Acid Containers and Cellular Networks in the Ascidian Tunic with Special Remarks on Ascidian Phylogeny

Author: Hirose, Euichi

Source: Zoological Science, 18(5) : 723-731

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.723

ZOOLOGICAL SCIENCE **18**: 723–731 (2001)

Acid Containers and Cellular Networks in the Ascidian Tunic with Special Remarks on Ascidian Phylogeny

Euichi Hirose*

Department of Chemistry, Biology & Marine Science, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

ABSTRACT—Tunic is a unique integument that contains cellulosic components and various types of free cells (tunic cells), and this tissue is exclusively found in the subphylum Urochordata (= Tunicata). In order to discuss the ascidian phylogeny, the presence or absence of the two characteristic types of tunic cells (tunic bladder cells and the tunic net cells) are examined in 65 ascidian species covering 11 families out of the 15 recognized ones. The tunic bladder cells are exclusively distributed in the species of Didemnidae, Holozoinae, Diazoninae, and Ascidiidae. The species of Corellidae have hemocytes, giant cells, that are very similar to the bladder cells, but these cells are not distributed in the tunic. The tunic pH of these species is usually acidic, because the tunic bladder cells contain strong acid in the vacuoles. The tunic net cells are found in Polyclinidae, Polycitorinae, and Didemnidae. The tunic rounding assay suggests that the tunic net cells may be involved in the contraction of the tunic at least in the species of Polyclinidae and Polycitorinae. If these types of tunic cells can be considered as synapomorphic characters, the character-state distribution suggests that the suborders Phlebobranchia and Aplousobranchia are not monophyletic groups.

INTRODUCTION

Tunicates (=urochordates) always produce extracellular matrix containing cellulose fibers outside the epidermis (De Leo *et al*., 1977; Van Daele *et al*., 1992; Okamoto *et al*., 1996; Hirose *et al*., 1999; Kimura *et al*., 2001). While the matrix forms feeding apparatus, called house, in appendicularians, it forms a leathery or gelatinous integument, called tunic, in the other tunicates (ascidians and thaliaceans). Tunic and house are very unique tissues among metazoans because of the cellulosic components, and they can be regarded as a synapomorph of tunicates. Therefore, the comparative study of the tunic morphology and functions would be important to discuss the tunicate phylogeny. For instance, ultrastructural studies of the tunic cuticle revealed the general stability of the character-state distribution (presence or absence) of the tunic cuticular protrusions within families or subfamilies in the class Ascidiacea (Hirose *et al*., 1997).

In ascidians, various types of cells (tunic cells) are distributed within the tunic, and they are involved in some events in the tunic, such as, pigmentation, phagocytosis, bioluminescence, photosynthetic symbiosis, and allorecognition between colonies. In this sense, the ascidian tunic is not merely a covering, but is a tissue performing various biological functions. Because the repertory of the cell types is different from species to species, the survey of the occurrence of a particular type of tunic cells would provide valuable information to discuss ascidian phylogeny.

Among various types of tunic cells, tunic bladder cells and tunic net cells have conspicuous cell morphology for their unique functions. The tunic bladder cells are large globular cells in which the bulk of cytoplasm is occupied by a large vacuole containing sulfuric acid (Cf. Goodbody, 1974; Hirose 1999; Hirose *et al*., in press). Stoecker (1978, 1980a, b, c) suggests that they are involved in chemical defense (*e.g.*, anti-predation and anti-fouling) in ascidians. The tunic net cells extend very long filopodia that contact with one another and form a cellular network in the tunic. This type of cells was referred as myocytes in *Diplosoma* species (Mackie and Singla, 1987) or elongated tunic cells in *Aplidium yamazii* (Hirose *et al*., 1994; Hirose and Ishii, 1995), and they function for conduction of impulses and for tunic contraction responding to wounding. In this study, the presence or absence of the tunic bladder cells and tunic net cells are examined in 65 species covering 11 families out of the 15 recognized ones in the class Ascidiacea. The respective 2 types of tunic cells are found in some limited groups of ascidians, suggesting some implications for ascidian phylogeny.

MATERIALS AND METHODS

Animals

The ascidians were collected in temperate and tropical shallow waters in Japan and Italy (see Table 1 for collection sites). The specimens were packed in a plastic container filled with seawater and immediately brought to the laboratory.

* Corresponding author: Tel. +81-98-895-8880;
FAX. +81-98-895-8576.
E-mail:euichi@sci.u-ryukyu.ac.jp

Table 1. Tunic pH and occurrence of tunic bladder cells and tunic net cells in ascidians

Species	Tunic pH[#]		Bladder cell	Net cell	Collection[†] site
	Electrode	Test paper			
Order Enterogona					
Suborder Aplousobranchia					
Polyclinidae					
Polyclininae					
Aplidium pliciferum	N	N	absent	present[4]	Shimoda
Aplidium yamazii	N	N	absent	present[4]	Shimoda
Aplidium sp. (Cf. *sagamiense*)	N	N	absent	present[4]	Ginowan
Aplidium sp.	N	N	absent*	present[4]	Chioggia
Polyclinum sp.	N	N	absent*	present[4]	Oh-hara
Euherdomaninae					
Pseudodistoma kanoko	N	N	absent	present[4]	Aka
Didemnidae					
Didemnum molle	A	SA	present[1]	?	Bise
Didemnum moseleyi	–	SA	present[1]	?	Shimoda
Didemnum proliferum	A	SA	present[1]	?	Shimoda
Didemnum pseudofulgens	A	SA	present*[1]	?	Chioggia
Didemnum sp.	–	N	absent	?	Shimoda
Diplosoma listrianum	SA	SA	present*	present	Chioggia
Diplosoma midori	SA	SA	present	present	Bise
Diplosoma mitsukurii	SA	SA	present	present	Shimoda
Diplosoma virens	SA	SA	present	present	Maeda
Lissoclinum bistratum	SA	SA	present	?	Bise
Leptoclinides madara	A	SA	present[1]	?	Shimoda
Tridemnum savignyi	SA	SA	present	?	Shimoda
Polycitoridae					
Polycitorinae					
Eudistoma gilboviride	N	N	absent	present[4]	Heya
Eudistoma parvum	N	N	absent	present[4]	Shimoda
Eudistoma sp. (Cf. *tokarae*)	N	N	absent*	present[4]	Oh-hara
Polycitor proliferus	N	N	absent	present[4]	Shimoda
Clavelininae					
Clavelina cyclus	–	N	absent	absent	Maeda
Clavelina lepadiformis	–	N	absent*	absent	Napoli
Clavelina miniata	–	N	absent	absent	Shimoda
Clavelina obesa	–	N	absent	absent	Bise
Clavelina sp. (Cf. *viola*)	–	N	absent	absent	Shimoda
Holozoinae					
Distaplia dubia	SA	SA	present	absent	Shimoda
Suborder Phlebobranchia					
Cionidae					
Diazoninae					
Rhopalaea sp.[$]	SA	SA	present	absent	Ginowan
Rhopalaea sp.[$]	SA	SA	present*	absent	Uehara
Cioninae					
Ciona intestinalis	N	N	absent	absent	Shimoda
Ciona savignyi	N	N	absent	absent	Shimoda
Perophoridae					
Perophora japonica	–	N	absent	absent	Shimoda
Ascidiidae					
Ascidia ahodori	SA	SA	present	absent	Shimoda
Ascidia archaia	–	SA	present	absent	Sesoko
Ascidia kreagra	SA	SA	present	absent	Gushikami
Ascidia sydneiensis	SA	SA	present	absent	Shimoda
Ascidia zara	N	N	present[2]	absent	Shimoda
Ascidiella aspersa	SA	SA	present*	absent	Sciacca

Continued to next page

Phallusia mammillata	N	N	present*[2]	absent	Napoli
Phallusia nigra	SA	SA	present	absent	Ginowan
Corellidae					
Corella japonica	SA	SA	absent*[3]	absent	Ohtsuchi
Corella sp. (Cf. *minuta*)	–	–	absent[3]	absent	Yamada
Order Pleurogona					
Suborder Stolidobranchia					
Botryllidae					
Botryllus primigenus	N	N	absent	absent	Shimoda
Botryllus scaralis	N	N	absent	absent	Shimoda
Botrylloides leachi	–	N	absent*	absent	Chioggia
Botrylloides simodensis	–	N	absent	absent	Shimoda
Styelidae					
Polyzoinae					
Metandrocarpa uedai	–	–	absent	absent	Shimoda
Polyandrocarpa misakiensis	–	–	absent	absent	Shimoda
Polyandrocarpa zorritensis	–	–	absent	absent	Shimoda
Polyzoa vesiculiphora	–	–	absent	absent	Shimoda
Symplegma reptance	N	N	absent	absent	Shimoda
Styelinae					
Cnemidocarpa Irene	N	N	absent	absent	Shimoda
Polycarpa cryptocarpa kuroboja	N	N	absent	absent	Shimoda
Polycarpa sp. (Cf. *reniformis*)	N	N	absent	absent	Maeda
Styela clava	N	N	absent*	absent	Ohtsuchi
Styela plicata	N	N	absent	absent	Shimoda
Pyuridae					
Halocynthia roretzi (C type)	N	N	absent*	absent	Ohtsuchi
Hartmeyeria orientaris	N	N	absent	absent	Shimoda
Herdmania momus (soft-type)	N	N	absent	absent	Shimoda
Pyura vittata	N	N	absent	absent	Shimoda
Pyura sp. (Cf. sacciformis)	N	N	absent	absent	Ginowan
Microcosmus sp. (Cf. *exasperatus*)	N	N	absent	absent	Ginowan
Microcosmus sp. (Cf. *savignyi*)	N	N	absent*	absent	Sciacca
Molgulidae					
Molgula manhattensis	N	N	absent*	absent	Chioggia
Molgula tectiformis	N	N	absent*	absent	Ohtsuchi

[#]: SA, < pH 3; A, pH 3–pH 6;N, > pH 6; –, not measured.
*: Tunic acid is stained with neutral red
[?]: Presence of tunic net cells could not be confirmed due to the spicules densely distributed.
[1]: Tunic bladder cells are not abundant, and usually form monolayer just beneath the tunic surface.
[2]: Vacuolar content of tunic bladder cells is not acidic.
[3]: Hemocytes (giant cells) contain strong acid.
[4]: Tunic shrinkage occurs.
[†]: Aka, Okinawa, Japan; Bise, Okinawa, Japan; Chioggia, Venezia, Italy; Ginowan, Okinawa, Japan; Gushikami, Okinawa, Japan; Heya, Okinawa, Japan; Maeda, Okinawa, Japan; Napoli, Italy; Oh-hara, Iriomote, Japan; Ohtsuchi, Miyagi, Japan; Sciacca, Sicilia, Italy; Sesoko, Okinawa, Japan; Shimoda, Shizuoka, Japan; Uehara, Iriomote, Japan.
[$]: These *Rhopalaea* species are probably the same species.

pH measurement

The pH in the tunic was measured with the needle-tip pH microelectrode (Orion Res. Inc., Massachusetts) or pH test paper (timol blue and phenol red; Advantec, Tokyo). The electrode was inserted into the tunic or the colonies, and the tunic specimens or the colonies were bruised on the pH test paper. The pH measurement could not conducted in some species that have a very thin tunic.

Microscopy

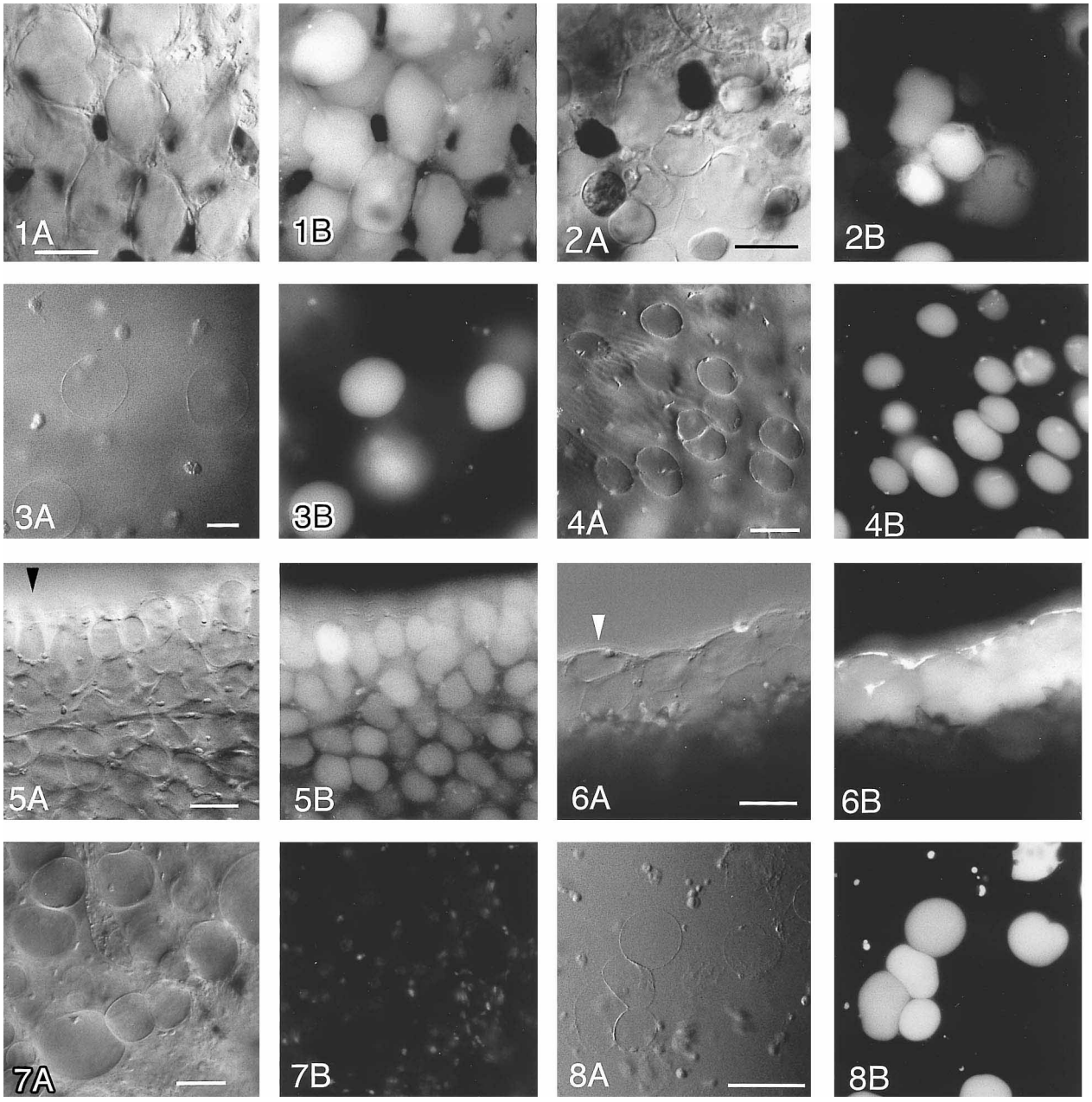
The unfixed pieces of tunic or colony were sectioned with a razor blade by hands. The sections (about 0.5 mm thick or less) were mounted with seawater and observed under an epifluorescence microscope equipped with Nomarski differential interference contrast optics. Some specimens were vitally stained with 10 μM LysoSensor Yellow/Blue DND60 (Molecular Probes Inc., Eugene, Oregon) in seawater for 30 min or more. The specimens were observed with violet light (380–425 nm) excitation. This dye is a fluorescent pH indicator that accumulates in acidic organelles. The color of the fluorescence that is emitted is pH dependent–predominantly yellow in acidic organelles and blue in less acidic organelles. The intensity of the fluorescence is also pH dependent: the intensity is stronger in a more acidic environment, whereas no fluorescence is emitted in neutral or basic environments. In the species collected in Italy, Ohtsuchi (Miyagi, Japan), and Iriomote (Okinawa, Japan), neutral red diluted in seawater (ca. 0.01%) was used for the vital staining of acidic organelles instead of LysoSensor.

Tunic rounding assay

This assay was carried out to assess the contractility of the tunic in some species that have tunic net cells. The colonies were sectioned into about 0.5 mm thick with a razor blade, and the fragments of zooids in the tunic slices were removed with forceps. Then, the tunic slices were placed in a petri dish filled with seawater, and incubated at room temperature for 12–24 hr.

RESULTS

The present study covers 65 species of ascidians, but the tunic pH could not be measured in some species because their tunic was too thin. Due to the unavailability of the live specimens, I could not examine any species of Agneziidae,



Figs. 1–8. Pair-images of the tunic slice vitally stained with LysoSensor (A, Nomarski differential interference contrast; B, epifluorescence). The specimens in Fig. 1–7 contain the tunic bladder cells. Scale bars, 20 μm for Fig 1, 2, 3, and 6; 50 μm for Fig 4, 5, 7, and 8.
Fig. 1. *Tridemnum savignyi.*
Fig. 2. *Distaplia dubia.*
Fig. 3. *Rhopalaea* sp.
Fig. 4. *Ascidia ahodori.*
Fig. 5. *Diplosoma virens*, a non-spiculate didemnid. There are many layers of the tunic bladder cells beneath the tunic surface (arrowhead).
Fig. 6. *Didemnum moseleyi*, a spiculate didemnid. There are only a few layers of the tunic bladder cells beneath the tunic surface (arrowhead).
Fig. 7. *Ascidia zara.* Fluorescence is not found in the bladders cells.
Fig. 8. Giant cells (hemocytes) of *Corella* sp.

Octacnemidae, Plurellidae, and Hexacrobylidae. The tunic pH and the presence or absence of the tunic bladder cells and tunic net cells are listed in Table 1.

Acidity of the tunic

The tunic pH was measured with a pH test paper and a needle-type pH electrode. The results from these methods, however, include some errors due to the contamination of seawater and hemolymph of the materials. In this report, the tunic pH was classified into 3 levels based on the measured value; strongly acidic (SA: <pH 3), acidic (A: pH 3–6), neutral (N: pH 6–8.5). The tunic pH was never more basic than the pH of seawater (ca. pH 8.5), so far studied.

As shown in Table 1, the tunic contains strong acid (level SA) in the species of particular taxa, *i.e.*, Didemnidae, Holozoinae, Diazoninae, Ascidiidae and Corellidae. Exceptionally, the tunic pH is almost neutral in *Ascidia zara* and *Phallusia mammillata* of Ascidiidae and *Didemnum* sp. of Didemnidae. The acidity is not so strong (level A) in some didemnids, *i.e.*, *Didemnum molle*, *D. moseleyi*, *D. proliferum*, and *Leptoclinides madara*, when the pH was measured with the pH electrode. In the other taxa, the tunic pH is almost neutral (level N) and, in most cases, slightly more acidic than seawater.

Acid containers in the tunic

Vital staining with LysoSensor Yellow/Blue or neutral red demonstrated the distribution of the acid in the tunic. In the all species having acidic tunic, the tunic contains giant, highly vacuolated cells that are often over 50 μm in diameter (Fig 1A–5A). A large vacuole occupies a great portion of the cytoplasm, and a thin layer of cytoplasm surrounds the vacuole. LysoSensor evenly stains the vacuolar lumen and emits strong yellow fluorescence (Fig. 1B–5B). Based on these characteristics, the tunic cells are concluded to be the tunic bladder cells that have been described in some ascidiids and didemnids (Cf. Goodbody, 1974). Neutral red also evenly stains the vacuolar lumen of the tunic bladder cells.

In the acidic tunic, the tunic bladder cells are usually distributed in close formation near the tunic surface and they often form a layer that is packed with the tunic bladder cells (Fig. 5). The amount of the tunic bladder cells is relatively

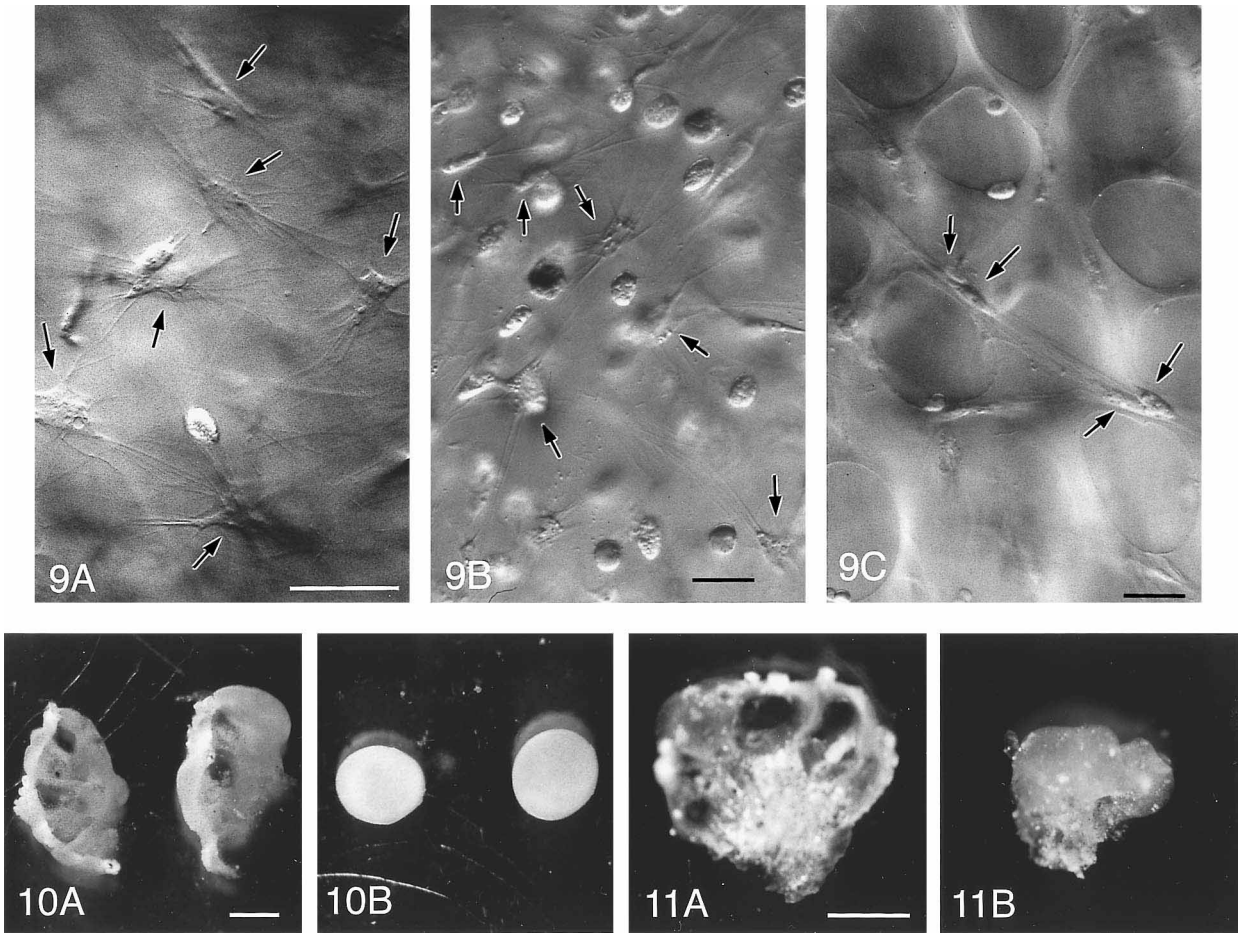


Fig. 9. Tunic net cells (arrows) in *Aplidium pliciferum* (A), *Eudistoma gilboviride* (B), *Diplosoma virens* (C). Scale bars, 20 μm.
Figs. 10 and 11. Tunic shrinkage in *Polycitor proliferus* (Fig. 10) and *Eudistoma parvum* (Fig. 11). A, Tunic slices freshly prepared. B, Tunic slices have shrunk into spheres after the incubation in seawater for 20 hr. Scale bar, 1 mm.

small in *D. molle*, *D. moseleyi*, *D. proliferum*, and *L. madara*. For instance, there are only one or two rows of the tunic bladder cells in *D. moseleyi* (Fig. 6). In these species, the acidity is relatively weak when the pH is measured with the pH electrode, and spicules are densely distributed throughout the tunic. In the neutral tunic of *Didemnum* sp., there are no tunic bladder cells, and the tunic is full of spicules. In the neutral tunic of Ascidia zara and *Phallusia mammillata*, there are many tunic bladder cells, but they do not contain acidic fluid (Fig. 7).

The tunic bladder cells were exclusively found in Didemnidae, Holozoinae, Diazoninae, and Ascidiidae. Although the tunic pH in *Corella japonica* is acidic (level SA), the vital staining with neutral red showed that there is no acid storage in the thin tunic. In *Corella japonica* and *Corella* sp. (Cf. *minuta*), large vacuolated cells are found in hemocoel, and they contain acid in the vacuoles (Fig. 8). These hemocytes are known as giant cells and their morphological characteristics are the same as those of the tunic bladder cells. The acidic pH in the tunic of *C. japonica* may be caused by the contamination of the contents of the giant cell. The giant cells are not found within the tunic in the two *Corella* species studied here.

Besides the tunic bladder cells, amoeboid cells contain round vesicles that emit blue or yellow fluorescence. The brightness of these fluorescence is much less than the fluorescence of the tunic bladder cells, indicating that the pH in the vesicles are less acidic than the pH in the vacuoles of the tunic bladder cells. Neutral red also stains some of the vesicles. This type of cells is found in the all species examined here, including non-acidic tunic, and thus, the vesicular contents have no effects on the tunic pH measured by the present method. Such fluorescent vesicles are also detected in epithelial cells of tunic vessels and some blood cells in the tunic vessels. No fluorescence was detected in acellular materials in the tunic; tunic cuticle, tunic matrix and hemolymph in the tunic vessels.

Tunic net cells and tunic rounding

Tunic net cells are exclusively found in all species of Polyclinidae and Polycitorinae, and some species of Didemnidae (Fig. 9). In spiculate didemnid species, since the spicules screen other structures in the tunic, I could not conclude whether they have tunic net cells or not. In non-spiculate didemnid, *Diplosoma* species, tunic net cells extend the filopodia through a crowd of tunic bladder cells (Fig. 9C).

Within 1-day incubation, the shrinkage of the tunic slices occurs in the species of Polyclinidae and Polycitorinae in which tunic net cells are distributed (Fig. 10 and 11). The extent of tunic shrinkage varies among species. For instance, the tunic slices of *Polycitor proliferus* are turned into spheres, whereas the tunic slices of *Aplidium* sp. (Cf. *sagamiense*) shrink in some extent but not become spheres. This assay was not performed in the species of Didemnidae, because the slicing of tunic is difficult due to the presence of spicules, and acid from the ruptured tunic bladder cells may damage the tunic net cells.

DISCUSSION

Tunic bladder cells

The tunic bladder cell is a large tunic cell in which a large vacuole occupies the bulk of the cytoplasm. This type of tunic cells had been described in several ascidians of the families Ascidiidae and Didemnidae as bladder cells, Blasenzellen, cellules vésiculeuses, and kalymmocytes (see Mackie and Singla, 1987). In the present study, the tunic bladder cell was found in the almost all species belonging to Didemnidae, Holozoinae, Diazoninae, and Ascidiidae, and it was never found in the other groups. Because the tunic bladder cell usually contains acid in the large vacuole, its occurrence is closely related with the acidity of the tunic. The tunic pH is always acidic in the species having bladder cells, except for *Ascidia zara* and *Phallusia mammillata* in which the tunic bladder cell does not contain acid in the vacuoles. In the species that do not have the bladder cells, tunic pH is always neutral, except for the species of Corellidae. In the corellid species, there is no acid storage in the tunic, but hemocytes, called giant cells, contain acid. The acidity of the tunic is supposed to be caused by the contamination of the contents of the giant cell in these species.

The acidic tunic does not mean that the pH is acidic in the any areas of the intact tunic. Vital staining with acid-sensitive dyes indicate that the pH in tunic matrix seems to be neutral, and the vacuoles of the bladder cells seems to be the only acid storage site that makes the tunic pH extremely acidic. When the pH is measured with the test paper or pH electrode, the tunic is bruised or injured by the insertion of the electrode. The measured pH is, therefore, the pH of the mixture of the aqueous components in the tunic, the surface seawater, and the acid leaked from the bladder cells. The hemolymph may be also contaminated in some cases.

The tunic acid would be an effective irritant against the predators. Moreover, Stoecker (1980b) indicated that the acid also protects the ascidians from bio-fouling. Many tunic bladder cells are always concentrated beneath the tunic surface, and this distribution pattern is consistent with the defensive function of the tunic acid. In *Phallusia nigra*, the pH of the tunic surface is also acidic when it was measured with a flat-surfaced pH electrode (Hirose *et al*., 2001). The acid should have essential functions in some species, because the bladder cells have to spend much energy to produce and maintain the acid. The bladder cells do not contain the acid in two ascidiid species, *Ascidia zara* and *Phallusia mammillata*. They would have lost the ability to produce the acid, and it may not be necessary for these species to have tunic acid for survival. In some didemnid ascidians, there are only one or two layers of the bladder cells beneath the tunic surface, while the tunic is full of spicules and there is no space for the bladder cells. In these species, the tunic pH measured with the electrodes is less acidic than other didemnids. Since the spicules have a protective function, the presence of the numerous spicules may cancel out the loss of protection due to smaller amount of the tunic acid.

Stoecker (1980b) reported the tunic pH in 35 ascidian species. Although the occurrence of acidic tunic in the limited families agree with the present results in the main, there are some inconsistencies in the subfamilies Euherdomaninae and Polycitorinae; Stoecker (1980b) reported that the tunic of *Pseudodistoma saxicavum* (Euherdomaninae), *Cystodytes dellechiajei* (Polycitorinae), and some *Eudistoma* spp. (Polycitorinae) is acidic, whereas the tunic of *Pseudodistoma kanoko* (Euherdomaninae), *Eudistoma* species (Polycitorinae), and *Polycitor proliferus* (Polycitoridae) is neutral and the bladder cells are not distributed in the tunic in the present study (see Table 1). Since Stoecker did not describe the acid storage site in the tunic, it is unknown whether these acidic species have the tunic bladder cells containing acid.

Tunic net cells

The tunic net cells extend long filopodia that connect with the filopodia of other tunic net cells, and they form a cellular network in the tunic. This type of cells was referred as myocytes in *Diplosoma* species (Mackie and Singla, 1987) or elongated tunic cells in *Aplidium yamazii* (Hirose *et al*., 1994). In the present study, tunic net cells were found only in some limited groups of aplousobranchians; Polyclininae, Euherdomaninae, Didemnidae, and Polycitorinae.

The contractility of the sliced tunic was assessed in the species having net cells, except for the didemnids. In the all species so far examined, the tunic slices always shrank during the 12–24 hr incubation in seawater, and the tunic slices became spheres in some species. The shrinkage of the sliced tunic is thought to be promoted by the contraction of the network of the tunic net cells in *Aplidium yamazii* (Hirose and Ishii, 1995), and it seems to be concerned with the wound healing of the tunic (Hirose *et al*., 1997). The comprehensive occurrence of tunic shrinkage suggests that the contractility promoting tunic shrinkage is a fundamental function of the tunic net cells. Because the extent of shrinkage was different among the species and among the specimens, the amount and distribution pattern of the net cells may be different from the species to species. Mackie and Singla (1987) indicated that the cellular network in the tunic is involved in the conduction of impulses in the tunic in *Diplosoma* species, and it is possible that the net cells in other species also have the same function.

Phylogenetic considerations

Tunic bladder cells and tunic net cells are found in the particular groups of ascidians, as described above. The present study could not cover the four families of the class Ascidiacea: Agneziidae, Plurellidae, Octacnemidae, and Hexacrobylidae. However, the character-state (presence or absence of these cell types) would be informative to discuss ascidian phylogeny, because of the general stability of the distribution within the families or subfamilies. The class Ascidiacea consists of the two orders, Enterogona and Pleurogona, and this system is supported by the 18S rDNA molecular phylogeny (Wada *et al*., 1992; Wada, 1998). Whereas tunic bladder cells and tunic net cells are found in some members of Enterogona that includes the suborder Aplousobranchia and Phlebobranchia, they are not found in Pleurogona so far studied here. Therefore, I suppose that neither tunic bladder cells nor tunic net cells would be distributed in the tunic of the common ancestors of Enterogona ascidians and Pleurogona ascidians, and these cell types are probably synapomorphs in some lineages of ascidians.

In the suborder Phlebobranchia, the tunic bladder cells are found in Diazoninae and Ascidiidae, they are not found in Cioninae, Corellidae, Perophoridae, and there are no data in Agneziidae, Octacnemidae, and Plurellidae. This indicates that Diazoninae and Ascidiidae may have a close relationship. The corellid species have giant cells as hemocytes. The giant cells are very similar in morphology to the tunic bladder cells and they also contain strong acid in their large vacuoles. Because some species of Ascidiidae are known to have the giant cells (Wuchiyama and Michibata, 1995), the family Corellidae may be related to Diazoninae and Ascidiidae. The tunic bladder cells and the giant cells may share a common precursor cells in hemocytes, while the homology of these cells should be carefully verified. Although the hemocytes named “giant cells” are also described in pyurid species (Cf. Dan-Shokawa *et al*., 1995), the pyurid’s giant cells are much different in morphology from the giant cells in Ascidiidae and Corellidae; they do not have large vacuoles containing strong acid and their cytoplasm is full of numerous small vesicles about 0.2–0.6 μm in diameter (Sawada *et al*., 1991; Ohtake *et al*., 1994). Therefore, the pyurid’s giant cells would have few relationships with the ascidiid and corellid’s giant cells and the tunic bladder cells described here.

The character state distribution is more complex in the suborder Aplousobranchia. The tunic bladder cells are found in Holozoinae of Polycitoridae and Didemnidae, whereas the tunic net cells are found in Polyclininae of Polyclinidae, Euherdomaninae of Polyclinidae, Polycitorinae of Polyclinidae and Didemnidae. Neither the bladder cells nor the net cells are found in Clavelininae of Polycitoridae. As a result, the character-states of the three subfamilies of the family Polycitoridae are different from one another. These results may suggest that the suborder Aplousobranchia may be a polyphyletic group, and the classification at the family level would be problematic. The presence of the tunic bladder cells in Holozoinae and Didemnidae may indicate the close phylogenetic relationship with some phlebobranchian groups bearing the bladder cells (and the giant cells). In Didemnidae, both of the two types of tunic cells are distributed, and this makes the phylogenetic discussion more complex. There may be two probable explanations for this condition; 1) the tunic net cells were acquired in the lineage that had the tunic bladder cells, and then the bladder cells were lost in this lineage except for in Didemnidae, or 2) the tunic bladder cells and tunic net cells were acquired in the different lineages, and the didemnid species acquired the tunic net cells (or the tunic bladder cells) independently. As described above, some spiculate didemnids contain fewer amounts of the bladder cells than other bladder

cell-bearing species (Fig. 6), and the bladder cells might be lost the ability to produce the acid in *Ascidia zara* and *Phallusia mammillata* (Fig. 7). Moreover, spiculate didemnids may not have the tunic net cells. These observations may support the occurrence of the loss of these tunic cells in some lineages. However, I suppose that the loss of these cell types did not occur so frequently, because the character-state distribution (presence or absence of the bladder cells and net cells) is stable within families or subfamilies.

Although the phylogenetic relationships among families and subfamilies are still uncertain, the genus *Ciona* is generally regarded as the most primitive among extant ascidians (Cf. Millar, 1966; Kott, 1969). *Ciona* is the only genus of the subfamily Cioninae, and neither the tunic bladder cells nor the tunic net cells are found in the two *Ciona* species. On the contrary, the tunic bladder cells are found in *Rhopalaea* sp. belonging to the subfamily Diazoninae that is the only sister subfamily of Cioninae. The species of Clavelininae, in which neither the bladder cells nor the net cells are distributed, are generally regarded to have primitive characteristics among aplousobranchian species. On the other hand, the didemnid species that have both of the two cell types are thought to be an advanced group (Kott, 1969).

The phylogenetic analyses based on the 18S rDNA sequence indicated that the Enterogona ascidians are more closely relate to the members of the class Thaliacea (salps, doliolids, and pyrosomas) than to the Pleurogona ascidians including stolidobranchians (Wada, 1998; Swalla *et al.*, 2000). However, these analyses do not include any data of the species of the suborder Aplousobranchia. The occurrence of the tunic bladder cells spreads over the Enterogona, and this may support the close relationships between the phleboranchians and aplousobranchians. It should be also noted that the tunic net cells are found in the all of three genera in Pyrosomata (Thaliacea) but not in salps and doliolids (Hirose *et al.*, 1999; Hirose *et al.*, unpublished). It would be possible that the pyrosomas share a common ancestor with some aplousobranchians having the tunic net cells.

As for the taxa so far examined in this study, I temporarily propose two possible trees of ascidian phylogeny on the basis of the presence or absence of the tunic bladder cells and the tunic net cells (Fig. 12). These trees also include following assumptions; 1) the families or the subfamilies are monophyletic, 2) the common ancestor of ascidians has neither tunic net cells nor tunic bladder cells, 3) the lineage of stolidobranchians was branched before the occurrence of diversification in Enterogona (Cf. Wada, 1998; Swalla *et al.*, 2000), 4) some aplousobranchians share a common ancestor with colonial species of Diazoninae (Cf. Kott, 1969). In one tree, the net cell is supposed to be acquired in the lineage that had acquired the bladder cells (Fig. 12A), and the two types of tunic cells are supposed to be acquired in the different lineages in the other tree (Fig. 12B). In either tree, both of the suborders Phlebobranchia and Aplousobranchia are not monophyletic, suggesting the necessity of reconsideration about the phylogenetic relationships among the families/subfamilies in the order Enterogona. In addition, pyrosomas may be closely related with some groups of aplousobranchians that have the tunic net cells.

These phylogenetic trees include the many assumptions, and the data set of the tunic cells, on which the trees are based, does not cover the four families. Moreover, the observation in only one species represents the character distribution in some taxa, such as Diazoninae, Perophoridae, Holozoinae, and Euherdomaninae. These phylogenetic trees, however, can point out the problems of the traditional phylogenetic system. Since the molecular phylogeny suggests that thaliaceans are closely related to Enterogona (Wada, 1998; Swalla *et al.*, 2000), the phylogeny in this order is very important to understand the evolutionary course of the subphylum Tunicata and to estimate the ancestral forms of the chordates. As well as the traditional approach, molecular phylogenetic study is surely needed especially in aplousobranchians, for better understanding of the tunicate phylogeny.

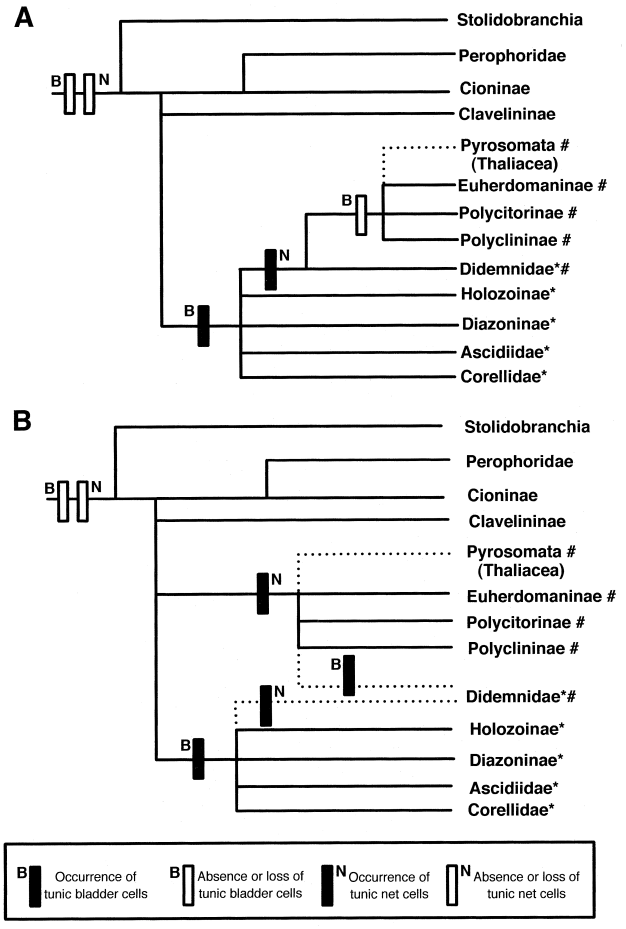


Fig. 12. Two possible trees of ascidian families and subfamilies studied here on the basis of the presence or absence of the tunic bladder cells and the tunic net cells with some assumptions (see text). The tree A supposes that the two types of tunic cells were acquired in the same lineage, and the tree B supposes that the two types were acquired in the different lineages. Asterisk, the presence of the tunic bladder cells; #, the presence of the tunic net cells.

ACKNOWLEDGMMENTS

The present study was partly supported by the grant from the Showa Seitoku Memorial Foundation and by the bilateral programs for scientist exchange promoted by JSPS and CNR. I thank Prof. T. Nishikawa (Nagoya Univ.) and Prof. R. Brunetti (Univ. Padova) for their valuable advises on the ascidian taxonomy and identification. I also thank the staff of the laboratories of Dr. R. De Santis (Stazione Zoologica "A. Dohrn"), Prof. N. Parrinello (Univ. of Palermo), Prof. P. Burighel (Univ. of Padova), Shimoda Marine Research Center (Univ. of Tsukuba), Ohtsuchi Marine Research Institute (Univ. of Tokyo), and Tropical Biosphere Research Center (Sesoko and Iriomote Station; Univ. of the Ryukyus) for their hospitality and providing materials.

REFERENCES

Dan-Shokawa M, Morimoto M, Mishima H, Kaneko H 1995. Characterization of coelomocytes of the ascidian *Halocynthia roretzi* based on phase-contrast, time-lapse video and scanning electron microscopic observations. Zool Sci, 12: 289–301

De Leo G, Patricolo E, & D'Ancona Lunetta G 1977. Studies on the fibrous components of the test of *Ciona intestinalis* Linnaeus. I. Cellulose-like polysaccharide. Acta Zool. (Stockh.) 58: 135–141

Goodbody I 1974. The physiology of ascidians. Adv Mar Biol, 12: 1–149

Hirose E, Ishii T, Saito Y, Taneda Y 1994. Seven types of tunic cells in the colonial ascidian *Aplidium yamazii* (Polyclinidae, Aplousobranchia): Morphology, classification, and possible functions. Zool Sci 11: 737–743

Hirose E, Ishii T 1995. Microfilament contraction promotes rounding of tunic slices: An integumentary defense system in the colonial ascidian *Aplidium yamazii*. Biol. Bull. 189: 29–35

Hirose E, Ishii T. & Taneda Y 1997. Two modes of tunic cuticle formation in a colonial ascidian *Aplidium yamazii*, responding to wounding. Dev. Comp. Immunol. 21: 25–34

Hirose E, Lambert G, Kusakabe T, Nishikawa T 1997. Tunic cuticular protrusions in ascidians (Chordata, Tunicata): A perspective of their character-state distribution. Zool Sci 14: 683–689

Hirose E, Kimura S, Itoh T, Nishikawa J 1999. Tunic morphology and cellulosic components of pyrosomas, doliolids, and salps (Thaliacea, Urochordata). Biol Bull 196: 113–120

Hirose E, Yamashiro H, Mori Y 2001. Properties of tunic acid in the ascidian *Phallusia nigra* (Ascidiidae, Phlebobranchia). Zool Sci 18: 309–314

Kimura S, Ohshima C, Hirose E, Nishikawa J, Itoh T 2001. Cellulose in the House of the Appendicularian, *Oikopleura rufescens*. Protoplasma 216: 71–74

Kott P 1969. Antarctic Ascidiacea. Antarct Res Ser Washington 13: 1–239

Mackie GO, Singla CL 1987. Impulse propagation and contraction in the tunic of a compound ascidian. Biol Bull 173: 188–204

Millar RH 1966. Evolution in ascidians. In "Some contemporary studies in marine science", Ed by H Barnes, George Allen & Unwin Ltd., London, pp 519–534

Ohtake S, Abe T, Shishikura F, Tanaka K 1994. The phagocytes in hemolymph of *Halocynthia roretzi* and their phagocytic activity. Zool Sci 11: 681–691

Okamoto T, Sugiyama J, Itoh T 1996. Structural diversity of ascidian cellulose. Wood Res 83: 27–29

Sawada T, Fujikura Y, Tomonaga S, Fukumoto T 1991. Classification and characterization of ten hemocytes types in the tunicate *Halocynthia roretzi*. Zool Sci 8: 939–950

Stoecker D 1978. Resistance of a tunicate to fouling. Biol Bull, 155: 615–626

Stoecker D 1980a. Chemical defenses of ascidians against predators. Ecology, 61: 1327–1334

Stoecker D 1980b. Relationships between chemical defense and ecology in benthic ascidians. Mar Ecol Prog Ser, 3: 257–265

Stoecker D 1980c. Distribution of acid and vanadium in *Rhopalaea birkelandi* Tokioka. J Exp Mar Biol Ecol, 48: 277–281

Swalla BJ, Cameron CB, Corley LS, Garey JR 2000. Urochordates are monophyletic within the deuterostomes. Syst Biol 49: 52–64

Van Daele Y, Revol J-F, Gaill F, Goffinet G 1992. Characterization and supramolecular architecture of the cellulose-protein fibrils in the tunic of the sea peach (*Halocynthia papillosa*, Ascidiacea, Urochordata). Biol Cell 76: 97–96

Wada H, Makabe KW, Nakauchi M, Satoh N 1992. Phylogenetic relationships between solitary and colonial ascidians, as inferred from the sequence of the central region of their respective 18S rDNAs. Biol Bull 183: 448–455

Wada H 1998. Evolutionary history of free-swimming and sessile lifestyles in urochordates as deduced from 18S rDNA molecular phylogeny. Mol Biol Evol 15: 1189–1194

Wuchiyama J, Michibata H 1995. Classifications, based on autonomous fluorescence of the blood cells of several ascidians that contain high levels of vanadium. Acta Zool 76: 51–55

(Received February 19, 2001 / Accepted April 4, 2001)