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Zebrafish *Leopard* gene as a component of the putative reaction-diffusion system

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Abstract

It has been suggested, on a theoretical basis, that a reaction-diffusion (RD) mechanism underlies pigment pattern formation in animals, but as yet, there is no molecular evidence for the putative mechanism. Mutations in the zebrafish gene, *leopard*, change the pattern from stripes to spots. Interestingly each allele gives a characteristic pattern, which varies in spot size, density and connectivity. That mutations in a single gene can generate such a variety of patterns can be understood using a RD model. All the pattern variations of *leopard* mutants can be generated in a simulation by changing a parameter value that corresponds to the reaction kinetics in a putative RD system. Substituting an intermediate value of the parameter makes the patterns similar to the heterozygous fish. These results suggest that the leopard gene product is a component of the putative RD mechanism.

Keywords: Zebrafish leopard gene; Putative reaction-diffusion system

1. Introduction

In 1952, Turing proposed a hypothetical molecular mechanism called the reaction-diffusion (RD) system, which can develop periodic patterns from an initially homogeneous state (Turing, 1952). Turing suggested that the RD system provides the positional information required in developing embryos (Turing, 1952). The RD model and its descendants have been proposed to account for many patterning phenomena in morphogenesis which are difficult to understand from simple morphogen gradient systems (Meinhardt, 1982; Murray, 1989). The pattern formation of animal pigmentation is a prime example. Many animal species have a characteristic coat patterns on the skin. The spatial scale of the coat pattern is much greater than the size of individual cells in the skin. However, in most cases, animals do not have any internal structure that look similar to the coat patterns. Therefore, the positional information for the coat pattern must be generated autonomously in the skin. A RD mechanism provides an effective solution to this problem. Many mathematical studies have shown that the RD system is able to produce most of the animal coat patterns (Murray, 1989; Koch and Meinhardt, 1994). However, as yet, there is no molecular evidence for the hypothetical mechanism.

We have previously reported that the pigment patterning of the tropical angelfish *Pomacanthus* moves continuously as predicted by the RD model (Kondo and Asai, 1995). The way in which the pattern changes with time clearly shows that some dynamic mechanism, such as a RD system, is maintained actively in the skin of the fish. This finding immediately suggested the possibility to identify the molecules related to the patterning with zebra danio, which is convenient for molecular genetic investigation (Meinhardt, 1995).

In this paper, we present evidence that the stripe pattern of zebra danio is produced with the same mechanism as that in *Pomacanthus*, and that the leopard gene is one of the core elements of a common dynamic mechanism of fish pattern formation, which is probably a reaction-diffusion system.

2. Results

2.1. Mechanism for the pigment patterning may be common

In order to investigate whether or not the dynamic char-

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acter of the skin pattern found in *Pomacanthus* (Kondo and Asai, 1995) is common, we observed the pattern alterations in several phylogenetically distant fish and reptiles. Fig. 1 shows some examples. A catfish, *Plecostomus*, has spots on its ventral skin. Spotted or striped patterns are the typical 2D pattern of Turing system (Murray, 1989). When the fish grows the division or insertion of spots occurs to keep the size of each spot constant, as the model predicts. (Fig. 1a–j) Similar pattern alterations are also detected in *Rajiformes*, *Anguilliformes*, *Characiformes*, *Siluriformes* and *Perciformes* and *Gekkota* (reptile) (data not shown). Therefore, it seems likely that the mechanism for the skin patterning is common to wide variety of lower vertebrate. A similar pattern alteration is observed in zebrafish. The adult pattern of wild type zebrafish emerges as three horizontal lines; the fourth and fifth stripes are then added the most ventral position (Kirschbaum, 1975; Johnson et al., 1995). In some mutants (Haffter et al., 1996), the stripe patterns contain disorders (branch or disruption), and pattern alteration during growth is often observed. This is very similar to the case of *Pomacanthus* (Kondo and Asai, 1995) (Fig. 1k–m). Another type of pattern change, line insertion, is occasionally observed in very old and large zebrafish (Fig. 1n). As observed in the juvenile of *Pomacanthus*, (Kondo and Asai, 1995) a thin white line is inserted at the widest regions of black bands (Fig. 1o). From these observations, we conclude that the common underlying mechanism for skin patterning can be studied using zebrafish.

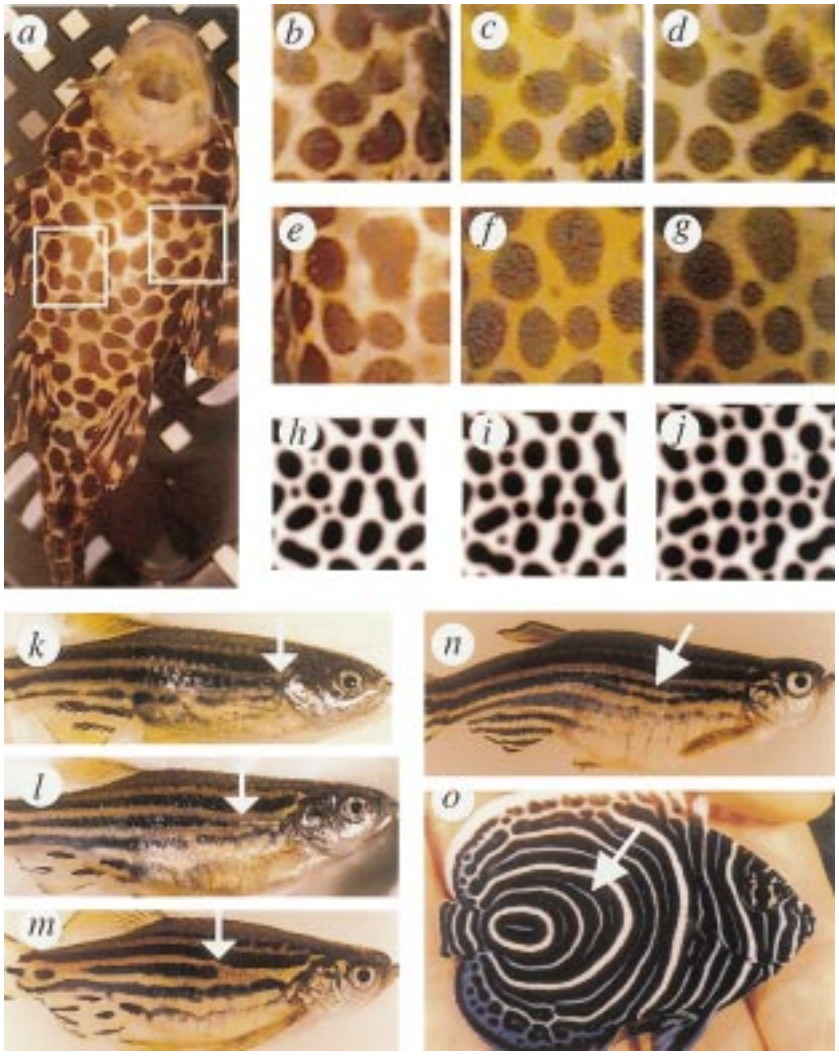

Fig. 1. Examples of the dynamic pattern alterations that fit the RD model. (a) The ventral pattern of a catfish, *Plecostomus*. (b–g) Pattern alterations of the framed area of (a), which occurred within two months. When the fish grow, division and insertion of new spots occurred to maintain the spot size of spacing. The catfish was purchased from a pet shop in Tokushima, and raised using standard methods. (h–j) RD model simulation of a spot pattern on a growing field. The pattern alteration exhibited by the catfish is reproduced. Both the spot division and insertion are seen in this simulation just as with the real fish. Equations and the growing condition are identical to that explained in Fig. 3 legend. The initial condition is a random pattern of both factors. The parameters are $a = 0.08$, $b = 0.08$, $c = 0.28$, $d = 0.03$, $e = 0.1$, $f = 0.15$, $g = 0.05$ and $P = 0.5$. (k–m) Growth dependent pattern change of a mutant zebrafish (leo^{+}/leo^{td15}) Age of the fish is 60, 120, and 240 days, respectively. The anterior end of the second line was at the branchial mantle at 60 days, then retreated posteriorly. The third line moved up slightly to fill the space where the second line disappeared. (n,o) White line insertion occurred in old zebrafish and juvenile of *Pomacanthus*.

2.2. A RD model to simulate the pigment pattern of zebrafish

From the large-scale mutagenesis in the zebrafish (Kimmel, 1989; Mullins and Nusslein-Volhard, 1993; Mullins et al., 1994; Haffter and Nusslein-Volhard., 1996), many mutant lines with abnormal pigmentation patterns have been isolated (Haffter et al., 1996; Kelsh et al., 1996). These may contain the genes that participate in the putative patterning mechanism. A promising candidate is the *leopard* gene. Fig. 2 shows the homozygous fish of four different alleles of *leopard* gene. Interestingly, the spot size, density and connectivity of mutant alleles are different from others and very easy to distinguish. How can the mutations in a single gene generate such a variety of 2D patterns? And what is the role of the gene? In the following mathematical model we conjecture possible answers to these questions (Kondo and Asai, 1995).

$$\frac{\partial u}{\partial t} = x(u,v) - du + D_u \nabla^2 u$$

$$\frac{\partial u}{\partial t} = y(u,v) - gv + D_v \nabla^2 v$$

$$x(u,v) = au - bv + c$$

$$y(u,v) = eu - f$$

$$0 \le x, y \le P$$

P is the upper limit of the synthesis of u and v. 'f' is the threshold of the inhibitor production.

The equation above describes a reaction-diffusion system used in this study. Where u and v are the concentration of materials called activator and inhibitor respectively. Other parameters represent the kinetics of each reaction (a, b, c, d, e, f, g) and the diffusion rates (D_u, D_v) in this hypothetical system (see Fig. 3a). If a mutation changes the structure of the enzyme that catalyses one of the reactions in this system, the corresponding parameter will be changed. The 2D pattern calculated from the equation is sensitive to such changes in the parameter values (Meinhardt, 1982; Murray, 1989). Therefore it is a reasonable assumption that the *leopard* gene is involving in determining the value of a parameter.

2.3. All the pattern variations of leopard mutants can be generated in a simulation by changing a parameter value

To determine which parameter can best model the patterning of the *leopard* mutants, simulations are carried out with sequentially changed parameters (Fig. 3b). The initial conditions and the number of iteration are identical for all simulation. In order to take into account the growth of the skin of the fish, the scale of space was gradually increased. The patterns at the left most columns correspond to the wild type fish. The parameters for them are selected for the formation of striped patterns. One of the parameters is changed stepwise in each line. The resulting patterns show the possible mutant phenotypes, which would occur when the parameter values are changed by the mutation. As shown in Fig. 3, changing each parameter produces a characteristic sequence of patterns. However, all the allelic variations of *leopard* mutants appear in the third line (parameter c). The relationship between the phenotypes and the c parameter is shown in Fig. 4. By enlarging the c parameter from 0.04 to 0.44, the striped pattern gradually changes to meandered lines, fused spots, isolated spots and then small spots.

2.4. Heterozygous patterns and the corresponding parameter values

Not only the patterns of homozygous fish but also the patterns of all the heterozygous fish are found in the series of simulation. In most case, the c values of the heterozygous fish are intermediate to parental homozygous fish. The leo^{+}/leo^{tw28} has twisted lines with branches and white spots, which does not occur in either homozygous parent. The c

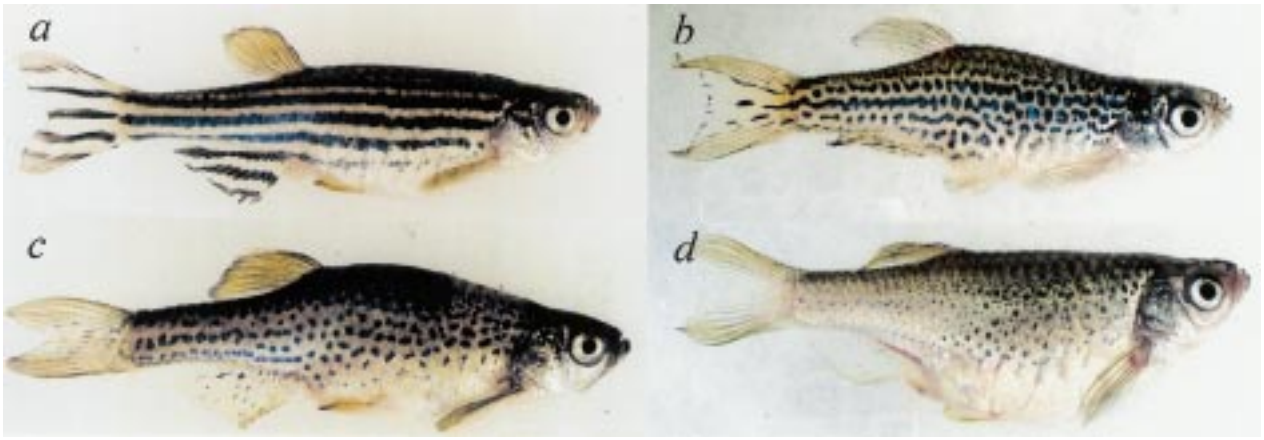

Fig. 2. Pigment patterns of the homozygous zebrafish of four different alleles of *leopard* gene. All the pictures were taken with fully-grown (5–6 months) females. (a) + / + ; (b) leo^{tw28}/leo^{tw28}; (c) leo^{t1}/leo^{t1}; (d) leo^{tq270}/leo^{tq270}.

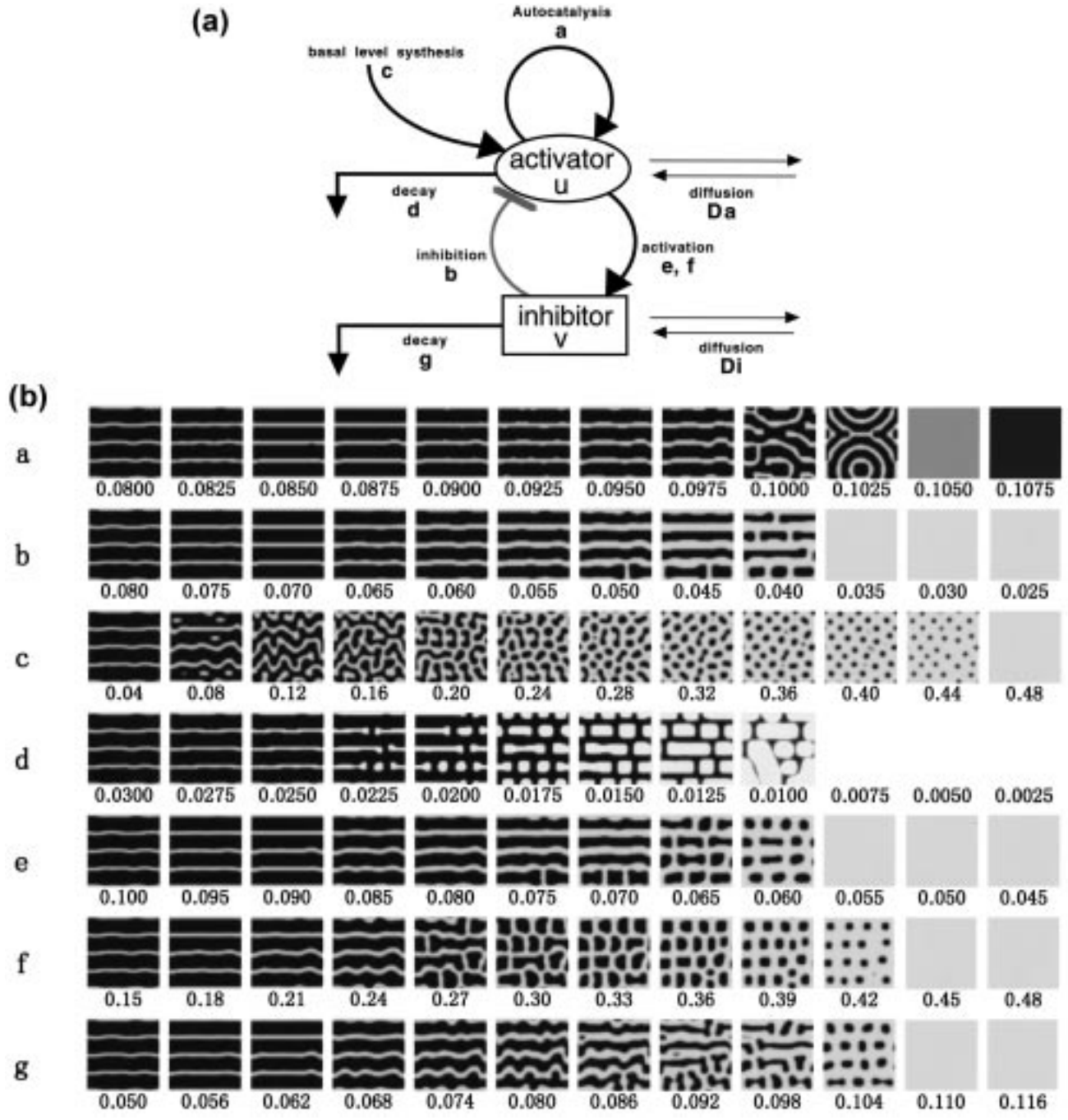

Fig. 3. (a) A schematic representation of the reaction-diffusion system. (b) Results of a simulation study. The left most columns are standard simulations corresponding to the wild type ($a = 0.08$, $b = 0.08$, $c = 0.04$, $d = 0.03$,$e = 0.1$, $f = 0.15$, $g = 0.05$ and $P = 0.5$). In each line, one of the parameter is changed sequentially. Only the direction that the white areas come wider is shown. Field size is 64×64. The concentration of activator is shown by gray scale. (light; high concentration, dark; low concentration) The starting pattern is a random pattern with three thin lines. Cells of relatively high activator concentration were put to form three horizontal lines. In spite of the pattern variety in adult, all the mutant fish have the identical juvenile pattern of three thin lines of melanocytes. 20 000 iterations used. To simulate the body growth, the diffusion constants are linearly decreased to a half of the starting value. All the details of simulations and the software used will be open at our HP when this paper is published.

values of the homozygous parents are 0.04 and 0.20–024, and the intermediate value, 0.08–0.12, gives the pattern of the heterozygous (leo^{+}/ leo^{tw28}) fish. In the case of leo^{+}/ leo^{tq270}, which has large spots, the corresponding c value (0.32–0.36) is also intermediate to that of homozygous parents (0.04 and 0.44). These results strongly suggest that the RD mechanism (or some equivalent dynamic mechanism) makes the pigment pattern of zebrafish, and leopard gene product is one of the core components. Concerning the c value of the heterozygous pattern, leo^{tl}

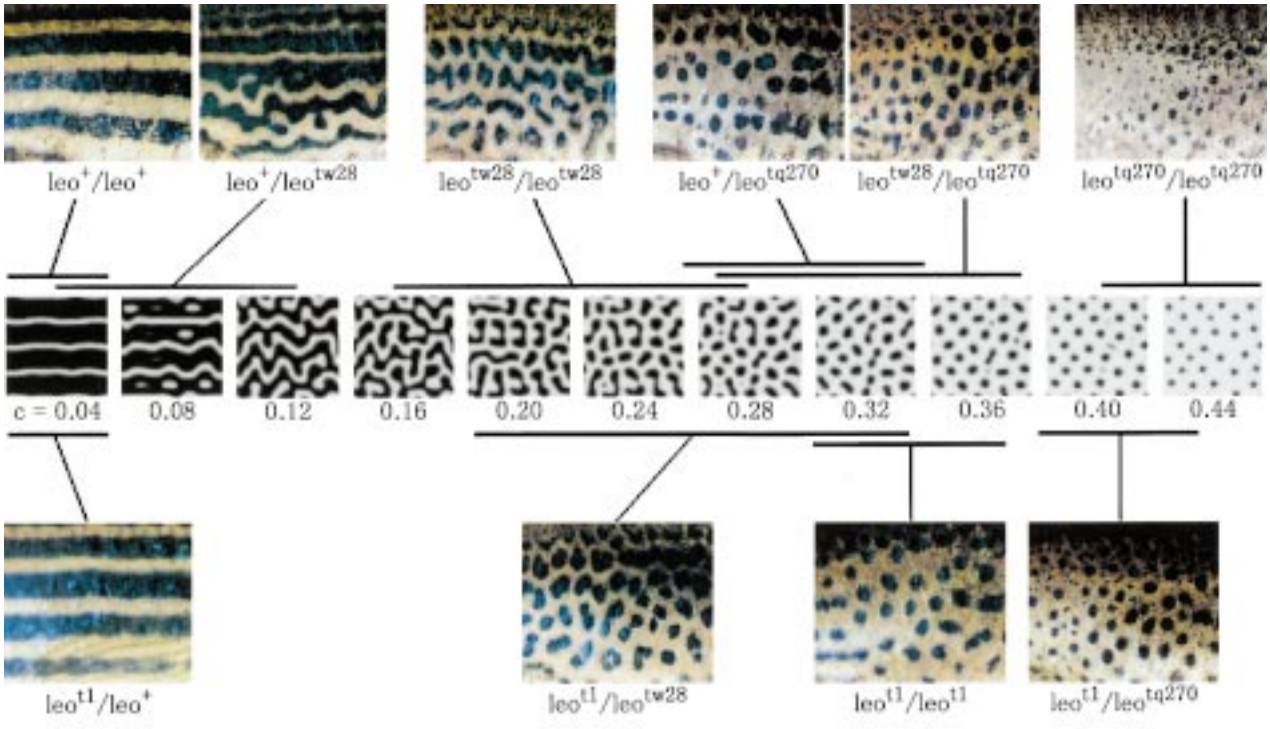

Fig. 4. Relationship between the mutant pattern and the c value of the RD system. Pictures are taken from the middle part of the fully-grown females. Horizontal bar shows the fluctuations within the genotypes.

alleles behave differently. The *c*-values of leo^{t1} containing heterozygous fish are close to that of homozygous fish of the counter alleles. The reason for this difference may result from the type of mutations. leo^{tw28} and leo^{tq270} are made by ENU mutagenesis and supposed to be point mutations, while leo^{t1} is a spontaneous one. Future molecular cloning of *leopard* gene should settle this issue.

3. Discussion

As shown above, the mechanism of pigment pattern formation may be common in lower vertebrates, and using the zebrafish mutants, the molecular level dissection of the mechanism is possible in combination with the computational analysis. Although our RD model explains the mutant patterns very well, it may not be an exclusive one. With the Gierer–Meihardt model, Meinhardt reported that the 'saturation of autocatalysis' controls the shift from stripes to spots (Meinhardt, 1989). It may be possible with other RD models as well to find parameters that generate all the patterns of *leopard* mutants because they share the common basic principles, 'short range activation and long range inhibition'(Meinhardt, 1982; Murray, 1989). The corresponding reaction that is controlled by the parameters would differ depending on the type of RD model. If our model is close to the real mechanism, the *leopard* gene may regulate the kinetics of activator synthesis, which is independent to the activator concentration (Fig. 3a). Only the cloning of the *leopard* gene and the following molecular analysis will allow a full molecular interpretation of the results.

In addition to reaction-diffusion, other mathematical models (Murray, 1989; Maini et al., 1991) have been proposed to explain biological pattern formation, for example the chemotactic model and the neural net model. Although it is not possible to rule out those alternatives from their dynamical properties alone, it seems they are unlikely because of the following observations. (1) In case of *Plecostomus*, the migration of melanocytes does not contribute to the pattern formation. The melanocytes distribute with a uniform density, and the pattern results from the size variations of the melanocytes (Fig. 5). Therefore it is difficult to apply a chemotactic model to the fish skin patterning. (2) A neural net model (Swindale, 1980) requires a network of neurons with extensive interconnectivity within the patterning field itself. However, in vertebrates the neural cells of the skin have connections only to the CNS – so such a network does not exist in the skin. The growth dependent change of the pattern on *Pomacanthus* and Plecostoms suggests that the pattern is formed in the skin.

The real molecular mechanism of the pigment patterning may be much more complex in comparison with the simplified theoretical models. Therefore it would be impossible to fully understand the mechanism without identifying the related molecules and genes. As shown in this report, the pigment pattern of zebra fish is an ideal subject for studying the dynamic pattern formation that can generate patterns

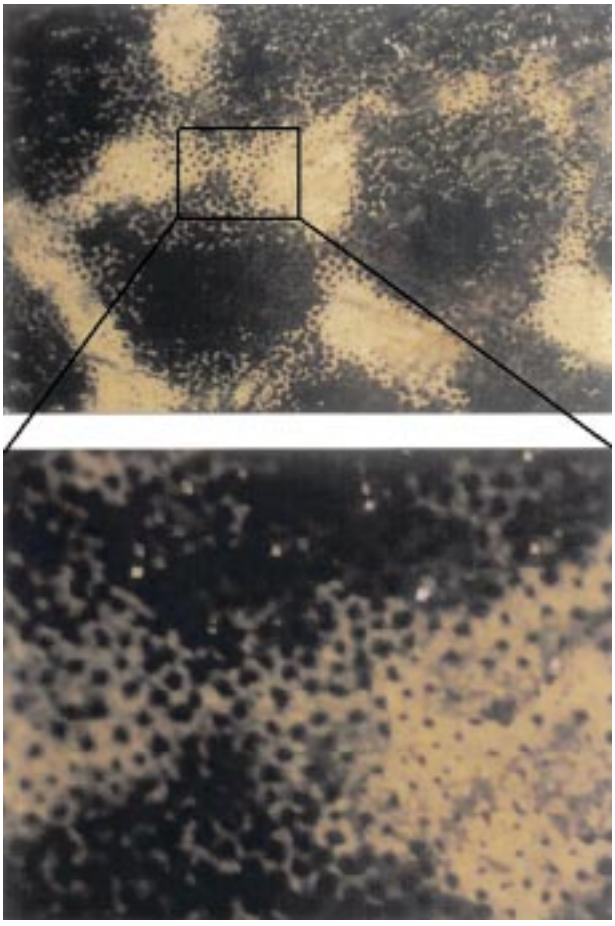

Fig. 5. Distribution of the melanocytes in the belly of Plecostomus. Melanocytes distribute uniformly. The difference in the size of melanocytes makes the pattern.

without any pre-pattern. The cloning of *leopard* gene will enable the interactive cooperation between the molecular and the theoretical biology in the eventual solution to this problem.

4. Experimental procedures

4.1. Fish

Mutant lines of zebra danio (leo^{t1}, leo^{tw28}, leo^{tq270}) (Haffter et al., 1996) are obtained from the zebrafish stock center of Max–Planck Institute (Tuebingen). Fish are maintained and bred as described in 'The zebrafish book'. The catfish Plecostomus was purchased from a local pet shop and maintained by the standard method. The pictures of the fish are taken after paralyzed with MS222 as described in the zebrafish book.

4.2. Simulations

The mathematical analysis was done with a workstation Gateway 2000 (G6-266). The program was coded with Delphy (Borland Co.). The source code and the compiled program used in this report will be provided on request.

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