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Innate sexuality determines the mechanisms of telomere maintenance

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ABSTRACT Recently, telomere length has been shown to be differentially regulated in asexually and sexually reproducing planarians. In addition, it was found that asexual worms maintain telomere length somatically during reproduction by fission or when regeneration is induced by amputation, whereas sexual worms only achieve telomere elongation through sexual reproduction. We have established an experimental bioassay system to induce switching from asexual to sexual reproduction in planarians, that is, sexualization. In this study, the relationship between the reproductive mode and telomere maintenance was investigated using innate asexually reproducing worms, innate sexually reproducing worms, and experimentally sexualized worms. Here, we show that innate asexual planarians maintain telomere length during cell division and that innate sexual planarians exhibit telomere shortening. However, experimental sexualized worms maintain telomere length during cell division. These results indicate that innate sexuality is linked to the mechanism of telomere maintenance.

KEY WORDS: *telomere length, innate sexuality, planarian*

Prokaryotes maintain an intact genome by asexual reproduction and therefore do not have a fixed lifespan. However, eukaryotic organisms that reproduce sexually produce offspring that have completely new genomes due to recombination of the parental genomes. In these organisms, cell lineages have a defined lifespan, as do the organisms themselves (Hug 2006; Williams 1957), which is linked to telomere shortening during each round of eukaryotic DNA replication. However, some eukaryotic animals can also reproduce asexually; therefore, asexual species, such as planarians, may not have a fixed lifespan.

The planarian is a useful model animal for investigating the relationship between the reproductive mode and predetermined lifespan. Many planarian species have strains with different modes of reproduction: exclusively asexual, exclusively sexual, or switching between them seasonally (Hyman, 1951). Although sexual planarians do not reproduce asexually (i.e., spontaneous fission), they can regenerate all tissues after amputation, including germ cells and somatic cells of the genital organs (Nodono *et al.,* 2012). The mechanism controlling lifespan in these species has not been elucidated, but many extrinsic factors, including oxidative stress and regulation of energy production, and intrinsic factors, such as maintenance of telomere length, are expected to be involved. It was recently shown in the planarian *Schmidtea mediterranea* that somatic telomere maintenance differs between asexually and sexually reproducing animals. Telomere length is maintained somatically during asexual reproduction by fission or when regeneration is induced by amputation, whereas sexually reproducing animals can only achieve telomere elongation by sexual reproduction (Tan *et al.,* 2012). This difference is reflected in the expression of alternatively spliced forms of the telomerase enzyme protein subunit. Therefore, asexually reproducing animals must have telomere-maintenance mechanisms that allow telomere maintenance to occur somatically.

We have established an experimental bioassay system to induce sexual switching in the planarian *Dugesia ryukyuensis* OH strain (Kobayashi *et al.,* 1999), which has been maintained under laboratory conditions for more than 20 years through only asexual reproduction. Sexual reproduction can be induced by feeding with sexually mature planarian. We termed this experimentally sexualized worm "acquired sexual (AqS)" worm. When 2 AqS worms mate, they produce both exclusively asexually and sexually reproducing offspring. We termed innate asexual (AS) and innate sexual (InS) worms, respectively. Recently, we compared InS and AqS worm's stem cell capability by transplantation into X-ray irradiated AS re-

Abbreviations used in this paper: AqS, acquired sexual; AS, asexual; InS, innate sexual.

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cipients, revealing that only InS worms can autonomously initiate a sexual state and AqS worms do not acquire the sexual state initiation capability like InS worms, although AqS worms can maintain the induced sexual state (Nodono and Matsumoto, 2012; Nodono *et al.,* 2012). Therefore, these 2 sexually reproducing worms with different innate reproductive mode enable us to test the relationship between innate sexuality and telomere maintenance. In this study, we investigated whether sexually reproducing animals can only achieve telomere elongation through sexual reproduction.

Results

Telomere FISH analysis in D. ryukyuensis

We compared the karyotypes and morphological features of *D. ryukyuensis* planarian worm populations for different reproductive modes (AS vs. InS). The karyotypes of these worms included some chromosomal changes, including polyploidy and heteroploidy. The most typical triploid karyotypes of AS worms and InS worms are shown in Fig. 1A. These karyotypes are identical and do not show any translocations. FISH analysis of the $(TAAGGG)_4$ sequence in triploid organisms of the *D. ryukyuensis* OH strain (Fig. 1B) showed that the chromosomal ends were fluorescently labeled.

Telomere maintenance in asexual planarians

To investigate whether AS worms maintain their telomere length, we performed Southern hybridization by using the telomere probe $(TAAGGG)_4$. Telomere signals for 3 independent AS strains were broad and smeared, with an average maximum length of 34.83 kb (SD = 0.76), an average minimum length of 13.17 kb (SD = 0.76), and a mean length of 24.00 kb, (SD = 0.75) (Fig. 2A). In addition, to determine whether telomere length was dependent on the period since hatching, telomere length was compared between newborn AS worms, AS worms maintained over 2 years with repeated amputation and regeneration, and OH worms (Fig. 2B). A mean telomere length of 24.00 kb was observed in all 3 populations.

Telomere maintenance in innate sexual planarians

Telomere length was compared between newborn InS worms, InS worms maintained for 2 years with repeated amputation and regeneration, and OH worms. Telomere length in InS worms depends on the period after hatching (mean length in newborn InS worms, 18.0 kb; mean length in 2 year-old InS worms, 14.5 kb; Fig. 3A). Statistical analysis showed that the range of telomere sizes in InS worms was wider than that in AS worms (AS worms, 1.036; InS worms, 8.060; $n = 7$ for both) and the telomeres were significantly longer in AS worms than in InS worms (mean length in AS worms, 23.67 kb; mean length in InS worms, 18.89 kb; $n = 7$; two-tailed t-test, $P < 0.003$).

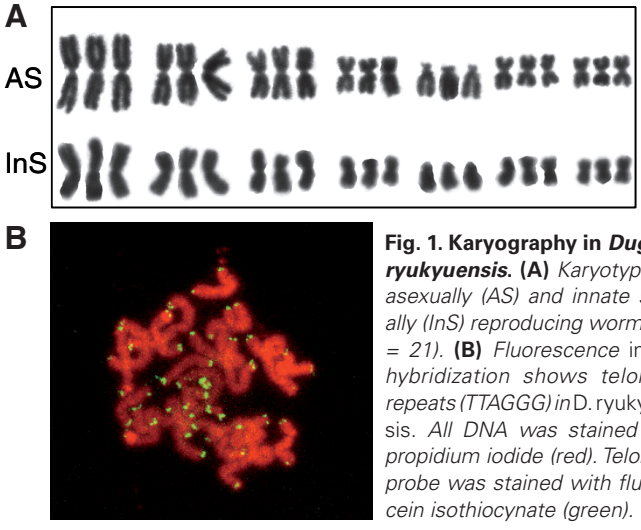

Fig. 1. Karyography in *Dugesia ryukyuensis*. (A) *Karyotypes of asexually (AS) and innate sexually (InS) reproducing worms (3n = 21).* **(B)** *Fluorescence* in situ *hybridization shows telomere repeats (TTAGGG) in* D. ryukyuensis. *All DNA was stained with propidium iodide (red). Telomere probe was stained with fluorescein isothiocynate (green).*

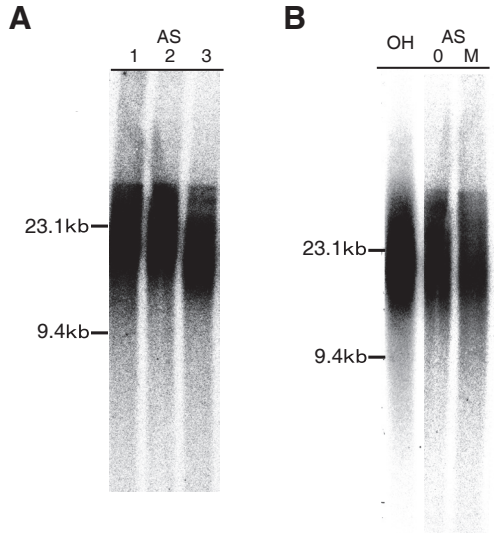

Fig. 2. Telomere length in asexual (AS) planarians. (A) *Telomere length in 3 independent experimentally sexualized (AqS) strains.* **(B)** *Effect of aging on telomere length in AqS worms. 0, newborn worms; M, worms maintained with repeated amputation and regeneration for 2 years; OH, the* D. ryukyuensis *fissiparous OH strain.*

Telomere length in InS worms shortened depending on the age, which led us to investigate the ability to maintain the telomere length during cell cycle. InS worms had been amputated and regenerated up to 12 times to activate the somatic cell cycle and the telomere length of InS worms regenerated 4, 8, and 12 times was examined (Fig. 3B). Worms that regenerated 4 times maintained a telomere length similar to that of asexually reproducing worms. However, the worms that regenerated 8 and 12 times had shorter telomeres. Repeated amputation and regeneration for 12 times shortened the InS worm's telomere by 200 bp (Fig. 3C). These data show that telomeres shorten in InS planarians by approximately 16.7 bp per regeneration.

Telomere maintenance in acquired sexual planarians

To investigate whether sexually reproducing animal can only achieve telomere elongation through sexual reproduction, telomere length was compared between newly sexualized AqS worms, AqS worms maintained for over 2 years with repeated amputation and regeneration, and OH worms. The telomere signals in 3 independent AqS strains were broad and smeared (Fig. 4A), with an average maximum length of 32.33 kb (SD = 2.31), an average minimum length of 15.33 kb (SD = 0.29), and a mean length of 23.83 kb (SD = 1.23). These telomeres are similar in length to telomeres in AS worms (mean length, 24.00 kb). Statistical analysis showed that telomere length was not significantly different between AqS worms at several period after sexualization and in AS worms (mean length in AS worms, 24.00 kb; mean length in AqS worms, 23.83

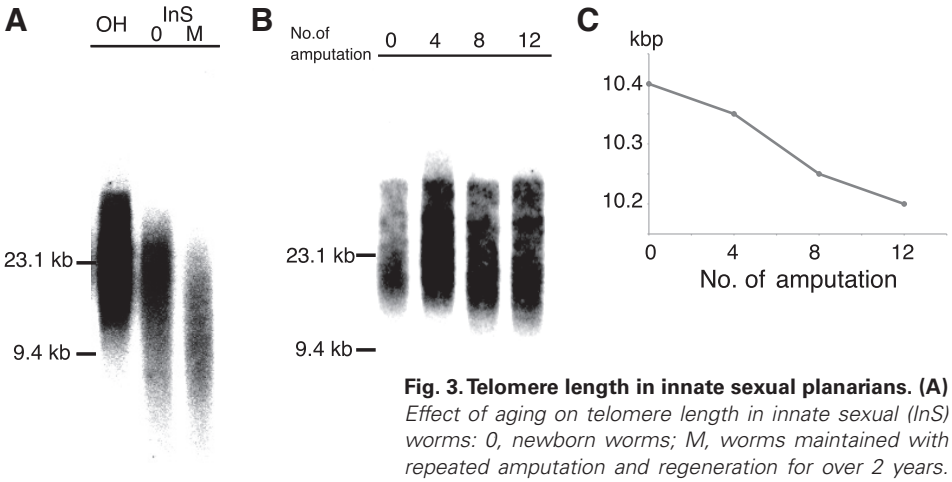

Fig. 3. Telomere length in innate sexual planarians. (A) *Effect of aging on telomere length in innate sexual (InS) worms: 0, newborn worms; M, worms maintained with repeated amputation and regeneration for over 2 years.* **(B)** *Effect of the number of amputation and regeneration cycles on telomere length in InS worms.* **(C)** *Graph showing data from Fig. 3B. The x-axis is the minimum length of telomere.*

kb; $n = 3$; two-tailed t-test, $P < 0.85$). The telomere length did not differ between newly sexualized worms and worms with repeated amputation and regeneration (Fig. 4B).

Discussion

There are 3 reproductive strategies in planarians: exclusively asexual, exclusively sexual, and switching between the asexual and sexual modes (Kenk, 1937; Kobayashi and Hoshi, 2002). By comparing telomere length in the 3 reproductive modes (AS, InS, and AqS) in *D. ryukyuensis*, we found that AS worms can maintain telomere length during regeneration (Fig. 2) and that telomeres shorten during InS worm regeneration (Fig. 3). These data support previous reports that telomere maintenance differs in asexual worms and sexual worms in *S. mediterranea* (Tan *et al.,* 2012). Our results show that the telomere length in AS worms that have reproduced by fission and regeneration for over 20 years is the same as that in AS newborn worms (Fig. 2B). However, it is not known whether switching the reproductive mode, resulting in the production of offspring by fertilization and embryogenesis, can help maintain telomere length. In mice and cattle, it has been demonstrated that a telomere-elongation program is initiated at the morula to blastocyst transition that establishes a specific telomere length set point during embryogenesis (Schaetzlein *et al.,* 2004). AqS worms that have transformed from the asexual to the sexual reproductive mode by our method provide a useful experimental model to address this issue. In this study, we observed that AqS worms can maintain their telomere length almost as well as AS worms (Fig. 4). Therefore, the ability to produce germ cells and sexual organs is not linked to telomere maintenance.

However, it is not known whether the rate of change in telomere length is associated with lifespan. In birds and mammals, telomere length shortens more slowly in long-lived species than in short-lived ones (Haussmann, *et al.,* 2003). For example, the maximum lifespan of zebra finches is 5 years, of tree swallows is 11 years, and of Adélie penguins is 20 years, and in these species, the rate of change in telomere length is 515 bp/year, 391 bp/year, and 235 bp/year, respectively. In this study, InS worms showed a relationship between regeneration number and the telomere length, but AS worms did not. Then, AS worms might not have a fixed life-span.

Neoblasts are proliferating cells in planarian bodies, comprising approximately 30% of their entire body (Baguñà, 1981; 2012). If it is assumed that the neoblasts in the half-body remaining after fission can proliferate and differentiate to resynthesize the entire body, then these neoblasts should each undergo cell division about 3 times. Therefore, neoblast telomeres should shorten by approximately 5 bp per cell division. In *S. mediterranea*, sexual and asexual strains can be distinguished by the presence of a chromosomal translocation only in asexually reproducing worms (Newmark and Sánchez Alvarado, 2002). Planarians containing this translocation reproduce by transverse fission and do not show differentiation for a germ line or somatic copulatory apparatus; planarians lacking this translocation are hermaphrodites that do not reproduce asexually. Asexually reproducing worms can produce offspring only by fission and regeneration, and only sexual reproducing worms can produce differentiated germ cells and perform fertilization. *S. mediterranea* worms with different reproductive modes can be distinguished by a chromosome translocation, i.e., both asexual and sexual strains are different. However, in *D. ryukyuensis,* the chromosome patterns are not different in both strains (Fig.1A). And worms can naturally switch the mode of reproduction to either sexual or asexual. AqS worms, which are transformed from asexual to sexual worms, produce offspring that reproduce either asexually or sexually. In addition, AqS worms can reproduce by genetic cross-mixing (Kobayashi *et al.,* 2008).

Recently, we reported that neoblasts from InS worms but not AqS worms have the autonomous capability to form individuals that can reproduce sexually (Nodono and Matsumoto 2012; Nodono *et*

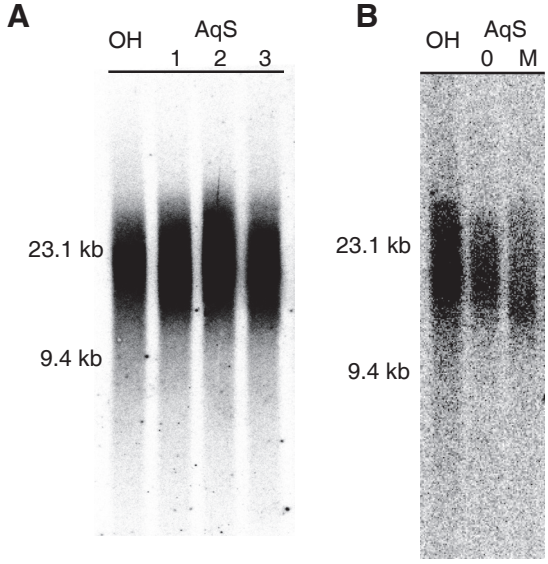

Fig. 4. Telomere length in acquired sexual planarians. (A) *Telomere length in 3 independent AqS strains. 0, newborn worms; M, worms maintained with repeated amputation and regeneration for 2 years.* **(B)** *Effect of aging on telomere length in experimentally sexualized (AqS) worms.*

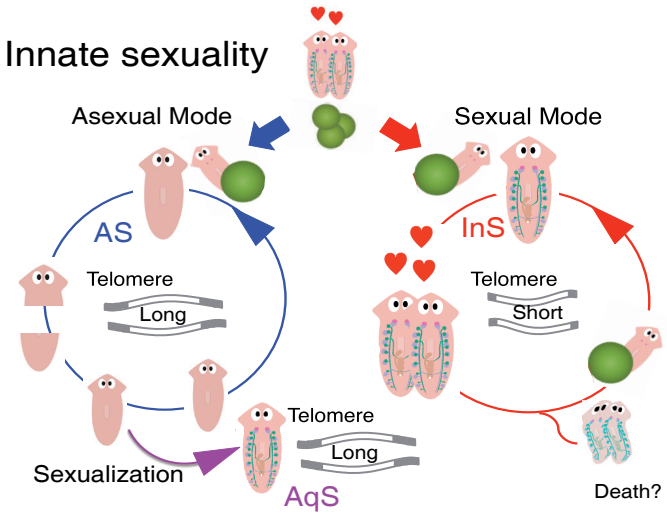

Fig. 5. Schematic of the relation of telomere length and innate sexuality.

al., 2012). In the 2 types of planarians that produce sexual organs, cellular abilities differ according to the sexual mode they have at birth (i.e., their innate sexuality). In this study, we showed that AqS worms can maintain telomere length and that InS worms cannot maintain telomere length. Therefore, the ability to maintain telomere length is determined by the mode of reproduction at birth (Fig.5).

In *S. mediterranea*, the capacity to maintain telomere length is determined by the expression level and alternative splicing of the telomerase enzyme protein subunit (Tan *et al.,* 2012). *D. ryukyuensis* is a useful model species for studying switching of reproductive modes. Studying the mechanism of telomerase gene expression in *D. ryukyuensis* (AS, AqS, and InS) could improve our understanding of the relationship between the reproductive mode and lifespan.

Materials and Methods

Planarian strains and culture

The planarian *D. ryukyuensis* OH strain was provided by Dr. S. Ishida of Hirosaki University. AS worms and InS worms were cultured as previously described (Kobayashi *et al.,* 2009). All the AqS worms were obtained by feeding OH worms with *Bdellocephala brunnea* worms (Kobayashi *et al.,* 1999).

Chromosome preparation and fluorescence in situ *hybridization*

To prepare metaphase spreads of mitotic chromosomes for imaging, a previously published hybridization protocol was followed (Kobayashi *et al.,* 2008). The preparations were dried overnight and stored at –80°C. The samples were stained with Giemsa stain or used for fluorescence *in situ* hybridization (FISH). Studies on the Planariidae *Polycelis tenuis* and the Dendrocoelidae *Dendrocoelum lacteum* have indicated that the planarian telomere sequence is a repeated TTAGGG sequence (Joffe *et al.,* 1996, 1998). A previously published hybridization protocol was followed (Suzuki, *et al.,* 1999), using the labeled telomere probe $(TTAGGG)_4$.

DNA preparation and Southern blot analysis

Planarian genomic DNA was prepared using the Wizard SV Genomic DNA preparation system (Promega, USA) according to the manufacturer's instructions. For Southern blot analysis, genomic DNA, completely digested by *Hinf*I, was separated on a 0.5% agarose gel and hybridized with the telomere probe $(TTAGGG)_4$ by using previously described protocols (Tan *et al.,* 2012).

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