SYSTEMATIC STATUS OF *HALICHONDRIA JAPONICA* (KADOTA) (DEMOSPONGIAE, HALICHONDRIDA) FROM JAPAN

SAYUMI HOSHINO*, MASATSUNE TAKEDA** & YOKO WATANABE**

*Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 3-23-1 Hyakunincho, Shinjuku-ku, 169-0073 Tokyo, Japan
**National Science Museum 3-23-1 Hyakunincho, Shinjuku-ku, 169-0073 Tokyo, Japan
E-mail: shoshino@kahaku.go.jp

ABSTRACT

Halichondria japonica (Kadota, 1922) is a thick and broadly encrusting sponge commonly found on intertidal rocky shores around Japan and also recorded from Korea. Two morphotypes, one orange and one yellow, are here compared on the basis of their skeletal composition and arrangement, larval morphology, and the ribosomal DNA sequences. We proved that the two morphotypes represent two distinct species that should be classified within the genus *Hymeniacidon* and not *Halichondria*. We suggest *Hymeniacidon japonica* (Kadota, 1922) as the revised specific assignation for the bright orange morphotype of "*Halichondria japonica*" (Kadota, 1922). The definite specific assignation for these species must await a taxonomic comparison with other *Hymeniacidon* species from the Pacific.

KEY WORDS

Halichondria japonica, *Hymeniacidon*, systematics, larval morphology, DNA analysis.

INTRODUCTION

Halichondria japonica (Kadota, 1922) is one of the most common sponge species in Japan. This species had been referred to the genus *Reniera* Nardo, 1847 (Chalinidae, Haplosclerida) by the original author, and later transferred to the genus *Halichondria* Fleming, 1828 by UTINOMI (1962) without discussion. We guess that this transfer is due to disagreement with diagnostic characters of *Reniera*, which has a reticulate skeleton of oxeote spicules. The genus *Halichondria* is characterized by having mainly oxeote spicules, a layer of tangential spicule bundles in the ectosome, and a disordered loose reticulation in the endosome. However, it is apparent that *H. japonica*, having only stylote spicules instead of oxeote spicules, differs generically from the other *Halichondria* species.

During the systematic study of *Halichondria japonica*, we recognized two different color morphotypes. These two morphotypes can be found living sympatrically and are easily distinguished in the field by their color and texture.

The authors examined these two morphotypes based on the external general morphology, spicule character and arrangement, and sequence of two regions of nuclear DNA [the internal transcribed spacer 2 (ITS2) and partial sequences of the 28S ribosomal DNA]. Then the systematic status of *H. japonica* was reevaluated.

MATERIALS AND METHODS

Material

In the original description of *Halichondria japonica*, no mention was made of its type designation and depository by KADOTA (1922), who prepared the description, presumably based on specimens from Aburatsubo, in the southwestern part of the Miura Peninsula facing Sagami Bay, on the Pacific coast of central Japan. Kadota's types of this species might no longer exist. So in this study, fresh specimens were collected from a rocky shore at Aburatsubo, and the external color of each specimen was recorded by using COLOR CHART (Dainippon Ink & Chemicals, Inc.) and expressed with 4 numbers, each number of which indicates the percentage of Cyan, Magenta, Yellow, and Black (CMYK). After a close examination of external characters, a small piece cut off from each specimen was fixed in 99 % ethanol for DNA analysis, and the bulk was fixed in 70 % ethanol for histological observation.

Morphology

Spicules slides were prepared following standard techniques (HOSHINO, 1980). To observe the spicule arrangement, some parts of each specimen were dried or embedded in Paraffin Wax. Vertical and tangential sections were made by handsection. The length and width of fifty spicules were measured for 24 and 8 specimens from the orange and yellow types, respectively.

Developmental observation

For a preliminary observation of larval morphology, specimens were collected once a month from May to July 2002 at Tateyama, on the southwestern coast of the Boso Peninsula, faced to the Sagami Sea. The specimens were fixed with Bouin's fixative, dehydrated through ethanol series, and embedded in Paraffin Wax. Series of 8 μm sections were made and stained with Mayer's haematoxyline and eosine.

DNA samples, extraction and analysis

Total genomic DNA was extracted from 9 specimens of the orange type, 7 specimens of the yellow type, 5 species of the genus *Halichondria*, *Hymeniacidon* sp., *Suberites ficus* and *Spirastrella* sp. by using the CTAB method (HOEZEL, 1998). The region including ITS2 (approximately 200 bps) and 3' end of 28S (approximately 200 bps) were amplified using the primers ITS3 and EP2 (CHOMBARD *et al.,* 1998). The PCR reaction was performed using following program. The first cycle is 4 min at 94° C, 2 min at 57° C and 2 min at 72° C followed by 30 cycles each consisting of 1 min at 94° C, 1 min at 57° C and 1 min at 72° C. The final cycle is 4 min at 72° C. Each PCR product was purified using Microcon YM-100 following the manufacturer's recommendations, and sequenced by using a BigDye Terminator cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) on ABI 310 automated sequencer. Sequences from other sponges deposited in GenBank, from Tetractinomorpha orders, and 3 genera of Halichondrida were used to draw the phylogenetic identity of these morphotypes within the Halichondriidae. Sequences were aligned using a sequence alignment program ClustalX (1.64b) (THOMPSON *et al.,* 1994, 1997). Distance analysis and Maximum-Parsimony and Maximum-likelihood (ML) analysis were applied to the aligned sequences using PAUP 4.0 b10 (SWOFFORD, 2001).

RESULTS

Morphology

Growth form is thickly and broadly encrusting. They were found in exposed areas, tide pools or narrow crevices between rocks. The orange type is bright reddish

orange (C 10 - 30, M 80, Y 80 - 100, K 0) with soft texture (Fig. 1a), and the yellow type is yellowish orange (C 0 - 10, M 30 - 60, Y 80 - 100, K 0) with relatively tough texture (Fig. 1e).

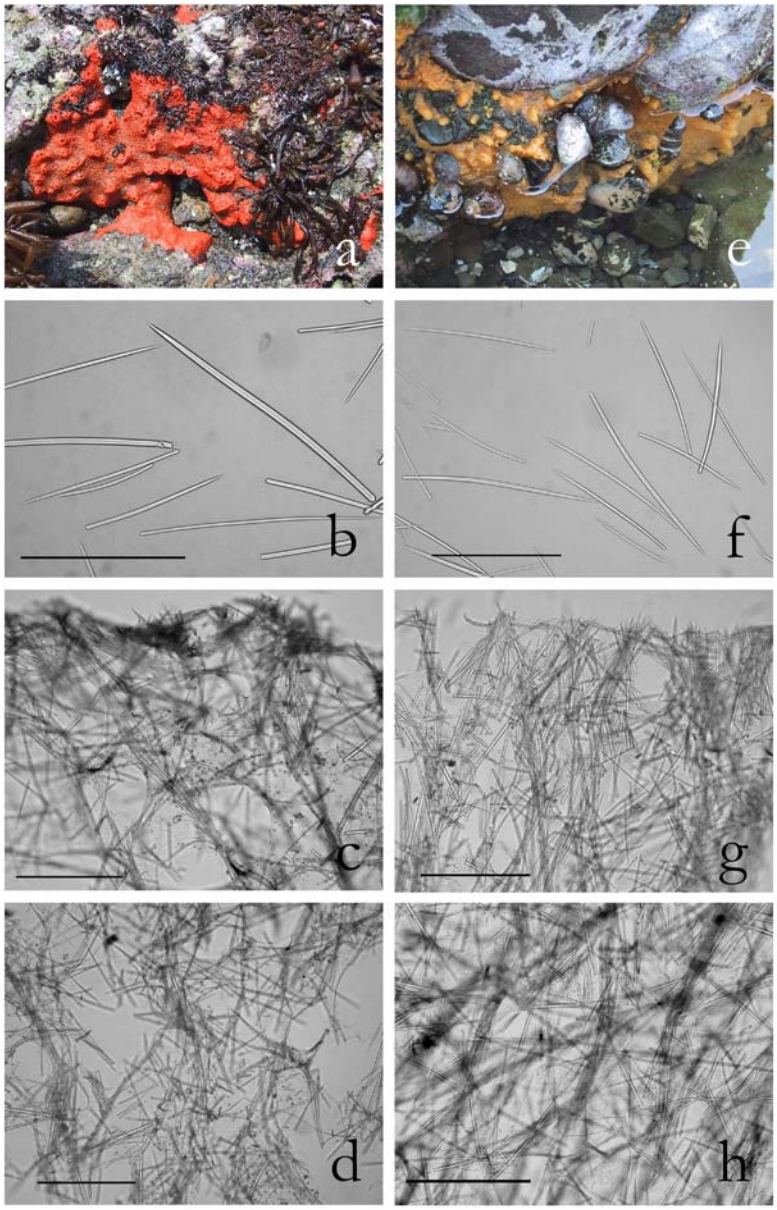

Fig. 1. Orange (**a-d**) and yellow (**e-h**) types of *Halichondria japonica*. **a** and **e,** specimens in the field. **b** and **f,** spicules. **c** and **g,** spicule arrangement in the ectosome. **d** and **h,** spicule arrangement in the endosome. Scale bars for b and f = 200 μm; c, d and g, h = 300 μm.

In both types, oscula (1 - 1.5 mm in diameter) are opened on each apex of conical or cylindrical processes (0.5 - 2 cm in height) that are irregularly scattered on the surface. The spiculation consists of only styles which are straight or slightly curved, 140.0 - 370.0 μm long and 2.4 - 10.1 μm wide for the orange type (Fig. 1b), and 142.5 - 374.7 μm long and 2.5 - 10.5 μm wide for the yellow type (Fig. 1f). The ectosome consists of thin membrane containing a few rows of styles arranged in a loose reticulation. In the endosome, a few or several rows of styles form tracts, which are arranged perpendicularly to the ectosomal membrane (Figs 1c, d, g, h). As for spicule morphology and its arrangement, no distinct differences between the two types were found.

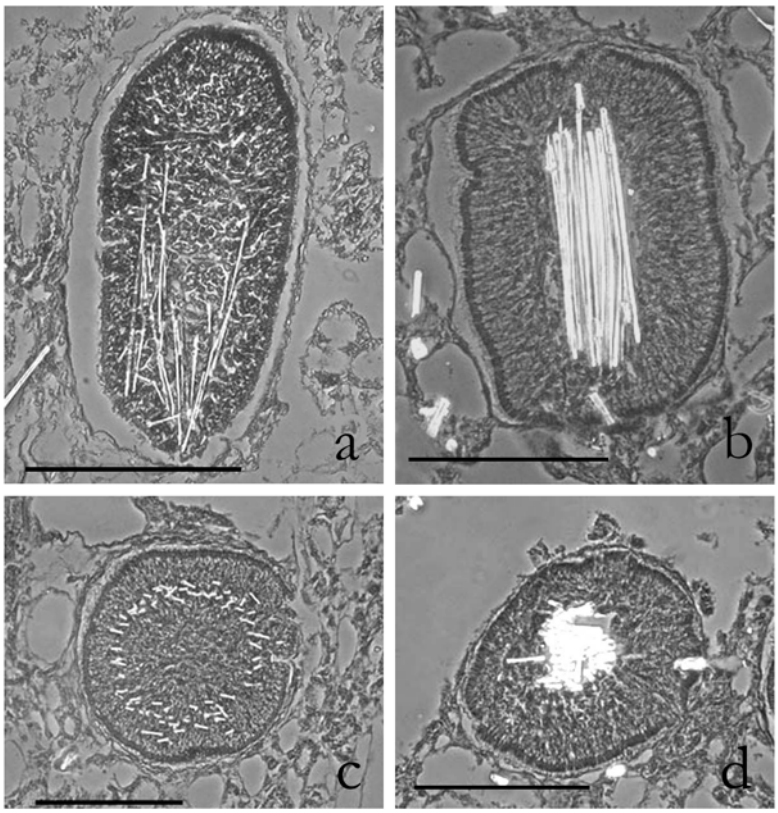

Fig. 2. Larvae of orange (**a, b**) and yellow (**c, d**) types. **a** and **c,** Longitudinal section. **b** and **d,** Cross section. Scale bar = 100 μm.

Preliminary results of developmental study

It is quite clear that larvae of the two types are parenchymella with spicules. Their spicule arrangements differ from each other. Both types reproduce during summer, from late May to early August in central Japan. Fig. 2 shows cross and longitudinal

sections of the orange and yellow type's larvae, respectively. In the orange type, spicules are arranged circularly on the cross section, and radially from the posterior side on the longitudinal section, while in the yellow type, they are accumulated in the center on both cross and longitudinal sections.

DNA analysis

Partial sequences of 28S rDNA were first analyzed to select the taxa for the analysis of ITS2, because the ITS2 sequences are too variable to align between distantly related taxa. Then sequences of ITS2 region were analyzed.

Fig. 3a shows the neighbour-joining tree reconstructed from the partial sequences of 28S rDNA. A maximum likelihood analysis provided a similar topology. Each morphotype studied constitutes a monophyletic clade, and each clade is supported by a high bootstrap value. They cluster with *Hymeniacidon* sequences, and not *Halichondria* sequences.

Fig. 3b shows the neighbour-joining tree derived from the distance analysis of the ITS2 sequences. A larger number of each type's samples was used in the ITS2 analysis. Both orange and yellow types constitute monophyletic clades and both clades are supported by very high bootstrap values. A maximum likelihood analysis also provided the same topology as for the two types.

DISCUSSION AND CONCLUSIONS

Each morphotype was clearly distinguished by its color in life, reddish orange and yellowish orange. However distinct differences between them could not be found for spicules and their arrangements. Their larvae, a parenchymella for the two types, had a very distinct spicule arrangement between the two types. DNA analysis reveals that both with 28S ribosomal DNA and ITS2 sequences each morphotype constitutes a solid monophyletic clade supported by a high bootstrap value. We concluded that the orange and yellow types represent two distinct species.

The skeletal features of these two species (only styles among their spicules, loose ectosomal skeleton and ascending spicule tracts) classify these two species into the genus *Hymeniacidon* rather than the genus *Halichondria* (*sensu* VAN SOEST *et al.,* 1990; DIAZ *et al.,* 1991).

In the DNA sequence comparison, (both for 28S and ITS2) the sequence of *Hymeniacidon heliophila*, obtained from Genbank, is identical with some specimens of the orange type. Therefore, the results of DNA analysis also support that both orange and yellow types should be referred to the genus *Hymeniacidon*.

The color in life of *Halichondria japonica* was recorded as bright orange in the original description. Therefore we propose that the orange morphotype should be referred to as *Hymeniacidon japonica* (Kadota, 1922).

H. japonica and *H. heliophila* are very similar to each other, in the external morphology, live color, spicules and their arrangement. The results of DNA sequence comparison and their morphological similarity suggest that these two are conspecific. However, the specific assignation of the present species must await further taxonomic comparison with other *Hymeniacidon* species within the Pacific Fauna.

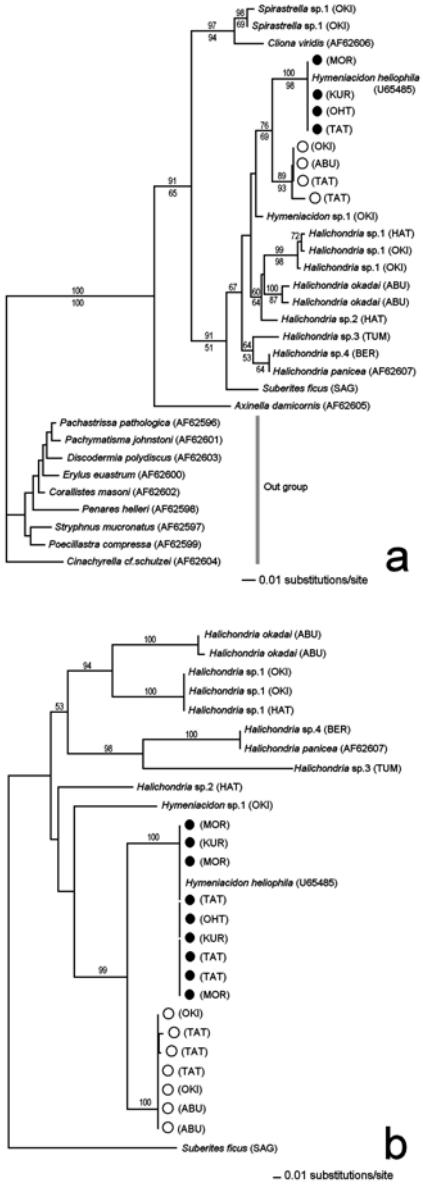

Fig. 3. Orange type (●) and yellow type (○). Sampling locality or accession number of GenBank are indicated in parentheses. **a,** the neighbour-joining tree reconstructed from partial sequences of 28S rDNA. Percentage of 2000 bootstrap replicates supporting a branching pattern is given above the tree. Numbers below branches are percentages of 1700 bootstrap replicates by ML analysis. **b,** The neighbour-joining tree reconstructed from ITS2. The results of 2000 NJ bootstrap replicates are shown above the branches. Distances were calculated by using the Kimura 2-parameter method.

ACKNOWLEDGEMENTS

We would like to thank all the members of the Division of Mammals and Birds, Department of Zoology, National Science Museum, Tokyo, for technical support for DNA analysis. Special thanks are due to Prof. Rob W.M. van Soest, Zoologisch Museum, University of Amsterdam, for his useful suggestions.

REFERENCE

CHOMBARD C., BOURY-ESNAULT N., TILLIER S., 1998 - Reassessment of Homology of Morphological Characters in Tetractinellid Sponges Based on Molecular Data. *Syst. Biol.,* **47** (3): 351-366.

DIAZ M.C., SOEST R.W.M. VAN, POMPONI S.A., 1991 - A Systematic Revision Of The Central Atlantic Halichondrids (Demospongiae, Porifera). Part I: Evaluation Of Characters And Diagnosis Of Genera. In J. Reitner, H. Keupp (eds), *Fossil and Recent Sponges*. Springer-Verlag, Berlin, Heidelberg: 134-149

HOEZEL A.R., 1998 - Molecular Genetic Analysis of Populations. A practical approach. Oxford University Press, Oxford, New York, Tokyo, 445 pp.

HOSHINO T., 1980 – A guide to identification of principal fouling organism. (4) Sponges (Calcareous sponges and Demosponges). *Mar. Fouling,* **2**: 53-58. in Japanese

KADOTA J., 1922 - Observations on two new species of the Genus *Reniera* of monoaxonid sponges *Zool. Mag. Tokyo,* **34**: 700-711, pls. 20, 21. in Japanese.

SOEST R.W.M. VAN, DIAZ M.C., POMPONI S.A., 1990 - Phylogenetic Classification Of The Halichondrids (Porifera, Demospongiae). *Beaufortia,* **40** (2):15-62.

SWOFFORD D.L., 2001 - 'PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0.' Sinauer Associates, Sunderland, Massachusetts, USA.

THOMPSON J.D., HIGGINS D.G., GIBSON T.J., 1994 - CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.,* **22**: 4673-4680.

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 Res.,* **25**: 4876-4882.

UTINOMI T., 1962 - Coloured Illustrations of Seashore Animals of Japan. Hoikusha Publ. Co., Ltd., Osaka, Japan, 168 pp. in Japanese.