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Ascidian Peribranchial Epithelium Cells May Possess the Information of Visceral Pattern

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Abstract

In the budding ascidian *Polyandrocarpa misakiensis*, chimeric zooids are induced by the fusion of two different adult body fragments. If each body fragment was derived from a different visceral pattern zooid (cf, normal body pattern zooid and situs inversus or reversed zooid), some types of chimeric zooids were known to be produced. Using two different color phenotypes, white-spot strain and spotless one, it is confirmed that chimeric zooids are surely produced. In this study, we made chimeric zooids between body fragments with reversed white-spot and ones with normal spotless, and analyzed offspring of the chimeric zooids. The chimeric zooids produced blastozooids with new combination of color pattern and visceral pattern, that is, blastozooids with normal white-spot or ones with reversed spotless. In blastogenic generation, palaeal budding, a bud consists of two envelopes of cell layers: the epidermal and the peribranchial epithelium. Previously, we found that the epidermal epithelium is the carrier of the color pattern. Therefore, our results strongly suggest that the information of determining of visceral pattern (L-R axial polarity) exists in the peribranchial epithelium in asexual reproduction i.e., palaeal budding.

Keywords : ascidian chimeras/ visceral pattern/ color pattern/ offspring/ positional information/ L-R axial polarity/ bilateral symmetry

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Introduction

In animals, two axial polarities—the anterior-posterior and dorsoventral axial polarities—are most important in the pattern formation of the development of both sexual and asexual reproduction. For a long time it was thought that if these two axial polarities are determined in a developing animal body, the last left-right (L-R) axial polarity is determined automatically. However, in the nearly 20 years since Levin *et al.* (1995) reported the asymmetrical gene expression of *Nodal* in chick embryo, the number of molecular analyses of L-R axial polarity in the development of sexual reproduction in vertebrates has increased (Yost 1995; Danos and Yost 1996; Hyatt *et al.* 1996). Most recently, it was reported that planar cell polarity is likely to be the initial key for bilateral symmetry in vertebrate sexual development (Hashimoto *et al.* 2010; Song *et al.* 2010). The current understanding of L-R axial polarity and its determination in sexual reproduction in vertebrates are advanced, but there is little information

about the determination of the L-R axis in asexual reproduction, especially in invertebrates.

In some colonial or budding ascidians, situs inversus viscerum (SIV) or reversed blastozooid is induced experimentally from the normal body pattern blastozooid (Sabbadin 1956; Oda and Watanabe 1981, 1982; Nakauchi and Sugino 1984). SIV is rarely found in the field specimens (Sabbadin 1956; Watanabe and Newberry 1976). An SIV zooid is always induced experimentally through asexual reproduction, i.e., budding. However, an SIV zooid produces only situs normale viscerum (SNV) oozoids through sexual reproduction. The reversed polarity of the L-R axis in blastozooids is maintained only through asexual reproduction; i.e., the L-R body axial polarity may be established not only by its genome but also by something else. It is worth studying the ‘something else’ in the mechanisms underlying the determination of L-R axial polarity using colonial or budding ascidians.

A budding ascidian, *Polyandrocarpa misakiensis*,

has significant pallear budding, regeneration and chimera formation abilities. Kawamura and Watanabe (1982b) revealed the relation between a parent and its buds in their body axes in pallear budding. Posterior determination in pallear budding was reported by Kawamura (1984a,b), and mitotic activity in pallear budding was demonstrated by Kawamura and Nakauchi (1986). The role of the epidermis and mesenchyme in pallear budding was pointed out by Kawamura and Watanabe (1987). Multipotent epithelial cells are most important in both pallear budding and regeneration (Kawamura *et al.* 2008). Studies of the regeneration of ascidian body fragments (Taneda 1985; Ishii *et al.* 1998) and chimera formation with two ascidian body fragments (Taneda 1985, 1986, 1987; Ishii *et al.* 1994, 1997) were also carried out. Kaneko *et al.* (2010) revealed the importance of retinoic acid in gut regeneration. In this way, many studies were carried out using *P. misakiensis*' high ability of pallear budding, regeneration and chimera formation.

SIV blastozooids are easily induced in *P. misakiensis* experimentally (Oda and Watanabe 1981, 1982; Kawamura and Watanabe 1982c, 1983). Once the SIV blastozooids are induced, they always produce SIV blastozooids in pallear budding. In addition, in *P. misakiensis*, two strains with different color patterns are well known (Ishii *et al.* 1993; Ishii and Saito 2012). The white-spot strain (WSS) has a large white spot on its dorsal area between the branchial and atrial siphons. The spotless strain (SLS) has a fine white band on the same area. These two color strains are good markers for chimera bodies derived from WSS and SLS fragments.

The present study is a continuation of the Ishii *et al.* (1994) investigation. They reported that chimeras formed between SNV and SIV body fragments. In that study, double-zooid monsters and single-zooid chimeras were exhibited. The double-zooid monsters were of three types: type 1 monsters had two branchial siphons and two atrial siphons, type 2 had one branchial siphon and two atrial siphons, and type 3 had three branchial siphons and one atrial siphon (Ishii *et al.*, 1994). The single-zooid chimeras had one branchial siphon and one atrial siphon. The single-zooid chimeras were similar to

normal blastozooids.

Ishii *et al.* (1994) reported the offspring of the single-zooid chimeras but not the offspring of the double-zooid monsters. In the present study, the offspring of double-zooid monsters were analyzed. Some double-zooid monsters produced blastozooids with a new combination of color pattern and visceral pattern; that is, blastozooids with an SNV white-spot or with the SIV spotless. Following our analysis of the offspring of the double-zooid monsters, we discuss the L-R axial polarity in chimeras and related information in the determination of L-R axial polarity in asexual reproduction.

Materials and Methods

Animals

Blastozooids of both the spot-less strain (SLS) and white-spot strain (WSS) of the tunicate *P. misakiensis* were used. *P. misakiensis* was first described by Watanabe and Tokioka (1972). Both SLS and WSS colonies were reared on glass slides in culture boxes immersed in Nabeta Bay near the Shimoda Marine Research Center, University of Tsukuba (Shizuoka Prefecture, Japan). Each strain has maintained its respective color pattern through asexual reproduction, i.e., pallear budding.

In *P. misakiensis*, SIV is induced experimentally (Oda and Watanabe 1981, 1982; Kawamura and Watanabe 1982c, 1983), and an SIV blastozooid always produces SIV blastozooids; SNV blastozooids produce only SNV blastozooids. In the present study, SLS blastozooids with the SNV pattern and WSS blastozooids with the SIV pattern of body polarity were used.

Chimera formation and offspring analysis

The details of the chimera formation procedure are provided by Ishii *et al.* (1994). Briefly, each half of each transected blastozooid of SLS with SNV and WSS with SIV was combined to be a chimera. When the chimera formation succeeded, we analyzed the offspring of the chimera and determined whether each offspring

Table 1. The four chimera combinations and results

Chimera combination	Results	Type of double-zooid monsters
A ⁺ A ⁻	double-zooid monsters	Type 1 (two branchial and two atrial siphons)
P ⁺ P ⁻	double-zooid monsters	Type 2 (one branchial and two atrial siphons)
A ⁺ P ⁻	double-zooid monsters or single-zooid chimeras	Type 3 (three branchial and one atrial siphons)
P ⁺ A ⁻	double-zooid monsters or single-zooid chimeras	Type 3 (three branchial and one atrial siphons)

⁺, white-spot strain = always SIV; ⁻, spotless strain = always SNV; A, anterior fragment; P, posterior fragment.

was SLS or WSS and SNV or SIV.

In all four chimera combinations, double-zooids monsters were observed (Table 1). Ishii *et al.* (1994) reported the results of chimera formation and the offspring derived from only single-zooid chimeras. In the present study, we analyzed the offspring of those double-zooid monsters. This study is thus a continuation of Ishii *et al.* (1994).

Results

Table 1 also shows the results of the four chimera combinations, modified from Ishii *et al.* (1994). As reported in Table 1 in Ishii *et al.* (1994), with 444 challenges and the four chimera combinations, 81 successful chimeras were obtained (Ishii *et al.* 1994). However, among the 81 successful chimeras, only 23 chimeric zooids were recognized by their body pattern; that is, seven single-zooid chimeras and 16 double-zooid monsters were observed (details in the Table 2 in Ishii *et al.* 1994). As shown in Table 1, three types of double-zooid monster (types 1, 2, and 3) were detected. All of the type 1 double-zooid monsters died before budding, as did the single type 2 double-zooid monster. However, two of the 13 type 3 double-zooid monsters produced offspring (Figs. 1, 2).

The chimera formations combined anterior fragments of the WSS with SIV and posterior fragments of the SLS with SNV, resulting in double-zooid monsters (malformed) or single-zooid chimeras. As was shown by Ishii *et al.* (1994), the offspring of the single-zooid chimeras grew into WSS with SNV and SLS with SNV; that is, all of the offspring had the SNV pattern. However, the offspring of only one of the 13 malformed double-zooid monsters derived from the combination of anterior fragments of the WSS with SIV and posterior fragments of the SLS with SNV grew into the WSS with SIV, WSS with SNV, and SLS with SNV (Fig. 1A–D). We observed similar results in chimera formations from the combined anterior fragments of the SLS with SNV and posterior fragments of the WSS with SIV, resulting in double-zooid monsters (malformed) or single-zooid chimeras.

As also shown by Ishii *et al.* (1994), the offspring of the single-zooid chimeras grew into the SLS with SNV and the WSS with SNV; that is, all of the offspring showed the SNV pattern. However, the offspring of another one of the 13 malformed double-zooid monsters derived from the combination of anterior fragments of SLS with the SNV and posterior ones of WSS with the SIV grew into SLS with SNV, SLS with SIV, and WSS with SIV (Fig. 2A–D).

Discussion

Blastozooids derived from malformed double-zooid monsters involved new combinations of color

pattern and visceral pattern. Each of the 13 malformed double-zooid monsters derived from the combination of anterior fragments of the WSS with SIV or the SLS with SNV and posterior fragments of the SLS with SNV or the WSS with SIV, respectively, produced blastozooids with a new combination (Fig. 3) and also with the original combination. The developmental pattern is transmitted from parent to pallear bud (Izzard 1973), and a bud consists of two envelopes of cell layers: the outer one is the epidermis and the inner one is the peribranchial epithelium. It was confirmed that all of the organs of blastozooids are derived from peribranchial epithelium (Kawamura and Watanabe 1982a; Kawamura and Nakauchi 1984).

After the theory of positional information was proposed by Wolpert (1969, 1971), many works were carried out concerning that theory. Positional information must be carried by something. The polarized information of the A-P axial polarity or the L-R axial polarity (bilateral asymmetry) may exist only in a cell layer that can provide a certain value. Therefore, positional information of bilateral asymmetry also exists in either the epidermis or the peribranchial epithelium. We found that the epidermis is the carrier of the color patterns (Ishii *et al.* 1993). If positional information of bilateral asymmetry exists in the epidermis, the color pattern should always coincide with the visceral pattern. However, new combinations of color pattern and visceral pattern were observed in the present study. This strongly suggests that positional information of bilateral asymmetry exists in the peribranchial epithelium in asexual reproduction i.e., pallear budding. In vascular budding in *Botryllus schlosseri*, positional information of bilateral asymmetry is seen to be conveyed by the wall of the vessels i.e., epidermal extrusions of the zooids (Sabbadin *et al.* 1975). Both the peribranchial epithelium in the present study and the wall of the vessels of the vascular bud in *Botryllus schlosseri* are cell sheets. It is reasonable that a cell sheet has positional information and that both of these cell sheets have morphogenesis ability.

Single-zooid chimeras derived from anterior fragments of the WSS with SIV and posterior fragments of the SLS with SNV produced only blastozooids with the SNV body pattern of the WSS and SLS (Ishii *et al.* 1994). Similar phenomena were seen in the budding of the same study's single-zooid chimeras derived from anterior fragments of the SLS with SNV and posterior fragments of the WSS with SIV. They produced only blastozooids with the SNV body pattern of the SLS and the WSS (Ishii *et al.* 1994). In those single-zooid chimeras, the L-R axial polarity returned from reversed to normal. It may be difficult for the reversed axial polarity in L-R to be maintained throughout the chimera formation. However, the malformed double-zooid monsters derived from the combination of anterior fragments of the WSS with SIV and posterior fragments

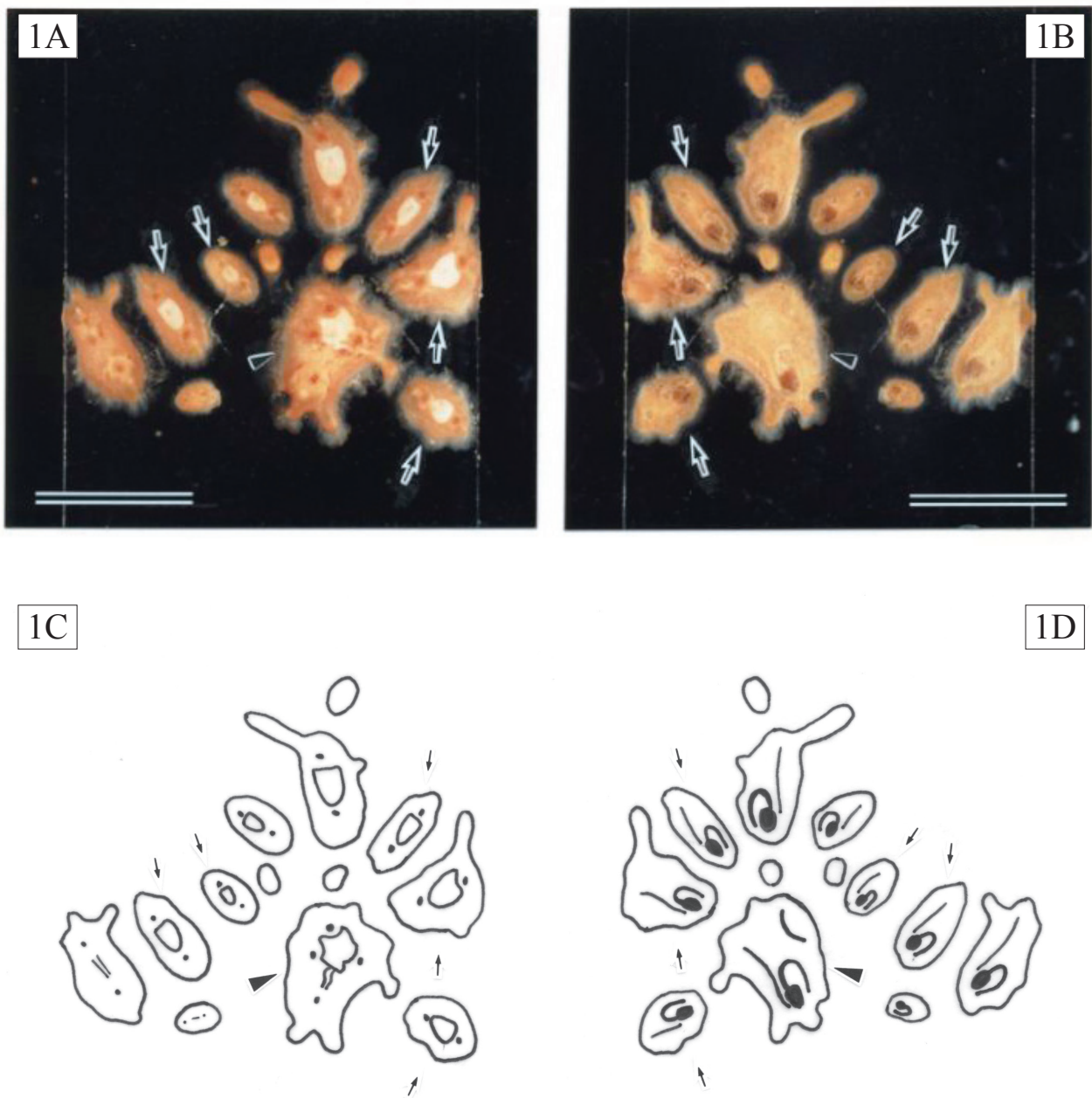


Fig. 1. Offspring of the only one of 13 malformed double-zooid monsters (type 3 monster, three branchial and one atrial siphons) obtained from the chimera combination A^+P^- , 160 days after treatment. Arrowhead indicates the parent chimera. Arrows indicate white-spot (WSS) blastozooids with SNV (new combination of color pattern and visceral pattern). A: Dorsal view. B: Ventral view through glass slide (Scale bars: 10 mm). C: Tracing of A. D: Tracing of B.

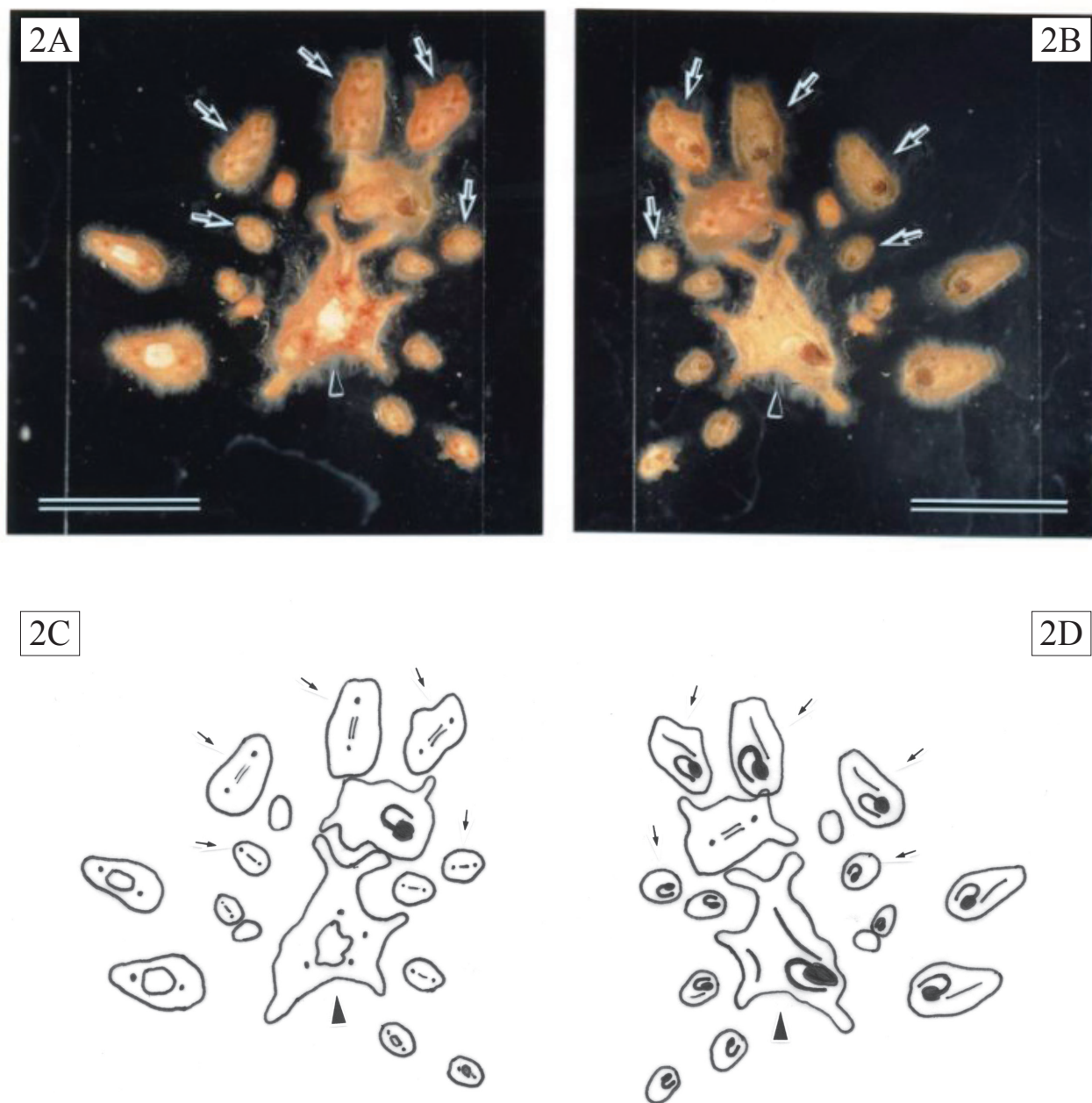


Fig. 2. Offspring of the only one of 13 malformed double-zooid monsters (type 3 monster, three branchial and one atrial siphons) obtained from chimera combination P^+A^- , 160 days after treatment. Arrowhead indicates the parent chimera. Arrows indicate spotless (SLS) blastozooids with SIV (new combination of color and visceral patterns). A: Dorsal view. B: Ventral view through glass slide (Scale bars: 10 mm). C: Tracing of A. D: Tracing of B.

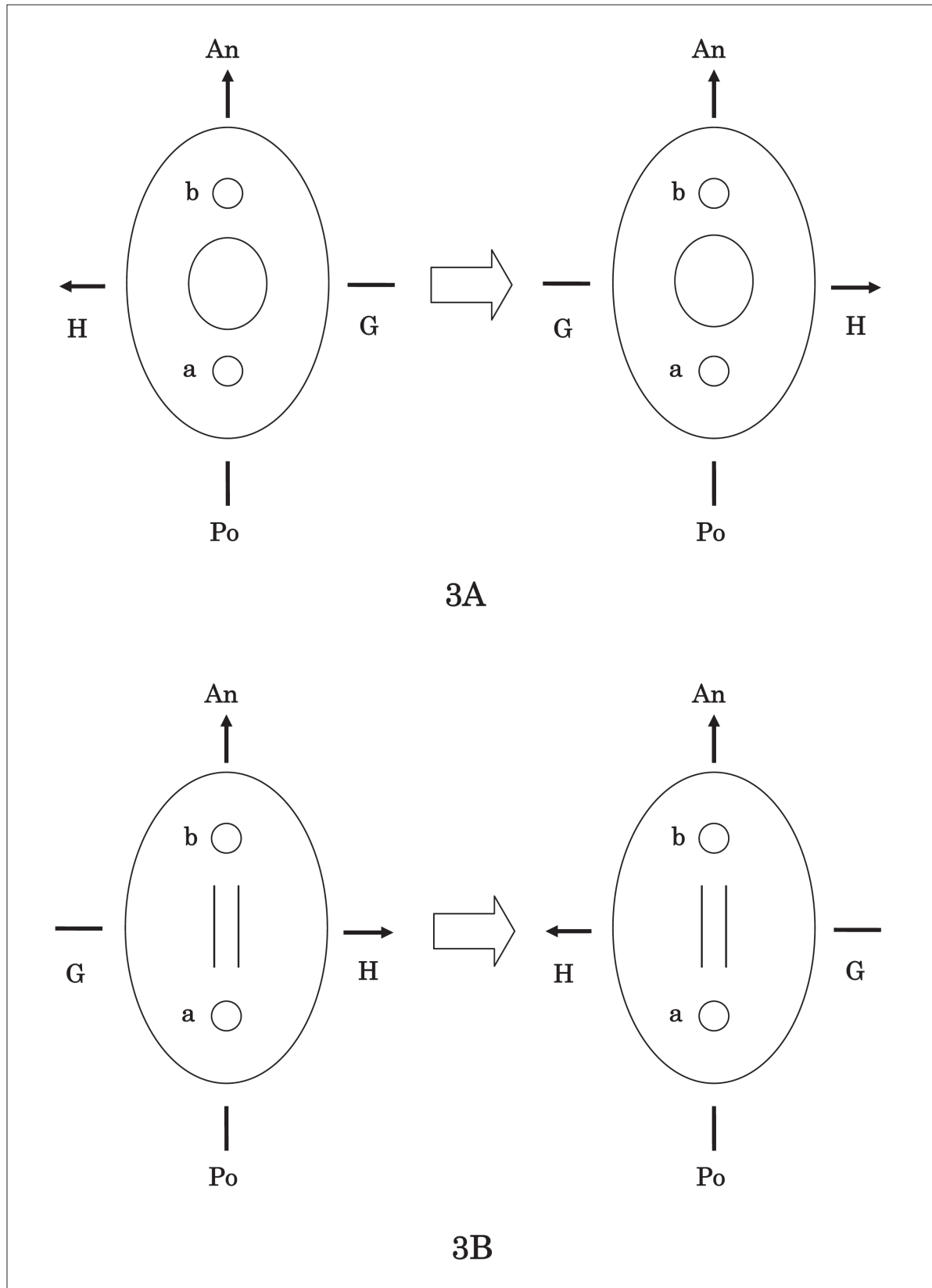


Fig. 3. Diagrammatic representation of new combination of color pattern and visceral pattern in the offspring of each malformed double-zooid monster. A: The case in Fig. 1. The white-spot strain (WSS) with normal visceral pattern (SNV) was newly produced from the original reversed visceral pattern. Left side, original combination. Right side, new combination. B: The case in Fig. 2. The spotless strain (SLS) with reversed visceral pattern (SIV) was newly produced from the original normal visceral pattern. Left side, original combination. Right side, new combination. a, atrial siphon; An-Po, anteroposterior axis; b, branchial siphon; G, gut side of body; H, heart side of body; H-G, heart-gut axis.

of the SLS with SNV reproduced blastozooids of three types: WSS with SIV, WSS with SNV, and SLS with SNV (Fig. 1A–D).

Similar results were seen in double-zooid monsters derived from anterior fragments of the SLS with SNV and posterior fragments of the WSS with SIV, which produced blastozooids with the SLS with SNV, the SLS with SIV and the WSS with SIV (Fig. 2A–D). In those double-zooid monsters, reversed axial polarities in the L-R axial polarity were maintained throughout the chimera formation and transmitted to the next generations. The blastozooids with new combinations of color and visceral patterns were born only in the contact area of the body fragment fusion and inherited their mothers' epidermis and peribranchial epithelium, which originated in different body fragments.

However, the double-zooid monsters in the present study did not produce all of the combinations of color and visceral patterns. The malformed double-zooid monsters derived from the combination of anterior fragments of the WSS with SIV and posterior fragments of the SLS with SNV did not produce blastozooids with the SLS with SIV, and the malformed double-zooid monsters derived from the combination of anterior fragments of the SLS with SNV and posterior fragments of the WSS with SIV also did not produce blastozooids with the WSS with SNV. That is, the offspring of the malformed double-zooid monsters derived from the combination of anterior fragments of the WSS with SIV and posterior fragments of the SLS with SNV were not derived from spotless epidermis and reversed bilateral peribranchial epithelium, and the offspring of the malformed double-zooid monsters derived from the combination of anterior fragments of the SLS with SNV and posterior fragments of the WSS with SIV were also not derived from white-spot epidermis and normal bilateral peribranchial epithelium. The value of bilateral information in the contact area of the body fragment fusion, in which opposite values are obtained, is not clear. However, it is likely that the information is different between the border of the fusion of the epidermis and the peribranchial epithelium in each of the malformed double-zooid monsters.

Our present analysis of the offspring of ascidian chimeras is the first to suggest that positional information of the L-R axial polarity in ascidian asexual reproduction i.e., palleal budding, is carried by the peribranchial epithelium. The nature of the polarity is still unknown. However, a simple gradient of a substance does not support the polarity. Further analyses are needed to elucidate the L-R axial polarity in asexual reproduction.

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