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# Minute Protrusions of Ascidian Tunic Cuticle: Some Implications for Ascidian Phylogeny

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**ABSTRACT**—Fine structure of the ascidian tunic cuticle was studied by transmission and scanning electron microscopy in 26 species belonging to 10 families. In 11 species of 8 families, the cuticular surface is ornamented with numerous minute protrusions that are papillate in shape and usually up to 100 nm in height. The present results in addition to the previous ones [1] gave us more information about morphology of protrusions and their distribution in 51 species ranging over 13 families out of the 15 ones of ascidians. It reveals a general tendency that the protrusions occur in the limited families and related species have the protrusions of similar size. Thus, the protrusions proved to have some features of certain phylogenetic significance in many cases. Brief references were made to the taxonomic position of such problematic genera as *Pterygascidia* in the Cionidae and *Sorbera* in the Hexacrobylidae on the basis of the present study.

## INTRODUCTION

The tunic is a gelatinous or leathery integument which is peculiar to the animals belonging to the subphylum Urochordata (=Tunicata). The ascidian tunic is composed of ground substance (tunic matrix) overlaid by a thin cuticle. The surface of the tunic cuticle is sometimes, though never always, specialized to have numerous minute protrusions, which are mostly papillate in shape and up to 100 nm high. Such a structure was firstly reported by Katow and Watanabe [2] and Milanesi *et al.* [3] in two species of the family Botryllidae. Then, Hirose *et al.* [1] detected the protrusions in 18 species of ascidians belonging to 5 families, out of the 25 species of 9 families, and they suggested that the protrusions have some implications for ascidian phylogeny.

To examine this suggestion closely, we should have more information. In this study, fine structure of tunic cuticle was newly described in 26 species of 10 families. Thus, we now get information about the protrusions from 51 species ranging over 13 families, although not yet examined specimens of the two remaining families (Octacnemidae and Plurellidae). On the basis of the information, we will give a comment on the phylogenetic significance of the stated structure.

## MATERIALS AND METHODS

### Animals and prefixation

Specimens of *Leptoclinides echinatus*, *Clavelina viola*, *Ascidia ahodori*, *Ascidia zara*, *Ascidia* sp. (cf. *tapni*) and *Pyura mirabilis* were collected in Shimoda, Shizuoka Pref., and fixed in a solution containing 2.5% glutaraldehyde, 0.45 M sucrose and 0.1 M cacodylate (pH 7.4). *Ascidia gemmata* and *Chelyosoma siboja* were collected in Asamushi, Aomori Pref., and fixed in 2.5% glutar-

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aldehyde-seawater. Specimens of the following species were fixed in 10% formol-seawater or 10% formol-water: *Syndiazona grandis* collected off Minabe, Wakayama Pref.; *Adagnesia vesiculiphora* off Fukushima Pref.; *Corella* sp. (cf. *japonica*) in Kuroshima, Okinawa Pref.; *Eugyrioides glutinans* off Oga Pen.; *Molgula manhattensis* in the Nagoya Harbor, Aichi Pref.; *Molgula tectiformis* in the Mutsu Bay; *Chelyosoma yezoense* and *Cnemidocarpa clara* from Otsuchi, Iwate Pref.; *Cnemidocarpa irene, Polycarpa cryptocarpa kroboja* and *Polycarpa maculata* taken off the Oki Island, Shimane Pref.; *Ciona edwardsi* and *Ciona roulei* [cf. 4] collected around Banyuls-sur-Mer (France); *Ciona intestinalis* in Napoli (Italy); and *Sorbera unigonas* during the Iucal cruise from N. Atlantic (47°30′N, 9°35′W; 4217–4366 m deep). *Pterygascidia longa* was collected off Zamami, Okinawa, and fixed in 70% ethanol. The paratype specimens of *Polyandrocarpa stolonifera,* fixed in 70% ethanol, are deposited in the Shimoda Marine Research Center.

### *Electron microscopy*

Samples of tunic, several millimeters square in size, were cut off from the above-listed specimens. Samples from the specimens in the fixative containing cacodylate were washed with 0.45 M sucrose solution buffered with 0.1 M cacodylate (pH 7.4), while samples from the specimens in the fixatives without cacodylate were washed with filtrated seawater. Then, the samples were post-fixed with 0.1% osmium tetroxide solution buffered with 0.1 M cacodylate (pH 7.4) for 1.5 hours, and afterward, dehydrated through an ethanol series. For scanning electron microscopy (SEM), they were dried in a critical point dryer, sputter-coated with Au-Pd, and examined by Hitachi S-570 scanning electron microscope at 15 to 20 kV. For transmission electron microscopy (TEM), they were cleared in n-butyl glycidyl ether, and embedded in a low viscosity resin [5]. Thin sections were doubly stained, and examined by Hitachi HS-9 transmission electron microscope at 75 kV.

## RESULTS

Tunic cuticle is an electron dense layer covering tunic matrix, and its structure varies from species to species. In some species, the cuticle forms minute protrusions. Sometimes there is a layer of moderate electron density or a zone of the mixture of tunic matrix and cuticular material under the cuticle, and it is called "subcuticle" by De Leo *et al.* [6]. Subcuticle is almost indistinguishable from the cuticle, when they are both of high electron density and thick, as seen in some solitary species in the Corellidae and Styelidae. Thickness of the cuticle and subcuticle is revealed variable in different species, and in different parts of tunic in a specimen. When the cuticle and/or subcuticle are thick, the tunic is usually hard, and leathery or cartilaginous. The protrusions shown in the present study are papillate in shape without exception.

In the suborder Aplousobranchia, two species are studied. In *Leptoclinides echinatus* the cuticle is flat and thin, about 10 nm thick, and its surface shows a fuzzy appearance (Fig. 1A). In *Clavelina viola* the cuticular surface has minute protrusions, about 30–40 nm high (Fig. 1B).

In the suborder Phlebobranchia, only 2 species out of the 13 studied species have minute protrusions. In the family Cionidae, *Syndiazona grandis* and three *Ciona* species have a flat cuticle accompanied with a subcuticle, but no protrusions (Fig. 1C). Thickness of the subcuticle ranges from about 30 nm (in *C. edwardsi*) to more than 300 nm (in *C. roulei*). On the other hand, *Pterygascidia longa* of the same family has minute cuticular protrusions, about 60 nm in height (Fig. 1D). In the Ascidiidae, the four species of *Ascidia* have a flat cuticle without any protrusions; its thickness is about 20–30 nm (in *A. ahodori, A.* sp. (cf. *tapni*) and *A. zara*) or more than 100 nm (in *A. gemmata*). In the Agnesiidae, *Adagnesia vesiculiphora* has minute cuticular protrusions, only about 20 nm high (Fig. 1E). In the Corellidae, all the three species examined here have no minute protrusions. *Corella* sp. (cf. *japonica*) has a thin cuticle, only about 30 nm, while the two species of *Chelyosoma* have a thick one, indistinguishable from the underlying subcuticle (Fig. 1F).

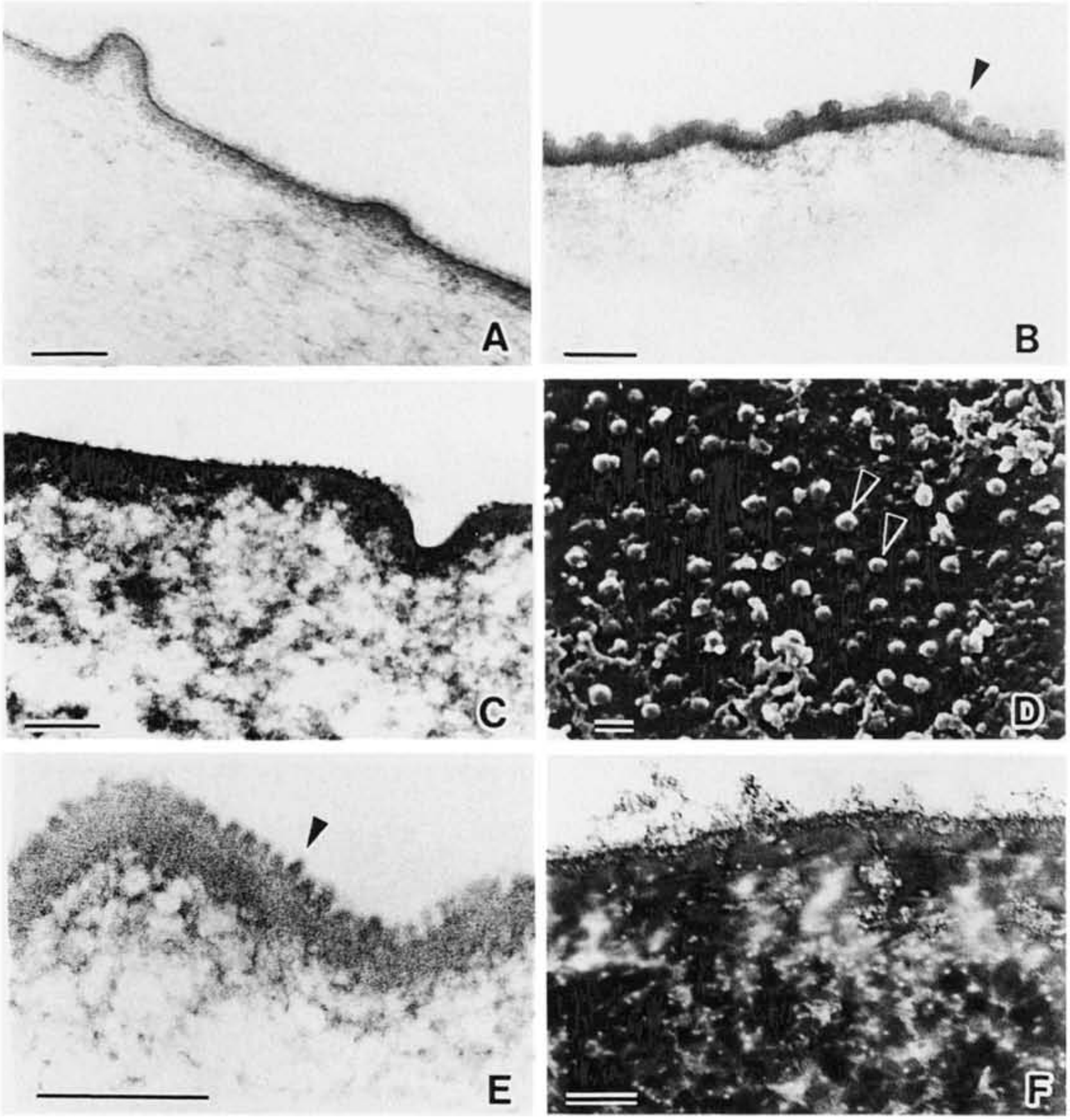
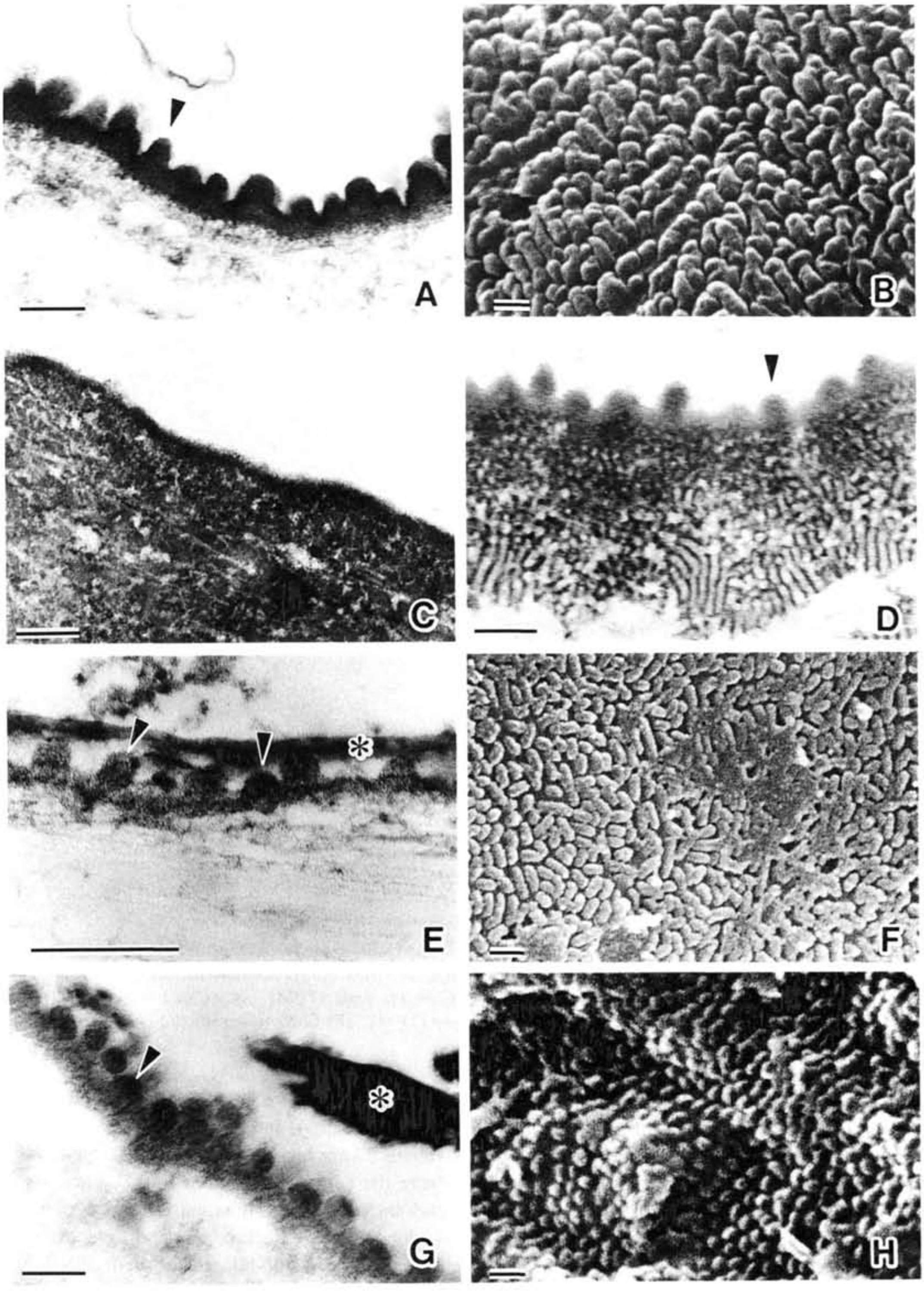


FIG. 1. Fine structure of the tunic cuticle in the order Enterogona. Tunic matrix positions in lower part of each figure in TEM. (A) *Leptoclinides echinatus* (TEM). (B) *Clavelina viola* (TEM). (C) *Ciona roulei* (TEM). (D) *Pterygascidia longa* (SEM). (E) *Adagnesia vesiculiphora* (TEM). (F) *Chelyosoma siboja* (TEM). Arrowheads indicate minute protrusions. Scale bars, 0.2 μm.

In the suborder Stolidobranchia, the minute cuticular protrusions are found in all the examined species excluding some solitary ones in the Styelidae. A strange styelid, *Polyandrocarpa stolonifera,* which propagates by stolonic budding but without any functional connection among the zooids [see 7], has the protrusions, 100 nm or less in height. Among the 6 examined solitary styelids, *Cnemidocarpa clara* has the protrusions about 100 nm (Fig. 2A, B), and *Styela clava* has smaller ones (about 30 nm high) and a thick subcuticle underlying the cuticle. The other four solitary styelids lack the protrusions; the tunic is leathery or cartilaginous, with the thick cuticle and subcuticle (Fig. 2C). In *Pyura mirabilis* of the Pyuridae, minute protrusions are about 100 nm high, and the sub-


A
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cuticle has a complex structure (Fig. 2D). In the Molgulidae, the protrusions are found in all of the 3 examined species. They are about 40–50 nm high (in *Molgula manhattensis* and *M. tectiformis*) or 30 nm (in *Eugyrioides glutinans*) (Fig. 2E, F), and they are rather lower than those seen in the other species in the Stolidobranchia.

In the suborder Aspiraculata, we could examine only a signle species, *Sorbera unigonas*. The cuticle has minute protrusions, about 80 nm in height (Fig. 2G, H). The protrusions are similar in size and shape to those usually seen in the Stolidobranchia.

## DISCUSSION

Table 1 represents a summation of the present results and the previous ones [1] in terms of the presence or absence and height of the minute cuticular protrusions. So far as these results are

TABLE 1. Presence and height of minute cuticular protrusions of ascidian tunic

| Species | Solitary (S) or Colonial (C) | Approx. height of protrusions (nm) |
|---|---|---|
| Order Enterogona | | |
| Suborder Aplousobranchia | | |
| Family Polyclinidae | | |
| *Aplidium pliciferum* | C | 30 |
| *A. yamazii* | C | 60 |
| Family Didemnidae | | |
| *Diplosoma mitsukurii* | C | absent |
| *Didemnum moseleyi* | C | absent |
| **Leptoclinides echinatus* | C | absent |
| Family Polycitoridae | | |
| *Clavelina miniata* | C | 30 |
| **C. viola* | C | 30–40 |
| *Polycitor proliferus* | C | 40 |
| Suborder Phlebobranchia | | |
| Family Cionidae | | |
| **Syndizona grandis* | C | absent |
| *Ciona savignyi* | S | absent |
| **C. edwardsi* | S | absent |
| **C. intestinalis* | S | absent |
| **C. roulei* | S | absent |
| **Pterygascidia longa* | S | 60 |
| Family Perophoridae | | |
| *Perophora japonica* | C | absent |
| *P. multiclathrata* | C | absent |
| Family Ascidiidae | | |
| *Ascidia sydneiensis* | S | absent |
| **A. ahodori* | S | absent |
| **A. gemmata* | S | absent |
| **A. zara* | S | absent |

FIG. 2. Fine structure of the tunic cuticle in the order Pleurogona. (A, B) *Cnemidocarpa clara* (A: TEM, B: SEM). (C) *Cnemidocarpa irene* (TEM). (D) *Pyura mirabilis* (TEM). (E) *Molgula manhattensis* (TEM). (F) *Molgula tectiformis* (SEM). (G, H) *Sorbera unigonas* (G: TEM, H: SEM). Arrowheads indicate minute protrusions, asterisks debris. Scale bars, 0.2 μm.

TABLE 1. (continued)

| Species | Solitary (S) or Colonial (C) | Approx. height of protrusions (nm) |
|---|---|---|
| **A.* sp. (cf. *tapni*) | S | absent |
| Family Agnesiidae | | |
| **Adagnesia vesiculiphora* | S | 20 |
| Family Corellidae | | |
| **Chelyosoma siboja* | S | absent |
| **C. yezoense* | S | absent |
| **Corella* sp. (cf. *japonica*) | S | absent |
| Order Pleurogona | | |
| Suborder Stolidobranchia | | |
| Family Botryllidae | | |
| *Botryllus primigenus* | C | 100 |
| *B. scalaris* | C | 100 |
| *B. sexiens* | C | 100 |
| *B. schlosseri* | C | 100 |
| *Botrylloides fuscus* | C | 100 |
| *B. lentus* | C | 100 |
| *B. simodensis* | C | 100 |
| *B. violaceus* | C | 100 |
| Family Styelidae | | |
| *Metandrocarpa uedai* | C | 100 |
| *Polyandrocarpa misakiensis* | C | 100 |
| **Polyandrocarpa stolonifera* | C | 100 |
| *Polyzoa vesiculiphora* | C | 100 |
| Symplegma reptans | C | 100 |
| **Cnemidocarpa clara* | S | 100 |
| **C. irene* | S | absent |
| **Polycarpa cryptocarpa kroboja* | S | absent |
| **P. maculata* | S | absent |
| *Styela plicata* | S | absent |
| **S. clava* | S | 30 |
| Family Pyuridae | | |
| *Halocynthia roretzi* (Type A) | S | 50[a] |
| *Herdmania momus* | S | 100 |
| **Pyura mirabilis* | S | 100[b] |
| Family Molgulidae | | |
| **Eugyrioides glutinans* | S | 30 |
| **Molgula manhattensis* | S | 40–50 |
| **M. tectiformis* | S | 40–50 |
| Suborder Aspiraculata | | |
| Family Hexacrobylidae | | |
| **Sorbera unigonas* | S | 80 |

* species firstly examined for the protrusions in this study.

[a] protrusions are not papillate.

[b] subcuticle has complex structure.

concerned, the shape of the protrusions is always papillate only except in *Halocynthia roretzi,* which has irregular-shaped ones [1]. This table shows clearly such a general tendency that the protrusions occur in the limited families and the related species have the protrusions of similar size. Thus, we may reasonably conclude that the mentioned features are of certain phylogenetic significance. On the other hand, we cannot make any references to the function of the protuberances.

In the suborder Aplousobranchia, the protrusions are found in the Polyclinidae and Polycitoridae. The protrusions are about 50 nm or less in height. In contrast, no protrusions are found in the Didemnidae.

In the suborder Phlebobranchia, most species lack the protrusions. Exceptionally, however, we could find them in *Pterygascidia longa* in the Cionidae and *Adagnesia vesiculiphora* in the Agnesiidae. This fact reminds us of Kott's longheld opinion that "Ciallusiinae" represented by the genus *Pterygascidia* should be included as a subfamily in the family Agnesiidae, which contains *Adagnesia* as a member of the other subfamily Agnesiinae [ref., 8]. Kott [9] paid attention to the similarity in the mantle musculature between these two subfamilies delimited by herself (for discussions on the taxonomic position of *Pterygascidia* in terms of macroscopical morphology, see Tokioka [10]). Further examinations of Kott's opinion are expected on the basis of more information about the protrusions from the related species.

Among the 25 examined species of the suborder Stolidobranchia, only the four solitary species in the Styelidae lack the protrusions. It seems to be curious that *Cnemidocarpa clara* and *Styela clava* have the protrusions, while the respective congeners *C. irene* and *S. plicata* lack them. It should be noted that such inconsistencies are limited to the subfamily Styelinae in the Styelidae. The lack of the protrusions in the four species may be related to the fact that the cuticle is rather thicker in these species than the others in this suborder. In the other stolidobranchians, the protrusions are papillate and about 100 nm high. Exceptionally, they are of smaller size in *Styela clava* and the three molgulid species, or are not papillate in *Halocynthia roretzi.* It is unclear about the phylogenetic significance of these exceptions at present.

There is a hot controversy on the systematic position of the family Hexacrobylidae in the suborder Aspiraculata. Monniot *et al.* [11] separated this family from the class Ascidiacea and established for it a new class Sorberacea of the subphylum Urochordata (=Tunicata). The reason for this treatment was that they considered such features of this family to be quite unique among tunicates, as the virtual lack of the branchial sac, the existence of the "cordon nerveux dorsal", and the histological peculiarities of the gut [also see 12]. On the contrary, Kott has been holding the opinion that the family Hexacrobylidae should be regarded as very closely related (or possibly even joinable) to the family Molgulidae of the suborder Stolidobranchia and therefore never mertis a different suborder. Kott [13] regarded the unique features of this group mentioned by Monniot *et al.* as due merely to a high adaptation to its deep-sea life, and claimed that its basic morphological features were shared with the Molgulidae, such as the presence of a kidney, the arrangement of gonads, the thin but tough and fibrous tunic, etc. On the other hand, Nishikawa [14] supported the traditional treatment of the Hexacrobylidae as the single constituent of the suborder Aspiraculata, although acknowledging the uniqueness of this group strongly claimed by Monniot *et al.* The present study revealed that the protrusions found in *Sorbera unigonas* were similar in shape and size to those of the stolidobranchian species rather than of the aplousobranchian and phlebobranchian ones. This fact may favor Kott's view, but it does not necessarily exclude the other two taxonomic treatments if we regard the mentioned similarity as a convergent nature. On the other hand, a closer examination shows that the protrusions of *S. unigonas* are much higher than those of the examined species in the Molgulidae (about 80 nm in the former, instead of 30–50 nm in the latter). This might suggest the somewhat distance of phylogenetic position between *Sorbera* and the Molgulidae. Accordingly, we prefer the tranditional systematic treatment of this group at present. More information should be gained as to the fine structure of tunic in some other species of the Molgulidae and Hexacrobylidae.

As shown above, some features seen in the fine structure of tunic cuticle have certain phylogenetic significance. We expect that more attentions will be paid to the fine structures in terms of the plylogeny of not only ascidians but also the other tunicates.

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