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Biology of *Porphyra pulchella* sp. nov. from Australia and New Zealand

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Porphyra pulchella sp. nov. Ackland, West, Scott and Zuccarello was obtained at Mimosa Rock National Park, New South Wales; Westgate Bridge, Victoria, Australia; and Waihau Bay, North Island, New Zealand. It occurs mainly in mangrove habitats and is very small (± 1 mm) in field collections. In laboratory culture at 21 ± 2°C tiny blades (0.5-3.0 mm) reproduced exclusively by archeospores liberated from vegetative cells of the upper sector of the blades. The archeospores displayed amoeboid and gliding motility once discharged. At 14 ± 2°C the blades grew to 25 mm and produced longitudinal spermatangial streaks mixed with 'phyllosporangial' streaks. The discharged 'phyllospores' showed amoeboid motility and germinated forming asexual blades. A conchocelis phase with typical bangiophycidean pit connections was observed in blade cultures after 8-10 weeks at 14 ± 2°C. Conchocelis filaments produced conchosporangia and these released amoeboid conchospores that developed into archeosporangiate blades. Molecular data indicate that all 3 isolates are genetically identical.

Key Words: Australia, molecular phylogeny, New Zealand, *Porphyra pulchella* sp. nov. SSU rDNA, TEM

INTRODUCTION

Porphyra is a red algal genus that comprises over 130 species, many of which form a major component of rocky intertidal habitats (Yoshida 1997). Representatives of this genus are cosmopolitan, spanning temperate and cold water systems, with particularly high abundance recorded in the North Pacific Ocean (Mumford and Cole 1977). Whilst most *Porphyra* species are thought to be regionally confined, distribution of these species has not been thoroughly established (e.g. Nelson *et al.* 2001, 2003; Broom *et al.* 2004).

The commercial importance of *Porphyra* has generated much interest in its life history and systematics. Research has focused on the effects of environmental factors such as photoperiod, light intensity, temperature and nutrients, on the morphological and reproductive development of the species in culture (e.g. Frazer and Brown 1995; Nelson and Knight 1996; Kim and Notoya 1997;

Knight and Nelson 1999; Ruangchuay and Notoya 2003). The establishment of optimal developmental conditions for *Porphyra* species has not only expanded our knowledge on how to enhance cultivar yields, but has provided a means of assessing the impacts of harvesting wild *Porphyra* populations (Schiel and Nelson 1990). Furthermore, these studies have improved our understanding of the diversity within this genus.

Species of *Porphyra* are typically recognized by a biphasic life cycle in which there is an alternation between a macroscopic, gametophytic leafy blade phase and a microscopic, sporophytic filamentous phase called the 'conchocelis' (Drew 1949; Kurogi 1972; Nelson *et al.* 2001). It is, however, well established that many variations in the life history of *Porphyra* species exist. Conway and Wylie (1972) described *P. subtumens* (now *Pyrophyllon subtumens* (J. Agardh ex R.M. Laing) W.A. Nelson) as an asexual species, lacking the alternation of the diploid conchocelis phase. Similarly, populations of *P. sanjuanensis* V. Krishnamurthy lack the ability to sexually reproduce (Lindstrom and Cole 1990).

Despite the current knowledge on *Porphyra* life histo-

ries, the taxonomy of this genus remains problematic. *Porphyra* species are morphologically simple and subsequently have few morphological characters for species identification (Kunimoto *et al.* 1999; Broom *et al.* 2002). Moreover, morphological plasticity caused by environmental change can hamper the identification of species limits (Conway and Wylie 1972; Hannach and Waaland 1989). Species discrimination based on morphological analysis is a traditional technique now thought to be unreliable when used independently (Kunimoto *et al.* 1999; Nelson *et al.* 2003). Molecular techniques are accurate indicators of phylogenetic diversity now commonly used in combination with morphological data to identify taxa (Broom *et al.* 1999; Broom *et al.* 2002; Kunimoto *et al.* 1999; Nelson *et al.* 2001; Nelson *et al.* 2003). These studies have revealed that similarities in morphology do not necessarily infer close relationships, yet morphological variation is not always an indicator of phylogenetic divergence.

Species identifications based on nuclear SSU (18S) rDNA have unveiled a greater diversity of *Porphyra* than was previously recognised using morphological criteria alone (Broom *et al.* 1999; Nelson *et al.* 1998, 2001, 2003; Oliveira and Ragan 1994; Oliveira *et al.* 1995). The SSU rDNA locus is a slowly evolving gene (Hillis and Dixon 1991) that has been used to infer systematic relationships of red algal specimens at an ordinal and familial level (Saunders and Kraft 1994; Bailey and Freshwater 1997; Ragan *et al.* 1994). Whilst it is too conservative to be used to distinguish organisms at an interspecies level, the final third (3 end) of the SSU rDNA locus in *Porphyra* comprises variable domains which differ amongst species. These regions have proved useful for the discrimination of *Porphyra* entities at a species level (Broom *et al.* 1999; Kunimoto *et al.* 1999). Moreover, small subunit rDNA sequencing analyses have been used to reassess the taxonomic status of *Porphyra* species previously identified on the basis of morphological characters. Nelson *et al.* (2003) used this technique to establish that *Pyrophyllon subtumens*, *P. cameronii* (W.A. Nelson) W.A. Nelson and *Chlidophyllon kaspar* (W.A. Nelson and N.M. Adams) W.A. Nelson, originally placed in the genus *Porphyra* (order Bangiales), are members of the order Erythropeltidales. The results of this study signify that the morphological and anatomical characters originally used to identify these *Porphyra* species are a result of evolutionary convergence.

In this paper, we have described a new species of *Porphyra* (*P. pulchella*) based on morphology and life his-

tory in culture, TEM of the conchocelis phase and molecular analysis of the nuclear SSU rDNA locus. Two Australian populations of *P. pulchella* (isolates 3924 and 4422) were morphologically and genetically contrasted to a morphotype from New Zealand and previously described *Porphyra* entities, to confirm the taxonomic status of this species. The Australian isolates are especially distinct from most other *Porphyra* species because of the very small blade size (1-2 mm) seen in field collections and the unusual habitat, i.e., mangroves of temperate coastlines.

MATERIALS AND METHODS

Field collections

Foliose thalli (1-2 mm) of *Porphyra* (holotype isolate 3923) were collected on 16th December, 1998 from pneumatophores of the mangrove *Avicennia marina* (Forsk.) Vierh. at Nelson's Lagoon, Mimosa Rock National Park, New South Wales, Australia (36°41′ S, 149°59°E). A second collection (paratype isolate 4422) was made 6th April 2004 from a band of *Caloglossa vieillardii* (Kützing) Setchell on wood pilings at Westgate Bridge, Port Phillip Bay, Victoria, Australia (37°49′S, 144°53′E). A morphotype also examined, was collected 1st August 2003 from Waihau Bay east, North Island, New Zealand (37°37′S, 177°55′E). Terminology for reproductive structures and life history phases of *Porphyra* is in accordance with Nelson *et al.* (1999).

Culturing the blade phase

Thalli were given two mild osmotic shocks to kill various colourless flagellates. Blades were placed in a 60×15 mm Petri dish containing deionised water for 10 seconds, then transferred to Modified Provasoli's Medium [MPM/2 (West 2005), 10 ml of enrichment per litre of sterilised 30 psu natural seawater] for one minute, before receiving a second osmotic shock for ten seconds. Blades were then incubated in a 60×15 mm Petri dish containing MPM/2 treated with 25 μg/ml antibacterial ciprofloxacin hydrochloride (ciprofloxacin, Sigma Chemical Co. Pty. Ltd, St Louis, MO) for 48 hours to reduce bacterial contamination. Petri dishes were maintained in a controlled environment culture room at 21 ± 2°C, 12:12 LD photoperiod and 10-20 μmol photons m^{-2} s^{-1} of cool white fluorescent lighting. Clean blades were transferred into Pyrex[TM] (#3250, Corning Glass Works, Corning, NY, USA) 500 ml storage dishes and 60×15 mm Petri dishes containing 250 ml and 10 ml of medium,

respectively, and maintained in the above conditions. Archeospore discharge was induced by incubating blades with archeosporangia in fresh medium.

Archeospores were pipetted into 500 ml storage dishes, 60 × 15 mm Petri dishes and 10 ml plastic six-welled plates (Iwaki SciTech Div., Asahi Techno Glass, Funabashi, Japan) containing MPM/2 medium. A coverslip (22 × 22 mm or 22 × 50 mm, #1) was placed in each dish as a substratum for archeospore settlement.

Dishes were maintained in a controlled environment culture room or controlled environment E-36L plant growth chambers (Percival Scientific Inc., Perry, Iowa, USA) set to the desired photoperiod and temperature. Desired irradiance levels were obtained by covering fluorescent lamps with dark plastic window screen (1 mm mesh) and using cardboard boxes to elevate dishes closer to the light source. Dishes were placed on New Brunswick™ Model G2 rotary shakers (New Brunswick Scientific Co., New Brunswick, New Jersey, USA) set at approximately 80 rpm.

Culturing the conchocelis phase

Conchocelis filament tufts were treated in the same way as blades to eliminate colourless flagellates and reduce bacterial contamination, and cultured in the same light and temperature regime.

Sterilised mollusc shell pieces (area 1.0-1.5 cm^2, thickness 0.5-1.0 mm) were inoculated with five conchocelis filament tufts (0.2-0.5 mm in diameter) and placed in a 60 × 15 mm Petri dish containing MPM/2. Conchocelis filaments were secured to shells using a coverslip (22 × 22 mm, #3) and 2 mm glass rods. The coverslip and glass rods were removed after conchocelis filaments attached to the shell pieces.

All Petri dishes were sealed with parafilm to minimise evaporation. The medium was renewed every 3-10 days. Blades and conchocelis filaments were examined using a Zeiss GFL bright field compound microscope and a Zeiss dissecting microscope with a Zeiss LCD 1500 fiber optic light source. Photomicrographs were taken with a Zeiss MC 100 35 mm camera using Ektachrome 200 colour film. The transparencies were scanned with an Epson FilmScan 200 using Photoshop 5.0 software on a Macintosh G4 computer.

Transmission electron microscopy (TEM)

Conchocelis filaments were removed from culture dish bottoms and fixed for 90 minutes at ambient temperature in 2.5% glutaraldehyde or 1.5% paraformaldehyde in a 0.1 M phosphate buffer solution (pH 6.8) with 0.25 M sucrose. Following buffer rinses, samples were post-fixed in the same buffer for 90 min in 1% OsO$_4$ at ambient temperature, rinsed thoroughly in distilled H$_2$O, left in 50% acetone for 30 minutes and stored in a 70% acetone-2% uranyl acetate solution at ambient temperature for 4 hours. Samples were then dehydrated in a graded acetone series, infiltrated and embedded in EmBed 812 (Electron Microscopy Sciences, P.O. Box 251, 321 Morris Rd., Fort Washington, PA 19034) and polymerized at 70°C for 3 days. Thin sections were cut with an RMC MT6000-XL ultramicrotome, stained with lead acetate and viewed with a Zeiss EM 109 electron microscope.

Molecular phylogeny

DNA extraction followed a modified Chelex extraction method (Zuccarello *et al.* 1999). Amplification of an approximately 900 bp region of the nuclear-encoded small subunit of ribosomal RNA (SSU), corresponding to the final third of the molecule (Saunders and Kraft 1994; Broom *et al.* 1999) followed the procedure in Broom *et al.* (1999). All PCR products were electrophoresed in 1-2% agarose to check product size and sequenced following procedures in Zuccarello *et al.* (1999).

Sequences were assembled using the computer software supplied with the ABI sequencer, and aligned with Clustal X (Thompson *et al.* 1997). All sequences were compiled in Se-Al version2a11 (Rambaut 1996). Phylogenetic relationships were inferred with PAUP* 4.0b10 (Swofford 2002). Outgroups and related sequences used were selected from GenBank deposits and the accession numbers are indicated in Fig. 26. Outgroups used were *Erythrocladia* sp., *Erythrotrichia carnea* (Dillwyn) J. Agardh and *Smithora naiadum* (C.L. Anderson) G.J. Hollenberg.

Maximum-parsimony trees (MP) were constructed in PAUP*, using the heuristic search option, 500 random sequence additions, TBR branch swapping, unordered and unweighted characters, gaps treated as missing data. The program Modeltest version 3.6 (Posada and Crandall, 1998) was used to find the model of sequence evolution that best fits each data set by a hierarchical likelihood ratio test ($\alpha = 0.01$) (Posada and Crandall, 2001). When the best sequence evolution model had been determined, maximum-likelihood was performed in PAUP* using the estimated parameters (substitution model, gamma distribution, proportion of invariable sites) (5 random additions).

Support for individual internal branches was deter-

mined by bootstrap analysis (Felsenstein 1985), as implemented in PAUP*. For MP bootstrap analysis, 1000 bootstrap data sets were generated from resampled data (5 random sequence additions), for ML bootstrap analysis 100 bootstrap data sets were generated (1 random sequence addition).

RESULTS

All observations were made on specimens (isolates 3923 and 4422) grown in unialgal culture.

Porphyra pulchella J.C. Ackland, J.A. West, J. Scott and G.C. Zuccarello sp. nov.

Description: Laminae roseo-rubrae, stipiti carentes, rotundae vel ovatae (0.5-3 mm ad 21 ± 2°C) aut lineari-lanceolatae (10-25 mm ad 14 ± 2°C), in maturitate marginibus modice undulatis praebentibus thallis aspectum leviter undulatum; laminae monostromaticae 19-21 μm crassae. Divisiones cellularum diffusae marginales. Cellulae marginales uni- vel biseriatae, quadratae (20-24 μm) vel elongatae, 20-24 μm latae, 40-48 μm longae; cellulae centrales vegetativae demum irregulariter dispositae, polygonales (19-24 μm); cellulae basales irregulariter dispositae, polygonales et demum elongatae, cellulis centralibus vegetativis plerumque majores, chloroplastis singulis stellatis pyrenoide unica. Archaeosporangia secus marginem superiorem laminae prodientia, regulariter disposita, aliquantum quadrata (19-22 μm). Archaeosporae liberatae 10-14 μm in diametro, motum amoeboideum et prolabentem exhibentes. Planta monoica; spermatangia strias conspicuas elongatas pallidas secus laminam superiorem se praebentia cum striis phyllosporangiorum putatorum mixtas; 32 divisione [a/4, b/4, c/2] facta, 4-6 μm in diametro, dimissa immota. Phyllosporangia obscure roseo-rubrae, cellulis spermatangialibus majora, singula vel bina terna quaternave aggregata; phyllosporae 14-16 μm in diametro, motum amoeboideum exhibentes.

Conchocelis, praebens se caespitem densum e filamentis tenuibus ramosis uniseriatis constantem, includere se in conchas potest. Divisiones cellularum intercalares. Cellulae breves vel elongatae, 9-12 μm latae 32-40 μm longae, conjunctionibus primariis fovearum junctae; chloroplasti singuli elongati torti parietales pyrenoide unica vel pluribus. Conchosporangia obscure roseo-rubra, filamenta uniseriata et fasciculos 2-12-cellulares facta, a superficie visa isodiametra, brevia vel elongata, 10-27 μm lata 7-27 μm longa. Conchosporae liberatae 10-14 μm in diametro, motum amoeboideum exhibentes.

Blades pinkish-red, lacking stipe, round to ovate (0.5-3 mm at 21 ± 2°C) or linear-lanceolate (10-25 mm at 14 ± 2°C). Margins of mature blades moderately undulate giving thalli a slightly ruffled appearance. Blades monostromatic, 19-21 μm thick. Cell divisions diffuse and marginal. Marginal cells in one or two rows, quadrate (20-24 μm) to elongate, 20-24 μm wide and 40-48 μm long; central vegetative cells becoming irregularly arranged, polygonal (19-24 μm); basal cells irregularly arranged, polygonal and becoming elongate, mostly larger than central vegetative cells, chloroplasts single and stellate with one pyrenoid. Archeosporangia along the upper blade margin, regularly arranged, relatively quadrate (19-22 μm). Liberated archeospores 10-14 μm in diameter, exhibiting amoeboid and gliding motility. Monoecious; spermatangia conspicuous, elongate, pale streaks along the upper blade, intermixed with streaks of presumed phyllosporangia; 32 spermatangia formed by division [a/4, b/4, c/2], 4-6 μm in diameter, immotile when discharged. Phyllosporangia dark pinkish-red, larger than spermatangial cells, single or divided into groups of 2-4; phyllospores 14-16 μm in diameter and exhibiting amoeboid motion. Conchocelis pinkish-red dense tuft of fine, branched, uniseriate filaments with the ability to embed in mollusc shells. Cell divisions intercalary. Cells short to elongate, 9-12 μm wide and 32-40 μm long, connected by primary pit connections; chloroplast single, elongate, twisted and parietal with one or more pyrenoids. Conchosporangia dark pinkish-red, formed as uniseriate filaments and in clusters of 2-12 cells, isodiametric in surface view, short to elongate, 10-27 μm wide and 7-27 μm long. Liberated conchospores 10-14 μm in diameter, exhibiting amoeboid motion.

Holotype specimen: Blades (NSW 722255), conchocelis (NSW722445), Royal Botanic Gardens, Mrs. Macquaries Road, Sydney, NSW 2000, Australia. Collection data: 16 xii 1998, on pneumatophores of the mangrove *Avicennia marina* at Nelson's Lagoon, Mimosa Rock National Park, New South Wales (36°41′ S, 149°59′E).

Holotype culture: CCAP 1379/3. Culture Collection of Algae and Protozoa, SAMS Research Services Ltd., Dunstaffnage Marine Laboratory, Dunbeg, Argyll, PA37 1QA, UK. Blade and conchocelis phases of isolate 3923 are included.

Paratype: NSW 722446, Royal Botanic Gardens, Mrs. Macquaries Road, Sydney, NSW 2000, Australia. Collection data: 6 iv 2004, in a band of *Caloglossa vieillardii* (Kützing) Setchell on wood pilings at Westgate Bridge, Port Phillip Bay, Victoria (37°49′S, 144°53′E).

New Zealand material: <u>Collection date</u>. 1 viii 2003, dense patches in sand on rocks in stream, Waihau Bay, North Island, (37°37′S, 177°55′E), collected by Tracy Farr. The collection number of the herbarium sheet and DNA sample is ASD 154. It is deposited and registered at Te Papa (with a WELT number).

Etymology: Latin *pulchellus* = small and beautiful (Stern 1973).

Development of the gametophytic blade phase in culture

Young blades were round to ovate and matured into ovate blades at 21 ± 2°C (Fig. 1) and to linear-lanceolate blades at 14 ± 2°C (Fig. 2) that were moderately ruffled at the margin. In culture, blades were pinkish-red in colour. Thalli were monostromatic, each cell containing a single, stellate chloroplast with one pyrenoid, a vacuole and a nucleus. The small size of blades was a distinguishing feature of this species. They grew up to 3 mm in diameter at 21 ± 2°C and up to 25 mm in length at 14 ± 2°C. Blades attached to a substratum by a mass of branched rhizoids (Fig. 3). Rhizoids developed from irregularly arranged, slightly elongated polygonal basal cells at the base (Fig. 4). Vegetative cells were smaller than basal cells and mostly polygonal and irregularly arranged, becoming quadrate to rectangular and regularly aligned along the thallus margin (Fig. 5). In mature thalli, marginal vegetative cells were commonly grouped in pairs.

The development of archeosporangia and subsequent mass release of archeospores readily occurred in blades maintained at 21 ± 2°C. Thalli ranging from 1-3 mm in length and as young as 16 days developed archeosporangia from up to 14 rows of vegetative cells located at the blade apex. During sporangium development, the vacuoles of differentiating cells became reduced in size. Archeosporangia thus appeared a darker pinkish-red than vegetative cells (Fig. 6). Prior to discharge, the cell wall enclosing each archeosporangium disintegrated. Archeospores remained surrounded only by a plasma membrane and were loosely positioned in the extracellular polysaccharide matrix. The entire mass release of archeospores occurred in approximately 25 minutes, leaving behind the colourless cell wall with depressions where the archeospores were originally positioned (Fig. 7). Extracellular matrix was extruded from the thallus during spore release. Discharged archeospores displayed a pulsing amoeboid motility, whereby fat pseudopodia randomly extended and retracted from the cell (Fig. 8).

Amoeboid archeospores travelled at < 8 μm min^{-1} and remained dynamic for up to 24 hours. Mucilage was secreted from all amoeboid cells (Fig. 8). Occasionally archeospores exhibited gliding motility. These cells remained in contact with the slide as they translocated at a relatively constant velocity of 0.27-0.4 μm s^{-1} (Fig. 9). Archeospores settled and developed a single digitate rhizoid within 24 hours of release. All germinating spores developed into asexual blades. Development of archeosporangia and subsequent mass release of archeospores was induced by transferring blades from 14 ± 2°C to 21 ± 2°C, 16L:8D or 12L:12D hours and 40 μmol m^{-2} s^{-1}, for 48-72 hours.

Male sexual reproductive structures developed in 10-20 mm long monoecious thalli, after 5-6 weeks at 14 ± 2°C. Spermatangial sori were conspicuous, as pale, golden streaks 3-7 cells across, up to 3 mm long at the blade apex (Fig 10). Spermatangial packets were 16-20 μm wide and 22-28 μm long, and were divided into 32 cells (4 x 4 x 2). These packets were intermixed with larger, dark pink-red phyllosporangia. Phyllosporangia were commonly undivided but did occur in packets of two and four, 16-22 μm wide and 20-28 μm long (Figs 10 and 11). Spermatia (4-6 μm in diameter) and phyllospores (14-16 μm in diameter) were released simultaneously following the degeneration of the surrounding cell walls (Fig. 12). Discharged spermatia were slightly deformed in shape but remained immotile.

Phyllospores were amoeboid following their release. All phyllospore germlings developed into blades (Fig 13). Conchocelis filaments occasionally developed in cultures of blades bearing spermatangia and phyllosporangia, after 8-10 weeks at 14 ± 2°C. Whilst this indicates the presence of female reproductive structures (carpogonia), we did not observe any carpogonia or zygotosporangia between phyllosporangia and spermatangia.

Development and TEM observations of the conchocelis phase in culture

The conchocelis comprised a densely branched network of narrow, uniseriate filaments in tight, spherical tufts that were free floating or anchored to a substrate. Vegetative filament cells were 9-12 μm in diameter and 32-40 μm in length (Fig. 14). The single chloroplast in each cell was rose to purple coloured, elongate, twisted and parietal with one or more pyrenoids.

Conchosporangia were variable in form. Entire vegetative filaments commonly differentiated into conchosporangial branches (Fig. 14). These branches were 10-27 μm
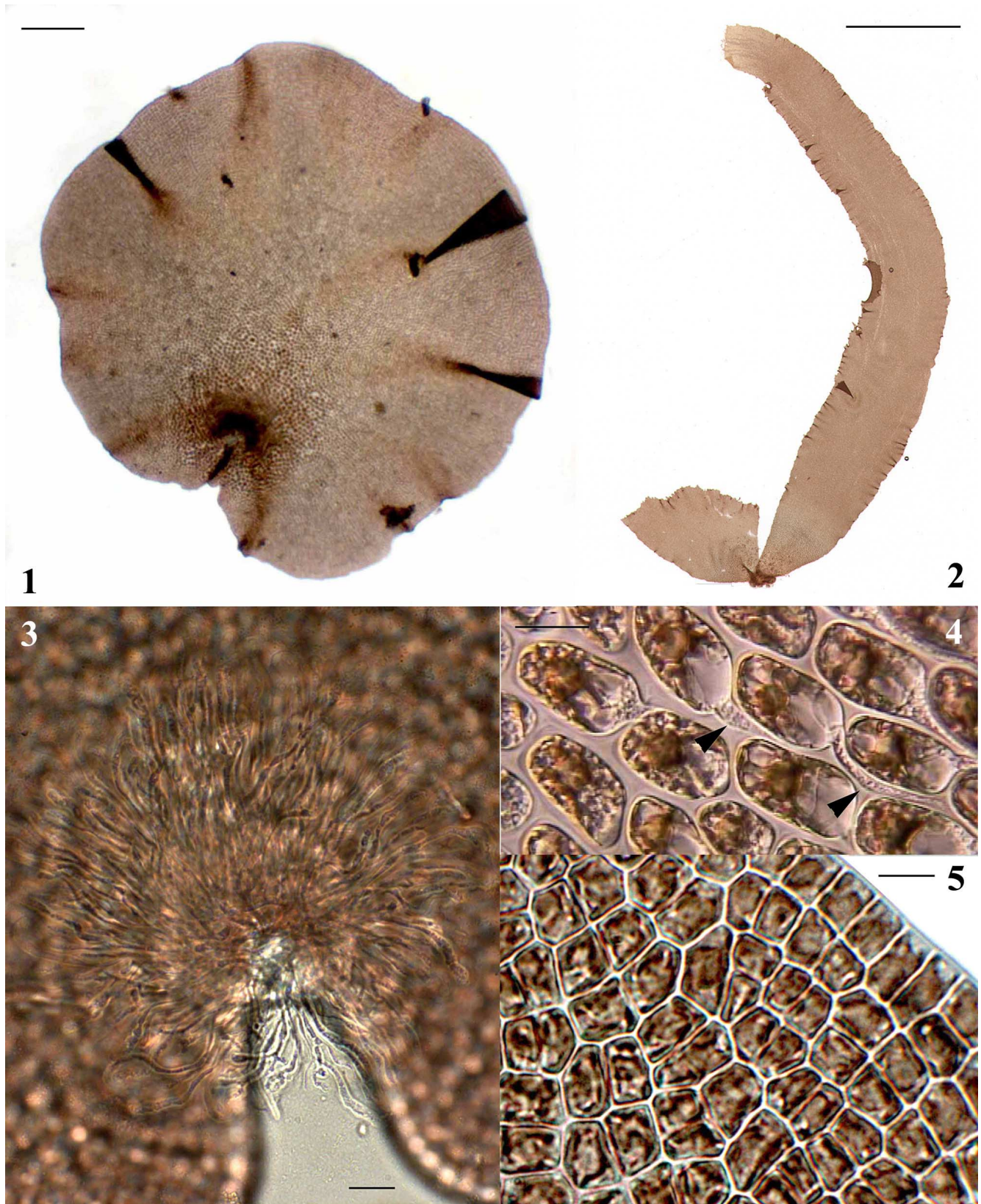
Fig. 1. Small 2 mm orbiculate blade formed at 14°C. Scale bar = 0.2 mm.
Fig. 2. Lanceolate 20 mm blade with spermatangia and phyllosporangia in linear streaks at the apical end. Scale bar = 5 mm.
Fig. 3. Basal attachment with multiple rhizoids. Scale bar = 20 μm.
Fig. 4. Rhizoids (arrowheads) developing from basal ends of cells near the blade base. Scale bar = 15μm.
Fig. 5. Elongate meristematic cells at blade margin and more quadrate meristematic cells away from the margin. Scale bar = 15 μm.
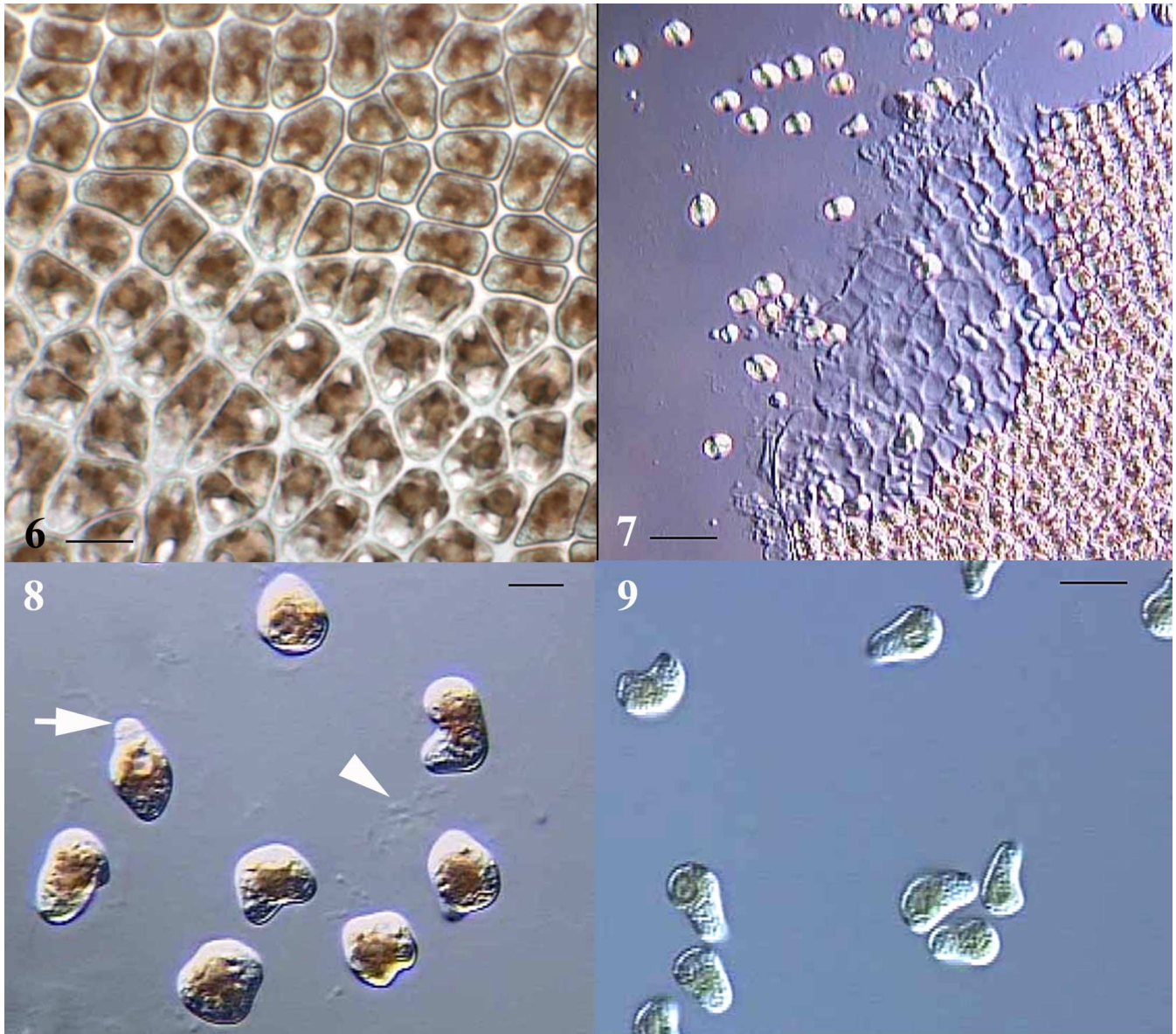
Fig. 6. Transition zone of larger vacuolate vegetative cells below and somewhat smaller developing archeosporangial cells with reduced vacuoles above. Scale bar = 10 μm.

Fig. 7. Discharged archeospores and empty sporangial walls visible. Scale bar = 30 μm.

Fig. 8. Freshly discharged archeospores have short fat pseudopodia (arrow) that move continuously in all directions. Faint strands of mucilage (arrowhead) are visible. Scale bar = 8 μm.

Fig. 9. Archeospores in contact with a slide displaying smooth gliding movement through the medium. Scale bar = 10 μm.

in diameter with transverse to oblique cross walls. Conchosporangia also occurred in large clusters of up to 12 sporangia, irregularly distributed amongst vegetative filaments (Figs 14, 15). The cells were usually shorter (7-27 μm) than those of vegetative cells (32-40 μm), appeared a darker pinkish-red and contained reduced vacuoles and a single chloroplast that occupied a large portion of the cell. Prior to conchospore discharge, the cell wall at the apex of conchosporangial filament broke down. Conchospores appeared slightly elongated as they successively passed through the filament before discharging at the filament apex. Discharged conchospores were pinkish-red, approximately 10-14 um in diameter and displayed an amoeboid motion similar to that of archeospores and phyllospores. All germinating conchospores developed into blades. The development of these blades was often aberrant. Many conchospore germlings underwent irregular cell divisions and exhibited a high cell death rate. Blades consequently developed into irregular shapes (Fig. 16). Some thalli produced
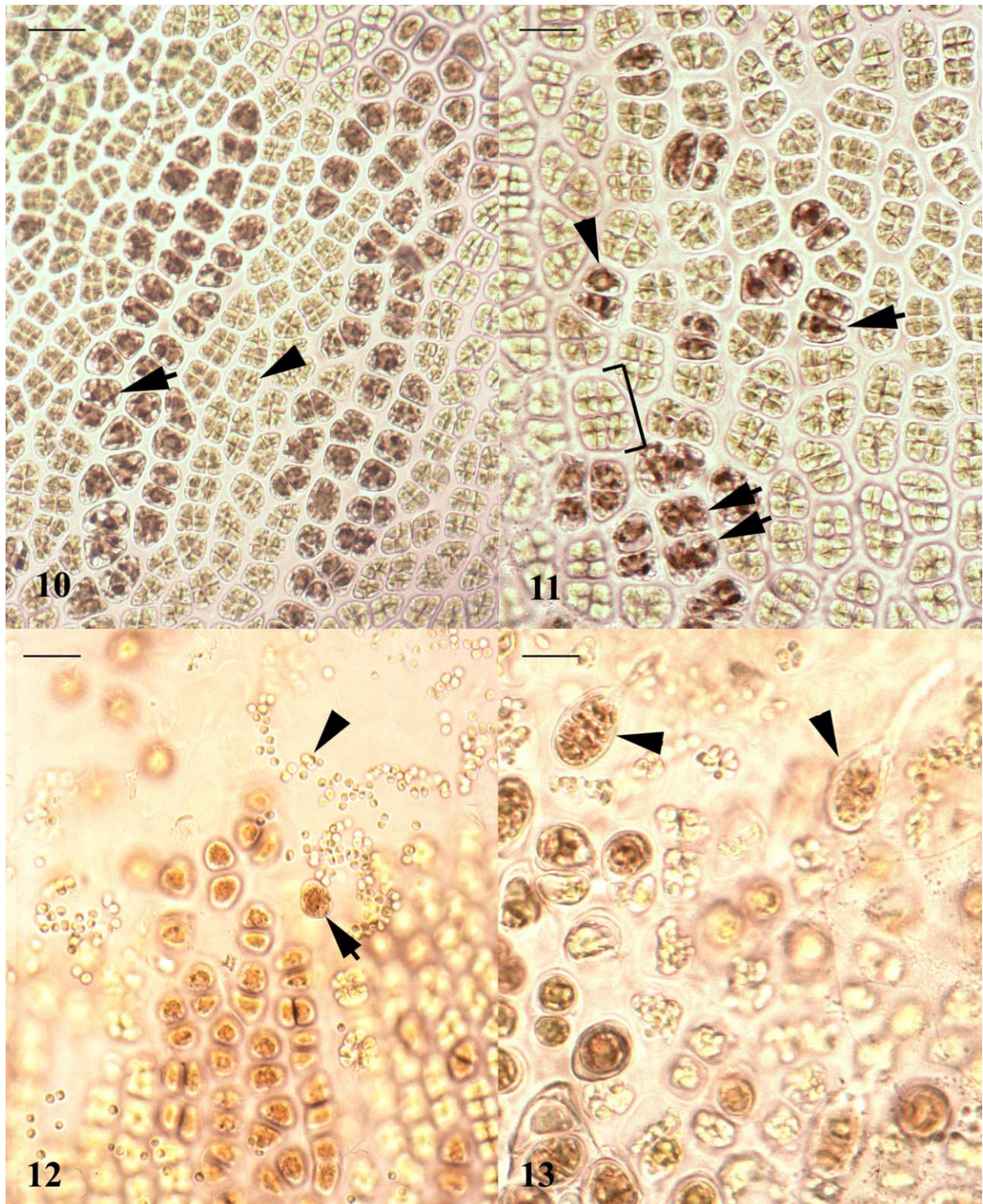
Fig. 10. Linear streaks of alternating spermatangia (arrowhead) and phyllosporangia (arrow) in blade grown at 14°C. 16 spermatangia are formed in two layers (32 total) from each vegetative cell. Single or double phyllospores are formed from each vegetative cell. Scale bar = 30 μm.

Fig. 11. Spermatangial packet (bracket) with 16 spermatia. Phyllosporangial packets of 2 (single arrow, arrowhead) or 4 (double arrow). Scale bar = 20 μm.

Fig. 12. Discharged spermatia (arrowhead) and phyllospores (arrow). Spermatia are non-motile and phyllospores are amoeboid. Scale bar = 30 μm.

Fig. 13. Phyllospores still in the old blade matrix and showing the bipolar germination (arrowheads) forming new blades (not conchocelis). Scale bar = 12 μm.
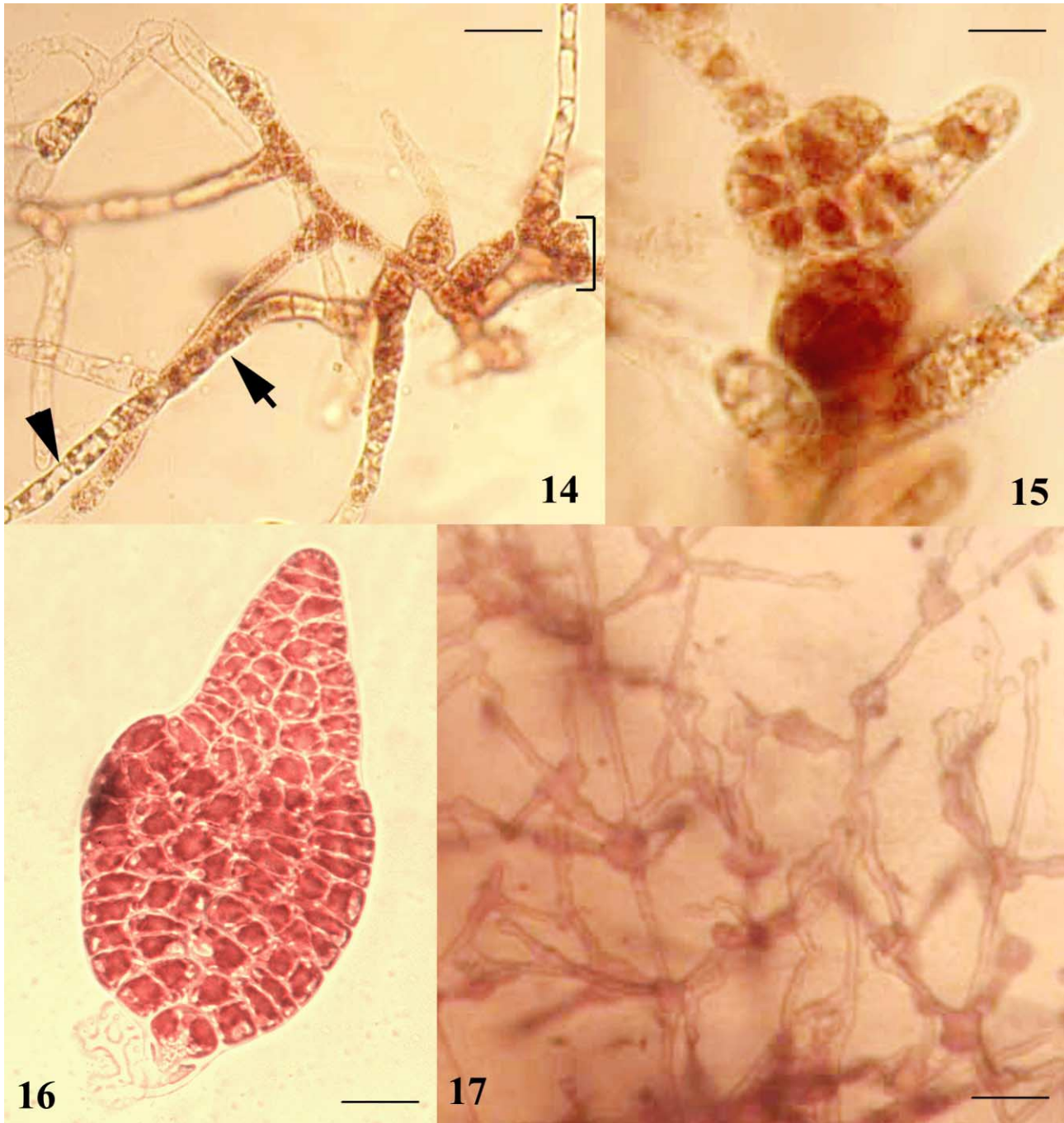
Fig. 14. Conchocelis filaments growing free in culture. Vegetative filaments (arrowhead), intercalary conchosporangial filaments (arrow) and conchosporangial clusters (bracket) are seen. Scale bar = 30 μm.

Fig. 15. A cluster of conchosporangial cells. Scale bar = 15 μm.

Fig. 16. Conchosporeling blade with two colour and cell patterns in upper and lower sectors. Scale bar = 35 μm.

Fig. 17. Conchocelis filaments growing in a mollusk shell matrix. Scale bar = 50 μm.

patches of vegetative cells that were lighter in colour compared to other vegetative cells of the same blade. Despite this unusual development, mature blades reproduced asexually by archeospores when grown at 20-22°C.

As is typical of *Porphyra* species, the conchocelis fila-

ments had the ability to burrow into calcium carbonate shell matrices. Eleven days after placing the conchocelis filaments on mollusk shell fragments, filaments had attached and begun penetration. By six weeks, vegetative filaments appeared on the same plane as calcium carbonate crystals of the shell matrix, indicating that the fila-

ments had burrowed into the shell. After three months, an intricate network of filaments bearing conchosporangia had developed throughout the shell matrices (Fig. 17). Some conchosporangial filaments emerged from the shell surface and eventually liberated conchospores that germinated, forming blades. Conchocelis filaments also reproduced asexually by fragmentation.

TEM preparation often resulted in the disruption of conchocelis filament cell walls. Cell wall breakage permitted a greater influx of fixatives and embedding resins. The cellular content of disrupted cells was thus better preserved than that of cells with intact walls. TEM of conchocelis vegetative filament cells revealed that the cells were connected by a primary pit connection (Figs. 18 and 19). The nucleus of each cell contained a prominent nucleolus and condensed chromatin (Fig. 20). Although mitochondria and Golgi bodies were scarce the cis-region of each Golgi complex was always associated with a mitochondrion (Fig. 21). Floridean starch granules were relatively small and abundant in the cytoplasm of each cell (Fig. 21). The chloroplast thylakoids were typically unstacked and covered by disk-shaped phycobilisomes (Fig. 22). The pyrenoids had inconspicuous, phycobilisome-free thylakoids winding through their matrix and small plastoglobuli frequently occurred at the periphery of pyrenoids and within several regions of the chloroplast stroma (Fig. 22).

Figure 23 represents a diagram summarising the life cycle of *Porphyra pulchella* in culture as we currently understand it.

Phylogenetic analysis

The partial sequence of the 18S gene consisted of 840 characters, 165 of which were parsimony-informative. The evolutionary model least-rejected by the hierarchical likelihood test was: TrN (Tamura and Nei 1993) (substitution rate matrix: a = 1.0, b = 1.65, c = 1.0, d = 1.0, e = 5.07, f = 1.00) plus proportion of invariable sites set to 0.599 and a gamma distribution of 0.432. The MP analysis produced 60 MP trees of 453 steps. Maximum Likelihood (ML) analysis produced one tree of *-ln* L score of 3502.2473. Both tree topologies were similar and the MP strict consensus topology is shown in Fig. 24.

Sequences of ribosomal DNA PCR amplification products from the Australian and New Zealand *Porphyra* samples were compared. Sequences from the blades and 'conchocelis' filaments of isolate 3923 were identical, confirming that both phases were of the same species. Blades of isolates 3923 and 4422 shared identical

sequences. These samples form a very supported clade with a sample from Waihau Bay, North Island, New Zealand. The tree topology is congruent with other studies showing a polyphyletic *Porphyra* (Broom *et al.* 2004; Nelson *et al.* 2005). No sequences in GenBank had sequences identical to *P. pulchella*. *Porphyra pulchella* forms a sister group relationship with *P. pseudolinealis*, although this relationship is only poorly supported (< 70%).

DISCUSSION

The conchocelis phase in the one culture isolate (3923) of *Porphyra pulchella* occurred without any evidence of sexual reproduction in the blades grown for many months at 20 ± 2°C. Molecular data (Fig. 25) clearly verifies its genetic link with the blade phase. Conchospores developed frequently and spore germination produced blades that reproduced by archeospores. The early stages of conchospore germination often showed a pattern of cell shape and size aberrant in the lower and upper sectors of the blade. We have no evidence of what is occurring but the pattern suggests that meiotic segregation occurred in a manner similar to that in *P. yezoensis* Ueda (Miura and Ohme-Takaki 1994).

Porphyra pulchella displayed a striking difference in blade size and reproduction at different temperatures. At the higher temperature (20 ± 2°C) in two weeks, small blades (0.5-3 mm) developed that reproduced with asexual archeospores. At 14 ± 2°C the blades take 5 weeks to mature reaching 20-25 mm before reproducing with spermatangia and phyllosporangia. This indicates that *P. pulchella* may be adapted to warmer coastal waters that allow more rapid reproduction and spore dissemination.

Kim and Notoya (2003) investigated the life history of *Porphyra koreana* M.S. Hwang and I.K. Lee, observing that archeospore formation began at 2 weeks when blades were 1.5 mm long in 15-25°C in long days (14:10 LD) and in short days (10:14LD) archeospore formation began at 4 weeks in 20-25°C and at 5 weeks in 15°C. At 20°C spermatia and zygotospores began release at 7-8 weeks in long days. In short days at 15-25°C no sexual structures developed for the entire culture period of 18 weeks. Blades reached only 4 mm at 20-25°C but grew to 60 mm in 5-10°C. Archeospores were formed in all conditions. In contrast to *P. koreana*, at higher temperatures only archeospores formed in *P. pulchella* and archeospores, spermatia and phyllospores were present in lower temperatures.
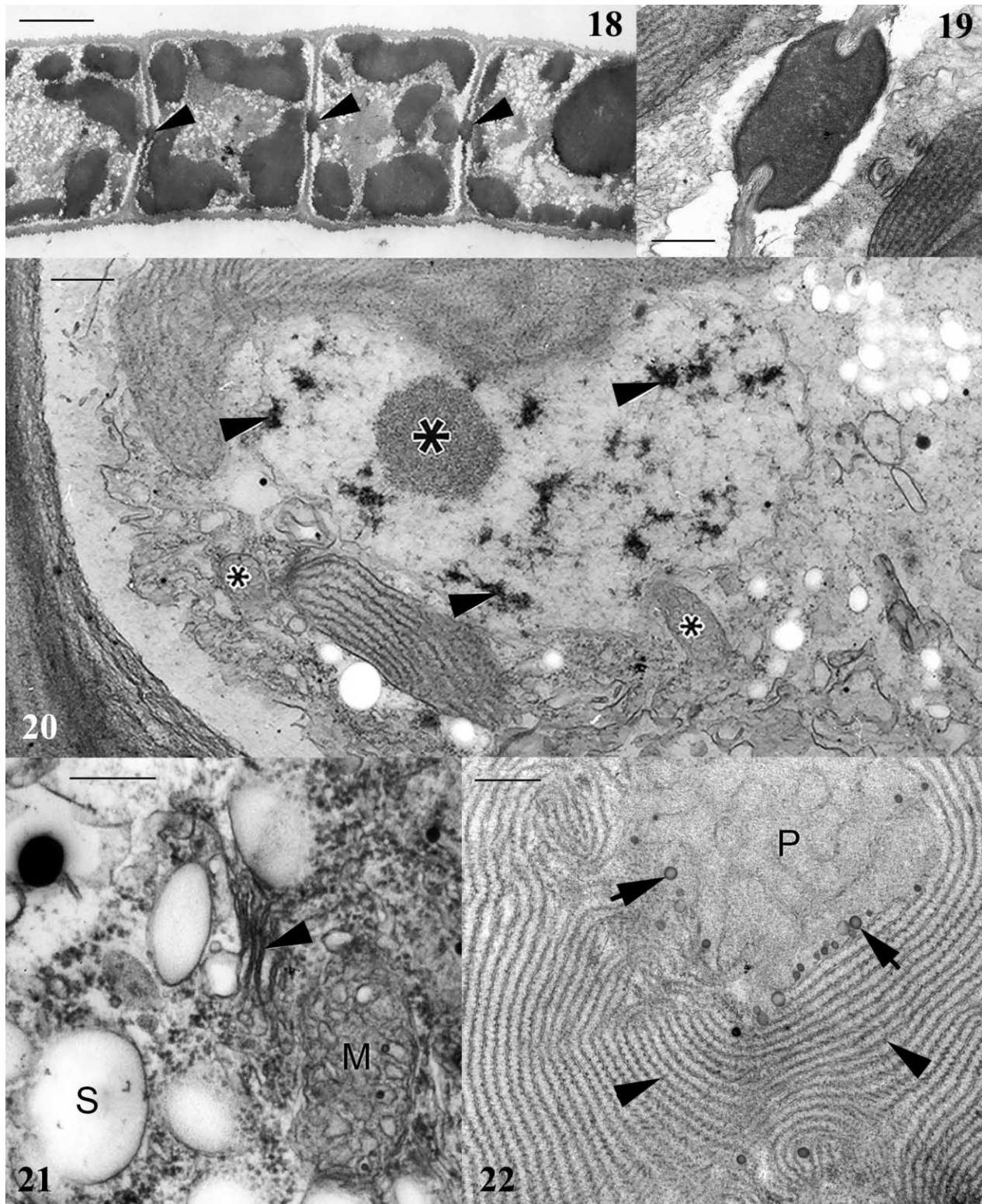
Figs 18-22. TEM of conchocelis phase. **Fig. 18.** Low magnification TEM of several cells connected by pit plugs (arrowheads). Scale bar = 4.5 μm. **Fig. 19.** High magnification TEM of pit plug between two vegetative filament cells. Although difficult to see in this image, pit plugs of *Porphyra* and Bangia possess a singe, thin cap but no cap membrane. Scale bar = 0.25 μm. **Fig. 20.** Nucleus with prominent nucleolus (large asterisk) and electron-dense chromatin (arrowheads). Mitochondria (small asterisks). Scale bar = 0.11μm. **Fig. 21.** Mitochondrion (M) is associated with the cis-region of the Golgi complex (arrowhead) in conchocelis cells. Abundant starch granules (S) can be seen in the cytoplasm of cells. Scale bar = 0.37μm. **Fig. 22.** Unstacked chloroplast thylakoids covered by disc-shaped phycobilisomes (arrowheads). The phycobilisome-free pyrenoid thylakoids and plastoglobuli (arrows) are more readily apparent in this micrograph. Scale bar = 1.32 μm.
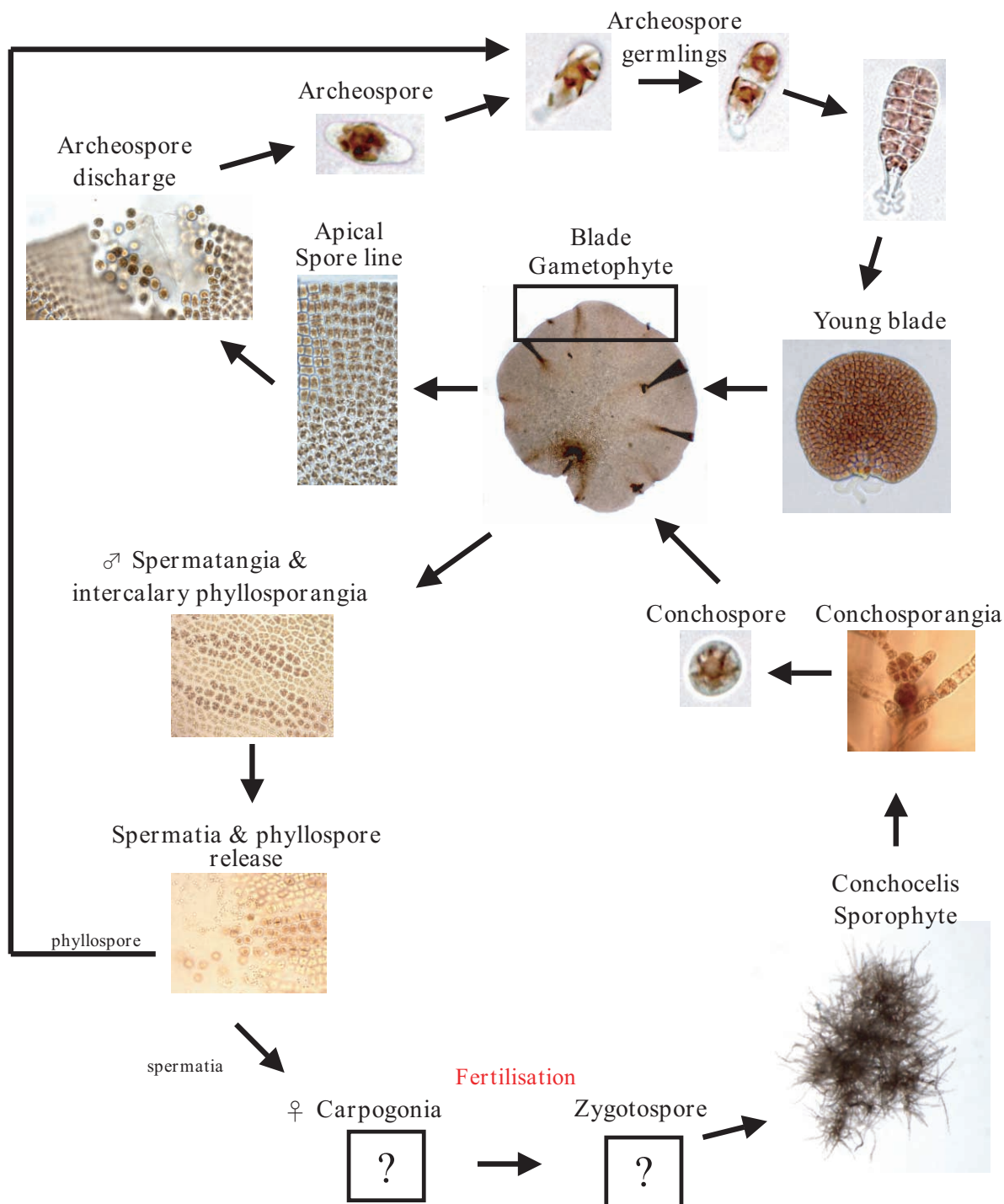
Fig. 23. Life history diagram of *P. pulchella*.

Notoya *et al.* (1993) investigated the life histories of *Porphyra lacerata* A. Miura and *P. suborbiculata* Kjellman. Archeospores formed within 1-3 weeks at higher temperatures (25°C) and blades remained small without sexual reproduction whereas at lower temperature (15-20°C) spermatia and zygotospores developed on blades in 4-5 weeks. These patterns appear similar to that seen for *P. pulchella*.

In the paper describing *P. koreana* as a new species Hwang and Lee (1994) provide a table of general characters showing the basic differences among the species, *P. koreana*, *P. kinositae*, *P. tenera*, *P. lacerata* and *P. kuniedae*.

The apparent lack of carpogonia in *P. pulchella* suggests that other conditions may be needed to have fully functional sexual blades. Certainly the spermatia and the phyllosporangia look normal and are released normally.
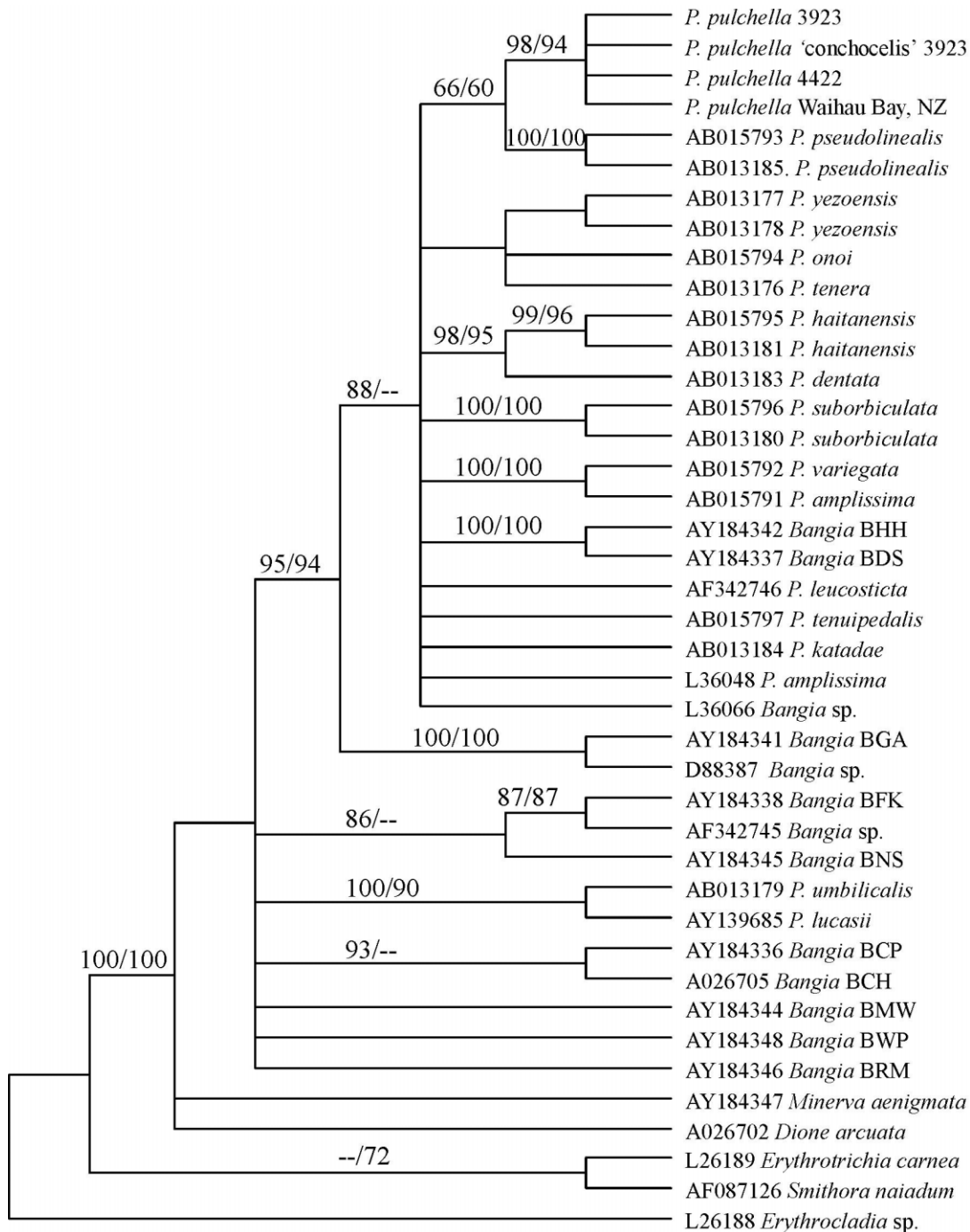
Fig. 24. Strict consensus of Maximum Parsimony trees based on partial 18S data of various *Porphyra* samples. Genbank samples given with their accession numbers. Branches showing bootstrap support ≥ 50% shown (before diagonal MP analysis; after diagonal ML analysis). Erythropeltidales selected as the outgroup.

We observed over 2000 phyllospore germlings that formed only blades (no conchocelis filaments). These were clearly not obtained from sexual ferlilization.

Phyllospores, archeospores and conchospores display a distinct amoeboid movement. This has been reported briefly in *Porphyra dioica* (Holmes and Brodie 2004) and in *Porphyra yezoensis* (Ueki *et al.* 2005). Spore motility is

common to most red algae that have been investigated with time lapse videomicroscopy (Pickett Heaps *et al.* 2001). Ackland *et al.* (2006) provides evidence that pseudopodia involved in amoeboid movement are regulated by the cytoskeletal components actin and myosin.

Other workers have seen amoeboid motility of monospores in *Colaconema caespitosum* (J. Agardh) Jackelman,

Stegenga and J.J. Bolton [as *Audouinella botryocarpa* (Harvey) Woelkerling)] (Guiry *et al.* 1987), monospores of *Erythrotrichia* (Rosenvinge 1927), carpospores and tetraspores of *Liagora harveyana* Zeh and *Helminthora stackhousei* (Clemente) Cremades and Pérez-Cirera (Cunningham *et al.* 1993, Guiry 1990). Although it is a relatively slow process, spore movement appears necessary for substrate selection and attachment prior to germination. The mechanism of amoeboid movement in red algae has not been fully investigated.

The complete lack of spermatium motility in *Porphyra pulchella* corresponds to our observations on other red algae such as *Bostrychia* and *Murrayella* (Pickett-Heaps *et al.* 1998, McBride and West 1999b, Wilson *et al.* 2002, 2003).

Molecular phylogeny of *Porphyra* is being slowly investigated to resolve the species complexes. *Porphyra suborbiculata*, *P. koreana, P. lacerata* and several other smaller species have morphological and reproductive features similar to those of *P. pulchella* but insufficient molecular data are available to clearly indicate their overall relationships.

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