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Phylogeny of Botryllid Ascidians

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Summary. It has been suggested that the past classificatory criteria for botryllid ascidians are unsatisfactory. One reason for that is the absence of studies on the phylogeny of this group. Fourteen botryllids collected from the vicinity of the Shimoda Marine Research Center, University of Tsukuba, have been cultured for several years, and their morphology observed throughout life, life history, sexual and asexual reproduction, and colony specificity. On the basis of this long-term observational data and the analysis of ascidian 18S rDNA sequences, we tried to elucidate the phylogenetic relationship among these botryllids. From this study, it is suggested that botryllids might be separated into two groups; the group of botryllids with only four stigmatal rows, *B. primigenus* and *B. communis*, and the other group consisting of the majority of botryllids. The decisive characteristics that divide the major group are not clear.

Key words. Phylogeny, Botryllid, Morphology, Brooding organ, Allorecognition

Introduction

It is considered that there are two genera, *Botryllus* and *Botrylloides*, in the family Botryllidae. According to the past definitions of these two genera (Van Name 1945; Berrill 1950; Tokioka 1953), species of the genus *Botryllus* have the following characteristics in common: 1) blastozooids are arranged in oval or star-shaped systems, 2) in a mature zooid, the ovary lies anterior or dorsal to the testis, and 3) there is no brooding organ and embryos develop in the peribranchial cavity. In the genus *Botrylloides*, the common characteristics are as follows: 1) blastozooids are arranged in ladder systems, 2) the ovary is situated posterior to the testis, and 3) the embryo develops in a sac-like incubatory pouch formed as an outgrowth of the body wall. However, recently it has been suggested that these criteria are unsatisfactory (Monniot and Monniot 1987).

In the vicinity of the Shimoda Marine Research Center (SMRC), University of Tsukuba, there are at least 14 species of botryllid ascidians: *Botryllus scalaris*, *Botryllus sexiens* (cf. Saito et al 1981a), *Botryllus primigenus*, *Botryllus communis*, *Botryllus schlosseri* (cf. Tokioka 1953), *Botrylloides simodensis*, *Botrylloides violaceus* (cf. Saito et al 1981b), *Botrylloides lentus*, *Botrylloides fuscus*, *Botrylloides lenis* (cf. Saito and Watanabe 1985), and four undescribed species (in preparation for publishing), which were tentatively named *Botryllus delicatus*, *Botryllus puniceus*, *Botryllus horridus*, and *Botryllus promiscuus*, respectively. We have cultured many colonies of these 14 botryllids in the bay near SMRC for several years, and have accumulated much information on their morphology, life history, sexual and asexual reproduction, and colony specificity. Here, on the basis of those data and their 18S rDNA sequences, we elucidate the phylogenetic relationship among botryllids. We then define new classificatory criteria for the family Botryllidae. In addition to morphological characteristics, we paid attention mainly to the following four characteristics: 1) sexual reproduction, 2) the morphology of brooding organs for embryos, 3) allorejection in colony specificity, and 4) vascular system formation.

Inconsistencies in the Past Classificatory Criteria

In B. primigenus, B. communis, *B. schlosseri*, and *B. horridus,* zooids are arranged into star-shaped systems, and the ovary is situated anterior to the testis. The remaining species have ladder-shaped systems. In five species of the group having ladder-shaped systems, *B. scalaris*, *B. puniceus*, *B. sexiens*, *B. delicatus*, and *B. promiscuus,* the ovary is situated anterior to the testis, but in another five species of this group, *B. simodensis*, *B. lentus*, *B. fuscus*, *B. lenis*, and *B. violaceus*, the ovaries are posterior to the testes. As the arrangement of gonads is not related to the arrangement of zooids, the shape of zooidal arrangement is not a suitable marker for the classification of botryllids. In the past criteria, sac-like brooding organs are unique to *Botrylloides*. However, *B. primigenus*, *B. communis*, *B. delicatus*, and *B. promiscuus* also form sac-like brooding organs (Mukai 1977); the ovary in these species is situated anterior to the testis like *Botryllus*. This means that the sac-like brooding organ is not a good marker for the classification either. At present, it seems that only the arrangement of gonads in a zooid may be a useful characteristic for distinguishing *Botryllus* and *Botrylloides*. The genera of undescribed species in the present work were assigned under this criterion.

Variation in Sexual Reproduction and Brooding Organs

As shown in Table 1, all *Botryllus* species are ovoviviparous, though they have a variety of brooding organs. Both viviparous and ovoviviparous species exist among *Botrylloides* species, all of which have sac-like brooding organs. In

Botrylloides, the viviparity is more highly-developed in species with longer breeding periods. However, the mode of sexual reproduction, viviparous or ovoviviparous, might be an indecisive marker.

The brooding organ is formed from either atrial epithelium (peribranchial wall) or branchial epithelium (Table 1). The formation of brooding organs is very important to the strategy of sexual reproduction in botryllids. Therefore, in the formation of brooding organs, the choice between atrial epithelium and branchial epithelium might be a significant event in the evolution of botryllids. Though only two species that form brooding organs from branchial epithelium are known now, the origin of brooding organs will likely be a significant decisive characteristic.

Table 1. The mode of sexual reproduction and embryo brooding

Mode of sexual reproduction	Breeding period	Brooding organ	Origin of brooding organ	Species name
Ovoviviparous	About 1 week	No organ	None	*B. scalaris* *B. puniceus* *B. horridus*
		Septum-like organ	Branchial epithelium	*B. sexiens*
		Sac-like organ	Branchial epithelium	*B. delicatus*
		Cup-like organ	Atrial epithelium	*B. schlosseri*
		Sac-like organ	Atrial epithelium	*B. primigenus* *B. promiscuus* *B. simodensis**
Viviparous	10-12 days	Sac-like organ	Atrial epithelium	*B. lentus**
	2 weeks	Sac-like organ	Atrial epithelium	*B. fuscus**
	3 weeks	Sac-like organ	Atrial epithelium	*B. lenis**
	> 1month	Sac-like organ	Atrial epithelium	*B. violaceus**

* Species belonging to the genus *Botrylloides*

Variety of Allorejection Mechanisms in Colony Specificity

Colony specificity is a kind of allorecognition manifested by fusibility between colonies; two colonies either fuse to form a single mass or reject each other at the contact area. Allorejection begins as if to interrupt the progress of fusion. There are a variety of allorejection mechanisms expressed at the onset of allorejection. Among botryllids, five types of allorejection reactions have been shown (Saito et al. 1994). Species which exhibit rejection at an earlier stage of the fusion process may be more advanced. Therefore, this feature might become a good marker for botryllid classification. In particular, the timing of rejection in relation to the fusion of ampullae (vascular vessels) may be an important defining feature.

Special Features of Botryllids with Four Stigmatal Rows

Species having only four of stigmatal rows are unique in their morphology and life history. *B. primigenus* and *B. communis* belong to this group, and it is considered that they might be the synonym of *Botryllus tuberatus* (cf. Ritter and Forsyth 1917). There are many significant differences between this group and the other group, having more than five stigmatal rows (Table 2). The bud of the former group discards the peduncular vessel, which is connected with the mother zooid, and makes two new vessels connected to the vascular network. However, in the latter group, the peduncular vessel is used as a connecting vessel, and only one connecting vessel is newly formed. Additionally, in the former group, larval ampullae degenerate after attachment and the colonial vascular system is then derived from newly formed ampullae. Conversely, for species with more than five stigmatal rows, the larval ampullae are used to make the colonial vascular system (Mukai and Sugimoto 1978). The differences shown in Table 2 strongly suggest that botryllids with four stigmatal rows might be unique and potentially separated from other botryllids early in their evolution.

Table 2. Differences between botryllids with 4 stigmatal rows and other botryllid

	4 stigmatal rows	More than 5 rows
Connection of zooid to vascular network	2 vessels	2 vessels
a) Peduncular vessel	degenerate	Remains as a connecting vessel
b) Newly formed connecting vessel	Endostyle connecting vessel & visceral connecting vessel	Endostyle connecting vessel
Larval ampullae	8 ampullae Degenerate after larval attachment (newly formed ampullae construct the first colonial vascular system)	8 ampullae (Most species) Form the first colonial vascular system after larval attachment
Vascular budding	usually	rarely
Shape of testis	Hemispheric testis	Rosette-like testis formed from several lobes
Gamete release	Release eggs and sperm at the same time	Release eggs before sperm release

Phylogenetic Relationship among Botryllids

The Phylogenetic relationship among botryllids is shown schematically (Figure 1). Both parsimony and maximum-likelihood analysis using 18S rDNA sequence data support monophyly for the family Botryllidae, with *B. scalaris* as the basal species among six Japanese botryllids (*Botryllus scalaris*, *B. sexiens*, *B. primigenus*, *Botrylloides simodensis*, *B. fuscus*, and *B. violaceus*). Further, the genus

Botrylloides is monophyletic (Cohen et al. 1998). However, a variety of characteristics suggest the genus *Botryllus* is polyphyletic. With regard to the formation of brooding organs, it is unclear which tissue, atrial epithelium or branchial epithelium, was first used to form those organs in the evolution of botryllids. We think the species using branchial epithelium might branch off earlier than the species using atrial epithelium, because the former show more primitive allorejection reactions. Two types of allorejection were found in the group that shows allorejection after the fusion of ampullae (Shirae et al. 1999). The first is a fast rejection that occurs soon after the fusion (*B. scalaris* and *B. puniceus*), and the other is a slow rejection that occurs several hours after the fusion (*B. sexiens* and *B. delicatus*).

In conclusion, it may be better that the group having four stigmatal rows is divided from the group having more than five stigmatal rows. However, we could not find any reasonable markers to divide the latter group into two or three.

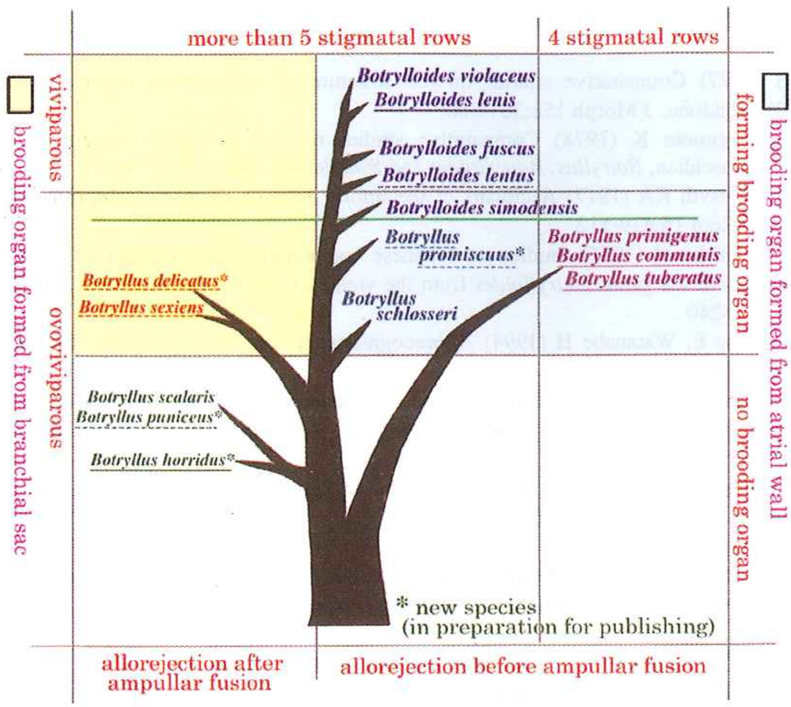

Fig. 1. Phylogenetic tree in botryllids with special features. The allorejection mechanisms of the species underlined with broken lines were observed, but not reported. In the species underlined with solid lines, allorejection has not yet been observed. The allorejection mechanisms of other species have previously been reported.

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