lower than those in *K. ballantinum* de Salas (0.6%) and *K. veneficum* (0.3–1.1%) (Supplementary Material S1).

### 3.5. ITS2 transcript

The secondary structure of the ITS2 transcript of *Karlodinium* species was determined based on the 5.8S–28S LSU interactions (Keller et al., 2009) (Fig. 7). The structure showed typically four conserved helices (I–IV). The average length of ITS2 in *K. australae*...
was 211 bp. Secondary structure comparisons of ITS2 from three Karlodinium species (K. australae, K. armiger Bergholtz, Daugbjerg and Moestrup and K. veneficum) revealed high structural conservation. The universal motif of pyrimidine–pyrimidine (U–U) mismatch was found in helix II (Fig. 7).

Pairwise comparison of the consensus ITS2 transcript of Karlodinium australae to its sister taxa, K. armiger, showed the presence of four HCBCs: two in helix I (both U–G $\rightarrow$ C–G), one of each in helix III (G–C $\rightarrow$ G–U) and helix IV (G–U $\rightarrow$ G–C), and 16 SNPs were found. No CBCs were observed between the two species. When compared to the type species, K. veneficum, three CBCs were identified: one situated in helix III (U–A $\rightarrow$ C–G) and two in helix IV (both G–C $\rightarrow$ U–A); seven HCBCs, one in helix I (U–G $\rightarrow$ C–G), one in helix II (G–C $\rightarrow$ G–U), three in helix III (two G–C $\rightarrow$ G–U and one C–G $\rightarrow$ U–G), two in helix IV (A–U $\rightarrow$ G–U and G–U $\rightarrow$ A–U), and 23 SNPs were found.

4. Discussion

4.1. Karlodinium bloom in the West Johor Strait

On February 11, 2014, a mass mortality of $\sim$50,000 caged fishes, involving over four species, occurred along the West Johor Strait, Malaysia. A phytoplankton bloom, with patches of water
discoloration, had been witnessed by the farm operators a week prior to the incident (February 6). It is postulated that the Karlodinium austral e cell densities capable of causing the fish kills had been much higher than our observations, because our samples were collected two weeks after the incident (February 20–21), partly due to poor information dissemination. In spite of that, our results showed that the phytoplankton composition was dominated by K. austral e (70–100%), with a mean cell density of 1.25 × 10⁶ cells l⁻¹. The bloom concentration of K. veneficu m associated with previous fish kills was ~6 × 10⁷ cells l⁻¹ (Deeds et al., 2002), and it is capable of forming intense blooms of up to 10⁸ cells l⁻¹ (Place et al., 2012).

Several studies have recognized that, in lieu of regular seasonal blooms, the timing of some dinoflagellates blooms is unpredictable (Train er et al., 2010; Smayda and Trainer, 2010). We theorize that there is a potential link between the outbreak of Karlodinium austral e blooms and the influx of nutrients into the West Johor Strait. This phenomenal, unprecedented bloom was likely

Fig. 5. Karlodinium austral e from the West Johor Strait. (A–C) LM. (A) Cell showing numerous peripheral chloroplasts and dorsal position of nucleus (n). (B) Auto-fluorescent peripheral chloroplasts. (C) Cells showing cell outline and nuclei in the epicone (n). (D–H) SEM. (D) Ventral view of cell showing ventral pore (arrow) and a tube-shaped peduncle-like structure in the sulcus (arrowhead). (E) Ventral view of cell; note the straight apical groove (arrow). (F) Dorsal view of cell showing the extent of straight apical groove (arrow). (G) Cell with flagella. (H) Close-up of alveolae and trichocysts (arrow). Scale bars: 10 μm (A–C); 2 μm (D–G); 1 μm (H).
triggered by anthropogenic nutrients from nearby areas, including land-based and riverine inputs and disturbances caused by ongoing coastal reclamation and maintenance dredging activities (near Stn 2, Fig. 1), which might affect the nutrient dynamics in the strait. These anthropogenic activities may contribute to excess nutrient enrichment that favours the phytoplankton directly, as well as stimulating the growth of its algal prey (cf. Adolf et al., 2008, 2009). The species is known as a mixotroph, consuming cryptophyte prey (de Salas et al., 2005). It is not surprising that the *K. veneficum* bloom may be promoted by the abundance of the cryptophyte prey, as demonstrated by several studies on a closely related species, *K. veneficum* (summarized in Burkholder et al., 2008).

During the outbreak, the area experienced a three-day neap tide, when the water remained stagnant, with very little water exchange. This condition was envisaged to initiate the bloom. The highest concentration of *Karlodinium australis* cells was observed in the innermost station (Stn 6), where a marina is situated. This is in agreement with Garcés et al. (2006), who also showed that cells trend to concentrate in harbours, i.e. areas where waters are less turbulence. At the same time, water currents in the strait move inwards (Goyal and Rathod, 2011), which might also carry the cell population towards Stn 6.

The basic necropsy findings presented here link the toxic *Karlodinium australis* cells to the acute fish kills observed in the West Johor Strait. It is well known that some *Karlodinium* species kill fish, but not all species are capable of producing karlotoxins. Among the ten known species, only *K. armiger*, *K. conicum* de Salas and *K. veneficum* were found to be ichthyotoxic (Garcés et al., 2006; Bachvaroff et al., 2008, 2009; Mooney et al., 2009). The ability of *K. australis* to produce toxins has not yet been elucidated, but the necropsy evidence of dead fish examined in this study show similarities to those reported in naturally occurring or experimental fish kills due to karlotoxins (Deeds et al., 2002, 2006; Kempton et al., 2002). Karlotoxins primarily damage the fish gill epithelia (Deeds et al., 2006; Place et al., 2012), and this was observed in our study.

Other than causing fish mortality, one of the congeners, KmTx2, was found to be toxic to mammalian epithelial cells, neurons, fibroblasts, cardiac myocytes and lymphocytes (Deeds et al., 2004b). The toxins have never been associated with human skin irritation; however, we eye-witnessed this during the sampling survey (Fig. 2). Dermatological problems such as swollen skin and rashes were developed after handling the dead fishes and plankton net samples. Analyses of the ichthyotoxicity and dermal toxicity of *Karlodinium australis* cells are thus pivotal and urgently needed.

### 4.2. Identity of the bloom species responsible for the fish kills in the West Johor Strait

The systematics of unarmored dinoflagellates have undergone several taxonomic modifications, and several species were transferred to the genus *Karlodinium* Larsen. The erection of the genus was based primarily on morphological and ultrastructure

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**Fig. 6.** Phylogenetic inference of *Karlodinium* species based on the large subunit rDNA sequences. The tree is rooted using Takayama tuberculata and T. acrotrocha. Bootstraps value >50% are shown in the order: maximum parsimony (MP)/maximum likelihood (ML)/Bayesian (BI). Branches marked in thick line indicated bootstraps values of 100% for MP/ML/BI. Taxa in boldface indicate isolates from this study.
reassessments, particularly on the character of the complex cell covering, the amphiesma; these are strongly supported by the phylogenetic data and pigment characterization (Daugbjerg et al., 2000). Prior to 2005, there were four known Karlodinium species, viz. K. armiger, K. australic, K. veneficum and K. vitiligo (Ballantine) Larsen (Bergholtz et al., 2005; de Salas et al., 2005). At the same time, Karlodinium micrum (Leadbeater and Dodge) Larsen was synonymized as K. veneficum (Bergholtz et al., 2005), based on the lack of morphological difference and the low genetic divergence of the LSU rDNA marker. Five more Karlodinium species were later described, i.e. K. antarcticum, K. ballantinum, K. conicum, K. corrugatum de Salas and K. decipiens (de Salas et al., 2008). The
most recent, *Gyrodinium circum* Paulmier (*Paulmier et al., 1995*), was revised to *K. circums* (Paulmier) Siano and Zingone (*Siano et al., 2009*); hence, the genus comprises ten species. The cells collected during this study were ovoid and small. SEM observation revealed typical *Karlodinium* characteristics, such as a straight apical groove and the presence of a ventral pore. Based on the outer morphology and the chloroplast numbers, we designated the species as *K. austral*e. Precise identification of species in this genus has been difficult due to their morphological plasticity. Bergholtz et al. (2005) claimed that sample preparation prior to electron microscopy might produce artefacts; morphological characteristics such as amphiaesa vesicles tend to deform during the fixation process. de Salas et al. (2008) also suspected that culture artefacts might distort the morphology of these delicate dinoflagellates. Hence, a total-evidence approach sensu McManus and Katz (2009), applying both detailed morphological assessment and molecular evidence, was used to support the species identity of this tiny dinoflagellate. In this study, we adopted the single-cell PCR technique, applied to field samples, and found efficient amplification of the targeted genetic markers (LSU and ITS rDNA), with nucleotide sequences successfully obtained for subsequent molecular characterizations. The sensitivity of PCR allows the amplification of minute amounts of nucleic acid from a single cell. The single-cell PCR technique is useful when the sample amount is limited, and is a powerful molecular technique for diagnosis or detection at the single-cell level (e.g. Bolch, 2001; Ki et al., 2005; Kai et al., 2006; Auinger et al., 2008). The phylogeny of *Karlodinium* based on LSU rDNA was well resolved, and the phylogenetic position of *K. austral*e is in accordance with that of Bergholtz et al. (2005) and de Salas et al. (2008). The species is genetically distinct from other *Karlodinium* species based on its sequence divergence, forming a sister group with *K. armiger* in our phylogenetic analyses. In addition, we applied another biological species marker, the secondary structure of ITS2 of the nuclear-encoded ribosomal RNA gene, to confirm the species identity. The CBCs in the secondary structure of ITS2 have proven useful for delimiting biological species (e.g. Coleman, 2003; Müller et al., 2007). As demonstrated by Müller et al. (2007), the presence of at least one CBC is a good indicator that two organisms belong to distinct species, while the absence of CBCs is not necessarily an indicator that they belong to the same species. The species in this study is readily distinguished from the toxic *Karlodinium veneficum*, with a difference of three CBCs. It is noteworthy that pairwise comparisons of CBCs in the ITS2 transcribes of *K. austral*e in this study, and its sister species *K. armiger*, showed four HBCBs, although no CBCs were found. It should be noted that when the structural features are compared among the *Karlodinium* ITS2 transcribes, helix IV showed the most variable base-pairing interaction (CBCs and HBCBs) among species. The identification of *Karlodinium austral*e in this study was supported not only by morphological evidence, but also by molecular data. This is the first report of *K. austral*e in Malaysia, and the first to associate it with a fish-kill event in the world. This ichthyotoxic species warrants a thorough long-term study, as it threatens not only the economy but also the welfare of local fishermen and breeders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhal.2014.10.005.

References


