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Morphological and molecular evidence for two new species of *Padina* (Dictyotales, Phaeophyceae), *P. sulcata* and *P. calcarea*, from the central Indo-Pacific region

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Two new species of *Padina* – 1. *Padina sulcata* sp. nov. and 2. *P. calcarea* sp. nov. – from Malaysia, Indonesia and Palau were described based on morphological and molecular phylogenetic observations. *Padina sulcata* was a three-layered species characterised by a covering of thick fibrous hairs from the base to the middle of the inferior surface (away from the in-rolled margin) of the thallus; conspicuous equally spaced hairlines that alternated between both frond surfaces; and broad, indusiate oogonial and tetrasporangial sori that occupied nearly the entire fertile zones, and the fertile zones were separated by sterile zones of equal width. *Padina calcarea* was a two-layered species characterised by a bright yellow inferior surface and a thick calcification on the superior surface (facing to the in-rolled margin), which imparted a strikingly whitish color; inconspicuous hairlines were confined to the inferior surface; and indusiate tetrasporangial sori were just above the hairlines and were found only on the inferior surface. Molecular phylogenetic analyses used chloroplast *rbc*L and mitochondrial *cox*3 gene sequences and revealed that the two new species each form strongly supported clades that were genetically distant. *Padina calcarea* formed an isolated clade that made an early divergence; whereas, *P. sulcata* showed a sister relationship to *P. ryukyuana*, indicating a more recent divergence. *Padina calcarea* was very similar to the Hawaiian *P. melemele* in gross appearance, particularly in the bright orange to yellow color of the inferior thallus surface and the heavy calcification on the superior surface. However, they were distinguished mainly by the position and arrangement of reproductive sori that were found on the inferior surface and located just above the hairlines in *P. calcarea* but that were found on the superior surface between the hairlines of the opposite surface in *P. melemele*. Molecular phylogenetic analyses did not reflect the morphological similarity of the two species because they occupied two distantly related clades.

Key Words: *cox*3, Dictyotales, Molecular analyses, *Padina sulcata*, *Padina calcarea*, Phaeophyceae, *rbc*L, Taxonomy

INTRODUCTION

The genus *Padina* Adanson is the only brown algal genus, aside from the recently described *Newhousia imbricata* Kraft, G.W. Saunders, I. A. Abbott & Haroun from Hawaii (Kraft *et al.* 2004), that calcifies itself to any significant extent. *Padina* typically has a fan-shaped thallus that grows from an in-rolled margin of meristematic cells. Some species, however, have a '*Vaughaniella*' stage of creeping rhizomes with a single apical cell from which the fan-shaped thalli develop (Børgesen 1951; Cribb 1951; De Clerck & Coppejans 1997). Thalli are two or more cell layers thick (up to 20 layers in some species) and erect to decumbent depending on the species. Most members of *Padina* are widely distributed in warm-temperate to tropical seas, occurring in lower-intertidal to deeply subtidal habitats.

There are currently 37 species recognised in the genus (Guiry & Guiry 2011), of which about 24 species are distributed in the Pacific Ocean, with about 16 recorded from the Indian Ocean (Silva *et al.* 1996; Guiry & Guiry 2011). Recently, Kraft (2009) proposed a new species (*P. condominium* Kraft from Lord Howe I., Australia) based on morphology of monoecious gametophytes. However, taxonomy of a genus that is anatomically based can be extremely uncertain because of morphological plasticity. Also, inconsistent use of taxonomic terminology (Trono 1969), uncertainty concerning diagnostic characters for species definition and the absence of DNA sequence data present difficulties in this genus.

Several studies, using chloroplast-encoded *rbc*L and mitochondrial encoded *cox*3 sequences as well as meticulous morphological observations, have documented species-level taxonomy of *Padina* from Japan, Hawaii, Southeast Asia

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and the Mediterranean Sea. These have resulted in the proposal of several new diagnostic characters for species delimitation as well as the description of ten new species (Ni-Ni-Win *et al.* 2008, 2010, 2011a, 2011b). Eight of the new species are from the Indo-Pacific regions (*P. fasciata* Ni-Ni-Win, M. Uchimura & H. Kawai, *P. ishigakiensis* Ni-Ni-Win, M. Uchimura & H. Kawai, *P. macrophylla* Ni-Ni-Win, M. Uchimura & H. Kawai, *P. maroensis* Ni-Ni-Win, I. A. Abbott & H. Kawai, *P. okinawaensis* Ni-Ni-Win, S. Arai & H. Kawai, *P. terricolor* Ni-Ni-Win, S. Arai, M. Uchimura & H. Kawai, *P. undulata* Ni-Ni-Win, S. Arai & H. Kawai and *P. usoehtunii* Ni-Ni-Win & H. Kawai), and the remaining two are from the Mediterranean Sea (*P. ditristromatica* Ni-Ni-Win & H. Kawai and *P. pavonicoides* Ni-Ni-Win & H. Kawai). In addition, four species have been newly recorded from Japan (*P. melemele* I. A. Abbott & Magruder, *P. moffittiana* I. A. Abbott & Huisman, *P. sanctae-crucis* Børgesen and *P. thivyae* Doty & Newhouse). These studies indicate high species diversity in the subtropical to warm-temperate North Pacific, particularly in southern Japan. Based on morphological and molecular evidence, we report here the occurrence of two new species from Malaysia, Indonesia and Palau.

MATERIAL AND METHODS

Morphological observations

Padina specimens used in this study were newly collected from Indonesia, Malaysia and Palau (Table 1). Selected voucher specimens used for morphological and molecular studies were deposited in the Herbarium of the Graduate School of Science, Hokkaido University (SAP); the herbarium of the Kobe University Research Center for Inland Seas; and the National Herbarium of the Netherlands in Leiden (L). Type specimens of *P. australis* Hauck (L 0055591), *P. distromatica* Hauck (L 0055592), *P. dubia* Hauck (L 0055593), *P. somalensis* Hauck (L 0055595), *P. tetrastromatica* Hauck (L 0055597; Hauck No. 68), *P. haitiensis* Thivy [Herbarium of University of Michigan (MICH), Taylor 20987], *P. perindusiata* Thivy (MICH, Taylor 1356) and *P. japonica* (SAP 9268), loaned from L, MICH, and SAP, were also examined. Specimens were hand sectioned for anatomical observations and the sections mounted on glass slides in Karo syrup/seawater. Photomicrographs were taken using a VB-7010 Digital Camera (Keyence, Tokyo, Japan) attached to a BX-51 microscope (Olympus, Tokyo, Japan). Herbarium abbreviations follow Thiers (2011).

Molecular phylogenetic analysis

DNA extraction, PCR amplifications and sequencing were carried out as specified in Ni-Ni-Win *et al.* (2008). Six new *rbc*L sequences and five new *cox*3 sequences were deposited at DDBJ (Table 1) and combined with previously published sequences (Ni-Ni-Win *et al.* 2008, 2010). *Stypopodium* sp. and *Lobophora* sp. (Dictyotales) were used as out-groups in the *rbc*L, *cox*3 and concatenated *rbc*L + *cox*3 analyses (Table 1). Sequences were aligned with Clustal X (Thompson *et al.* 1997) and then manually adjusted by eye. In order to check the positions/clusters of the specimens attributed to a species, as well as for checking tree topology, three alignments using each data set of *rbc*L and *cox*3 and their combined data set were created to construct phylogenetic trees. Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI) analyses. With the aid of KAKUSAN3 (Tanabe 2007), 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 corrected Akaike information criterion (Akaike 1974) for ML analysis and the Bayesian information criterion (Schwarz 1978) for the BI analysis. The selected models in each codon position of each gene for ML and BI are as follows: ML [*cox*3 (GTR+G for first codon position, HKY85+G for second codon position and J1+G for third codon position), *rbc*L (GTR+G for first codon position, GTR+G for second codon position and TVM+G for third codon position), *cox*3+*rbc*L (GTR+G for *cox*3 first codon position, HKY85+G for *cox*3 second codon position, J1+G for *cox*3 third codon position, GTR+G for *rbc*L first codon position, GTR+G for *rbc*L second codon position and TVM+G for *rbc*L third codon position)] and BI [*cox*3 (GTR+G for first codon position, F81+G for second codon position and K2P+G for third codon position), *rbc*L (GTR+G for first codon position, JC+G for second codon position and GTR+G for third codon position), *cox*3+*rbc*L (GTR+G for *cox*3 first codon position, F81+G for *cox*3 second codon position, GTR+G for *cox*3 third codon position, GTR+G for *rbc*L first codon position, GTR+G for *rbc*L second codon position and JC+G for *rbc*L third codon position)]. The ML analysis was performed by the likelihood ratchet method (Vos 2003). For the ML tree search, 1000 sets of 25% site-upweighted data were created using the pgresampleseq command in Phylogears 1.5.2009.12.29 (Tanabe 2009), and the ML trees with the upweighted data were estimated using Treefinder (Jobb *et al.* 2004) with application of the best-fit model. The robustness of the resulting phylogenies was tested by bootstrap analysis (Felsenstein 1985) using 1000 replications in ML analysis. Bayesian analyses with the selected evolutionary models were done using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The Bayesian analyses were initiated with a random starting tree, and they ran four chains of Markov chain Monte Carlo iterations simultaneously for 100,000,000 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 & Drummond 2009); a consensus topology and posterior probability values were calculated with the remaining trees.

RESULTS

Molecular phylogenetic analyses

The *rbc*L alignment consisted of 36 sequences (1319 characters per sequence) representing 20 *Padina* species, including six new sequences of the central Indo-Pacific specimens and two out-group taxa (Table 1). The *cox*3

Table 1. Origin of specimens used in this study and their DDBJ accession numbers.[1]

Species	Specimens code	Origin (collection date; collector)	Voucher no.[2]	DDBJ code for *rbc*L	DDBJ code for *cox*3
Padina arborescens Holmes	NAG1	Taira, Nagasaki, Japan (4 Jul. 2006; S. Arai)	SAP105578	AB358905	AB358940
P. australis Hauck	OKI1	Urazoko, Okinawa I., Japan (4 Oct. 2004; T. Hanyuda)	SAP105580	AB358906	AB358941
	MYA1	Ngapali beach, Thandwel (Sandoway), Myanmar (3 May 2006; Ni-Ni-Win)	—	AB489913	AB489954
	AUS1	Newcastle, NSW, Australia (2005; H. Kawai)	—	AB489914	AB512565
P. boryana Thivy	MYA4	Setsei, Kyaikkhami, Mon State, Myanmar (20 Apr. 2005; Mya Kyawt Wai)	65542 in KURCIS[3]	AB512527	AB512568
	KEP7	Bidadari, Kepulauan Seribu, Indonesia (9 Sep. 2005; S.G.A. Draisma)	L0609513	AB512529	AB512570
P. calcarea sp. nov.	GAM1	Cape Besir, Besir Bay, West-Papua, Gam I., Indonesia (27 Nov. 2007; S.G.A. Draisma)	L SGAD 0712219	**AB671198**	**AB671208**
	BAT1	Gegenlol Bay, Batanta I., Indonesia (27 Nov. 2007; S.G.A. Draisma)	L SGAD 0712254	**AB671199**	**AB671209**
	WAI1	Yenweres Bay, Wai I. (a.k.a. Jerief Isl.), Indonesia (5 Dec. 2007; S.G.A. Draisma)	L SGAD 0712521	**AB671200**	—
	BAB1	Back Reef (N 07°57.271′, E 134°37.661′), north of Babeldaob I., Palau (31 Mar. 2009; E. Verheij and W. F. Prud'homme van Reine)	SAP111112	**AB671201**	**AB671210**
	BAB2	Back Reef (N 07°57.271′, E 134°37.661′), north of Babeldaob I. (31 Mar. 2009; E. Verheij and W.F. Prud'homme van Reine)	L0821280	**AB671202**	**AB671211**
P. crassa Yamada	NAG2	Taira, Nagasaki, Japan (4 Jul. 2006; S. Arai)	SAP105581	AB358908	AB358943
P. fasciata Ni-Ni-Win, M. Uchimura & H. Kawai	OKI2	Awase, Okinawa I., Japan (19 Nov. 2006; S. Arai)	SAP106507	AB489915	AB489955
P. fraseri (Greville) Greville	AUS2	Flat Rock, Northern New South Wales, Australia (16 Dec. 2006; J. Phillips)	65550 in KURCIS	AB548389	AB548397
P. japonica Yamada	NAG3	Taira, Nagasaki, Nagasaki Pref., Japan (4 Jul. 2006; S. Arai)	SAP105583	AB358910	AB358942
P. melemele I.A. Abbott & Magruder	HAW1	BISH700753, Hawaii	BISH700753	AB358918	AB358947
P. minor Yamada	IRI3	Iriomote I., Okinawa Pref., Japan (21 Nov. 2006; S. Arai)	SAP105611	AB358920	AB358948
	KEP2	Kotok Besar, Kepulauan Seribu, Indonesia (16 Sep. 2005; S.G.A. Draisma)	L0609547	AB489922	AB489956
P. moffittiana I.A. Abbott & Huisman	HAW2	Maro Reef, Hawaii (21 Jun. 2006; R. Moffitt)	IA31668	AB358927	AB358951
P. okinawaensis Ni-Ni-Win, S. Arai & H. Kawai	OKI5	Awase, Okinawa I., Okinawa Pref., Japan (19 Nov. 2006; S. Arai)	SAP106474	AB489923	AB489959
P. pavonica (Linnaeus) Thivy	SPA2	Mallorca, Spain (22 Jun. 2006; S.G.A. Draisma)	L SGAD 0606012	AB512545	AB512586
	SIC1	South East Sicily, Italy (13 Jul. 2007; H. Kawai)	—	AB512546	AB512587
	SIC2	Catania, Sicily, Italy (12 Jul. 2007; H. Kawai)	—	AB512547	AB512588
	AKY1	Akyaka, Turkey (22 Apr. 2008; H. Kawai)	—	AB512548	AB512589
	CAN2	Cannes, France (16 Apr. 2008; H. Kawai)	—	AB512551	AB512592
P. ryukyuana Y.P. Lee & Kamura	OKI6	Awase, Okinawa I., Okinawa Pref., Japan (19 Nov. 2006; S. Arai)	SAP105631	AB358929	AB358953
P. sanctae-crucis Børgesen	OKI7	Haemida Beach, Okinawa I., Japan (27 May 2007; M. Uchimura)	SAP106512	AB489935	AB489969
P. sulcata sp nov.	MAL7	Pulau Rusukan Kecil, Pulau Labuan, Malaysia (Jun. 1998; M. Masuda and S-M. Phang)	MAL7	**AB671206**	**AB671214**
	MAL8	Pulau Sapangar, Kota Kinabalu, Sabah, Malaysia (7 Jun.; M. Masuda and S-M. Phang)	MAL8	**AB671207**	—
	KEP10	Sepa, Kepulauan Seribu, Indonesia (14 Sep. 2005; S.G.A. Draisma)	L0609512	**AB671203**	**AB671212**
	KEP11	Panjang, Kepulauan Seribu, Indonesia (14 Sep. 2005; S.G.A. Draisma)	L0609516	**AB671204**	—
	KEP12	Semak Daung, Kepulauan Seribu, Indonesia (17 Sep. 2005; S.G.A. Draisma)	L0609544	**AB671205**	**AB671213**
P. terricolor Ni-Ni-Win, M. Uchimura & H. Kawai	OKI8	Awase, Okinawa I., Okinawa Pref., Japan (19 Nov. 2006; S. Arai)	SAP106500	AB489944	AB489973
P. tetrastromatica Hauck	MAL2	Desaru, Johor, Malaysia (22 May 2005; P-E. Lim)	—	AB512554	AB512595
P. thivyae Doty & Newshouse	TAN1	Tanega I., Kagoshima Pref., Japan (2 Oct. 2005; S. Arai)	SAP105633	AB358931	AB358954
P. undulata Ni-Ni-Win, S. Arai & H. Kawai	OKI11	Awase, Okinawa I., Okinawa Pref., Japan (19 Nov. 2006; S. Arai)	SAP106493	AB489949	AB489976

Table 1. Continued

Species	Specimens code	Origin (collection date; collector)	Voucher no.[2]	DDBJ code for *rbc*L	DDBJ code for *cox*3
Stypopodium sp.	—	Awase, Okinawa I., Okinawa Pref., Japan (15 Dec. 2004; S. Arai)	—	AB358936	AB358955
Lobophora sp.	—	Awase, Okinawa I., Okinawa Oref., Japan (15 Dec. 2004; S. Arai)	65551 in KURCIS	AB548390	AB548398

[1] Bold letters indicate new sequences generated in the present study.
[2] IA, the herbarium of I. Abbott; BISH, the Herbarium of Bishop Museum; SAP, the Herbarium of the Graduate School of Science, Hokkaido University; L, Nationaal Herbarium Nederland, Universiteit Leiden branch.
[3] KURCIS serial numbers refer specimen numbers of the voucher specimens housed in the herbarium of the Kobe University Research Center for Inland Seas.

alignment consisted of 35 sequences (748 characters per sequence) representing 20 *Padina* species, including five new sequences and two out-group taxa (Table 1). Thirty-three sequences representing 20 *Padina* species and two out-group taxa were available for both *rbc*L and *cox*3 and were used for combined analyses (2067 characters per combined sequences). All sequences were unambiguously aligned, and no gaps were present. ML and BI analyses using these three alignments showed similar tree topologies, except for some clades that received low support. A ML tree of the combined analysis, together with branch support values from both ML and BI analyses, is shown in Fig. 1. The newly generated DNA sequences differed substantially from any previous published *rbc*L and *cox*3 sequences, but they were assigned to two strongly supported *Padina* clades in all molecular phylogenetic analyses. Because these specimens were morphologically (see below) and genetically distinct from each other and from any described species included in the analyses, they were considered new species: *Padina sulcata* sp. nov. and *Padina calcarea* sp. nov. The specimens of the *P. sulcata* clade, all collected from western Indonesia and Malaysia, showed very low sequence divergence, measured as uncorrected *p* distances, (0–0.23% in *rbc*L and 0–0.40% in *cox*3). *Padina sulcata* was consistently sister to *P. ryukyuana* Lee & Kamura, with sequence divergence of 1.1–1.36% in *rbc*L and 3.49–3.90% in *cox*3. The specimens of the *P. calcarea* clade, collected from eastern Indonesia and Palau, also showed very low sequence divergence (0–0.07% in *rbc*L and 0–0.94% in *cox*3); however, these specimens had no close relationship with any of the other *Padina* species included in this study.

Morphological observations

Padina sulcata Ni-Ni-Win, S.G.A. Draisma & H. Kawai sp. nov.

Figs 2–9

DESCRIPTION: Thalli bilayered at the involute margins and three-layered proximally; the inferior surface thickly covered with fibrous hairs at the base and up to 1–2 cm distally. Hairlines concentric, conspicuous, arranged in alternating sequence and equally spaced when viewed on both surfaces of the frond. Gametophytes dioecious; reproductive bodies located mainly on the inferior surface; mature oogonial and tetrasporangial sori indusiate, broad, occupying most of the fertile zone that bordered by sterile zones of equal width; antheridial sori nonindusiate, forming broken lines that located just above the hairlines.

HOLOTYPE: L 0609512 in L, male gametophyte from Pulau Sepa, Kepulauan Seribu, Java, Indonesia (S 5°34′32″, E 106°34′48″), collected by Stefano G. A. Draisma and Willem F. Prud'homme van Reine (14 September 2005).

ETYMOLOGY: From the Latin adjective 'sulcatus', meaning 'furrowed' or 'grooved' (Stearn 1983, p. 524), in reference to the furrows of the hairlines on the thallus surfaces.

SPECIMENS EXAMINED: **Malaysia**: Pulau Bakkungan, Sandakan (leg. S-M. Phang, 15 May 1998, PSM3367), Pulau Rusukan Kecil, Pulau Labuan (leg. M. Masuda and S-M. Phang, June 1998, NNW20, female gametophyte), Pulau Sapangar, Kota Kinabalu, Sabah (7 June 1998, NNW21, tetrasporophyte), Pulau Tiga (site 3), Kota Kinabalu, Sabah (7 June 1998, PSM 4311), Pantai Penarik, Terengganu (leg. S-M. Phang, 11 January 2009, PSM11071), Pantai Teluk Bidara, Terengganu (31 October 2009, PSM11063); **Indonesia**: Sepa, Kepulauan Seribu (leg. S.G.A. Draisma, 14 September 2005, L 0609512, female gametophyte), Panjang, Kepulauan Seribu (14 September 2005, L 0609516, two plants, both male gametophytes), Semak Daun, Kepulauan Seribu (17 September 2005, L 0609544, sterile).

HABITAT: Intertidal and subtidal to 2 m deep.

MORPHOLOGY: The thalli were erect, semicircular or circular to flabelliform, rigid in texture, with entire margins, up to 10 cm wide and 8 cm tall, shallowly split into several fan-shaped lobes but sometimes deeply incised, lightly to moderately calcified on the inferior surface while heavily calcified on the superior surface except at the hairlines, and attached by a stupose base with a short stipe (Figs 2, 3). Holdfast and basal 1–2 cm of the frond were thickly covered by long fibrous hairs confined to the inferior surface (Figs 2, 3). *Vaughaniella* stages were usually present. The thallus was composed of two layers at the involute margin and three layers at the other portions, 90–95 μm thick at the margin and 125–130 μm proximally (Figs 4, 5). Hairlines were concentric, conspicuous and forming broad, slightly depressed lines on the inferior surface (Fig. 6), but those on the superior surface formed narrow lines that often emerged from the thallus surface by rupturing the cuticle layer (Fig. 7). Hairlines were arranged in alternate sequence between the surfaces and equally spaced from each other, 2–3 mm apart on each surface (Fig. 8).

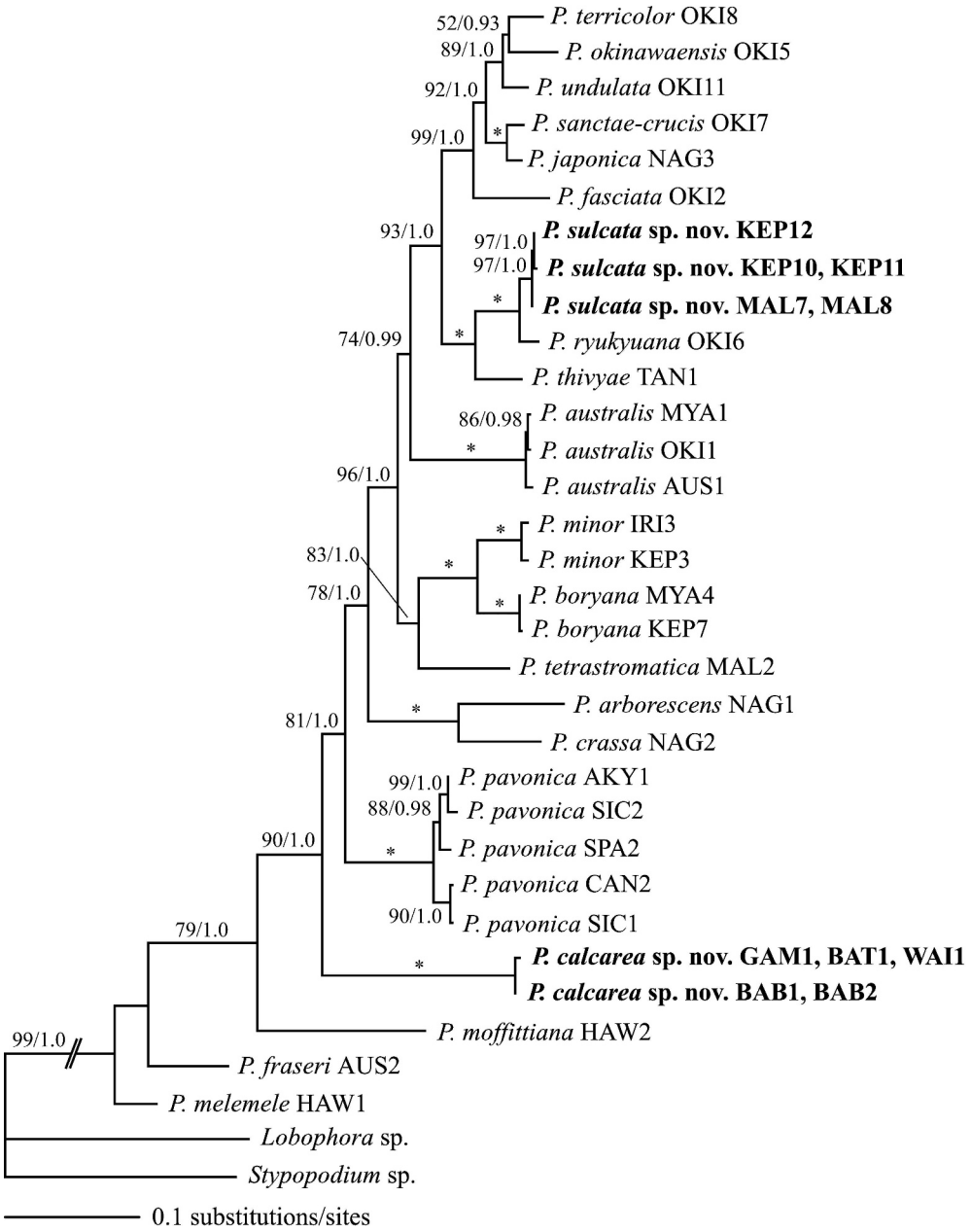


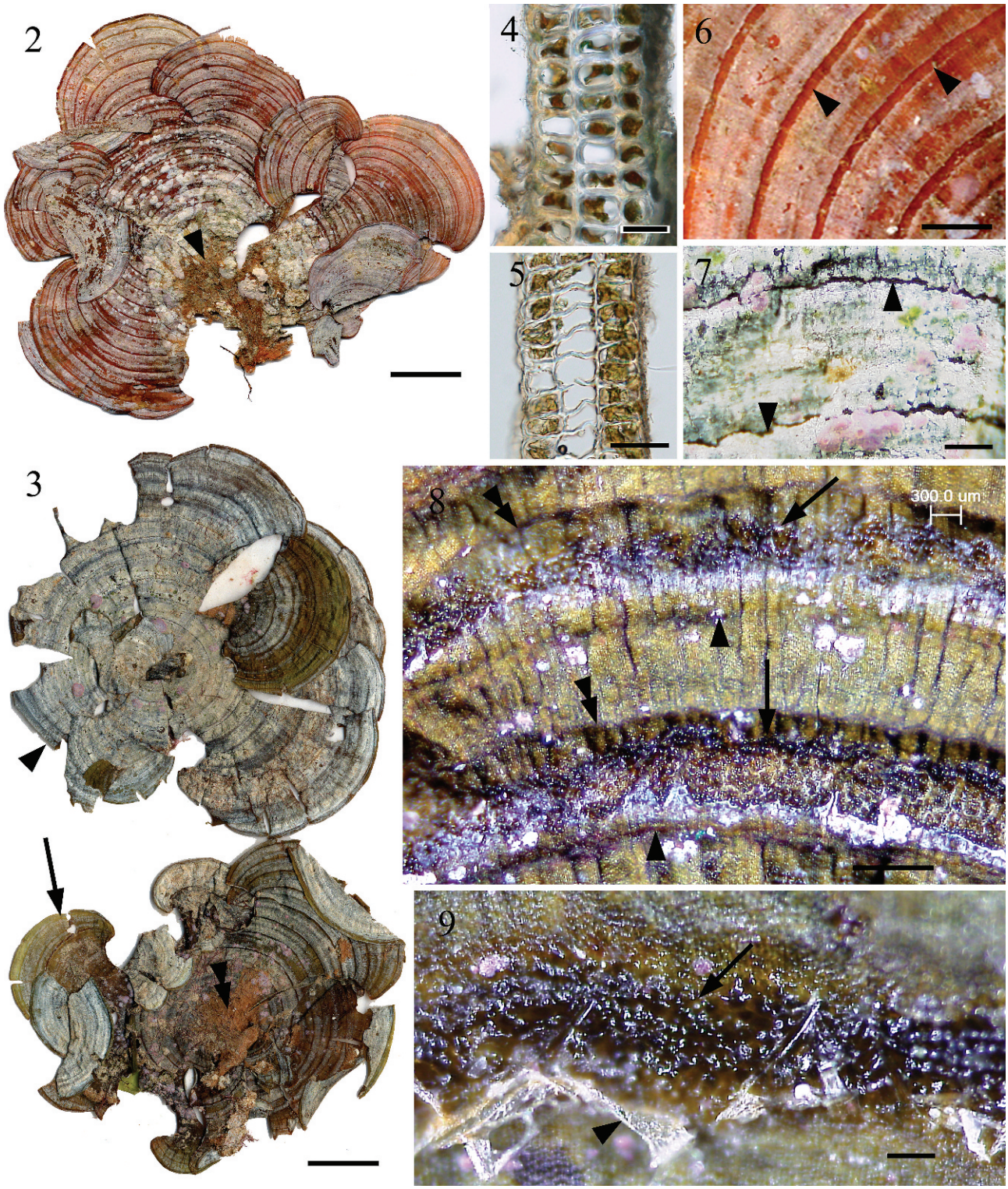
Fig. 1. Maximum likelihood (ML) tree based on the combined *rbc*L+*cox*3 gene sequences. Numbers at each node indicate bootstrap values (> 50%) for ML (left) and posterior probabilities for BI (right). Asterisks indicate 100% bootstrap values and 1.0 posterior probabilities.

Gametophytes were dioecious. Oogonia and tetrasporangia, in indusiate sori (Fig. 9), were rather broad (*c.* 1 mm), and they occupied almost the entire fertile zones; fertile zones were separated by sterile zones of equal width (i.e. fertile and sterile zones were of equal width and separated on both sides by hairlines when the two surfaces were viewed superimposed). Sori generally formed continuous lines that were distally adjacent to the hairlines (Figs 8, 9; i.e. sori located just above the hairlines), but sometimes sori formed small patches below the hairlines (Fig. 9). Sori were mainly found on the inferior surface, and they rarely occurred on the superior surface. Both oogonia and tetrasporangia were obovate, 60–65 μm wide, 82–85 μm long, and 85–90 μm wide, 120–125 μm long, respectively. Antheridial sori were nonindusiate; they usually formed discontinuous lines or patches just above the hairlines, but occasionally they occurred below the hairlines on the inferior surface.

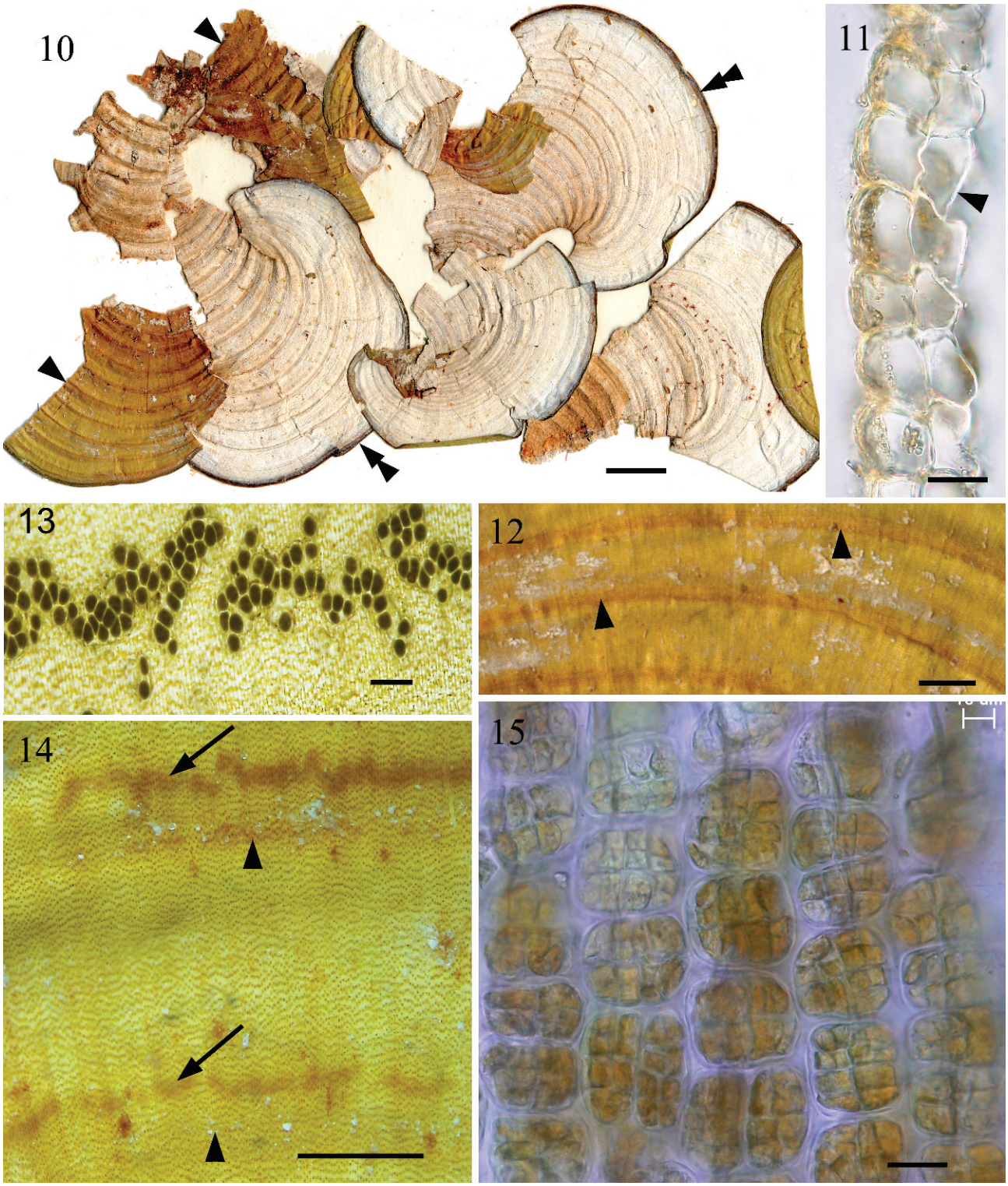
Padina calcarea sp. nov. Ni-Ni-Win, S.G.A. Draisma, W.F. Prud'homme van Reine & H. Kawai

Figs 10–15

DESCRIPTION: Thalli bright orange or bright yellow on the inferior surface, with heavy and thick calcification on the superior surface; blades bilayered throughout. Concentric hairlines inconspicuous, located only on the inferior surface. Gametophytes dioecious; reproductive sori forming



Figs 2–9. Morphology of *Padina sulcata* Ni-Ni-Win, S.G.A. Draisma & H. Kawai sp. nov.
Fig. 2. Habit of male gametophyte (holotype) from Indonesia with thick fibrous hairs at the base (arrowhead). Scale bar = 1 cm.
Fig. 3. Habit of female gametophytes (NNW20), showing the superior (arrowhead) and inferior (arrow) surfaces with thick fibrous hairs at the base (double arrow). Scale bar = 1 cm.
Fig. 4. Transverse section of basal portion of the thallus (NNW20). Scale bar = 50 μm.
Fig. 5. Transverse section of middle portion (NNW20). Scale bar = 50 μm.
Fig. 6. Surface view of the inferior surface with broad hairlines (arrowheads; holotype). Scale bar = 2 mm.
Fig. 7. Surface view of the superior surface with hairlines (arrowheads; holotype). Scale bar = 1 mm.
Fig. 8. Surface view of the inferior surface of tetrasporophyte (NNW21), showing the relationship of the alternating hairlines (arrowhead on the inferior surface; double arrowhead on the superior surface) and tetrasporangial sori (arrows). Scale bar = 1 mm.
Fig. 9. Detail of surface view of tetrasporangial sori (arrow) with indusium (arrowhead; NNW21). Scale bar = 300 μm.



Figs 10–15. Morphology of *Padina calcarea* Ni-Ni-Win, S.G.A. Draisma, W.F. Prud'homme van Reine & H. Kawai sp. nov. (Holotype).
Fig. 10. Habit of male gametophyte, showing the inferior (arrowheads) and superior (double arrowheads) surfaces with heavy calcification. Scale bar = 1 cm.
Fig. 11. Transverse section of middle portion of the thallus removing calcium layer on the superior surface (arrowhead). Scale bar = 25 μm.
Fig. 12. Surface view of the inferior surface of the thallus with hairlines (arrowheads). Scale bar = 2 mm.
Fig. 13. Surface view of tetrasporangial sori. Scale bar = 200 μm.
Fig. 14. Surface view of the inferior surface of the thallus, showing antheridial sori (arrows) and inconspicuous hairlines (arrowheads). Scale bar = 1 mm.
Fig. 15. Detail of surface view of antheridial sori. Scale bar = 20 μm.

broken lines or patches, distally close to the hairlines, found only on the inferior surface; oogonial and tetrasporangial sori indusiate; antheridial sori nonindusiate. The species resembles *P. melemele* in the color and thick calcification of thallus but can be distinguished by the somewhat thinner thallus, heavier calcification on the superior surface and the position and arrangement of the reproductive sori.

HOLOTYPE: SAP111112 in SAP, from Back Reef, north of Babeldaob I., Palau (N 07°57.271′, E 134°37.661′), collected by Eric Verheij and Willem F. Prud'homme van Reine (31 March 2009).

ETYMOLOGY: The species epithet is the Latin adjective for calcified and refers to the heavy calcification on the thallus surface.

SPECIMENS EXAMINED: **Indonesia**: Cape Besir, Besir Bay, West-Papua, Gam Is. (leg. S.G.A. Draisma, 27 November 2007, L SGAD 0712219), Gegenlol Bay, Batanta Is. (27 November 2007, L SGAD 0712254), Yenweres Bay, Wai Is. (a.k.a. Jerief Isl.; 5 December 2007, L SGAD 0712521), lagoon, Wai Is. (P. Jerief; 11 December 2007, L SGAD 0712684); **Palau**: Back Reef (N 07°57.271′, E 134°37.661′), north of Babeldaob I. [leg. E. Verheij and W.F. Prud'-homme van Reine, 31 March 2009, L0821280, female and male gametophytes (isotypes)].

HABITAT: Subtidal, from *c.* 1–26 m deep.

Morphology: The thalli were erect, semicircular or flabelliform, with entire margins, up to 8 cm wide and 5 cm tall, negligibly or lightly calcified on the inferior surface while thickly calcified on the superior surface, and attached by a stupose base with a short stipe (Fig. 10). The inferior frond surface was bright orange or bright yellow; whereas, the superior surface was strikingly white because of thick calcification (Fig. 10). The thallus was composed of two cell layers throughout, 45–50 μm thick marginally and 50–60 μm thick proximally (Fig. 11). Both cell layers were of equal height (Fig. 11). The calcium layer on the superior surface was almost 1.5 times thicker than the thallus, measuring about 60–75 μm thick across the whole of the thallus. Concentric hairlines were inconspicuous, spaced 2.5–3.5 mm apart and present only on the inferior surface (Fig. 12).

Gametophytes were dioecious; oogonial and tetrasporangial sori forming in broken lines were distally close to the hairlines and located only on the inferior surface (Fig. 13). They were both surrounded/covered by a transparent indusium. Both oogonia and tetrasporangia were more or less obovate, 55–70 μm wide and 75–90 μm long and 65–75 μm wide and 90–100 μm long, respectively. Antheridial sori were nonindusiate, found only on the inferior surface and forming in patches or broken lines that were distally adjacent to the hairlines (Figs 14, 15).

Thalli of this species were very similar to those of *P. melemele*, as both species have blades that were bright yellow/orange inferiorly and white with heavy calcification superiorly, as well as producing inconspicuous hairlines that were restricted to the inferior surface (Fig. 10). The two species were distinguished by blade thicknesses (45–60 μm in *P. calcarea* vs. 50–80 μm in *P. melemele*), degree of calcification on the superior surface (60–75 μm thick in *P. calcarea* vs 25–30 μm in *P. melemele*), the position of reproductive sori (on the inferior thallus surface in *P. calcarea* v. the superior surface in *P. melemele*) and the conjunction of sori with hairlines (distally close to the hairlines in *P. calcarea* v. in the middle between hairlines of the inferior surface in *P. melemele*; Abbott 1996; Abbott & Huisman 2004; Ni-Ni-Win *et al.* 2008; this study).

DISCUSSION

Molecular phylogenetic analyses using chloroplast-encoded *rbc*L and mitochondrial *cox*3 sequences have provided strong evidence for the occurrence of two undescribed species, *P. sulcata* sp. nov. and *P. calcarea* sp. nov, in Malaysia, Indonesia and Palau. Molecular phylogenetic analyses have shown consistently that *P. sulcata* is sister to *P. ryukyuana*, which was originally reported from subtropical Japan. The two species shared a rigid thallus texture and the structure of conspicuous alternating hairlines between both surfaces (the hairs arrange in broad depressed lines on the inferior surface but narrow lines that often emerge by rupturing the cuticle layer on the superior surface). However, they differed in the number of cell layers composing the thallus; both species have three-layered thalli with a two-layered involute margins, but *P. ryukyuana* was sometimes four-layered thick at the base; whereas, *P. sulcata* never had a four-layered base. They also differed by the presence (*P. ryukyuana*) or absence (*P. sulcata*) of small groups of rhizoid-like hairs on the inferior thallus surface and the arrangement of alternating hair lines between both surfaces (equally spaced in *P. sulcata* v. unequally spaced in *P. ryukyuana*). Finally, as for the structure and arrangement of the reproductive sori, *P. sulcata* had broad sori located just above the hairlines of the inferior surface, which were occupying most of the fertile zone when both surfaces are viewed together; whereas, *P. ryukyuana* had narrow sori arranged distally far from the hairlines or in the middle of fertile zones (Lee & Kamura 1991; this study). *Padina sulcata* can be distinguished from the previously reported three-layered species, as well as from the other members of the genus, by the structure and arrangement/position of mature reproductive sori, which are rather broad, distally very close to the hairlines of the inferior surface (i.e. sori located just above the hairlines of the inferior surface) and occupying nearly the whole fertile zone. In *P. sulcata*, fertile and sterile zones are of equal breadth and separated by the hairlines when the two surfaces are viewed superimposed (i.e. hairline of the superior surface is offset by half the distance between adjacent hairlines on the inferior surface and vice versa).

To date, only *P. boergesenii* Allender & Kraft (with the exception of some portions where some of the second transverse divisions fail to take place), *P. fraseri* (Greville) Greville and *P. tristromatica* Hauck have been reported to be three cell layers thick throughout the entire thallus, except for the involute margin, which is two layers thick in all *Padina* species apart from some multilayered species, such as *P. arborescens* and *P. crassa* Yamada (Greville

1830; Levring 1942; Allender & Kraft 1983; Womersley 1987; Lee & Kamura 1991). Among them, *P. fraseri*, collected from northern New South Wales, Australia, whose morphology agrees well with the description reported by Womersley (1987), was included in the molecular analyses and was distantly related to *P. sulcata*. *Padina sulcata* also differed from *P. fraseri* in other morphological characters, particularly in the relative thicknesses of the cell layers (cells of the central layer were taller than those of the surface layers in *P. fraseri*; whereas, all three cell layers were of almost the same height in *P. sulcata*), the position of the reproductive sori (on the superior surface in *P. fraseri* v. on the inferior surface in *P. sulcata*) and the arrangement of oogonial and tetrasporangial sori between hairlines (in rows of one to three in *P. fraseri* v. a single row in *P. sulcata*; Womersley 1987; this study). Although *P. boergesenii* and *P. tristromatica* were not included in the present molecular analyses, they were morphologically different from *P. sulcata* in certain characters, namely, thickness of cell layers (the inferior surface cell layer was thicker than the superior surface cell layer, and the central cell layer was slightly shorter than the surface cell layers in *P. boergesenii* and *P. tristromatica*) and the absence of an indusium (Allender & Kraft 1987 for *P. boergesenii* and Levring 1942 and Meneses & Hoffmann 1994 for *P. tristromatica*).

In our molecular analyses, *P. calcarea* is resolved as an early divergence and not closely related to any of the *Padina* species in the present study. As mentioned previously, *P. calcarea* is morphologically most similar to *P. melemele,* particularly in the bright orange to yellow color of the inferior surface of the thallus and heavy calcification of the superior surface, but molecular analyses did not reflect their morphological similarity, positioning them in distantly related clades. Their anatomical differences seem relatively minor and might well have been taken for phenotypic variations in a single species were it not for the molecular data. Such features as the position and arrangement of reproductive sori, differences in blade thickness (although more samples are need to be measured) and degree of superior surface calcification show measurable discontinuities, however, and thus must serve to differentiate the two species when DNA sequences are unavailable.

Based on recent studies (Ni-Ni-Win *et al.* 2008, 2010, 2011a, 2011b), several morphological attributes of *Padina* species retain or gain renewed emphasis in the taxonomy of the genus. The number of cell layers composing the thallus and the position of hairlines on one surface or both surfaces of the thallus have been regarded as taxonomically important features, both singly or in combination. Thus, among the bilayered species, only *P. boryana* Thivy (although its thallus is occasionally three layers thick at the base), *P. melemele* and the new *P. calcarea* have hairlines on only one surface (inferior surface) of the thallus (Taylor 1960; Abbott 1996; Abbott & Huisman 2004; Ni-Ni-Win *et al.* 2008; this study), all the rest having hairlines on both surfaces (Abbott & Huisman 2003; this study). *Padina boryana* is clearly distinct from *P. calcarea* in both *rbc*L and *cox*3 and can be easily distinguished by its drab coloration, light calcification, presence of *Vaughaniella* stages, occasional three layers at the base of the thallus in contrast to usual two-layered thallus in *P. calcarea* and absence of an indusium as opposed to the presence in *P. calcarea* (Taylor 1960; this study).

The finding of two new *Padina* species in the central Indo-Pacific complements recent studies (Ni-Ni-Win *et al.* 2008, 2010, 2011a) that have also demonstrated greater diversity in even well-studied regions than had been suspected. As the warm-water areas of the world are among some of the least well known phycologically, we anticipate that an even higher diversity of *Padina* species remains to be uncovered.

Distribution of two new species is confined to the central Indo-Pacific: *Padina sulcata* was collected from Indonesia and Malaysia, whereas *P. calcarea* was found in Indonesia and Palau. However, this geographical distribution may likely to be a result of limited sampling. Greater sample collections from the rest of Indian and Pacific Oceans as well as the Atlantic Ocean may probably extend the distribution of these two new species.

The molecular phylogeny of the present study did not show any clear geographic structure as was found in the previous studies of Ni-Ni-Win *et al.* (2010, 2011a, 2011b; Fig. 1). The Indo-Pacific species were distributed among the clades of the phylogenetic tree. This result may be caused by limited sampling in the South Pacific and Indian Oceans, apart from the Andaman Sea and Southeast Asia regions. Meanwhile, the Mediterranean specimens formed a monotypic clade, *P. pavonica*, which was nested within the Indo-Pacific clades. However, more worldwide samples, and especially from the Mediterranean Sea, should be included in phylogenetic analyses before any geographical conclusions are made.

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REFERENCES

Abbott I.A. 1996. New species and notes on marine algae from Hawai'i. *Pacific Science* 50: 141–156.

Abbott I.A. & Huisman J.M. 2003. New species, observations, and a list of new records of brown algae from the Hawaiian Islands. *Phycological Research* 51: 173–185.

ABBOTT I.A. & HUISMAN J.M. 2004. *Marine green and brown algae of the Hawaiian Islands*. Bishop Museum Press, Honolulu. 259 pp.

AKAIKE H. 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* 19: 716–723.

ALLENDER B.M. & KRAFT G.T. 1983. The marine algae of Lord Howe Island (New South Wales): the Dictyotales and Cutleriales (Phaeophyta). *Brunonia* 6: 73–130.

BØRGESEN F. 1951. Some marine algae from Mauritius. Additions to parts previously published. III. *Kongelige Danske Videnskabernes Selskab, Biologiske Meddelelser* 18: 1–44.

CRIBB A.B. 1951. Invalidation of the genus *Vaughaniella*. *Nature* 168: 302.

DE CLERCK O. & COPPEJANS E. 1997. Notes on the *Dictyota vieillardii* and *D. adnata* (Dictyotaceae, Phaeophyta). *Taxon* 46: 33–36.

FELSENSTEIN J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

GREVILLE R.K. 1830. *Algae britannicae, or descriptions of the marine and other inarticulated plants of the British islands, belonging to the order Algae*; with plates illustrative of the genera. McLachlan & Stewart; Baldwin & Cradock, Edinburgh. 218 pp.

GUIRY M.D. & GUIRY G.M. 2011. *Algaebase*. Worldwide electronic publication. National University of Ireland, Galway. Available at: http://www.algaebase.org (15 February 2011).

JOBB G., VON HAESELER A. & STRIMMER K. 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evolutionary Biology* 4: 18.

KRAFT G.T., SAUNDERS G.W., ABBOTT I.A. & HAROUN R.J. 2004. A uniquely calcified brown alga from Hawaii: *Newhousia imbricata* gen. et sp. nov. (Dictyotales, Phaeophyceae). *Journal of Phycology* 40: 383–394.

KRAFT G.T. 2009. *Algae of Australia. Marine benthic algae of Lord Howe Island and the southern Great Barrier Reef, 2. Brown algae*. Australian Biological Resources Study and CSIRO Publishing, Canberra. 364 pp.

LEE Y.P. & KAMURA S. 1991. *Padina ryukyuana* Lee et Kamura, a new marine brown alga from southern Japan. *Korean Journal of Phycology* 6: 91–96.

LEVRING T. 1942. Einige Meeresalgen von den Inseln San Ambrosio und San Felix. *Botaniska Notiser* 1942: 60–62.

MENESES I. & HOFFMANN A.J. 1994. Contribution to the marine algal flora of San Felix Island, Desventuradas Archipelago, Chile. *Pacific Science* 48: 464–474.

NI-NI-WIN, HANYUDA T., ARAI S., UCHIMURA M., ABBOTT I.A. & KAWAI H. 2008. Three new records of *Padina* in Japan based on morphological and molecular markers. *Phycological Research* 56: 288–300.

NI-NI-WIN, HANYUDA T., ARAI S., UCHIMURA M., PRATHEP A., DRAISMA S.G.A., SOE-HTUN U. & KAWAI H. 2010. Four new species of *Padina* (Dictyotales, Phaeophyceae) from the western Pacific Ocean, and reinstatement of *Padina japonica*. *Phycologia* 49: 136–153.

NI-NI-WIN, HANYUDA T., ARAI S., UCHIMURA M., PRATHEP A., DRAISMA S.G.A., PHANG S-M., ABOTT I.A., MILLAR A.J.K. & KAWAI H. 2011a. A taxonomic study of the genus *Padina* (dictyotales, phaeophyceae) including the description of four new species from Japan, Hawaii and the Andaman sea. *Journal of Phycology* 47: 1193–1209.

NI-NI-WIN, HANYUDA T., DRAISMA S.G.A., FURNARI G., MEINESZ A. & KAWAI H. 2011b. *Padina ditristromatica* sp. nov. and *Padina pavonicoides* sp. nov. (Dictyotales, Phaeophyceae), two new species from the Mediterranean Sea based on morphological and molecular markers. *European Journal of Phycology* 46: 327–341.

RAMBAUT A. & DRUMMOND A.J. 2009. *Tracer*. Available at: http://beast.bio.ed.ac.uk/tracer.

RONQUIST F. & HUELSENBECK J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

SCHWARZ G. 1978. Estimating the dimension of a model. *Annals of Statistics* 6: 461–464.

SILVA P.C., BASSON P.W. & MOE R.L. 1996. Catalogue of the benthic marine algae of the Indian Ocean. *University of California Publications in Botany* 79: 1–1259.

STEARN W.T. 1983. *Botanical Latin*. David & Charles, Newton Abbot. 566 pp.

TANABE A.S. 2007. *KAKUSAN*: a computer program to automate the selection of a nucleotide substitution model and the configuration of a mixed model on multilocus data. *Molecular Ecology Notes* 7: 962–964.

TANABE A.S. 2009. *Phylogears Version 1.5.2009.12.29*. Software distributed by the author. Available at: http://www.fifthdimension.jp.

TAYLOR W.R. 1960. *Marine algae of the eastern tropical and subtropical coasts of the Americas*. University of Michigan Press, Ann Arbor. 870 pp.

THIERS B. 2011. *Index Herbariorum: A global directory of public herbaria and associated staff*. New York Botanical Garden's Virtual Herbarium [continuously updated]. Available at: http://sweetgum.nybg.org/ih.

THOMPSON J.D., GIBSON T.J., PLEWNIAK F., JEANMOUGIN F. & HIGGINS D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876–4882.

TRONO G.C. JR. 1969. The marine benthic algae of the Caroline Islands, II. Phaeophyta and Rhodophyta. *Micronesica* 5: 25–119.

VOS R.A. 2003. Accelerated likelihood surface exploration: the likelihood ratchet. *Systematic Biology* 52: 368–373.

WOMERSLEY H.B.S. 1987. *The marine benthic flora of southern Australia. Part II*. South Australian Government Printing Division, Adelaide. 484 pp.

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