MORPHOTAXONOMY AND GENETIC AFFINITIES OF FIVE NOVEL, POTENTIALLY FISH-KILLING, AUSTRALIAN GYMNODINIOID DINOFLAGELLATES.

by

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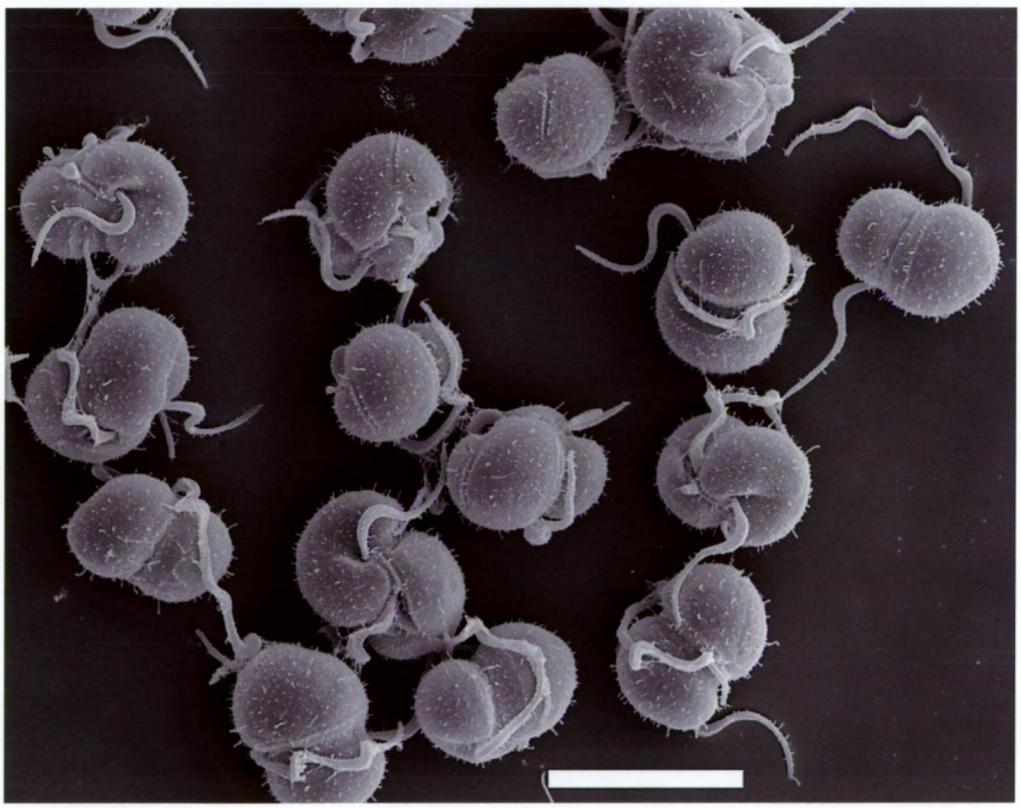

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STATEMENT OF CO-AUTHORSHIP

While several chapters in this dissertation have more than one author, the majority of the work was carried out by its primary author, Miguel de Salas.

Chapters 2-5 have both Dr. Christopher J.S. Bolch and Associate Professor Gustaaf M. Hallegraeff as co-authors, as they were involved in the design and development of this project, as mentors and supervisors. In addition, though the primary author wrote the articles, Dr. Bolch and Assoc. Prof. Hallegraeff helped proofread many drafts and re-write small sections of these chapters.

Chapter 4 has three more co-authors in addition to those above, which helped out with the project in the following ways:

Dr. Lizeth Botes, in Cape Town, South Africa, contributed a South African sequence, pigment profile and light micrographs of *Takayama helix*, as well as proofreading the manuscript arising from Chapter 4.

Dr. Simon Wright, of the Australian Antarctic Division, Kingston, Tasmania, Australia, performed HPLC pigment analyses of dinoflagellates presented throughout this thesis on a collaborative basis. His co-authorship of Chapter 4 is a recognition of his help in this field.

Geraldine Nash, also of the Australian Antarctic Division, trained the author in SEM preparation and use of the scanning electron microscope. Her co-authorship of Chapter 4 is a recognition of her help throughout this project.

Miguel Félix de Salas, July 2004

ABSTRACT

Water samples from several southern Australian locations (Tasmania, Victoria, New South Wales, South Australia and Western Australia) have revealed a range of previously undescribed gymnodinioid dinoflagellates. Five new species are described in this study, from field samples and laboratory cultures, with the use of light microscopy, scanning and transmission electron microscopy, pigment analysis and sequencing of the large subunit ribosomal gene (LSU rDNA).

Karenia umbella de Salas, Bolch & Hallegraeff sp. nov., has a straight apical groove typical of the genus. This species is morphologically similar to *K. longicanalis* but has an asymmetrically shaped hypocone, irregularly shaped chloroplasts, and is ornamented by 8 radial furrows on the epicone surface. *Karenia asterichroma* de Salas, Bolch & Hallegraeff sp. nov. is a dorso-ventrally flattened unarmoured dinoflagellate resembling *K. brevis*. Unlike other known *Karenia* species, the chloroplasts of *K. asterichroma* radiate from a central pyrenoid, and its nucleus is located in the centre of the epicone. While the ichthyotoxicity of *K. asterichroma* is yet to be confirmed, it was associated, together with *K. umbella* and further undescribed *Karenia* species, with the recent mass mortality in May 2003 of over 100,000 salmonoids in an aquaculture facility in southern Tasmania.

A new, potentially ichthyotoxic genus, *Takayama* de Salas, Bolch, Botes et Hallegraeff gen. nov. is described with two new species: *T. tasmanica* de Salas, Bolch et Hallegraeff, sp. nov., and *T. helix*, de Salas, Bolch, Botes et Hallegraeff, sp. nov., isolated from Tasmanian (Australia) and South African coastal waters. The new *Takayama* species have sigmoid apical grooves and close affinities to the other fucoxanthin-containing genera *Karenia* and *Karlodinium*.

A new mixotrophic species in the toxigenic genus *Karlodinium, K. australe* de Salas, Bolch & Hallegraeff sp. nov., is morphologically similar to *Gyrodinium corsicum* and *Karenia digitata* but has an anteriorly located nucleus and a different amphiesmal pattern. *K. australe* lacks the hexagonal arrays of plug-like structures below the amphiesma that define the genus, and

its chloroplasts contain pyramidal rather than lenticular pyrenoids. Thus the definition of *Karlodinium* needs to be emended, as it is too narrow, and currently excludes *K. australe.*

Pigment analyses of these species, all from previously well studied fish-killing groups, have determined that they contain fucoxanthin and its derivatives as the main accessory pigments. The pigment gyroxanthin-diester, was present in all *Karenia* species tested, but its presence was variable in *Karlodinium* and *Takayama*. Furthermore, phylogenetic analysis of these and other gymnodinioid species show that all the fucoxanthin-containing, fish-killing species in the genera *Karenia*, *Karlodinium* and *Takayama* form a well-defined, monophyletic clade within the Gymnodiniales.

The description and detailed characterisation of these five new species will help design more effective monitoring tools, such as molecular probes, for the early detection of these potentially fish killing dinoflagellates.

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A number of people have contributed to this project.

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I thank Lizeth Botes for contributing ideas and South African micrographs, pigment profiles and sequences of *Takayama helix*.

Judi Marshall contributed many live samples collected during fish-kills, and liaised effectively with the aquaculture industry to organise the delivery of samples to our lab. *Karenia asterichroma* was described from such samples. I would also like to thank Judi for teaching me most of what I know of culturing techniques, use of laboratory equipment, and for generally keeping the lab running smoothly. Judi, you don't get the recognition, but you have been as much a supervisor to many of us as any that are so in an official capacity.

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This thesis is dedicated to Esteban de Salas, my father.

Although he passed away before this work was complete, he was always my strongest encouragement to achieve.

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CHAPTER 1:
BACKGROUND AND CONTEXT OF THIS STUDY. PROBLEMS IN UNARMOURED DINOFLAGELLATE TAXONOMY

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1.1. INTRODUCTION

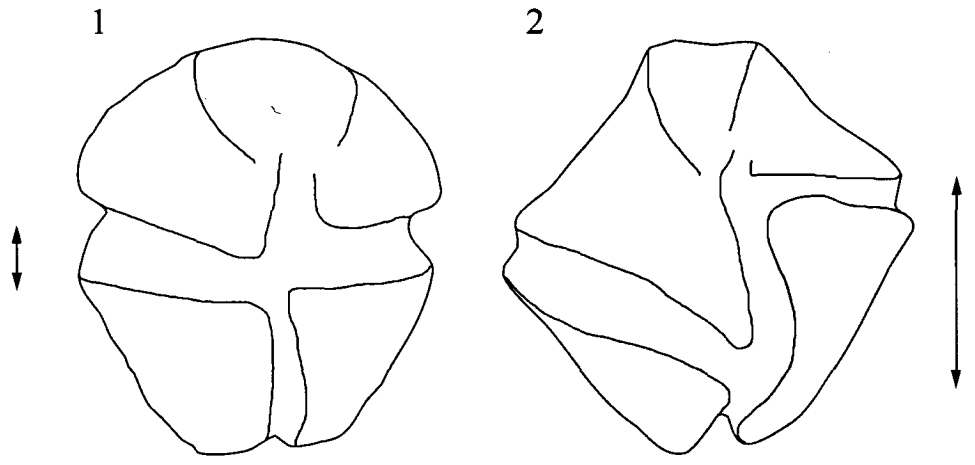
This chapter introduces the traditional approach to unarmoured dinoflagellate taxonomy, following the classification of Kofoid & Swezy (1921), as well as its apparent problems. A review of morphological characters of taxonomic importance as well as other tools used for taxonomy is provided in the context of our current understanding of this group. New toxic genera arising from a recent revision of unarmoured dinoflagellate taxonomy (Daugbjerg *et al.* 2000) are explained, in addition to examples of their fish-killing capabilities, in particular in the Australian situation. This discussion leads to the development of the context and scope of this study, as well as the aims of this dissertation.

1.2. BACKGROUND TO THIS STUDY

1.2.1. Use of morphology in the identification of unarmoured dinoflagellate species

Morphology remains the primary means of identifying and describing species of unarmoured dinoflagellates. Gymnodinioid dinoflagellates have been traditionally assigned to one of two main genera, following the classification of Kofoid & Swezy (1921): *Gymnodinium* (type species *G. fuscum* F. Stein), if the degree of cingulum displacement is less than 20% or 1/5 of the total cell length (Fig. 1.1), and *Gyrodinium* (type species *G. spirale* (Berg) Kofoid & Swezy), if the degree of cingulum displacement is more than 20% (Fig. 1.2). Other morphological characters, such as the pigment profiles, internal structure of the cells, and surface features such as apical grooves, have traditionally been used to discriminate only between species within these genera. This classification system using the girdle displacement as the main criterion to discriminate between the two genera has been maintained until quite recently (Dodge 1982; Larsen 1994; 1996).

One common problem with this classification has been that many unarmoured dinoflagellate species have cingular displacements that are borderline, and in some species a clonal culture can exhibit a variation of morphotypes belonging to different genera (Kimball & Wood 1965).



Figures 1.1-1.2: Schematic representations of unarmoured dinoflagellate genera following the traditional classifications of Kofoid and Swezy (1921). Arrows mark extent of girdle displacement:

Fig. 1.1: Genus *Gymnodinium* (*G. chlorophorum*). Girdle displacement < 20%.

Fig. 1.2: Genus *Gyrodinium* (*G. instriatum*). Girdle displacement >20%.

1.2.2. Methods other than morphology

1.2.2.1. Pigments

Pigment analysis has been used in the past as a complementary taxonomic tool, to differentiate between groups of dinoflagellates (Jeffrey *et al.* 1975). It is now generally accepted that pigment profiles are useful for discrimination of dinoflagellates at the generic level (Daugbjerg *et al.* 2000). As an example, while most photosynthetic dinoflagellates have chlorophyll *a* and *c*, as well as peridinin as the major carotenoid, some, such as *Lepidodinium viride* Watanabe *et al.* (1987), instead contain chlorophylls *a* and *b*, as well as violaxanthin and zeaxanthin, giving the cells a bright green, instead of reddish-brown colour. Furthermore, the genera *Karenia* G. Hansen & Moestrup and *Karlodinium* J. Larsen have been defined for species whose major carotenoids are fucoxanthin, and fucoxanthin derivatives instead of peridinin (Daugbjerg *et al.* 2000).

Recently a new pigment has been characterised, gyroxanthin diester (Björnland *et al.* 2000), from cultures of *Karlodinium micrum* (Leadbeater & Dodge) J. Larsen. It has since been shown that this pigment also occurs in several *Karenia* species (Hansen *et al.* 2000a; Haywood 2001; Botes *et al.* 2003) and

proposals have been made to use it as a marker for *Karenia* in routine monitoring of surface waters (Schofield *et al.* 1999; Ornolfsdottir *et al.* 2003; Staehr & Cullen 2003).

It has also recently been shown that most of the species in the genera *Karenia* and *Karlodinium*, which form a clade based on their pigment composition, also cluster together in phylogenetic analyses of partial rDNA sequences (Daugbjerg *et al.* 2000; Botes *et al.* 2003). Furthermore, the support for the fucoxanthin-containing clade within the dinoflagellates is very high, when tested with bootstrap (Daugbjerg *et al.* 2000; Haywood *et al.* 2004).

1.2.2.2. Lectins

Lectins are proteins on the cell surface which bind to specific glycolipids, glycoproteins and polysaccharides (Sharon & Lis 1989). They have been used to identify dinoflagellates, as they bind to sugars in cell membranes and flagellar systems (Costas & Lopez-Rodas 1994; Costas *et al.* 1996).

Lectins have been used to discriminate between a range of organisms without distinct morphological characters (Rhodes *et al.* 1995; Rhodes 1998). However, Haywood (2001) found lectin binding patterns inconsistent with results obtained through ultrastructure, rDNA sequencing, pigment analysis and external morphology, and also that stages of the cell-cycle and growth phase had an important effect in lectin-binding, and that replication of results wasn't always possible.

1.2.2.3. Sterols

Karenia brevis (Davis) G. Hansen & Moestrup, *K. mikimotoi* (Miyake & Kominami *ex* Oda) G. Hansen & Moestrup and *Karlodinium micrum* have been shown to be closely related in their sterol composition (Leblond & Chapman 2002), which is represented by a simple profile combining primarily (24*S*)-4α-methyl-5α-ergosta-8(14),22-dien-3β-ol (ED) and its 27-*nor* isomer (NED). They have very different sterol profiles to species of other unarmoured and armoured dinoflagellate genera, and it is possible that sterols could be used as an extra tool to discriminate between dinoflagellate genera in routing monitoring. However, at present, the sterols of *Karenia* species other than *K. brevis* and *K. mikimotoi* have not been examined.

1.2.2.4. Sequencing of ribosomal genes

Since the discovery of thermostable polymerases for use in the polymerase chain reaction (PCR, Saiki *et al.* 1988), the amount of sequences available for genetic comparisons has been increasing exponentially. The genes most commonly amplified in dinoflagellates have historically been ribosomal genes because, being present as multiple copies in each cell, amplification is generally easy (Fensome *et al.* 1999). Use of the gene coding for the small subunit RNA (SSU rDNA), which is approximately 1800 bases long, is restricted to comparison in a larger scale, such as between different species, genera and families (Hillis & Dixon 1991), as this gene is fairly conserved. The small amount of variability present in this gene is offset by the ease of alignment of its sequences, which allows the comparison of distantly related taxa (Saunders *et al.* 1997).

In contrast to the SSU rDNA, the large subunit ribosomal gene (LSU rDNA) is nearly twice as long, approximately 3400 bases. As in the SSU rDNA, the LSU is composed of core conserved regions interspersed with more variable domains (Michot *et al.* 1984). The variable regions in the LSU rDNA are far more variable than even the entire SSU gene, so that as little as 700-1000 bases from the domains D1, D2 and D3, at the beginning of the gene, provide sufficient information for the construction of meaningful phylogenetic trees (Fensome *et al.* 1999). Because of the need to sequence shorter fragments to obtain as much usable information, many authors, such as Scholin *et al.* (Scholin *et al.* 1994a; Scholin *et al.* 1994b; Scholin & Anderson 1996), Bolch *et al.* (1999), Botes *et al.* (2003), Daugbjerg *et al.* (2000), Hansen *et al.* (2000a), Haywood *et al.* (2004), Judge *et al.* (1993), Lenaers et al. (1989), MacKenzie *et al.* (2004), Walsh *et al.* (1998), Wilcox (1998), Yeung *et al.* (1996) and Zardoya *et al.* (1995), have used the LSU ribosomal gene in preference to the small subunit.

Sequences of the 5.8S gene and flanking internal transcribed spacer (ITS regions have been used by authors in the past (Adachi *et al.* 1994; 1996; Penna & Magnani 1999; de Salas *et al.* 2001), as the high variability of this fragment makes it possible to determine relationships between closely related taxa and even within species (Fensome *et al.* 1999). In comparison to both the SSU and

LSU genes sequences of this fragment are not as common in GeneBank, and therefore not easily obtainable for comparisons.

1.2.3. Recent revisions and new genera

A recent revision of the major genera of unarmoured dinoflagellates (Daugbjerg *et al.* 2000) has redefined the genera *Gymnodinium* and *Gyrodinium* in an attempt to make them monophyletic. This revision has also erected three new genera for several groups previously not separated from the main cluster of *Gymnodinium* / *Gyrodinium*:

- *Akashiwo* G. Hansen & Moestrup, for the species previously known as *Gymnodinium sanguineum* Hirasaka, which has peridinin as the main carotenoid and an apical groove that is a clockwise spiral,
- *Karenia* G. Hansen & Moestrup (type species *Karenia brevis* (Davis) G. Hansen & Moestrup), for species whose major carotenoids are fucoxanthin or its derivatives, and which have a straight apical groove (Fig. 1.3),
- *Karlodinium* J. Larsen (type species *Karlodinium micrum* (Leadbeater & Dodge) J. Larsen), for species containing fucoxanthin or fucoxanthin derivatives as the main accessory pigments, a straight apical groove, a ventral pore, and an amphiesma with arrays of plug-like structures in a hexagonal pattern (Fig. 1.4).

1.2.4. Morphology revisited

Daugbjerg *et al.*'s (2000) revision has encompassed a redefinition of which morphological characters are of taxonomic importance, and which ones are not. When examined in conjunction with characters such as rDNA sequences, pigment profiles and ultrastructure, the degree of girdle displacement is shown to be of little or no taxonomic value, as it is highly variable even within clonal strains. However, other external features like the apical groove have proven to be of taxonomic importance at the generic level. Ultrastructural characters such as the flagellar root system, the pusule and the nuclear envelope, and biochemical characters, such as the photosynthetic pigment profile and rDNA

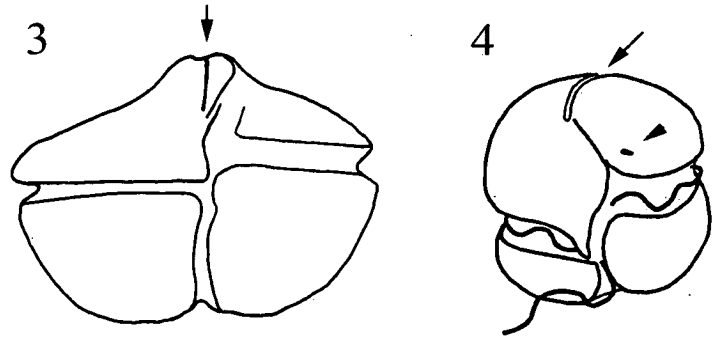
sequences have also shown they can be of help in resolving gymnodinioid dinoflagellates at the generic level (Haywood 2001).

Characters considered of taxonomic importance in this group at the generic level at present include:

- surface features such as:
  - apical grooves (Takayama 1985),
  - ventral pores (Daugbjerg *et al.* 2000)
  - amphiesmal vesicle patterns
- Ultrastructural features such as:
  - Flagellar root system (Hansen 2001)
  - Pusule (Hansen *et al.* 2000b)
  - Nuclear envelope
- Presence or absence of chloroplasts,

1.2.5. Fish-killing gymnodinioids

As a result of Daugbjerg *et al.*'s (2000) revision it became apparent that the main fish-killing unarmoured dinoflagellates clustered into a defined group of closely related species, with fucoxanthin (or its derivatives) as the main carotenoid and being genetically close.



Figures 1.3 – 1.4: New fish-killing dinoflagellate genera erected by Daugbjerg *et al.* (2000).
Fig. 1.3: New genus *Karenia* (*K. brevis*). Note linear apical groove (arrow).
Fig. 1.4: New genus *Karlodinium* (*K. micrum*, adapted from Daugbjerg *et al.* 2000). Note linear apical groove (arrow) and ventral pore (arrowhead).

The best studied of these species, *Karenia brevis*, has been known to be responsible for recurrent blooms in the Gulf of Mexico since it was first described (Davis 1948). Its close relative *K. mikimotoi* has been reported [under several different names: *Gyrodinium cf. aureolum* (Partensky *et al.* 1988), *Gymnodinium nagasakiense* (Takayama & Adachi 1984)] as a fish killer from northern Europe (Tangen 1977) and Japan (Takayama & Adachi 1984), for a period spanning several decades. A third species transferred to *Karenia* based on its morphology is *K. brevisulcata* (Chang) G. Hansen & Moestrup, which was responsible for a massive bloom in Wellington Harbour, N.Z (Chang 1999; Chang *et al.* 2001), that killed most other marine life, ranging from fish to invertebrates and even seaweeds and seagrasses. This bloom also produced respiratory irritation symptoms in people exposed to toxins carried by the wind in the form of aerosols. The closely related genus *Karlodinium* has recently been described (Daugbjerg *et al.* 2000) for one well known and two poorly studied species: *K. micrum*, *K. veneficum* (Ballantine) J. Larsen and *K. vitiligo* (Ballantine) J. Larsen, respectively. These species have been known to cause marine life mortalities since the 1950s (Ballantine 1956; Braarud & Heimdal 1970).

Although three species of *Karenia* were known at the time of Daugbjerg *et al.*'s (2000) revision of unarmoured dinoflagellate taxonomy, only two had been widely studied, *K. brevis* and *K. mikimotoi*. Since then awareness of this fish-killing genus has expanded, and the appearance of papers describing new species in this genus is accelerating:

- *Karenia digitata* Yang, Takayama, Matsuoka & Hodgkiss killed wild and cultured fish in Hong Kong and Japan, and fishing boats coming into Hong Kong harbour reported their live catch was killed before they entered the high cell-density area (Yang *et al.* 2000).
- Shortly after the *K. digitata* bloom, another *Karenia* species bloomed in Hong Kong harbour, without causing fish-kills: *K. longicanalis* Yang, Hodgkiss & Hansen (Yang *et al.* 2001).

- Blooms of two species caused marine life mortalities and human health problems in South Africa: *K. bicuneiformis* Botes, Sym & Pitcher, and *K. cristata* Botes, Sym & Pitcher (Botes *et al.* 2003).
- Toxicity of shellfish in New Zealand in 1993, which contained brevetoxin and new brevetoxin analogues, resulted in the isolation and description of three new species: *Karenia papilionacea* Haywood & Steidinger., *K. bidigitata* Haywood & Steidinger, and *K. selliformis* Haywood, Steidinger & Mackenzie. (Haywood *et al.* 2004). The latter species also caused massive fish kills in Chile (Clement *et al.* 2001; Haywood *et al.* 2004).

Finally a group of ichthyotoxic species of distinct morphology but uncertain taxonomic status, composed of *Gymnodinium pulchellum* Larsen and the closely related *Gyrodinium acrotrochum* Larsen and *Gyrodinium cladochroma* Larsen (Larsen 1994; 1996) have been blamed for fish kills in Australia (Hallegraeff 2002), Japan (Takayama 1985), and north America (Steidinger *et al.* 1998).

1.2.5.1. Fish kills in Australia

In Australia, unarmoured dinoflagellates are thought responsible for a number of marine life mortality events both in natural situations and artificial, aquaculture enterprises.

Port Phillip Bay blooms of *G. pulchellum*-like species in the 1950s caused massive marine life mortalities (Hallegraeff 2002). This species has also been reported from Tasmanian waters (Hallegraeff 2002).

In Murdunna, Tasmania, a bloom of *Karenia cf. mikimotoi* is thought to have caused the mortality of approximately 1,000 aquacultured rainbow trout (*Oncorhynchus mykiss* Walbaum) in December 1989 (Hallegraeff 2002).

Several *Karenia* species bloomed again in south-eastern Tasmania in May 2003, this time killing approximately 100,000 aquacultured Atlantic salmon (*Salmo salar* L.)

Natural fish kill events caused by *Karlodinium micrum* (Leadbeater & Dodge) G. Hansen & Moestrup occur almost annually in the Swan River, Western

Australia (2001-2003), and have also been recorded in the Murray River (W.A., 1999), in Lake Illawarra (1991 & 2000, Hallegraeff 2002) and Sydney Harbour, New South Wales (this study). Because of its similarity to much publicised and worked-on *Pfiesteria*, awareness of *Karlodinium* blooms and associated fish-kills is expanding, and blooms of this species have now been reported all over the world.

Karenia brevis- like species have been reported from southern Australia, including Western Australian coastal waters, Port Lincoln (South Australia) and Gippsland Lakes (Victoria) (Hallegraeff 2002). However, they have so far not been reported in association with fish-kills. *K. mikimotoi* has been reported by Larsen (1994) as common during the austral summer in Hobson's Bay, Victoria.

Throughout this study some taxa are referred to as 'fish-killing' and 'ichthyotoxic', where their toxicity to marine life is generally accepted and usually has been replicated in the lab. Most species of *Karenia* as well as *Karlodinium micrum* and *Gymnodinium pulchellum* fall in this group. Other species where taxonomic confusion exists, or their fish-killing capability has not been confirmed in the laboratory are referred to as 'potentially ichthyotoxic' and 'potentially fish-killing'. An example of this would be species newly isolated from natural fish kills, or others, like *Gyrodinium acrotrochum* or *G. cladochroma*, where taxonomic confusion with *Gymnodinium pulchellum* may cause uncertainty.

1.3. SCOPE AND CONTEXT OF THIS STUDY

This study arose when water samples collected from various southern Australian locations, especially during fish kills or visible blooms or 'red tides' were found to contain a range of unusual and undescribed gymnodinioid dinoflagellates. In particular several species were isolated from the recently erected genus *Karenia*, one new species in the new genus *Karlodinium*, and two new species, clearly congeneric and morphologically similar to Larsen's (1994; 1996) species *Gymnodinium pulchellum*, *Gyrodinium acrotrochum* and *G. cladochroma*. There is a consistent trait of fish killing ability associated with species in the genera, *Karenia*, *Karlodinium*, and also the group of species

related to *Gymnodinium pulchellum* (Steidinger *et al.* 1998; Chang 1999; Yang *et al.* 2000; Deeds *et al.* 2002; Hallegraeff 2002). As such the definitive identification of Australian species in these genera, and the clear and unambiguous characterisation of novel taxa is a clear necessity for effective monitoring.

1.4. AIMS OF THIS STUDY

- To characterise as fully as possible several unusual or new species belonging to unarmoured, fish-killing dinoflagellate genera.
- To determine whether the recently erected genera *Karenia* and *Karlodinium* are valid and can be separated on the basis of morphological and biochemical characters.
- To identify if fish killing dinoflagellates of the fucoxanthin-containing genera *Karenia* and *Karlodinium* form a monophyletic clade within the gymnodinioid dinoflagellates.

1.5. STRUCTURE OF THIS THESIS

This dissertation is composed of several stand-alone papers that are presented in chapters 2 to 5, linked by a common introduction, a recapitulation of the main ichthyotoxic and potentially ichthyotoxic unarmoured dinoflagellate species found in Australia (Chapter 6), and their genetic relationships, and a common conclusion (Chapter 7).

1.6. PUBLICATIONS ARISING FROM THIS THESIS

Chapter 2.

DE SALAS, M. F., BOLCH, C. J. S., AND HALLEGRAEFF, G. M. 2004. *Karenia umbella* sp. nov. (Gymnodiniales, Dinophyceae), a new, potentially ichthyotoxic dinoflagellate species from Tasmania, Australia. *Phycologia* **43**: 166-175.

Chapter 3.

DE SALAS, M. F., BOLCH, C. J. S., AND HALLEGRAEFF, G. M. 2004. *Karenia asterichroma* sp. nov. (Gymnodiniales, Dinophyceae), a new dinoflagellate

species associated with finfish aquaculture mortalities in Tasmania, Australia. *Phycologia* **43**: 624-631.

Chapter 4.

DE SALAS, M. F., BOLCH, C. J. S., BOTES, L., NASH, G., WRIGHT, S. W., AND HALLEGRAEFF, G. M. 2003. *Takayama* (Gymnodiniales, Dinophyceae) gen. nov., a new genus of unarmoured dinoflagellates with sigmoid apical grooves, including the description of two new species. *Journal of Phycology* 39: 1233-1246.

Chapter 5.

DE SALAS, M. F., BOLCH, C. J. S., AND HALLEGRAEFF, G. M. *Karlodinium australe* (Gymnodiniales, Dinophyceae) sp. nov., a new potentially ichthyotoxic unarmoured dinoflagellate from lagoonal habitats of south-eastern Australia. *Phycologia* (submitted July 12th, 2004).

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CHAPTER 2:

KARENIA UMBELLA SP. NOV. (GYMNODINIALES, DINOPHYCEAE), A NEW, POTENTIALLY ICHTHYOTOXIC DINOFLAGELLATE FROM SOUTHERN AUSTRALIA [1]

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and

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ABSTRACT: A new, potentially ichthyotoxic gymnodinioid dinoflagellate, *Karenia umbella* de Salas, Bolch & Hallegraeff, is described from southern Australian coastal waters. This species is characterised by light and electron microscopy of field samples and laboratory cultures, as well as large subunit ribosomal DNA sequences and HPLC pigment analyses of two cultured strains. This new *Karenia* species has a straight apical groove and contains fucoxanthin derivative carotenoid pigments, typical of the genus. *Karenia umbella* differs from its closest relative *K. longicanalis* in its larger size, its longer epicone with a finger like sulcal intrusion, asymmetrically shaped hypocone, irregularly shaped chloroplasts, and the presence of 8 radial furrows on the epicone surface. It differs from *K. digitata* in its significantly larger size and the shape of the epicone and hypocone. Genetically, this species is distinct from the dorsoventrally flattened *Karenia* species *K. mikimotoi*, *K. brevis* and *K. brevisulcata*.

[1] **de Salas, M. F., Bolch, C. J. S. & Hallegraeff, G. M.** (2004) *Karenia umbella* sp. nov. (Gymnodiniales, Dinophyceae), a new, potentially ichthyotoxic dinoflagellate species from Tasmania, Australia. *Phycologia* 43: 166-175.

2.1. INTRODUCTION

Fish kills caused by difficult to identify, small (10-30 μm size) gymnodinioid dinoflagellates were first reported from Florida in 1844 (as *Gymnodinium breve* Davis 1948). However, in the past three decades such events have increasingly impacted on fisheries and aquaculture operations, for example Norway [*Karenia mikimotoi* (Miyake & Kominami ex Oda) G. Hansen & Moestrup as *Gyrodinium aureolum* Hulburt (Braarud & Heimdal 1970; Hansen *et al.* 2000)], Japan [*Gymnodinium nagasakiense*, Takayama & Adachi (Takayama & Adachi 1984)], New Zealand [*G. brevisulcatum* Chang (Chang 1999) and three other *Gymnodinium breve* look-alikes (Haywood *et al.* 1996)], Chile (Carreto *et al.* 2001), and Hong Kong [*K. digitata* Yang *et al* (Yang *et al.* 2000) and *K. longicanalis* Yang *et al* (Yang *et al.* 2001)]. It has long been recognised that these species constitute a well-defined taxonomic grouping within the polyphyletic gymnodinioid dinoflagellates, because they possess fucoxanthin- derived carotenoid pigments rather than the typical dinoflagellate carotenoid peridinin (Björnland & Tangen 1979; Tangen & Björnland 1981; Steidinger 1990). Based on molecular sequencing of the large subunit ribosomal RNA gene (LSU rDNA), combined with evidence from ultrastructure, details of the flagellar apparatus and morphology of the apical groove, Daugbjerg *et al.* (2000) created the new genus *Karenia* G. Hansen & Moestrup [type species: *Karenia brevis* (Davis) G. Hansen & Moestrup] to include these ichthyotoxic dinoflagellates with straight apical grooves. Other small ichthyotoxic, fucoxanthin derivative-containing species were moved to the new genus *Karlodinium* J. Larsen [e.g. for *Gymnodinium galatheanum* Braarud *sensu* Kite & Dodge (1988), with a short, straight apical groove, amphiesmal plugs and a ventral pore). The generic affinities and taxonomic revision of *Gymnodinium pulchellum* Larsen (with sigmoid apical groove) and other species with sigmoid apical grooves has been resolved and will be detailed by us in a separate communication (de Salas *et al.* 2003).

In the present work we describe a new, medium-sized gymnodinioid dinoflagellate, *Karenia umbella* de Salas, Bolch & Hallegraeff sp. nov., which was first associated with the mortality of approximately 1000 caged rainbow trout (*Oncorhynchus mykiss*) at a salmonid fish farm in Murdunna, on the

Tasman Peninsula (Fig. 2.1), south – eastern Tasmania, in December 1989. A further more serious mortality event involving 100,000 Atlantic salmon (*Salmo salar* L.) recurred at a neighbouring Tasmanian site in May 2003. Earlier scanning electron micrographs of field material of the causative organism were reproduced in Hallegraeff (1991, Fig.10C, D) as a species related to *Gymnodinium mikimotoi* Miyake & Kominami *ex* Oda, and a LSU rDNA sequence of an earlier culture was reported by Bolch (1999, as *Gyrodinium* sp. 2, strain Gy2DE, since lost from culture). Regular sampling in waters off eastern Tasmania have shown the species to be present in a number of other locations (Fig. 2.1), and here we characterise the organism based on field samples and unialgal cultures, by light and scanning electron microscopy, sequencing of the LSU rDNA gene and HPLC pigment analysis. We compare our new species with authentic culture material of *K. brevis, K. mikimotoi* and *K. brevisulcata* (Chang) G. Hansen & Moestrup. *Karenia umbella* is similar to the poorly characterised *K. longicanalis* (Yang, *et al.* 2001), whose description is based on preserved field samples only, and we discuss the differences that support the separation of the two species.

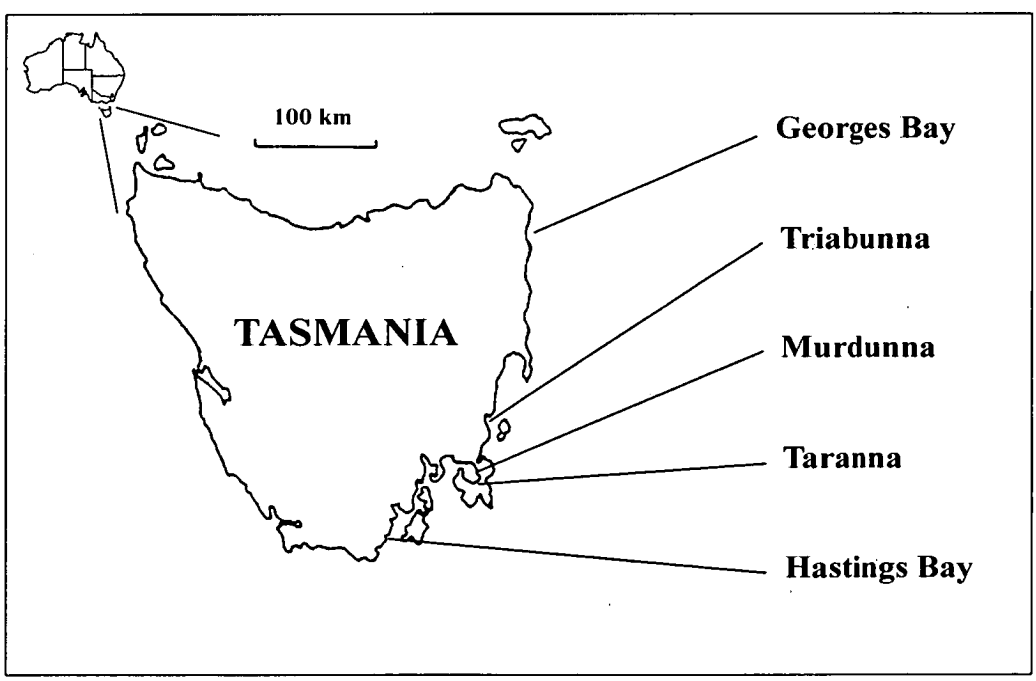


Figure 2.1. Localities in Tasmania, Australia, where *K. umbella* has been collected.

2.2. MATERIALS AND METHODS

2.2.1. Culture conditions

Cells of *Karenia umbella* were isolated from 20 µm plankton net samples collected from Murdunna, Taranna, and Triabunna, in south eastern Tasmania (see Fig. 2.1). Single cells were isolated with a micropipette into 28‰ GSe medium (Blackburn *et al.* 2001). Cultures were maintained in this medium, at 17°C, with a 12:12 L:D photoperiod of 100 µmol photosynthetically active radiation m^{-2} s^{-1}, supplied by cool white fluorescent lights. *Karenia mikimotoi* (strain CCMP429) and *K. brevis* (strain CCMP718) were obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, Maine, USA. *Karenia brevisulcata* was obtained from Cawthron Institute, Nelson, New Zealand. These and other cultures used in this study are detailed in Table 2.1.

2.2.2. Light microscopy

Live cells of *Karenia umbella* were examined and photographed using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss, Göttingen, Germany) equipped with bright field and differential interference contrast illumination, and both a Canon Powershot G1 digital camera (Canon, Tokyo, Japan) and a Carl Zeiss

Table 2.1. Strains and samples used in this study

Species	Locality	Date	Isolator	Strain code
Field samples				
K. umbella	Murdunna	4.12.1989		
K. umbella	Georges Bay	15.3.2002		
K. umbella	Port Arthur	17.10.2001		
K. umbella	River Derwent	27.2.2002		
Cultures:				
K. umbella	River Derwent	04.1997	C.J. Bolch	GY2DE
K. umbella	Triabunna	15.3.2002	M. de Salas	KULV01
K. umbella	Taranna	17.10.2001	M. de Salas	KUTN05
K. brevisulcata	New Zealand		Lincoln Mackenzie	
Karlodinium micrum	Perth, WA, Australia	11.03.2001	M. de Salas	KDMSR01
G. aureolum	Adelaide, SA	1.02.2000	M. de Salas	GAAD01

Axiocam Hr digital camera. Cell length, width, and degree of girdle displacement were measured on 50 individual live cells in mid-logarithmic growth phase.

2.2.3. Scanning electron microscopy

One millilitre of *Karenia umbella* culture or field sample was fixed by adding an equal volume of 4% OsO_4 solution prepared at the same salinity as the sample. Fixed cells were concentrated by gentle centrifugation (Bolch *et al.* 1999). Cells were rinsed in culture medium, followed by deionised water, after which they were allowed to settle on glass coverslips coated with 1 mg ml^{-1} poly-L-lysine hydrochloride (Marchant & Thomas 1983). Dehydration was accomplished using a graded ethanol/acetone series (10%, 30%, 50%, 70%, 90% and 100% ethanol in water, followed by two 100% dry acetone steps), after which samples were critical-point dried with liquid carbon dioxide and sputter-coated with gold. Samples were observed using a JEOL 35C scanning electron microscope (JEOL, Tokyo, Japan).

2.2.4. DNA extraction, PCR amplification and sequencing

Cultures were grown to mid-logarithmic phase and approximately 10 ml pelleted by gentle centrifugation. Total DNA was extracted by the gentle lysis method (Bolch *et al.* 1998). Extracted DNA was used as a template to amplify a fragment of the large subunit ribosomal gene approximately 1400 bp long, using the primers D1R (Scholin *et al.* 1994a) and 28:1483R (Daugbjerg, *et al.* 2000). PCR amplifications were performed in volumes of 50µl, as described in Bolch (2001), but with an annealing temperature of 60°C for the first 10 cycles and 56°C for a further 25. Amplification products were checked by electrophoresing through 1% agarose gels stained with ethidium bromide and visualised under ultraviolet light. Successful reactions were purified using QIAquick PCR purification columns (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and sequenced in both directions using a Beckman-Coulter Dye Terminator Sequencing Kit (Beckman-Coulter, Fullerton, CA, USA), according to the manufacturers instructions. Sequencing reactions were electrophoresed on a Beckman-Coulter CEQ2000 capillary electrophoresis sequencer. Primers D1R, D2C and D3Ca (Scholin *et al.* 1994b)

were used to determine the nucleotide sequence of approximately 900 bp of the amplified fragment. All sequence electropherograms were examined visually and base-calling errors corrected manually. Both forward and reverse sequences were aligned and conflicts resolved by manual inspection.

2.2.5. Sequence alignment and phylogenetic analyses

Sequences were aligned using ClustalX (Thompson *et al.* 1997), and alignments were refined by hand. Inserts and deletions were coded by construction of a character matrix in which gaps were coded as single insertion or deletion events regardless of length. Phylogenetic analysis was carried out using PAUP 4.0* (Swofford 1999). An outgroup consisting of *Akashiwo sanguinea* (Hirasaka) G. Hansen & Moestrup, *Gymnodinium aureolum* (Hulbut) G. Hansen & Moestrup, *G. chlorophorum* Elbrächter & Schnepf, *G. impudicum* (Fraga & Bravo) G. Hansen & Moestrup, *G. cf. placidum* Herdman, *G. palustre* Schilling, *G. fuscum* F. Stein, *G. catenatum* Graham, *G. nolleri* Ellegaard & Moestrup and *G. microreticulatum* Bolch was used in the analysis, representing the major gymnodinioid groups for which sequences are available. Distance, parsimony and likelihood were all used as criteria to construct trees, with no significant difference in the arrangement of ingroup species. Bases were treated either as equal, unweighted characters, or with transitions having twice the weight of transversions, with no noticeable difference in the result. Bootstrap analyses were carried out (1000 replicates) with all three criteria.

2.2.6. Pigment analysis

Approximately 10 ml of culture in mid-logarithmic phase was filtered gently onto 45-mm Whatman GF/F glass fibre filters (Whatman, Maidstone, U.K.) and snap frozen in liquid nitrogen for storage. For extraction of pigments the filters were cut into approximately 4 mm^2 sections and placed in 10 ml syringes containing 6 ml of methanol, sonicated for 30 seconds and filtered (0.45 µm) into HPLC vials. Pigments were analysed by the method of Zapata *et al.* (2000) using a Waters 626 pump (Waters, Milford, MA, USA), a Gilson 232 autoinjector (with the sample stage refrigerated at -10°C; Gilson, Midleton, WI, USA), a Waters Symmetry C8 column (150 x 4.6mm, 3.5 µm packing, at 30.0°C) and a Waters 996 diode array detector. Peaks were integrated using

Waters Millennium software and identified by comparison of their retention times and spectra with those of mixed standards obtained from known cultures (Jeffrey & Wright 1997), coinjected with each batch of samples. In addition, gyroxanthin-diester (DHI Bioproducts, Denmark) was also co-injected, as this pigment is known from *Karenia* species (Hansen *et al.* 2000).

2.3. OBSERVATIONS

Karenia umbella* de Salas, Bolch & Hallegraeff, *sp. nov.

Figures 2.2 – 2.14

DIAGNOSIS: Cellulae ovatae, dorsiventraliter parum complanatae, 29-42 µm longae, 21-32 µm latae. Epiconus conicus vel hemisphericus, 8 striis superficiebus radiantibus ornatus. Hypoconis lobus dexter lobo sinistro longior. Extrema cingulo mediano a cellulae longitudine 20% semota. Sulcus ut parva projectura digitiformis in epiconum invadens. Canalis apicalis rectus latus e positione parum dextrorsum sub apicem sulcii supra apicem epiconi ad dimidum lateris dorsalis epiconi extensus. Nucleus sphaericus vel ellipsoidus, centralis, capsula circumcinctus. Chloroplasti peripherales circa 20, flavovirentes, multilobati pyrenoide praediti.

Cells ovate, slightly dorsoventrally flattened, 29-42 µm long (average 35.85 ± 3.37 µm), 21-32 µm wide (average 26.61 ± 2.90 µm). Epicone conical or hemispherical, adorned with 8 radiating furrows. Hypocone truncated and uneven, with the right lobe longer than the left lobe. Cingulum deeply incised and wide, displaced approximately 20 % of total cell length (average 20.3 ± 1.36 %). Sulcus wide, extending as a finger-like protrusion into the epicone at a 45° angle. A straight, broad apical groove extends from slightly to the right and below the sulcal apex, over the apex of the epicone, and approximately halfway down the dorsal epicone. A large round or ellipsoidal nucleus is central, surrounded by a capsule, and is closer to the dorsal surface. The cell contains approximately 20 peripherally located, irregular, shallow, multi-lobed and strap- shaped chloroplasts.

HOLOTYPE: Figure 2.7, from strain KUTN05 isolated from Taranna, Tasmania, Australia, in October 2001. Culture deposited in the University of Tasmania Harmful Algae Culture Collection.

ETYMOLOGY: named from the Latin *umbella*, meaning parasol, after the radial furrows in the epicone which resemble the radial spokes in a parasol.

DISTRIBUTION: East coast of Tasmania, from George's Bay in the north east to Hastings Bay in the far south (Fig. 2.1). Also observed in field samples in Western Australia (Swan River) and cultured from South Australia (Port Lincoln).

DESCRIPTION: *Karenia umbella* is a medium sized dinoflagellate known from both field samples and laboratory cultures. Average measurements are provided in Table 2.2, in comparison with its close relatives *K. longicanalis*, *K. digitata* and *K. mikimotoi*. Under the light microscope *K. umbella* cells have a distinctive shape, shown in Figures 2.2 – 2.4. The epicone is conical and tapering, with a blunt apex (Fig. 2.2), shrinking to hemispherical in stressed cells (Fig. 2.4). The hypocone is truncated, but asymmetrical, and the right lobe is always longer than the left lobe (Fig. 2.4). An intrusion of the sulcus into the epicone is obvious as a finger-like projection angled approximately 45° relative to the sulcus (Fig. 2.2). A straight apical groove extends from slightly below and to the right of the sulcal intrusion into the epicone, through the cell apex, to approximately halfway down the dorsal epicone (Fig. 2.2). The sulcus is broad and deep towards the antapex, but narrow and shallow between the two terminal ends of the cingulum (Fig. 2.2). Chloroplasts are irregular and multi-lobed, situated around the periphery of healthy cells (Fig. 2.5). Chloroplasts of stressed cells, such as cells that have spent some time under a coverslip, become disc-shaped. Chloroplasts possess single, lenticular pyrenoids (Fig. 2.6). A spherical pusule (not shown) surrounded by sac shaped vesicles has been observed under high magnification. The nucleus can be spherical or ellipsoidal in shape. It is normally located close to the center of the cell, but closer to the dorsal surface. It is surrounded by a thick bilayered envelope (Fig. 2.4). Cultured cells of this species spend much of their time on the bottom of the culture vessel swimming slowly; only a small proportion of cells actively swim through the medium.

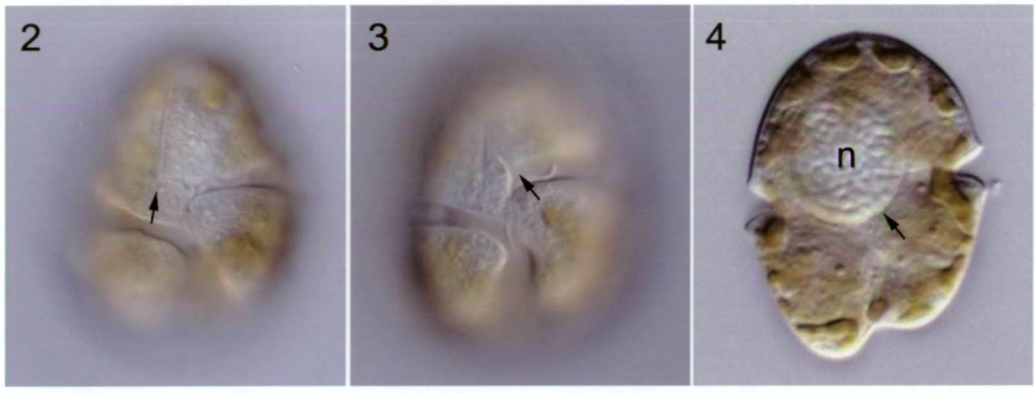
Under the SEM, the cell surface appears composed of numerous, small amphiesmal vesicles (Figs 2.7, 2.11). The apical groove is clearly displayed, being shallow and wide (Fig. 2.7, 2.8). The bend at the ventral end can be seen

in Figures (Figs 2.7, 2.9). Eight shallow furrows extend radially around the apex, and almost touch the cingulum. The six furrows in the dorsal surface are deeper and more easily seen than the two in the ventral surface. The sulcal intrusion can be seen as a cleft or furrow extending from the junction of the sulcus and the cingulum (Figs 2.7, 2.9). A clustered pattern of pores is sometimes present on the ventral epicone, hypocone, or both (Figs 2.9, 2.10, 2.12).

A schematic representation of *K. umbella* in comparison with *K. longicanalis*, *K. digitata* and *K. mikimotoi* drawn to the same scale (Figs 2.13-2.20) provides a comparison of the relative average size of these species.

PIGMENTS: The photosynthetic and accessory pigments of *K. umbella* include chlorophylls *a*, c_2 and c_3. No chlorophyll c_1 was detected. The following carotenoids were identified, reported here as a percentage of chlorophyll *a*: fucoxanthin (38.4%), 4-keto-19'-hexanoyloxyfucoxanthin (16.6%), 19'-hexanoyloxyfucoxanthin (17.1%), a 19'- hexanoyloxyfucoxanthin- like pigment (14.9%), diadinoxanthin (13.9%), diatoxanthin (4.5%), gyroxanthin diester (8.9%). A pigment chromatogram of *Karenia umbella* is provided in Figure 2.21.

MOLECULAR SYSTEMATICS: The LSU rDNA sequences of *K. umbella* diverge approximately 7.4% from those of *K. mikimotoi*, 6.4 % from *K. brevis*, and 6.3 % from *K. brevisulcata*. Trees resulting from bootstrap analyses of distance, likelihood and parsimony criteria presented minor differences in the arrangement of some outgroup species with respect to others, but in all cases *K. umbella* formed a distinct group, clearly within the genus *Karenia*. A parsimony analysis of *K. umbella*, related species of *Karenia* and other gymnodinioids produced the tree shown in Figure 2.22. Based on LSU rDNA sequences, the genus *Karenia* is monophyletic, with *K. umbella* somewhat divergent from the dorsoventrally flattened species *K. mikimotoi, K. brevis,* and *K. brevisulcata*.

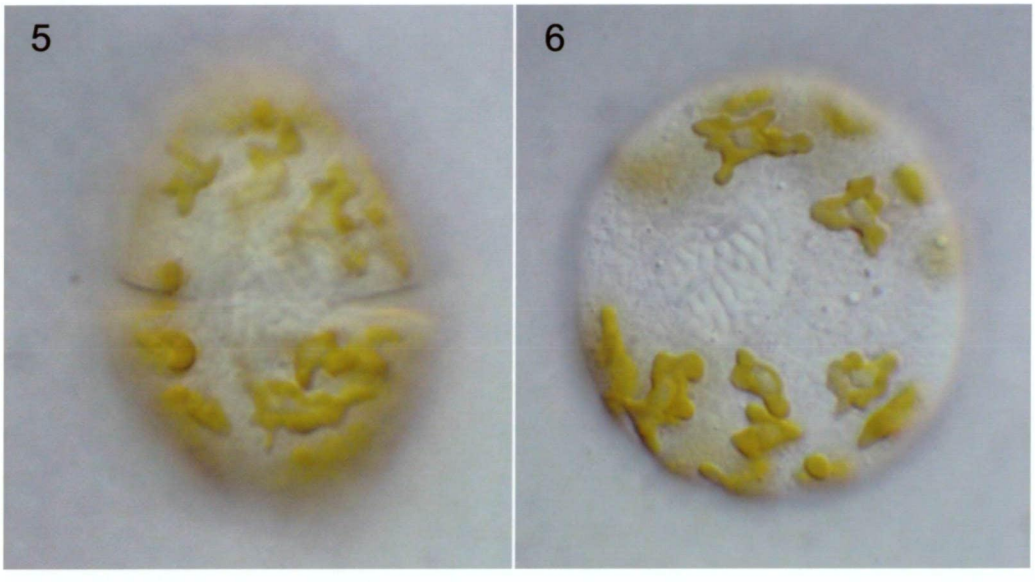


Figures 2.2 – 2.4: Light micrographs of *Karenia umbella*. Scale bars = 10 µm.

Fig. 2.2: Ventral view of a cultured cell of *K. umbella* with a surface focus, showing the apical groove (arrow).

Fig. 2.3: Surface focus of another cultured cell of *K. umbella* showing an intrusion of the sulcus into the epicone at an angle of 45° (arrow).

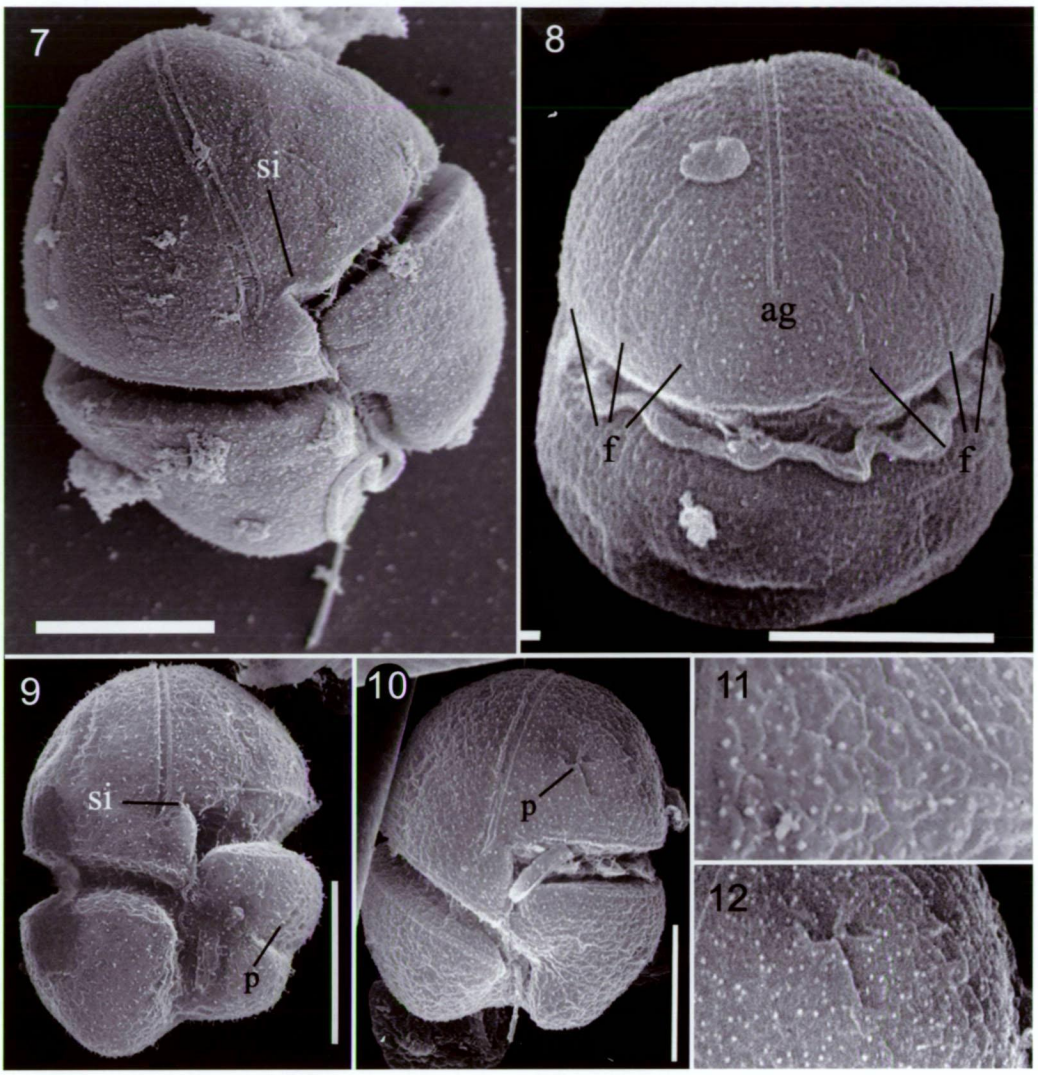
Fig. 2.4: Cross section view of a cultured cell of *K. umbella*, showing the spherical nucleus (n). Note the thick bilayered envelope surrounding the nucleus.



Figures 2.5, 2.6: Light micrographs of chloroplasts of *Karenia umbella*. Scale bars = 10µm.

Fig. 2.5: Subsurface dorsal view of a healthy cell showing chloroplast shape and peripheral arrangement.

Fig. 2.6: Stressed cell showing pyrenoids inside the chloroplasts (arrows).



Figures 2.7 – 2.12: *Karenia umbella,* SEMs. Scale bars = 10 µm.

Fig. 2.7: Ventral view of a cultured cell (strain KUTN05) showing the sulcal intrusion into the epicone (si).

Fig. 2.8: Apical-dorsal view of a, showing the straight apical groove (ag) only descending about ½ the length of the dorsal epicone, and at least six of the shallow furrows (f) radiating from the apex after which the species is named. Please note that the angle of the specimen makes the apical groove appear to descend further than it does (see Fig. 2.14).

Fig. 2.9: Ventral view of a field-collected cell (from Murdunna) showing the sulcal intrusion (si) into the epicone and pores (p) on the left ventral hypocone. Note the right lobe of the hypocone is longer than the left lobe.

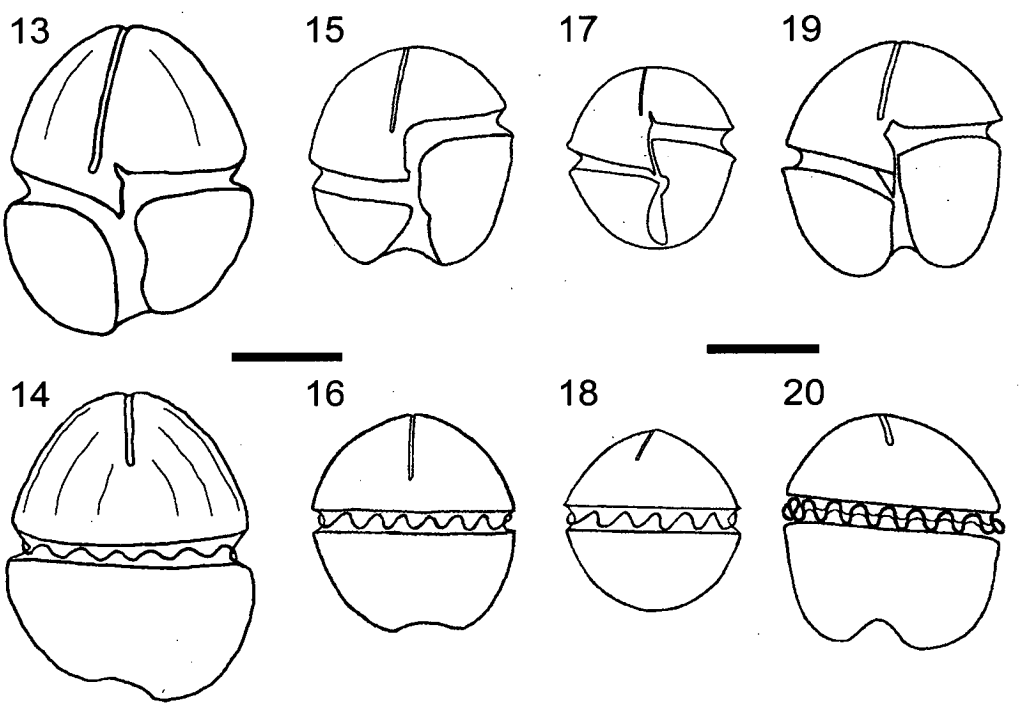
Fig. 2.10: Ventral view of another field-collected cell (from Murdunna) showing the pores (p) in the left ventral epicone.

Fig. 2.11: Detail of another field-collected cell (from Murdunna) showing the pattern of amphiesmal vesicles that compose the cell membrane.

Fig. 2.12: Detail of pores on the cell shown in Figure 10.

2.4. DISCUSSION

Karenia umbella is morphologically similar to *K. longicanalis*, but differs in size, the shape of the epicone and hypocone, its finger-like sulcal intrusion, and the presence of epiconal furrows. The shape of the epicone in *K. longicanalis* is described as hemispherical (Yang, *et al.* 2001), whereas *K. umbella* has an epicone that is conical and truncated (Figs 2.2, 2.7, 2.13), and only appears hemispherical if cells are stressed (Figs 2.4, 2.8-2.10). *Karenia umbella* has a clear, shallow finger-like extension of the sulcus into the epicone at an angle of approximately 45° (Figs 2.3, 2.7, 2.9), whereas the sulcus of *K. longicanalis* does not continue beyond the epicingular border (Yang, *et al.* 2001). A finger –



Figures 2.13 – 2.20: Schematic representations (to same scale) of four different but closely related *Karenia* species. The upper drawing of each pair (Figs 13, 15, 17 and 19) represents the ventral surface, and the lower drawing (Figs 14, 16, 18 and 20) the corresponding dorsal surface. Scale bars = 10 μm. Figures 2.15-2.20 adopted from Yang *et al.* (2001).

Figs 2.13, 2.14: *K. umbella.*

Figs 2.15, 2.16: *K. longicanalis.*

Figs 2.17, 2.18: *K. digitata.*

Figs 2.19, 2.20: *K. mikimotoi.*

like extension of the sulcus into the epicone is described in *K. digitata* (Yang, *et al.* 2000), but the smaller size, overall rounded shape, and rounded hypocone of this species clearly separate it from *K. umbella* (compare Figures 2.13 and 2.14 to 2.17 and 2.18). A consistent character in *K. umbella* is the asymmetrical shape of the hypocone, with the left lobe shorter than the right lobe (Figs 2.4, 2.7, 2.9).

Yang *et al.* (2001) described *K. longicanalis* as having approximately 30 globular chloroplasts, whereas *K. umbella* possesses only about 20 and they are irregularly shaped, long, shallow and multi-lobed (Fig. 2.5). The shape of these varies in *K. umbella* from distinctly strap shaped (but with lobes) in healthy cells, to shallow, irregular discs in stressed cells. However, the number of chloroplasts in a clonal culture was found to be highly variable, from as few as one per cell for aberrant, small cells (possibly gametes) to as many as 20 in large, healthy motile cells. The photosynthetic pigments of *K. umbella*, notably the possession of fucoxanthin derivatives and gyroxanthin- diester, classify this species with *K. mikimotoi* and *K. brevis* (Tangen & Björnland 1981; Björnland *et al.* 1984; Steidinger 1990). The nucleus of *K. umbella* is not large compared to the overall size of the cell (Fig. 2.4, 2.5), and can be spherical or elongated

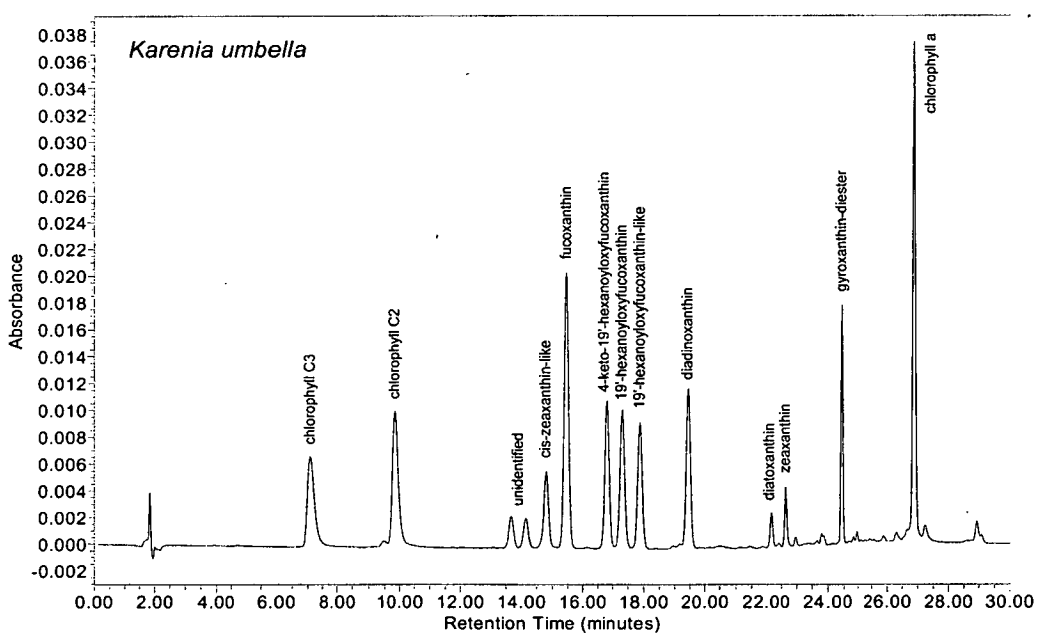


Figure 2.21: Pigment chromatogram of *Karenia umbella* strain KUTN05, from Taranna, Tasmania, Australia.

along the longitudinal axis. Its location is normally near the center of the cell, or sometimes slightly displaced towards the epicone or hypocone. It is situated closer to the dorsal surface of the cell. In comparison, the nucleus of *K. longicanalis* is described as being large and round, and located in the center of the cell. This is stated to be a constant character in this species when compared to the variable shape and position in species such as *K. mikimotoi* and *K. brevisulcata*. *Karenia longicanalis* is also claimed to have a 'nuclear capsule' (*sensu* Hansen 1993), a feature apparent under the light microscope also in *K. umbella* (Fig. 2.4). However, other *Karenia* species such as *K. brevis*, which have a spherical nucleus surrounded by an apparent capsule have been shown by TEM to possess a normal nuclear envelope as present in the generic description of *Karenia* (Steidinger 1990).

Although cell size is an important taxonomic character in dinoflagellates, it can be variable, both in field populations and laboratory cultures. For example, both *K. mikimotoi* and *K. brevisulcata* are known to produce subpopulations of both small and large cells, in which the length - width ratio changes (Partensky, *et al.* 1988; Chang 1999). *Karenia longicanalis* cells range from 17 to 35 μm long (average 26 μm), and 10 to 22.5 μm wide (average 21 μm, Yang, *et al.* 2001). This is significantly smaller than *K. umbella*, which ranges from 29 to 42 μm long (35.85 ± 3.37 μm) and 21 to 32 μm wide (26.61 ± 2.90 μm). Full measurements are provided in Table 2.2. Figures 2.13-2.20 show the comparative shapes and sizes of *K. umbella*, *K. longicanalis*, *K. digitata*, and *K. mikimotoi*.

A puzzling feature of *K. umbella* is the occasional presence of several deep pores in the left ventral hypocone, or epicone, or both (Figs 2.9, 2.10, 2.12). Similar pores, numbering 4 to 6, have been reported in the left lateral hypocone of *K. brevis* (Steidinger 1990), and *K. mikimotoi* (as *Gymnodinium nagasakiense*, Takayama & Adachi 1984, Plate II, Fig. 4). A ventral pore in the epicone is also noted as a defining feature of the genus *Karlodinium* Larsen (Daugbjerg, *et al.* 2000). However, in *Karenia umbella*, this feature is not easily preserved upon fixation for SEM, and its taxonomic value is uncertain. We are confident, however, that these pores are not a fixation artefact, because they occur consistently in the same location on the cell surface, though their

numbers are variable. The overall outline of *K. umbella* can be reminiscent of *K. mikimotoi*, but *K. umbella* has a longer epicone, and a clearly uneven hypocone, and is only slightly dorsoventrally flattened, unlike *K. mikimotoi*.At the time of writing of this paper, LSU rDNA sequences were only available for *K. mikimotoi* and *K. brevis*; no sequences were published with the description of either *K. longicanalis* or *K. digitata*. With the increasing availability of LSU rDNA sequences of gymnodinioid dinoflagellates that are difficult to identify, descriptions of new species should ideally be based on a combination of morphotaxonomy (LM and SEM), ultrastructure (transmission electron microscopy), molecular characters (e.g. DNA sequences), and biochemical features, such as chloroplast pigments, toxins (e.g. brevetoxin) and fatty acid characterisation. Without such additional data, the descriptions of the Hong Kong species *K. longicanalis* and *K. digitata* are incomplete and hamper further studies of gymnodinioid dinoflagellates. On the basis of available data, *K. umbella* and the poorly characterised *K. longicanalis* seem to be different species. However, it remains possible that DNA sequencing data and reexamination of live material of *K. longicanalis* may demonstrate synonymy with *K. umbella* and require amendment of the description of *K. longicanalis*.

Further work needs to be undertaken in order to ascertain whether *K. umbella* produces brevetoxins or other toxic chemicals that might account for its implication in fish kills in Tasmania.

2.5. ACKNOWLEDGEMENTS

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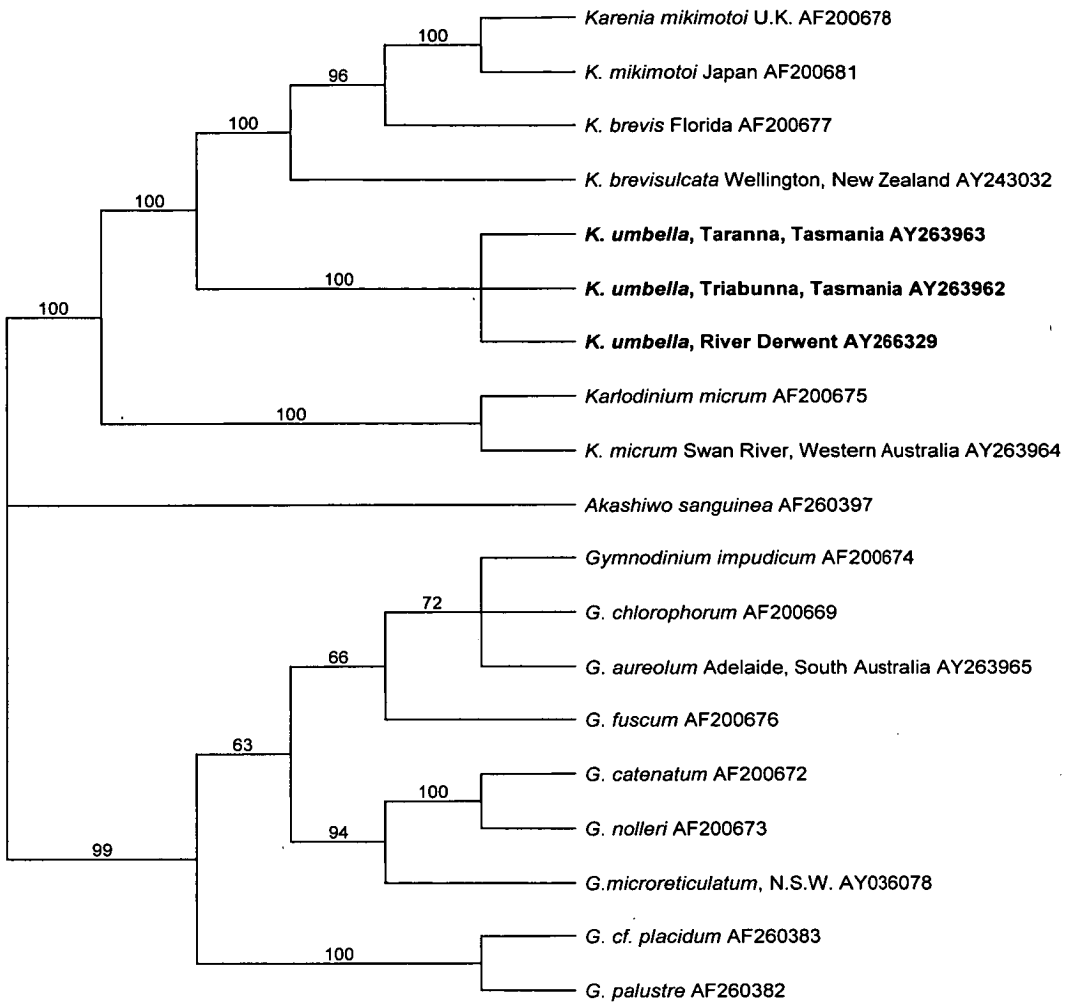

Figure 2.22: Bootstrap consensus tree maximum parsimony searches of *Karenia umbella* (in bold type) and closely related species. *Akashiwo sanguinea* and the true *Gymnodinium* spp. were all used as the outgroup. GeneBank Accession numbers are given for all sequences. Bootstrap values are shown for all branches.

Table 2.2: A comparison of cell parameters of *Karenia umbella* with other *Karenia* species.

Parameter	*Karenia umbella*	*Karenia longicanalis* [1]	*Karenia digitata* [2]	*Karenia mikimotoi* [3]
Cell length (μm)	29-42 (35.85 ± 3.37, $n = 50$)	28.85 ± 5.51 (n = 50)	21.46 ± 2.96 (n = 50)	18 – 37
Cell width (μm)	21-32 (26.61 ± 2.90, $n = 50$)	21.07 ± 4.31 (n = 50)	18.25 ± 2.54 (n = 50)	14 – 35
Length/Width ratio	1.03-1.53 (1.35 ± 0.10, $n = 50$)	1.23 ± 0.11 (n = 50)	1.18 ± 0.082 (n = 50)	1.2 ± 0.1 [4]
Thickness	4/5 of cell width	slightly flattened	slightly flattened	1/3 – 3/4 of width
Girdle displacement (% of cell length)	20.3 ± 1.36 (n = 20)	22.06 ± 1.6 % (n = 6)	24.87 ± 1.9 (n = 9)	11 – 25% but mostly 14 – 20%
Sulcus extension	short, finger-like, 45° angle	no extension	short, finger-like	small, wedge-shaped
Apical groove	linear, wide & shallow, about ½ down dorsal side	linear, long, thick about 2/3 down dorsal side	linear, short, thin 1/3- 1/2 down dorsal side	linear, short, thick, 1/3 down dorsal side
Nucleus	round, to ellipsoidal, anterior to subcentral.	round, central	round, subcentral	ellipsoidal or reniform, left
Chloroplasts	multilobed and flattened; peripheral, up to 20	round, up to 30	shapeless, numerous	shapeless, 10-20

[1] Yang *et al.* 2001

[2] Yang *et al.* 2000

[3] Takayama & Adachi 1984

[4] Partenski *et al.* 1988

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CHAPTER 3:

KARENIA ASTERICHROMA SP. NOV. (GYMNODINIALES, DINOPHYCEAE), A NEW DINOFLAGELLATE SPECIES ASSOCIATED WITH FINFISH AQUACULTURE MORTALITIES IN TASMANIA, AUSTRALIA [1]

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Christopher J. S. Bolch

and

Gustaaf M. Hallegraeff

ABSTRACT: A new species of unarmoured dinoflagellate is described from Tasmanian field samples and laboratory cultures: *Karenia asterichroma* de Salas, Bolch & Hallegraeff sp. nov. This species is characterised by the use of light and scanning electron microscopy and sequencing of the large subunit ribosomal gene. *Karenia asterichroma* is a dorso-ventrally flattened cell resembling *K. brevis*, although it differs from this species by 4.5% in the D1-D3 regions of its LSU rDNA sequence. Unlike other *Karenia* species, the epicone of *K. asterichroma* has concave sides and its chloroplasts radiate from a central, putative pyrenoid. The nucleus is located in the centre of the epicone and the apical groove is long, extending ventrally to near a broad sulcal extension into the epicone. Partial LSU rDNA sequences indicate a relationship with *K. bicuneiformis*. While the ichthyotoxicity of *K. asterichroma* is yet to be confirmed, it was associated (with three other *Karenia* species) with a recent mass mortality of over 100,000 caged salmonids at an aquaculture operation in southern Tasmania, Australia.

[1] **de Salas, M.F., Bolch, C.J.S., and Hallegraeff, G.M.** (2004) *Karenia asterichroma* sp. nov. (Gymnodiniales, Dinophyceae), a new dinoflagellate species associated with finfish aquaculture mortalities in Tasmania, Australia. *Phycologia* **43**: 624-631

3.1. INTRODUCTION

At the time of Daugbjerg *et al.*'s (2000) revision of unarmoured dinoflagellate genera, only three species of *Karenia* G. Hansen & Moestrup had been formally described, Since then, the study of potentially ichthyotoxic gymnodinioids has intensified, several new species have been described [such as *Karenia digitata* Yang *et al.* (Yang *et al.* 2000), *K. longicanalis* Yang *et al.* (Yang *et al.* 2001), *K. cristata* Botes *et al.* and *K. bicuneiformis* Botes *et al.* (Botes *et al.* 2003), *K. umbella* de Salas *et al.* (de Salas *et al.* 2004), *K. bidigitata* Haywood *et al.*, *K. papilionacea* Haywood *et al.*, *K. selliformis* Haywood *et al.* (Haywood *et al.* 2004)], and it is clear that the genus harbours numerous undescribed species. While many are morphologically similar to already-described species [e.g. *K. umbella* to *K. longicanalis* (de Salas *et al.* 2004); *K. papilionacea* to *K. brevis* (Davis) G. Hansen *et* Moestrup (Haywood *et al.* 2004)], some are also clearly distinct from anything previously described [e.g. *K. brevisulcata* (Chang) G. Hansen *et* Moestrup (Chang 1999) and *K. digitata* Yang *et al.* (Yang *et al.* 2000)]

In May 2003, the death of 100,000 caged mature Atlantic salmon (*Salmo salar* L.) at aquaculture facilities at Parsons Bay and Port Arthur, Tasmania, Australia (Fig. 3.1), coincided with a dense mixed bloom of five distinct dinoflagellates belonging to the genus *Karenia*. Of the five morphotypes present, only one could be assigned to a known species, *K. umbella* (de Salas *et al.* 2004; see also Chapter 2), that had been associated with previous fish kills at a nearby location (Murdunna, Fig. 3.1). Despite intensive effort, most of the *Karenia* morphotypes in this bloom could not be cultured and properly characterised, including two non-flattened, *Karenia* morphotypes similar to *K. umbella* and *K. longicanalis*. Those that could be cultured could only be maintained in tissue culture vessels at very low cell densities with periodic re-isolation needed.

Despite the culturing difficulties, we were able to grow enough material to undertake a detailed morphological and genetic analysis of some morphotypes. Here we present a description of a new, potentially ichthyotoxic dinoflagellate, *Karenia asterichroma* sp. nov., supported by LM and SEM analysis and

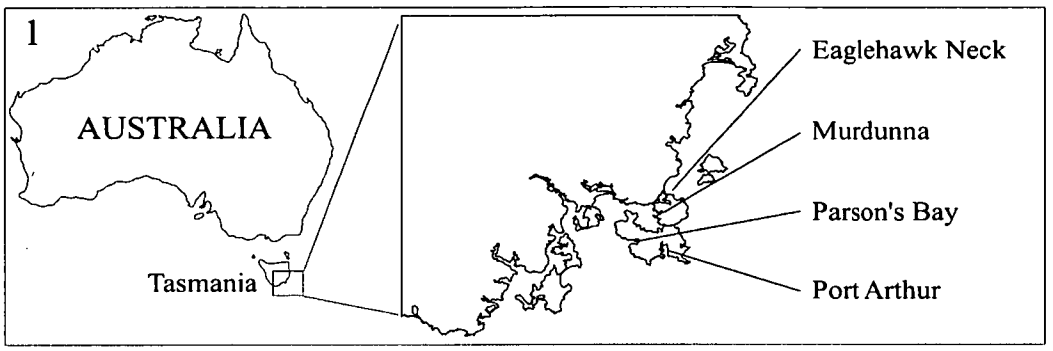


Figure 3.1: Map of Tasmanian (Australia) locations mentioned in this study.

comparison of partial LSU rDNA sequences with other existing *Karenia* species.

3.2. MATERIALS AND METHODS

3.2.1. Strain isolation and culture maintenance

Clonal cultures of *Karenia asterichroma* were established by isolation of vegetative cells from seawater samples using micropipette manipulation and cell washing. Cultures were maintained in GSe medium (Blackburn *et al.* 2001) of 28‰ salinity at 17° C under 100 μmol m^{-2} s^{-1} PAR illumination, supplied by cool white fluorescent lights, with a photoperiod of 12:12 L:D. Cultures used for morphological and genetic comparisons were from the authors' collections (University of Tasmania). *Karenia brevis* (Davis) G. Hansen & Moestrup (CCMP718) was obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, Maine, USA. *Karenia brevisulcata* (CAWD82) was obtained from Cawthron Institute, Nelson, New Zealand.

3.2.2. Light and Scanning electron microscopy

Live cells of laboratory cultures and field samples were examined and photographed using a Zeiss Axioskop 2+ microscope equipped with bright field and differential interference contrast illumination, and Zeiss Axiocam HRc digital camera (Carl Zeiss, Göttingen, Germany). Cell length and width were measured on 20 individual live cells in mid-logarithmic growth phase.

For scanning electron microscopy, cells were fixed with equal volumes of 4% osmium tetroxide made up in culture medium, processed and mounted as

described in Chapter 2 and examined in a JEOL JSM 840 scanning electron microscope (JEOL, Tokyo, Japan).

3.2.3. DNA extraction, PCR amplification and cycle sequencing

Total DNA was extracted from approximately 5 ml of culture by a modified gentle lysis, phenol/chloroform method (Bolch *et al.* 1998) This was used as a template to amplify a fragment of the large subunit (LSU) ribosomal RNA gene approximately 900 bases long, using the primers D1R and D3Ca (Scholin *et al.* 1994). Reagent concentrations and PCR reaction conditions are detailed in Chapter 2. Completed reactions were checked for successful amplification by electrophoresis of 5 µl aliquots through a 1% agarose gel followed by staining with ethidium bromide and examination under UV light. Reaction products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturers instructions and product concentration measured using a Bio-Rad SmartSpec^{tm} 3000 spectrophotometer (Bio-Rad, Hercules, CA, U.S.A.). Alternatively with field samples, two to four single cells were placed into a 200 µl PCR tube, and PCR was carried out as explained above.

Approximately 50 fmol of PCR product was sequenced in both directions using either the forward or reverse amplification primer and a Beckman-Coulter Dye Terminator Sequencing Kit according to the manufacturers instructions (Beckman-Coulter, Fullerton, CA. U.S.A.). Sequencing reactions were electrophoresed on a Beckman-Coulter CEQ2000 capillary electrophoresis sequencer. A consensus DNA sequence was determined by alignment of the forward and reverse sequences and correction of automated base-call errors by visual inspection of the electropherograms.

3.2.4. Sequence alignment and phylogenetic analysis

Corrected partial LSU-rDNA sequences were aligned to comparable existing *Karenia* species sequences held by the authors or obtained from GenBank, and those from the related genera, *Karlodinium* J. Larsen and *Takayama* de Salas *et al.*, using ClustalX (Thompson *et al.* 1997). A range of taxa (Table 3.1) representing the gymnodinioid, peridinioid, prorocentroid and gonyaulacoid

Table 3.1: Origin (where known) and GenBank accession numbers of sequences used in phylogenetic analyses.

Sequence name	Origin	Accession number
Akashiwo sanguinea	Japan	AF260397
Alexandrium catenella	U.S.A.	AF200667
Ceratium fusus		AF260390
Gymnodinium aureolum	South Australia	AY263965
G. catenatum		AF200672
G. chlorophorum		AF200669
G. falcatum	South Australia	AY320049
G. fuscum		AF200676
G. impudicum		AF200674
G. microreticulatum	Eastern Australia	AY036078
G. nolleri		AF200673
G. palustre		AF260382
G. cf. placidum		AF260383
Heterocapsa rotundata		AF260400
H. triquetra		AF260401
Karenia asterichroma	Tasmania	AY590123
K. bidigitata	New Zealand	U92251
K. brevis	U.S.A	AF200677
K. brevisulcata	New Zealand	AY243032
K. mikimotoi	U.K.	AF200678
K. mikimotoi	Japan	AF200681
K. papilionacea	Tasmania	AY590124
K. papilionacea	New Zealand	U92252
K. umbella	Triabunna, Tasmania	AY263962
K. umbella	Taranna, Tasmania	AY263963
Karlodinium micrum	Norway	AF200675
K. micrum	Western Australia	AY263964
Peridinium cinctum		AF260394
P. pseudolaeve		AF260395
Prorocentrum micans		AF260377
P. rhathymum		AF260378
Scrippsiella trochoidea		AF260393
Takayama helix	Tasmania	AY284950
Takayama sp.	New Zealand	U92254
T. tasmanica	Tasmania	AY284948
Woloszynskia pseudopalustris		AF260402

lineages were also included in the alignments, with *Alexandrium catenella, A. tamarense, Ceratium fusus* and *C. tripos* as the outgroup. Phylogenetic analyses using minimum evolution, maximum parsimony and likelihood approaches were carried out using PAUP 4.0* (Swofford 1999) and MrBayes (Huelsenbeck & Ronquist 2001). Bases were treated either as equal, unweighted characters, or with transitions having twice the weight of transversions. Analyses were carried out both with and without the addition of a binary character matrix coding for inserts and deletions, and including or excluding the variable domains (equivalent to bases 387-492 and 556-675 of the *K. asterichroma* sequence, accession number AY590123). Assessment of support for branches in trees from distance and parsimony analyses was estimated with bootstrap using 1000 replicates. For MP analysis, starting trees were obtained via simple stepwise addition, and a tree-bisection-reconnection branch swapping algorithm was used. Alternatively Bayesian analysis was carried out using MrBayes (Huelsenbeck & Ronquist 2001), with the following parameters: 2.5 million generations, 4by4 nucleotide substitution, general time-reversible model, with gamma distributed among-site rate variation, sampled every 1000 generations, with the consensus tree drawn using the last 2000 trees.

3.3. OBSERVATIONS AND RESULTS

Karenia asterichroma de Salas, Bolch & Hallegraeff sp. nov.

Figures 3.2 – 3.11

DIAGNOSIS: Cellulae dorsiventraliter complanatae, 30-40 µm longae, 23-42 µm latae, 17-25 µm crassae. Epiconus acutus vel truncatus, lateribus concavis sed interdum rotundatis. Hypoconus saepe truncatus et sulco incisus, sed interdum semicircularis. Cingulum profunde incisum marginibus elevatis, per circiter 1/6 longitudinis cellulae dislocatum. Sulcus latus, in epiconum profunde extendens sub forma incisura truncatae. Canalis apicalis rectus, per circiter ½ longitudinis epiconi dorsalis descendens. Nucleus parvus sphaericus in centro epiconi. Chloroplasti 10-20, elongati, stellatim radiantes e corpore rotundato in hypocono.

Dorsoventrally flattened cells, 30 – 40 μm long (average 34.7 ± 2.8 μm), 32 – 42 μm wide (average 35.2 ± 3.9 μm), 17-25 μm thick (average 21.6 ± 2.3 μm). Epicone acute to truncated, with concave sides but sometimes rounded. Hypocone often truncated and incised by the sulcus, but sometimes semicircular in outline. Cingulum deeply incised, with raised margins, displaced approximately 1/6 of the cell length. Sulcus wide, extending deeply into the epicone as a truncated indentation. Apical groove straight, and extending approximately 1/2 of the way down the dorsal epicone. Nucleus small and spherical, in the center of the epicone. Chloroplasts 10 – 20, elongated, radiating in a star shape from a round body in the hypocone.

HOLOTYPE: Figure 3.11, a schematic representation of typical cell shapes from laboratory cultures and field samples.

ETYMOLOGY: named *asterichroma* (Greek asteris – star and chroma – colour) after the distinctive radiating chloroplast arrangement evident in live cells.

DISTRIBUTION: Cells have been observed in field samples from the Tasman Peninsula, Tasmania, Australia (Fig. 3.1), off Eaglehawk Neck, Port Arthur and Parsons Bay.

DESCRIPTION: *Karenia asterichroma* is a medium to large unarmoured dinoflagellate (see comparison with other morphologically similar *Karenia* species, Table 3.2). Under the light microscope, cells of *K. asterichroma* appear large and approximately pentagonal or sometimes hexagonal. They are clearly dorsoventrally flattened, and have a deeply incised cingulum that often has prominently raised margins (Fig. 3.2). The epicone is normally truncated and notched at the apex, with concave sides (Fig. 3.2). In stressed cells it can assume a more rounded shape. A linear apical groove extends from the right of the intercingular region of the sulcus (Fig. 3.3) to approximately half way down the dorsal epicone (Fig. 3.4). A wide but shallow swelling or carina is apparent in the ventral epicone surface (Fig. 3.3). The sulcus is narrow in the intercingular region, invading the epicone as a shallow, wide extension (Fig. 3.3). It is wider in the hypocone than in the intercingular region. The hypocone is normally truncated and incised by the sulcus (Figs 3.2, 3.5-3.7), but can become rounded when cells are under stress. The right hypocone lobe is often

longer than the left (Figs 3.2, 3.5-3.7), but not always so. A variable number (10-20) of elongated, tear-drop or sometimes wedge-shaped chloroplasts radiate from a putative pyrenoid near the center of the cell (Figs 3.5-3.7). The spherical nucleus is surrounded by a bi-layered envelope, is situated near the centre of the epicone, and is small relative to the overall cell size (Figs 3.2 and 3.5).

Under the scanning electron microscope, the epicone can be acute or rounded, and an amphiesmal vesicle pattern can be discerned, as can the shallow wide sulcal extension into the epicone. The deeply incised apical groove (Figs 3.8 and 3.9) is long and positioned on a raised area or carina (Fig. 3.9). It extends from a point approximately level with the top of the sulcal extension (Fig. 3.8), passes directly over the cell apex, and extends approximately half-way down the dorsal epicone (Fig. 3.10). The deeply incised cingulum with raised edges can be seen in Figs 3.8-3.10, and an amphiesmal vesicle pattern can be seen in Fig. 3.10.

A diagrammatic representation of a typical cell of *K. asterichroma*, in comparison to morphologically similar *K. bicuneiformis*, *K. brevis*, and *K. mikimotoi* (Miyake & Kominami *ex* Oda) G. Hansen & Moestrup, is provided in Figs 3.11-3.14. Non-flattened species like *K. umbella, K. longicanalis* and *K. digitata*, as well as very small species (*K. brevisulcata*) were not included in this comparison as there is little potential for confusion with *K. asterichroma*. *K. bidigitata* was not included as its morphology and size are indistinguishable from *K. bicuneiformis*.

MOLECULAR SYSTEMATICS: The LSU rDNA sequence of *K. asterichroma* differs by 3.9% from *K. bidigitata*, by 4.5% from *K. brevis* and 5.5%, from *K. mikimotoi*. Divergence from the other fucoxanthin-containing dinoflagellate genera is much higher, differing by 17.4% from *Karlodinium micrum* (Leadbeater & Dodge) J. Larsen, and 13.1% from *Takayama tasmanica* de Salas *et al.* Maximum parsimony (MP) heuristic analysis comparing *K. asterichroma* with fucoxanthin- and peridinin- containing gymnodinioids and, using an outgroup composed of *Alexandrium* and *Ceratium*, resulted in 5 equally parsimonious trees of length 2243. These trees differed in the

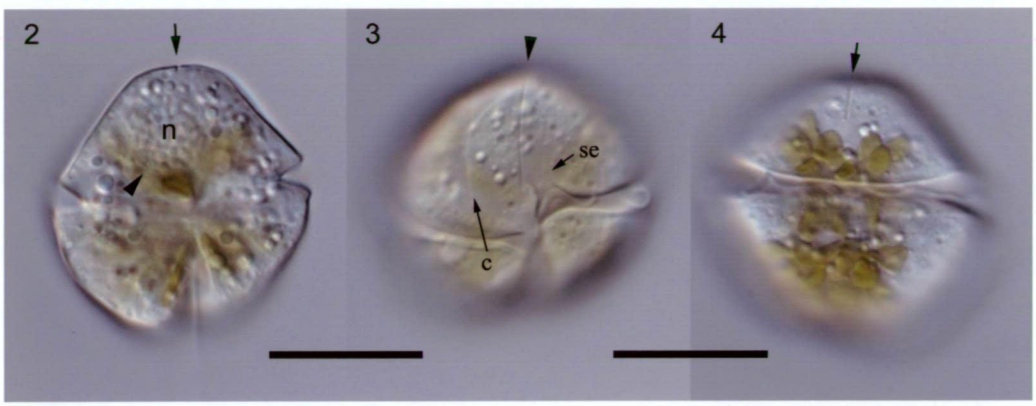
Table 3.2: Comparisons between *Karenia asterichroma*, and other *Karenia* species of similar morphology.

Parameters \ Species	***Karenia asterichroma***	***K. bicuneiformis*** [I]	***K. brevis*** [II]	***K. mikimotoi*** [III]
Cell length µm	30 – 40 (34.77, $n = 20$)	34 – 39 (36.25)	18 – 40	18 – 37
Cell width µm	30 – 42 (35.23, $n = 20$)	31 – 36 (33.83)	15 – 70	14 – 35
Cell thickness µm	17 – 25 (21.59, $n = 20$)	~ 5	flattened – no data	1/3 – 3/4 of width
Sulcus extension	long & wide, truncated	present	shallow and short	small, wedge-shaped
Apical groove	long, ½ down dorsal side	extending briefly onto dorsal surface	short on dorsal side	linear, short, thick, 1/3 down dorsal side
Carina	wide and shallow	absent	pronounced, nose-like	absent
Nucleus	spherical, near cell apex	oval, in left hypocone	round, in left hypocone	ellipsoidal or reniform, left sided
Chloroplasts	10-20, elongated, radiating from central body.	variable, disc-shaped when fixed.	peripheral	shapeless, 10-20

[I] Botes *et al.* (2003)

[II] Takayama (1990)

[III] Takayama (1984)

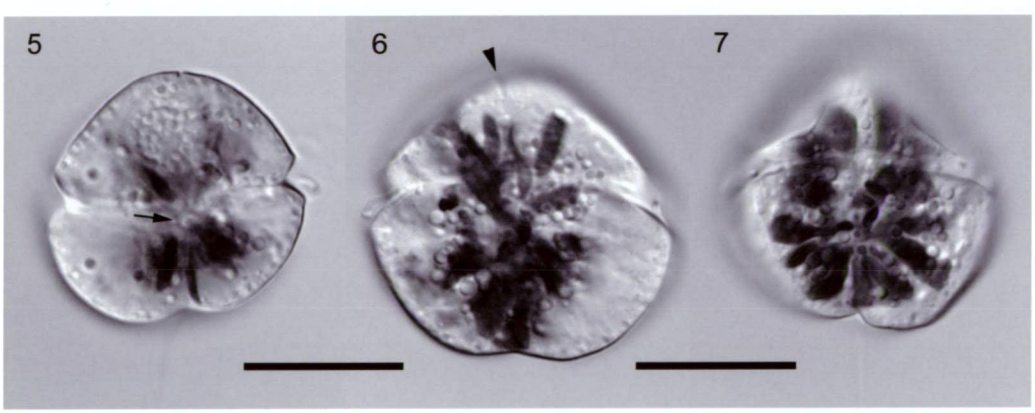


Figures 3.2-3.4: Light micrographs of *Karenia asterichroma* from Parsons Bay, Tasmania.

Fig. 3.2: Central focus of cell in ventral view. Note notch made by the apical groove (arrow), spherical anterior nucleus (n) bordered by thick bilayered envelope (arrowhead), and longer right hypocone lobe.

Fig. 3.3: Ventral view of cell in surface focus. Note apical groove (arrowhead), groove at the edge of the shallow swelling or carina (c) and sulcal extension into epicone (se).

Fig. 3.4: Dorsal view of cell in surface focus. Note dorsal extent of apical groove (arrow). Scale bars = 20 μm.

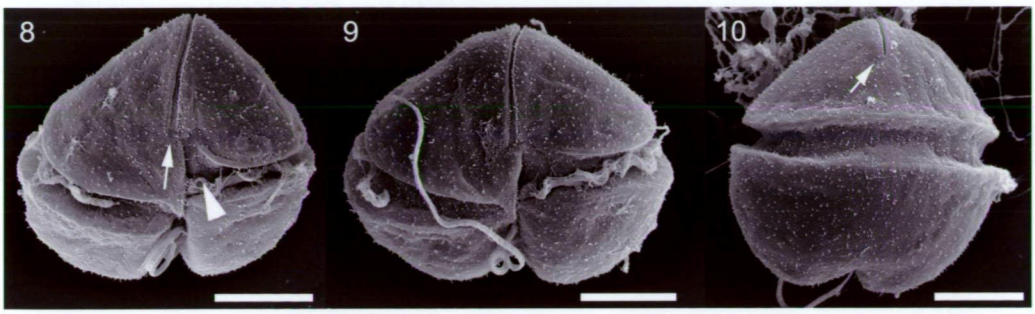


Figures 3.5-3.7: Light micrographs of *Karenia asterichroma* taken with a blue filter to enhance chloroplast contrast.

Fig. 3.5: Ventral view of cell in central focus. Note spherical putative pyrenoid (arrow), at the centre of chloroplast radiation.

Fig. 3.6: Dorsal view of cell in subsurface focus. Note apical groove (arrowhead), and chloroplast arrangement.

Fig. 3.7: Dorsal view of another cell in subsurface focus. Note recurring radial arrangement, but different chloroplast shape with wider distal ends. Scale bars = 20 μm.



Figures 3.8-3.10: Scanning electron micrographs of *Karenia asterichroma.*

Fig. 3.8: ventral view of cell with a straight and deeply incised apical groove (arrow), and a well developed, wide extension of the sulcus into the epicone (arrowhead).

Fig. 3.9: ventral view of another cell showing a swelling on the ventral epicone.

Fig. 3.10: Dorsal view of cell showing extent of apical groove on dorsal epicone (arrow), and deeply incised cingulum with raised margins (arrowheads). Scale bars = 10 μm.

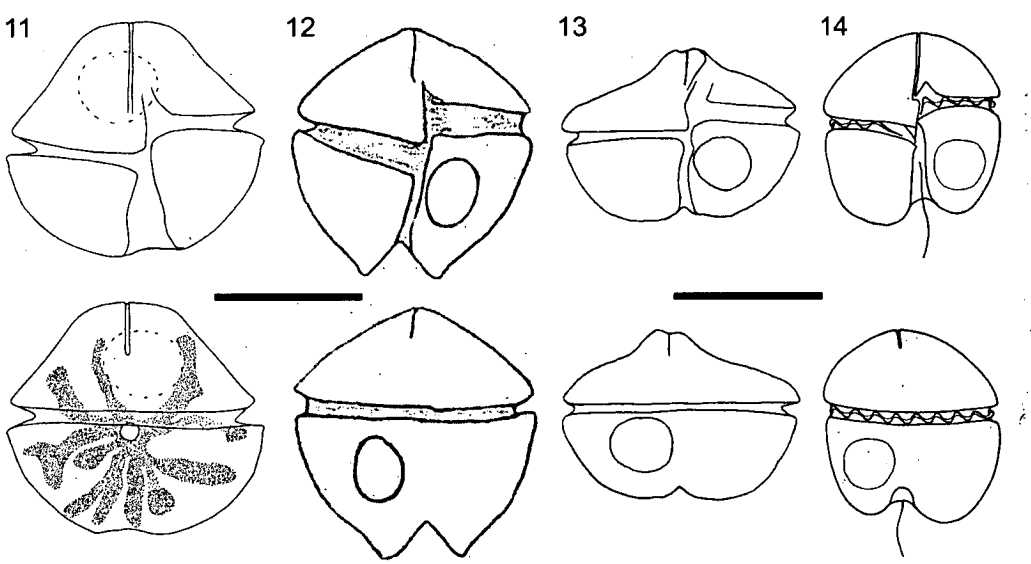
placement of taxa in the basal position within *Karenia*: three placed *K. papilionacea* as the most basal *Karenia*, whereas the remaining two placed *K. umbella* in this position. As bootstrap analysis showed slightly higher support for trees with *K. papilionacea* in the basal position, the MP tree with this topology is shown in Fig. 3.15. Bootstrap values from 1000 MP replicates are shown at the branch points of those branches not collapsed in the bootstrap consensus tree. MP analysis excluding the variable domains in D2 and D3 adequately resolved the major groups, but produced lower bootstrap support values within genera. Using equal weights for all characters produced the same branch order as analyses using different weights for transitions and transversions. Similarly, the addition of a binary character matrix to represent insertions and deletions did not change tree topology, and only slightly altered branch lengths. The results of these analysis are not shown, as they are not significantly different to Figure 3.15. Phylogenetic analyses using minimum evolution (distance), maximum likelihood (ML) and maximum parsimony (MP) criteria, consistently placed *Karenia asterichroma* within the *Karenia* clade, always paired with *K. bidigitata*, although bootstrap support for the *K. asterichroma / bidigitata* clade was low (54%). The genus *Karenia* was maintained as a monophyletic group with high bootstrap support (100%) in all analyses. The log-likelihood of the trees produced by Bayesian analysis stabilised after 15000 generations. A majority-rule consensus tree generated from the final 2000 trees showed the same branch order and topology to the

MP bootstrap consensus tree (Fig. 3.15), therefore the results of the Bayesian analysis are not shown.

3.4. DISCUSSION

Karenia asterichroma is morphologically somewhat similar to both *K. brevis* and *K. mikimotoi* in the degree of flattening of the cells, colour, and general cell shape, however, *K. brevis* and *K. mikimotoi* both have a nucleus located in the left hypocone (Steidinger *et al.* 1978; Takayama & Adachi 1984) compared to the anterior-central, nucleus of *K. asterichroma* (Fig. 3.2).

K. bidigitata is the most closely related species in terms of LSU-rDNA sequences, however it has a nucleus located in the lower left hypocone and is dorsoventrally flattened to a much greater extent than *K. asterichroma* (Botes



Figures 3.11 – 3.14: Diagrammatic comparisons of *Karenia asterichroma* with other *Karenia* species.

Fig. 3.11: Ventral and dorsal surface detail of *K. asterichroma*. Note chloroplast arrangement typical of this species.

Fig. 3.12: *K. bicuneiformis* (from Botes *et al.* 2003). Note position of the nucleus in comparison with *K. asterichroma.*

Fig. 3.13: *K. brevis* CCMP718. Note apical carina and position of the nucleus.

Fig. 3.14: *K. mikimotoi* (from Takayama & Adachi 1984). Note average size differences. Scale bars = 20 μm.

et al. 2003). Both *K. brevis* and *K. mikimotoi* contain numerous peripheral chloroplasts with individual pyrenoids (Steidinger *et al.* 1978; Takayama & Adachi 1984), which are clearly different from the radiating chloroplasts of *K. asterichroma* (Figs 3.5-3.7). The chloroplasts of *K. asterichroma* radiate from a small body located just posterior to the nucleus (Fig. 3.5), which appears to be a centrally located pyrenoid. Such an arrangement has not been described before in the genus *Karenia*, or the closely related *Karlodinium* (Daugbjerg *et al.* 2000), however, at least one species from a closely related genus, *Takayama tasmanica*, has a similar arrangement (Chapter 4; de Salas *et al.* 2003). The chloroplast morphology of *K. bicuneiformis* is not detailed in its description (Botes *et al.* 2003), however, the description of *K. bidigitata* (Haywood *et al.* 2004) refers to chloroplasts that are peripheral or evenly distributed throughout the cell.

K. asterichroma, K. bicuneiformis and *K. bidigitata* all have a distinctive hypocone (rounded in *K. asterichroma*, Fig. 3.11, but W-shaped in *K. bicuneiformis,* and *K. bidigitata,* Fig. 3.12) and epicone (rounded and concave-sided in *K. asterichroma*, Fig. 3.11, and conical in *K. bicuneiformis,* and *K. bidigitata,* Fig. 3.12), features that make them easily identifiable. The size of *K. asterichroma* is significantly larger than the average sizes of *K. brevis* or *K. mikimotoi* (Takayama & Adachi 1984; Takayama 1990). However, the average size of *K. asterichroma* falls within the range of sizes exhibited by these species. In comparison, *K. bicuneiformis,* and *K. bidigitata* are slightly larger than *K. asterichroma* (Botes *et al.* 2003). Given the wide variation in size of dinoflagellates within wild populations (Chang 1999; Hansen *et al.* 2000) and even within our clonal cultures, small variations in size alone are of little taxonomic value. We are confident, however, that the much larger average size of *K. asterichroma* in comparison with flattened *K. brevis*, *K. brevisulcata* and *K mikimotoi* is of help when trying to discriminate between these species. The cingulum of *K. asterichroma* is distinctive, very deeply incised, and often has raised edges (Fig. 3.2). The shallow, wide swelling on the ventral epicone of *K. asterichroma* (Fig. 3.3) may be homologous to the carina that is present in a much more pronounced form in *K. brevis* (Steidinger & Joyce 1973; Takayama 1981), and to an lesser extent

in *K. papilionacea* (Haywood *et al.* 2004). This structure has not been described in other species of the genus *Karenia,* although the apical crest of *K. cristata* (Botes *et al.* 2003) may also be homologous to the carina of *K. brevis*.

The wide sulcal extension of *K. asterichroma* (Fig. 3.3) resembles that of *K. brevis* (Takayama 1981), and *K. papilionacea* (Haywood *et al.* 2004) but is more pronounced. By comparison, *K. bicuneiformis* and *K. bidigitat,* both have a narrow, pointed sulcal extension (Botes *et al.* 2003). The apical groove of *K. asterichroma*, which extends on the epicone along most of the length of the ventral side (Fig. 3.3) to approximately half of the length of the dorsal side (Figs 3.4, 3.10, 3.11) is longer than in *K. bicuneiformis* and *K. bidigitata* (Fig. 3.12), *K. brevis* (Fig. 3.13), *K. mikimotoi* (Fig. 3.14), *K. brevisulcata* (Chang 1999), *K. digitata* (Yang *et al.* 2000) and *K. papilionacea* (Haywood *et al.* 2004), all of which have grooves that only extend a short distance down the dorsal epicone. However, the apical groove is shorter than in species such as *K. longicanalis* (Yang *et al.* 2001) and *K. umbella* (Chapter 2; de Salas *et al.* 2004), in which the dorsal extension is closer to two thirds of the epicone surface.

The two recently described species *K. bicuneiformis* Botes *et al.* and *K. bidigitata* Haywood *et al.* are morphologically indistinguishable from each other and distinct from other *Karenia* species (Botes *et al.* 2003; Haywood *et al.* 2004), strongly suggesting their synonymity. However, deffinitive data demonstrating that the New Zealand *K. bidigitata* sequence (GenBank U92251) was derived from a species synonymous with South African *K. bicuneiformis* is not yet available, Until the synonymy of these two species can be clarified, we refer to the New Zealand sequence used in genetic comparisons as *K. bidigitata.*

The increasing number of *Karenia* species and LSU-rDNA data available for comparison make it possible to better understand the taxonomy and phylogeny of this genus. The phylogeny of *Karenia* species illustrated in Fig. 3.15 includes several sequences not previously analysed together and supports the monophyletic status of *Karenia* and the fucoxanthin-containing genera. The close relationship of the genera *Karenia, Karlodinium* and *Takayama* is

evidenced not only by their close LSU rDNA sequences, but also their similar pigment profiles, as all species so far analysed contain fucoxanthin and its derivatives as their main carotenoids (Björnland & Tangen 1979; Hansen *et al.* 2000; Botes *et al.* 2003; de Salas *et al.* 2003; de Salas *et al.* 2004). The genus *Karenia* is restricted to species whose major carotenoid is fucoxanthin or its derivatives, rather than peridinin (Daugbjerg *et al.* 2000). Due to the slow growth rate and low cell maximum density of *K. asterichroma* cultures we have not yet been able to obtain enough material for pigment analysis and therefore have relied on the clear morphological affinities with others in the genus, such as *K. brevis*, *K. mikimotoi* (Takayama 1981; Takayama & Adachi 1984; Takayama 1985), *K. bidigitata* (Haywood *et al.* 2004) and *K. bicuneiformis* (Botes *et al.* 2003). Our generic assignment of this new species is strongly supported by phylogenetic analysis of partial sequences of the LSU gene, therefore we feel that our decision to place *K. asterichroma* within *Karenia* is well justified.

While molecular data is of increasing importance in dinoflagellate systematics, we believe that calls for taxonomy of all organisms to be based exclusively on molecular data (Tautz *et al.* 2003) are highly premature. Such an approach is likely to be misleading and prone to over- or under-estimation of the taxonomic complexity, especially within groups of organisms where the level of molecular divergence and diversity is poorly characterised. The value of morphological, pigment, toxin composition and ultrastructural data should not be discounted and we support the retention of morphological characters (Dunn 2003) as part of a polyphasic approach to taxonomy of dinoflagellates.

The on-site recognition of *Karenia* species by aquaculture staff is important for the management and mitigation of impacts of blooms on caged aquaculture stock. In addition, *Karenia* species are typically fragile and field samples often do not survive transport to specialist laboratories, or survive standard fixatives without significant loss of cells or changes to cell shape and structure. Aquaculture facilities must therefore continue to rely on microscopic examination for routine detection and monitoring. For these reasons alone, we feel that characterisation of species should still include

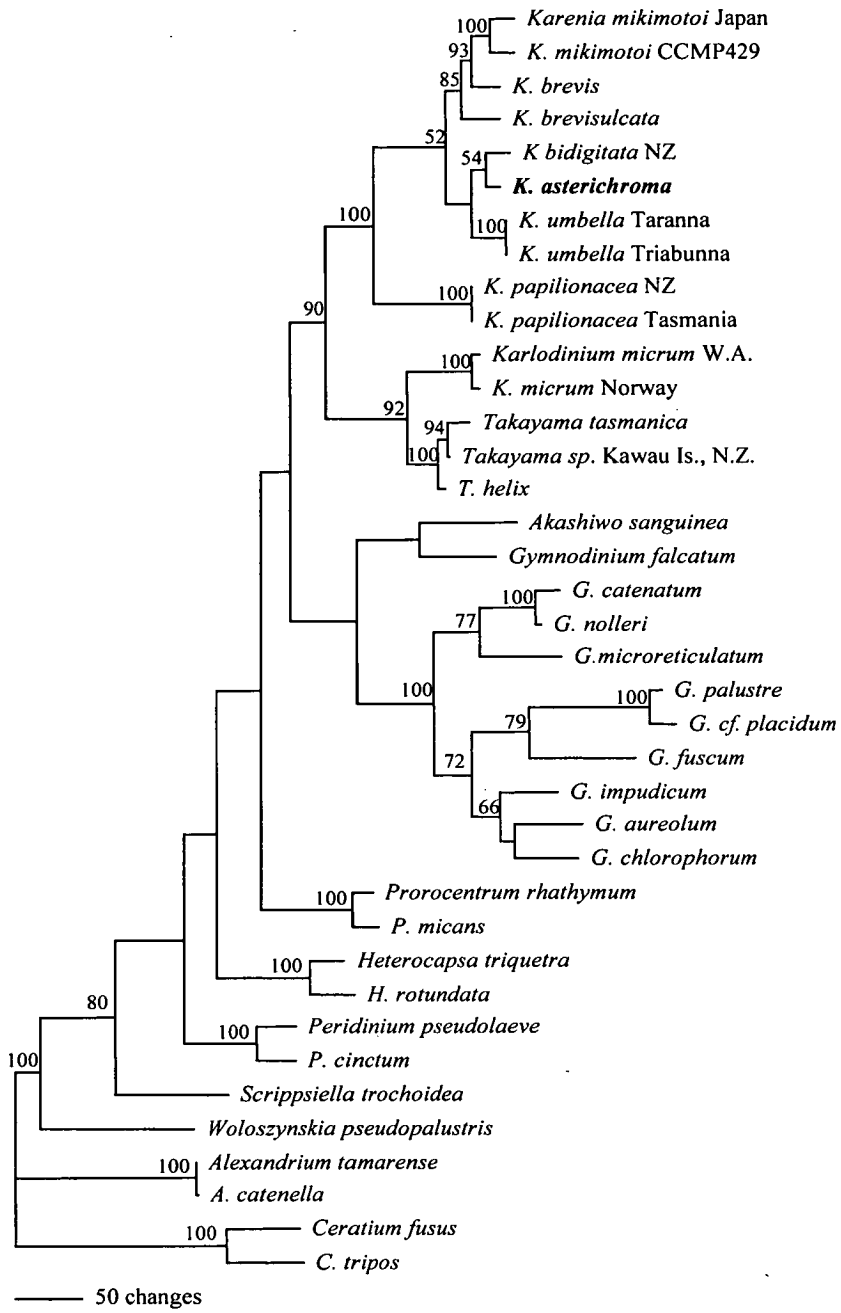


Figure 3.15: Proposed phylogeny derived of MP analysis of *Karenia*, *Karlodinium* and *Takayama* sequences, with representatives from the main gymnodinioid, prorocentroid, peridinioid and gonyaulacoid groups. Outgroup was composed of *Alexandrium catenella, A. tamarense, Ceratium fusus* and *C. tripos*. Tree was one of 5 equally parsimonious trees, of length = 2243, consistency index = 0.514, and retention index = 0.671. Support values were derived from a bootstrap analysis of 1000 replicates using maximum parsimony as the search criterion. Minimum evolution and Bayesian analyses produced the same ingroup topology.

morphology, but ideally be supported by other data such as sequencing of ribosomal RNA genes. Such detailed characterisation then forms an ideal base for the development of additional detection an differentiation techniques based on biochemical or molecular techniques.

3.5. ACKNOWLEDGEMENTS

The authors would like to thank Australian Biological Resources Study (ABRS) for partially funding this work. We would like to thank Dr. Judith-Anne Marshall and Tassal Ltd. (Nubeena, TAS) for organising collection and delivery of various field samples during the May 2003 *Karenia* bloom. We would also like to thank Lincoln Mackenzie and the Cawthron Institute (Nelson, New Zealand), for providing a comparison culture of *Karenia brevisulcata*. Finally we would like to thank Geraldine Nash, of the Australian Antarctic Division, for assistance with scanning electron microscopy.

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CHAPTER 4:

TAKAYAMA GEN. NOV. (GYMNODINIALES, DINOPHYCEAE), A NEW GENUS OF UNARMOURED DINOFLAGELLATES WITH SIGMOID APICAL GROOVES, INCLUDING THE DESCRIPTION OF TWO NEW SPECIES[1]

Miguel Félix de Salas, Christopher J. S. Bolch, Lizeth Botes, Geraldine Nash,

Simon W. Wright, and Gustaaf M. Hallegraeff [2]

ABSTRACT: A new, potentially ichthyotoxic genus, *Takayama* de Salas, Bolch, Botes & Hallegraeff gen. nov. is described with two new species isolated from Tasmanian (Australia) and South African coastal waters: *T. tasmanica* de Salas, Bolch & Hallegraeff, sp. nov., and *T. helix*, de Salas, Bolch, Botes & Hallegraeff, sp. nov. The genus and two species are characterised by light and electron microscopy of field samples and laboratory cultures, as well as large subunit ribosomal rDNA sequences and HPLC pigment analyses of several cultured strains. The new *Takayama* species have sigmoid apical grooves and contain fucoxanthin and its derivatives as the main accessory pigments. *T. tasmanica* is similar to the previously described species *Gymnodinium pulchellum* Larsen, *Gyrodinium acrotrochum* Larsen and *G. cladochroma* Larsen in its external morphology, but differs from these in having two ventral pores, a large, horseshoe-shaped nucleus and a central pyrenoid with radiating chloroplasts that pass through the nucleus. It contains gyroxanthin-diester and a gyroxanthin- like accessory pigment, both of which are missing in *T. helix*. *Takayama helix* has an apical groove that is nearly straight while still being clearly inflected. A ventral pore or slit is present. It has numerous peripheral, strap-shaped, and spiralling chloroplasts with individual pyrenoids, and a solid ellipsoidal nucleus. The genus *Takayama* has close affinities to the genera *Karenia* and *Karlodinium*.

[1] **de Salas M.F., Bolch C.J.S., Botes L., Nash G., Wright S.W. & Hallegraeff G.M.** (2004) *Takayama* (Gymnodiniales, Dinophyceae) gen. nov., a new genus of unarmoured dinoflagellates with sigmoid apical grooves, including the description of two new species. *Journal of Phycology* **39: 1233-1246.**

4.1. INTRODUCTION

Fish-killing, unarmoured, gymnodinioid dinoflagellates with sigmoid apical grooves have been reported since the 1980s, as *Gymnodinium* type-'84 K (Onoue *et al.* 1985) and as *Gymnodinium* sp. 1 (Takayama 1985). The first species described with this character was *Gymnodinium pulchellum* Larsen (1994), from a Port Phillip Bay field sample, which was considered to be most likely identical to the ichthyotoxic taxa of Takayama's (1985), and Onoue *et al.* (1985). Fish mortalities have been attributed to *Gymnodinium pulchellum* –like species in Australia (Larsen 1994; Hallegraeff 2002), Japan (Onoue *et al.* 1985), and the U.S.A. (Steidinger *et al.* 1998). Two new species morphologically similar to G. *pulchellum* were also described by Larsen in 1996 from field samples collected in Port Phillip Bay as *Gyrodinium acrotrochum* Larsen and *Gyrodinium cladochroma* Larsen.

Until recently, unarmoured dinoflagellate taxonomy was based exclusively on morphological and cytological features, such as set out by Kofoid and Swezy (1921). However, a revision by Daugbjerg *et al.* (2000) combined large subunit (LSU) ribosomal DNA (rDNA) sequences, ultrastructural characters and chloroplast pigment composition to divide the large heterogeneous genus *Gymnodinium* (*sensu lato*) into four genera: *Gymnodinium sensu stricto*, with a horseshoe shaped apical groove and peridinin as the main carotenoid; *Akashiwo,* with a clockwise spiral apical groove and peridinin; *Karenia,* with a straight apical groove and fucoxanthin; and *Karlodinium,* with a short, straight apical groove, a ventral pore, and fucoxanthin. However, the status of gymnodinioids with sigmoid grooves such as *Gymnodinium pulchellum* was not resolved, as no cultures of *G. pulchellum* -like organisms were available at the time.

We have recently isolated *G. pulchellum*-like species from several locations in southern Australia and South Africa (Fig. 4.1) and established cultures of two species with sigmoid grooves. Based on morphological, ultrastructural and pigment analyses and LSU rDNA sequences we create a new genus: *Takayama* de Salas, Bolch, Botes & Hallegraeff gen. nov. for these two new species: *Takayama tasmanica* de Salas, Bolch & Hallegraeff sp. nov., and *Takayama helix* de Salas, Bolch, Botes & Hallegraeff sp. nov. We also

transfer *Gymnodinium pulchellum*, *Gyrodinium acrotrochum* and *G. cladochroma* to the genus *Takayama*.

4.2. MATERIALS AND METHODS

4.2.1. Culture conditions

Australian cells of *T. tasmanica* and *T. helix* were isolated from plankton net (20 µm mesh) samples collected in the Derwent estuary and in North West Bay, in south-eastern Tasmania (Fig. 4.1). Single cells were isolated with a micropipette into 28‰ GSe medium (Blackburn *et al.* 1989). Cultures were maintained in this medium, at 17°C, with a 12:12 L:D photoperiod of 100 µmol m^{-2} s^{-1}, supplied by cool white fluorescent lights. Cultures used in this study are detailed in Table 4.1. South African *T. helix* was isolated from False Bay at Gordon's Bay, in south-western South Africa. (Fig 4.1) in the same manner as the Australian isolates but were maintained in F_2 medium (Guillard and Ryther 1962) at 18°C.

4.2.2. Light microscopy

Live cells of *T. tasmanica* and *T. helix* were examined and photographed with bright field and differential interference contrast using a Zeiss Axioskop 2 Plus microscope (Zeiss, Gottingen, Germany) connected to a Canon Powershot G1 digital camera (Canon, Tokyo, Japan). Cell length, width, and degree of girdle displacement were measured on 50 individual cells in mid-logarithmic phase.

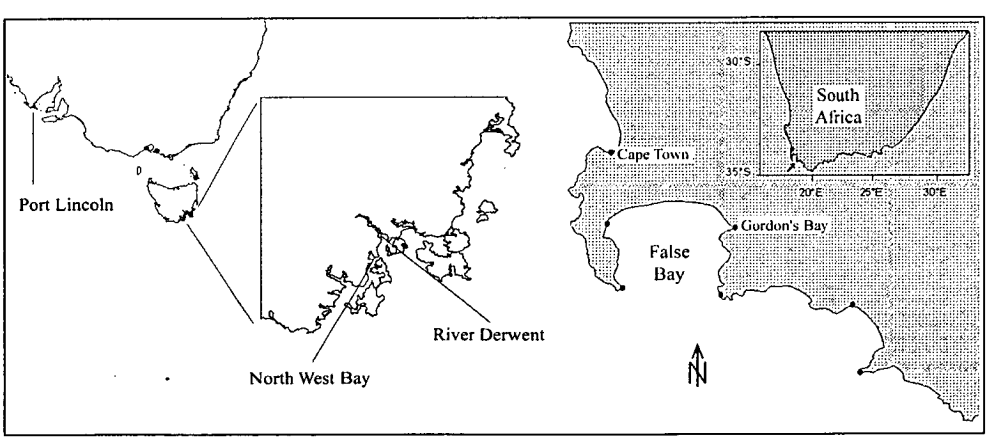


Fig. 4.1: Map of Australia and South Africa showing locations where *T. tasmanica* and *T. helix* have been isolated.

Table 4.1: Cultures and field samples used in this study.

	Field Samples			
Species	Locality		Date	
T. tasmanica	Port Arthur, Tasmania, Australia		17.10.2001	
T. tasmanica	St. Helens, Tasmania		24.5.2002	
T. tasmanica	Tuggerah Lakes, New South Wales, Australia		12.10.2002	
T. helix	River Derwent, Tasmania		19.3.2002	
T. helix	Port Arthur, Tasmania		17.10.2001	
T. helix	Port Lincoln, South Australia		5.4.2002	
	Cultures			
Species	Locality	Date	Isolated by	Strain code
T. tasmanica	River Derwent	7.2.2001	M. de Salas	TTDW01
T. tasmanica	River Derwent	3.5.2001	M. de Salas	TTDW03
T. helix	North West Bay, Tasmania	14.5.2001	M. de Salas	THNWB01
T. helix	North West Bay	14.5.2001	M. de Salas	THNWB02
T. helix	False Bay, Western Cape, South Africa	1998	L. Botes	CTCC19

4.2.3. Scanning electron microscopy

Cells of the Australian isolates of *T. tasmanica* and *T. helix* were fixed by addition of equal volumes of 4% osmium tetroxide (OsO_4) solution prepared in culture medium. Samples were mounted, critical point dried and sputter-coated as described in Chapter 2. Cells were observed using a JEOL 35C and a JSM 840 scanning electron microscope. Scanning electron micrographs of the South African isolate of *T. helix* were obtained according to Botes *et al.* (2002).

4.2.4. Transmission electron microscopy

Cells of *T. tasmanica* and *T. helix* were fixed for 1 hour in a solution containing 2% OsO_4 and 2.5% glutaraldehyde made in GSe culture medium. After rinsing twice in culture medium and twice in distilled water, cells were dehydrated in an ethanol-acetone series (10 %, 30%, 50%, 70%, 80%, 90%, 95% ethanol in double distilled water, 100% ethanol, and 2 steps in 100% dry acetone) and embedded in Spurr's resin. Sections of 60 nm thickness were taken using a Reichert Ultracut E microtome, mounted on Formvar coated

grids, stained with uranyl acetate (Hansen *et al.* 2000) and observed using an Phillips CM 100 transmission electron microscope.

4.2.5. DNA extraction, PCR amplification and sequencing

Cultures were grown to mid-logarithmic phase and approximately 10 ml pelleted by gentle centrifugation. Total DNA was extracted by the gentle lysis, phenol/chloroform method (Bolch *et al.*, 1998). Extracted DNA was used as a template to amplify a fragment of the large subunit ribosomal gene approximately 1400 base pairs long, using the primers D1R (Scholin *et al.* 1994) and 28:1483R (Daugbjerg *et al.* 2000). Details of reaction conditions and post-treatment can be found in Chapter 2. Primers D1R and D3Ca (Scholin *et al.* 1994) were used to determine the nucleotide sequence of approximately 900 bp of the amplified fragment. All sequence electropherograms were examined visually and base-calling errors corrected manually. Both forward and reverse sequences were aligned and conflicts resolved by manual inspection.

4.2.6. Sequence alignment and phylogenetic analyses

Sequences were aligned using ClustalX (Thompson *et al.* 1997), and alignments were refined by hand. Inserts and deletions were coded by construction of a character matrix in which gaps were coded as single insertion or deletion events regardless of length. Phylogenetic analysis was carried out using PAUP 4.0* (Swofford, 1999). An outgroup consisting of *Alexandrium catenella, A. tamarense, Ceratium fusus* and *C. tripos* was used in the analysis. Additionally, sequences of all main gymnodinioid genera previously dealt with in Daugbjerg *et al.* (2000), such as *Karenia*, *Karlodinium*, *Akashiwo*, *Woloszynskia*, as well as a cross section through freshwater and marine gymnodinioids and closely related genera such as *Heterocapsa*, *Scrippsiella*, *Peridinium*, and *Prorocentrum* were used in the analysis, to provide a thorough comparative framework. Distance, parsimony and likelihood were all used as criteria to construct trees, with no significant difference in the arrangement of ingroup species. Bases were treated either as equal, unweighted characters, or with transitions having twice the weight of

transversions, with no noticeable difference in the result. Bootstrap analyses were carried out (1000 replicates) with all three criteria.

4.2.7. Pigment composition

Approximately 10 ml of culture in mid-logarithmic phase were filtered gently onto 45-mm Gelman GF/F glass fibre filters and snap frozen in liquid nitrogen for storage. Pigments were extracted as described in Chapter 2, and analysed by the modified HPLC method of Zapata *et al.* (2000). Peaks were integrated using Waters Millennium software and identified by comparison of their retention times and spectra with those of mixed standards obtained from known cultures. A standard of gyroxanthin- diester (DHI Bioproducts, Denmark) was also compared, as this pigment is known from *Karenia* and *Karlodinium* species (Hansen *et al.*, 2000).

4.3. RESULTS

Takayama de Salas, Bolch, Botes & Hallegraeff gen. nov.

Dinoflagellata inarmata cum fucoxanthin aut oriundis ex fucoxanthin pro pigmentis principalibus accessoriisque. Canalis apicalis sigmoides.

Unarmoured dinoflagellates with fucoxanthin or its derivatives as the major accessory pigments. Sigmoid apical groove.

ETYMOLOGY: Named after Dr. Haruyoshi Takayama, whose work first drew attention to the importance of apical grooves in unarmoured dinoflagellate taxonomy.

TYPE SPECIES: *Takayama tasmanica* de Salas, Bolch & Hallegraeff, sp. nov.

OTHER SPECIES IN GENUS:

Takayama helix de Salas, Bolch, Botes & Hallegraeff sp. nov.

Takayama pulchella (Larsen) de Salas, Bolch & Hallegraeff comb. nov., [Basionym: *Gymnodinium pulchellum* (Larsen, 1994, Fig 58, page 32)]

Takayama acrotrocha (Larsen) de Salas, Bolch & Hallegraeff comb. nov., [Basionym: *Gyrodinium acrotrochum* (Larsen, 1996, Fig. 35, page 342)]

Takayama cladochroma (Larsen) de Salas, Bolch & Hallegraeff comb. nov.

[Basionym: *Gyrodinium cladochroma* (Larsen, 1996, Fig. 37, page 343)]

Takayama tasmanica de Salas, Bolch & Hallegraeff sp. nov.

Figs 4.2 – 4.15, 4.38, 4.39.

Cellulae obovatae, leniter dorsiventraliter complanatae, 16-27 μm longae, 14-26 μm latae, 10-20 μm crassae. Epiconus hemisphaericus. Hypoconus truncatus incisus. Cingulum profunde incisum, latum, per 1/4 longitudinis cellulae totius dislocatum. Sulcus in hypocono latior quam in regione intercingulari, in epiconum breviter invasus. Canalis apicalis sigmoideus, e puncto infra dexteram extensionis sulcalis ascendens, epiconum transcendens, apicem cellulae circumiens, et per quasi 2/3 longitudinis epiconi dorsalis descendens. Partem tubiformem regio intercingularis sulci exhibens. Chloroplasti 7-10, e pyrenoide centrali per nucleum radiantes et in peripherio ramificantes. Nucleus sine capsula, maximam partem epiconi implens, latera dorsum apicemque pyrenoidis cingens.

Cells obovate in outline, slightly dorsoventrally flattened, 16 – 27 μm long, 14 – 26 μm wide, 10 – 20 μm thick. Epicone hemispherical. Hypocone truncated and incised. Cingulum deeply excavated and wide, displaced about 25 % of total cell length. Sulcus wider in the hypocone than the intercingular region, extending shortly into the epicone. Apical groove sigmoid, extending from below and to the right of the sulcal extension, across the epicone and around the cell apex, to approximately 2/3 of the way down the dorsal

epicone. Tube like structure in the intercingular region of the sulcus. Chloroplasts 7 – 10, radiating from a central pyrenoid and through the nucleus, branching peripherally. Nucleus without a capsule, filling most of the epicone, surrounding the pyrenoid laterally, dorsally and apically.

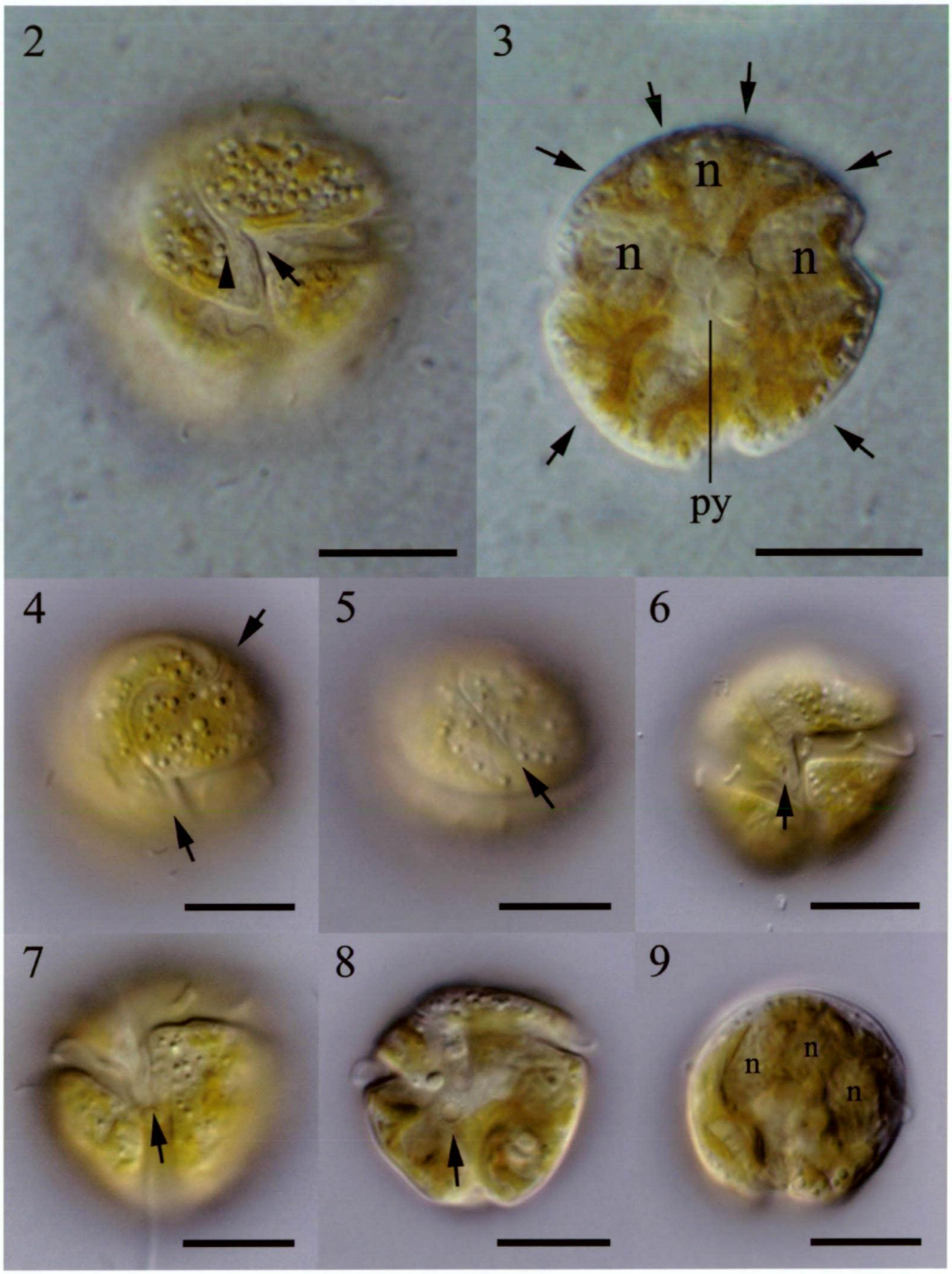
HOLOTYPE: Figs 38, 39 of culture TTDE01, isolated from the River Derwent, Tasmania, Australia. Culture deposited into the University of Tasmania Harmful Algae Culture Collection.

TYPE LOCALITY: Estuary of the River Derwent, Tasmania, Australia (Fig. 4.1)

ETYMOLOGY: named after the island of Tasmania, in south eastern Australia, where the species was first isolated.

DISTRIBUTION: North- and south-eastern Tasmania.

DESCRIPTION: *T. tasmanica* is a medium sized dinoflagellate. Measurements of 50 cells, compared with *T. helix, T. pulchella, T. acrotrocha* and *T. cladochroma* are given in Table 4.2. Under the light microscope the cell outline appears obovate to almost spherical (Figs 4.3, 4.6). The epicone is hemispherical, and comprises approximately 1/3 of the cell length. The hypocone is truncated and deeply incised by the sulcus. The sulcus itself is wide, but narrows in the intercingular region, and extends shortly into the epicone as a finger-like projection (Fig. 4.2). The cingulum is displaced approximately 1/4 of the total cell length. A sigmoid apical groove skirts around the apex of the cell (Figs 4.4, 4.5) and descends 2/3 of the length of the dorsal epicone, angled towards the right side of the cell (Fig. 4.5). A pore is visible below the ventral termination of the apical groove (Fig. 4.6). A tube-shaped structure is evident inside and along the sulcus (Fig. 4.7) Chloroplasts radiate from a central pyrenoid, through the nucleus, branching irregularly underneath the cell surface (Figs 4.3, 4.8). In stressed cells the chloroplasts become rod shaped and their number is more obvious. A spherical pusule is surrounded by sac or tear-drop shaped vesicles (Fig. 4.8). The nucleus is large and cup shaped (Fig. 4.9), and occupies most of the epicone. Chloroplasts pass through it as they radiate out of the central pyrenoid, which surrounds the nucleus apically, dorsally and laterally.



Figures 4.2-4.9: Light micrographs of *Takayama tasmanica*. Scale bars = 10 µm.

Fig. 4.2: Ventral surface view of cell showing sulcal extension into epicone (arrow) and indistinct origin of apical groove (arrowhead).

Fig. 4.3: Cross section focus of the same cell showing central pyrenoid (py) surrounded by nucleus (n), and irregular chloroplasts branching under the surface (arrows).

Fig. 4.4: Apical view showing shape and extent of sigmoid apical groove (arrows).

Fig. 4.5: Dorsally tilted apical view showing dorsal extent of apical groove (arrow)

Fig. 4.6: Ventral view of cell showing ventral pore or slit adjacent to the ventral termination of the apical groove (arrow).

Fig. 4.7: Ventral view of cell tilted towards antapex. Note tube-shaped structure within the sulcus (arrow).

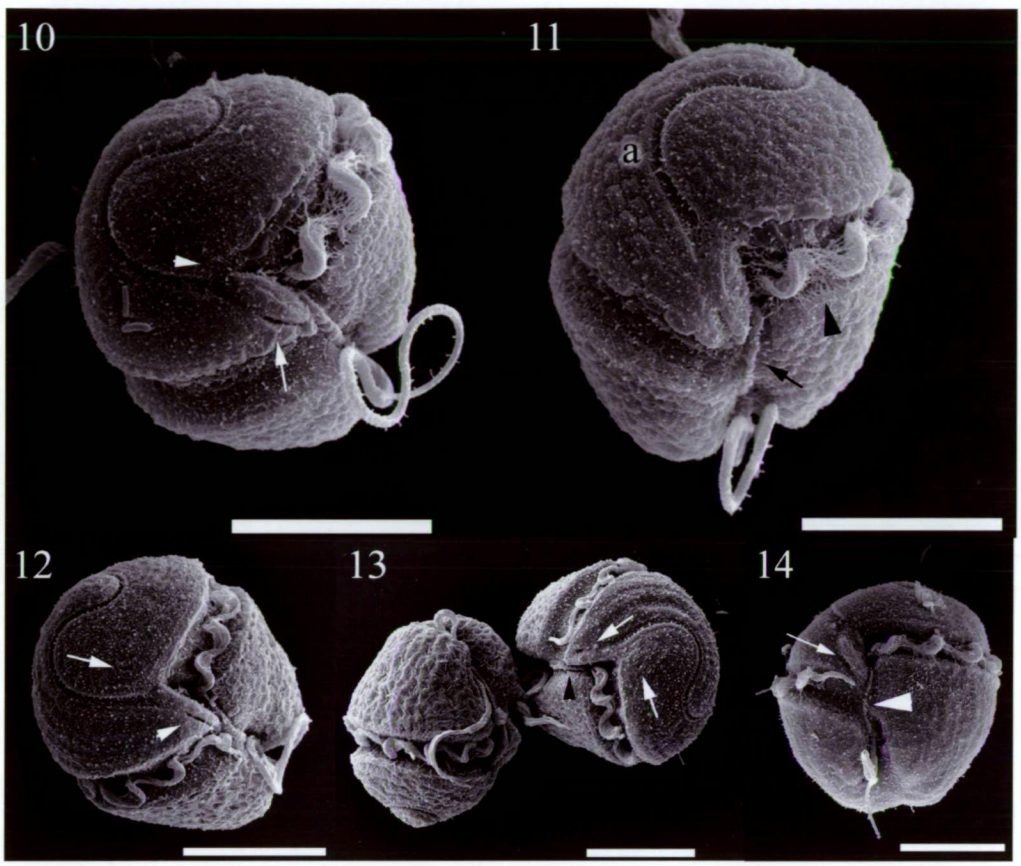
Fig. 4.8: Subsurface view of same cell showing spherical pusule (arrow) with tear-drop shaped vesicles.

Fig. 4.9: Subsurface apical view of cell showing dorsal distribution of the nucleus (n) around central pyrenoid.

Using SEM, the sigmoid apical groove can be seen to skirt around the cell apex, but does not pass directly over it (Figs 4.10, 4.11). The ventral termination of the apical groove becomes shallower (Fig. 4.10) and has no clearly defined starting point. The area that lies between the apical groove and the sulcus is swollen and rod-like (Figs 4.10-4.14). Often a slit, also visible as a pore, is present in the lower part of the apical groove (Figs 4.10, 4.12, 4.14). Another pore, situated in the left ventral epicone just above the sulcus (Figs 4.12, 4.13), is visible in some (but not all) cells. In well preserved samples, the apical groove appears as a cleft shaped incision into the cell surface (Figs 4.10-4.12). The cell surface itself is composed of amphiesmal vesicles that are rectangular in the sulcus and areas adjacent to the apical groove, but polygonal elsewhere (Figs 4.10, 4.11). The sulcus can be seen to intrude into the epicone at an angle (Figs 4.10, 4.14), but this is not obvious in all cells, as the intrusion is short and shallow. A tube-like appendage that occupies the intercingular region is visible in Figs 4.10 - 4.14.

Using TEM of ultra-thin sections, the central pyrenoid is revealed to be surrounded by a starch cap (Fig. 4.15). The shape of the nucleus, which lacks a nuclear capsule or envelope chambers, is illustrated in Fig 4. It surrounds the central pyrenoid anteriorly and laterally. The chloroplasts appear to be engulfed by the nucleus as they radiate to the cell surface.

PIGMENT COMPOSITION: The photosynthetic and accessory pigments of *T. tasmanica* (Fig 4.16) include chlorophyll *a* and chlorophylls c_2 and c_3; no chlorophyll c_1 was detected. The following carotenoids were identified, quantified as a percentage of chlorophyll *a*: 19'-butanoyloxyfucoxanthin (5.7%), fucoxanthin (75.8%), violaxanthin (1.8%), diadinoxanthin (20.9%), diatoxanthin (4.5%), a gyroxanthin– like pigment (11.5%), and gyroxanthin-diester (0.5%).



Figures 4.10-4.14. Scanning electron micrographs of *Takayama tasmanica.* Scale bars: 10 µm.

Fig. 4.10: Ventral view of cell showing sigmoid apical groove, sulcal intrusion into epicone (arrowhead), and pore or slit on cingular end of apical groove (arrow).

Fig. 4.11: Ventral view of another cell showing transverse flagellum (arrowhead), tube like structure in sulcus (arrow), and arrangement of amphiesmal vesicles in parallel rows to apical groove (a).

Fig. 4.12: Ventral view of cell showing slit in cingular end of apical groove (arrowhead) and ventral pore in epicone (arrow).

Fig. 4.13: View of pair of cells showing tube like structure in sulcus (arrowhead) and pair of ventral pores in same position as C (arrows).

Fig. 4.14 Ventral view of hypocone showing swollen flap between apical groove and sulcus, pore in cingular end of apical groove (arrow pointing to both), and tube-like structure located between the two flagellar insertion points (arrowhead).

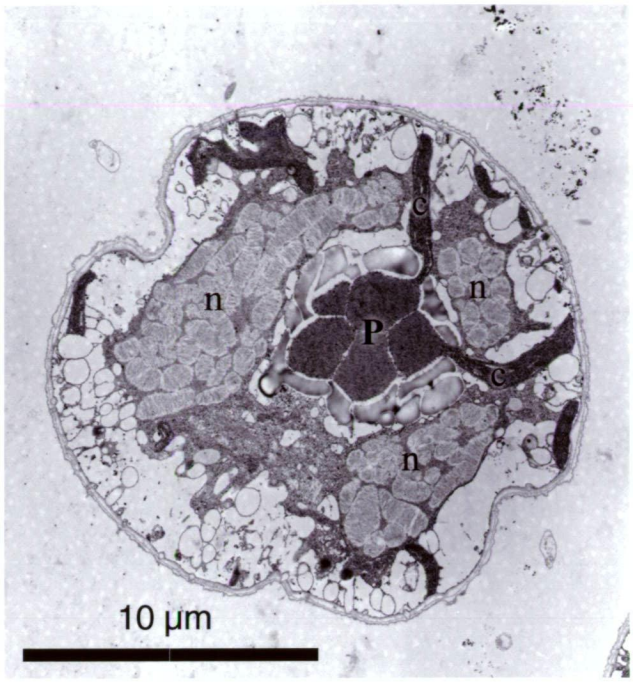


Fig. 4.15. TEM cross section of *Takayama tasmanica* (clonal culture TTDW01), showing central pyrenoid (p), enveloping nucleus (n), and chloroplasts passing through openings in the nucleus on their way to the cell periphery (c).

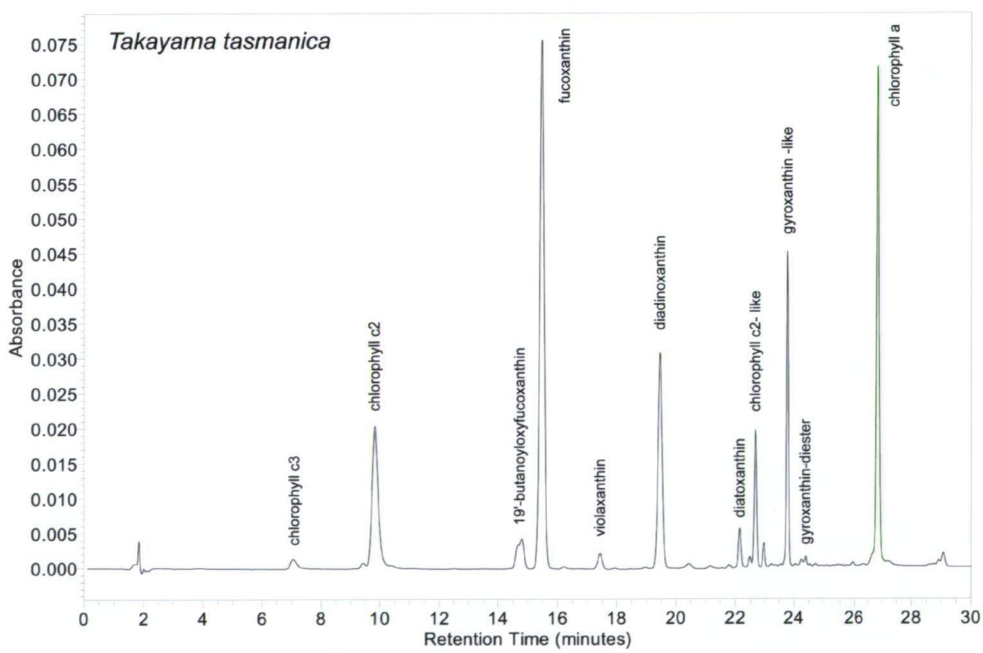


Figure 4.16: HPLC pigment chromatogram of *Takayama tasmanica* strain TTDE01, from the River Derwent, Tasmania, Australia. Note the presence of large amounts of a gyroxanthin-like pigment and trace amounts of gyroxanthin-diester.

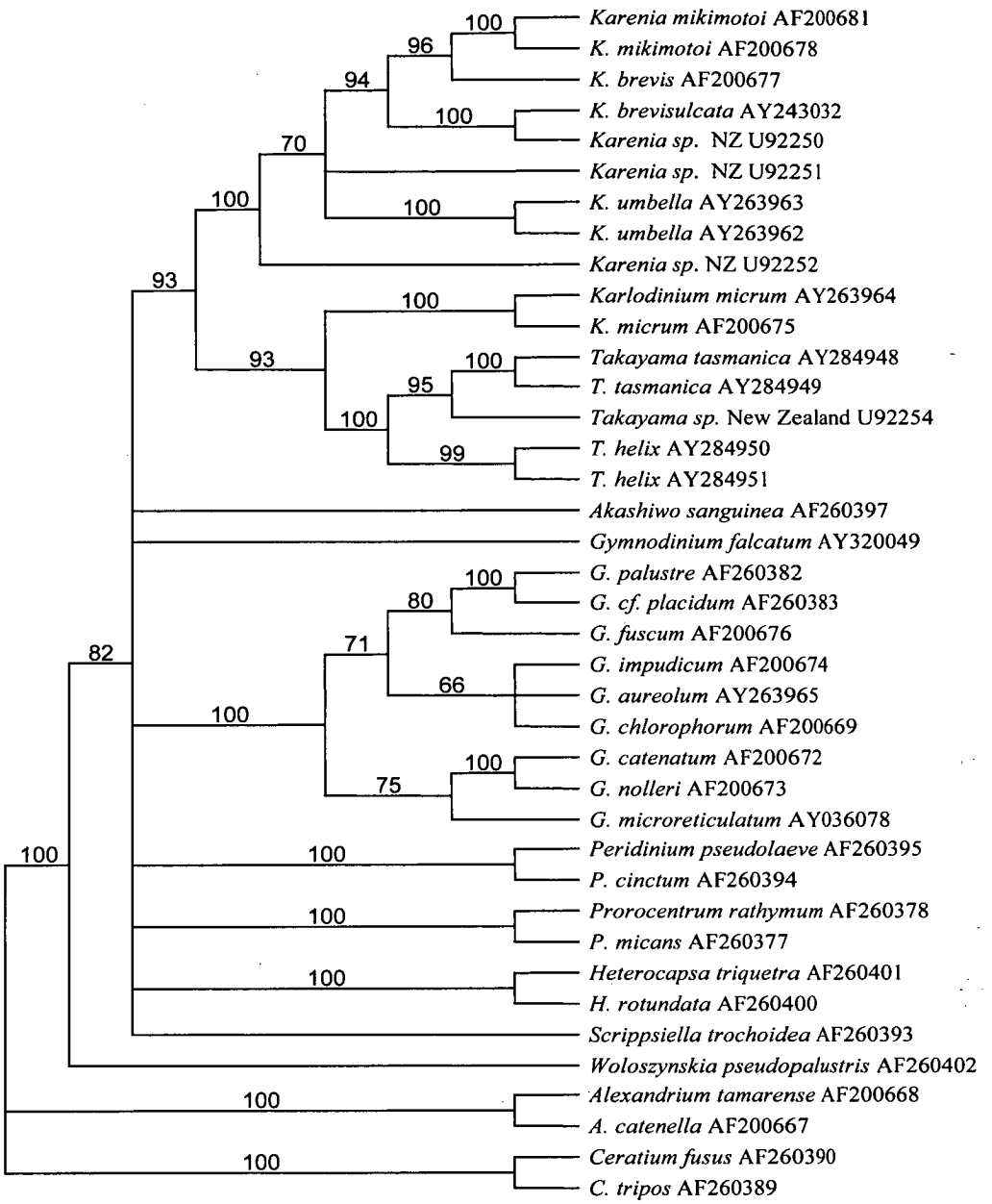


Fig. 4.17. Bootstrap analysis of 1000 heuristic searches using maximum parsimony search criterion. Tree shows phylogeny of *Takayama,* compared with *Karenia* and *Karlodinium*, with all other gymnodinioid groups, and an outgroup composed of *Alexandrium* and *Ceratium*. Numbers next to branching points indicate bootstrap support for groups.

Table 4.2: Comparison of measurements and morphological characteristics the two new species of *Takayama* with *T. pulchella*, *T. acrotrocha* and *T. cladochroma*

Parameter	***Takayama tasmanica***	***Takayama helix***	***Takayama pulchella****	***Takayama acrotrocha****	**Takayama cladochroma***
Cell length (μm)	16-27 (mean 22.8 ± 2.8 n = 50)	17-45 (mean 28.2 ± 4.6 n = 50)	16-25	22-27	18-22
Cell width (μm)	14-26 (mean 19.8 ± 2.7 n = 50)	11-31 (mean 22.3 ± 3.9 n = 50)	11-16	18-22	17-19
Length / width ratio	1.00-1.37 (1.15 ± 0.08 n = 50)	0.98 -1.74 (1.28 ± 0.15 n = 50)	1.45 – 1.56	1.22	1.06 – 1.16
Girdle displacement % total cell length	21-33 (mean 25.8 ± 2.4)	21-28 (mean 24.62 ± 2.75)	~23 % (from published figures)	25 - 80	25
Sulcal extension	finger-like, angled 45° to the rest of the sulcus	finger-like, angled 45° to the rest of the sulcus	short, finger-like	none	small- blunt
Apical groove	sigmoid, curving around the cell apex. Descending sideways 2/3 down dorsal side	shallowly sigmoid, but passing through the cell apex. Descending 1/3 down the dorsal epicone	sigmoid, counter-clockwise, encircling the cell apex	sigmoid, curves around the apex	sigmoid, curving around apex, and extending shortly on dorsal side
Nucleus	large and multi-lobed, enveloping the central pyrenoid	ellipsoidal or round, centred in dorsal epicone or slightly to the left	large, located on the left part of the cell	Large, fills up most of the epicone	on left side of the cell
Chloroplasts	7-10, radiating though the nucleus and branching under the surface. Pyrenoids centrally located inside the nucleus	numerous, peripheral, arranged in spiralling bands	several, irregularly shaped, with pyrenoids	mostly in hypocone, disc shaped, with pyrenoids	few, large and branched, with conspicuous pyrenoids

(*Larsen 1994, 1996)

MOLECULAR DATA: The large subunit ribosomal DNA (LSU rDNA) sequence of *T. tasmanica* diverges approximately 3.79%. from *T. helix* from both Australia and South Africa, 3.4% from a *Takayama* species from Kawau Is., New Zealand, and also diverges from other close relatives such as *Karlodinium micrum* and *Karenia umbella* by 11.5% and 14.9%, respectively. A phylogenetic reconstruction of *Takayama*, *Karenia* and *Karlodinium* (Fig. 4.17) shows that the genus *Takayama* forms a distinct lineage related to *Karenia* and *Karlodinium*. These genera are, in turn, clearly defined taxa, with *Takayama* positioned closer to *Karlodinium* than *Karenia*.

Takayama helix de Salas, Bolch, Botes & Hallegraeff sp. nov.

Figs 4.18-4.33, 4.40, 4.41.

Cellulae rhomboideae ad fere circulares, 17-45 μm longae, 11-31 μm latae, 9-25 μm crassae. Epiconus late conicus ad hemisphaericus. Hypoconus truncatus incisus. Cingulum profunde incisum, latum, per quasi 1/4 longitudinis cellulae totius dislocatum. Sulcus latus sed in regione intercingulari latior quam in hypocono, in hypoconum breviter invasus. Canalis apicalis leniter sigmoideus, e puncto infra sinistram extensionis sulcalis ascendens, apicem cellulae transcendens, et per quasi 1/3-1/2 longitudinis epiconi dorsalis descendens. Partem tubiformem regio intercingularis sulci exhibens. Chloroplasti multi, peripherales, elongati, in zonis spiralibus dispositi, pyrenoides discretas continentes. Nucleus grandis solidusque, plerumque ellipsoideus sed forma locoque varius.

Cells rhomboidal to almost circular in outline, 17 – 45 μm long, 11 – 31 μm wide, 9 – 25 μm thick. Epicone broadly conical to hemispherical. Hypocone truncated and incised. Cingulum deeply excavated and wide, displaced about 25 % of total cell length. Sulcus wide, but narrower in the intercingular region than in the hypocone, extending shortly into the epicone. Apical groove shallowly sigmoidal, extending from below and to the left of the sulcal extension, over the cell apex, to approximately 1/3 to 1/2 of the way down the dorsal epicone. Tube- like structure present in the intercingular region of the

sulcus. Chloroplasts numerous, peripheral, elongated and shallow, arranged in spiralling bands and containing individual pyrenoids. Nucleus large and solid, normally ellipsoidal, but with variable shape and position.

HOLOTYPE: Figs 40, 41 of culture THNWB01, isolated from North West Bay, Tasmania, Australia. Culture deposited into the University of Tasmania Harmful Algae Culture Collection.

TYPE LOCALITY: North West Bay, Tasmania, Australia (Fig. 4.1)

SYNONYMS: *Gymnodinium* sp. 6; (Takayama, 1998, Plate 6, Figs 8, 9)

ETYMOLOGY: named after the Greek *helix*, spiral, after the spiralling arrangement of chloroplasts and surface furrows in the epicone.

DISTRIBUTION: East coast of Tasmania from the North East to the far south, Port Lincoln (South Australia – Fig. 4.1), South Africa (Fig. 4.1), and Japan.

DESCRIPTION: *Takayama helix* is a small to medium sized dinoflagellate known from field samples and laboratory cultures. Average dimensions in comparison with close relatives *T. tasmanica*, *G. pulchellum*, *G. acrotrochum* and *G. cladochroma* are given in Table 4.2.

Cells of *T. helix* have a distinctive appearance under the light microscope. The cell outline is elliptical or rhomboidal, with the epicone conical or hemispherical and the hypocone truncated and incised (Figs 4.24, 4.25). The cingulum and sulcus are deeply excavated. The sulcus is broad posteriorly but narrow between the two terminal ends of the cingulum, and extends into the epicone as a finger-like protrusion at a variable angle (Fig. 4.18). The apical groove extends from below and to the right of the sulcal extension (Fig. 4.18), and passes near and to the left of the apex (Figs 4.20, 4.22), and extends approximately 1/3 to 1/2 way down the dorsal side (Figs 4.19, 4.22). The groove is sigmoid, shaped like a shallow, open 'S' (Figs 4.20, 4.22), but clearly inflected and never straight. Chloroplasts are thin, shallow and elongated, and are arranged in spiralling bands, especially in the epicone (Figs 4.19, 4.21). The nucleus is of variable shape and size, but usually large and elongated, located in the epicone or almost centrally, being sometimes longer in the left part of the cell (Fig. 4.24). A spherical pusule (Fig. 4.25),

surrounded by sac-shaped vesicles is present below the sulcal surface on the right central hypocone.

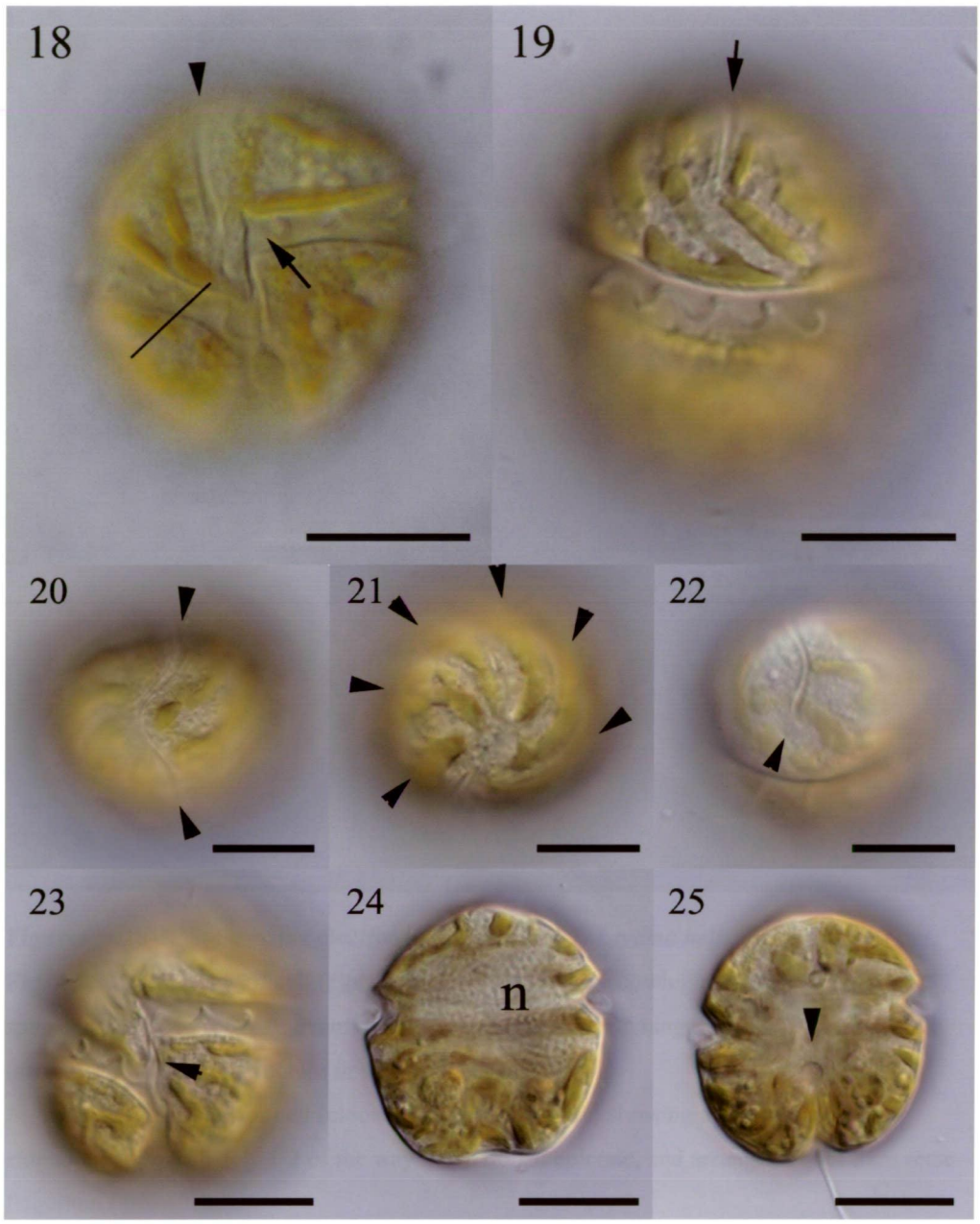
Scanning electron microscopy of the apical groove shows clearly its sigmoid shape from the ventral side across the apex to the dorsal side (Figs 4.26-4.30). Spiralling surface impressions are often visible that parallel the spiralling chloroplasts (Figs 4.26, 4.28, 4.30). The apical groove almost connects with the cingulum, and the epicone surface between it and the sulcus is swollen (Figs 4.26, 4.27, 4.30). A slit, at times also appearing as a pore, is found in the shallow ventral end of the groove (Fig. 4.26, 4.30). This feature is also visible in light micrographs of live material (Fig. 4.18). The region of epicone between this pore and the sulcus is swollen (Fig. 4.26, 4.30).

Transmission electron microscope images provide the best detail of the location and shape of the peripherally located chloroplasts (Fig. 4.31). Chloroplasts are arranged in bands and located peripherally, with individual pyrenoids (Figs 4.31, 4.32). The pusule, in Figure 4.33 damaged due to fixation, is located adjacent to the sulcus.

Cultures of this species form a mucus matrix within which they spend most of their time.

PIGMENT COMPOSITION: Both Tasmanian and South African *T. helix* were extracted for pigment analysis. As the results were identical, only Tasmanian results are shown. The photosynthetic and accessory pigments of *T. helix* (Fig. 4.34) include chlorophyll *a* and chlorophylls c_2 and c_3. No chlorophyll c_1 was detected. The following carotenoids were identified, quantified as a percentage of chlorophyll a: 19'-butanoyloxyfucoxanthin (13.7%), fucoxanthin (109.1%), 19'-hexanoyloxyfucoxanthin (5.7%), diadinoxanthin (42.8%), and diatoxanthin (28.6%).

MOLECULAR DATA: LSU rDNA sequences of Tasmanian and South African *T. helix* were identical. Both of them diverge approximately 3.79%. from *T. tasmanica*, 2.14% from *Takayama sp.* from Kawau Is., New Zealand (Genebank Accession No. U92254), and 11.34% and 12.99% from *Karlodinium micrum* and *Karenia umbella*, respectively. The phylogenetic



Figures 4.18-4.25. Light micrographs of *Takayama helix*. Scale bars = 10 µm.

Fig. 4.18: Ventral view of cell in surface focus showing sulcal intrusion into epicone (arrow), apical groove (arrowhead), and pore in cingular end of apical groove (line).

Fig. 4.19: Dorsal view showing spiralling arrangement of chloroplasts that give the species its name, and apical groove extending approximately 1/3 down dorsal surface (arrow).

Fig. 4.20: Apical view of cell in surface focus showing shape of apical groove.

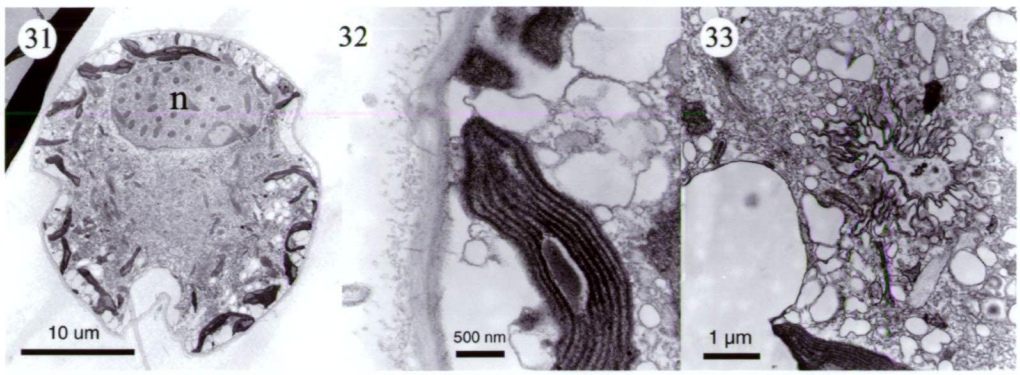
Fig. 4.21: Subsurface focus of cell in apical view. Arrows mark spiralling chloroplasts.

Fig. 4.22: Dorsal view of cell tilted apically showing apical groove (arrow)

Fig. 4.23: Ventral view of cell showing tube-shaped structure in the sulcus (arrowhead).

Fig. 4.24: Central focus of cell showing shape and size of a typical nucleus (n).

Fig. 4.25: Subsurface ventral view of cell showing spherical pusule (arrowhead).



Figures 4.31-4.33: Transmission electron micrographs of *Takayama helix* (clonal culture TTNWB01).

Fig. 4.31: Cross section through cell showing ellipsoidal, anteriorly located nucleus (n) with nucleolus, and peripheral chloroplasts.

Fig. 4.32: Chloroplast detail showing individual lenticular pyrenoid.

Fig. 4.33: Detail of pusule adjacent to sulcus.

reconstruction (using the LSU rDNA sequences) of *Takayama*, *Karenia*, *Karlodinium* and other gymnodinioids displayed in Fig. 4.17 shows that *T. helix* is closely related to *T. tasmanica*, and forms part of a well defined group within the *Karenia* – *Karlodinium* generic complex. However, the group formed by *T. helix* and *T. tasmanica* is clearly separated from *Karenia* and *Karlodinium*.

4.4. DISCUSSION

The taxonomic affinities of the three species with sigmoid grooves described by Larsen (1994; 1996): *Takayama pulchella*, *T. acrotrocha* and *T. cladochroma* were left unresolved in Daugbjerg *et al*'s (2000) revision of gymnodinioid dinoflagellates. The other fucoxanthin containing gymnodinioids were given separate generic status (*Karenia, Karlodinium*); however, determination of the genetic affinities of *T. pulchella* (and related species) had not been attempted in the absence of ultrastructure and LSU rDNA sequences.

From the new data presented here, comprising cell morphology, chloroplast pigment composition and LSU rDNA sequences, the creation of a new genus for species with sigmoid grooves that contain fucoxanthin (and its derivatives) as its main carotenoids is justified. Pigment analysis confirms that the genus *Takayama* is closely related to the fucoxanthin-containing

genera *Karenia* and *Karlodinium*. This is corroborated by LSU rDNA sequences, which show that dinoflagellates with sigmoid grooves, including *Takayama tasmanica, T. helix* (from Australia and South Africa), and a GeneBank sequence (Accession number U92254) referred to here as *Takayama* sp, from Kawau Island, New Zealand, form monophyletic clade that clusters close to *Karlodinium* while remaining distinct.

Two species of *Takayama* examined in detail in this study, *T. tasmanica* and *T. helix*, exhibit morphological features that indicate their close taxonomic relationship. One of these is the sigmoid or clearly inflected apical groove intermediate between the straight grooved genera (*Karenia*, *Karlodinium*), and the loop shaped species in *Gymnodinium sensu stricto* (Daugbjerg, *et al.*, 2000). Another recurring feature is a tube shaped structure which lies along the sulcus in the intercingular region, and is also documented by Steidinger *et al.* (1998). This structure may be homologous with the putative peduncle of *Karlodinium* (Taylor, 1992). There is also a swollen structure which occurs between the proximal extreme of the apical groove and the sulcus, and a pore or slit exists in the proximal extreme of the apical groove. Both of these characters occur both in *T. tasmanica* and *T. helix*.

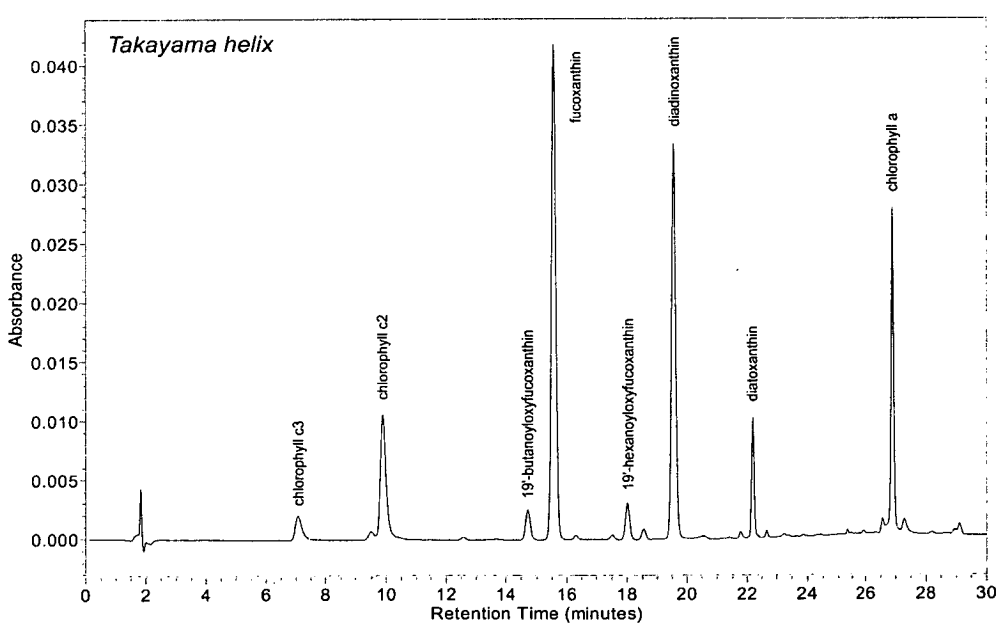


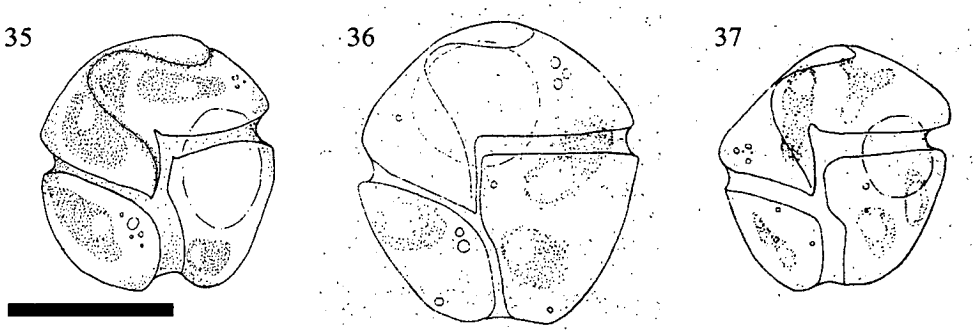
Figure 4.34: HPLC pigment chromatogram of *Takayama helix* strain THNWB01, from North West Bay, Tasmania. Note the absence of gyroxanthin- diester or gyroxanthin- like pigments.

Despite the range of similarities between *T. tasmanica* and *T. helix*, the following differences justify their discrimination as separate species: *T. tasmanica* has a clear 'S' shaped apical groove, like that previously described in *T. pulchella*, *T. acrotrocha* and *T. cladochroma* (Onoue, *et al.*, 1985; Takayama, 1985; Larsen, 1994; 1996; Steidinger, *et al.*, 1998), while *T. helix* has a shallowly sigmoid groove, unlike any previously described species. However, apical grooves like the one present in *T. helix* have been illustrated before from samples in Japan (Takayama, 1998, Plate 6, Figs 8, 9). *T. tasmanica* has a central pyrenoid surrounded by starch, whereas *T. helix* has individual pyrenoids inside peripherally located chloroplasts. The chloroplasts of *T. tasmanica* radiate out from the central pyrenoid and penetrate through the nucleus, which occupies much of the cell and surrounds the pyrenoid laterally and anteriorly. The nucleus of *T. helix* is also large, ellipsoidal in shape in some cells, longitudinally elongated and displaced towards the left side of the cell in others, but always solid (spherical or ovoid) rather than cup-shaped. LSU rDNA sequences of *T. tasmanica* and *T. helix* differ by a larger amount (3.79%) to widely accepted separate species within other genera, such as *Karenia brevis – K. mikimotoi* (2.81%), and *Gymnodinium catenatum – G. nolleri* (2.46%). Additionally, *T. tasmanica* produces small amounts of gyroxanthin –diester (Fig. 5), a pigment typical of *Karenia* and *Karlodinium* species (Bjørnland *et al.* 2000; Ornolfsdottir *et al.* 2003). As well as gyroxanthin- diester, *T. tasmanica* produces large amounts of a gyroxanthin-like pigment distinct from the former. Neither Tasmanian nor South African *T. helix* produce gyroxanthin-diester or the gyroxanthin-like pigment present in *T. tasmanica*.

Takayama tasmanica is morphologically similar to *T. pulchella* (as described by Larsen, 1994), *T. acrotrocha* and *T. cladochroma* (Larsen, 1996), three Port Phillip Bay (Victoria, Australia) species illustrated for comparison in Figures 4.35-4.37. The sulcal intrusion into the epicone of *T. tasmanica* (Figs 4.2, 4.10) is similar to *T. pulchella* and *T. cladochroma* (Figs 4.35, 4.37), however, both these species have a solid nucleus in the left side of the cell, unlike the centrally located, cup-shaped nucleus of *T. tasmanica* (Figs 4.3, 4.9, 4.15). Like *T. tasmanica*, *T. acrotrocha* (Fig. 4.36) has a nucleus that

occupies most of the epicone (Larsen, 1994), but the chloroplasts are disc-shaped and possess individual pyrenoids, and there is no sulcal intrusion into the epicone like that of *T. tasmanica.*

Previously published accounts of species with sigmoid grooves include Takayama's (1985) '*Gymnodinium* sp. 1', which appears to have a sulcal intrusion, and as such is probably either *T. pulchellum* or *T. cladochroma*, and Onoue's (1985, Fig. 1) '*Gymnodinium* type-'84 K', which is not described in sufficient detail to assess its precise identity. However, Fukuyo's (1990) description of this organism does not include a sulcal intrusion and illustrates a centrally located nucleus, which makes it closest to *T. acrotrocha.* Carrada *et al.* (1991) illustrate an organism with a sigmoid groove and a short sulcal intrusion, likely to be either *T. pulchella* or *T. cladochroma.* Since many of the features that identify species in the genus relate to nucleus and chloroplast details, scanning electron micrographs alone are not sufficient to distinguish between species of *Takayama.* For example Steidinger *et al.*'s (1998) report of fish kills in Florida (U.S.A) caused by *T. pulchella* could instead refer to *T. cladochroma*, as both species have a left-sided nucleus, which is reported (but



Figures. 4.35-4.37. Previously described sigmoid-grooved gymnodinioid species, represented at the same scale (after Larsen 1994; 1996). Scale bar 10 μm.

Fig. 4.35: Holotype of *T. pulchella.* Note sharp sulcal intrusion into epicone, chloroplasts with individual pyrenoids and left sided nucleus.

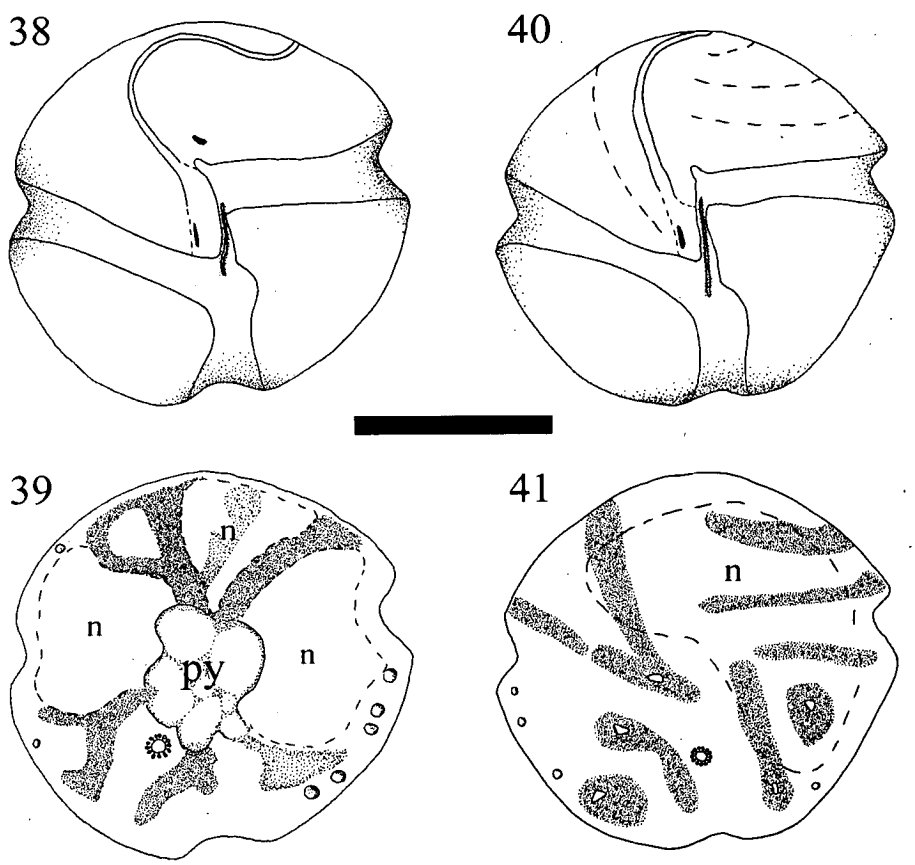
Fig. 4.36: Holotype of *T. acrotrocha.* Note lack of sulcal intrusion, anterior nucleus, and disc-shaped chloroplasts with individual pyrenoids.

Fig. 4.37: Holotype of *T. cladochroma.* Note truncated sulcal intrusion into epicone, peripheral chloroplasts with individual pyrenoids, and left sided nucleus.

not illustrated) in their paper. Steidinger *et al.*'s (1998, p. 434, Figs 2, 5, 6) SEMs show cells that have a distinct inflection of the sulcus at the point of origin of the longitudinal flagellum, and a truncated sulcal intrusion, characters which are represented in *T. cladochroma* (Larsen, 1996 p. 348 Fig. 37), but not *T. pulchella* (Larsen, 1994 p. 32, Fig. 58). A common feature of these illustrations is the presence of an apical groove that occupies less of the epicone surface than *T. tasmanica*. The presence of a tube-like structure lying along the intercingular region of the sulcus has also been mentioned by Steidinger *et al.* (1998) for US material. Such a structure is present both in *T. tasmanica* and *T. helix* (Figs 4.7, 4.11, 4.23, 4.26), and may be a feature of all species in the genus. While easily visible by SEM, its recognition can be difficult under a light microscope, and as such we prefer not to include this feature in the generic diagnosis. The presence of a ventral pore above and to the left of the sulcal intrusion in *T. tasmanica* (Figs 4.12, 4.13), though difficult to preserve for electron microscopy, has also been shown by Carrada *et al.* (1991). This character has not been seen in *T. helix*, but appears to be easily obscured or overgrown, and *T. helix* produces copious amounts of mucus. As its presence is difficult to verify, and almost impossible to see under the light microscope, we have not included it in the species or genus diagnoses. However, it should be noted that its position is almost identical to the ventral pore that is a diagnostic character of *Karlodinium micrum* (Daugbjerg *et al.*, 2000). Features such as the tube- like structure present in *T. tasmanica*, *T. helix*, and documented by Steidinger *et al.* (1998), and the *Karlodinium*-like ventral pore (Figs 4.12, 4.13), support the findings of the sequencing work (Fig. 4.17) that show *Takayama* to be the closest genus to *Karlodinium*.

In conclusion, the exact identity of the species described by Larsen (1994; 1996), *T. acrotrocha, T. cladochroma* and *T. pulchella*, will remain unresolved until new material or cultures of all morphotypes from the type locality (Port Phillip Bay) are available. Re-description of these species is essential, as the level of detail present in the Latin (and English) diagnoses is insufficient. For example, features essential for the discrimination between species in the genus, such as sulcal intrusions into the epicone, and relative

shape, size and position of chloroplasts and pyrenoids are excluded from the diagnosis, and only mentioned in the discussion. The only major difference between *T. cladochroma* and *T. pulchella* is the presence of branched chloroplasts in the former species, which are described simply as 'irregular' in the latter. This character can be misleading, since cells placed under a microscope can change the shape of their chloroplasts, which tend to shrink and assume a globular or disc-shaped form.



Figures 4.38 - 4.41: Schematic representations of *Takayama*. Scale bar 10 μm.

Fig. 4.38: Ventral view of *T. tasmanica*.

Fig. 4.39: Arrangement of nucleus (n – bounded by dashed line), pyrenoids (py – bounded by solid line) and chloroplasts (stippled) in *T. tasmanica*.

Fig. 4.40: Ventral view of *T. helix*.

Fig. 4.41: Chloroplast arrangement and nucleus (n) position in *T. helix*.

Morphological examination and sequencing of the LSU rDNA from cultures isolated from Port Phillip Bay should provide a clear understanding of the genetic affinities of all formally described species with sigmoid apical grooves. The close morphological affinity of Larsen's (1994, 1996) species justifies their transfer to the genus *Takayama*.

4.5. ACKNOWLEDGEMENTS

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CHAPTER 5:

KARLODINIUM AUSTRALE SP. INEDIT. (GYMNODINIALES, DINOPHYCEAE), A NEW POTENTIALLY ICHTHYOTOXIC UNARMOURED DINOFLAGELLATE FROM LAGOONAL HABITATS OF SOUTH-EASTERN AUSTRALIA [1]

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and

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ABSTRACT: A new species in the toxigenic genus *Karlodinium, K. australe* de Salas, Bolch & Hallegraeff sp. nov., is described from coastal lagoons and enclosed bay habitats in Tasmania and south-eastern Australia. This mixotrophic species is characterised with the use of light and electron microscopy, pigment analysis and sequencing of the large subunit ribosomal gene from field samples and laboratory cultures. *Karlodinium australe* has a straight, short apical groove and a ventral pore typical of the genus, but is almost twice the size (18-20 um long) of other species in the genus. It is morphologically similar to *Gyrodinium corsicum* and *Karenia digitata* but has an anteriorly located nucleus and a different amphiesmal pattern. *Karlodinium australe* has a partial LSU sequence that differs by 7.2% from that of *K. micrum*, and its pigment profile does not include gyroxanthin-diester. Unlike the type species of the genus, *K. micrum*, *K. australe* has no hexagonal array of plug-like structures below the amphiesma, and its chloroplasts contain pyramidal rather than lenticular pyrenoids.

[1] **de Salas, M. F., Bolch, C. J. S. & Hallegraeff, G. M.** *Karlodinium australe* sp. nov. (Gymnodiniales, Dinophyceae), a new potentially ichthyotoxic unarmoured dinoflagellate from lagoonal habitats of south-eastern Australia. *Phycologia* (submitted July 04)

5.1. INTRODUCTION

The dinoflagellate genus *Karlodinium* J. Larsen was erected by Daugbjerg *et al.* (2000) based on a combination of morphological (linear apical groove, ventral pore), ultrastructural (internal lenticular pyrenoids, hexagonal arrays of plug-like structures in the amphiesma), genetic and pigment (fucoxanthin and its derivatives) characteristics.

The first description of a *Karlodinium*-like species was by Ballantine (1956), who described two cultures isolated from the Plymouth region of southern England as *Gymnodinium vitiligo* and *G. veneficum*. Independently Braarud (1957) described *Gymnodinium galatheanum* based on formalin-preserved material from Walvis Bay, Namibia. However, the original sketch is believed to be optically reversed (Daugbjerg *et al.* 2000), and the quality of the micrographs and diagrams is insufficient to resolve the identity of this organism. A decade later *Woloszynskia micra* Leadbeater & Dodge (1966) was described in detail from cultures established from the English Channel near Plymouth, which Daugbjerg *et al.* (2000) used as the interim basionym of their new genus *Karlodinium*, as *K. micrum*. Ballantine's (1956) species were also transferred to this genus as *K. vitiligo* and *K. veneficum*. The close morphological similarity between *K. veneficum, K. vitiligo* (culture now lost) and *K. micrum* (Ballantine 1956; Daugbjerg *et al.* 2000; Leadbeater and Dodge 1966), render the status of *K. micrum* uncertain until sequences of *K. veneficum* can be produced. An ultrastructural examination of this species from the Plymouth culture collection suggests that it is indistinguishable from *K. micrum* (J. Larsen & Moestrup, pers. comm.). If this is the case, the name *K. veneficum* will take precedence over *K. micrum* [*Gymnodinium veneficum* (Ballantine 1956) was described earlier than *Woloszynskia micra* (Leadbeater and Dodge 1966)]. However, as the synonymy between these two species has not been formally established at the time of writing, we use the name *Karlodinium micrum* throughout this study.

Björnland and Tangen (1979) characterised the fucoxanthin derivative accessory pigments of an Oslofjord culture, initially referred to as *Gyrodinium* sp. 'A', that they later interpreted to be *Gymnodinium galatheanum*. This culture was subsequently synonymised to *Karlodinium*

micrum by Daugbjerg *et al.* (2000). The name *Gyrodinium galatheanum* has also been used for this species (Taylor 1992).

Gyrodinium corsicum, a species described by Paulmier *et al.* (1995) from waters in Corsica, France, closely resembles *Karlodinium micrum* in its apical groove and identically located ventral pore, but details of its amphiesma are not clear, and neither cultures nor DNA sequences exist, therefore its status remains uncertain.

Karlodinium micrum is most likely a widespread species, though probably often overlooked because of its size. It has been reported in Australia (Hallegraeff 2002), North America (Deeds *et al.* 2002), southern Africa (Tengs *et al.* 2001) and Europe (Björnland and Tangen 1979). In Australian waters it is widespread from cool temperate waters (River Derwent), to subtropical Sydney Harbour (Fig. 5.1), and Western Australia (Hallegraeff 2002).

Many occurrences of *K. micrum* have been associated with fish mortalities (Deeds *et al.* 2002; Kempton *et al.* 2002), and at least two such blooms have been recorded in Australia. Fish mortalities in Lake Illawarra, N.S.W., in 1991, and more recently in the Swan River estuary, in 2001, are recorded by Hallegraeff (2002). Of the two other species in the genus, *K. veneficum* is toxic to fish, whereas *K. vitiligo* is described as non-toxic (Ballantine 1956), however, this may represent physiological variations within the same taxon. *G. corsicum* has been responsible for natural fish-kills in both the Spanish and French Mediterranean coasts (Delgado and Alcaraz 1999; Garcés *et al.* 1999; Paulmier *et al.* 1995), where winter and early spring blooms of this species recurred every winter between 1994 and 1999 (Garcés *et al.* 1999). A number of species in closely related genera are also known fish-killers, such as *Karenia brevis* (Davis) G. Hansen & Moestrup, *K. brevisulcata* (Chang) G. Hansen & Moestrup, *K. digitata* Yang *et al.*, *K. mikimotoi* (Miyake & Kominami *ex* Oda) G. Hansen & Moestrup, and *Takayama pulchella* (Larsen) de Salas *et al.* (Chang 1999; de Salas *et al.* 2003; Hallegraeff 2002; Steidinger *et al.* 1998; Yang *et al.* 2000).

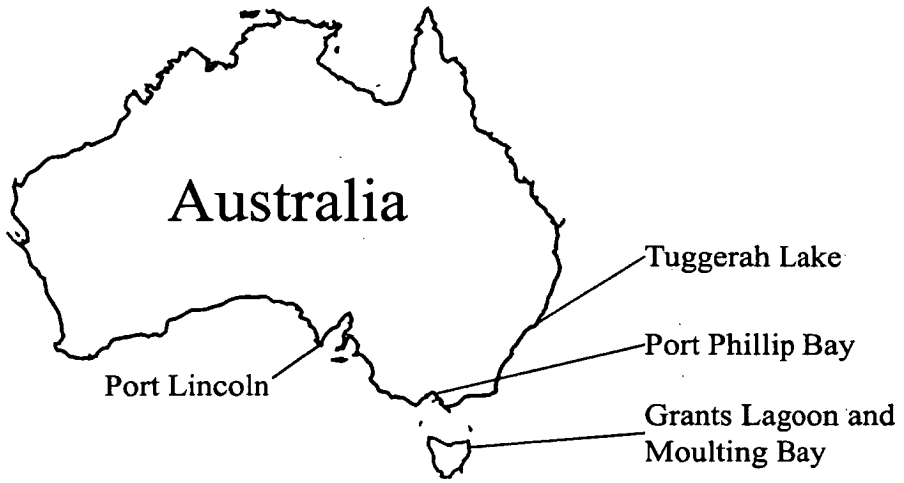

Figure 5.1: Distribution of *Karlodinium australe* in Australian waters

Regular sampling in enclosed waterways along Australia's south-east coast (Tuggerah Lakes, NSW, Port Phillip Bay, VIC, Grants Lagoon and Moulting Bay, TAS – Fig. 5.1) has produced isolates of a new mixotrophic *Karlodinium* species. This species is morphologically similar to *Gyrodinium corsicum* and *Karenia digitata*, and is described in this study as *Karlodinium australe* de Salas, Bolch *et* Hallegraeff sp. nov.

5.2. MATERIALS AND METHODS

5.2.1. Strain isolation and culture maintenance

Clonal cultures of *Karlodinium australe* were isolated from vegetative cells in seawater samples by the use of a micromanipulator. Table 5.1 lists field samples and cultures used in this study. Established cultures were maintained in GSe medium (Blackburn *et al.* 2001), at 28‰ salinity and 17° C. They were incubated at 100 µmol PAR m^{-2} s^{-1}, supplied by cool white fluorescent lights, with a photoperiod of 12:12 L:D.

Feeding experiments were conducted in GSe culture medium, at the same conditions described above. Cultures of *K. australe* were fed with clonal cultures of *Rhodomonas salina* obtained from the CSIRO collection of microalgae, Hobart, Tasmania, Australia.

Comparative cultures of *Karlodinium micrum* were isolated by the authors from several Australian locations, and a Norway isolate (CCMP 415) was obtained from the Provasoli-Guillard Centre for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, Maine, USA.

5.2.2. Light microscopy

Live cells of laboratory cultures and field samples of *K. australe* were suspended in a drop of fluid, clear gel made with 4% w/w carrageenan in culture medium. Carrageenan was found to set to a clear gel, unlike agar and agarose, both of which solidify to an optically opaque gel. Thus it was found to satisfactorily immobilise the cells without interfering with microscopy. Samples were examined and photographed using a Zeiss Axioskop 2+ microscope equipped with bright field and differential interference contrast illumination, and Zeiss Axiocam HRc digital camera (Carl Zeiss, Göttingen, Germany). Cell length, width, and degree of girdle displacement were measured on 20 individual live cells in mid-logarithmic growth phase.

5.2.3. Scanning and transmission electron microscopy

Cells were fixed for SEM with equal volumes of 4% osmium tetroxide dissolved overnight in culture medium, and processed as outlined in Chapter 2. Mounted samples were examined in a JEOL JSM 840 (Jeol, Tokyo, Japan) scanning electron microscope.

Approximately 2 ml of culture were fixed for TEM as explained in de Chapter 4, and observed in a Phillips CM 100 transmission electron microscope (Phillips, Eindhoven, Holland).

5.2.4. DNA extraction, PCR amplification and cycle sequencing

Approximately 5 ml of autotrophically grown culture was extracted by the gentle lysis, phenol/chloroform method (Bolch *et al.* 1998). Extracted DNA was used as a template to amplify approximately 900 bases of the large subunit ribosomal gene (LSU rDNA), encompassing the D1, D2 and D3 domains. Primers, reaction conditions and cycle sequencing parameters are described in detail in Chapter 2.

5.2.5. Sequence alignment and phylogenetic analysis

Corrected sequences were aligned to other *Karlodinium*, *Karenia* and *Takayama* sequences (Table 5.2) using ClustalX (Thompson *et al.* 1997). Phylogenetic analyses using minimum evolution (ME) and maximum parsimony (MP) search criteria were carried out using PAUP 4.0 (Swofford 1999), and Bayesian analysis (likelihood) was carried out using MrBayes (Huelsenbeck and Ronquist 2001), both as described in Chapter 3.

5.2.6. Pigment composition

Approximately 10 ml of autotrophically grown culture were centrifuged gently at 1000 rpm and the resulting pellet was transferred to a 1.5ml microcentrifuge tube. This was centrifuged at 2000 rpm and the supernatant removed. The resulting pellet was snap frozen in liquid nitrogen for storage. The thawed pellet was resuspended in 300µl of methanol, sonicated at 50W for 30 seconds and centrifuged at 13,000 rpm for 4 minutes. The supernatant was transferred into HPLC vials. Pigments were analysed by the modified HPLC method of Zapata *et al.* (2000). Peaks were integrated using Waters Millennium software and identified by comparison of their retention times and spectra with those of mixed standards obtained from known cultures. A standard of gyroxanthin-diester (DHI Bioproducts, Denmark) was also compared, as this pigment is known from *Karlodinium micrum* (as *Gyrodinium* sp. 'A', Björnland *et al.* 2000).

5.3. RESULTS

Karlodinium australe de Salas, Bolch et Hallegraeff, sp. nov.

Figs 5.2-5.17

DIAGNOSIS: Cellulae parvae inarmatae, ovales, 19 - 26 µm longae 16 - 22 µm latae. Epiconus hemisphaericus apice retusus. Hypoconus hemisphaericus. Sulcus latus non profundus, epiconum invadens sub forma projecturae digitiformis. Porus ventralis super sulci extensionem et in sinistrum ejusdem. Cingulum per circiter quartem partem longitudinis cellulae dislocatum. Canalis apicalis brevis rectus et supra latus dorsale epiconi tantum breviter extendens. Nucleus magnus anteriorque. Chloroplasti aliquot irregulariter dispersi per peripheriam cellulae, pyrenoideis pyramidalibus internis. Structurae amphiesmales obturamentis similes in hexagono dispositae nullae.

Table 5.1: Field samples and strains used in this study.

Species	Locality	Date	Collector / Isolator	Strain code
Field Samples				
Karlodinium australe	Grants Lagoon, Tasmania, Australia	15-04-2002	M. de Salas	
K. australe	Tuggerah Lake, N.S.W., Australia	23-10-2002	N.S.W. Fisheries	
K. australe	Moulting Bay, Tasmania, Australia	30-03-2003	M. de Salas	
K. australe	Port Lincoln, South Australia	29-05-2003	C. Wilkinson	
K. australe	Port Phillip Bay, Victoria, Australia	11-02-2004	R. Coello	
Karlodinium micrum	Lake Illawarra, N.S.W.	06-1991	N.S.W. E.P.A.	
K. micrum	Swan River, Western Australia	11-03-2001	W. Hosja	
K. micrum	Canning River, Western Australia	29-04-2003	W. Hosja	
K. micrum	River Derwent, Tasmania, Australia	21-02-2003	M. de Salas	
K. micrum	Sydney Harbour, N.S.W., Australia	8-07-2003	N.S.W. E.P.A.	
Cultures				
Karlodinium australe	Grants Lagoon, Tasmania, Australia	15-04-2002	M. de Salas	KDAGT03
K. australe	Tuggerah Lake, N.S.W., Australia	23-10-2002	M. de Salas	KDATL05
K. australe	Port Phillip Bay, Victoria, Australia	11-02-2004	M. de Salas	KDAPP01
Karlodinium micrum	Swan River, Western Australia	11-03-2001	M. de Salas	KDMP01
K. micrum	River Derwent, Tasmania, Australia	21-02-2003	M. de Salas	KDMDE01
K. micrum	Sydney Harbour, N.S.W., Australia	8-7-2003	M. de Salas	KDMSH01
K. micrum	Norway	07-1976	K. Tangen	CCMP415

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K. australe	Tuggerah Lake, N.S.W., Australia	23-10-2002	N.S.W. Fisheries	
K. australe	Moulting Bay, Tasmania, Australia	30-03-2003	M. de Salas	
K. australe	Port Lincoln, South Australia	29-05-2003	C. Wilkinson	
K. australe	Port Phillip Bay, Victoria, Australia	11-02-2004	R. Coello	
Karlodinium micrum	Lake Illawarra, N.S.W.	06-1991	N.S.W. E.P.A.	
K. micrum	Swan River, Western Australia	11-03-2001	W. Hosja	
K. micrum	Canning River, Western Australia	29-04-2003	W. Hosja	
K. micrum	River Derwent, Tasmania, Australia	21-02-2003	M. de Salas	
K. micrum	Sydney Harbour, N.S.W., Australia	8-07-2003	N.S.W. E.P.A.	
Cultures				
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K. australe	Tuggerah Lake, N.S.W., Australia	23-10-2002	M. de Salas	KDATL05
K. australe	Port Phillip Bay, Victoria, Australia	11-02-2004	M. de Salas	KDAPP01
Karlodinium micrum	Swan River, Western Australia	11-03-2001	M. de Salas	KDMP01
K. micrum	River Derwent, Tasmania, Australia	21-02-2003	M. de Salas	KDMDE01
K. micrum	Sydney Harbour, N.S.W., Australia	8-7-2003	M. de Salas	KDMSH01
K. micrum	Norway	07-1976	K. Tangen	CCMP415

Table 5.2: Origin (where known) and GenBank accession numbers of sequences used in phylogenetic analyses.

Species	Origin	Accession number
Karenia mikimotoi	English Channel	AF200678
K. brevis	Florida	AF200677
K. brevisulcata	New Zealand	AY243032
K. selliformis	New Zealand	U92250
K. cristata	South Africa	AY525907
K. bidigitata	New Zealand	U92251
K. asterichroma	Tasmania	AY590123
K. papilionacea	New Zealand	U92252
K. umbella	Tasmania	AY263963
Karlodinium micrum	Norway	AF200675
K. micrum	Western Australia	AY263964
K. australe	New South Wales	to be submitted
K. australe	Tasmania	to be submitted
Gymnodinium sp. Corsica	Corsica, France	AF318249
Takayama helix	Tasmania	AY284950
Takayama sp.	New Zealand	U92254
T. tasmanica	Tasmania	AY284948
Akashiwo sanguinea	Japan	AF260397
Gymnodinium catenatum	-	AF200672
G. chlorophorum	-	AF200669
G. fuscum	-	AF200676
Woloszynskia pseudopalustris	-	AF260402
Alexandrium catenella	California	AF200667

Small unarmoured dinoflagellate cells, oval in outline, 19 - 26 µm long, and 16 – 22 µm wide. Epicone hemispherical and notched at the apex. Hypocone hemispherical. Sulcus shallow and wide, invading the epicone as a finger-like projection. Ventral pore above and to the left of the sulcal extension. Cingulum displaced approximately ¼ of the cell length. Apical groove short and straight, extending only briefly onto the dorsal side of the epicone. Nucleus large and anterior. Several chloroplasts distributed irregularly through the cell periphery, with individual pyramidal pyrenoids. Amphiesmal plug-like structures in hexagonal configuration absent.

HOLOTYPE: Figure 5.17, schematic representation of ventral and dorsal views of a cell from strain KDMGTL, from Grants Lagoon, Tasmania (Fig. 5.1).

ETYMOLOGY: Named *australe* (Latin: southern) after the localities where this species has been found, all within south-eastern Australia.

DISTRIBUTION: Cells have been observed in field samples from Moulting Bay and Grants Lagoon, (north-eastern Tasmania), Port Phillip Bay (Victoria), Port Lincoln (South Australia) and Tuggerah Lakes (N.S.W. – Fig. 5.1). Table 5.1 lists sites where *Karlodinium australe* and *K. micrum* have been recorded in Australia.

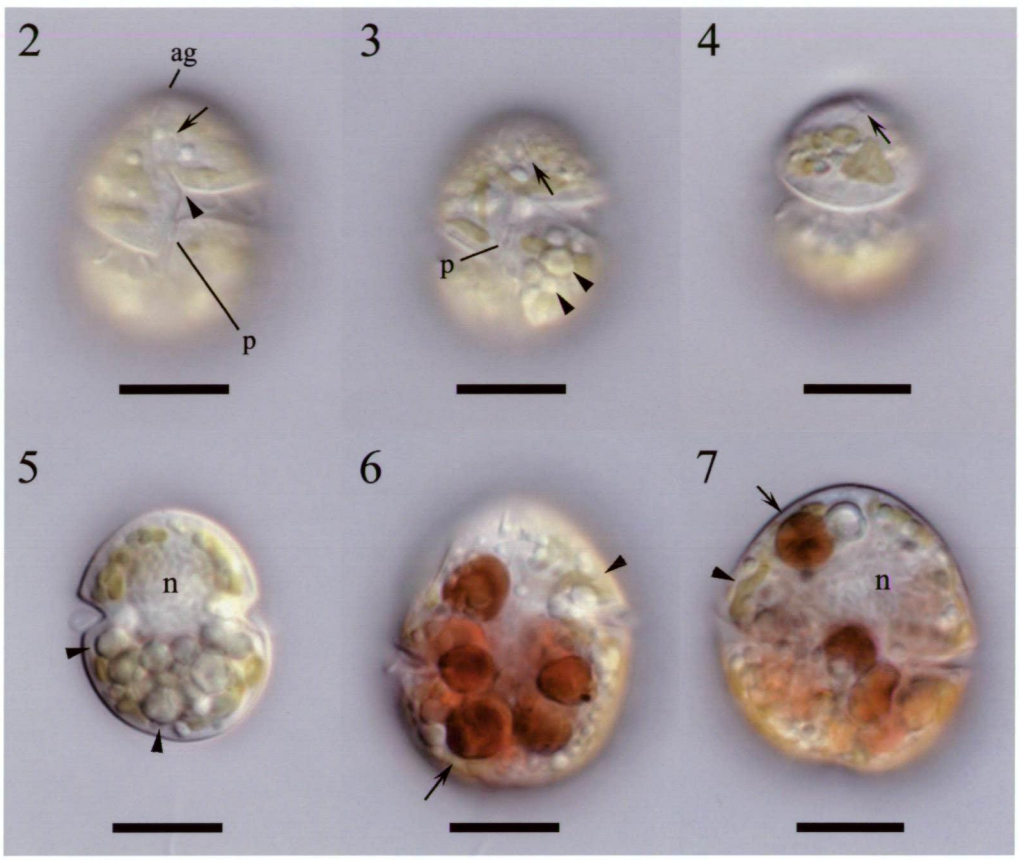
DESCRIPTION: *Karlodinium australe* is a small unarmoured dinoflagellate known from field samples and established cultures. Average measurements in comparison with *Karlodinium micrum*, *K. veneficum* and *Karenia digitata* are presented in Table 5.3. Clonal cultures of *K. australe* isolated from wild populations and grown autotrophically in GSe medium grew slowly and after a number of divisions cell numbers in a culture stabilised at low concentration (10^2 – 10^3 cells ml^{-1}). Attempts to culture this species to higher cell concentrations have so far failed.

Under the light microscope, cells of *K. australe* appear oval in shape, and cultured cells from aged cultures often have numerous refractive lipid bodies that obscure other cell contents. The epicone is hemispherical (Figs 5.2-5.7), and often notched at the apex by the apical groove (Figs 5.4, 5.4) The hypocone is rounded but not significantly truncated by the sulcus. Both the sulcus and cingulum are shallow in the ventral surface (Figs 5.2 , 5.3), but the cingulum is well excavated on the sides of the cell (Fig. 5.5). The sulcus invades the epicone as a finger-like protrusion and the cingulum is displaced approximately ¼ of the total cell length (Fig. 5.2). A ventral pore is present in the epicone above and to the left of the sulcal intrusion, sometimes appearing reniform (Fig. 5.2), sometimes as a long slit (Fig. 5.3). An apical groove extends from shortly above and to the right of the sulcal extension to terminate a short way down the dorsal side (Figs 5.2, 5.4). The nucleus is anterior and occupies much of the epicone (Figs 5.5, 5.7). Refractive lipid bodies are distributed irregularly throughout the cell, but are generally larger and more abundant in the hypocone (Figs 5.3, 5.5).

Table 5.3: Comparison of morphological characters of *Karlodinium australe*, in comparison with *K. micrum*, *Gyrodinium corsicum* and *Karenia digitata*.

Morphological character	*Karlodinium australe*	*Karlodinium micrum* [1]	*Gyrodinium corsicum* [3]	*Karenia digitata* [5]
Cell length (µm)	19-26 (21.81 ± 1.85, n = 30)	14 – 17 9-15 [2]	17-24 15-24 [4]	10-26.3 (21.5 ± 3.0)
Cell width (µm)	16-22 (18.92 ± 1.78, n = 30)	10-14 8-14 [2]	12-16 12-18 [4]	10-22 (18.2 ± 2.5)
Girdle displacement (% cell length)	25.1 ± 2.2	20	32-34	24.9 ± 1.9
Sulcal extension in epicone	Short and fold-like or long and finger-like	shallow, short	narrow slit	long, finger-like
Apical groove	linear, wide, extending very shortly onto dorsal epicone	short and linear	deflected ventrally towards the sulcus, extending dorsally 1/3 of the epicone length	linear, short, wide at ends but narrow in the middle; extends 1/3 – 1/2 of the length of dorsal epicone
Nucleus	round and anterior	median to posterior, round [2]	central, round	posterior
Chloroplasts	several (6-10) ribbon-shaped and irregularly distributed peripherally, with pyramidal pyrenoids	usually two, one in epicone and one in hypocone, with several lenticular pyrenoids	approximately 15, peripheral	10 – 20, irregular

[1] Braarud (1957)
[2] Leadbeater & Dodge (1966)
[3] Paulmier *et al.* (1995)
[4] Garces *et al.* (1999)
[5] Yang *et al.* (2000)



Figures 5.2-5.7: Light micrographs of *Karlodinium australe*. Scale bars = 10 µm.

Fig. 5.2: Surface focus of cell in ventral view showing an extension of the sulcus into the epicone, visible as a fine groove (arrow). A ventral pore (arrowhead), and the apical groove (ag) are also visible.

Fig. 5.3: Subsurface focus of cell showing apical groove (ag), and ventral pore shaped as a long slit (arrow). Arrowheads mark the position of some refractive lipid bodies within the hypocone.

Fig. 5.4: Dorsal view of cell tilted towards the apex showing dorsal extension of the apical groove (arrow).

Fig. 5.5: Cross section through cell showing the anterior position of the nucleus (n) and refractive bodies (arrowheads).

Fig. 5.6: Subsurface ventral view of cell after feeding overnight on *Rhodomonas salina*. Note light yellow-green chloroplasts (arrowhead) and red food vacuoles (arrow).

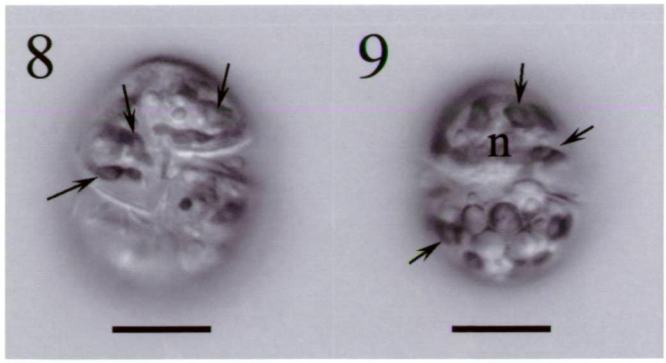
Fig. 5.7: Cross section view of cell after feeding. Note unchanged position of the nucleus (n), chloroplasts (arrow) and anterior position of recently formed food vacuole (arrow), where some internal features of the *Rhodomonas* cell are still visible.

Phagotrophy was observed when autotrophically grown cultures of *K. australe* were offered live *Rhodomonas salina* cells as prey. Cells exposed to *Rhodomonas* near the beginning of the light period (early morning) did not ingest any prey by the end of this period. However after the following dark period, *K. australe* cells possessed a variable number (1 - >10) of red food vacuoles (Figs. 5.6, 5.7), indicating cells of this species may only feed at night. When cells are grown without prey, chloroplasts normally number 6-8, although aberrant cells with 1 or no chloroplasts are regularly observed in culture. Chloroplasts are distributed throughout the cell, and their distribution and morphology are variable. They are irregularly shaped but often elongated (Figs 5.8 – 5.9). Pyramidal pyrenoids are visible as triangular pale areas by light microscopy (Figs 5.8 and 5.9).

Under the scanning electron microscope (SEM), the ventral pore is readily apparent (Fig. 5.10), and the short, thick flagella and short, linear apical groove are evident (Figs 5.10-5.12). The sulcal extension into the epicone, which appears finger-like by light microscopy, appears like a fold under the SEM (Figs 5.10, 5.11). A thick tube-like structure lying along the intercingular region of the sulcus is visible in Fig. 5.11. The apical groove extends onto the dorsal surface for a short distance (Fig. 5.12). A pattern of hexagonal amphiesmal vesicles is evident, that elongate along the edge of the cingulum (Fig. 5.13). No pores are visible on the surface of the cell except the single, large ventral pore above and to the left of the sulcal extension into the epicone (Figs 5.10 and 5.11).

Transmission electron microscopy of this species reveals an irregular nucleus without a nuclear capsule, containing at least two nucleoli (Fig. 5.14). Peripherally located chloroplasts contain individual pyrenoids that are pyramidal in shape (Fig. 5.14). Tangential sections taken just inside the cell surface show the polygonal structure of the outer layer of amphiesmal vesicles, directly overlying a pattern of parallel bands with no evidence of amphiesmal plugs (Fig. 5.15, 5.16).

A diagrammatic representation (Figs 5.17 and 5.18) illustrates the close morphological affinity between, and relative size of *Karlodinium australe* compared to the types species *K. micrum*.



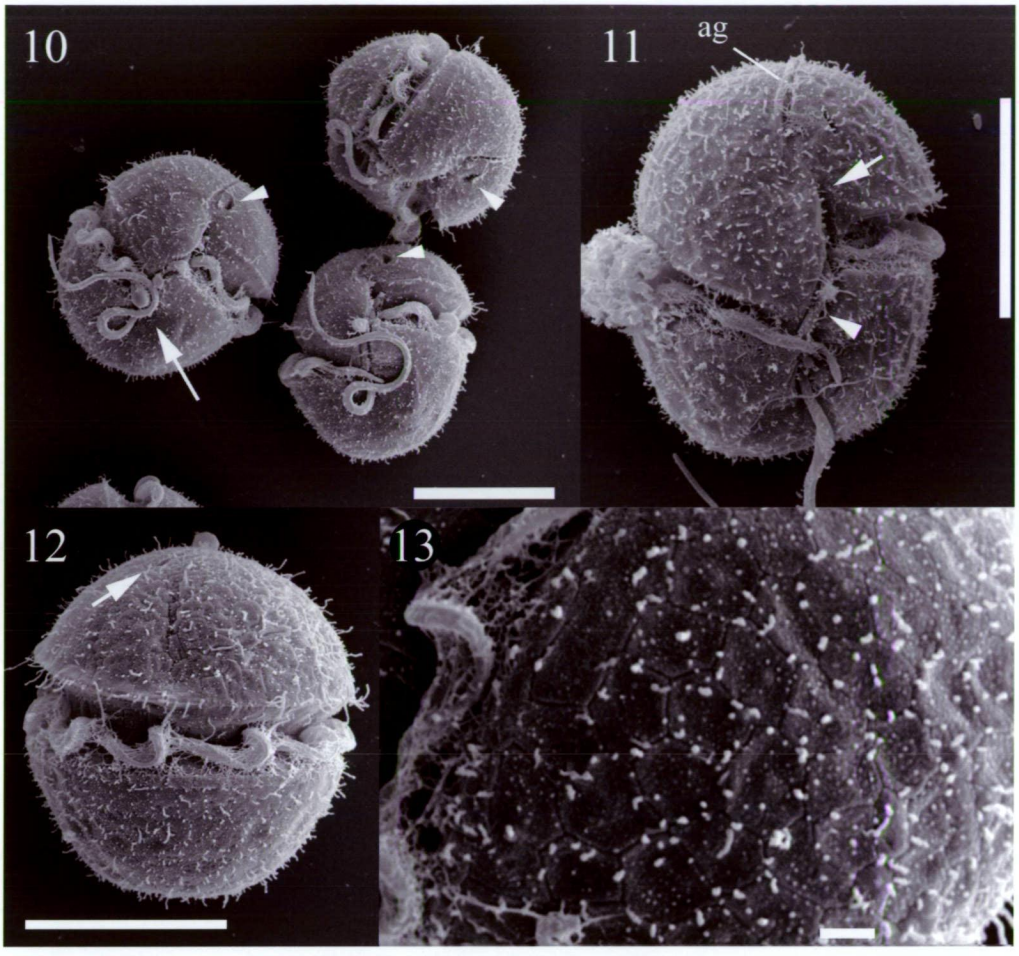
Figures 5.8-5.9: Extracted blue channel (from RGB file) of light micrographs of *Karlodinium australe,* highlighting chloroplast detail. Scale bars = 10 μm.

Fig. 5.8: Subsurface focus of cell in ventral view, showing irregular, ribbon-shaped chloroplasts with triangular (pyramidal) pyrenoids (arrows).

Fig. 5.9: Dorsal view of cell in subsurface focus showing dorsal distribution of chloroplasts (arrows mark pyramidal pyrenoids) and anterior position of the nucleus (n).

MOLECULAR SYSTEMATICS: *Karlodinium australe* LSU rDNA sequences differ by approximately 7.2% from its closest relative, *K. micrum*, by 8.6% from *Takayama tasmanica*, and by 12% - 15% from members of the genus *Karenia*. Phylogenetic analysis using Bayesian inference methods (Fig. 4.19) clearly show *K. australe* to cluster with *K. micrum* and a rDNA sequence from an unspecified *Gymnodinium* species isolated from Corsica (which is consistent with *K. micrum*), and distinct from the other two fucoxanthin-containing genera *Karenia* and *Takayama* included in the analysis. MP analysis produced several equally parsimonious trees, but its bootstrap consensus tree does not show branch length. Therefore the tree shown in Fig. 4.19 is that derived by Bayesian inference, as this method allows the production of a phylogram showing branch lengths.

PIGMENTS: The photosynthetic pigments of *K. australe* (Figure 5.20) include chlorophylls *a*, c_1 and c_2; no chlorophyll c_3 was detected. The following diagnostic carotenoids were identified, reported here as a percentage of chlorophyll *a*: 19'-butanoylfucoxanthin (trace), fucoxanthin (29.9%), 19'-hexanoyloxyfucoxanthin (23%), diadinoxanthin (15%), and diatoxanthin (53%). No gyroxanthin diester was detected.



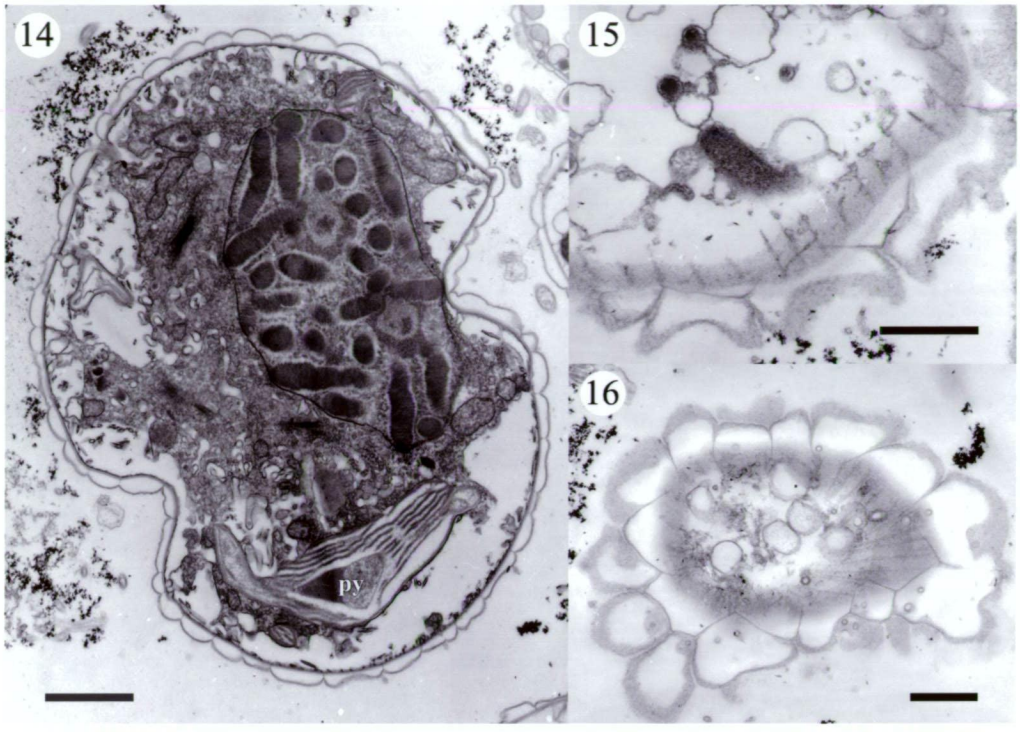
Figures 5.10-5.13: Scanning electron micrographs of *Karlodinium australe*.

Fig. 5.10: Three cells in ventral view, showing thick flagella (arrow) and prominent ventral pores (arrowheads). Scale bar = 10 µm.

Fig. 5.11: Ventral view of cell showing straight apical groove (ag), extension of the sulcus into the epicone (arrow), and tubular peduncle-like structure along the sulcus (arrowhead). Scale bar = 10 µm.

Fig. 5.12: Dorsal view of cell showing extent of apical groove (arrow). Scale bar = 10 µm.

Fig. 5.13: Detail of the amphiesma of *K. australe* composed of polygonal vesicles that elongate into approximate rectangles (arrows) on the edges of the cingulum. Scale bar = 1 µm.



Figures 5.14-5.16: Transmission electron micrographs of *Karlodinium australe*.

Fig. 14: Cross section through a cell of *K. australe*. Note chloroplast with pyramidal (triangular in cross section) pyrenoid (py). Scale bar = 2 µm.

Fig. 5.15: Tangential section through the amphiesma. Parallel bands of microtubules are evident but note the absence of hexagonal arrays of plug-like structures. Scale bar = 1 µm.

Fig. 5.16: Section taken immediately under the outer layer of the amphiesma. Note polygonal pattern of amphiesmal vesicles and absence of plug-like structures in hexagonal arrays that are found in *K. micrum*. Scale bar = 1 µm.

5.4. DISCUSSION

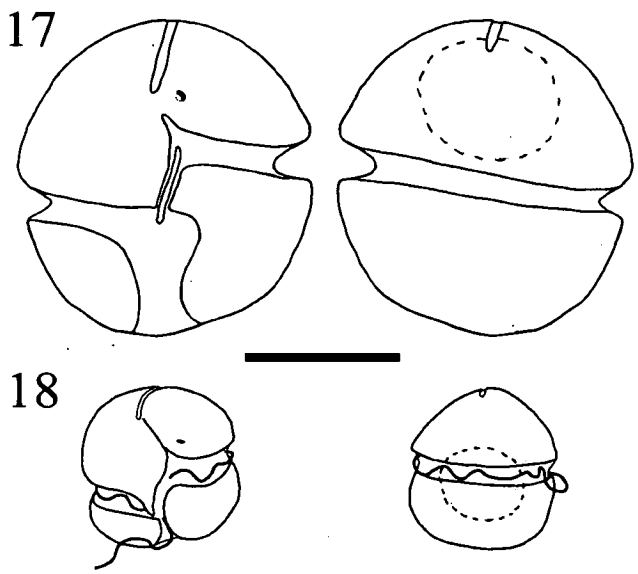
A species found so far only in lagoons and enclosed bays (Fig. 1), *Karlodinium australe* appears at first glance very similar to *Karenia digitata* (Yang *et al.* 2000) and *Gyrodinium corsicum* (Paulmier *et al.* 1995). However, some important differences are apparent. *K. australe* displays the ventral pore typical of *Karlodinium* (Figs 5.17, 5.18), that although present also in *Gyrodinium corsicum* and *Takayama tasmanica* (Paulmier *et al.* 1995; de Salas *et al.* 2003), has never been documented among *Karenia* species. *K. australe* also has an anteriorly placed nucleus (Fig. 5.17), compared to the posterior nucleus of *Karenia digitata* and the central nucleus of *G. corsicum*. *K. australe* displays a pattern of strongly developed, approximately hexagonal

amphiesmal vesicles, a feature not present in *K. digitata* (Yang *et al.* 2000), or *G. corsicum* (Paulmier *et al.* 1995).

The type species of *Karlodinium*, *K. micrum*, is much smaller than *K. australe*, and has a sub-central nucleus (Daugbjerg *et al.* 2000; Leadbeater and Dodge 1966). *K. micrum* also normally has two large chloroplasts, one in the epicone and one in the hypocone, with internal lenticular pyrenoids (Daugbjerg *et al.* 2000). In contrast, *K. australe* has several irregularly distributed, elongated chloroplasts, containing pyramidal pyrenoids. The taxonomic status of *Gyrodinium corsicum* is likely to change in the future, as this species is morphologically consistent with *Karlodinium*, though it has not been studied in sufficient detail (ultrastructure, pigments, or molecular sequences) to warrant its transfer to this genus at this time.

LSU rDNA sequences of *Gyrodinium corsicum* are not yet available, but a sequence named *Gymnodinium* sp. Corsica (Fig. 5.19) is consistent with to *Karlodinium micrum* (*sensu* Daugbjerg *et al.* 2000). *K. australe* is genetically distinct from *K. micrum*, but clearly belongs within the genus *Karlodinium* (Fig. 5.19).

HPLC pigment analysis of autotrophically grown cultures of *K. australe* indicate that this species contains fucoxanthin, as well as 19'-hexanoyloxyfucoxanthin, and 19'-butanoylfucoxanthin as the main carotenoid pigments (Fig. 5.20). Autotrophically grown cultures of *K. australe* did not produce gyroxanthin-diester, a pigment typical of *Karlodinium micrum* (Björnland *et al.* 2000) and *Karenia* species (Botes *et al.* 2003; Daugbjerg *et al.* 2000; de Salas *et al.* 2004). Other gyroxanthin-like pigments have been reported in the closely related species *Takayama tasmanica* (de Salas *et al.* 2003), but these were not present in cultures of *K. australe*. This suggests that monitoring for fish-killing dinoflagellates using gyroxanthin-diester (Ornolfsdottir *et al.* 2003; Schofield *et al.* 1999; Staehr and Cullen 2003) would not detect this and other potentially ichthyotoxic gymnodinioid dinoflagellates.



Figures 5.17-5.18: Diagrammatic comparison between *Karlodinium australe* and *K. micrum* drawn on the same scale.

Fig. 5.17: Ventral and dorsal diagrams of *K. australe*, showing position of the nucleus and surface features.

Fig. 5.18: Ventral and dorsal diagrams of *K. micrum* (adapted from Daugbjerg et al. 2000). Note average size difference. Scale bar = 10μm.

All morphological, genetic and biochemical data indicate that *K. australe* clearly belongs in the genus *Karlodinium*, however, two ultrastructural features conflict with the diagnosis of this genus as circumscribed by Larsen in Daugbjerg *et al.* (2000). Firstly, the genus *Karlodinium* is described as possessing chloroplasts with lenticular pyrenoids (Daugbjerg *et al.* 2000), yet *K. australe* possesses pyramidal pyrenoids (Fig. 5.14) very similar to those described for *Karenia mikimotoi* and *K. brevis* (Hansen *et al.* 2000; Steidinger *et al.* 1978). Secondly, *Karlodinium* as currently described is has arrays of plug-like structures immediately below the amphiesma, as illustrated by Leadbeater & Dodge (1966, Figs. 19, 20 and 22), and Daugbjerg *et al.* (2000, Figs. 8 and 9). We could find no evidence of these plugs in *K. australe,* although it did possess similar parallel, tubular bands to *K. micrum* immediately under the amphiesmal vesicles (Figs. 5.15, 5.16). The genus *Karlodinium* was circumscribed from observations only one species but our

work indicates that the generic diagnosis is too narrow and needs to be emended to include species without plug-like amphiesmal structures.

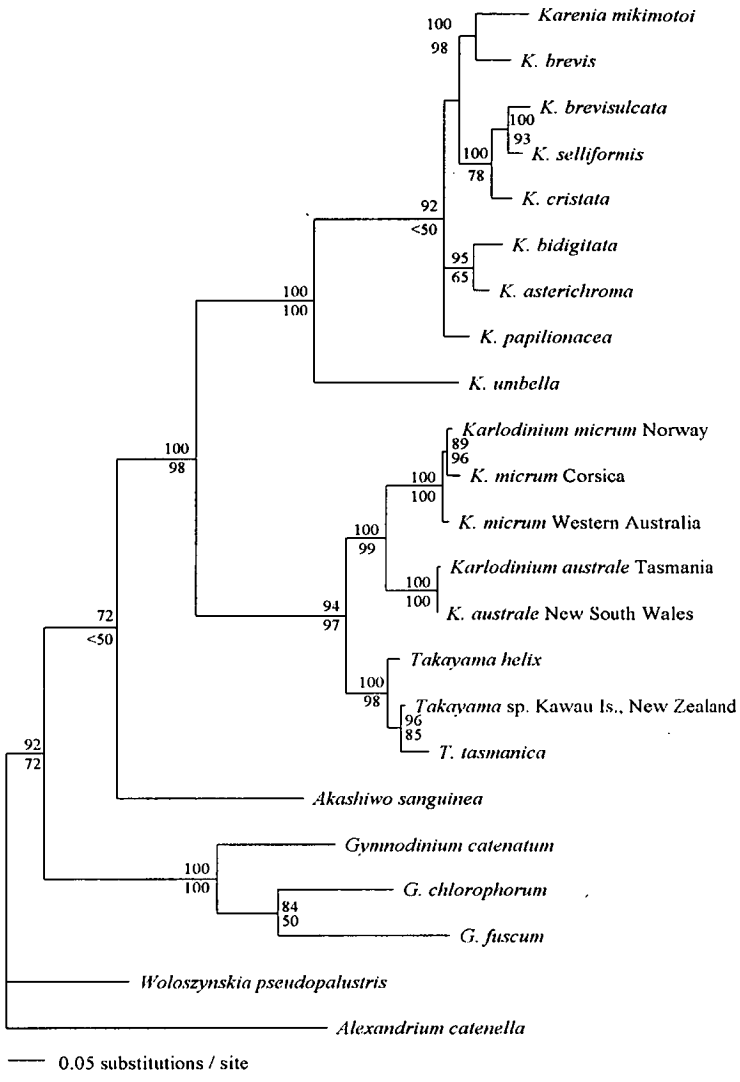


Figure 5.19: Proposed Bayesian inferred phylogeny of fucoxanthin-containing dinoflagellates with an outgroup representing other unarmoured and armoured dinoflagellates. Support values above nodes were derived from a Bayesian analysis of 150,000 generations (excluding first 50,000) using a general time-reversible evolution model with gamma distributed among-site rate variation. Support values below nodes are derived from MP bootstrap search using 1000 replicates. Tree length in Bayesian tree = 1163, consistency index = 0.672 and retention index = 0.684. Tree length in MP bootstrap tree = 1164, CI = 0.672 and RI = 0.684.

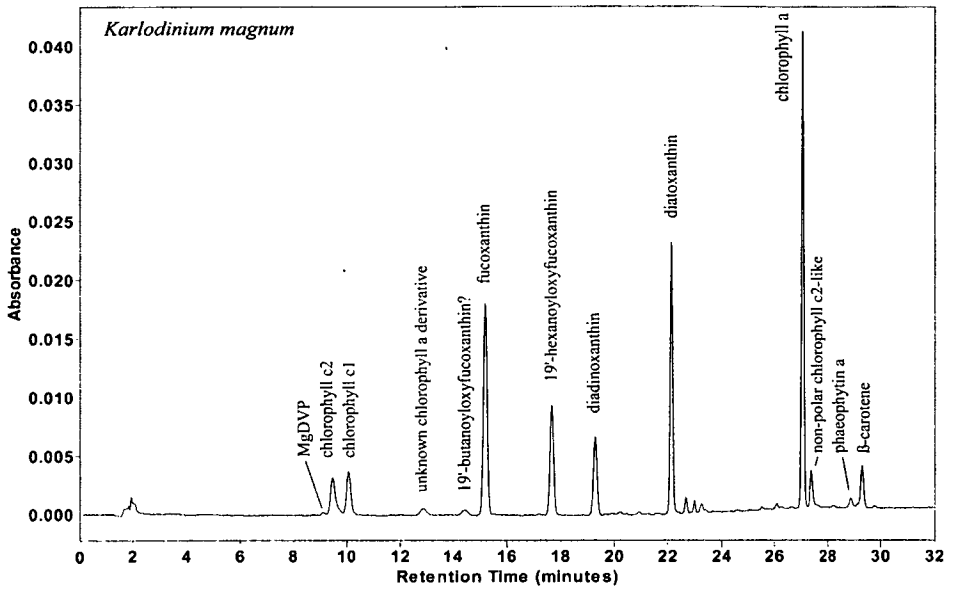


Figure 5.20: HPLC pigment chromatogram of an autotrophically grown culture of *Karlodinium australe*. Note the absence of gyroxanthin-diester or gyroxanthin-like pigments.

The clear morphological similarity between *Karlodinium australe* and *Gyrodinium corsicum* and between *K. australe* and *Karenia digitata* has made it apparent that *Gyrodinium corsicum* and *K. digitata* are morphologically very close to each other. Some morphological details visible in SEM are identical, notably the suture-like rows of pores encircling the mid hypocone and just below the cingulum, and the epicone just above it (Paulmier *et al.* 1995, Figs 10-12; Yang *et al.* 2000, Figs 2 and 4). However, no ventral pore is visible in Yang *et al.*'s (2000) illustrations of *K. digitata*, and the dorsal extent of its apical groove surpasses that of both *Karlodinium australe* and *Gyrodinium corsicum*. Despite these apparent differences, there is a strong possibility that both of these species are congeneric within *Karlodinium*.

Mixotrophy exhibited by *Karlodinium australe* is not surprising, as this character has been reported from other dinoflagellate species, including *K. micrum* (Legrand *et al.* 1998; Li *et al.* 2000a, b; Smalley and Coats 2002). However, while *K. micrum* is capable of growing well when kept under strictly autotrophic conditions (facultative mixotrophy, Li *et al.* 2000a), *K. australe* growth stagnates when kept in culture with no prey, and this new taxon may be an almost obligate mixotroph. This species has a thick, tube-

like structure located along the sulcus, which appears homologous with the putative peduncle of *K. micrum* (Taylor 1992), also reported from *Takayama* species (de Salas *et al.* 2003; Steidinger *et al.* 1998), and *Karenia* species (Haywood *et al.* 2004).

Other species of *Karlodinium*, notably *K. micrum* (Deeds *et al.* 2002), *K. veneficum* (Ballantine 1956), and *Gyrodinium corsicum* (Delgado and Alcaraz 1999; Garcés *et al.* 1999; Paulmier *et al.* 1995) are strongly ichthyotoxic, and while *Gyrodinium corsicum* is so far confined to the Mediterranean sea (Delgado and Alcaraz 1999; Garcés *et al.* 1999; Paulmier *et al.* 1995), *K. micrum* appears to be a cosmopolitan species, and is widespread in Australia (Table 5.1). The widespread distribution of *K. australe* in this country and its close affinity to problem species such as *Gyrodinium corsicum*, *Karenia digitata* and *Karlodinium micrum* mean that development of culturing protocols to allow assessment of its ichthyotoxicity are important research priorities.

5.5. ACKNOWLEDGEMENTS

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CHAPTER 6:
FURTHER POTENTIALLY ICHTHYOTOXIC UNARMOURED DINOFLAGELLATES FROM TASMANIAN AND AUSTRALIAN WATERS

Miguel Félix de Salas

ABSTRACT: In addition to the new species characterised in Chapters 2-5, several previously described species of fish-killing dinoflagellates in *Karenia* and *Karlodinium* were encountered during the course of this study. *Karlodinium micrum* was a common bloom former from subtropical to temperate waters, responsible for several fish-kills. *Karenia papilionacea* was found in high numbers in north-eastern Tasmania, South Australia and Western Australia. *Karenia mikimotoi* was found in low numbers in Port Lincoln, South Australia, during the late austral autumn. Four undescribed *Karenia* species of distinct morphology were isolated from field samples, but they could not be cultured or characterised in sufficient detail for their formal description.

6.1. INTRODUCTION

Several unarmoured dinoflagellate species belonging to the known fish-killing genera *Karenia* and *Karlodinium* were identified during this study, both from field samples and laboratory cultures obtained from these. Some of these have been formally described by other authors and are presented in this chapter for the sake of completeness. Several new species could not be cultured and characterised in enough detail to warrant their formal description in stand-alone papers, however enough detail was obtained to illustrate them in this chapter. Two previously described species of *Karenia*, and one of *Karlodinium* are presented here, as well as three (possibly four) undescribed species in the genus *Karenia*.

6.2. MATERIALS AND METHODS

6.2.1. Culture conditions

Samples were collected from many southern Australian locations using a 20 µm plankton net. Clonal strains were established by isolating single cells with a micropipette and rinsing three times in sterile GSe/2 (Blackburn *et al.* 2001) medium. Established cultures were maintained in GSe/2 medium at 35 ‰ salinity, at a constant temperature of either 17° or 20° C, with a 12:12 L:D photoperiod of 100 µmol m^{-2} s^{-1}, supplied by cool white fluorescent lights.

6.2.2. Light microscopy

Live cells from both established cultures and field samples were suspended in a drop of 4% fluid carrageenan gel (Chapter 5), and observed using differential interference contrast illumination in a Zeiss Axioskop 2 Plus microscope (Carl Zeiss, Gottingen, Germany). Cells were photographed using either a Canon Powershot G1 digital camera (Canon, Tokyo, Japan) or a Zeiss Axiocam Hr digital camera (Carl Zeiss, Gottingen, Germany). Cell length, width and girdle displacement were averaged when possible from measurements of at least 20 cells in mid-exponential growth phase.

6.2.3. Scanning electron microscopy

Cultures were fixed for SEM with equal volumes of 4% osmium tetroxide (OsO4), mounted, dehydrated and coated as outlined in Chapter 2. The resulting preparations were observed in a JEOL JSM 840 scanning electron microscope (JEOL, Tokyo, Japan).

6.2.4. DNA extraction, PCR amplification and cycle sequencing

For cultured strains, approximately 5 ml of autotrophically grown culture were extracted by gentle lysis (Bolch *et al.* 1998). Extracted DNA was used as a template to amplify approximately 900 bases of the large subunit ribosomal gene (LSU rDNA), encompassing the D1, D2 and D3 domains.

For field samples of unculturable species (*Karenia* sp. 'A' and *Karenia* sp. 'D'), four cells were isolated with a micropipette, rinsed three times in sterile medium, and added to PCR tubes containing ready-to-go PCR reaction mix. Tubes were held in ice during the isolation process and transferred to the thermal cycler immediately afterwards (Bolch 2001). Primers, reaction conditions and cycle sequencing parameters are described in detail in Chapter 2.

6.2.5. Sequence alignment and phylogenetic analysis

Corrected sequences were aligned to other gymnodinioid sequences (Table 6.1) using ClustalX (Thompson *et al.* 1997), and alignments were refined by eye. Sequences of some undescribed species have not been submitted to GenBank as work on these is still in progress. Phylogenetic analyses using maximum parsimony (MP) and distance search criteria were carried out using PAUP 4.0 (Swofford 1999), both including and excluding the hypervariable domain (equivalent to bases 568-675 of the *Karenia brevis* sequence, accession number AF200677). Bayesian analysis of likelihood was carried out using MrBayes (Huelsenbeck and Ronquist 2001), searching for 1.2 million generations, using a general time-reversible model, with gamma shaped among-site rate variation; the consensus tree was drawn excluding the first 200,000 generations, to allow the –Ln to stabilise.

Table 6.1: Origin (where known) and GenBank accession numbers of sequences used in phylogenetic analyses.

Sequence name	Origin (where known)	Accession number
Akashiwo sanguinea	Japan	AF260397
Alexandrium catenella	U.S.A.	AF200667
Gymnodinium aureolum Adel	Adelaide, South Australia	AY263965
G. aureolum S1	U.S.A.	AF200670
G. catenatum	Vigo, Spain	AF200672
G. chlorophorum	Sylt, Germany	AF200669
G. falcatum	South Australia	AY320049
G. fuscum	La Trobe, Australia	AF200676
G. impudicum	Naples, Italy	AF200674
G. microreticulatum	Eastern Australia	AY036078
G. nolleri	Øresund, Denmark	AF200673
G. palustre	---	AF260382
G. cf. placidum	Isefjord, Denmark	AF260383
G. uncatenum	River Derwent, Tas.	not submitted
G. uncatenum	North West Bay, Tas.	not submitted
Karenia asterichroma	Tasmania	AY590123
K. bidigitata	New Zealand	U92251
K. brevis	U.S.A	AF200677
K. brevisulcata	New Zealand	AY243032
K. cristata	South Africa	AY525907
K. mikimotoi	U.K.	AF200678
K. mikimotoi	Australia	AF200679
K. papilionacea	Tasmania	AY590124
K. papilionacea	New Zealand	U92252
K. selliformis	Chile	AF318247
K. selliformis	New Zealand	U92250
K. umbella	Triabunna, Tasmania	AY263962
K. umbella	Taranna, Tasmania	AY263963
Karenia sp. 'A'	Parsons Bay, Tasmania	not submitted
Karenia sp. 'B'	Parsons Bay, Tasmania	not submitted
Karenia sp. 'C'	Parsons Bay, Tasmania	not submitted
cf. Karenia sp. 'D'	Perth, Western Australia	not submitted
Karlodinium micrum	Norway	AF200675
K. micrum	New Zealand	U92257
K. micrum	Western Australia	AY263964
K. australe	Grants Lagoon, Tasmania	not submitted

K. australe	Tuggerah Lake, N.S.W.	not submitted
Takayama helix	N.W.B., Tasmania	AY284950
T. helix	Port Arthur, Tas.	AY284951
T. tasmanica	River Derwent, Tasmania	AY284948
T. tasmanica	Tuggerah Lake, N.S.W.	AY284949
Takayama sp.	Kawau Is., New Zealand	U92254
Woloszynskia pseudopalustris	---	AF260402

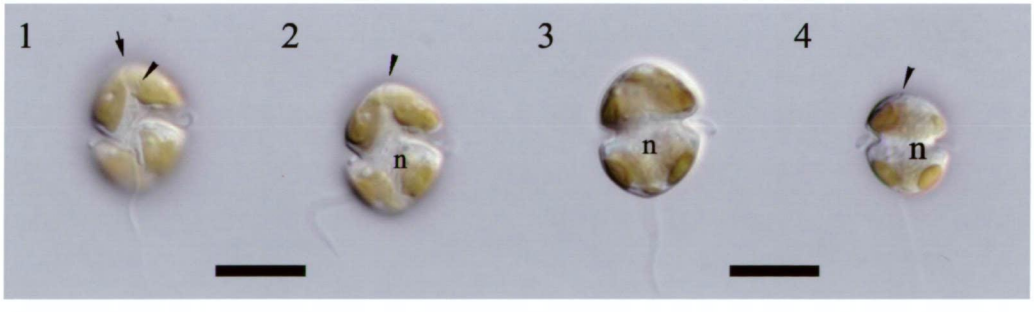
6.3. RESULTS:

6.3.1. *Karlodinium micrum* (Leadbeater & Dodge) J. Larsen

Daugbjerg *et al.* 2000, Figs 3-10

Morphology

Cells of *K. micrum* in Australia were on average 12 µm long and 10 µm wide. The sulcus extends into the epicone. The cingulum is displaced up to 25% of the total cell length. Apical groove short and straight. Ventral pore present, to the left of the sulcal extension. Nucleus generally central but sometimes slightly displaced apically or antapically. Chloroplasts normally 4 but up to 8, peripheral with individual pyrenoids. Figures 6.1-6.5 illustrate some of the typical morphological features of this species.



Figures 6.1-6.4: Light micrographs of *Karlodinium micrum* from the River Derwent, Tasmania. Scale bars = 10 µm.

Fig. 6.1: Ventral view in surface focus showing linear shaped apical groove (arrow) and ventral pore in epicone (arrowhead).

Fig. 6.2: Subsurface focus of the same cell showing subcentral position of the nucleus (n) and apical notch (arrow).

Fig. 6.3: Cross section focus through same cell showing position of the nucleus (n).

Fig. 6.4: Dorsal view of cell showing nucleus position (n) and brief dorsal extension of the apical groove (arrowhead).

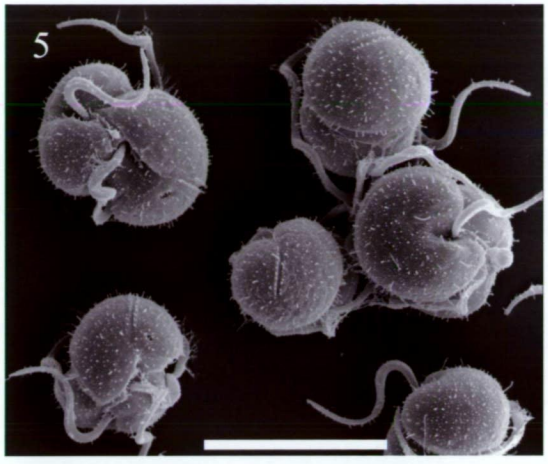


Figure 6.5: Scanning electron micrographs of *Karlodinium micrum* from Sydney Harbour, N.S.W. Scale bar = 10 µm.

Distribution and abundance

Karlodinium micrum has been found throughout southern Australian waters, from the Swan river in Western Australia to Sydney Harbour, New South Wales. It regularly blooms in the Swan river estuary, causing fish kills with widespread media attention. Blooms of this species have been recorded in Lake Illawarra (N.S.W.) in 1993, and Sydney Harbour in 2002. This species has been found in low numbers in Tasmanian, Victorian and South Australian waters. Table 5.1 in Chapter 5 lists Australian field samples containing this species and cultures isolated for this study.

Genetic relationships

The LSU sequence of Australian isolates of *K. micrum* is almost identical to the published sequence of Norway and New Zealand isolates. It is closely related to *Karlodinium australe*, and forms a monophyletic clade with the other fucoxanthin-containing genera *Karenia* and *Takayama* (Figs 6.26-6.31).

6.3.2. *Karenia papilionacea* Haywood *et* Steidinger

Haywood *et al.* 2004, Fig 2 (e)-(h), Fig. 3, Fig. 6

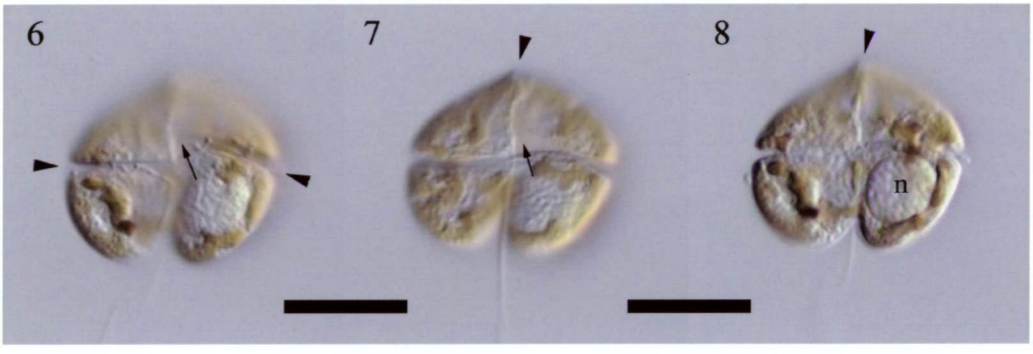
Morphology

Small to medium sized, dorsoventrally flattened dinoflagellate, 18-32 µm long, 18-48 µm wide, and 8-15 µm thick. Cells are ventrally concave, with the left and right sides of the cell angled towards the ventral surface. Apical

carina is present, with apical groove that extends shortly into dorsal surface. Hypotheca strongly bilobed, with deep sulcal excavation. Cingulum is premedian to median and often displaced by one width. There is a broad, open extension of the sulcus into the epicone. Nucleus spherical in left hypotheca. Chloroplasts are variable in number and size, yellow-green, reniform to round, and located peripherally, containing individual pyrenoids. Figures 6.6 – 6.11 show the typical morphology of cells encountered in Australia.

Distribution and abundance

In Australia cells of *K. papilionacea* have been recorded from the Swan River (W.A.) to Port Lincoln (S.A.) and eastern Tasmania (Table 6.2). Common



Figures 6.6-6.8: Light micrographs of a live field sample of *Karenia papilionacea* from Moulting Bay, Tasmania. Scale bars = 20 µm

Fig. 6.6: Surface focus of cell in ventral view showing open sulcal extension (arrow) into the epicone and little displaced cingulum (arrowheads).

Fig. 6.7: Same cell in subsurface focus, showing linear apical groove (arrowhead) and edge of the carina (arrow).

Fig. 6.8: Cross section focus through the same cell, showing apical notch (caused by apical groove) in the carina (arrowhead), and shape and position of the nucleus (n).

Table 6.2: Field samples containing *Karenia papilionacea* in Australian waters

Location	Date	Collector	Notes
Moulting Bay, TAS	31-03-2003	A.S.T. (Analytical services Tasmania	$9.6 \cdot 10^4$ cells L^{-1}
Ansons Bay, TAS	14-04-2003	I. Pearce	<1,000 cells L^{-1}
Port Lincoln, S.A.	31-05-2003	C. Wilkinson (S.A. Water)	
North West Bay, TAS	3-02-2003	M. de Salas	<100 cells L^{-1}
Parsons Bay, TAS	23-02-2003	Tassal	<100 cells L^{-1}

reports of *K. brevis* in Australian waters are likely to refer to this species. In Tasmania a bloom of $9.6 \cdot 10^4$ cells l^{-1} was recorded in Moulting Bay in late March 2003, and was found in low numbers throughout the austral summer and early autumn.

Genetic relationships

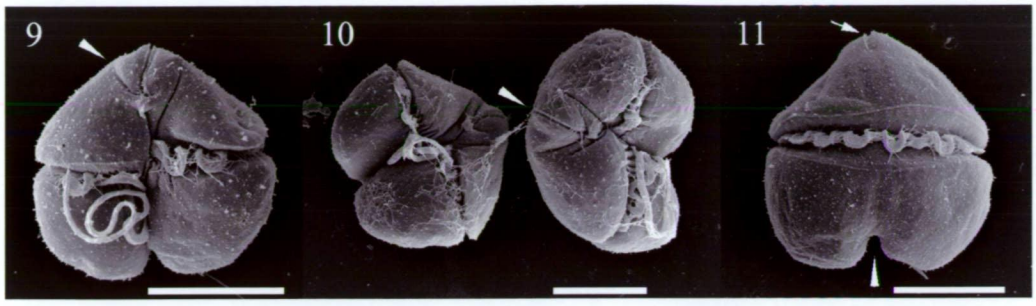
Phylogenetic analysis of the LSU rDNA sequence of *K. papilionacea* (Figs 6.26 – 6.31) show that Australian and New Zealand isolates of this species are genetically identical, and place this species clearly within the genus *Karenia*. However, it is not close to *K. brevis*, the species it most resembles morphologically. Different search criteria used in phylogenetic analyses conflict over the most basal placement within *Karenia*. While maximum parsimony and distance searches place *K. umbella* as the most primitive *Karenia* species (Figs. 6.26, 6.28, 6.30), some likelihood searches place *K. papilionacea* in this position (Fig. 6.31).

6.3.3. *Karenia mikimotoi* (Miyake & Kominami *ex* Oda) G. Hansen & Moestrup

Takayama 1984, Figs 1, 2

Morphology

Dorsoventrally flattened dinoflagellates, 20 – 30 µm long, 15-25 µm wide, and 12-17 µm thick. Epicone conical to hemispherical. Hypocone a truncated hemisphere, obviously indented by the sulcus. The cingulum is premedian, and displaced approximately 20% of the total cell length. The sulcus extends into the epicone as a wedge shaped notch. A narrow, linear apical groove originates to the right of the sulcal axis, and extends approximately 1/3 of the way down the dorsal epicone. The nucleus is rounded to elongated, often reniform, and situated in the left part of the cell, mostly within the left hypocone lobe. Chloroplast numbers are variable, but most commonly 10-15, elongated and irregularly shaped. Chloroplasts contain single, individual pyrenoids which are normally pyramidal in shape. Figures 6.12-6.14 show the typical morphology of culture material from the Australasian region (New Zealand).

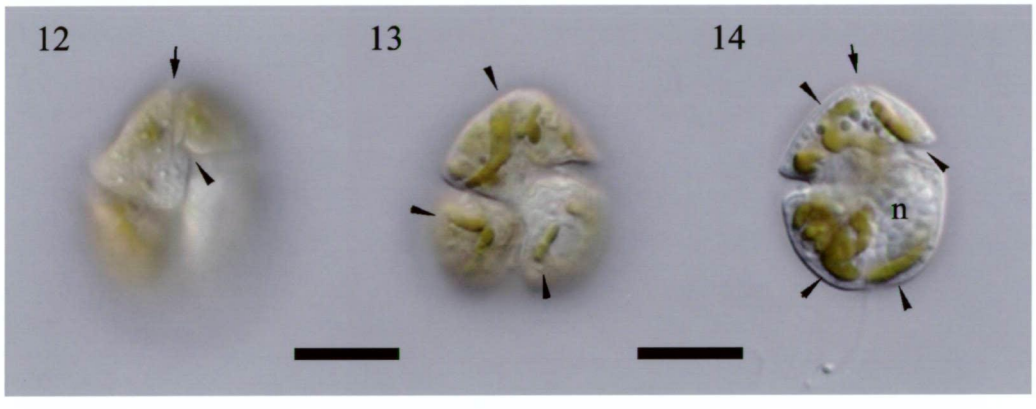


Figures 6.9-6.11: Scanning electron micrographs of *Karenia papilionacea* from Moulting Bay, Tasmania. Scale bars = 10 µm.

Fig. 6.9: Ventral SEM of cell showing apical carina (arrowhead).

Fig. 6.10: Pair of cells in ventral view. Note apical path of the apical groove (arrowhead).

Fig. 6.11: Dorsal SEM of cell showing dorsal extent of apical groove (arrow), and pronounced sulcal indentation (arrowhead)



Figures 6.12 – 6.14: Light micrographs of *Karenia mikimotoi* strain CAWD63, from the Cawthron Institute, New Zealand. Scale bar = 10 µm.

Fig. 6.12: Surface focus of cell in ventral view. Arrow points to linear apical groove. Note distinctive shape of sulcal extension into epicone (arrowhead)

Fig. 6.13: Subsurface focus of the same cell. Note position and shape of the chloroplasts (arrowheads).

Fig. 6.14: Central focus of cell. Note position of the nucleus (n) in the left hypocone lobe, notch caused by the apical groove (arrow) and peripheral chloroplasts (arrowheads).

Distribution and abundance

Karenia mikimotoi is a common species in the phytoplankton flora of temperate southern Australia (Hallegraeff 2002), but uncommon in Tasmania. Larsen (1994) reports this species in Port Phillip Bay, especially Hobson's Bay during the austral summer. Blooms of this species have been a regular feature in the Gippsland Lakes (G. Hallegraeff, pers. comm.), and low cell

numbers are regularly detected in Port Lincoln, South Australia (Clinton Wilkinson, SA Water, pers. comm.).

Genetic relationships

Australian *Karenia mikimotoi* is genetically identical to material from New Zealand, Norway, Denmark, United Kingdom, and Japan, varying by no more than 2 base pairs in the >900 bp sequence. long D1-D3 section of the LSU rDNA (Daugbjerg *et al.* 2000). The closest sequence in terms of pairwise differences to *K. mikimotoi* was found to be *K. brevis*. Trees of proposed phylogenies of this and related species are illustrated in Figures 6.26 – 6.31.

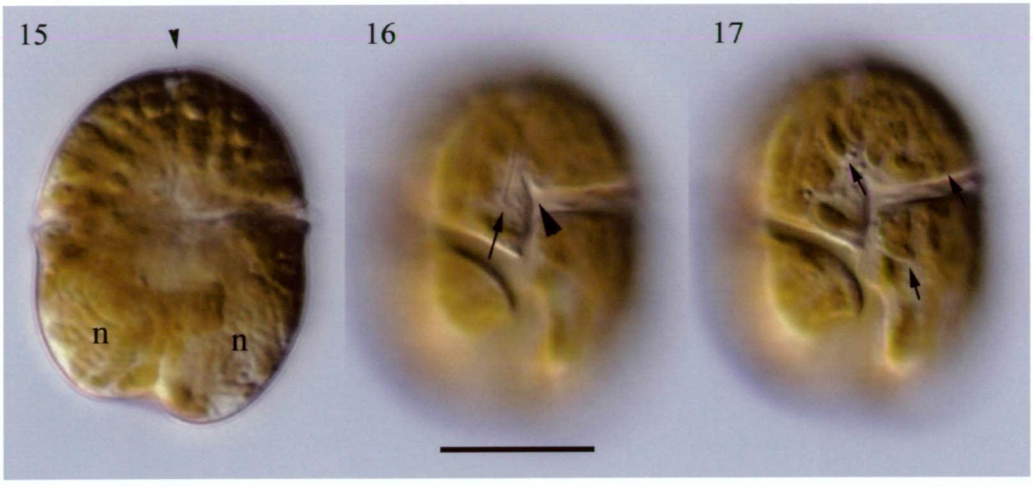
6.3.4. *Karenia* species 'A' (antapical nucleus)

Morphology

Medium sized, globular unarmoured dinoflagellate, with no dorsoventral flattening. Length 38 μm, width 32 μm. Epicone hemispherical or elongated with a hemispherical apex. Hypocone truncated and incised deeply by the sulcus. Apical groove long and straight. Sulcus wide and invading the epicone as a finger-like projection. Cingulum displaced about ¼ of the cell length. Chloroplasts numerous and irregularly shaped, mostly globular. Nucleus very large, occupying most of the hypocone. Figures 6.15-6.17 show the internal and external morphology of cells of this species from the Parsons Bay, May 2003 fish kill.

Distribution and abundance

Cells of this species have only been recorded from south-eastern Tasmania (Table 6.3), where a bloom containing this and four other species (*K. umbella, K. asterichroma, Karenia* sp. 'B' and *Karenia* sp. 'C') was responsible for the mortality of approximately 100,000 Atlantic salmon (*Salmo salar* L.). During the austral summers of 2003 and 2004, low background numbers of this species were present in many SE Tasmanian locations, but the bloom of May 2003 (approximately 10^5 cells l^{-1} total *Karenia* concentration) occurred very suddenly, possibly indicating an offshore origin. Cells of this species could not be cultured using standard media formulations [GSe and GSe/2 (Blackburn *et al.* 2001), F and F/2 (Guillard 1983) and K (Keller *et al.* 1987].



Figures 6.15-6.17: Light micrographs of *Karenia sp.* 'A' from Parsons Bay, Tasmania. Scale bar = 20 µm.

Fig. 6.15: Cross section view of cell showing position of the nucleus at the cell antapex (n), and apical notch in the path of the apical groove (arrowhead).

Fig. 6.16: Ventral view of cell in surface focus showing long, linear apical groove (arrow) and extension of the sulcus onto the epicone surface (arrowhead).

Fig. 6.17: Subsurface focus of same cell, showing peripheral distribution of globular chloroplasts with individual pyrenoids (arrows).

Table 6.3: Distribution of *Karenia* sp. 'A' in Tasmanian waters

Location	Date	Collector	Notes
Pirates Bay, TAS	10-05-2003	M. de Salas	
Parsons Bay, TAS	13-05-2003	TASSAL	$1 \cdot 10^5$ cells L^{-1} total *Karenia*
Port Arthur, TAS	14-05-2003	TASSAL	
North West Bay, TAS	3-02-2004	M. de Salas	<100 cells L^{-1}
River Derwent, TAS	5-03-2004	G. Wallace	<100 cells L^{-1}
Parsons Bay, TAS	16-03-2004	M. de Salas	<100 cells L^{-1}
Pirates Bay, TAS	16-03-2004	M. de Salas	<100 cells L^{-1}

Genetic relationships

The LSU rDNA sequence of this species (obtained by performing PCR on single cells from field samples) differs by approximately 4.8% from its closest relative, *K. umbella*, Phylogenetic trees of known sequences of *Karenia* species place the *K. umbella* and *Karenia* sp. 'A' together in a well-supported clade (Figs 6.26-6.31), either basally within the genus, or with only *K. papilionacea* basal to them.

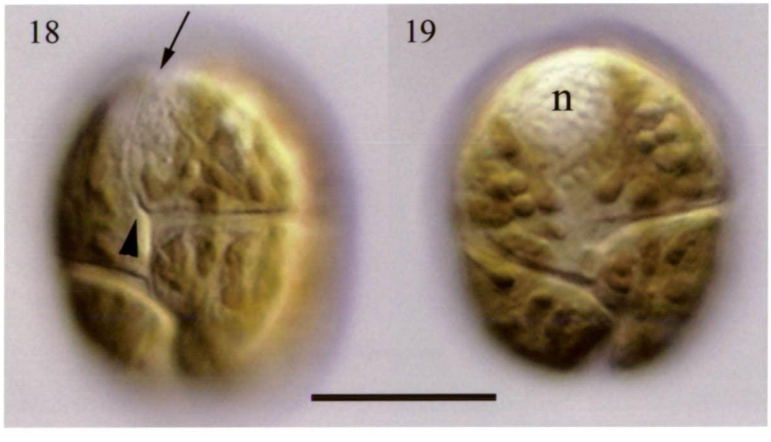
6.3.5. *Karenia* sp. 'B': (apical nucleus)

Morphology

Medium sized, globular unarmoured dinoflagellate, with no dorsoventral flattening. Length 35 µm, width 30 µm. Epicone hemispherical or elongated with a hemispherical apex. Hypocone hemispherical but sometimes truncated. Apical groove long and straight. Sulcus wide and invading the epicone as a finger-like projection. Cingulum displaced about ¼ of the cell length. Chloroplasts numerous and irregularly shaped, mostly globular. Nucleus small, located at the apex of the cell. Figures 6.18-6.19 show the surface and internal morphology of cells from the May 2003 fish-kills.

Distribution and abundance

Karenia sp 'B' cells have been discovered in water samples from south-eastern Tasmania (Table 6.4), where it bloomed in May 2003 (see *Karenia* sp. 'A' section). During the austral summers of 2003 and 2004, low background numbers of this species were present in many SE Tasmanian locations. In both years *Karenia* sp 'A' was more abundant, with only half to 1/4 the cell numbers of *Karenia* sp. 'B'. Cells of this species could not be cultured using standard media formulations (GSe and GSe/2, F and F/2, and K).



Figures 6.18-6.19: Light micrographs of *Karenia* sp. 'B' from Parsons Bay, Tasmania. Scale Bar = 20 µm.

Fig. 6.18: Surface focus of cell in ventral view. Note linear apical groove (arrow) and sulcal extension into epicone (arrowhead).

Fig. 6.19: Subsurface focus of another cell in ventral view showing apical location of the nucleus (n).

Table 6.4: Distribution of *Karenia* sp. 'B' in Tasmanian waters

Location	Date	Collector	Notes
Pirates Bay, TAS	10-05-2003	M. de Salas	
Parsons Bay, TAS	13-05-2003	Tassal	$1 \cdot 10^5$ cells L^{-1} total *Karenia*
Port Arthur, TAS	14-05-2003	Tassal	
River Derwent, TAS	18-03-2004	M. de Salas	<100 cells L^{-1}

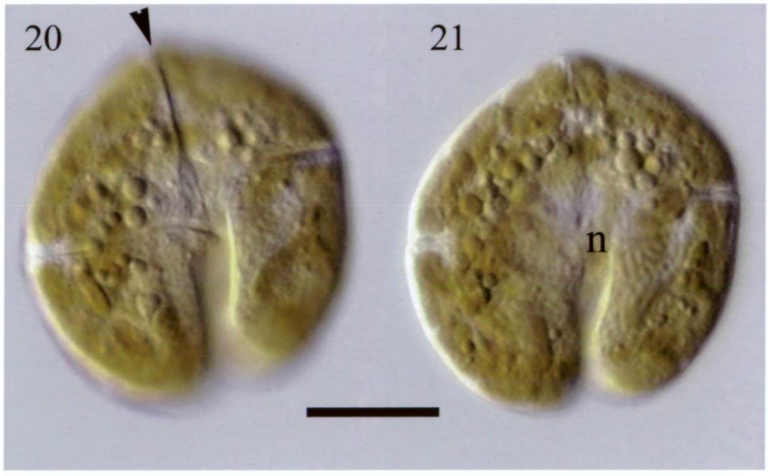
Genetic relationships

Karenia sp. 'B' could not be cultured, and attempts to sequence single cells from field samples failed.

6.3.6. Karenia sp. 'C': (disc shaped)

Morphology

Large, disc-shaped, extremely dorsoventrally flattened cells, 50 µm long and 50 µm wide. Epicone with long, straight apical groove. Hypocone incised by the sulcus. Nucleus centrally located. Chloroplasts numerous and evenly distributed through the cell. Figures 6.20-6.21 show the morphology of cells of this species from the May 2003 fish-kill.



Figures 6.20-6.21: Light micrographs of *Karenia* sp. 'C' from Parsons Bay, Tasmania. Scale Bar = 20 µm.

Fig. 6.20: Surface focus of cell in ventral view. Note the displacement of the cingulum, indentation of the sulcus, and the linear apical groove (arrowhead).

Fig. 6.21: Subsurface focus of same cell showing the central position of the nucleus (n), and the pronounced indentation of the sulcus.

Distribution and abundance

Uncommon species found in southern Tasmania (Table 6.5) during the late austral summer and early autumn. Low numbers of cells were present during the May 2003 fish kill in Parsons Bay, Tasmania, and again in March 2004.

Genetic relationships

Karenia sp. 'C' could not be cultured and attempts to sequence single cells from field samples failed.

6.3.7. cf. *Karenia* sp. 'D' (flat with uneven hypocone)

Morphology

Cells of this species are dorsoventrally flattened, 18 µm long and 18 µm wide. Epicone is disc-shaped with a long, slightly curved apical groove. Hypocone is truncated and incised, with left lobe significantly shorter than right lobe. There no a long linear apical groove. The nucleus is anteriorly placed, but extends into the left hypocone lobe. There are approximately ten chloroplasts which are sausage-shaped and contain individual pyrenoids. Figures 6.22-6.25 show the morphology cells in a field sample from the Swan River (W.A.).

Distribution and abundance

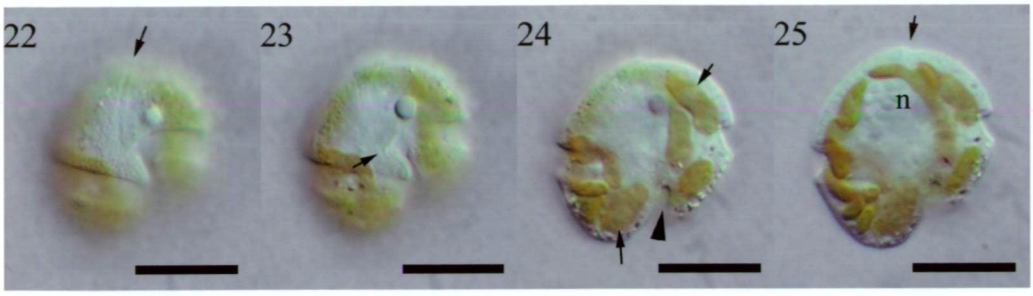
This species has been found in lagoon and estuary habitats in the Swan River (W.A.) Ansons Bay and Grant's Lagoon (northern Tasmania – Table 6.6). They are uncommon and have proven impossible to culture. They have a very characteristic swimming behaviour, looping around in the same space several times before swimming in a straight line and resuming circling, reminiscent of the typical swimming behaviour of *Scrippsiella* spp.

Genetic relationships

Although this species could not be cultured, PCR was successful on single cells from field samples. Phylogenetic analyses (Figs 6.26-6.31) place this

Table 6.5: Distribution of *Karenia* sp. 'C' in Tasmanian waters

Location	Date	Collector	Notes
Pirates Bay, TAS	10-05-2003	M. de Salas	Very rare
Parsons Bay, TAS	13-05-2003	Tassal	Rare
Parsons Bay, TAS	16-03-2004	M. de Salas	one cell only



Figures 6.22-6.25: Light micrographs of *cf. Karenia* sp. D. Scale bars = 10µm.

Fig. 6.22: Surface focus of cell in ventral view. Note long apical groove (arrow).

Fig. 6.23: Subsurface focus of same cell. Sulcus intrudes sideways into epicone.

Fig. 6.24: Deeper subsurface view showing deep sulcus (arrowhead) and chloroplasts with individual pyrenoids (arrows).

Fig. 6.25: Cross section through cell showing apical notch (arrow) and anterior nucleus (n). Nucleus extends into left hypocone (not shown)

Table 6.6: Distribution of *cf. Karenia* sp. 'D' in Australian waters

Location	Date	Collector	Notes
Swan River, W.A.	27-04-2003	W. Hosja	Rare
Ansons Bay, TAS	14-04-2003	I. Pearce	<100 cells L^{-1}
Grants Lagoon, TAS	14-04-2003	I. Pearce	<100 cells L^{-1}

species within the clade formed by *Karenia*, *Karlodinium* and *Takayama*, but different analyses place it either as basal to *Takayama* and *Karlodinium* (which form a well supported subclade), or as basal to the whole group.

6.4. DISCUSSION

This chapter circumscribes several species encountered during the course of this study, and which have proven or potential fish-killing ability. Some of these species had previously been described by other authors, and others could not be cultured, and thus were not characterised in sufficient detail for their formal description in this study. However, it was possible to obtain DNA from several of these species, which allows for their identification through genetic means.

The fish-killing dinoflagellate *Karlodinium micrum* has been responsible for the largest proportion of dinoflagellate- caused fish-kill events reported in Australia. It is abundant in locations such as the Swan River (Western Australia), where they bloom almost annually, and in the New South Wales coast, where blooms are not uncommon. *K. micrum* is a widespread species

that is most likely cosmopolitan (Deeds *et al.* 2002; Hallegraeff 2002; Kempton *et al.* 2002). Because of its small size and fast swimming behaviour, this species is difficult to identify, and is probably often reported as *Gymnodinium estuariale* or *G. simplex*. It is genetically closely related to *Karlodinium australe* (though to a lesser degree than species in the ralated genera *Karenia* and *Takayama* are to each other), and morphologically similar to *Gyrodinium corsicum* and *Karenia digitata.*

A larger, dorsoventrally flattened fish-killing dinoflagellate is *Karenia mikimotoi,* which has been reported from Hobsons Bay (Victoria) by Larsen (1994), and has been found in low numbers in Tasmanian and South Australian waters. Although genetically identical, isolates from New Zealand, Japan, Australia and Europe are quite variable in shape, size and physiology (Hansen *et al.* 2000). However, a high variability is shown within natural populations of this species, and a large proportion of the geographic variability may be more reflective of natural variation within populations than between populations.

The recently described species *Karenia papilionacea* (Haywood *et al.* 2004) was found to be common in southern Australian waters, from Western Australia to Tasmania. The almost morphologically indistinguishable *Karenia brevis* (by its old name of *Gymnodinium breve*) has often been reported in Australian waters. However, the real *Karenia brevis* (Davis) G. Hansen & Moestrup, from Florida, has not been confirmed outside of this region and Japan, and Australian reports are probably misidentifications of *Karenia papilionacea* or other morphologically similar, flattened species. A natural bloom of 9.6 10^4 cells l^{-1} of this species caused no problems for marine life in Moulting Bay, north eastern Tasmania. No toxicity was associated with this bloom, and cultures grown in the laboratory tested negative for brevetoxin 2 and 3 when analysed by liquid chromatography – mass spectroscopy (P. McNabb, Cawthron Institute, N.Z., pers. comm.).

Two non-flattened *Karenia* species, present in high numbers during a multi-species bloom in May 2003, could not be cultured. *Karenia* sp. 'A' (which is morphologically similar to *K. umbella*, but with an antapical nucleus) and *Karenia* sp. 'B' (only distinguished under the light microscope by its

anteriorly placed nucleus) were the most abundant and second most abundant species during the bloom, respectively. *Karenia* species in this bloomed reached a combined density of 10^5 cells l^{-1} (Chapter 2, Chapter 5, de Salas *et al.* 2004). Although attempts to sequence *Karenia* sp. 'B' failed, a partial LSU rDNA sequence was obtained for *Karenia* sp. 'A' by performing PCR on single-cell isolates, which shows that genetically the closest relative of this species is the morphologically almost-indistinguishable *K. umbella*. It is likely that the group of non-flattened, elongated *Karenia* species formed by *K. umbella, Karenia* sp. 'A', *Karenia* sp. 'B', and *K. longicanalis* forms a single monophyletic clade that diverged early on, as in most phylogenetic analyses the flattened species form a distinct separate clade. Moreover, the morphology of the non-flattened clade is closer to that of the closely related genera *Karlodinium* and *Takayama*.

The sequence of *Karenia* sp. 'A' obtained from a field sample allows the design of genetic probes to distinguish this species from the virtually identical *Karenia* sp. 'B', *K. umbella* and *Karenia longicanalis*, once sequences are obtained for all of these species.

The last novel species identified in this chapter is *Karenia* sp. 'D', a flattened cell with a characteristic swimming behaviour. This is a species that according to the phylogenies proposed in figures 6.26-6.31 does not belong to any of the fucoxanthin-containing genera *Karenia*, *Karlodinium* and *Takayama* as currently circumscribed. This species appears to be basal in the group, either a close relative of *Karlodinium* and *Takayama*, or basal in the whole clade. It could be a link between the mostly flattened *Karenia* species, which are probably highly derived, and the non-flattened *Karlodinium* and *Takayama*, which are likely more primitive. Morphologically this cell is closer to *Karenia*, with an evident linear or bow-shaped apical groove, though its dorsal extent is unclear. Attempts to culture this species have failed, and this may reflect a form of obligate mixotrophy more extreme than that shown by *Karlodinium australe*.

The range of new and undescribed species encountered during the course of this study has made clear that the fucoxanthin-containing dinoflagellates are a highly diverse group and that we have only begun to expose its diversity. Of

notable importance to our understanding of the evolutionary history of this group is the position of *cf. Karenia* sp. 'D', which may be an ancestral species within the *Karenia-Karlodinium-Takayama* clade.

While the economic importance and human health implications of the fucoxanthin-containing dinoflagellate genera are widely recognised, one sign of the limits of our knowledge of this group is that every location sampled for this study contained potentially toxic gymnodinioid species that were previously undescribed. The combination of our limited awareness of the diversity in this group (species of which were always present in low numbers in water samples) and our limited knowledge of their optimal growth conditions prevents the accurate prediction of potentially toxic gymnodinioid blooms. Consequently the determination of which species in this group are toxic, their degree of toxicity (to establish regulatory limits for aquaculture operations), and their physiological characterisation to establish bloom conditions are future research priorities.

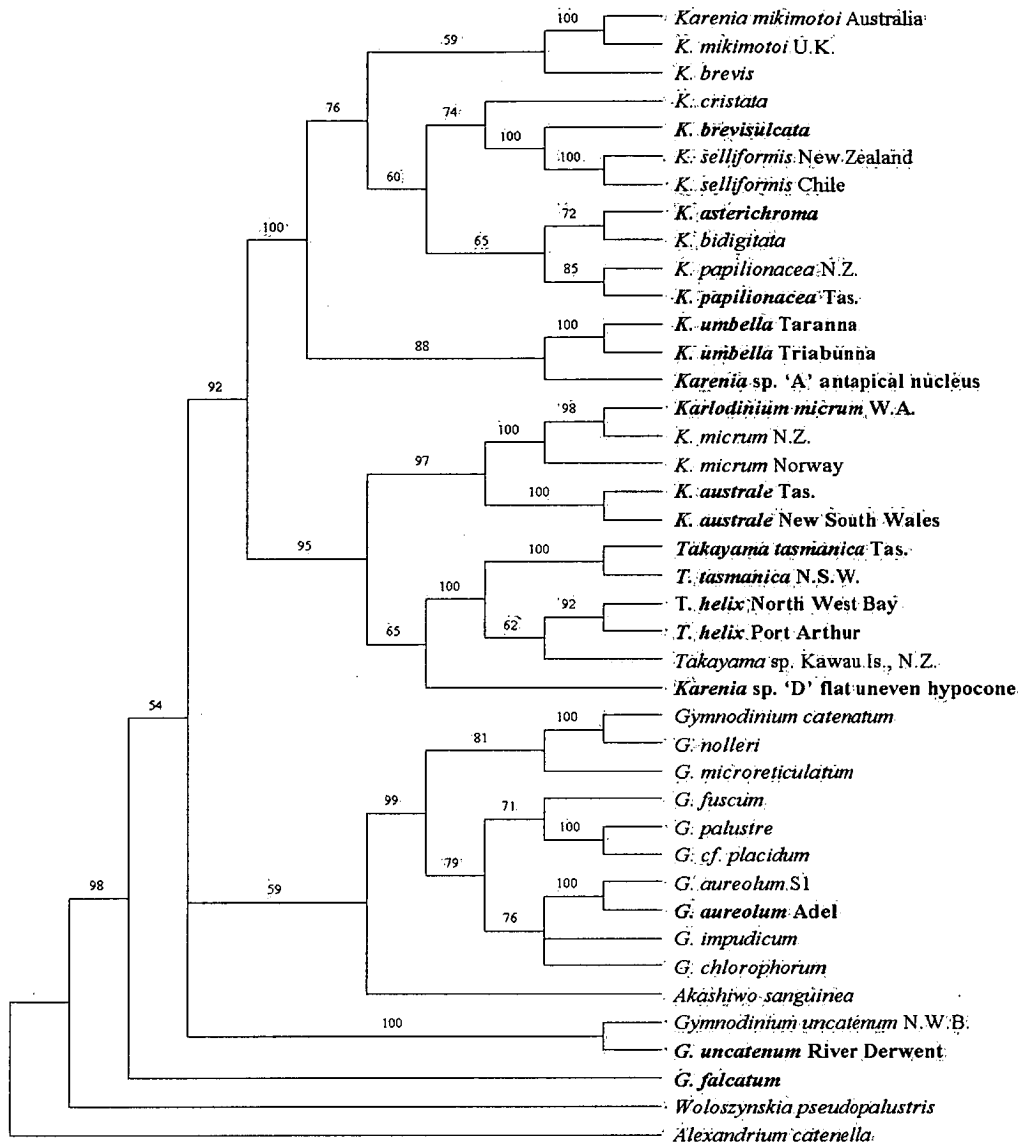


Figure 6.26: Minimum evolution (distance) bootstrap analysis of 1000 replicates using the entire D1-D3 sequence of the LSU rDNA. Outgroup = *Alexandrium catenella.* Values on branches indicate bootstrap support for groups. Tree length = 1949, consistency index = 0.514, retention index = 0.702. -Ln l = 10637.716.

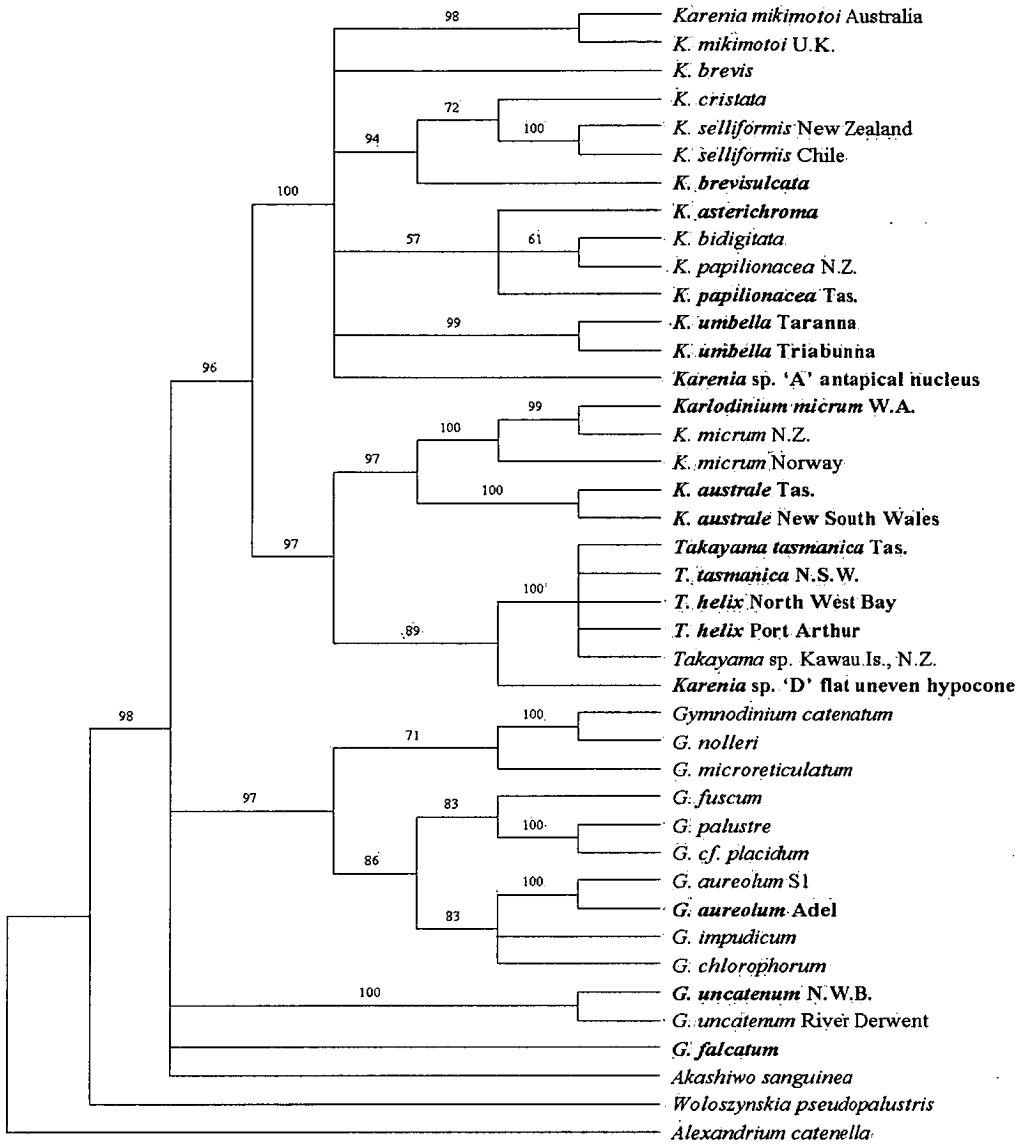


Figure 6.27: Minimum evolution (distance) bootstrap analysis of 1000 replicates of the D1-D3 region of the LSU rDNA, excluding the hypervariable domain. Outgroup = *Alexandrium catenella*. Values on branches indicate bootstrap support for groups. Tree length = 1369, consistency index = 0.519, retention index = 0.699. –Ln l = 7751.656.

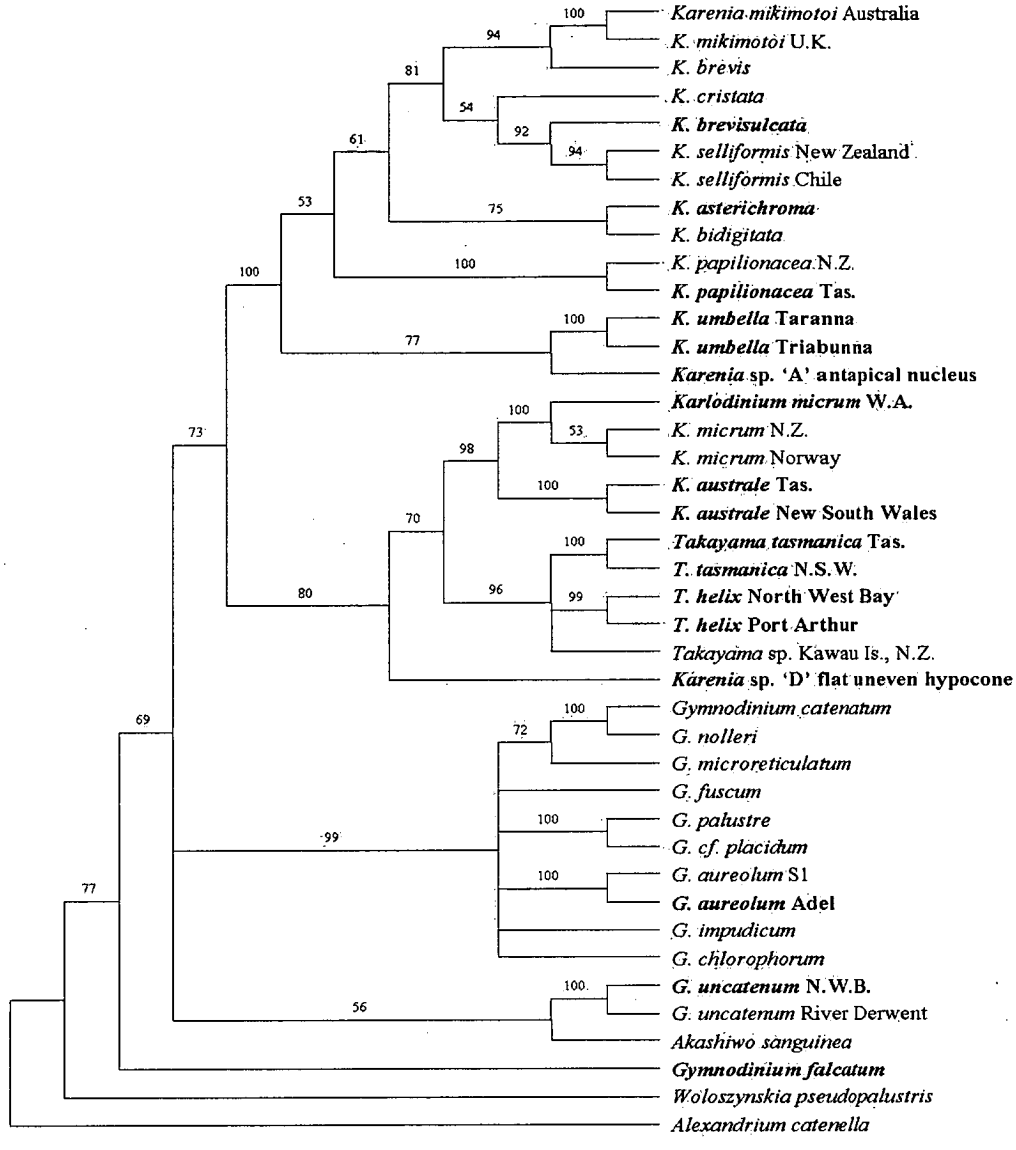


Figure 6.28: Maximum parsimony bootstrap analysis of 1000 replicates using the entire D1-D3 sequence of the LSU rDNA. Outgroup = *Alexandrium catenella*. Values on branches indicate bootstrap support for groups. Tree length = 1974, consistency index = 0.504, retention index = 0.694. –Ln l = 10655.125.

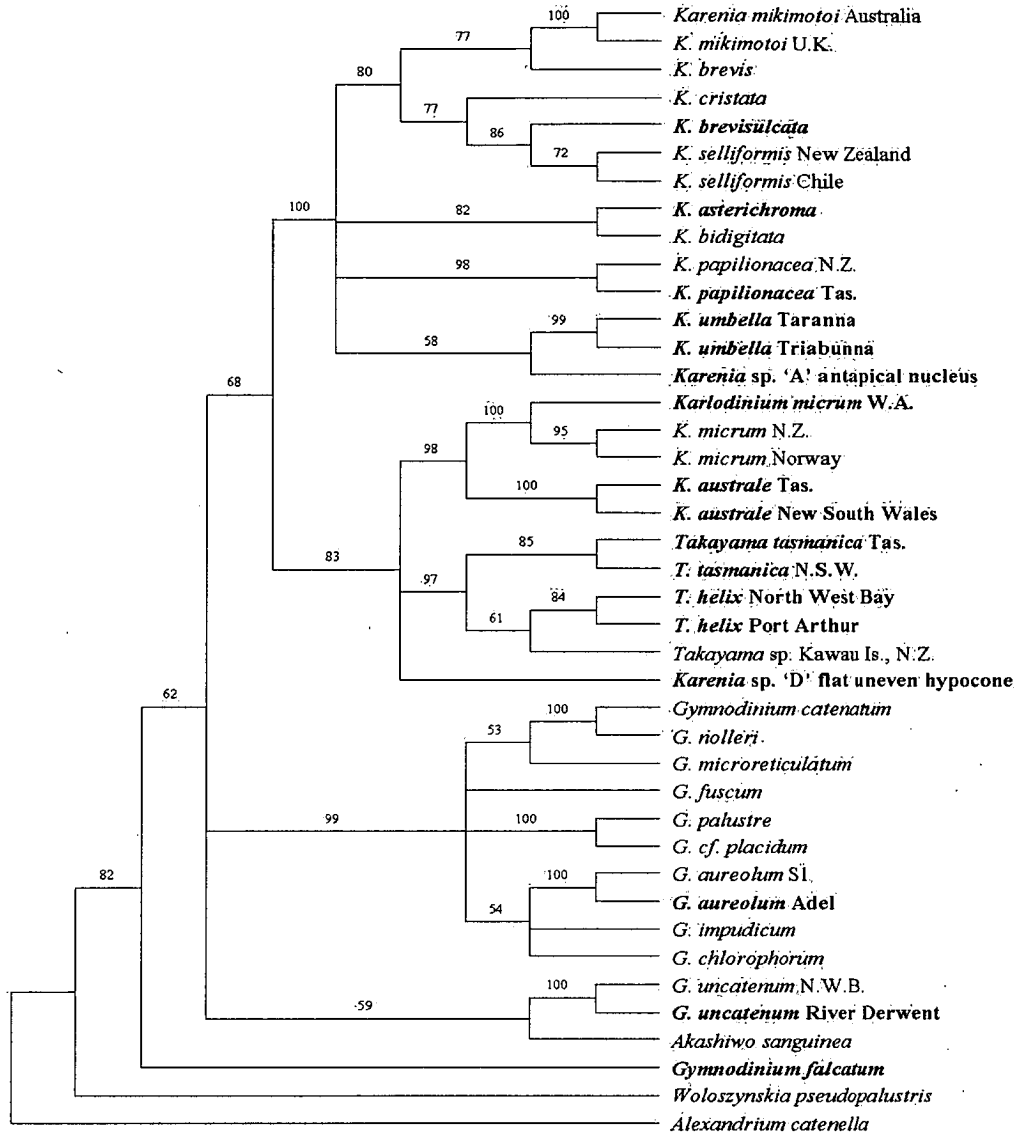


Figure 6.29: Maximum parsimony bootstrap analysis of 1000 replicates of the D1-D3 region of the LSU rDNA, excluding the hypervariable domain. Outgroup = *Alexandrium catenella*. Values on branches indicate bootstrap support for groups. Tree length = 1317, consistency index = 0.540, retention index = 0.723. –Ln l = 7541.298.

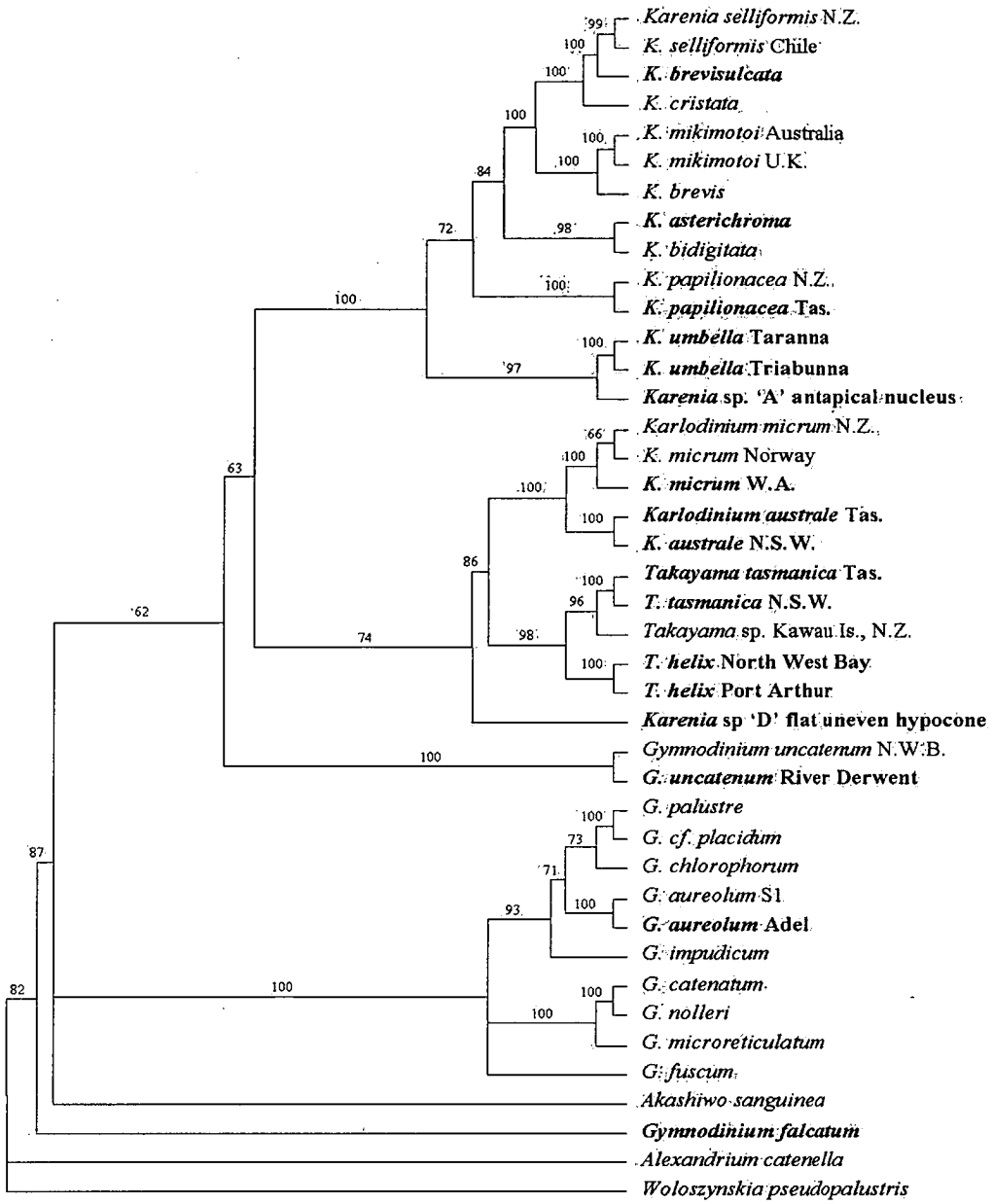


Figure 6.30: Proposed Bayesian inferred phylogeny of the main unarmoured dinoflagellate genera using the entire D1-D3 sequence of the LSU rDNA. Outgroup = *Alexandrium catenella*. Support values of branches were derived from a Bayesian analysis of $1.2 \cdot 10^6$ generations (excluding first $2 \cdot 10^5$ generations) using a general time-reversible evolution model with gamma distributed among-site rate variation. Tree length = 1942, consistency index = 0.516 and retention index = 0.704. –Ln l = 10609.237.

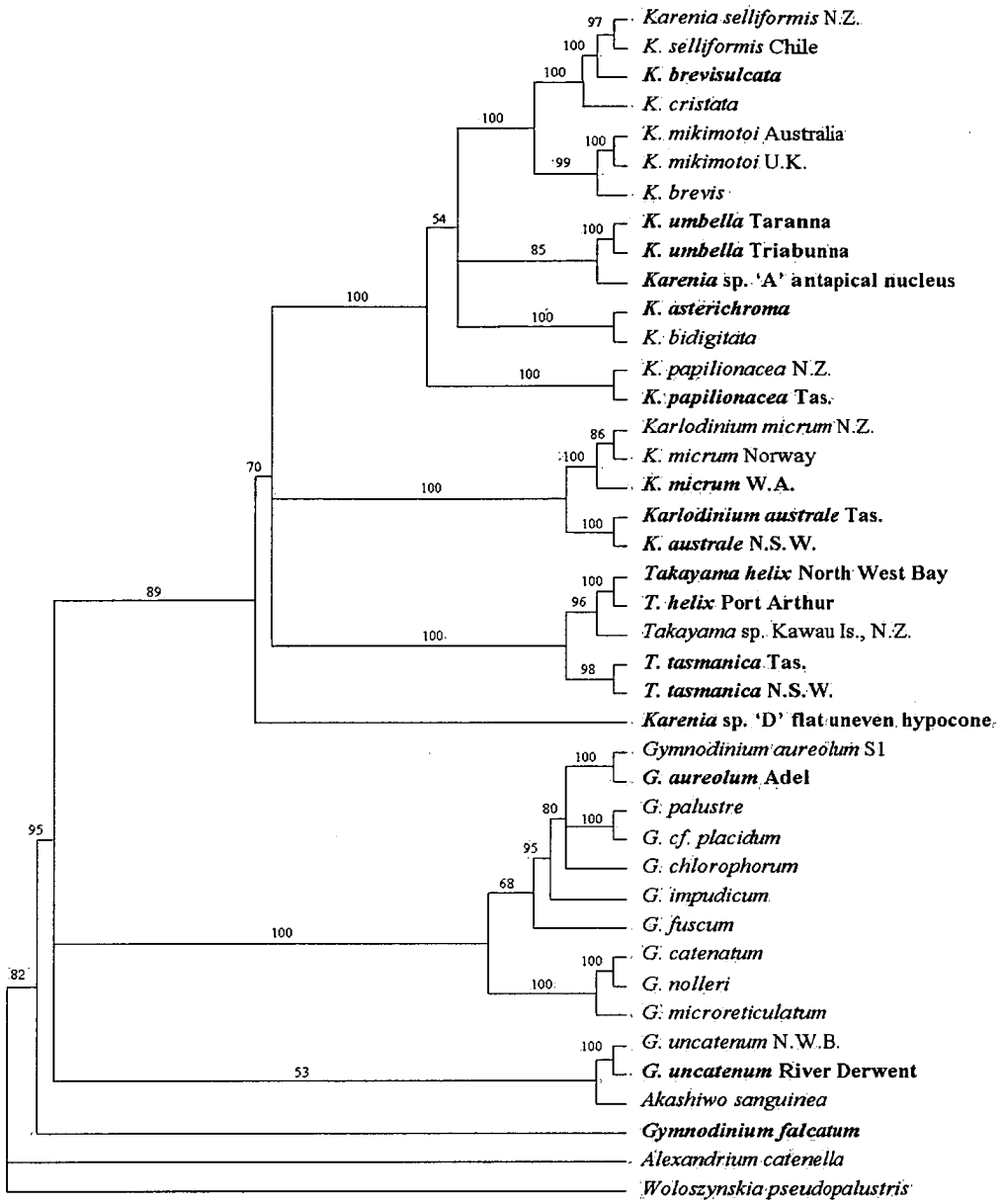


Figure 6.31: Proposed Bayesian inferred phylogeny (obtained using the D1-D3 region of the LSU rDNA, but excluding the hypervariable domain) of the main unarmoured dinoflagellate genera. Outgroup = *Alexandrium catenella*. Support values of branches were derived from a Bayesian analysis of $1.2 \cdot 10^6$ generations (excluding first $2 \cdot 10^5$ generations) using a general time-reversible evolution model with gamma distributed among-site rate variation. Tree length = 1947, consistency index = 0.515 and retention index = 0.703. –Ln l = 10625.359.

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CHAPTER 7:

CONCLUDING REMARKS ON AUSTRALIAN FISH-KILLING, UNARMOURED DINOFLAGELLATES, WITH PARTICULAR EMPHASIS ON THE FUCOXANTHIN-CONTAINING GENERA *KARENIA*, *KARLODINIUM* AND *TAKAYAMA*

Miguel Félix de Salas

7.1. AIM: TO CHARACTERISE AS FULLY AS POSSIBLE SEVERAL UNUSUAL OR NEW SPECIES BELONGING TO UNARMOURED, FISH-KILLING DINOFLAGELLATE GENERA

The primary aim of this study was to characterise as fully as possible several previously undescribed unarmoured dinoflagellate species discovered in Tasmanian waters.

In fulfilling this aim, the study, as detailed in Chapters 2-5, has employed morphology (in the form of light and electron microscopy), sequencing of part of the large subunit ribosomal RNA gene (LSU rDNA) and pigment analysis to describe five new species which were located in the waters of Australia.

The first two of these newly described species, in the recently erected, ichthyotoxic genus *Karenia* have been described from type localities in south-eastern Tasmania.

The first new species described during this research project, *K. umbella* (described in Chapter 2), is a non-flattened species morphologically similar to *K. longicanalis* (and also two new, undescribed species reported in Chapter 6). *K. umbella* differs from other non-flattened *Karenia* species in that it has been found to have an approximately central nucleus, an uneven hypocone (the right lobe longer than the left), has 8 radial furrows on the epicone surface, and a sulcal extension into the epicone. *K. umbella* (reported as *K. cf. mikimotoi*) has been associated with aquaculture fish kills in 1989 and again in 2003 (discussed in detail in Chapter 2).

The second new species described in this research project has been named *K. asterichroma* (described in Chapter 3). *K. asterichroma* is a flattened *Karenia* species morphologically resembling *K. brevis* and *K. mikimotoi*. While it was found to be present in the water in low numbers during a fish kill in 2003, its toxicity has not been established at this time. This species is distinct from other *Karenia* species in its anterior nucleus and its chloroplast arrangement. While other known *Karenia* species exhibit chloroplasts with internal pyrenoids, *K. asterichroma* has a central pyrenoid agglomeration from which chloroplasts radiate towards the surface.

A third new species belonging to the toxic genus *Karlodinium*, *K. australe,* has been described in Chapter 5. This species has been located in Grants Lagoon in north-eastern Tasmania, and has also been found in several locations in Australia's east coast. *Karlodinium australe* is a large species when compared to *K. micrum*, and has an anterior nucleus and an evident finger-like sulcal extension into the epicone. Unlike *K. micrum*, the chloroplasts of *K. australe* have internal pyrenoids that are pyramidal rather than lenticular. *K. australe* also lacks hexagonal arrays of plug-like structures in the amphiesma which are a defining feature of the genus *Karlodinium*. This research also established *K. australe* as a mixotrophic species that can prey on cryptomonads, and can ingest numerous cryptomonad cells in quick succession, accumulating a large number (>10) of food vacuoles (as shown in Chapter 5). Mixotrophy may be an obligate requirement of this species, as its culture in the absence of prey is difficult and slow.

Because our knowledge to date of the genus *Karlodinium* is limited, it is likely that an increase in awareness of this genus will show as much species diversity and morphological variability (such as chloroplast morphology) as in the genera *Karenia* and *Takayama*.

An important finding of this research is the two morphological characters of *K. australe* that are inconsistent with the current generic diagnosis of the genus *Karlodinium*. Despite this apparent conflict, *K. australe* is clearly identifiable as a *Karlodinium* species when considering its gross morphology, pigment profile, and LSU sequence. Consequently, this study suggests that the description of the genus *Karlodinium* needs to be emended in light of differing characters in this new species.

In addition to the three new species described from the fish-killing genera *Karenia* and *Karlodinium*, this study has erected a new genus of fish-killing dinoflagellates. The description of the new genus *Takayama* (in Chapter 4) has clarified the dubious taxonomic position of *Gymnodinium pulchellum* and other sigmoid-grooved, unarmoured dinoflagellate species. This new genus has been described with two new species: *T. tasmanica* and *T. helix*, from south-eastern Tasmania. In addition to the newly described species, three previously described, sigmoid grooved species in the unarmoured genera

Gymnodinium and *Gyrodinium* have been transferred to the new genus: *Takayama acrotrocha* (=*Gyrodinium acrotrochum*), *T. cladochroma* (=*Gyrodinium cladochroma*) and *T. pulchella* (=*Gymnodinium pulchellum*).

The new species *Takayama tasmanica* is morphologically similar to three previously described species (*Takayama acrotrocha, T. cladochroma,* and *T. pulchella*). However, this new species differs from any previously described by having a central pyrenoid from which branched chloroplasts radiate to the periphery. The second new species in this genus, *Takayama helix*, is morphologically distinct from all other species in this genus. While other species of *Takayama* have an apical groove with a pronounced inflection (clearly 'S' shaped), that of *T. helix* is nearly straight. In comparison with *T. tasmanica*, *T. helix* has peripheral chloroplasts with internal lenticular pyrenoids. Despite these differences, both of these species are shown to be congeneric by their pigment profiles, LSU sequences, and several recurring morphological features (discussed in Chapter 4).

Although the two new species of *Takayama* have not been implicated in fish kills during the course of this study, other species in this genus and most other fucoxanthin-containing dinoflagellate species are well-known fish killers. As a result, it can be assumed that any newly described species in these genera may exhibit the same toxicity.

7.2. AIM: TO DETERMINE WHETHER THE RECENTLY ERECTED GENERA *KARENIA* AND *KARLODINIUM* ARE VALID AND CAN BE SEPARATED ON THE BASIS OF MORPHOLOGICAL AND BIO-CHEMICAL CHARACTERS.

The determination of whether the genera *Karenia* and *Karlodinium* are valid as currently described and can be distinguished on the basis of their morphological and biochemical characters is of particular relevance because the genus *Karlodinium* is described from only one well known species (*K. micrum,* because *K. veneficum* appears to be synonymous). Therefore characters considered of taxonomic importance for *Karlodinium* at the generic level may instead simply reflect variation between species. Similarly the

genus *Karenia* was described based only on two species (*K. brevis* and *K. mikimotoi*) and may pose similar problems.

Our increasing awareness of the genus *Karenia* has grown at a faster rate than that of the other fucoxanthin-containing dinoflagellate genera, and at the time of writing contains eleven formally described species (*K. asterichroma, K. bicuneiformis / bidigitata, K. brevis, K. brevisulcata, K. cristata, K. digitata, K. longicanalis, K. mikimotoi, K. papilionacea, K. selliformis* and *K. umbella*). Eight of these species have been described in the time since the genus *Karenia* was erected, and they all have linear apical grooves, as well as fucoxanthin pigments or their derivatives. Up to this point, external morphology alone has consistently been sufficient [both in this study (Chapters 2, 3) and for other authors] for discriminating *Karenia* species at the generic level.

As a result, this study has determined that the genus *Karenia* is valid as currently described and its species can be identified on the basis of morphological and biochemical characters. Moreover, this genus can be identified exclusively on the basis of morphological characters visible under the light microscope.

In comparison to *Karenia*, the definition of the genus *Karlodinium* contains two morphological characters (lenticular pyrenoids and amphiesmal plugs) which this study has shown are absent from the new species *Karlodinium australe* (Chapter 5). Although this genus was erected based on two species, *K. micrum* and *K. veneficum*, these two species now appear to be synonymous (J. Larsen, pers. comm.). As a result, the shared characters between *K. micrum* and *K. veneficum* do not necessarily reflect consistent morphology at the generic-level, but rather at the species level.

Some features of the genus *Karlodinium* as currently described have been shown in this study to be consistent across species (see Chapter 5), for example fucoxanthin or its derivatives as the principal carotenoids (but not necessarily gyroxanthin-diester), a short, linear apical groove and a ventral pore above and to the left of a finger-like sulcal extension onto the ventral

epicone surface. As a result, this study suggests that these characters should be the focus of a necessary re-description of the genus *Karlodinium*.

7.3. AIM: TO IDENTIFY WHETHER FISH KILLING DINOFLAGELLATES OF THE FUCOXANTHIN-CONTAINING GENERA *KARENIA* AND *KARLODINIUM* FORM A MONOPHYLETIC CLADE WITHIN THE GYMNODINIOID DINOFLAGELLATES.

The final aim of this study was to establish whether the genera *Karenia* and *Karlodinium* (both of which have fucoxanthin as the main carotenoid pigment) form a monophyletic clade when analysed with new sequence information. Sequences are now available for several new *Karenia* and one new *Karlodinium* species, described after the two genera were first shown to form a clade within the Gymnodiniales.

This study found that not only do *Karenia* and *Karlodinium* species form a well-supported clade within this group (Chapters 2, 3, 6), but the new genus *Takayama*, described in Chapter 4, forms a third component of this clade that also contains fucoxanthin as the main carotenoid. Phylogenetic analyses show consistently that this new genus is more closely related to *Karlodinium* than to *Karenia*. Perhaps the sigmoid apical grooves of the genus *Takayama* represent an intermediate morphology between the linear- grooved genera *Karenia* and *Karlodinium*, and the horseshoe-shaped grooves of the genus *Gymnodinium sensu stricto*.

In conclusion, this study has shown an unexpectedly high degree of diversity within the monophyletic clade of dinoflagellates composed of the genera *Karenia*, *Karlodinium* and *Takayama*. These genera share some common characters, such as their toxicity, and a different pigment composition to other photosynthetic dinoflagellates (fucoxanthin and its derivatives rather than peridinin), while each possess a range of distinctive morphological characters that allow their differentiation using microscopy. While tools such as ultrastructure, pigment analyses and molecular sequencing are essential for discriminating between species within *Karenia, Karlodinium* and *Takayama*, they are ultimately unnecessary for assigning species to the three existing genera.

7.4. FUTURE RESEARCH

Determination of specific toxicity: Some species such as *Karlodinium micrum* and most *Karenia* are well known fish-killers. Others like *Karenia brevis* have long been recognised as the causative organisms of Neurotoxic Shellfish Poisoning (NSP) outbreaks, as well as killing fish. NSP has been recorded in many locations worldwide, including Australia and New Zealand, however, the real *K. brevis*, which is the only species so far conclusively linked to NSP outbreaks, has never been confirmed from outside the general area of the Gulf of Mexico. As many *Karenia* species remain to be described (see for example Chapter 6), there is a strong possibility that one or more of these may be responsible for occurrences of NSP outside the USA. As such effective monitoring and early warning systems for NSP will not be possible until the causative organisms have been identified and their toxicity assessed.

Independently of brevetoxin production, most of the species in the genera *Karenia, Karlodinium* and *Takayama* are capable of killing fish. Some preliminary results suggest that species in these genera are capable of producing reactive oxygen species and ichthyotoxic free fatty acids (J.A. Marshall, pers. comm.). Few species in this group have bloomed in high concentrations without detrimentally affecting marine life, except perhaps *Karenia longicanalis*. However, its bloom in Hong Kong Harbour closely followed one of *Karenia digitata* that caused massive mortalities.

This high occurrence of toxic species warrants the testing of the fish-killing ability of all species newly described in this group. An efficient monitoring program is indispensable for a viable aquaculture industry in locations that are susceptible to blooms of these species. Parameters such as optimum bloom conditions and minimum harmful concentration of cells in the water must be elucidated if such a monitoring program is to be implemented.

Research to establish both growth optima and ichthyotoxicity is ongoing.

Revisiting generic descriptions: The definition of the genus *Karlodinium* explicitly excludes species that lack lenticular internal pyrenoids and amphiesmal plugs. It has been made evident by this study that at least one

species in this genus does not correspond to the current description of the genus, and this description needs to be emended.

Development of RT-PCR assays: The extreme morphological similarity of some of the species in this group, for example *Karenia brevis* and *K. papilionacea*; *K. longicanalis*, *K. umbella*, *Karenia* sp. 'A' and *Karenia* sp. 'B'; or *Takayama* species except *T. helix*, makes their precise identification difficult. As such the development of straightforward assays for their presence or abundance is a clear priority. While rDNA targeted fluorescent probes are being developed for New Zealand *Karenia* species, their use has limitations. Whole-cell hybridisation, which can be used to estimate cell numbers in samples, suffers from problems of cell-membrane permeability or other issues affecting fluorescence uptake that may be cell-cycle dependent. Probes that work on cell extracts, while being more reliable, are not accurate at estimating cell concentrations, and only have a limited usefulness for detection.

The use of real-time quantitative PCR to detect these species seems to offer the best prospects of successful detection, combining reliability and replicability with the potential to estimate cell numbers in samples once the assay has been calibrated. As ribosomal DNA sequences can be easily obtained by conducting PCR on single cells from field samples, sequences can be obtained for unculturable species that would otherwise be impossible to work with. The flexibility and ease of design of both primers and probes allows the specificity of probes to be customised, potentially from detecting single species to closely related groups of species (i.e. non-flattened *Karenia* species – *K. umbella* and others), a whole genus, or all fucoxanthin-containing genera.

7.5. CONCLUSION

This study has made a substantial contribution to our current knowledge of the principal group of toxic and fish killing unarmoured dinoflagellates. This increased awareness must be put into practice to advance the research agenda developed in section 7.4. Only in this way can a comprehensive and effective early warning system be developed to identify species that have both economic and human health implications. Such an early warning system is

indispensable for a smoothly operating fin- and shellfish aquaculture industry, and is critical if human outbreaks of Neurotoxic Shellfish Poisoning are to be prevented.

The description and detailed characterisation of the five new species presented in this work will help design more effective monitoring tools, such as molecular probes, for the early detection of these potentially fish killing dinoflagellates.