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Taxonomic revision of the agaraceae with a description of *Neoagarum* gen. nov. and reinstatement of *Thalassiophyllum*

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Abstract

We confirmed the monophyly of the Agaraceae based on phylogenetic analyses of 6 mitochondrial and 6 chloroplast gene sequences from *Agarum*, *Costaria*, *Dictyoneurum* and *Thalassiophyllum* species as well as representative species from other laminarialean families. However, the genus *Agarum* was paraphyletic, comprising two independent clades, *A. clathratum/A. turneri* and *A. fimbriatum/A. oharaense*. The latter clade was genetically most closely related to *Dictyoneurum* spp., and morphologically the species shared a flattened stipe bearing fimbriae (potential secondary haptera) in the mid to upper portion.

The phylogenetic position of *Thalassiophyllum* differed between the two datasets: in the chloroplast gene phylogeny *Thalassiophyllum* was included in the *A. clathratum/A. turneri* clade, but in the mitochondrial gene phylogeny, it formed an independent clade at the base of the Agaraceae, the same position it took in the phylogeny when the data from both genomes were combined despite a larger number of bp being contributed by the chloroplast gene sequences. Considering the remarkable morphological differences between *Thalassiophyllum* and other Agaraceae, and the molecular support, we conclude that *Thalassiophyllum* should be reinstated as an independent genus. *Dictyoneurum reticulatum* was morphologically distinguishable from *D. californicum* due to its midrib, but because of their close genetic relationship, further investigations are needed to clarify species level taxonomy. In summary, we propose the establishment of a new genus *Neoagarum* to accommodate *A. fimbriatum* and *A. oharanese* and the reinstatement of the genus *Thalassiophyllum*.

Introduction

Traditionally the order Laminariales has been characterized by a heteromorphic life history alternating between large parenchymatous sporophytes with an intercalary growth zone and filamentous, oogamous gametophytes (Kylin 1916, Sauvageau 1916, Fritsch 1945, Bold & Wynne 1985, Kawai 2014). Genera were traditionally classified into four families: Alariaceae Setchell et Gardner 1925, Chordaceae Dumortier 1822, Laminariaceae Bory

1827, and Lessoniaceae Setchell et Gardner 1925. Later, Kawai and Kurogi (1985) added the Pseudochordaceae. Using a molecular phylogenetic approach with 18S rDNA sequencing, Boo et al. (1999) confirmed the monophyly of derived Laminariales including members of the Laminariaceae, Lessoniaceae and Alariaceae and suggested that the Pseudochordaceae was sister to the laminarialean lineage that leads, through the Chordaceae, to the derived Laminariales. Kawai & Sasaki (2001 '2000') added Akkesiphycaceae Kawai et Sasaki 2001 to the Laminariales on the basis of *rbc*L gene, 18S and ITS rDNA sequences and extended the definition of the order to include a taxon with plano-anisogamy (Kawai 1986). Yoon et al. (2001) using *rbc* spacer and ITS rDNA sequences showed a strong monophyly of the genera *Agarum, Costaria, Dictyoneurum* and *Thalassiophyllum,* that *Egregia* was the sister taxon to the remaining derived Laminariales, and suggested the necessity of systematic revision of the taxa. Lane et al. (2006) reexamined the phylogeny of derived Laminariales using ITS and 26S rDNA, *rbc*L-*rbc*S and *nad*6 gene sequences and suggested that derived Laminariales consists of three major lineages roughly corresponding to Alariaceae ('Group-1'), a newly proposed Costariaceae C.E.Lane, C.Mayes, Druehl et G.W.Saunders ('Group-2'), and Laminariaceae/Lessoniaceae ('Group-3'). As to the notion of the basalmost position of *Egresia*, Lane et al. (2006) concluded that this was an artifact

of biased taxon sampling. Subsequently Kawai et al. (2013) described a new family Aureophycaceae based on the recently described species *Aureophycus aleuticus* H.Kawai, T.Hanyuda, M.Lindeberg et S.C.Lindstrom (Kawai et al. 2008). They suggested that Aureophycaceae represents the first diverged lineage in the derived Laminariales on the basis of their multigene molecular phylogeny and the reproductive morphology of *A. aleuticus*. Thus, at present, the Laminariales consists of three basal families and four derived families. Silberfeld et al. (2014) pointed out that the family Agaraceae Postels et Ruprecht 1840 (as Agaroideae) has priority over Costariaceae (Lane et al. 2006), and we use Agaraceae in the present study. Species of the Agaraceae are distributed in cool waters of the North Pacific, with at least one species of *Agarum* also found in the Northwest Atlantic (Boo et al. 2011).

Lane et al. (2006) included four genera, *Agarum, Costaria, Dictyoneurum* and *Thalassiophyllum* in Agaraceae (as Costariaceae). In the genus *Agarum,* four to five species have been recognized: *A. clathratum* Dumortier (= *A. cribrosum* Bory de Saint-Vincent), *A. fimbriatum* Harvey, *A. oharaense* Yamada (Yamada 1961), *A. yakishiriense* Yamada (Yamada [1962], but as a form of *A. clathratum* in Yamada [1974] or as a subspecies in Boo et al. [2011]), and *A. turneri* Postels et Ruprecht (Klochkova 1998). Miyata &

Yotsukura (2005) reported the genetically distant relationship between *A. oharaense* and *A. cribrosum* (= *A. clathratum*) using *rbc* spacer and ITS2 rDNA sequences. Later, in a molecular phylogenetic study using ITS rDNA, *cox*1, *cox*3 and *rbc* spacer sequences, Boo et al. (2011) observed *Thalassiophyllum clathrus* (S.G. Gmelin) Postels et Ruprecht to be included in the clade of *Agarum* spp. and therefore synonymized *Thalassiophyllum* with *Agarum*, thereby recognizing 6 species in *Agarum*. However, they did not include *Dictyoneurum*, another genus of Agaraceae, nor *A. oharaense* in their analyses.

The genus level as well as species level taxonomy of *Dictyoneurum californicum* Ruprecht and *D. reticulatum* (D.A. Saunders) P.C. Silva (= *Dictyoneuropsis reticulata* (D.A.Saunders) G.M.Smith) has been controversial (Setchell 1896, Smith 1944, Lane et al. 2006). Therefore, we examined the taxonomy of the entire Agaraceae (all currently recognized species except *A. yakishiriense*), including *Dictyoneurum* spp. and *A. oharanese,* by analyzing DNA sequences of multiple chloroplast and mitochondrial genes and by reexamining the morphology of representative specimens.

MATERIALS AND METHODS

Morphological observations. The gross morphology of sporophytes (i.e., blade shape, presence/absence of a midrib, splitting and perforations; stipe shape, presence/absence of

splitting and fimbriae or secondary haptera, etc.), of representative taxa of Agaraceae (*Agarum* spp., *Costaria costata*, *Dictyoneurum* spp. and *Thalassiophyllum clathrus*) was examined using field-collected specimens and voucher specimens including the type specimen of *Agarum oharaense* (in SAP, the Herbarium of the Graduate School of Science, Hokkaido University) and specimens of *D. reticulatum* (UC 936291 and UC 1260995 in the University Herbarium, University of California, Berkeley). General collections of *Agarum* spp. and *Dictyoneurum* spp. housed in the UC and UBC (The University of British Columbia) herbaria as well as the Phycology Herbarium of the Moss Landing Marine Laboratories were also examined (Appendix S1 in the Supporting Information).

Molecular phylogeny. Genomic DNA was extracted from fresh or silica gel-dried algal tissue of field-collected specimens and unialgal culture strains housed in the Kobe University Macroalgal Culture Collection (KU-MACC; Table S1 in the Supporting Information) using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) or Blood & Cell Culture DNA Mini Kit (QIAGEN) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications of the chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, *rbc*L and mitochondrial *cox*1, *cox*3, *nad*2, *nad*4, *nad*5, *nad*6 protein-coding genes, and nuclear

ITS2 rDNA region and 28S rRNA gene were carried out using the KOD FX (ToYoBo, Osaka, Japan) PCR enzyme and the TaKaRa PCR Thermal Cycler Dice (Takara Bio, Kusatsu, Japan). Primers used for PCR and/or sequencing are listed in Table S2 in the Supporting Information. After PEG purification (Lis 1980), PCR products were sequenced using the CE DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and the CEQ8000 DNA analysis system (Beckman Coulter) according to the manufacturer's instructions, or were sequenced by a DNA sequencing service (FASMAC, Atsugi, Japan). The almost complete chloroplast and mitochondrial genomes of *Agarum clathratum* (KU-d14287), *A. oharaense* (KU-d13142) and *Thalassiophyllum clathrus* (KU-d4860) were sequenced using the Ion Torrent (200 bp reads): After DNA extraction and purification by QIAGEN Genomic-tips, 1 µg of DNA was sheared and subjected to sequencing library construction using the Ion Xpress Plus Fragment Library Kit (Life Technologies, Carlsbad, CA, USA) according to the Ion Torrent PGM protocol with some modifications. The DNA concentration of the resulting individual libraries was quantified with a Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) using Qubit high-sensitivity assay reagents. After template amplification (emulsion PCR) and enrichment using Ion OneTouch 200 Template Kit v.2 with OneTouch2 System (Life Technologies), each sample was loaded

onto an Ion 318 chip and sequenced using the Ion PGM 200 bp Sequencing Kit (Life Technologies) according to the manufacturer's instructions. After removing low-quality reads, resultant clean reads were assembled by a Torrent Assembler plugin (SPAdes). The contigs derived from the chloroplast and mitochondrial genomes were detected using BLASTn searches (e-value threshold: E^{-4}) against the NCBI nucleotide database, and used for the subsequent sequence analysis.

The molecular phylogenetic analyses used published and newly determined sequence data for the Laminariales (Tables S1 and S3 in the Supporting Information). Alignments were prepared using the program MAFFT v.6 (Katoh and Toh 2008) and then manually adjusted prior to phylogenetic analyses. Numbers of phylogenetically informative sites in the gene sequences were calculated by DIVEIN (Deng et al. 2010). Substitution saturation at the third codon position in the gene sequences was calculated by DAMBE v.6.1.17 (Xia 2013) using default parameters. Molecular phylogenetic trees for three datasets (dataset 1: 27 OTUs, 6 chloroplast genes, total 8,473 bp; dataset 2: 27 OTUs, 6 mitochondrial genes, total 6,193 bp; dataset 3: 27 OTUs, combined 6 mitochondrial genes and 6 chloroplast genes, total 14,666 bp) were constructed by maximum likelihood (ML) and Bayesian (BI) analysis. Furthermore, for reference, ML trees based on the individual DNA sequences of

12 genes (27 OTUs), the *cox*3 gene sequences (29 OTUs, 718 bp) including the data of Boo et al. (2011) and the ITS2 rDNA sequences (18OTUs, 306 bp; 19 OTUs, 299 bp), and 28S rRNA gene sequences (7 OTUs, 1173 bp) were constructed. For ML analysis, we used RAxML GUI v.1.31 (Silvestro and Michalak 2012), conducting 10,000 Rapid Bootstrap searches followed by an ML search, with the GTR + G model for each codon position of each organellar gene or for each position of each nuclear region. We compared the ML trees with the GTR + G model based on the first and second codon positions and that based on the third codon position, and the tree topologies were essentially the same (data not shown). Bayesian analysis was run using MrBayes v.3.2.2 (Ronquist et al. 2012), with the GTR + G model for each codon position of each gene. The Bayesian analysis was initiated with a random starting tree, and four chains of Markov chain Monte Carlo (MCMC) iterations were run simultaneously for10,000,000 or 15,000,000 generations, keeping one tree every 100 generations. The first 25,000 or 37,500 trees sampled were discarded as 'burn-in', based on the stationarity of ln L as assessed using Tracer v.1.6 (Rambaut and Drummond 2013). Consensus topology and posterior probability values were calculated from the remaining trees. Shimodaira–Hasegawa tests (SH test, Shimodaira & Hasegawa 1999) and approximately unbiased tests (AU test; Shimodaira 2002) were performed based on the 12

gene sequences with RAxML and CONSEL (Shimodaira & Hasegawa 2001) to test tree topologies.

RESULTS

Morphological observations. *Agarum oharaense* (Fig. 1, a and b) and *A. fimbriatum* (Fig. 1, c and d) shared a flattened stipe and secondary haptera (fimbriae); *A. oharaense* has a decumbent habit whereas *A. fimbriatum* does not. *Agarum yakishiriense* has a flattened stipe and fimbriae in its mid to basal portion (Fig. 1e). *Thalassiophyllum clathrus* has a peculiar asymmetric developmental pattern: only one side of the meristematic margin of the young sporophyte continues to develop, forming an inrolled blade that extends to form fan-shaped blades (Fig. 1f). Examination of the gross morphology of *Dictyoneurum* and *Dictyoneuropsis* specimens housed in the UC and UBC herbaria showed that *Dictyoneurum californicum* and *D. reticulatum* were distinguishable by the presence/absence of a midrib as previously described. *Dictyoneurum californicum* lacks a midrib and is reticulated throughout the entire thallus (Fig. 1, g–i, k). A large proportion of the specimens housed in UC herbarium had characteristic splitting of the blade into two blades following the formation of a longitudinal slit (Fig. 1, g–i). In contrast, *D. reticulatum* has a midrib (Fig. 1j) and generally lacks such slits. One of the two specimens labeled as isotypes of *D.*

reticulatum (as *Costaria reticulata* D.A.Saunders) sent by De Alton Saunders to Setchell had the characteristic morphology of *D. reticulatum*, with a midrib (UC 936291; Fig. 1j), but the other specimen (UC 1260995; Fig. 1k) lacked a midrib and therefore is morphologically assignable to *D. californicum*.

Molecular phylogeny based on 6 mitochondrial genes. The ratio of phylogenetically informative sites in 6 individual genes ranged from 24.9% (*cox*1) to 39.7% (*nad*5) (average, 32.4%) across all 27 OTUs (Table S4 in the Supporting Information). In the Maximum Likelihood (ML) molecular phylogenetic tree based on the concatenated 6 mitochondrial genes (*cox*1, *cox*3, *nad*2, *nad*4, *nad*5 and *nad*6 genes; total 6,193 bp; Figs. 2 and S1 in the Supporting Information), the four derived families of the order (i.e., Aureophycaceae, Agaraceae, Alariaceae and Laminariaceae/Lessoniaceae) each formed monophyletic clades supported respectively by high bootstrap (MLB)/posterior probability (PP) values (87–98 % in ML and 0.99–1.0 in BI). *Aureophycus* diverged before the members of the other three families. In contrast, the statistical support for the branching order of the other three families was low (53–64 % in ML).

Members of the family Agaraceae (*Agarum, Costaria, Dictyoneurum* and *Thalassiophyllum*) formed monophyletic clades supported by high MLB values. Within the Agaraceae, *Thalassiophyllum clathrus* was sister to the remaining genera, and species in the genus *Agarum* were separated into two distinct clades supported by full statistical values. *Agarum clathratum*, the type of the genus, grouped with *A. turneri*, whereas *A. fimbriatum* and *A. oharaense* formed a clade that was sister to *Dictyoneurum reticulatum* and *D. californicum*. *Costaria costata* was sister to the clade containing *Dictyoneurum* spp., *A. oharaense* and *A. fimbriatum*, although the MLB value was moderate (69%). Phylogenetic relationships within Agaraceae based on the individual mitochondrial genes were similar to those based on concatenated sequences of 6 mitochondrial genes (Fig. S2, g-l in the Supporting Information). The genus *Agarum* was polyphyletic in five of six trees, and *Thalassiophyllum* was located at the basal position in five of six trees. The sequence divergences among the specimens of *D. californicum* and *D. reticulatum* were 0.24-0.88% in the 6 mitochondrial genes.

Molecular phylogeny based on 6 chloroplast genes. The ratios of phylogenetically informative sites in the individual chloroplast genes ranged from 8.7% (*rbc*L) to 27.4%

(*psb*C; average, 18.5%) across all 27 OTUs (Table S4). Molecular phylogeny based on the 6 chloroplast genes (*atp*B, *psa*A, *psa*B, *psb*A, *psb*C and *rbc*L; total 8,473 bp; Figs. 3 and S3 in the Supporting Information) gave tree topologies similar to those of the 6 mitochondrial genes (Fig. 2) but differed in the branching order of first diverging taxa and relationships among *A. clathratum, A. turneri* and *T. clathrus*. Within the family Agaraceae, *A. clathratum* formed a clade with *T. clathrus,* and *A. turneri* was sister to the clade of *A .clathratum* and *T. clathrus*. The relationships among *A. fimbriatum/A. oharaense, D. reticulatum/D. californicum* and *Costaria costata* were basically the same as with the mitochondrial genes, and *C. costata* was sister to the others although the MLB value was low (58%). Phylogenetic relationships within Agaraceae based on the individual chloroplast genes were similar to those based on concatenated sequences of 6 chloroplast genes (Fig. S2, a-f). *Thalassiophyllum* formed a clade with *A. clathratum* and/or *A. turneri*. The sequence divergences among the specimens of *D. californicum* and *D. reticulatum* were 0–0.57% for the 6 chloroplast genes. The index of the substitution saturation (Iss) values for the third codon position was significantly smaller than the critical index of substitution saturation (Iss.c) values for all genes examined, indicating that the influence of saturation at the third codon position is not significant (data not shown).

Molecular phylogeny based on the combined 6 mitochondrial and 6 chloroplast genes. Molecular phylogeny based on combined sequences of the 6 mitochondrial genes and the 6 chloroplast genes (Figs. 4 and S4 and Supporting Information) gave essentially the same tree topology as that of the concatenated 6 mitochondrial genes alone, and all nodes connecting the genera and species of the Agaraceae were supported by higher MLB values (≥97%). Especially the MLB value of the node connecting the clade of *Dicytoneurum*/*Agarum oharanese*/*A. fimbriatum*/*Costaria* with other agaracean members significantly improved from 58(chloroplast)/69(mitochondria) to 97%. SH and AU tests comparing the tree topologies of Figures 3 and 4 rejected the hypothesis that *Thalassiophyllum clathrus* forms a clade with *Agarum clathratum.* P-values were 0.009 (SH test) and 0.007 (AU test).

Molecular phylogeny based on mitochondrial cox3 gene. Molecular phylogeny based on mitochondrial *cox*3 gene sequences supported the polyphyly of the genus *Agarum* and the basal position of the genus *Thalassiophyllum* within Agaraceae (Fig. S5 in the Supporting Information).

Molecular phylogeny based on nuclear ITS2 rDNA and 28S rDNA sequences. Molecular phylogeny based on the sequence of ITS2 rDNA (Fig. S6 in the Supporting Information) supported the independence of *Agarum oharaense*/*A. fimbriatum* from *A. clathratum* and *A. turneri*, although the MLB values were low (50–52 %). The relationship between *Thalassiophyllum clathrus* and *A. clathratum*/*A. turneri* was somewhat different between the trees using one and two species of Laminariaceae (*Laminaria digitata* in Fig. S6a and *L. digitata* and *Saccharina japonica* in Fig. S6b) as outgroups in the analyses. In the former tree using only *L. digitata* as outgroup, *Thalassiophyllum* was nested in the *A. clathratum*/*A. turneri* clade, whereas independent in the latter tree adding *S. japonica* as a second outgroup taxon, although the MLB values were low (<81 %). In anyway, the resolution of the molecular phylogeny based on ITS2 rDNA sequences was considered to be low, because *S. japonica* occurred in a clade with *C. costata* when both *L. digitata* and *S. japonica* were used as outgroups (Fig. S6b), and the monophyly of Agaraceae was not supported.

The resolution of the molecular phylogeny based on 28S rDNA sequences was also considered to be low (Fig. S7, a and b in the Supporting Information). When two outgroup taxa (*Ecklonia radiata* and *L. digitata*) were included in the analysis, the monophyly of Agaraceae was not supported (Fig. S7b).

DISCUSSION

The molecular phylogenetic analyses using multigene mitochondrial and chloroplast DNA sequences revealed that *Agarum* species were paraphyletic, forming two independent clades comprising an *A. clathratum/A. turneri* clade and an *A. fimbriatum/A. oharaense* clade. The *A. fimbriatum/A. oharaense* clade was sister to *Dictyoneurum* spp., and *Costaria* was sister to the clade of *A. fimbriatum/A. oharaense* and *Dictyoneurum* spp. in both mitochondrial and chloroplast gene trees. The phylogenetic position of *Thalassiophyllum* differed between the analyses based on mitochondrial and chloroplast genes: *Thalassiophyllum* was independent from the two *Agarum* clades in the tree of mitochondrial genes but was included in the *A. clathlatus/A. turneri* clade in the tree of chloroplast genes. It is difficult to resolve the phylogenetic relationships between *Agarum* and *Thalassiophyllum* based solely on available genetic data, even using the relatively long, multigene concatenated sequences. However, in the combined gene phylogeny of all 12 mitochondrial and chloroplast genes, the tree topology agreed with that suggested in the mitochondrial gene phylogney despite a larger number of bp being contributed by the chloroplast genome.

Boo et al. (2011) suggested synonymizing *Thalassiophyllum* with *Agarum,* because *T. clathrus* was included in the *Agarum* clade. However, we consider that this result was an artifact of the inappropriate selection of *Costaria* as outgroup. In the present analyses including more laminarialean taxa covering all derived families, adding *Dictyoneurum* spp., and using taxa of sister families as outgroups, the paraphyly of *Agarum* as well as the more distant relationship of *Thalassiophyllum* became clearer.

Agarum fimbriatum and *A. oharaense* resemble *A. clathratum* and *A. turneri* in blade gross morphology, sharing a midrib and perforations (Table 1). However, *A. fimbriatum* and *A. oharaense* have characteristic fimbriae (secondary haptera in *A. oharaense*) on both sides of the mid to upper part of the flattened stipe (Fig. 1, a and b), frequently isolated from basal haptera. In contrast, *A. clathratum* and *A. turneri* have terete or somewhat compressed stipes and lack such fimbriae although *A. yakishiriense* has a flattened stipe. *Agarum fimbriatum* and *A. oharaense* tend to have undulations at the margins of the blades whereas *A. clathratum* and *A. turneri* generally have entire (smooth) margins. *Agarum clathratum* and *A. turneri* form haptera radially so that the thalli grow more or less perpendicular to the substrate, but *A. oharaense* tends to grow in a decumbent fashion, and *A. oharaense* may

secondarily form haptera from the mid to upper portion of the stipe, which initially appear as fimbriae.

Boo et al. (2011) detected no differences in *rbc* spacer sequences between Asian *A. clathratum* (A. *clathratum* subsp. *clathratum*) and *A. yakishiriense*, whereas *A. clathratum* from the North Atlantic, American North Pacific, and Kamchatka shared the same sequence and were separate from them. This result contrasted with the data from ITS2 rDNA, *cox*1 and *cox*3, in which *A. yakishiriense* was independent from the others. We consider that the closer relationship implied by *rbc* spacers is due to insufficient resolution of the region for discriminating phylogenetic relationships among the taxa. On the other hand, despite the very close phylogenetic relationship between *A. yakishiriense* and Japanese *A. clathratum*, there is no sign of hybrid formation in the sequence data because no individuals showing intermediate types between the two taxa were observed in the nuclear ITS2 rDNA tree.

Agarum yakishiriense (as *A. rugosum* f. *rishiriense* in Yamada [1974]) resembles *A. fimbriatum* and *A. oharasense* in having stipes flattened through their length and forming secondary haptera in their mid to upper portions, thus making it distinct from *A. clathratum* and *A. turneri*, whose stipes are terete at the basally portion and form radial haptera solely at the base. Further study is needed to clarify the taxonomic position of this species.

The genus level as well as species level taxonomy of *Dictyoneurum californicum* and *Dictyoneuropsis reticulata* has been controversial. *Dictyoneurum reticulata* was first described as *Costaria reticulata* D.A.Saunders 1895, but Setchell (1896) considered that the species was conspecific with *Dictyoneurum californicum*. Later, Smith (1944) distinguished *C. reticulata* from *D. californicum* chiefly by the presence of a midrib, and he proposed the establishment of a new genus *Dictyoneuropsis* for *C. reticulata*. Lane et al. (2006) reported that they found no sequence difference in the rDNA ITS region between the two taxa. They also referred to Setchell and Gardner (1925) who stated that there was little difference in blade morphology, and suggested merging *Dictyoneuropsis reticulata* with *Dictyoneurum californicum*. Silva (in Pedroche et al. 2008) held that the species were different and proposed the combination of *Dictyoneurum reticulatum* (D.A.Saunders) P.C.Silva.

Examination of the gross morphology of *Dictyoneurum/Dictyoneuropsis* specimens housed in UC, UBC and the Moss Landing Marine Laboratories confirmed that *D. californicum* and *D. reticulatum* are distinguishable by the presence/absence of a midrib, as previously described (Saunders 1895, Smith1944, Abbott & Hollenberg 1976). The blade of *Dictyoneurum californicum* lacks a midrib and is reticulated over its entire surface. A large portion of the specimens have the characteristic division of the blade into two blades

following the formation of a longitudinal slit, whereas *D. reticulatum* has a midrib, and relatively few specimens have such slit.

Saunders (1895) did not designate a holotype for *Costaria reticulata*. G. M. Smith (1942, p. 652), when transferring the species to his new genus *Dictyoneuropsis*, stated "Type locality 'Monterey Bay near Pacific Grove, Cal.' Type specimen in Farlow Herbarium, Harvard University." The Farlow Herbarium (FH) has five Saunders specimens, two of which are part of their type collection (FH00805599 and FH00805600). These have "Type" on the handwritten labels, are dated August 1894 and are from Pacific Grove, California. All the FH specimens have a midrib, as described by Saunders (1895). We choose FH00805600 (Fig. 5) as lectotype as it is the specimen that most closely matches the illustration in Saunders (1895). Other Saunders specimens with the same locality and date are in UC. One of these (UC1260995) has a label identical to the Farlow specimens and is labeled “Type”. This specimen, however, lacks a midrib and is attributable to *Dictyoneurum californicum*. The International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) (http://www.iapt-taxon.org/nomen/main.php) has made a provision for just such cases. Article 9.14. states, "When a type (herbarium sheet or equivalent preparation) contains parts belonging to more than one taxon (see Art. 9.11), the name must

remain attached to the part (specimen as defined in Art. 8.2) that corresponds most nearly with the original description or diagnosis." We think that UC1260995 represents a lapsus committed during the preparation of many specimens, and it has no standing as type material of *C. reticulata*. The other UC specimen (UC936291) is accompanied by a letter from Saunders to Setchell (dated 10 May 1895, after publication of the species in February 1895) that identifies the specimen, which was trimmed and remounted with a UC label, as a duplicate specimen of *C. reticulata* and asks Setchell's opinion about it. Although Setchell labeled it *D. californicum*, he attached a note: "This, fide Saunders, is the *Costaria reticulata* Saunders". Paul Silva, a student of Smith, annotated both specimens as isotypes of *C. reticulata* in 1951. However, only UC 936291 should be considered an isotype of that species.

Dictyoneurum is characterized by the peculiar process of vegetative division of the sporophytes into two individuals: a longitudinal slit is formed in the transition zone of the blade (growth zone), and during the growth of the blade the splitting broadens both upwards and downwards to separate the blade and stipe, and eventually dividing the thallus into two individuals. Such a process is less common in *D. reticulatum,* which has a midrib, but still does occasionally occur as illustrated in Smith (1942). However, considering their close

phylogenetic relationship, it is possible that they form hybrids that show this intermediate morphology.

Although Saunders & Druehl (1993) and Lane et al. (2006) reported that *D. californicum* and *D. reticulatum* had identical DNA sequences, they were genetically independent in our analyses based on 10 of 12 gene sequences (except for *psa*A and *psb*A) with sequence divergences of 0.18–0.57% for the other 4 chloroplast genes (Fig. S2, a,c,e,f) and 0.24–0.88% for the 6 mitochondrial genes (Fig. S2, g–l). However, the genetic divergence among the local populations of each morphological type has not yet been elucidated, and further study is needed to clarify their species level taxonomy. The use of microsatellite markers as employed by Geoffroy et al. (2015) to differentiate two sympatric species of "*Pylaiella littoralis*" in northern France offers a promising approach to emulate.

The monophyly of Agaraceae is confirmed in the present molecular phylogeny based on both chloroplast and mitochondrial genes. The taxa in this family share simple anatomical features, lacking specialized mucilage-producing cells and frequently having perforated blades. The mechanism producing the perforations is not clear, but it is noteworthy that the characteristic longitudinal splitting of the thallus at the transition zone in *Dictyoneurum* may possibly occur by the same mechanism.

In conclusion, we consider that it was inappropriate to merge *Thalassiophyllum* with *Agarum* as done by Boo et al. (2011). Furthermore, as indicated in analyses of both chloroplast and mitochondrial gene sequences, *A. fimbriatum* and *A. oharaese* do not belong in *Agarum*. We propose the establishment of the new genus *Neoagarum* to accommodate them. Species in the new genus *Neoagarum* are distinguishable from *Agarum* by the presence of fimbriae, which become secondary haptera in *A. oharaense*, in the mid to upper part of the stipe and by DNA sequences.

Description and diagnosis

Neoagarum H.Kawai et T.Hanyuda **gen. nov.**

Type species: *Agarum fimbriatum* Harvey 1862 (*J. Proc. Linn. Soc. Botany* 6:*166*).

Sporophyte with holdfast comprising narrow, branched haptera. Stipe simple, compressed, often forming fimbriae, which can become secondary haptera, on the mid to upper part of the stipe. Blade relatively thin, flat or bullate, with a narrow to broad midrib and perforations in lateral wings. Sori forming broad patches on both surfaces of blade. The genus resembles *Agarum* in gross morphology but is distinguished by occurrence of

fimbriae and by the DNA sequences of chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, *rbc*L and mitochondrial *cox*1, *cox*3, *nad*2, *nad*4, *nad*5, *nad*6 genes.

Type species: *Neoagarum fimbriatum* (Harvey) H.Kawai & T.Hanyuda comb. nov.

Basionym: *Agarum fimbriatum* Harvey 1862 (*Jour. Proc. Linn. Soc. Botany* 6: 166)

Additional species: *Neoagarum oharaensis* (Yamada) H.Kawai, T.Hanyuda & M. Miyata comb. nov.

Basionym: *Agarum oharaense* Yamada 1961 (*Bull. Res. Counc. Islael, Sect. D, Botany* 10(D): 121–3)

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Figure legends

Fig. 1. Sporophyte morphology of selected agaracean species. a, b. Type of *Agarum oharaense*. a. Gross morphology. b. Secondary haptera (fimbriae) formed in upper part of the flat stipe (arrow). Collected by I. Ohno at Ohara, Chiba, Japan on May 1932. SAP044797. c, d. *Agarum fimbriatum*. c. Gross morphology. d. Fimbriae formed in upper part of the flat stipe (arrow). Collected by E. Y. Dawson at Santa Catalina I., CA, USA on 1 December 1948. SAP037255. e. Type of *Agarum yakishiriense* Yamada. Flattened stipe has fimbriae (arrow) in its mid to basal portions. f. *Thalassiophyllum clathrus*. Field-collected young sporophyte showing inrolled meristematic margin of the blade (arrow). Collected by H. Kawai on 6 August 2015 at Avacha Bay, Kamchatka, Russia. g. Habit of *Dictyoneurum californicum* showing longitudinal slit in the transitional zone (arrow). h. Lectotype specimen of *D. californicum* photographed by I. Yamada at Komarov Botanical Institute. Arrow shows longitudinal slit. i. Herbarium specimen of *D. californicum* showing longitudinal slits (arrows). UC 936296. Collected by M. A. Howe in July 1892. j. Isotype of *Dictyoneurum reticulatum.* Note midrib (arrow). UC 936291. Collected by D.A. Saunders.

k. Specimen of *D. californicum* (without midrib) originally misidentified as *D. reticulatum*. UC 1260995. Collected by D.A. Saunders.

Fig. 2. Maximum likelihood (ML) tree based on the concatenated DNA sequences of mitochondrial *cox*1, *cox*3, *nad2*, *nad*4, *nad*5 and *nad*6 genes. Numbers on the branches indicate bootstrap values (%) from ML analysis (left) and posterior probabilities from Bayesian analysis (right). Asterisk (*) indicates 100 % bootstrap (ML) and 1.00 posterior probability (Bayesian) values. Only bootstrap values >50% and posterior probabilities >0.90 are shown.

Fig. 3. Maximum likelihood (ML) tree based on the concatenated DNA sequences of chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C and *rbc*L genes. Numbers on the branches indicate bootstrap values (%) from ML analysis (left) and posterior probabilities from Bayesian analysis (right). Asterisk (*) indicates 100 % bootstrap (ML) and 1.00 posterior probability (Bayesian) values. Only bootstrap values >50% and posterior probabilities >0.90 are shown.

Fig. 4. Maximum likelihood (ML) tree based on the concatenated DNA sequences of mitochondrial *cox*1, *cox*3, *nad2*, *nad*4, *nad*5,*nad*6 and chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, *rbc*L genes. Numbers on the branches indicate bootstrap values (%) from ML analysis (left) and posterior probabilities from Bayesian analysis (right). Asterisk (*) indicates 100 % bootstrap (ML) and 1.00 posterior probability (Bayesian) values. Only bootstrap values >50% and posterior probabilities >0.90 are shown.

Fig. 5. Scanned image of the lectotype specimen of *Dictyoneurum reticulata* housed in Farlow Herbarium (FH00805600).

Fig. S1. Bayesian consensus tree based on the concatenated DNA sequences of mitochondrial *cox*1, *cox*3, *nad*2, *nad*4, *nad*5 and *nad*6 genes. Numbers on branches indicate posterior probabilities from Baysian analysis. Only posterior probabilities >0.90 are shown and asterisk (*) indicates 1.00.

Fig. S2. Maximum likelihood (ML) trees based on individual chloroplast (a-f) and mitochondrial (g-l) gene sequences. Numbers on the branches indicate bootstrap values

from ML analysis. Asterisk (*) indicates 100 % bootstrap values. Only bootstrap values >50% are shown. a. *atp*B (1,163 bp). b. *psa*A (1,928 bp). c. *psa*B (1,691 bp). d. *psb*A (972 bp). e. *psb*C (1,307 bp). f. *rbc*L (1,412 bp). g. *cox*1 (1,519 bp). h. *cox*3 (720 bp). i. *nad*2 (758 bp). j. *nad*4 (1,449 bp). k. *nad*5 (811 bp). l. *nad*6 (936 bp).

Fig. S3. Bayesian consensus tree based on the concatenated DNA sequences of chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C and *rbc*L genes. Numbers on branches indicate posterior probabilities from Baysian analysis. Only posterior probabilities >0.90 are shown and asterisk (*) indicates 1.00.

Fig. S4. Bayesian consensus tree based on the concatenated DNA sequences of mitochondrial *cox*1, *cox*3, *nad2*, *nad*4, *nad*5, *nad*6 and chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, *rbc*L genes. Numbers on the branches indicate posterior probabilities. Asterisk (*) indicates 100 % bootstrap (ML) values. Only posterior probabilities >0.90 are shown and asterisk (*) indicates 1.00.

Fig. S5. Maximum likelihood (ML) tree based on the DNA sequences of mitochondrial *cox*3 gene. Numbers on the branches indicate bootstrap values from ML analysis (left) and

posterior probabilities from Bayesian analysis (right). Asterisk (*) indicates 100 % bootstrap (ML) and 1.00 posterior probability (Bayesian) values. Only bootstrap values >50% and posterior probabilities >0.90 are shown.

Fig. S6. Maximum likelihood tree based on the sequence of nuclear ITS2 rDNA region. a. Analysis using *Laminaria digitata* as outgroup. 18 OTUs, 306 bp. b. Analysis using *L. digitata* and *Saccharina japonica* as outgroups.19 OTUs, 299 bp. Numbers on the branches indicate MLB values. Only values >50% are shown.

Fig. S7. Maximum likelihood tree based on the sequence of nuclear 28S ribosomal DNA region. a. 6 OTUs, 1,173 bp. b. 7 OTUs, 1,173 bp. Numbers on the branches indicate MLB values. Only values >50% are shown.

Appendix S1. List of herbarium specimens of agaracean species examined for gross morphology in the present study.

Table S1. Sources of samples and sequence data used for molecular analyses of chloroplast and mitochondrial genes, including their database accession numbers. Sample codes in [KU-###] correspond to KU-MACC (Kobe University Macroalgal Culture Collection) strain code, and [KU-d###] corresponds to silica gel-dried specimens housed at Kobe University Research Center for Inland Seas.

Table S2. List of primers used for PCR and sequencing.

Table S3. Sources of samples and sequence data used for molecular analyses of nuclear DNA sequences, including their database accession numbers. Sample codes in [KU-###] correspond to KU-MACC (Kobe University Macroalgal Culture Collection) strain code, and [KU-d###] corresponds to silica gel-dried specimens housed at Kobe University Research Center for Inland Seas.

Table S4. Comparison of number and ratio of phylogenetically informative sites in mitochondrial and chloroplast gene sequences used in the present study.

Table 1. Comparisons of morphological features in genera of Agaraceae.

	Agarum	*Neoagarum gen. nov.*	*Thalassiophyllum*	*Costaria*	*Dictyoneurum*
Blade morphology	Symmetric, elliptical to ovate, flat or bullate with perforations	Symmetric, elliptical to ovate, flat or bullate with perforations	Asymmetric, inrolled at the lower portions, fan-shaped, with perforations	Symmetric, linear to ovate with 5 percurrent longitudinal narrow ribs, bullate in regions between ribs, frequently with perforations	Symmetric, linear, with or without midrib; forming longitudinal splitting in the transition zone, without perforations
Stipe morphology	Terete at least at the base, forming haptera radially and basally	Flattened, sometimes decumbent, forming haptera at the base, and fimbriae or secondary haptera in mid/upper portion	Terete, branched, twisted, forming haptera radially at the basal part	Terete at the base, forming haptera radially and basally	Flattened, branched, decumbent, forming secondary haptera in mid/upper portion
Distributional range	Northern Pacific (Korea and Japan to Washington); NW Atlantic	Northern Pacific (Japan and Alaska to Mexico)	Northern Pacific (Kurile to Aleutian Islands)	Northern Pacific (Korea and Japan to California)	NE Pacific (Alaska to British Columbia to Mexico)