A TAXONOMIC STUDY OF THE GENUS *PADINA* (DICTYOTALES, PHAEOPHYCEAE) INCLUDING THE DESCRIPTIONS OF FOUR NEW SPECIES FROM JAPAN, HAWAII, AND THE ANDAMAN SEA[1]

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A taxonomic study of the genus *Padina* from Japan, Southeast Asia, and Hawaii based on morphology and gene sequence data (*rbc*L and *cox*3) resulted in the recognition of four new species, that is, *Padina macrophylla* and *Padina ishigakiensis* from Ryukyu Islands, Japan; *Padina maroensis* from Hawaii; and *Padina usoehtunii* from Myanmar and Thailand. All species are bistratose and morphologically different from one another as well as from any known taxa by a combination of characters relating to degree of calcification; the structure, position, and arrangement of hairlines (HLs) and reproductive sori; and the presence or absence of rhizoid-like groups of hairs and an indusium. Molecular phylogenetic analyses demonstrated a close relationship between *P. ishigakiensis*, *P. macrophylla*, *P. maroensis*, and *Padina australis* Hauck. The position of *P. usoehtunii*, however, was not fully resolved, being either sister to a clade comprising the other three new species and *P. australis* in the *rbc*L tree or more closely related to a clade comprising several other recently described species in the *cox*3 tree. The finding of the four new species demonstrates high species diversity particularly in southern Japan. The following characters were first recognized here to be useful for species delimitation: the presence or absence of small rhizoid-like groups of hairs on the thallus surface, structure and arrangement of HLs on both surfaces either alternate or irregular, and arrangement of the alternating HLs between both surfaces in equal or unequal distance. The evolutionary trajectory of these and six other morphological characters used in species delineation was traced on the phylogenetic tree.

Padina Adans. is, other than *Newhousia imbricata* (Kraft et al. 2004), the only brown algal genus that is calcified. The plants are typically fan shaped with an inrolled margin of meristematic cells by which the growth is initiated. Some species, however, show an uncalcified *Vaughaniella* stage (creeping rhizomes, also known as *Dictyerpa* stage) with a single apical cell from which the erect thalli develop (Børgesen 1951, Cribb 1951, De Clerck and Coppejans 1997). Thalli are two or more cell layers thick (up to 20 layers) and erect to decumbent depending on the species. The life cycle is isomorphic diplohaplontic with an alternation of haploid gametophytes and diploid sporophytes. Gametophytes are mostly dioecious. *Padina* species are widely distributed in warm temperate to tropical coastal areas where they can be found from the lower intertidal to deep subtidal zones.

According to the AlgaeBase database (Guiry and Guiry 2010, http://www.algaebase.org/), 37 species are currently recognized worldwide, and 11 species have been reported from Japan (Yoshida et al. 2000, Ni-Ni-Win et al. 2008). Recently, Ni-Ni-Win et al. (2010) described four new species (*Padina okinawaensis* Ni-Ni-Win, S. Arai et H. Kawai; *Padina undulata* Ni-Ni-Win, S. Arai et H. Kawai; *Padina terricolor* Ni-Ni-Win, M. Uchimura et H. Kawai; and *Padina fasciata* Ni-Ni-Win, M. Uchimura et H. Kawai) from Ryukyu Is., Japan, on the basis of morphology and molecular evidence. *Padina okinawaensis* was also found in Hawaii, Indonesia, and Thailand. The authors also demonstrated the distinctness of *Padina sanctae-crucis* Børgesen and *Padina japonica* Yamada, which until then were considered conspecific (Gaillard 1975, Abbott and Huisman 2003, 2004), and reported the occurrence of the former species in southern Japan.

Taxonomy of *Padina* species has mainly been based on the thallus morphology. This is notoriously difficult because of their considerable morphological plasticity (e.g., thallus shape, size, color), inconsistent usage of taxonomic terminology (Trono 1969), and lack of understanding of diagnostic characters for species delimitation, as well as the absence of DNA sequence data. To date, there have been some studies using DNA sequences including *Padina* species, but most did not address the intrageneric taxonomy (Lee and Bae 2002, Hoshina et al. 2004, De Clerck et al. 2006, Bittner et al. 2008, Phillips et al. 2008). Only few studies dealt with species level taxonomy of *Padina* (Ni-Ni-Win et al. 2008, 2010). The chloroplast-encoded large subunit of the RUBISCO gene (*rbc*L) has been extensively used in molecular phylogenetic studies of brown algae and has been demonstrated to be a useful molecular marker by many authors (Siemer et al. 1998, Draisma et al. 2001, Lee and Bae 2002, Cho et al. 2004, Hoshina et al. 2004, De Clerck et al. 2006, Lane et al. 2006, Cho et al. 2007, Bittner et al. 2008, Ni-Ni-Win et al. 2008, 2010, Phillips et al. 2008). Similarly, some authors proposed the mitochondrial cytochrome oxidase subunit 3 (*cox3*) as a marker for the studies of intra- and interspecific genetic diversity of Phaeophyceae due to its maternal inheritance and higher evolutionary rate (Kato et al. 2005, Kogame et al. 2005, Uwai et al. 2006). Accordingly, in this study, *rbc*L and *cox3* are used as molecular markers combined with morphological observations to clarify the classification of *Padina* species, evaluate taxonomically important morphological characters, assess the evolution of morphological characters, and interpret the phylogenetic relationships among the species as well as their biogeography.

MATERIALS AND METHODS

Morphological observations. *Padina* specimens were collected mainly in Japan, Southeast Asia and Hawaii (Fig. 1; Table S1 in the supplementary material). Morphological observations were performed mostly on the same specimens used for molecular analyses (Table S1). Representative voucher specimens used for the morphological observations are deposited in the Herbarium of the Graduate School of Science, Hokkaido University (SAP); Leiden Nationaal Herbarium (L); and herbarium of the Kobe University Research Center for Inland Seas (KURCIS). Type specimens of *P. australis* Hauck (L0055591), *Padina distromatica* Hauck (L0055592), *Padina dubia* Hauck (L0055593), *Padina somalensis* Hauck (L0055595), *Padina tetrastromatica* Hauck (L0055597) (Hauck No. 68), *Padina haitiensis* Thivy (Taylor 20987), *Padina perindusiata* Thivy (Taylor 1356), and *P. japonica* (SAP9268) loaned from Leiden Nationaal Herbarium, Herbarium of University of Michigan, and SAP were also examined. For anatomical observations, specimens were hand-sectioned and micrographed using a VB–7010 Digital Camera (Keyence, Tokyo, Japan) attached to a BX–51 microscope (Olympus, Tokyo, Japan). Sections were mounted on glass slides in Karo syrup/seawater. About 10–20 specimens of each species were sectioned in three places (basal, middle, and margins) to determine the number of cell layers composing the thallus. For the measurements of the sizes of reproductive structures, 20 mature oogonia and tetrasporangia from 10 to 15 specimens each were randomly selected and measured.

Molecular phylogenetic analysis. DNA extraction, amplification (PCR) of *rbc*L and *cox3* regions, and sequencing followed Ni-Ni-Win et al. (2008). DNA sequences are deposited in DNA Data Bank of Japan. The sequence AB096907 assigned to *Padina* sp. in Hoshina et al. (2004) was downloaded from GenBank. To check positions/clusters of specimens assigned to a single species as well as for congruence in tree topology, three alignments using each data set of *rbc*L and *cox3* and their combined data were created for the construction of phylogenetic trees. *Dictyota dichotoma* and *Stypopodium* sp.
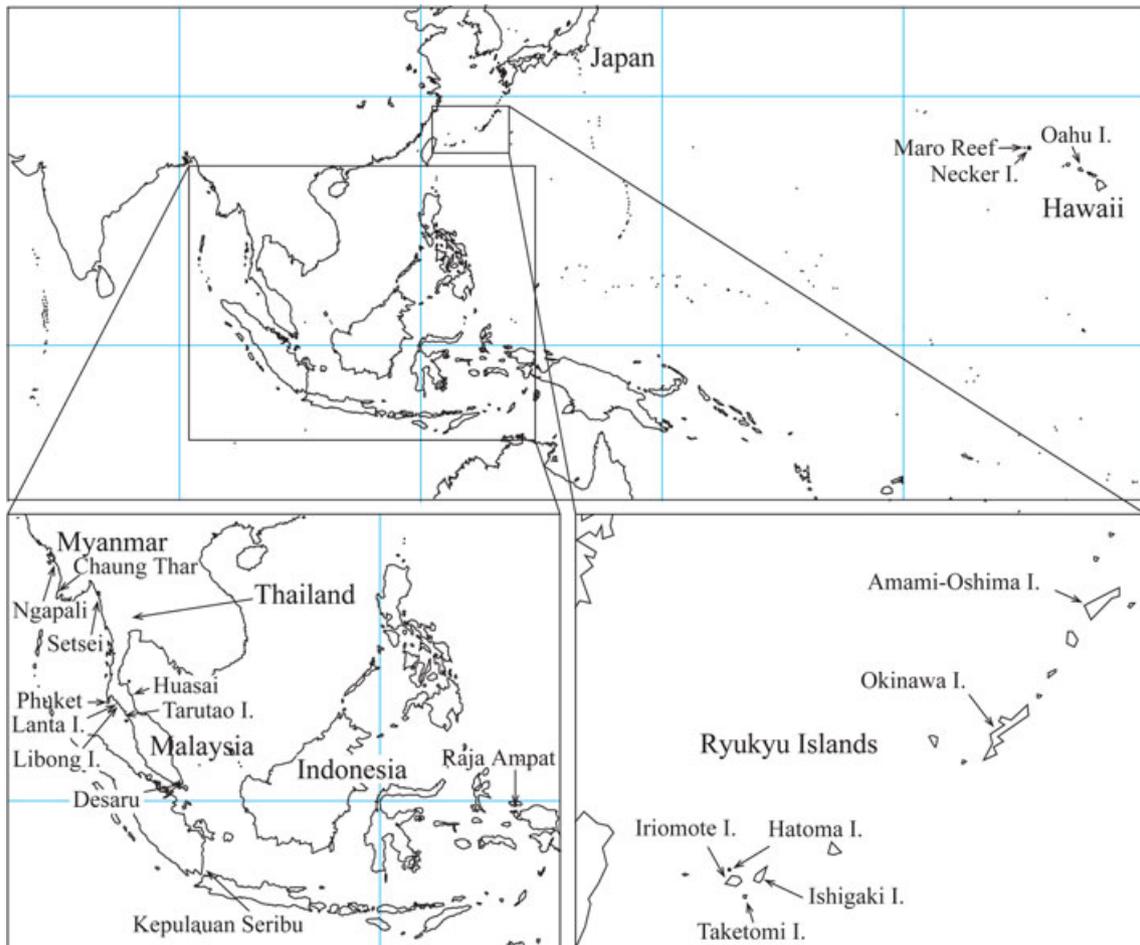
FIG. 1. Map indicating the collection sites of the specimens used in the present study.

(Dictyotales) were used as outgroups (Table S1). Sequences were aligned with Clustal X (Thompson et al. 1997) and then manually adjusted. Phylogenetic trees were inferred using maximum-likelihood (ML) and Bayesian inference (BI) methods. ML analyses of each data set of *rbc*L and *cox*3 were carried out using PAUP* version 4.0b10 (Swofford 2002). Modeltest v.3.06 (Posada and Crandall 1998) was used to find the optimal model of sequence evolution to fit the data. The Akaike information criterion selected a GTR+I+G and a TVN+I+G model for the *rbc*L and *cox*3, respectively. ML analyses were performed using the best-fit model with estimated parameters (gamma distribution and proportion of invariable sites). A heuristic search consisted of 100 replicates with tree bisection reconnection (TBR) branch-swapping. ML analysis of the combined data set was performed using the likelihood ratchet method (Vos 2003) with the best-fit evolutionary model in each codon position of each gene (six partitions) by comparing different evolutionary models via the corrected Akaike information criterion (Akaike 1974) implemented in KAKUSAN3 (Tanabe 2007). For the ML tree search, 1,000 sets of 25% site-upweighted data were created using the pgresampleseq command in Phylogears 1.5.2009.12.29 (Tanabe 2009), and the ML tree with the upweighted data was estimated using Treefinder (Jobb et al. 2004) with application of the best-fit model. Bootstrap analyses (Felsenstein 1985) were carried out to find support for individual internal branches in a heuristic search option with 100 replicates and 10 random additions under

the TBR branch-swapping algorithm. For Bayesian analyses of all data sets, the best-fit evolutionary model in each codon position of each gene was determined for each data set by comparing different evolutionary models via the Bayesian information criterion (Schwarz 1978) with the aid of KAKU-SAN3 (Tanabe 2007). Bayesian analyses with the selected evolutionary models were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) with a random starting tree and four chains of Markov chain Monte Carlo iterations ran simultaneously for 1×10^6 generations, keeping one tree every 100 generations. The first 10,000 trees sampled were discarded as ''burn-in,'' based on the stationarity of ln L as assessed using Tracer version 1.4.1 (Rambaut and Drummond 2009); a consensus topology and posterior probability values were calculated with the remaining trees. Morphological character mappings were performed using parsimony reconstruction implemented in the MacClade version 4.05 computer program (Maddison and Maddison 2002), as well as parsimony and ML reconstructions implemented in the Mesquite version 2.73 (Maddison and Maddison 2010) to confirm the ancestral character states.

RESULTS

Molecular phylogenetic analyses. The combined *rbc*L + *cox*3 alignment consisted of 41 sequences representing 21 *Padina* species and two outgroup taxa

and was 2,053 bp in length. ML and BI analyses using this alignment showed an identical tree topology, and the ML tree is shown in Figure 2. ML trees inferred from each data set of *rbc*L and *cox*3 are respectively shown in Figures S1 and S2 (in the supplementary material). The phylogenetic trees inferred from separate and combined data were highly congruent, differing only in the position of some nodes that received little or no support. *Padina moffittiana* I. A. Abbott et Huisman formed basal followed by *Padina melemele* I. A. Abbott et Magruder in *rbc*L, but *P. melemele* was basal in *cox*3. The combined phylogeny (Fig. 2) supported the *cox*3 topology (Fig. S2) where the branching order was *P. melemele*, *P. moffittiana*, *Padina pavonica*, the *Padina crassa* + *Padina arborescens* clade, and then the other *Padina* species.

In all analyses using separate and combined data sets, four well-supported clades (i.e., Clades A, B, C, and D) were recognized, morphologically corresponding to unknown taxa (see below). Japanese specimens collected from many localities in the Okinawa Islands formed two different clades and

were tentatively named clade A (=*P. macrophylla* sp. nov.) and clade C (=*P. ishigakiensis* sp. nov.). All specimens belonging to clade A had identical sequences in both *rbc*L and *cox*3. Similarly, specimens belonging to clade C showed identical sequences in *rbc*L but slightly variable sequences in *cox*3, with sequence divergence of 0.14%–1.2%. Hawaiian specimens collected from Maro Reef and Necker I. showed identical sequences and formed an independent clade named clade B (=*P. maroensis* sp. nov.) sister to clade A. Specimens collected from Myanmar and Thailand formed a statistically well-supported clade, which was named clade D (=*P. usoehtunii* sp. nov.). Clades A, B, and C together were sister to *P. australis* in all analyses, and this clade was sister to clade D in the *rbc*L and combined trees, but without support in the ML combined tree. The *cox*3 tree, however, supported clade D to be sister to the clade consisting of *P. okinawaensis*, *P. terricolor*, *P. undulata*, *P. sanctae-crucis*, *P. japonica*, *P. fasciata*, *Padina thivyae* Doty et Newhouse, and *Padina ryukyuana* Y. P. Lee et Kamura (Fig. S2). Sequence divergences among clades A, B, C, D and
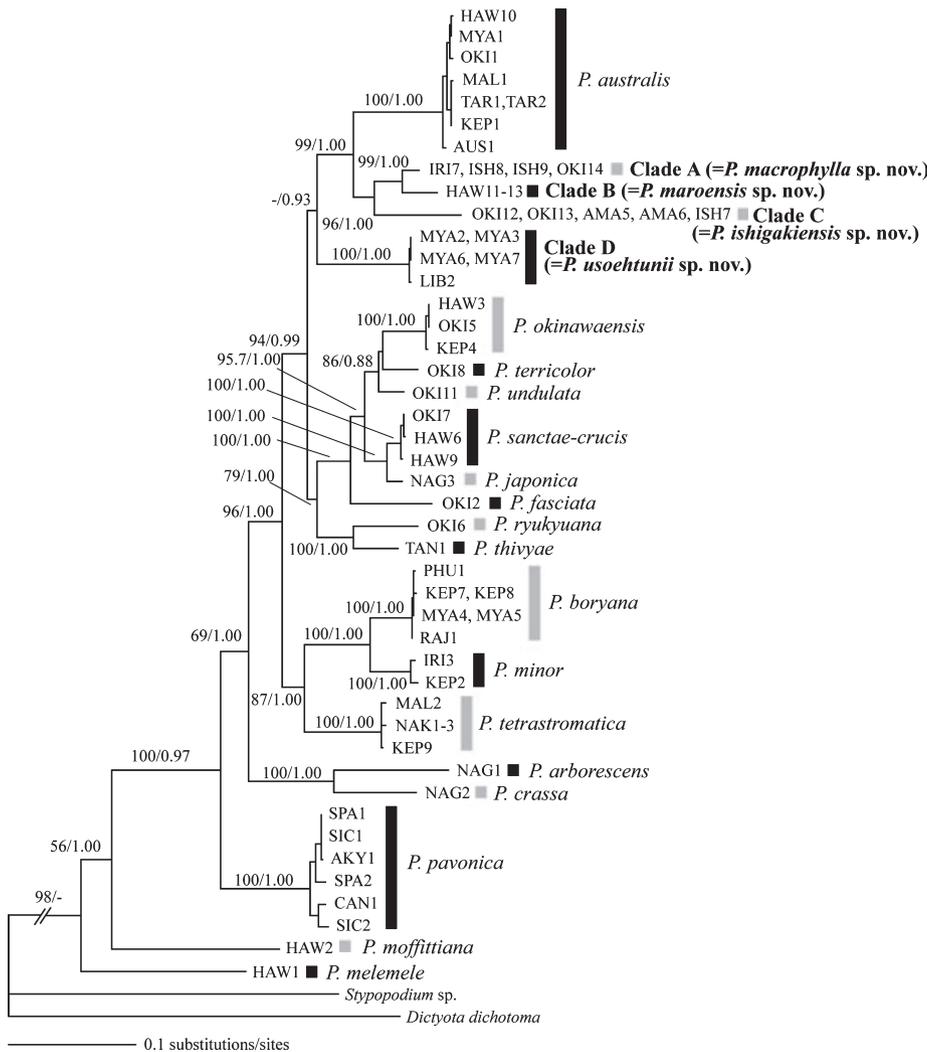

FIG. 2. Maximum-likelihood (ML) tree based on the combined *rbc*L + *cox*3 gene sequences. Numbers at each node indicate bootstrap values (>50%) for ML (left) and Bayesian posterior probabilities (>0.80) (right).

P. australis ranged from 1.44% to 5.16% in *rbc*L and 6.90%–16.80% in *cox*3.

Four recently described species, *P. okinawaensis, P. terricolor, P. undulata,* and *P. fasciata,* together with *P. japonica* and *P. sanctae-crucis* formed a clade with high support. This clade was sister to a *P. ryukyuana + P. thivyae* clade in all analyses but was only supported in the combined analysis. The sequence AB096907 assigned to *Padina* sp. in Hoshina et al. (2004) grouped with the *P. tetrastromatica* specimens (Fig. S1).

Here, we prefer to use the name *P. tetrastromatica* even though it was considered a synonym of *Padina antillarum* (Kütz.) Piccone by Wynne (1998). We wish to retain the name *P. tetrastromatica* until specimens from the respective type localities have been compared using sequence data.

Sequence divergences within the *P. tetrastromatica* clade were very small (0.07%–0.6% in *rbc*L), suggesting a single species. *Padina boryana* Thivy specimens from Thailand, Myanmar, and Indonesia formed a highly supported clade, which was consistently sister

to *Padina minor* Yamada. Together, these two species formed a sister clade to *P. tetrastromatica* in all analyses, but without support in the *cox*3 tree.

Morphological observations.
Padina macrophylla Ni-Ni-Win, M. Uchimura et H. Kawai **sp. nov.** (Fig. 3) (clade A, in Figs. 2, S1, and S2).

Thalli magni, usque ad 30 cm lati et 25 cm alti, utraque superficie parum vel modice calcificati. Thalli dioecii, soris indusiatis tetrasporangialibus oogonialibusque vulgo lineas plusminusve continuis facientibus. Species haec *P. maroensi* similis sed thallo minus calcificato, lineis pilorum superficie superiore inconspicuis, loco sororum reproductivorum in superficie thalli, et distantia inter lineas pilorum majore distinguenda. Sequentiae nucleotidorum propriae AB512539 (*rbc*L), AB512580 (*cox*3).

Thalli large, up to 30 cm wide and 25 cm high, slightly to moderately calcified on both surfaces. Thalli dioecious; indusiate tetrasporangial and oogonial sori normally forming more or less continuous lines. The species resembles *P. maroensis,* but distin-
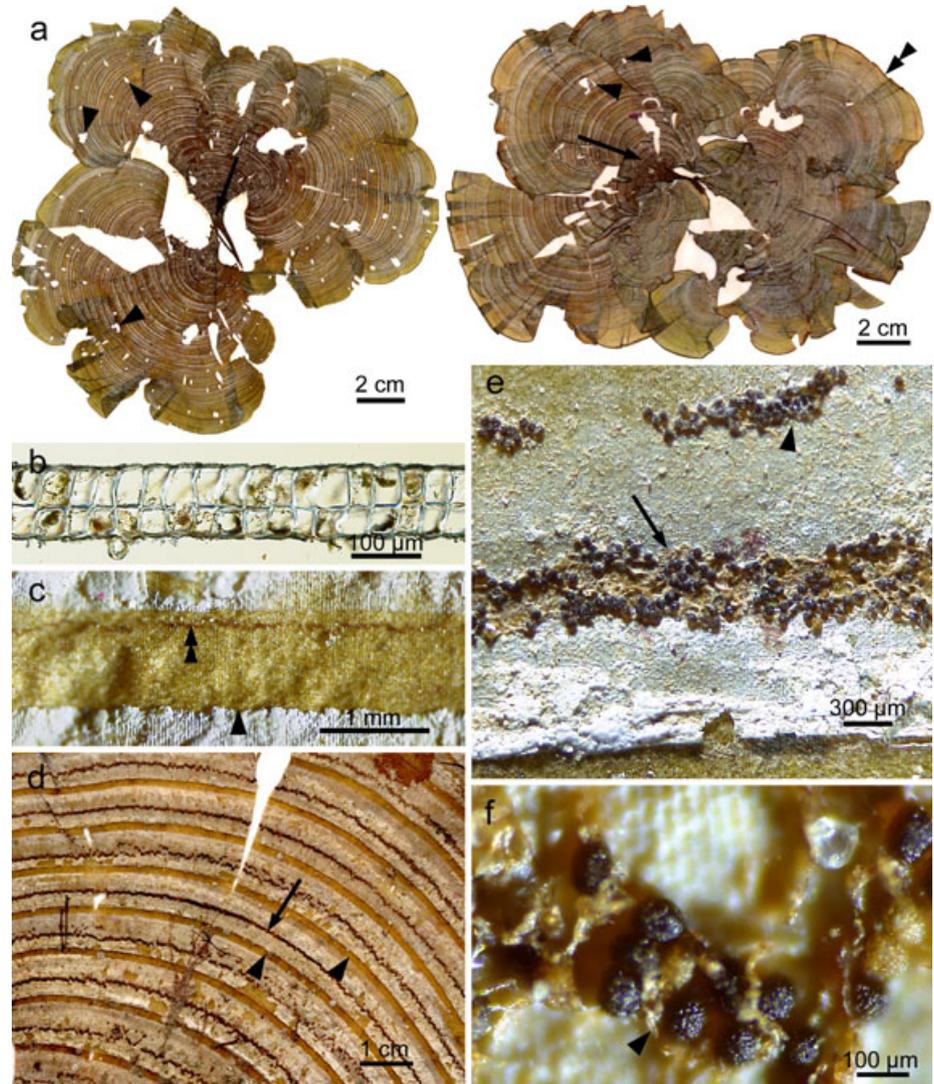

FIG. 3. Morphology of *Padina macrophylla* sp. nov. (a) Habit of tetrasporophyte (left) and male gametophyte (right), showing inferior and superior surfaces (double arrowhead) with tears and pores (arrowhead) and long fibrous hairs at the base (arrow). (b) Transverse section of middle portion of the thallus. (c) Surface view of broad, depressed line (arrowhead) with red, narrow hairline (double arrowhead) on inferior surface of the thallus. (d) Surface view of inferior surface of the thallus, showing relationship of hairlines (arrowhead) and tetrasporangial sori (arrow). (e) Surface view of tetrasporangial sori (arrow) with additional sori (arrowhead). (f) Surface view of tetrasporangia with indusium (arrowhead).

guished by having less calcified thallus, inconspicuous hairlines of superior surface, the location of reproductive sori on thallus surface, and larger distance between hairlines. Representative DNA sequences of the type specimen; AB512539 (*rbc*L), AB512580 (*cox*3).

Holotype: SAP107787, Figure 3, Akasaki, Ishigaki I., Okinawa Prefecture, Japan, collected by M. Uchimura (May 30, 2007).

Habitat: Subtidal zone of 5–15 m deep.

Etymology: The species epithet originates from the conspicuously large size of the thalli.

Specimens examined: Hinai, Iriomote I., May 27, 2007 (SAP107793); Aashioya, May 30, 2007 (SAP107785); Akahashi, May 30, 2007 (SAP107786); Akasaki, Ishigaki I., May 30, 2007 (SAP107787 [holotype], 107788); Sesoko I., June 26, 2007 (SAP107797); Sesoko I., June 26, 2007 (SAP107798, 107799); Shuwabo, Oura Wan, June 21, 2007 (SAP107800); Genka, June 22, 2007 (SAP107791); Nakohi, June 22, 2007; Awase, June 25, 2007 (SAP107789); Agonoura, June 27, 2007; Kudakakita, June 28, 2007; Miyagi, June 29, 2007 (SAP107795); Hamada, Okinawa I., July 7, 2007 (SAP107792); Akaogi, July 28, 2007; Ikomo, July 30, 2007; Saneku, July 30, 2007 (SAP107796); Doran, July 31, 2007 (SAP107790); Ikema, Amami-Oshima I., Okinawa Pref., Japan, July 31, 2007 (SAP107794) (leg. M. Uchimura).

Morphology: The erect thalli are circular or flabelliform, relatively large up to 30 cm in width, 25 cm in height, yellowish to pale brown in color, shallowly to deeply split into several fan-shaped lobes, and attached by stupose base with short stipe (Fig. 3a). Many small holes and tears are found on old thalli (Fig. 3a). Calcification is light to moderate on both surfaces except for the area of HLs (Fig. 3a). The thallus is composed of two cell layers throughout, 85–90 μm thick at the margin, 90–95 μm in the middle (Fig. 3b), and 100–110 μm in the basal portion. Cells of the superior (SP) surface layer are 1.2 times as tall as those of the inferior (IF) layer (Fig. 3b). Concentric HLs are conspicuous on the IF surface while inconspicuous on the SP surface (Fig. 3, a, c, and d), and alternating between both surfaces of the thallus in unequal distance, resulting in wide and narrow glabrous zones. They are formed as a reddish-brown line at the upper end of a broad, depressed line (1 mm wide) on the IF surface (Fig. 3c), whereas narrow HL on the SP surface of the thallus. The distance between two HLs is rather wide, measured ∼7–10 mm apart on each surface (Fig. 3d).

The species is dioecious. Both oogonial and tetrasporangial sori are mainly found on the IF surface of the thallus and formed continuous or discontinuous lines located nearly in the middle between two HLs (Fig. 3d). Sometimes, small groups or patches of tetrasporangial sori are found on the SP surface of the thallus. Additional sori are occasionally observed as patches beside regular line on the IF surface (Fig. 3e). They are protruded from the upper cuticle layer and situated on the surface of the thallus (Fig. 3, e and f) and covered with a persistent indusium (Fig. 3f). Both oogonia and tetrasporangia are obovate, measuring 85 ± 3.1 μm wide, 105 ± 2.0 μm long and 100 ± 2.2 μm wide, and 136 ± 3.9 μm long, respectively. Antheridial sori are found only on the IF surface and formed discontinuous lines or patches without an indusium.

Padina macrophylla resembles *P. maroensis* in appearance and also shows the closest phylogenetic relationship to this species in both the *rbc*L and *cox*3 analyses. However, the former differs from the latter in (i) less calcification on both surfaces of the thallus, whereas the latter is heavily calcified on the SP surface; (ii) inconspicuous HLs on the SP surface, but conspicuous HLs on both surfaces in the latter; (iii) having wider space between HLs than the latter; (iv) the arrangement of reproductive sori (both oogonial and tetrasporangial sori) in continuous lines in the former, but in broken lines or patches in the latter; and (v) their location placed on the thallus surface, whereas those are half immersed in the cuticle layer in the latter.

Padina maroensis Ni-Ni-Win, I. A. Abbott et H. Kawai **sp. nov.** (Fig. 4, a–e) (clade B, in Figs. 2, S1, and S2).

Thalli utraque superficie modice vel dense calcificati. Lineae pilorum concentricae conspicuae, in utraque superficie alternantes, inaequidistantes. Soris indusiatis tetrasporangialibus in utraque superficie thalli prodientibus, areolas parvas vel lineas interruptas epidermide immersas facientibus. Species haec *P. moffittianae* similis sed thallo majus calcificato, loco sororum tetrasporangialorum minus profundo in cuticulo, et lineis pilorum latis depressis in superficie inferiori lineisque pilorum angustis non depressis in superficie superiori distinguenda. Sequentiae nucleotidorum propriae AB512541 (*rbc*L), AB512582 (*cox*3).

Thalli moderately to heavily calcified on the inferior and superior surfaces. Concentric hairlines conspicuous, alternating between both surfaces in unequal distance. Indusiate tetrasporangial sori formed on both surfaces of the thallus, forming small patches or broken lines embedded in epidermis. The species resembles *P. moffittiana* but distinguished by the heavier thallus calcification, shallower location of tetrasporangial sori in cuticle, and having broad depressed hairlines of inferior surface and narrow undepressed hairlines of superior surface. Representative DNA sequences of the type specimen: AB512541 (*rbc*L), AB512582 (*cox*3).

Holotype: SAP108066, 23.6 N, 164.6 W, Maro Reef, Hawaii, collected by Mr. Robert Moffitt (June 21, 2006), Figure 4, a, b, d, and e.

Habitat: Subtidal, attached on lobster traps below 20 m deep.

Fig. 4. Morphology of *Padina maroensis* sp. nov. and *Padina ishigakiensis* sp. nov. (a–e) *P. maroensis* sp. nov. (a) Habit of tetrasporophytes, showing inferior and superior surfaces (arrowhead) of the thalli with long fibrous hairs at the base (arrow). (b) Surface view of inferior surface of the thallus, showing relationship of hairlines on inferior (arrowheads) and superior surfaces (double arrowhead) and tetrasporangial sori (arrows). (c) Surface view of superior surface of the thallus with narrow hairlines (arrowheads). (d) Detail of surface view of tetrasporangial sori with cuticular indusium (arrowhead). (e) Surface view of inferior surface of the thallus, showing relationship of broad, depressed lines (double arrowheads) with red, narrow hairlines (arrowheads) on inferior surface and tetrasporangial sori (arrow). (f–j) *P. ishigakiensis* sp. nov. (f) Habit of tetrasporophyte. (g) Transverse section of middle portion. (h) Surface view of inferior surface, showing relationship of hairlines (arrowheads) on inferior surface and tetrasporangial sori (arrow). (i) Detail of surface view of tetrasporangial sori with indusium (arrowhead). (j) Transverse section of tetrasporangial sori, showing obovate tetrasporangia.
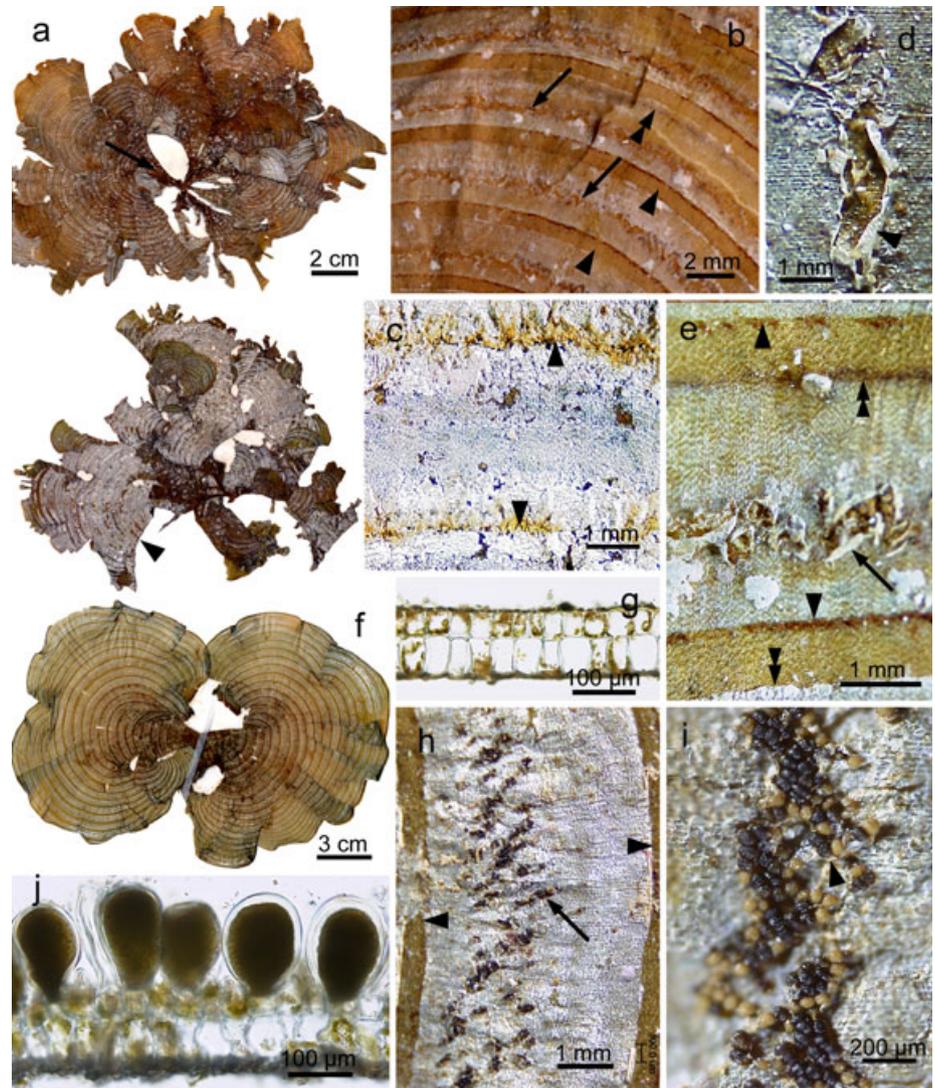
Etymology: The species epithet originates from the type locality.

Specimens examined: From lobster traps, 23.6 N, 164.3 W, Necker I., June 30, 2001 (NNW1 [No. 65536 in KURCIS]); from lobster traps, 23.6 N, 164.6 W, Maro Reef, Hawaii, June 21, 2006 (SAP108066, 108067; NNW2, 3 [No. 65537, 65538 in KURCIS]) (leg. Robert Moffitt).

Morphology: The erect thalli are flabelliform, mostly with fimbriate margin, relatively large up to 20 cm in diameter, shallowly to deeply split into several fan-shaped lobes, yellowish to dark brown or reddish brown in color, and attached by a stupose base with a short stipe (Fig. 4a). IF surface of the thallus is slightly to moderately calcified, whereas the SP surface is heavily calcified forming white color (Fig. 4a). Long fibrous hairs cover the base along the stipe to 1–2 cm upward of the thallus (Fig. 4a). The thallus is composed of two cell layers throughout, 95–100 μm thick at the base and 80–95 μm thick at the other portions. Concentric HLs are conspicuous and formed a reddish-brown line at the upper end of a broad, depressed line (0.5–0.7 mm wide) on the IF surface (Fig. 4, b and e), whereas a narrow HL emerged from the cuticle layer on the SP surface (Fig. 4c). They are alternating between both surfaces in unequal distance (3.5–5 mm apart on each surface) (Fig. 4b) and separated by wide fertile and narrow sterile zones. Tetrasporangial sori are positioned mainly on the IF surface but sometimes on the SP surface and formed small patches or broken lines, which are connected and forming a continuous line located nearly in the middle between two HLs (Fig. 4, b and e). The mature tetrasporangial sori are situated deeply in the cuticle layer (Fig. 4, d and e) and surrounded with a persistent cuticular indusium (Fig. 4, d and e). Gametophytes are unknown.

The species resembles *P. moffittiana* in overall morphology. They were both collected from deep water (28–30 m depth) from Necker I. and Maro Reef, Hawaii, and originally identified as *P. moffittiana*

due to similar thallus structure. However, the new species differs in several morphological features. *P. maroensis* is moderately to heavily calcified on the IF and SP surfaces, respectively, whereas *P. moffittiana* is not or slightly calcified on both surfaces. In *P. maroensis*, HLs on the IF surface of the thallus are broad depressed (Fig. 4, b and e), whereas those on the SP surface are narrow undepressed (Fig. 4c), but those on both surfaces of the thallus in *P. moffittiana* are narrow-depressed. Tetrasporangial sori are broader and located much deeper in the cuticle layer in *P. moffittiana* than those in *P. maroensis*. *P. maroensis* not closely related to *P. moffittiana* (Figs. 2, S1, and S2).

Padina ishigakiensis Ni-Ni-Win, S. Arai, M. Uchimura et H. Kawai **sp. nov.** (Fig. 4, f–j) (clade C, in Figs. 2, S1, and S2).

Thalli interdum in superficie inferiori gregibus pilorum laxe dispositis. Lineae pilorum concentricae conspicuae, in utraque superficie alternantes, inaequidistantes. Thalli dioecii, soris indusiatis tetragonialibus oogonialibusque in utraque superficie lineas discontinuas vel areolas irregulares inter lineas pilorum facientibus. Species haec *P. australi* similis sed lineis pilorum ad distantias inaequales dispositis, soris reproductivis inter lineas pilorum irregulariter sparsis vel longe a lineis pilorum locatis, et gametophytis dioeciis distinguenda. Sequentiae nucleotidorum propriae AB512534 (*rbc*L), AB512575 (*cox*3).

Thalli sometimes with groups of hairs loosely occurring on the inferior surface. Concentric hairlines conspicuous, occurring alternating between both surfaces of the thallus in unequal distance. Thalli dioecious; both indusiate tetrasporangial and oogonial sori forming discontinuous lines or patches irregularly spreading between hairlines on both surfaces of the thallus. The species resembles *P. australis*, but distinguished by the arrangement of alternating hairlines at unequal distance, reproductive sori irregularly spread between hairlines or distant from hairlines, and dioecious gametophytes. Representative DNA sequences of the type specimen: AB512534 (*rbc*L), AB512575 (*cox*3).

Holotype: SAP107778, Figure 4f, Hunakoshi, Ishigaki I., Okinawa Prefecture, Japan, collected by M. Uchimura (May 29, 2007).

Habitat: Subtidal up to 10 m deep.

Etymology: The epithet originates from the type locality.

Specimens examined: Awase, November 19, 2006 (leg. S. Arai) (SAP107774–7); June 25, 2007 (leg. M. Uchimura) (NNW17, 18 [No. 65552, 65553 in KURCIS]); Genka, June 22, 2007 (NNW19, 20 [No. 65554, 65555 in KURCIS]); Yagachikita, Okinawa I., June 22, 2007 (SAP107783); Hunakoshi, Ishigaki I., May 29, 2007 (SAP107778–80); Moba, Kuro I., Okinawa Pref., Japan, June 3, 2007 (leg. M. Uchimura) (SAP107784).

Morphology: The erect thalli are semicircular or flabelliform with entire margin, up to 15 cm wide and 12 cm tall, rarely split into fan-shaped lobes, grayish or dark brown, attached by a stupose base with a stipe of up to 2.5 cm in length and 0.6 cm in width (Fig. 4f). Small groups of hairs are sometimes found on the IF surface of the thallus. IF surface of the thallus is lightly to moderately calcified (Fig. 4, f, h, and i), whereas the SP surface is moderately to heavily calcified. The thallus is composed of two cell layers throughout the whole body, 85–90 μm thick at the margin, 90–100 μm at the middle (Fig. 4g) and 120–125 μm at the base. Cells of the SP layer are 1.5 times taller than those of the IF layer (Fig. 4g). Concentric HLs are alternating between both surfaces and arranged in unequal distance. The distances between HLs are 2–5 mm on each surface (Fig. 4h). They form reddish-brown lines at the upper end of broad, depressed lines (0.3–0.5 mm wide) on the IF surface (Fig. 4, f and h), whereas narrow lines on the SP surface of the thallus.

The species is dioecious. Tetrasporangial and oogonial sori are formed discontinuous lines or patches, which are irregularly spreading between HLs (Fig. 4h) or sometimes far from the HLs. They are located on both surfaces but mainly on the IF surface and covered with an indusium (Fig. 4i). Both oogonia and tetrasporangia are obovate and 77.3 ± 2.4 μm wide, 123.0 ± 2.4 μm long and 93.3 ± 2.5 μm wide, and 142.9 ± 2.3 μm long (Fig. 4j), respectively. Antheridial sori are formed discontinuous lines or patches nearby HLs distally on the IF surface and without an indusium.

Padina ishigakiensis is similar to *P. australis* in the thallus structure but differs in the arrangement of alternating HLs (unequal distance in *P. ishigakiensis* vs. equal distance in *P. australis*), in the arrangement of reproductive sori (irregularly spreading between HLs or far from the HLs in *P. ishigakiensis* vs. close to HLs distally in a regular distance in *P. australis*), and in the reproductive system (dioecious in *P. ishigakiensis* vs. monoecious in *P. australis*). The species is distinguishable from the other three new species by the grayish thalli and the arrangement of reproductive sori irregularly spreading between HLs.

Padina usoehtunii Ni-Ni-Win et H. Kawai **sp. nov.** (Fig. 5) (clade D, in Figs. 2, S1, and S2).

Thalli pilis densis fibrosis a basi ad medium vestiti. Lineae pilorum concentricae in utraque superficie alternantes, inaequidistantes. Sori tetrasporangiales lineas latas continuas 1–1.5 mm latas facientes, sine indusio, in parte distali ad lineas pilorum in superficie inferiori proximi. Species haec distincta lineis angustis alternantibus in spatiis 0.4–0.6 mm et 3.0–3.5 mm longis inter ambas superficies dispositis et soris tetrasporiangialibus latioribus in parte distali ad lineas pilorum proximis. Sequentiae nucleotidorum propriae AB512559 (*rbc*L), AB512597 (*cox*3).
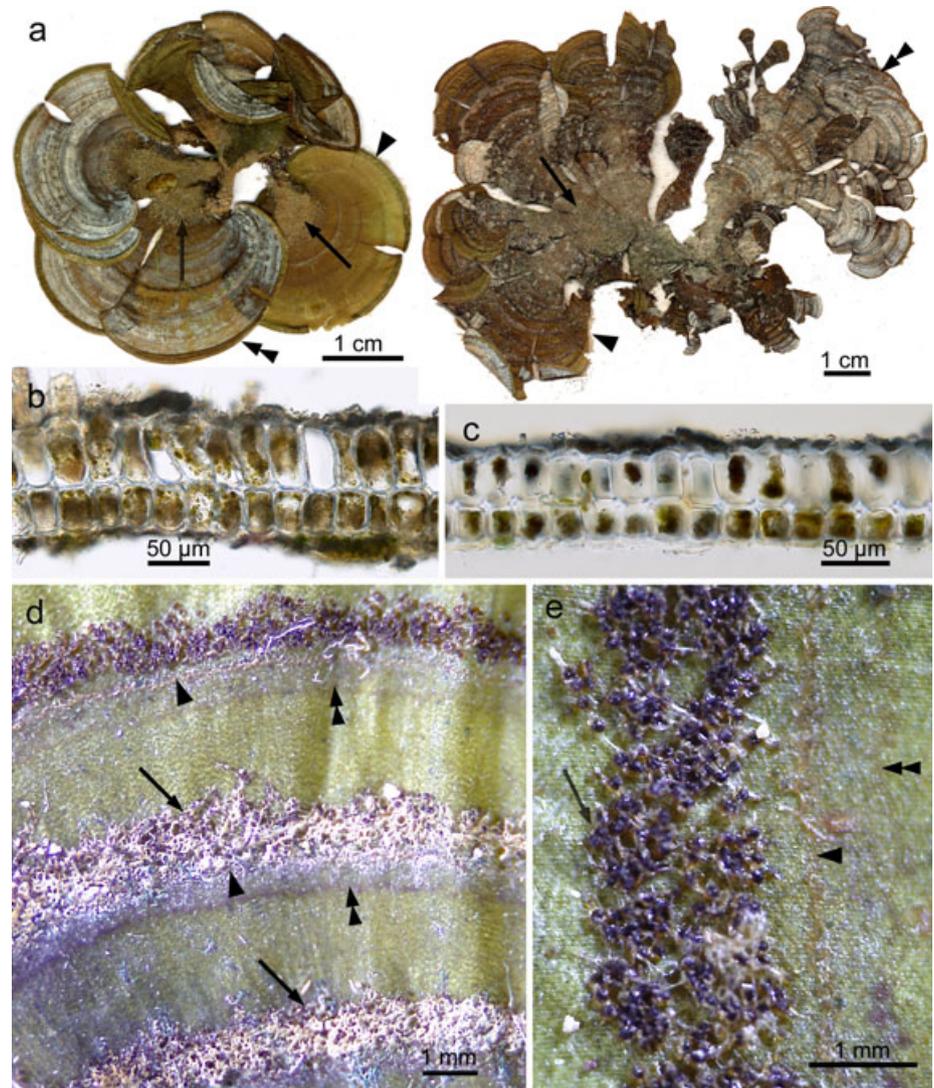
FIG. 5. Morphology of *Padina usoehtunii* sp. nov. (a) Habit of tetrasporophytes from Thailand (left) and Myanmar (right), showing inferior (arrowheads) and superior (double arrowheads) surfaces of the thalli with fibrous hairs (arrows) at the base. (b) Transverse section of the base. (c) Transverse section of middle portion. (d) Surface view of inferior surface of the thallus, showing relationship of hairlines on inferior (arrowheads) and superior (double arrowheads) surfaces, and tetrasporangial sori (arrows). (e) Detail of surface view of tetrasporangial sori (arrow) distally adjacent to hairlines (arrowhead: on inferior surface, double arrowhead: on superior surface).

Thalli with thick fibrous hairs from the basal to the middle part of the thallus. Concentric hairlines alternating between both surfaces of the thallus in unequal distance. Tetrasporangial sori forming broad continuous lines of 1–1.5 mm wide, without an indusium, distally very close to hairlines on the inferior surface. The species is distinctive in having thin alternating hairlines arranged in repeated intervals of 0.4–0.6 mm and 3.0–3.5 mm between both surfaces, and broader tetrasporangial sori distally very close to hairlines. Representative DNA sequences of the type specimen: AB512559 (*rbc*L), AB512597 (*cox*3).

Holotype: SAP107801, Figure 5a (right), Chaung Thar beach, Pathein, Myanmar, collected by Ni-Ni-Win (April 2, 2005).

Habitat: Intertidal.

Etymology: The species epithet originates from Myanmar phycologist Prof. U Soe-Htun.

Specimens examined: Chaung Thar beach, Pathein, April 2, 2005 (SAP107801, NNW4 [No. 65539 in KURCIS]); Ngapali beach, Thandwel, Myanmar, May 5, 2006 (leg. Ni-Ni-Win) (NNW5, 6 [No. 65540, 65541 in KURCIS]); Ko Lanta I., Krabi Province, July 17, 2002 (leg. A. Prathep); Ko Libong, Trang Province, Thailand, October 17, 2005 (leg. B. Nichachucherd).

Morphology: The erect thalli are reniform with entire margin when young, flabelliform when aged, up to 9 cm wide and 7 cm tall, shallowly to deeply split into several fan-shaped lobes, growing as cluster, dark green or yellowish brown to dark brown in color and attached by a stupose base (Fig. 5a). Fibrous hairs thickly cover from the base to the middle portion of the thallus (Fig. 5a). IF surface of the thallus is slightly calcified while the SP surface is heavily calcified forming white color (Fig. 5a). The thallus is composed of two cell layers throughout the whole body, 90–100 μm thick at the basal portion (Fig. 5b) and 75–85 μm thick in the other portions (Fig. 5c). Cells of the SP layer are slightly taller than those of the IF layer (Fig. 5, b and c).

Concentric HLs are alternating between both surfaces of the thallus and arranged in unequal distance, resulting in repeated intervals of 0.4–0.6 mm and 3.0–3.5 mm between both surfaces (i.e., forming narrow sterile and wide fertile zones) (Fig. 5d). They are very thin and placed on the thallus surface (Fig. 5, d and e). The distance between two HLs is 2–3.5 mm on each surface (Fig. 5d). Tetrasporangial sori are rather broad (1–1.5 mm wide) and formed continuous lines without an indusium (Fig. 5, d and e). They are situated distally and very close to HLs only on the IF surface (Fig. 5, d and e). Gametophytes are unknown.

Morphological evolution. Ancestral state reconstruction using Mesquite and MacClade softwares showed similar results, and the evolution of a number of morphological characters along the phylogenetic tree of the combined *rbc*L + *cox*3 data implemented in the MacClade is shown in Figure 6. Members of the genus have been generally divided into groups based on the number of cell layers. As shown in Figure 6a, the majority of the taxa are distromatic throughout the thallus. Taxa with more than two cell layers throughout the entire thalli (i.e., *P. arborescens* and *P. crassa*) formed a monophyletic clade. A thallus with two to three layers evolved a single time (*P. boryana*), whereas a thallus with two to four layers probably evolved three times independently within the genus *Padina*. The calcification of the IF surface of the thallus is absent or negligible in the basal taxa (i.e., *P. moffittiana*, *P. melemele*, *P. crassa*, *P. arborescens*, and *P. pavonica*), but common in other taxa, except for *P. japonica* in which the IF surface is also uncalcified (Fig. 6b). Figure 6c illustrates that gametophytes of most species are dioecious and only two phylogenetically distant species, *P. australis* and *P. pavonica*, have monoecious gametophytes. Gametophytes of *P. maroensis* and *P. usoehtunii* have not been found. The occurrence of a *Vaughaniella* stage probably evolved three times independently and was lost again in the *P. thivyae* lineage (Fig. 6d). The occurrence of an indusium, a hyaline cover over or surrounding sporangial sori, has been lost two times in *Padina* evolutionary history, and in *P. australis*, it is present in female gametophytes and absent in male gametophytes and tetrasporophytes (Fig. 6e). Figure 6f illustrates that the formation of groups of rhizoid-like hairs on the thallus surface probably evolved two times independently. All taxa show HLs on both surfaces of the thallus, except for the two unrelated taxa *P. melemele* and *P. boryana* in which HLs are found only on the IF surface (Fig. 6g). In taxa with HLs on both surfaces, they are alternately positioned, except in the clade of multilayered species (*P. arborescens* and *P. crassa*) where they are positioned irregularly. The arrangement of alternating HLs at equal or unequal distance between both surfaces has each arisen multiple times independently (Fig. 6h). In *Padina*, the arrangement

of reproductive sori is generally related to the HLs. The two early diverging species, *P. melemele* and *P. moffittiana*, are characterized by reproductive sori arranged in the middle between HLs, which evolved once again in the clade consisting of *P. macrophylla* and *P. maroensis* (Fig. 6i). In most species, reproductive sori are arranged just above the HLs. The two other types of sori arrangement (i.e., on both sides of HLs and irregularly spreading between HLs) have each evolved two times independently.

DISCUSSION

Molecular phylogenetic analyses using chloroplast *rbc*L and mitochondrial *cox*3 gene sequences revealed the existence of four undescribed species of *Padina*. They formed independent, statistically well-supported clades in *rbc*L, *cox*3, and combined *rbc*L + *cox*3 analyses and showed a close relationship with *P. australis* in all analyses, except in the *cox*3 tree where clade D (=*P. usoehtunii*) showed a closer relationship with a clade comprising *P. sanctae-crucis* as well as several other recently described species, but with moderate support. Sequence divergence among the four undescribed species and *P. australis* was 1.4%–5.0% in *rbc*L and 6.9%–16.8% in *cox*3. These values are comparable to those between other different species of *Padina* (Ni-Ni-Win et al. 2008, 2010), supporting their genetic separation.

Detailed morphological analysis demonstrated that the newly described species were also morphologically distinguishable from one another as well as from other *Padina* species. A morphological comparative overview of the four newly described species and the closely related *P. australis* is given in Table 1. All four new species are bistratose throughout the thallus. Among the 37 currently recognized species of *Padina*, 18 species are reported to have a bistratose thallus structure (Ni-Ni-Win et al. 2010), of which 11 species were included in this study. All molecular analyses separated these eleven bistratose species from the four new species. A morphological comparison of the seven unsampled bistratose species and the four newly described bistratose species is given in Table 2. These seven species differ from the four new species in the arrangement of alternating HLs. The position of oogonial and tetrasporangial sori occurring between HLs (Levring 1940, Taylor 1960) is a common feature of *P. haitiensis* Thivy, *P. perindusiata* Thivy, *Padina plumbea* (Aresch.) Levring, *P. macrophylla*, and *P. maroensis*. However, *P. haitiensis* differs by the heavy calcification on both surfaces, structure of HLs on both surfaces, and position of tetrasporangial sori only on the IF surface (Taylor 1960, examination of the type specimen by Ni-Ni-Win). Likewise, after thorough examination of the type material, *P. perindusiata* differs by the presence of 2–3 rows of tetrasporangial sori only on the IF surface (Taylor 1960), while only a single row of tetrasporangial sori was found on both
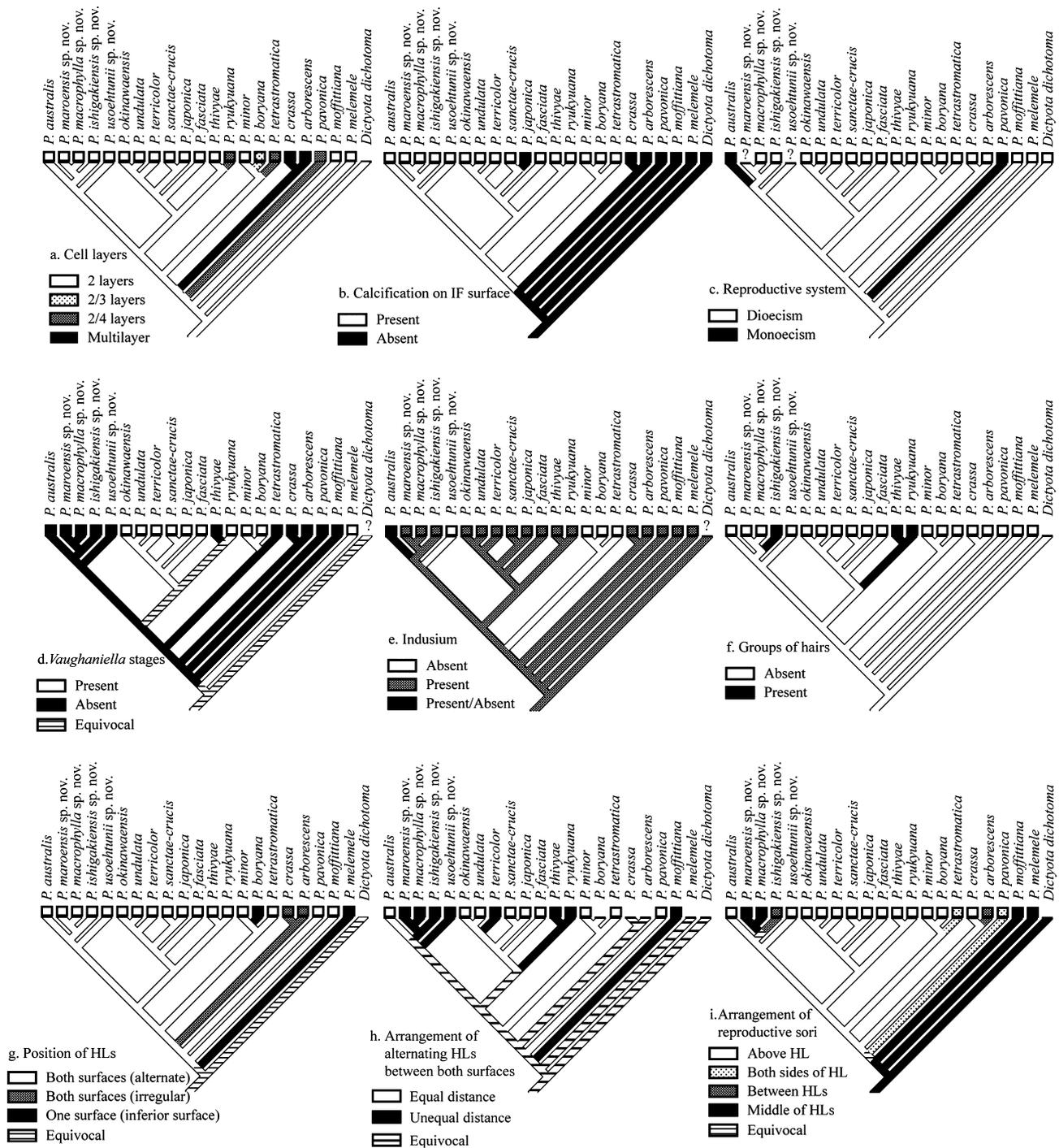
FIG. 6. Character mapping of nine representative taxonomic features of *Padina* spp. onto the phylogenetic tree inferred from *rbc*L + *cox*3 gene sequences. The boxes under each terminal taxon name indicate the state observed in that taxon. If a character is absent in a taxon, no box is shown, and if the state is unknown, a question mark is shown. (a) Number of cell layers constituting the thalli. (b) Degree of calcification on inferior surface. (c) Dioecism or monoecism of gametophytes. (d) Presence or absence of *Vaughaniella* stage. (e) Presence or absence of indusium. (f) Presence or absence of groups of hairs. (g) Position of hairlines. (h) Arrangement of the alternating hairlines between both surfaces of the thallus. (i) Arrangement of reproductive sori. HL, hairline.

surfaces (mainly on the IF surface) in *P. macrophylla* and *P. maroensis*. Moreover, *P. perindusiata* and *P. plumbea* have successive fertile zones (Levring 1940, Taylor 1960, Abbott and Huisman 2003, Ni-Ni-Win et al. 2010), but *P. macrophylla* and *P. maroensis* have alternative fertile zones. *Padina jonesii* Tsuda is similar to *P. ishigakiensis* in having small groups of rhizoids on the IF surface of the thallus but differs in the arrangement of alternating HLs, the position and arrangement of tetrasporangial sori, and the

TABLE 1. Comparison of morphological features among *Padina australis* Hauck and four new species of *Padina*.

Characters	Clade A (*Padina macrophylla* sp. nov.)	Clade B (*Padina maroensis* sp. nov.)	Clade C (*Padina ishigakiensis* sp. nov.)	Clade D (*Padina usoehtunii* sp. nov.)	*P. australis* Hauck
Vegetative characters					
Thallus					
Size	Up to 30 cm wide and 25 cm tall	Up to 20 cm wide and 16 cm tall	Up to 15 cm wide and 12 cm tall	Up to 9 cm wide and 7 cm tall	Up to 9 cm wide and 7 cm tall
Color	Yellowish to pale brown	Dark brown or reddish brown	Grayish to dark brown	Yellowish green or yellowish brown	Yellowish or dark brown
Shape of margin	Entire	Fimbriate	Entire	Entire	Entire
Calcification on IF/SP surfaces	Light/moderate	Moderate/heavy	Light to moderate/heavy	Light/heavy	Light/light to moderate
Small groups of hairs irregularly spreading on the thallus surface	Absent	Absent	Present	Absent	Absent
Fibrous hairs at the base or basal portion	Absent	Present	Absent	Present (thickly covered from the base to the middle portion of the thallus)	Absent
Hairlines					
Arrangement of alternating HLs between both surfaces	Unequal	Unequal	Unequal	Unequal (alternating very closely and widely)	Equal
Distance between HLs on each surface	7–10 mm	3.5–5.0 mm	2–5 mm	2.0–3.5 mm	3–4 mm
Hairlines (IF/SP surfaces)	Conspicuous/inconspicuous	Conspicuous/conspicuous	Conspicuous/moderate	Conspicuous/conspicuous	Conspicuous/conspicuous
Structures (IF/SP surfaces)	Broad, depressed/narrow	Broad, depressed/narrow	Broad, depressed/narrow	Narrow/narrow	Broad/narrow
Reproductive characters					
Reproductive system	Dioecious	Unknown	Dioecious	Unknown	Monoecious
Sporangial sori					
Position (surface)	Both, mainly on IF	Both, mainly on IF	Both, mainly on IF	IF	IF
Structure	Narrow	Narrow	Narrow	Broad	Narrow
Number in row between HLs	One[a]	One	Many	One	One
Arrangement; position	Continuous lines; in the middle of HLs	Broken lines or patches; nearly in the middle of HLs	Broken lines or patches; irregularly spreading between HLs	Continuous lines; just above HLs	Continuous lines; just above HLs
Location	On thallus surface	Half immersed in the cuticle layer	On thallus surface	On thallus surface	On thallus surface
Indusium	Present	Present (cuticular)	Present	Absent	Present (female gametophyte)/absent (male gametophyte and tetrasporophyte)
Reference	Herein	Herein	Herein	Herein	Hauck 1887, herein

IF, inferior; SP, superior; HLs, hairlines.
[a]Sometimes with additional broken line.

TABLE 2. Comparison of morphological features among four newly described bistratose species of *Padina* and seven unsampled bistratose species.

Characters	Clade A (=*Padina macrophylla* sp. nov.)	Clade B (=*Padina maroensis* sp. nov.)	Clade C (=*Padina ishigakiensis* sp. nov.)	Clade D (=*Padina usoehtunii* sp. nov.)	*Padina distromatica*	*Padina elegans*	*Padina fernandeziana*	*Padina haitiensis*	*Padina jonesii*	*Padina perindusiata*	*Padina plumbea*
Vegetative characters											
Thallus											
Shape of margin	Entire	Fimbriate	Entire	Entire	Entire	Split	Split	Split	–	–	–
Calcification on IF/SP surfaces	Moderate/moderate	Moderate/heavy	Light to moderate/heavy	Light/heavy	Light/moderate	No/light	No/light	Heavy/heavy	Heavy/heavy	Light/light	Light/moderate
Small groups of hairs	Absent	Absent	Present	Absent	Absent	Absent	Absent	Absent	Present	Absent	Absent
Hairlines											
Arrangement of alternate HLs between both surfaces	Unequal	Unequal	Unequal	Unequal	–	Equal	Equal	Equal	Equal	Irregular	Irregular
Hairlines (IF/SP surfaces)	Conspicuous/inconspicuous	Conspicuous/conspicuous	Conspicuous/conspicuous	Conspicuous/conspicuous	Conspicuous/inconspicuous	Conspicuous/conspicuous	Conspicuous/Inconspicuous	Conspicuous/conspicuous	Conspicuous/inconspicuous	Conspicuous/conspicuous	Inconspicuous/inconspicuous
Structure (IF/SP)	Broad, depressed/narrow	Broad, depressed/narrow	Broad, depressed/narrow	Narrow/narrow	–	–	–	Narrow, depressed/narrow, depressed	–	Narrow/narrow	–
Reproductive characters											
Sporangial sori											
Position (surface)	Both, mainly on IF	Both, mainly on IF	Both, mainly on IF	IF	IF	SP	IF	IF	IF	IF	SP
Number in row between HLs	One[a]	One	Many	One	Two	One	Two	One	One	2–3	–
Arrangement; position	Continuous lines; in the middle of HLs	Broken lines or patches; nearly in the middle of HLs	Broken lines or patches; irregularly spreading between HLs	Continuous lines; just above HLs	Abutting HLs	Continuous or broken lines; above HLs	Continuous or broken lines; abutting HLs	Broken lines; in the middle of HLs	Continuous lines; above HLs	Continuous or broken lines; between HLs	Broken lines or patches; between HLs
Oogonia											
Shape	Obovate	–	Obovate	–	–	–	–	–	–	–	–
Size: wide × long (µm)	85 ± 3.13 × 105 ± 2.03	–	77.3 ± 2.36 × 122.95 ± 2.39	–	–	–	–	–	–	–	–
Tetrasporangia											
Shape	Obovate	Obovate	Obovate	Obovate	–	Ovoid	–	–	–	–	–
Size: wide × long (µm)	100 ± 2.22 × 136.25 ± 3.93	–	93.25 ± 2.45 × 142.9 ± 2.27	–	–	50–100 × 80–120	60–75 × 90–120	80–110 × 80–110	75 × 75	170 × 170	30–35 × 45–55
Location	On thallus surface	Half immersed in the cuticle layer	On thallus surface	On thallus surface	–	–	–	On thallus surface	–	On thallus surface	–
Fertile zone	Alternate[b]	Alternate[b]	Alternate[b]	Alternate[b]	Successive[c]	Alternate[b]	Successive[c]	Alternate[b]	Alternate[b]	Successive[c]	Successive[c]

TABLE 2. Continued.

Characters	Clade A (=Padina macrophylla sp. nov.)	Clade B (=Padina mareensis sp. nov.)	Clade C (=Padina ishigakiensis sp. nov.)	Clade D (=Padina usoehtunii sp. nov.)	Padina distromatica	Padina elegans	Padina fernandeziana	Padina haitiensis	Padina jonesii	Padina perindusiata	Padina plumbea
Indusium	Present	Present (cuticular)	Present	Absent	Absent	Present	Present	Present	Absent	Present	Present
Reference	Herein	Herein	Herein	Herein	Hauck 1887, examination of type specimen	Womersley 1987	Levring 1941	Taylor 1960, examination of type specimen	Tsuda 1972	Taylor 1960, examination of type specimen	Levring 1940

IF, inferior; SP, superior; HLs, hairlines.
aSometimes with additional broken line.
bFertile zones are separated by sterile zones when both surfaces are viewed together.
cSterile zones are absent.

absence of an indusium (Tsuda 1972). *P. distromatica* Hauck and *Padina fernandeziana* Skottsb. et Levring are distinguishable from these four new species in the position and arrangement of tetrasporangial sori, and in the presence of successive fertile zones (Hauck 1887, Levring 1941). *Padina elegans* Koh ex Womersley differs from the four new species in the position of tetrasporangial sori only on the SP surface and the arrangement of alternating HLs in equal distance between both surfaces (Womersley 1987).

This study indicates that some of the morphological characters, namely, shape, size, color, and thickness of the thallus, have been shown to be highly variable within the species and are subject to environmental conditions and age of the individual (personal observations of Ni-Ni-Win, data not shown). Other characters were considered stable within the species regardless of environmental conditions and age, namely, the number of cell layers, presence or absence and degree of calcification, presence or absence of *Vaughaniella* stage, monoecism or dioecism, the position and arrangement of HLs and sporangial sori, and presence or absence of an indusium. In addition, the present study demonstrates that the presence or absence of groups of rhizoid-like hairs on the thallus surface and the structure and arrangement of HLs and reproductive sori are also stable characters within species.

To further explore the phylogenetic implications of some traditional morphological characters, these characters were mapped onto the molecular tree (Fig. 6), which is the best estimate of the *Padina* phylogeny currently available. Members of the genus have traditionally been grouped based on the number of cell layers constituting the thallus (e.g., two-layered, 2/3-layered, 2/4-layered, and multilayered species). However, only multilayered species formed a monophyletic clade, and the 2/3-layered clade contained only a single species (Fig. 6a). A two-layered thallus throughout is probably the plesiomorphic state in *Padina*, a 2/3-layered and a multilayered thallus have each evolved a single time, and a 2/4-layered thallus evolved three times. In brown algae, calcification of the thallus is only known in *Padina* and in the recently described monotypic genus *Newhousia* (Kraft et al. 2004). In *Padina*, all members are lightly to heavily calcified on the SP surface of the thallus, but the calcification on the IF surface may be absent in some species. Figure 6b shows that the early diverging taxa are not or negligibly calcified on the IF surface and all other taxa are commonly calcified on the IF surface to varying degrees, except for *P. japonica*. Therefore, the absence of obvious calcification in the IF surface in *P. japonica* is considered a secondarily evolved feature. As in many species of Dictyotales, gametophytic plants are much rarer than sporophytic plants in *Padina*. All but two *Padina* species are dioecious. The two monoecious species

P. australis and *P. pavonica* are not sister species (Fig. 6c). Monoecism in *Padina* is considered a derived feature that has evolved at least two times independently in the genus (Fig. 6c). The *Vaughaniella* stage was originally not recognized as a stage in the life cycle of (some) *Padina* species, and the genus *Vaughaniella* was erected by Børgesen (1950) for these prostrate rhizomes. Cribb (1951), however, discovered that these prostrate rhizomes gave rise to fan-shaped thalli, which were identified by him as *Padina commersonii* Bory (=*P. boryana*). In species without this stage, fan-shaped thalli develop directly from the germlings of zygotes and tetraspores. The presence of a *Vaughaniella* stage may be the ancestral state in *Padina* because it appears in the first taxon to branch off, that is, *P. melemele* (Fig. 6d). It is subsequently lost in its sister lineage and then gained again in at least two sublineages. An indusium covering or surrounding reproductive sori was also reported in some other Dictyotales (e.g., *Dictyota* [known as involucrum] and *Lobophora*). The presence of an indusium may be considered the ancestral state in *Padina* because it has been determined in most species, but lost two times in the *Padina* lineage (Fig. 6e). Support for the ancestral state of an indusium also comes from the putative sister genus of *Padina*, that is, *Distromium* Levring (Bittner et al. 2008). *Distromium* has more features in common with the basal *Padina* species. It is bistratose throughout, is probably dioecious (oogonia are unknown, antheridial sori are only known for one species), and has indusiate sori. However, variation of the presence and absence of indusium was recognized in *P. australis* for the first time in the present study. In *P. australis,* indusiate sori are present in female gametophytes and absent in male gametophytes and tetrasporophytes. A careful examination on this character is recommended here because it is one of the most important characters for species delimitation.

The formation of small groups of rhizoid-like hairs on the IF surface of the thallus has been reported in *P. ryukyuana* (Lee and Kamura 1991), *P. thivyae* (Tsuda 1972), and the unsampled *P. jonesii* (Tsuda 1972) and was also observed in the new species *P. ishigakiensis*, although varying in degree. All other species lack this feature, including the early diverging ones (Fig. 6f). The formation of HLs on the thallus surfaces, in concentric lines or small turfs, is a common feature in the order Dictyotales. But their position on one surface or both surfaces of the thallus is one of the important characters to delimit the species in the genus *Padina*. In addition, their arrangement (alternate or irregular) is also useful to differentiate among the species that have HLs on both surfaces. All but two *Padina* species show HLs on both surfaces. The irregular arrangement of the HLs between both surfaces (i.e., some parts of HL appear on the IF surface while the other parts of the same HL develop on the SP surface) is restricted to the clade of multilayered species (Fig. 6g), indicating the taxonomic significance of this feature for the differentiation of *P. arborescens* and *P. crassa* from the other *Padina* species. Among the species in which HLs are arranged alternately between both surfaces, the arrangement of the HLs can be at equal or unequal distance between both surfaces. Alternating HLs at unequal distance is considered to be the ancestral state, and an equal arrangement a derived state (Fig. 6h). However, both states have been gained and lost multiple times, and although useful for *Padina* species identification, it is not phylogenetically informative. The arrangement of reproductive sori is generally related to the HLs and useful for species delineation in *Padina*. The arrangement of reproductive sori in the middle between HLs appears to be the ancestral state from which the ''above HL'' type evolved one time and the two other types (on both sides of HLs, and irregularly spreading between HLs) each evolved two times (Fig. 6i).

Most character states show at least some degree of homoplasy. Only multilayered species and species with an irregular arrangement of the alternating HLs between both surfaces formed a monophyletic clade (Fig. 6, a and g) (both *P. arborescens* + *P. crassa*). Species can be delineated and identified with a combination of characters, but the characters are of limited value for inferring phylogenetic relationships within *Padina*, due to high morphological convergence.

This study and two recent studies (Ni-Ni-Win et al. 2008, 2010) revealed the occurrence of eight new species of *Padina*. Seven were recorded from subtropical North Pacific regions (i.e., *P. fasciata, P. ishigakiensis, P. maroensis, P. macrophylla, P. okinawaensis, P. undulata,* and *P. terricolor*) and two from the eastern Indian Ocean (i.e., *P. okinawaensis* and *P. usoehtunii*). In addition, four previously known species were newly recorded for Japan (i.e., *P. melemele, P. moffittiana, P. sanctae-crucis,* and *P. thivyae*). This brings the total number of *Padina* species for Japan to 18, indicating high species diversity, particularly in southern Japan. Before 2008, only eight *Padina* species were recorded in Japan. In the past, *Padina* taxonomy was notoriously difficult due to the morphological plasticity of the gross morphology, which was traditionally used in identifications. Hence, it is considered that the recently and currently newly described species have been overlooked or placed under the names of different species as a result of a similar overall morphology and a lack of understanding of diagnostic characters for species delimitation. This is certainly the case for *P. melemele* and *P. fasciata* in Japan and *P. maroensis* in Hawaii, which were kept as *P. boryana, P. minor,* and *P. moffittiana,* respectively. Moreover, the lack of sound molecular data to support the recognition of morphological discontinuities is one of the reasons that hampered the creation of a stable species classification.

Eight out of nine *Padina* species reported for Hawaii [i.e., *P. australis, P. boryana, Padina gymnospora* (Kütz.) Sond., *P. melemele, P. moffittiana, P. okinawaensis, P. thivyae,* and *P. sanctae-crucis*] were also reported for Japan (for *P. gymnospora,* Ni-Ni-Win, Takeaki Hanyuda, Stefano G. A. Draisma, Hiromori Shimabukuro, and Hiroshi Kawai unpubl. data), indicating biogeographic affinities between the two regions. However, we doubt previous reports of *P. boryana* in Japan (Yamada 1931 [as *P. commersonii*], Tanaka and Nozawa 1962 [as *P. commersonii*], Yoshida et al. 2000). *P. boryana* was originally reported from Tonga Is. (Taylor 1966) and has worldwide temperate–tropical distribution (Silva et al. 1996), but was not found in our collections from Japan, despite extensive sample collections in a wide range of localities. Moreover, meticulous reexaminations of all *Padina* specimens kept in SAP by Ni-Ni-Win revealed that all records of *P. boryana* actually represented different species, mainly *P. minor* or *P. melemele,* and sometimes other species. It is considered that previous records of *P. boryana* in Japan were based on the specimens of different species. Morphologically, *P. boryana* shares most characters with *P. minor,* and it is difficult to distinguish between them. The only two characters to differentiate *P. boryana* from *P. minor* are (i) the number of cell layers of the thallus (two layers from marginal to middle and three layers at the base in *P. boryana* vs. two layers throughout in *P. minor*) and (ii) the position of HLs (only on the IF surface of the thallus in *P. boryana* vs. alternating between both surfaces in *P. minor,* but HLs on the SP surface are sometimes difficult to detect). Molecular phylogenetic analyses also confirmed their close relationship; they were always sister taxa with high support (Figs. 2, S1, and S2).

Our molecular phylogeny did not reveal any clear geographic structuring. There was a separate Mediterranean clade, but this clade was monotypic (*P. pavonica*) and nested within Indo-Pacific clades. Although *P. pavonica* has been reported from Southeast Asia (Silva et al. 1996), it was not found in our extensive collections from Indonesia and peninsular Southeast Asia, nor in those from Japan and Hawaii (from where there are no previous reports). The distribution of *P. pavonica* might therefore be restricted to the Mediterranean and Atlantic, but a much more comprehensive sampling is necessary to confirm or reject its presumed worldwide distribution. *P. australis* and *P. okinawaensis* appear to have the widest distribution according to our sampling and occur in all our sampled subregions: Hawaii, Japan, Indonesia, and peninsular Southeast Asia. But many species seem to have a very restricted distribution. Whether this is real or an effect of limited sampling cannot be concluded. Therefore, to get a clear picture of the actual geographic distribution of species as well as to understand phylogeography of the species within the genus *Padina,* a comprehensive sampling from its worldwide distribution range to cover all reported species is necessary in further investigations.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Maximum-likelihood (ML) tree based on *rbc*L gene sequences.

Figure S2. Maximum-likelihood (ML) tree based on *cox*3 gene sequences.

Table S1. Origin of specimens used in this study and their DNA Data Bank of Japan (DDBJ) accession numbers.

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