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Genetic Similarity of the Hainan Medaka Populations Collected from Hyper- and Hypo-osmotic Environments in Northern Vietnam

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Abstract – Ricefishes of the genus *Oryzias*, including Japanese medaka (*O. latipes*), are known as excellent model organisms for studies in various fields of science. Some species of the genus inhabit brackish water, and such species are recognized to be useful to investigate physiological phenomena in seawater. However, only a limited number of species have been recorded from brackish waters. In addition, there is no information about the genetic relationship among populations inhabiting sites with different salinities. Here we report the discovery of *Oryzias* fish in two locations near Haiphong, northern Vietnam, a brackish mangrove planting area and a freshwater pond. A phylogenetic analysis using mitochondrial 12S and 16S ribosomal RNA (rRNA) gene sequences indicated that the fish from the two localities are the same species, Hainan medaka, *O. curvinotus*. Population genetic analysis using the mitochondrial 12S and 16S rRNA gene sequences revealed a close genetic relationship between the two populations. These results suggest that *O. curvinotus* is adaptable to both hyperosmotic and hypoosmotic environments. Due to its osmotic adaptability and ease of rearing in the laboratory, this species is expected to become a model for marine environmental and toxicological studies, as well as for studies of osmotic adaptation mechanisms.

Key words – *Oryzias curvinotus*, population genetics, molecular phylogenetics, euryhalinity, environmental sciences

1. Introduction

Small fish species have been used in various fields including environmental science and toxicology because they are easily cultured in small aquaria in the laboratory. However, most model species are stenohaline freshwater fish and not suitable for experiments in seawater. In contrast, marine fish are generally large and not suitable for laboratory use. Thus, new model species are needed for marine environmental studies. We expect that the genus *Oryzias* (Beloniformes: Adrianichthyidae), a group of small fish including the euryhaline species can be used as such a model species (Inoue and Takei 2002, 2003; Kinoshita et al. 2009).

The genus *Oryzias* contains more than 20 species, all of which have been thought to be primarily inhabiting fresh water (Takehana et al. 2005; Parenti 2008; Kinoshita et al. 2009) except for two brackish-water species, Indian medaka (*O. dancena*) and Javanese medaka (*O. javanicus*) (Yusof et al. 2012). Other than *Oryzias*, the family Adrianichthyidae is dominated by freshwater fishes. The dominance of freshwater fishes in this family is of evolutionary interest because other families in the order Beloniformes include seawater fishes, such as the sauries, flying fishes, and halfbeaks (Setiamarga et al. 2008).

The freshwater habitat of a species does not directly imply that it lacks the ability to survive in seawater. For example, it has been experimentally demonstrated that Japanese medaka (*O. latipes*) adapts to seawater after a short acclimation period in half-diluted seawater (Inoue and Takei 2002, 2003). In addition, there is a record of Japanese medaka bred in salt-water ponds (Yanagishima and Mori 1957). Thus, Japanese

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medaka is potentially euryhaline, even though its major habitat is fresh water.

The genus *Oryzias* is divided into three major groups, i.e., the *O. latipes* species group, *O. javanicus* species group, and *O. celebensis* species group (Kinoshita et al. 2009). This classification was proposed based on karyotype analyses but was later confirmed by phylogenetic analyses using the mitochondrial 12S and 16S and nuclear tyrosinase gene sequences (Takehana et al. 2005). Japanese medaka is included in the *O. latipes* group, and Indian and Javanese medaka are in the *O. javanicus* group. That is, the Japanese medaka is distantly related to the two brackish-water species. These phylogenetically distant species share euryhalinity, indicating that other *Oryzias* species may also have adaptability to high salinity. However, no scientific study has examined the natural distribution of *Oryzias* species in hyperosmotic conditions, except for the three species mentioned above.

We have studied the distribution of *Oryzias* species in Asia to obtain good experimental models that can be used in salt water and to understand the evolutionary background of the euryhalinity of this genus. In this study, we report the discovery of *Oryzias* fish in a mangrove planting area in Bang La, Do Son, Vietnam, where the salinity is greater than 20 ppt, and also from a freshwater pond in a rice paddy area away from the coast. We identified the fish from both localities as the Hainan medaka, *O. curvinotus*, by phylogenetic analysis using mitochondrial 12S and 16S ribosomal RNA (rRNA) gene sequences that have been used as genetic markers for *Oryzias* fishes in a previous study (Takehana et al. 2005). In addition, genetic differentiation was not detected between the two populations, suggesting that Hainan medaka inhabits a range of environments including both fresh water and hyperosmotic brackish water.

2. Materials and Methods

Fish sampling

The *Oryzias* fishes are easily caught because they prefer to swim near the surface of shallow water. Thirty-nine fish were collected at a mangrove planting area facing the open sea at Bang La, Do Son, Vietnam (20°42'24.80"N, 106°45'04.05"E) (Fig. 1) using a hand net. Sixty-six fish were collected from a pond in a rice paddy area in Dai Hop, Vietnam (20°41'46.35"N, 106°42'22.74"E) (Fig. 1). The salinity at the sampling points, measured using the salinity meter S-10E (Atago, Tokyo, Japan) were 22 ppt (Bang La) and 0 ppt (Dai Hop). Fish samples
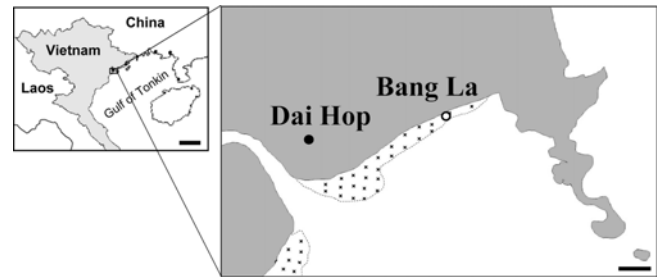
Fig. 1. Map of the sampling locations. In the right panel, "x" surrounded by a hatched line represents a mangrove planting area, and gray area indicates land. White and black circles indicate the sampling points at Bang La and Dai Hop, respectively. Bars in the left and right panels indicate 100 km and 1 km, respectively

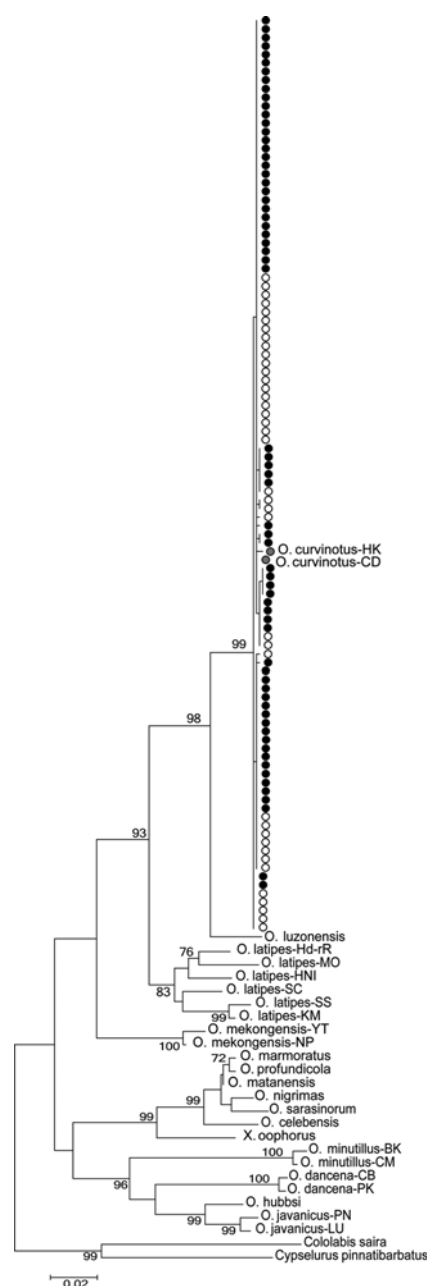were kept in 99.5% ethanol until use.

DNA sequencing

Total DNA was extracted from a piece of caudal fin using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The partial sequences of the nuclear tyrosinase gene (725 bp) and mitochondrial 12S and 16S rRNA genes (386 and 471 bps, respectively) were amplified according to the methods of Takehana et al. (2005). Variations in these sequences are supposed to be neutral for osmotic adaptation. The sequences of the amplified fragments were determined using an ABI3130 Sequencer (Life Technologies, Carlsbad, CA, USA).

Phylogenetic analysis

The sequences obtained in this study and corresponding regions (partial tyrosinase gene sequence and mitochondrial 12S and 16S rRNA gene sequences) of *Oryzias* species obtained from the DDBJ/EMBL/GenBank database (Takehana et al. 2005) were aligned using MAFFT v6 (Katoh and Toh, 2008). Maximum likelihood (ML) trees were constructed with MEGA v6 (Stamatakis 2006) using the Tamura-Nei model and bootstrap analysis was conducted with 1,000 replications.

Population genetics analyses

Haplotype diversity (h) within each locality was calculated using Arlequin v3.5 (Excoffier et al. 2010). A statistical parsimony network indicating the relationships and their distribution was assembled using TCS v1.21 (Clement et al. 2000). The difference in the haplotype frequencies between the two localities was examined using the exact test of population differentiation (Raymond and Rousset 1995) implemented in Arlequin, based on the Markov Chain Monte Carlo method

with 100,000 steps, discarding 10,000 steps as a burn-in. The genetic differentiation between the two localities was assessed using the unbiased fixation index (F_{ST}) and the significance was tested using 100,000 permutations (Weir and Cockerham 1984).
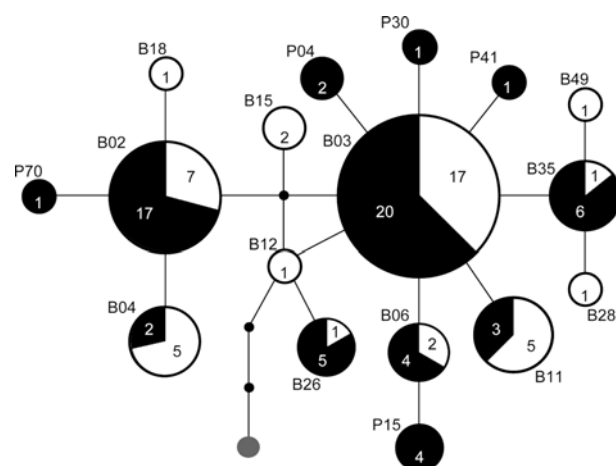
3. Results

Phylogenetic analysis

In the phylogenetic tree based on the 12S and 16S rRNA genes, the fish of the two locations formed a single clade with the *O. curvinotus* sequences obtained from the database (Fig. 2) and this clade was supported by a high bootstrap value (100%). Thus, all of the fish collected in this study were confirmed to be *O. curvinotus*. The fish collected from the two locations did not form separate clusters within the clade. We also attempted phylogenetic analysis using the tyrosinase gene sequences but there was not enough resolution to separate even *O. curvinotus* from *O. luzonensis* (data not shown). Thus, the tyrosinase gene sequence was not used for further analysis.

Population differentiation

Of the 857 nucleotides of the partial 12S+16S rRNA gene sequences, 17 were variable. We identified 17 distinct 12S+16S

Fig. 2. Maximum likelihood tree of sequences of the *Oryzias* fish sampled in the present study and previous studies. For the details regarding the samples from previous studies, see Takehana et al. (2005). The tree was constructed using MEGA (v.6) based on 12S and 16S mitochondrial rRNA gene sequences. Numbers at the nodes indicate bootstrap values; values greater than 70 are indicated. White and black circles represent the fish from Bang La and Dai Hop, respectively. Gray circles indicate Hainan medaka reported in a previous study. BK, Bangkok (Thailand); CB, Chidambaram (Indoa); CD, Caudien (Vietnam); CK, Chiang Mai (Thailand); HK, Sam A Tsuen (Hong Kong); KM, Kunming (China); LU, locality unknown; MO, Mooka (Japan); NP, Nakhon Phanom (Thailand); SC, Sokcho (Korea); SS, Samsan (Korea); YT, Yang Talat (Thailand); PK, Phuket (Thailand); PN, Penang (Malaysia). HNI and Hd-rR represent inbred strains

Fig. 3. The statistical parsimony network of the Hainan medaka *Oryzias curvinotus* obtained in this study. The areas of the circles are approximately proportional to the frequency of the haplotypes. White and black sectors indicate the relative frequencies of specimens from Bang La and Dai Hop, respectively. Numbers with P or B are the names of haplotypes. Numbers in the circles are the number of fish with the haplotype. Haplotypes that were not obtained in this study are indicated by dots. The gray circle represents Hainan medaka from Hong Kong reported in a previous study (Takehana et al. 2005)

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rRNA gene haplotypes from the 105 specimens. The haplotype diversity was similar for the Bang La and Dai Hop specimens, 0.853 ± 0.036 and 0.828 ± 0.029 ($h \pm SD$), respectively. Seven of the 17 haplotypes were common to both localities and 5 specific haplotypes were obtained from each locality. In the parsimony network analyses (Fig. 3), none of the major haplotypes (consisting of six or more specimens) was specific to a single locality, and minor location-specific haplotypes differed from shared haplotypes by 1 substitution, except for one haplotype (B15 in Fig. 3) that differed by two substitutions. The genetic differentiation between the two locations was not significant based on the exact test of population differentiation between the two locations ($p = 0.052$). The F_{ST} value between the two localities was -0.00377, and this estimate of differentiation was not significant ($p = 0.465$).

4. Discussion

An enormous number of fish have been identified from freshwater and seawater habitats worldwide. However, most species inhabit either freshwater or seawater, and a limited number of euryhaline species can inhabit both environments (McCormick et al. 2013). In hypoosmotic freshwater environments, fish must cope with overhydration and ion-deficiency, whereas they must deal with dehydration and ion overload in hyperosmotic seawater. Thus, fish must differently regulate ions and water under hyper- and hypoosmotic conditions (Takei et al. 2007; McCormick et al. 2013), and this explains the limited number of euryhaline species. Among *Oryzias* fishes, Indian and Javanese medaka are euryhaline (Inoue and Takei 2002, 2003). The two euryhaline species co-exist around estuaries in Peninsular Malaysia, but the habitats of the two species are essentially separated by salinity; Indian medaka mainly inhabits hypoosomotic areas and Javanese medaka is found in hyperosmotic areas (Yusof et al. 2012). Thus, these two species avoid the bidirectional regulation of water and ions by preferentially inhabiting regions characterized by a particular salinity range despite their adaptability to both conditions (Inoue and Takei 2002).

In this study, we demonstrated that all of the *Oryzias* fish found at a mangrove planting area and a freshwater pond in northern Vietnam are Hainan medaka, *O. curvinotus*. As the osmolality of teleost fish is almost one-third that of seawater (Takei et al. 2007), the former habitat is hyperosmotic and the latter hypoosmotic for Hainan medaka. Genetic differentialtion was not detected between the two populations, suggesting

that they are not only the same species, but also genetically similar. These results indicate that Hainan medaka can inhabit both hyperosmotic and hypoosmotic environments and the isosmotic point does not limit the habitat of this species. Thus, this species is highly flexible with respect to the direction of ion and water regulation. Although the mechanisms of long-term adaptation to hyperosmotic and hypoosmotic environments have been studied in detail in teleosts (Evans 2008; McCormick et al. 2013), information about adaptation to fluctuated osmolality is still limited. For example, atrial natriuretic peptide (ANP) has been suggested to be involved in the immediate response to the sudden increase of environmental osmolality in eels (Takei and Hirose 2002) but *Oryzias* fishes seem to have a different regulatory system (Inoue et al. 2012). Hainan medaka may offer an interesting model for studies of adaptation to salinity fluctuation. Due to its salinity adaptability and ease of rearing in the laboratory, this species may become a useful experimental animal to examine the effects of toxic chemicals and pollutants in salt water.

Among the three species groups in the genus *Oryzias*, Hainan medaka belongs to the *O. latipes* species group. In this group, Japanese medaka has the potential to adapt to seawater, as mentioned above (Inoue and Takei 2002, 2003). Hainan medaka was adaptable to hyper- and hypoosmotic conditions in this study, indicating that the *O. latipes* species group may consist of euryhaline species, although it is still unknown whether the Philippine medaka (or Luzon medaka) *O. luzonensis* is euryhaline. The *O. javanicus* species group also contains Indian and Javanese medaka inhabiting brackish areas. Thus, euryhalinity may be a common characteristic of the genus. If so, the Beloniformes consists entirely of euryhaline fishes. The only exception may be the species that belong to the *O. celebensis* species group. *O. marmoratus*, a member of this group, has been shown experimentally to be a stenohaline freshwater fish (Inoue and Takei 2002; 2003). The phylogenetic position of Hainan medaka, i.e., its close relationship with Japanese medaka, is also advantageous owing to the abundance of scientific information, including genome sequence, the knowledge of germ cell maturation, and developmental stages, for Japanese medaka.

Acknowledgments

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