Discovery of a novel brown algal genus and species *Setoutiphycus delamareoides* (Phaeophyceae, Ectocarpales) from the Seto Inland Sea, Japan

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Abstract

A novel brown algal genus and species *Setoutiphycus delamareoides* (Ectocarpales *sensu lato*) is described from the Seto Inland Sea, Japan. The species is similar to *Delamarea attenuata* in gross morphology and anatomy, but distinctive in having longer thalli with rare branching, shorter cortical cells, and occurrence of branches at the tip of plurilocular zoidangia. Erect thalli were epilithic, solitary or caespitose, filiform, simple or rarely branched, attenuated towards the base, blunt at the tip, with uneven surface, yellowish brown in color, up to about 15 cm in height and 2 mm in diameter, parenchymatous, solid when young and later becoming hollow, composed of 1–2 layers of large colorless inner cells, subcortical cells, barrel-shaped large cortical cells of up to 100 μm in height and up to 60 μm in diameter, and phaeophycean hairs. Plurilocular and unilocular zoidangia developed on the same thallus, the former conical to lanceolate, sometimes branched at the tip and projected from the cortical cells, and the latter ovate. Each cell contained many discoidal chloroplasts with projected pyrenoids. In the molecular phylogeny using concatenated DNA sequences of mitochondrial *cox*1, *cox*3 and chloroplast *atp*B, *psa*A, *psb*A and *rbc*L genes, the novel alga nested in the clade composed of ectocarpalean genera of diffuse growth, with parenchymatous thalli with multiple chloroplasts, but was distinctive. Therefore, we propose the establishment of a new genus and species *Setoutiphycus delamareoides gen. & sp. nov.* for the novel alga, and provisionally place it in Chordariaceae, Ectocarpales *sensu lato*. As to the biogeography, the Seto Inland Sea repeatedly dried by sea level regression during the glacial periods and the present sea level has recovered after the LGM of ca. 10,000 years ago. Therefore, it is unlikely that the evolution of the genus and species occurred within this area, and its isolated distribution may be explained as a remnant population that survived in the refugia in southern Japan during LGM. It is possible that the species also has a broader distributional range, at least in Japan, such as northern Honshu, but if not, the species can be endangered due to the raise of seawater temperature in the area by global climate change, because the Seto Inland Sea is enclosed at its northern end, so the population cannot spread to colder more northern coasts.

Introduction

The higher rank taxonomy of brown algae (Phaeophyceae) has been considerably revised in the last few decades, especially in certain orders, by the application of life history studies and molecular phylogenetic analyses [1,2,3,4,5]. Ectocarpales and its related orders, comprising taxonomic groups of relatively small, soft thalli and showing heteromorphic life histories, are an example. Traditionally, members of Ectocarpales *sensu lato* have been classified in independent orders within Ectocarpales *sensu stricto*, Chordariales, Dictyosiphonales and Scytosiphonales, or Ectocarpales *s.l.* including Ectocarpales *s.s.*, plus Chordariales, Dictyosiphonales and Scytosiphonales, based on the thallus constructions (filamentous, or pseudoparenchymatous/haplostichous, or parenchymatous/polystichous) and number of chloroplasts per cell (single or multiple). However, life history studies suggested indistinct boundaries separating those orders, and molecular phylogenetic analyses did not support the monophyly of those orders except for Scytosiphonales. Therefore, it was proposed to classify them in a single order Ectocarpales *s.l.*, including

Scytosiphonales as a family (Scytosiphonaceae), and merging many genera that used to be placed in Dictyosiphonales and Chordariales into the family Chordariaceae [6,7]. As a result, currently more than a hundred genera are included in Chordariaceae, which is an exceptionally great number in the brown algae, and their morphology, including the basic thallus architecture, is highly diverse. On the other hand, a new monotypic family was proposed primarily based on molecular phylogenetic data (*i.e.*, Petrospongiaceae[8]). Also, the taxonomic status of some families is still unclear because their diagnostic characters are not consistent, and phylogenetic resolution of the molecular phylogenetic studies is insufficient. Therefore, family level taxonomic delineation of Ectocarpales is still rather confused and substantial revisions are needed.

Among them, genus and species level taxonomy of the ectocarpalean members with terete, parenchymatous thalli having multiple chloroplasts have been relatively well documented because of their easily recognizable macroscopic thalli, distinctive anatomical features applicable to taxonomic comparisons, and the substantial number of life history studies using unialgal cultures elucidating their life histories and early development. Most of their genera were described by the 1960's, and no additional taxa at genus level have been published for several decades, excluding the proposals of new a genus for known species originally described in a different genus[2,4,9,10,11]. However, recently in the western part of the Seto Inland Sea, Japan, which has a unique geography but has had only limited taxonomic surveys, we collected an undescribed brown alga with unique morphological features. Here we describe, based on morphology and molecular phylogenetic studies, a novel member among the terete parenchymatous species belonging to Ectocarpales *s.l.*, and discuss the family level taxonomy.

Results

Morphological studies

This novel brown alga appeared as a spring annual growing on hard substrates of sandy bottoms of the upper subtidal zone, together with various annuals such as *Acrothrix gracilis* Kylin, *Cutleria multifida* (Turner) Greville, *Striaria attenuata* (Greville) Greville, *Tinocladia crassa* (Suringar) Kylin, *Ulva intestinalis* Linnaeus, etc. (Fig. 1a). Erect thalli were epilithic or growing on dead shells, solitary or caespitose, filiform, simple or rarely branched, attenuated towards the base, blunt at the tip, surface rough yellowish brown in color, up to about 15 cm in height and up to 2 mm in diameter (Figs. 1b, 2a, b). The thalli were parenchymatous, solid when young and later becoming hollow, composed of one to two layers of large colorless inner cells, subcortical cells and barrel-shaped or short clavate cortical cells, and terminal and lateral phaeophycean hairs (Fig. 2c–h). Cortical cells measured up to 100 μm in height and up to 60 μm in diameter. Plurilocular and unilocular zoidangia were formed on the same thallus, developed among the cortical cells at the distal end of subcortical cells (Fig. 2h-k). Plurilocular zoidangia were formed by subdivisions of initial cells (Fig. 2i), conical to lanceolate, often branched at the tip (Fig. 2j), becoming longer than the cortical cells (Fig. 2i), up to 120 μm in height and up to 72 μm in diameter. Unilocular zoidangia were ovate, up to about 60 μm in height and up to 50 μm in diameter (Fig. 2k). Each cell

contained many discoidal chloroplasts with projected pyrenoids. In the cortical cells, chloroplasts were parietal at the distal end (Fig. 2d, i).

Molecular phylogeny

Molecular phylogenies based on concatenated mitochondrial *cox*1, *cox*3 and chloroplast *atp*B, *psa*A, *psb*A and *rbc*L gene sequences (7,696 bp) showed identical tree topologies by Maximum Likelihood (ML, Fig. 3) and Bayesian (BI) analyses. The novel alga nested in the clade composed of *Trachynema, Delamarea, Cladothele*, and *Punctaria* supported by full bootstrap/posterior priority values, although the supports for the nodes connecting the novel alga with other genera were low (clade 1). This clade was sister to the clade of *Striaria* and *Asperococcus* supported by full bootstrap/p.p. values (clade 2).

In the molecular phylogeny based on *rbc*L gene sequences covering a large portion of the genera in Ectocarpales, the novel alga was also included in the clade composed of *Cladothele, Delamarea, Hecatonema, Punctaria* and *Trachynema* supported by medium and high bootstrap/p.p. values (95%/1.00), but the relationships among the genera were not resolved (clade 1; Fig. 4). The novel alga was nested in the clade of *Punctaria latifolia* Greville, *Hecatonema* sp. and *Punctaria plantaginea*, but the statistical supports for the nodes connecting them were low. *Hecatonema* has filamentous thalli, but the sequenced specimen was likely a gametophytic stage of *Punctaria* sp.[12]. Clade 1 was sister to clade 2 as in the phylogenetic tree based on six genes, but the statistical support was low. In contrast, some of the ectocarpalean genera such as *Coelocladia, Litosiphon, Pogotrichum* and *Stictyosiphon* with parenchymatous terete thalli and many chloroplasts with projected pyrenoids, having similar basic thallus constructions as the novel alga, showed distant phylogenetic relationships with these genera, and were scattered in the *rbc*L tree (Fig. 4).

Discussion

In morphology, the novel alga was most similar to *Delamarea attenuata* Hariot. They shared terete rough thalli with an attenuated basal portion and blunt tip, thallus architecture composed of large inner cells and a cortical layer developing large clavate or barrel-shaped cortical cells, and the occurrence on the erect thalli of both unilocular and plurilocular zoidangia among large cortical cells. However, the novel alga was distinctive in having longer thalli with rare branching, shorter cortical cells, and occurrence of branches at the tips of the plurilocular zoidangia. *Cladothele, Punctaria* and *Trachynema, Striaria* and *Asperococcus*, which showed close phylogenetic relationships in our molecular phylogeny based on the concatenated DNA sequences of six genes, shared similar thallus morphological features: terete parenchymatous thalli of diffuse growth bearing unilocular and plurilocular zoidangia among large cortical cells, and terminal and lateral phaeophycean hairs. Although *Punctaria* spp. have foliose thalli, their juvenile thalli show similar terete stages with apical and opposite lateral hairs, which are common to those genera. Our multigene molecular phylogeny, including most of the related genera, showed that the novel alga is distinct from any of those genera. Therefore, for this novel alga we propose the establishment of a new genus and species, *Setoutiphycus delamareoides gen. & sp. nov.*. In contrast, *rbc*L

of other ectocarpalean genera with similar thallus architecture (*e.g.*, *Coelocladia*, *Litosiphon*, *Pogotrichum* and *Stictyosiphon*) showed distant phylogenetic relationships with the clade, suggesting convergent evolution of the thallus architecture in Ectocarpales.

Small brown algae having parenchymatous thalli and multiple chloroplasts with projected pyrenoids have been generally classified in Dictyosiphonales[13,14,15] or in Ectocarpales *s.l.* including Ectocarpales *s.s.*, Dictyosiphonales, Chordariales and Scytosiphonales[9,16]. Within these orders, members were placed in the families Asperococcaceae, Adenocystaceae, Coelocladiaceae, Delamareaceae, Punctariaceae, Striariaceae, etc.[10,16,17]. For *Delamarea* and *Cladothele*, a new family Delamareaceae comprised of *Cladothele*, *Coelocladia*, *Delamarea* and *Stschapovia* was proposed based on the anatomical similarity of possessing large cortical cells (paraphyses)[18]. Later, a new order Delamareales was proposed for the family, assuming an isomorphic life history alternating between macrothalli forming plurilocular gametangia (gametophyte) or unilocular zoidangia (sporophyte) [19]. However, *Coelocladia arctica* Rosenvinge and *Delamarea attenuata* were shown by unialgal culture studies to have heteromorphic life histories[20,21], and since then the order has not been cited.

Because monophyly of the families with parenchymatous thalli in Dictyosiphonales or Ectocarpales *s.l.*, was not supported in molecular phylogenetic studies[6,22], expansion of Chordariaceae to include members that used to be classified in independent families such as Punctariaceae and Striariaceae was proposed[6]. In contrast, small brown algae having parenchymatous thalli and a single chloroplast with projected pyrenoids have been classified in Scytosiphonales[23], and in spite of this unique cytological feature and highly supported monophyly, they are classified as a family nested in Ectocarpales.

However, currently roughly 140 genera are included in Ectocarpales *s.l.*, more than 100 of which are of Chordariaceae, and these numbers are exceptionally great, considering the genetic divergence within each order of the Phaeophyceae[2,5,7,11] (Supplementary Information 4). The DNA sequence divergence of *rbc*L genes ranges from about 5–15% within each brown algal order. However, divergence was less than 10% in Ectocarpales *s.l.* and not especially high compared with other orders (Supplementary Information 4). In our molecular phylogeny, monophyly of several genera of former members of Dictyosiphonales (*i.e.*, Delamareaceae [*Delamarea*, *Cladothele*], Punctariaceae [*Punctaria*, *Trachynema*] and Striariaceae [*Striaria*, *Asperococcus*] sharing the following morphological features was supported: Parenchymatous, terete or foliose thalli of diffuse growth, with terminal and lateral (often opposite) phaeophycean hairs; normally forming both unilocular and lanceolate to ovoid plurilocular zoidangia; cells with many discoid chloroplasts with projected pyrenoids. Remarkably, in spite of its close morphological similarity with these genera, *Coelocladia* was shown to have phylogenetic relationship instead with the clade of *Cladothele*, *Delamarea*, *Punctaria*, *Setoutiphycus*, and *Trachynema*. Indeed, an independent family Coelocladiaceae has been proposed for *Coelocladia*, based on the unique morphology of the plurilocular zoidangia showing a clustered or crown-like appearance and the occasional sympodial branching of the primary filament[21]. In contrast, *Dictyosiphon*, the type of Dictyosiphonaceae and Dictyosiphonales, does

have parenchymatous terete thalli, but the genus is unique in showing apical growth by a single apical cell, and forming only unilocular zoidangia embedded in the subcortical and peripheral layers[9].

Although the number of taxa we examined were rather limited, our multigene molecular phylogeny based on six genes showed considerable improvement of the phylogenetic resolution of families within Ectocarpales *s.l.*. Therefore, we expect that the application of multigene molecular phylogeny to additional taxa will give clues for obtaining a better taxonomy of the family. As to the taxonomy of the three families traditionally used for the genera comprising the clade including *Setoutiphycus* (*i.e.*, Delamareaceae A.D.Zinova 1953, Punctariaceae (Thuret) Kjellman 1880, Striariaceae Kjellman 1890), Punctariaceae has taxonomic priority. Therefore, it is possible to reappraise Punctariaceae for the lineage in reorganizing current Chordariaceae by subdividing it to several monophyletic lineages sharing distinctive morphological features. However, for the moment, we suspend any taxonomic treatment, and provisionally place *Setoutiphycus* in Chordariaceae, Ectocarpales *s.l.*.

As to the biogeography of *Setoutiphycus delamareoides*, at present it has been only found from the western end of the Seto Inland Sea, Japan, and it is possibly endemic to the region (Supplementary Information 1). Similarly, an endemic red alga *Neorhodomela enomotoi* Masuda & Kogame was described from the Seto Inland Sea, and has not been reported from any other coasts[24]. Members of *Neorhodomela* are cool-temperate or cold-water species, and the localities of the species appear to represent the southern limits of their distributional ranges[24]. In spite of the low latitude (34° N) and short distance from the main flow of the Kuroshio Warm Current, water temperatures at the locality are relatively low (monthly average is from 9.5 to 26.5°C; Wanishi 2004), because of the enclosed geography of the area. The Seto Inland Sea repeatedly dried due to sea level regression during the glacial periods. Therefore, the history of the flora is rather recent, since the present sea level recovered after the LGM of only ca. 10,000 years ago[25]. Therefore, it is unlikely that the evolution of the genus and species occurred within this area. It has been noted that the macroalgal flora of the area is more similar to the cool-temperate Pacific coast of northern Honshu (Tohoku region) than that of the adjacent areas in Pacific Shikoku and Kyushu where the water temperature is higher[26]. This is explained as the result of separation of the populations that survived in the refugia in southern Japan during LGM[27]: during the northward expansion of the populations after the LGM, some of them survived in the Seto Inland Sea[26,27,28]. Therefore, it is possible that *S. delamareoides* has a broader distributional range, at least in Japan, such as northern Honshu. However, if not, the species can be endangered by the rise of seawater temperature in the area due to global climate change[29,30], because the Seto Inland Sea is enclosed at its northern end, so the population cannot spread to colder northern coasts.

Description And Diagnosis

Setoutiphycus gen. nov. H. Kawai & T. Hanyuda

Typus: *Setoutiphycus delamareoides* H. Kawai & T. Hanyuda

Erect thalli, filiform, rarely branched, attenuated towards the base, blunt at the tip, parenchymatous, solid when young and becoming hollow with age, composed of large colorless inner cells and barrel-shaped cortical cells and phaeophycean hairs. Plurilocular and unilocular zoidangia formed on the same thallus at the end of subcortical cells among the large cortical cells. Plurilocular zoidangia conical to lanceolate, often branched at the tip. Unilocular zoidangia ovate. Each cell containing many discoidal chloroplasts with projected pyrenoids.

The new genus resembles *Delamarea* in gross morphology and anatomy, but differs in the longer, rarely branched thallus and shorter cortical cells. The species differs from *Cladothele* in the epilithic habit and rare branching, and from *Trachynema* in having large cortical cells. Nucleotide sequences of mitochondrial *cox*1 and *cox*3, chloroplast *atp*B, *psb*A and *rbc*L genes are also distinctive.

Setoutiphycus delamareoides sp. nov. H. Kawai & T. Hanyuda Figs. 1, 2.

Erect thalli, epilithic, solitary or caespitose, filiform, simple or rarely branched, attenuated towards the base, blunt at the tip, yellowish brown in color, up to about 15 cm in height, up to about 2 mm in diameter, parenchymatous, solid when young and becoming hollow with age, composed of 1–2 layers of large colorless inner cells, barrel-shaped cortical cells, and phaeophycean hairs. Cortical cells measure up to 100 μm in height and up to 60 μm in diameter. Plurilocular and unilocular zoidangia on the same thallus, at the end of cortical cells, among the cortical cells. Plurilocular zoidangia conical to lanceolate, often branched at the tip, projected from the cortical cells, up to 120 μm in height and up to 72 μm in diameter. Unilocular zoidangia ovate, up to ca. 60 μm in height and up to ca. 50 μm in diameter. Each cell containing many discoidal chloroplasts with projected pyrenoids. Nucleotide sequences of mitochondrial *cox*1 and *cox*3, chloroplast *atp*B, *psb*A and *rbc*L genes are also distinctive.

Holotypus

SAP115639, Suo-Oshima (33.9407 N 132.4016 E), Yamaguchi, Japan, 10, April 2017.

Etymology: The genus name refers to the original locality. The specific epithet refers to the morphological features of the thallus.

Methods

Specimens were collected at Suo-Oshima, Yamaguchi, Japan by SCUBA diving (Supplementary Information 1). Portions of the specimens were quickly dried in silica-gel and used for molecular analyses. For anatomical observations, cross and longitudinal sections were made by hand using a razor blade. Photomicrographs were taken with a VB–7010 Digital Camera (Keyence, Tokyo, Japan) attached to an Axioplan microscope (Zeiss, Oberkochen, Germany).

DNA extractions were made from field-collected specimens rapidly desiccated in silica-gel (KU-d5871, -d17414, -d17417, -d17418) and unialgal culture strains housed in the Kobe University Macroalgal Culture Collection (KU-MACC, KU-1881) (Supplementary Information 2). Genomic DNA was extracted using a

DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications of the mitochondrial *cox*1, *cox*3 and chloroplast *atp*B, *psa*A, *psb*A and *rbc*L were carried out using the KOD FX (ToYoBo, Osaka, Japan) and the TaKaRa PCR Thermal Cycler Dice (Takara Bio, Otsu, Japan). Primers used for PCR and sequencing were as described in Supplementary Information 2. The profiles of PCRs were as described in Supplementary Information 3. The profiles of PCRs were as follows: initial denaturation at 94°C for 2 min; 30–35 cycles of denaturation at 98°C for 10 s, annealing at 50°C–46°C for 30 s, and extension at 68°C for 30 s; and a final extension at 72°C for 7 min. After PEG purification[31], PCR products were sequenced by a DNA sequencing service (FASMAC, Atsugi, Japan). For molecular phylogenetic analyses, published and newly determined DNA sequences were used (Supplementary Information 1). Molecular phylogenetic trees for each dataset (dataset 1: 28 OTUs, six genes, total 7,696 bp; dataset 2: 84 OTUs, *rbc*L gene, 1,413 bp) were constructed by Maximum Likelihood (ML) and Bayesian analysis (BI). The species of *Ectocarpus* and *Pylaiella* for dataset 1, and the species of *Asterocladon* for datasets 2 were selected as the outgroups. Alignments were prepared using the program MAFFT v.6[32] and then manually adjusted prior to phylogenetic analyses. Molecular phylogenetic trees were constructed by Maximum Likelihood (ML) and Bayesian analysis (BI). RAxML-NG v.1.0.0[33] was used for ML analyses. The best-fit substitution model for each codon position of each gene was selected by ModelTest-NG v.0.1.7[34]. To find the best tree, 1,000 tree searches using 500 random and 500 parsimony-based starting trees were performed. Bootstrap searches were performed with 1,000 replicates. Bayesian analyses were run using MrBayes v.3.2.2[35]. With the aid of the Kakusan4 program[36], the best-fit substitution model for each codon position of each gene was selected. The Bayesian analyses were initiated with a random starting tree and ran four chains of Markov chain Monte Carlo iterations simultaneously for 10,000,000 generations, keeping one tree every 100 generations. The first 25,000 trees sampled were discarded as 'burn-in', based on the stationarity of ln L as assessed using Tracer v.1.7.1[37]. A consensus topology and posterior probability values were calculated from the remaining trees.

Declarations

Author contribution statement

H. K. collected the specimens and made morphological observations and wrote the paper, T. H. did the molecular phylogeny.

Competing interests

The authors declare no competing financial interests.

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Figures
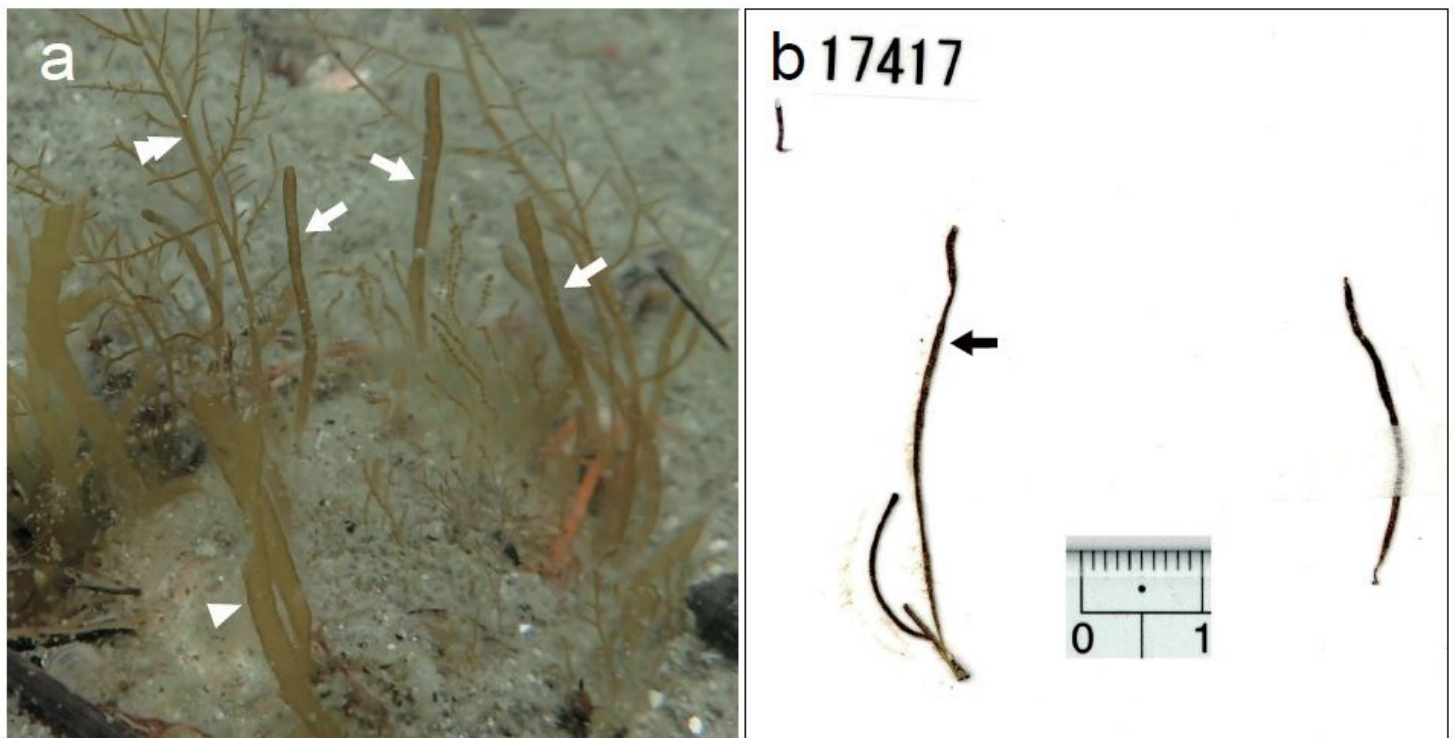

Figure 1

Habit and type specimen of Setoutiphycus delamareoides (Suo-Oshima, 12 April 2017). a, Underwater photograph showing habit of S. delamareoides (arrows) growing mixed with Acrothrix gracilis (double arrowhead) and Striaria attenuata (arrowhead). b, Holotype specimen (arrow; SAP115639; silica-gel dried specimen housed as KU-d17417).
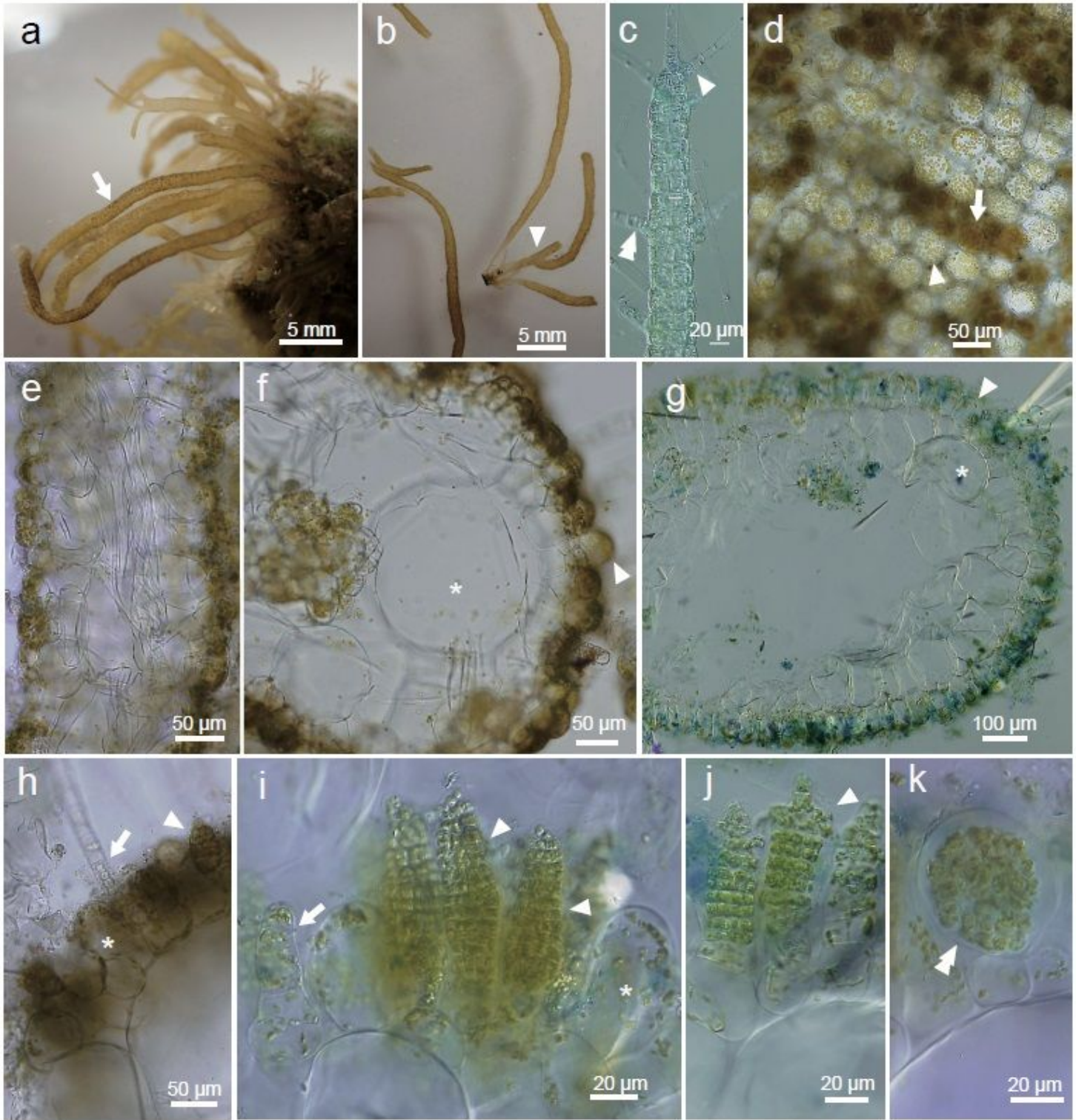
Figure 2

Morphology and anatomy of Setoutiphycus delamareoides (Suo-Oshima, 12 April 2017). a, Erect thalli (arrow) growing on shell of dead barnacle. Reproductive structures are recognizable as dark dots. b, Caespitose erect thalli showing a rare branch (arrowhead). c, juvenile thallus with apical (arrowhead) and lateral (double arrowhead) phaeophycean hairs. d, Surface view of fertile erect thallus showing cortical cells containing peripheral discoid chloroplasts with projected pyrenoid (arrowhead) and plurilocular

zoidangia (arrow). e, Longitudinal section showing large inner cells and pigmented cortical layer composed of cortical cells. f, Cross section of young solid thallus. Asterisk shows large inner cell. g, Cross section of developed hollow thallus. Asterisk shows large inner cell. Arrowhead shows barrel-shaped cortical cell. h, Cortical cells (asterisk) and phaeophycean hair (arrow). i, Plurilocular zoidangia (arrowhead) formed among cortical cells (asterisk). Arrow shows premature plurilocular zoidangium. j, Plurilocular zoidangium forming branches at the tip (arrowhead). k, Unilocular zoidangium (double arrowhead) borne on subcortical cell. a, b, d−f, h: Fresh specimens. c, g, i-k: Preserved in corn syrup and stained with Cotton Blue.
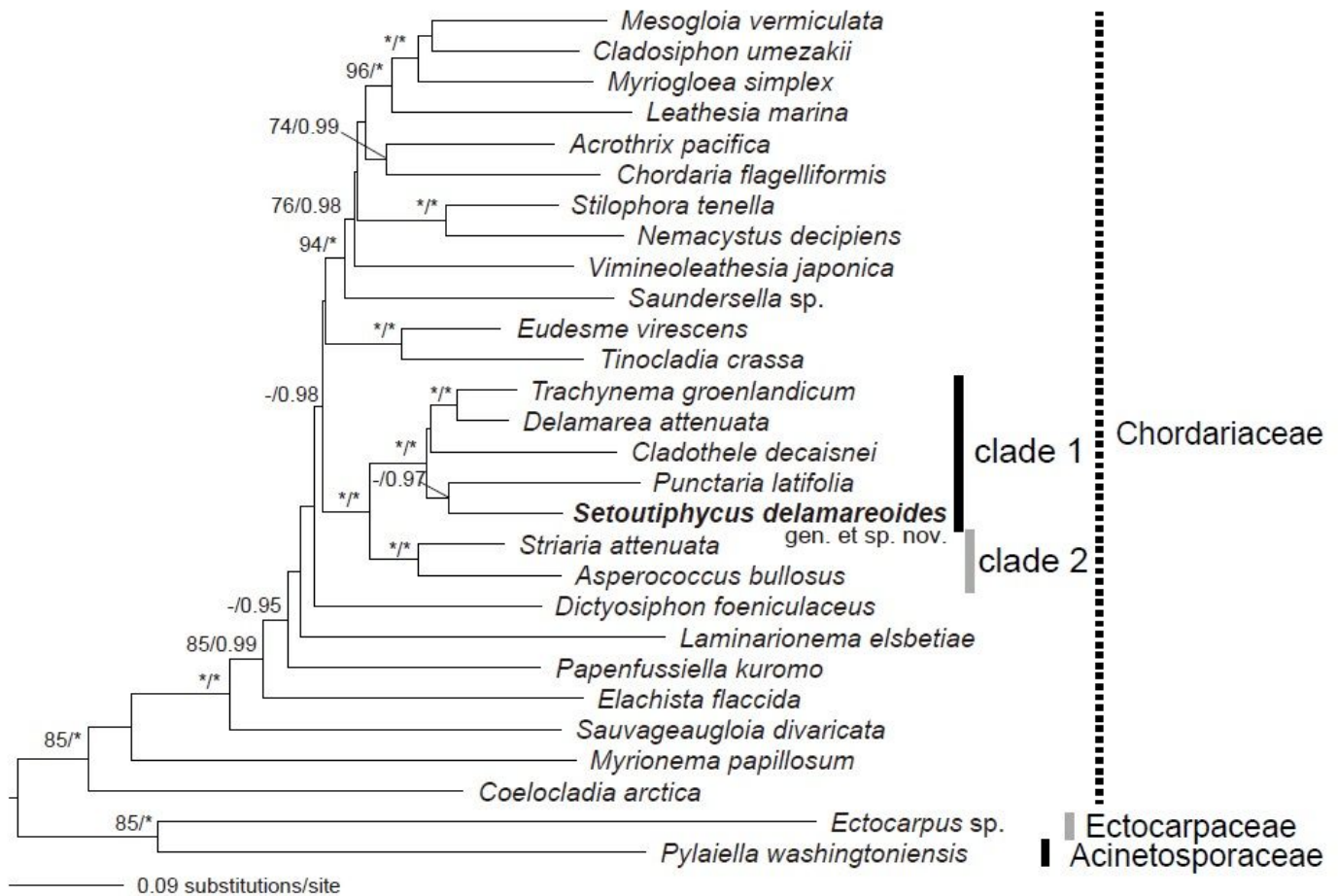

Figure 3

Maximum Likelihood molecular phylogeny of selected ectocarpalean species including Setoutiphycus delamareoides gen. & sp. nov. based on concatenated DNA sequences of mitochondrial cox1 and cox3 and chloroplast atpB, psaA, psbA and rbcL genes (7,696 bp). Numbers on branches indicate bootstrap values (%) from ML analysis (left) and posterior probabilities from Bayesian analysis (right). Asterisks (*) indicate 100% bootstrap (ML) and 1.00 posterior probability (Bayesian) values. Only bootstrap values >70% and posterior probabilities >0.90 are shown.
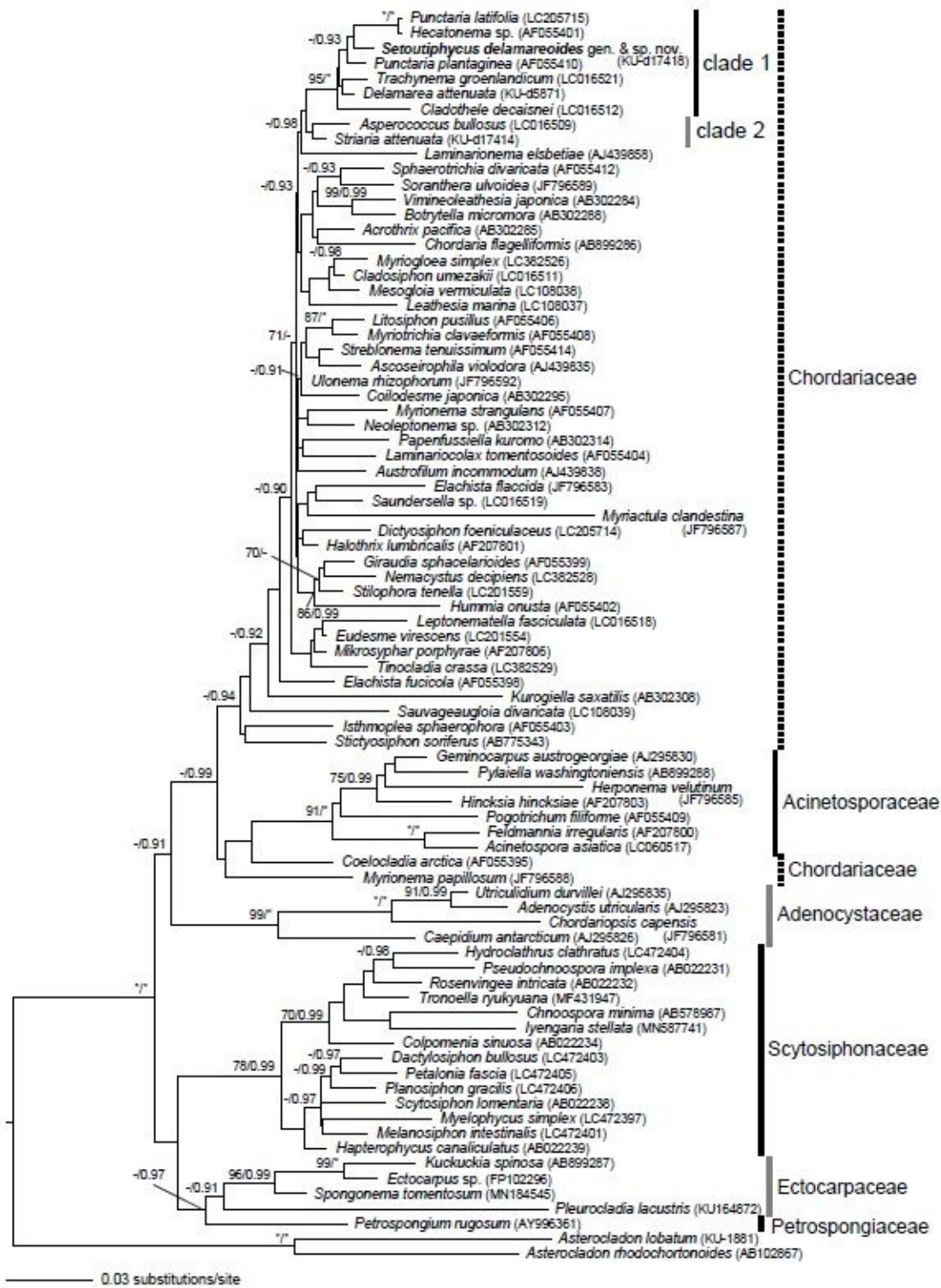
Figure 4

Maximum Likelihood molecular phylogeny of ectocarpacean species including Setoutiphycus delamareoides gen. & sp. nov. based on rbcL genes DNA sequences (1,413 bp). Numbers on branches indicate bootstrap values (%) from ML analysis (left) and posterior probabilities from Bayesian analysis (right). Asterisks (*) indicate 100% bootstrap (ML) and 1.00 posterior probability (Bayesian) values. Only bootstrap values >70% and posterior probabilities >0.90 are shown.

Supplementary Files

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- SetoutiphyusMSsi2021feb17rev.pdf