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Medaka as a model organism for research in experimental biology

Kiyoshi Naruse[1], Mitsuru Sakaizumi[2] and Akihiro Shima[1]

1) Laboratory of Radiation Biology, Zoological Institute, School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, 2) Department of Environmental Science, Faculty of Science, Niigata University, Niigata 950-21, Japan.

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Abstract The medaka, *Oryzias latipes*, has been widely used as an experimental animal in Japan, because of its relatively short life cycle (2 to 3 months), high fecundity (laying over 10 eggs daily) and small size (saving space for large-scale breeding). In this review, we will describe the development of the medaka as an experimental animal based on its characteristics, availability of several experimental methodologies, mutant and inbred strains, and availability of other species of the genus *Oryzias* and related genera. We also describe advantages and disadvantages of the medaka compared with other small freshwater fish such as zebrafish, platyfish and swordtail.

1. Characteristics of the medaka as an experimental animal

1) Essential characteristics of the medaka as an experimental animal

Because the medaka is an inhabitant of stagnant water in the Temperate Zone, aeration and thermostability are not necessary for the breeding of medaka, and relatively large numbers of fish can be kept in small-scale facilities. The medaka can survive at low temperature (4°C) in winter. This differs from the cases of other small tropical freshwater fish such as zebrafish and platyfish. Males and females can be easily distinguished by external morphology. The medaka is oviparous and females lay about 10 eggs every day under temperature- and light-controlled conditions (Robinson and Rugh, 1943; Egami, 1954). Embryos hatch around 7 days after fertilization at 25°C. Newly hatched fry grows to adult size in 3 months and the next generation can be obtained. The egg chorion is transparent, so we can observe all stages of embryonic development under a binocular microscope. Artificial insemination can be carried out under the isotonic conditions (Yamamoto, 1933, 1944). Using this method, we can obtain synchronously developing embryos. Sexuality of newly hatched fry is committed but not determined (Yamamoto, 1953, 1958), such that the sex of the offspring can be controlled even after hatching.

The genome size (1.7 pg) of the medaka is about one-third that of mice. The chromosome number for a diploid individual is 48, an exception being the fish from the China-West Korean population, where it is 2n = 46 as a result of Robertsonian centric fusion (Uwa *et al.*, 1988). Fourteen species of the genus *Oryzias* have been reported to date and 12 out of 14 species are maintained in Japan (Naruse *et al.*, 1993; Iwamatsu *et al.*, 1993). All species are tropical except *Oryzias latipes*. *Xenopoecilus saracinorum*, which is a relative of the genus *Oryzias*, is also maintained at Shinshu University. All these species are potential candidates for additional experimental models.

2) Experimental methods and characteristics of medaka strains

Several unique methods and strains have been established using the characteristics described above. Several inbred strains have been established by brother-sister mating (Hyodo-Taguchi and Egami, 1985). Over 60 visible mutant strains were isolated and are maintained at the Bioscience Center of Nagoya University (Tomita, 1992). Methods for producing transgenic fish (Ozato *et al.*, 1986) or chimeric fish were established using artificial insemination and manipulation of embryos. Chromosome manipulation methods using artificial insemination with UV-irradiated sperm and application of heat shock or hydrostatic pressure to embryos have also been established (Naruse *et al.*, 1985; Naruse and Shima, 1989). We can control the sexuality by administering steroid hormones via fish food (Yamamoto, 1953, 1958, 1975) or by dipping fertilized eggs in steroid hormone-containing solution. Using the artificial control of sex, we can separate the effects of genetic sex from the those of phenotypic sex. Hybrid female fish obtained by crossing *O. latipes* and *O. curvinotus* lay diploid eggs, not haploid

eggs. In addition, all eggs from one such female were genetically identical (Sakaizumi *et al.*, 1993). The specific-locus method has also been established using the medaka and we can induce mutations by gamma-rays or ENU (Shima and Shimada, 1988, 1991). Induced or spontaneous mutants could be isolated by gynogenesis or F2 inbreeding (Shimada and Shima, in preparation). Furthermore, the AP-PCR fingerprinting method has been successfully combined with the medaka specific-locus method, yielding a new model system for detecting mutations on a DNA basis (Kubota *et al.*, 1992, 1994).

2. Medaka strains and their use

1) Visible mutant strains

The genotypes of over 60 visible mutant strains of medaka (Tomita, 1992) can be easily identified using a binocular microscope. For example, the *r* gene (Aida, 1921) and the *lf* gene (Wada *et al.*, submitted) are linked to the sex determination locus. Using these sex-linked genes, we can identify the sex with a binocular microscope at 3 days post-fertilization (Yamamoto, 1975; Wada *et al.*, submitted). All visible mutants isolated to date are autosomal recessive except those due to mutations at the *r, lf, Va, Da,* and *Si* loci. Four linkage groups of visible mutant loci have been reported (Yamamoto and Oikawa, 1973; Tomita, 1985); however, recent success in application of the AP-PCR fingerprinting method (Kubota *et al.*, 1992, 1994) yielded 28 linkage groups (Wada *et al.*, submitted). All the visible mutant strains were derived from spontaneous mutants occurring in wild populations or culture stocks. These strains are maintained at the Bioscience Center of Nagoya University. Several radiation- or chemical-induced mutant strains are also being maintained at the Laboratory of Radiation Biology, School of Science, The University of Tokyo (Shima and Shimada, in preparation).

2) Wild stocks and inbred lines

Genetic differences in the wild population of the medaka have been studied extensively (Sakaizumi *et al.*, 1983; Sakaizumi, 1985, 1986; Matsuda *et al.*, 1993). Wild stocks of the medaka have been preserved at the Laboratory of Radiation Biology, School of Science, The University of Kyoto, as a genetic resource under subsidy from the Ministry of Education, Science and Culture, Japan (Shima *et al.*, 1985a,b). Analysis of protein polymorphism showed that wild populations of *Oryzias latipes* are divided into 4 groups: Southern population, Northern population, East-Korean population and China-West Korean population. These four populations are characterized by population-specific allozymic variations and differences in mitochondrial DNA. The Northern population which is distributed along the coast of the Sea of Japan, is genetically homogeneous; very few genetic variations of proteins and mitochondrial sequences have been observed (Sakaizumi, 1986; Matsuda *et al.*, 1993). One inbred strain, HNI, has been established from the Northern population. The Southern population is distributed along the Pacific coast extending to Iwate Prefecture and is genetically quite variable. Within the Southern population we detected region-specific genetic variations in both proteins and mitochondrial sequences. All inbred strains except HNI belong to the Southern population (Table 1). The East-Korean population extends from the Sea of Japan side of Korea to the southern part of the Korean peninsula (Sakaizumi and Jeon, 1987). The China-West Korean population is widely distributed, ranging from Yunnan to the western coast of the Korean peninsula (Sakaizumi and Jeon, 1987; Uwa *et al.*, 1988). Although these four populations have large sequence differences in their mitochondrial DNA, they could be freely interbred.

Nucleotide divergence of the mitochondrial cytochrome b sequence is about 10% between the Northern and Southern populations (Matsuda *et al.*, 1993). This large genetic difference can be advantageous in genome mapping studies. When we randomly selected 1 kbp single-copy genomic DNA fragments from the medaka genome and analyzed the RFLPs with 10 restriction endonucleases using them as probes, we always detected RFLPs between HNI and other inbred strains (Kurihara *et al.*, 1992). A genetic linkage map comprised of 170 loci and spanning over 2480 contiguous centimorgans has been established by PCR-based DNA fingerprinting, allozyme analysis and observation of coloration pattern using HNI and other inbred strains derived from the Southern population (Wada *et al.*, submitted).

3) The genus Oryzias and relatives

Fourteen species of the genus *Oryzias* have been identified to date. Twelve out of 14 are maintained at several laboratories in Japan (Table 2) (Yamamoto, 1975; Naruse *et al.*, 1993; Iwamatsu *et al.*, 1993). Karyological analysis revealed that

Table 1. Strain distribution of polymorphic variants

Strain	Origin	Mutant genes	Protein locus						
			Acp	*Adh*	*Iddh*	*Odh*	*Pgm*	*Sod*	*Ldh-A*
HO4C	cultivated	b R	*b*	*a*	*a*	*c*	*b*	*b*	*c*
HO5	cultivated	b R	*b*	*a*	*a*	*b*	*b*	*b*	*c*
HB11	Wild (Chiba)	B R of	*b*	*a*	*b*	*b*	*b*	*b*	*a*
HB12	Wild (Chiba)	B R	*b*	*a*	*b*	*b*	*b*	*b*	*a*
HB32	Wild (Chiba)	B R	*b*	*a*	*a*	*b*	*b*	*b*	*c*
HNI	Wild (Niigata)	B R	*a*	*b*	*b*	*b*	*a*	*a*	*c*
Hd-rR	Stock in Nagoya University	b r (female) b R/r (male)	*b*	*a*	*a*	*b*	*b*	*b*	*d*

Acp, acid phosphatase; *Adh*, alcohol dehydrogenase; *Iddh*, L-iditol dehydrogenase; *Odh*, octanol dehydrogenase; *Pgm*, phosphoglucomutase; *Sod*, superoxide dismutase; *Ldh-A*, lactate dehydrogenase-A.

the genus *Oryzias* can be divided into three groups, the mono-armed chromosome group, the bi-armed chromosome group and the fused chromosome group (Uwa, 1986). *O. latipes* belongs to the bi-armed chromosome group. Three other species (*O. curvinotus, O. luzonensis* and *O. mekongensis*) also belong to this bi-armed chromosome group. The monophyly of this group is supported by phylogenetic analyses suing both the mitochondrial 12s ribosomal RNA gene sequences and the bi-armed chromosome group-specific repetitive sequences (Naruse *et al.*, 1992; Kusama *et al.*, 1993; Matsuda *et al.*, 1993). Hybrid males obtained by crossing *O. latipes* and *O. curvinotus* are sterile, although such hybrid females are fertile. These hybrid females spawn diploid eggs, as a result of failing to undergo meiosis. All eggs from one female have identical genotypes (Sakaizumi *et al.*, 1993). Using chromosome manipulation and taking advantage of this phenomenon, trigenomic allo-triploids or amphi-diploids have been produced (Kurita *et al.*, 1992, 1993). These experiments indicate the potential usefulness of the related species.

The mono-armed chromosome group consists of *O. melastigma, O. javanicus* and *O. minutillus.* This group is widely distributed over Pakistan, India, the Malaysian Peninsula, Borneo, Java,

Table 2. List of species of the suborder Adrianichthyoidei maintained in Japan and their chromosome numbers

Group name	Species	Chromosome numbers
Mono-armed chromosome group	*Oryzias melastigma*	48
	O. javanicus	48
	O. minutillus	28, 30, 32, 34, 40, 42
Bi-armed chromosome group	*O. mekongensis*	48
	O. latipes	46 or 48
	O. curvinotus	48
	O. luzonensis	48
Fused chromosome group	*O. celebensis*	36
	O. nigrimas	36
	O. marmoratus	42
	O. matanensis	42
Unclassified	*O. profundicola*	?
	Xenopoecilus saracinorum	?

Lombok and the southern part of Sulawesi. The fused chromosome group (*O. nigrimas, O. matanensis, O. marmoratus,* and *O. celebensis*) is found only in Sulawesi (Uwa, 1986; Naruse *et al.*, 1993). Most species of the genus *Oryzias* in Sulawesi are endemic to individual lakes located in central or south Sulawesi. Phylogenetic analysis of the cytochrome b gene sequence indicates that these species are monophyletic (Naruse *et al.*, 1993). In addition, phylogenetic analysis using the 12s ribosomal RNA gene sequence showed that two species of the genus *Xenopoecilus* are related to the fused chromosome group (Naruse *et al.*, in preparation). These results suggest that reconsideration on the erection of the two genera, *Oryzias* and *Xenopoecilus,* might be necessary.

3. The medaka and other freshwater fish used as model systems

There are several freshwater fish which have been used as model systems. Platyfish, *X. maculatus,* and swordtail, *X. helleri*, have been used for studying genetic carcinogenesis. In this system, melanoma was induced in hybrid fish obtained by crossing platyfish and swordtail. The melanoma-inducing locus (*Tu*) was revealed to be sex-linked, and the *Tu* gene has already been isolated and characterized as encoding the tyrosine kinase receptor (Wittbrodt *et al.*, 1989). Linkage relationships of enzyme loci are also well studied using interspecific hybrids (Morizot, 1990). Variations or modifier genes of the sex-determination system in the genus *Xiphophorus* have been reported (Kallman, 1984). Fish of the genus *Xiphophorus* are ovoviviparous. With regard to this point, fishes of the genus *Xiphophorus* differ significantly from the medaka. It is not possible to rear eggs from ovoviviparous fish *in vitro* and the analysis of early embryonic development is therefore difficult. We speculate that it would be very difficult to produce transgenic fish or chimeric fish using this species.

The zebrafish, *Danio rerio,* is oviparous and belongs to the family Cyprinidae. Its generation time is similar to that of the medaka, although embryonic development is faster than that of the medaka, with embryos hatching between 2 to 3 days post-fertilization at 28°C. Chorions of zebrafish embryos are soft, and blastomeres at early cleavage stages are larger than those of the medaka. Therefore, the manipulation of embryos is easier (Westerfield, 1993). Many mutant strains have been produced using gamma-irradiation or chemicals as mutagens (Westerfield, 1993; Solnica-Kretzel *et al.*, 1994). Many developmental analyses using mutants and/or embryo manipulations have been reported (Kimmel *et al.*, 1990; Warga and Kimmel, 1990; Hatta *et al.*, 1991). Chromosome manipulation methods are also established (Streisinger *et al.*, 1981). On the other hand, genetic analyses of the wild population and domesticated stock or phylogenetic analyses of related species have been rather poor. However, recently, the Darjeeling strain was collected from the wild population of zebrafish. This strain differs genetically from the domesticated stocks such as Oregon AB strain (Johnson *et al.*, 1994). A genetic linkage map has been established using the Darjeeling and Oregon AB strains. This map consists of 401 RAPDs and 13 simple sequence repeats spaced at an average interval of 5.8 cM (Postlethwait *et al.*, 1994), and will be a powerful aid for position-based cloning of genes. Zebrafish researchers have been most generous in sharing their findings, techniques, strains and insights, and this knowledge has been compiled in the Zebrafish Book (Westerfield, 1993) and well-organized network among researchers.

4. Concluding remarks

When we compare the medaka with other experimental model systems, knowledge about genome structure is poor. Although many visible mutant strains have been reported, there are only a few studies about these mutants on the molecular level. Construction of a detailed linkage map of the medaka genome must be achieved as soon as possible. More cDNA and genes of interest must be cloned. Establishment of insertional mutagenesis and gene targeting systems is required. There are several reports on the presence of retroposons (Naruse *et al.*, 1992; Kido *et al.*, 1991) or Tc1-like element in the fish genome (Radice *et al.*, 1994). These elements seem to be good candidates for establishing a transposon tagging system. The establishment of embryonic stem cell lines and cell transplantation methods should also accelerate the developmental study of the medaka. This might be an appropriate time for medaka researchers to organize our research efforts, emulating the successful and well-organized network achieved by our colleagues researching zebrafish.

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