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Genetic diversity of *Oryzias* fishes based on mitochondrial DNA variation

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CONTENT

ABSTRACT

The genus *Oryzias* is distributed from India to the Far East. Eleven species have been identified. Karyological analyses have demonstrated that the genus *Oryzias* has been divided into three groups, the monoarmed, the biarmed, and the fused chromosome group. Biochemical analysis of enzyme polymorphisms within the genus *Oryzias* has provided support for these groupings. The biarmed chromosome group includes *O. latipes*, *O. curvinotus*, *O. luzonensis*, and *O. mekongensis*. Allozymic analysis demonstrate that four genetically distinct populations exist in the *O. latipes*; the Northern Population, the Southern Population, the East Korean Population, and the China-West Korean Population. The genetic diversity estimated among these four populations are large enough to be considered as characteristic of interspecific comparisons.

Mitochondrial DNA (mtDNA) is a valuable molecule for understanding the evolutionary relationships among individuals, populations, and species. Mitochondrial DNA is inherited maternally and nonrecombine. The nucleotide of the genome substitute rapidly in contrast to the nuclear genome. The rate is about 1-2% sequence divergence per lineage per million years, or perhaps 5-10 times faster that typical single-copy nuclear DNA.

In this study I investigated genetic diversity of *Oryzias* fishes based on mitochondrial DNA variation. The main objectives of the present study were to survey mtDNA polymorphism in Japanese wild population of medaka, and to presume phylogenetic relationships among the species in the biarmed chromosome group.

The phylogenetic reconstructions are based on a restriction fragment length polymorphisms (RFLPs) of mtDNA in Korean and Japanese population. To investigate the origin of the Japanese two

populations of medaka, I compared nucleotide sequence of mitochondrial control region and cytochrome *b* gene of four species in the biarmed chromosome group.

Basic results in the present study are as follows.

(1) Mitochondrial DNAs of the Japanese wild population of medaka are divided into three clusters (A to C). Cluster B was subdivided into 11 subclusters. The average nucleotide diversities among these three clusters are 8.9% (A versus B), 8.4% (A versus C), and 7.3% (B versus C). Cluster C is miner cluster.

(2) Mitochondrial DNAs of Korean wild population were separated into two clusters. The average nucleotide diversity between two clusters from Korea was 8.9%.

(3) The distribution patterns of five clusters and subclusters in medaka revealed strong geographical associations.

(4) Nucleotide sequences of complete mitochondrial control region from two Japanese populations have tandemly repeat sequence.

(5) *Oryzias mekongensis* had no such a tandem repeat sequences in the control region, whereas other three species had a tandemly repeat sequences.

(6) The phylogenetic analysis using nucleotide sequences of complete mitochondrial cytochrome *b* gene indicated monophyly of Japanese major two clusters.

(7) A sister relationship between *O. curvinotus* and *O. luzonensis* is also indicated.

The results of mtDNA analysis suggested a scenario of speciation process in the biarmed chromosome group and intraspecific relationships in *O. latipes*. *Oryzias mekongensis* had first diverged from a common ancestor of the biarmed chromosome group. Next, *O.*

curvinotus- *O. luzonensis* and *O. latipes* were separated. Next, in *O. latipes*, the China-West Korean Population, the East Korean Population, and Japanese populations was divided. In the time, *O. curvinotus* and *O. luzonensis* are separated. Finally, Japanese two population was separated into the Northern Population and the Southern Population.

GENERAL INTRODUCTION

The medaka, *Oryzias latipes*, is an egg-laying freshwater fish native to East Asia. Its small size, ease of breeding and shortness of generation time make it a useful experimental animal for genetic research (Yamamoto, 1975).

An outstanding ecological characteristic of this fish is that it inhabits marches, ponds and irrigation canals amid rice fields in flat alluvial lowlands. Typical habitats of this species are still waters in lowlands. This means that they cannot survive either in seawater or in swift streams in the mountains. Thus, local populations of this species are confined to their own watershed and isolated from one another.

Because of the proximity of Japan to the Asian continent, its freshwater fish fauna is closely related to that of Asia. Because of its isolation, however, many endemic species are found. In the Pleistocene the islands of Japan were connected by land bridges not only with one another but also with the continent in consequence of the lowering of the sea level. Medaka are found in China, Korea, the southern three major islands of Japan, and many smaller islands near them. This interrupted range suggests the possibility of local genetic differentiation.

As for geographic variation in medaka, *O. latipes*, some meristic characters have been studied (Egami, 1953; Egami and Yoshino, 1958; Yamamoto, 1975). The counts of anal fin-rays collected at 125 localities revealed that the mean value for populations along the Sea of Japan was smaller than for those along the Pacific. Recently, geographic variation in biochemical characters of medaka was demonstrated in proteins (Sakaizumi *et al.*, 1980; Sakaizumi *et al.*, 1983). These results indicate four genetically distinct populations; the Northern Population from the northern coast of the Sea of Japan, the

Southern Population from western and southern Japan, the West Korean Population from western and southern Korea, and the China-West Korean Population from China and western Korea. These four assemblages can be distinguished by means of many unique alleles and Nei's coefficients of genetic distance are no less than 0.30 (Sakaizumi, 1986a; Sakaizumi and Joen, 1987; Sakaizumi *et al.*, 1983). These findings suggest that the wild populations of the medaka are genetically differentiated with respect to region and that the large intraspecific variation may be another important tool for genetic research with the medaka. These strains would be the best materials for linkage and mapping studies, and with the success of such strains the usefulness of the medaka in the laboratory would be further enhanced.

The genus *Oryzias* is distributed from India to the Far East. Eleven species have been identified (Uwa and Magtoon, 1986; Yamamoto, 1975). The phylogenetic relationships among eight species have been studied by karyological (Magtoon and Uwa, 1985) and biochemical analysis (Sakaizumi, 1985b). From the results of these studies, the genus *Oryzias* has been divided into three groups which are based on karyological characteristics. The first group is the monoarmed group, which includes *O. melastigma* and *O. javanicus*. The second group is the biarmed group, which includes *O. latipes*, *O. curvinotus*, *O. luzonensis*, and *O. mekongensis*. The last group is the fused chromosome group. *O. celebensis* and *O. minutillus* are members of this group (Magtoon and Uwa, 1985). Biochemical analysis of enzyme polymorphisms within the genus Oryzias has provided support for these groupings (Sakaizumi, 1985b).

Animal mtDNA is a circular molecule usually ranging in size from 14,000 to 26,000 base pairs (bp). It is present in high copy

number per cell making it relatively easy to purify (Brown, 1983). Generally, mtDNA is homoplasmic, that is, all the molecules are identical in an organism, meaning that any tissue can be used as a source. The rate of nucleotide substitution in the mtDNA of higher vertebrates is approximately 5-10 times that of the nuclear genome, enhancing the resolution capacity for population-level studies (Brown, 1983; Moritz *et al.*, 1987). Mitochondrial DNA is maternally transmitted and is not subject to recombination (Avise and Vrijenhoch, 1987; Gylensten and Wharton, 1985; Hutchingson-III *et al.*, 1974).

In philopatric fish species these characteristics have the effect of generating more genetic divergence in the mitochondrial than in the nuclear genome. The extent of genetic divergence among both segments of the genome is determined by the interaction of processes such as natural selection and genetic drift, which promote divergence and gene exchange which reduces it. When populations are reasonably large and selection pressures weak, gene frequency divergence in the nuclear genome can be suppressed by modest flow arising from the movement of individuals of either sex. Thus, in the case where females are philopatric and males disperse during spawning no divergence in the nuclear genome will occur. However, this same pattern of gene exchange permits divergence of the mitochondrial genome because of its maternal inheritance.

Maternally inherited mtDNA is also more likely to show differences among populations than nuclear DNA because its smaller effective population size (1/4 that of the bisexually inherited diploid nuclear genome if the sex ratio is 1:1) means that it is more susceptible to reductions in genetic variability due to population bottlenecks (Birky *et al.*, 1983; Nei and Tajima, 1981). Moreover, assuming equal

migration ability of both sexes, four times as much interchange among populations is required to prevent divergence in the mitochondrial, as opposed to the nuclear genome in populations which are now genetically similar. Conversely, four times as much migration is required to erode existing divergence in mtDNA haplotype frequencies as to erode gene frequency differences at nuclear loci.

Recently, complete nucleotide sequence of the mitochondrial DNA genomes of fish species are also reported (carp; Chang *et al.*, 1994, rainbow trout; Zardoya *et al.*, 1995, and Atlantic cod; Johansen and Bakke, 1996)

In the present theses, I investigate mtDNAs of medaka and those of its related species. The main purpose of this study is to survey mtDNA polymorphisms and to inferred migration history in Japanese wild population of medaka. This paper consists of five parts.

The main purpose of the Part I is to survey mtDNA polymorphism in Japanese wild population of medaka, that have large intraspecific divergences. The phylogenetic reconstructions are based on a restriction fragment length polymorphisms (RFLPs) of mtDNA. These results revealed 63 mtDNA haplotypes and showed that the mtDNAs of Japanese medaka were divided into three clusters, which were subdivided into some subclusters. The distribution of mtDNA haplotypes indicated strong geographical associations. The migration events inferred from the distribution are discussed.

The main purpose of the Part II is to survey mtDNA polymorphism in Korean wild population of medaka, that have large intraspecific divergences. The phylogenetic reconstructions are based on a RFLPs of mtDNA. These results revealed seven mtDNA haplotypes and showed that the mtDNAs of Korean medaka were

divided into two clusters. The distributions of mtDNAs strong geographical association and suggest recent migration events.

A sequence on the major noncoding region of mtDNA, located between the tRNA-Pro and tRNA-Phe genes, are called the control region. The main objectives of the Part III are to compare nucleotide sequence of the control region in the Northern Population with that in the Southern Population, and, more generally, to evaluate empirically the utility of hyper-variable control region sequence in population studies. Comparison between two nucleotide sequences of the control region indicates that the region of medaka is consist of two blocks, highly conserved and the hyper-variable blocks. The hyper variable blocks in medaka contain tandemly repeated sequences. However *O. mekongensis* do not have such tandemly repeated sequence in the region.

In the Part IV, I obtained nucleotide sequences of the complete mitochondrial cytochrome *b* gene in order to investigate the origin of the Japanese two populations of medaka. Dendrograms based on the nucleotide sequences of this gene suggest close relationship between *O. curvinotus* and *O. luzonensis*, monophyletic relation of *O. latipes*, and monophyletic relationship of Japanese two populations.

The objectives of the Part V are to compare nucleotide sequences of mitochondrial cytochrome *b* gene in the Northern Population with those in the Southern Population, to elucidate relationships in the Southern Population, and, more generally, to evaluate empirically the utility of the cytochrome *b* gene sequence in population studies of *O. latipes*. A phylogenetic inference suggests some relationships in mtDNAs of the cluster B's subclusters. It was not clear from RFLPs analysis.

In addition, we can presume migration history of Japanese wild population of medaka.

Part I.
Geographic variation and diversity in the mitochondrial DNA of the medaka, *Oryzias latipes*, as determined by restriction endonuclease analysis

INTRODUCTION

The medaka, *Oryzias latipes*, is an egg-laying freshwater fish native to East Asia. Its small size, ease of breeding and shortness of generation time make it a useful experimental animal for genetic research. It inhabits marshes, ponds, and brooks amid rice fields in alluvial plains. As with other freshwater fish, it is thought that land is a barrier to the migration of *O. latipes*, and that local populations of this species are thus confined to their own watershed and isolated from one another. Because this species has little commercial value, it seems that its natural distribution has not been disturbed by human action (e.g., by fish breeding and discharge *etc.*).

Geographic variations in the biochemical characters of medaka have been demonstrated in the allozymes encoded in the nuclear genome (Sakaizumi *et al.*, 1983). The results of such analyses show that the Japanese wild populations of medaka are divided into two genetically different groups: the Northern Population from the northern coast of the Sea of Japan, and the Southern Population distributed in eastern, western, and southern Japan. The Southern Population is further divided into 5-7 subgroups. The genetic diversity estimated between the two populations is large enough to be considered characteristic of interspecific comparisons. However, male and female progeny from crosses between the two populations are fully fertile (Sakaizumi, 1986; Sakaizumi *et al.*, 1992).

Allozymes are coded by a different allele at a given locus in the nuclear genome. In contrast to the nuclear DNA, the rapid pace of mitochondrial DNA (mtDNA) nucleotide substitution, coupled with the special mode of maternal nonrecombining inheritance, offers advantages for phylogenetic analysis. Thus, the mtDNA of various

genera and species has been studied (reviewed in Avise, 1991; Meyer, 1993).

The main purpose of the present study was to survey mtDNA polymorphism in the Japanese wild population of medaka, which has large intraspecific divergences. The phylogenetic reconstructions are based on the restriction fragment length polymorphisms (RFLPs) of the mtDNA. This analysis revealed 63 mtDNA haplotypes and showed that the mtDNAs of Japanese medaka were divisible into three clusters, which were further divided into subclusters. The distribution of mtDNA haplotypes indicated strong geographical associations. The migration events inferred from the distribution are discussed.

MATERIALS AND METHODS

Sample collection

In the years 1979 to 1991, we collected wild specimens of *O. latipes* at 188 different sites in Japan (Fig. 1). The collection sites are listed in Table 1. One fish was analyzed at most of the sites; two were examined at the sites with an asterisk (*).

Five inbred strains were also examined. The HNI strain of the medaka was established from the wild population of Niigata. The Hd-rR and the HO5 strains were established from orange-red stocks. The HB32C and the HB12C strains were established from wild stocks (Hyodo-Taguchi and Egami, 1985).

DNA extraction

The head, intestines and fins were removed and discarded from adult medaka. The remaining tissue was placed in 500 μl of 100 mM EDTA, 50 mM Tris (pH 8.0), 100 mM NaCl, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml of Proteinase K, then minced and

incubated at 55°C overnight. The homogenate was extracted twice with buffer-equilibrated phenol, once with a 1:1 mixture of phenol:chloroform, and once with chloroform. DNA was precipitated with isopropyl alcohol, rinsed with ethanol, and resolved in TE buffer (1 mM EDTA, 10 mM Tris pH 8.0).

Isolation of mtDNA for probe

Approximately 50 grams orange-red medaka (about 200 individuals) were homogenized twice by a whirling blender for 10 sec, then the mtDNAs were prepared by the SDS-phenol method. Mitochondria and crude mtDNAs were prepared as described by Yonekawa *et al.* (1978). Mitochondrial DNAs were further purified by CsCl-ethidium bromide density-gradient centrifugation at 36,000 rpm for 40 h. The fractions containing closed circular and open circular mtDNAs were collected separately.

Restriction analysis

The isolated closed circular mtDNA was used as a radioactive probe in Southern blotting to detect mtDNA restriction fragments obtained by restriction endonuclease digestions from total cellular DNA. Six restriction enzymes, *Sma* I, *Bgl* II, *Xba* I, *Dra* I, *Pst* I, and *Eco* RV were used for RFLPs analysis. The restriction fragments were assigned molecular weights in comparison to a size standard of lambda phage DNA digested with *Eco* T14I. Each distinct restriction fragment pattern produced by any of the six endonucleases was assigned an upper-case letter code in alphabetical order of the detection (A, B, etc.). Thus, each individual was finally assigned a six-letter composite mtDNA haplotype.

Analysis of data

Percentages of nucleotide sequence divergence (p value) between mtDNA haplotypes were estimated from the shared restriction fragment (Nei and Li, 1979). Relationships among mtDNA haplotypes were assessed by unweighted pair group method using arithmetic averages (UPGMA) and neighbor joining (NJ) clustering using the NEIGHBOR program, version 3.5c of PHYLIP (Felsenstein, 1993).

RESULTS

Mitochondrial DNA haplotypes of wild population of medaka

Polymorphisms in the cleavage fragment patterns of medaka mtDNA were revealed by the six restriction enzymes. The fragment patterns revealed by each enzyme are illustrated in Figure 2. Five different fragment patterns were found for *Sma* I (A-E), six for *Bgl* II and *Xba* I (A-F), eight for *Dra* I and *Pst* I (A-H), and 10 for *Eco* RV (A-J).

Altogether, 63 mtDNA haplotypes were recognized, as presented in Table 2. Each haplotype was designated with a #number representing a haplotype. Thirty haplotypes were found in unique sites. Fifteen haplotypes shared two sites. Six haplotypes shared three sites. Three haplotypes shared four sites. Nine haplotypes shared more than six sites (Table 2).

Genetic distance between mtDNA haplotypes and phylogenetic analysis

The estimated pairwise sequence divergence among haplotypes ranged from 0.1 to 12.4 % (Appendix I). A phylogenetic tree based on these sequence divergences was constructed by the UPGMA method (Fig. 3). The tree showed that the 63 mtDNA haplotypes found among the Japanese wild populations were divisible into three clusters:

haplotypes #1 to #7 (cluster A), haplotype #46 (cluster C), and the other 55 haplotypes (cluster B). Cluster C was more closely related to cluster B than to cluster A. Cluster A was further separated into two subclusters (A-1 and A-2) and cluster B into 11 subclusters (B-1 to B-11). We accepted a depth of 0.48% as a standard of subclusters, except for subcluster B-11. In subcluster B-11, it was clear that haplotype #22 was closely related to haplotypes #59, #60, and #61.

The estimated nucleotide sequence divergence among clusters ranged from 5.5% to 11.4%. Between clusters A and B, the range (mean ± standard deviation) was 7.0%-11.4% (8.9±0.87%); between clusters A and C, 8.1%-8.6% (8.4±0.18%); and between clusters B and C, 5.5%-11.2% (7.3±1.14%).

The intra-cluster divergence in cluster B is two times as large as that in cluster A (Fig. 3). Specifically, the estimated pairwise sequence divergence in cluster A and cluster B ranged from 0.3 to 1.5% (mean: 0.8±0.37%) and from 0.1 to 5.0% (mean: 1.5±0.66%), respectively.

We constructed an NJ tree using the same data set. The results also demonstrated that the mtDNA haplotypes were divisible into three clusters and that clusters A and B could be subdivided into two and ten subclusters, respectively. The patterns of clustering revealed by the UPGMA and NJ methods were essentially identical.

Geographic distributions of the mtDNA haplotypes

Figure 4 shows the geographic distribution of the mtDNA haplotypes of each subcluster. The data analysis revealed strong geographical associations for the mtDNAs of clusters and subclusters. The features of the haplotypes of each (sub)cluster are as follows.

Cluster A was separated into two subclusters (A-1 and A-2). The mtDNA haplotypes of cluster A were distributed in northern Japan

along the Sea of Japan. The mtDNAs of subcluster A-1 had a large distribution area. Haplotype #1 in particular had a large distribution area. The mtDNAs of subcluster A-2 were found only in three sites at the western part of the Noto Peninsula.

Cluster B was divided into 11 subclusters (B-1 to B-11). The mtDNA haplotypes of cluster B were found in southern Japan along the Pacific coast southward from Iwate Prefecture and along the Sea of Japan westward from Kyoto Prefecture. The mtDNAs of subcluster B-1 were found only in the San-in district. Most of the mtDNAs of subcluster B-2 were found mainly in the western region of the Seto Inland Sea. The mtDNAs of subcluster B-3 were found at the Pacific coast. The mtDNAs of subcluster B-4 were found in the central of part Honshu island, and also in the Kanto district. The mtDNAs of subcluster B-5 were mainly distributed at the western edge of Honshu. However, five specimens from the Kanto district also had mtDNAs of subcluster B-5. All five of the haplotypes found in the Kanto district were haplotype #29. The mtDNAs of subcluster B-6 were distributed in the southeastern area of Kyushu and at the western edge of Honshu. The mtDNA haplotype of subcluster B-7 was a minor haplotype in this study. Two specimens from two sites (No. 67 and 68) showed haplotype #12. The mtDNAs of subcluster B-8 had a large distribution area and a large number of haplotypes. The haplotypes were found mainly in the eastern region of the Seto Inland Sea district. In addition, the haplotypes were found in the Kanto district (haplotype #55) and at the western edge of Honshu (#48 and #56). The mtDNAs of subcluster B-9 had two distribution areas. The two areas were a range from the Kanto district to the Tohoku district and northern Kyushu. The mtDNAs found in the two areas were different. Haplotypes #15, #16,

and #17 were found in the Kanto-Tohoku district. Haplotypes #23 and #24 were found in northern Kyushu. The mtDNA haplotype of subcluster B-10 was a minor haplotype, haplotype #14 from one site, Kainan (No. 109). The mtDNAs of subcluster B-11 were found in the western area of Kyushu. The medaka from the Ryukyu Islands also had the mtDNA of the subcluster B-11. We found only haplotype #60 in the southern region of the distribution range.

Cluster C had only one haplotype, #46. The mtDNA of two specimens found at two sites in the Kanto district showed haplotype #46.

Haplotypes of the inbred strains of medaka

In this study, five inbred strains were analyzed. The results showed that all inbred strains except for the HNI strain shared the same haplotype, #32. The HNI strain was haplotype #1. This result coincides with the mtDNA haplotype of the wild fish captured at Niigata (Site No. 16).

DISCUSSION

Marked intraspecific diversity among mtDNAs

Intraspecific mtDNA divergences of 7.4% in anchovy (Magoulas *et al.*, 1996) and 8.5% in sunfish (Avise *et al.*, 1984) have been reported as the highest degrees of intraspecific divergences. In the medaka, the pairwise comparisons between haplotypes of clusters A, B and C demonstrated large divergence; 7.3%-12.4%. In particular, the mean sequence divergence between clusters A and B is 9.0%. The large divergence among these three clusters suggests that the events separating these clusters are very old.

The rate of base substitution of mammalian mtDNA has been estimated to be 2.0% per million years (Brown *et al.*, 1979). At this rate, cluster A and cluster B would have shared a common ancestor 4-5 million years ago (sequence divergence estimated approximately 8-10%).

Concordance between mtDNA haplotypes and allozyme genotypes in the medaka

Allozymic variations have been studied at 21 loci in Japanese wild populations of the medaka collected at 53 sites, and the Japanese wild population of medaka was divided into two genetically distinct groups, the Northern Population and the Southern Population. Allozymic analyses demonstrated that the Northern Population is less variable than the Southern Population (Sakaizumi *et al.*, 1983).

The rigid isolation between the mtDNAs of cluster A and those of cluster B coincides perfectly with the previously defined two population ranges— A is associated with the Northern Population and B with the Southern Population. The characteristics of cluster A's mtDNAs also show lower intra-cluster polymorphism than do cluster B's mtDNAs (Fig. 3). Such concordance between mtDNA haplotypes and allozyme genotypes would be expected if the Northern Population and the Southern Population had evolved in complete isolation in the past.

Mitochondrial DNA haplotype of cluster C

Haplotype #46 in cluster C is a rare variant in the Japanese wild population of medaka. This cluster is more closely related to cluster B than to cluster A (mean estimated divergence 7.7 versus 8.9), but divergent from cluster B (pairwise divergence ranged from 5.8% to 12.1%). The fish with this mtDNA haplotype have an allozymic

genotype similar to that of the fish with haplotypes of cluster B (the Southern Population type; data not shown), despite the such large sequence divergence in mtDNA estimated. Thus, we suspect that the "old" mtDNA haplotypes which diverged in the ancestor of the medaka may have persisted in a limited area (the Kanto district). Examinations of the frequency and distribution, and detailed molecular analysis are in progress to elucidate the origin and dispersal of this haplotype.

Migration of the Japanese wild population of medaka

In the present study, we divided the mtDNA haplotypes in Japanese medaka into three clusters, and clusters A and B were subdivided into two and 11 subclusters, respectively. The distribution pattern of haplotypes of each subcluster showed a strong geographical association and unique distribution pattern (Fig. 4). The distribution pattern presumably reflects the migration history of Japanese wild medaka. We are especially mindful of the distributions of single haplotypes and those of haplotypes in a subcluster. We can thus propose three migration scenarios from the distribution patterns of mtDNA haplotypes. These scenarios are "to the Tohoku district," and "to the Ryukyu Islands," and "to the Kanto district." These scenarios are based on a hypothesis that migration occurred from a region where we found high variation in mtDNAs to a region with low variation in mtDNAs.

All specimens found in the Tohoku district at the sea of Japan coast had mtDNA of subcluster A-1, those at the Pacific coast had mtDNA of subcluster B-9. In the southern regions of the Tohoku district, we found mtDNAs of more than one subcluster. This observation leads to a scenario in which the medaka in the southern region of the Tohoku district have recently expanded their range into the northern region.

We also propose a scenario from the distribution pattern of mtDNAs of the subcluster B-11 in western Kyushu and the Ryukyu Islands. Subcluster B-11 consists of four haplotypes (Fig. 3). Haplotype #60, of these four haplotypes, is found in the southern part of western Kyushu and the Ryukyu Islands. The other three haplotypes were found in the northern part of western Kyushu. This observation leads to a scenario in which the medaka in the southern part of the region have recently expanded their range into the Ryukyu Islands.

In the Kanto district, two features were notable; the high frequency of the subcluster B-9 mtDNA haplotypes, and a small number of haplotypes from each other subcluster of cluster B. Regarding the first feature, we found that mtDNA haplotypes of subcluster B-9 were common in the Kanto district, and that the mtDNA haplotypes had split distribution ranges, i.e., the Kanto to the Tohoku district and northern Kyushu (Site No. 151, 152, and 161). In addition, there were no haplotypes common to both ranges; haplotypes #15,#16, and #17 in the Kanto and Tohoku district, and haplotypes #23 and #24 in northern Kyushu. This distribution pattern of the subcluster B-9 mtDNAs suggest that it was long ago when the medaka with the haplotype #15, #16, or #17 colonized the Kanto district. Regarding the second feature, we found five other mtDNA haplotypes in the Kanto district (haplotypes #20, #21, #29, #46, and #55). These five mtDNAs are classifiable into four (sub)clusters (subclusters B-4, B-5, and B-8, and cluster C). The four haplotypes of the three subclusters were also found in western Japan. Haplotypes #20 and #21 were found in the Tokai district, haplotype #29 at the western edge of Honshu, and haplotype #55 in the Kinki district. The sole haplotype of the cluster C was found only in the Kanto district. Thus, haplotypes other than those

of subcluster B-9 and cluster C were also found west of the Kanto district. Therefore, we suspect that the low variation of haplotypes in the four subclusters is the result of a recent expansion eastward (to the Kanto district). These two features lead to a scenario in which at least two migration events occurred in the Kanto district; first, medaka with mtDNAs of subcluster B-9 expanded their range into the Kanto district. Then, other haplotypes diverged in western Japan, and introgressed to the Kanto district recently. Consequently, the medaka found in the Kanto district are a "mixed population."

In this study, mtDNA RFLPs analysis suggests three clusters and two and 11 subclusters for wild populations of medaka, which have strong geographical associations. The geographical distributions of the mtDNA haplotypes suggest three migration events. However, this analysis also suggests the necessity for more research concerning the Southern Population. The phylogenetic relationships among the subclusters of cluster B, the separate distribution origin of haplotypes in subcluster B-9 and the origin of cluster C, and the migration histories in the San-in district and the Seto-Inland Sea area are not yet clear. Phylogenetic analysis using nucleotide sequence information is necessary to elucidate these issues. We are currently identifying the nucleotide sequences of mitochondrial cytochrome *b* gene. This project may clarify the relationships among the clusters and subclusters.

Table 1 Site numbers, collection site and mtDNA haplotype numbers.

Collection site	Haplotype	Collection site	Haplotype
1*. Higakubo, Momoishi	7	50. Takada, Mobara	46
2. Sakaenuma, Kamikita	1	51. Shimoiino, Futtu	15
3*. Mena, Higashidori	1, 4	52. Kuzuma, Kisarazu	15
4. Koyanagi, Aomori	5	53. Kawado-cho, Chiba	15
5. Minori-cho, Hirosaki	1	54. Hagiyamashinden, Sakura	21
6*. Fukkoshi, Noshiro	1, 4	55. Chiyoda-ku, Tokyo	17
7. Nakadai, Ootate	1	56. Setagayaku, Tokyo	20
8. Teramura, Yuwa	1	57. Kamiimai, Toyoda	19
9. Tsutsumi, Yokote	1	58. Ryoke, Nagano	15
10. Deto-machi, Honjo	1	59. Nakatsuna, Oomachi	15
11. Kisakata, Kisakata	1	60. Kogawa, Suwa	16
12. Shimokawa, Tsuruoka	1	61. Fukushima, Suwa	16
13. Senjudo, Yamagata	1	62. Shibusaki, Suwa	18
14. Sanjogata, Inawashiro	1	63. One, Kobuchizawa	16
15. Kamo-Utashiro, Ryoutsu	1	64. Shinden, Fijiyoshida	16
16. Nabekata, Niigata	1	65. Nanbara, Iida	18
17. Yoneoka, Jyoetsu	1	66*. Kawahara, Odawara	32
18*. Honkaihotsu, Oshima	2, 5	67. Odoi, Kannami	12
19. Okisaki, Himi	1	68. Oya, Shizuoka	12
20. Kawashiri, Nanao	3	69. Ninomiya, Iwata	32
21. Yuwaku-machi, Kanazawa	3	70. Kozakai, Kozakai	33
22. Iburibashi-machi, Kaga	3	71. Juroku-cho, Ogaki	20
23. Kaminoda, Sabae	1	72. Kose, Saori	20
24. Maruyama, Tsuruga	1	73. Kamaganiji, Nagashima	19
25. Kogasaki, Obama	1	74. Ishinden, Tsu	32
26. Ichiba, Maizuru	1	75. Kuzaki, Toba	47
27. Suzu, Miyazu	1	76. Sakazaki, Isobe	16
28. Kotsubo, Ine	1	77. Tategami, Ago	32
29*. Miyajima, Toyooka	6	78. Funatsu, Umiyama	42
30*. Yunokawa, Hakodate	16	79. Sano, Shingu	42
31. Saichi-moriai, Kesennuma	16	80. Minachi, Hongu	51
32. Akogi, Ichinoseki	15	81. Kujinokawa, Kushimoto	57
33*. Gamo, Sendai	16	82. Tanoi, Hikigawa	57
34*. Obama, Soma	15, 16	83. Moto-machi, Tanabe	57
35. Taira-fujima, Iwaki	17	84. Musata, Wakayama	55
36. Kawawada, Mito	16	85. Ikedashimo-cho, Izumi	63
37. Higashi-kinokura, Naka	16	86. Samita, Kawai	50
38. Kashima, Urizura	15	87. Ota-cho, Ueno	49
39. Kamisaruuchi,Kawachi	16	88. Kiko-cho, Ueno	55
40*. Imai, Mibu	15, 29	89. Kamigori, Ueno	63
41. Sayado, Mooka	46	90. Nodayama-cho, Hikone	55
42. Katori, Sawara	29	91. Oto, Kinomoto	55
43. Kamishinshikushinden, Nagareyama	16	92. Nakasuji, Ayabe	55
44. Kita-ku, Tokyo	29	93. Kaibara, Kaibara	55
45. Hasuda, Hasuda	29	94. Ikawadani-cho, Kobe	62
46. Shimogosoya, Yoshimi	55	95. Kusumoto, Higashiura	55
47. Hinatashinden, Tatebayashi	29	96. Torikaiura, Goshiki	52
48. Kamiyokota, Ogawa	15	97. Tugi, Himeji	54
49. Harayokoji, Narutou	17	98. Kanoharada, Himeji	55

Table 1 Continued

Collection site	Haplotype
99. Futaomote, Ikeda	55
100. Tsushima, Okayama	55
101. Yata, Saeki	55
102. Koigakubo, Tessei	55
103. Hatajiki, Miyoshi	53
104. Takasugi-cho, Miyoshi	55
105. Inokuchi, Kamiura	55
106. Siwaishinden, Yoshiumi	55
107. Ato, Yasuura	53
108. Ikushima-cho, Takamatsu	56
109. Kokubi-cho, Tokushima	31
110. Tomioka-cho, Anan	11
111. Tai, Yuki	58
112. Okugawachi, Hiwasa	48
113. Asakawa, Kainan	14
114. Doi, Aki	32
115. Asakura, Kochi	32
116. Nii, Tosa	32
117. Saga, Tosasaga	32
118. Gudo, Nakamura	33
119. Kozukushi, Sukumo	32
120. Fussaki, Misho	55
121. Uchiko, Uchiko	55
122. Dogo, Matsuyama	55
123. Ozu, Iwakuni	43
124. Usanagi, Hirao	39
125. Koigahama, Kudamatsu	32
126. Daido, Hofu	36
127. Kiwa, Ube	36
128. Ozuki, Kotsuki	26
129. Yamakawa, Sanyo	30
130. Yoshida, Yamaguchi	38
131. Uga, Toyoura	26
132. Kawatana, Toyoura	56
133. Kandakami, Hohoku	45
134. Igami, Yuya	27
135. Higashifukagawa, Nagato	29
136. Tamae, Hagi	37
137. Nago, Abu	30
138. Esaki, Tamagawa	26
139. Toda, Masuda	26
140. Chiwa, Hamada	34
141. Tsuchi, Goutsu	44
142. Kawado, Sakurae	8
143. Nagahisa, Oota	35
144. Yawata-cho, Matsue	34
145. Oshinozu-cho, Yonago	34
146. Tsuma, Tsuma	34
147. Koyama-cho, Tottori	35
148. Honjo, Iwami	41
149. Shimazu, Amino	34
150. Misaka, Omiya	34
151. Shitaru, Kamiagata	23
152. Komoda, Izuhara	23
153. Yamada, Hisayama	48
154. Taku, Munakata	28
155. Imai, Yukuhashi	25
156. Minato, Shiida	25
157. Mikekado, Buzen	25
158. Kusaji, Bungotakada	25
159. Kamegawa, Beppu	40
160. Ikeda, Saeki	33
161. Mushika-cho, Nobeoka	24
162. Uwae, Takanabe	36
163. Tonokori-cho, Saito	10
164. Shioji, Miyazaki	9
165. Kihara, Kiyotake	10
166. Takamatsu, Kushima	10
167. Minamikata, Kushima	13
168. Kawahigashi, Higashikushira	10
169. Kamiobaru, Kushira	10
170. Noma, Nakatane	10
171. Oasato, Kikai	60
172. Taira, Nago	60
173. Odon, Gushikami	60
174. Sato, Sato	60
175. Suguchiike, Sato	60
176. Oura, Oura	60
177. Yamada, Hiyoshi	60
178. Takae-cho, Sendai	60
179. Ichihino, Hiwaki	60
180. Euchi, Takaono	61
181. Kotsunagi, Minamata	61
182. Taraki, Taraki	61
183. Tomioka, Reihoku	60
184. Shimomiyabaramyo, Kazusa	60
185. Tsunehiro, Kashima	61
186. Arita, Arita	59
187. Sakioka-cho, Sasebo	22
188. Mukata, Fukue	22
Inbred strains	
HNI	1
HO5	32
HB12C	32
HB32C	32
Hd-rR	32

Asterisk (*) shows the locality where we have examined two individuals.

Table 2 Mitochondrial DNA haplotypes, fragment patterns (indicated by six letters), number of sites in which the haplotype was found, and number of individuals detected. Enzyme order: *Sma* I, *Bgl* II, *Xba* I, *Dra* I, *Pst* I, *Eco* RV.

	Fragment patterns	No. of locarities	No. of individuals		Fragment patterns	No. of locarities	No. of individuals
#1	AAAAAA	22	22	#33	DDBDCD	3	3
#2	AAAABA	1	1	#34	DDBDCF	6	6
#3	ABAABA	3	3	#35	DDBDCG	2	2
#4	ACAAAA	2	2	#36	DDBDFB	3	3
#5	BAAAAA	2	2	#37	DDBDFD	1	1
#6	BBAABA	1	2	#38	DDBDGB	1	1
#7	BCAAAA	1	2	#39	DDBECD	1	1
#8	BDBDCF	1	1	#40	DDBEGD	1	1
#9	BDBDFB	1	1	#41	DDBGCF	1	1
#10	BDBDFD	6	6	#42	DDCDCB	2	2
#11	BDBDGD	1	1	#43	DDEECD	1	1
#12	BDDDCD	2	2	#44	DDEDCF	1	1
#13	BDEDFD	1	1	#45	DDECCB	1	1
#14	BFBGGB	1	1	#46	DDFHIC	2	2
#15	CDBBCB	10	10	#47	DEBDCB	1	1
#16	CDBBDB	13	15	#48	EDBBGD	2	2
#17	CDBBDD	3	3	#49	EDBCGB	1	1
#18	CDBCDB	2	2	#50	EDBDCB	1	1
#19	CDBDCB	2	2	#51	EDBDCD	1	1
#20	CDBDCD	3	3	#52	EDBDEB	1	1
#21	CDBDDD	1	1	#53	EDBDFB	2	2
#22	CDEDFJ	2	2	#54	EDBDGA	1	1
#23	DDBBCD	2	2	#55	EDBDGB	19	19
#24	DDBBFB	1	1	#56	EDBDGD	2	2
#25	DDBCCA	4	4	#57	EDBDHB	3	3
#26	DDBCCB	4	4	#58	EDBFGD	1	1
#27	DDBCCE	1	1	#59	EDEBFI	1	1
#28	DDBCFA	1	1	#60	EDEDFH	11	11
#29	DDBCFB	6	6	#61	EDEDFI	4	4
#30	DDBCFD	2	2	#62	EDEDGB	1	1
#31	DDBCGB	1	1	#63	EEBDGB	2	2
#32	DDBDCB	10	11				

Fig. 1 Location of collection sites for medaka.
The numbers refer to the locations in Table 1.

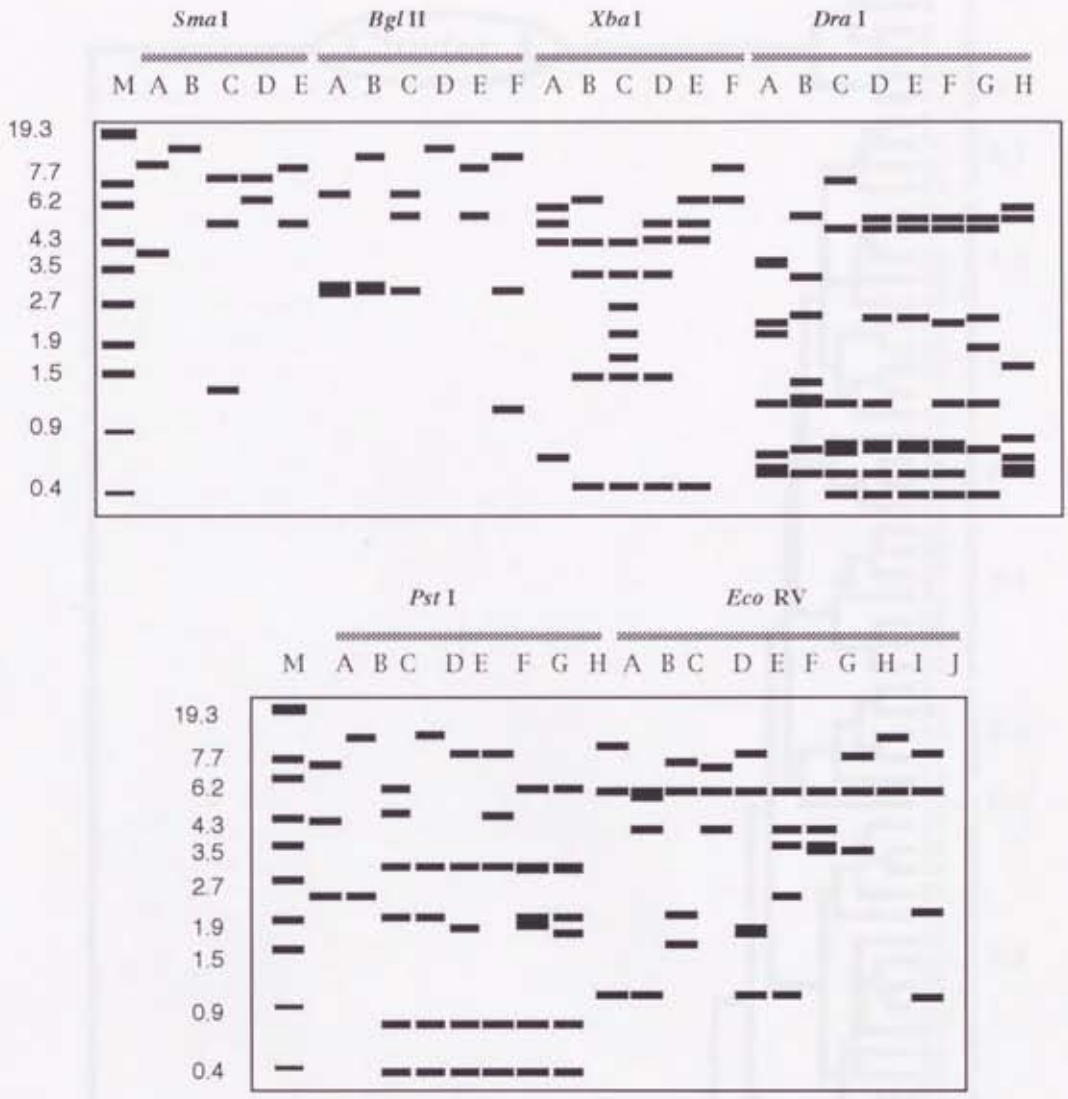

Fig. 2 Diagram of the restriction patterns revealed by each of six restriction enzymes.

Restriction fragment length polymorphisms of medaka mtDNAs were digested with six endonucleases. M shows DNA size marker, lambda phage DNA digested with *Eco* T14I. The numbers indicate fragment length (Kb).

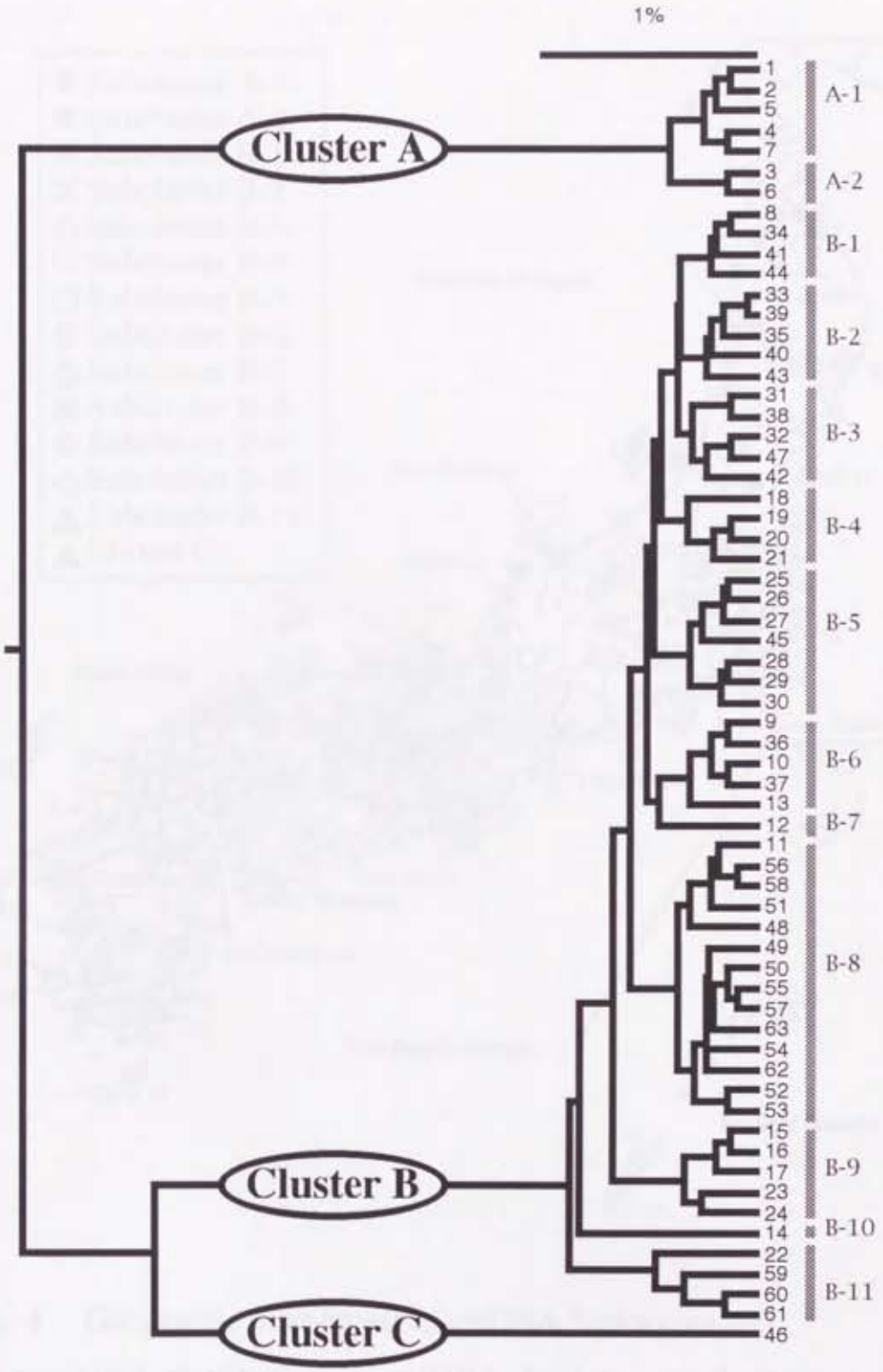

Fig. 3 Phylogenetic tree
The UPGMA phenogram of 63 mtDNA haplotypes found among Japanese medaka. The phenogram was derived from a matrix of percentage nucleotide sequence divergence estimates based on the restriction fragment length polymorphisms among the haplotypes (Appendix 1).

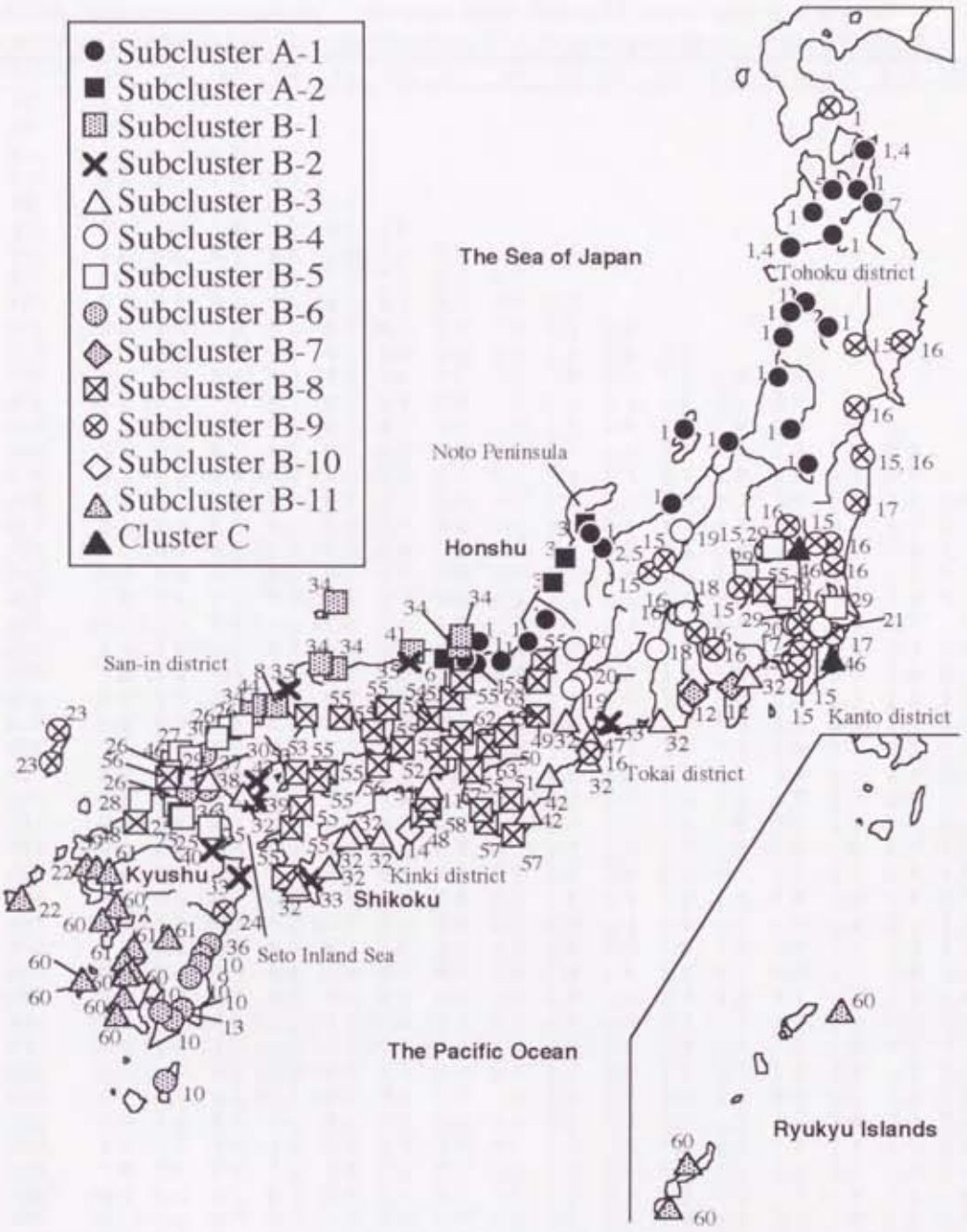

Fig. 4 Geographic distribution of mtDNA haplotypes

Geographical distribution of mtDNA haplotypes of clusters and subclusters inferred from the UPGMA analysis. Numbers indicate the haplotype numbers. One symbol represents one fish, except for the ten sites which are marked with an asterisk (*) in Table 1.

Appendix I Percentage of pairwise sequence divergence among the 63 composite mtDNA haplotypes (*p* values). Estimates were derived from a matrix of shared restriction fragments and the numbered mtDNA haplotypes are defined in Table 1.

Haplotype	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16
#2	0.3															
#3	0.8	0.5														
#4	0.3	0.7	1.0													
#5	0.3	0.7	1.3	0.7												
#6	1.3	0.9	0.4	1.4	0.9											
#7	0.7	1.1	1.4	0.3	0.3	1.0										
#8	9.0	8.8	8.6	8.8	7.9	7.5	7.8									
#9	8.7	8.6	8.3	8.6	7.6	7.2	7.5	0.7								
#10	9.8	9.6	9.4	9.6	8.5	8.1	8.3	0.9	0.4							
#11	10.0	9.9	9.6	9.9	8.7	8.3	8.6	0.8	1.1	0.8						
#12	10.0	9.9	9.6	9.9	8.7	8.3	8.6	0.8	1.1	0.8	0.7					
#13	8.5	8.3	8.1	8.3	7.4	7.0	7.2	1.3	0.8	0.4	1.2	1.2				
#14	10.2	10.1	9.9	10.1	9.0	8.6	8.8	1.6	1.7	2.2	1.2	2.1	2.8			
#15	9.2	9.1	8.8	9.1	9.1	8.7	9.0	1.4	1.5	2.0	1.9	1.9	2.6	2.4		
#16	9.1	9.0	8.7	9.0	9.0	8.6	8.8	1.9	1.7	2.2	2.1	2.4	2.8	2.7	0.3	
#17	10.1	10.0	9.8	10.0	10.0	9.6	9.9	2.1	2.2	1.8	1.7	2.0	2.3	3.3	0.7	0.3
#18	8.8	8.7	8.5	8.7	8.7	8.3	8.6	1.7	1.4	2.0	1.9	2.2	2.6	2.4	1.3	0.9
#19	9.1	9.0	8.7	9.0	9.0	8.6	8.8	0.8	0.8	1.3	1.2	1.2	1.8	1.7	0.5	0.9
#20	10.1	10.0	9.8	10.0	10.0	9.6	9.9	0.9	1.3	0.9	0.8	0.8	1.3	2.2	0.9	1.3
#21	10.0	9.9	9.6	9.9	9.9	9.5	9.8	1.4	1.4	1.0	1.0	1.3	1.5	2.4	1.3	0.9
#22	7.9	7.8	7.5	7.8	7.8	7.4	7.6	2.0	1.4	1.6	2.6	2.6	1.2	3.6	1.9	2.1
#23	10.1	10.0	9.8	10.0	10.0	9.6	9.9	1.5	1.9	1.4	1.4	1.4	2.0	2.9	0.7	1.0
#24	9.0	8.8	8.6	8.8	8.8	8.5	8.7	1.8	1.0	1.4	2.3	2.3	2.0	2.9	0.7	0.8
#25	7.8	7.6	7.4	7.6	7.6	7.2	7.5	1.3	1.6	1.8	1.8	1.8	2.5	2.7	1.8	2.3
#26	8.8	8.7	8.5	8.7	8.7	8.3	8.6	1.1	1.1	1.6	1.6	1.6	2.2	2.1	1.3	1.8
#27	8.8	8.7	8.5	8.7	8.7	8.3	8.6	1.4	1.8	2.0	1.9	1.9	2.6	2.8	1.9	2.4
#28	7.6	7.5	7.2	7.5	7.5	7.1	7.4	1.8	1.2	1.4	2.3	2.3	1.9	3.4	2.3	2.6
#29	8.7	8.6	8.3	8.6	8.6	8.2	8.5	1.6	0.8	1.2	2.1	2.1	1.7	2.7	1.8	2.0
#30	9.8	9.6	9.4	9.6	9.6	9.2	9.5	1.8	1.2	0.8	1.6	1.6	1.3	3.4	2.3	2.6
#31	9.0	8.8	8.6	8.8	8.8	8.5	8.7	1.5	1.6	2.1	1.1	2.0	2.7	1.6	1.7	1.9
#32	9.0	8.8	8.6	8.8	8.8	8.5	8.7	0.7	0.7	1.1	1.1	1.1	1.6	1.6	0.9	1.3
#33	10.0	9.9	9.6	9.9	9.9	9.5	9.8	0.8	1.1	0.8	0.7	0.7	1.2	2.1	1.3	1.8
#34	9.1	9.0	8.7	9.0	9.0	8.6	8.8	0.3	1.1	1.3	1.2	1.2	1.8	2.0	1.3	1.7
#35	10.1	10.0	9.8	10.0	10.0	9.6	9.9	0.7	1.3	1.1	1.1	1.1	1.6	2.2	1.4	1.9
#36	8.8	8.7	8.5	8.7	8.7	8.3	8.6	1.1	0.4	0.8	1.6	1.6	1.2	2.1	1.3	1.5
#37	9.9	9.8	9.5	9.8	9.8	9.4	9.6	1.3	0.8	0.4	1.1	1.1	0.8	2.7	1.8	2.0
#38	9.1	9.0	8.7	9.0	9.0	8.6	8.8	1.0	1.1	1.6	0.7	1.5	2.1	1.2	1.3	1.4
#39	11.3	11.2	10.9	11.2	11.2	10.8	11.0	1.0	1.3	0.9	0.9	0.9	1.4	2.3	1.5	2.0
#40	11.4	11.3	11.0	11.3	11.3	10.9	11.2	1.4	1.8	1.3	0.5	1.3	1.8	1.8	1.9	2.1
#41	10.1	10.0	9.8	10.0	10.0	9.6	9.9	0.7	1.6	1.8	1.7	1.7	2.3	1.6	1.7	2.2
#42	10.1	10.0	9.8	10.0	10.0	9.6	9.9	1.2	1.2	1.7	1.6	1.3	2.2	2.1	1.4	1.8
#43	9.8	9.6	9.4	9.6	9.6	9.2	9.5	1.4	1.8	1.4	1.3	1.3	1.0	2.9	2.0	2.6
#44	8.0	7.9	7.6	7.9	7.9	7.5	7.8	0.7	1.6	1.8	1.7	1.7	1.3	2.5	1.7	2.2
#45	7.8	7.6	7.4	7.6	7.6	7.2	7.5	1.6	1.6	2.2	2.1	2.1	1.7	2.7	1.8	2.3
#46	8.6	8.5	8.2	8.5	8.5	8.1	8.3	8.5	7.2	7.1	8.3	9.5	7.0	11.2	7.8	7.6
#47	9.1	9.0	8.7	9.0	9.0	8.6	8.8	1.0	1.1	1.6	1.5	1.5	2.1	1.7	1.3	1.7
#48	10.2	10.1	9.9	10.1	10.1	9.8	10.0	1.9	2.3	1.9	0.9	1.8	2.4	2.3	1.0	1.2
#49	9.0	8.8	8.6	8.8	8.8	8.5	8.7	1.5	1.6	2.1	1.1	2.0	2.7	1.6	1.7	1.9
#50	9.0	8.8	8.6	8.8	8.8	8.5	8.7	0.7	0.7	1.1	1.1	1.1	1.6	1.6	0.9	1.3
#51	10.0	9.9	9.6	9.9	9.9	9.5	9.8	0.8	1.1	0.8	0.7	0.7	1.2	2.1	1.3	1.8
#52	9.0	8.8	8.6	8.8	8.8	8.5	8.7	1.5	0.7	1.1	1.1	2.0	1.6	1.6	1.7	1.6
#53	8.8	8.7	8.5	8.7	8.7	8.3	8.6	1.1	0.4	0.8	1.6	1.6	1.2	2.1	1.3	1.5
#54	8.0	7.9	7.6	7.9	7.9	7.5	7.8	1.2	1.6	1.8	0.8	1.7	2.3	1.6	1.7	1.9
#55	9.1	9.0	8.7	9.0	9.0	8.6	8.8	1.0	1.1	1.6	0.7	1.5	2.1	1.2	1.3	1.4
#56	10.1	10.0	9.8	10.0	10.0	9.6	9.9	1.2	1.6	1.1	0.3	1.1	1.6	1.6	1.7	1.9
#57	9.1	9.0	8.7	9.0	9.0	8.6	8.8	1.0	1.1	1.6	0.9	1.5	2.1	1.4	1.3	1.4
#58	9.0	8.8	8.6	8.8	8.8	8.5	8.7	1.5	1.9	1.4	0.6	1.4	2.0	1.9	2.0	2.2
#59	8.6	8.5	8.2	8.5	8.5	8.1	8.3	2.8	2.2	2.1	3.1	3.1	1.6	4.8	1.7	1.9
#60	8.6	8.5	8.2	8.5	8.5	8.1	8.3	2.1	1.5	1.4	2.3	2.3	1.0	3.8	2.7	2.9
#61	8.5	8.3	8.1	8.3	8.3	7.9	8.2	2.0	1.4	1.3	2.2	2.2	0.8	3.7	2.6	2.8
#62	8.0	7.9	7.6	7.9	7.9	7.5	7.8	1.5	1.6	2.1	1.1	2.0	1.6	1.6	1.7	1.9
#63	9.2	9.1	8.8	9.1	9.1	8.7	9.0	1.4	1.5	2.0	1.0	1.9	2.6	1.3	1.6	1.8
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16

Appendix I Continued

Haplotype	#17	#18	#19	#20	#21	#22	#23	#24	#25	#26	#27	#28	#29	#30	#31	#32
#18	1.4															
#19	1.3	0.7														
#20	0.9	1.1	0.3													
#21	0.6	0.7	0.7	0.3												
#22	2.3	1.9	1.2	1.4	1.6											
#23	0.7	2.3	1.3	0.9	1.4	2.7										
#24	1.2	2.0	1.3	1.8	2.0	2.0	0.7									
#25	2.6	1.1	1.1	1.3	1.8	2.1	1.6	1.9								
#26	2.3	0.7	0.7	1.1	1.6	2.2	1.4	1.4	0.4							
#27	2.7	1.3	1.2	1.4	1.9	2.2	1.7	2.0	0.4	0.5						
#28	2.8	1.3	1.6	1.8	2.0	1.6	2.1	1.4	0.4	0.8	0.8					
#29	2.6	0.9	1.1	1.6	1.8	1.8	1.9	1.0	0.8	0.4	0.9	0.4				
#30	2.1	1.3	1.6	1.1	1.3	2.0	1.4	1.4	0.9	0.8	1.0	0.5	0.4			
#31	2.4	0.8	1.0	1.5	1.7	2.7	1.8	1.8	0.7	0.3	0.8	1.1	0.7	1.1		
#32	1.8	1.1	0.3	0.7	1.1	1.7	0.9	0.9	0.7	0.3	0.8	1.1	0.7	1.1	0.7	
#33	1.4	1.6	0.7	0.3	0.7	1.9	0.6	1.4	0.9	0.7	1.0	1.3	1.1	0.8	1.1	0.3
#34	1.9	1.5	0.7	0.8	1.2	1.8	1.0	1.3	0.8	0.7	0.9	1.3	1.1	1.3	1.0	0.3
#35	1.8	1.7	0.8	0.7	1.1	2.0	0.9	1.5	1.0	0.8	1.1	1.4	1.3	1.1	1.2	0.4
#36	2.0	1.3	0.7	1.1	1.3	1.3	1.4	0.6	1.1	0.7	1.3	0.8	0.4	0.8	1.1	0.3
#37	1.6	1.8	1.1	0.7	0.9	1.4	1.0	1.0	1.3	1.1	1.4	0.9	0.8	0.4	1.6	0.7
#38	1.9	1.2	0.7	1.0	1.2	2.1	1.3	1.3	1.1	0.7	1.2	1.6	1.1	1.6	0.3	0.3
#39	1.6	1.8	0.8	0.5	0.9	2.1	0.7	1.6	1.0	0.9	1.1	1.5	1.3	0.9	1.3	0.5
#40	1.7	1.9	1.2	0.8	1.0	2.6	1.1	2.0	1.4	1.3	1.6	2.0	1.8	1.3	0.8	0.8
#41	2.4	2.0	1.0	1.2	1.7	2.3	1.5	1.8	1.3	1.1	1.4	1.8	1.6	1.8	1.5	0.7
#42	2.3	1.6	0.8	1.2	1.6	2.2	1.4	1.4	1.2	0.8	1.3	1.7	1.2	1.7	1.2	0.4
#43	2.1	2.3	1.3	0.9	1.3	1.6	1.1	2.1	1.5	1.3	1.6	2.1	1.8	1.4	1.8	0.9
#44	2.4	2.0	1.0	1.2	1.7	1.4	1.5	1.8	1.3	1.1	1.4	1.8	1.6	1.8	1.5	0.7
#45	2.9	1.1	1.1	1.6	2.1	1.8	1.9	1.9	0.8	0.4	0.9	1.2	0.8	1.2	0.7	0.7
#46	7.5	7.4	7.6	7.5	7.4	5.8	6.7	5.9	6.4	6.5	6.5	5.5	5.7	5.5	6.7	6.7
#47	2.2	1.5	0.7	1.0	1.5	2.1	1.3	1.3	1.1	0.7	1.2	1.6	1.1	1.6	1.0	0.3
#48	0.8	2.4	1.7	1.3	1.5	3.2	0.8	1.6	2.7	2.4	2.8	3.4	3.1	2.6	1.9	1.9
#49	2.4	0.8	1.0	1.5	1.7	2.7	2.4	2.4	1.3	0.8	1.4	1.8	1.3	1.8	0.4	1.2
#50	1.8	1.1	0.3	0.7	1.1	1.7	1.5	1.5	1.3	0.8	1.4	1.8	1.3	1.8	1.2	0.4
#51	1.4	1.6	0.7	0.3	0.7	1.9	1.1	2.0	1.4	1.3	1.6	2.0	1.8	1.3	1.7	0.8
#52	2.1	1.4	1.0	1.5	1.4	1.7	2.4	1.5	2.2	1.7	2.3	1.8	1.3	1.8	1.2	1.2
#53	2.0	1.3	0.7	1.1	1.3	1.3	2.0	1.1	1.8	1.3	1.9	1.3	0.9	1.3	1.7	0.8
#54	2.1	1.7	1.0	1.2	1.4	2.0	2.1	2.4	1.3	1.7	1.7	1.8	2.2	2.4	1.2	1.2
#55	1.9	1.2	0.7	1.0	1.2	2.1	1.9	1.9	1.7	1.2	1.8	2.2	1.7	2.2	0.8	0.8
#56	1.5	1.7	1.0	0.7	0.8	2.3	1.5	2.4	1.9	1.7	2.0	2.4	2.2	1.8	1.2	1.2
#57	1.9	1.2	0.7	1.0	1.2	2.1	1.9	1.9	1.7	1.2	1.8	2.2	1.7	2.2	1.0	0.8
#58	1.8	1.7	1.3	0.9	1.1	2.7	1.8	2.8	1.9	1.7	2.0	2.4	2.2	1.8	1.2	1.5
#59	1.8	3.6	2.6	2.4	2.7	1.6	1.8	1.4	3.4	3.6	3.6	2.9	3.0	2.9	4.2	2.8
#60	2.8	2.7	1.9	1.8	2.0	1.0	2.8	2.4	2.6	2.7	2.7	2.1	2.2	2.1	3.2	2.1
#61	2.7	2.6	1.8	1.6	1.8	0.9	2.7	2.3	2.5	2.6	2.6	1.9	2.1	1.9	3.1	2.0
#62	2.4	1.7	1.0	1.5	1.7	1.7	2.4	2.4	2.2	1.7	2.3	2.8	2.2	2.8	1.2	1.2
#63	2.3	1.6	1.0	1.4	1.6	2.5	2.3	2.3	2.1	1.6	2.2	2.7	2.1	2.7	1.2	1.2
	#17	#18	#19	#20	#21	#22	#23	#24	#25	#26	#27	#28	#29	#30	#31	#32

Appendix I Continued

Haplotype	#33	#34	#35	#36	#37	#38	#39	#40	#41	#42	#43	#44	#45	#46	#47	#48
#34	0.4															
#35	0.3	0.3														
#36	0.7	0.7	0.8													
#37	0.4	0.8	0.7	0.4												
#38	0.7	0.7	0.8	0.7	1.1											
#39	0.1	0.6	0.5	0.9	0.5	0.8										
#40	0.5	0.9	0.8	1.3	0.9	0.4	0.4									
#41	0.8	0.3	0.7	1.1	1.3	1.0	1.0	1.4								
#42	0.8	0.8	0.9	0.8	1.2	0.8	0.9	1.3	1.2							
#43	0.5	1.0	0.9	1.3	0.9	1.3	0.4	0.8	1.4	1.4						
#44	0.8	0.3	0.7	1.1	1.3	1.0	1.0	1.4	0.7	1.2	0.6					
#45	1.1	1.1	1.3	1.1	1.6	1.1	1.3	1.8	1.6	1.2	0.9	0.7				
#46	6.5	6.8	6.7	5.8	5.7	6.8	6.4	6.5	7.5	7.8	6.3	6.7	6.4			
#47	0.7	0.7	0.8	0.7	1.1	0.7	0.8	1.2	1.0	0.8	1.3	1.0	1.1	7.6		
#48	1.5	2.0	1.9	2.4	2.0	1.4	1.7	1.2	2.5	2.4	2.2	2.5	3.1	8.6	2.3	
#49	1.7	1.6	1.8	1.7	2.2	0.8	1.9	1.4	2.1	1.7	2.4	2.1	1.3	8.5	1.6	1.3
#50	0.8	0.8	0.9	0.8	1.3	0.8	1.0	1.4	1.2	0.9	1.4	1.2	1.3	8.5	0.8	1.3
#51	0.5	0.9	0.8	1.3	0.9	1.2	0.6	1.0	1.4	1.3	1.0	1.4	1.8	8.3	1.2	0.9
#52	1.7	1.6	1.8	0.8	1.3	0.8	1.9	1.4	2.1	1.7	2.4	2.1	2.2	7.5	1.6	1.3
#53	1.3	1.2	1.4	0.5	0.9	1.2	1.4	1.9	1.7	1.3	2.0	1.7	1.8	7.4	1.2	1.8
#54	1.4	1.3	1.5	1.7	1.9	0.8	1.6	1.1	1.8	1.7	2.1	1.8	2.2	8.5	1.6	1.0
#55	1.2	1.2	1.3	1.2	1.7	0.4	1.4	0.9	1.6	1.3	1.9	1.6	1.7	8.6	1.2	0.9
#56	0.8	1.3	1.2	1.7	1.3	0.8	1.0	0.6	1.8	1.7	1.4	1.8	2.2	8.5	1.6	0.6
#57	1.2	1.2	1.3	1.2	1.7	0.7	1.4	1.2	1.6	1.3	1.9	1.6	1.7	8.6	1.2	1.2
#58	1.1	1.6	1.5	2.0	1.6	1.0	1.3	0.8	2.1	2.0	1.8	2.1	2.2	8.5	1.9	0.8
#59	2.7	2.9	2.8	2.3	2.2	3.4	3.0	3.6	3.7	3.5	2.5	2.4	3.0	7.1	3.4	1.6
#60	2.0	2.2	1.8	1.6	1.5	2.6	2.2	2.7	2.8	2.7	1.7	1.8	2.2	7.1	2.6	2.6
#61	1.8	2.1	2.0	1.5	1.4	2.4	2.1	2.6	2.7	2.6	1.6	1.6	2.1	7.0	2.4	2.4
#62	1.7	1.6	1.8	1.7	2.2	0.8	1.9	1.4	2.1	1.7	1.4	1.2	1.3	8.5	1.6	1.3
#63	1.6	1.5	1.7	1.6	2.1	0.8	1.8	1.3	2.0	1.6	2.3	2.0	2.1	9.9	0.8	1.3
	#33	#34	#35	#36	#37	#38	#39	#40	#41	#42	#43	#44	#45	#46	#47	#48

Appendix I Continued

Haplotype	#49	#50	#51	#52	#53	#54	#55	#56	#57	#58	#59	#60	#61	#62
#50	0.7													
#51	1.1	0.3												
#52	0.7	0.7	1.1											
#53	1.1	0.3	0.7	0.3										
#54	0.7	0.7	0.8	0.7	1.1									
#55	0.3	0.3	0.7	0.3	0.7	0.3								
#56	0.7	0.7	0.3	0.7	1.1	0.4	0.3							
#57	0.6	0.3	0.7	0.6	0.7	0.6	0.2	0.6						
#58	0.7	0.9	0.6	0.9	1.4	0.7	0.6	0.2	0.8					
#59	3.2	2.1	2.0	2.1	1.6	2.4	2.6	2.4	2.6	2.8				
#60	2.4	1.4	1.3	1.4	1.0	1.8	1.9	1.8	1.9	2.1	1.1			
#61	2.3	1.3	1.2	1.3	0.9	1.6	1.8	1.6	1.8	2.0	0.7	0.4		
#62	0.7	0.7	1.1	0.7	1.1	0.7	0.3	0.7	0.6	0.9	2.1	1.4	1.3	
#63	0.7	0.7	1.0	0.7	1.0	0.7	0.3	0.7	0.5	0.9	3.1	2.3	2.2	0.7
	#49	#50	#51	#52	#53	#54	#55	#56	#57	#58	#59	#60	#61	#62

Part II.
Mitochondrial DNA variation in the Korean wild population of the medaka, *Oryzias latipes*

INTRODUCTION

The medaka, *Oryzias latipes*, is an egg-laying freshwater fish native to Japan, China and Korea. It inhabits marshes, ponds, and brooks amid rice fields in alluvial plains. As with other freshwater fish, land is considered a barrier to migration, thus local populations of this species are confined to their own watershed and isolated from one another. Because this species has little commercial value, it seems that its natural distribution has not been disturbed by human action.

Geographic variation in biochemical characters of Korean medaka has been demonstrated in allozymes encoded in the nuclear genome (Sakaizumi and Joen, 1987). The results showed that the wild populations of the Korean medaka are divided into two genetically different groups: the East Korean Population from the coast of the Sea of Japan, and the China-West Korean Population distributed in western Korea and China. These two groupings can be distinguished by means of unique alleles at the *ACP*, *AMY*, *CK-A*, *GPI-A*, *LDH-A*, *LDH-C*, *PGM*, *SOD*, and *TF* loci, and the Nei's coefficients of genetic distance (*D*) based on 16 loci are no less than 0.7.

Karyological studies demonstrated that specimens belonging to the China-West Korean population had a diploid chromosomal number of 46 including a large metacentric pair. The karyotype closely related to those from east and southwestern China. By contrast, specimens diagnosed as the East Korean population showed a diploid chromosomal number of 48 without these large chromosomes (Uwa and Joen, 1987; Uwa *et al.*, 1988). Geographic distribution of these two chromosomal forms is similar to that of two allozymically distinguished populations, namely, the 2n=46 form (*O. latipes sinensis*) in western Korea and the 2n=48 form in eastern and southern Korea

(Kim and Lee, 1992; Kim and Moon, 1987; Uwa and Joen, 1987; Uwa *et al.*, 1988).

Allozymes are coded by a different allele at a given locus in the nuclear genome. In contrast to nuclear DNA, the rapid pace of mitochondrial DNA (mtDNA) nucleotide substitution, coupled with the special mode of maternal nonrecombinant inheritance, offers advantages for phylogenetic analysis (reviewed in Avise, 1991; Billington and Hebert, 1991; Meyer, 1993).

The main purpose of this study was to survey mtDNA polymorphism in Korean wild population of medaka that have large intraspecific divergences. The phylogenetic reconstructions are based on a restriction fragment length polymorphisms (RFLPs) of mtDNA. The findings revealed seven mtDNA haplotypes and showed that the mtDNAs of Korean medaka were divided into two clusters. The distributions of mtDNAs strong geographical association and suggest recent migration events.

MATERIALS AND METHODS

Sample collection

From 1986 to 1989, we collected wild specimens of *O. latipes* at 60 different localities in Korea (Fig. 1). Collection localities are listed in Table 1. The specimens of wild population at Beijing and Shanghai in China were from wild stocks housed at the Faculty of Science, University of Tokyo, and the specimens of wild population at Kunming in China was from the Faculty of Science, Shinshu University.

DNA extraction

Head, intestine and fins were removed and discarded from adult medaka. The tissue was placed in 500 μl of 100 mM EDTA, 50 mM

Tris (pH 8.0), 100 mM NaCl, 1% SDS, and 100 μg/ml of Proteinase K. The tissue was minced in a 1.5 ml tube and incubated at 55°C over night. The homogenate was extracted twice with buffer-equilibrated phenol, once with a 1:1 mixture of phenol:chloroform, and once with chloroform. DNA was precipitated with isopropyl alcohol, rinsed with ethanol, and resolved in TE buffer (1 mM EDTA, 10 mM Tris pH 8.0).

Isolation of mtDNA for probe

About 200 individuals of orange-red medaka (approximately 50 grams) were homogenized twice by a whirling blender for 10 sec, then the mtDNAs were prepared by the SDS-phenol method. Mitochondria and crude mtDNAs were prepared as described by Yonekawa *et al.* (1978). Mitochondrial DNAs were further purified by CsCl-ethidium bromide density-gradient centrifugation at 36,000 rpm for 40 h. The fractions containing closed circular and open circular mtDNAs were collected separately.

Restriction analysis

The isolated closed circular mtDNA was used as a radioactive probe for Southern blotting to detect mtDNA restriction fragments obtained by restriction endonuclease digestions from total cellular DNA. Restriction enzymes *Apa* I, *Dra* I, *Eco* RI, *Eco* RV, *Hin* dIII, *Pst* I, *Sma* I, and *Xba* I were used for RFLPs analysis of 17 specimens from 16 localities in Korea and 3 Chinese specimens, and 2 restriction enzymes, *Apa* I and *Eco* RV, for analyzing 69 specimens from 44 localities in Korea. Restriction fragments were assigned molecular weights by comparing to *Eco* T14I lambda phage DNA size marker. Each distinct restriction fragment pattern produced by any of the eight endonucleases was assigned a capital letter code in alphabetical order of

detection (A, B, etc.). Thus, each individual was finally assigned an eight-letter composite mtDNA haplotype.

Analysis of data

Percentages of nucleotide sequence divergence (*p* value) between mtDNA haplotypes were estimated from the shared restriction fragment (Nei and Li, 1979). Relationships among mtDNA haplotypes were assessed by UPGMA clustering using the NEIGHBOR program in the 3.5c version of PHYLIP (Felsenstein, 1993).

RESULTS

Mitochondrial DNA haplotypes of wild population of Korean medaka

We used 8 restriction enzymes to analyze cleavage fragment patterns of mtDNA from 17 Korean medaka. Fragment patterns in each enzyme are illustrated in Fig 2. In Korean medaka, two different fragment patterns were found for *Dra* I, *Hin* dIII, *Pst* I, and *Sma* I (A-B), three for *Apa* I, *Eco* RI and *Eco* RV (A-C), and four was for *Xba* I (A-D).

In the mtDNA from the 17 Korean medaka, we recognized 7 mtDNA haplotypes as presented in Table 1. Each haplotype was designated with a #number representing a haplotype (#1-#7). In addition, 69 other specimens were analyzed with two restriction enzymes, *Apa* I and *Eco* RV. The two enzymes revealed four *Apa* I/*Eco* RV types. The mtDNA haplotype #1 and #2 were *Apa* I/*Eco* RV type AA, the #3 was AB, the #4 was BA, and #5, #6, and #7 were CC type.

Mitochondrial DNA haplotypes of wild population of Chinese medaka

In five enzymes, we found some "China-specific" fragment patterns: fragment pattern D for *Apa* I, C for *Dra* I, D and E for *Eco* RV, C for *Hin* dIII, and E for *Xba* I.

The two Chinese medaka captured at Beijing and at Shanghai had mtDNA haplotype #3 and #8, respectively. The *Apa* I/*Eco* RV types of these two Chinese specimens were AB and AD. The mtDNA haplotype #8 was only different from haplotype #3 in a fragment pattern for *Eco* RV. The specimen from Kunming had specific fragment patterns for five enzymes. The mtDNA haplotype was designated as haplotype #9, and the *Apa* I/*Eco* RV type of Kunming specimen was DE (Table 1).

Genetic distance and phylogenetic analysis

Estimated pairwise sequence divergence among haplotypes ranged from 0.2 to 12.9 % (Table 2). A phylogenetic tree based on these sequence divergences was constructed by the UPGMA method (Fig. 3). The tree showed that mtDNA haplotypes from Korean and Chinese wild populations were divided into two clusters: (1) haplotype #1 to #4, #8 and #9 (cluster A) and (2) haplotype #5 to #7 (cluster B). In the cluster A, haplotype #9 was markedly different from the other five haplotypes.

Estimated pairwise nucleotide sequence divergence between two clusters ranged from 6.7% to 12.9% (mean: 8.5%). Estimated pairwise sequence divergence in cluster A and in cluster B ranged from 0.3 to 5.0% (mean: 2.1±1.73%) and from 0.2 to 0.9% (mean: 0.6±0.35%), respectively. The average estimated sequence divergence among haplotype #9 and other haplotypes of cluster A were 4.37%.

Geographic distributions of the mtDNA haplotypes in Korean medaka

Figure 4 shows the geographic distribution of mtDNA *Apa* I/*Eco* RV type in Korea. Overall, the data analysis revealed strong geographical associations for the *Apa* I/*Eco* RV types.

The mtDNA haplotypes of cluster A (*Apa* I/*Eco* RV type AA, AB, and BA) were found in the western Korea (Fig. 4). The *Apa* I/*Eco* RV type AA and BA were found in the northern, Han River drainage area. The BA type was mainly found in the southern, Kum River drainage area.

The mtDNAs of cluster B (*Apa* I/*Eco* RV type CC) were mainly found in freshwaters flowing into the East Sea and the South Sea. In addition, the specimens from seven localities in the West Sea coast also had the *Apa* I/*Eco* RV type CC.

DISCUSSION

Large intraspecific diversity among mtDNAs

Intraspecific mtDNA divergences of 7.4% in anchovy (Magoulas *et al.*, 1996) and 8.5% in sunfish (Avise *et al.*, 1984) have been reported as the highest degrees for intraspecific divergences. In the Korean medaka, the pairwise comparisons between haplotypes of cluster A and B, demonstrate a large divergence, 6.7%-12.9%. This large divergence suggests that the event separating these clusters is very old.

The rate of base substitution of mammalian mtDNA has been estimated to be 2.0% per million years (Brown *et al.*, 1979). At this rate, cluster A and cluster B could have shared a common ancestor approximately 4.3 million years ago (average divergence between cluster A and B: 8.5%).

Agreement of geographic distribution among mtDNA haplotypes, allozyme genotypes, and chromosomal types in Korean medaka

Allozymic analyses in Korean wild populations demonstrated two genetically different groups in the Korean Peninsula (Sakaizumi and Joen, 1987): the China-West Korean Population and the East Korean Population. Furthermore, karyological and morphological studies also indicated differences between the two populations (Chen *et al.*, 1989; Kim and Lee, 1992; Kim and Moon, 1987; Uwa and Joen, 1987; Uwa *et al.*, 1988). Kim and Lee (1992) have described that medaka with a diploid chromosomal number of 46 are distributed in the Kangwha Island, Han River, Kum River, Tongjin River, Mangyong River, Yongsan River, and Somjin River.

On the other hand, we can also divided mtDNA haplotypes into two groups, cluster A and B. The distribution ranges of the mtDNA types in two clusters coincide perfectly with the previously defined two population ranges; A associated with the China-West Korean Population (2n, 46) and B with the East Korean Population (2n, 48). The mtDNAs of the medaka from west Korea more closely related to those from Kunming than those from east Korea, whereas there is approximately 2,600 km between Kunming and west Korea. The medaka from Kunming also had a diploid chromosomal number of 46 and were defined by allozymic analysis as the China-West Korean Population. Such agreement between independent elements of the genome might be expected if the China-West Korean Population and the East Korean Population had evolved in complete isolation at some time in the past.

Geographic variation of Korean wild population of medaka

Since the distribution pattern of the mtDNA in the Korean wild populations agrees with those of other markers, the mtDNA types may reflect the population structure in the Korean wild population of medaka.

UPGMA analysis divides the seven Korean mtDNA haplotypes (four mtDNA *Apa* I/*Eco* RV types) into two clusters (Fig. 2). Geographic distributions in mtDNA *Apa* I/*Eco* RV types of these two clusters suggest three geographical areas and three (sub)populations of Korea: the Han River basin (the Han River Subpopulation), the Kum River, Mangyong River, and Yongsan River basin (the Kum River Subpopulation), and the southern and eastern Korea (the Eastern Korean Population).

Each mtDNA type was primarily distributed in one geographical area. The *Apa* I/*Eco* RV AA and BA were distributed in the Han River basin, the AB primarily in the Kum River, Mangyong River, and Yongsan River basin, and the CC in the South Sea coast and eastern Korea. However, the AB type was also distributed in the north area (Locality No. 5), and the CC type in the western coast (Locality No. 3, 5, 14, 16, 17, 21, 22).

Furthermore, in the Somjin River basin, we found the mtDNAs of the two *Apa* I/*Eco* RV types belonged to different clusters. Downstream, we found the CC type (cluster B) and upperstream, the AB type (cluster A). The distribution pattern of mtDNA types is inconsistent with the present river course.

These distribution patterns of the mtDNA types may reflect a migration history in the Korean wild populations of medaka. Thus, the Han River Subpopulation, the Kum River Subpopulation, and the East Korean Population may have been geographically isolated from each

other at some time in the past, and subsequently these three (sub)populations evolved by natural selection and/or genetic drift. However, these assumptions are inconsistent with present distribution patterns of mtDNA types in western Korea and in the Somjin River. These contradictions can be explained by two hypotheses: (i) recent migration events from south to north in the west Korea and (ii) a stream capture occurred in the Somjin River.

Table 3 Locality numbers, fragment patterns (indicated by eight letters and *Apa* I/*Eco* RV types), mtDNA haplotype numbers, and collection localities. Enzyme order: *Apa* I, *Dra* I, *Eco* RI, *Eco* RV, *Hin* dIII, *Pst* I, *Sma* I, and *Xba* I. The locality numbers with an asterisk (*) show two specimens which have identical mtDNA types.

Locality	Fragment patterns		Haplotype	
Number	8 enzymes	*Apa* I/*Eco* RV	Number	Locality name
1*	AAAAAAAA	AA	#1	Sokmo, Samsan, Kanghwa, Kyonggi
2		AA		Taeso, Changhowon, Ichon, Kyonggi
3		CC		Puk, Taebu, Ongjin, Kyonggi
4		AA		Songam, Taebu, Ongjin, Kyonggi
5		AB		Haechang, Paltan, Hwasong, Kyonggi
5	CBBCBBBB	CC	#5	Haechang, Paltan, Hwasong, Kyonggi
6		BA		Sokpo, Ujong, Hwasong, Kyonggi
7		BA		Hwasan, Ujong, Hwasong, Kyonggi
8		AA		Machyang, Ujong, Hwasong, Kyonggi
9*		AA		Nae, Taedok, Ansong, Kyonggi
10	BACAAAAC	BA	#4	Suksong, Osong, Pyongtaik, Kyonggi
11		AA		Pyongami, Saingguk, Umsong, Chungchongbuk
12	AAABAAAC	AB	#3	Pugang, Puyong, Chongwon, Chungchongbuk
13		AA		Haingjong, Tangjin, Tangjin, Chungchongnam
13	AAAAAABA	AA	#2	Haingjong, Tangjin, Tangjin, Chungchongnam
14	CBBCBBBB	CC	#5	Taegi, Wonbuk, Sosan, Chungchongnam
14		CC		Taegi, Wonbuk, Sosan, Chungchongnam
15		AA		Shingok, Kuhang, Hongsong, Chungchongnam
16	CBBCBBBB	CC	#5	Chinjuk, Chongso, Poryong, Chungchongnam
16		CC		Chinjuk, Chongso, Poryong, Chungchongnam
17	CBBCBBBB	CC	#5	Taechon, Taechon, Poryong, Chungchongnam
17		CC		Taechon, Taechon, Poryong, Chungchongnam
18*	AAABAAAC	AB	#3	Shinkwan, Misong, Okku, Chollabuk
19		AB		Paikgu, Paikgu, Kimje, Chollabuk
20	AAABAAAC	AB	#3	Tongsan, Chochon, Wanju, Chollabuk
20		AB		Tongsan, Chochon, Wanju, Chollabuk
21	CBBCBBBB	CC	#5	Teahang, Sannae, Puan, Chollabuk
21		CC		Teahang, Sannae, Puan, Chollabuk
22*		CC		Sokpo, Chinso, Puan, Chollabuk
23*		AB		Chodong, Songnae, Kochang, Chollabuk
24		AB		Kalma, Taesan, Kochang, Chollabuk
25		AB		Yongsan, Shinpyong, Imshil, Chollabuk
26		AB		Tugok, Imshil, Imshil, Chollabuk
27		AB		Kunpyong, Tunnam, Imshil, Chollabuk

Table. 3 Continued

Locality Number	Fragment patterns 8 enzymes	*Apa* I/*Eco* RV	Haplotype Number	Locality name
28		AB		Chechon, Chusaing, Namwon, Chollabuk
29		AB		Ipsok, Yonggwang, Yonggwang, Chollanam
30		AB		Hampyong, Hampyong, Hampyong, Chollanam
31		AB		Yongdong, Tashi, Naju, Chollanam
32*		CC		Pojon, Chisan, Chindo, Chollanam
33		CC		Popyong, Haenam, Haenam, Chollanam
34*		CC		Upho, Hyonsan, Haenam, Chollanam
35		CC		Shinwol, Pukil, Haenam, Chollanam
36	CBBCBBBD	CC	#7	Yonghwa, Shinjon, Kangjin, Chollanam
37		CC		Mandok, Toam, Kangjin, Chollanam
38		CC		Chongdo, Wando, Wando, Chollanam
39*		CC		Kwansan, Yaksan, Wando, Chollanam
40*		CC		Suyang, Anryang, Changhung, Chollanam
41*		CC		Pyokgyo, Hoechon, Posong, Chollanam
42*		CC		Tokam, Sunchon, Chollanam
43		AB		Chichon, Kwangui, Kurye, Chollanam
44*		CC		Kyechon, Kumnam, Hadong, Kyongsangnam
45		CC		Songnae, Konyang, Sachon, Kyongsangnam
46		CC		Pangji, Sanam, Sachon, Kyongsangnam
47		CC		Nanum, Samdong, Namhae, Kyongsangnam
48		CC		Taedok, Kosong, Kosong, Kyongsangnam
49	CBBCBBBB	CC	#5	Pojon, Maam, Kosong, Kyongsangnam
49		CC		Pojon, Maam, Kosong, Kyongsangnam
50		CC		Sanyang, Tongbu, Koje, Kyongsangnam
51		CC		Imyong, Chinjon, Changwon, Kyongsangnam
51	CBBCBBBC	CC	#6	Imyong, Chinjon, Changwon, Kyongsangnam
52		CC		Kangju, Pobsu, Haman, Kyongsangnam
53		CC		Hwajon, Ilgwang, Yangsan, Kyongsangnam
54		CC		Myongsan, Sosaing, Ulju, Kyongsangnam
55		CC		Kugil, Yangbuk, Wolsong, Kyongsangbuk
56*		CC		Hungan, Uichang, Yongil, Kyongsangbuk
57*		CC		Shinpyong, Yonghae, Yongdok, Kyongsangbuk
58	CBBCBBBB	CC	#5	Sachonjin, Sachon, Myongju, Kangwon
58*		CC		Sachonjin, Sachon, Myongju, Kangwon
59		CC		Chonghak, Sokcho, Kangwon
60*		CC		Yongchon, Tosong, Kosong, Kangwon
61	AAABAAAC	AB	#3	Beijing
62	AAADAAAC	AD	#8	Shanghai
63	DCBECABE	DE	#9	Kunming

Table 4 Numbers of common and different fragments, upper matrix (common, different), and percentage of pairwise sequence divergence among the nine composite mtDNA haplotypes (p values), lower matrix.

	1	2	3	4	5	6	7	8	9
haplotype 1		25, 3	22, 8	23, 8	7, 38	7, 36	5, 41	11, 25	24, 6
haplotype 2	0.325		20, 11	21, 11	8, 35	8, 33	6, 38	12, 22	22, 9
haplotype 3	0.941	1.376		21, 10	4, 42	6, 36	3, 43	11, 23	24, 4
haplotype 4	0.902	1.316	1.207		6, 40	8, 34	5, 41	10, 27	23, 8
haplotype 5	7.872	6.913	11.17	8.845		21, 6	23, 2	6, 33	6, 40
haplotype 6	7.626	6.663	8.339	6.789	0.75		20, 7	6, 31	8, 34
haplotype 7	9.877	8.597	12.874	9.877	0.237	0.908		7, 30	5, 41
haplotype 8	4.441	3.783	4.177	5.024	7.932	7.646	6.825		11, 25
haplotype 9	0.661	1.049	0.448	0.902	8.845	6.789	9.877	4.441	

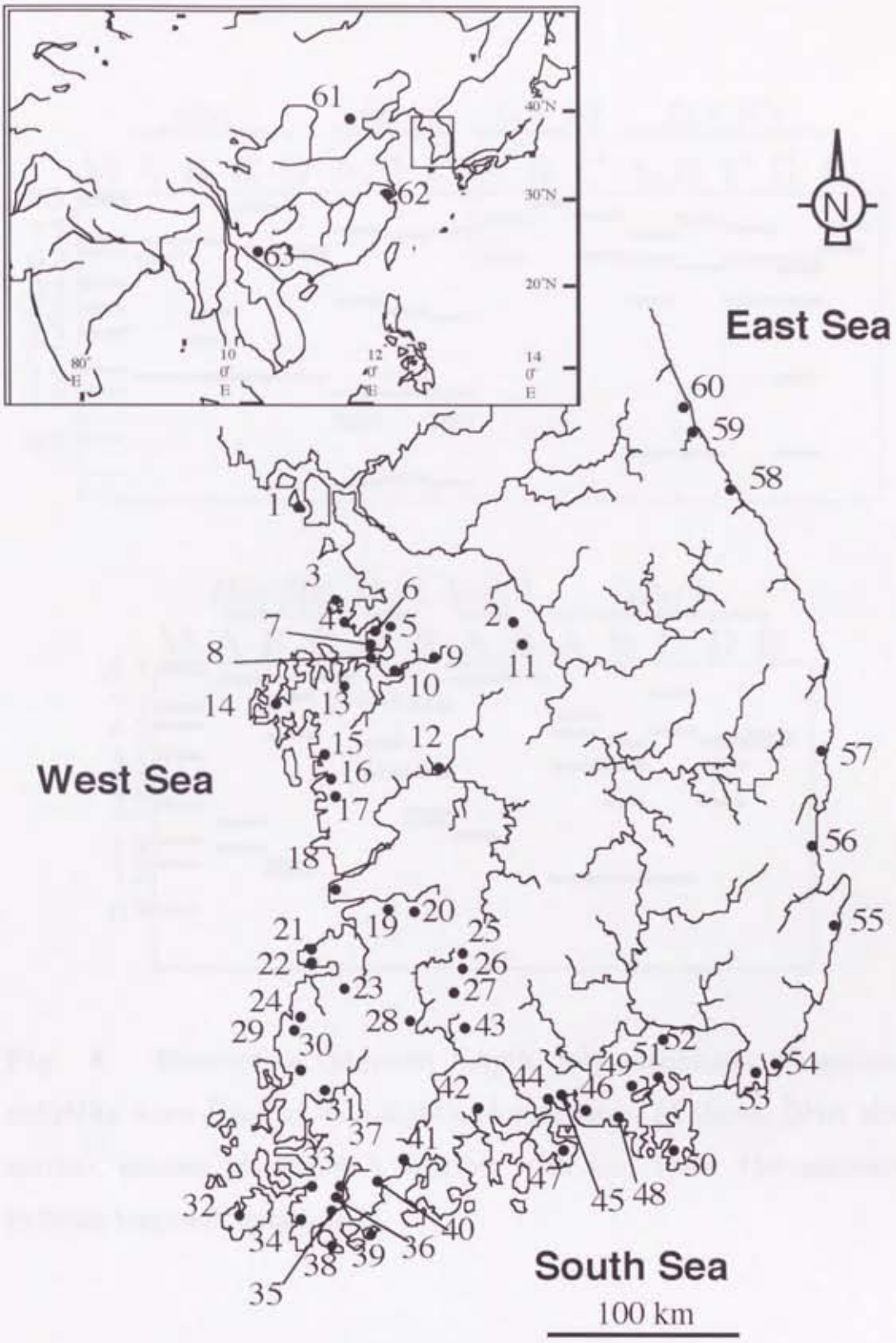

Fig. 5 Collection localities for medaka. The numbers refer to locations in Table 3.

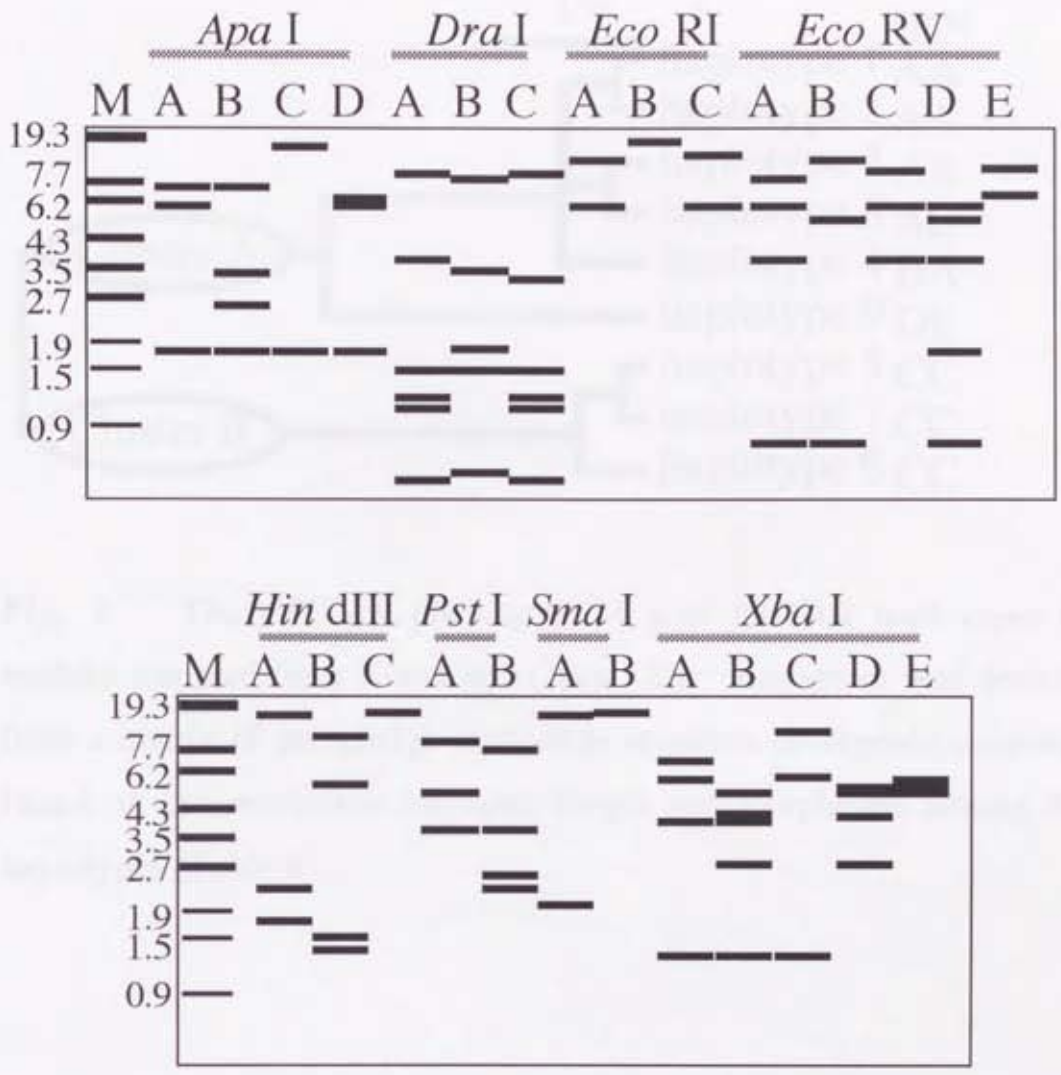

Fig. 6 Restriction fragment length polymorphisms of medaka mtDNAs were digested with eight endonucleases. M shows DNA size marker, lambda phage DNA digested with *Eco* T14I. The numbers indicate fragment length (Kb).

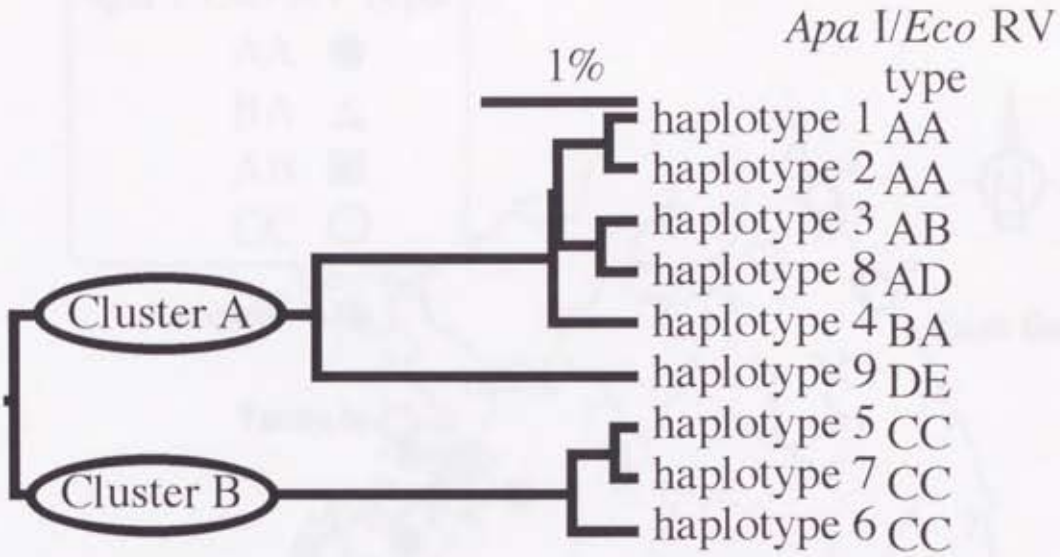

Fig. 7 The UPGMA phenogram of nine mtDNA haplotypes of medaka sampled from Korea and China. The phenogram was derived from a matrix of percentage nucleotide sequence divergence estimates based on the restriction fragment length polymorphisms among the haplotypes (Table 4).

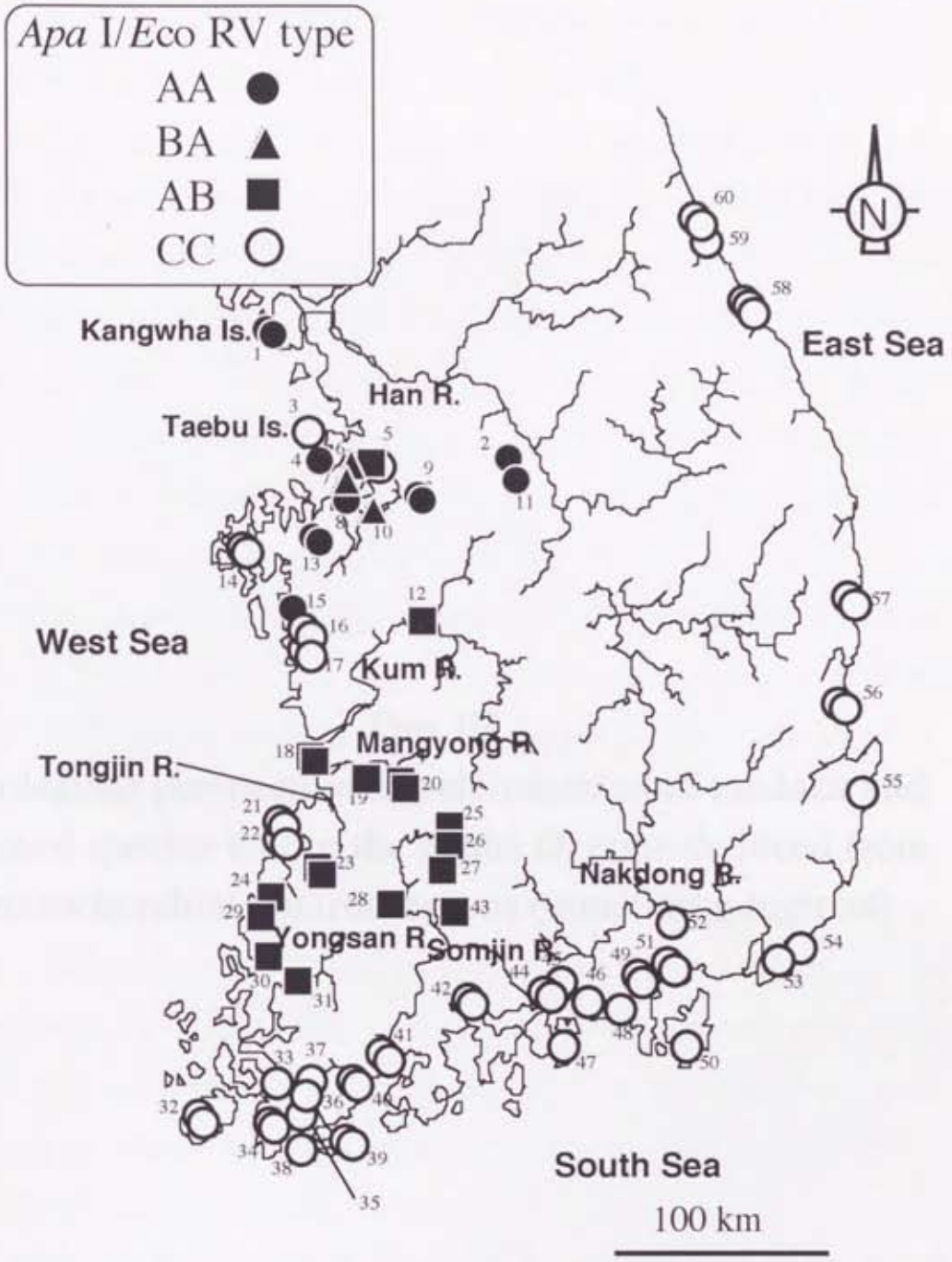

Fig. 8 Geographical distribution of mtDNA *Apa* I/*Eco* RV types. Numbers indicate the locality number. One symbol represents one fish.

Part III.
Molecular perspective on relationships of medaka and related species within the genus *Oryzias* deduced from mitochondrial control regions (noncoding regions)

INTRODUCTION

Mitochondrial DNA (mtDNA) has been widely used as a marker for evolutionary and population studies because of its compact size, nearly complete maternal inheritance, and fast evolutionary rate (Brown *et al.*, 1979). Mitochondrial genes, because of their maternal inheritance, are expected to provide a more sensitive tool for detecting population subdivision than nuclear genes (Birky *et al.*, 1989). Observations of mtDNA gene diversity thus provide some of the best information for studying levels of gene flow among fish populations (Ovenden, 1990).

Numerous studies of mtDNA haplotype diversity in fish have been conducted using restriction-fragment-length polymorphisms (RFLPs) of the entire mitochondrial genome (Avise *et al.*, 1988; Graves *et al.*, 1992). Recently, the use of nucleotide sequence data, rather than RFLPs, has been encouraged, primarily because of the grater sensitivity of sequencing in detecting variants (Bartlett and Davidson, 1991; Beckenbach, 1991; Carr and Marshall, 1991a; Carr and Marshall, 1991b; Finnerty and Block, 1992). Polymerase chain reaction techniques make it feasible to target particular gene segments carrying the highest density of intraspecific variation in large numbers of individuals (Kocher *et al.*, 1989).

Many studies of mammalian mtDNA have focused on the major noncoding region, located between the tRNA-Pro and tRNA-Phe genes, because of its supposedly rapid rate of evolution (Hoelzel *et al.*, 1991). This region, often called the control region, includes transcriptional promoters for both strands, the heavy strand replication origin, and the displacement or D-loop region (Chang and Clayton, 1986; Clayton, 1982). Because of reduced functional constraints, some portions of the

control region evolve much faster than the average mitochondrial sequence (Brown, 1985). The segments directly adjacent to the flanking tRNAs often show the highest rates of base substitutions and insertion/deletion events (Saccone *et al.*, 1987).

Although large variations in the size of fish mitochondrial genomes are known (Billington and Hebert, 1991), the extent to which this represents expansion of unique control-region sequences is poorly understood. The exact size of the control region is known for a few fish species (reviewed in Lee *et al.*, 1995).

The genus *Oryzias* is distributed from India to the Far East. Eleven species have been identified (Uwa and Magtoon, 1986; Yamamoto, 1975). The eight species for which data on karyotype have been descried can be divided into three chromosomal groups, the monoarmed, the biarmed, and the fused chromosome group. The biarmed chromosome group includes *O. latipes*, *O. curvinotus*, *O. luzonensis*, and *O. mekongensis*. (Uwa, 1986). The division of species of *Oryzias* by karyology coincides with divisions suggested by electrophoretic studies (Sakaizumi, 1985b; Sakaizumi, 1986a).

Geographic variation in biochemical characters of medaka has been demonstrated in allozymes (Sakaizumi *et al.*, 1980; Sakaizumi *et al.*, 1983). These results indicate four genetically distinct populations; the Northern Population from the northern coast of the Sea of Japan, the Southern Population from western and southern Japan, the West Korean Population from western and southern Korea, and the China-West Korean Population from China and western Korea. These four groups can be distinguished by means of many unique alleles and Nei's coefficients of genetic distance are no less than 0.30 (Sakaizumi, 1986a; Sakaizumi and Joen, 1987; Sakaizumi *et al.*, 1983). The genetic

diversity estimated between the two populations is large enough to be considered as characteristic of interspecific comparisons. However, male and female progeny from crosses between two populations are fully fertile (Sakaizumi, 1986a; Sakaizumi *et al.*, 1992).

Furthermore, mtDNAs of the medaka have divided into three clusters (Part I). The results suggest 10% nucleotide sequence divergence between the Northern Population and the Southern Population.

The main objectives of the present study are to compare nucleotide sequence of control region in the Northern Population with that in the Southern Population, and, more generally, to evaluate empirically the utility of hyper-variable control region sequence in population studies.

MATERIALS AND METHODS

Materials

For construction of mitochondrial genomic library, we used two wild stocks of the medaka, *Oryzias latipes*. One was wild stock of Maizuru, from the Northern Population. The other was wild stock of Himeji, from the Southern Population. For PCR amplification, we used other two populations of medaka and other three species of genus *Oryzias*. Two *O. latipes* were wild medaka of Tosong and Kunming, from the East Korean Population and China-West Korean Population, respectively. Three species were belong to biarmed chromosome group, *O. curvinotus*, *O. luzonensis*, *O. mekongensis*.

Isolation of mtDNA

Mitochondrial DNAs was isolated from two strains of wild stocks; Himeji and Maizuru. About 200 individuals of medaka (approximately

50 grams) were homogenized twice by a whirling blender for 10 sec, then the mtDNAs were prepared by the SDS-phenol method. Mitochondria and crude mtDNAs were prepared as described by Yonekawa *et al.* (1978). Mitochondrial DNAs were further purified by CsCl-ethidium bromide density-gradient centrifugation at 36,000 rpm for 40 h. The fractions containing closed circular and open circular mtDNAs were collected separately.

Construction of mtDNA genomic library

Eighty micrograms of the closed circular mtDNA isolated from two wild strains were digested with restriction endonuclease *Xba* I (TOYOBO). Enzyme digestion conditions were as specified by the manufacturer. The mtDNA digested was separated in a 0.7% agarose gel electrophoresis. Each of the fragments was cut out of the gel, and purified using Ultrafree C3HV cartridge (MILLIPORE), phenol extraction, and ethanol precipitation. The fragment purified was resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). About 0.2 μg of each particular fragment digested was ligated into pUC119. The ligation was performed using DNA ligation Kit (TAKARA) at 16°C over night. Five microliters of the each ligation reaction were mixed with 50 μl of *Escherichia coli* JM109 Competent Cells (TAKARA) in a 14 ml sterilized tube (Falcon 2059) on ice, which was then stood for 30 min. The tube was immersed into 42°C water bath for 90 s and the cell suspension was incubated for one hour at 37°C adding one ml L-broth (Bacto-trypton 10 g, Bacto-yeast extract 5 g, NaCl 5 g, and distilled water 1000 ml). The transformation mixture was plated on LA plates (Bacto-trypton 10 g, Bacto-yeast extract 5 g, NaCl 5 g, Bacto-agar 15 g, and distilled water 1000 ml) containing carbenicillin 2 Na salt (60 μg/ml) with 50 μl 6% Bluo-Gal (BRL) and

100 μl 23.8 g/ml IPTG (BRL). After incubation for over night at 37°C, white colonies were collected from each of the fragments cloned, and subjected to a 20 ml LB/CB culture and purification by the modified alkaline lysis method (Birnboim and Doly, 1979).

In mtDNAs of Maizuru strains, the second fragment was subcloned with *Pst* I. By contrast, in mtDNAs of Himeji strains, the third fragment was subcloned with *Bam* HI.

Sequencing

Sequence data were obtained by BcaBEST™ Dideoxy Sequencing Kit (TAKARA). Hydrolink™ Long Ranger (AT Biochem) 6% gel containing 7 M urea was used as a sequence gel. All inserts were sequenced using M13 Primer M4 and M13 Primer RV (TAKARA). The sequence data were compared to the complete mtDNA sequence of carp (the accession number X61010; Chang *et al.*, 1994). Homology search was performed using DNASIS™-Mac (HITACHI). We estimated location of six *Xba* I sites of Maizuru strains and five *Xba* I sites of Himeji strains, respectively. Furthermore, to comparison of tandem repeat sequence, we sequenced using Cy-b, Fd primer (5'-CCC TAT TCT ACA CAC CTC TAA ACA ACG-3').

Isolation of total DNA

Head, intestine and fins were removed and discarded from adult medaka. The tissue was placed in 500 μl of 100 mM EDTA, 50 mM Tris (pH 8.0), 100 mM NaCl, 1% SDS, and 100 μg/ml of Proteinase K. The tissue was minced in a 1.5 ml tube and incubated at 55°C for over night. The homogenate was extracted twice with buffer-equilibrated phenol, once with a 1:1 mixture of phenol:chloroform, and once with chloroform. DNA was precipitated with isopropyl alcohol, rinsed with

ethanol, and resolved in TE buffer (1 mM EDTA, 10 mM Tris pH 8.0).

PCR amplification

We designed two PCR primers to amplify variable region of the control region; Fa (5'-CTC CCA A(GA)G C(CT)A (GA)GA TTC TAA-3'), Fb (5'-CCA TTT TTG CCT AGT ACA CCT CG-3'), RVa (5'-(CT)TA TCA CTG CTG AGT TCC-3'), RVb (5'-TGC ATG GAC GAG GTG TAC TAG GCA A-3'). Double-stranded PCR amplifications were carried out in 25 μl reactions containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.25 μmol each of the heavy- and light-strand primers, and 1.25 unit of *Taq* DNA polymerase (TOYOBO). To this mixture was added 1 μl of the template DNA (<0.1 μg total cellular DNA). The DNA was amplified in a Program Temp Control System PC-800 (ASTEC) on the following step cycle profile: strand denaturation at 94°C for 1.5 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 2 min, repeated for 30 cycles. Preliminary denaturation at 94°C for 2 min before the first cycle.

The double-stranded amplification products formed were separated by gel electrophoresis on a 2% agarose. The fragments amplified were visualized under long wave UV light.

RESULTS

Construction of complete genomic library of mtDNA

We constructed two complete mitochondrial genomic libraries from two strains. One library was derived form Maizuru medaka belonging to the Northern Population. The library contained six

recombinant clones (insert; 5.9, 5.4, 4.2, 0.7, 0.5, 0.1 kbp). The other library was derived form Himeji medaka belonging to the Southern Population, and consist of five recombinant clones (insert; 6.7, 4.2, 3.8, 1.6, 0.5 kbp).

The nucleotide sequences of both ends of inserts were obtained. Then we estimated the position of *Xba* I fragments and that of *Xba* I sites relative to the carp complete mitochondrial sequence, and we concluded that complete mitochondrial genomic library was constructed.

In these two libraries, a clone of insert length 0.5 kbp was only identical, and three *Xba* I sites were identical out of eight *Xba* I sites.

Two sequences of the control region in medaka

The control region was included in second clone from the Maizuru and in third clone from the Himeji. Therefore, we subcloned these clones with *Pst* I and *Bam* HI, and obtained two nucleotide sequences of the control region (Fig. 9). The sizes of these control regions were 1,374 bp and 1,302 bp.

Figure 9 shows aligned sequences of the mitochondrial control region of two populations of medaka. From comparison between the nucleotide sequence of Maizuru and that of Himeji, we noticed that medaka control region separated into two blocks. One block was "highly conserved," and the other block is "hyper-variable." The hyper-variable region had tandemly repeated sequences. Variation in size is mainly due to the tandemly repeated sequences. The tandem repeats sequence in the control region of Maizuru was 20 copies of an 11 bp (TGC ATG TGC GT). By contrast, that of Himeji had 10 copies of an 11 bp (TGC ATG CGC GT). One substitution (T to C) was observed at seventh nucleotide in the 11 bp.

Length variation in the control region of Oryzias

We designed PCR primers to amplify hyper-variable region of medaka control region, and we amplified four species of *Oryzias*, included four populations of *O. latipes* (Fig. 10). We found length variation of the hyper-variable blocks in the control region of *Oryzias* species. In particular, *O. mekongensis* had extremely short hyper-variable block among other biarmed species of *Oryzias*.

Sequencing analysis using Cyt-b, Fd primer demonstrated that the biarmed chromosome group of *Oryzias* had tandemly repeat sequences in control region, expect for *O. mekongensis*. The species had no tandemly repeat sequences in the region (Fig. 11).

DISCUSSION

Length of mitochondrial genome

Mitochondrial genomes of fish vary little in size, with a range of 15.2-19.8 kbp reported among the 47 species analyzed (Table 1 in Billington and Hebert, 1991). Some of the smaller size values are likely under-estimates, as they were obtained in studies that employed techniques which did not permit the detection of restriction fragments under 0.5 kbp. In this study, we constructed complete mtDNA library and estimated insert length of recombinant clones. The estimation was performed by electrophoresis and relating to the carp complete mitochondrial sequence. The shortest insert length of recombinant clone is 114 bp. Our estimation was hence reliable. We estimated that the lengths of medaka mtDNAs of two populations are 16.8 kbp. This length are similar to that of the mtDNAs of carp (16,575 bp;Chang *et al.*, 1994), human (16,569; Anderson *et al.*, 1981), mouse (16,295; Bibb *et al.*, 1981), chicken (16,775; Desjardins and Morais, 1990),

bovine (16,338; Anderson *et al.*, 1982), fin whale (16,398; Arnason *et al.*, 1991), and rat (16,298; Gadaleta *et al.*, 1989), but shorter than that of *X. laevis* (17,553; Roe *et al.*, 1985).

Length of control region

A large number of nucleotide sequence of fish mitochondrial control regions has been reported (reviewed in Lee *et al.*, 1995). The size of these control regions varies from 856 to 1,500 bp. The sizes of medaka control regions are also in the range. Variation in size is mainly due to large insertions of unique sequence, although tandemly repeated sequences are found in several species (Lee *et al.*, 1995). These length variations of control region lead to length variations of whole mitochondrial genomes.

Tandemly repeated sequences of control region are reported in a variety of vertebrates, including fishes (Brown *et al.*, 1993; Brown *et al.*, 1996; Lee *et al.*, 1995; Miracle and Campton, 1995; Mundy *et al.*, 1996; Stewart and Baker, 1994; Wenink *et al.*, 1994). These reports have indicated a variation of character of tandem repeat sequence (length, copy number, and position in control region), and revealed existence of heteroplasmy. In Lee *et al.* (1995), repetitive sequences have been found most often near the end of D-loop strand. In the medaka, however, tandem repeats are found after the 3' end of the $tRNA^{Pro}$ gene. This location of tandem repeats is similar to Atlantic cod and sturgeon (Buroker *et al.*, 1990; Lee *et al.*, 1995).

Conserved sequence blocks (CSBs)

Many of the conserved sequence blocks (CSBs) observed in mammals are also found among fishes. In particular, the mammalians CSB-D is present in all the fish species studied in Lee *et al.* (1995). In the medaka control regions, a central conserved region is conserved

between two populations and contained in the "highly conserved block." Homology between central conserved region of carp and that of medaka is 51.2%. In addition, GTGGG-box which is common in euteleosts expect for gadids, is also conserved in medaka (Fig. 9).

The CSB-D is 17 nucleotide sequence. The CSB-D sequence is highly conserved between medaka and carp. There are three nucleotide differences. Furthermore, the CSB-D is also conserved between medaka and other fishes' control region reported, suggesting that it contains functions critical for mitochondrial metabolism. Although a number of different approaches have been tried (Mignotte *et al.*, 1987; Saccone *et al.*, 1987), the function of this central conserved region is not understood.

Phylogenetic insight

We can amplify hyper-variable and highly conserved blocks of medaka mitochondrial control region, using Fa-RVb primers and Fb-RVa primers, respectively. Although we can not obtain a length variation from amplification of highly conserved block in the biarmed chromosome group of *Oryzias* species, we can detect a length variation of hyper-variable block in the control region (Fig. 10). The length of *O. mekongensis* is very short, and sequence analysis shows the species has no tandem repeat sequences (Fig. 11). These results suggest that *O. mekongensis* diverge first from common ancestor of the biarmed chromosome group of *Oryzias*. This suggestion is coincided with dendrogram to show karyotypic relationships in the biarmed chromosome group (Uwa, 1991). Moreover, a highly repetitive interspersed sequence analysis using OLR1 suggests that the nucleotide sequences and/or the number of copies of OLR1-related sequences in the genome of *O. mekongensis* are different from those in the other

three species (Naruse *et al.*, 1992). Nucleotide sequence of this region may be particularly useful of reconstruction of phylogenetic relationships among three other species within the biarmed chromosome group.

Electrophoresis of hyper-variable block also shows intraspecific variation in *O. latipes*. There are length variations among four populations of the medaka (Fig. 10). These length variations suggest differences of copy number in tandem repeat sequences among four populations. Furthermore, intra-populational variations are also demonstrate in two populations of Japan; the Northern Population and the Southern Population. In particular, the Northern Population would have large intra-populational differences of copy numbers in the tandem repeat. Nucleotide sequence of the hyper-variable block is probably the most useful for studies of intraspecific variation.

In addition, we notice that probability of heteroplasmy in mtDNA of *O. latipes*. Although tandemly repeated sequences in the control region of fishes have reported by many authors, length heteroplasmy of mtDNA have been only found in some species (reviewed in Billington and Hebert, 1991; Lee *et al.*, 1995). Moreover, it is uncertain if heteroplasmic individuals transmit variations to their offspring (Mulligan and Chapman, 1989). In this study, two inbred strains of *O. latipes* (the HNI and the Hd-rR strains) also show probabilities of length heteroplasmy. These inbred strains are appropriate for studying transmission genetics of length heteroplasmy.

```
                10         20         30         40         50
Himeji     CTATTCTCTG CCGAGCGCTG CCATTCGTAG CTTC-AAACA T-GTCTGAGC
Maizuru    CTATTCTCTG CCGAGCTCCG CCATTCATGA TTCCCAAATA TCATGGGGGC

                60         70         80         90        100
Himeji     TGTAAA-GAC A-TTCTTCCG GCCCCCAACA CGT-GCATGC GCGTTGCATG
Maizuru    TGTAAAAGAC ACCCCCCCCG ---------- CGTTGCATGT GCGTTGCATG

               110        120        130        140        150
Himeji     CGCGTTGCAT GCGCGTTGCA TGCGCGTTGC ATGCGCGTTG CATGCGCGTT
Maizuru    TGCGTTGCAT GTGCGTTGCA TGTGCGTTGC ATGTGCGTTG CATGTGCGTT

               160        170        180        190        200
Himeji     GCATGCGCGT TGCATGCGCG TTGCATGCGC GTTGCATGCG CGTTGCAT--
Maizuru    GCATGTGCGT TGCATGTGCG TTGCATGTGC GTTGCATGTG CGTTGCATGT

               210        220        230        240        250
Himeji     ---------- ---------- ---------- ---------- ----------
Maizuru    GCGTTGCATG TGCGTTGCAT GTGCGTTGCA TGTGCGTTGC ATGTGCGTTG

               260        270        280        290        300
Himeji     ---------- ---------- ---------- ---------- -------AAC
Maizuru    CATGTGCGTT GCATGTGCGT TGCATGTGCG TTGCATGTGC GTTGCATGAC

               310        320        330        340        350
Himeji     CCAAAACGGC CTAGT--GAA CAAGCGTGTG TTTGATGCGC ACGCGTGTGA
Maizuru    CTAAAAGGGC CTACTACTTA CAAG--TGCG TTCAGTGCAC GCGCGTGT-A

               360        370        380        390        400
Himeji     ACGTGCGCAA AACGTTCACA CAACACTC-A AAAATGTGCG TCGCCGGGCT
Maizuru    -----CGCAA AACGTTCACA -AACGCTCGC AAAATGTACC TCCCCGGGCT

               410        420        430        440        450
Himeji     CTGCCAATAT AGTGCCGAGT ACTTCCAAAA AGTCCCAAAA AAGTCCCAAA
Maizuru    CTGCCAAAAT AGTGCCGAGC ACTTCCAAAA AGTCCCAAAA AAGTCCCAAA

               460        470        480        490        500
Himeji     ATACATATAT GTATTATCCC CATATGTGGT TTTAACCATT TTTGCCTAGT
Maizuru    ATACATATAT GTATTATCCC CATGAATGGT TTTAACCATT TTTGCCTAGT

               510        520        530        540        550
Himeji     ACACCTCGTC CATGCAAGTC AATTATATTT ACCCCGCGCT CCAGGCCGCA
Maizuru    ACACCTCGTC CATGCAAGTC AATTATATTT ACCCCGCGCT CCAGGCCGCA

               560        570        580        590        600
Himeji     GAGCATACAC CTACGATTGG TGTATTTAGC ACAAGTGTGC CTCAGCTAGT
Maizuru    GAACATACAC CTACGATTGG TGTATTTAAC ACAAGTGTGC CTCAGCTAGT

               610        620        630        640        650
Himeji     TTCAAGTCGT CAGCATCCTT CCTTCAATTG TTATTTAATG TAGTAAGAGC
Maizuru    TTCAAGTCAT CAACATCCTT CCTTCAATTG TTATTTAATG TAGTAAGAGA

               660        670        680        690        700
Himeji     CCACCATCAG TTGATTTCTT AATGTTAACG GTTATTGAAG GTGAGGGACA
Maizuru    CCACCATCAG TTGATTTCTT AATGTCAACG GTTCTTGAAG GTGAGGGACA
```

Fig. 9 Aligned sequences of the mitochondrial control region of two populations of medaka. Himeji belong to the Southern Population, Maizuru the Northern Population. Hyphen (-) denote gaps inserted to maximize similarity among the sequences. The most conserved sequence block (CSB) in fish, corresponding to mammalian CSB-D, is marked, and GTGGG box is also marked.

```
                GTGGG box   720         730          CSB-D          750
Himeji      AAAATCGTGG GGGTTTCACT TCTTGAATTA TTCCTGGCAT TTGGCTCTAC
Maizuru     AAAATCGTGG GGGTTTCACT TCTTGAATTA TTCCTGGCAT TTGGCTCTAC

              760         770         780         790         800
Himeji      ATCTCAAGGC CATATAGTTT CTCGTCTCTC ACACTTTCAC TGGCCCTGAC
Maizuru     ATCTCAAGGC CATTTAGTTT CTCGTCTCTC ACACTTTCAC TGGCCCTGAC

              810         820         830         840         850
Himeji      ATTGGTTAAT GGTGGAGTAC ATACTCCTCG TTACCCACCA AGCCGAGCGT
Maizuru     ATTGGTTAAT GGTGGAGTAC ATACTCCTCG TTACCCACCA AGCCGAGCGT

              860         870         880         890         900
Himeji      TCTTTCTAAT GGGCAGGGGG TTCTCTTTTT TTTTTCCTTT CAATTTGCAT
Maizuru     TCTTTCTAAT GGGCAGGGGG TTTTTTTTTC TTTTTCCTTT CAATTTGCAT

              910         920         930         940         950
Himeji      TTCACAGTGC ATACAGACCT TGTTGACAAG GTTGAACATT TAGAACTCGG
Maizuru     TTCACAGTGC ATACAGACCT TGCTGACAAG GTTGAACATT TAGAACTCGG

              960         970         980         990        1000
Himeji      CCGCAAAGAA TATGGTGAGT TATTGTAAGA TATTAACAGA TGAATTGCAT
Maizuru     CCGCAAAGAA TATGGTGAGT TATTTTAAGA TATTAACAGA TGAATTGCAT

             1010        1020        1030        1040        1050
Himeji      AACTGATATC AAGAGCATAA ATGGCCAAAT GAATCTAGGA ACTTCCTATT
Maizuru     AACTGATATC AAGAGCATAA ATAGCTAAAT TAATCTAGGA ACTTCCTATT

             1060        1070        1080        1090        1100
Himeji      ATTTCGACCC CCGGCTTCTG CGGGCAAACC CCCCTACCCC CCTATACTAG
Maizuru     ATTTCGACCC CCGGCTTCCG CGCGCAACCC CCCCTACCCC CCAATACTAG

             1110        1120        1130        1140        1150
Himeji      TAAAAGCTCT ACATTCCTGC AAACCCCCCG GAAACAGGAA AGCCCCTACT
Maizuru     TAAGAGCTCT GCATTCCTGC AAACCCCCCG GAAACAGGAA AGCCCCTACT

             1160        1170        1180        1190        1200
Himeji      AATATTTTTT ACCTCCCTAA ATTGTGTGTA TTTACATTAT TTGTAATATT
Maizuru     AATATTTTTT CCCTCCCTAA ATTATGCGTA TTTACATTAT TTGTAATATT

             1210        1220        1230        1240        1250
Himeji      GCAAAAGCTA GCGTAGCTTA ACTAAAGCAT GACACTGAAG ATGTTAAGAT
Maizuru     GCAAAAGCTA GCGTAGCTTA ACTAAAGCAT AACACTGAAG ATGTTAAGAC

             1260        1270        1280        1290        1300
Himeji      AAACCTTAGA TTGGTTTCGC AAGCACAAAA GTTTGGTCCT GACTTTTCTA
Maizuru     AAACCTTAGA CTGGTTTCGC AAGCACAAAA GTTTGGTCCT GACTTTTCTA

             1310        1320        1330        1340        1350
Himeji      TCAACTCTAG CTAAACTTAC ACATGCAAGT ATCCGCAGTC CCGTGAGAAT
Maizuru     TCAACTATAG CTAAACTTAC ACATGCAAGT ATCCGCAATC CCGTGAGAAT

               1360        1370        1380        1390        1400
Himeji      GCCCTACAGT TTCCTTAATG GAAACAAGGA GCTGGTATCA GGCACAATAT
Maizuru     GCCCGACAGT TTCCTTAATG GAAACAAGGA GCTGGTATCA GGC.......

             1410        1420        1430        1440        1450
Himeji      AATGCC.... .......... .......... .......... ..........
Maizuru     .......... .......... .......... .......... ..........
```

Fig. 9 Continued

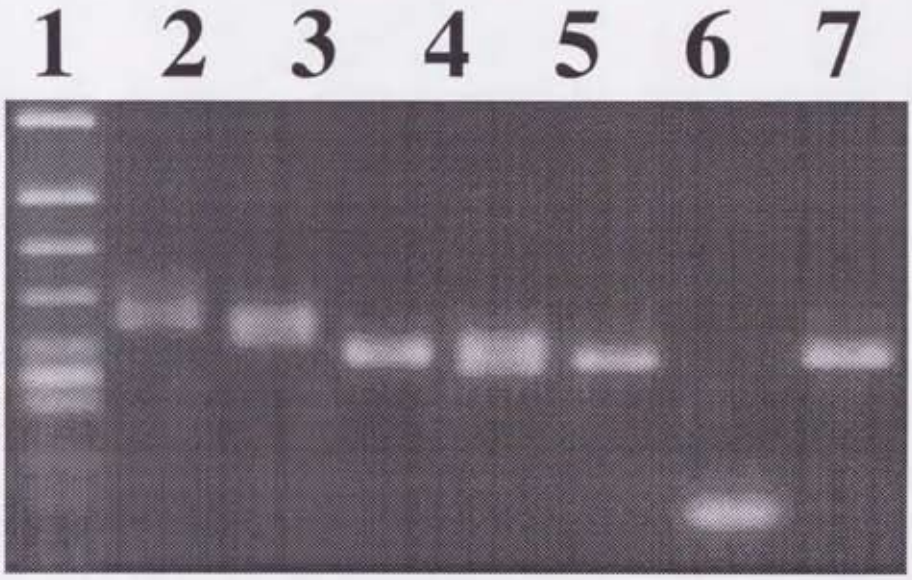

Fig. 10 PCR amplification products for *Oryzias* species. Amplification involved Fa and RVb primers resolved on a 2.0% agarose gel. Line 1 are size marker, phi X174 DNA digested with *Hin* cII. Lanes 1-4 depict *Oryzias latipes*, lane 1; the Northern Population (the HNI strain), lane 2; the Southern Population (the Hd-rR strain), lane 3, the China-West Korean Population (Kunming), and lane 4; the East Korean Population (Tosong). Line 5, 6, and 7 depict *O. curvinotus*, *O. mekongensis*, and *O. luzonensis*.

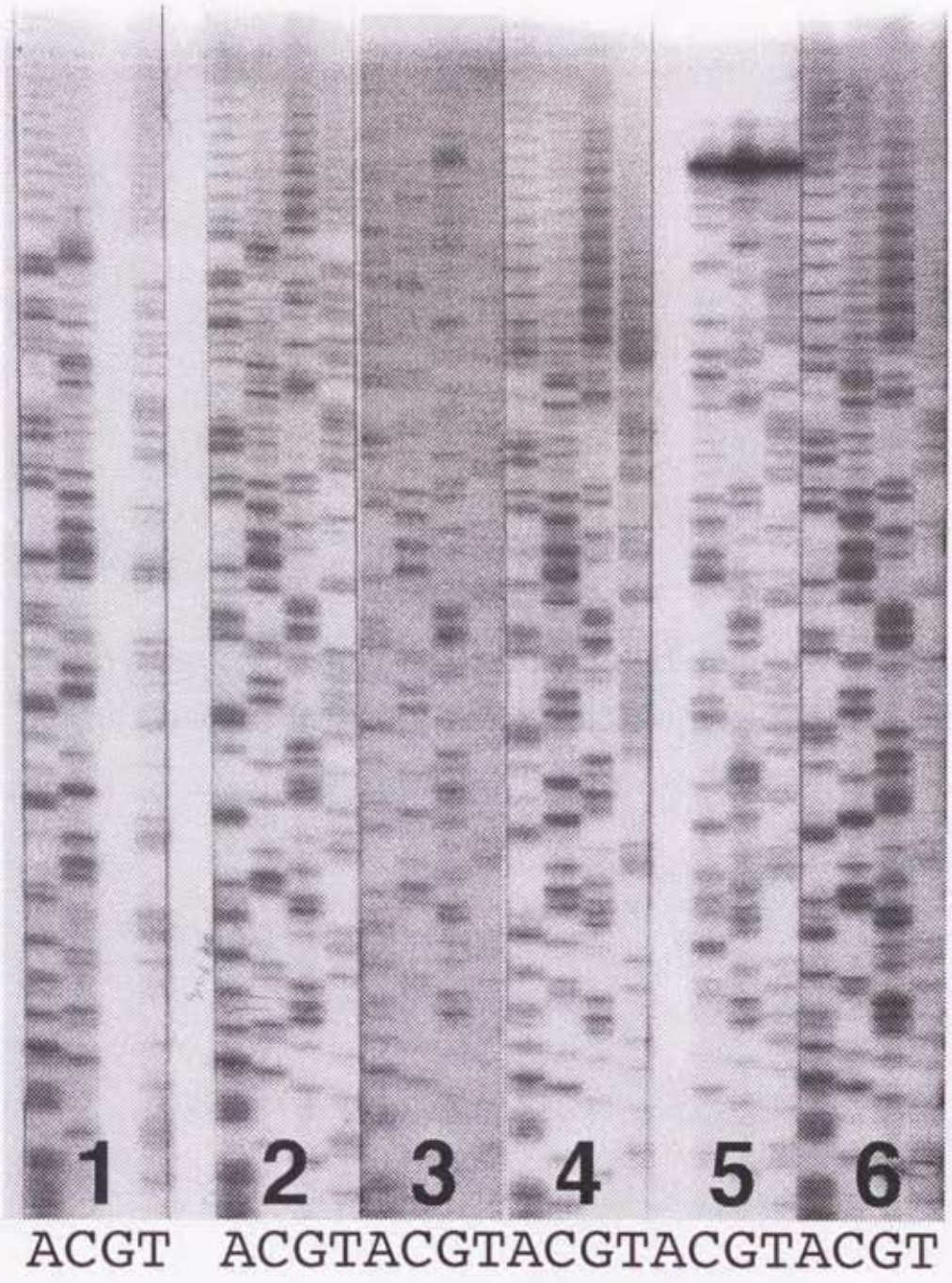

Fig. 11 Autoradiography showing the results obtained when direct sequencing from PCR was achieved. Sequences where obtained using the Cyt-b, Fd. The gel is loaded left to right as follows: 1 = HNI strain, the Northern Population of *O. latipes*, 2 = Hd-rR strain, the Southern Population of *O. latipes*, 3 = *O. curvinotus*, 4 = Kunming, the China-West Korean Population of *O. latipes*, 5 = *O. mekongensis*, 6 = Tosong, the East Korean Population of *O. latipes*.

Part IV.

A phylogeny of medaka and related species within the genus *Oryzias* inferred from complete sequences of the mitochondrial cytochrome *b* gene

INTRODUCTION

Mitochondrial DNA (mtDNA) is a valuable molecule for understanding the evolutionary relationships among individuals, populations, and species. Until recently such understanding was achieved mainly by restriction analysis because that technique is simpler than conventional cloning and sequencing (Harrison, 1989; Wilson *et al.*, 1985). With the advent of the polymerase chain reaction (PCR) (White *et al.*, 1989), it became possible to obtain mtDNA sequences directly from many taxa; these sequences can be aligned across greater time spans than can restriction maps (Kocher *et al.*, 1989). However, short sequences amplified in this manner could not resolve ancient evolutionary relationships (Kocher *et al.*, 1989).

The cytochrome *b* gene was chosen as a phylogenetic probe because it may be easier to align a protein-coding sequence that has evolved over the period spanning the origin of mammalian orders than to align either mitochondrial rDNA or noncoding sequences from distant relatives. This particular gene, together with a mitochondrial rDNA, has been found valuable for addressing even deeper phylogenetic questions, such as the origin of tetrapods (Meyer and Wilson, 1990).

Furthermore, the protein-coding gene we have chosen has an advantage over those studied before by cloning and sequencing from a taxonomically graded series of mammalian mitochondria. Cytochrome *b* is the only one of the best known of 9-10 proteins that make up complex III of the mitochondrial oxidative phosphorylations system (Hatefi, 1985), and it is the only one of them encoded by the mitochondrial genome. Complex III transfers electrons from dihydroubiquinone to cytochrome *c*, and this reaction is coupled to

translocation of protons across the mitochondrial inner membrane (Hatefi, 1985). Cytochrome *b* is believed to contain both redox centers, Qo and Qi, involved in electron transfer (Hatefi, 1985; Howell and Gilbert, 1988). Mutational and evolutionary studies have facilitated the development of a model structure of cytochrome *b* as well as the definition of the sites of electron transfer and inhibitor action (Howell and Gilbert, 1988). Our knowledge of structure-function relationships in this protein enhances the utility of its gene for evolutionary investigations.

The genus *Oryzias* is distributed from India to the Far East. Eleven species have been identified (Uwa and Magtoon, 1986; Yamamoto, 1975). The phylogenetic relationships among eight species have been studied by karyological (Magtoon and Uwa, 1985) and biochemical analysis (Sakaizumi, 1985b). From the results of these studies, the genus *Oryzias* has been divided into three groups which are based on karyological characteristics. The first group is the monoarmed group, which includes *O. melastigma* and *O. javanicus*. The second group is the biarmed group, which includes *O. latipes*, *O. curvinotus*, *O. luzonensis*, and *O. mekongensis*. The last group is the fused chromosome group. *O. celebensis* and *O. minutillus* are members of this group (Magtoon and Uwa, 1985). Biochemical analysis of enzyme polymorphisms within the genus *Oryzias* has provided support for these groupings (Sakaizumi, 1985b).

A dendrogram to show karyotypic relationships in the biarmed chromosome group have demonstrated by Uwa (1991). This result suggests that *O. mekongensis* diverge first from common ancestor of the biarmed chromosome group of the *Oryzias*. Although, we can not obtain a length variation from amplification of highly conserved block

in the biarmed chromosome group of *Oryzias* species, we can detect a length variation of the "hyper-variable block" in the control region. The length of *O. mekongensis* is very short, and sequence analysis shows the species has no tandem repeat sequences (Part III). Moreover, a highly repetitive interspersed sequence analysis using OLR1 suggests that the nucleotide sequences and/or the number of copies of OLR1-related sequences in the genome of *O. mekongensis* are different from those in the other three species (Naruse *et al.*, 1992).

In the biarmed chromosome group, intraspecific diversity of *O. latipes* has been well studying. During the course of a survey of electrophoretic mobilities of proteins from this species, it has been found that four genetically distinct populations exist; the Northern Population, the Southern Population, East Korean Population, and Chine-West Korean Population. These four groups can be distinguished by means of many unique alleles and Nei's coefficients of genetic distance are no less than 0.30 (Sakaizumi, 1986a; Sakaizumi and Joen, 1987; Sakaizumi *et al.*, 1983). In order to investigate the origin of the Japanese wild populations of medaka, especially that of the Northern Population, the genetic relationship between fish in Japan and those in the Korea peninsula and China have been studied (Sakaizumi, 1986a). A UPGMA dendrogram based on Nei's *D* among 21 populations of *O. latipes* from Japan and China shows closely relationship between the Chinese Population and the Southern Population. No other data show relationships among four populations of *O. latipes*.

We have surveyed intraspecific divergence of mtDNAs in medaka using restriction fragment length polymorphisms (Part I and Part II). These results indicate that large intraspecific diversities are exist in medaka. However, these results also suggest limitation of this method.

These results have been obtained in studies that employed techniques which do not permit the detection of restriction fragments under 0.5 kbp.

In this study, we hence obtained nucleotide sequences of the mitochondrial cytochrome *b* gene in order to investigate the origin of the Japanese two populations of medaka. Dendrogram based on the nucleotide sequences of this gene suggest close relationship between *O. curvinotus* and *O. luzonensis*, monophyletic relation of *O. latipes*, and monophyletic relationship of Japanese two populations.

MATERIALS AND METHODS

Template preparation for PCR

We used seven specimens of four species in *Oryzias*; *O. mekongensis*, *O. curvinotus*, *O. luzonensis*, and *O. latipes*. In *O. latipes*, we used four specimens representing four populations. A specimen from the HNI strain is representative of the Northern Population, the Hd-rR strain is representative of the Southern Population, the wild stocks of Tosong represent the East Korea Population, and those of Kunming represent the China-West Korea Population.

Head, intestine and fins were removed and discarded from adult medaka. The tissue was placed in 500 μl of 100 mM EDTA, 50 mM Tris (pH 8.0), 100 mM NaCl, 1% SDS, and 100 μg/ml of Proteinase K. The tissue was minced in a 1.5 ml tube and incubated at 55°C for over night. The homogenate was extracted twice with buffer-equilibrated phenol, once with a 1:1 mixture of phenol:chloroform, and once with chloroform. DNA was precipitated with isopropyl alcohol, rinsed with

ethanol, and resolved in TE buffer (1 mM EDTA, 10 mM Tris pH 8.0).

Polymerase Chain Reaction (PCR)

Total cytochrome *b* gene was amplified using two primers: Cyt-b, Fa (5'-AGG ACC TGT GGC TTG AAA AAC CAC-3') and Cyt-b, RVa (5'-T(TC)C GAC (TC)(TC)C CG(AG) (TA)TT ACA AGA CCG-3'). Double-stranded PCR amplifications were carried out in 25 μl reactions containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.25 μmol each of the heavy- and light-strand primers, and 1.25 unit of *Taq* DNA polymerase (TOYOBO). To this mixture was added 1 μl of the template DNA (<0.1 μg total cellular DNA). The DNA was amplified in a Program Temp Control System PC-800 (ASTEC) on the following step cycle profile: strand denaturation at 94°C for 1.5 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 2 min, repeated for 30 cycles. Preliminary denaturation at 94°C for 2 min before the first cycle.

The double-stranded amplification products formed were separated by gel electrophoresis on a 1% agarose. The fragments amplified were visualized under long wave UV light and excised from the gel. DNA was purified from excised agarose bands using Ultrafree C3HV cartridge (MILLIPORE), phenol extraction, and ethanol precipitation. The PCR product purified was resuspended in TE buffer.

Cloning of PCR product

Fifty micro grams of PCR product was directly ligated with 50 ng pT7Blue(R) T-vector (Novagen). The ligation was performed using 5X ligation buffer (0.25 M Tris pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% polyethylene glycol-8000) and 10 unit T4 DNA ligase

(BRL) at 16°C over night. *E. coli* JM109 Competent Cells (TAKARA) were used for transformation. The recombinant clones were purified by the modified alkaline lysis method (Birnboim and Doly, 1979).

Sequencing

Sequence data were obtained by BcaBEST™ Dideoxy Sequencing Kit (TAKARA). Hydrolink™ Long Ranger (AT Biochem) 6% gel containing 7 M urea was used as a sequence gel. In all cases, M13 Primer M4 and M13 Primer RV (TAKARA) were used as sequence primer. Furthermore, we designed seven primers for sequencing of the cytochrome *b* gene; Cyt-b, Fb (5'-CAA ATA TCA TTT TGA GGG GCC ACT GT), Cyt-b, Fc (5'-CGA CAA AGT ATC CTT CCA CCC TTA CTT), Cyt-b, Fd (5'-CCC TAT TCT ACA CAC CTC TAA ACA ACG), Cyt-b, Fe (5'-CTC GTC AGT TGC ACA CAT CTG CCG), Cyt-b, RVb (5'-ACT GAA AAT CCC CCT CAA ATT CAT TG), Cyt-b, RVc (5'-CCT CCA AGT TTG TTT GGA ATT GAT CGT AG), and Cyt-b, RVd (5'-GCA TGT ATA TTC CGG ATT AGT CAG CCG TA).

Phylogenetic analysis

Relationships among the nucleotide sequences of the mitochondrial cytochrome *b* gene were inferred using maximum parsimony, maximum likelihood, UPGMA, and neighbor-joining method. Phylogenetic analysis was performed using programs in Version 3.5c of PHYLIP (Felsenstein, 1993). SEQBOOT was used to generate 100 bootstrapped data sets that were resampled versions of the input data set. Parsimony trees were constructed from bootstrapped data sets using DNAPARS program, then a consensus tree was printed out using CONSENSE program.

DNADIST program used to compute matrixes of pairwise distances using Kimura's two-parameter model (Kimura 1980) for nucleotide changes, in which the transition / transversion ratio was set at 2.0. NEIGHBOR program used the pairwise distance matrixes to construct UPGMA and neighbor-joining trees.

The program of fastDNAml were used to reconstruct Maximum likelihood dendrogram. The transition / transversion ratio was set at 2.0.

RESULTS & DISCUSSION

Nucleotide sequences of cytochrome b gene

The complete mitochondrial cytochrome *b* sequences of seven individuals were amplified via PCR method. A single DNA fragment of approximately 1500 bp resulted from Cyt-b, Fa-RVa primer set, with no apparent differences in size being evident among species. The PCR products were cloned and sequenced using T-vectors. The DNA sequence of the all individual was determined from two independent clones, because cloned material amplified via PCR typically contains mutations (Saiki *et al.*, 1988).

We obtained seven mitochondrial cytochrome *b* sequences from seven specimens. The alignment of this gene was easy, because no insertions or deletions were observed in the nucleotide sequences (Fig. 12).

Phylogenetic analysis

We inferred phylogenetic relationships among fishes of the biarmed chromosome group using complete nucleotide sequence of mitochondrial cytochrome *b* gene 1146 bp. Figure 13 shows

phylogenetic tree based on these nucleotide sequences seven *Oryzias* specimens from the present study, using neighbor-joining (NJ), UPGMA, maximum likelihood (ML), and maximum parsimony (MP) method.

The NJ tree (Fig. 13*A*) shows that the cytochrome *b* sequences in *Oryzias* are divided into three groups: (1) *O. mekongensis*, (2) *O. curvinotus* and *O. luzonensis*, and (3) *O. latipes*.

Figure 13*B* shows dendrogram on the *Oryzias* cytochrome *b* gene, using UPGMA method. The UPGMA analysis indicates that a root of *Oryzias* mitochondrial cytochrome *b* gene lies between *O. mekongensis* and other species. Tree topology is mainly consistent with those of NJ tree. However, relationships in four populations of *O. latipes* are different from that of NJ tree.

A dendrogram of ML methods is perfectly coincide with that of NJ method (Fig. 13*C*).

Parsimony analysis of the *Oryzias* cytochrome *b* genes shows bootstrap values more than 70%, expect for one position (Fig. 13*D*). The tree topology of MP tree is concordant with that of UPGMA tree. The bootstrap supports of forming three groups in the biarmed chromosome group were 100% and 73.5%, and were highly reliable. Furthermore, the bootstrap supports of a monophyly of the Japanese two populations (97.0%). Relationships among the East Korean Population, the China-West Korean Population, and the Japanese two populations were not clear (the bootstrap supports 31.8%).

Perspective on relationships in the biarmed chromosome group

A dendrogram based on the mitochondrial cytochrome *b* gene using UPGMA analysis suggest that *O. mekongensis* diverge first from

a common ancestor of the biarmed chromosome group of *Oryzias*. This suggestion is also supported by karyological analysis (Uwa, 1991), southern analysis of OLR1(Naruse *et al.*, 1992), and tandem repeat sequence of mitochondrial control region (Part III).

Moreover, four dendrogram in this study indicate closely relationship between *O. curvinotus* and *O. luzonensis*. These results agree with a dendrogram to show karyotypic relationships described in Uwa (1991).

Origin of Japanese wild population of medaka

In this study, four dendrograms indicate that Japanese two populations of medaka are a monophyletic group. In addition, a monophyletic Japanese population's clade was supported with a bootstrap value of 97%.

We can assume that a common ancestor of *O. latipes* had divided into three groups; the China-West Korean Population, the East Korean Population, and a common ancestor of Japanese two populations. Then the common ancestor of Japanese two populations had separated into two populations; the Northern Population and the Southern Population.

```
                    10          20          30          40          50
O. mekongensis ATGGCAAATC TTCGGAAAAC CCACCCCCTT CTAAAAATCG CTAATGATGC
O. curvinotus  ATGGCCAATC TTCGAAAAAC CCATCCCCTA TTAAAAATCG CAAACGATGC
O. luzonensis  ATGGCCAACC TTCGAAAAAC TCATCCCCTA TTAAAAATCG CAAACGATGC
Northern P.    ATGGCTAACC TTCGAAAAAC CCACCCCCTA TTAAAAATTG CAAACGATGC
Southern P.    ATGGCCAACC TTCGAAAAAC CCACCCCCTG TTAAAAATTG CAAACGATGC
Western P.     ATGGCCAACC TTCGAAAAAC CCACCCTCTA TTAAAAATCG CAAACGATGC
Eastern P.     ATGGCCAACC TTCGAAAAAC CCACCCCCTT TTAAAAATCG CAAACGATGC

                    60          70          80          90          100
O. mekongensis CTTAGTAGAC CTACCAGCCC CATCAAACAT CTCCGTCTGA TGAAACTTTG
O. curvinotus  CCTCATTGAT CTCCCAGCCC CGTCGAACAT TTCAGTTTGA TGAAACTTCG
O. luzonensis  CCTAGTTGAT CTTCCCGCCC CCTCAAACAT TTCAGTTTGA TGGAACTTCG
Northern P.    CCTAGTTGAC CTCCCAGCCC CTTCAAACAT CTCAGTTTGA TGAAACTTTG
Southern P.    TCTAGTTGAC CTTCCAGCCC CTTCGAACAT TTCAGTTTGA TGAAACTTTG
Western P.     CCTAGTAGAC CTTCCAGCCC CCTCAAACAT TTCAGTTTGA TGAAACTTTG
Eastern P.     CCTAGTCGAC CTCCCGGCCC CATCAAACAT TTCAGTTTGA TGAAACTTTG

                    110         120         130         140         150
O. mekongensis GGTCCTTGCT AGGGCTCTGT CTAGCTGCAC AAATCGTTAC TGGCTTATTC
O. curvinotus  GATCTCTTCT AGGACTTTGT TTGGCCGCCC AGATCGTTAC GGGCCTTTTC
O. luzonensis  GCTCCCTTCT AGGGCTTTGT TTAGCCGCCC AAATCGTGAC GGGCCTTTTC
Northern P.    GTTCTCTTCT TGGGCTCTGT TTGGCCGCCC AAATCGTCAC AGGCCTATTT
Southern P.    GTTCTCTTCT CGGACTTTGT TTAGCCGCCC AAATCATCAC GGGCCTTTTT
Western P.     GGTCACTCCT TGGGCTCTGT CTGGCCGCCC AAATCATTAC CGGCCTTTTT
Eastern P.     GGTCTCTTCT TGGGCTTTGT CTGGCCGCCC AAATCATTAC CGGCCTTTTT

                    160         170         180         190         200
O. mekongensis CTTGCAATAC ATTATACCTC AGACATTGCA ACAGCATTCT CATCAGTTGC
O. curvinotus  CTTGCAATAC ATTATACATC AGGCATTGCC ACAGCATTTT CATCTGTCGC
O. luzonensis  CTTGCAATAC ACTATACCTC AGACATTGCC ACAGCCTTTT CATCTGTTGC
Northern P.    CTTGCCATGC ATTATACATC TGATATTGCC ACAGCATTCT CATCAGTTGC
Southern P.    CTTGCCATAC ATTATACATC CGACATCGCC ACAGCATTCT CATCAGTTGC
Western P.     CTTGCCATAC ATTATACATC TGACATCGCC ACAGCCTTTT CATCAGTTGC
Eastern P.     CTTGCCATGC ATTATACGTC TGACATCGCC ACAGCATTTT CATCTGTTGC

                    210         220         230         240         250
O. mekongensis CCACATCTGC CGAGACGTGA ACTACGGCTG ACTTATTCGA AACATGCATG
O. curvinotus  ACATATTTGA CGTGATGTCA ACTACGGCTG ACTAATCCGA AACATGCATG
O. luzonensis  ACACATTTGT CGTGACGTCA ATTACGGCTG ACTAATCCGC AACATACATG
Northern P.    ACACATCTGC CGGGATGTTA ACTACGGCTG ACTAATCCGT AATATGCATG
Southern P.    ACACATCTGC CGGGATGTTA ACTACGGCTG ACTAATCCGG AATATACATG
Western P.     ACACATCTGC CGAGATGTTA ACTACGGCTG ATTAATCCGA AATATGCACG
Eastern P.     ACATATTTGC CGGGATGTTA ACTACGGCTG ACTAATCCGT AATATACACG

                    260         270         280         290         300
O. mekongensis CCAACGGAGC CTCTTTCTTC TTCATCTGCA TCTACCTTCA TATTGGTCGG
O. curvinotus  CAAACGGTGC TTCCTTTTTC TTCATTTGCA TCTACCTGCA CATCGGACGG
O. luzonensis  CAAACGGTGC TTCTTTTTTC TTCATCTGCA TCTACCTGCA CATCGGGCGG
Northern P.    CAAACGGCGC TTCTTTTTTC TTCATCTGTA TCTACTTGCA CATTGGTCGA
Southern P.    CAAACGGCGC TTCTTTTTTC TTCATCTGCA TTTACCTTCA CATTGGGCGA
Western P.     CAAACGGTGC TTCTTTTTTC TTCATCTGTA TCTACTTACA CATCGGACGG
Eastern P.     CAAACGGTGC TTCTTTTTTC TTCATCTGTA TTTATCTGCA TATCGGACGA
```

Fig. 12 Sequence of *Oryzias* mitochondrial cytochrome *b* genes. The DNA sequences of *O. mekongensis*, *O. curvinotus*, *O. luzonensis*, and four population of medaka: Northern P.; the Northern Population. Southern P.; the Southern Population, Western P.; the China-West Korean Population, and Eastern P.; the East Korean Population.

	310	320	330	340	350
O. mekongensis	GGCCTATATT	ACGGCTCATA	CCTTTATAAA	GAGACCTGAA	ACGTGGGTGT
O. curvinotus	GGCCTGTATT	ATGGATCATA	CTTATATAAA	GAGACATGAA	ATGTAGGTGA
O. luzonensis	GGCTTGTACT	ACGGTGCCTA	CTTGTATAAA	GAGACATGGA	ACGTGGGTGT
Northern P.	GGCTTATACT	ATGGTTCCTA	CTTATACAAG	GAAACATGAA	ACGTTGGTGT
Southern P.	GGCTTGTACT	ACGGATCCTA	CTTATACAAG	GAAACATGAA	ATGTCGGTGT
Western P.	GGGCTCTATT	ATGGGTCCTA	TTTGTACAAG	GAAACATGAA	ACGTTGGTGT
Eastern P.	GGGTTATATT	ATGGATCCTA	TTTGTATAAA	GAAACATGAA	ACGTTGGCGT

	360	370	380	390	400
O. mekongensis	GATCCTCCTT	CTCCTTGTAA	TAATAACCGC	CTTCGTAGGG	TACGTACTGC
O. curvinotus	TGTCCTTCTC	CTCCTAGTGA	TAATGACTGC	TTTCGTAGGC	TACGTCCTAC
O. luzonensis	CGCCCTTCTT	CTACTTGTAA	TGATGACTGC	TTTCGTAGGC	TACGTCCTGC
Northern P.	TATTCTTCTT	CTGCTTGTAA	TAATAACAGC	TTTCGTAGGC	TATGTCCTAC
Southern P.	AATTCTTCTT	CTACTAGTAA	TAATAACGGC	TTTCGTAGGT	TACGTTTTAC
Western P.	GATCCTCCTC	CTACTTGTTA	TAATAACAGC	CTTCGTGGGA	TATGTCCTTC
Eastern P.	AATTCTTCTT	CTACTCGTTA	TAATGACAGC	TTTCGTGGGC	TATGTATTAC

	410	420	430	440	450
O. mekongensis	CCTGAGGACA	AATATCATTT	TGAGGGGCCA	CCGTAATTAC	TAACCTCCTA
O. curvinotus	CCTGAGGACA	AATGTCATTC	TGAGGGGCTA	CCGTCATTAC	CAACCTTTTA
O. luzonensis	CTTGAGGTCA	AATATCGTTC	TGAGGTGCCA	CCGTTATTAC	CAACCTTTTG
Northern P.	CCTGAGGACA	AATATCATTC	TGGGGGGCCA	CTGTAATTAC	CAACCTACTG
Southern P.	CCTGAGGACA	AATATCATTT	TGAGGAGCCA	CTGTAATCAC	CAACCTCTTG
Western P.	CCTGAGGACA	AATATCATTC	TGAGGGGCTA	CTGTAATTAC	TAATCTACTA
Eastern P.	CCTGAGGACA	GATGTCATTC	TGGGGGGCCA	CTGTAATTAC	CAACCTCCTT

	460	470	480	490	500
O. mekongensis	TCCGCCATTC	CTTATGTTGG	TAATGCTTTA	GTACAATGAA	TTTGAGGCGG
O. curvinotus	TCAGCCATCC	CCTACATTGG	AAACGCCCTA	GTTCAATGGA	TCTGAGGCGG
O. luzonensis	TCCGCTATTC	CCTACATTGG	AAACGCCCTA	GTCCAGTGGA	TTTGGGGCGG
Northern P.	TCTGCTGTCC	CTTACATTGG	CAACGCCCTC	GTCCAATGGA	TTTGAGGGGG
Southern P.	TCTGCCGTCC	CTTACGTTGG	CAACGCCTCC	GTCCAATGAA	TTTGAGGAGG
Western P.	TCTGCCGTCC	CCTACATTGG	CAACGCCTTC	GTCCAATGAA	TTTGAGGGGG
Eastern P.	TCTGCCGTTC	CTTATGTTGG	CAACGCCTTA	GTCCAATGAA	TTTGGGGTGG

	510	520	530	540	550
O. mekongensis	CTTTTCAGTA	GACAATGCAA	CCCTAACTCG	ATTCTTTGCA	TTCCACTTCC
O. curvinotus	ATTTTCAGTA	GACAACGCCA	CCCTTACCCG	GTTCTTTGCC	TTCCATTTCC
O. luzonensis	CTTTTCAGTA	GATAATGCCA	CACTTACCCG	ATTTTTTGCC	TTCCATTTCC
Northern P.	ATTTTCAGTA	GATAATGCCA	CACTCACCCG	GTTCTTTGCT	TTCCACTTCC
Southern P.	ATTTTCAGTA	GATAACGCCA	CACTTACCCG	ATTCTTTGCC	TTCCATTTCC
Western P.	TTTCTCAGTA	GACAACGCCA	CACTCACCCG	ATTCTTCGCC	TTCCATTTTC
Eastern P.	CTTCTCAGTA	GATAACGCCA	CACTCACCCG	ATTCTTCGCT	TTCCATTTCC

	560	570	580	590	600
O. mekongensis	TTCTACCATT	TATTATTGCT	GCCGCAACAG	TTGTCCACCT	CATCTTCCTC
O. curvinotus	TCCTCCCCTT	TGTAATTGCT	GCTGCTACAG	TTGTACATCT	TATTTTCCTG
O. luzonensis	TCCTCCCCTT	TGTAATCGCT	GCTGCTACAA	TTGTTCATCT	TATTTTCCTG
Northern P.	TCCTTCCCTT	CGTAATTGCC	GCCGCAACAA	TCGTCCATCT	AATCTTCCTT
Southern P.	TCCTCCCCTT	CGTAATTGCC	GCCGCAACAG	TTGTTCATCT	AATTTTTCTT
Western P.	TTCTCCCATT	CGTAATTGCC	GCCGCAACAG	TCGTTCACCT	AATTTTCCTT
Eastern P.	TCTTTCCCTT	CGTTATTGCC	GCCGCAACAA	TCGTTCATTT	AATTTTCCTT

Fig. 12 Continued

	610	620	630	640	650
O. mekongensis	CATGAAACAG	GATCAAATAA	CCCTACAGGA	CTGAACTCCG	ACTCAGATAA
O. curvinotus	CACGAGACAG	GGTCGAATAA	CCCAACGGGT	CTAAACTCAG	ACTCTGACAA
O. luzonensis	CATGAAACAG	GGTCCAATAA	CCCCACAGGT	CTAAACTCCG	ACTCTGATAA
Northern P.	CACGAAACGG	GATCAAACAA	CCCAACCGGC	CTCAATTCAG	ACTCTGACAA
Southern P.	CACGAAACAG	GTTCAAACAA	CCCAACCGGC	CTCAATTCAG	ACCCCGACAA
Western P.	CACGAAACAG	GGTCCAATAA	CCCAACAGGG	CTCAACTCAG	ACTCTGACAA
Eastern P.	CACGAAACAG	GATCAAACAA	CCCAACTGGT	CTCAACTCAG	ACTCTGACAA

	660	670	680	690	700
O. mekongensis	AATCTCTTTC	CACCCCTATT	TCTCGTACAA	AGATCTCCTG	GGCTTTGCTG
O. curvinotus	AGTGTCTTTC	CACCCCTACT	TTTCTTATAA	AGATCTTCTA	GGCTTTGCTG
O. luzonensis	AGTGTCTTTC	CACCCTTACT	TTTCTTATAA	AGATCTCCTA	GGCTTTGCTG
Northern P.	AGTCTCCTTC	CACCCTTACT	TCTCTTATAA	AGATCTTTTA	GGCTTTGCTG
Southern P.	AGTCTCCTTC	CACCCTTACT	TTTCCTATAA	AGACCTTTTA	GGGTTTGCTG
Western P.	GGTGTCGTTT	CACCCCTATT	TCTCTTACAA	AGACCTCCTA	GGCTTTGCGG
Eastern P.	AGTATCCTTC	CACCCTTACT	TTTCTTACAA	AGATCTCCTG	GGCTTTGCTG

	710	720	730	740	750
O. mekongensis	CCTTGCTAGT	AGCCCTAATC	TCCTTAGCAC	TCTTCTCCCC	AAACTTGCTT
O. curvinotus	CCCTGCTAGT	AGCCCTTATC	TCTTTAGCCC	TCTTCTCCCC	TAATCTACTT
O. luzonensis	CCCTATTAGT	GGCCCTCATC	TCTCTGGCCC	TCTTCTCCCC	CAACCTGCTC
Northern P.	CCTTGCTAGT	AGCCTTAATT	TCTCTAGCAC	TATTCTCCCC	CAACCTGCTT
Southern P.	CCTTGCTAGT	AGCCTTAATT	TCCCTGGCGC	TTTTCTCCCC	CAACCTGCTT
Western P.	CGTTACTGGT	GGCCCTAATC	TCTTTAGCAC	TCTTTTCGCC	AAATCTGCTC
Eastern P.	CCTTGCTAGT	AGCCCTAATC	TCCTTAGCAC	TCTTCTCCCC	AAACTTGCTT

	760	770	780	790	800
O. mekongensis	GGAGACCCCG	ACAACTTCAC	CCCTGCTAAC	CCTTTAGTTA	CCCCTCCCCA
O. curvinotus	GGAGATCCTG	ATAACTTTAC	CCCTGCTAAT	CCTTTAGTAA	CTCCACCTCA
O. luzonensis	GGGGACCCTG	ACAACTTCAC	CCCTGCCAAC	CCTTTAGTGA	CCCCACCTCA
Northern P.	GGAGACCCCG	ATAACTTCAC	CCCTGCTAAC	CCGTTAGTAA	CCCCTCCTCA
Southern P.	GGAGACCCAG	ACAACTTCAC	CCCTGCCAAC	CCGCTAGTTA	CTCCCCCTCA
Western P.	GGAGACCCCG	ACAACTTCAC	CCCTGCCAAC	CCGCTAGTTA	CGCCTCCCCA
Eastern P.	GGAGACCCCG	ACAACTTCAC	CCCTGCTAAC	CCTTTAGTTA	CCCCTCCCCA

	810	820	830	840	850
O. mekongensis	CATCAAACCC	GAATGATATT	TCCTATTTGC	TTACGCCATT	CTACGGTCAA
O. curvinotus	CATCAAACCT	GAGTGATACT	TCCTGTTCCC	TTACGCCATT	CTACGATCCA
O. luzonensis	CATCAAGCCC	GAGTGATATT	TCCTATTTGC	TTACGCCATT	TTACGATCCA
Northern P.	CATTAAACCC	GAATGATATT	TCCTATTTGC	TTACGCTATT	CTACGATCAA
Southern P.	CATCAAGCCT	GAATGATACT	TCCTATTTGC	CTACGCCATT	CTACGATCAA
Western P.	TATTAAGCCC	GAATGATACT	TCTTATTTCG	TTACGCAATT	CTACGCTCAA
Eastern P.	CATCAAACCC	GAATGATATT	TCCTATTTGC	TTACGCCATT	CTACGGTCAA

	860	870	880	890	900
O. mekongensis	TTCCAAACAA	ACTGGGAGGA	GTCCTAGCTT	TACTGGCCTC	CATTCTAGTA
O. curvinotus	TCCCAAACAA	GCTTGGCGGG	GTTCTAGCCC	TATTAGCCTC	TATTCTAGTT
O. luzonensis	TCCCTAACAA	ACTTGGGGGG	GTCTTAGCCC	TGTTGGCCTC	CATTCTTGTT
Northern P.	TTCCAAACAA	ACTTGGAGGC	GTTCTAGCCT	TATTAGCCTC	TATCCTAGTT
Southern P.	TTCCAAATAA	ACTTGGAGGG	GTCCTAGCCC	TATTAGCCTC	TATTCTAGTA
Western P.	TTCGAAATAA	ACTGGGGGGA	GTACTAGCTC	TATTAGCCTC	CATTCTTGTT
Eastern P.	TTCCAAACAA	ACTGGGAGGG	GTCCTGGCTT	TACTGGCCTC	CATTCTAGTA

Fig. 12 Continued

```
                      910         920         930         940         950
O. mekongensis  TTATTCTTAG TTCCCATCCT CCACACCTCT AAACAACGAA GCCTTACATT
O. curvinotus   CTCTTTCTTG TCCCTATCCT GCACACATCA AAACAACGAA GCCTAACATT
O. luzonensis   CTCTTTCTTG TCCCCATCCT GCACACATCG AAGCAACGAA GCCTAACATT
Northern P.     CTATTCCTAG TCCCTATCCT ACACACATCC AAACAACGAG GACTAACATT
Southern P.     CTATTCCTGG TCCCTATCCT ACACACCTCT AAACAACGAA GCCTTACGTT
Western P.      CTATTTCTGG TCCCCATCCT CCACACTTCA AAACAACGAA GTCTTACATT
Eastern P.      TTATTCTTAG TTCCCATCCT CCACACCTCT AAACAACGAA GCCTTACATT

                      960         970         980         990        1000
O. mekongensis  TCGACCCTTC ACCCAATTCC TTTTCTGGCT CCTAGTGGCA GACGTTTTGG
O. curvinotus   CCGACCCCTA ACCCAATTCC TCTTCTGATT GCTAGTCGCC GATGTAATAA
O. luzonensis   CCGACCTTTT ACCCAACTCC TCTTCTGATT ACTAGTGGCC GATGTAATGA
Northern P.     CCGACCTTTC ACCCAATTCC TTTTCTGACT CTTAGTAGCA GACGTAGTGG
Southern P.     TCGACCTTTC ACCCAATTCC TTTTCTGACT CCTAGTAGCA GACGTGATGG
Western P.      CCGACCTTTC ACCCAATTTC TCTTTTGACT CCTCGTAGCA GACGTAATGG
Eastern P.      TCGACCCTTC ACCCAATTCC TTTTTTGGCT TCTAGTGGCA GACGTAATAG

                     1010        1020        1030        1040        1050
O. mekongensis  TCCTTACCTG AATCGGCGGT ATGCCAGTAG AACACCCATT CATCATCATC
O. curvinotus   TTTTAACCTG AATTGGGGGT ATGCCTGTAG AACACCCTTA CATTATCATT
O. luzonensis   TTCTAACCTG AATCGGGGGC ATGCCGGTAG AACACCCGTA TATTATTATT
Northern P.     TTTTAACCTG AATCGGTGGT ATACCTGTAG AACATCCCTT TATTATCATC
Southern P.     TTTTAACCTG AATTGGCGGA ATGCCCGTAG AACACCCATT TATTATCATT
Western P.      TGTTAACCTG AATTGGAGGA ATACCTGTAG AACACCCATT TATTATTATT
Eastern P.      TACTGACCTG AATTGGAGGC ATACCTGTAG AACACCCATT TATTATCATT

                     1060        1070        1080        1090        1100
O. mekongensis  GGCCAAGTTG CCTCCTTCCT GTATTTCATG CTTTTCTTAG TTTTAGCCCC
O. curvinotus   GGACAAGTCG CATCCTTCAT TTATTTTTCC CTTTTTCTAG TCATGGCGCC
O. luzonensis   GGGCAAGTCG CATCCTTAAT TTATTTCTCC CTCTTTCTAG TTTTAGCCCC
Northern P.     GGTCAAGTCG CATCTTTTCT TTATTTCTTC CTCTTTCTTG TTATAACACC
Southern P.     GGTCAAATCG CATCTTTTCT TTATTTTTCC CTCTTTCTTA TTATAGCACC
Western P.      GGTCAAATCG CGTCTTTTAT ATACTTTTCC CTCTTTCTTG TTATAGCACC
Eastern P.      GGTCAAATCG CATCTTTTCT TTATTTCTCT CTCTTTCTTG TAATAGCACC

                     1110        1120        1130        1140        1150
O. mekongensis  AACAGCTGGA TGACTAGAAA ATAAAGTTCT AAAATGACAA TGCATT....
O. curvinotus   TATGGCCGGC CTACTAGAAA ACAAAGTCTT AAAATGACAA TGCATT....
O. luzonensis   CATGGCCGGC GTACTAGAAA ACAAAGTCTT AAAATGGCAA TGCACT....
Northern P.     AGCGGCAGGA TGACTAGAAA ATAAAGTCTT AAAATGACAA TGCACG....
Southern P.     AGCGGCGGGA TGACTAGAAA ATAAAGTCTT AAAATGACAA TGCACG....
Western P.      AGCAGCAGGG TGACTAGAAA ACAAAGTCTT AAAATGACAA TGCATG....
Eastern P.      AACAGCAGGA TGACTAGAAA ATAAAGTCTT AAAATGACAA TGCATG....
```

Fig. 12 Continued

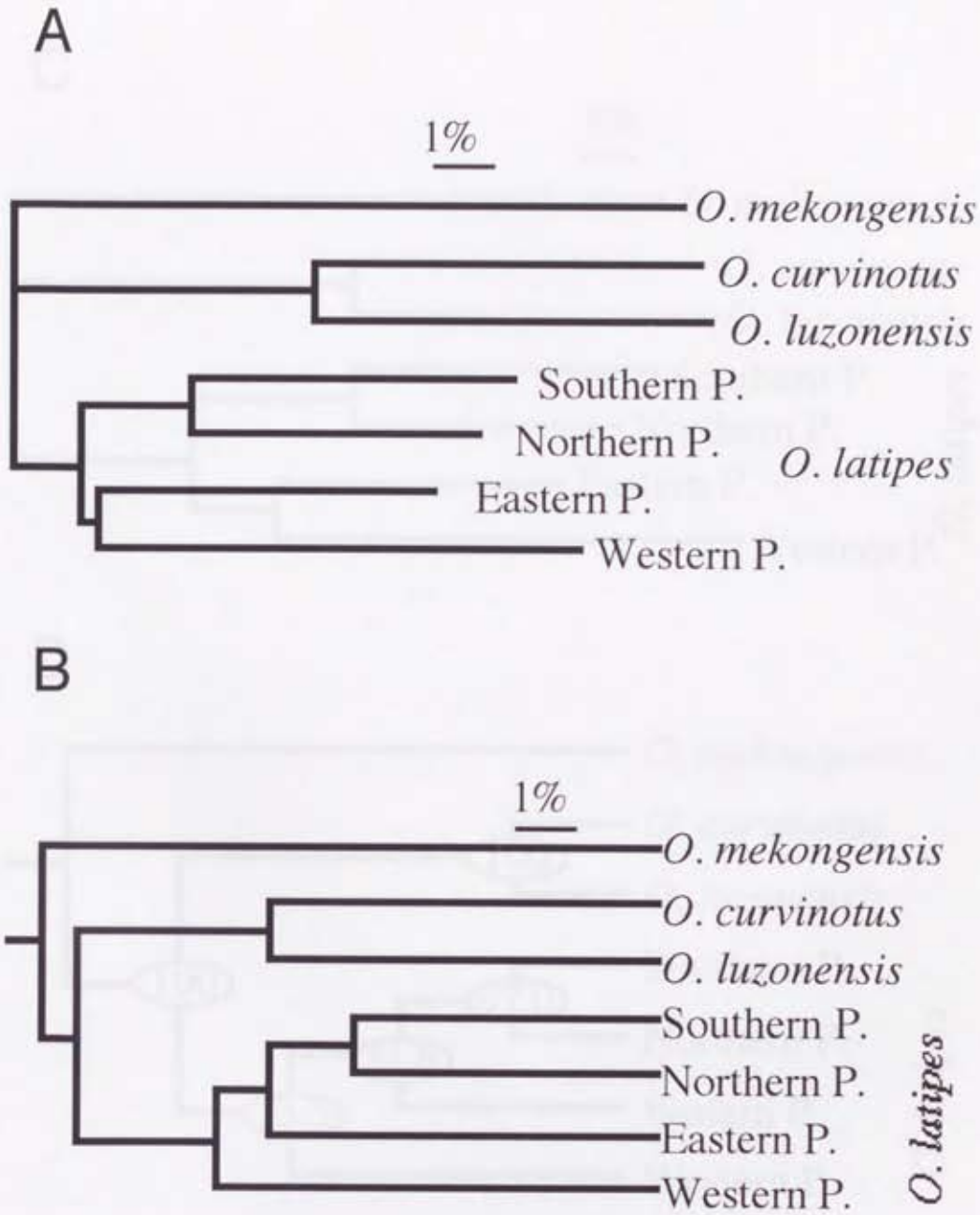

Fig. 13 Phylogenetic trees estimated by NJ (*A*), UPGMA (*B*), ML (*C*), and MP (*D*) methods based on the 1146 nucleotide sequences of the cytochrome *b* gene. In the MP tree, the numbers on the node indicate the bootstrap value.

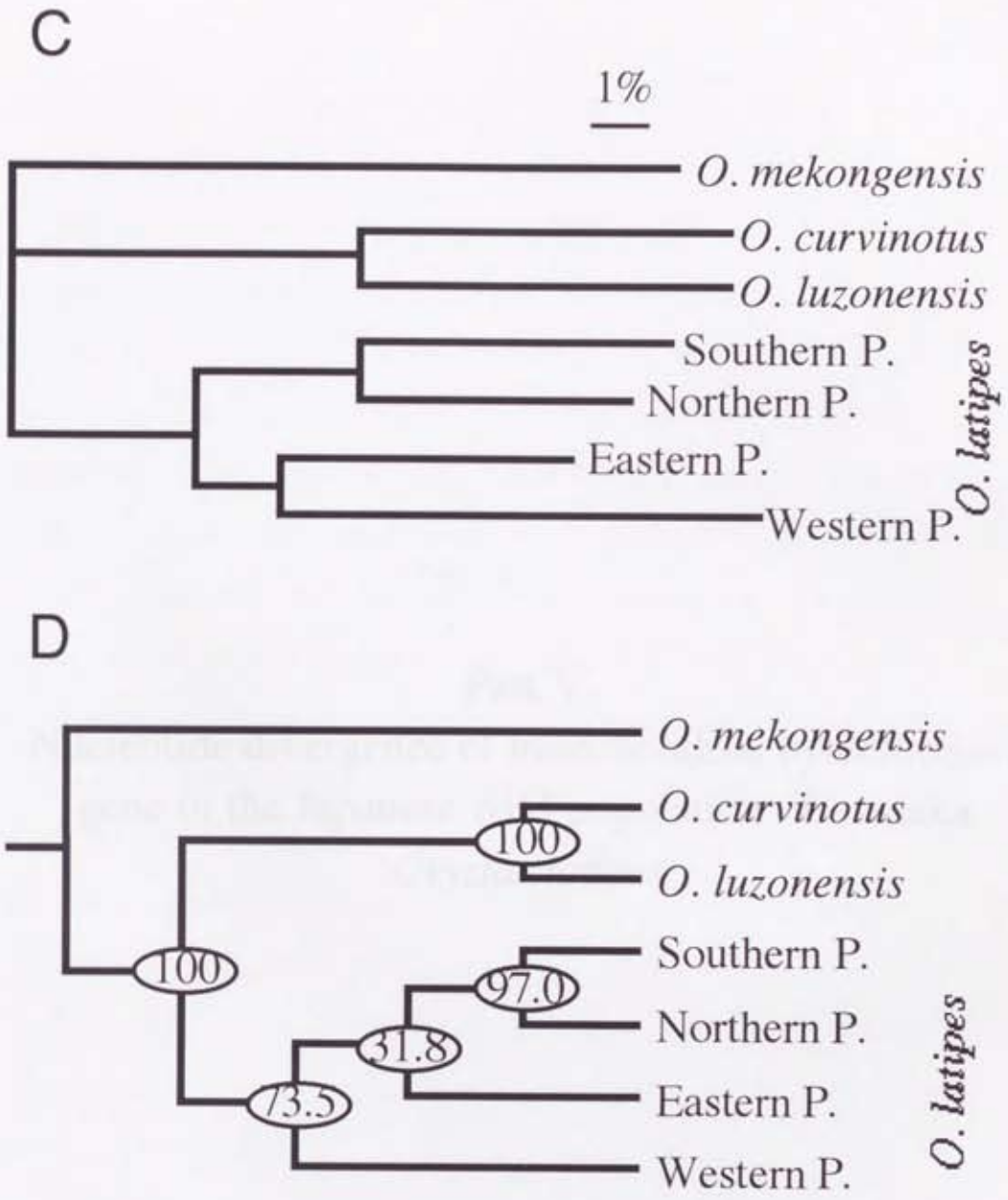

Fig. 13 Continued

Part V.
Nucleotide divergence of mitochondrial cytochrome *b* gene in the Japanese wild population of medaka, *Oryzias latipes*

INTRODUCTION

Mitochondrial DNA (mtDNA) is a valuable molecule for understanding the evolutionary relationships among individuals, populations, and species. Until recently such understanding was achieved mainly by restriction analysis because that technique is simpler than conventional cloning and sequencing (Harrison, 1989; Wilson *et al.*, 1985). With the advent of the polymerase chain reaction (PCR) (White *et al.*, 1989), it became possible to obtain mtDNA sequences directly from many taxa; these sequences can be aligned across greater time spans than can restriction maps (Kocher *et al.*, 1989). However, short sequences amplified in this manner could not resolve ancient evolutionary relationships (Kocher *et al.*, 1989).

The cytochrome *b* gene was chosen as a phylogenetic probe because it may be easier to align a protein-coding sequence that has evolved over the period spanning the origin of mammalian orders than to align either mitochondrial rDNA or noncoding sequences from distant relatives. This particular gene, together with a mitochondrial rDNA, has been found valuable for addressing even deeper phylogenetic questions, such as the origin of tetrapods (Meyer and Wilson, 1990).

Geographic variation in biochemical characters of medaka has been demonstrated in allozymes (Sakaizumi *et al.*, 1980; Sakaizumi *et al.*, 1983). These results indicate four genetically distinct populations; the Northern Population from the northern coast of the Sea of Japan, the Southern Population from western and southern Japan, the West Korean Population from western and southern Korea, and the China-West Korean Population from China and western Korea. These four groups can be distinguished by means of many unique alleles and Nei's

coefficients of genetic distance are no less than 0.30 (Sakaizumi, 1986a; Sakaizumi and Joen, 1987; Sakaizumi *et al.*, 1983).The genetic diversities estimated among the four populations are large enough to be considered as characteristic of interspecific comparisons. However, male and female progeny from crosses between two populations are fully fertile (Sakaizumi, 1986a; Sakaizumi *et al.*, 1992).

Intraspecific variation of the Japanese wild population of medaka is also demonstrated using mtDNA restriction fragment length polymorphisms (RFLPs). The analysis revealed a large number of mtDNA haplotypes that form three distinct clusters (clusters A, B and C). Furthermore, cluster A consists of seven haplotypes that is subdivided into two subclusters, and cluster B has 55 haplotypes that is subdivided into 11 subclusters. Cluster C consists of only one haplotype that found in two localities of the Kanto district. The geographic distributions of mtDNA haplotypes in clusters A and B appear fully concordant with the previously described ranges of the Northern Population and the Southern Population defined by allozymes. Moreover, distributions of mtDNA haplotypes in subclusters show strong geographical associations (Part I).

The objectives of the present study were to compare nucleotide sequences of the mitochondrial cytochrome *b* gene in the Northern Population with those in the Southern Population, to elucidate relationships in the Southern Population, and, more generally, to evaluate empirically the utility of the cytochrome *b* gene sequence in population studies of *O. latipes*.

MATERIALS AND METHODS

DNA sources

Total genomic DNA from 16 specimens of the Japanese wild population of medaka , Momoishi (haplotype #7, subcluster A-1 in Part I), Kanazawa (#3, subcluster A-2), Toyooka (#6, subcluster A-2), Sakurae (#8, subcluster B-1), Hirao (#39, subcluster B-2), Tosa (#32, subcluster B-3), Saori (#20, subcluster B-4), Buzen (#25, subcluster B-5), Nagato (#29, subcluster B-5), Kanoya (#10, subcluster B-6), Himeji (#55, subcluster B-8), Nagano (#15, subcluster B-9), Sendai (#16, subcluster B-9), Sasebo (#22, subcluster B-11), Kazusa (#60, subcluster B-11), Mooka (#46, cluster C), were from a previous study (Part I). The DNA sequences for cytochrome *b* were from Par IV for the HNI strain (haplotype #1, subcluster A-1, in Part I). The HNI strain had been established from wild population of Niigata (Hyodo-Taguchi and Egami, 1985)

Cytochrome b sequence

Mitochondrial sequences containing the cytochrome *b* gene were isolated via PCR. Primers for amplification and sequencing are follows, Cyt-b, Fa (5'-AGG ACC TGT GGC TTG AAA AAC CAC-3'), Cyt-b, Fb (5'-CAA ATA TCA TTT TGA GGG GCC ACT GT), Cyt-b, Fc (5'-CGA CAA AGT ATC CTT CCA CCC TTA CTT), Cyt-b, Fd (5'-CCC TAT TCT ACA CAC CTC TAA ACA ACG), Cyt-b, RVa (5'-T(TC)C GAC (TC)(TC)C CG(AG) (TA)TT ACA AGA CCG-3'), Cyt-b, RVb (5'-ACT GAA AAT CCC CCT CAA ATT CAT TG), and Cyt-b, RVc (5'-CCT CCA AGT TTG TTT GGA ATT GAT CGT AG). Typically the entire cytochrome *b* gene was amplified with the flanking tRNA primers Cyt-b, Fa and Cyt-b, RVa. Condition for amplification was as described in Part IV. Double-stranded DNA was purified by using Ultrafree C3LGC cartridge (MILLIPORE). Nucleotide sequences were obtained by using cycle sequence

Data analysis

Relationships among the nucleotide sequences of the mitochondrial cytochrome *b* gene were inferred using maximum parsimony, maximum likelihood, UPGMA, and neighbor-joining method. Phylogenetic analysis was performed using programs in Version 3.5c of PHYLIP (Felsenstein, 1993). SEQBOOT was used to generate 100 bootstrapped data sets that were resampled versions of the input data set. Parsimony trees were constructed from bootstrapped data sets using DNAPARS program, then a consensus tree was printed out using CONSENSE program.

DNADIST program used to compute matrixes of pairwise distances using Kimura's two-parameter model (Kimura 1980) for nucleotide changes, in which the transition / transversion ratio was set at 2.0. NEIGHBOR program used the pairwise distance matrixes to construct UPGMA and neighbor-joining trees.

The fastDNAml were used to reconstruct Maximum likelihood dendrogram. The transition / transversion ratio was set at 2.0.

RESULTS & DISCUSSION

DNA sources

Sample choice for sequencing was based on mtDNA RFLPs analysis of Japanese wild populations. We picked up a haplotype that represents a subcluster, and which shared more localities than other haplotypes in the subcluster. We except subcluster B-2 and B-7, that are found in a few localities.

Nucleotide sequences of cytochrome b gene

The mitochondrial sequences containing the cytochrome *b* gene of 17 individuals were amplified via PCR method. A single DNA fragment of approximately 1500 bp resulted from Cyt-b, Fa-RVa primer set, with no apparent differences in size being evident among species. The PCR products were sequenced directly, using six primers.

We obtained 16 partial mitochondrial cytochrome *b* sequences and complete cytochrome *b* gene sequence of the HNI strain (Part IV). The alignment of this gene was easy, because no insertions or deletions were observed in the nucleotide sequences (Fig. 14).

Phylogenetic analysis

We inferred phylogenetic relationships in the 17 medaka using 505 nucleotide sequence of the mitochondrial cytochrome *b* gene. Figure 15 shows phylogenetic tree based on these nucleotide sequences of 17 medaka from the present study, using neighbor-joining (NJ), UPGMA, maximum likelihood (ML), and maximum parsimony (MP) method.

The NJ tree (Fig. 15*A*) shows that the medaka cytochrome *b* sequences are divided into three clusters, clusters A, B and C. In cluster B, we found that subcluster B-11 has large divergence form other members of cluster B. This topology was similar to that of UPGMA and ML method. We noticed long blanch length of subcluster B-11 in NJ and ML dendrogram (Fig. 15*B* and 15*C*).

Parsimony analysis of the medaka cytochrome *b* genes shows seven bootstrap values more than 70% (Fig. 15*D*). A monophyletic clade of clusters A and B was supported with a bootstrap value of 100% and of 96.0%, respectively. A monophyletic clade of subclusters A-1, B-5 and B-11 was also supported with a bootstrap value of 100%, 70.7%, and 98.9%, respectively. A sister group relationship between

subclusters B-4 and B-9 was supported with a bootstrap value of 97.8%. In cluster B, a sister group relationship among eight subclusters except for subcluster B-11 was supported with a bootstrap value of 73.6%. In cluster A, we found high bootstrap values. A sister group relationship between the cytochrome *b* sequence of subcluster A-1 and that of Kanazawa was supported with a bootstrap value of 88.2%.

Perspective on relationships in the Southern Population

RFLPs analysis of mtDNA in wild population of medaka reveled three clusters. The geographic distributions of mtDNA haplotypes in clusters A and B appear fully concordant with the previously described ranges of the Northern Population and the Southern Population defined by allozymes.

RFLPs analysis revealed 11 subclusters in cluster B. These relationships, however, have been unclear. Analyses using partial cytochrome *b* gene sequence indicate some relationships among cluster B's subclusters. Four dendrograms in this study indicate that subcluster B-11 is first divided from common ancestor of cluster B. Furthermore, a sister group relationship among subclusters B-2, B-6, and B-8 is found in all four dendrograms, whereas its bootstrap support is low (43.5%) in bootstrapping MP tree. Moreover, a sister group relationship between subclusters B-4 and B-9 is supported by a bootstrap value 97.8% in MP tree. Although ML dendrogram support this sister grouping, dendrograms using distance methods are not support this sister group. We suppose that longer blanch length of Saori (#20 subcluster B-4) than that of other haplotypes of the cluster B (excepted for the subcluster B-11) leads to the contradiction between distance methods and other methods.

```
                        10         20         30         40         50
A-1, #1 (HNI)       GACAAAGTCT CCTTCCACCC TTACTTCTCT TATAAAGATC TTTTAGGCTT
A-1, #7 (Momoishi)  GACAAAGTCT CCTTCCACCC TTACTTCTCT TATAAAGATC TTTTAGGCTT
A-2, #3 (Kanazawa)  GACAAAGTCT CTTTTCACCC TTACTTCTCT TATAAAGATC TTTTAGGCTT
A-2, #6 (Toyooka)   GACAAAGTTT CTTTCCATCC TTACTTATCT TATAAAGATC TTTTAGGCTT
B-1, #8 (Sakurae)   GACAAAGTCT CCTTCCACCC TTACTTCTCC TATAAAGACC TTTTAGGATT
B-2, #39 (Hirao)    GACAAAGTAT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-3, #32 (Tosa)     GACAAAGTCT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-4, #20 (Saori)    GACAAAGTCT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-5, #25 (Buzen)    GACAAAGTCT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-5, #29 (Nagato)   GACAAAGTCT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-6, #10 (Kanoya)   GACAAAGTAT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-8, #55 (Himeji)   GACAAAGTAT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-9, #15 (Nagano)   GACAAAGTCT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-9, #16 (Sendai)   GACAAAGTCT CCTTCCACCC TTACTTTTCC TATAAAGACC TTTTAGGGTT
B-11, #22 (Sasebo)  GAGAAAGTCT CCTTCCACCC CTACTTTTCC TATAAAGACC TTTTAGGATT
B-11, #60 (Kazusa)  GACAAAGTCT CCTTCCACCC CTACTTTTCC TATAAAGACC TTTTAGGGTT
C, #46 (Mooka)      GATAAAGTCT CTTTCCACCC TTACTTTTCT TATAAAGACC TTTTAGGCTT

                        60         70         80         90        100
A-1, #1(HNI)        TGCTGCCTTG CTAGTAGCCT TAATTTCTCT AGCACTATTC TCCCCCAACC
A-1, #7 (Momoishi)  TGCTGCCTTG CTAGTAGCCT TAATTTCTCT AGCACTATTC TCCCCCAACC
A-2, #3 (Kanazawa)  TGCTGCCTTG CTAGTAGCCT TGATTTCTCT CGCACTATTC TCCCCCAACC
A-2, #6 (Toyooka)   TGCTGCCTTA CTAGTGGCCT TAATTTCCCT AGCGCTATTC TCCCCCAACC
B-1, #8 (Sakurae)   TGCTGCCTTG CTAGTAGCCT TAATTTCCCT AGCGCTTTTC TCCCCCAACC
B-2, #39 (Hirao)    TGCTGCCTTG CTAGTAGCCT TAATTTCCGT AGCGCTTTTC TCCCCCAACC
B-3, #32 (Tosa)     TGCTGCCTTG CTAGTAGCCT TAATTTCCCT GGCGCTTTTC TCCCCCAACC
B-4, #20 (Saori)    TGCTGCCTTG CTAGTAGCCT TAATCTCCCT GGCGCTTTTC TCCCCCAACC
B-5, #25 (Buzen)    TGCTACCTTG CTAGTAGCCT TAATTTCCCT AGCGCTTTTC TCCCCCAACC
B-5, #29 (Nagato)   TGCTACCTTG CTAGTAGCCT TAATTTCCCT AGCGCTTTTC TCCCCCAACC
B-6, #10 (Kanoya)   TGCTGCCTTG CTAGTAGCCT TAATTTCCCT AGCGCTTTTC TCCCCCAACC
B-8, #55 (Himeji)   TGCTGCCTTG CTAGTAGCCT TAATTTCCCT AGCGCTTTTC TCCCCCAACC
B-9, #15 (Nagano)   TGCTGCCTTG CTAGTAGCCT TAATTTCCCT AGCGCTTTTC TCCCCCAACC
B-9, #16 (Sendai)   TGCTGCCTTG CTAGTAGCCT TAATTTCCCT AGCGCTTTTC TCCCCCAACC
B-11, #22 (Sasebo)  TGCTGCCCTG CTAGTAGCCT TGATCTCCCT AGCGCTTTTC TCCCCCAACC
B-11, #60 (Kazusa)  TGCTGCCCTG CTAGTAGCCT TGATTTCCCT AGCACTTTTC TCCCCCAACC
C, #46 (Mooka)      TGCTGCCTTG CTAGTAGCCT TAATTTCCTT AGCACTCTTC TCCCCAAATC
```

Fig. 14 Sequences of *O. latipes* cytochrome *b* genes. The 504 nucleotide sequences of 17 specimens are shown. Subcluster, haplotype number (#number), and collected locality name (or strain) are also shown.

```
                          110         120         130         140         150
A-1, #1(HNI)              TGCTTGGAGA CCCCGATAAC TTCACCCCTG CTAACCCGTT AGTAACCCCT
A-1, #7 (Momoishi)        TGCTTGGAGA CCCCGATAAC TTCACCCCTG CTAACCCGTT AGTAACCCCT
A-2, #3 (Kanazawa)        TGCTTGGAGA CCCCGATAAC TTCACCCCTG CTAACCCATT AGTAACCCCT
A-2, #6 (Toyooka)         TGCTAGGAGA CCCCGATAAC TTCACCCCTG CTAACCCATT AGTAACCCCT
B-1, #8 (Sakurae)         TGCTTGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTTACCCCT
B-2, #39 (Hirao)          TGCTTGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTTACTCCC
B-3, #32 (Tosa)           TGCTCGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTTACTCCG
B-4, #20 (Saori)          TGCTTGGAGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AATTACTCCC
B-5, #25 (Buzen)          TGCTTGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTTACCCCC
B-5, #29 (Nagato)         TGCTTGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTTACCCCT
B-6, #10 (Kanoya)         TGCTTGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTTACTCCC
B-8, #55 (Himeji)         TGCTTGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTTACTCCC
B-9, #15 (Nagano)         TGCTTGGAGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AATTACTCCC
B-9, #16 (Sendai)         TGCTTGGAGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AATTACTCCC
B-11, #22 (Sasebo)        TGCTTGGGGA CCCAGACAAC TTCACCCCTG CCAACCCGCT AGTCACCCCC
B-11, #60 (Kazusa)        TGCTTGGGGA CCCAGACAAC TTTACCCCTG CCAACCCGCT AGTCACTCCC
C, #46 (Mooka)            TGCTTGGAGA TCCCGACAAC TTCACCCCAG CTAACCCGCT GGTTACACCT

                          160         170         180         190         200
A-1, #1(HNI)              CCTCACATTA AACCCGAATG ATATTTCCTA TTTGCTTACG CTATTCTACG
A-1, #7 (Momoishi)        CCTCATATTA AACCCGAATG ATATTTCCTA TTTGCTTACG CTATTCTACG
A-2, #3 (Kanazawa)        CCTCACATTA AACCCGAATG ATATTTCCTA TTTGCTTACG CTATTCTACG
A-2, #6 (Toyooka)         CCTCACATTA AGCCCGAATG ATATTTCCTA TTTGCTTACG CTATCCTACG
B-1, #8 (Sakurae)         CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-2, #39 (Hirao)          CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-3, #32 (Tosa)           CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-4, #20 (Saori)          CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-5, #25 (Buzen)          CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-5, #29 (Nagato)         CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-6, #10 (Kanoya)         CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-8, #55 (Himeji)         CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-9, #15 (Nagano)         CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-9, #16 (Sendai)         CCTCACATCA AGCCTGAATG ATACTTCCTA TTTGCCTACG CCATTCTACG
B-11, #22 (Sasebo)        CCTCACATCA AGCCTGAATG ATATTTCCTA TTTGCTTACG CCATTCTACG
B-11, #60 (Kazusa)        CCTCACATCA AGCCTGAATG ATATTTCCTA TTTGCTTACG CCATTCTACG
C, #46 (Mooka)            CCTCACATCA AGCCCGAATG ATATTTCCTG TTTGCTTACG CCATTCTACG

                          210         220         230         240         250
A-1, #1(HNI)              ATCAATTCCA AACAAACTTG GAGGCGTTCT AGCCTTATTA GCCTCTATCC
A-1, #7 (Momoishi)        ATCAATTCCA AACAAACTTG GAGGCGTTCT AGCCTTATTA GCCTCTATCC
A-2, #3 (Kanazawa)        ATCAATTCCA AACAAACTTG GAGGCGTCCT AGCCTTATTA GCCTCTATCC
A-2, #6 (Toyooka)         ATCAATTCCA AATAAACTTG GAGGCGTACT AGCCTTATTA GCCTCTATCC
B-1, #8 (Sakurae)         ATCAATTCCA AATAAACTTG GAGGGGTCCT AGCCCTATTA GCCTCTATTC
B-2, #39 (Hirao)          ATCAATTCCA AATAAACTTG GAGGGGTCCT AGCCCTATTA GCCTCTATTC
B-3, #32 (Tosa)           ATCAATTCCA AATAAACTTG GAGGGGTCCT AGCCCTATTA GCCTCTATCC
B-4, #20 (Saori)          ATCAATTCCA AATAAACTTG GAGGAGTCCT AGCCCTATTA GCCTCTATTC
B-5, #25 (Buzen)          ATCAATTCCA AATAAACTTG GGGGGGTCCT AGCCCTATTA GCCTCTATTC
B-5, #29 (Nagato)         ATCAATTCCA AATAAACTTG GAGGGGTTCT AGCCCTATTA GCCTCTATTC
B-6, #10 (Kanoya)         ATCAATTCCA AATAAACTTG GAGGGGTCCT AGCCCTATTA GCCTCTATTC
B-8, #55 (Himeji)         ATCAATTCCA AATAAACTTG GAGGGGTCCT AGCCCTATTA GCCTCTATTC
B-9, #15 (Nagano)         ATCAATTCCA AATAAACTTG GAGGAGTCCT AGCCCTATTA GCCTCTATTC
B-9, #16 (Sendai)         ATCAATTCCA AATAAACTTG GAGGAGTCCT AGCCCTATTA GCCTCTATTC
B-11, #22 (Sasebo)        ATCAATTCCA AATAAACTTG GGGGAGTCCT AGCTCTGTTG GCCTCCATTC
B-11, #60 (Kazusa)        ATCAATTCCA AATAAACTTG GAGGGGTCCT AGCTCTGTTG GCCTCTATTC
C, #46 (Mooka)            ATCAATCCCA AACAAACTTG GGGGCGTCCT GGCCCTATTA GCCTCTATTC
```

Fig. 14 Continued

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                          260         270         280         290         300
A-1, #1(HNI)              TAGTTCTATT  CCTAGTCCCT  ATCCTACACA  CATCCAAACA  ACGAGGACTA
A-1, #7 (Momoishi)        TAGTTCTATT  CCTAGTCCCT  ATCCTACACA  CATCCAAACA  ACGAGGACTA
A-2, #3 (Kanazawa)        TAGTTCTATT  CCTAGTCCCC  ATCCTACACA  CATCCAAACA  ACGAGGCCTA
A-2, #6 (Toyooka)         TAGTTCTGTT  CCTAGTCCCC  ATCTTACACA  CATCCAAACA  ACGAGGCCTG
B-1, #8 (Sakurae)         TAGTTCTATT  CCTGGTCCCC  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-2, #39 (Hirao)          TAGTACTATT  CCTGGTCCCT  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-3, #32 (Tosa)           TAGTACTATT  TCTGGTCCCT  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-4, #20 (Saori)          TAGTACTATT  CCTGGTCCCT  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-5, #25 (Buzen)          TAGTACTATT  CCTGGTCCCC  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-5, #29 (Nagato)         TAGTACTATT  CCTGGTCCCC  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-6, #10 (Kanoya)         TAGTACTATT  CCTGGTCCCT  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-8, #55 (Himeji)         TAGTACTATT  CCTGGTCCCT  ATTCTACACA  CCTCTAAACA  ACGAAGCCTT
B-9, #15 (Nagano)         TAGTACTATT  CCTGGTCCCC  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-9, #16 (Sendai)         TAGTACTATT  CCTGGTCCCC  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-11, #22 (Sasebo)        TGGTATTATT  CCTGGTCCCC  ATCCTACACA  CCTCTAAACA  ACGAAGCCTT
B-11, #60 (Kazusa)        TAGTATTATT  CCTGGTCCCC  ATCCTGCACA  CCTCTAAACA  ACGAAGCCTT
C, #46 (Mooka)            TAGTACTATT  TCTCGTCCCT  ATTCTACACA  CCTCTAAACA  ACGTAGCCTT

                          310         320         330         340         350
A-1, #1(HNI)              ACATTCCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCTTAG  TAGCAGACGT
A-1, #7 (Momoishi)        ACATTCCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCTTAG  TAGCAGACGT
A-2, #3 (Kanazawa)        ACATTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
A-2, #6 (Toyooka)         ACATTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-1, #8 (Sakurae)         ACGTTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-2, #39 (Hirao)          ACGTTTCGAC  CTTTCACCCA  ACTCCTTTTC  TGACTCCTGG  TAGCAGACGT
B-3, #32 (Tosa)           ACATTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-4, #20 (Saori)          ACGTTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-5, #25 (Buzen)          ACGTTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-5, #29 (Nagato)         ACGTTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-6, #10 (Kanoya)         ACGTTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-8, #55 (Himeji)         ACATTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-9, #15 (Nagano)         ACGTTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-9, #16 (Sendai)         ACGTTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCAGACGT
B-11, #22 (Sasebo)        ACATTTCGAC  CTTTCACCCA  ATTCCTTTTT  TGACTCCTAG  TAGCGGACGT
B-11, #60 (Kazusa)        ACATTTCGAC  CTTTCACCCA  ATTCCTTTTC  TGACTCCTAG  TAGCGGACGT
C, #46 (Mooka)            ACATTTCGAC  CTTTTACTCA  GTTCCTTTTC  TGACTTCTCG  TAGCAGACGT

                          360         370         380         390         400
A-1, #1(HNI)              AGTGGTTTTA  ACCTGAATCG  GTGGTATACC  TGTAGAACAT  CCCTTTATTA
A-1, #7 (Momoishi)        AATGGTTTTA  ACCTGAATCG  GCGGTATACC  TGTAGAACAT  CCCTTTATTA
A-2, #3 (Kanazawa)        AATGGTTTTA  ACCTGAATTG  GTGGTATGCC  TGTAGAACAT  CCCTTTATTA
A-2, #6 (Toyooka)         AATGGTTTTA  ACCTGAATTG  GTGGTATGCC  TGTAGAACAC  CCATTTATTA
B-1, #8 (Sakurae)         AATGGTTTTA  ACCTGAATTG  GCGGAATGCC  AGTAGAACAC  CCATTTATTA
B-2, #39 (Hirao)          AATGGTTTTA  ACCTGAGTTG  GTGGAATGCC  CGTAGAACAC  CCATTTATTA
B-3, #32 (Tosa)           AATGGTTTTA  ACCTGAATTG  GTGGAATGCC  CGTAGAACAC  CCATTTATTA
B-4, #20 (Saori)          AATGGTTTTA  ACCTGAATTG  GCGGAATGCC  CGTAGAACAC  CCATTTATTA
B-5, #25 (Buzen)          AATGGTTTTA  ACCTGAATTG  GTGGAATGCC  CGTAGAACAC  CCATTTATTA
B-5, #29 (Nagato)         AATGGTTTTA  ACCTGAATTG  GTGGAATGCC  CGTAGAACAC  CCATTTATTA
B-6, #10 (Kanoya)         AATGGTTTTA  ACCTGAATTG  GTGGAATGCC  CGTAGAACAC  CCATTTATTA
B-8, #55 (Himeji)         AATGGTTTTA  ACCTGAATCG  GTGGGATGCC  CGTAGAACAC  CCATTTATTA
B-9, #15 (Nagano)         AATGGTTTTA  ACCTGAATTG  GCGGAATGCC  CGTAGAGCAC  CCATTTATTA
B-9, #16 (Sendai)         AATGGTTTTA  ACCTGAATTG  GCGGAATGCC  CGTAGAACAC  CCATTTATTA
B-11, #22 (Sasebo)        AATGGTTTTA  ACCTGAATTG  GTGGAATGCC  TGTGGAACAT  CCCTTTATTA
B-11, #60 (Kazusa)        AATGGTTTTA  ACCTGAATTG  GAGGAATGCC  CGTAGAACAC  CCATTTATTA
C, #46 (Mooka)            TATAGTTTTA  ACCTGAATTG  GTGGAATGCC  TGTAGAACAC  CCGTTTATTG
```

Fig. 14 Continued

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                          410        420        430        440        450
A-1, #1(HNI)              TCATCGGTCA AGTCGCATCT TTTCTTTATT TCTTCCTCTT TCTTGTTATA
A-1, #7 (Momoishi)        TCATCGGTCA AATCGCATCT TTTCTTTATT TCTTCCTCTT TCTTGTTATA
A-2, #3 (Kanazawa)        TCATCGGTCA AATCGCATCC TTTCTTTATT TCTTCCTCTT TCTTGTTATA
A-2, #6 (Toyooka)         TCATCGGTCA AATCGCATCT TTTCTTTATT TCTTCCTCTT TCTTGTTATA
B-1, #8 (Sakurae)         TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATTATA
B-2, #39 (Hirao)          TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATTATA
B-3, #32 (Tosa)           TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTTTT TCTTATCATA
B-4, #20 (Saori)          TTATTGGCCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATTATA
B-5, #25 (Buzen)          TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATCATA
B-5, #29 (Nagato)         TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATCATA
B-6, #10 (Kanoya)         TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATTATA
B-8, #55 (Himeji)         TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATTATA
B-9, #15 (Nagano)         TTATTGGCCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATTATA
B-9, #16 (Sendai)         TTATTGGCCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTATTATA
B-11, #22 (Sasebo)        TCATTGGTCA AGTTGCATCT TTTCTTTATT TTTCCCTCTT TCTTGTTATA
B-11, #60 (Kazusa)        TCATTGGTCA AATCGCATCT TTTCTTTATT TTTCCCTCTT TCTTGTTATA
C, #46 (Mooka)            TTATTGGCCA AATCGCATCT TTCCTCTATT TCTCCCTCTT TCTTATTATA

                          460        470        480        490        500
A-1, #1(HNI)              ACACCAGCGG CAGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
A-1, #7 (Momoishi)        ACACCAGCGG CAGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
A-2, #3 (Kanazawa)        GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
A-2, #6 (Toyooka)         GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-1, #8 (Sakurae)         GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-2, #39 (Hirao)          GCACCAGCGG CAGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-3, #32 (Tosa)           GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-4, #20 (Saori)          TCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-5, #25 (Buzen)          GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-5, #29 (Nagato)         GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-6, #10 (Kanoya)         TCACCAGTGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-8, #55 (Himeji)         GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-9, #15 (Nagano)         TCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAGT GACAATGCAC
B-9, #16 (Sendai)         TCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-11, #22 (Sasebo)        GCACCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
B-11, #60 (Kazusa)        ACGCCAGCGG CGGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC
C, #46 (Mooka)            GCACCAGCGG CAGGATGACT AGAAAATAAA GTCTTAAAAT GACAATGCAC

                          510        520        530        540        550
A-1, #1(HNI)              GAGA...... .......... .......... .......... ..........
A-1, #7 (Momoishi)        GAGA...... .......... .......... .......... ..........
A-2, #3 (Kanazawa)        GAGA...... .......... .......... .......... ..........
A-2, #6 (Toyooka)         GAGA...... .......... .......... .......... ..........
B-1, #8 (Sakurae)         GAGA...... .......... .......... .......... ..........
B-2, #39 (Hirao)          GAGA...... .......... .......... .......... ..........
B-3, #32 (Tosa)           GAGA...... .......... .......... .......... ..........
B-4, #20 (Saori)          GAGA...... .......... .......... .......... ..........
B-5, #25 (Buzen)          GAGA...... .......... .......... .......... ..........
B-5, #29 (Nagato)         GAGA...... .......... .......... .......... ..........
B-6, #10 (Kanoya)         GAGA...... .......... .......... .......... ..........
B-8, #55 (Himeji)         GAGA...... .......... .......... .......... ..........
B-9, #15 (Nagano)         GAGA...... .......... .......... .......... ..........
B-9, #16 (Sendai)         GAGA...... .......... .......... .......... ..........
B-11, #22 (Sasebo)        AAGA...... .......... .......... .......... ..........
B-11, #60 (Kazusa)        AAGA...... .......... .......... .......... ..........
C, #46 (Mooka)            AAGA...... .......... .......... .......... ..........
```

Fig. 14 Continued

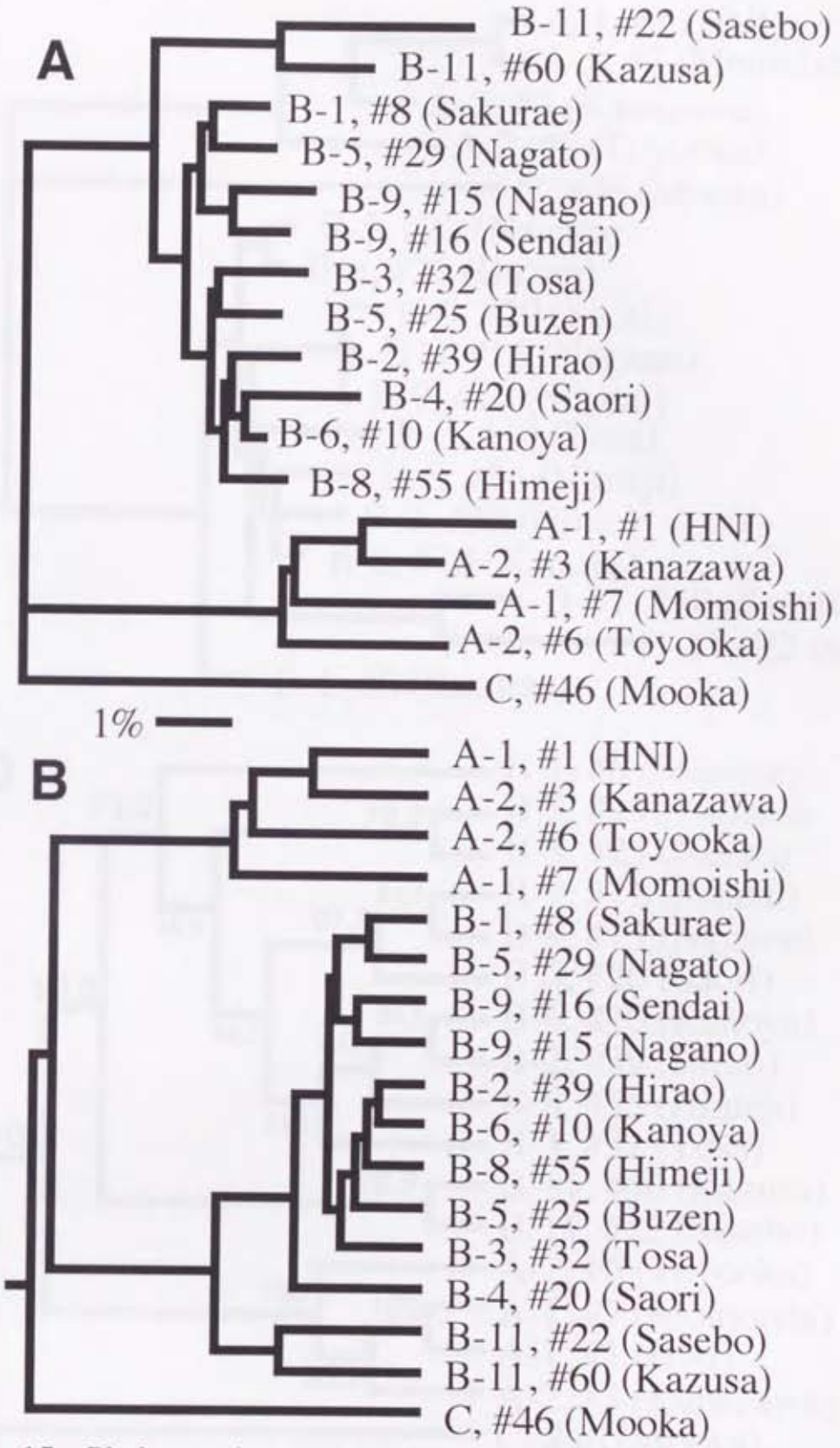

Fig. 15 Phylogenetic trees estimated by NJ (*A*), UPGMA (*B*), ML (*C*), and MP (*D*) methods based on the 504 nucleotide sequences of the cytochrome *b* gene. In the MP tree, the numbers for each interior branch is the bootstrap value.

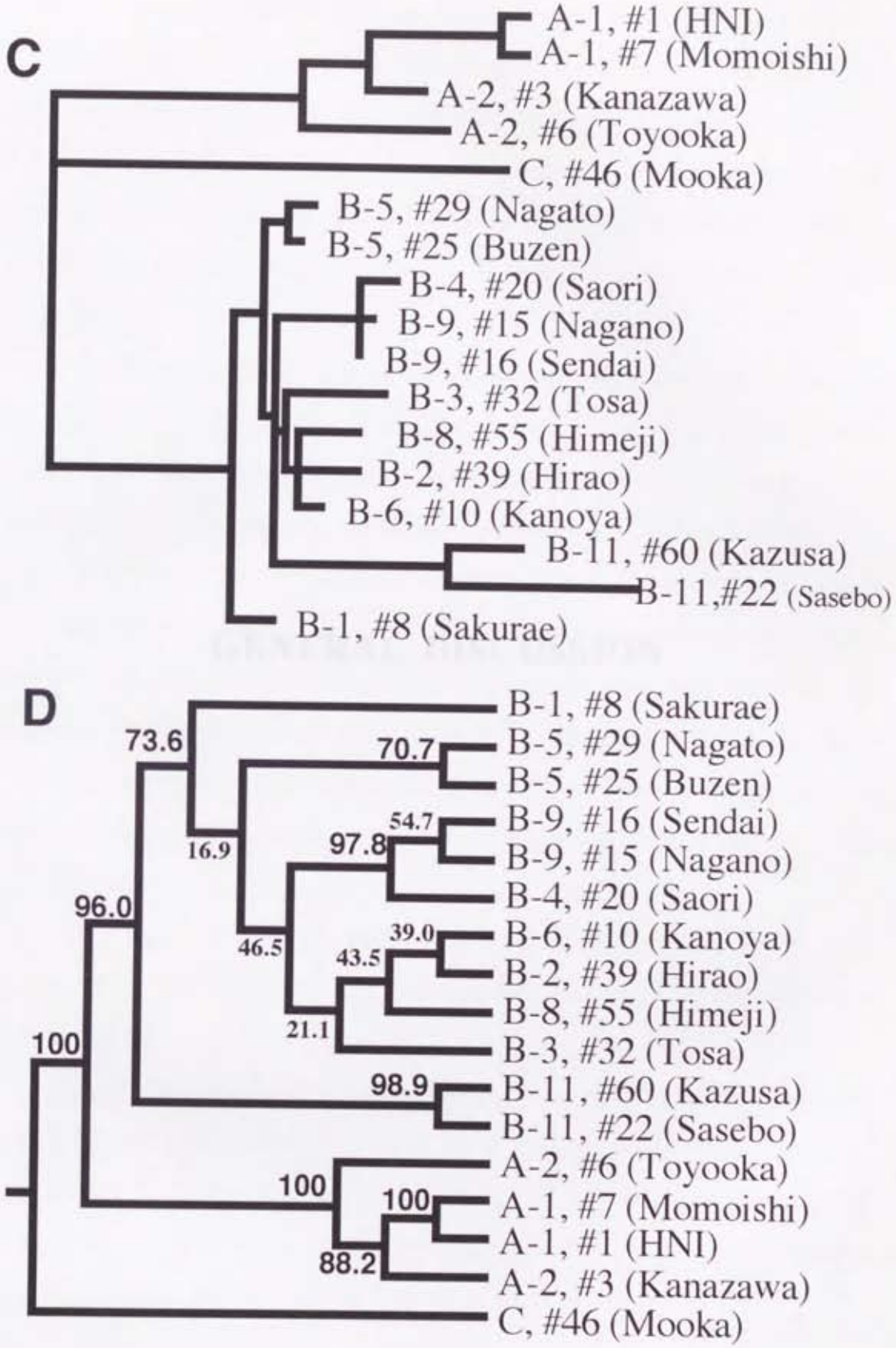

Fig. 15 Continued

GENERAL DISCUSSION

I investigated mtDNAs of the medaka, *Oryzias latipes*, and related species within the genus *Oryzias*. Analyses of restriction fragment length polymorphisms (RFLPs) and comparison of nucleotide sequences in the cytochrome *b* gene and major noncoding region of mtDNA (control region) revealed inter- and intra-specific relationships of medaka and related species. Points of results in the present study are as follows.

(1) Mitochondrial DNAs of the Japanese wild population of medaka are divided into three clusters (A to C). Cluster B was subdivided into 11 subclusters. The average nucleotide diversities among these three clusters are 8.9% (A versus B), 8.4% (A versus C), and 7.3% (B versus C). Cluster C is miner cluster (Part I).

(2) Mitochondrial DNAs of the Korean wild population of medaka were separated into two clusters. The average nucleotide diversity between two clusters from Korea was 8.9% (Part II).

(3) These distribution patterns of five clusters and a large number of subclusters in medaka revealed strong geographical associations (Part I and Part II).

(4) Nucleotide sequences of complete mitochondrial control region from two Japanese populations have tandemly repeat sequence (Part III).

(5) *O. mekongensis* had no such tandem repeat sequences in the control region (Part III).

(6) The phylogenetic analysis using the nucleotide sequences of complete mitochondrial cytochrome *b* gene indicated monophyletic group of Japanese two populations (Part IV).

(7) Nucleotide sequence of partial cytochrome *b* gene suggested relationships among subclusters in cluster B of mtDNA in the Japanese medaka (Part V).

Concordance with allozymic analysis

Overall the data analysis reveals perfectly concordance between distribution patterns of mitochondrial haplotypes and those of allozymic genotypes. Japanese mitochondrial clusters A and B are associated with the Northern Population and the Southern Population, respectively. Korean mitochondrial clusters A and B are associated with the China-West Korean Population and the East Korean Population, respectively. Such concordance might be expected if the four populations of medaka had evolved in complete isolation in the past. Estimated sequence divergences between Japanese two clusters are approximately 9%. I presume that the Japanese two clusters share a common ancestor in 4.5 million years ago. Furthermore, mtDNA divergences between Korean two clusters are also 9%. We hence estimate a same divergence time.

Japanese one cluster, cluster C is rare variant. The fish with this mtDNA haplotype have allozymic genotype similar to that of the fish with haplotypes of cluster B (the Southern Population type; data not shown), despite the large sequence divergence in mtDNA estimated. Thus, we suspect that the "old" mtDNA haplotypes which diverged in the ancestor of the medaka may have persisted in a limited area (the Kanto district). Examination of the frequency and distribution, and detailed molecular analysis are in progress to elucidate the origin and dispersal of this haplotype.

Japanese mitochondrial clusters, cluster A and cluster B are subdivided into two and 11 subclusters. Haplotypes belonging cluster A

have low divergence. In subclusters of cluster B, by contrast, haplotypes belonging this cluster have large divergence. The characteristics are also concordant with allozymic characteristics. In addition, distribution patterns of these subclusters are similar to those of subpopulations inferred from allozymes. Although, the phylogenetic relationships among these subclusters are not clear form RFLPs analysis, the RFLPs analyses on the medaka mtDNA have revealed that the mtDNA characteristics represent population characteristics of the medaka.

From sequence phylogenetic relationships within the genes

Phylogenetic relationships within the genus *Oryzias* are inferred from nucleotide sequence data. The inferences are based on noncoding region and cytochrome *b* gene in mitochondrial genome. I compared nucleotide sequences of two regions for four species belonging to biarmed chromosome group and four populations of *O. latipes*. These results suggest that *O. mekongensis* first divided from a common ancestor of the biarmed chromosome group, and that second divergence occurred between *O. latipes* clade and *O. curvinotus-O. luzonensis* clade. These inferences are coincide with those from karyological and allozymic analyses (Magtoon and Uwa, 1985; Sakaizumi, 1985b).

Furthermore, phylogenetic analysis using complete nucleotide sequence of cytochrome *b* gene, revealed monophyletic grouping of Japanese two populations. This is the first report for supporting monophyletic relationship between the Southern Population and the Northern Population. A dendrogram to show relationships among four populations in medaka, is inferred form Nei's *D* estimated from allele frequencies (Sakaizumi and Joen, 1987). However, such large

divergence among these four populations may lead unreliable phylogenetic relationships. For instance, RFLPs analysis also can not infer mtDNA relationships among these four populations, since I can not detect shared fragment. From this result, I assume a common ancestor of the Japanese wild population of medaka.

Migration history of Japanese medaka

RFLPs analysis can not reveal relationships among subclusters in cluster B, that is associated with the Southern Population. The relationships among these subclusters inferred from partial nucleotide sequence of the mitochondrial cytochrome *b* gene. Phylogenetic tree based on this sequence support four phylogenetic relationships among these subclusters.

(a) Subcluster B-11 first divided from a common ancestor of cluster B. The present distribution rage of the medaka with subcluster B-11's mtDNAs is western Kyushu to the Ryukyu Islands.

(b) A sister group relationship of subclusters B-4 and B-9 is supported. These two subcluster's mtDNAs distributes in eastern Japan.

(c) Haplotypes of subclusters B-2, B-3, B-6, and B-8 are closely related. These haplotypes are found in medaka distributed around the Seto Inland Sea.

(d) Phylogenetic analyses suggest relationships among subclusters of cluster B. The order of their division are follow: first subcluster B-11, next B-1, next B-5, and finally, subcluster B-4 and -9, and subcluster B-2, -3, -6, and -8.

These four relationships of subclusters, coupled with distribution patterns indicated in Part I, offer some presumption for migration

history of Japanese wild population of medaka in the Southern Population.

A population which now lives in western Kyushu was divided first from a common ancestor of the Southern Population. Following separation of medaka with an ancestral mtDNA haplotype of subcluster B-1, medaka which had an ancestral mtDNA of subcluster B-5 was divided. At last, a common ancestor of medaka with ancestral mtDNA of subcluster B-2, -3, -4, -6, -8, and -9 was separated into two populations: medaka with ancestral mtDNA of subcluster B-2, -3, -6, and -7, and medaka with ancestral mtDNA of subcluster B-4 and -9.

The population which now lives in western Kyushu recently expanded their range into the Ryukyu Islands. A part of population living in eastern Japan also recently expanded their range into the Tohoku district. In the Kanto district, medaka which had mtDNAs differentiated in other district (subcluster B-3; the Nankai district, B-5; the San-in district, and B-8; the Kinki district), were recently expanded their range into the Kanto district.

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