**Gracilaria** species (Gracilariaceae, Rhodophyta) from southeastern Australia, including a new species, *Gracilaria perplexa* sp. nov.: Morphology, molecular relationships and agar content

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**SUMMARY**

Select species of the agarophyte *Gracilaria* were studied from southeastern Australia. The morphology and anatomy of species is described and molecular relations are inferred based on plastid and mitochondrial DNA sequence data. Agar yields and qualities are determined for each species. *Gracilaria chilensis*, found in Tasmania and Victoria, is morphologically and molecularly similar to *G. chilensis* from New Zealand and Chile and has low agar yields of 11–16%. *Gracilaria cliftonii* from Victoria, has high crude agar yield (52%) and is molecularly uniform. *Gracilaria perplexa* sp. nov., known only from Botany Bay, New South Wales, has an agar yield of 39%. The agar of *G. perplexa* is unusual in requiring the addition of 0.1 mol L\(^{-1}\) NaCl for alcohol precipitation and is cold-water (25°C) soluble because of the very high sulfate ester content. Molecular phylogeny shows that *G. perplexa* is closely related to *Gracilaria preissiana* from western Australia, but differs from the latter in its reduced branching and narrower more terete axes.

**Key words:** agar content, cox2–3 spacer, *Gracilaria*, *Gracilaria perplexa*, Gracilariaceae, RuBisCo spacer.

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**INTRODUCTION**

*Gracilaria* is the third largest genus in the Rhodophyta, with over 150 described species. It is cosmopolitan, with the greatest standing stocks and numbers of species occurring in temperate seas. *Gracilaria* species are commercially important in the production of the phycocolloid agar (Armisen 1995). Many recent studies have thus been directed toward resolving their taxonomy and suitability for commercial exploitation. The full potential for utilization of *Gracilaria* is unlikely to be realized without the establishment of sound taxonomic criteria for identifying species (Bird 1995). Progress in this activity over the past 40 years has been slow and has mostly come from comparative morphological studies of Chang and Xia (1963), Yamamoto (1978), Edelstein et al. (1978), Bird and McLachlan (1982), Fredericq and Hommersand (1989a,b), Abbott et al. (1991) and Withell et al. (1994). These investigations have established the importance of spermatangial structures, gonimoblast placentation, traversing-filament orientation and carposporophyte anatomy in delineating species and relatedness. At the same time, major monographic surveys of the *Gracilaria* floras of Japan (Ohmi 1958; Yamamoto 1978), China (Zhang and Xia 1992), Asian countries (Trono et al. 1983; Dinh 1992), New Zealand (Nelson 1987), Australia (Withell et al. 1994; Womersley 1996), and Atlantic North America (Fredericq and Norris 1992) were conducted, which further clarified species concepts and biogeographic distribution.

The first extensive anatomical and taxonomic study of the genus *Gracilaria* in Australia was made by May (1948), who dealt mostly with terete species and described nine species, 24 forms, one variety, and five incertae sedis taxa. She separated the species into two main groups (*Macro cystideae* and *Microcystideae*) based on thallus transverse sections. *Macro cystideae* have an abrupt transition between peripheral and medullary cells and generally have dense granular cell contents, whereas *microcystideae* have no sharp boundary between the two tissue types, the cells becoming gradually smaller toward the periphery. May (1948) disregarded reproductive structures entirely, relying exclusively on vegetative and habit features to distinguish taxa. She regarded cystocarp shape and size, thallus color and size and cell-wall thicknesses as not particularly valuable for the delineation of the species she studied. It was not until 46 years later that comparable broad-scale studies on Australian *Gracilaria* were again attempted (Withell et al. 1994; Womersley 1996).

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Withell et al. (1994) emphasized spermatangial morphology, carposporophyte anatomy, and tetrasporangial configurations, as well as vegetative and habit features, in a detailed treatment of nine of the 21 Australian species that they recognized. They described two new species, re-named another, and newly recorded a further four species for Australia. Like May (1948), Withell et al. (1994) found the type of transition in cell size from cortex to medulla (whether abrupt or gradual) to be consistent within species. Characteristics such as habit and cystocarpic features varied considerably in some species, but were highly uniform in others.

The identification of slender, terete and irregularly branched *Gracilaria* species has been a constant problem for taxonomists, particularly as many populations are only rarely reproductive in the field. Laboratory culture to obtain missing life history stages, particularly the male gametophytes that so often elude detection even in well-known species, is one approach to complete anatomical characterization. Another promising approach is the comparative analysis of nucleotide sequences of selected genomic DNA fragments such as the small subunit of ribosomal RNA (18S rDNA) (Bird et al. 1992, 1994), the ribosomal internal transcribed spacer (Goff et al. 1994) and the rbcL-rbcS intergenic spacer (RuBisCo spacer) (Destombe and Douglas 1991; Goff et al. 1994). Another promising genetic region is the mitochondria encoded intergenic spacer, and parts of flanking genic regions, between the cytochrome oxidase 2 and cytochrome oxidase 3 genes (cox 2–3 spacer) (Zuccarello et al. 1999a). These molecular tools, when combined with anatomical data, are capable of resolving taxonomic problems at virtually every taxonomic level from strains and populations to classes and phyla. The present study has aimed to analyze samples of *Gracilaria* from southeastern Australia taxonomically and molecularly, and to characterize their agars, in order to increase our understanding of this potentially commercially important genus in Australia.

**MATERIALS AND METHODS**

**Anatomical methods**

Anatomical observations were made on a previously unnamed species from the Sydney region (designated *Gracilaria perplexa* sp. nov.), *Gracilaria chilensis* Bird, McLachlan et Oliveira (Marion Bay, Glenelg River, Phillip Island, Hobson’s Bay) and *Gracilaria cliftonii* Withell, Millar et Kraft (Portland, Portsea). The anatomy of *G. cliftonii* is detailed extensively by Withell et al. (1994) and Womersley (1996; as *Gracilaria ramulosa* J. Agardh), and as the material of the present study matches these descriptions very precisely, its morphology is only briefly considered.

Fresh, dried and formalin-fixed tissues were hand-sectioned, stained in 1% acidified aniline-blue solution and mounted on glass slides in 20% Karo syrup. Anatomical features were photographed using a Zeiss C35 camera and a Zeiss MC 100 camera on a Zeiss GFL bright field microscope. Low-power habit photographs were taken using a Nikon F801 Camera on a Wild dissecting microscope.

**Culture methods**

Unialgal culture methods are described in West and Zuccarello (1999). Culture isolates were all maintained at 20–26°C, 12 : 12 LD daily photoperiods, 10–15 µmol photons m⁻² s⁻¹ coolwhite fluorescent lighting, MPM/2 culture medium (30% salinity; McBride and West 1999). For faster growth and reproduction, cultures were placed under 20–25 µmol photons m⁻² s⁻¹ on a rotary shaker (70 r.p.m).

**Molecular phylogeny methods**

Samples used for molecular analysis are listed in Table 1. Field samples were cleaned of surface epiphytes or debris, and portions of each specimen analyzed were pressed as a voucher.

DNA extractions followed the Chelex protocol of Goff and Moon (1993) as outlined in Zuccarello et al. (1999b). A fresh or dried thallus tip of approximately 3–5 mm was used.

RuBisCo spacer amplification followed the procedures in Zuccarello et al. (1999b) using the rbcF1 forward and rbcR3M2 reverse primers. Cox2–3 spacer amplification followed the procedures in Zuccarello et al. (1999a). The internal transcribed spacer 1 region was amplified as outlined in Zuccarello et al. (1999c). Polymerase chain reaction (PCR) products were checked by electrophoresing in a 1–2% agarose gel, stained in ethidium bromide, visualized under UV light and photographed.

The PCR products were prepared for sequencing by polyethylene glycol (PEG) precipitation. A total of 45 µL of crude PCR product was added to 45 µL of 2X PEG precipitation mix (26.7% PEG 8000, 0.6 mol L⁻¹ Na-acetate (pH 5.2) and 6.5 mmol L⁻¹ MgCl₂), briefly vortexed and left at room temperature for 10 min. The precipitated DNA was pelleted by centrifugation for 15 min at 9447 g. The supernatant was gently removed by pipette, leaving the DNA pellet in the tube. The DNA was washed twice with 1 mL of 100% ethanol and centrifuged for 15 min after each wash. The residual ethanol was then removed and the DNA pellet dried at room temperature. The pellet was then resuspended in 20–40 µL of H₂O.

Automated sequencing was performed on an ABI Prism 377 DNA Sequencer (Perkin-Elmer) after cycle sequencing of the purified PCR product with dye-labeled
Table 1. Samples used for phylogenetic study

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Culture/ sample no.</th>
<th>RuBisCo GenBank no.</th>
<th>cox2–3 GenBank no.</th>
<th>ITS GenBank no.</th>
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ITS, internal transcribed spacer. NA, not applicable, sequences retrieved from GenBank. The 4-digit numbers refer to J. A. West culture numbers. Sample numbers beginning with E indicate DNA extractions of field-collected material.


Gracilaria perplexa sp. nov.

dideoxynucleotides. Cycle sequencing was performed on a P-E 2400 Thermal Cycler (Perkin Elmer), and samples cleaned up by the ethanol precipitation method following the manufacturer’s recommendations. Sequences were assembled using the computer software supplied with the sequencer. All sequences were aligned visually in SEQPUP (free software distributed by D. G. Gilbert, Biology Department, Indiana University, Bloomington, IN, USA).

Phylogenetic relationships were inferred with PAUP*4.0b6-8 (Swofford 2001). *Ptilophora prolifera* was used as an outgroup. Maximum parsimony (MP) trees were constructed using the heuristic search option, 500 random sequence additions, tree bisection-reconnection (TBR) branch swapping, unordered unweighted characters, gaps treated as missing data. The program MODElTEST version 3.06 (Posada and Crandall 1998) was used to find the model of sequence evolution that best fitted each data set by a hierarchical likelihood ratio test (λ = 0.05) (Posada and Crandall 2001). When the best sequence evolution model had been determined, maximum likelihood (ML) and distance searches were performed in PAUP* using the estimated parameters (substitution model, gamma distribution, proportion of invariant sites, and transition/transversion ratio). Distance trees were constructed using neighbor-joining reconstruction (NJ). Maximum likelihood was also used to construct the most likely tree from the data set (5 random additions). Support for individual internal branches was determined by bootstrap analysis (Felsenstein 1985), as implemented in PAUP*, and a decay index (Bremer 1988). For bootstrap analysis, 1000 bootstrap data sets were generated from resampled data (5 random sequence additions), for both the MP and NJ analysis. Decay indices, on a strict consensus of the most parsimonious trees, were calculated with AUTODECAY version 4.0.2 (Eriksson 1998).

Polysaccharide methods

Both field and cultured materials were used for polysaccharide analyses. The field material was cleaned by hand to remove epiphytes or debris. The cleaned material was then dried in vacuo for 24 h at 60°C. The dried material was then ground into powder using liquid nitrogen and a mortar and pestle.

The powdered specimen was hydrated overnight in distilled water (ca 1 g in 100 mL). The seaweed suspension was then heated in a water bath for 1 h at
95–100°C, with occasional stirring. The extraction mixture was centrifuged to separate the insoluble algal material from the extracted water-soluble polysaccharide. The pellet was re-suspended and the extraction was repeated twice. The successive polysaccharide solutions (supernatants) from each sample were combined, heated to ca 90°C, and filtered successively through a Whatman no. 541 filter paper, Whatman no. 542 filter paper and Whatman GF/A glass microfibre filter. The filter, funnel and solution were kept hot (>60°C) throughout the filtration process. The refined polysaccharide solution was then heated to >60°C, placed on a magnetic stirrer with rapid stirring, and precipitated by adding 3 vols of hot (>60°C) 2-propanol. The precipitate was collected by centrifugation, washed with 60% 2-propanol and left to dehydrate in 99.5% 2-propanol. After drying overnight at 60°C in vacuo over P₂O₅, the yield of crude polysaccharide was determined as a percentage of the dry weight of the algal sample.

Films for Fourier transform infrared (FTIR) spectroscopy analysis were prepared by evaporating aqueous solutions of the polysaccharide (2.5 mL of 4 mg mL⁻¹ solution) at 60°C in plastic dishes (3.2 cm diameter) (Liao et al. 1993). The spectra were recorded on a Perkin-Elmer Series 2000 FTIR spectrophotometer in transmittance mode (16 scans, collected at a resolution of 4 cm⁻¹).

RESULTS

Morphology

The following species have been identified from the eight sampling sites studied; unialgal isolates were made from a single individual from most sampling sites (culture numbers are shown in Table 1):


Type Locality: Penco, Bahía Concepción, Chile (Romo, 1.ix.1982); holotype and isotypes in NRCC, 9131 (a) and (b).

Distribution: Chile, from Bahía Herradura to Río Maullín; New Zealand, North, South, Stewart and...
Producing numerous laterals of varying lengths with slightly constricted at the base. Branching becomes more sparingly branched thalli are gener-

Branches on more sparingly branched thalli are gener-

Morphology: Thalli dark brown to black, 20–21 cm high, irregularly branched, and generally stringy in appearance, fronds terete and slender throughout, laterals of varying length, acutely pointed and basally unconstriicted. Primary axes are up to 850 µm in diameter, branching to three orders, with some of the ultimate laterals hooked (Fig. 2a), numerous primary axes arising from a discoid holdfast.

Vegetative Anatomy: Cortex-to-medulla transition gradual, with cells increasing to 230 µm in diameter towards the center of the frond (Fig. 2d), cortex is two to three cells thick, outer cells anticlinally elongated and the inner cells subsisodiametric. Surface cortical cells 7–10 µm broad by 12–15 µm in length. A subcortex of one or two cell layers overlies a medulla of seven to nine cells.

Reproduction: Sessile cystocarps scattered radially on all branch orders except the lower main axes (Fig. 2b). Cystocarps beaked, shaped like depressed spheres, basally constricted and reaching 800–1000 µm in width (Fig. 3d,f), scattered radially on all branch orders except for lower main axes, traversing cells or filaments descending into cystocarp floor and growing outwardly into the lower pericarp. Carpospores pyriform to sub-

Vegetative Anatomy: Cortex-to-medulla transition is gradual with cell diameters increasing to 290 µm towards center of the thallus (Fig. 3c). Cortex is two to three cells thick, outermost cells being anticlinal and elongated. Cortical cells are 7.5 µm in breadth and 15 µm in length, with the innermost cells isodiametric. Subcortical layer is one or two cells thick and surrounds a medulla consisting of 9–10 cells.

Reproduction: Field plants only known to reproduce vegetatively. Culture isolate 3948 did not reproduce, whereas isolate 3947 formed all reproductive phases. Plants in culture became tetrasporic after about 30 days at 25°C, 10–20 µmol photons m⁻² s⁻¹, 30% salinity, the tetraspores germinating into dioecious gametophytes that bore gametes after about 28 days. Sessile cystocarps depressed spheres with pointed beaks, basally constricted, reaching 800–1000 µm in width (Fig. 3d,f), scattered radially on all branch orders except for lower main axes, traversing cells or filaments descending into cystocarp floor and growing outwardly into the lower pericarp. Carpospores pyriform to sub-

Phillip Island, Victoria (38°28'S, 145°14'E) (Fig. 1) Habitat: Plants attached to shells scattered across a muddy/sandy substratum on a sheltered intertidal flat at Woody Point, Phillip Island. (G. Kraft and B. Stevens, 19.iv.1999, MELU A042867).

Morphology: Thalli light green and terete throughout, 16–20 cm in length, and stringy in appearance, branching irregular to three orders, mature primary axes to 900 µm in diameter, first-order branches occasionally having long slender extensions, all laterals with acute apices and lacking basal constrictions.

Vegetative Anatomy: Cortex-to-medulla transition gradual with cell diameters increasing to 210 µm towards the thallus center, cortex two to three cells thick, having anticlinally elongated outermost cells, cortical cells 7–9 µm in breadth by 12–15 µm in length, with the innermost cells isodiametric; subcortical layer one to two cells thick surrounding a medulla of 9–10 cells.

Isolate 3967 did not reproduce in culture.

Hobson’s Bay, Victoria (37°50'S, 144°53'E) (Fig. 1) Habitat: Attached to muddy substrata near mangroves in intertidal zone, Stoney Creek backwash, Hobson’s Bay, Spotswood, Melbourne. (J. West, 25.iii.1999, MELU A042869).
Morphology: Mature field plants, not reproductive, similar in morphology and length to those from Marion Bay, Glenelg River and Phillip Island. Isolates of tetrasporophyte in culture (3954) reproduced as described.
Fig. 3.  a–f. *Gracilaria chilensis* from Marion Bay, Tasmania (MELU A042865, A042866).  a. Non-reproductive plant grown for 6 months in culture from a 3.5-mm long excised branch tip.  b. Male gametophyte grown for 6 months in culture from a tetraspore.  c. Branch cross-section of male gametophyte showing *Textorii*-type spermatangial conceptacles (arrows) and a gradual cortex-to-medulla transition.  d. Pyriform cystocarps on a female gametophyte grown from a tetraspore in culture.  e. Detail of *Textorii*-type male conceptacles showing clavate paraphyses (arrows) between adjacent clusters of spermatangial mother cells.  f. Cross-section of cystocarp showing broad basal placentation with downwardly penetrating peripheral traversing filaments (arrow head) irregularly contoured small-celled central sterile carposporophyte tissue, and terminal ovoid carposporangia (arrows).
**Gracilaria perplexa** Byrne et Zuccarello sp. nov., Bare Island, New South Wales (34°00′S, 151°14′E) (Fig. 1)

Froentes teretes, ad 15 cm [latae], varie ramosae, usque ad quadruplo subdichotomae, apicibus acutis, interdum arcuatae; axes primarii 1.5–1.7 mm in diam., cortex 30–40 µm lato, e stratis cellularum 2–4 constanti, cellulis hyalinis dispersis (basibus trichoma-
tum) instructo, abrupte in medullam transitri, cellulis medullosis polygonis, ad 250–550 µm in diam. Generatio typi Polysiphonae. Gametophyta dioica; cystocarpi globosi, rostrate, ad basin leviter constricta, ad 1.3 mm in diam., solitaria vel interdum aggregata, filamentis nutritios ad fundum loculi cystocarpii crescentibus; carposporangia subsphaerica, ad 25 µm in diam., pericarpium 220–270 µm crasso, e stratis cellularum 7–10 constanti; spermatangia in conceptaculis compositis (typi Polycavernosae) 50–85 µm lati in subcortice vel medulla exterio-ri disposita; tetrasporangia cruciatim decussata, 17–26 × 26–29 µm, in cortice exterio-
dispersa.

Locus Typicus: Bare Island (lat. aust. 33°59′, long. orient. 151°13′, Fig. 1), La Perouse, Botany Bay, New South Wales, Australia.


Distributio: E loco typico et Kurnell, NSW (lat. aust. 34°00′; long. orient. 151°12′) tantum cognita.

Terete shoots to 15 cm, branching variable, sub-
dichotomous to four orders, acute tips, sometimes arcuate, primary axes 1.5–1.7 mm diam., cortex 30–40 µm wide, two to four cell layers, with scattered hyaline cells (hair cell bases). Transition to medulla abrupt. Medullary cells, polygonal, to 250–320 µm diam. *Polysiphonia*-type life history, gametophytes dioecious, cystocarps globose, rostrate, slightly constricted at base, to 1.3 mm diameter, solitary or sometimes aggregated; traversing filaments directed toward cystocarp cavity floor, carposporangia subspheri-
cal to 25 µm diameter, pericarp, 220–270 µm thick with 7–10 cell layers; spermatangia borne in compound conceptacles 50–85 µm across (*Polycavernosa*-type) in subcortex/outer medulla; tetrasporangia cruci-at-decussate, 17–26 × 26–29 µm scattered in outer cortex.

Type Locality: Bare Island (33°59′S, 151°13′E, Fig. 1), La Perouse, Botany Bay, New South Wales, Australia.


Distribution: Known only from type locality and Kurnell, New South Wales (34°00′S, 151°12′E).

Etymology: The species designation ‘*perplexa*’ indicates the frequent difficulties in resolving the charac-
teristics of many *Gracilaria* species. Molecular analysis has proven essential as evidence of the difference between many species.

Morphology: Mature thallus burgundy to light green, cartilaginous, cylindrical to subcylindrical, reaching 15 cm in length (Fig. 4a), fronds extremely variable with subdichotomous branches to four orders, basally constricted. Primary axes to 1.5–1.7 mm in diameter. On some plants, branching arcuate and apically attenuated, becoming more dense distally. Attachment by a lacerate holdfast.

Vegetative Anatomy: Cortex-to-medulla transition abrupt with cell diameters reaching 250–400–550 µm towards thallus center (Fig. 4f). Cortex surface with scattered hyaline cells of undetermined function (pos-
sibly basal remnants of hair cells), two to four cells thick and 30–40 µm wide. Cortical cells 10–20 µm in diameter, quadrate to spherical in outline. Medulla reaches 1500–1600 µm in diameter.

Reproduction: Gametophytes dioecious, cystocarps solitary or, less commonly, aggregated radially on all but the lower main axes. Cystocarps to 1.3 mm in diameter, globoide, rostrate, and slightly constricted at the base (Fig. 4b). Traversing cells or filaments are directed exclusively towards cystocarp cavity floor (Fig. 4d,e), sterile gonimoblast cells subsidiometric with relatively thick walls, carposporangia subspheri-
cal, to 25 µm in diameter, pericarp 220–270 µm wide and 7–10 cell layers thick; spermatangia produced by spermatangial mother cells, which form a branch system covering the entire inner surface of the com-
pound (>4 cavities) conceptacles (*Polycavernosa*-type), 50–85 µm across within the subcortex and outer medulla (Fig. 4c); Tetrasporangia cruciate-decussate, 17–26 × 26–29 µm, scattered in outer cortex (Fig. 4g).

Remarks: Entity 3903 was isolated from Bare Island, New South Wales on 6.xii.1998. The *Polysiphonia*-type sexual life-history was completed in culture. A second collection from Bare Island was made on 29.iii.2000. It constitutes a distinct new species of *Gracilaria* in the Australia flora. The large subunit of ribulose bisphos-
phate carboxylase was sequenced for this species (culture isolate 3903, GenBank Accession no. AY131306) and, when added to large rbcL data, it is shown to be closely related to *Gracilaria preissiana* (Sonder) Womersley from Western Australia (Hommer-
sand, M., 2002 personal communication). We com-
pared the morphological characters of *G. preissiana* with *G. perplexa* (Table 2). *Gracilaria preissiana* has wider and often flattened branches and is more fre-
cently branched than *G. perplexa*. 
Fig. 4. a–g. *Gracilaria perplexa* Byrne et Zuccarello, sp. nov. from Bare Island, Sydney (MELU 042862). a. Field-collected female gametophyte showing lacerate holdfast (arrow). b. Basally constricted cystocarps on a female gametophyte grown in culture. c. Cortex of a male gametophyte showing the many-chambered *Polycavernosa*-type spermatangial conceptacles. d. Sterile gonimoblast tissue and descending traversing filaments (arrowheads). e. Cross-section of cystocarp showing descending traversing filaments (arrowheads) and anticlinally elongated basal sterile tissue (star). f. Cross-section of thallus showing gradual cortex to medulla transition. g. Cruciate tetrasporangia (arrow) embedded in the cortex.
Gracilaria cliftonii Withell, Millar et Kraft

Gracilaria ramulosa J Agardh (Agardh 1876: 471; Womersley 1996: 18, pl. 1, figs 1,3; De Toni 1900: 439; Sonder 1881: 22).


Type Locality: Swan River at Fremantle.


Distribution: Cottesloe, Western Australia, to Walkerville, Victoria, and around Tasmania (Womersley 1996).

Portland (38°20’S, 141°36’E) (Fig. 1)

Material Examined: Plants at 2-m depths attached to stones and shells debris in sandy substrata in a dense population in the intertidal area of Portland (K. Byrne and B. McDonald, 18.iv.1999).

Morphology: Thalli burgundy to dark red, 20–25 cm high, cartilaginous and terete throughout (Fig. 5a). Branching subdichotomously to five orders, with primary axes (to ca 2 mm in diameter), laterals with attenuate to acute apices. Attachment is by an encrusting holdfast.

Vegetative Anatomy: Cortex-to-medulla transition gradual, with cells increasing to 250 μm in diameter towards frond center (Fig. 5b). Cortex two to four cells thick, outer cells anticlinally elongated and inner cells subsisodicametric. Surface cortical cells are up to 12.5 μm broad and 12.5 μm in length.

Reproduction: Cystocarps are sessile and scattered radially on all branch orders except for the lower main axes (Fig. 5e). Cystocarps are strongly beaked, globose, basally constricted, and reach 1700 μm in width (Fig. 5c). Traversing cells or filaments both descend into the cystocarp floor and radiate into the mid to lower pericarp (Fig. 5d). Pericarp is 12–14 cells and

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Table 2. Morphological comparison of Gracilaria preissiana and Gracilaria perplexa

<table>
<thead>
<tr>
<th></th>
<th>Gracilaria preissiana</th>
<th>Gracilaria perplexa</th>
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<tbody>
<tr>
<td><strong>Thallus</strong></td>
<td>Erect to 24 cm. Lower axes and major branches are subterete or compressed. Upper branches are terete or subterete with apices acute. Branching irregular, dichotomously divide, many short branchlets and short, undivided lateral arising from blade margins. Obtuse apices, all blades linear to slightly flagellate.</td>
<td>Erect to 13 cm, subcylindrical to terete throughout. Branching irregular with subdichotomous acute apices, widely spaced to three or four orders throughout. Branches occasionally basally constricted. Thallus is cartilaginous. Both discoid and lacerate holdfasts have been observed.</td>
</tr>
<tr>
<td><strong>Stipe width and length</strong></td>
<td>2 cm long (0.5) 0.1–2.2 (2.7) mm diam.</td>
<td>1 cm long (1.0) 1.2–1.5 (2.0) mm wide</td>
</tr>
<tr>
<td><strong>Lower branches</strong></td>
<td>2.5–4.0 (5.8) mm wide and 0.3–0.5 (0.6) mm thick</td>
<td>1.5–1.7 mm diam.</td>
</tr>
<tr>
<td><strong>Lower axes</strong></td>
<td>1.7–2.3 (2.6) mm wide and 1.3–1.9 mm thick</td>
<td>Abruption</td>
</tr>
<tr>
<td><strong>Major branches</strong></td>
<td>1.4–1.8 (2.1) mm wide and 1.3–1.6 mm thick</td>
<td>(1) 2–4 cells thick and (25) 30–37 (40) μm wide</td>
</tr>
<tr>
<td><strong>Cortex–medulla transition</strong></td>
<td>Abrupt</td>
<td>Isodiametric</td>
</tr>
<tr>
<td><strong>Cortex</strong></td>
<td>1–2 cells thick</td>
<td>(900) 1500–1600 μm in width</td>
</tr>
<tr>
<td><strong>Cortex cell shape</strong></td>
<td>Hemispherical to ovoid</td>
<td>Polygonal-sub spherical</td>
</tr>
<tr>
<td><strong>Cortex cell size</strong></td>
<td>(7) 9–17 μm long by (6) 8–14 (16) μm diam.</td>
<td>(95) 250–320 (550) μm diam.</td>
</tr>
<tr>
<td><strong>Medulla</strong></td>
<td>6–9 cells broad</td>
<td>Present</td>
</tr>
<tr>
<td><strong>Medulla cell size</strong></td>
<td>(65) 90–180 (240) μm diam.</td>
<td>Dioecious</td>
</tr>
<tr>
<td><strong>Medulla cell shape</strong></td>
<td>Polyanal-sub spherical</td>
<td>Spermatangia globose 4–5 μm, Polycavernosa-type conceptacles in sub cortex to outer medulary regions, 50–85 μm across, consisting of &gt;4 cavities</td>
</tr>
<tr>
<td><strong>Hair and basal cells</strong></td>
<td>Abundant except in pressed specimens</td>
<td>Cystocarps solitary, globoid and slightly rostrate</td>
</tr>
<tr>
<td><strong>Gametophytes</strong></td>
<td>Dioecious, occasionally bisexual</td>
<td>Exclusively directed towards floor of cystocarp cavity</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>Spermatangia globose to ovoid, 2–4 μm, Polycavernosa-type conceptacles in cortex and medulla, 100–250 μm deep by 115–200 μm wide</td>
<td></td>
</tr>
<tr>
<td><strong>Female cystocarps</strong></td>
<td>Cystocarps, solitary ostiolate</td>
<td>Cystocarps solitarily, globoid and slightly rostrate</td>
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<tr>
<td><strong>Traversing filaments</strong></td>
<td>Common to abundant, from gonimoblast cells penetrating deep into gametophyte tissue basal to carpophorophyte, and lower third of pericarp</td>
<td>Exclusively directed towards floor of cystocarp cavity</td>
</tr>
<tr>
<td><strong>Cystocarp size</strong></td>
<td>(0.5) 1.0–1.6 mm high by (0.7) 1.6–2.0 mm wide</td>
<td>To 1.3 mm wide</td>
</tr>
<tr>
<td><strong>Cystocarp base</strong></td>
<td>Sometimes constricted at base</td>
<td>Slightly constricted at base</td>
</tr>
<tr>
<td><strong>Cystocarp position</strong></td>
<td>Occurring on upper third of thallus</td>
<td>Occurring on secondary branches and above</td>
</tr>
<tr>
<td><strong>Pericarp tissue</strong></td>
<td>240–360 μm wide and 11–17 cells thick</td>
<td>220–270 μm wide and 7–10 cell layers thick</td>
</tr>
<tr>
<td><strong>Carpophore shape and size</strong></td>
<td>Obovoid, globose or oval, 26–38 μm by 20–27 μm</td>
<td>Subspherical, up to 25 μm diam.</td>
</tr>
<tr>
<td><strong>Carpophore origin</strong></td>
<td>Borne terminally on gonimoblast filaments</td>
<td>Borne terminally on gonimoblast filaments</td>
</tr>
<tr>
<td><strong>Tetrasporangia</strong></td>
<td>Cruciate divided, 28–40 μm long by 25–30 μm wide, in outer cortex, mainly in laterals and smaller branches</td>
<td>Cruicately decussately divided, 26–29 μm long by 17–26 μm wide, scattered in outer cortex, borne on all branches</td>
</tr>
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†From Withell et al. (1994).
Gracilaria perplexa sp. nov.

250–300 µm thick. Carposporangia are ovoid, reaching 37–40 µm in diameter. Males and tetrasporophytes were not observed.

Portsea (38°19'S, 144°43'E) (Fig. 1)
Material Examined: Plants at ca 2-m depth attached to shells and rocks, scattered and arising individually under the Portsea pier. (K. Byrne and N. Watt, 25.iv.1999, MELU A042861, A042860, A042872).

Morphology, Vegetative Anatomy and Reproduction:
As described for the Portland specimens.

Remarks: The material studied conforms to recent descriptions of G. cliftonii by Withell et al. (1994) and Womersley (1996). Neither isolate (3957, 3984) grew satisfactorily in laboratory culture.

Fig. 5. a–e. Gracilaria cliftonii from Portland, Victoria (MELU A042860, A042861, A042872). a. Field-collected sterile thallus. b. Cross-section of thallus showing extremely gradual cortex-to-medulla transition. c. Rostrate cystocarp on a field-collected thallus. d. Traversing filaments (arrows) entering the pericarp from peripheral gonimoblast tissue. e. Cross-section of mature cystocarp.
Phylogenetic analysis

All sequence data has been deposited in GenBank (Table 1).

The plastid-encoded RuBisCo spacer produced an unambiguously aligned data set of 317 characters with 83 potentially parsimony-informative characters. All phylogenetic reconstruction methods (MP, NJ and ML) produced similar tree topologies. Maximum parsimony produced seven equally most parsimonious trees of 285 steps (Consistency index (CI) = 0.6807). The topology of the ML tree (–ln likelihood score = 1650.736) is shown in Fig. 6.

The three samples of G. chilensis from Marion Bay, Phillip Island and Glenelg River, and the GenBank sequence of G. chilensis from New Zealand, formed a well-supported group with Gracilaria tenuistipitata. The relationship between these three G. chilensis samples and the G. chilensis from New Zealand is not resolved in the most parsimonious tree (only 2 bp difference between all samples). Gracilaria tenuistipitata differs by 7–9 bp from the G. chilensis samples. The first ribosomal intergenic spacer reveals some base pair variation between these G. chilensis samples (Table 3), although no pattern is obvious.

The three samples of G. cliftonii form a well-supported group, with a possible sister relationship, although without bootstrap support, to Gracilaria eucheumatoides and Gracilaria secundata.

Gracilaria perplexa samples formed a well-supported group, distinct from the other Australian species, with G. preissiana as its sister species.

Cox2–3 spacer region

The mitochondrial-encoded cox2–3 spacer produced an unambiguously aligned data set of 362 characters with 107 potentially parsimony-informative characters. All phylogenetic reconstruction methods (MP, NJ and ML) produced similar tree topologies. Maximum parsimony produced 24 equally most parsimonious trees of 307 steps (CI = 0.7655). The topology of the ML tree (–ln likelihood score = 1682.763) is shown in Fig. 7.

The relationships inferred for the cox2–3 spacer region shows similar tree topology to the RuBisCo tree, although there are fewer taxa upon which to base phylogenetic inferences since there are no cox2–3 spacer regions for Gracilaria species in GenBank. Some of the species relationships at the termini of the branches are more resolved or better supported due to the known higher mutation rate of this spacer compared with the RuBisCo spacer (Zuccarello and West 2002). Although the cox2–3 sequence has not been determined for the New Zealand G. chilensis, the trees strongly support the clade of the three Australian samples, as previously shown for the RuBisCo tree. Similarly, G. tenuistipitata forms a close relationship to the G. chilensis samples, in this case a well-supported sister relationship.

The three samples of G. cliftonii form a strongly supported clade. Gracilaria secundata Harvey and Gracilaria sp.1. (‘verrucosa’-like) form a sister group with these samples. The sister group relationship with the Philippine species might be expected from the ‘verrucosa’-type males of both species (Withell et al. 1994; fig. 5h).
Gracilaria perplexa sp. nov.

Again, G. perplexa forms a separate well-supported clade containing G. preissiana as a well-supported sister species. Samples of G. perplexa were collected on three occasions at Bare Island (10.xii.1998, 22.vi.1999 and 10.iii.2000) and at Kurnell (29.iii.2000); all samples had identical cox2–3 spacer sequences.

Polysaccharides

Physical properties

The polysaccharide of G. perplexa differed from that of the other taxa analyzed in requiring the addition of 0.1 mol L\(^{-1}\) NaCl to the extract for alcohol precipitation and being soluble in H\(_2\)O at 25°C. The polysaccharide preparations of the other samples showed the characteristic agar property of being insoluble in H\(_2\)O at 40°C. In 0.5% (w/v) solution, their gelation was ion-independent, of good clarity and thermoreversible. The crude polysaccharide yields of samples from five Gracilaria populations differ substantially. Gracilaria cliftonii produced an exceptionally high yield (52%) of crude polysaccharide, exceeding that of G. perplexa (39%). Samples of G. chilensis from Marion Bay, Phillip Island and Glenelg River all produced low crude polysaccharide yields of between 11 and 16%.

Fourier transform infrared spectroscopy

The FTIR spectra of polysaccharide preparations from all Gracilaria populations were recorded. The diagnostic regions (1400–700 cm\(^{-1}\)) for three preparations are shown in Fig. 8 for comparison. For all preparations, except that of G. perplexa, the FTIR patterns obtained were characteristic of agars, showing essentially no absorption peaks over the range 850–800 cm\(^{-1}\) that are indicative of various equatorial and axial sulfate esters at significant levels. The spectra also consistently show a very weak band of absorption at ca 1250 cm\(^{-1}\), reflecting very low levels of total sulfate ester (Stancioff and Stanley 1969). Strong bands at 932.75 and 892.05 cm\(^{-1}\), characteristic of agar biopolymers, demonstrate the presence of 3,6-anhydrogalactosyl and unsulfated 3-linked galactopyranosyl residues, respectively (Whyte et al. 1985).

The preparation from G. perplexa exhibits an intense absorption band at 850 cm\(^{-1}\) (Fig. 8), denoting that the polysaccharide is heavily sulfated at the C(O)\(_4\) position of the 3-linked galactosyl residues.
DISCUSSION

The characterization of *Gracilaria* species is largely based on gross plant morphology and the developmental morphologies of vegetative and reproductive structures (Withell et al. 1994; Womersley 1996). In a genus as large and morphologically plastic as *Gracilaria* obviously is, it would seem inevitable that the relatively few consistent features that classically distinguish species are going to show much overlap and ambiguity, particularly in cases where field populations are seldom if ever represented by all life history stages. This is particularly true of the filiform types that make up the bulk of dominant *Gracilaria* species in virtually every world region. Withell et al. (1994) focused mainly on eastern and western Australian members of the genus, on a number of particularly morphologically distinctive taxa, and paid relatively little attention to the much more widespread terete forms, of which some 15 are named in their appendix of currently accepted Australian *Gracilaria* species. The research reported here has concentrated on these ambiguous types that make up the *Gracilaria* populations indigenous to Victoria, Tasmania and parts of New South Wales.

To break morphological ambiguity, plasticity and incomplete expression of life history phases that so often characterize field populations of *Gracilaria*, molecular, polysaccharide and laboratory culture techniques were jointly applied. The RuBisCo spacer and cox2–3 spacer have been applied by other workers to *Gracilaria* and *Textorii* type spermatangial structures (Fig. 3c,f) distinctive of *G. chilensis* and which distinguish it from a number of similar-appearing taxa, including several Australian representatives designated by May (1948) as various forms of *G. confervoides* (= *G. verrucosa*). We conclude from these results that *G. chilensis* in Australia is characteristic of habitats in which large salinity fluctuations are the norm, such as the estuary of the Glenelg River, the sloughs of Marion Bay, and the intertidal mudflats of wave-sheltered northern Phillip Island and Hobsons Bay. As pointed out by Womersley (1996), these environments are typical of *G. chilensis* as described previously from both Chile (Bird et al. 1986) and New Zealand (Nelson 1987). The Marion Bay population, in particular, shows great promise as a potential feed stock for abalone mariculture, growing in dense aggregates of unattached, vegetatively fragmenting fronds in slack water subject to wide salinity fluctuations. Its assignment to *G. chilensis* (plants from Marion Bay previously had been regarded as likely forms of *G. secundata* and were filed under that name in MELU) has a number of important implications, not least of which is that, far from being unique to Tasmania, this species is native to Victoria and thus would not constitute an exotic import subject to special Environmental Protection Agency and Australian Quarantine and Inspection Service regulations if its cultivation were to be undertaken by the abalone industry in Victoria.

Molecular evidence indicates that *G. perplexa*, from central New South Wales, is a sister species to *G. preissiana* from Western Australia. Comparison of the morphology and anatomy of these two species (Table 2) show that these two species can be readily distinguished by their gross morphology, with *G. preissiana* being more compressed (flattened) on the main and lower axes and having many short undivided laterals arising from blade margins. The restricted range of *G. perplexa* to Botany Bay probably has more to do with the lack of effort in collecting in the lower intertidal/shallow subtidal in this region than a restricted range of this species.

Polysaccharides and Fourier transform infrared spectroscopy

The agar yields of most *Gracilaria* species have been reported to be over the range of 10–30%. The exceptionally high yield of crude agar preparation obtained from *G. cliftonii* was thus surprising, although its quality remains to be accurately analyzed. In contrast,
the three samples of *G. chilensis* produced agars at the low end of the yield range. It should be noted, however, that different agarophytes vary significantly in the optimum conditions for their agar extraction. The extraction protocol adopted in this study represents only one approach, by no means optimized for each species. Furthermore, *G. chilensis* is established as an agarophyte of commercial importance, based on its agar yield, quality and growth performance in field culture (e.g. Pickering et al. 1993).

All the polysaccharide preparations obtained, except that from *G. perplexa*, showed physicochemical properties characteristic of agars. Interestingly, no polysaccharide could be obtained from the *G. perplexa* precipitation process without the addition of NaCl. This necessary addition of NaCl, together with the FTIR spectrum and cold-water solubility of the preparation from *G. perplexa*, initially suggested that it contained κ-β-carrageenan when compared with infrared data established by Liao et al. (1993), or a highly sulfated agar (Whyte et al. 1985). Anatomical features confirmed that the species could only be a *Gracilaria*, a genus not known to produce any cell-wall hydrocolloid other than agar. Confirmation of the agar identity of the polysaccharide from *G. perplexa* can readily be achieved by using $^{13}$C nuclear magnetic resonance spectroscopy (Usov 1984). Agars with high levels of sulfation have been recorded from several species of *Gracilaria* in recent literature, such as *Gracilaria edulis* by Villanueva and Montano (1999). Like that of *G. perplexa*, these agars have heavy sulfation at the C(O)$\alpha$ position of the 3-linked galactosyl residues. Fourier transform infrared spectroscopy, a very useful technique for qualitative and semiquantitative comparisons of the 3,6-anhydrogalactosyl residues and sulfate esters (Stancioff and Stanley 1969; Rochas et al. 1986), was employed in this study for rapid screening of the cell-wall polysaccharides, although testing of other quality parameters is needed for overall assessment of the agar samples.

In conclusion, our study integrates several biological/molecular approaches in this study of the commercially important genus, *Gracilaria*, in southeastern Australian waters. We have shown that *G. chilensis* is common in Victorian waters, *G. cliftonii* produces the highest agar yield of the investigated species, and we have described a new species *G. perplexa* Byrne et Zuccarello from New South Wales.

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REFERENCES
