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Morphological and molecular phylogenetic studies of a red alga, *Halymenia durvillei*, (Halymeniaceae, Halymeniales) from Indo-Pacific

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Abstract—Morphological and molecular phylogenetic studies were made on recently collected *Halymenia* plants widely from Japan, the Philippines, Indonesia, Malaysia and Thailand. Although the external morphology (branching pattern, blade width, or degree of dentation) was very variable, no special differences were found in their vegetative and reproductive structures. The features are close to *Halymenia durvillei*. Our *rbc*L gene sequence analysis has shown that the branched *Halymenia* plants are all included in a distinct, monophyletic clade, separate from those including the foliose plants. The branched plants studied here are, therefore, concluded to belong in a single species, *Halymenia durvillei*, irrespective of their great external variations.

As *Halymenia microcarpa* clearly fall within the range of external variations of *H. durvillei*, it was concluded to be synonymous with *H. durvillei*. The taxonomic interrelationship among the four varieties (var. *formosa*, var. *ceylanica*, var. *denudata* and var. *edentata*) remained unresolved, although apprently encompassed within the morphological range of *H. durvillei*.

Key words: Halymeniaceae, *Halymenia durvillei*, Indo-Pacific, morophology, *rbc*L gene, Rhodophyta, taxonomy

Introduction

Halymenia durvillei is one of the commonest *Halymenia* species found along the southeastern Asian coasts. This alga was first described by Bory de Saint-Vincent (1828) from New Ireland, Papua New Guinea and has been reported from various localities around the Pacific and the Indian Oceans (Guiry et al. 2005). *Halymenia durvillei* is characterized by its comparatively dark red color, robust blades with non gelatinous texture, toothed margin besides repeatedly branched blades (Weber van Bosse 1921). However, it is very difficult to distinguish the alga from other related species such as *H. microcarpa* (Montagne) Silva (in Silva et al. 1987) from the Philippines or *H. venusta* Boergesen (1932) from India, probably due to its gross morphological variations. The distinction of the varieties of *H. durvillei* proposed by Weber-van Bosse (1921) such as var. *ceylanica* (Harvey ex Kutzing) Weber-van Bosse, or var. *denudata* Weber-van Bosse is also unclear.

Recently, based on the study of the type material and many other plants of *Halymenia durvillei* from the Philippines, De Smedt et al. (2001) reported that the type material of *H. microcarpa* and *H. venusta* had no differences that warrant recognition on the species level, and concluded that they are both conspecific with *H. durvillei*. Similarly, the four varieties recognized by Weber-van Bosse (1921), var. *formosa* (Harvey ex Kutzing) Weber-van Bosse, var. *ceylanica* (Harvey ex Kutzing) Weber-van Bosse, var. *denudata* Weber-van Bosse and var. *edentata* Weber-van Bosse, were all considered to be growth forms that fall within the morphological range of *H. durvillei* (De Smedt et al. 2001).

In this study, to clarify the range of morphological variations of *Halymenia durvillei* and its taxonomic relationships with the allied taxa, we made morphological and molecular phylogenetic studies on recently collected *Halymenia* plants widely from Japan, the Philippines, Indonesia, Malaysia and Thailand. On these results, the validity of the taxonomic revisions made by De Smedt et al. (2001) was also discussed.

Materials and Methods

The branched *Halymenia* plants used in the present study: Japan: Kagoshima, 26. vi. 2002, sterile (KWGSH53, 54), 5. vi. 2003, tetrasporangial (KWGSH72), Yonaguni Island, 7. iii. 2000, sterile (SAP092235). The Philippines: Bolinao, 24. xi. 2004, tetrasporangial (KWGSH11). Malaysia:

Langkawi, 16. v. 1998, cystocarpic, terasporangial (SAP090438, 090439); Sandakan, 18. v. 1998, cystocarpic (SAP090441); Kota Kinabal, 3. vi. 1998, spermatangial (SAP090444), cystocarpic (SAP090445); Johor, 6. vi. 1999, cystocarpic, cystocarpic (SAP090448, KWGSH80). Indonesia: Bintan Island, 24. v. 2005, cystocarpic, tetrasporangial, spermatangial, sterile (KWGSH40, 41, 42, 43). Thailand: Koh Ra, 17. i. 1997, spermatangial (KL7601); Koh Nui, 4. iv. 1997, tetrasporangial (KL7611); Ao Tang Khen, 10. v. 1997, cystocarpic, sterile (KL7621, 7622); Khao Bae Na, 14. iii. 1998, tetrasporangial (KL7701). Most of these plants were collected by the first author, while the Thai plants were collected by Dr. K. Lewmanomont.

For comparison, two foliose *Halymenia* plants (Indonesia: Bintan Island, 24. v. 2005, KWGSH31, the Philippines: Bolinao, 24. xi. 2004, KWGSH10) collected by the first author were added in the molecular phylogenetic study.

The herbarium specimens with SAP numbers are housed in the herbarium of Faculty of Science, Hokkaido University, Japan, KWGSH specimens in the herbarium of Faculty of Agriculture, Kyushu University, Japan and KL specimens in the herbarium of Faculty of Fisheries, Kasetsart University, Thailand, respectively.

Small portions dissected from the herbarium specimens were resoaked and prepared for microscopic observations. Sections were made by hand with a razor blade and stained with 1% cotton blue in 50% glycerol/seawater.

Total DNA was extracted from 11 samples of *Halymenia* using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol. PCR amplification and sequencing of the chloroplast-encoded *rbc*L gene were performed as in Wang et al. (2000). The *rbc*L sequences were aligned manually and no insertion-deletion mutations were detected. Sequences of 10 species of the *Halymenia*ceae were downloaded from GenBank and included in the alignment (see Wang et al. 2001 and Kawaguchi et al. 2002). *Gelidiella ligulata* Dawson (GenBank accession no.: AB017678) and *Sebdenia monardiana* (Montagne) Berthold (U21600) were used as outgroups for the analysis. The alignment is available from the second author upon request.

The maximum parsimony (MP) method was used to construct phylogenetic tree. Parsimony analysis was performed with PAUP 4.0 b10 (Swofford 2002). All sites were treated as unordered and equally weighted. Heuristic search option with random addition of sequences (100 replicates) and tree-bisection-reconnection branch swapping algorithm (TBR) was used for tree searching. Bootstrap analysis based on 2000 re-samplings of the data set (Felsenstein 1985) was calculated (10 random additions, TBR, Full heuristic search option).

Results

External morphology

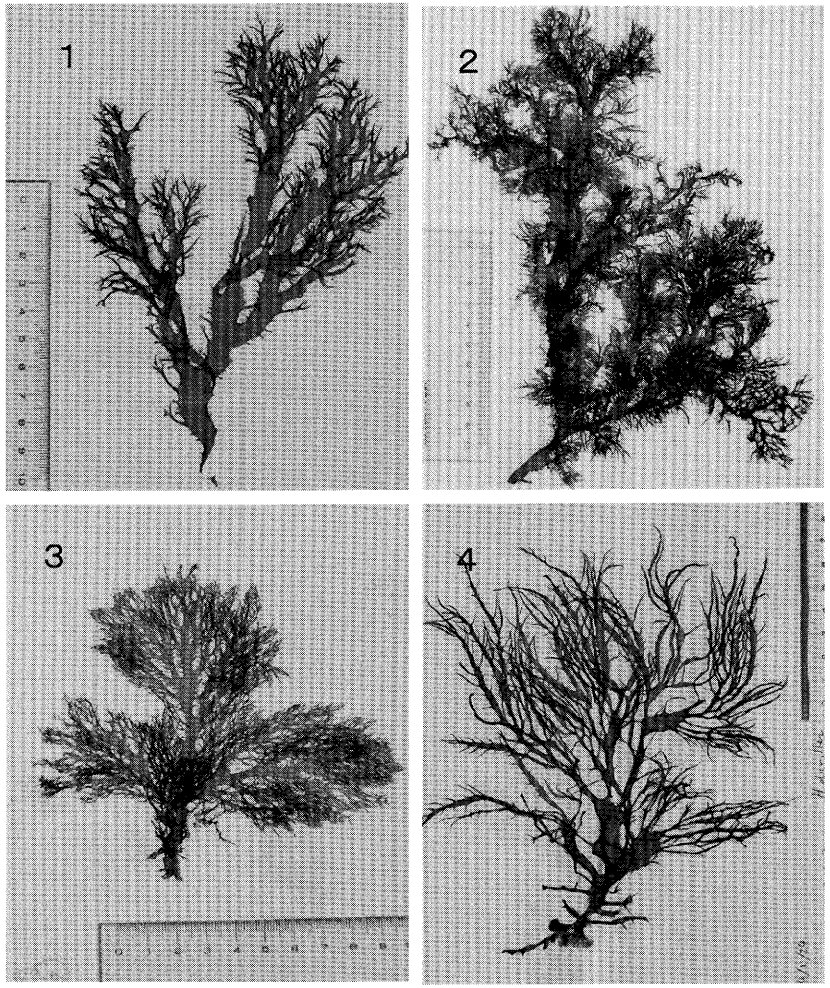
Japanese specimens: Erect blades are up to 18 cm long, 1.5 cm wide; Blades with a cuneate base are repeatedly subdichtomously branched, becoming narrower upwards. Numerous short lateral branchlets are formed from the margins of the branches. The branches and the branchlets are beset with spine-like proliferations along the margins and on the surfaces (Figs. 1–3). Philippine specimen: Erect balde with a cuneate base is up to 17 cm long, 1.1 cm wide. Erect blades are subdichotomously or trichotomously branched, becoming narrower upwards. The branches are beset with marginal spines (Fig. 4). Malaysian specimens: Erect blades with a cuneate base are up to 50 cm long, 5 cm wide. In some plants, the blades are lacking percurrent axes, and are dichotomously or subdichotomously (or trichotomously) branched with narrow axils, forming irregularly pinnate branchlets. In others, percurrent axes are obvious and beset with dense or sparse lateral branchlets. In both types of blades, short spines are occasionally produced from the margins and/or on the surfaces (Figs. 5–8). Indonesian specimens: Erect blades are up to 24 cm long, 2 cm wide. Erect blades with a cuneate base are repeatedly subdichotomously branched, becoming narrower upwards. Lateral branchlets are densely or sparsely produced from the margins of the branches. The branches and the branchlets are beset with marginal spines. Surface spines were not observed (Figs. 9–12). Thai specimens: Erect blades are up to 18 cm long. Erect blades with a cuneate or linear base are repeatedly subdichotomously branched. The branches reach 1.8 cm wide in the widest part, and become narrower upwards, or remain to be linear up to 0.5 cm wide. Lateral branchlets, dense or sparse, are formed in a pinnate or distichous manner. The branches and the lateral branchlets are beset with marginal spines. Surface spines are occasionally found (Figs. 13–16).

Vegetative and reproductive structures

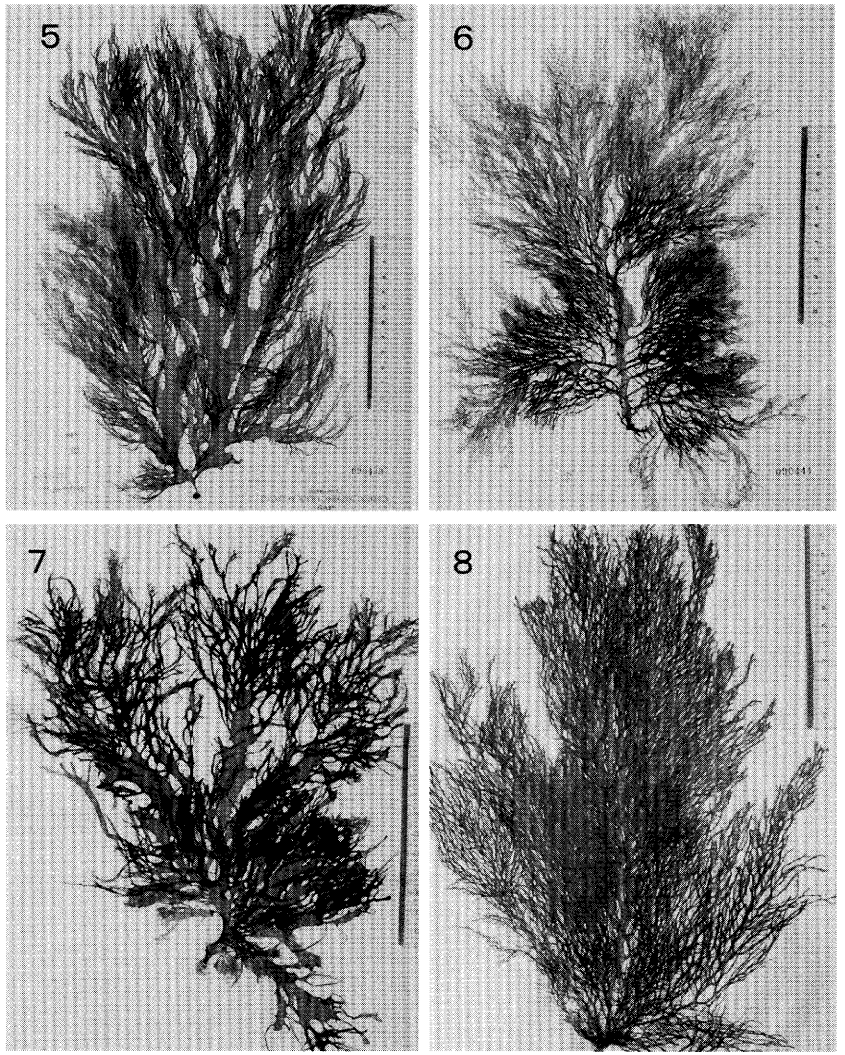
No special differences in the vegetative and reproductive structures were found among the specimens investigated in the present study.

The blades consist of a comparatively dense medulla and a compact cortex; the medullary filaments are mainly running vertically or obliquely from cortex to cortex, or sometimes periclinally oriented; the cortex consists of an outer layer of 3 or 4 rounded cells tightly packed in vertical rows and an inner layer of 3 or 4 larger, polygonal to stellate cells connected to each other by secondary pit-connections; the outermost cortical cells are usually highly elongated (Figs. 17–20)

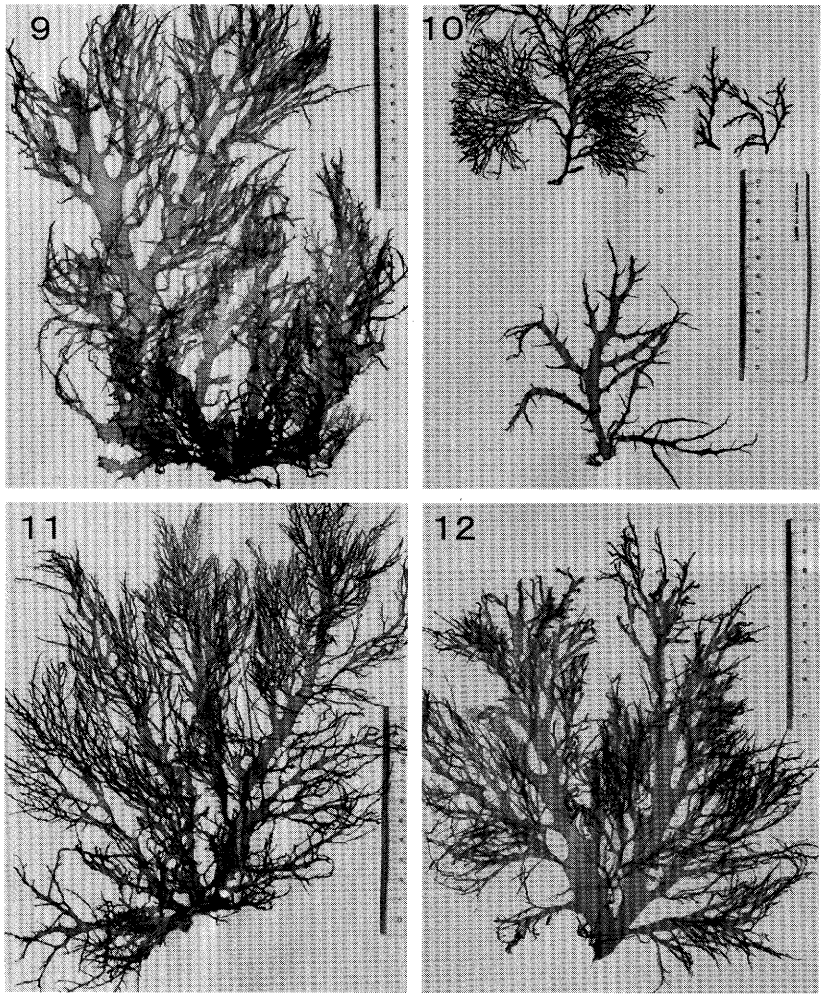
Gametophytes are dioecious. Carpogonial branches were not clarified. From the surface view, auxiliary cell ampullae



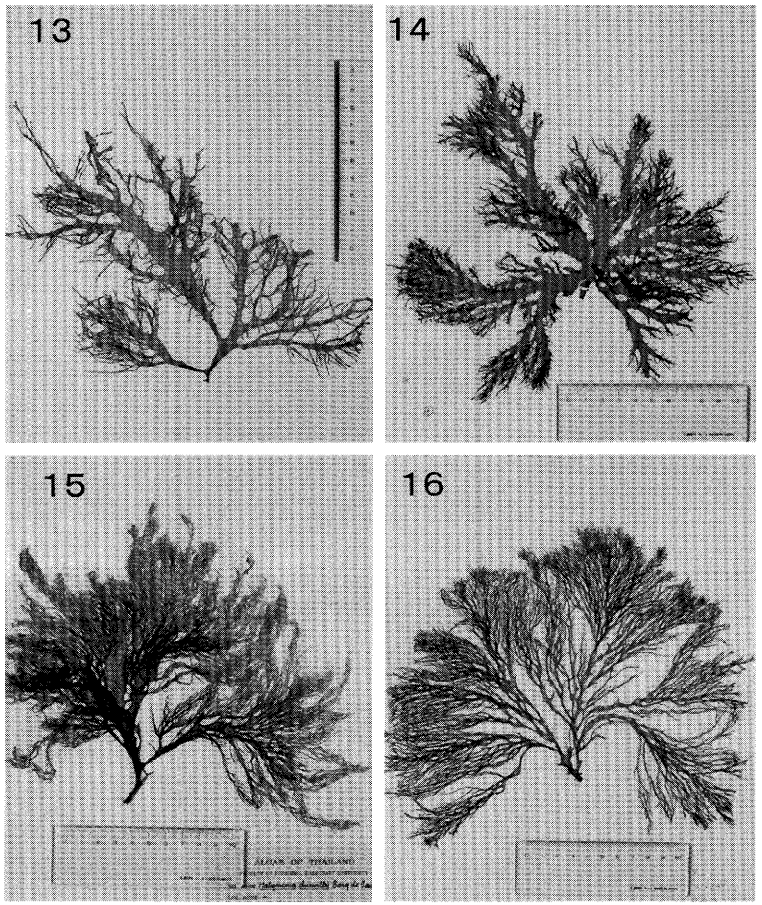
Figs. 1–4. Herbarium specimens of *Halymenia* from Japan and the Philippines. Fig. 1. KWGSH72 (Japan), Fig. 2. KWGSH54 (Japan), Fig. 3. SAP092235 (Japan), Fig. 4. KWGSH11 (the Philippines).



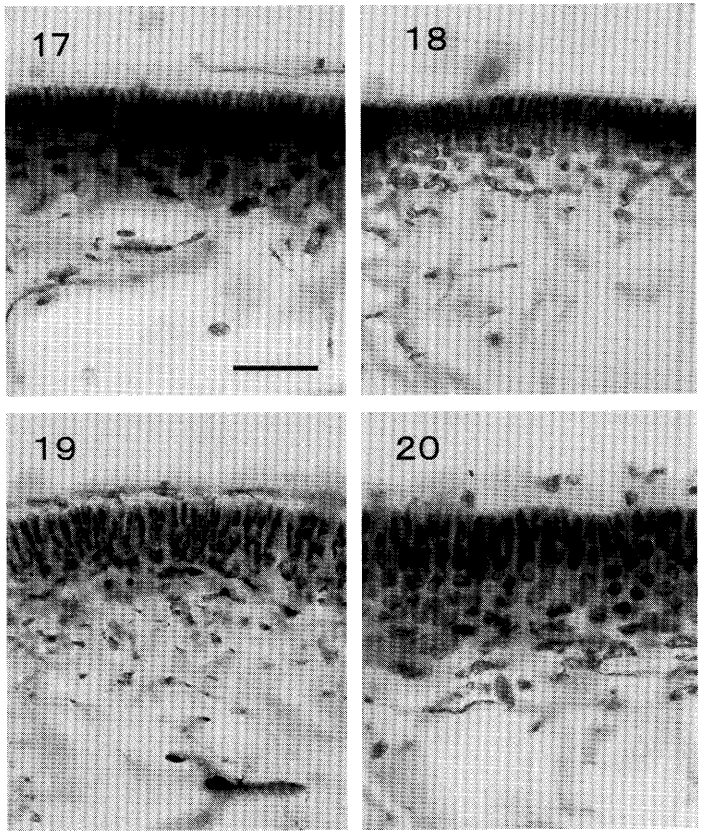
Figs. 5–8. Herbarium specimens of *Halymenia* from Malaysia. Fig. 5. SAP090439, Fig. 6. SAP090444, Fig. 7. SAP090438, Fig. 8. KWGSH80.



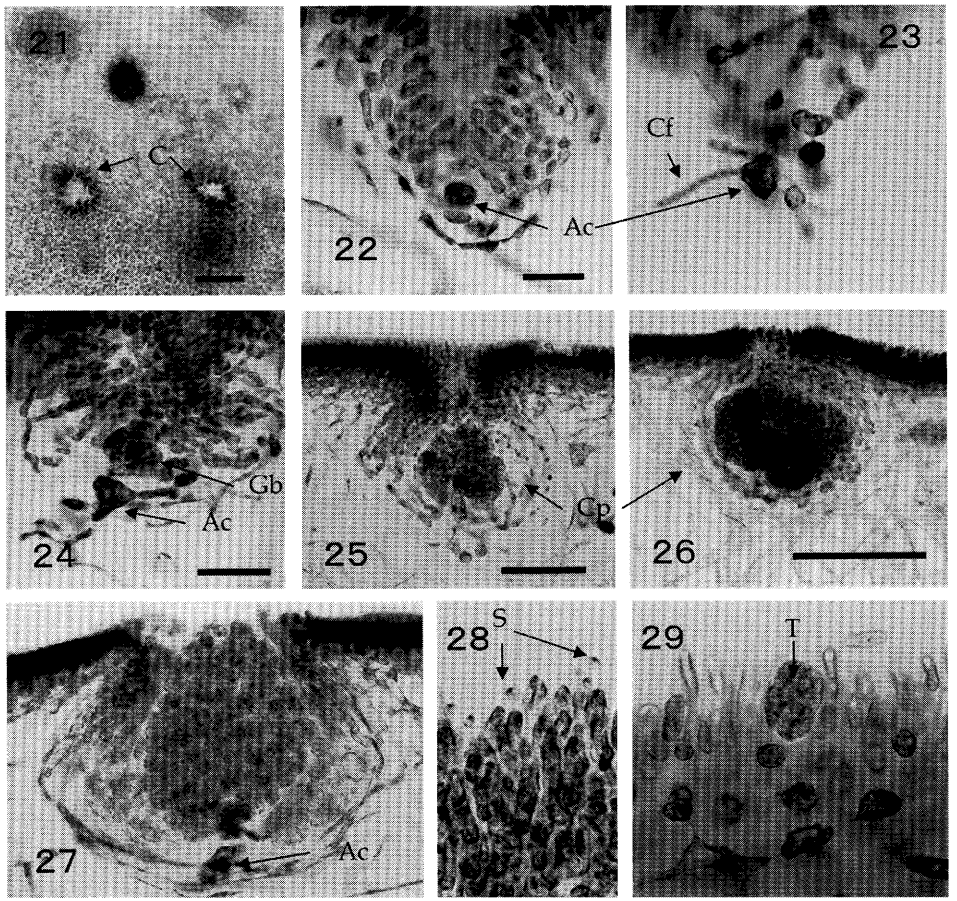
Figs. 9–12. Herbarium specimens of *Halymenia* from Indonesia. Fig. 9. KWGSH42, Fig. 10. KWGSH40, Fig. 11. KWGSH43, Fig. 12. KWGSH41.



Figs. 13–16. Herbarium specimens of *Halymenia* from Thailand. Fig. 13. KL7611, Fig. 14. KL7701, Fig. 15. KL7622, Fig. 16. KL7621.



Figs. 17–20. Vegetative construction of *Halymenia* specimens from Japan, Malaysia, the Philippines and Thailand. Cross sections were indicated. Fig. 17. Japanese specimen (KWGSH72), Fig. 18. Malaysian specimen (KWGSH80), Fig. 19. Philippine specimen (KWGSH11), Fig. 20. Thai specimen (KL7611). Scale bar in Fig 17=50 μm, applying also to Figs. 18–20.



Figs. 21–29. Reproductive structures of *Halymenia* specimens from Malaysia. Surface view and cross sections were indicated. Fig. 21. Surface view of carpostoma (C) (SAP090438), Fig. 22. Auxiliary cell ampulla with auxiliary cell (Ac) (SAP090438), Fig. 23. Incoming connecting filament (Cf) attached to auxiliary cell (Ac)(SAP090438), Fig. 24. Developing gonimoblasts (Gb) from auxiliary cell (Ac)(SAP090448), Figs.25, 26. Developing cystocarp (Cp)(SAP090448), Fig. 27. Mature cystocarp with auxiliary cell (Ac) in the bottom (SAP090448), Fig. 28. Formation of spermatangium (S)(SAP090444), Fig. 29. Formation of tetrasporangium (T)(SAP090439). Scale bars in Figs. 21, 22, 24, 25, 26=50 μm, 20 μm, 50 μm, 100 μm, 150 μm, respectively. Scale bar in Fig. 22 applying also to Figs. 23, 27–29.

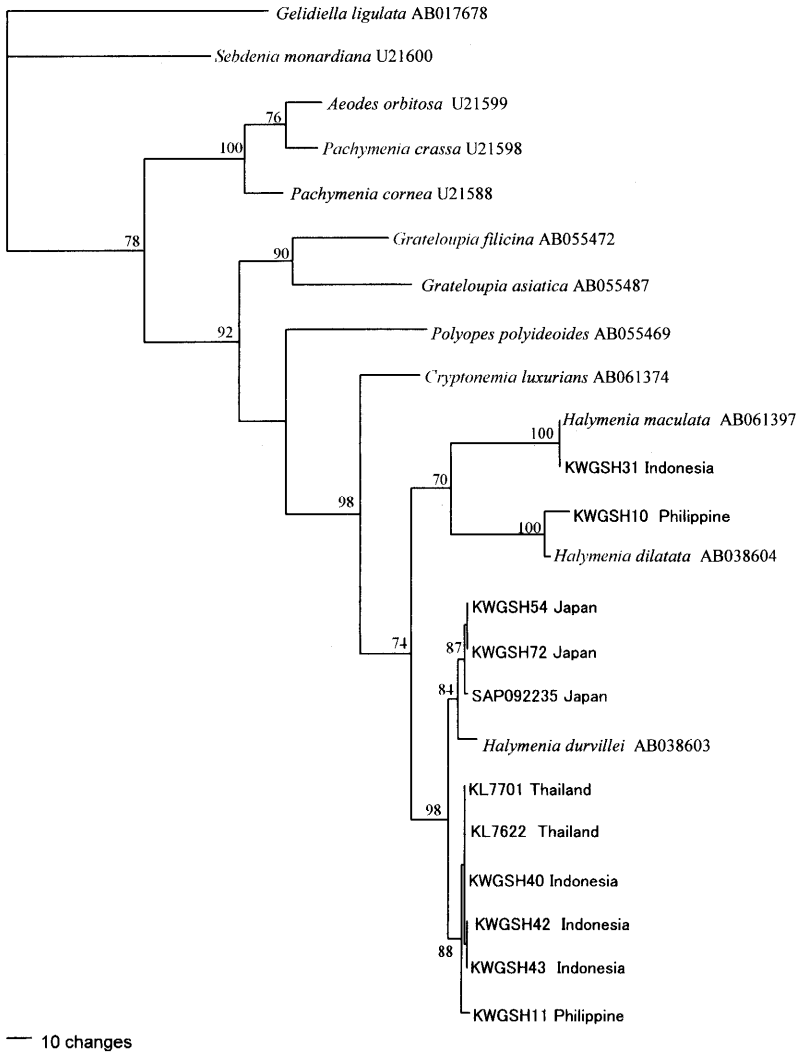
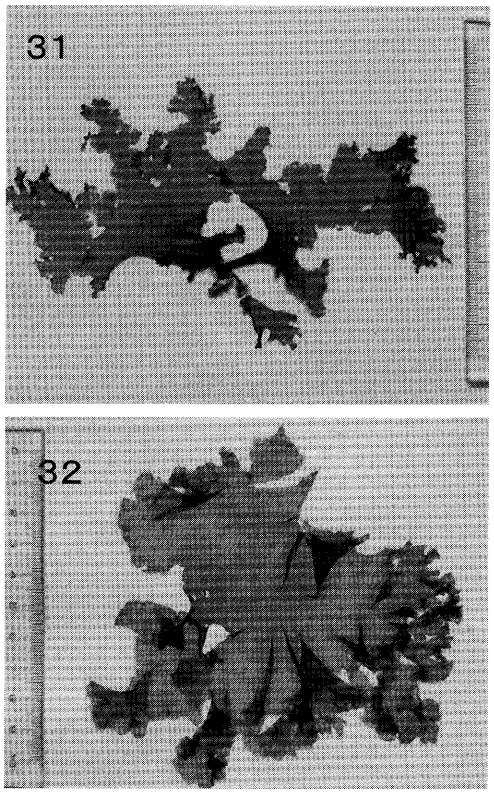


Fig. 30. Maximum parsimony tree for *rbc*L sequences of 11 *Halymenia* specimens (with KWGSH, SAP and KL numbers), 10 *halymenia*cean species and two outgroups downloaded from GenBank (with AB and U numbers).

are recognizable by the carpostoma on the thallus surface (Fig. 21). Auxiliary cells are formed in the bottom of cup-shaped ampullae branched to the third order (Fig. 22). Early post-fertilization events were not clarified, but connecting filaments in contact with auxiliary cells were frequently observed (Fig. 23). The auxiliary cells with an attached connecting filament produced gonimoblasts toward the blade surface (Fig. 24). During the gonimoblast development, ampullary cells become elongated and remain as a loose net work of filaments surrounding the developing carposporophyte (Figs. 25, 26). Mature cystocarps were spherical to pear-shaped, 180–230 μm in diameter and deeply submerged in the medulla (Fig. 27). Spermatangia are scattered over the blade and formed from the outermost cortical cells (Fig. 28). Tetrasporangia are cut off from the cortical cells in the third or fourth layer from the surface. Mature tetrasporangia are broadly ellipsoidal in shape, 17–20 μm wide by 28–32 μm long, cruciately or decussately divided (Fig. 29).

*rbc*L analysis

Phylogenetic relationships of plants of *Halymenia* and other genera within the Halymeniaceae inferred from *rbc*L gene sequences are shown in Fig. 30. The 14 species of *Halymenia* including the three species from GenBank were all included within the *Halymenia-Cryptonemia* clade, separate from the *Aeodes-Pachymenia* clade, the *Grateloupia* clade and the *Polyopes* clade. Within the *Halymenia-Cryptonemia* clade, the deeply clefted foliose plant from Indonesia (Fig. 31) formed a first subclade, together with *H. maculata* from Malaysia. Another foliose plant without deep clefts from the Philippines (Fig. 32) formed a second subclade, together with *H. dilatata* from Japan. The remaining nine plants with repeatedly branched blades formed a third subclade, together with *H. durvillei* from Malaysia.



Figs. 31, 32. Herbarium specimens of foliose *Halymenia* from Indonesia and the Philippines. Fig. 31. KWGSH31, Fig. 32. KWGSH10.

Discussion

The branched plants of *Halymenia* investigated here from Japan, Malaysia, the Philippines, Indonesia and Thailand showed a very wide range of gross morphological variations. In some plants, the width is not exceeding 1 cm, while others reach 5 cm in width. The branching patterns also varied greatly from plants having a percurrent axis with pinnately or distichously arranged laterals to those with repeatedly dichotomosly or subdichotomously branched blades. On the contrary, no special differences were found in their vegetative and reproductive features. This fact suggests that these plants may all belong to a single species and that the wide external variations found among them be growth forms in different environmental conditions.

Our *rbc*L gene sequence analysis of the 11 *Halymenia* plants has shown that, irrespective of their great external variations, all the nine branched plants are included in a distinct, monophyletic clade together with *H. durvillei* from Malaysia (Fig. 30). As the remaining plants are apparently encompassed within the morphological range of the analyzed plants, all the branched *Halymenia* plants studied here are considered to belong in a single species, *H. durvillei*. One of the two foliose plants is morphologically identified as *H. maculata* by its dark red color, heavily dissected margins and the conspicuous surface spots (maculae) (Kawaguchi et al. 2002). Another foliose plant without deep clefts is identified as *H. dilatata* by its pinkish red color, rounded shape and the soft-gelatinous texture (Kawaguchi and Lewmanomont 1999). Our *rbc*L gene sequence analysis also supported our identification, as is clear in the phylogenetic tree (Fig. 30).

The type material of *Halymenia microcarpa* (De Smedt et al. 2001, Fig. 2B) is an apical fragment which is undistinguished from the apical parts of our material (ex. KWGSH11, Fig. 4, KWGSH40, Fig. 10). This alga should be treated under the synonymy of *H. durvillei*, in agreement with the conclusion by De Smedt et al. (2001). The type material of the four varieties of *H. durvillei*, var. *ceylanica*, var. *formosa*, var. *denudata* and var. *edentata* (De Smedt et al. 2001, Fig. 2C, Fig. 3D, Fig. 3C, Fig. 3B) apparently fall within the range of external variations of *H. durvillei* clarified in this study. However, the interrelatioship among the four varieties is at present still unclear, in disagreement with the conclusion by De Smedt et al. (2001). To make a final decision on the taxonomic status of the four varieties, more comprehensive, molecular phylogenetic study would be necessary.

As for *H. venusta*, no molecular data are available to us. Any taxonomic decisions on this alga would also be pending until such data have been shown.

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References

Boergesen, F. 1932. Some Indian Rhodophyceae, especially from the shores of the Presidency of Bombay. II. Bull. Misc. Inform. Kew 1932: 113–134.

Bory de Saint-Vincent, J. B. 1828. *In* Duperrey, L. I., Voyage autour du monde, Botanique, Cryptogamie, pp. 1–300, Bertrand, Paris.

De Smedt, G., De Clerck, O., Leliaert, F., Coppejans, E. and Liao, L. M. 2001. Morphology and systematics of the genus *Halymenia* C. Agardh (Halymeniales, Rhodophyta) in the Philippines. Nova Hedwigia 73: 293–322.

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

Guiry, M. D., Rindi, F. and Guiry, G. M. 2005. AlgaeBase version 4.0. Worldwide electronic publication, National University of Ireland,Galway. http://www. algaebase.org.

Kawaguchi, S. and Lewmanomont, K. 1999. Morphology and culture study of a red alga, *Halymenia dilatata* Zanardini, from Vietnam and Japan. *In* Abbott, I, A. (ed.), Taxonomy of economic seaweeds with special reference to some Pacific species. Vol. VII. pp. 147–161. A publication of the California Sea Grant College System.

Kawaguchi, S., Lewmanomont, K. and McDermaid, K. 2002. Morphology of *Halymenia maculata* J. Agardh from Vietnam. *In* Abbott, I. A. and McDermid, K. (eds.), Taxonomy of economic seaweeds with special reference to some Pacific species. Vol. VIII. pp. 259–266. A publication of the California Sea Grant College Program.

Kawaguchui, S., Wang, H. W., Horiguchi, T. Lewis, J. A. and Masuda, M. 2002. Rejection of *Sinkoraena* and transfer of some species of *Carpopeltis* and *Sinkoraena* to *Polyopes* (Rhodophyta, Hallymeniaceae). Phycologia 41: 619–635.

Silva, P. C., Menez, E. G.. and Moe, R. L. 1987. Catalogue of the benthic marine algae of the Philippines. Smithsonian Contrib. Mar. Sci. 27: 179 pp.

Swofford, D. L. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sinauer Associates, Sunderland, Massachusetts.

Wang, H. W., Kawaguchi, S., Horiguchi, T. and Masuda, M. 2000. Reinstatement of *Grateloupia catenata* (Rhodophyta, *Halymenia*ceae) on the basis of morphology and *rbc*L sequences. Phycologia 39: 228–37.

Wang, H. W., Kawaguchi, S., Horiguchi, T. and Masuda, M. 2001. A morphological and molecular assessment of the genus *Prionitis* J. Agardh (*Halymenia*ceae, Rhodophyta). Phycologica Res. 49: 251–261.

Weber-van Bosse, A. 1921. Liste des algues du Siboga II. Rhodophyceae, Protoflordeae, Nemalionales, Cryptonemiales. Brill, Leiden.