



Faculty of Resource Science and Technology

**Species Diversity of the Ichthyotoxic Dinoflagellates Kareniaceae
(Gymnodiniales) in Malaysia Waters Based on Morphology and
Molecular Analysis, and Their Toxicologic Effect towards the Brine
Shrimp (*Artemia* sp.) and Fish Fingerling (*Lates calcarifer*)**

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Analysis, and Their Toxicologic Effects towards the Brine Shrimp (*Artemia*
sp.) and Fish Fingerling (*Lates calcarifer*)

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Karenaceae is a known family of naked dinoflagellates associated with harmful algal blooms (HABs) worldwide is initially made up of three genera namely *Karenia*, *Karlodinium* and *Takayama*, and four recently established genera *Asterodinium*, *Brachidinium*, *Gertia*, and *Shimiella*. In Malaysia, *Karlodinium australe* had reported as the culprit in mass fish mortality in West Johor Straits in 2014 and the recurrence a year later had caused greater loss of mariculture. The aims of this study were to investigate the species diversity of Karenaceae in Malaysia waters by morphological and molecular means, to investigate the survival of fish exposed to different cell densities and cell conditions of *Karlodinium australe*, and to examine the toxigenic effect of family Karenaceae towards the brine shrimp *Artemia* sp. and fish. A total of 59 strains of Karenaceae were established from the waters of Peninsular Malaysia and Malaysian Borneo. Ten Karenaceae species, *Karenia mikimotoi*, *Karlodinium armiger*, *K. australe*, *K. azanzae*, *K. ballantini*, *K. decipiens*, *K. gentienii*, *K. veneficum*, *K. zhouanum* and *Takayama acrotricha*, were confirmed morphologically by light and scanning electron microscopy, and identified by key features such as cell shape, cell size, apical groove, ventral pore, and nucleus placement with the well-described species. Molecular phylogeny of internal transcribed spacer (ITS1–5.8S–ITS2) and large-subunit (LSU) ribosomal DNA were further confirmed the identity of the ten species above revealing polyphyletic clade of *Karenia*, *Takayama* and *Karlodinium* species. ITS2 secondary-structure transcript was applied to further support the species delineation of the *Karlodinium* species with presence of the Compensatory Base Changes (CBCs) and Hemi-Compensatory Base Changes (hCBCs) between the closely related species. Bioassay was conducted by exposing brine shrimp *Artemia* sp. to each Karenaceae species in this study. Naupliar mortality was observed at initial 6-h of exposure

and mortality rates increased to 50% after 24-h exposure. The 100% mortality of naupliar was observed after 48 hours in *K. australe* (cell density: 2.3×10^3 cells mL⁻¹), *K. azanzae* (2.3×10^3 cells mL⁻¹), *K. veneficum* (2.8×10^3 cells mL⁻¹), *K. armiger* (2.3×10^3 cells mL⁻¹), *K. gentienii* (2.7×10^3 cells mL⁻¹), and *T. acrotrocha* (3.0×10^3 cells mL⁻¹). In addition, further bioassay was conducted by exposing 1-inch fingerling seabass *Lates calcarifer* to *K. australe* in different cell densities and conditions. Fish mortality was observed at the first 10 min exposure at cell densities of 2.4×10^3 cells mL⁻¹, 2.1×10^3 cells mL⁻¹, and 1.8×10^3 cells mL⁻¹, and 100% mortality occurred within 24-h at cell densities of 2.4×10^3 cells mL⁻¹, 2.1×10^3 cells mL⁻¹, 1.8×10^3 cells mL⁻¹, 1.7×10^3 cells mL⁻¹, and 1.5×10^3 cells mL⁻¹. The result in this study showed *K. mikimotoi*, *K. armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. veneficum*, and *T. acrotrocha* were lethal to brine shrimp, and *K. australe* is toxic and harmful to fish. The presence of ten Kareniceae species in this study is the first record on high diversity family Kareniaceae in Malaysia waters, where two species were recorded in Johor and Perak waters, five species recorded in Sarawak waters and seven species recorded in Sabah waters.

Keywords: Bioassay, first record, ITS, Kareniaceae, *Karenia*, *Karlodinium*, LSU, morphology, phylogeny, *Takayama*, toxicity, Malaysia

Kepelbagaian Spesies ‘Ichthyotoxic’ Dinoflagellat Kareniaceae (Gymnodiniales) di Perairan Malaysia berdasarkan Analisa secara Morfologi dan Molekular, dan Kesan Toksikologik Mereka terhadap Udang Air Masin (Artemia sp.) dan Anak Ikan (Lates calcarifer)

ABSTRAK

Kareniaceae merupakan keluarga dinoflagellat atekat terkenal dikaitkan dengan fenomena ledakan alga merbahaya (HAB) di seluruh dunia yang pada awalnya terdiri daripada tiga genera, Karenia, Karlodinium dan Takayama, dan genera yang baru ditubuhkan iaitu Asterodinium, Brachidinium, Gertia, dan Shimiella. Di Malaysia, Karlodinium australe telah dilaporkan sebagai penyebab kematian ikan secara besar-besaran di Selat Johor, 2014 dan kejadian berulang setahun kemudian telah menyebabkan kerugian besar dalam marikultur. Tujuan kajian ini adalah untuk menyiasat kepelbagaian spesies Kareniaceae di perairan Malaysia dengan cara morfologi dan molekul, untuk menyiasat kelangsungan hidup ikan yang terdedah kepada kepadatan sel dan keadaan sel K. australe yang berbeza, serta mengkaji kesan bertoksin keluarga Kareniaceae terhadap udang air masin Artemia sp. dan ikan. Sebanyak 59 strain sel Kareniaceae telah diperoleh dari perairan Semenanjung Malaysia dan Borneo Malaysia. Sepuluh spesies Kareniaceae, Karenia mikimotoi, Karlodinium armiger, K. australe, K. azanzae, K. ballantinum, K. decipiens, K. gentienii, K. veneficum, K. zhouanum dan Takayama acrotrocha, telah dianalisa secara morfologi oleh pencahayaan dan pengimbasan mikroskop elektron, dan dikenalpasti melalui ciri utama seperti bentuk sel, saiz sel, apikal alur, liang ventral dan penempatan nukleus. Filogeni menggunakan ‘internal transcribed spacer’ (ITS1–5.8S–ITS2) dan DNA ribosom subunit besar (LSU) selanjutnya mengesahkan identiti sepuluh spesies di atas dengan mendedahkan hubungan polifiletik spesies Karenia, Takayama dan Karlodinium. Transkrip struktur sekunder ITS2 digunakan untuk menyokong persempadanan spesies Karlodinium dengan

kehadiran 'compensatory base-changes' (CBCs) dan 'hemi-compensatory base changes' (hCBCs) antara spesies yang berkait rapat. Bioassai dijalankan dengan mendedahkan udang air masin Artemia sp. kepada setiap spesies Kareniaceae dalam kajian ini. Kematian nauplius diperhatikan pada awal 6-jam pendedahan dan kadar kematian meningkat kepada 50% selepas pendedahan 24-jam. Kematian 100% nauplius diperhatikan setelah 48-jam di K. australe (kepadatan sel: 2.3×10^3 sel mL^{-1}), K. azanzae (2.3×10^3 sel mL^{-1}), K. veneficum (2.8×10^3 sel mL^{-1}), K. armiger (2.3×10^3 sel mL^{-1}), K. gentienii (2.7×10^3 sel mL^{-1}), dan T. acrotrocha (3.0×10^3 sel mL^{-1}). Di samping itu, bioassai dilanjutkan dengan mendedahkan 1-inci anak ikan siakap Lates calcarifer kepada K. australe dalam kepadatan dan keadaan sel yang berbeza. Kematian ikan diperhatikan pada pendedahan 10-minit pertama dalam kepadatan sel 2.4×10^3 sel mL^{-1} , 2.1×10^3 sel mL^{-1} , dan 1.8×10^3 sel mL^{-1} , dan kematian 100% berlaku di dalam 24-jam pendedahan dalam kepadatan sel 2.4×10^3 sel mL^{-1} , 2.1×10^3 sel mL^{-1} , 1.8×10^3 sel mL^{-1} , 1.7×10^3 sel mL^{-1} , dan 1.5×10^3 sel mL^{-1} . Hasil kajian ini telah menunjukkan K. mikimotoi, K. armiger, K. australe, K. azanzae, K. ballantinum, K. decipiens, K. gentienii, K. veneficum, dan T. acrotrocha adalah berbahaya kepada udang air masin, dan K. australe adalah toksik dan berbahaya kepada ikan. Kehadiran sepuluh spesies Kareniaceae di dalam kajian ini adalah rekod pertama kepelbagaian tinggi keluarga Kareniaceae di perairan Malaysia, dua spesies direkod di perairan Johor dan Perak, lima spesies direkod di perairan Sarawak dan tujuh spesies direkod di perairan Sabah.

Kata kunci: Bioassai, rekod pertama, ITS, Kareniaceae, Karenia, Karlodinium, LSU, morfologi, filogeni, Takayama, ketoksikan, Malaysia

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENT	ii
ABSTRACT	iii
ABSTRAK	v
TABLE OF CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xx
CHAPTER 1 INTRODUCTION	1
1.1 Study background	1
1.2 Problem statement	8
1.3 Objectives	10
1.4 Chapter Summary	11
CHAPTER 2 LITERATURE REVIEW	12
2.1 Taxonomical history of family Kareniaceae	12
2.2 Morphological characterisation of family Kareniaceae	17
2.2.1 Genus <i>Karenia</i>	17

2.2.2	Genus <i>Karlodinium</i>	21
2.2.3	Genus <i>Takayama</i>	27
2.3	Molecular characterisation of family Kareniaceae	29
2.4	ITS2 secondary structure	33
2.5	Toxicity of family Kareniaceae (Gymnodiniales)	36
2.5.1	Lethality assay	37
2.5.2	Worldwide bloom events	40
2.6	Kareniaceae blooms in Malaysia and Southeast Asia	45
CHAPTER 3 MATERIALS AND METHODS		48
3.1	Sampling sites and water sample processing	48
3.2	Single-cell isolation, culture establishment and maintenance	49
3.3	Morphological characterisation	53
3.3.1	Light microscopy (LM)	53
3.3.2	Scanning electron microscopy (SEM)	53
3.4	Molecular characterisation	54
3.4.1	Genomic DNA extraction, gene amplification, purification and sequencing	54
3.4.2	Taxon sampling and phylogenetic analysis	56
3.4.3	ITS2 secondary structure modelling and sequence-structure phylogeny	56
3.5	Lethality assay of <i>Lates calcarifer</i> fingerlings	57
3.5.1	Fish husbandry and acclimatisation	57

3.5.2	Toxicity testing in different <i>Karlodinium australe</i> cell densities and cell condition	57
3.6	Lethality assay of brine shrimp	58
3.6.1	Brine shrimp cyst hatching and decapsulation	58
3.6.2	Toxicity testing towards brine shrimp	58
3.7	Data analysis	59
CHAPTER 4 RESULTS		60
4.1	Species diversity	60
4.2	Species diagnoses	61
4.2.1	Morphology of <i>Karenia mikimotoi</i>	67
4.2.2	Morphology of <i>Karlodinium armiger</i>	69
4.2.3	Morphology of <i>Karlodinium australe</i>	71
4.2.4	Morphology of <i>Karlodinium azanzae</i>	73
4.2.5	Morphology of <i>Karlodinium ballantinum</i>	75
4.2.6	Morphology of <i>Karlodinium decipiens</i>	77
4.2.7	Morphology of <i>Karlodinium gentienii</i>	79
4.2.8	Morphology of <i>Karlodinium veneficum</i>	81
4.2.9	Morphology of <i>Takayama acrotrocha</i>	83
4.3	Phylogenetic inferences	85
4.4	ITS2 sequence-structure information	89

4.5	Toxicity screening of Kareniaceae in brine shrimp lethality	93
4.6	Acute toxicity of <i>Karlodinium australe</i> on <i>Lates calcarifer</i> fingerling	94
4.7	Micropredation of <i>Karlodinium</i>	98
CHAPTER 5 DISCUSSION		100
5.1	Species diversity and geographical distribution	100
5.2	Morphological characterisation Kareniaceae	102
5.3	Species boundaries among closely related <i>Karlodinium</i> species	104
5.4	ITS2 secondary structure information of <i>Karlodinium</i>	105
5.5	Acute toxicity of <i>Karlodinium australe</i> towards <i>Lates calcarifer</i> fingerlings	108
5.6	Toxicity screening of Kareniaceae in brine shrimp lethality	110
5.7	Mode of action of <i>Karlodinium</i> in lethality assay	114
CHAPTER 6 CONCLUSION AND RECOMMENDATIONS		117
6.1	Conclusion	117
6.2	Recommendations	118
REFERENCES		119
APPENDICES		151

LIST OF TABLES

	Page
Table 3.1 List of Kareniaceae strains established and used in this study, with localities, and isolation dates	49
Table 3.2 Primer used to amplify LSU rDNA and ITS of Kareniaceae in this study	54
Table 3.3 Composition of PCR mastermix used in this study	55
Table 3.4 PCR thermal cycling steps used in this study	55
Table 4.1 Kareniaceae species list, distribution and number of species found in this study	61
Table 4.2 Morphometric descriptions of <i>Karenia mikimotoi</i> , <i>Karlodinium armiger</i> , <i>Karlodinium australe</i> , <i>Karlodinium azanzae</i> , <i>Karlodinium ballantinum</i> , <i>Karlodinium decipiens</i> , <i>Karlodinium gentienii</i> , <i>Karlodinium veneficum</i> and <i>Takayama acrotrocha</i> in Malaysian coastal waters obtained in this study. All measurements were taken by LM and SEM	62
Table 4.3 Number of CBC found in the comparison of ITS2 transcript among <i>Karlodinium veneficum</i> and <i>Karlodinium australe</i> with close related <i>Karlodinium</i> species	92
Table 4.4 Number of HCBC found in the comparison of ITS2 transcript among <i>Karlodinium veneficum</i> and <i>Karlodinium australe</i> with close related <i>Karlodinium</i> species	93

LIST OF FIGURES

		Page
Figure 2.1	Morphological characteristic of <i>Karenia</i> . (A) Ventral view, (B) Dorsal view, (C) Arrangement of nucleus (nu, bounded by solid line), apical groove (ag), chloroplasts (ch), transverse flagellum (tf), sulcus at hypocone (sh), longitudinal flagellum (lf), sulcus at middle (sm) and cingulum (ci). Adapted from Yang et al. (2001)	18
Figure 2.2	Diagram of <i>Karenia</i> morphology ventral view drawn to the same scale with nucleus (n, bounded by dash line). (A) <i>Karenia asterichroma</i> (De Salas et al., 2004), (B) <i>Karenia bicuneiformis</i> (Botes et al., 2003), (C) <i>Karenia brevis</i> (De Salas et al., 2004), (D) <i>Karenia brevisulcata</i> (Chang, 1999), (E) <i>Karenia concordia</i> (Chang & Ryan, 2004), (F) <i>Karenia cristata</i> (Botes et al., 2003), (G) <i>Karenia hui</i> (Cen et al., 2024), (H) <i>Karenia longicanalis</i> (De Salas et al., 2004), (I) <i>Karenia mikimotoi</i> (Takayama & Adachi, 1984), (J) <i>Karenia papilionacea</i> (Haywood et al., 2004), and (K) <i>Karenia selliformis</i> (Haywood et al., 2004). Scales, 20 µm	21
Figure 2.3	Morphological characteristics of <i>Karlodinium</i> . (A) Ventral view, (B) Arrangement of nucleus (bounded by dash line), and chloroplasts (stippled). Scale bar, 5 µm. Adapted from Bergholtz et al. (2006)	22
Figure 2.4	Diagram of <i>Karlodinium</i> morphology ventral view drawn to the same scale with nucleus (n, bounded by dash line). (A) <i>Karlodinium</i>	26

antarcticum (De Salas et al., 2008), (B) *Karlodinium armiger* (Bergholtz et al., 2006), (C) *Karlodinium australe* (De Salas et al., 2005), (D) *Karlodinium azanzae* (Benico et al., 2020), (E) *Karlodinium ballantini* (De Salas et al., 2008), (F) *Karlodinium conicum* (De Salas et al., 2008), (G) *Karlodinium corrugatum* (De Salas et al., 2008), (H) *Karlodinium corsicum* (Paulmier et al., 1995), (I) *Karlodinium decipiens* (De Salas et al., 2008), (J) *Karlodinium digitatum* (Yang et al., 2000), (K) *Karlodinium elegans* (Cen et al., 2020), (L) *Karlodinium gentienii* (Nézan et al., 2014), (M) *Karlodinium jejuense* (Li et al., 2018), (N) *Karlodinium veneficum* (Daugbjerg et al., 2000), (O) *Karlodinium vitiligo* (Ballantine, 1956), and (P) *Karlodinium zhouanum* (Luo et al., 2018). Scales, 20 μm

Figure 2.5 Morphological characteristic of *Takayama*. (A) Ventral view, (B) 27
Position of nucleus (n, bounded by dash line), pyrenoid (py, bounded by solid line), and chloroplasts (shaded). Scale bar, 10 μm . Adapted from De Salas et al. (2003)

Figure 2.6 Diagram of *Takayama* morphology ventral view drawn to the same 29
scale with nucleus (n, bounded by dash line). (A) *Takayama tuberculata* (De Salas et al., 2008), (B) *Takayama tasmanica* (De Salas et al., 2008), (C) *Takayama helix* (De Salas et al., 2008), (D) *Takayama pulchella* (Larsen, 1994), (E) *Takayama acrotrocha* (Larsen, 1996), and (F) *Takayama cladochroma* (Larsen, 1996). Scales, 10 μm

Figure 2.7	Structure of nuclear ribosomal RNA consisted of SSU (18S), ITS and LSU (28S). Adapted from Kauserud (2023)	31
Figure 2.8	Homologous modelling of ITS2 secondary structures consisted of four helices. Adapted from Kocot & Santos (2009)	35
Figure 3.1	Map showing sampling locations in this study at Peninsular Malaysia: Perak (Marina Island) and Johor (Johor Bahru), and East Malaysia: Kuching, Sarawak (Batang Salak, Santubong, Sematan and Telaga Air) and Kota Kinabalu, Sabah (Jesselton Point, KK Fish Market, Sabah Park, Sepanggar Bay and Tuaran)	49
Figure 3.2	Experimental design of fingerlings <i>Lates calcarifer</i> lethality assay. (A) Exposure to different cell concentrations of <i>Karlodinium australe</i> . (B) Exposure to different cell conditions of <i>Karlodinium australe</i>	58
Figure 3.3	Experimental design of brine shrimp <i>Artemia</i> sp. lethality assay to <i>Karenia mikimotoi</i> , <i>Karlodinium armiger</i> , <i>Karlodinium azanzae</i> , <i>Karlodinium australe</i> , <i>Karlodinium ballatinum</i> , <i>Karlodinium decipiens</i> , <i>Karlodinium gentienii</i> , <i>Karlodinium veneficum</i> , <i>Takayama acrotrocha</i> , including starvation and fed control	60
Figure 4.1	Map showing species distribution and species diversity of family Kareniaceae in the coastal waters of Peninsular Malaysia and East Malaysia	61
Figure 4.2	<i>Karenia mikimotoi</i> . (A-E) LM. Live cells showing apical groove (arrowhead) and nucleus (N). (D-E) SYBR Safe-stained cells with an	68

ellipsoid nucleus (N) located on the left side of hypocone nearing the edge of cell. (F-H) SEM. Straight apical groove (arrowhead) in apical (F), ventral (G) and dorsal (H) views. Scales, 5 μ m

- Figure 4.3 *Karlodinium armiger*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, spherical nucleus (N) located at the centre of the cell. (F-I) SEM. Straight apical groove (arrowhead) extends from ventral to dorsal and ventral pore (vp) in apical view (F) and ventral views (G-I). Scales, 5 μ m 70
- Figure 4.4 *Karlodinium australe*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, elongated spherical nucleus (N) located at the centre of the cell. (F-I) SEM. Straight apical groove (arrowhead) extends from ventral to dorsal and ventral pore (vp) in apical view (F) and ventral views (G-I). Scales, 5 μ m 72
- Figure 4.5 *Karlodinium azanzae*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with an elongated or spherical nucleus (N) located at the centre of the cell. (F-H) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F), ventral (G) and dorsal (H) views. Scales, 5 μ m 74
- Figure 4.6 *Karlodinium ballantinum*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR 76

- Safe-stained cells with a large, spherical nucleus (N) located at the centre of the cell. (F-H) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F) and ventral (G-H) views. Scales, 5 μ m
- Figure 4.7 *Karlodinium decipiens*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a spherical nucleus (N) located at the centre of the cell. (F-H) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F), ventral (G-H) dorsal views. Scales, 5 μ m 78
- Figure 4.8 *Karlodinium gentienii*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, ellipsoid nucleus (N) located at the centre of the cell. (F-I) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F), ventral (G) extending to dorsal views (H-I). Scales, 5 μ m 80
- Figure 4.9 *Karlodinium veneficum*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, spherical nucleus (N) located at the centre of the cell. (G-H) SEM. Sigmoid apical groove (arrowhead) extends to dorsal in ventral view (G), and apical views (H). Scales, 5 μ m 82
- Figure 4.10 *Takayama acrotrocha*. (A-F) LM. Live cells showing apical groove (arrowhead) and nucleus (N). (D-F) SYBR Safe-stained cells with a large, transversely ellipsoid nucleus (N) occupies the whole epicone. (F-H) SEM. Sigmoid apical groove (arrowhead) extend from 84

proximal end of the girdle around the cell apex in ventral view (G) and apical view (F). Scales, 5 μ m

- Figure 4.11 Phylogram of LSU rDNA consensus tree for maximum parsimony (MP), maximum likelihood (ML), and Bayesian Inference (BI) tree rooted with *Gyrodinium dominans*. Nodal supports are bootstrap values of MP, ML, and posterior probability of BI; which only values >50% supports are indicated. The strains and species in this study are in bold. Dash represents support values <50 87
- Figure 4.12 Phylogram of ITS1-5.8S-ITS2 rDNA consensus tree for maximum parsimony (MP), maximum likelihood (ML), and Bayesian Inference (BI) tree rooted with *Prorocentrum micans*. Nodal supports are bootstrap values of MP, ML, and posterior probability of BI; which only values >50% supports are indicated. The strains and species in this study are in bold. Dash represents support values <50 88
- Figure 4.13 ITS2 RNA transcripts of *Karlodinium veneficum* with closely related species, viz. *Karlodinium ballantinum*, *Karlodinium gentienii* and *Karlodinium zhouanum*. Shaded rectangles indicate CBCs or HCBCs 90
- Figure 4.14 ITS2 RNA transcripts of *Karlodinium australe* with closely related species, viz. *Karlodinium azanzae* and *Karlodinium armiger*. Shaded rectangles indicate CBCs or HCBCs 91
- Figure 4.15 Survival of brine shrimp *Artemia* sp. exposed to *Karenia mikimotoi* (KMSPBUD5), *Karlodinium armiger* (KARMJPGC5), *Karlodinium australe* (KAJBL05, KABSUF2), *Karlodinium azanzae* 94

(KAZSPBGD9), *Karlodinium ballatinum* (KBTAUB3), *Karlodinium decipiens* (KDSPBLB11), *Karlodinium gentienii* (KGKKGD3), *Karlodinium veneficum* (KVTAUD8), and *Takayama acrotrocha* (TYTAUF4). *Tetraselmis chuii* was used as fed control. Data points represent triplicate mean $\pm$ SD

- Figure 4.16 Survival of seabass *Lates calcarifer*. (A) Exposure of *Lates calcarifer* to *Karlodinium australe* Johor strain (KAJB05) at five cell concentrations: 1.5×10^3 cells mL⁻¹, 1.7×10^3 cells mL⁻¹, 1.8×10^3 cells mL⁻¹, 2.1×10^3 cells mL⁻¹ and 2.4×10^3 cells mL⁻¹. (B) Exposure of *Lates calcarifer* to *Karlodinium australe* Sarawak strain (KATAL01) at five cell concentrations: 0.6×10^3 cells mL⁻¹, 1.0×10^3 cells mL⁻¹, 1.2×10^3 cells mL⁻¹, 1.4×10^3 cells mL⁻¹ and 1.8×10^3 cells mL⁻¹. GPM medium was used as a starvation control. Data points represent triplicate mean $\pm$ SD 96
- Figure 4.17 Survival of seabass *Lates calcarifer* exposed to *Karlodinium australe* Sarawak strain (KATAL01) of four cell conditions: live cells, sonicated cell, culture filtrate and sonicated cell filtrate. GPM medium was used as a control. Data points represent triplicate mean $\pm$ SD 97
- Figure 4.18 Observation of brine shrimp *Artemia*. (A–C) Cells were attached onto immobile nauplii in the exposure towards *Karlodinium australe*, (D–E) *Karlodinium azanzae* and (F–H) *Karlodinium armiger*. (C) The peduncle protruded from *Karlodinium australe* cell and 99

penetrated the dead nauplii (arrowhead) to feed by sucking out the content. Scales, 20 μm

LIST OF ABBREVIATIONS

µm	Micrometre
µmol	Micromole
BI	Bayesian inference
bp	Base pair
CBC	Compensatory base change
D1-D3	Domain 1 to domain 3
ddH ₂ O	Deionised water
dNTP	Deoxyribonucleotide triphosphate
GPM	General purpose medium
HAB	Harmful algal bloom
HCBC	Hemi-compensatory base change
ITS	Internal transcribed spacer
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
LM	Light microscopy
LSU	Large subunit ribosomal
ML	Maximum likelihood
MP	Maximum parsimony

NCBI	National Center for Biotechnology Information
Nm	Nanometre
PAUP	Phylogenetic analysis using parsimony
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
SEM	Scanning electron microscopy
sp.	Species
TBR	Tree-bisection reconnection
Vp	Ventral pore

CHAPTER 1

INTRODUCTION

1.1 Study background

Sudden high biomass of phytoplankton in the aquatic ecosystem was a criterion for blooms formation which worsening water quality, development of hypoxia and anoxia condition in the water, toxicity, mortality of halobios, and modification in food web (Paerl et al., 2001). The phrase ‘harmful algal blooms’(HABs) covers a various set of events has two common features that are caused by microalgae and have adverse effect to human activities (Zingone & Oksfeldt Enevoldsen, 2000; Kazmi et al., 2022). Non-HABs usually cause water discolouration without known detrimental effect can be beneficial for the activities aquaculture and wild fisheries (Hallegraeff, 2003), whereas HABs often associated with adverse impact on environment health, human health as well as socio-economic factor (Kyewalyanga, 2016; Käse & Geuer, 2018). The term “red tide” often used to describe bloom event with water discolouration irrespective of any particular colour as red is the most common pigment (Okaichi, 2004; Guy, 2005). HABs can be classified into three types; first, harmless water discolouration that caused indiscriminate death of fish and invertebrates by anoxia condition, second, non-toxic to human but deteriorated or clogged the gills of fish and invertebrates in intensive aquaculture systems, and third, trophic transfer of potent toxins produced by causative species to human via consumption of shellfish that resulted in gastrointestinal and neurological illnesses (Hallegraeff, 2003).

Most causative species of HABs belong to six different microalgae groups including cyanophytes, diatoms, dinoflagellates, haptophytes, raphidophytes and pelagophytes (Zingone & Oksfeldt Enevoldsen, 2000). Amongst the mentioned microalgae groups,

blooms attributed by diatom, dinoflagellate and cyanobacteria had gained researchers' concern due to the high biomass, long period and toxin produced (Sha et al., 2021). The need to identify the causative phytoplankton species had become apparent following the growing activities in aquaculture sectors, recurrence of harmful algae bloom event including reported toxicity of certain phytoplankton species (Tomas & Hasle, 1997).

One of dinoflagellate families well-known for causing HAB events worldwide is family Kareniaceae. The three most common genera of Kareniaceae, *Karenia*, *Karlodinium*, and *Takayama* have been identified as containing HAB-causative species due to their ability of producing toxins and harmful substances that not just kill marine life, fish, and other animals, but also cause respiratory illnesses in humans and neurotoxic shellfish poisoning (NSP)(Zhang et al., 2022a). Among all the species in Kareniaceae, the longest history of widespread distribution with detrimental impacts of marine life was shared by two species of the genus *Karenia* which were *Karenia mikimotoi* and *K. brevis*. *K. mikimotoi* is a worldwide bloom-forming dinoflagellate species and had been observed occurring along the coast of Pacific, Atlantic, Indian Ocean, and even in temperate coastal waters (Li et al., 2019). The earliest verified record of *K. mikimotoi* red tide was in the year 1934 occurring in Japan had caused mortality to the fish and shellfish in the coastal of Gokasho Bay, Honshu (Oda, 1935). In the year 1966, *K. mikimotoi* bloom had resulted in brown water and fish mortality along the Norwegian coast (Braarud, 1970). From 1988 to 1989, the blooms of *K. mikimotoi* in South Africa had resulted in mortalities of chitons, limpets, abalone, periwinkles, whelks, black mussels, alikreukel, octopus and starfish, as well as posing health risk by causing irritations to the eye, nose and lung in bathers and fishermen (Horstman et al., 1991).

Likewise, *K. brevis* had the lengthiest documented history of ichthyotoxic bloom with the initial verified record of red tides in the Gulf of Mexico since the year 1648 (Magaña et al., 2003). The occurrence of red tides on the lower West Coast of Florida from late 1946 to 1947 had caused fatalities to mass number of marine animals (Gunter et al., 1948), and in April 1947, water discolouration and fish mortality were also observed in the Gulf of Mexico off the Florida Keys, and *K. brevis* was reported to be associated with these bloom episodes (Davis, 1948; Galtsoff, 1954). *K. brevis* also had been reported forming red tides from the West Atlantic, Spain, Greece, Japan and New Zealand (Davis, 1948). Mass occurrence of *K. brevis* in Greek Gulf of Saronikos caused massive fish kill in the year 1978 (Satsmadjis & Friligos, 1983). Mass occurrence of *K. brevis* in Greek Gulf of Saronikos caused massive fish kill in the year 1978 (Satsmadjis & Friligos, 1983). In the 21st century, the blooms of *K. brevis* still occurring in few areas in three West Florida Shelf estuaries such Caloosahatchee River, Charlotte Harbor, and Tampa Bay from 2020 to 2023 (Hall et al., 2024).

Apart from genus *Karenia*, genus *Karlodinium* is also well-known for fish-killing bloom event and among *Karlodinium* species, *Karlodinium veneficum* had the earliest history of mass occurrence. Braarud (1957) had documented that *K. veneficum* (formerly known as *Glenodinium galatheanum*) was first discovered in Walvis Bay, Namibia during a Danish Deep-sea Expedition in 1950 and subsequent studies from 1964 to 1967 had reported *K. veneficum* as the cause of red tide that occurred in the bay at the same time with mass fish mortality event. In the year 1994, recurrent fish kills in Alfacs Bay, Spain occurred during winter bloom were attributed to the dinoflagellate species of genus *Karlodinium*, identified as *K. veneficum* and *K. armiger* (Garcés et al., 2006). *K. veneficum* had been associated with fish kill incidence in a *Sparus aurata* gilthead seabream aquaculture farm during bloom events in Chesapeake Bay since 1996 (Deeds et al., 2006). The recurrent blooms in 1997

and 1999 also resulted in mortalities of cultured fish (Deeds et al., 2006). In South Carolina brackish water ponds, a dinoflagellate bloom associated to fish kill event in 2002 was identified to be caused by *K. veneficum* (Kempton et al., 2002). Fish mortalities were reported of co-occurred with *K. veneficum* bloom in 2003 in the upper Swan River and Canning River estuaries, Australia (Adolf et al., 2015). In 2006, an intense bloom of *K. veneficum* was reported in Neuse River estuary, north Carolina and this bloom event was linked to four subsequent fish mortalities (Hall et al., 2008).

Since the 1990s, *Takayama* species have rarely been accountable for forming blooms, as series of HAB phenomenon in Kareniaceae family was mostly caused by species of *Karenia* and *Karlodinium* (Zhang et al., 2022a). Genus *Takayama* was first found as bloom-forming dinoflagellate during occurrence of red tide occurred in Kagoshima Bay, Japan, in 1984 and the bloom posed a severe threat to yellowtail fish farm in the area (Onoue et al., 1985). The bloom of *Takayama pulchella* in Indian River, Florida in 2013 was associated with mass fish kills (Tang et al., 2012). In Singapore coastal water, *T. acrotrocha* was reported of forming bloom but no fish kills occurred during the bloom (Tang et al., 2012). During a cruise survey in Haizhou Bay, China, a bloom caused by *Takayama* sp. was detected in the year 2020 (Zhang et al., 2022a).

Kareniaceae was described in 2005 as a naked dinoflagellate family in the order of Gymnodiniales (Bergholtz et al., 2006). The diversity within Kareniaceae family is poorly understood because of their small in size, minimal morphological divergence, alongside low abundance in non-bloom periods (Zhang et al., 2022b). Kareniaceae was originally composed of three gymnodinioid genera *Karenia*, *Karlodinium*, and *Takayama* (Daugbjerg et al., 2000; Bergholtz et al., 2006; de Salas et al., 2008). To date, a total of 33 species are accepted taxonomically in family Kareniaceae that consists of *Karenia*, *Karlodinium*, and

Takayama (Guiry & Guiry, 2023). Ten species of genera *Karenia* are *Karenia asterichroma*, *K. bicuneiformis*, *K. brevis*, *K. brevisulcata*, *K. concordia*, *K. cristata*, *K. longicanalis*, *K. mikimotoi*, *K. papilionacea*, and *K. selliformis*. For genera *Karlodinium*, 16 species are *Karlodinium antarcticum*, *K. armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. conicum*, *K. corrugatum*, *K. corsicum*, *K. decipiens*, *K. digitatum*, *K. elegans*, *K. gentienii*, *K. jejuense*, *K. veneficum*, *K. vitiligo*, and *K. zhouanum*. In genera *Takayama*, the seven species are *Takayama acrotrocha*, *T. cladochroma*, *T. helix*, *T. pulchella*, *T. tasmanica*, *T. tuberculata*, and *T. xiamenensis*.

Genera *Karenia* and *Karlodinium* which belong to the Kareniaceae family are known for having the most number of toxic species among the naked dinoflagellates and many of these species had frequently attributed to algal blooms in the coastal zones all over the world (Escobar-Morales & Hernández-Becerril, 2015). Given that many Kareniaceae species are potentially harmful because many of its species produce ichthyotoxin, for instance, brevetoxin karlotoxin, and karmitoxin has making this family one of the most ecologically significant families within Gymnodiniales order considering the social, economic, and public health risks posed by this toxigenic family (Zingone & Oksfeldt Enevoldsen, 2000; Steidinger et al., 2008). An endotoxin known as brevetoxin (PbTx) was documented in the mass mortality event of marine mammals such as bottlenose dolphins and manatees along the eastern Florida coasts (Fire et al., 2015) and can be transferred in the food webs when upper trophic levels consumed brevetoxin-contaminated shellfish or exposed to aerosolised PbTx (Naar et al., 2007; Watkins et al., 2008). Aerosolised PbTx posed fatalities threats to the aquatic invertebrates, fish, sea turtle, seabirds, marine mammals (Lowenstine, 2008), as well as terrestrial organisms such as coyote, domestic dog, green tree frogs and ground squirrels (Castle et al., 2013; Buttke et al., 2018). An amphidinol-like compounds class

called karlotoxin (KmTx) were produced by mixotrophic *Karlodinium* species (Van Wagoner et al., 2010) had demonstrated the activities of hemolytic, cytotoxic, antifungal and ichthyotoxic (Deeds et al., 2006; Mooney et al., 2010). Apart from karlotoxins, a new toxin of polyhydroxy-polyene related to amphidinols and KmTx called karmitoxin was recently characterised and reported of exhibiting nanomolar cytotoxicity, as well as immobility and mortality of organism (Rasmussen et al., 2017; Binzer et al., 2020). Among all the species of family Kareniaceae, only four species, *K. brevis*, *K. veneficum*, *K. conicum* and *K. armiger* had been confirmed of producing toxin until now (Baden et al., 2005; Mooney et al., 2009; Mooney et al., 2010; Rasmussen et al., 2017). PbTx was characterised in the early 1970s and frequently associated HAB along the eastern coast of Florida caused by *K. brevis* (Baden et al., 2005; Fire et al., 2015). KmTx was first described in 1996 following massive fish mortalities at HyRock fish farm, Maryland due to *K. veneficum* bloom (Deeds et al., 2002b). *K. conicum* was identified to be KmTx producer during the screening of KmTx production and had caused mortality in the assay of larval sheepshead minnow (Mooney et al., 2009; Mooney et al., 2010). Karmitoxin was characterised from *K. armiger* (Rasmussen et al., 2017) and had reported of causing death to the copepod and sheepshead minnow fish larvae (Rasmussen et al., 2017; Binzer et al., 2020).

Over the centuries, taxonomy was typically defined as using morphological approaches such as the phenotypic traits of organisms (Ott et al., 2022). Genus *Karenia* is highly variable morphologically, but the shared features such as flattened dorsoventrally body, elliptical or pentagonal in cell shape, has straight apical groove, and some does possess apical carina (Botes et al., 2003; de Salas et al., 2004b; Haywood et al., 2004; Escobar-Morales & Hernández-Becerril, 2015). Morphologically, *Karenia* and *Karlodinium* both exhibit a straight apical groove on the epicone, but the presence of ventral pore distinguishes

Karlodinium species apart from *Karenia* species (Bergholtz et al., 2006; de Salas et al., 2008). Genera *Karlodinium* also has pentagonal or hexagonal amphiesma vesicles on the cell surface (Bergholtz et al., 2006; Nézan et al., 2014; Li & Shin, 2018). Meanwhile, genera *Takayama* is morphologically distinct from *Karenia* and *Karlodinium* by the form of the apical groove, which is sigmoid in *Takayama* species, and straight in *Karenia* and *Karlodinium* species (de Salas et al., 2003; Bergholtz et al., 2006).

Species identification solely based on morphological criteria can be quite challenging due to the difficulty in obtaining the morphology and morphometric data (Wayne et al., 2007). Species characterisation was better with the combination of morphological heterogeneity along with genetic analyses for detailed classification in the taxonomy of phytoplankton (Monaco & Prouzet, 2015). To overcome the struggle in taxonomy using traditional morphology, DNA-based identification system was necessary to identify species (Stern et al., 2012). Since 1990s, the ribosomal (rDNA) genes were the most common used and the most reliable in determining the phylogenetic relationships among dinoflagellate species (Ott et al., 2022). DNA markers such as 18S rDNA, 28S rDNA, and Internal Transcribed Spacer (ITS) regions had been commonly used to identify HAB species including the species of family Kareniaceae (Zhang et al., 2022b). For an instance, initially *Karenia digitata* was described primarily using morphology observation but, when using DNA sequence data, the species was revised to *Karlodinium digitatum* (Cen et al., 2020). Despite of the useful auxiliary information provided by DNA sequence data, no specific DNA marker had been developed for overall Kareniaceae family as of now, thus, becoming a hindrance to study the species diversity of Kareniaceae in the natural settings (Zhang et al., 2022b). 5.8S rRNA gene was flanked by two ITS regions called ITS1 and ITS2 (Stern et al., 2012; Hoggard et al., 2018). The conservative characteristics of ITS2 secondary structure

including structure form, structural motifs, cleavage sites and base-pair interactions can be utilised to accurately delineate species (Zhang et al., 2020). The highly conserved ITS2 secondary structure can be assessed from the presence of evolutionary conserved compensatory base change (CBC) (Cao et al., 2022).

In Malaysia, a species from genus *Karlodinium* of the toxigenic family Kareniaceae namely as *Karlodinium australe* had elevated researchers' interest after the first reported bloom at a fish farm in West Johor Strait (Lim et al., 2014). The bloom of *K. australe* had caused death to the wild and caged fishes in the aquaculture farm (Lim et al., 2014). The recurrent bloom of harmful dinoflagellate *K. australe* in West Johor Straits in 2015 had caused even severe unfavourable impact to the caged fish and wild population, including demersal fishes and crustacean (Teng et al., 2016b). The incident of *K. australe* blooms have alerted researchers and public concern on the food safety and human health. However, due to the unknown status on the distribution of toxigenic *Karlodinium* in Malaysia, regular monitoring and mitigations are quite challenging as the knowledge on *Karlodinium* is in scarce. Therefore, the present study focuses on determining the species diversity of family Kareniaceae and understanding the possible causal mechanism of fish-killing during the past blooms, as well as providing knowledge on future HABs monitoring and mitigation.

1.2 Problem statement

Red tide outbreak causes a great suffering to the environment health, public health, tourism sector and the affected country's economy. Kareniaceae family is widely known for forming a series of bloom events associated with halobios mortality since 1940s. Extensive research had been conducted in different countries, including monitoring and documentation of various species. To date, only 16 species (*Karenia bicuneiformis*, *K. brevis*, *K. brevisulcata*, *K. concordia*, *K. cristata*, *K. longicanalis*, *K. mikimotoi*, *K. papilionacea*, *K.*

selliformis, *Karlodinium armiger*, *K. conicum*, *K. corsicum*, *K. digitatum*, *K. gentienii*, *K. veneficum* and *Takayama cladochroma* are being listed as toxic species in IOC-UNESCO Taxonomic Reference List of Harmful Microalgae (Iwataki, 2024). The discovery of Kareniaceae in Malaysia waters is sufficient to alarm us of the presence of potential toxin producers especially *Karlodinium* that have potential toxicity effect towards the aquatic organism in our waters for future prevention measures.

The outbreak of *K. australe* in Johor Strait, Malaysia was one of the worst HAB phenomenon in the country due to the association of farmed and wild fish, crustacean as well as bringing an estimated loss of over 30 tonnes fish to the aquaculture farm. In the past years, the occurrence of harmful dinoflagellate of family Kareniaceae in Malaysia have been documented, including *Karlodinium veneficum* in Tebrau Strait and *K. australe* in Johor Strait. Despite the global research concerning toxigenic family Kareniaceae, the diversity and distribution of this family in Malaysia remain unearth. The occurrence of two species *Karlodinium* may indicate there is high diversity of the toxigenic family Kareniaceae in Malaysian waters. The research of genus *Karlodinium* from Kareniaceae family in Malaysia had been focused mainly on species description using morphology and molecular approaches and the combination of these data can be used for species delineation in Malaysia waters. The study of toxicity of Kareniaceae, especially *Karlodinium* is still in scarce and limited, since the first bloom of *Karlodinium australe* is reported of attributing to the death of aquatic life in 2014 in Johor Straits, Malaysia. Moreover, the exact causal fish-killing mechanism of *K. australe* is under discussion in Malaysia.

In addition, a comprehensive research on species diversity of Kareniaceae in Malaysia waters has not been conducted. Likewise, the distribution of toxigenic family Kareniceae in Malaysia waters is yet to be discovered. Previous studies in Malaysia focus

more on investigate *K. australe* only after the bloom events. Thus, no study on the species diversity and distribution of Kareniaceae were conducted to investigate this family in Malaysia.

1.3 Objectives

This study's purpose was to give an insight concerning the toxigenic family Kareniaceae in Malaysia waters, as well as to describe the species of Kareniceae using morphology and molecular approaches. Toxicity screening was studied in brine shrimp *Artemia* sp. lethality assay. This is the first report on species diversity and distribution of Kareniaceae in the Malaysia waters. This study is also part of the survey of Harmful Algal Blooms (HABs) species diversity investigation project in Malaysia Borneo waters.

The objectives of this study are listed as below:

- i. To determine the species diversity of Kareniaceae in Malaysia waters by morphological and molecular means, including ITS2 sequence-structure;
- ii. To investigate the survival of fish fingerling *Lates calcarifer* exposed to different cell densities and cell conditions of *Karlodinium australe*; and
- iii. To examine the toxigenic effect and micropredation of family Kareniaceae towards the brine shrimp *Artemia* sp.

1.4 Chapter Summary

This dissertation consisted of five chapters. **Chapter 1** introduced short overview of the research study, emphasising on the impact of Kareniaceae to the environment, problem statements and three objectives of the study **Chapter 2** consisted of the literature review, including the taxonomy of family Kareniaceae, the occurrence of harmful algal blooms (HABs) in Malaysia caused by Kareniaceae associated with mass fish kills, and *in vivo* assay conducted previously for toxicity testing of Kareniaceae. **Chapter 3** included the details on field sampling, methodologies in morphological and molecular characterisation, including experimental bioassay in this study. **Chapter 4** provided the results in this study. A total of ten species of Kareniaceae were reported in this study, namely as *Karenia mikimotoi*, *Karlodinium armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, *K. veneficum*, *K. zhouanum*, and *Takayama acrotrocha*. Out of ten species, eight excluding *K. australe* and *K. veneficum* were firstly reported in Malaysian waters. **Chapter 5** was discussed the diversity and distribution of Kareniaceae in Malaysian waters. The species delineation of *Karlodinium* was further supported by the ITS2 transcripts, revealing the presence of CBCs and hCBCs. The toxicity of Kareniaceae was proven through fingerlings seabass *Lates calcarifer* and brine shrimp *Artemia* sp. lethality assay. Lastly, **Chapter 6** summarised the conclusion and recommendation of this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomical history of family Kareniaceae

Dinoflagellates are a diverse group of microscopic unicellular eukaryotes that mostly present in the euphotic zone of water bodies, from freshwater bodies to the open oceans (Falkowski, 2012; De Vernal et al., 2013). To understand the highly ecological diversity of dinoflagellates, it was necessary to comprehend the morphology-based taxonomy and classification, including the molecular phylogenetic hypotheses (Hoppenrath, 2017). Traditionally, dinoflagellates had been classified into two groups which were armored (thecate) and unarmoured (athecate or naked) dinoflagellates (Fernando, 2020). In athecate dinoflagellate taxonomy, species identification is mainly determined by a blend of several physical traits such as cell shape and size, nucleus shape and location, chloroplasts shape and colour, pyrenoid, apical groove and ventral pore, and features formed on cell surface (Daugbjerg et al., 2000; Larsen, 2002; Bergholtz et al., 2006).

The dynamic nature of biological classification and the significance of assimilating molecular and morphology data were necessary to precisely define species boundaries and comprehend the evolutionary relationships in taxonomy. For almost two decades, detailed revisions on morphological characters combined with molecular phylogenetic studies of athecate dinoflagellate taxa had resulted in the descriptions of new genera, including the genera within family Kareniaceae, for instances, *Karenia* Gert Hansen & Moestrup, *Karlodinium* J. Larsen, *Takayama* M. F. Salas, Bolch, Botes & Hallegraeff (Hoppenrath, 2017). One of the valuable taxonomic characters that led to these new genera descriptions was the apical structure complex or apical groove (Daugbjerg et al., 2000). Henceforth in

2005, the composition of three genera *Karenia*, *Karlodinium*, and *Takayama* were later described a naked dinoflagellate family in the order of Gymnodiniales called Kareniaceae family (Daugbjerg et al., 2000; Bergholtz et al., 2006; de Salas et al., 2008).

The taxonomical of genus *Karenia* had gone through several re-evaluations and amendments following the improvement in the understanding of this genus. Naked dinoflagellates of genus *Karenia* were previously classified under genus *Gymnodinium* (Caruana & Amzil, 2018), and some species had complex histories of classification (Brand et al., 2012). Genus *Karenia* G.Hansen & Moestrup was established in the year 2000 (Daugbjerg et al., 2000), and was initially consisted of three species called *Karenia brevis* (Davis, 1948), *K. mikimotoi* (Oda, 1935) and *K. brevisulcata* (Chang, 1999). Genus *Karenia* had the earliest species description by a single species called *Karenia brevis*, which was previously named genus *Gymnodinium breve* (Davis, 1948) for a long time, renamed to *Ptychodiscus brevis* (C.C.Davis) K.A.Steidinger, and since the re-evaluation of establishment of genus *Karenia*, it is now referred to as *K. brevis* (Brand et al., 2012).

Subsequent research had led to the discovery of other species known as *K. mikimotoi*, and previously named as *Gymnodinium mikimotoi* before genus *Karenia* was established from three species (Oda, 1935; Bergholtz et al., 2006). The historical taxonomic of *K. mikimotoi* became a state of turmoil, since the *K. mikimotoi* strain isolated from European waters was identified as *Gyrodinium cf. aureoleum* (Hulburt, 1957; Gentien, 1998). Strains discovered from different areas of Japan were named as *Gymnodinium* sp., *G. sp. 1* and *G. type-'65* which later were re-examined and re-described as *K. mikimotoi* (Fukuyo et al., 2002). In 1984, another strains isolated from Japanese waters were named as *Gymnodinium nagasakiense*, owing to the dissimilar morphology traits as *G. mikimotoi* when observed under light microscope (Takayama & Adachi, 1984), and *Gyrodinium nagasakiense*, due to

the similarity in cingular displacement with European *G. cf. aureolum* (Tangen, 1977; Takayama & Adachi, 1984; Partensky et al., 1988). Molecular study was performed and based on the data, *G. nagasakiense* and *G. nagasakiense* were later identified to be the identical with *G. mikimotoi* (Hansen et al., 2000). An in-depth morphology, molecular and pigment study on the European *G. cf. aureolum* and *G. mikimotoi* were performed and had reached a consensus that European *G. cf. aureolum* was conspecific with *G. mikimotoi* (Hansen et al., 2000; Hansen, 2001; Tang et al., 2008).

Following the first two species of genus *Karenia*, *K. brevisulcata* was first discovered in New Zealand waters and previously named *Gymnodinium brevisulcatum* (Chang, 1999). *K. brevisulcata* (= *G. brevisulcatum*) was also referred to as *G. mikimotoi* complex species because the morphological traits were much closer to *G. mikimotoi* than *G. breve* (Chang, 1999). The re-evaluation of species within genus *Gymnodinium* by Daugbjerg et al. (2000) had emphasised on the presence of straight apical groove as the unique trait possessed by all *Karenia* species which was morphologically distinct than that of *Gymnodinium sensu stricto*, thus, separating *Karenia* species from genus *Gymnodinium*. In 2024, a new *Karenia* species called *K. hui* had been described from China (Cen et al., 2024). As of now, the genus *Karenia* is consists of eleven recognised species such as *Karenia asterichroma*, *K. bicuneiformis*, *K. brevis*, *K. brevisulcata*, *K. concordia*, *K. cristata*, *K. hui*, *K. longicanalis*, *K. mikimotoi*, *K. papilionacea*, and *K. selliformis* (Guiry & Guiry, 2023).

On the other hand, genus *Karlodinium* J. Larsen was established in the year 2000 with three species proposed (Daugbjerg et al., 2000; Bergholtz et al., 2006). *K. veneficum* was one of the pioneer species in genus *Karlodinium* and had taxonomic confusion (Ballantine, 1956). *K. veneficum* was initially described as *Gymnodinium galatheanum* after the first discovery in 1950 during a Danish Deep-sea Expedition in Walvis Bay, Namibia,

South Africa (Braarud, 1957). The external morphology of *G. galatheanum* was described under different name, *Gyrodinium* sp. A (Bjørnland & Tangen, 1979). The features such as nucleus, cytoplasm, and array of plug-like structure of *G. galatheanum* were similar with *Gymnodinium micrum* (Kite & Dodge, 1988). *K. veneficum* isolated from English Channel waters in 1958 was initially identified as *Woloszynskia micra* which then was renamed to *G. micrum* (Leadbeater & Dodge, 1966). *K. veneficum* isolated in 1950 from Devonport, Australia was named as *G. veneficum* and had determined its toxicity towards marine life as important character to distinguish *G. veneficum* (Ballantine, 1956). An unarmoured dinoflagellate isolated from Cape Cod, was described as *Gyrodinium estuariale*, but no mention on the apical groove (Hulburt, 1957). The *G. estuariale* strain was very similar to *Gymnodinium vitiligo* and *G. veneficum*, only differ in the displacement of cingulum (Ballantine, 1956). However, using cingulum displacement as valid generic trait was being questioned by Gardiner et al. (1989) since it can be influenced by cultural factors. An ultrastructural examination by Bjørnland & Tangen (1979) had found *G. galatheanum*, *Gyrodinium* sp-A, *G. estuariale*, *G. micrum*, and *W. micra* were synonymous with *G. veneficum*. The splitting of large genus *Gymnodinium* into four genera, including *Karlodinium* as a genus consisted of fish-killing species, hence, re-classify *G. veneficum* into *K. veneficum*, considering the previous record on fish kill events (Daugbjerg et al., 2000). Bergholtz et al. (2006) made a comparison study between *K. veneficum* and *K. micrum* had deduced the similarities in morphology and eventually, the findings had led to the amendment of *K. veneficum* as *Karlodinium* type species and demoted *K. micrum* as synonym.

Another species with taxonomical history that had been described into genus *Karlodinium* was *K. vitiligo*. The original description was *Gymnodinium vitiligo* since the

isolation from Knap Buoy region off Plymouth, England in 1949 (Ballantine, 1956). Although *K. vitiligo* (= *G. vitiligo*) was not documented of being harmless by causing detrimental effects to marine life like *K. veneficum* (= *G. veneficum*), both species were very much similar in respect morphology but not physiological (Ballantine, 1956). Another *Karlodinium* species discovered before the establishment of genus *Karlodinium* in 2000 was *K. corsicum*. *K. corsicum* was formerly named as *Gyrodinium corsicum* after the discovery during bloom associated with fish kill event in Corsica, 1994 (Paulmier et al., 1995). Because *K. corsicum* (= *G. corsicum*) fit the criteria of fish-killing in genus *Karlodinium*, (Daugbjerg et al., 2000), it was revised and re-classified to *K. corsicum* by Siano et al. (2009). Since the establishment of genus *Karlodinium*, there were 16 species of genus *Karlodinium* that had been described called *Karlodinium antarcticum*, *K. armiger*, *K. australe*, *K. azanzae*, *K. ballantini*, *K. conicum*, *K. corrugatum*, *K. corsicum*, *K. decipiens*, *K. digitatum*, *K. elegans*, *K. gentienii*, *K. jejuense*, *K. veneficum*, *K. vitiligo*, and *K. zhouanum* up to now (Guiry & Guiry, 2023).

Since 1980s, athecate dinoflagellates with sigmoid apical groove had been reported of causing fish kill events, and was known as *Gymnodinium* type-'84 K and *Gymnodinium* sp. 1 (Onoue et al., 1985; Takayama, 1985). The first species that had been described with sigmoid apical groove was *T. pulchella* (= *Gymnodinium pulchellum*), a species isolated from the shore in the vicinity of Melbourne, Australia between 1985 and 1986 (Larsen, 1994). Larsen (1994) documented the *G. pulchellum* was identical to *G. type '84 K* and *G. sp. 1*, in morphology and in physiology because they were responsible for fish kills of their areas. Two new species, *T. acrotrocha* (= *Gyrodinium acrotrochum*) and *T. cladochroma* (= *G. cladochroma*) were also described by Larsen (1996) during phytoplankton surveying in the vicinity of Melbourne, Australia between 1995 and 1986. In the year 2000, genus *Takayama*

M.F.De Salas, Bolch, Botes & Hallegraeff was established following the revision of taxa and the erection of naked dinoflagellates family Kareniaceae by Daugbjerg et al. (2000). In 2003, two Australian species were isolated from Tasmania, Australia and coastal waters of South Africa known as *Takayama tasmanica* and *T. helix* (de Salas et al., 2003). Upon the establishment of genus *Takayama*, the previously described species were revised to *Takayama tasmanica*, *T. helix*, *T. pulchella*, *T. acrotrocha*, and *T. cladochroma* (de Salas et al., 2003). Up to the present time, seven species had been described into genus *Takayama* called *Takayama acrotrocha*, *T. cladochroma*, *T. helix*, *T. pulchella*, *T. tasmanica*, *T. tuberculata*, and *T. xiamenensis* (Guiry & Guiry, 2023).

Kareniaceae family was a distinct evolutionary lineage of naked dinoflagellates established with three genera *Karenia*, *Karlodinium*, and *Takayama* that shared morphological traits between the genera such as fucoxanthin and derivatives, also supported by bootstrap values in molecular phylogeny (de Salas et al., 2003; Bergholtz et al., 2006). Recently, another four genera, *Brachidinium*, *Asterodinium*, *Gertia* and *Shimiella* (Henrichs et al., 2011; Benico et al., 2019; Takahashi et al., 2019; Ok et al., 2021) were suggested to be included into the Kareniaceae family. However, the inclusion of these later three taxa was debateable due to the variations in morphological traits and the absence of fucoxanthin or derivatives (Zhang et al., 2022b).

2.2 Morphological characterisation of family Kareniaceae

2.2.1 Genus *Karenia*

Genera *Karenia* is naked dinoflagellates with no distinct cell wall plates (Brand et al., 2012). The species of *Karenia* were morphological variable but sharing the same traits such as dorso-ventrally flattened body, elliptical or pentagonal in cell shape, has straight apical groove, and some does possess apical carina (Botes et al., 2003; de Salas et al., 2004b;

Haywood et al., 2004; Escobar-Morales & Hernández-Becerril, 2015). Figure 2.1 showed the morphological characteristics of genus *Karenia*.

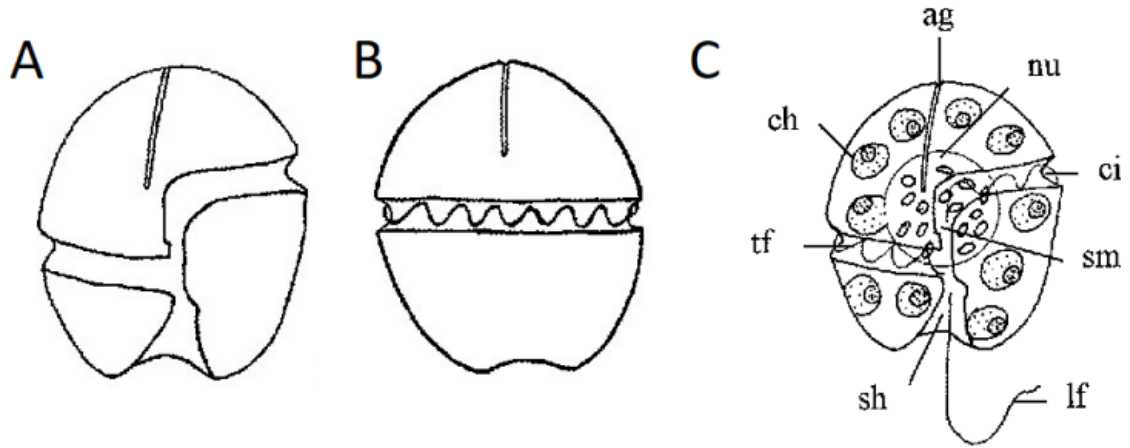


Figure 2.1: Morphological characteristic of *Karenia*. (A) Ventral view, (B) Dorsal view, (C) Arrangement of nucleus (nu, bounded by solid line), apical groove (ag), chloroplasts (ch), transverse flagellum (tf), sulcus at hypocone (sh), longitudinal flagellum (lf), sulcus at middle (sm) and cingulum (ci). Adapted from Yang et al. (2001)

K. asterichroma is medium to medium to large-sized and body is dorso-ventrally flattened. The epicone is truncated and has concave notch at the apex. The concave notch is formed from carina of the ventral epicone surface. The hypocone is truncated and slitted by the sulcus. A linear apical groove extends into the sulcus from intercingular region halfway down the dorsal epicone (De Salas et al., 2004a).

K. bicuneiformis is medium to large-sized and has dorso-ventrally flattened body. The epicone is conical and hypocone has two lobes. The sulcus extends into the epicone but does not widen into the hypocone. A linear apical groove starts extending from sulcus extension briefly to the dorsal side (Botes et al., 2003).

K. brevis size is medium to large-sized and the body is moderately compressed dorso-ventrally. The epicone is conical and the hypocone has slight excavation. The sulcus

intrusion is open-ended into the epicone, adjacently to the apical groove. The apical carina possesses is clearly bulging from both ventral and dorsal sides. A short and linear apical groove extends from sulcus intrusion to the dorsal epicone (Davis, 1948; Haywood et al., 2004).

K. brevisulcata is medium to large-sized and has dorso-ventrally flattened body. The epicone is hemispherical with apical carina on the apex, and the hypocone with no central excavation. The sulcus intrusion is closed and right deflected into the epicone. A short and linear apical groove extends to the dorsal side (Haywood et al., 2004).

K. concordia sizes ranged from medium to large and has dorso-ventrally flattened body. The epicone is conical to broadly conical and from lateral view, the epicone usually arches dorso-ventrally frontward attributing to the pointy apex. The sulcus extension is almost hidden beneath cell membrane flap. The linear apical groove centrally slits the epicone and forming an elongated apical crest. The apical groove extends from sulcus intrusion crossing the apex to the dorsal side (Chang et al., 2008).

K. cristata is medium to large-sized and body is dorso-ventrally flattened. The epicone and hypocone are dissimilar in shape as the hypocone is asymmetric with the larger and more rounded right lobe. The sulcus extends into the epicone and widen crossing the antapex. The linear apical groove starts from the sulcus intrusion and elevates into a crest on the apex (Botes et al., 2003).

K. hui is medium to large-sized and has dorso-ventrally flattened body. The epicone is pointed and flattened. The hypocone is hemispherical and is equally or slightly longer than the epicone. The sulcus is deep and straight. The linear apical groove extends from the widely open sulcus intrusion to the dorsal epicone, forming apical crest (Cen et al., 2024).

K. longicanalis is medium to large-sized and has slightly dorso-ventrally flattened body. The epicone is hemispherical and the hypocone is hemispherical to slightly truncated. The cell antapex has indentation or concavity. No sulcus intrusion is present and linear, long apical groove descends down the dorsal side of the epicone (Haywood et al., 2004; Iwataki et al., 2022).

K. mikimotoi ranges from medium to large-sized and the body is dorso-ventrally flattened. The epicone is roundish conical and the hypocone is hemispherical with antapical notch. The sulcus extends from the antapex into the sulcus intrusion. A narrow, linear apical groove extends slightly above the sulcus intrusion to the dorsal side of epicone (Hansen et al., 2000; Iwataki et al., 2022).

K. papilionacea is medium to large-sized and is moderately compressed at dorso-ventral. The body is ventrally concave and dorsally convex. The epicone has a pointed apical carina on the cell apex while the hypocone is bi-lobed and centrally excavated. The intrusion of the sulcal into the epicone is open. A very short, linear apical groove bisects the carina and extends to the dorsal of epicone (Haywood et al., 2004).

K. selliformis ranges medium to large-sized and the body is flattened dorsoventrally. The epicone is a flattened hemisphere or slightly conical. The hypocone has two lobed and centrally excavated. The epicone is much smaller than the hypocone. The sulcus extension is narrow but open into the epicone. The linear apical groove extends across the cell apex to the dorsal side (Haywood et al., 2004; Iwataki et al., 2022).

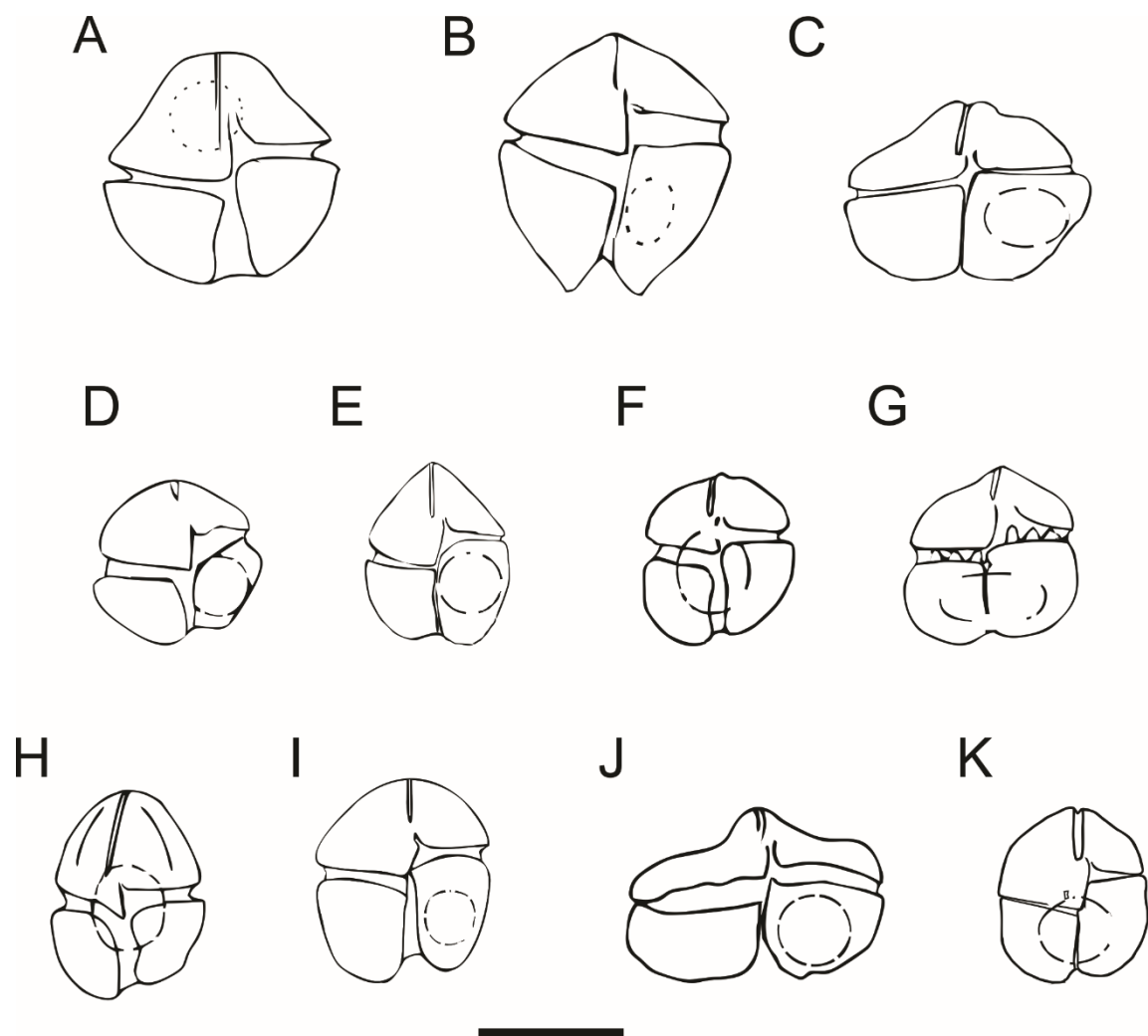


Figure 2.2: Diagram of *Karenia* morphology ventral view drawn to the same scale with nucleus (n, bounded by dash line). (A) *Karenia asterichroma* (De Salas et al., 2004), (B) *Karenia bicuneiformis* (Botes et al., 2003), (C) *Karenia brevis* (De Salas et al., 2004), (D) *Karenia brevisulcata* (Chang, 1999), (E) *Karenia concordia* (Chang & Ryan, 2004), (F) *Karenia cristata* (Botes et al., 2003), (G) *Karenia hui* (Cen et al., 2024), (H) *Karenia longicanalis* (De Salas et al., 2004), (I) *Karenia mikimotoi* (Takayama & Adachi, 1984), (J) *Karenia papilionacea* (Haywood et al., 2004), and (K) *Karenia selliformis* (Haywood et al., 2004). Scales, 20 μ m.

2.2.2 Genus *Karlodinium*

In respect of morphology, *Karenia* and *Karlodinium* were morphologically similar because the species of both genus possessed a straight apical groove on the epicone (Daugbjerg et al., 2000). The key morphology character that distinguished *Karlodinium* species from *Karenia* species was the presence of ventral pore in *Karlodinium* species which

was absent in *Karenia* species (Daugbjerg et al., 2000; De Salas et al., 2008). The morphology traits of *Karlodinium* possesses plug-like structures and amphiesma vesicles on the cell surface (Bergholtz et al., 2006; Nézan et al., 2014; Li & Shin, 2018; Benico et al., 2020). (Bergholtz et al., 2006). The amphiesma vesicles forms pentagonal or hexagonal configuration on the cell surface (Bergholtz et al., 2006; Li & Shin, 2018). Figure 2.2 showed the morphological characteristics of genus *Karlodinium*.

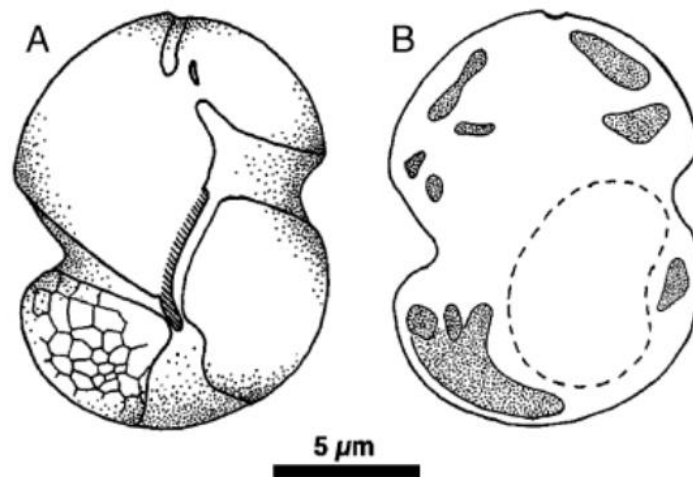


Figure 2.3: Morphological characteristics of *Karlodinium*. (A) Ventral view, (B) Arrangement of nucleus (bounded by dash line), and chloroplasts (stippled). Scale bar, 5 μm. Adapted from Bergholtz et al. (2006)

K. antarcticum is small to medium-sized with elongated and ovoid body. The epicone is conical and smaller than the hypocone. The hypocone is conical or conoidal, and not truncated by the sulcus. The sulcus is tube-shaped structure. A small inconspicuous pore is positioned on the ventral side, and a very long apical groove is extending from ventral to halfway down the dorsal of the epicone (De Salas et al., 2008).

K. armiger size is small to medium and body is slightly asymmetrically ovoid. The epicone is conical and the hypocone is hemispherical, slightly larger than the epicone. The

sulcus intrusion into the epicone is narrower than hypocone. An elongated ventral pore is present on the epicone. The linear apical groove extends briefly from sulcus intrusion to the dorsal side of epicone (Bergholtz et al., 2006; Garcés et al., 2006).

K. australe is small to medium-sized and body has oval outline. The epicone is hemispherical with notch at the apex. The hypocone is hemispherical but not significantly truncated by the sulcus. The sulcus intrudes the epicone as a finger-like protrusion. A reniform or long slit ventral pore is present. A linear apical groove extends briefly from sulcus extension to the dorsal side of epicone (De Salas et al., 2005a).

K. azanzae is medium to large-sized and has ovoid to spherical body. The epicone and hypocone are hemispherical. The sulcus is narrow, shallow and pointed extension protruding into epicone. A ventral pore is present on the epicone. A linear and short apical groove extends from the sulcus intrusion to the dorsal of epicone (Benico et al., 2020).

K. ballantinum is small in sized and has ellipsoidal body. The epicone is hemispherical to conoidal, and the hypocone is hemispherical or slight flattened but not truncated by the sulcus. The sulcus is tube-shaped structure. The ventral pore is absent or obscure. The linear apical groove is very short and extending less than halfway down the dorsal (De Salas et al., 2008).

K. conicum is medium to large-sized and has pentagonal outline. The epicone is larger than the hypocone. The epicone is acute and conical, and the hypocone is rectangular and flattened on the antapex due to the truncation of the sulcus. The sulcus is tube-shaped structure. The ventral pore is large and obvious. The short, linear apical groove extends briefly to the dorsal (De Salas et al., 2008).

K. corrugatum is small in sized and has biconical to pentagonal outline. The epicone is conical with parallel configurations. The hypocone is conical or hemispherical. The narrow, thread-like sulcus extends into the epicone. The ventral pore is positioned away from the nucleus. The linear apical groove starts just above the cingulum extending to the dorsal epicone (De Salas et al., 2008).

K. corsicum size is small to medium range and body is in oval outline. The epicone and hypocone is almost equal in size and symmetrical. The sulcus extends as a narrow slit into the epicone. A kidney-shaped ventral pore is present on the epicone. The conspicuous apical groove extends from sulcus intrusion to the dorsal side of epicone (Paulmier et al., 1995).

K. decipiens is small to medium-sized and body is slightly dorso-ventral flattened. The epicone and hypocone are hemispherical. The sulcus is narrow with tube-shaped structure in the intercingular region and extends shortly into the epicone as small fold. A ventral pore is present as a conspicuous slit-like above the sulcus. A long, linear apical groove is extending from sulcus intrusion to the dorsal side of epicone (De Salas et al., 2008).

K. digitatum is medium to large-size and has globular body. The epicone is hemispherical or broadly conical. The hypocone is hemispherical and antapex is not concave. The sulcus invades the epicone slightly as a finger-like protrusion. The short, linear apical groove is wide at both ends but narrow at middle, and extending from sulcus intrusion down the dorsal side of epicone (Yang et al., 2000).

K. elegans is small to medium in size. The epicone is conical and hypocone is hemispherical. The sulcus extends into the epicone as a finger-like protrusion with a tube-shaped structure in intercingular region. A slit-like ventral pore is present. The linear and

deep apical groove starts from the sulcus intrusion extending halfway down the dorsal epicone (Cen et al., 2021).

K. gentienii is small-sized and body is slightly flattened dorso-ventrally. The epicone is conical, and the hypocone is hemispherical and not truncated by the sulcus. A ventral pore is elongated. The sulcus is wide in hypocone and become narrower in the intercingular region with tube-shaped structure. The sulcus intrudes epicone as a finger-like protrusion. The linear apical groove is directed obliquely, extending from the sulcus intrusion to the dorsal epicone (Nézan et al., 2014).

K. jejuense is relatively small in size. The epicone is conical to hemispherical, and the hypocone is hemispherical and not truncated by the sulcus. The ventral pore is absent. The sulcus intrudes into the epicone as a finger-like appearance with tube-shaped structure. A short and linear apical groove is directed obliquely, starting ventrally from the sulcus intrusion to the dorsal epicone (Li & Shin, 2018).

K. veneficum is small to medium in size and body has oval outline. The epicone is conical to hemispherical and the hypocone is hemispherical. The sulcus as a finger-like appearance intrudes the epicone with tube-shaped structure in intercingular region. The ventral pore is deep and elongated on the epicone. The linear apical groove descends down the dorsal epicone (Ballantine, 1956; Bergholtz et al., 2006; Garcés et al., 2006).

K. vitiligo is relatively small-sized, and body is broadly oval. Both epicone and hypocone are hemispherical, but the epicone is slightly smaller than the hypocone. The sulcus extends to the epicone and hypocone. Ventral pore and apical groove presence are unknown (Ballantine, 1956).

K. zhouanum is relatively small in size. The epicone is conical and the hypocone is hemispherical. As a finger-like protrusion, the sulcus intrudes into the epicone with tube-shaped structure along the intercingular region. No ventral pore is present. The linear apical groove extends from the sulcus intrusion to the dorsal epicone (Luo et al., 2018).

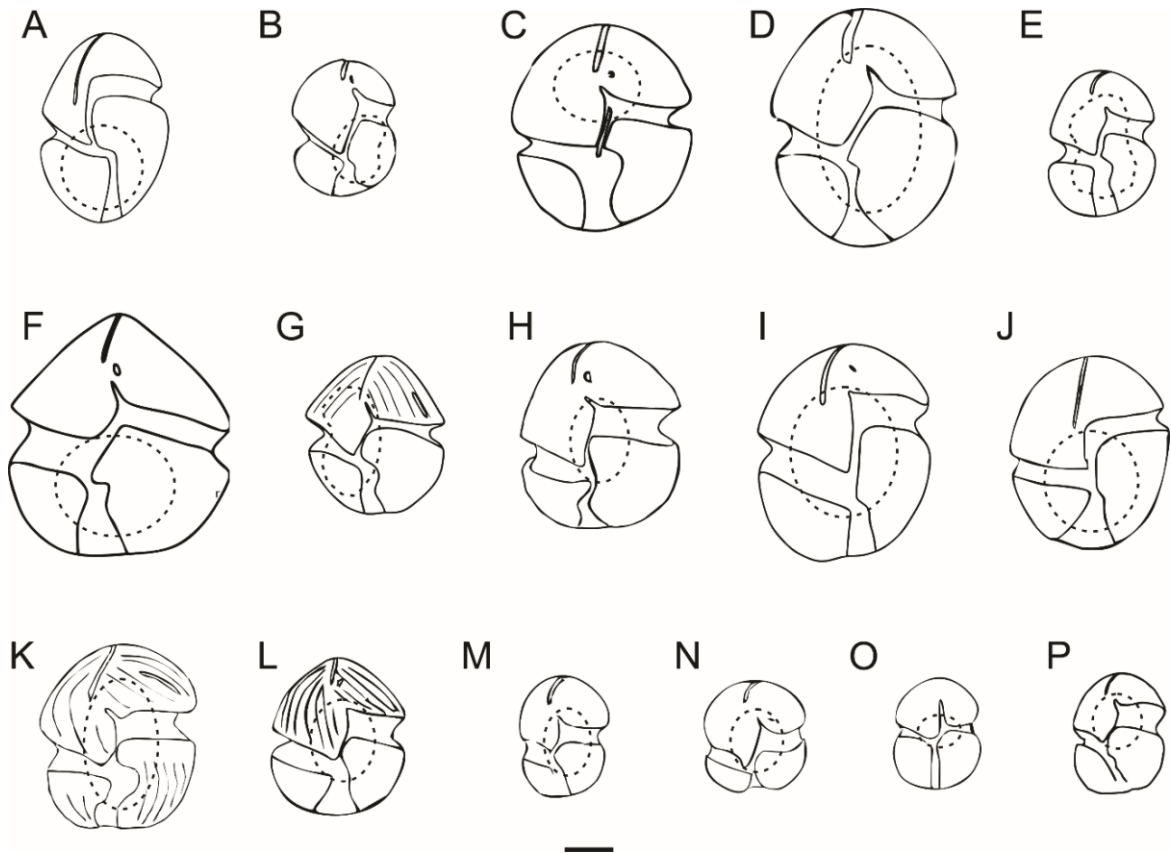


Figure 2.4: Diagram of *Karlodinium* morphology ventral view drawn to the same scale with nucleus (n, bounded by dash line). (A) *Karlodinium antarcticum* (De Salas et al., 2008), (B) *Karlodinium armiger* (Bergholtz et al., 2006), (C) *Karlodinium australe* (De Salas et al., 2005), (D) *Karlodinium azanzae* (Benico et al., 2020), (E) *Karlodinium ballatinum* (De Salas et al., 2008), (F) *Karlodinium conicum* (De Salas et al., 2008), (G) *Karlodinium corrugatum* (De Salas et al., 2008), (H) *Karlodinium corsicum* (Paulmier et al., 1995), (I) *Karlodinium decipiens* (De Salas et al., 2008), (J) *Karlodinium digitatum* (Yang et al., 2000), (K) *Karlodinium elegans* (Cen et al., 2020), (L) *Karlodinium gentienii* (Nézan et al., 2014), (M) *Karlodinium jejuense* (Li et al., 2018), (N) *Karlodinium veneficum* (Daugbjerg et al., 2000), (O) *Karlodinium vitiligo* (Ballantine, 1956), and (P) *Karlodinium zhouanum* (Luo et al., 2018). Scales, 5 μ m

2.2.3 Genus *Takayama*

Genera *Takayama* was morphologically distinguishable by the form of apical which was sigmoid in *Takayama* species, but straight in *Karenia* and *Karlodinium* species (de Salas et al., 2003; Bergholtz et al., 2006). The sigmoidal apical groove extended from the proximal end of the girdle and encircling the cell apex (Larsen, 1994; Larsen, 1996; de Salas et al., 2003; de Salas et al., 2008; Gu et al., 2013). Through scanning electron microscopy, wart-like surface that composed of large amphiesma vesicles with convex surface can be seen (de Salas et al., 2008). Figure 2.3 showed the morphological characteristics of *Takayama*.

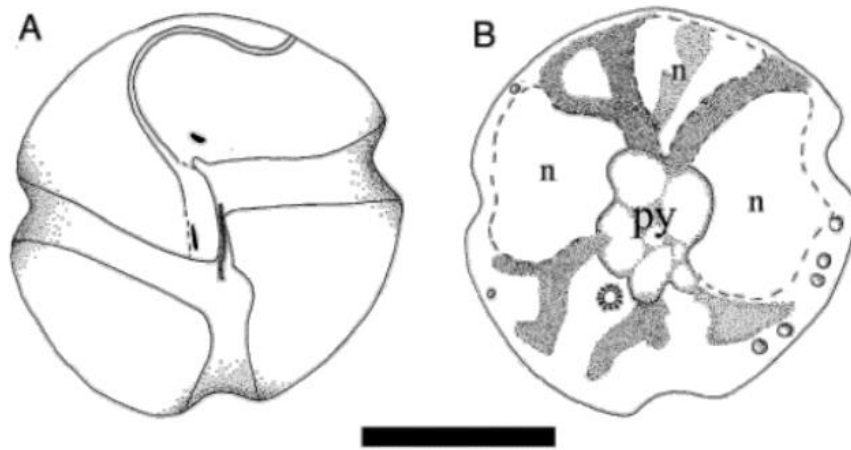


Figure 2.5: Morphological characteristic of *Takayama*. (A) Ventral view, (B) Position of nucleus (n, bounded by dash line), pyrenoid (py, bounded by solid line), and chloroplasts (shaded). Scale bar, 10 μ m. Adapted from de Salas et al. (2003)

T. tuberculata is medium to large-sized, and body is slightly dorso-ventrally flattened. The epicone is hemispherical with flattened apex. The hypocone is rectangular and slits by the sulcus. The sulcus is wide and has a tube-shaped structure within the cingulum region that extends shortly into the epicone. On the apex, a deeply sigmoid apical groove is extending down the dorsal epicone (de Salas et al., 2008).

T. tasmanica is medium to large-sized, and body slightly dorso-ventrally flattened with hemispherical epicone and truncated hypocone. The hypocone also slits by the sulcus which is wider in the hypocone than intercingular region than extends shortly into the epicone. At the apex, the sigmoid apical groove extends from sulcus extension across the epicone and around the cell apex (de Salas et al., 2003).

T. helix is medium to large-sized, and body is rhomboidal to almost circular. The epicone is broadly conical to hemispherical and the hypocone is truncated and slits by the sulcus. The sulcus is wider in hypocone than intercingular region and is extending shortly into the epicone. The cell apex has a shallow, sigmoid apical groove extending from sulcus extension and around the cell apex (de Salas et al., 2003).

T. pulchella is medium to large-sized and body is slightly flattened. The epicone and hypocone are about similar in size. The sulcus becomes wider towards the antapex, with a short, finger-like intrusion into the epicone. The sigmoid apical groove is encircling the cell apex from the proximal end of girdle in counter-clockwise, starting from the sulcus extension (Larsen, 1994; de Salas et al., 2003).

T. acrotrocha is medium to large-sized. The epicone is almost hemispherical and the hypocone is being somewhat flattened and a slight indented antapically. The sigmoid apical groove curves around the apex (Larsen, 1996; de Salas et al., 2003).

T. cladochroma is medium to large-sized and body is obliquely oval in longitudinal optical section. The epicone and hypocone are about similar in size. The sulcus is wider in hypocone than in intercingular region and extends to the slightly excavated antapex. The apical groove is sigmoid and curving around the apex near the proximal end of the girdle (Larsen, 1996; de Salas et al., 2003).

T. xiamenensis is medium to large-sized and body is slightly compressed. The epicone and hypocone are about equal in size. The epicone is conical or hemispherical and the hypocone is truncated, slits by the sulcus. The sulcus as a finger-like protrusion is extending into the epicone. The apical groove is sigmoidal and originated near the cingulum proximal end across the left side of cell apex (Gu et al., 2013).

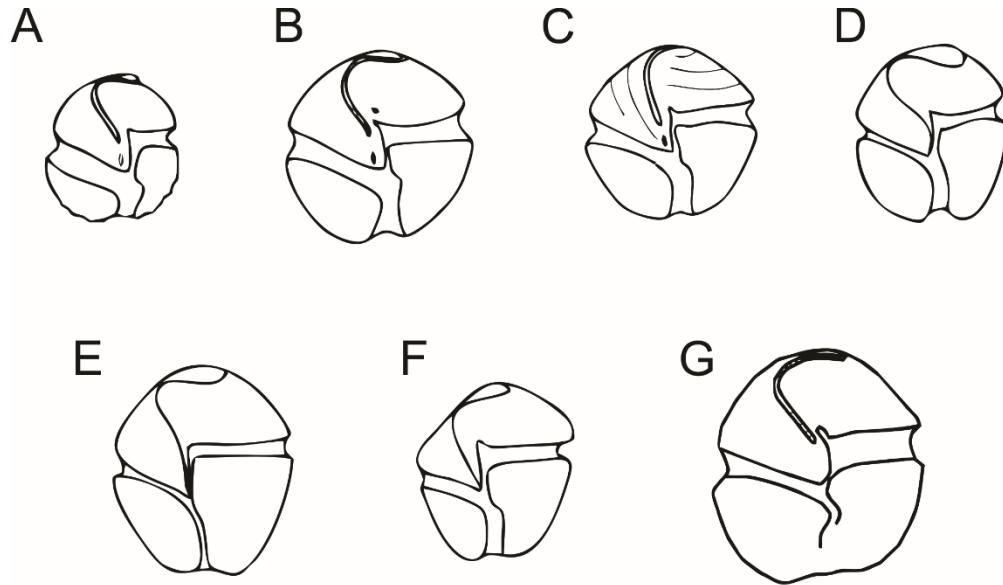


Figure 2.6: Diagram of *Takayama* morphology drawn to the same scale. (A) *T. tuberculata* ventral surface view and dorsal view with nucleus position. (B) *T. tasmanica*. (C) *T. helix*. (D) *T. pulchella* (Larsen 1994). (E) *T. acrotrocha* (Larsen 1996). (F) *T. cladochroma* (Larsen 1996). Scale bar, 10 μ m.

2.3 Molecular characterisation of family Kareniaceae

Morphological systematics did help in identifying and comparing species, however, as Kareniaceae species were relatively small and identical in morphology, inferring species using microscopy identification was almost impossible (Friedheim, 2016; Zhang et al., 2022b). Naked dinoflagellates were delicate and prone to deform or even cell lysis during fixation (Krock et al., 2009), hence, the identification of *Karenia*, *Karlodinium* and *Takayama* species often required live samples as preserved samples may be ambiguous

especially for *Takayama* species (de Salas et al., 2003; Bergholtz et al., 2006). The fragile nature of athecate dinoflagellates can cause difficulty in obtaining the data for morphology morphological features and morphometric data, hence, the species identification of naked dinoflagellates can be a struggle (Wayne et al., 2007). Thus, by using the combination of morphological heterogeneity and genetic analyses were a better approaches for providing detailed information to characterise species (Monaco & Prouzet, 2015). Species characterisation was better with the combination of morphological heterogeneity along with genetic analyses for detailed classification in the taxonomy of phytoplankton (Monaco & Prouzet, 2015).

As doing taxonomy using traditional morphology can be quite challenging, it was necessary to use DNA-based identification system to overcome the challenges for species identification (Stern et al., 2012). Genetic information was more reliable than external phenotypic evaluation hypothetically because genetic was the fundamental basis for the exterior phenotypes (Zhang et al., 2018). Molecular information such as nucleotide DNA and protein amino acid sequences can be utilised for exploring phylogenetic and evolutionary relationships between homologous genes, organelles, or organisms (Foth, 2007; Yang & Rannala, 2012; Zhang et al., 2018). Phylogenetic inferences were exclusively used for demonstrating the relationships between species in systematics and taxonomy (Yang & Rannala, 2012). When comparing closely related organism, DNA sequences were more likely to be impacted by evolution changes than protein sequences (Baldauf, 2003). Nuclear ribosomal DNA (rDNA) was used extensively for molecular studies and genetic analyses (Park, 2007). The most common and reliable genes in determining the phylogenetic relationships among dinoflagellate species was the ribosomal (rDNA) genes (Ott et al., 2022). The rDNAs such as 5S, 5.8S, 18S, and 28S/26S/25S were the housekeeping genes

and being heavily utilised in molecular studies as they were the most conserved genes encoded in eukaryotic organism (Wang et al., 2023). The 18S, 5.8S and 28S rDNA genes were typically arranged in tandem repeats, with internal transcribed spacers 1 (ITS1) and internal transcribed spacers 2 (ITS2) in between of them (Prasad et al., 2008; Tatonova et al., 2017). ITS1 and ITS2 were separated by 5.8S rDNA and flanked by 18S and 28S rDNA (Bower et al., 2008). Figure 2.4 showed the nuclear ribosomal RNA structure.

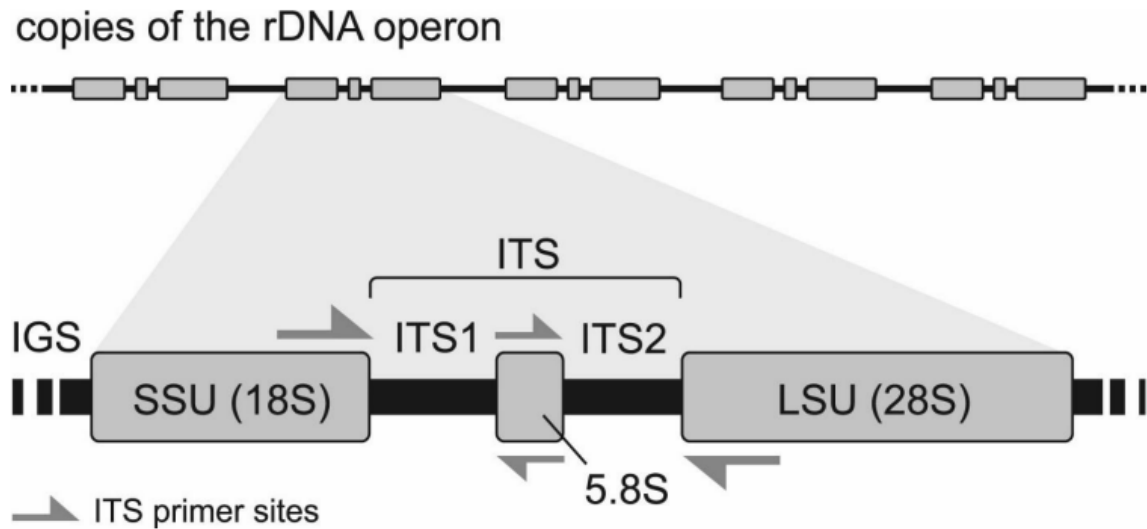


Figure 2.7: Structure of nuclear ribosomal RNA consisted of SSU (18S), ITS and LSU (28S). Adapted from Kauserud (2023)

The rDNA 18S small-subunit (SSU), Internal Transcribed Spacer (ITS), and 28S large-subunit (LSU) regions were the most commonly used gene markers for identifying the species of family Kareniaceae (Zhang et al., 2022b). The SSU, ITS, and LSU rDNA regions were very effective for molecular identification (Demirel, 2016). Among these three regions, SSU was a poor marker with lower species discrimination, worst topology outcome and less efficient in determining phylogenetic relationships (Demirel, 2016). SSU also had disadvantage in terms of the length and quality of reference sequences available in the

nucleotide database (Demirel, 2016). Hence, LSU and ITS regions were mostly preferred for molecular studies due to their high efficiency and performance in species identification, discrimination, tree topology and even application (Demirel, 2016; Ott et al., 2022).

LSU D1–D3 region had both conserved and divergent domains (Wuyts et al., 2001) that can be used to define dinoflagellates species at higher taxonomic level (Lumbsch et al., 2001; Sonnenberg et al., 2007; Ott et al., 2022). LSU region delineated closely related species in phylogenetic analyses, however, LSU was less variable as the taxonomic resolution at species level and diversity may be restricted compared to the ITS region (Sonnenberg et al., 2007; Porras-Alfaro et al., 2014). LSU regions were beneficial in distinguishing dinoflagellate species and examining heterogeneity in sequences among various strains of the same species (Scholin et al., 1994; Daugbjerg et al., 2000). Several Kareniaceae species were described based on LSU phylogenetic inferences, for instances, *K. cristata*, *K. bicuneiformis*, *K. asterichroma*, *K. mikimotoi*, *K. brevis*, *K. brevisulcata*, *K. hui*, *K. papilionacea*, *K. selliformis*, *K. longicanalis*, *K. veneficum*, *K. vitiligo*, *K. australe*, *K. armiger*, *K. antarcticum*, *K. ballantinum*, *K. conicum*, *K. corrugatum*, *K. decipiens*, *K. gentienii*, *K. zhouanum*, *K. jejuense*, *K. azanzae*, *K. elegans*, *K. corsicum*, *T. acrotrocha*, *T. cladochroma*, *T. helix*, *T. pulchella*, *T. tasmanica*, *T. xiamenensis*, and *T. tuberculata* (Daugbjerg et al., 2000; Botes et al., 2003; de Salas et al., 2003; Haywood et al., 2004; De Salas et al., 2004a; de Salas et al., 2004b; de Salas et al., 2005a; Bergholtz et al., 2006; de Salas et al., 2008; Gu et al., 2013; Nézan et al., 2014; Luo et al., 2018; Wang et al., 2018; Siano et al., 2009; Li & Shin, 2018; Benico et al., 2020; Cen et al., 2021; Cen et al., 2024).

Apart from LSU, ITS was also a powerful genetic marker used in the phylogenetic analyses to classify the taxonomy within Kareniaceae species. ITS was a useful tool for taxon-specific studies of dinoflagellate taxonomy (Stern et al., 2012). ITS regions had higher

accuracy of classification than LSU region at lower taxonomic or genus level (Porrás-Alfaro et al., 2014). The genetic marker, ITS was derived from the rapid evolving genomic region positioned between the ribosomal RNA genes (Fathy et al., 2023), and the relative high nucleotide substitution rate enabling ITS region to resolve the taxonomic ambiguity of species (Kowalska et al., 2019). ITS markers consisted of two high variable section, ITS1 and ITS2, and both had been used widely for the studies on ecological genetic, phylogenetic analyses and evolutionary analyses at various taxonomic levels of organism due to the low intraspecific and high interspecific variability (Hall et al., 2010; Dai et al., 2012; Buathong et al., 2017). ITS regions were typically divergent among species but remain homogenous within individuals due to the concerted evolution (Bower et al., 2008). In the recent decade, ITS molecular phylogeny had been utilised to define some new Kareniaceae species, particularly in the genus *Karlodinium*, such as *K. zhouanum*, *K. jejuense*, *K. azanzae*, and *K. elegans* (Luo et al., 2018; Li & Shin, 2018; Benico et al., 2020; Cen et al., 2021).

2.4 ITS2 secondary structure

Discriminate different species of the same genus was the most challenging situation due to the strong interrelation between the species (Müller et al., 2007). The nuclear ribosomal ITS2 was more conserved compared to ITS1 (Schultz et al., 2005). ITS2 regions provided sufficient resolution to delimitate species (Moestrup & Daugbjerg, 2007). ITS2 regions was utilised for predicting and distinguishing the closely related species with clade separation into subclades (Müller et al., 2007; Coleman, 2009). The prediction of species can be accurate by analysing the presence of evolutionary conserved compensatory base pair changes in ITS2 (Stern et al., 2012). The species were inferred by analysing the homology modelling via the presence of CBCs (Compensatory Base Changes), a modified double

stranded pair, or hCBC (hemi-Compensatory Base Change), a single-nucleotide polymorphism (Coleman, 2003; Müller et al., 2007).

The relevance of ITS2 regions was grounded on the secondary structures (Meron et al., 2012). The analyses of ITS2 secondary structure revealed of four double-stranded helices or hair-pin like structures that were separated by single-stranded regions (Kocot & Santos, 2009). Helices were the most common elements of ITS2 secondary structure (Caisová & Melkonian, 2014). Among the four helices, helices I and IV were the most evolutionary variable, mainly being utilised to do comparison among species and subspecies (Coleman, 2009). For instance, the comparison of structural features between *Karlodinium* ITS2 secondary structures had revealed helix IV as the most variable base-pairing interaction of CBCs and HCBCs of the species (Lim et al., 2014). Whereas helices II and III were more conserved in sequences and varied from the ITS2 structures of other eukaryotic (Coleman, 2009). Figure 2.5 showed homologous modelling of ITS2 secondary structures.

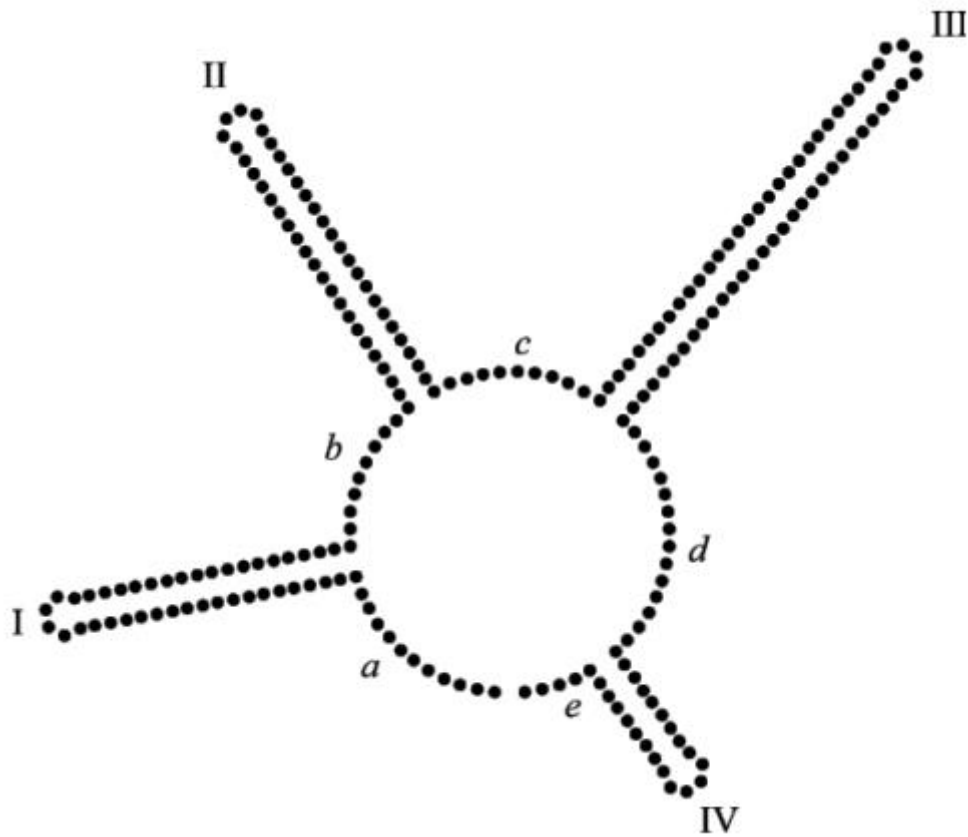


Figure 2.8: Homologous modelling of ITS2 secondary structures consisted of four helices. Adapted from Kocot & Santos (2009)

Previously, the presence of at least one CBC was necessary to delimit biological species (Coleman, 2003; Müller et al., 2007). An example of using ITS2 in the phylogenetic inference within family Kareniaceae, in particular genus *Karlodinium* was documented by Lim et al. (2014). ITS2 secondary structure was utilised in the study to support the species identity of *K. australe*. When comparing *K. australe* with *K. veneficum* and *K. armiger* respectively, in *K. veneficum* three CBCs were detected, however, in *K. armiger* only four HCBCs were found and no CBC detected (Lim et al., 2014). Hence, absence of CBC was not necessary an indication of two organism belonged of the same species and HCBC can also be used for species delimitation (Lim et al., 2014).

2.5 Toxicity of family Kareniaceae (Gymnodiniales)

The genus *Karlodinium* is known as a cosmopolitan, toxic unarmoured dinoflagellate that often associated with harmful algal blooms (HABs) in coastal waters with extensive ecological impacts (Place et al., 2012; Liu et al., 2020). *Karlodinium veneficum* was discovered for the first time in Malaysian waters in 2009 at Tebrau Strait during assembling dinoflagellates as species inventory (Tan et al., 2013). A field survey conducted in Aman Island, the northern Malacca Strait had identified the occurrence of *K. australe* at low cell densities 2.0×10^4 cells L⁻¹, however no fish fatalities were observed (Razali et al., 2015).

In 2014, *K. australe* bloomed at West Johor Strait had cause mass fish killed event with cell densities recorded reaching 2.34×10^6 cells L⁻¹ (Lim et al., 2014). This bloom had affected the cage-fish cultures along the coasts of Tanjung Kupang, Johor where the fish deaths grew progressively large over the following three days and persisted for a week, killing approximately four tonnes of fish, including wild fishes in the surrounding area.

The subsequent bloom in 2015 of *K. australe* with the greatest cell densities recorded of 2.0×10^8 cells L⁻¹, had resulted in a bigger loss of more than 30 tonnes of fish cultured were affected, excluding the deaths of demersal wild fish and crustaceans throughout the West Johor Strait (Teng et al., 2016a). According to the fish farm operators, the cage-cultured fish exhibit abnormal behaviour such as leaping out of cages aggressively at night, between 10 PM to 1 AM and installation of aeration system did not appear to improve fish conditions.

The recent algal bloom in the mariculture areas of Johor Strait had documented *Karlodinium* spp. as one of the bloom contributors, with cell densities ranging from $3.5\text{--}6.6 \times 10^3$ cells L⁻¹, however, no mussel or fish mortality were reported during the bloom event (Razali et al., 2022).

2.5.1 Lethality assay

The naked dinoflagellates of family Kareniaceae including genera *Karenia*, *Karlodinium*, and *Takayama* were three closely related genera that had been responsible for harmful effects to marine life such as fish and molluscs worldwide (De Salas et al., 2005b). Hence, various bioassays had been conducted to determine the potency of toxic species by using different organisms such as microalgae, brine shrimp, copepods, shellfish or fish (Hégaret & Mardones, 2023). Apart from marine organisms, mammalian as well as erythrocytes was used extensively to assess the haemolytic properties of causative species extracts (Hégaret & Mardones, 2023). Several studies in the past had conducted bioassays by exposing Kareniaceae species to the marine life organism to document the harmful effects caused by causative species on the survivorship of tested organisms.

For genera *Karenia*, several species such as *K. brevis*, *K. mikimotoi*, *K. brevisulcata*, and *K. selliformis* had went through toxicity testing. In an assay to the stone crab larvae, the *K. brevis* caused mortality of the larvae and reversed swimming behaviour (Gravinese et al., 2019). The recent exposure experiment of *K. brevis* cell densities had documented to be highly toxic to the survival of red porgy *Pagrus pagrus* larvae (Litaker et al., 2022). *K. brevis* was not only tested on marine life, but also tested in mouse bioassay as representative test organism for epidemiological investigation to estimate public health risk from consuming molluscs contaminated in bloom events (McFarren et al., 1965). *K. brevis* was also assessed for the ichthyotoxicity to juvenile Chinook salmon *Oncorhynchus tshawytsch*, snapper *Pagrus auratus* and marine invertebrate larvae, and was found to be highly toxic to juvenile salmon compared to juvenile snapper. but no harmful effect was observed in the invertebrate larvae (Shi et al., 2012). Meanwhile, the toxicity testing of *K. mikimotoi* was only toxic to abalone larvae, but non-toxic to finfish (Shi et al., 2012). As *K. mikimotoi* was recognised

and considered of being toxic, bioassay was conducted to the extracts of contaminated mussel *Perna indica* and injected to mice, however, the mouse bioassay had negative results (Robin et al., 2013). Dang et al. (2015) had carried out toxicity testing of *K. mikimotoi* to the cladoceran *Moina mongolica*, copepod *Pseudodiaptomus annandalei*, and crustacean *Artemia salina* to assess the effects to the grazers. The exposure experiment of *K. mikimotoi* had been haemolytic to the grazers by decreasing the survival rate of the three tested grazers (Dang et al., 2015). In the invertebrate and finfish larvae bioassay, *K. brevisulcata* was highly lethal to mussel, sea urchin, abalone, sea slug, and oyster larvae (Shi et al., 2012). Shi et al. (2012) also tested the toxicity of *K. brevisulcata* to the juvenile salmon *O. tshawytsch* and snapper *P. auratus*, but the lethal effects was lesser compared to in the invertebrate larvae. In addition, mouse bioassay was conducted to detect the toxicity of *K. selliformis* extracts and resulted in symptoms observed in the mice such immediate jumping upon injection, stretching, hyperactive, stiff tail, slowed movements, hind legs paralyse, breathing difficulties, exophthalmoses, and eventually death (Amel et al., 2009).

Within genus *Karlodinium*, *K. armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. conicum*, *K. corsicum*, *K. gentienii*, and *K. veneficum* had been tested for the ichthyotoxicity in several bioassays. Preliminary test by using *Artemia franciscana* had tested the toxicity of *K. armiger* in different temperature, and resulted in higher toxicity occurred in 25 °C compared to at 15°C (Bergholtz et al., 2006). Assay to the copepod *Acartia tonsa* by exposing to *K. armiger* had caused rapid paralysis and mortality in mortality assay (Rasmussen et al., 2017; Berge et al., 2012). Besides, when *K. armiger* cells were exposed to the blue mussel *Mytilus edulis*, the survival of blue mussel was negatively affected and the response of blue mussels was reduced, for instances, shells cannot completely close and mantle draw in very slow upon touching (Binzer et al., 2018). The conducted assay of *K.*

australe to the rotifers *Brachionus plicatilis*, brine shrimp *Artemia salina*, and larval marine medaka *Oryzias melastigma* had impacted their survivorship (Song et al., 2020). In addition, *K. azanzae* was extremely fatal to the marine zooplankton such as calanoid copepod, harpacticoid copepod, polychaete trochophore and bivalve veliger in exposure experiment (Benico et al., 2020). A fish bioassay using erythrocytes of rainbow trout *Oncorhynchus mykiss* had little to non haemolytic activity by *K. ballantinum* cells (Mooney et al., 2010). The extracted toxin compound from *K. conicum* was exposed to sheepshead minnow larvae and was found to be highly toxic (Mooney et al., 2010). A long-term experiment of *K. corsicum* to copepods *A. grani* had significant adverse effects on the survival rates by causing mortality (Da Costa et al., 2005). The assay of *K. elegans* culture to brine shrimp *A. salina* and marine medaka *O. melastigma* had no adverse effect (Cen et al., 2021). The haemolytic effect of *K. gentienii* to horse blood was documented in a preliminary assay (Nézan et al., 2014). Additionally, gill damage as sub-lethal effects was documented in the exposure test of *K. veneficum* to the larval sheepshead minnow *Cyprinodon variegatus* (Adolf et al., 2015). *K. veneficum* was highly toxic to the brine shrimp *Artemia salina*, rotifer *Brachionus plicatilis* and juvenile *Oryzias melastigma* in the bioassay (Yang et al., 2019). The toxin compound from *K. veneficum* was extracted and found to be highly toxic to sheepshead minnow larvae in exposure test (Mooney et al., 2010).

In the genus *Takayama*, exposure experiments have only been previously documented for *T. acrotrocha*, *T. tasmanica*, and *T. xiamenensis* (Tang et al., 2012; Lim et al., 2018; Lü et al., 2022). *T. acrotrocha* was tested on fingerlings of Asian sea bass (*Lates calcarifer*) and sheepshead minnows (*Cyprinodon variegatus*), but it did not exhibit ichthyotoxicity towards these two fish species (Tang et al., 2012). An assay on the ingestion of *T. tasmanica* had recorded mixotrophic *T. tasmanica* with an average equivalent spherical

diameter (ESD) of 24.3 μm feeding on dinoflagellates *Akashiwo sanguinea* (ESD: 30.8 μm), *Alexandrium tamarense* (ESD: 31.2 μm), *Coolia malayensis* (ESD: 30.4 μm), *Coolia canariensis* (ESD: 32.8 μm), *Gambierdiscus caribaeus* (ESD: 67.4 μm), *Gymnodinium catenatum* (ESD: 33.9 μm), *Levanderina fissa* (ESD: 31.2 μm) and *Lingulodinium polyedra* (ESD: 38.2 μm), which were much larger than *T. tasmanica* cell sizes (Lim et al., 2018). Lastly, the acute toxicity of *T. xiamenensis* was tested in bioassays using brine shrimp *A. salina*, and marine medaka *O. melastigma* had resulted in negative results (Lü et al., 2022).

2.5.2 Worldwide bloom events

Genera Kareniaceae, *Karenia*, *Karlodinium*, and *Takayama* had HAB-causative species that also toxin producers which not only kill marine life, fish, and other animals, but also cause human respiratory illnesses and neurotoxic shellfish poisoning (NSP)(Zhang et al., 2022a). The first *K. mikimotoi* bloom was observed in Gokasho Bay and Ago Bay, Japan, in 1934 and recorded of causing deaths to the fish and shellfish coastal waters (Oda, 1935). In the year 1966, *K. mikimotoi* had recorded of causing water discolouration in Norwegian coast and killing fish along the coast (Braarud, 1970). Since 1950s, the blooms of *K. mikimotoi* occurring in several countries were increasing in trend compared to the previous decades. From the year 1981 to 1985, *K. mikimotoi* had red tide occurrences in the Korean coastal waters (Park et al., 2013). An outbreak of *K. mikimotoi* was reported bloom in the Ria of Pontevedra, north-west coast of Spain, 1989 (Jiménez et al., 1992). A bloom of *K. mikimotoi* associated with fish kill event had occurred in Indian waters in the year 1989 (D'Silva et al., 2012). Between the year 1988 and 1989, the death of chitons, limpets, abalone, periwinkles, whelks, black mussels, alikreukel, octopus and starfish were observed co-occurred in *K. mikimotoi* blooms in South Africa (Horstman et al., 1991). Horstman et al. (1991) also documented the *K. mikimotoi* had negatively affecting the bathers' and

fishermen's health such as irritation of eye, nose and lung. In the year 2009, the outbreak of *K. mikimotoi* along the coasts of England had harmful effect to the marine life including dogfish (Shutler et al., 2015). In 1998, *K. mikimotoi* blooms were first hit Hong Kong waters (Lu & Hodgkiss, 2004). According to Lu & Hodgkiss (2004), the red tide had caused huge losses to about two thirds (estimated 1000 out of 1500) of the mariculture farms and estimated financial loss was HK\$315, 000, 000 which equivalent to 40 million USD. Following the first bloom in coastal waters of China, the bloom areas of *K. mikimotoi* was observed to spread from Guangdong province to Tianjin city, Zhejiang, Fujian, Hebei and Jiangsu provinces (Baohong et al., 2021). *K. mikimotoi* bloom in China became frequently in the East China Sea, including Changjiang River estuary, and the coastal of Zhejiang and Fujian Provinces at almost every year since 2002 (Li et al., 2017).

Another *Karenia* species with the longest history of bloom recorded was *K. brevis*. *K. brevis* also had outbreak with devastating impacts to the fish and human health respiratory distress in the West Atlantic coast, Greece, Japan, and coastal areas bordering the Gulf of Mexico (Davis, 1948; Satsmadjis & Friligos, 1983; Magaña et al., 2003). The red tide *K. brevis* was recorded in 1987 along the waters of North Carolina and post-bloom event had reported many cases of NSP in human (Tester et al., 1988). In Saronikós Gulf, Greece, *K. brevis* red tide had caused massive fish kill in the year 1978 (Satsmadjis & Friligos, 1983). In Omura Bay, Japan, *K. brevis* blooms had been recorded since the 1970s (Iizuka, 1975; Iizuka, 1978). *K. brevis* had been a major HAB-causative species in the Gulf of Mexico (Steidinger & Penta, 1999). Based on historical assessment of *K. brevis* by Magaña et al. (2003), *K. brevis* was recorded of causing red tides in several areas in Gulf of Mexico for more than 350 years. The first recorded of *K. brevis* bloom was in 1648 in Yucatan, Mexico and associated with massive fish kill event (De Cogolludo, 1688; Magaña et al., 2003). From

1792 to 1995, fish kill associated with *K. brevis* bloom events were observed in several areas in Mexico, and human health risks including dry cough and throat irritation to human were also reported (De Tejada, 1940; Nuñez-Ortega, 1879; Cortés-Altamirano et al., 1995). In Texas, *K. brevis* bloom events had occurred yearly between 1997 to 2002, and massive fish kills were reported with the blooms (Villareal et al., 2001; Magaña et al., 2003). Meanwhile in Florida, *K. brevis* red tides was observed since 1844 and massive fish kill was recorded (Ingersoll, 1881). The bloom events from 1946 to 1947 had caused mortalities of marine animals (Gunter et al., 1948), All of the bloom episodes of *K. brevis* in Florida waters from 1844 until 1947 had recorded massive fish kill (Davis, 1948; Galtsoff, 1954; Taylor, 1917; Magaña et al., 2003).

In Gulf of Gabes, Tunisia, there was a red tide in 2009 attributed to *K. selliformis* and shellfish contamination was recorded (Feki et al., 2013). A massive bloom in 2020 by *K. selliformis* had extensive yellow sea foam areas on the surface of the ocean off the Kamchatka Peninsula coast in Russia (Orlova et al., 2022). Orlova et al. (2022) had documented the public health risks faced by the surfers of Khalaktyrsky beach during the bloom such as having red and swollen eyes, short vision loss, dry cough, sinus discomfort, light-headedness, nausea, diarrhoea, ocular burn, irritation of skin and increasing in blood bilirubin levels. Post-bloom had worsened the situation when dead marine fauna were found stranded by the shore, massive mortalities of benthic marine organisms on the seabed, and abnormal behaviour of spotted seals were recorded (Orlova et al., 2022).

Red tide phenomenon of other species within genus *Karenia* were also reported occurring worldwide. *K. cristata* blooms were first reported in False Bay, South Africa from the year 1988 to 1989 (Horstman et al., 1991). Subsequent *K. cristata* bloom in Gariep Nature Reserve (previously known as Hendrik Verwoerd Dam Nature Reserve), South

Africa were associated with massive abalone mortality and human respiratory illnesses such as eye, nose, and irritation to the throat and skin (Botes et al., 2003). Also, Botes et al. (2003) had recorded subsequent blooms attributed to *K. bicuneiformis* in Gordon's Bay, South Africa in 1995 and 1997, yet no harmful effects were observed during the bloom. In 1998, *K. brevisulcata* bloom were associated with massive marine life mortality and human respiratory distress in east coast of the North Island, New Zealand, and the adverse impacts were also reported along Wairarapa coast and spread north as far as Hawke Bay (Chang et al., 2001). In 1998, *K. longicanalis* were reported causing red tides in Victoria Harbour, Hong Kong and southwestern coastal waters of Hong Kong Island, but no detrimental impacts to marine life recorded (Yang et al., 2001). In addition, *K. concordia* was reported of causing a bloom in Hauraki Gulf, New Zealand, and had caused massive fish mortalities in the year 2002 (Chang et al., 2008).

Besides genus *Karenia*, genus *Karlodinium* is recognised for causing fish mortalities during red tides. Within genus *Karlodinium*, *K. veneficum* had recorded the earliest mass occurrence of all *Karlodinium* species. From 1964 to 1967, *K. veneficum* was reported of causing red tides Walvis Bay, Namibia and mass fish mortality was observed at the same time (Pieterse & Van der Post, 1967). Subsequent blooms of *K. veneficum* in 1984 were recorded from Oslofjord, Norway and along the southern coast of Norway (Bjørnland & Tangen, 1979; Dahl & Yndestad, 1985). In 1994, a HAB-associated with fish kills events in Alfacs Bay, Spain were caused by two dinoflagellate species of genus *Karlodinium*, *K. veneficum* and *K. armiger* (Garcés et al., 2006). From 1996 to 1999, *K. veneficum* blooms in Chesapeake Bay in mid-Atlantic region had caused fish mortality in a *Sparus aurata* gilthead seabream aquaculture farm (Deeds et al., 2006). In 2002, *K. veneficum* bloom associated with fish kill event was reported in South Carolina brackish water ponds

(Kempton et al., 2002). In Australia, blooms of *K. veneficum* were never reported in Swan River estuary, however, since 2002, series of blooms attributed to *K. veneficum* had occurred periodically until 2012 (Place et al., 2012; Adolf et al., 2015). According to Adolf et al. (2015), fish mortalities were observed during *K. veneficum* bloom in the upper Swan River and Canning River estuaries, Australia in the year 2003. In North Carolina, four subsequent fish mortalities was reported in Neuse River estuary associated with *K. veneficum* bloom in the year 2006 (Hall et al., 2008).

Apart from *K. veneficum*, there were several *Karlodinium* species that had been reported of HAB associated with fish kill events such as *K. corsicum*, *K. digitata*, *K. australe*, *K.* and *K. elegans*. A bloom of *K. corsicum* occurred in saltwater lake of Diana, France in 1994 had reported event of fish kill (Paulmier et al., 1995; Siano et al., 2009). Between 1995 and 1997, *K. digitata* blooms had caused fish kills along the coasts of western Japan and farmed fish in the inland Sea of Japan (Baba et al., 1997; Yang et al., 2000). In China, 1998, *K. digitata* had reported of bloom in 1998 and caused huge losses to the Hong Kong aquaculture industry (Lee et al., 2011). The recurrent bloom of *K. digitata* in 2009 had cause massive fish mortalities in Fujian province aquaculture fish farm (Cen et al., 2020). Since the first description in 2005, Australian *K. australe* was never reported of causing problems in Australian waters (de Salas et al., 2005a; Hallegraeff et al., 2021). A decade ago, *K. australe* was first reported of causing bloom associated with mass mortality of fish in an aquaculture farm in Johor Straits, Malaysia (Lim et al., 2014). A year later, *K. australe* bloom had recurred along Johor Straits, Malaysia and caused even greater impacts to the marine life and economic losses (Teng et al., 2016b). To add, a harmful algal bloom in 2019 caused by *K. elegans* had led to the cage-cultured fish mortality located in Fujian province of China (Cen et al., 2021).

Within the family Kareniaceae, most HAB events were attributed to the species of *Karenia* and *Karlodinium* (Zhang et al., 2022a) while *Takayama* species was seldomly accountable for causing red tides. The first time of recognising genus *Takayama* as bloom-forming dinoflagellate was during a red tide occurred in Kagoshima Bay, Japan in 1984 (Onoue et al., 1985). The first reported bloom of *Takayama* species had caused adverse impact to the yellowtail farm within the affected area (Onoue et al., 1985). The subsequent bloom in Florida, 2013 had identified *Takayama pulchella* as the culprit and the bloom was associated with massive fish death (Tang et al., 2012). Apart from that, *T. acrotrocha* bloom was reported in Singapore coastal waters but no fish mortalities was observed during the bloom (Tang et al., 2012). The recent bloom of *Takayama* species was in Haizhou Bay, China in the year 2020 which was detected during a cruise survey (Zhang et al., 2022a).

2.6 Kareniaceae blooms in Malaysia and Southeast Asia

Since the 1970s, harmful algae blooms (HABs) were observed in Southeast Asia region and had been a continuous concern (Yñiguez et al., 2021). In Southeast Asia (SEA), there were few countries such as Philippines, Singapore, and Malaysia that had been reported of having HABs including Kareniaceae blooms (Tang et al., 2012; Tan et al., 2013; Lim et al., 2014; Leong et al., 2015; Teng et al., 2016b). The species of family Kareniaceae is known as a cosmopolitan, toxic unarmoured dinoflagellate that often associated with HAB events in coastal waters with extensive ecological impacts (Place et al., 2012; Liu et al., 2020).

The occurrence of *Takayama* sp. during bloom associated with fish kill event in Philippines coastal waters was reported in the year 2002 (Yñiguez et al., 2021). In 2016, *Takayama* sp. was reported to be co-occurred with a bloom that caused mass mortality of farmed milkfish in Balinao, northwestern Philippines (Benico et al., 2010). The recurring

blooms in 2017 and 2018, in which the latter was concurred with mass fish mortalities were attributed to *Takayama* sp. (Benico et al., 2018). In Balinao-Anda, Pangasinan province in Philippines, high biomass of *K. mikimotoi* was reported occasionally but no fish kills were observed (Yñiguez et al., 2021).

In the year 2004 and 2007, *T. acrotrocha* was reported of having moderate bloom in Singapore coastal waters and patches of water discolouration were observed, but no fish mortality or other harmful effects was reported during the bloom event (Tang et al., 2012). In 2009, post-bloom sample in Singapore waters had identified the culprit as *K. australe* (Leong et al., 2015). According to Leong et al. (2015), the coastal waters of Singapore was progressively affected by algal blooms and few species within family Kareniaceae were reported in bloom events occurring during field observation between 2012 and 2014. Whereas in 2011 and 2014, *K. mikimotoi* was recorded of causing patches of water discolouration along East Johor Strait, Singapore (Leong et al., 2015). The bloom in Tuas, Singapore had recorded *T. xiamenensis* as the most dominant HAB-causative species in the year 2014 (Leong et al., 2015).

In Malaysia coastal waters, the first Kareniaceae species found was *K. veneficum* in 2009 at Tebrau Strait during the collection of dinoflagellates for species inventory (Tan et al., 2013). Between 2011 and 2013, a harmful microalgae assemblage was conducted in Aman Island, the northern Malacca Strait had discovered the first occurrence of *K. australe* in Malaysia waters at cell densities 2.0×10^4 cells L⁻¹, but no fish kill were observed (Razali et al., 2015). In 2014, *K. australe* had reported bloomed at West Johor Strait, Malaysia causing massive death of fish with recorded cell densities reaching 2.34×10^6 cells L⁻¹ (Lim et al., 2014). As reported by Lim et al. (2014), *K. australe* bloom had negatively impacted the cultured fish along the coasts of Tanjung Kupang, Johor and the number of fish kills had

increased for three days until a week. About four tonnes of fish, including wild fishes in the surrounding area was killed during the *K. australe* bloom event (Lim et al., 2014). The bloom of *K. australe* recurred in 2015 with the highest cell densities recorded of 2.0×10^8 cells L⁻¹, and had caused even greater loss for approximately more than 30 tonnes of caged-fish died, exclusive of the mortality of wild demersal fish and crustaceans along the West Johor Strait (Teng et al., 2016a). According to the witness or aquaculture operators, the fish demonstrated abnormal behaviour, for instances, aggressively jumping out of cages at night and aeration system installed did not improve the fish conditions. In the year 2020, the recent bloom in the aquaculture areas of Johor Strait had reported *Karlodinium* spp. as one of the bloom contributors, with cell densities ranging from 3.5 to 6.6×10^3 cells L⁻¹, but, no mortality of mussel or fish were observed in the bloom event (Razali et al., 2022).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling sites and water sample processing

Samples were collected from 11 locations located in Peninsular Malaysia and East Malaysia (Figure 3.1). Water samples were collected using a 20- μm mesh-size and 10- μm mesh-size plankton net. The water samples were kept in a cooler box and brought back to the laboratory. Upon reaching the laboratory, the water samples were processed using 23- μm and 35- μm mesh-size, and the retain cells were incubated in a 48-well culture plate.

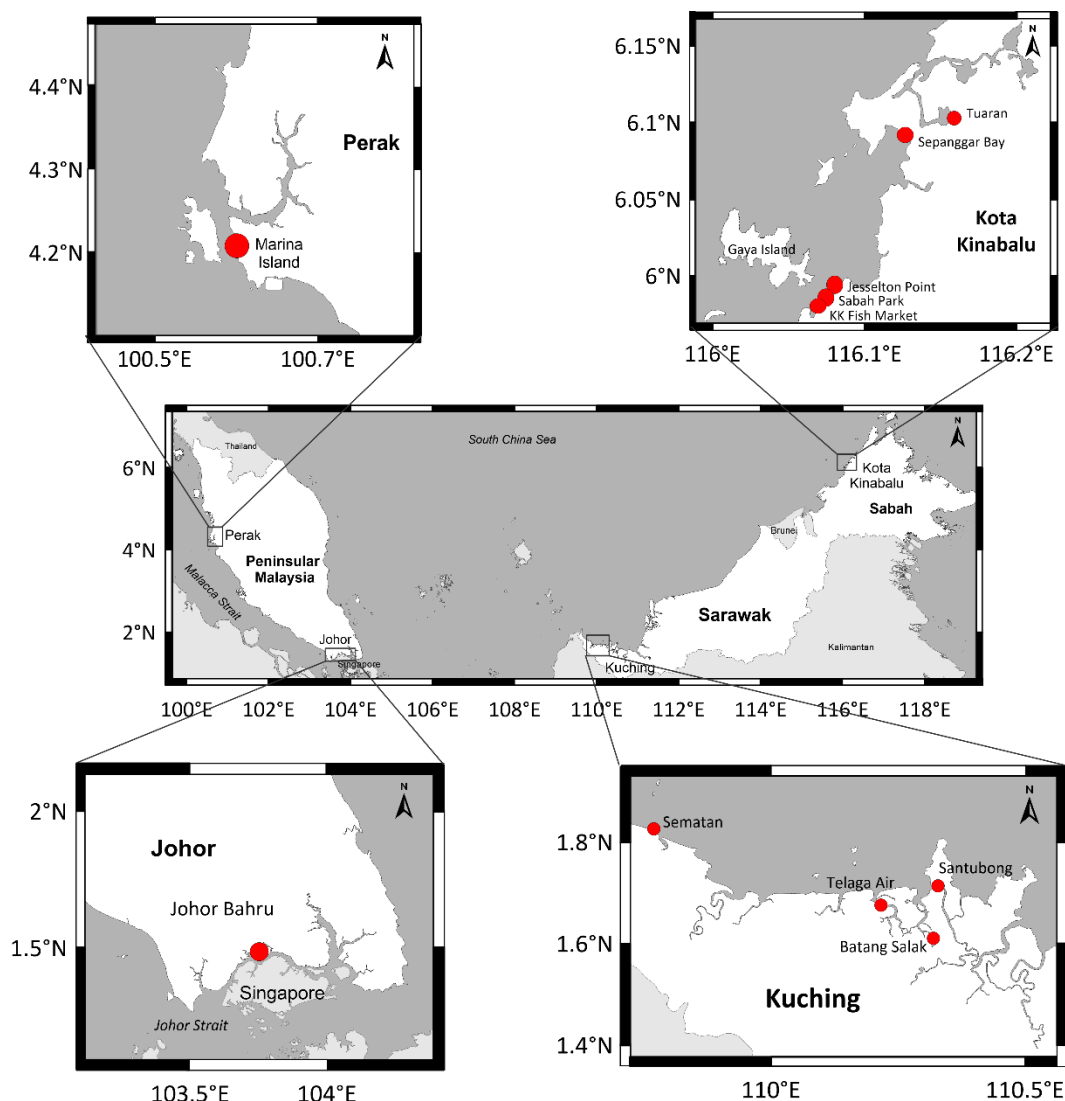


Figure 3.1: Map showing sampling locations in this study at Peninsular Malaysia: Perak (Marina Island) and Johor (Johor Bahru), and East Malaysia: Kuching, Sarawak (Batang Salak, Santubong, Sematan and Telaga Air) and Kota Kinabalu, Sabah (Jesselton Point, KK Fish Market, Sabah Park, Sepanggar Bay and Tuaran)

3.2 Single-cell isolation, culture establishment and maintenance

Single-cell isolation (Hoshaw & Rosowski, 1973) in 96-well plate was carried out using sterile Pasteur pipette under an inverted microscope Olympus IX71 (Olympus, Tokyo, Japan). The isolated cells were monitored daily and established into clonal cultures when reaching about 100 cells. The established clonal cultures were maintained in ES-DK medium (Kokinos & Anderson, 1995) and General Purpose Medium (GPM) (Loeblich, 1975), pH

range of 7.8 to 8.0, salinity of 30 PSU, and kept in growth chamber with temperature-controlled at 25°C under 12:12 light:dark photocycle and light intensity 100 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ (de Salas *et al.*, 2005; Kon *et al.*, 2017). Kareniaceae strains established and used in this study are listed in Table 3.1.

Table 3.1: List of Kareniaceae strains established and used in this study, with localities, and isolation dates

Strain	Locality	Coordinates (Latitude, Longitude)	Isolation date
KATAA02	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	24 August 2018
KATAL01	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	24 August 2018
KAJBL01	Johor Straits, Johor	1°28'03.500"N 103°46'48.000"E	15 April 2019
KAJBL02	Johor Straits, Johor	1°28'03.500"N 103°46'48.000"E	15 April 2019
KAJBL03	Johor Straits, Johor	1°28'03.500"N 103°46'48.000"E	15 April 2019
KAJBL04	Johor Straits, Johor	1°28'03.500"N 103°46'48.000"E	15 April 2019
KAJBL05	Johor Straits, Johor	1°28'03.500"N 103°46'48.000"E	15 April 2019
KAJBL06	Johor Straits, Johor	1°28'03.500"N 103°46'48.000"E	15 April 2019
KAJBL07	Johor Straits, Johor	1°28'03.500"N 103°46'48.000"E	15 April 2019
KABSG6	Batang Salak, Sarawak	1°36'31.500"N 110°19'36.800"E	11 April 2022
KBTAUB10	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	12 July 2022
KBTAUB3	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	12 July 2022
KBTAUB4	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	12 July 2022
KBTAUB5	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	12 July 2022

Table 3.1 continued

KBTAUB6	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	12 July 2022
KBTAUB7	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	12 July 2022
KBTAUB9	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	12 July 2022
KVLML01	Marina Island, Perak	4°12'21.200"N 100°35'48.900"E	5 April 2019
KVLML02	Marina Island, Perak	4°12'21.200"N 100°35'48.900"E	5 April 2019
KVLML03	Marina Island, Perak	4°12'21.200"N 100°35'48.900"E	5 April 2019
KVLML04	Marina Island, Perak	4°12'21.200"N 100°35'48.900"E	5 April 2019
KVLML05	Marina Island, Perak	4°12'21.200"N 100°35'48.900"E	5 April 2019
KZSTL01	Sematan, Sarawak	1°49'38.2"N 109°45'28.6"E	17 July 2018
KVTAUD7	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	17 May 2021
KVTAUD8	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	17 May 2021
KZTAUC7	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	17 May 2021
KGKKGD3	KK Fish Market, Sabah	5°59'0.006"N 116°4'20.443"E	2 September 2022
KARMJPGC5	Jesselton Point, Sabah	5°59'24.777"N 116°4'47.280"E	2 September 2022
KASKUF2	Sabah Park, Sabah	5°59'8.820"N 116°4'28.858"E	2 September 2022
KASKUF3	Sabah Park, Sabah	5°59'8.820"N 116°4'28.858"E	2 September 2022
TYTAUB2	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 February 2022
TYTAUB4	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 February 2022
TYTAUB6	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 February 2022
TYTAUF4	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 February 2022

Table 3.1 continued

KBTALB8	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 July 2022
KBTALC8	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 July 2022
KBTALD8	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 July 2022
KBTALD9	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 July 2022
KBTALE2	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	21 July 2022
KBSB703	Santubong, Sarawak	1°42'58.900"N 110°19'40.700"E	26 June 2018
KBSBL01	Santubong, Sarawak	1°42'58.900"N 110°19'40.700"E	26 June 2018
KBSBL02	Santubong, Sarawak	1°42'58.900"N 110°19'40.700"E	26 June 2018
TXTAL01	Telaga Air, Sarawak	1°40'35.800"N 110°12'35.600"E	23 August 2018
TXTAL02	Telaga Air, Sarawak	1°40'35.800"N 110°12'35.600"E	23 August 2018
TXTAL03	Telaga Air, Sarawak	1°40'35.800"N 110°12'35.600"E	17 August 2018
TXTAL04	Telaga Air, Sarawak	1°40'35.800"N 110°12'35.600"E	23 August 2018
TXTAL05	Telaga Air, Sarawak	1°40'35.800"N 110°12'35.600"E	23 August 2018
TYKKLF10	Tuaran, Sabah	6°06'13.100"N 116°09'35.000"E	27 September 2018
KABSUF2	Batang Salak, Sarawak	1°36'31.500"N 110°19'36.800"E	30 June 2022
TYTAUF6	Telaga Air, Sarawak	1°40'35.500"N 110°12'35.800"E	4 April 2022
KZJPGF6	Jesselton Point, Sabah	5°59'24.777"N 116°4'47.280"E	4 September 2022
KASPBGF11	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	4 September 2022
KAZSPBGD9	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	4 September 2022
KMSPBUD5	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	4 September 2022

Table 3.1 continued

KASPBLB2	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	7 September 2022
KASPBLB7	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	7 September 2022
KASPBLB8	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	7 September 2022
KASPBLB9	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	7 September 2022
KASPBLC2	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	7 September 2022
KDSPBLB11	Sepanggar Bay, Sabah	6°5'27.597"N 116°7'40.796"E	7 September 2022

3.3 Morphological characterisation

3.3.1 Light microscopy (LM)

Live Kareniaceae cells of about five to seven days of cultures were observed using a light microscope Olympus BX51 (Olympus, Tokyo, Japan) equipped with a Lumenera Infinity3-3UR microscopy camera (Lumenera, Ottawa, Canada). Cell micrographs were captured to record the cell shape and morphometric measurements. Cells were stained with 0.1% SYBR® Safe DNA Gel stain (Invitrogen, Massachusetts, United States) and examined under epifluorescence with 470–490 nm excitation and 510–550 nm emission to observe position and shape of the nucleus, and chloroplast arrangement.

3.3.2 Scanning electron microscopy (SEM)

Clonal culture of Kareniaceae was preserved for Scanning Electron Microscopy (SEM) with 1% acidic Lugol's solution and 1% glutaraldehyde overnight, and stored in 4°C (Nezan *et al.*, 2014). The fixed samples were dehydrated with a graded series of ethyl alcohol concentration (10%, 30%, 50%, 75%, 90%, 95% and 100%) and critical-point-dried. Dried

samples were mounted on stub and coated with gold-palladium using a JEOL JFC-1600 magnetron sputter coating instrument (JEOL, Japan) before viewing under a JEOL JSM-6510 analytical SEM (JEOL, Japan).

3.4 Molecular characterisation

3.4.1 Genomic DNA extraction, gene amplification, purification and sequencing

The cells in the exponential phase were harvested by centrifugation (3,500 g, 10 min) and the genomic DNA was extracted according to DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) protocol. The large subunit ribosomal RNA gene (LSU rDNA) was amplified using primer pair, D1R and D3Ca (Scholin *et al.*, 1994; Table 3.2) and the internal transcribed spacer 1 (ITS1) region was amplified using primer pair, SDINOITSF and Smalldino ITS2R (this study, Table 3.2).

Table 3.2: Primer used to amplify LSU rDNA and ITS of Kareniaceae in this study.

Primer	Gene	Sequence (5'-3')	Annealing temperature (°C)	Direction
D1R	LSU	ACC CGC TGA ATT TAA GCA TA	52.0	Forward
D3Ca	LSU	ACG AAC GAT TTG CAC GTC AG	56.0	Reverse
SDINOITSF	ITS	TCG TAA CAA GGT TTC CGT AGG TG	56.7	Forward
Smalldino ITS2R	ITS	GGT ACT TGT TTG CTA TCG GTC TCG	57.4	Reverse

Polymerase Chain Reaction (PCR) was conducted to amplify and sequence the ITS region and LSU rDNA by preparing PCR master mix (Table 3.3). PCR was performed in thermal cycling steps (Table 3.4) using a Mastercycler® nexus GX2 thermocycler (Eppendorf, Hamburg, Germany) with 35 total cycles.

Table 3.3: Composition of PCR master mix used in this study

Reagents	Stock concentration	Final concentration	Volume (μL)
ddH ₂ O	-	-	15.1
Buffer	10×	5×	5.0
MgCl ₂	25 μM	1.5 μM	1.5
dNTP	25 μM	0.2 μM	0.2
Forward primer	25 μM	1.5 μM	0.5
Reverse primer	25 μM	1.5 μM	0.5
DNA template	-	1-100 ng	2.0
<i>Taq</i> polymerase	5U μL^{-1}	1U μL^{-1}	0.2
Total volume	25.0		

Table 3.4: PCR thermal cycling steps used in this study

Gene	Steps	Temperature ($^{\circ}\text{C}$)	Duration
LSU	Initial Denaturation	94.0	4 min
	Denaturation	94.0	35 s
	Annealing	51.5	50 s
	Elongation	72.0	35 s
	Final Elongation	72.0	7 min
	Hold	4.0	∞
ITS	Initial Denaturation	94.0	4 min
	Denaturation	94.0	35 s
	Annealing	56.0	50 s
	Elongation	72.0	35 s
	Final Elongation	72.0	7 min
	Hold	4.0	∞

The amplicons were run for gel electrophoresis in 75V for 25 min and viewed using a stand-alone E-Box gel documentation imaging (Vilber, Marne-la-Vallée, France). The amplicons were purified using Promega Wizard® PCR Preps DNA Purification System (Madison, Wisconsin, United States) and outsourced for sequencing by Apical Scientific Sdn. Bhd. (Selangor, Malaysia).

3.4.2 Taxon sampling and phylogenetic analysis

The sequences of SSU, LSU and ITS rDNA obtained were evaluated by Basic Local Alignment Search Tool (BLAST) in GenBank nucleotide database (NCBI) and multiple aligned using Clustal-X (Thompson *et al.*, 1997) with related sequences. For LSU dataset, outgroups selected were *Gyrodinium dominans* Hulburt, *G. rubrum* (Kofoid & Swezy) Y.Takano & T.Horiguchi and *G. spirale* (Bergh) Kofoid & Swezy; while in SSU and ITS dataset, outgroups selected were *Prorocentrum micans* Ehrenberg, *P. minimum* (Pavillard) Schiller and *P. shikokuense* Hada.

Phylogenetic analyses were inferred using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). PAUP* ver. 4.0b.10 (Swofford, 2011) was used to perform Maximum Parsimony (MP) and Maximum Likelihood (ML). MP analysis was performed using heuristic searches of 1,000 random-addition replications and branch-swapping with tree-bisection reconnection (TBR). The bootstrap analysis was carried out with 1,000 bootstrap replications and 100 random additions of sequences run per bootstrap replicate. ML analysis was performed using heuristic searches and branch-swapping with 100 random addition replications in TBR. Bayesian inference (BI) analysis was performed with MrBayes 3 (Huelsenback & Ronquist, 2001). The best-fit model of ML and BI were calculated by the Akaike information criterion using jModelTest 2.1.10 (Darriba *et al.*, 2012). Phylogenetic trees were visualized in FigTree v1.4.3 (Rambaut, 2006) and phylogenetic trees for each marker were constructed by BI analysis.

3.4.3 ITS2 secondary structure modelling and sequence-structure phylogeny

ITS2 secondary structure of *Karlodinium* species were annotated from the ITS sequences based on the 5.8S–28S interaction (proximal stem). The ITS2 secondary structure of *Karlodinium* species were predicted using the free energy minimization in RNAstructure

v6.4. The ITS2 RNA transcripts were modelled by homology modelling workflow (Wolf *et al.*, 2005), using the ITS2 Database (Koetschan *et al.*, 2012; Merget *et al.*, 2012). The ITS2 secondary structure was illustrated in VARNA (Darty *et al.*, 2009). Eight sequences of *Karlodinium* species modelled were four of *K. australe*, *K. ballantini*, *K. veneficum* and *K. zhouanum* from Sarawak, and four of *K. armiger*, *K. azanzae*, *K. decipiens* and *K. gentienii* from Sabah.

The multiple sequence-structure alignment of *Karlodinium* ITS2 was generated in an ITS sequence structure-specific scoring matrix (Seibel *et al.*, 2006) in 4SALE v1.7 (Seibel *et al.*, 2006, 2008). A compensatory base change (CBC) and hemi-compensatory base change (HCB) were identified in 4SALE (Wolf *et al.*, 2005; Seibel *et al.*, 2006, 2008).

3.5 Lethality assay of *Lates calcarifer* fingerlings

3.5.1 Fish husbandry and acclimatisation

About 1-inch fingerlings of cultured Asian seabass *Lates calcarifer* were provided by Fisheries Research Institute Santubong. The fingerlings were kept in aerated aquariums filled with natural seawater of 30 PSU and fed daily. The fingerlings were acclimatised and starved within 24-h prior to fish bioassay.

3.5.2 Toxicity testing in different *Karlodinium australe* cell densities and cell condition

The lethality assay was conducted in a 24-h static test (Grant *et al.*, 2014; Svendsen *et al.*, 2018) or terminated when all the fish died. The exposure to different cell densities was carried out by exposing fingerlings seabass *L. calcarifer* to five different cell densities of *K. australe* Sarawak strain (KATAL01), and five different cell densities of *K. australe* Johor strain (KAJB05). A starvation control was prepared by exposing fingerlings seabass *L.*

calcarifer to only GPM medium. All the treatments in the cell densities experiments were conducted in triplicates.

In the exposure experiments to different cell conditions of *K. australe*, *K. australe* Sarawak (KATAL01) of cell concentrations 2.5×10^3 cells mL⁻¹ was prepared in four treatments that were live cells, sonicated cells, culture filtrate and sonicated cell filtrate. The fingerlings seabass *L. calcarifer* were exposed in four treatments, and a control with only GPM medium. All the treatments in the cell conditions experiment was conducted in triplicates.

3.6 Lethality assay of brine shrimp

3.6.1 Brine shrimp cyst hatching and decapsulation

Hatching bottle was filled with sterile filtered natural seawater of 30 PSU, equipped with aeration and fully illuminated. About 0.2 g of brine shrimp cysts was weighed and mixed in the hatching bottle containing sterile seawater. The hatching was monitored at 12-h interval to observe any signs of hatching or decapsulation. Once hatching, the newly hatched brine shrimp was observed swimming towards the light. The newly hatched brine shrimps were transferred into a new bottle containing sterile seawater via adjustable stopper.

3.6.2 Toxicity testing towards brine shrimp

The bioassay for toxicity screening was conducted with brine shrimps *Artemia* sp., 48-h after hatching in sterile filtered natural seawater of salinity 30 PSU. The lethality was run by exposing *Artemia* sp. to ten treatments in triplicate: *K. mikimotoi* (KMSPBUD5), *K. armiger* (KARMJPGC5), *K. australe* (KAJBL05, KABSUF2), *K. azanzae* (KAZSPBGD9), *K. ballantinum* (KBTAUB3), *K. decipiens* (KDSPBLB11), *K. gentienii* (KGKKGD3), *K. veneficum* (KVTAUD8), and *T. acrotrocha* (TYTAUF4). Ten *Artemia* nauplii were isolated

using glass Pasteur pipette and transferred into each well. Chlorophyte *Tetraselmis chuii* was used as fed control and starvation control with only filtered seawater. The number of dead nauplii in each treatment was counted and the survival of nauplii throughout exposure was determined. Nauplii was recorded dead when no motility observed and failure in responding to external stimulation.

3.7 Data analysis

The percentages of fish survival in different cell densities and cell conditions were determined by plotting the percentages of fish mortality against time of exposure in the analysis of XY table performed in GraphPad Prism ver. 9.0.0. Percentage of brine shrimp survival was determined by plotting the percentage of brine shrimp mortality against time of exposure in the analysis of XY table performed in GraphPad Prism ver. 9.0.0. The interaction between nauplii and Kareniaceae cells were observed and recorded using an inverted microscope Olympus CKX53 (Olympus, Tokyo, Japan) equipped with microscopy camera.

CHAPTER 4

RESULTS

4.1 Species diversity

Genera *Karenia*, *Karlodinium* and *Takayama* were found at all sampling locations (Figure 4.1). A total of 10 species were described and identified from the coastal waters of Peninsular Malaysia and East Malaysia: one of *Karenia*, eight of *Karlodinium* and one of *Takayama*. This study revealed the Kareniaceae family had high diversity in Malaysian coastal waters (Figure 4.1). Among the locations, Sabah had the highest species diversity with eight species observed viz. *K. mikimotoi*, *K. armiger*, *K. australe*, *K. azanzae*, *K. decipiens*, *K. gentienii*, *K. zhouanum* and *T. acrotrocha*, followed by Sarawak of five species which were *K. australe*, *K. ballantinum*, *K. veneficum*, *K. zhouanum* and *T. acrotrocha*. The lowest species diversity was recorded at two locations, Johor and Perak where only one species was found, *K. australe* and *K. veneficum* respectively. Out of all the recorded species, only *K. australe* was mostly present at all locations investigated, except in Perak.

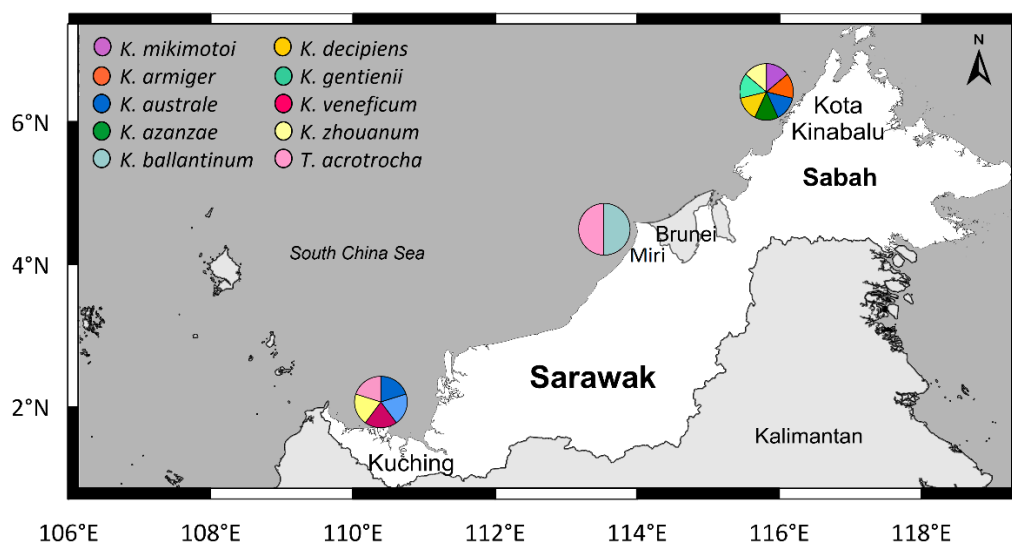


Figure 4.1: Map showing species distribution and species diversity of family Kareniaceae in the coastal waters of Peninsular Malaysia and East Malaysia

A total of 59 strains of Kareniaceae were found in the coastal waters of Malaysia (Table 4.1). Five strains of *K. veneficum* were found in Perak. Seven strains of *K. australe* were found in Johor. In Sarawak, 32 strains consisted of *K. australe*, *K. ballantinum*, *K. veneficum*, *K. zhouanum*, and *T. acrotrocha* were found. Meanwhile in Sabah, 15 strains of *K. mikimotoi*, *K. armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, *K. zhouanum* and *T. acrotrocha* were found.

Table 4.1: Kareniaceae species list, distribution and number of species found in this study

Genera	Species	Perak	Johor	Sarawak	Sabah
<i>Karenia</i>	<i>K. mikimotoi</i>	-	-	-	+
<i>Karlodinium</i>	<i>K. armiger</i>	-	-	-	+
	<i>K. australe</i>	-	+	+	+
	<i>K. azanzae</i>	-	-	-	+
	<i>K. ballantinum</i>	-	-	+	+
	<i>K. decipiens</i>	-	-	-	+
	<i>K. gentienii</i>	-	-	-	+
	<i>K. veneficum</i>	+	-	+	-
	<i>K. zhouanum</i>	-	-	+	+
<i>Takayama</i>	<i>T. acrotrocha</i>	-	-	+	+
Total species		5	7	32	15

4.2 Species diagnoses

Ten species of Kareniaceae were observed in this study and nine of them were identified as *Karenia mikimotoi*, *Karlodinium armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, *K. veneficum* and *Takayama acrotrocha*, as unfortunately the culture of *K. zhouanum* died off upon completion this study. The species morphotype descriptions of the remaining nine morphotypes are presented below (Table 4.2).

Table 4.2: Morphometric descriptions of *Karenia mikimotoi*, *Karlodinium armiger*, *Karlodinium australe*, *Karlodinium azanzae*, *Karlodinium ballantinum*, *Karlodinium decipiens*, *Karlodinium gentienii*, *Karlodinium veneficum* and *Takayama acrotrocha* in Malaysian coastal waters obtained in this study. All measurements were taken by LM and SEM

Species	Length (µm)	Width (µm)	Cell shape in ventral view	Nucleus	Sulcal intrusion	Ventral pore	Apical groove	Reference(s)
<i>K. mikimotoi</i>	22.1–27.4 (25.0±1.6, <i>n</i> =30)	17.2–23.9 (21.0±2.3, <i>n</i> =30)	Broadly ovoid and dorso-ventrally flattened, with conical epicone and two-lobed hemispherical hypocone	Ellipsoid, located on the left side of hypocone nearing the edge of cell	Present	Absent	Straight, wide, slightly above sulcal intrusion extending to dorsal epicone	In this study
<i>K. mikimotoi</i>	20.0–30.0 (24.8±0.4)	16.0–30.0 (20.9±0.3)	Broadly ovoid and flattened dorsal abdomen, with wide conical epicone and two-lobed flakes hypocone	Ellipsoid, located on the left side of hypocone nearing the edge of cell	Present	Absent	Straight, slightly above right side of the starting point of the sulcal intrusion extending to the dorsal epicone	Oda (1935); Haywood et al. (2004)
<i>K. armiger</i>	10.1–18.3 (13.9±2.4, <i>n</i> =30)	8.4–14.2 (11.4±1.8, <i>n</i> =30)	Ovoid, with slightly conical epicone and hemispherical hypocone	Large, spherical and centrally located	Present	Elongated (1.8–2.0 µm) to the left of apical groove	Straight, crossing apex, descending to dorsal epicone	In this study

Table 4.2 continued

<i>K. armiger</i>	12.0–22.0 (17.4±2.4, <i>n</i> =50)	8.0–18.0 (13.1±0.1, <i>n</i> =50)	Ovoid, with conical epicone and hemispherical hypocone	Large and centrally located	Present	Elongated (1 µm) to the left of apical groove	Straight, barely crossing apex, descending one- fourth down the dorsal epicone	Bergholtz et al. (2005)
<i>K. australe</i>	13.7–21.5 (18.6±2.4, <i>n</i> =30)	11.7–18.9 (16.3±2.5, <i>n</i> =30)	Ovoid, with both hemispherical epicone and hypocone	Large, elongated spherical and centrally located	Present	Reniform (0.7– 1.0 µm) to the left of apical groove	Straight, wide at both ends but narrow in the middle, extending to dorsal epicone	In this study
<i>K. australe</i>	19.0–26.0 (21.8±1.9, <i>n</i> =30)	16.0–22.0 (18.9±1.8, <i>n</i> =30)	Ovoid, with both hemispherical epicone and hypocone	Large, irregular spherical and centrally located	Present	Reniform, above and to the left of the sulcal intrusion	Short and straight, extending only briefly onto the dorsal epicone	de Salas et al. (2005)
<i>K. azanzae</i>	18.4–25.4 (21.1±1.7, <i>n</i> =30)	15.7–21.1 (18.5±1.3, <i>n</i> =30)	Ovoid, with both hemispherical epicone and hypocone	Elongated or spherical and centrally located	Present	Slit-shaped (0.7–0.8 µm) to the left of apical groove	Straight, wide, extending down to dorsal epicone	In this study
<i>K. azanzae</i>	21.4–29.6 (25.3±2.3, <i>n</i> =71)	15.1–24.1 (19±2.1, <i>n</i> =66)	Ovoid, with both hemispherical epicone and hypocone	Elongated and centrally located	Present	Slit-shaped, located at the left side of the apical structure complex and above sulcal extension	Straight and short, briefly extends down into the dorsal side of the epicone	Benico et al. (2020)

Table 4.2 continued

<i>K. ballantinum</i>	10.2–15.9 (12.6±1.7, <i>n</i> =30)	8.3–12.8 (10.5±1.3, <i>n</i> =30)	Ovoid, with slightly conical epicone and hemispherical hypocone	Large, spherical and centrally located	Present	Elongated (0.4 µm) to the left of apical groove	Straight, wide at both ends but narrow in the middle, extending to dorsal epicone	In this study
<i>K. ballantinum</i>	11.0–18.0 (14.7±0.2, <i>n</i> =73)	8.0–14.0 (10.9±0.2, <i>n</i> =73)	Ellipsoid, with both hemispherical epicone and hypocone	Large and centrally located	Present	Absent or inconspicuous	Very short and linear, extending very briefly onto the dorsal epicone	de Salas et al. (2008)
<i>K. decipiens</i>	15.7–19.7 (18.0±1.1, <i>n</i> =30)	11.5–14.9 (13.6±0.8, <i>n</i> =30)	Ellipsoid, with both hemispherical epicone and hypocone	Spherical and centrally located	Present	Slit-like (0.5– 0.7 µm) to the left of apical groove	Straight, long and wide, extending halfway down to dorsal epicone	In this study
<i>K. decipiens</i>	18.0–25.0 (21.4±0.2, <i>n</i> =50)	13.0–29.0 (16.2±0.2, <i>n</i> =50)	Ellipsoid, a little dorsoventrally flattened, with both hemispherical epicone and hypocone	Large and centrally located	Present	Slit-like, located above the sulcus	Long, extending halfway down the dorsal epicone	de Salas et al. (2008)

Table 4.2 continued

<i>K. gentienii</i>	10.4–12.8 (11.5±0.6, <i>n</i> =30)	7.4–9.8 (8.9±0.5, <i>n</i> =30)	Ovoid, with conical and pointy epicone, and hemispherical hypocone	Large, ellipsoid and centrally located	Present	Elongated (0.3–0.4 µm) to the left of apical groove	Straight, wide, descending down the dorsal epicone	In this study
<i>K. gentienii</i>	13.5–18.9 (16.3±1.5, <i>n</i> =30)	11.5–16.8 (13.6±1.4, <i>n</i> =30)	Ovoid, with conical and hemispherical hypocone	Large, irregular shape and centrally located	Present	Elongated, (0.8–1.0 µm), above the sulcal intrusion, next to the base of the carina	Straight, descending one- sixth down the dorsal epicone	Nezan et al. (2014)
<i>K. veneficum</i>	9.9–12.5 (11.0±0.7, <i>n</i> =30)	8.0–10.5 (9.2±0.8, <i>n</i> =30)	Ovoid, with both hemispherical epicone and hypocone	Large, spherical and centrally located	Present	Elongated (0.8–1.0 µm) to the left of apical groove	Straight, wide, descending down the dorsal epicone	In this study
<i>K. veneficum</i> (K-0522)	8.0–18.0 (13.6±1.9, <i>n</i> =50)	8.0–14.0 (11.1±1.4, <i>n</i> =50)	Ovoid, with conical or hemispherical epicone and hemispherical hypocone	Large, spherical and centrally located	Present	Elongated (1.0 µm) to the left of the apical groove	Straight, descending one- seventh down the dorsal epicone	Bergholtz et al. (2005)

Table 4.2 continued

<i>T. acrotrocha</i>	12.5–16.5 (14.2±1.1, <i>n</i> =30)	10.6–15.4 (12.9±1.6, <i>n</i> =30)	Ovoid, with depressed ovoid epicone and depressed obovoid hypocone	Large, transversely ellipsoid	Present	Absent	Sigmoid, extending from the proximal end of girdle around the apex	In this study
<i>T. acrotrocha</i>	14.9–21.7	12.9–20.2	Spherical, episome semi-circular, hyposome somewhat flattened and slightly indented antapically	Fill up much of the episome	Present	Absent	Sigmoid, extend from the proximal end of the girdle around the apex	Larsen (1996); Cho et al. (2021)

4.2.1 Morphology of *Karenia mikimotoi*

Cells of *K. mikimotoi* were broadly ovoid with dorso-ventrally flattened body, the epicone was conical and slightly smaller than the hemispherical hypocone with two lobes (Figure 4.2 A–C). Cell size ranged from 22.1–27.4 μm in length (mean $25.0 \pm 1.6 \mu\text{m}$; $n = 30$) and 17.2–23.9 μm in width (mean $21.0 \pm 2.3 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.2 A–C), with numerous chloroplasts visible under epifluorescence microscopy (Figure 4.2 D–E). The ellipsoidal nucleus was situated at the left side of hypocone nearing the edge of cell (Figure 4.2 D–E). Straight and wide apical groove was situated slightly above the sulcal intrusion, extending to the dorsal of epicone, creating a slight indentation at the cell apex (Figure 4.2 A–C, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Sepanggar Bay ($6^{\circ}5'27.597''\text{N}$ $116^{\circ}7'40.796''\text{E}$), Sabah, Malaysia Borneo.

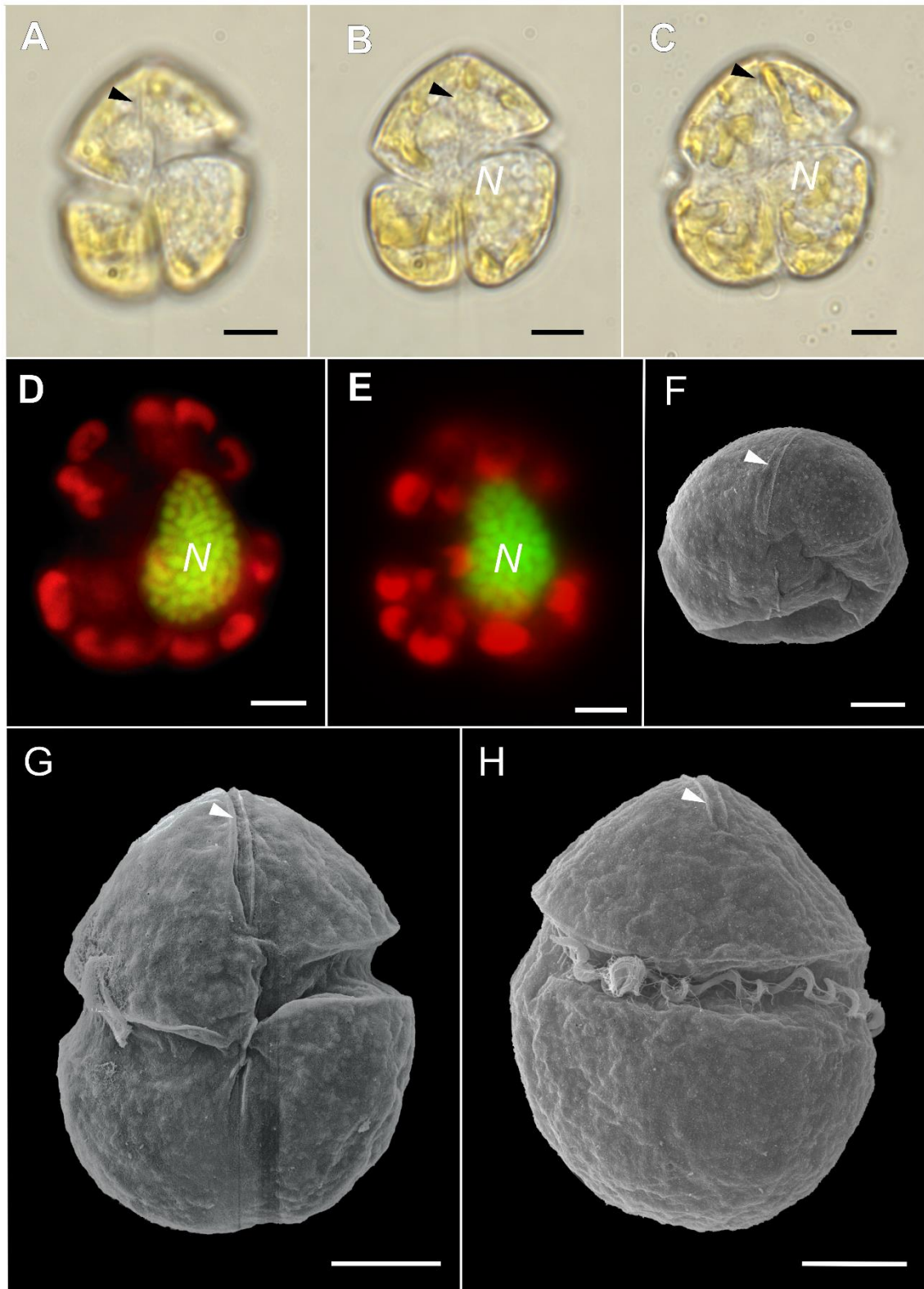


Figure 4.2: *Karenia mikimotoi*. (A-E) LM. Live cells showing apical groove (arrowhead) and nucleus (N). (D-E) SYBR Safe-stained cells with an ellipsoid nucleus (N) located on the left side of hypocone nearing the edge of cell. (F-H) SEM. Straight apical groove (arrowhead) in apical (F), ventral (G) and dorsal (H) views. Scales, 5 μ m

4.2.2 Morphology of *Karlodinium armiger*

Cells of *K. armiger* were ovoid with the epicone slightly conical, and slightly smaller than the hemispherical hypocone (Figure 4.3 A–C). Cell size ranged from 10.1–18.3 μm in length (mean $13.9 \pm 2.4 \mu\text{m}$; $n = 30$) and 8.4–14.2 μm in width (mean $11.4 \pm 1.8 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.3 A–C), with several chloroplasts visible under epifluorescence microscopy (Figure 4.3 D–E) surrounding the large, spherical nucleus situated at the centre of the cell (Figure 4.3 D–E). Straight and wide apical groove was situated above the sulcal intrusion, extending to the dorsal of epicone, creating a slight indentation at the cell apex (Figure 4.3 A–C, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Jesselton Point ($5^{\circ}59'24.777''\text{N}$ $116^{\circ}4'47.280''\text{E}$), Sabah, Malaysia Borneo.

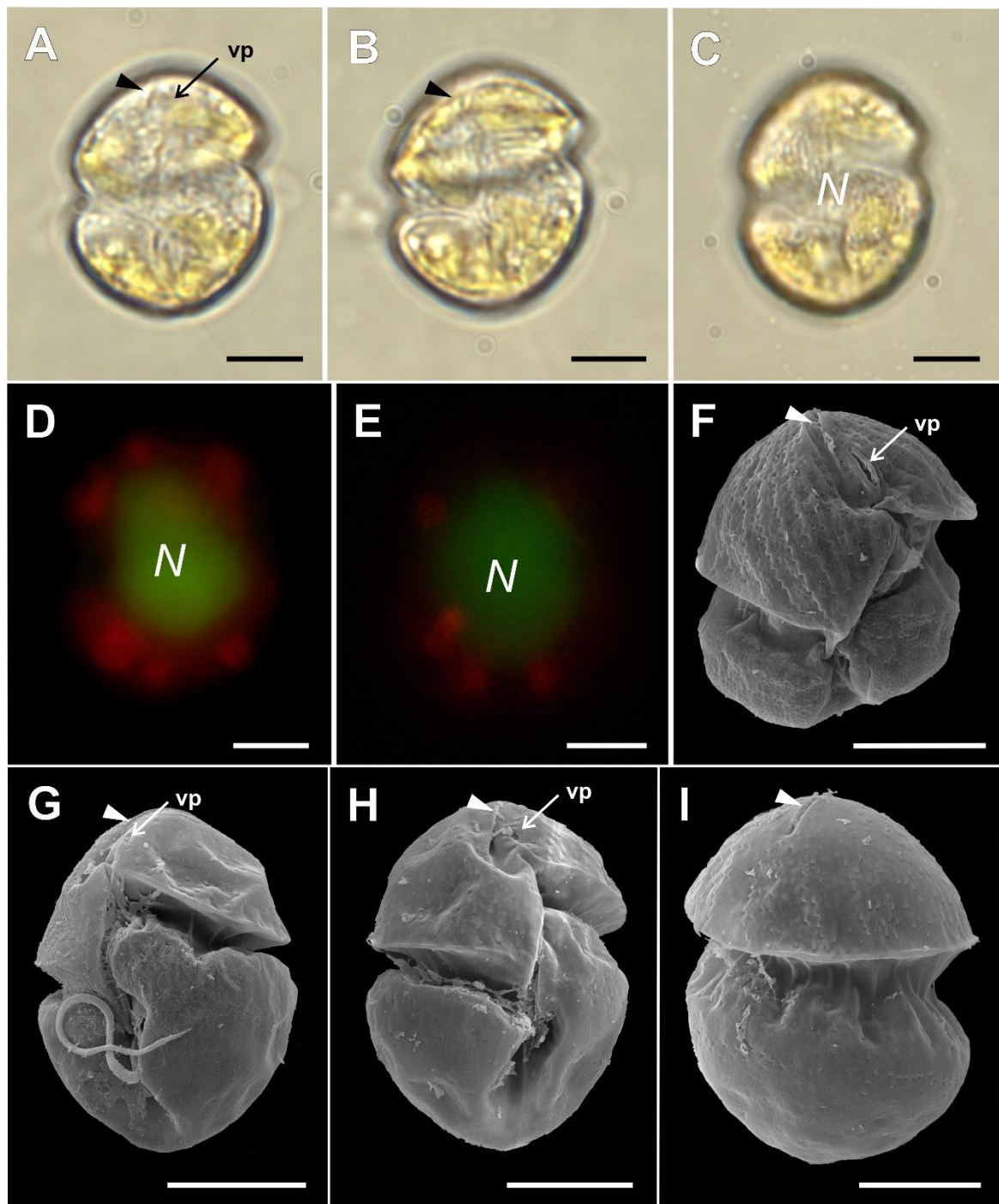


Figure 4.3: *Karlodinium armiger*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, spherical nucleus (N) located at the centre of the cell. (F-I) SEM. Straight apical groove (arrowhead) extends from ventral to dorsal and ventral pore (vp) in apical view (F) and ventral views (G-I). Scales, 5 μm

4.2.3 Morphology of *Karlodinium australe*

Cells of *K. australe* were ovoid with both epicone and hypocone were hemispherical (Figure 4.4 A–C). Cell size ranged from 13.7–21.5 μm in length (mean $18.6 \pm 2.4 \mu\text{m}$; $n = 30$) and 11.7–18.9 μm in width (mean $16.3 \pm 2.5 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.4 A–C), with several chloroplasts visible under epifluorescence microscopy (Figure 4.4 D–E) surrounding the large, spherical nucleus situated at the centre of the cell (Figure 4.4 D–E). Straight and wide apical groove situated above the sulcal intrusion was wide at both ends but getting narrower in the middle, and extending to the dorsal of epicone, creating a slight indentation at the cell apex (Figure 4.4 A–C, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Batang Salak ($1^{\circ}36'31.500''\text{N}$ $110^{\circ}19'36.800''\text{E}$), Sarawak, Malaysia Borneo. Telaga Air ($1^{\circ}40'35.800''\text{N}$ $110^{\circ}12'35.600''\text{E}$), Sarawak, Malaysia Borneo. Sabah Park ($5^{\circ}59'8.820''\text{N}$ $116^{\circ}4'28.858''\text{E}$), Sabah, Malaysia Borneo. Sepanggar Bay ($6^{\circ}5'27.597''\text{N}$ $116^{\circ}7'40.796''\text{E}$), Sabah, Malaysia Borneo. Johor Straits ($1^{\circ}28'03.500''\text{N}$ $103^{\circ}46'48.000''\text{E}$), Johor, Peninsular Malaysia

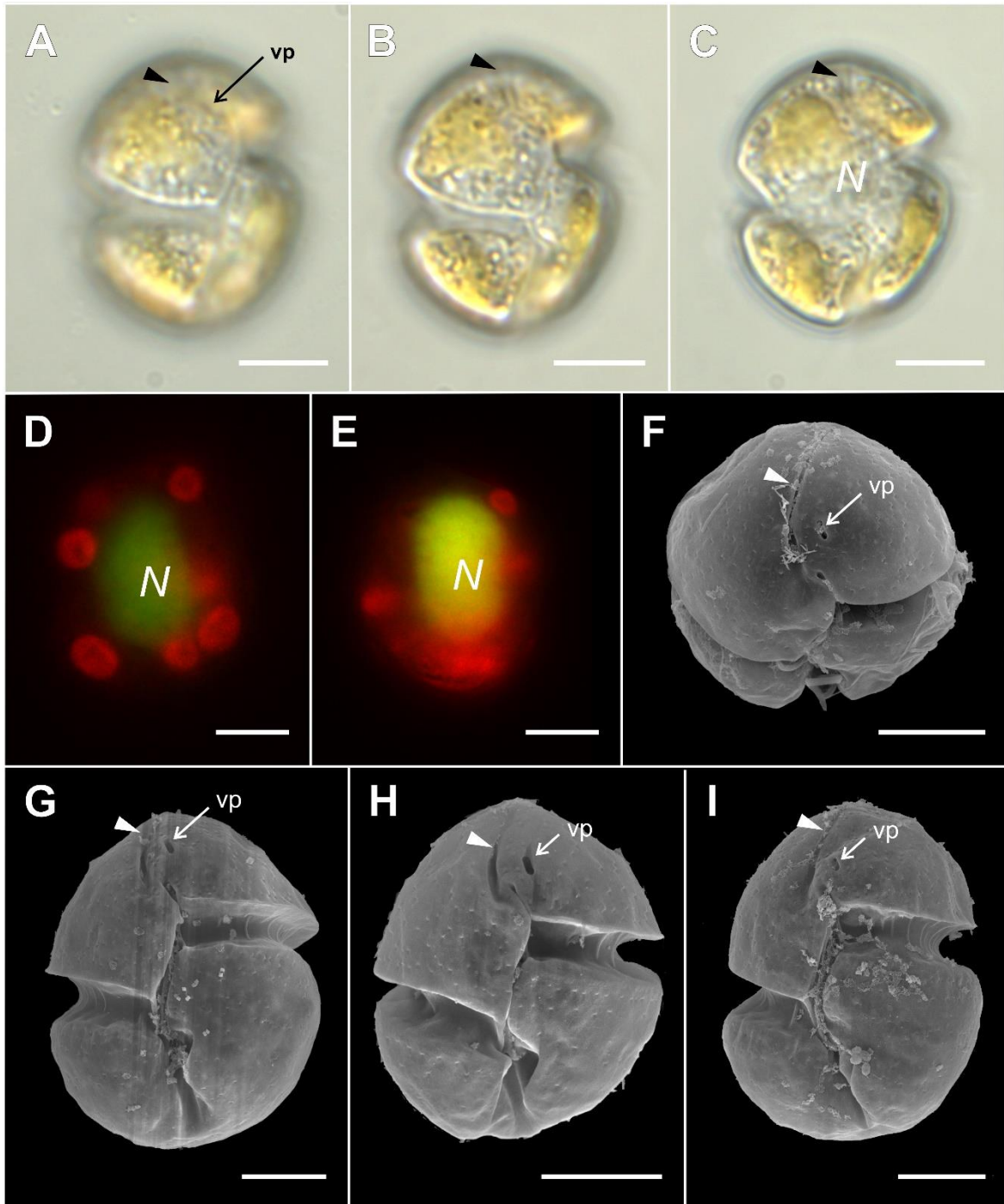


Figure 4.4: *Karlodinium australe*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, elongated spherical nucleus (N) located at the centre of the cell. (F-I) SEM. Straight apical groove (arrowhead) extends from ventral to dorsal and ventral pore (vp) in apical view (F) and ventral views (G-I). Scales, 5 µm

4.2.4 Morphology of *Karlodinium azanzae*

Cells of *K. azanzae* were ovoid with both epicone and hypocone were hemispherical (Figure 4.5 A–C). Cell size ranged from 18.4–25.4 μm in length (mean $21.1 \pm 1.7 \mu\text{m}$; $n = 30$) and 15.7–21.1 μm in width (mean $18.5 \pm 1.3 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.5 A–C), with numerous chloroplasts visible under epifluorescence microscopy (Figure 4.5 D–E) surrounding the elongated or spherical nucleus was situated at the centre to left edge of cell (Figure 4.5 D–E). The straight, wide apical groove extended from ventral at slightly above the sulcal intrusion to the dorsal of epicone, creating a slight indentation at the cell apex (Figure 4.5 A–C, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Sepanggar Bay ($6^{\circ}5'27.597''\text{N}$ $116^{\circ}7'40.796''\text{E}$), Sabah, Malaysia Borneo.

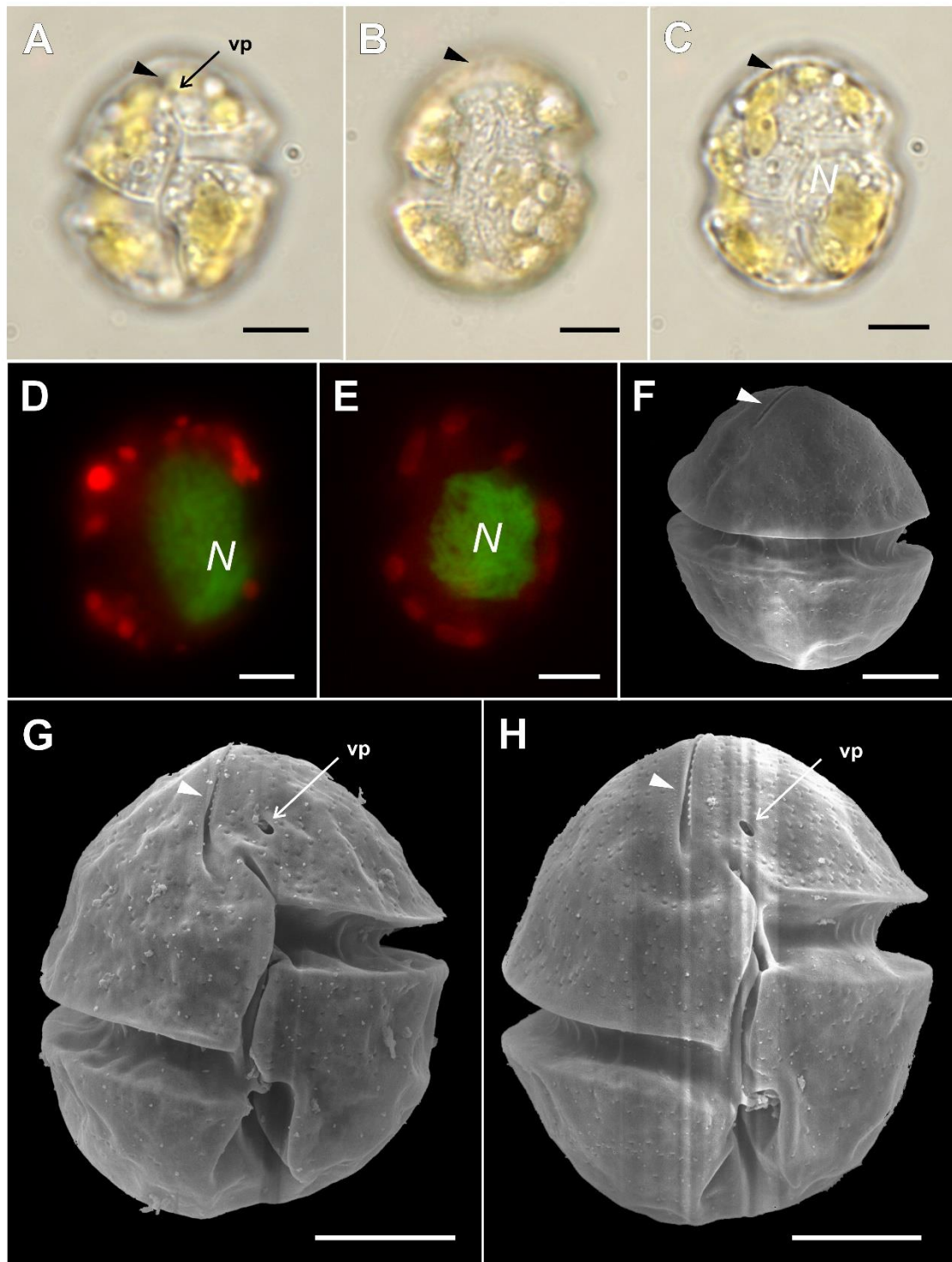


Figure 4.5: *Karlodinium azanzae*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with an elongated or spherical nucleus (N) located at the centre of the cell. (F-H) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F), ventral (G) and dorsal (H) views. Scales, 5 μm

4.2.5 Morphology of *Karlodinium ballantinum*

Cells of *K. ballantinum* were ovoid, with the epicone slightly conical, and slightly larger than the hemispherical hypocone (Figure 4.6 A–C). Cell size ranged from 10.2–15.9 μm in length (mean $12.6 \pm 1.7 \mu\text{m}$; $n = 30$) and 8.3–12.8 μm in width (mean $10.5 \pm 1.3 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.6 A–C), with numerous chloroplasts visible under epifluorescence microscopy (Figure 4.6 D–E) surrounding the elongated or spherical nucleus was situated at the centre to left edge of cell (Figure 4.6 D–E). The straight, wide apical groove was wide at both ends but narrow in the middle, extended from ventral at slightly above the sulcal intrusion to the dorsal of epicone, creating a slight indentation at the cell apex (Figure 4.6 A–C, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Santubong (1°42'58.900"N 110°19'40.700"E), Sarawak, Malaysia Borneo. Telaga Air (1°40'35.800"N 110°12'35.600"E), Sarawak, Malaysia Borneo.

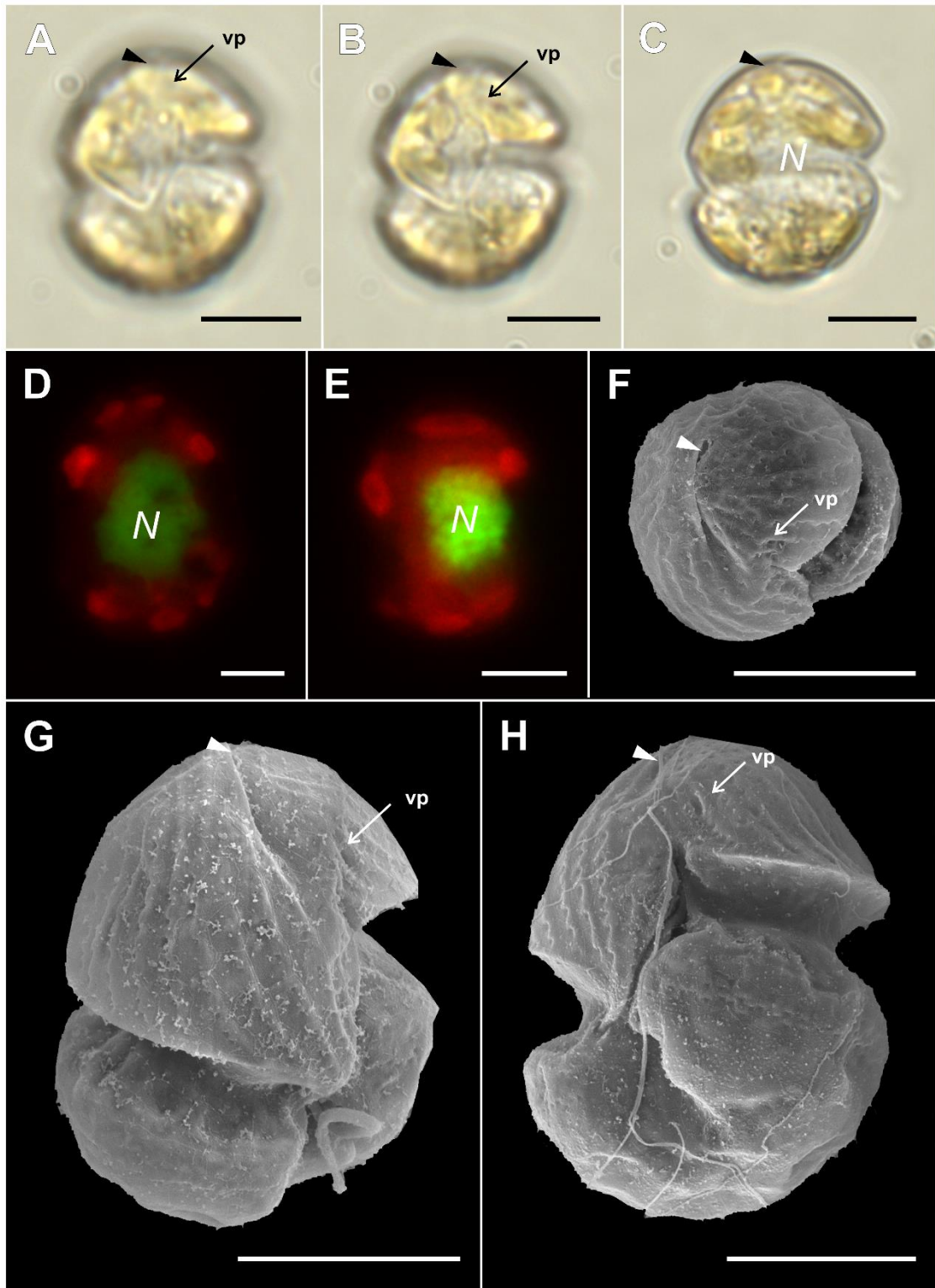


Figure 4.6: *Karlodinium ballantini*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, spherical nucleus (N) located at the centre of the cell. (F-H) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F) and ventral (G-H) views. Scales, 5 μm

4.2.6 Morphology of *Karlodinium decipiens*

Cells of *K. decipiens* were ellipsoid, with both epicone and hypocone were hemispherical, the hypocone was larger and elongated than epicone (Figure 4.7 A–C). Cell size ranged from 15.7–19.7 μm in length (mean $18.0 \pm 1.1 \mu\text{m}$; $n = 30$) and 11.5–14.9 μm in width (mean $13.6 \pm 0.8 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.7 A–C), with numerous chloroplasts visible under epifluorescence microscopy (Figure 4.7 D–E) surrounding the spherical nucleus was situated at the centre to left edge of cell (Figure 4.8 D–E). The straight, wide and long apical groove was extending below sulcus intrusion at ventral to the dorsal epicone, creating a slight indentation at the cell apex (Figure 4.7 A–B, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Sepanggar Bay ($6^{\circ}5'27.597''\text{N}$ $116^{\circ}7'40.796''\text{E}$), Sabah, Malaysia Borneo.

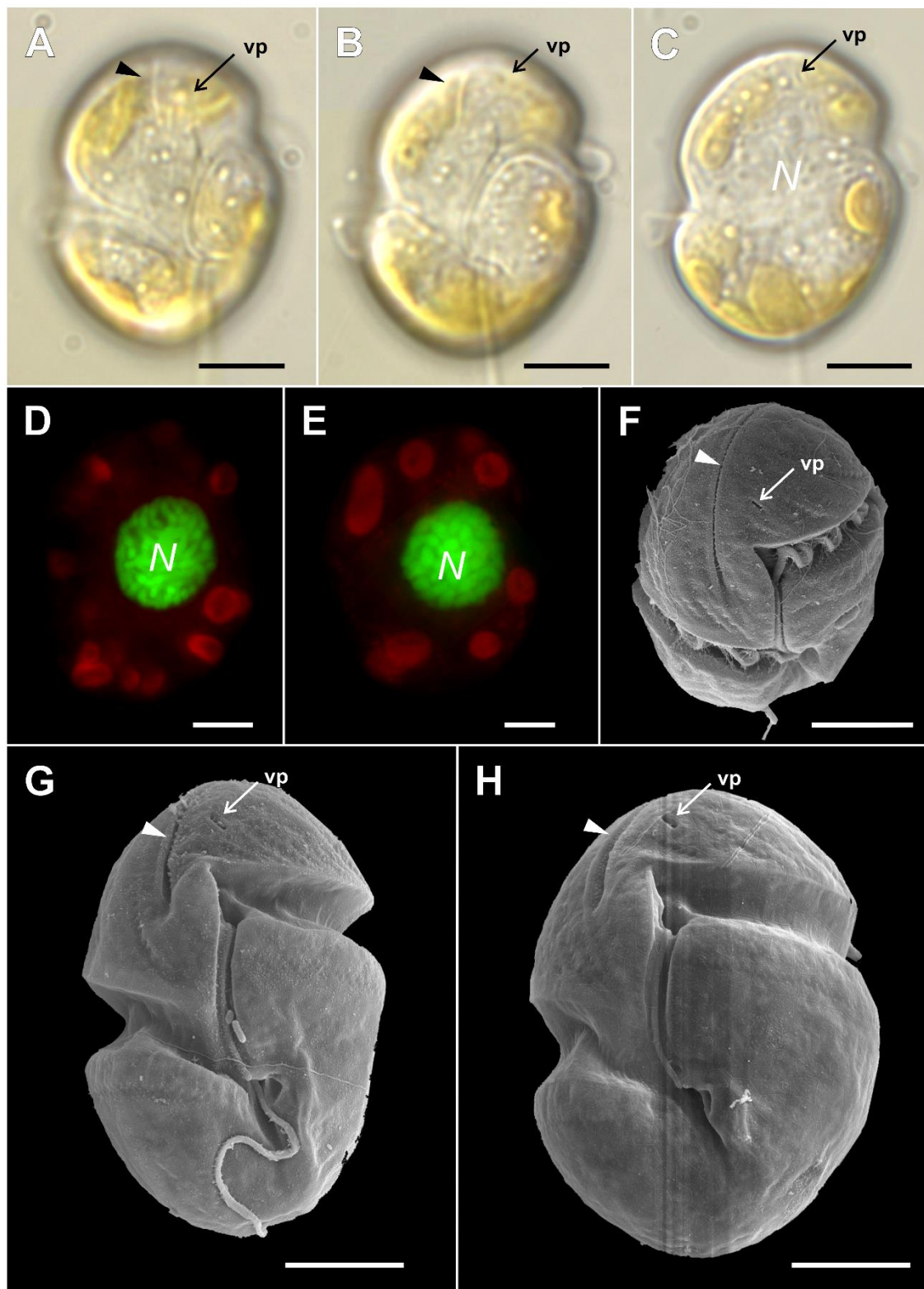


Figure 4.7: *Karlodinium decipiens*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a spherical nucleus (N) located at the centre of the cell. (F-H) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F), ventral (G-H) dorsal views. Scales, 5 µm

4.2.7 Morphology of *Karlodinium gentienii*

Cells of *K. gentienii* were ovoid, with epicone was conical with pointy end, and hypocone was hemispherical (Figure 4.8 A–C). Cell size ranged from 10.4–12.8 μm in length (mean $11.5 \pm 0.6 \mu\text{m}$; $n = 30$) and 7.4–9.8 μm in width (mean $8.9 \pm 0.5 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.8 A–C), with several chloroplasts visible under epifluorescence microscopy (Figure 4.8 D–E) surrounding the large, ellipsoid nucleus was situated at the centre of the cell (Figure 4.8 D–E). The straight, wide and long apical groove was extending below sulcus intrusion at ventral to the dorsal epicone, creating a slight indentation at the cell apex (Figure 4.8 A–B, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: KK Fish Market (5°59'0.006"N 116°4'20.443"E), Sabah, Malaysia Borneo

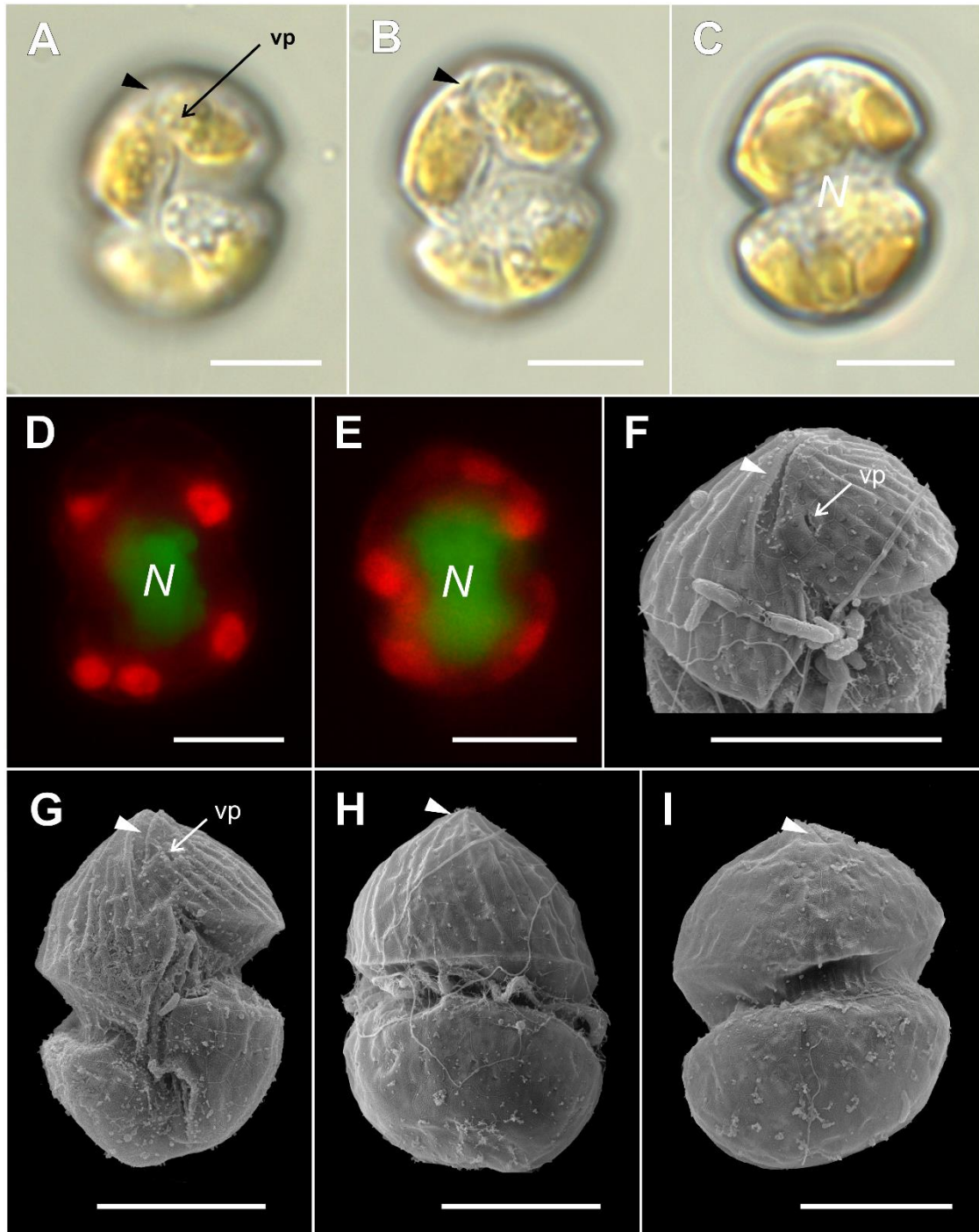


Figure 4.8: *Karlodinium gentienii*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, ellipsoid nucleus (N) located at the centre of the cell. (F-I) SEM. Straight apical groove (arrowhead) and ventral pore (vp) in apical (F), ventral (G) extending to dorsal views (H-I). Scales, 5 µm

4.2.8 Morphology of *Karlodinium veneficum*

Cells of *K. veneficum* were ovoid, hemispherical epicone was slightly larger than the hemispherical hypocone (Figure 4.9 A–C). Cell size ranged from 9.9–12.5 μm in length ($11.0 \pm 0.7 \mu\text{m}$; $n = 30$) and 8.0–10.5 μm in width ($9.2 \pm 0.8 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.9 A–C), with several chloroplasts visible under epifluorescence microscopy (Figure 4.9 D–E) surrounding the large, spherical nucleus was situated at the centre of the cell (Figure 4.9 D–E). The straight, wide and long apical groove was extending below sulcus intrusion at ventral to the dorsal epicone, creating a slight indentation at the cell apex (Figure 4.9 A–B, F–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Marina Island ($4^{\circ}12'21.200''\text{N}$ $100^{\circ}35'48.900''\text{E}$), Perak, Peninsular Malaysia. Telaga Air ($1^{\circ}40'35.800''\text{N}$ $110^{\circ}12'35.600''\text{E}$), Sarawak, Malaysia Borneo.

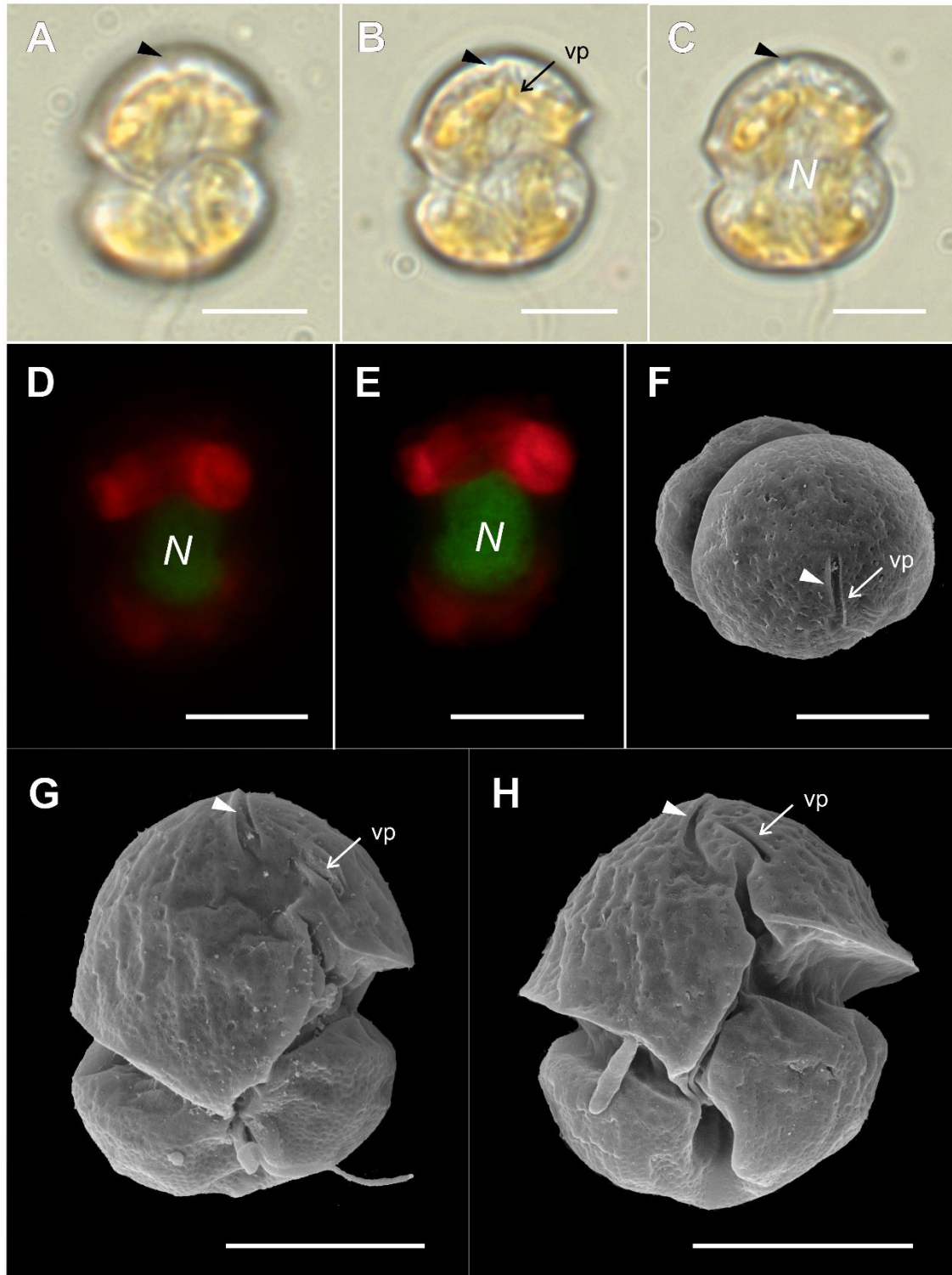


Figure 4.9: *Karlodinium veneficum*. (A-E) LM. Live cells showing apical groove (arrowhead), ventral pore (vp) and nucleus (N). (D-E) SYBR Safe-stained cells with a large, spherical nucleus (N) located at the centre of the cell. (G-H) SEM. Sigmoid apical groove (arrowhead) extends to dorsal in ventral view (G), and apical views (H). Scales, 5 μm

4.2.9 Morphology of *Takayama acrotrocha*

Cells of *T. acrotrocha* were ovoid, with the depressed ovoid epicone was larger than the depressed obovoid hypocone (Figure 4.10 A–C). Cell size ranged 12.5–16.5 μm in length ($14.2 \pm 1.1 \mu\text{m}$; $n = 30$) and 10.6–15.4 μm in width (mean $12.9 \pm 1.6 \mu\text{m}$, $n = 30$). The cells were yellowish green in colour (Figure 4.11 A–C), with several chloroplasts visible under epifluorescence microscopy (Figure 4.10 D–E), and the large, transversely ellipsoid nucleus occupied the epicone (Figure 4.10 D–E). The sigmoid apical groove extended from the proximal end of the girdle around the cell apex (Figure 4.10 A–C, G–H). GenBank accession no. (LSU rDNA domains D1-D3), and (ITS region).

Distribution: Telaga Air ($1^{\circ}40'35.800''\text{N}$ $110^{\circ}12'35.600''\text{E}$), Sarawak, Malaysia Borneo. Tuaran ($6^{\circ}06'13.100''\text{N}$ $116^{\circ}09'35.000''\text{E}$), Sabah, Malaysia Borneo.

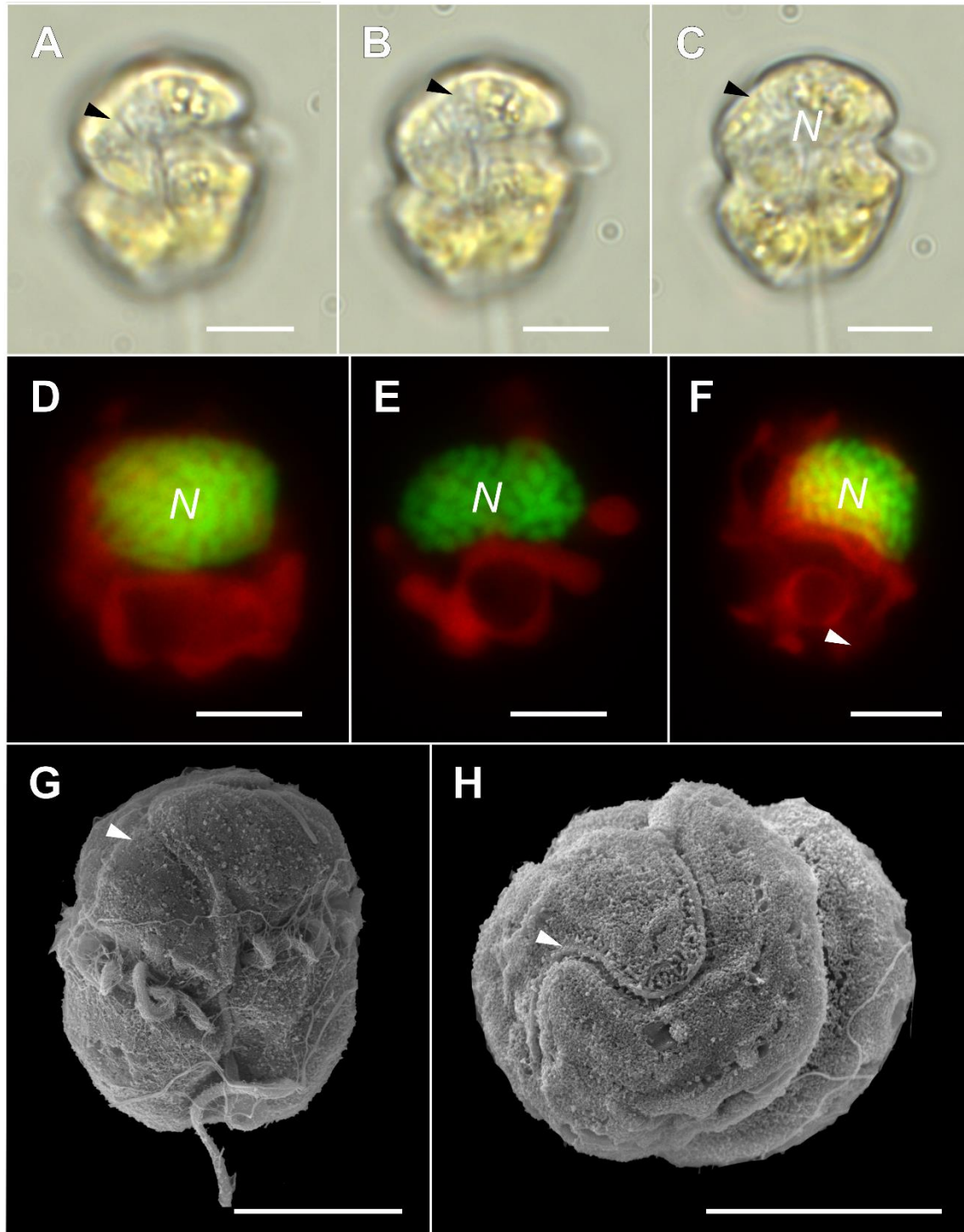


Figure 4.10: *Takayama acrotrocha*. (A-F) LM. Live cells showing apical groove (arrowhead) and nucleus (N). (D-F) SYBR Safe-stained cells with a large, transversely ellipsoid nucleus (N) occupies the whole epicone. (F-H) SEM. Sigmoid apical groove (arrowhead) extend from proximal end of the girdle around the cell apex in ventral view (G) and apical view (F). Scales, 5 μm

4.3 Phylogenetic inferences

Two nuclear rDNA markers, LSU (D1-D3) and ITS1 rDNAs were used in this study to address species delineation in the family Kareniaceae. GenBank accession numbers of the sequences used in this study and those retrieved from the GenBank nucleotide database were included in Appendix A, Table 1. The total length yielded by family Kareniaceae for the complete sequences of LSU rDNA and ITS1 rDNA were 754 bp and 513 bp respectively. Phylogenetic analysis of these two markers had resulted in similar tree topologies by MP, ML, and BI, with the BI trees shown for each marker (LSU, Figure 4.11; ITS1, Figure 4.12).

The phylogenetic analyses of the LSU and ITS1 rDNAs revealed distinct clades of genera *Karlodinium*, *Takayama* and *Karenia*. In both trees, *Karlodinium* was separated into three clades (LSU, Figure 4.11; ITS1, Figure 4.12). Clade I was comprised of *K. veneficum*, *K. conicum*, *K. corrugatum*, *K. ballantinum*, *K. gentienii* and *K. zhouanum* (ML bootstrap values/BI posterior probabilities values = 88/100% in LSU, 88/100% in ITS1) (LSU, Figure 4.11; ITS1, Figure 4.12). *K. veneficum* formed a sister clade with *K. conicum*, and paraphyletic clade with *K. corrugatum* in LSU, but polyphyletic clade with *K. ballantinum*, *K. gentienii* and *K. zhouanum* in both LSU and ITS1 (LSU, Figure 4.11; ITS1, Figure 4.12). *K. ballantinum* formed a monophyletic clade with *K. gentienii*, paraphyletic clade with *K. zhouanum* (junior synonym: *K. jejuense*) in LSU (Figure 4.11), however, *K. ballantinum* was paraphyletic to the sister groups of *K. gentienii* and *K. zhouanum* in ITS1 (Figure 4.12).

Clade II was composed of *K. australe*, *K. azanzae*, *K. digitatum* and *K. armiger* (99/100% in LSU, 100/100% in ITS1) (LSU, Figure 4.11; ITS1, Figure 4.12). *K. australe* and *K. azanzae* formed a sister clade in LSU and ITS1, which was paraphyletic to *K. digitatum* and *K. armiger* in LSU and ITS1 respectively (LSU, Figure 4.11; ITS1, Figure 4.12).

Clade III was made up of *K. decipiens* and *K. antarcticum* (100/100% in LSU) (Figure 4.11) where *K. decipiens* formed a monophyletic clade with *K. antarcticum*. Clade III of *Karlodinium* was closely related to *Takayama* clade (74/99% in LSU and 100/94% in ITS1) (LSU, Figure 4.11; ITS1, Figure 4.12). In LSU (Figure 4.11), *T. acrotrocha* (junior synonym *T. xiamenensis*) formed a sister clade with *T. helix*, but polyphyletic to the sister groups of *T. tasmanica* and *T. tuberculata*.

The monophyletic clade formed, viz *Karlodinium* and *Takayama* clades were closely related to *Karenia* clade (95/100% in LSU, 69/70% in ITS1) (LSU, Figure 4.11; ITS1, Figure 4.12). *K. mikimotoi* was a sister group of *K. brevis* in both LSU and ITS1 (LSU, Figure 4.11; ITS1, Figure 4.12). Outgroups were consistently branched off from the Kareniaceae clades.

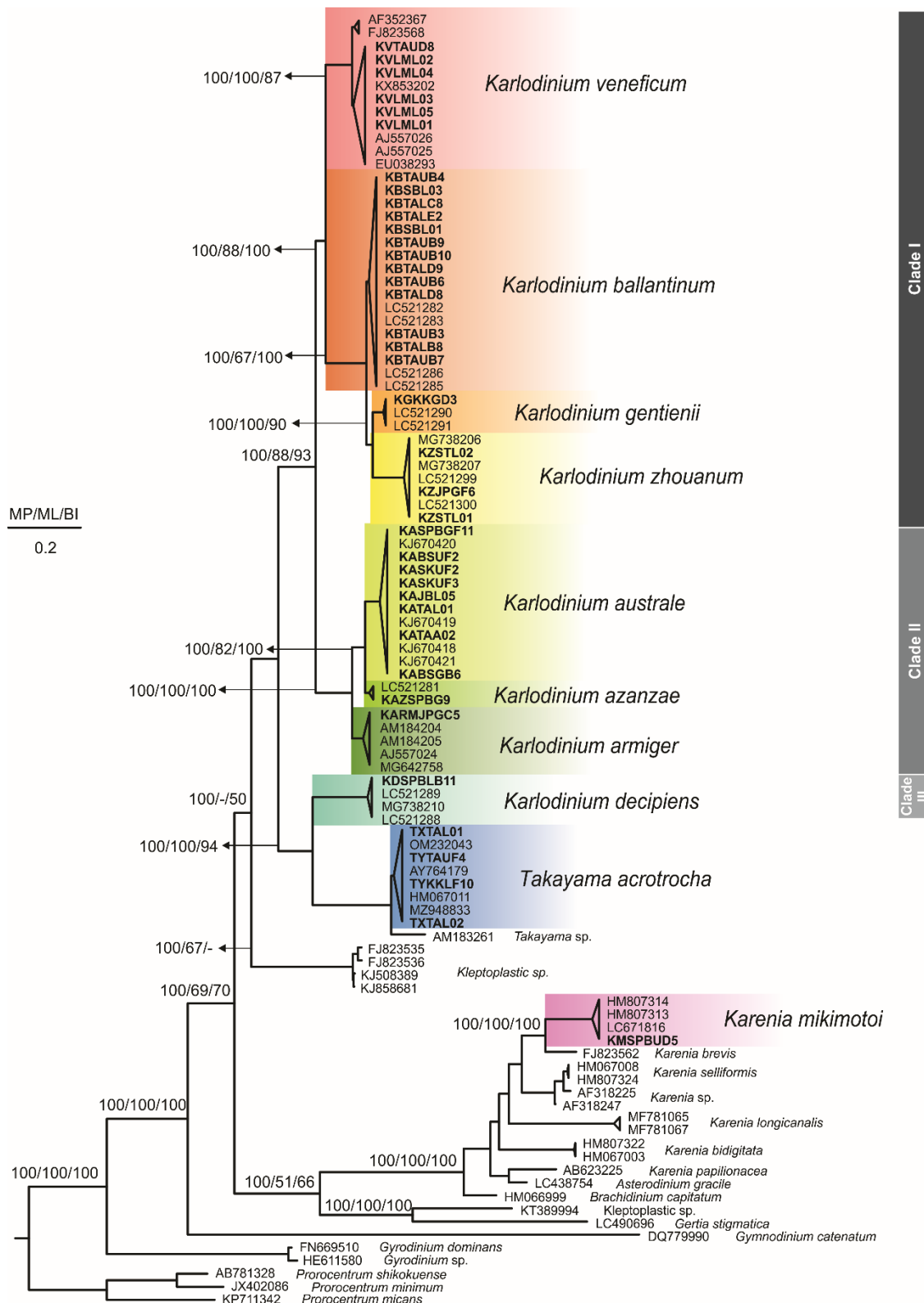


Figure 4.12: Phylogram of ITS1-5.8S-ITS2 rDNA consensus tree for maximum parsimony (MP), maximum likelihood (ML), and Bayesian Inference (BI) tree rooted with *Prorocentrum micans*. Nodal supports are bootstrap values of MP, ML, and posterior probability of BI; which only values >50% supports are indicated. The strains and species in this study are in bold. Dash represents support values <50

4.4 ITS2 sequence-structure information

The ITS2 RNA transcripts of *Karlodinium* Clade I comprised of *K. veneficum*, *K. ballantinum*, *K. gentienii* and *K. zhouanum* (Figure 4.13), as well as *Karlodinium* Clade II consisted of *K. australe*, *K. azanzae* and *K. armiger* (Figure 4.14) were illustrated. The comparison on the compensatory base changes (CBCs) and hemi-compensatory base changes (hCBCs) positions to the closely related species were mapped on the transcripts.

The pairwise structural comparison between *K. veneficum* and *K. ballantinum* showed (Figure 4.13) showed one CBC (in Helix III, C-G↔U-A), five hCBCs (in Helix I, C-G↔U-G, G-U↔A-U; Helix II, G-U↔A-U, U-G↔U-A; Helix III, U-G↔C-G). When comparing *K. veneficum* with *K. gentienii*, two CBCs (in Helix I, C-G↔U-A; Helix III, C-G↔U-A) and four hCBCs (in Helix I, C-G↔U-G; Helix II, G-U↔A-U, U-G↔U-A; Helix III, U-G↔C-G) were showed. The comparison between *K. veneficum* and *K. zhouanum* had revealed one CBC (in Helix I: C-G↔U-A) and six hCBCs (in Helix I, C-G↔U-G, G-U↔A-U, G-U↔A-U, U-G↔U-A; Helix III, A-U↔G-U, U-G↔C-G). When *K. ballantinum* was compared to *K. gentienii* (Figure 4.13), one CBC (in Helix I, C-G↔A-U) and no hCBCs showed. Pairwise structural comparison of *K. ballantinum* and *K. zhouanum* (Figure 4.13) revealed two CBCs (in Helix I, C-G↔U-A; Helix III, U-A↔C-G) and one hCBC (in Helix III, A-U↔G-U). When comparing *K. gentienii* and *K. zhouanum* (Figure 4.13), one CBC (in Helix III, U-A↔C-G) and no hCBC detected.

The comparison of ITS2 RNA transcript of *K. australe* to *K. azanzae* (Figure 4.14) detected in two hCBCs (in Helix I, G-U↔G-C; Helix II, U-G↔C-G). Meanwhile, the pairwise structural comparison between *K. australe* and *K. armiger* (Figure 4.14) had revealed four hCBCs (in Helix I, G-U↔G-C, U-G↔C-G; Helix III, G-C↔G-U; Helix IV,

G-U $\leftrightarrow$ G-C). When comparing *K. azanzae* to *K. armiger* (Figure 4.14), four hCBCs (in Helix I, U-G $\leftrightarrow$ C-G; Helix II, C-G $\leftrightarrow$ U-G; Helix III, G-C $\leftrightarrow$ G-U; Helix IV, G-U $\leftrightarrow$ G-C).

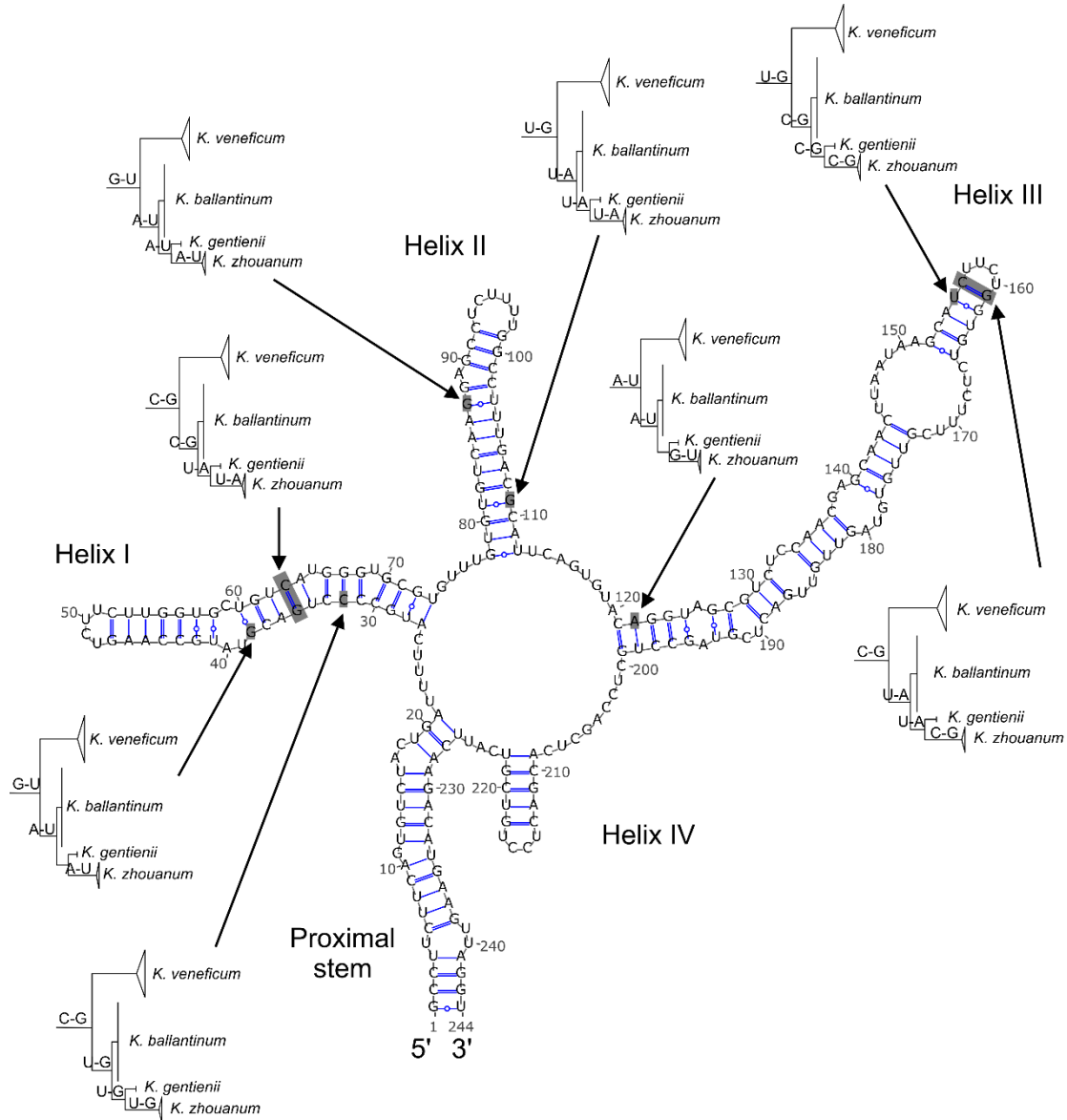


Figure 4.13: ITS2 RNA transcripts of *Karlodinium veneficum* with closely related species, viz. *Karlodinium ballantinum*, *Karlodinium gentienii* and *Karlodinium zhounum*. Shaded rectangles indicate CBCs or HCBCs

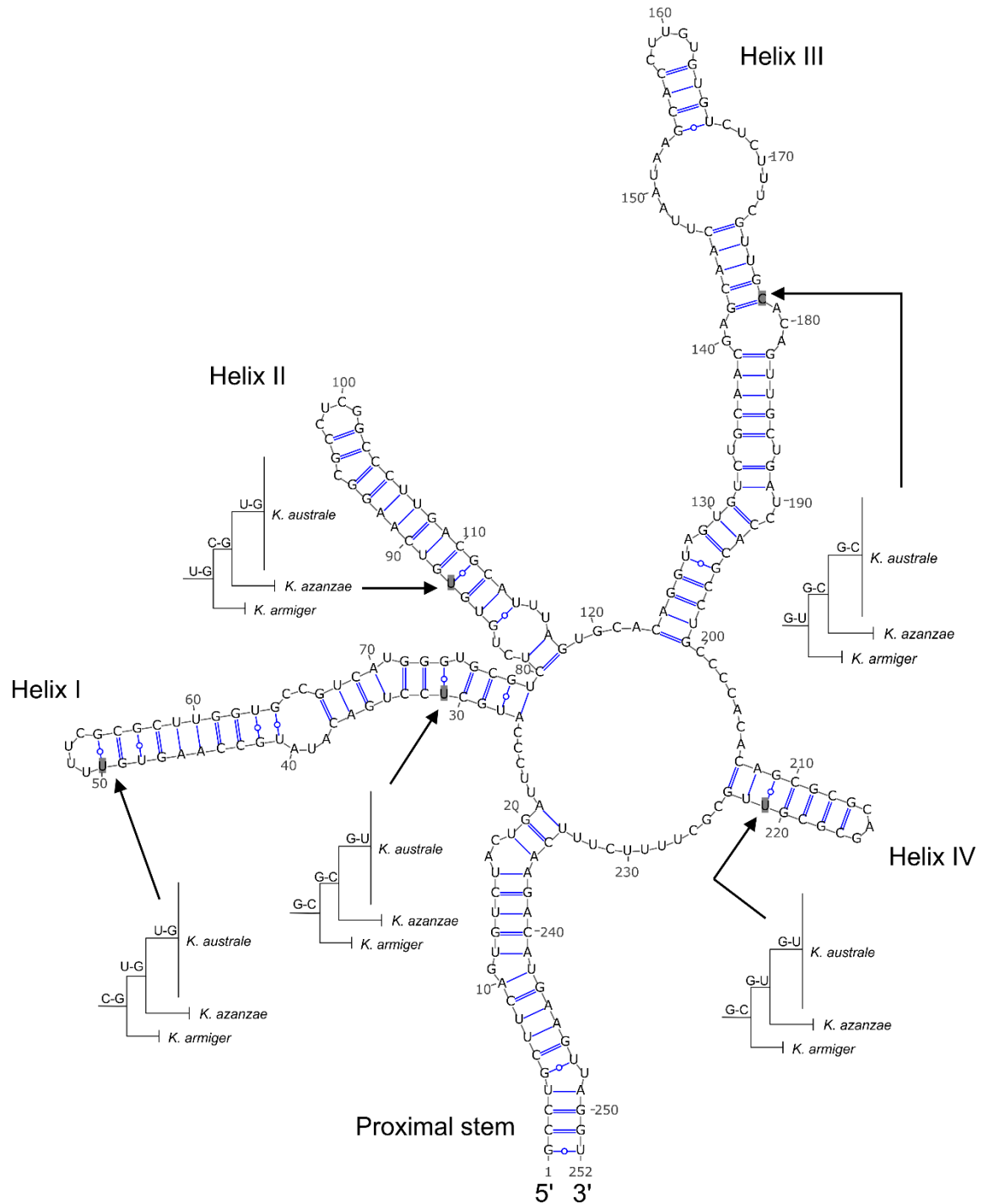


Figure 4.14: ITS2 RNA transcripts of *Karlodinium australe* with closely related species, viz. *Karlodinium azanzae* and *Karlodinium armiger*. Shaded rectangles indicate CBCs or HCBCs

Table 4.3: Number of CBC found in the comparison of ITS2 transcript among among *Karlodinium veneficum* and *Karlodinium australe* with close related *Karlodinium* species

ITS2 Transcript	No. of CBC			
	Helix I	Helix II	Helix III	Helix IV
<i>Karlodinium veneficum</i>				
<i>K. ballantinum</i>	-	-	1	-
<i>K. gentienii</i>	1	-	1	-
<i>K. zhouanum</i>	1	-	-	-
<i>Karlodinium australe</i>				
<i>K. azanzae</i>	-	-	-	-
<i>K. armiger</i>	-	-	-	-

Table 4.4: Number of HCBC found in the comparison of ITS2 transcript among *Karlodinium veneficum* and *Karlodinium australe* with close related *Karlodinium* species

ITS2 Transcript	No. of HCBC			
	Helix I	Helix II	Helix III	Helix IV
<i>Karlodinium veneficum</i>				
<i>K. ballantinum</i>	2	2	1	-
<i>K. gentienii</i>	1	2	1	-
<i>K. zhouanum</i>	2	2	2	-
<i>Karlodinium australe</i>				
<i>K. azanzae</i>	1	1	-	-
<i>K. armiger</i>	2	-	1	1

4.5 Toxicity screening of Kareniaceae in brine shrimp lethality

The lethality of nauplii *Artemia* sp. was observed in the treatments exposed to *K. mikimotoi* (KMSPBUD5), *K. armiger* (KARMJPGC5), *K. australe* (KAJBL05, KABSUF2), *K. azanzae* (KAZSPBGD9), *K. ballantinum* (KBTAUB3), *K. decipiens* (KDSPBLB11), *K. gentienii* (KGKKGD3), *K. veneficum* (KVTAUD8), and *T. acrotracha* (TYTAUF4). The nauplii of *Artemia* sp. undergone mortality in all treatments and starvation control, and no mortality was observed in the fed control (Figure 4.15). The death of naupliar was observed in initial 3-h exposure for both *K. australe* strain KAJBL05 and KABSUF2, followed by within 6-h for *K. azanzae* KAZSPBGD9 (Figure 4.15). After 12-h of exposure, naupliar death was observed in *K. veneficum* KVTAUD8, *K. armiger* KARMJPGC5, *K. gentienii* KGKKGD3 and *K. ballantinum* KBTAUB3 (Figure 4.15). Mortality of nauplii exposed to *K. decipiens* KDSPBLB11, *T. acrotracha* TYTAUF4 and *K. mikimotoi* KMSPBUD5 were observed latter at 24-h and 60-h of exposure respectively (Figure 4.15). Within 48-h of exposure, total mortality of nauplii was observed in *K. australe*, *K. azanzae*, *K. veneficum*, *K. armiger*, *K. gentienii*, and *T. acrotracha*, meanwhile mortality observed after 80-h exposure in *K. ballantinum*, *K. decipiens* and *K. mikimotoi* was about 70%, 30% and 20% correspondingly (Figure 4.15). The survival of *Artemia* nauplii in fed control remained during the experiment while unfed *Artemia* nauplii showed gradual decrease after 48-h of bioassay (Figure 4.15).

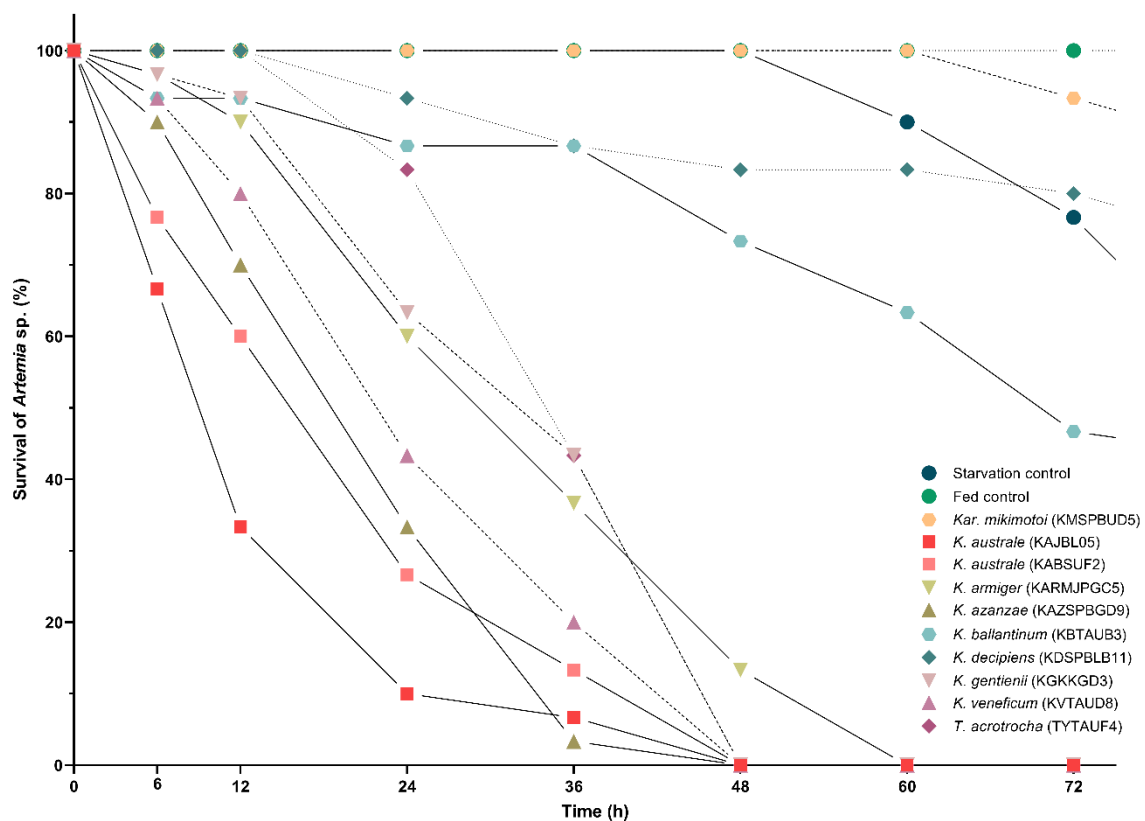


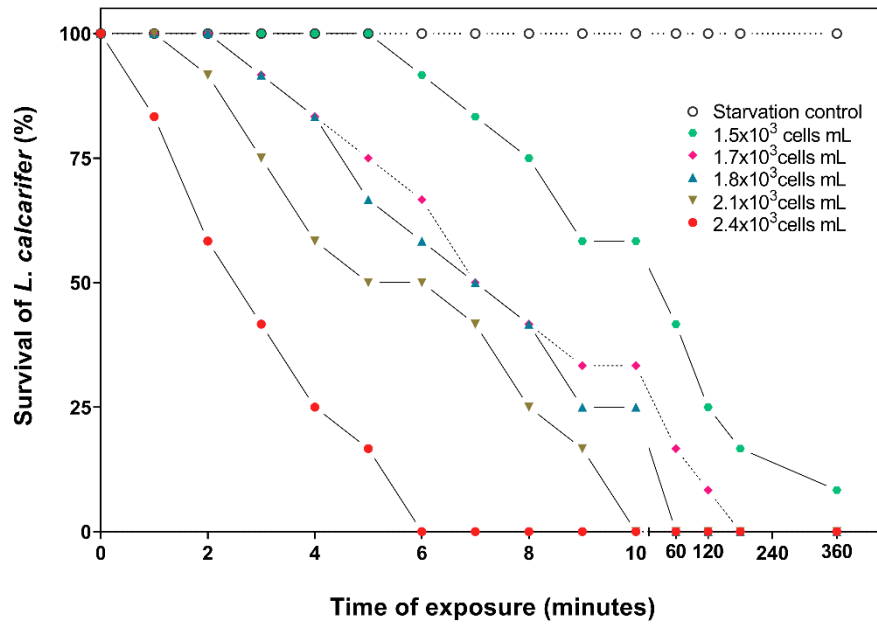
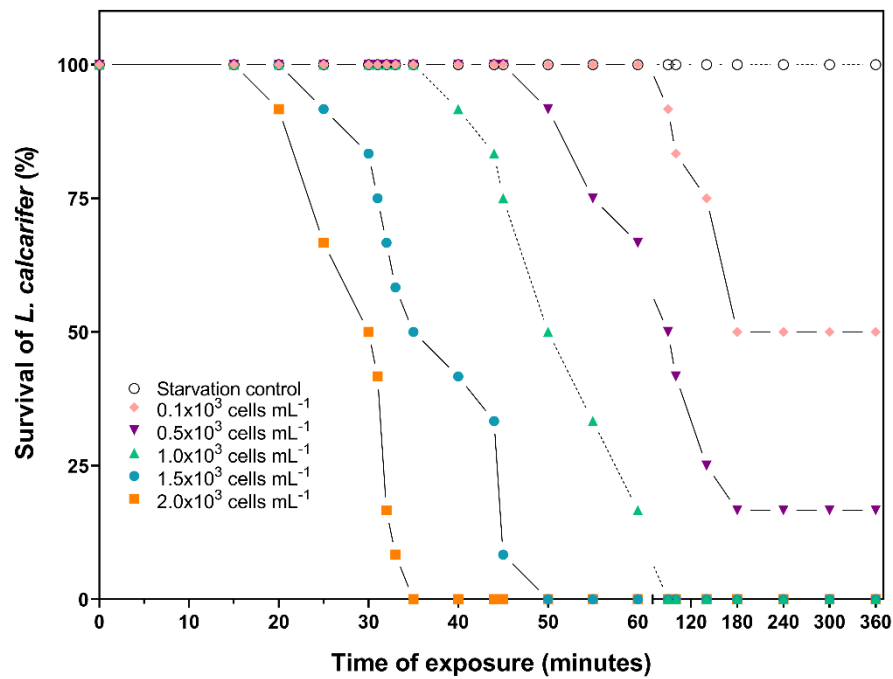
Figure 4.15: Survival of brine shrimp *Artemia* sp. exposed to *Karenia mikimotoi* (KMSPBUD5), *Karlodinium armiger* (KARMJPGC5), *Karlodinium australe* (KAJBL05, KABSUF2), *Karlodinium azanzae* (KAZSPBGD9), *Karlodinium ballantini* (KBTAUB3), *Karlodinium decipiens* (KDSPBLB11), *Karlodinium gentienii* (KGKKGD3), *Karlodinium veneficum* (KVTAUD8), and *Takayama acrotracha* (TYTAUF4). *Tetraselmis chuii* was used as fed control. Data points represent triplicate mean $\pm$ SD

4.6 Acute toxicity of *Karlodinium australe* on *Lates calcarifer* fingerling

In the fish bioassay of different cell concentrations *K. australe* Johor at 1.5×10^3 cells mL⁻¹, 1.7×10^3 cells mL⁻¹, 1.8×10^3 cells mL⁻¹, 2.1×10^3 cells mL⁻¹ and 2.4×10^3 cells mL⁻¹ respectively had resulted in total mortality of fingerlings seabass *L. calcarifer* in all treatments within 24-h exposure and no mortality was observed in starvation control (Figure 4.16A). The shortest time recorded for 25% fish mortality was approximately 2 min when exposed to cell concentration 2.4×10^3 cells mL⁻¹. A 100% mortality occurred in 6 min

exposure at cell concentration 2.4×10^3 cells mL⁻¹, followed by 10 min exposure at cell concentration 2.1×10^3 cells mL⁻¹, 1-h exposure at cell concentration 1.8×10^3 cells mL⁻¹, and about 4-h exposure at cell concentration 1.7×10^3 cells mL⁻¹. At cell concentration 1.5×10^3 cells mL⁻¹, a 100% fish mortality was observed after more than 3-h of exposure.

Meanwhile, in the fish bioassay of different cell concentrations *K. australe* Sarawak at 0.6×10^3 cells mL⁻¹, 1.0×10^3 cells mL⁻¹, 1.2×10^3 cells mL⁻¹, 1.4×10^3 cells mL⁻¹ and 1.8×10^3 cells mL⁻¹ respectively had recorded total mortality of fingerlings seabass *L. calcarifer* in all treatments and no mortality was observed in starvation control (Figure 4.16B). The earliest time recorded for 25% fish mortality was 4 min at cell concentration 1.8×10^3 cells mL⁻¹. A 100% fish mortality was observed upon 2-h exposure at cell concentration 1.8×10^3 cells mL⁻¹, and observed later than 24-h of exposure in other cell concentrations (1.4×10^3 cells mL⁻¹, 1.2×10^3 cells mL⁻¹, 1.0×10^3 cells mL⁻¹ and 0.6×10^3 cells mL⁻¹).

A**B**

Total mortality of fingerlings seabass *L. calcarifer* in every cell condition of *K. australe* was observed within 24-h exposure and no mortality was observed in the control (Figure 4.17). The first 25% mortality was observed in sonicated cell about 6 min exposure, followed by live cells about 10 min exposure, sonicated cell filtrate was about 35 min exposure, and culture filtrate was about 50 min exposure. 50% fish mortality of sonicated cell, live cells, sonicated cell filtrate and culture filtrate were about 8 min, 35 min, 40 min and 70 min respectively. 75% fish mortality of sonicated cell, live cells, sonicated cell filtrate and culture filtrate were about 10 min, 40 min, 50 min, and 80 min respectively. *K. australe* sonicated cells recorded the fastest 100% fish mortality at 15 min exposure, tailed by live cells at 45 min exposure, and sonicated cell filtrate needed 55 min exposure. The longest time taken for causing 100% fish mortality was at 90 min exposure of *K. australe* culture filtrate compared to the other cell conditions.

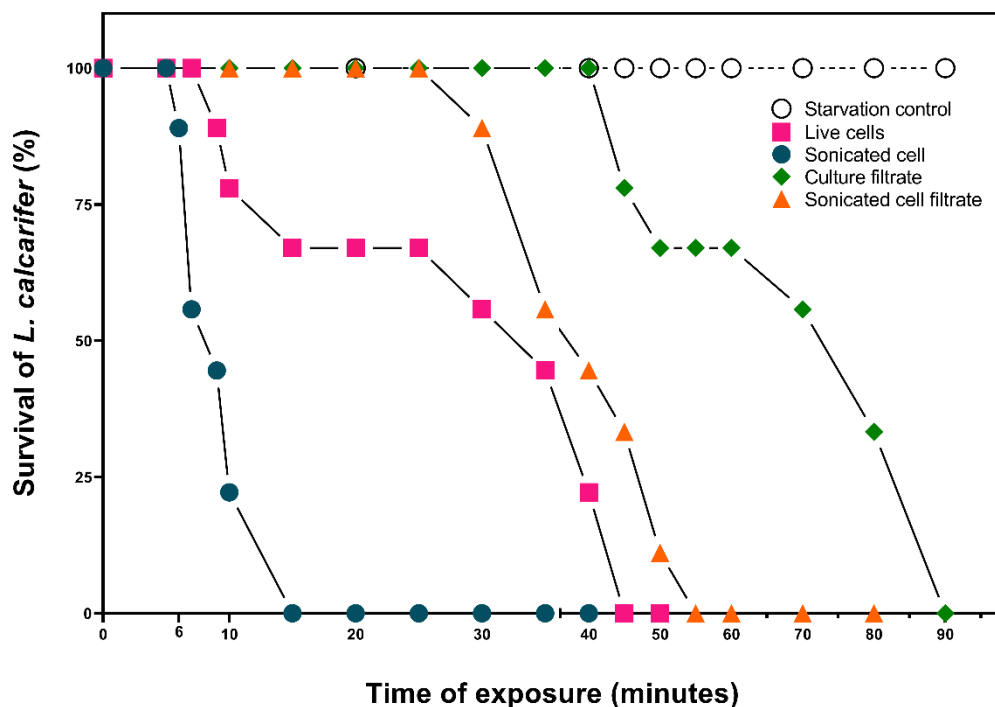


Figure 4.17: Survival of seabass *Lates calcarifer* exposed to *Karlodinium australe* Sarawak strain (KATAL01) of four cell conditions: live cells, sonicated cell, culture filtrate and sonicated cell filtrate. GPM medium was used as a control. Data points represent triplicate mean $\pm$ SD

4.7 Micropredation of *Karlodinium*

In the bioassay, the microscopic observation of brine shrimp exposed to *Karlodinium* had showed *K. armiger*, *K. azanzae*, and *K. australe* making direct contact with the body of naupliar (Figure 4.18). The cells of *K. armiger*, *K. australe*, and *K. azanzae* began to attach themselves to the naupliar after the naupliar became immobilised during lethality assay (Figure 4.18). The number of cells attaching onto the brine shrimp also had increased over time (Figure 4.18). The cells were heavily attached surrounding the upper body of naupliar, especially nearing the antenna and mandible compared to the lower body (Figure 4.18A, D—E). By examining using light microscopy, the cells of *K. armiger*, *K. australe* and *K. azanzae* had positioned themselves with their ventral side facing the naupliar (Figure 4.18B, G—H). A visible peduncle was revealed emerging beneath the sulcus intrusion of the cells (Figure 4.18C). The developed peduncle was penetrating the tissue of naupliar and sucking out the contents inside of naupliar (Figure 4.18C). The movement of the contents sucked was observed leaving the body of brine shrimp into the cells of *K. armiger*, *K. australe* and *K. azanzae* (Figure 4.18C).

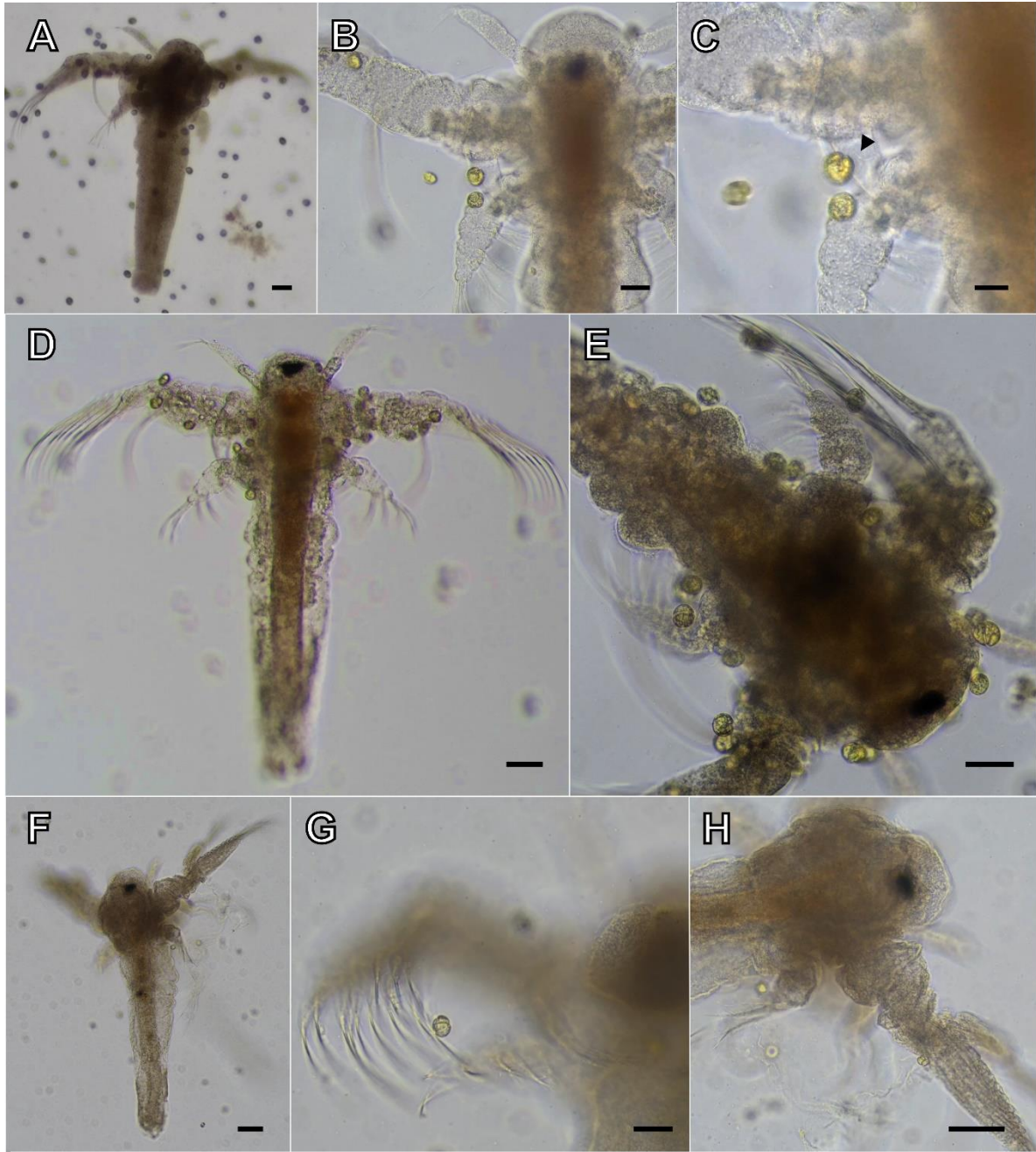


Figure 4.18: Observation of brine shrimp *Artemia*. (A–C) Cells were attached onto immobile nauplii in the exposure towards *Karlodinium australe*, (D–E) *Karlodinium azanzae* and (F–H) *Karlodinium armiger*. (C) The peduncle protruded from *Karlodinium australe* cell and penetrated the dead nauplii (arrowhead) to feed by sucking out the content. Scales, 20 μm

CHAPTER 5

DISCUSSION

5.1 Species diversity and geographical distribution

This study of family Kareniaceae distribution in Malaysian coastal waters had 10 species which were one *Karenia* (*K. mikimotoi*), eight *Karlodinium* (*K. armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, *K. veneficum* and *K. zhouanum*) and one *Takayama* (*T. acrotrocha*). Toxigenic species of Kareniaceae namely *K. mikimotoi*, *K. armiger*, *K. azanzae*, *K. australe*, *K. gentienii*, and *K. veneficum* were found in all locations sampled.

Among the Kareniaceae species reported in this study, *K. mikimotoi* had the lengthiest bloom-forming history in the worldwide (Li et al., 2019). This study is the first report on *K. mikimotoi* occurrence in the coastal waters of Borneo. *K. mikimotoi* was well-known of causing red tides linked with marine life mortalities. A precedent report on *K. mikimotoi* red tide was in the year 1934 in Japan, and was associated with mortalities of fish and shellfish (Oda, 1935). Since the earliest verified record, *K. mikimotoi* was reported of causing annual bloom from 1991 to 1995 in several areas in Japan such as Tanabe Bay, Hoketsu Bay, Suo-Nada, near Ie-shima islands, and Hiroshima Bay (Nakamura et al., 1995; Koizumi et al., 1996; Kimura et al., 1999; Matsuyama et al., 1999), and these bloom events had caused fish and shellfish mortalities, including great economic losses to the fisheries sector with amount over 10 million US\$ (Yanagi et al., 1995). *K. mikimotoi* had also reported in several countries in Asia regions such as in Vietnam (Larsen & Nguyen, 2004), in Korea (Park et al., 2013), in India (D'Silva et al., 2012; Hartman et al., 2014; Babu et al., 2016; Kumar et al., 2018), in China (Baohong et al., 2021; Zhang et al., 2023), including in

Singapore (Leong et al., 2015; Kok & Leong, 2019). Out of all the countries in Asia that had reported *K. mikimotoi* occurrences in the coastal waters, China was the most affected by the red tides of *K. mikimotoi*. Until the 21st century, the blooms of *K. mikimotoi* had turned into a yearly calamity by recurring over 120 times (Zhang et al., 2023), affecting Fujian Province, Zhejiang Province, Tianjin city, Hebei Province, Jiangsu Province (Baohong et al., 2021).

Out of eight *Karlodinium* species reported in this study, five species viz. *K. armiger*, *K. azanzae*, *K. australe*, *K. gentienii*, and *K. veneficum* were linked with mortalities in previous studies. *K. veneficum* was the most distribution species in genus *Karlodinium* and the occurrences of red tide were frequently associated with marine life mortalities. The first description of *K. veneficum* was dated back to 1956 in Devonport, England (Ballantine, 1956) and the distribution were documented in several countries such as Norway (Bjørnland & Tangen, 1979), South Africa (Braarud, 1957), Atlantic Ocean (Hulburt, 1957; Hall et al., 2008), Chile (Carreto et al., 2001), North America (Terlizzi et al., 2000; Li et al., 2001), Spain (Garcés et al., 2006), Australia (Place et al., 2012; Adolf et al., 2015), India (Naik et al., 2010), Korea (Park et al., 2011), German (Wang et al., 2011), and China (Wang et al., 2011; Dai et al., 2014). The blooms of *K. veneficum* were documented to cause detrimental impact to the aquatic life as they caused death of fish and shellfish of the affected areas, as well as huge losses to the aquaculture sector (Carreto et al., 2001; Deeds et al., 2002a). A previous study on *K. veneficum* had characterised toxin produced known as karlotoxin that also played a significant role in massive fish mortalities during red tides (Van Wagoner et al., 2010).

Since 1994, fish kill events had recurred in Alfacs Bay, Spain during winter seasons and the culprit was identified to be *K. armiger* (Garcés et al., 2006). The ichthyotoxicity of *K. armiger* was confirmed as it had caused mortality to bivalve *Tapes semidecussatus* in

toxicity assay (Garcés et al., 2006), and further study had acknowledged *K. armiger* as a karmitoxin producer (Rasmussen et al., 2017). Another *Karlodinium* species was *K. australe*, first isolated from south-eastern Australia lagoon in the year 2005 (de Salas et al., 2005a). At first, *K. australe* of Australian lagoon type locality was never reported of causing any trouble (de Salas et al., 2005a; Hallegraeff et al., 2021) until in 2014, *K. australe* was reported of having red tide occurrences in West Johor Straits, Malaysia that lead to a massive mortality of aquaculture finfish along the coast (Lim et al., 2014). The recurrent bloom of *K. australe* a year later had also killed mass number of cultured fish, as well as demersal wild fish and crustaceans at the impacted areas (Teng et al., 2016b). Meanwhile, *K. gentienii* was first described in France in 2014 following the event of massive mortality oysters along the French coasts (Nézan et al., 2014). The preliminary assay of *K. gentienii* had demonstrated haemolytic activity and further analyses was suggested for toxin and haemolytic compounds characterisation (Nézan et al., 2014). In addition, *K. azanzae* was first described in Philippines in 2020, and bioassay conducted had confirmed the lethality of *K. azanzae* to invertebrates, as well as suggested the involvement of bioactive substances produced assisting the killing (Benico et al., 2020).

5.2 Morphological characterisation Kareniaceae

The taxonomy of Kareniaceae was inferred from morphological variations such as cell shape, shape of apical groove, presence of ventral pore and position of nucleus which were used in the past studies to discriminate *Karenia*, *Karlodinium* and *Takayama* species (Daugbjerg et al., 2000; Larsen, 2002; Bergholtz et al., 2006; de Salas et al., 2008). In this study, *Karenia mikimotoi* is morphologically differ than *Karlodinium* and *Takayama* species. *K. mikimotoi* had dorsoventrally flattened body and possessed apical carina, those were similar traits shared with other *Karenia* species (Botes et al., 2003; de Salas et al.,

2004b; Haywood et al., 2004; Escobar-Morales & Hernández-Becerril, 2015). The cells of *K. mikimotoi* are easily distinguishable by light microscopy due to the broadly ovoid shape with conical epicone and two-lobed hemispherical hypocone, and larger cell size ranges from 22.1–27.4 μm long and 17.2–23.9 μm wide (Table 4.1, Figure 3.3) than other Kareniaceae species in this study. *K. mikimotoi* possess a straight apical groove that is similar in *Karlodinium* species, thus, genus *Karenia* was morphologically close to genus *Karlodinium* (Daugbjerg et al., 2000)

When differentiating genera *Karenia*, *Karlodinium* and *Takayama*, the presence of ventral pore was an exclusive trait of genus *Karlodinium* (Bergholtz et al., 2006; de Salas et al., 2008). Because *Karlodinium* species were morphologically similar and usually difficult to observe using light microscopy, the use of scanning electron microscopy (SEM) was required to observe morphological features such as apical grooves, apical pores, thin thecal plates, and scale, albeit the small sizes and delicateness meaning that they can be easily damaged throughout sampling and preservation processes (Botes et al., 2002; Zhang et al., 2022b). All *Karlodinium* species in this study exhibits similar morphological traits such as centrally large nucleus, has straight apical groove extending from ventral to dorsal epicone, including the presence of ventral pore to the left of apical groove. Another unique trait of *Karlodinium* was the amphiesma vesicles on the cell surface in the shape of pentagonal or hexagonal (Bergholtz et al., 2006; Nézan et al., 2014; Li & Shin, 2018). A detailed, magnified imaging in SEM has recognised pentagonal or hexagonal configuration of amphiesma vesicles on the *Karlodinium* cell surface (Figure 3.4—Figure 3.10).

In contrast, a previous study by de Salas et al. (2003) described the sigmoid apical groove as a typical characteristics of *Takayama* species and readily distinguishable from the other two genera. The sigmoid apical groove that begins from the proximal end of the girdle

around the apex distinguishes *Takayama acrotrocha* in light microscopy and SEM from the straight apical groove in *Karenia* and *Karlodinium* (Figure 3.11). *T. acrotrocha* also had large nucleus that occupied most part of the epicone (Lü et al., 2022). The large, transversely ellipsoid nucleus that fills up mostly of the epicone observed in *T. acrotrocha* is significantly different from the centrally located nucleus in *Karlodinium* species and nucleus nearing left edge of the cell in *Karenia* species (Table 4.1, Figure 3.11).

5.3 Species boundaries among closely related *Karlodinium* species

The substantial morphological variations of different species belonging to the single genus had led to various species descriptions (Annenkova et al., 2015). It is almost impossible to distinguish *Karlodinium* species to each another solely by light microscopy because the cells of these species are generally ovoid in shape and relatively small in sizes (Table 4.1). Cell size and shape were insufficient to define up to species level because several species within same genus might be of the same size and similar shape, thus, previously overlooked feature was emphasized such as unique shape of epicone (Jørgensen et al., 2004). *Karlodinium* species possess a range of dissimilar epicone shape, for instances, the cells of *K. gentienii* has a pointed, conical epicone and hemispherical hypocone (Table 4.1), *K. armiger* and *K. ballatinum* have slightly conical epicone and hemispherical hypocone (Table 4.1), whereas *K. australe*, *K. azanzae*, *K. decipiens*, and *K. veneficum* have hemispherical epicone and hypocone (Table 4.1).

Apart from epicone shape, morphological characteristics such as cell size, nucleus placement and apical groove length may differ amongst species of the same genus (Luo et al., 2018; Zhu et al., 2020). Despite this, the pattern in the cell size range can assist in determining taxa for close-related species had similar cell size range (Nakov et al., 2014). In our observation, *K. veneficum*, *K. ballatinum*, *K. gentienii*, and *K. armiger* are within

similar range in cell sizes from about 10–18 μm long and 7–14 μm wide, while *K. australe*, *K. azanzae*, and *K. decipiens* have wider range in cell sizes from about 14–25 μm long and 12–22 μm wide (Table 4.1). Here, the sizes of cells are slightly smaller than the original descriptions (Table 4.1) because light and SEM are carried out with *Karlodinium* cells that has reach early to mid-exponential phase, about five to seven days of culture.

The presence of ventral pore was more conservative in the genus *Karlodinium* that absence in other genera in the Kareniaceae family (Daugbjerg et al., 2000; Bergholtz et al., 2006), yet, it had been proposed that the ventral pore might be present or lacking in different *Karlodinium* species (de Salas et al., 2008). In the original description, the ventral pore in *K. ballantinum* was described as lacking or inconspicuous (de Salas et al., 2008). The visible ventral pore in *K. ballantinum*, on the other hand, is unquestionably demonstrated in our investigation (Figure 3.7). Each *Karlodinium* species studied here viz. *K. armiger*, *K. azanzae*, *K. australe*, *K. decipiens*, *K. gentienii*, *K. veneficum* including *K. ballantinum* exhibit the presence of ventral pore, lending credence to the notion that ventral pore is a key feature of *Karlodinium* species.

5.4 ITS2 secondary structure information of *Karlodinium*

A combination study of morphological characters and genetic studies particularly regarding small unarmoured species were essential for defining the species boundaries (Simon et al., 2009). Classifying species within the same genus can be the most challenging circumstance as they are strongly interrelated (Müller et al., 2007). The phylogenetic inference using ITS2 rDNA of *Karlodinium* species was previously introduced by Lim et al. (2014). In this study, ITS2 secondary structure was used to further support the notion of sequence heterogeneity and *Karlodinium* species lineages at lower taxonomic levels. Prediction using ITS2 region was used to distinguish closely related species by focusing on traits associated with clade

separation into two or more subclades, as well as providing a simpler sequence-based approach for evaluation of biological species level (Müller et al., 2007; Coleman, 2009).

The modelling of ITS2 secondary structure in *Karlodinium* have revealed highly structural conserved four universal helices (I–IV). Helices I and IV were the most evolutionary variable, particularly used for comparing species and subspecies, meanwhile helices II and III were more conserved in sequences and differed from other eukaryotic ITS2 structures (Coleman, 2009). The taxa were classified as different species when CBCs (Compensatory Base Changes), a modified double stranded helix of ITS2 sequence-structure alignment, or hCBC (hemi-Compensatory Base Change) was single-nucleotide polymorphism but preserved the helix pairings were present in the homology modelling (Coleman, 2003; Müller et al., 2007).

In molecular characterisation, *K. veneficum* and *K. ballatinum* are divided into two distinct species lineages as revealed in phylogenetic analysis (Figure 4.12–4.13) and further supported by the presence of one CBC, and five hCBCs when making comparison of their ITS2 transcripts (Figure 3.14). *K. veneficum* and *K. ballatinum* are similar morphologically as both have similar ovoid shape, hemispherical epicone and hypocone, however, substantial degrees of genetic diversity existed within morphologically identical species (Murray et al., 2012).

By having dissimilar epicone shape, *K. gentienii* is morphologically distinct than *K. ballatinum* and *K. veneficum*. Based on ITS2 secondary structure and CBCs analyses, *K. gentienii* is distinct from *K. ballatinum* and *K. veneficum* by having one CBC, no hCBC, and two CBCs, four hCBCs respectively (Figure 4.14). *K. zhouanum* was described of having conical epicone (Luo et al., 2018) similar with *K. gentienii* observed in this study. Although no morphological analysis is made on *K. zhouanum* in this study, the analysis on

ITS2 transcripts has revealed *K. zhouanum* is of different lineage by having of one CBC, no hCBC to *K. gentienii*, two CBCs, one hCBC to *K. ballantinum*, and one CBC, six hCBCs to *K. veneficum* (Figure 4.15).

From phylogenetic analysis, *K. australe*, *K. azanzae* and *K. armiger* form three distinct subclades (Figure 4.12–4.13). No CBC is revealed among *K. australe*, *K. azanzae* and *K. armiger*, still, *K. australe* has the presence of two hCBCs and four hCBCs to *K. azanzae* and *K. armiger* respectively, meanwhile *K. azanzae* reveals four hCBCs to *K. armiger* (Figure 4.15). Morphologically, *K. australe* and *K. azanzae* differ in cell size, which is bigger than that of *K. armiger*, but this study has observed weak genetic diversity in CBC and hCBC analysis of these three species. Weak genetic diversity but significant morphological differentiation among species could be the consequences of high level gene flow and historical introgressive in different environment inhabited (Hu et al., 2019).

Classifying species within the same genus can be the most challenging circumstance as they are strongly interrelated (Müller et al., 2007). CBCs information can be useful in evaluating species delineation but divergence of ITS2 sequences due to hybridisation and polyploidisation can led to misleading inference of true homology between taxa and accurate phylogenetic reconstruction (Alvarez & Wendel, 2003). A good indicator of distinct species was the presence of at least one CBC (Müller et al., 2007; Wolf et al., 2013) and this concept was ineffective in the absence of CBC (Sundaresan et al., 2019). As demonstrated in this study, the presence of hCBCs can be used as diagnostic feature of species distinctness when CBCs is absent. Therefore, using CBCs and hCBCs of ITS2 directly as marker for speciation can be regarded as secondary information of phylogenetic analysis.

5.5 Acute toxicity of *Karlodinium australe* towards *Lates calcarifer* fingerlings

24-h of exposure test with different *K. australe* cell densities was conducted to study the relationship of cell densities and time taken of fish mortality. The highest cell densities of *K. australe* tested in the fish bioassay was 2.4×10^3 cells mL⁻¹ and had resulted in a total fish mortality within 6 min of exposure. Despite the lowest cell density of *K. australe* at 0.1×10^3 cells mL⁻¹, fish mortality still occurs but at slower pace. This was further supported by Lim et al. (2014) and Song et al. (2020) that mentioned *K. australe* had stronger lethal effects as this species can cause massive fish death even at a relatively low cell density. The rapid response of fish mortality was proven in this study as the first 25% of overall fish mortality in the lethality assay had occurred underneath 15 min when treated with *K. australe*. An acute, rapid and harmful response to an organism was often associated with water-soluble toxin (Rosner et al., 2007). Genus *Karlodinium* was reported of producing potent ichthyotoxin that responsible for the mortalities of aquatic faunal and fish (Deeds et al., 2002b; Kempton et al., 2002; Place et al., 2012; Rasmussen et al., 2017). One of the ichthyotoxins produced by genus *Karlodinium* is karlotoxin, a unique polykeptide toxin that resembled amphidinol structurally (Krock et al., 2017). The amphoteric characteristic of karlotoxin was provided from the hydrophilic part that enabled the water solubility, while the lipophilic part caused lytic effect on the membrane of marine organisms (Van Wagoner et al., 2008; Krock et al., 2017). Previous study on karlotoxin isolated from *K. veneficum* was characterised as water-soluble toxin with few properties such as haemolytic, cytotoxic and ichthyotoxic (Peng et al., 2010). Overall fish mortality that occurred within 24-h of fish bioassay in this study had strongly suggested the involvement of water-soluble toxin in *K. australe*.

The fish bioassay in different cell conditions of *K. australe* was conducted to demonstrate the mode of action of *K. australe* towards the fish. The faster response of fish mortality recorded in the sonicated *K. australe* cells has demonstrated that burst *K. australe* cell is more lethal compared to live cells, and this furthermore proves the possibility of *K. australe* in producing water-soluble toxin. *K. australe* is an athecate dinoflagellate, thus, is more fragile and susceptible to release the produced toxin when the cell burst (Chow, 2011). The toxin released can enter the fish body either when fish consume *K. australe* live cells or directly absorbing toxin from the water induced by ruptured *K. australe* cell. Fish gills directly had contacted the toxin present in the water during oxygen absorption (Chovanec et al., 2003), or the toxin can be lethal prior to gradual ingestion of fish in the water (Hamdan et al., 2017).

In this study, mortality occurred despite the *K. australe* cell condition, as the fish were killed not only when exposed to the live cells but also after lysating or filtering the *K. australe* cells. The findings from the fish lethality assay in this study suggested the fish experienced mortality due to the release of toxin component originated from the inside of *K. australe* cells was released into the water column through cell rupture. The exact location of the toxin was unknown but lysis of cell might be prompted to release toxin inside the cells (Sheng et al., 2010). An lethality assay had identified on how karlotoxin easily released out of the cells affecting the fish viz. applying shear forces such as centrifugation, filtration, or pass through gill rackers (Peng et al., 2010). Although toxin produced by *K. australe* had not been characterised, the affected fish in *K. australe* bloom at Johor Straits had exhibited similar symptoms of fish exposed to high levels of karlotoxin (Lim et al., 2014). Therefore, present study had proven the involvement of toxin of *K. australe* in causing the fish mortality.

5.6 Toxicity screening of Kareniaceae in brine shrimp lethality

The species of family Kareniaceae were reported to be toxic species, as well as non-toxic species, and most of the toxic species were in genus *Karlodinium* (Ballantine, 1956; Adolf et al., 2007; Mooney et al., 2009; Place et al., 2012). The exposure test of Kareniaceae in this study had showed that *K. mikimotoi*, *K. armiger*, *K. azanzae*, *K. australe*, *K. ballantini*, *K. decipiens*, *K. gentienii*, *K. veneficum* and *T. acrotrocha* caused lethal effects to the brine shrimp.

Our result had revealed the toxicity of *K. mikimotoi* to the brine shrimp in the lethality assay. Although *K. mikimotoi* was identified of HAB-causative species and considered toxic (Zhang et al., 2022a), toxin released by *K. mikimotoi* was assessed in mouse bioassay had negative results, thus reported the killing mechanism of *K. mikimotoi* was due to the abnormal mucous secreted causing clogging of fish gills (Robin et al., 2013). However, recent study had suggested toxin involvement produced by *K. mikimotoi* cells made direct contact with the fish gills and stuffed the gills (Satake et al., 2002; Satake et al., 2005). A previous study reported that *K. mikimotoi* strains from different localities of Hong Kong, Japan and New Zealand had different ichthyotoxicity in viability assay on fish gill cells, where the assays had demonstrated different effects for each strains such as New Zealand strain did not had significant toxic effect, while Japanese strain was more toxic to the fish gill cells than Hong Kong strain (Lam et al., 2022). In another study, behavioural experiments were conducted to zebrafish *Danio rerio* larvae and had revealed *K. mikimotoi* to be significantly less lethal to zebrafish larvae in low abundance, however, neurotoxicity was still exhibited (Niu et al., 2021). Present study had displayed *K. mikimotoi* Malaysian Borneo strain was significantly less lethal to the brine shrimp in the lethality assay.

The ichthyotoxicity of *K. veneficum* was well-known even before the characterisation of karlotoxin in the 1950s (Place et al., 2012; Puglisi & Becerro, 2018) as this toxigenic species was commonly linked with HAB-associated fish kill events, either in the wild or in the aquaculture farms (Braarud, 1957; Garcés et al., 2006; Deeds et al., 2006; Kempton et al., 2002; Adolf et al., 2015; Hall et al., 2008). *In vivo* toxicity of *K. veneficum* had reported haemolytic activity to the human erythrocyte (Van Wagoner et al., 2010). *K. veneficum* exposure to the sheepshead minnows *Cyprinodon variegatus* larvae had observed sub-lethal gill damage (Adolf et al., 2015). The toxicity of *K. veneficum* was displayed through mortality of brine shrimp *A. salina*, rotifer *Brachionus plicatilis* and juvenile *Oryzias melastigma*, in the bioassay (Yang et al., 2019). However, there was a study reporting no effect on the copepod *Acartia tonsa* when exposed to *K. veneficum* and had suggested *K. veneficum* can be grouped into toxic strain and non-toxic (Berge et al., 2012). It was suggested that the toxin production in *K. veneficum* was highly variable, determined by genetic and under certain growth condition (Place et al., 2012). Our result had revealed that *K. veneficum* from Malaysian Borneo was capable of causing mortality in the brine shrimp bioassay.

In this study, *K. armiger* was highly lethal to the brine shrimp naupliar. *K. armiger* was reported to be lethal to the copepod *Acartia tonsa* by causing rapid paralysis and mortality in mortality assay (Rasmussen et al., 2017; Berge et al., 2012). Previous study had conducted assay on blue mussel *Mytilus edulis* had resulted in mortality when exposed to the *K. armiger* cells (Binzer et al., 2018). Not only causing mortality, but also reducing the response of blue mussels as the observation during the assay to *K. armiger* had documented the shells were unable to close completely and the mantle retracted very slow upon touching

(Binzer et al., 2018). The toxin involvement in mortality event attributed to *K. armiger* was verified with the characterisation of karmitoxin (Rasmussen et al., 2017).

K. australe had revealed a high lethal effect to the brine shrimp in the bioassay of this study. A decade ago, *K. australe* was associated to the HAB-associated fish kills in Malaysian aquaculture farm (Lim et al., 2014; Teng et al., 2016b). The assays conducted in previous study had documented the lethal effects of *K. australe* by causing mortalities to the rotifers *B. plicatilis*, brine shrimp *A. salina*, and marine medaka *O. melastigma* larvae (Song et al., 2020). The previous study also reported the comparison in lethal effects between *K. australe* and *K. veneficum*. *K. australe* was reported of having higher lethal potency compared to *K. veneficum* and this was due to the greater predation capacities in *K. australe* than in *K. veneficum* (Yang et al., 2019; Song et al., 2020). Similar with the variable lethality of *K. veneficum* strain based on geographic areas (Place et al., 2012), the variation may also existed in *K. australe* strains, and this was proven in this study where different strains of *K. australe* from different areas of the same region had exhibited similar toxicity.

The result from the lethality assay of this study had reported a highly lethal effect of *K. azanzae* to the brine shrimp. *K. azanzae* was previously reported to be extremely fatal to the other invertebrates such as copepod and bivalve (Benico et al., 2020). The high lethality of *K. azanzae* in this study had suggested the possibility of toxin involvement in killing brine shrimp. Previous study on *K. azanzae* also had theorised the capability of *K. azanzae* in producing bioactive substances which assisted in causing death of organism (Benico et al., 2020).

Meanwhile for *K. gentienii*, preliminary assay was done in previous study and had reported the haemolytic effect of *K. gentienii* to horse blood (Nézan et al., 2014). The lower haemolytic activity of *K. gentienii* than *K. armiger* had suggested the possibility of

producing ichthyotoxin which needed to be further investigated especially on the characterisation of the haemolytic compound (Nézan et al., 2014). The lethal effect of *K. gentienii* to the brine shrimp during the bioassay was verified in this study.

Despite genus *Karlodinium* being acknowledged as toxigenic dinoflagellates, there were few species viz. *K. ballantinum* and *K. decipiens* stated to be non-toxic species as there were no report on causing death of organism (Mooney et al., 2010), nonetheless, the death of brine shrimp in the bioassay of this study had revealed *K. ballantinum* and *K. decipiens* were capable of being toxic species. The previously conducted fish lethality assay by exposing *K. ballantinum* live cells to erythrocytes of rainbow trout *Oncorhynchus mykiss* had reported little to no haemolytic activity (Mooney et al., 2010), whereas the toxicity of *K. decipiens* to marine life had never been done apart from its morphological and molecular characterisation (de Salas et al., 2008). Although these two species were not reported hitherto to be toxigenic, the bioassay had demonstrated their lethal effects to brine shrimp likewise the other *Karlodinium* species of this study.

The toxicity of *Takayama acrotrocha* in this study was further supported by the mortality of brine shrimp in the bioassay. The authenticity of *Takayama* species as ichthyotoxic was up for debate as none species of genus *Takayama* had been verified (Tang et al., 2012). Previously, *T. acrotrocha* from Australian type locality was not associated with fish kill events (Larsen, 1996), and the fish bioassay of *T. acrotrocha* from Singapore also did not show fish mortalities (Tang et al., 2012). Another species from *Takayama* called *T. pulchella* was causing bloom in Gulf of Naples and had recorded a high cell density of 7.3×10^6 cells L⁻¹, yet no fish kills was observed in the laboratory assay to the *T. pulchella* isolated during the bloom event (Zingone et al., 2006). Other than that, fish and invertebrate mortalities reported in the Florida were caused by the red tide of *T. pulchella* and effort had

been made to demonstrate the ichthyotoxic of *T. pulchella* in mouse and fish bioassay, however, neither mortality nor signs of toxicity were observed in the tested organisms (Steidinger et al., 1998). Even though fish mortalities have been linked to *Takayama* species in the field, none of the *in vivo* ichthyotoxic were successful (Onoue et al., 1985; Steidinger et al., 1998). The ichthyotoxicity of *T. acrotrocha* was revealed in the brine shrimp lethality assay of this study to support the toxicity of genus *Takayama* of family Kareniaceae.

5.7 Mode of action of *Karlodinium* in lethality assay

Genus *Karlodinium* was known as almost obligate mixotrophic dinoflagellates (de Salas et al., 2005a) which combined two nutritional modes such as phototroph via photosynthesis, and heterotroph by acquiring food through predation (Stoecker et al., 2017; Li et al., 2022). *Karlodinium* relied on both phototroph and heterotroph modes for higher growth rates (Li et al., 2022), hence, was further categorised as mixotrophic *sensu stricto* as mixed nutrition forms were required (Schnepf & Elbrächter, 1992). *Karlodinium* was primarily photosynthetic dinoflagellates but may feed when there was changes in inorganic nutrients availability (Stoecker, 1998). Four of *Karlodinium* species viz. *K. veneficum*, *K. armiger*, *K. aculat* and *K. azanzae* were reported of having mixotrophic mode, but the remaining species were possible to be mixotrophic too as mixotrophic mode may be an important trait of all *Karlodinium* species (Yang et al., 2021).

It was suggested that mixotrophic mode to be one of the essential traits of all *Karlodinium* (Yang et al., 2021), and this nutritional mode of mixotroph was the indicator of the *Karlodinium* species being toxic (Sheng et al., 2010). The bioassay in this study had revealed that *K. armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, and *K. veneficum* led to the mortality of brine shrimp. Despite only *K. veneficum*, *K. conicum* and *K. armiger* had been verified of producing toxin to date (Mooney et al., 2009; Mooney

et al., 2010; Rasmussen et al., 2017; Binzer et al., 2020), the potential of remaining species of *Karlodinium* to be toxic species was also possible. This was because the presence of toxin produced by *Karlodinium* assisted in the role of prey capture, and was clearly demonstrated through prey immobilisation, started with slowing down the prey movement, stunning then ingesting the prey (Sheng et al., 2010). The bioassay of brine shrimp in this study had recorded that naupliar became immobile first before the *Karlodinium* cells approaching the naupliar and attaching themselves onto the body of naupliar, thus it was reasonable to state that contact micropredation was not the main causal killing mechanism of *Karlodinium*. However, the location of the toxin in the cells is yet to be known, and might be prompted to release either in close proximity or direct contact, whereas rupture of cells causing almost complete toxins release (Sheng et al., 2010). It was suggested that the rapid-acting paralytic toxin was directly transferred from the numerous extrusomes covering the surface of *Karlodinium* cells into the prey's nervous system, or through the tube developed during feeding (Bergholtz et al., 2006; Berge et al., 2012).

The uptake of food particles in *Karlodinium* had involved phagotrophy that can be classified into three feeding mechanisms such as direct engulfment, tube feeding and pallium feeding (Schnepf & Elbrächter, 1992; Yang et al., 2021). The mixotrophic nutritional strategy in *Karlodinium* was previously studied in *K. veneficum* and had documented the tube feeding (Yang et al., 2021). Throughout the lethality study, the mixotrophy of *K. armiger*, *K. australe* and *K. azanzae* was observed through the action of cells swarming surrounding brine shrimp naupliar. The action of swarming towards the prey was the pre-capture behaviour of *Karlodinium*, where the cells increased their swimming speed and surrounded the cells (Yang et al., 2021). This study also had observed a visible peduncle emerged slightly below the cell's sulcus intrusion, protruding the body of naupliar and

sucking out the contents from the naupliar. Tube feeding helped the feeder to suck out the inside contents of the prey into food vacuole (Berge et al., 2008a) via peduncle that protruded from the sulcus mid-ventral area connecting the predator and prey (Schnepf & Elbrächter, 1992). The prey size for tube feeders did not had an upper size limit, in simpler words, tube feeders had a wide spectrum of prey types (Berge et al., 2008a). The diverse range of prey types of *Karlodinium* was proven during bloom occurrence associated with the mortalities of fish and crustaceans, and in laboratory assay had revealed *Karlodinium* contact predation towards other dinoflagellates such as *Rhodomonas salina*, *Isochrysis galbana*, *Margalefidinium polykrikoides*, *K. mikimotoi*, zooplankton viz. *Brachionus pilicatilis* and *Artemia salina*, including *M. edulis* (Berge et al., 2008b; Teng et al., 2016b; Song et al., 2020).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study revealed species diversity of Kareniaceae family, especially genus *Karlodinium* in Malaysian coastal waters. Out of 36 described species of family Kareniaceae, a total of ten species had been discovered in Malaysian waters had been identified as *Karenia mikimotoi*, *Karlodinium armiger*, *K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, *K. veneficum*, *K. zhouanum* and *Takayama acrotrocha*. Out of the ten species, seven of them were reported for the first time in Malaysian coastal waters namely *K. mikimotoi*, *Karlodinium armiger*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, and *K. zhouanum*. All these species identification was on the basis of microscopic observation and molecular inferences of LSU and ITS. The detailed molecular of genus *Karlodinium* using sequence structure of phylogeny ITS2, presence of Compensatory Based Changes (CBCs) and hCBC had strongly supported the species boundaries from closely related species in genus *Karlodinium*.

In addition, this study accentuates the importance of investigation on understanding HAB-associated fish kill and, in this respect, a fish kill investigation on *Karlodinium australe* was applied. In this study, *K. australe* caused mortality to *L. calcarifer* fingerlings at cell concentration of 1.5×10^3 cells mL⁻¹, 1.7×10^3 cells mL⁻¹, 1.8×10^3 cells mL⁻¹, 2.1×10^3 cells mL⁻¹, and 2.4×10^3 cells mL⁻¹. The findings also had identified *K. australe* is a toxigenic naked dinoflagellate that caused *L. calcarifer* mortality despite of its cell conditions as live cells, sonicated cell or culture filtrate. Kareniaceae species in this study were confirmed for their lethal effects to the brine shrimp *Artemia* sp. when exposed to *K.*

australe (cell density: 2.3×10^3 cells mL⁻¹), *K. azanzae* (2.3×10^3 cells mL⁻¹), *K. armiger* (2.3×10^3 cells mL⁻¹), *K. gentienii* (2.7×10^3 cells mL⁻¹), *K. veneficum* (2.8×10^3 cells mL⁻¹), and *T. acrotrocha* (3.0×10^3 cells mL⁻¹). Lastly, the micropredation of *K. azanzae*, *K. australe* and *K. armiger* were proven in brine shrimp lethality assay.

6.2 Recommendations

For future study, it is recommended to construct a species tools interactive key for genus *Karenia*, *Karlodinium* and *Takayama* in order to get a better understanding on identifying species of the family Kareniaceae. Also, it is essential to study the life cycle of genus *Karenia*, *Karlodinium* and *Takayama*, and favourable environmental condition that may trigger their bloom to get better understanding on the bloom dynamic study of toxigenic family Kareniaceae. Additional study in the toxicity of genus *Karlodinium* is necessary to further investigate the involvement of each *Karlodinium* species in producing toxin, as well as to characterise the toxin produced. The increasing number of Kareniaceae species highlighten the importance and urgency to precisely and rapidly detect the presence of toxic species in the water. The occurrence of the bloom-forming family Kareniaceae emphasized the potential risk of harmful algal blooms (HABs) in Malaysia associated with halobios mortality and this calls the need for continuous HABs monitoring in the country.

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APPENDICES

Appendix A:

Table 1: List of LSU, ITS1 and ITS2 rDNA sequences of Kareniaceae used in this study.

Species	Strains	Locality	GenBank accession number		
			LSU	ITS	ITS2
<i>K. veneficum</i>	ICMB 260	Ebro River Delta, Alfacs Bay, Spain	-	-	JF906076
<i>K. veneficum</i>	IRTA-SMM-12-03	Alfacs Bay, Spain	-	-	MG642759
<i>K. veneficum</i>	GgaITSC36	North Carolina, Neuse River, United States of America	-	AF352367	-
<i>K. veneficum</i>	CCMP 1975	Maryland, United States of America	-	FJ823568	FJ823568
<i>K. veneficum</i>	CCMP415	-	-	AJ557026	-
<i>K. veneficum</i>	KM1 CSIC-1	-	-	AJ557025	-
<i>K. veneficum</i>	NMBjah047-1	-	-	EU038293	-

<i>K. veneficum</i>	GBJS52	Jogashima, Miura, Japan	LC438756	-	-
<i>K. veneficum</i>	Plymouth 103	Devonport, United Kingdom	DQ114466	-	-
<i>K. veneficum</i>	K3	Catalan Coast	KP790220	-	-
<i>K. veneficum</i>	KDMPT01	Swan River, Western Australia	AY263964	-	-
<i>K. veneficum</i>	SRE#7	Swan River, Western Australia	KP639236	-	-
<i>K. veneficum</i>	QUCCCM_SS2- 14	Arabian Gulf, Qatar	KX853188	-	KX853202
<i>K. conicum</i>	KDCSO15	Southern Ocean	EF469231	-	-
<i>Karlodinium</i> sp.	IFR797	Douarnenez Bay, France	KJ508385	-	-
<i>K. corrugatum</i>	KDGSO08	Southern Ocean	EF469233	-	-
<i>K. ballantinum</i>	LBK116	Manila Bay, Philippines	LC521285	LC521285	-
<i>K. ballantinum</i>	KDBMP01	Mercury Passage, Tasmania, Australia	EF469232	-	-
<i>K. ballantinum</i>	MC701-B1	Gulf of Naples, Italy	FJ024699	-	-
<i>K. ballantinum</i>	GBA078	Mutsu Bay, Japan	-	LC521282	-
<i>K. ballantinum</i>	GBMB64	Manila Bay, Philippines	-	LC521283	LC521283

<i>K. ballantinum</i>	LBK116	Manila Bay, Philippines	-	-	LC521285
<i>K. ballantinum</i>	GBNG13	Imari Bay, Japan	-	LC521286	LC521286
<i>K. ballantinum</i>	TIO1456	Fujian coastal waters, East China Sea	-	-	MW750292
<i>K. gentienii</i>	LUR99	Okinawa, Japan	LC521290	LC521290	LC521290
<i>K. gentienii</i>	GBJS47	Miura, Japan	LC521291	LC521291	-
<i>K. jejuense</i>	IFR10-074	Brest Bay, France	KJ508378	-	-
<i>K. jejuense</i>	LMBEV136	East China Sea, west of Jeju Island, Korea	KX519410	-	-
<i>K. jejuense</i>	LMBEV135	East China Sea, west of Jeju Island, Korea	KX519409	-	-
<i>K. zhouanum</i>	TIO397	Daya bay, South China Sea	MG737358	-	-
<i>K. zhouanum</i>	SHKL571	Suruga bay, Japan	LC521301	-	-
<i>K. zhouanum</i>	MNL527	Manila bay, Philippines	LC521300	LC521300	-
<i>K. zhouanum</i>	TIO401	Daya bay, South China Sea	-	MG738206	MG738206
<i>K. zhouanum</i>	TIO355a	Dalian, Yellow Sea	-	MG738207	MG738207
<i>K. zhouanum</i>	GBBUL63	Manila Bay, Philippines	-	LC521299	LC521299
<i>K. australe</i>	LBK117	Manila Bay, Philippines	-	-	LC521280

<i>K. australe</i>	F201ITS	Tanjung Kupang, West Johor Strait, Malaysia	-	KJ670418	KJ670418
<i>K. australe</i>	F202ITS	Tanjung Kupang, West Johor Strait, Malaysia	-	KJ670419	-
<i>K. australe</i>	F203ITS	Tanjung Kupang, West Johor Strait, Malaysia	-	KJ670420	-
<i>K. australe</i>	F204ITS	Tanjung Kupang, West Johor Strait, Malaysia	-	KJ670421	KJ670421
<i>K. australe</i>	F2-06LSU	Tanjung Kupang, West Johor Strait, Malaysia	KJ670423	-	-
<i>K. australe</i>	GT5	Singapore	DQ156228	-	-
<i>K. australe</i>	T02	Fuzhou, East China Sea	MG737364	-	-
<i>K. australe</i>	MNL529	Manila Bay, Philippines	LC521277	-	-
<i>K. australe</i>	KDAGT03	Grants Lagoon, Tasmania, Australia	DQ151559	-	-
<i>K. azanzae</i>	GBMB68	Manila Bay, Philippines	LC521281	LC521281	LC521281
<i>K. armiger</i>	GC-2 IRTA	Ebro River Delta, Alfacs Bay, Spain	-	AM184204	-
<i>K. armiger</i>	GC-3 IRTA	Ebro River Delta, Alfacs Bay, Spain	-	AM184205	-
<i>K. armiger</i>	GC-7 IRTA	Ebro River Delta, Alfacs Bay, Spain	-	AJ557024	-
<i>K. armiger</i>	K-0668	Alfacs Bay, Spain	-	MG642758	MG642758
<i>K. decipiens</i>	HMD537	Lake Hamana, Japan	-	LC521289	LC521289

<i>K. decipiens</i>	HK1	Hong Kong, South China Sea	-	MG738210	-
<i>K. decipiens</i>	MD482	Sagami Bay, Japan	-	LC521288	-
<i>K. decipiens</i>	Bilbao	Abra de Bilbao, Spain	EF469237	-	-
<i>K. decipiens</i>	GBASA74	Mutsu Bay, Japan	LC521287	-	-
<i>K. decipiens</i>	IFR10-069	Brest Bay, France	KJ508377	-	-
<i>K. digitatum</i>	KdLomme02	Korea	MZ358883	-	-
<i>K. digitatum</i>	PT-B-3	Pingtang coastal area, Fujian Province, China	MN134479	-	-
<i>K. digitatum</i>	PT-B-1	Pingtang coastal area, Fujian Province, China	MN134477	-	-
<i>K. antarcticum</i>	KDANSO10	Southern Ocean	EF469234	-	-
<i>T. acrotrocha</i>	TxURN1HY	Uranouchi Inlet, Kochi, Japan	LC535352	-	-
<i>T. acrotrocha</i>	GT15	Singapore	DQ656116	-	-
<i>T. acrotrocha</i>	MEL104	Haizhou Gulf, China	-	OM232043	-
<i>T. acrotrocha</i>	MC728-D5	Gulf of Naples, Italy	FJ024703	HM067011	HM067011
<i>T. acrotrocha</i>	C2	China	-	MZ948833	-
<i>T. helix</i>	TTNWB01	North West Bay, Tasmania, Australia	AY284950	-	-

<i>T. helix</i>	TTPA01	Port Arthur, Tasmania, Australia	AY284951	-	-
<i>T. tasmanica</i>	TTDE01	River Derwent, Tasmania, Australia	AY284948	-	-
<i>T. tuberculata</i>	TTBSO11.1	Southern Ocean	EF469230	-	-
<i>T. xiamenensis</i>	MC728-B4	Gulf of Naples, Italy	FJ024702	-	-
<i>T. xiamenensis</i>	GSXM03	Xiamen Harbor, East China Sea	KC485078	-	-
<i>T. xiamenensis</i>	TPXM	Xiamen, China	AY764178	AY764179	-
<i>Takayama</i> sp.	IEO-VGO341	Atlantic Coast, Spain	-	AM183261	-
<i>Takayama</i> sp.	IFR909	Quiberon Bay, France	KJ508388	-	-
<i>Takayama</i> sp.	IFR863	Quiberon Bay, France	KJ508387	-	-
<i>Kleptoplastic</i> sp.	KJ47-3-6	South China Sea	KT389994	KT389994	-
<i>Kleptoplastic</i> sp.	IFR11-015	Concarneau Bay, France	KJ508390	-	-
<i>Kleptoplastic</i> sp.	IFR11-001	Concarneau Bay, France	KJ508389	KJ508389	KJ508389
<i>Kleptoplastic</i> sp.	IFR10-474	Concarneau Bay, France	-	KJ858681	KJ858681
<i>Kleptoplastic</i> sp.	W5-1	-	-	FJ823535	FJ823535
<i>Kleptoplastic</i> sp.	RS24	Ross Sea, Antarctica	-	FJ823536	FJ823536

<i>K. mikimotoi</i>	K-0579	Øresund, Denmark	AF200682	-	-
<i>K. mikimotoi</i>	NOAA-2	Sarasota, Florida, United States of America	AY355460	-	-
<i>K. mikimotoi</i>	CAWD117	East Bay, Marlborough, New Zealand	-	HM807314	-
<i>K. mikimotoi</i>	CAWD63	Waimangu Point, New Zealand	-	HM807313	-
<i>K. mikimotoi</i>	KKsKm70	Katsurakoi Port, Kushiro, Hokkaido, Japan	-	LC671816	-
<i>K. mikimotoi</i>	KmURN9mM	Kochi, Japan	-	-	LC055227
<i>K. mikimotoi</i>	Daya Bay	Daya Bay, Guandong, China	-	-	JF683413
<i>K. brevis</i>	Jacksonville C3-5	United States of America	DQ847431	-	-
<i>K. brevis</i>	CCMP2281	Florida, United States of America	-	FJ823562	-
<i>K. brevisculata</i>	-	New Zealand	AY243032	-	-
<i>K. selliformis</i>	CAWD79	Foveaux Strait, New Zealand	KT020848	HM067008	-
<i>K. longicanalis</i>	YB01	Dongyu Pier, Daya Bay, off the South China Sea	-	MF781065	-
<i>K. longicanalis</i>	NJ01	Nanji Island, East China Sea	-	MF781067	-
<i>K. bidigitata</i>	CAWD92	Hawkes Bay, New Zealand	-	HM067003	-
<i>K. bidigitata</i>	CAWD80	Foveaux Strait, New Zealand	AY947662	-	-

<i>K. bidigitata</i>	CAWD81	Foveaux Strait, New Zealand	AY947663	HM807322	-
<i>K. cristata</i>	-	Gordon's Bay, South Africa	AY243963	-	-
<i>K. papilionacea</i>	KspNOM7H	Nomi Inlet, Kochi, Japan	LC055221	-	-
<i>K. papilionacea</i>	KspNOM4H	Nomi Inlet, Kochi, Japan	LC055219	-	-
<i>K. papilionacea</i>	GBCH6	Okinoshima Chiba, Japan	LC438755	-	-
<i>K. papilionacea</i>	KMY-2	Kumamoto, Yatsushiro Sea, Japan	-	AB623225	-
<i>Asterodinium gracile</i>	NG512	Imari Bay, Japan	LC438754	LC438754	-
<i>Brachidinium capitatum</i>	-	Port Aransas, Texas	-	HM066999	-
<i>Gertia stigmatica</i>	mdd472-kt	Sagami Bay, Japan	LC490696	LC490696	-
<i>Gymnodinium aureolum</i>	K-0303	Nivå Bugt, Denmark	AF200671	-	-
<i>Gymnodinium fuscum</i>	CCMP 1677	La Trobe, Australia	AF200676	-	-
<i>Gymnodinium nolleri</i>	DK4	Øresund, Denmark	AF200673	-	-
<i>Gymnodinium</i>	CCMP414	Ria de Vigo, Spain	DQ779990	-	-
<i>catenatum</i>					
<i>Gyrodinium dominans</i>	GDMS0704YD	Masan, South Korea	-	FN669510	-

<i>Gyrodinium dominans</i>	-	Denmark	AY571370	-	-
<i>Gyrodinium moestrupii</i>	HJ-2011	Saemankeum Bay, South Korea	-	HE611580	-
<i>Gyrodinium spirale</i>	-	Skagerrak, Denmark	AY571371	-	-
<i>Gyrodinium rubrum</i>	-	Kattegat, Denmark	AY571369	-	-
<i>Prorocentrum</i>	-	Ehime, Uwajiwa Bay, Japan	-	AB781328	-
<i>shikokuense</i>					
<i>Prorocentrum minimum</i>	D-127	Busan, South Korea	-	JX402086	-
<i>Prorocentrum micans</i>	HYMS-1211-1	Masan, South Korea	-	KP711342	-
