Texture-Based Screening to Identify Genes Involved in Reproductive Aging in *Caenorhabditis elegans*

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Manuscript submitted February 18, 2021; accepted May 12, 2021.
doi: 10.17706/ijbbb.2021.11.3.40-49

Abstract: Reproductive capacity declines with aging, thereby increasing errors in fertilization, chromosome segregation, and embryonic cleavage. Age-related changes in oocytes involve not only function but also appearance. We previously showed that the texture of the cytoplasm in *Caenorhabditis elegans* oocytes varies with their age. We quantitatively characterized this change by evaluating two image features: Mm Value (mean difference between the maximum and minimum intensities within each moving window) and Correlation (COR), which is based on the Gray Level Co-occurrence Matrix (GLCM). However, the association between genetics and changes in cytoplasmic texture with aging has not been clarified. Genes that cause texture changes similar to those observed during oocyte aging are candidates for involvement in the aging pathway. Here, to examine whether gene knockdown of specific genes can change the Mm Value or COR, we analyzed the cytoplasmic texture in images from the Worm Developmental Dynamics Database 2. This database stores differential interference contrast microscopy images of early *C. elegans* embryos with genes silenced by RNA-mediated interference. Of 316 target genes screened, we identified five genes (*smc-4*, F10C2.4, *tin-44*, *let-754*, *hmp-2*) whose knockdown decreased the Mm Value and one gene (*csr-1*) whose knockdown increased the COR. The five genes included genes involved in age-related function, such as chromosome segregation and mitochondrial function, and some genes that are not known to be related to aging. The gene that increased the COR is also involved in chromosome segregation. Our findings indicate that these six genes could potentially be involved in reproductive aging.

Key words: *Caenorhabditis elegans*, image texture analysis, Nomarski DIC microscopy, RNAi screening, statistical image processing, reproductive aging.

1. Introduction

Reproductive capacity declines with aging, thereby increasing errors in fertilization, chromosome segregation, and embryonic cleavage [1]-[3]. However, the mechanisms regulating reproductive aging remain incompletely understood.

Caenorhabditis elegans (*C. elegans*) is a leading model for studying aging because of its short lifespan (~3 weeks) and the evolutionary conservation of longevity pathways from *C. elegans* to humans [4]. In particular, *C. elegans* has been developed as a model for studying age-related decline in fertility [5].

Age-related changes are found not only in oocyte function but also in oocyte appearance [2], [5], [6]. We previously showed that the texture of the cytoplasm in *C. elegans* oocytes varies with age [7]. We

quantitatively characterized this change by measuring the Differential Interference Contrast (DIC) image features of Max-min Value (Mm Value) and *Correlation* (COR) (Fig. 1a and Fig. 1b). The Mm Value is defined as the mean of the difference between the maximum and minimum intensities within successive moving windows and is a measure of texture roughness [7]. The COR, which is based on Gray Level Co-occurrence Matrix (GLCM) [8], measures the gray level linear dependencies of pixels at specified positions relative to each other. To calculate the COR, we first define a spatial relationship by using the parameters distance d and angle θ. We then calculate the second-order joint probability $P(i, j \mid d, \theta)$ of two pixels with gray levels i and j ($0 \le i < G$, $0 \le j < G$). To calculate $P(i, j \mid d, \theta)$, we sum the number of pixels with paired intensities (i and j) in the defined spatial relationship. We then extract the image feature using GLCM [7].

When the cytoplasm of 1-day-old and 3-day-old *C. elegans* oocytes are compared, the Mm Value decreases with aging whereas the COR, at several levels of parameters, increases with aging [7]. However, the relationship between variances in texture and biological and genetic processes has not been clarified. We consider that genes whose knockdown causes texture changes similar to those seen in aging oocytes are candidates for involvement in the aging pathway, and that identification of such genes may lead to understanding of the mechanisms underlying oocyte aging.

Worm Developmental Dynamics Database 2 (WDDD2, https://wddd.riken.jp), an update of WDDD [9], shares four-dimensional DIC microscopy images of 33 wild-type and 1728 RNAi-treated *C. elegans* embryos. RNAi is a phenomenon in which specific genes are inactivated by introduction of double-stranded RNA with complementary sequences [10]. The RNAi-treated embryos represent the 316 embryonic genes that exhibited an embryonic lethal phenotype in 100% of offspring when silenced in the previous genome-wide screen [11].

RNAi screening using computational image processing or automatic screening system is one of the state-of-the-art technologies in biomedical research [12], [13]. RNAi screening based on morphological features from image processing is unbiased and high-throughput approach. Recently, we proposed a method to quantify the age-associated changes in the cytoplasmic texture of *C. elegans* oocytes [7]. This method provides a new opportunity to apply the computational image processing-based RNAi screening to studies of aging in *C. elegans* oocytes.

Here, to examine whether gene knockdown can decrease the Mm Value or increase the COR (as seen in oocytes with aging), we analyzed the cytoplasmic texture in the early embryo images in WDDD2. Morphological defects in oocytes correlate with a decreased embryo-hatching rate [5], [6]. We assumed that the cytoplasmic texture properties in the oocytes are reflected in the early embryo and screened for genes whose knockdown decreased the Mm Value or increased the COR in cytoplasmic regions of early embryos. We identified five genes whose knockdown resulted in a decreased Mm Value, and one gene whose knockdown led to increased COR, suggesting that the genes are involved in reproductive aging.

The remainder of this paper is organized as follows. We provide details of the methods in Section 2. In Section 3, we describe the results. First, we overview our computational experiments (3.1). Then, we screen genes using the Mm Value (3.2), investigate the interactions among the candidate genes identified by the Mm Value (3.3). Finally, we screen genes using the COR (3.4). In Section 4, we discuss the implementation details and the candidate genes. Finally, in Section 5, we conclude this work.

2. Methods

2.1. WDDD2

Four-dimensional DIC microscope images in the WDDD2 were recorded in multiple focal planes and at multiple time points (0.102 μm per pixel). In the RNAi screen, young adult worms were separately injected with each double-stranded RNA, and embryos were dissected from the worms immediately after fertilization.

2.2. Calculation of Image Features

We used the images at the first time point in embryo development in WDDD2 and at the focal planes where the Mm Value in the entire image was maximum. To detect the cytoplasmic region, we used local image entropy [14]. To calculate image features, random regions of 80 × 80 pixels were extracted from the cytoplasmic region. Image features of individual embryos were defined as the mean of those in the extracted four regions.

The Mm Value was calculated by moving the local window except for the border in the same way as in the previous study of oocytes [7]. We set the size of the moving window to 3 × 3 pixels. COR, a second-order statistical feature based on GLCM, was calculated by using the following equation and the co-occurrence matrix $P(i, j \mid d, \theta)$ of two pixels with gray levels i and j ($0 \le i < G$, $0 \le j < G$):

$$\mathrm{COR} = \frac{\sum_{i=0}^{G-1}\sum_{j=0}^{G-1} ijP(i,j) - \mu_x\mu_y}{\sigma_x\sigma_y} \quad (1)$$

where G is the number of gray levels, μ_x, μ_y, σ_x, and σ_y are the means and standard deviations in the x and y direction given by $P(i, j \mid d, \theta)$,

$$\mu_x = \sum_{i=0}^{G-1}\sum_{j=0}^{G-1} iP(i,j)\,,\ \mu_y = \sum_{i=0}^{G-1}\sum_{j=0}^{G-1} jP(i,j) \text{ and}$$

$${\sigma_x}^2 = \sum_{i=0}^{G-1}\sum_{j=0}^{G-1} P(i,j)(i-\mu_x)^2\,,\ {\sigma_y}^2 = \sum_{i=0}^{G-1}\sum_{j=0}^{G-1} P(i,j)\left(j-\mu_y\right)^2$$

The co-occurrence matrix defined is symmetric.

3. Results

3.1. Overview of Our Experiments

To identify genes whose expression might affect Mm Values and/or the COR in aging oocytes, we examined the differences in image features between RNAi-treated and wild-type embryos in WDDD2 (Fig. 1c). We used 33 datasets from wild-type embryos and 1728 datasets from RNAi experiments, where 316 embryonic essential genes were individually silenced.

3.2. RNAi Screening Using the Mm Value

Of the 316 genes tested, we detected five genes, F35G12.8 (*smc-4*), F10C2.4, T09B4.9 (*tin-44*), C29E4.8 (*let-754*) and K05C4.6 (*hmp-2*), whose knockdown significantly reduced the Mm Value compared with that of the wild type (Fig. 2), suggesting that they are involved in reproductive aging. No genes were found to significantly increase the Mm Value compared with that of wild type.

The *smc-4* gene encodes an ortholog of human SMC4 (structural maintenance of chromosomes 4) protein, a mitotic condensing subunit that acts with MIX-1 to enable chromosome segregation [15]. Chromosomal abnormalities are a major cause of age-related decline in oocyte quality [2]. In *C. elegans* mutants of the TGF-β Sma/Mab pathway, reproductive aging is delayed [16]. The *smc-4* gene is upregulated in the TGF-β mutant oocytes [13]. The condensin SMC declines in both mouse and human oocytes with aging [17], [18], suggesting that chromosome segregation is a key process determining oocyte quality that is conserved between worms

and mammals [13]. Knockdown of *smc-4* degrades germline and oocyte morphology and increases the rate of unfertilized oocytes and unhatched embryos [13]. In WDDD2, almost all *smc-4* RNAi worms exhibited embryonic lethality and some of them produced unfertilized oocytes.

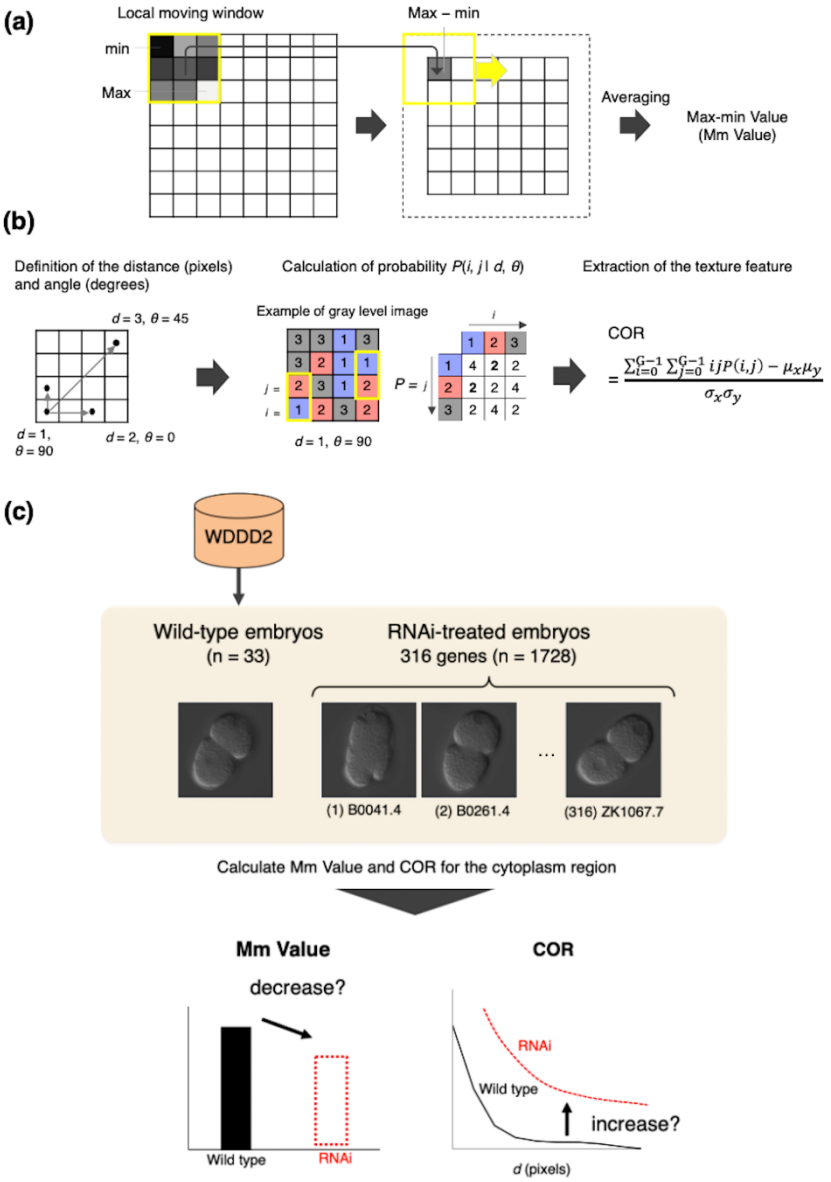

Fig. 1. Schema of image analysis methodologies. (a) Algorithm for calculating the max-min value (Mm value). (b) Algorithm for calculating the gray level co-occurrence matrix (GLCM). When the distance is 1 pixel and the direction is 90 degrees, the calculated number of pixels with $i = 1$ and $j = 2$ or $i = 2$ and $j = 1$ is 2. (c) Overview of our in silico experiments. The labels under the images refer to the individual genes. We screened for genes whose knockdown decreased the Mm Value or increased the COR in cytoplasmic regions of early embryos.

The F10C2.4 gene encodes the DNA polymerase delta complex subunit and is required for normal chromosome segregation [19]. Reduced expression of this gene may affect reproductive aging due to chromosome segregation errors, similar to *smc-4*.

The *tin-44* gene functions in transporting proteins into the inner mitochondrial membrane and matrix [20], [21]. Mitochondria have a pivotal role not only in energy metabolism but also in regulation of the rate of aging

[22]. *tin-44* RNAi worms exhibited a shorter lifespan than wild type [23], suggesting that mitochondrial dysfunction caused by RNAi of *tin-44* possibly affects aging.

The genes *let-754* encodes adenylate kinase [24], [25] and *hmp-2* encodes β-catenin and is required for proper cell migration [26]. Neither protein has been directly linked to aging. The genes *let-754* and *hmp-2* may be related to the aging pathway or may cause abnormalities in cytoplasmic textures via alternative pathways.

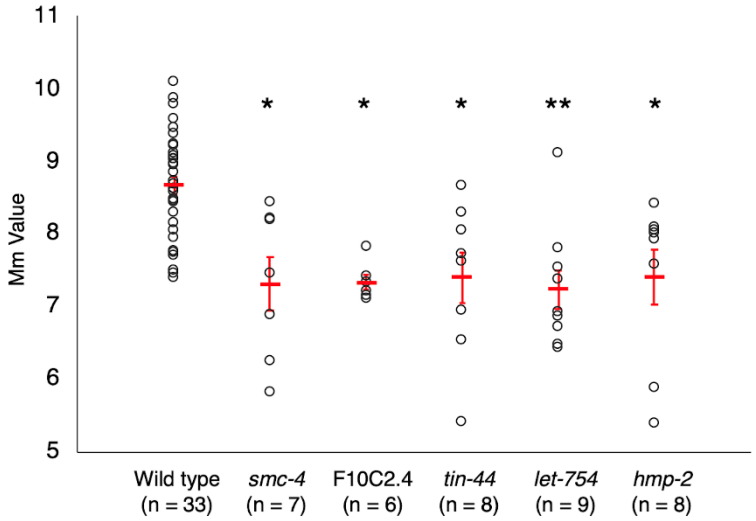

Fig. 2. Genes identified using the Mm value. When the five genes displayed were silenced in *C. elegans* embryos, the cytoplasmic region showed a significantly lower Mm Value than was seen in wild-type embryos. Red bars indicate mean values; error bars indicate SEM. Asterisks indicate statistical significance versus wild type (*P < 0.05, **P < 0.01; Dunnett's test).

3.3. Interaction among the Candidate Genes Identified by the Mm Value

We then automatically predicted the interactions (genetic interactions, co-expression, and physical interactions) among all five gene candidates identified by the Mm Value by using GeneMania software (http://www.genemania.org) [27]. In particular, we examined whether *let-754* and *hmp-2* interact with the three candidate genes that have known age-related functions (i.e., *smc-4*, F10C2.4, *tin-44*). From the network shown in Fig. 3, three genes, *hcp-3*, *zfp-1*, and *mut-7* have predicted simultaneous interactions with *smc-4*, F10C2.4, and *hmp-2*. Although *tin-44* was predicted to interact with *hmp-2* via two genes, the interaction was relatively weak. We found no association between *let-754* and the genes identified by the Mm Value. The results suggest that *smc-4*, F10C2.4, and *hmp-2* have high genetic interactions and *tin-44* and *let-754* have less interactions with other genes.

We then examined whether the knockdown of the five candidate genes shares any of the phenotype categories annotated by Piano *et al.* [28], which are available in the RNAi Database (http://www.rnai.org) [29]. We focused on cytoplasmic phenotypes and found that knockdown of *smc*-4, F10C2.4, and *hmp-2* was included in the category "Multiple cavities", which is described as "multiple vesicles, vacuoles, or cavities are seen during early embryogenesis". Both the shared phenotype category and the predicted gene interactions mentioned above suggest that *smc-4*, F10C2.4, and *hmp-2* have similar properties in terms of function and phenotype; therefore, *hmp-2* may function in an aging pathway related to chromosome segregation like *smc-4* and F10C2.4. In addition, out of the 27 genes in the "Multiple cavities" category, 13 genes were not included in WDDD2. These 13 genes might include gene(s) whose knockdown decreases Mm Value and have a related

function to *smc-4*, F10C2.4, and *hmp-2*.

The five genes whose knockdown reduced the Mm Value can be divided into three groups: a chromosome abnormality group (*smc-4*, F10C2.4, and *hmp-2*), mitochondrial function group (*tin-44*), and unknown group (*let-754*), based on the results of the gene interaction and phenotype category analyses. Therefore, multiple pathways may contribute to reductions in Mm Value.

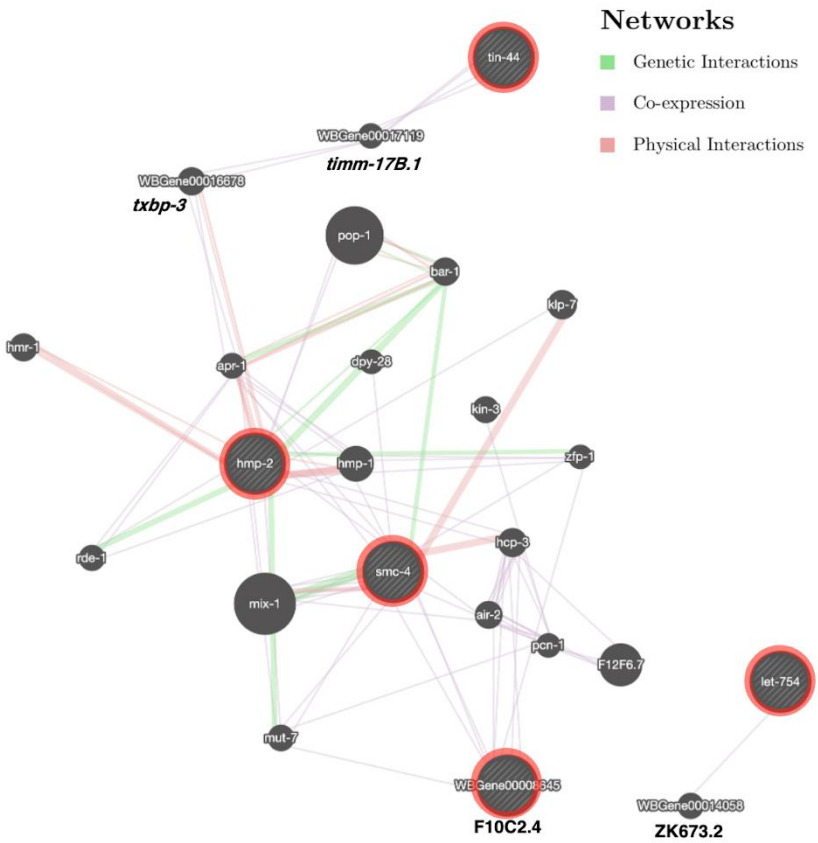

Fig. 3. Gene interactions among the genes whose knockdown decreased the Mm value. The genes identified by changes in Mm Value are highlighted by red circles.

3.4. RNAi Screening Using the COR

We then screened the RNAi-treated embryos' images in WDDD2 to find RNAi-treated embryos whose cytoplasmic regions displayed significantly higher COR than in wild type. The angle parameter of GLCM was set to θ = 0, 45, 90, and 135 degrees. For the 316 genes tested, the COR when the angle was set to 0, 45, or 135 degrees did not differ significantly between RNAi and wild-type embryos (data not shown). However, for two genes, *csr-1* and *gbp-1*, knockdown significantly increased and decreased the COR, respectively, compared with wild type when θ =90 (Fig. 4).

The *csr-1* gene encodes CSR-1, an Argonaute protein that is essential for chromosome segregation and embryo viability [30]. CSR-1 is a core component of P granules and acts with P granules to maintain germline integrity [31]. Because the COR increased when *csr-1* was silenced, *csr-1* is a candidate for involvement in oocyte aging.

Conversely, knockdown of *gpb-1* decreased the COR. The *gpb-1* gene encodes the heterotrimeric G protein β subunit, which is required for proper spindle orientation and regulation of egg laying [32], [33], but has not yet been shown to be directly related to reproductive aging. Further experiments are needed to clarify the associations between the decline of the COR and gene functions.

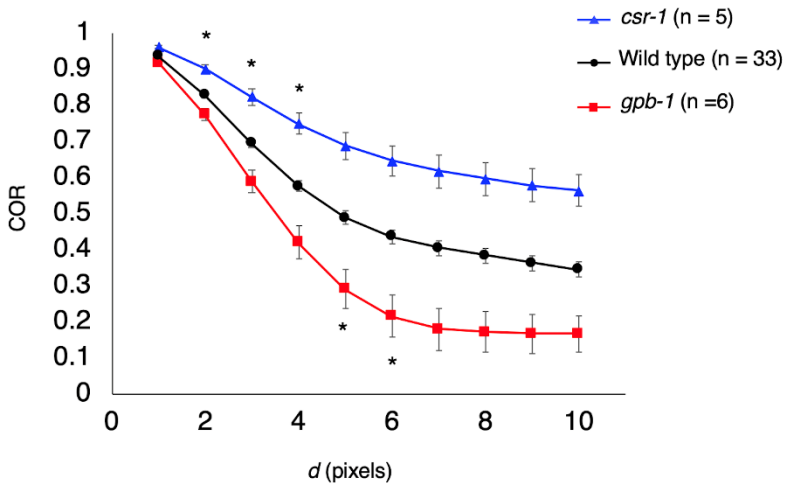

Fig. 4. Genes identified using the COR. *Correlation* (COR) is displayed as a function of distance *d* for two genes whose knockdown (in RNAi embryos) resulted in significantly increased or decreased COR compared with wild type. Error bars indicate SEM. Asterisks indicate statistical significance between the RNAi embryos and wild-type embryos (*$P < 0.05$, Dunnett's test).

4. Discussion

In our previous study, the Mm Value and the COR in oocytes changed simultaneously with aging [7]. However, in the current study, knockdown of specific genes changed the image features independently, suggesting that the image features can be used to characterize different properties of the age-associated changes in the cytoplasmic texture. In other words, texture changes caused by RNAi knockdown can be divided into two patterns, one that decreases the Mm Value and one that increases the COR, and the associated genes might function in different pathways involved in aging. The insulin/insulin-like growth factor pathway, a well-known pathway controlling the lifespan of *C. elegans*, and the TGF-β Sma/Mab pathway are considered to regulate reproductive lifespan independently [16], [34], [35]. Our results show that knockdown of *smc-4*, which functions in the TGF-β Sma/Mab pathway, decreased the Mm Value in cytoplasmic regions of *C. elegans* embryos, implying that the texture characterized by the Mm Value is involved in the TGF-β pathway. In contrast, the texture characterized by the COR is possibly involved in the insulin/insulin-like growth factor pathway although there is no direct experimental evidence to support this. The texture characterized by the Mm Value reflects the changing contrast in the DIC granules, which may be due to chemical modification or a difference in content quantity of the granules. A change in the COR reflects a change in the size of cytoplasmic granules [7]. Further experiments are needed to understand the association between gene function and the morphology of the cytoplasmic granules. Elucidation of this association may be helpful for understanding the aging pathways and mechanisms.

5. Conclusion

By screening images of RNAi-treaded embryos in WDDD2, we identified genes whose knockdown changes the cytoplasmic texture in early *C. elegans* embryos, mirroring the changes in oocytes with aging. Of 316 target genes, knockdown of five genes was found to significantly decrease the Mm Value and knockdown of one gene significantly increased the COR. These genes could potentially be involved in reproductive aging.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Momoko Imakubo, Jun Takayama and Shuichi Onami designed research; Momoko Imakubo and Shuichi Onami wrote the paper; Momoko Imakubo performed computational experiments; Koji Kyoda and Hiroya Itoga managed and prepared datasets; all authors had approved the final version.

Acknowledgment

This work was supported by Core Research for Evolutionary Science and Technology (CREST) Grant Number JPMJCR1511, Japan Science and Technology Agency (JST); JSPS KAKENHI Grant Number JP18H05412; and DECODE project, RIKEN Center for Biosystems Dynamics Research, Japan.

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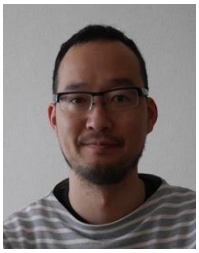

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