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A molecular-assisted floristic survey of crustose brown algae (Phaeophyceae) from Malaysia and Lombok Island, Indonesia based on *rbc*L and partial *cox*1 genes

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Abstract Studies on the crustose brown algae are relatively few despite a long history of studies conducted since the 1800s, with temperate species forming the bulk of these studies. There is a need for more focus on crustose brown algae particularly in the tropics as they are generally different from those in the temperate regions. Taxonomic confusion arising from morphological simplicity largely dependent on the reproductive structures and overlap in morpho-anatomical features among species necessitates the use of molecular techniques. This study is dedicated to a better understanding of the diversity of these understudied algae in the Indo–Malay region. Specimens collected from Peninsular Malaysia, Sabah (Borneo) and Lombok Island in Indonesia were identified using molecular markers from the plastid rubisco large subunit (*rbc*L) and mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) genes in tandem with morphology and anatomy. Three *Mesospora* spp., two putative *Diplura* spp. and the cosmopolitan *Neoralfsia expansa* were identified in this study, including a new record of *Mesospora negrosensis* for Malaysia. Despite their morpho-anatomical similarities, *Mesospora* and *Diplura* occur in widely divergent clades within the brown algae, the former in the Mesosporaceae in the Ralfsiales, the latter in an unclassified clade sister to the Ishigeales. All six species occurred both in Malaysia and Lombok Island except for *M. elongata* and *M. negrosensis*, respectively. The *rbc*L marker performed better in the elucidation of phylogeny among the brown algal orders, whereas *cox*1-5′ is more suited as a barcoding marker for species level identification.

Keywords *Diplura* · Diversity · Indo–Malay region · *Mesospora* · *Mesospora negrosensis* · *Neoralfsia expansa* · New record · Ralfsiales

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Introduction

Weber-van Bosse (1911, 1913) initiated studies on crustose brown algal taxa in the Indo–Malay region using materials collected during the Siboga Expedition. Five taxa were identified in her studies and were placed in two families: *Neoralfsia expansa* (J. Agardh) Lim et Kawai ex Cormaci et G. Furnari (as *Ralfsia expansa* J. Agardh), *Mesospora schmidtii* Weber-van Bosse, *Stragularia clavata* (Harvey) G. Hamel (as *Stragularia clavata* (Carmichael) Kjellman) and *S. polycarpa* Weber-van Bosse in the Ralfsiaceae while a putative species of *Lithoderma* was placed in the Lithodermataceae. In Malaysia, the crustose brown algae were first documented by Phang et al. (2007) in which only the genus *Ralfsia* was recorded and no details on the description or distribution of the species was given. Subsequently *N. expansa*, *M. schmidtii* and *Mesospora* sp. C were reported in two publications on Ralfsiales in Malaysia (Lim et al. 2007, 2008).

The Indo–Malay archipelago, located between the Indian and Pacific Ocean, is well known as a marine biodiversity hotspot (Hoeksema 2007). Yet, there are relatively few reports of crustose brown algal taxa from this enclave. The

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geographical coverage of the present study, which includes Peninsular Malaysia, Sabah (Borneo) and Lombok Island (Indonesia), is chosen for its location, the history of taxonomic work on crustose brown algal taxa and the manageable number of taxa. Lombok Island, which lies south to the equator (latitude 08° S), is also considered as a study site for a rough estimate of southern hemisphere crustose brown algal diversity. From the time of Weber-van Bosse's work until recently, only one new addition (i.e., *M. elongata* Poong, Lim et Phang; Poong et al. 2013) was made to the Indo–Malay crustose brown algal flora, clearly highlighting a need for this study.

A major challenge in the taxonomy of crustose brown algae is the difficulty in identifying species based solely on morphological and anatomical features. Like the red algae, their classification and taxonomy has largely relied on presence of reproductive structures. An instance of their hazardous identification is seen when Kain et al. (2010) misidentified the crustose form of *Colpomenia bullosa* (Saunders) Yamada and an unidentified species of *Ralfsia* as *Ralfsia verrucosa* (Areschoug) J. Agardh, and the misidentification was only realised upon conducting molecular analyses. Taxonomic and systematic studies on crustose brown algae began in the 1800s (e.g., Agardh 1847, p. 7), but the majority were based on conventional morphology description without the support of molecular data. Gene sequence data is currently used in combination with existing morphology observation to improve classification at higher taxonomic levels, estimates of species diversity, species delineation and knowledge of evolutionary relationships (Kawai et al. 2005; Ni-Ni-Win et al. 2011; Silberfeld et al. 2011; Tan et al. 2013). The current trend for floristic surveys, especially those involving taxa with simple or convergent morphologies, employed molecular techniques for more accurate identification (e.g., Cianciola et al. 2010; Kucera and Saunders 2012).

Our study aims to identify and document species of crustose brown algae in the Indo–Malay region by combining molecular data (using *rbc*L and partial *cox*1 sequences) and morphological observations, thus contributing to an improved understanding of the taxonomy, diversity and distribution of the tropical brown crusts from this region. Phylogenetic analyses of combined *rbc*L and *cox*1-5′ data were also conducted to infer the relationship among the identified crustose brown algal taxa.

Materials and methods

Collections of crustose brown algae were made from May 2009 to July 2012. Specimens from Peninsular Malaysia, Sabah (Borneo) and Lombok Island, Indonesia (Fig. 1) were collected in the field and air-dried prior to desiccation in silica gel. Voucher specimens were deposited in University of Malaya Seaweeds and Seagrasses Herbarium (KLU) while the culture strain of *Mesospora negrosensis* West et Calumpong was obtained from the Kobe University Macroalgal Culture Collection (KU-MACC) and used for DNA extraction. Eighty-seven crustose brown algal specimens were examined morphologically and sequenced using chloroplast *rbc*L and mitochondrial *cox*1-5′ molecular markers.

For anatomical studies, squash preparations of the brown crusts mounted on glass slides in corn syrup were observed under light microscope. Photomicrographs were taken using a DP72 digital camera attached to a BX51 microscope (Olympus, Japan).

Total DNA extractions were performed on ground tissue samples using the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology Inc., South Korea) following the manufacturer's instructions. Parameters for polymerase chain reaction (PCR) amplification and sequencing followed Poong et al. (2013). Primers used for *rbc*L amplification included: rbcFO, rbcF4 and rbcR2 (Kawai and Sasaki 2004); PRBF2, PRBF3, PRBR2, PRBR3 and RSPR (Kogame et al. 1999); NDrbcL2 and NDrbcL9 (Daugbjerg and Andersen 1997); RalR952 (Lim et al. 2007). RspBF2 (5′-TACGGTCGTGTTG TTTATGA-3′) and RspBR2 (5′-AGTCGCACCTGATTGAAT AC-3′) were newly designed for this study. Primers used for *cox*1-5′ amplification were 117F and 784R (Bittner et al. 2008); GazF2 and GazR2 (Lane et al. 2007) and L and H (Folmer et al. 1994). Amplification and sequencing of the *cox*1-5′ region was also conducted for some of the crustose brown algal taxa published in the study by Lim et al. (2007). PCR products were purified using LaboPass Gel & PCR purification kit (Cosmo Genetech, South Korea) while sequencing was undertaken by First Base Laboratories (Malaysia) with the same primers used for PCR amplification.

For molecular phylogenetic analyses, raw sequences were first assembled and edited via ChromasPro ver. 1.42 (Technelysium Pty. Ltd.), subsequently aligned using ClustalX v. 2.0.8 (Larkin et al. 2007) and then manually adjusted with Bioedit v. 7.0.9.0 (Hall 1999). Maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses were performed for each gene separately, and for the two genes combined. Combined analyses included 65 brown algal taxa plus three outgroup taxa (*Schizocladia ischiensis*, *Phaeothamnion confervicola* and *Tribonema aequale*) or only the 51 taxa (including *T. aequale* as outgroup) for which the sequences of both genes were determined to explore the influence of missing data. Crustose brown algal taxa used for molecular phylogenetic analyses of which sequences were newly generated for this study is listed in Table 1. Accession numbers of previously published taxa (both crustose brown and non-crustose brown) are given next to the species name in the combined ML phylogenetic tree (Fig. 2). Analyses of *rbc*L alignment alone included 68 taxa (using similar outgroups as the combined data set) and of *cox*1-5′ alignment alone, 50 taxa (including *Ishige okamurae* as outgroup). *I. okamurae* was used as outgroup in the *cox*1-5′ only analyses to improve resolution

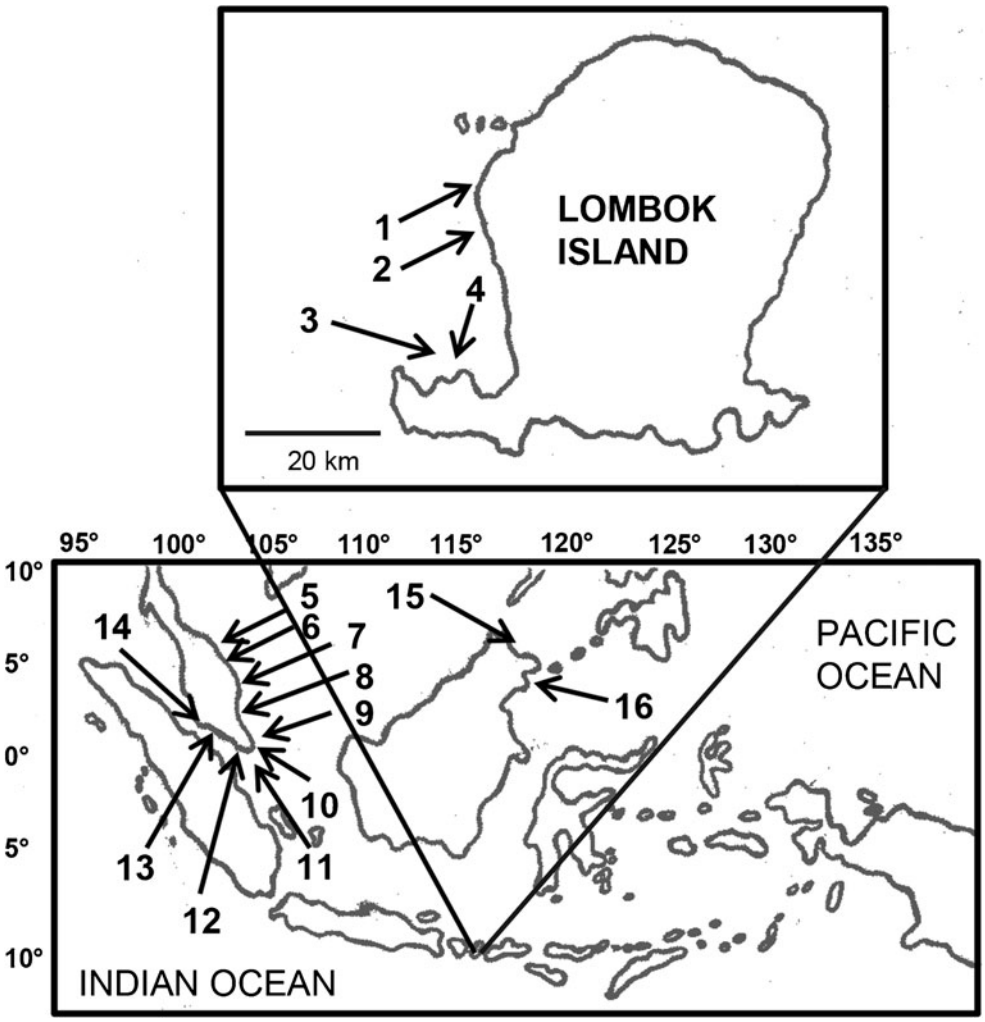

Fig. 1 Map indicating the collection sites of the specimens used in the present study (adapted from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm). *1* Nipah; *2* Batulayar; *3* Gili Genting; *4* Batukijok; *5* Pantai Chendering; *6* Pantai Kemasik; *7* Telok Kalong; *8* Teluk Sari; *9* Teluk Ramunia; *10* Pulau Che Kamat; *11* Pelabuhan Tanjung Langsat; *12* Pulau Merambong; *13* Pulau Besar; *14* Port Dickson; *15* Kampong Dandulit; *16* Semporna

within the brown algal taxa. The resulting phylogenies were screened for significant topological incongruency (conflicting relationships with supported nodes) to assess the feasibility of combining sequences from the two genes. The separate trees did not show any supported conflicting nodes, thus the focus was placed on the combined data set.

MP trees were constructed using PAUP 4.0b10 (Swofford 2002) under a heuristic search with 100 random sequence addition replicates and a tree bisection reconnection (TBR) branch-swapping algorithm with gaps treated as missing data. Bootstrap percentage (BP) was computed under a heuristic search method and TBR swapping with 1,000 replications and one random taxon additions to assess branch support.

Kakusan v.3 (Tanabe 2007) was used to determine the best-fit nucleotide substitution models for ML and BI analyses selected using the corrected Akaike information criterion (Akaike 1973) and the Bayesian information criterion (Schwarz 1978), respectively. ML trees were inferred using Treefinder v. October 2008 (Jobb et al. 2004) with BP generated from 1,000 resamplings to estimate robustness. BI analyses were conducted using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003), and the program was set to run four chains of Markov chain Monte Carlo iterations for 2,000,000 generations, keeping one tree for every 100 generations. The first 2,000 trees sampled were discarded as "burn-in" to ensure stabilization, based on the stationarity of log likelihood values in the first 100,000 generations as assessed using Tracer v.1.5 (http://tree.bio.ed.ac.uk/software/tracer/). The remaining trees were used to compute a consensus topology and calculate the posterior probability (PP) values. For the purpose of comparison with bootstrapping, nodes with Bayesian PP >0.95 (the node appears in greater than 95% of sampled trees) are implied as being strongly supported, between 0.90 and 0.95 as moderately supported, and <0.90 as weakly supported. ML BP and MP BP are described as strong (≥85%), moderate (70–85%) and weak (<70%). To assess the levels of intra- and interspecific variations in the *rbc*L and *cox*1-5′ sequences, uncorrected (*p*)

Table 1 List of crustose brown algal specimens used in the molecular phylogenetic analyses of which sequences were newly generated for this study (accession numbers in bold)

Taxa	Collection site, date of collection, voucher number or reference of *rbc*L/*cox*1-5′ sequences	Genbank accession number *rbc*L/*cox*1-5′
Diplura simplex Tanaka et Chihara	Lim et al. 2007/this study	AB250084/**KC847385**
Diplura sp. B	Lim et al. 2007/this study	AB250086/**KC847386**
Diplura sp. F	Pantai Dickson, Malaysia; 16 Dec. 2009; PSM12208	**KC847395/KC847374**
Diplura sp. F	Gili Genting, Lombok Island, Indonesia; 10 June 2010; PSM12222	**KC847396/KC847375**
Diplura sp. F	Pantai Chendering, Terengganu, Malaysia; 16 Feb. 2012; PSM12325	**KC847397/KC847376**
Diplura sp. F	Semporna, Sabah, Malaysia; 5 July 2012; PSM12359	**KC847398/KC847377**
Diplura sp. G	Pulau Che Kamat, Johor, Malaysia; 29 May 2009; PSM12172	**KC847399/KC847378**
Diplura sp. G	Gili Genting, Lombok Island, Indonesia; 10 June 2010; PSM12224	**KC847400/KC847379**
Diplura sp. G	Batulayar, Lombok Island, Indonesia; 8 June 2010; PSM12215	**KC847401/KC847380**
Diplura sp. G	Pantai Dickson, Malaysia; 30 July 2012 ; PSM12371	**KC847402/KC847381**
Mesospora schmidtii Weber-van Bosse	Telok Kalong, Terengganu, Malaysia; 17 Feb. 2012; PSM12317	**KC847387/KC847366**
Mesospora schmidtii	Semporna, Sabah, Malaysia; 1 July 2012; PSM12353	**KC847388/KC847367**
Mesospora sp. C	Lim et al. 2007/This study	AB250065/**KC847382**
Mesospora negrosensis West et Calumpong	KU1065	**KC847389/KC847368**
Mesospora negrosensis	Pulau Merambong, Johor, Malaysia; 24 August 2009; PSM12183	**KC847390/KC847369**
Mesospora negrosensis	Pantai Chendering, Terengganu, Malaysia; 16 Feb. 2012; PSM12326	**KC847391/KC847370**
Neoralfsia expansa (J. Agardh) Lim et Kawai ex Cormaci et G. Furnari	Lim et al. 2007/This study	AB250077/**KC847383**
Neoralfsia expansa	Lim et al. 2007/This study	AB250078/**KC847384**
Neoralfsia expansa	Pulau Besar, Melaka, Malaysia; 11 April 2010; PSM12254	**KC847392/KC847371**
Neoralfsia expansa	Gili Genting, Lombok Island, Indonesia, 10 June 2010; PSM12230	**KC847393/KC847372**
Neoralfsia expansa	Pantai Kemasik, Terengganu, Malaysia; 17 Feb. 2012; PSM12322	**KC847394/KC847373**

PSM and KU indicate reference code of vouchers at the University of Malaya Seaweeds and Seagrasses Herbarium (KLU) and of culture obtained from Kobe University Macroalgal Culture Collection (KU-MACC), respectively

pairwise genetic distances were estimated using PAUP 4.0b10 (Swofford 2002).

Results

Molecular phylogenetic analyses

The length of the *rbc*L, *cox*1-5′ and combined alignments were 1,349, 665 and 2,014 nucleotides (nt), respectively. The *rbc*L gene was successfully sequenced for all 87 specimens (100% success).The *rbc*L intraspecific genetic variation ranged from 0–0.075% in *M. elongata* to 0–4.67% in *N. expansa*, while interspecific variation ranged from 2.99% between *D. simplex* and *Diplura* sp. C to 17.96–18.41% between *Diplura* sp. G and *Diplura* sp. B. Amplification of *cox*1-5′ gene was successful for all but four specimens (95% success). The *cox*1-5′ intraspecific divergences ranged from 0–0.149% in *M. elongata* to 0–15.35% in *M. schmidtii* while interspecific divergences ranged from 0.149% between *D. simplex* and *Diplura* sp. B to 18.48–22.45% between *M. negrosensis* and *M. schmidtii*. All three methods of phylogenetic inference (ML, MP, BI) resulted in near-identical trees for all well-supported nodes for the four data sets (genes combined or separate, and all taxa included or a subset). Nonetheless, the concatenated data gave better resolution and clade support than each individual gene. Phylogenetic signal was virtually congruent between the more variable *cox*1-5′ gene and the conserved *rbc*L gene and mainly carried by chloroplastic information. Results indicated that missing *cox*1-5′ sequences in the combined data set did not affect the overall phylogeny with variations only in the position of certain clades with low or no support; thus, the ML tree inferred from the combined data set with all taxa included is depicted with support (Fig. 2).

The combined data set included 2,014 characters, of which 1,201 (60%) were variable sites and 1,019 (51%) were parsimony-informative sites. MP analysis resulted in four equally most parsimonious trees, and tree length was 8,178, consistency index (CI) was 0.2539 and retention index (RI) was 0.5444. The ML and BI trees were constructed using a GTR + gamma and SYM + gamma models, respectively. The topologies obtained in all three analyses (ML, MP and BI) were reasonably congruent at the interordinal level, although there was little resolution of

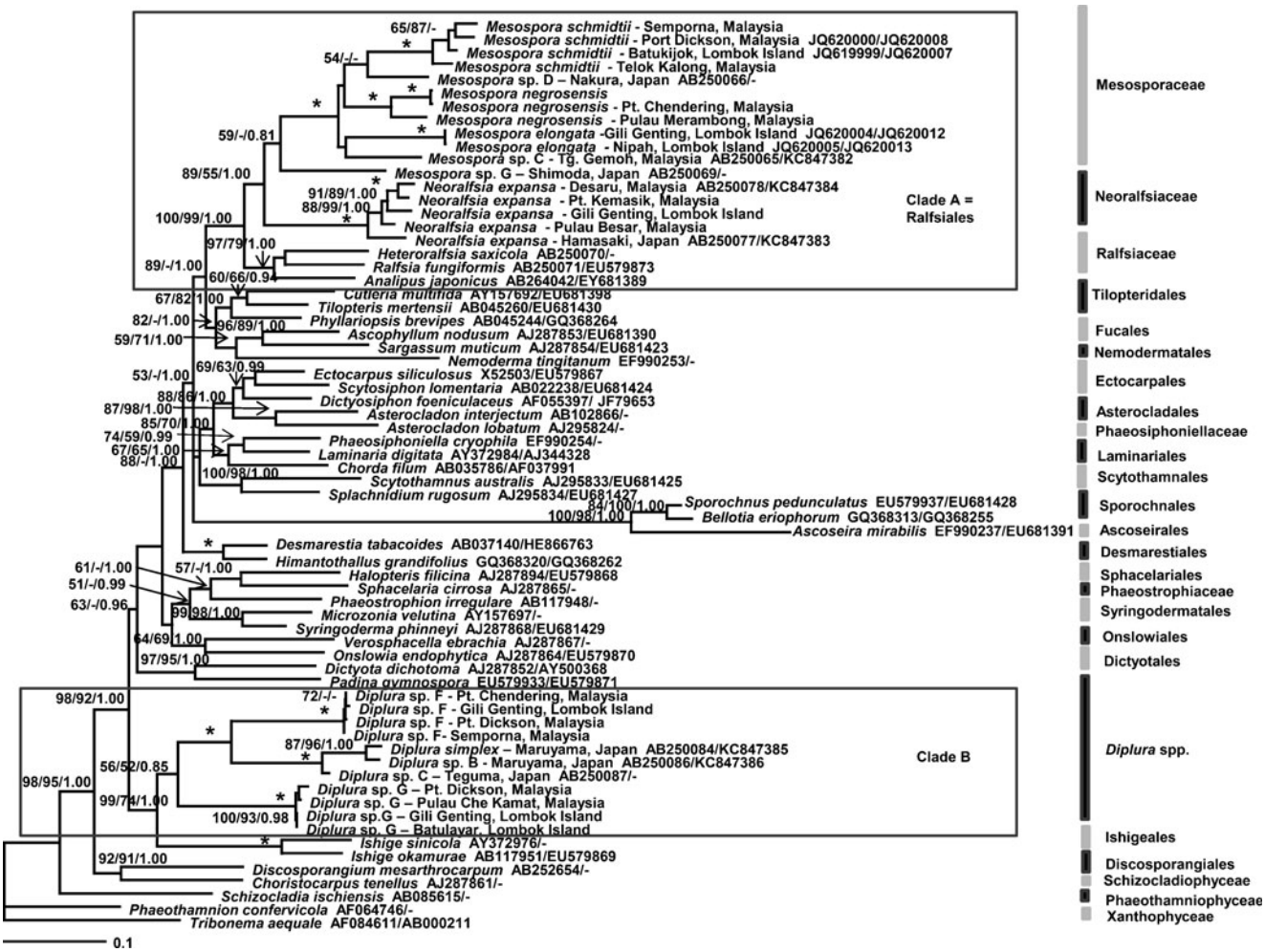

Fig. 2 ML phylogeny inferred based on the combined *rbc*L and partial *cox*1 data set. Numbers above each branch denote BP for ML (*left*), MP (*middle*) and PP for BI (*right*). *Asterisks* indicate 100% BP and 1.00 PP. *Dashes* indicate percentages of <50% (or that the node did not occur in the MP or BI tree). The –ln likelihood was 35,367.35; gamma distribution shape parameter (alpha)=0.3025804, nucleotide frequencies: *A*=0.25, *C*=0.25, *G*=0.25, *T*=0.25; and substitution model rate matrix: [TC=0.3666329, TA=0.2089154, TG=0.05988139, CA=0.0491223, CG=0.05342058, AG=0.2620275]. Scale bar=0.1 substitutions per site. Genbank accession numbers are given next to the species names for further information on the published taxa

relationships among the orders. The monophyly of all the brown algal orders was strongly to fully supported in all analyses (BP=98% [ML], 95% [MP]; PP =1.00).

For the *rbc*L only data set, 687 (51%) sites were variable and 546 (40%) sites were parsimony-informative out of the total 1,349 nucleotides. The number of parsimonious trees obtained was 28, the tree length was 4,605, and CI and RI indices were 0.2512 and 0.5695, respectively. As for the *cox*1-5′ data set, 509 (77%) nucleotides were variable and 469 (71%) nucleotides were parsimony-informative. Four most parsimonious trees were obtained, and tree length was 3,609, the CI index was 0.2502 and the RI index was 0.4849. Phylogenetic analyses using *rbc*L data gave a satisfactory resolution at the ordinal and familial level, whereas the use of *cox*1-5′ data alone was better suited for phylogeny inference at the species level. Intra- and interordinal relationships were poorly resolved in the *cox*1 trees which were within expectation as the mitochondrial-encoded gene was noted for its high evolutionary rate. This study also illustrated the feasibility of using *cox*1-5′ as a barcode marker for species of crustose brown algae.

Three species of *Mesospora* (*M. schmidtii*, *M. elongata* and *M. negrosensis*), two putative species of *Diplura* (*Diplura* sp. F and *Diplura* sp. G) and the monotypic genus, *N. expansa* were identified from the molecular analyses of the 87 specimens examined for this study (Table 2). *M. schmidtii* was the most common brown crusts found with 44 specimens, *M. negrosensis* (8), *M. elongata* (4), *N. expansa* (8), *Diplura* sp. F (14) and *Diplura* sp. G (9). The crustose brown algal taxa were not monophyletic but separated into two major clades (A and B). Clade A, which has strong to full support (BP=100% [ML], 99% [MP]; PP=1.00), corresponded to the order Ralfsiales and comprised of the families Mesosporaceae, Neoralfsiaceae and Ralfsiaceae. The family Mesosporaceae comprising *Mesospora*

Table 2 Floristic results of the six species identified in this study with information on the range of distribution and number of sequences

Species (number of specimens)	Range of collection (number of specimens per site)	Number of sequences	
		*rbc*L	*cox*1-5′
Mesospora schmidtii (*n*=44)	CHE (4), MER (10), POR (5), PTL(1), BAT (1), DAN (2), TKA (2), TKS (3), TKR (1), SEM (15)	44	44
Mesospora elongata (*n*=4)	NIP (1), GIL (2), BAT (1)	4	4
Mesospora negrosensis (*n*=8)	CHE(1), MER (2), CHD (3), SEM (2)	8	8
Neoralfsia expansa (*n*=8)	NIP (1), LAY(1), GIL(3), BES(1), KEM(1), CHE (1)	8	7
Diplura sp. F (*n*=14)	MER (1), POR (6), GIL (1), CHD (3), TKS (2), SEM (1)	14	11
Diplura sp. G (*n*=9)	CHE (1), MER (1), POR (3), LAY (2), GIL (1), SEM (1)	9	9

CHE Pulau Che Kamat, Johor, Malaysia; *MER* Pulau Merambong, Johor, Malaysia; *POR* Port Dickson, Malaysia; *PTL* Pelabuhan Tanjung Langsat, Johor, Malaysia, *SEM* Semporna, Sabah, Malaysia; *DAN* Kampong Dandulit, Sabah, Malaysia; *BES* Pulau Besar, Melaka, Malaysia; *CHD* Pantai Chendering, Terengganu, Malaysia; *KEM* Pantai Kemasik, Terengganu, Malaysia; *TKA* Telok Kalong, Terengganu, Malaysia; *TKS* Teluk Sari, Johor, Malaysia; *TKR* Teluk Ramunia, Johor, Malaysia; *NIP* Nipah, Lombok Island, Indonesia; *BAT* Batukijok, Lombok Island, Indonesia; *GIL* Gili Genting, Lombok Island, Indonesia; *LAY* Batulayar, Lombok Island, Indonesia

sp. C, *Mesospora* sp. D and the three species of *Mesospora* identified in the present study was resolved with maximum BP and PP when *Mesospora* sp. G, suspected to be a close relative of the genus, is omitted. Each of the *M. schmidtii*, *M. elongata* and *M. negrosensis* clades was resolved with full BP and PP support. The Mesosporaceae formed a sister relationship to the Neoralfsiaceae, and both were in turn resolved as sister to the Ralfsiaceae. Species of *Diplura* represented by Clade B, formed a sister relationship to Ishigeales with moderate to strong support (BP=99% [ML], 74% [MP]; PP=1.00), near the basal end of the phylogenetic tree. Surprisingly, *Diplura* sp. F is more closely related to *Diplura* spp. from Japan (BP=100% for both ML and MP; PP=1.00) than to *Diplura* sp. G in spite of their geographical distribution. Each of the *Diplura* sp. F and *Diplura* sp. G clades was resolved with full BP and PP support. However, the branch support for inclusion of all *Diplura* spp. was rather weak (BP=56% [ML], 52% [MP]; PP=0.85).

Morphological and anatomical observations

The diagnostic morpho-anatomical features of the crustose brown algae examined in the present study are summarised in Table 3. Species of *Mesospora* are generally characterized by their gelatinous thallus, loose organisation of vegetative filaments, single chloroplast in each cell, unilocular reproductive structures unaccompanied by paraphyses and plurilocular reproductive structures terminated by more than one sterile cell. At the species level, *Mesospora* spp. are distinguished based on the organisation of reproductive structures (Fig. 3a–f). The crusts of *N. expansa* are characteristically thicker than *Mesospora* and *Diplura* and a distinct delineation of the cortical and medullary layers are observed (Fig. 4a, b). Species of *Diplura* are recognised by their relatively thin thallus, multiple chloroplasts per cell and plurilocular structures terminated by a single sterile cell (Fig. 5a–c). The two putative species of *Diplura*, i.e., *Diplura* sp. F and *Diplura* sp. G, are barely distinguishable morphologically although unilocular reproductive structures were observed in *Diplura* sp. G (Fig. 5d) but not in *Diplura* sp. F. Morpho-anatomical characteristics of previously described *Diplura* spp. are included in Table 3 for comparison purpose.

Discussion

Our study indicates that the common crustose brown algae in the Indo–Malay region are species of *Mesospora*, *Diplura* and *Neoralfsia*. Despite global reports of crustose brown algae from the northern to southern hemisphere (e.g., Jaasund 1965; Fletcher 1987; Womersley 1987), the distribution of certain genera, or even species, are probably restricted to the colder ocean waters in the temperate, subpolar or subtropical regions. Members of the Ralfsiaceae (e.g., *Ralfsia fungiformis* (Gunnerus) Setchell et Gardner, *Analipus japonicus* (Harvey) Wynne and *Heteroralfsia saxicola* (Okamura et Yamada) Kawai; Fig. 2) which have been reported mostly from the temperate or colder water region (e.g., North America: Hollenberg 1969; Japan: Tanaka and Chihara 1980) were not encountered in our study. Species of *Mesospora* are the more common brown crusts found in this region surrounded by the warm waters of the eastern Indian Ocean and the South China Sea (e.g., West and Calumpong 1996; Krishnamurthy and Baluswami 1986). In contrast, Japan and Hong Kong recorded a high diversity of crustose brown algal taxa (e.g., Kaehler 1998; Tanaka and Chihara 1980) presumably due to the influence of the Pacific Ocean and the outcome of a dedicated study to this group of algae. *N. expansa* (previously known as *Ralfsia expansa*) have a cosmopolitan distribution and was reported in almost all continents (e.g., León-Alvarez and González-González 2003; Ribera et al. 1992; Rull Lluch 2002).

Table 3 Comparison of morphological characters among (1) genera and (2) species of crustose brown algae in the present study

Taxa	Relative comparison of thalli thickness and gelatinous feature (especially in fertile thallus)	Organisation of vegetative filaments	Distinct delineation of cortical and medullary layer	Plurilocular reproductive structures	Unilocular reproductive structures	Number of chloroplasts per cell
Mesospora	Thicker than *Diplura* but thinner than *Neoralfsia*. Gelatinous upon contact with water	Loosely adherent, with exception in certain species. Cell numbers of the vegetative filaments can be used for species level identification	No	Presence or absence is species dependent. Generally more common than unilocular reproductive structures	Presence or absence is species dependent. Position of the structure on the erect filament and number of stalk cells is useful for species level identification.	Single
M. schmidtii	Thicker than *Diplura* but thinner than *Neoralfsia*. Gelatinous upon contact with water	Loosely adherent, held together by the gelatinous matrix and joined at the basal portion	No	Initially uniseriate later biseriate, and terminated by 2–3 terminal cells (Fig. 3a)	Terminally inserted on up to 4 stalk cells, lateral and basal to the surrounding filaments. Paraphyses lacking (Fig. 3d)	Single
M. negrosensis	Thicker than *Diplura* but thinner than *Neoralfsia*. Gelatinous upon contact with water	Rather closely adherent especially the lower half portion of the vegetative filaments	No	Generally uniseriate, sometimes biseriate; terminated by 1–3 (usually 2) enlarged sterile terminal cells (Fig. 3b)	Terminally inserted on 1–2 stalk cells, lateral and basal to the surrounding filaments. Paraphyses lacking (Fig. 3e)[a]	Single
M. elongata	Thicker than *Diplura* but thinner than *Neoralfsia*. Gelatinous upon contact with water	Loosely adherent, held together by the gelatinous matrix and joined at the basal portion	No	Initially uniseriate later biseriate, and terminated by 2–4 terminal cells (Fig. 3c)	Terminally inserted on up to 8 stalk cells, lateral and basal to the surrounding filaments. Paraphyses lacking (Fig. 3f)	Single
Neoralfsia expansa (monotypic genus)	Thick crusts. Gelatinous upon contact with water (Fig. 4a)	Filaments are tightly adherent	Yes	Biseriate, terminated by a sterile terminal cell. Less common than unilocular structures	Terminally inserted on 3–6 stalk cells at the terminal end of erect filaments. Paraphyses are present (Fig. 4b)	Single
Diplura	Very thin. Slightly gelatinous upon contact with water (Fig. 5a)	Filaments are somewhat tightly adherent	No	Present in all species reported with varying structures among species	Species dependent. Not observed in the type species	Multiple
Diplura sp. F	Smooth and thin. Slightly gelatinous upon contact with water	Filaments are somewhat tightly adherent	No	Initially uniseriate later biseriate, and both filaments shared a sterile terminal cell (Fig. 5b)	Not observed	Multiple
Diplura sp. G	Smooth and thin. Slightly gelatinous upon contact with water	Filaments are somewhat tightly adherent	No	Initially uniseriate later biseriate, and both filaments shared a sterile terminal cell (Fig. 5c)	Terminally inserted on two stalk cells, lateral to the surrounding filaments. Paraphyses are present (Fig. 5d)	Multiple
D. simulans[b]	Gelatinous	Loosely held together by gelatinous matrix and readily separating under pressure	No	Uniseriate, single or mostly in pairs with a sterile terminal cell at the apex	Unknown and probably lacking	Several to many
D. simplex[c,d]	Smooth and thin, somewhat gelatinous	Tightly adherent, not so readily separated	No	Standing in two rows on each erect filament, reproductive filament biseriate bearing one sterile terminal cell	Terminally inserted on one to two stalk cells. Paraphyses are present	Several
Diplura sp. B[d]	Somewhat thin	Tightly adherent	No	Mostly uniseriate, single sterile terminal cell	Unknown	Several
Diplura sp. C[d]	Somewhat thin	Tightly adherent	No	Mostly uniseriate, single sterile terminal cell	Unknown	Several

Table 3 (continued)

Taxa	Relative comparison of thalli thickness and gelatinous feature (especially in fertile thallus)	Organisation of vegetative filaments	Distinct delineation of cortical and medullary layer	Plurilocular reproductive structures	Unilocular reproductive structures	Number of chloroplasts per cell
Diplura sp. "*australis*" [e]	Less gelatinous	Laterally coherent, separate only with considerable pressure	No	Usually uniseriate and in pairs, each reproductive filament has a single pale coloured sterile terminal cell	Absent	Several

[a] First report of unilocular reproductive structures in *Mesospora negrosensis*
[b] Data from Hollenberg (1969) and Abbott and Hollenberg (1976)
[c] Data from Tanaka and Chihara (1981)
[d] Data from Lim et al. (2007)
[e] Data from Buchanan (2005)

The crustose brown algae found in Malaysia and Lombok Island included *M. schmidtii*, *N. expansa*, *Diplura* sp. F and *Diplura* sp. G. Conversely, *M. negrosensis* was found in Malaysia but not on Lombok Island, and it was vice versa for *M. elongata*. A relatively higher diversity of crustose brown algae was recorded at the south-western and north-eastern parts of Peninsular Malaysia and the western part of Lombok Island. The specimens were predominantly epilithic in the intertidal zone, although some of the *N. expansa* crusts were epizoic and were found in the subtidal zone. This is the first report of *M. negrosensis* from Malaysia. This species has thus far been reported only from the Philippines (West and Calumpong 1996). DNA sequencing of *rbc*L and partial *cox*1 genes from our specimens matched those of the culture strain of *M. negrosensis* deposited in KU-MACC. Apart from that, the presence of *Diplura* spp. in Malaysia and Indonesia is also documented for the first time here, expanding the known range for this genus. Other members of this genus, i.e., *D. simulans* Hollenberg and *D. simplex* Tanaka and Chihara, were previously reported in North America (Hollenberg 1969), Mexico (Pedroche et al. 2008), Japan (Tanaka and Chihara 1981; Lim et al. 2007) and Hong Kong (Kaehler 1994), while an unidentified species, *Diplura* sp. "australis" has been documented in New Zealand (Buchanan 2005).

The two species of *Stragularia* reported by Weber-van Bosse (1913) from Indonesia are potentially species of a different genus because members of the family Scytosiphonaceae are rarely, if ever, reported from warmer water regions. The description (Weber-van Bosse 1913) given for *S. clavata* is too brief and incomplete for definite identification, whereas for *S. polycarpa*, the thallus construction and unilocular reproductive structures described and illustrated bear a slight resemblance to those of *Diplura* sp. G. However, four to five sporangia inserted on one or two stalk cells were described for *S. polycarpa* as opposed to one to two sporangia on a single stalk cell in *Diplura* sp. G. Furthermore, plurilocular reproductive structures, which are common in *Diplura* spp., were not observed in *S. polycarpa*. To our knowledge, there is no further mention of *S. polycarpa* in the literature since the first report by Weber-van Bosse (1913). Therefore, we refrain from making any conclusions on the taxonomic status of these two taxa, especially considering that they were initially reported from other locations in Indonesia which do not include Lombok Island.

Mesospora is regarded as a synonym of *Hapalospongidion* (Womersley 1987), but Poong et al. (2013) retained them as distinct genera pending molecular data from the generitype. The three genera identified in this study are distinguished based on thickness of thallus/crust, organization of vegetative filaments, reproductive structures and number of chloroplasts (Table 3). The thickness of crust decreased in the order of *Neoralfsia* > *Mesospora* > *Diplura*. Vegetative filaments in *Mesospora* spp. are generally loosely adhered to each other and are readily separated by slight pressure. Erect filaments in *Diplura* spp. are

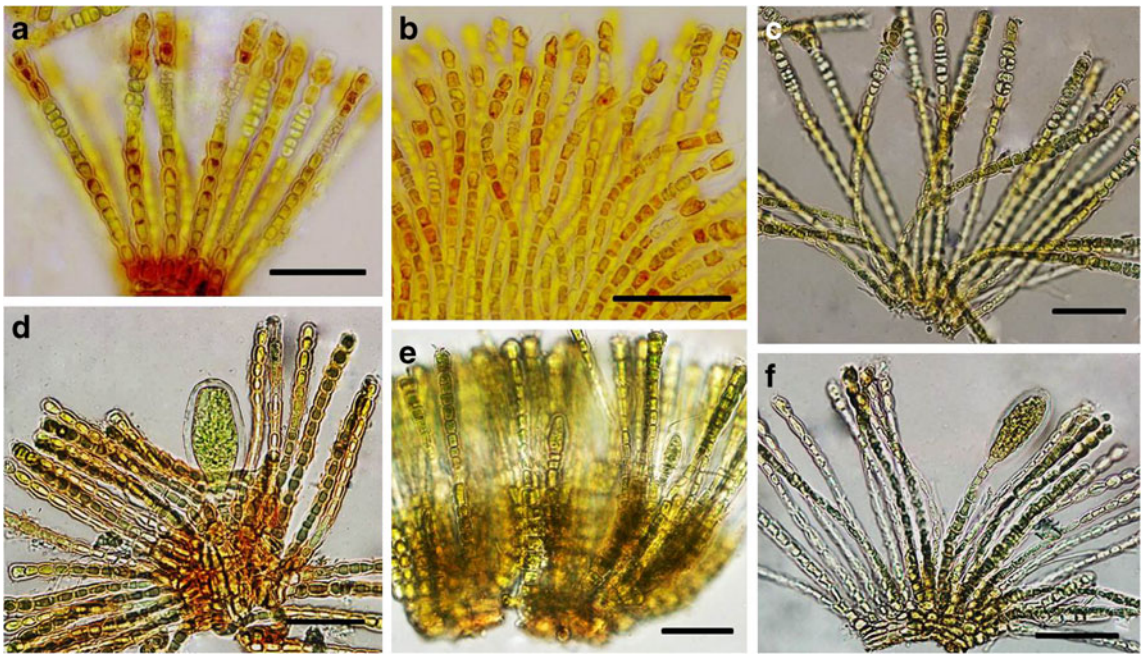

Fig. 3 *Mesospora* spp. **a–c** Plurilocular reproductive structures of *M. schmidtii* (voucher number: PSM 12203), *M. negrosensis* (voucher number: PSM 12183) and *M. elongata* (voucher number: PSM 12214) borne near the apex of erect filaments with sterile terminal cells, respectively. Scale bars=50 μm. **d–f** Unilocular reproductive structures of *M. schmidtii* (voucher number: PSM 12353), *M. negrosensis* (voucher number: PSM 12324) and *M. elongata* (voucher number: PSM12221) inserted lateral to the surrounding erect filaments, respectively. Scale bars=50 μm

more tightly adhered and are only partially separated by pressure whereas in *N. expansa*, erect filaments are tightly adhered to each other and are difficult to be separated even by applying pressure. *Mesospora* and *N. expansa* are reported to have a single chloroplast per cell while *Diplura* is known for its multiple chloroplasts per cell. Plurilocular reproductive structures are more commonly observed compared to unilocular reproductive structures for both *Mesospora* and *Diplura* but not for *N. expansa*.

Three species of *Mesospora* were collected in this study, and they can be distinguished based on several features. *M. schmidtii*, which is the type, differed from *M. elongata* in their number of cells and the number of stalk cells associated with the unilocular reproductive structures. The plurilocular reproductive structures of *M. negrosensis* are generally uniseriate, and its sterile terminal cells are characteristically enlarged. Unilocular reproductive structures for *M. negrosensis* were observed for the first time in this study although they were not completely matured. A recent study by Poong et al. (2013) compiled a detailed comparison of morpho-anatomical features among species of *Mesospora*.

Two species of *Diplura*, tentatively designated as *Diplura* sp. F and *Diplura* sp. G, were identified in our study, and unilocular reproductive structures were observed for *Diplura* sp. G. Hollenberg (1969) and Tanaka and Chihara (1981) did not observe unilocular reproductive structures for *D. simulans* and *D. simplex*, and these structures were first mentioned in *D. simplex* by Lim et al. (2007). Tanaka and Chihara (1981) proposed and distinguished *D. simplex* from *D. simulans* on the basis of the size, thickness and construction of the crusts. Although it is uncertain whether Tanaka and Chihara examined the authentic specimen of *D. simulans*, these features seemed

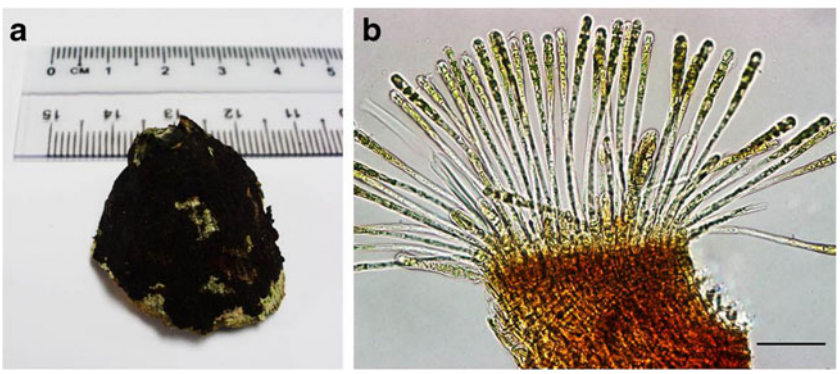

Fig. 4 *Neoralfsia expansa.* **a** Thallus in the form of thick crusts loosely adherent on rocks (voucher number: PSM 12322). **b** Unilocular reproductive structures with stalk cells among surrounding erect filaments (voucher number: PSM 12223). Scale bar=50 μm

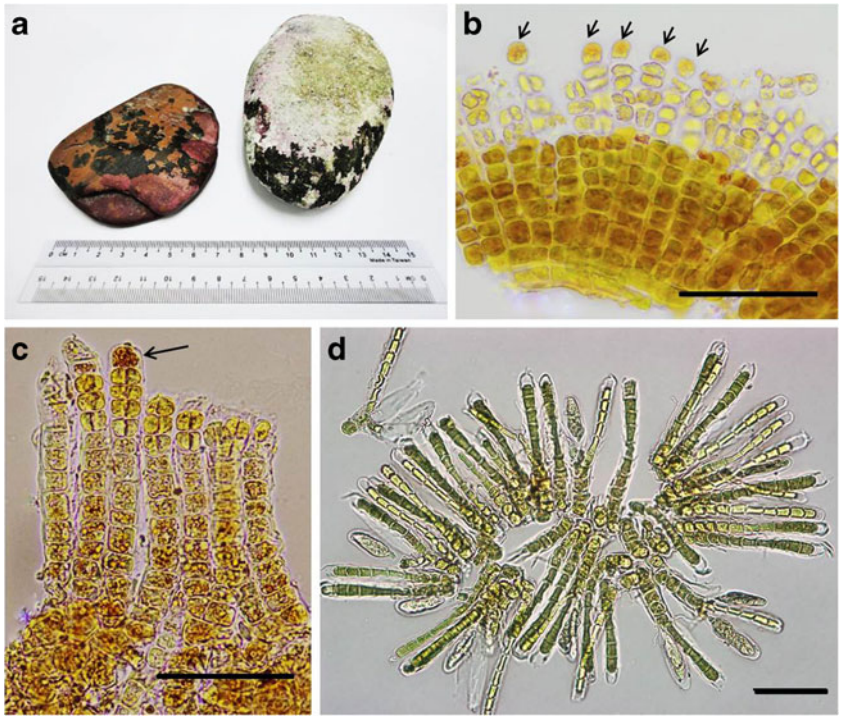

Fig. 5 *Diplura* spp. **a** Thin crusts growing on rocks (*left*: *Diplura* sp. G, voucher number: PSM 12193; *right*: *Diplura* sp. F, voucher number: PSM 12359). **b** Plurilocular reproductive structures of *Diplura* sp. F (voucher number: PSM 12208) terminated by a sterile terminal cell (marked with an arrow). Scale bar=50 μm. **c** Plurilocular reproductive structures of *Diplura* sp. G (voucher number: PSM 12224) terminated by a sterile terminal cell (marked with an arrow). Scale bar=50 μm. **d** Unilocular reproductive structures of *Diplura* sp. G (voucher number: PSM 12172) on stalk cells growing lateral to surrounding erect filaments. Scale bar=50 μm

insufficient for species delineation, and sequence data of *D. simulans* from the type locality is necessary for confirmation. Molecular sequencing allowed the separation of *Diplura* sp. F and *Diplura* sp. G when no single outstanding morphological feature is available to distinguish them despite the wide genetic differences (*rbc*L interspecific distance=12.92–15.66%; *cox*1 interspecific distance=19.37–20.42%). The low interspecific variation between *D. simplex* and the two undescribed species, *Diplura* sp. B and *Diplura* sp. C [*p* distance=3.36–5.27% (*rbc*L); 0.15% (*cox*1)] leads us to speculate that the three are probably conspecific.

Molecular data is essential in the notoriously challenging identification of crustose brown algae. Some species are stages of other taxa with heteromorphic life histories, particularly in the Scytosiphonaceae (Kain et al. 2010). The conventional method of identification up to genus and species level is based on the construction of thalli, life history patterns, number of chloroplasts, occurrence of sessile or stalked unilocular reproductive structures associated with multicellular paraphyses and plurilocular reproductive structures with sterile terminal cells. Although the position and organization of the reproductive structures are crucial for positive identification, collection of fertile specimens is often by chance, especially in the tropics where seasonality is not observed. Variation of terminology used by authors in describing the position of reproductive structures further complicates the identification process (León-Alvarez and Norris 2005). Additionally, there is a risk of misidentification due to confluence of thalli from two or more different species.

Our molecular analyses involve a larger taxon sampling in which more brown algal orders were included compared to the study by Lim et al. (2007). Although a number of taxa are missing in *cox*1 data, we decided to include them in the combined analyses since a study by Wiens (2009) demonstrated that the addition of missing taxa to a data set can be highly beneficial and improve phylogenetic accuracy and cases of decreased accuracy are limited. Although the *cox*1 marker was ineffective at resolving interfamilial and interordinal relationships, it was capable of assigning samples to genetic species. Combination of *rbc*L and *cox*1-5′ data is advocated for use in species identification and phylogenetic reconstruction of this group of algae.

Lim et al. (2007) were the first to dedicate a molecular study to the Ralfsiales as a whole to test their traditional classification. Most specimens originated from Japan, with only two taxa from Malaysia. Their circumscription of Ralfsiales excluded the families Neoralfsiaceae and Lithodermataceae which, along with Ralfsiaceae, were initially included in the order (Nakamura 1972). Molecular evidence by Reviers et al. (2007; Fig. 14.5, p. 278) indicated that Ralfsiaceae (Ralfsiales), Nemodermataceae (Nemodermatales) and Lithodermataceae are not monophyletic. Our findings concur with earlier results (Lim et al. 2007; Reviers et al. 2007) which showed that the brown crusts are not monophyletic. Specimens used in the present study were resolved in two major clades, clade A corresponding to the Ralfsiales and clade B which encompassed the *Diplura* spp. and which diverged much earlier and form a sister clade to the Ishigeales. Our circumscription of the Ralfsiales followed the approach used

by Lim et al. (2007) which included only the Ralfsiaceae, Mesosporaceae and Neoralfsiaceae.

Although the establishment of a new family as suggested by Lim et al. (2007) is necessary to accommodate species of *Diplura*, it is premature to do so at this stage as we await the publication of the gene sequences of the generitype, *D. simulans*. Further investigation of morpho-anatomical characters (and life history studies, if necessary) will help in understanding the evolutionary history of this early diverging group. As of now, the placement of this genus among the early lineages of brown algae is supported by the presence of several chloroplasts per cell. Putative *Diplura* spp. from Malaysia and Lombok Island displayed sister relationship with *D. simplex* and two other *Diplura* spp. from Japan. The phylogenetic relationship inferred for species of *Diplura* examined in this study mirrored the geographic location where these specimens were collected, i.e., samples from Japan, collectively formed a sister clade to Indo-Malaysian samples. *Diplura* sp. G, which was resolved as a sister to *Diplura* sp. F and Japanese *Diplura* specimens, may represent a separate but closely related genus; nonetheless, current data are insufficient to support this hypothesis.

More work is necessary on crustose brown algae, in particular sampling of genera that were previously assigned to the Ralfsiales such as *Jonssonia* Lund, *Acrospongium* Schiffner, *Symphyocarpus* Rosenvinge, *Sorapion* Kuckuck, *Zeacarpa* Anderson, Simons and Bolton and *Basispora* John and Lawson, for molecular studies in order to clarify their ambiguous taxonomic position. We anticipate the discovery of more crustose brown algal species from this region following the exposure to DNA sequencing, subsequently altering the makeup of the diversity of this under-represented group of brown algae as it was previously known from morphological descriptions.

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