Evolution of sex chromosomes in the order Aulopiformes

Kinya Ota

DOCTOR OF PHILOSOPHY

Department of Genetics

School of Life Science

The Graduate University for Advanced Studies

2001

Acknowledgments

I wish to express my sincere gratitude to my supervisor, Professor Takashi Gojobori for his continuous guidance and encouragement during all the stages of this work.

I thank Professors Yoshio Tateno, Toshimichi Ikemura, Hiroyuki Sasaki, Norihiro Okada, Hiroyuki Takeda and Naruya Saitoh for their useful comments on my work, serving on my supervisory committee. I would like to express my gratitude to Dr. Tatsuo Fukagawa for his technical advices. I also thank Drs. Koichi Ueno and Toru Kobayashi for their kind suggestion in the cytogenetic work and sample collection.

I wish to express my appreciation to Takashi Itoh, Yoshiyuki Suzuki, Ronald Chi Chiu Wang and Hwang Jung Shan for their valuable advices. I would also like to express my gratitude appreciation, Yumi Umehara, Akemi Mizuguchi, Hiroko Oizumi, Chie Iwamoto and Hiroaki Ono for their laboratory technical assistance.

I thank Captain Koji Yamada, Satoshi Fukuchi, Koji Hotta, Kosuke Hanada, Yoji Nakamura, Hisaya Suzuki, Noboru Kawaguchi, Kanazashi Tsuriguten, Captains Endo and Kubota for helping me in sample collections.

Finally, I would like to dedicate this paper to the late Dr. Susumu Ohno.

Abstract

In general, sex of fish is not so strongly controlled by the genetic sex determination system, because highly differentiated sex chromosomes are not observed in most fish species. The order Aulopiformes consists of both synchronous hermaphroditic and gonochoristic species. The synchronous hermaphroditic species tend to inhabit the deep-sea areas whereas the gonochoristic species inhabit shallow waters. There are a few cytogenetic reports that gonochoristic species have a heteromorphic sex chromosome (probably a part of ZW chromosomes) that is distinguishable from the autosomes under the microscope. On the other hand, hermaphroditic species do not appear to have heteromorphic chromosomes and they are known to have a special reproductive organ, ovotestis, which consists of both testis and ovary. Thus, the order Aulopiformes contains both sexualities.

This raises the immediate questions as follows: (1) Is the order Aulopiformes monophyletic or polyphyletic? If the former is true, is the ancestor of this order hermaphroditic or gonochoristic species? (2) Did the hetermorphic sex chromosome exist in the common ancestor? (3) Does a gonochoristic species really have an established ZW chromosome system? (4) Does a gonochoristic species have any sex chromosome-specific DNA sequence? To answer these questions, I conducted the following studies using fish in the order Aulopiformes.

First, I participated in a deep-sea trawling ship to collect a sufficient number of specimens, because hermaphroditic aulopiform species tend to

inhabit in the deep-sea areas. To elucidate the evolutionary process of the order Aulopiformes, I performed phylogenetic and cytogenetic analyses for the collected specimens. I found that hermaphroditic species were evolutionarily derived from gonochoristic species and that the common ancestor of aulopiform species must have had a female heteromophic sex chromosome (W chromosome). I also suggest the possibility that heteromorphic sex chromosomes disappeared from the genome of hermaphroditic species during evolution.

Second, clarifying the number of diploid chromosomes, I found that large-scale chromosome rearrangements have occurred in evolution. To investigate the large-scale chromosome rearrangement in more detail, I estimated the genome sizes of gonochristic and hermaphroditic species. The results indicated that the large-scale chromosome rearrangement in the aulopiform species have indeed occurred without any major changes in genome size.

Third, since the W chromosome is identified from all the gonochoristic aulopiform species investigated, the ZW chromosome system might have been conserved among the gonochoristic species. But, I could not identify the Z chromosome in gonochoristic aulopiform species studied under the microscope, because morphological differences were not clear between Z chromosome and the autosomes. Thus, I employed Fluorescence *in-situ* hybridization (FISH) to identify Z chromosome in *Aulopus japonicus.* Using 5S rDNA as a probe, I was able to identify Z chromosome clearly in this species. My FISH analysis indicates that this species has highly

differentiated Z and W sex chromosomes both in size and the number of repetitive sequences.

Finally, I made an attempt to isolate any female-specific sequences in *Aulopus japonicus* by use of 5S rDNA. In general, 5S rDNA and its adjacent non-transcripted intergenic spacer (NTS) form a 5S rDNA cluster. I conducted PCR amplification and sequencing of the 5S rDNA cluster in this species. I then found that the 5S rDNA sequences existed in both female and male individuals. However, I found that there were two different NTS types, "type A" and "type B", which were highly divergent from each other. To confirm the existence of both "type A" and "type B" of NTS in this species, I performed PCR amplification of the NTS regions using "type A" and "type B"-specific primers, indicating that male and female individuals, indeed, possess both of "type A" and "type B" sequences. Although the precise locations of the "type A" and "type B" clusters on Z and W chromosomes are unknown, I pointed out a possibility that there is an intervening sequence between the "type A" and "type B" clusters. If it is the case, the intervening sequence can be amplified by PCR. Hence, I performed PCR amplification using NTS sequences primer in the hope that the intervening sequence may contain female-specific DNA segments. As a result, I could successfully amplify a 6kb-long fragment of female specificity at last. Sequencing the fragment, I found that it contained a "mapk-like" sequence.

In conclusion, although the previous studies have implied that fish do not have the highly differentiated sex chromosomes in general, the present study clearly indicated that gonochoristic aulopiform species have the highly

differentiated sex chromosomes, leading me to the proposal that this order of fish may be comparable to birds and mammals in the view point of the evolutionary process of the sex chromosomes.

Contents

Chapter 1

Introduction

1.1 Evolutionary study of the sex chromosomes in fish species and other vertebrates

The XX female / XY male system is one of several sex determining systems in vertebrates. The other systems include the female heterogamety, in which a male has two copies of Z chromosome and a female has a single Z and a small heteromorphic W chromosome. Reptiles, amphibians and fishes do not have cytologically distinguishable sex chromosomes, and sex may be determined either by genes located somewhere in the genome or even by an environmental stimulation (Graves and Reed 1992).

For the fish species in particular, there is no simple model of the chromosomal sex determination system. Both of the XY and ZW chromosome systems have been reported in some species, and the XYY and ZWW sex chromosome systems have been demonstrated in others (Uyeno and Miller 1971; Filho et al. 1980). Moreover, the occurrences of their sex chromosome systems do not correspond to their phylogenetic relationships in most cases. For instance, both of the XY and ZW chromosome systems are

observed in each lineage of Cypriniformes, Cyprinidontiformes and Syngnathiformes (Ojima 1987). Thus, it has been considered that the sex chromosome systems of fish are less conservative than those of mammals and birds at least at the cytogenetic level.

It has been already known that the sex-determining genes are located on the sex chromosomes in mammals and birds (Graves and Shetty 2001). Therefore, to study the process and mechanism of sex chromosome evolution in vertebrates, we can compare the sex chromosomes of mammals with those of birds at the cytogenetic and molecular levels (Nanda et al. 1999). There are reports that several genes are located on the sex chromosomes in fish species (Nakayama et al. 1994; Reed et al. 1995; Delvin et al. 1998; Forbes et al. 1994). However, we cannot compare these fish with other vertebrates (mammals and birds), because, there is no guarantee that the sequence has been conserved in the same linage owing to the instability of sex chromosomes of fish. The question, then, is whether or not we can find a fish species that has conservative sex chromosomes and sex specific genes.

There are two reports that describe cytogenetic features in the order Aulopiformes (Chen and Ebeling, 1974; Nishikawa and Sakamoto 1978). Chen and Ebeling (1974) investigated a cytogenetic feature in one aulopiform species (*Synodus lucioceps*). They observed a heteromorphic chromosome in one specimen of this species. But, they did not check whether the specimen was male or female. Nishikawa and Sakamoto (1978) have observed that two aulopus species (*Saurida elongata* and *Saurida*

undosquamis) have female heteromorphic sex chromosomes. The three heteromorphic chromosomes have the same morphological feature at the cytogenetic level. Therefore, it is possible that the heteromorphic chromosome is conserved among gonochoristic aulopiform species. Thus, aulopiform species provide me with useful information to study sex chromosome evolution of vertebrates.

1.2 Sexuality and sex chromosomes in the order Aulopiformes

Various species of fish inhabit in a large variety of ecological habitats. Each species has acquired its own reproductive strategy to adapt to its habitats. Sexuality in particular represents an important aspect of the reproductive strategies. In the fish species, it is known that various types of sexuality exist such as synchronous hermaphroditism, protandrous and protogynous hermaphroditism and gonochrorism (Baroiller et al. 1999). Such a large variety of sexuality was not observed in other vertebrates.

The order Aulopiformes consists of synchronous hermaphroditic and gonochoristic species. The synchronous hermaphroditic species tends to inhabit the deep-sea, whereas the gonochoristic species inhabits shallow waters.

There are a few cytogenetic reports that gonochoristic species have a heteromorphic sex chromosome (probably part of ZW chromosomes) that is

distinguishable from the autosomes under the microscope (Chen and Ebeling, 1974; Nishikawa and Sakamoto 1978). It is known that hermaphroditic species have a special reproductive organ, ovotestis, which consists of both testis and ovary (Nakazono and Kuwamura 1987). Since the order Aulopiformes contains of both sexualities as mentioned above, we should consider whether the heteromorphic sex chromosomes of the three species mentioned above (1.1) are derived from a common ancestor or not. If the occurrence of heteromorphic sex chromosomes does not correspond to the phylogenetic relationships, the sex chromosome of aulopiform species also have not been conserved as other sex chromosomes of fish species. If the heteromorphic sex chromosomes are derived from a common ancestor, this order probably gives us very interesting opportunity in studying evolution of sex chromosomes, because we can compare this order with other vertebrate species.

1.3 Aims of this study

The order Aulopiformes has interesting points in both sexuality and sex chromosomes. In this study, especially I focused on sex chromosome evolution in the order Aulopiforomes, because it seems that we can compare this order with other vertebrates in an evolutionary process and a mechanism of sex chromosomes. Thus, in this study, I have two aims.

The first aim of this study is to elucidate the evolution of the sex chromosome in the order Aulopiformes. To reveal sex chromosome

evolution in this order, I conducted phylogenetic and cytogenetic analyses. I also used several molecular techniques to reveal structure of the sex chromosome and tried to identify sex specific sequences in aulopiform species.

The last aim is to compare these species with other vertebrate species in sex chromosomes at the molecular level and to consider sex chromosome evolution in vertebrates.

Chapter 2

Sample Collection and preparation of chromosome slides

2.1 Introduction

It is important to prepare good slides of methaphase chromosomes in order to observe sex chromosomes under the microscope. It is also necessary to obtain a sufficient number of specimens to conduct reliable experiments. In the case of dealing with mammals and birds, it easier to obtain a sufficient number of specimens, because the tools and technique have been well developed.

But, in the case of deep-sea fish, the basic technique is not well established for sample collection and preparation of chromosome slides. Indeed, although several deep-sea fishes were investigated at the cytogenetic levels (Chen 1969), there has been no report on the study of sex chromosomes at the molecular level. For hermaphroditic aulopiform species that inhabit in the deep-sea, I have established the basic technique and conditions in sample collection and preparation of chromosome slides. In this chapter I will explain the technique.

2.2 Location of sampling and equipment of the trawl ship

Aulopiform species inhabit in the Suruga Bay, Japan. In order to collect samples of these species, I participated in trawling on ship in two areas (depth 50-200m) in the Suruga Bay several times (Fig 2.1). The trawl ship was equipped with large winches (Fig 2.2) and trawl nets (Fig 2.3). Using these equipments, a large amount of fishes were collected (Fig 2.4), from which I could select appropriate species for my study. To collect shallow water species, I worked with local fishermen in Izu and Wakayama Peninsular, Japan. Some of these samples are kept in an aquarium system with sterilization lamps and a cooling machine in the lab. Figs 2.5 and 2.6 show pictures of the aulopiform species used in this study.

2.3 Chromosome preparation by using cell culture technique

In the case of deep-sea fish, it is difficult to keep living specimens for a long time. Thus, to prepare chromosome slides, I performed cell culture for *Chlorophthalmus albatrossis* as follows. I first removed the pectoral and pelvic fins from the right side of the fish body on the trawling ship. These tissues were kept in physiological saline (NaCl: 8g, KCl: 0.45g,$CaCl_2$: 0.2g $NaHCO_3$: 0.02g per 1 liter distilled water) at 4℃. Coming back to the laboratory, I performed aseptic manipulation for the tissues.

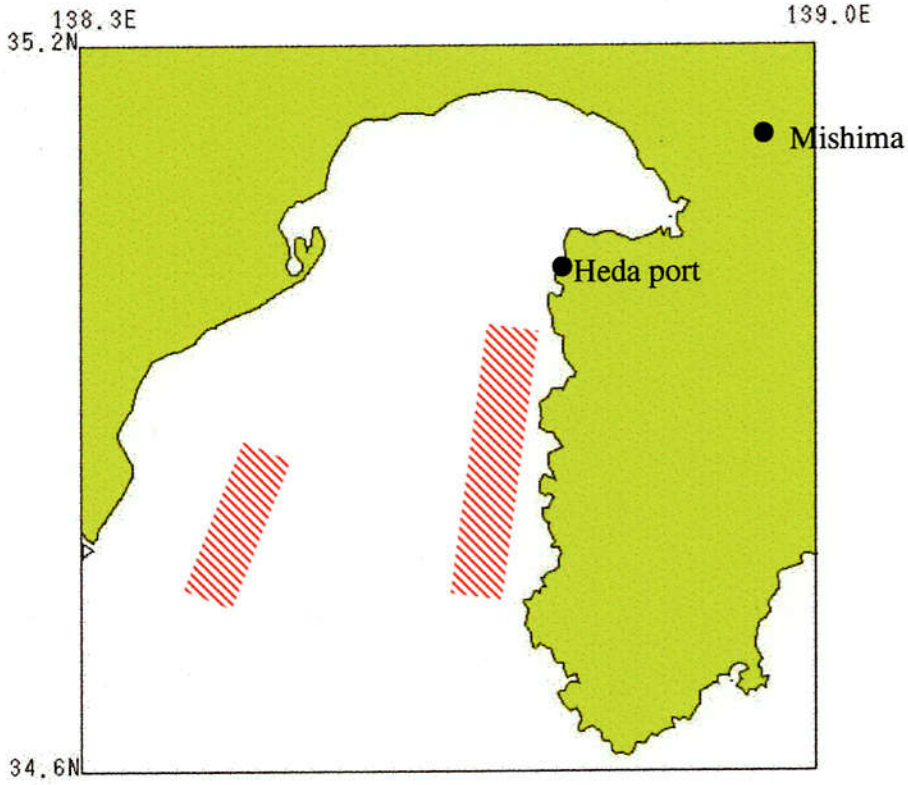


Fig. 2.1 Map of sampling area within the Suruga bay.

▨ indicated sampling areas.

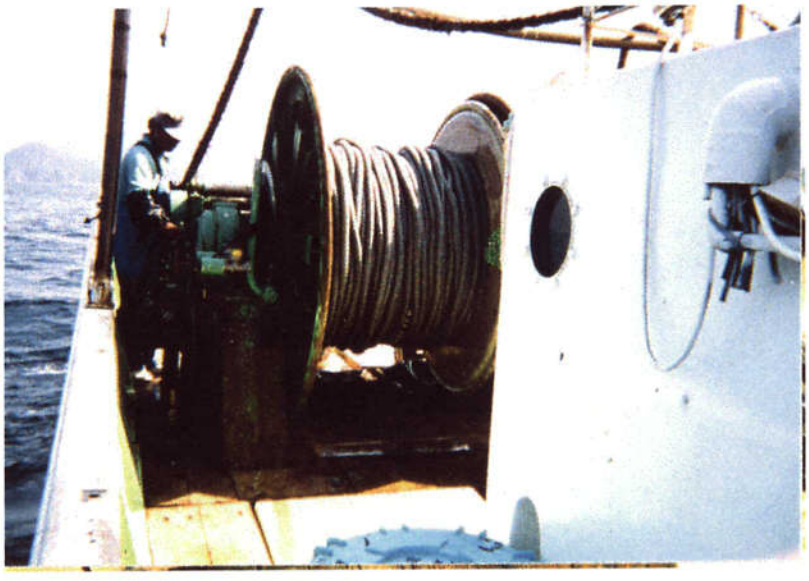


Fig. 2.2. The winch for deep-sea trawling.

Fig. 2.3 The trawl net for deep-sea trawling.

Fig. 2.4 Captured fish species by deep-sea trawling.

Fig. 2.5 The collected samples of hermaphroditic aulopiform species.

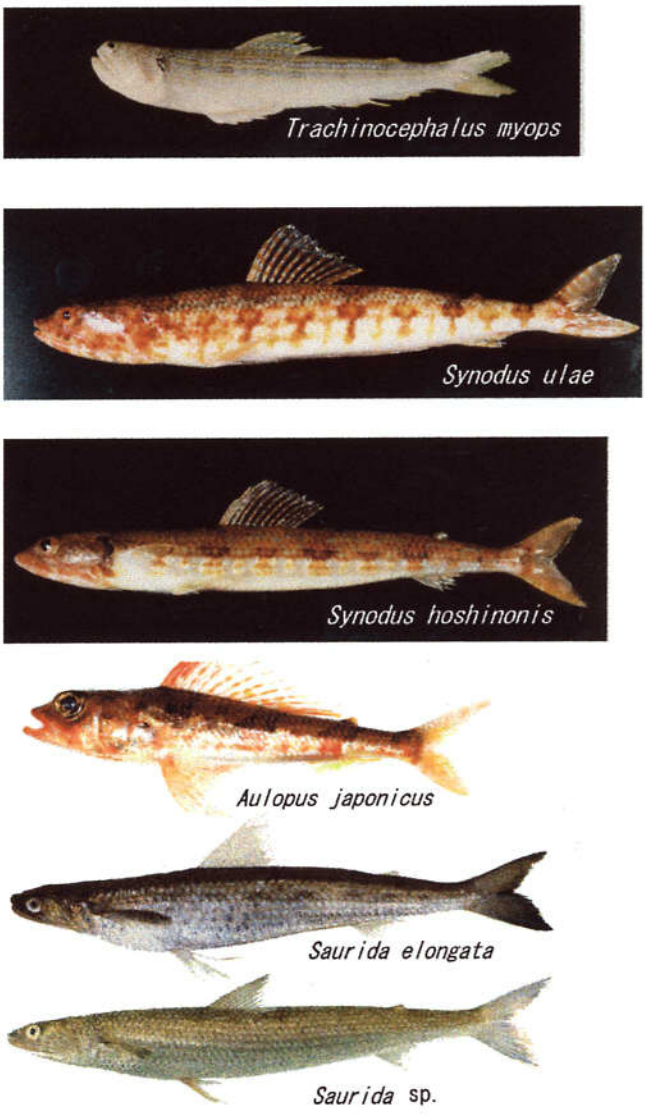


Fig. 2.6 The collected samples of gonochoristic aulopiform species.

These tissues were then washed by sodium hypochlorite solution and rinsed by phosphate buffered saline solution (NaCl: 8g, KCl: 0.2g, $NaHPO_4$: 1.15g, KH_2PO_4 per 1 liter distilled water) three times. The tissues were next cultured on 3cm culture dishes with the Alpha Modification of Eagle's medium (Alpha-MEM) at 22 - 24 ℃. The Alpha-MEM contained streptomycin sulfate (0.1g), penicillin (hundred thousand unit) and fetal bovine serum (10 %) in 1 liter of the medium.

However, these cells could not survive more than two weeks and could not grow up to a sufficient number of cells for this study (Fig. 2.7).

2.4 *In vivo* chromosome preparation on board of the trawl ship

Since I could not obtain a sufficient number of cells in the cell culture, I conducted chromosome preparations *in vivo* on a trawl ship.

To increase the number of metaphase cells, I performed an intraperitoneal injection of colchichine solution in collected specimens on boat. To maintain the specimens intact, I kept them in ice during transfer from the sampling area to the laboratory. At the laboratory, I performed the following experimental procedures at a low temperature. First, the kidney was removed and minced. Then, kidney cell suspension was treated by hypotonic solution (75 mM KCl) for 30 minute, and fixed with methanol and acetic acid (3 :1 volume). Chromosome slides were finally prepared.

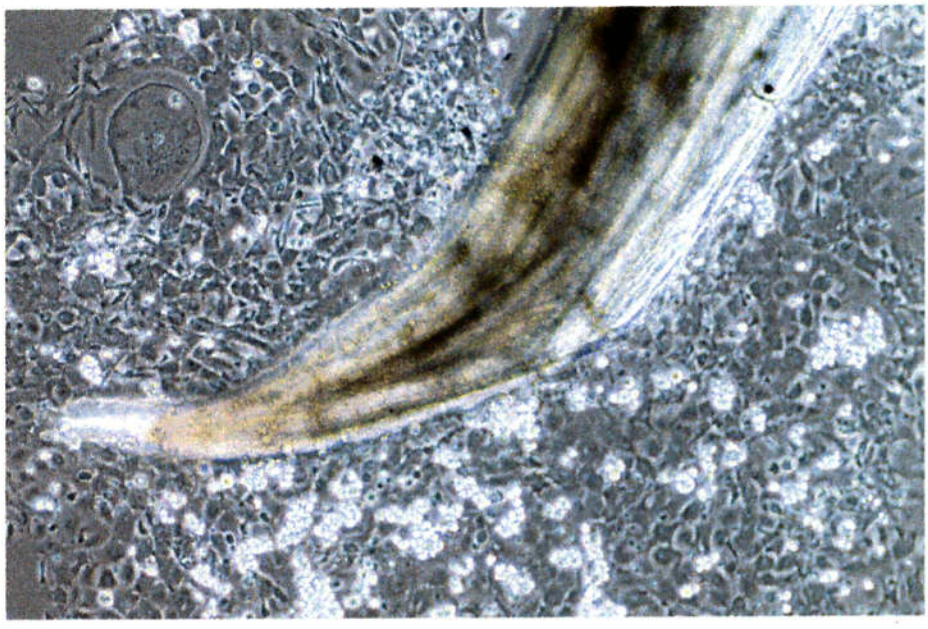


Fig. 2.7 Cultured epithelial cells and fibroblasts of *Chlorophtalmus albatrossis.*

To increase the number of chromosome slides on boat, I also devised a tool. It consists of a small centrifugal machine, a 20 V battery, an electrical transformer, pipettes and some reagents (Fig 2.8). Using the tool, I could conduct the experimental procedures, as described above, on boat. The *in vivo* technique provided me with suitable materials for the experiments.

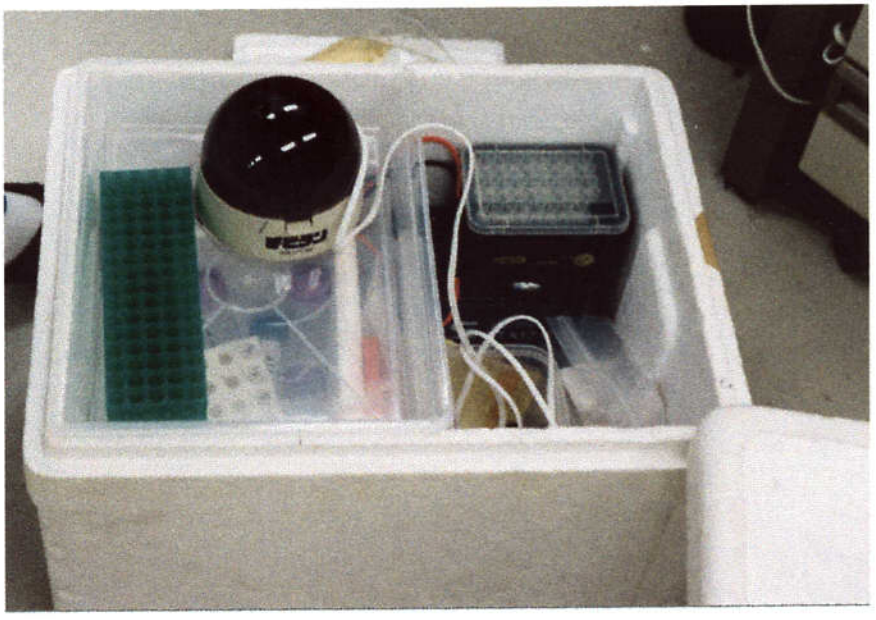


Fig. 2.8 The implement to prepare chromosome slides. The implement consists of a small centrifugal machine,a 20 V battery, a electrical transformer, pipettes and some reagents.

Chapter 3

Phylogenetic and cytogenetic analyses in the order Aulopiformes

3.1 Introduction

According to Ohno's hypothesis (1967), heteromorphic sex chromosomes such as XY and ZW chromosomes might have emerged from autosomes. This hypothesis is thought to be applicable to vertebrates in general. Although these kinds of heteromorphic sex chromosomes are widely observed in vertebrates, some species lack such chromosomes. There are two mutually exclusive explanations. One is that in such species, heteromorphic sex chromosomes have not appeared yet in evolution. The alternative explanation is that heteromorphic sex chromosomes have secondarily disappeared. There is little literature in which the disappearance process of heteromorphic sex chromosomes is discussed, because it is usually difficult to investigate the disappearance process particularly in higher vertebrates. Thus, it is of particular interest to know which explanation is more reasonable in order to understand the evolutionary process of sex chromosomes. Fish is one of the most

interesting groups to study such an evolutionary issue, because fish lack a firmly established system of sex chromosomes (Ohno, 1974). Hence, I decided to investigate the evolution of sex chromosomes in fish.

The order Aulopiformes consists of 13 families, in which 42 genera are composed of 219 species (Nelson, 1994). This order has a characteristic feature of synchronous hermaphroditic and gonochoristic species: The former tends to inhabit the deep-sea and the latter inhabits shallow waters. There are a few cytogenetic reports that gonochoristic species have heteromorphic sex chromosomes that are distinguishable from autosomes under the microscope (Chen and Ebeling 1974; Nishikawa and Sakamoto 1978). In morphological and ecological studies, Smith (1975) proposed that the gonochoristic group of Aulopiformes was evolutionarily derived from hermaphroditic species. Smith's proposal appears to be supported by the fact that hermaphroditic species are dominant in number while gonochoristic species are found in fewer numbers. According to Smith's proposal, it is assumed that heteromorphic sex chromosomes are derived from the genome of hermaphroditic species. However, Smith's proposal should be evaluated more carefully in which there is novaryid evidence that a minor group was derived from a dominant group.

Therefore, it is important to examine a phylogenetic relationship between gonochoristic and hermaphroditic groups of Aulopiformes by both molecular and cytogenetic methods, because it gives us a unique opportunity to elucidate the evolution of sex chromosomes. Thus, I conducted molecular phylogenetic and cytogenetic analyses of six species in the order

Aulopiformes.

3.2. Materials and methods

3.2.1 Sample collection and gonadal sexing

I collected 50 individual samples in six species of the order Aulopiformes. Two species, *Chlorophthalmus albatrossis* and *Chlorophthalmus* sp. were trawled in deep basins of Suruga gulf in Shizuoka prefecture, Japan. Four species, *Saurida elongata*, *Synodus ulae*, *Synodus hoshinonis* and *Trachinocephalus myops*, were captured off Wakayama prefecture, Japan. All of the samples were sexed by macroscopic examination of the gonads.

3.2.2 Phylogenetic analysis

I determined the nucleotide sequences of the complete mitochondrial cytochrome b gene for three synchronous hermaphroditic species (*Chlorophthalmus albatrossis* and *Chlorophthalmus* sp. *Chrorophthalmus nigromarginatu*) and five gonochoristic species (*Saurida* sp. *Saurida elongata*, *Synodus ulae*, *Synodus hoshinonis*, and *Trachinocephalus myops*). Total blood was diluted in TNES-urea buffer (Asahida et al. 1996). After several days to weeks at room temperature, 20 – 60 μl proteinase K was added and the solution was incubated for one to four hours at 60℃. This

was followed by two phenol-chloroform and two chloroform extraction. After precipitation with two volumes of ethanol, the pellet was rinsed in 70% ethanol, moderately dried and dissolved in TE buffer (10mM Tris-HCl [pH 8.0], 1mM EDTA).

Cytochrome b gene regions were amplified by polymerase chain reaction(PCR). The primers used were AJG (CAAAAACCATCGTTGTAA-TTCAACT), H5 (GAATTYTRGCTTTGGGAG), eso-f-472 (YTDTCYGCMGT-CCCMTACGT), eso-R-768 (RTTVGCBGGSGARAAGTT), eso-r-286 (CCBCG-DGCRATGTGHATATA) and eso-r-960 (ARCGDGGMYTWAYWTTCCG). The thermal cycle profile was as follows; denaturation for 10 sec at 94℃, annealing for 10 sec at 48-52℃, and extension for 12 sec at 72℃. PCR products were purified by filtration kit (Mo Bio Laboratories) and were used for direct cycle sequencing with dye-labeled terminators (Applied Biosystems). The primers used were the same as those for PCR. Labeled fragments were analyzed on a Model 310 DNA sequencer (Applied Biosystems).

Sequence alignment was performed by ClustalX (Thompson et al. 1997), and this alignment was improved by visual inspection (Fig 3.1). The total number of nucleotide sites for the alignment was 1137, which contains a gap of three nucleotides in the two hermaphroditic species. A phylogenetic tree was reconstructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987). In the tree, I used a published sequence data of aulopiform species (NC_002674 *Aulopus japonicus*). I also used 10 teleost fishes as outgroups. DDBJ/EMBL/Genbank accession numbers for the representative sequences

```
Salvelinus alpinus              ATGGCCAACCTCCGAAAAACCCACCCACTCCTAAAAATTGCTAATGACGC
Brachymystax lenok              ATGGCTAACCTCCGAAAAACCCACCCCCTCCTAAAAATTGCTAATGACGC
Salmo salar                     ATGGCCAACCTCCGAAAAACTCACCCGCTCCTAAAAATTGCTAATGACGC
Oncorhynchus keta               ATGGCCAACCTCCGAAAAACCCACCCTCTCCTAAAAATCGCTAATGACGC
Gadus morhua                    ATGGCCAGCCTTCGGAAAACCCATCCAATCCTAAAAATTGCTAATAGCGC
Amphilophus alfari              ATGGCCAGCCTCCGAAAAACTCACCCCCTCCTAAAAATCGCAAATGACGC
Paraneetroplus siebold          ATGGCCAACCTCCGAAAAACCCACCCCCTCCTAAAAATCGCAAATGACGC
Tomocichla tuba                 ATGGCCAGCCTCCGAAAAACCCACCCTCTCCTAAAAATCGCAAATGACGC
Poeciliopsis monacha            ATGGCTAGCCTACGAAAATCCCACCCCCTACTAAAAATTGCAAACAACGC
Saurida elongata                ATGGCCATCCTCCGAAAGACACACCCCCTCATGAAAATTGCAAACGACGC
Chlorophthalmus albatrossis     ATGGCC----CTCCGAAAAACCCACCCTTTATTGAAAATCGCAAACGACGC
Chlorophthalmus sp              ATGGCC----CTCCGAAAAACTCATCCCTTACTGAAAATCGCAAACGACGC
Trachinocephalus myops          ATGGCAAGCCTGCGAAAAACCCACCCCCTACTAAAGATTGCAAACGGCGC
Synodus ulae                    ATGGCAAGCCTACGTAAAACCCACCCCCTCCTAAAAATCGCAAACGGTGC
Synodus hoshinonis              ATGGCAAGCCTACGTAAAACCCACCCGCTCCTAAAAATCGCAAACGGCGC
Sebastes umbrosus               ATGGCAAGTCTACGAAAGACACACCCCCTCCTCAAAATCGCAAACAATGC
                                *****    ** ** **  * ** **  *  * ** ** ** **    **

Salvelinus alpinus              ACTAGTCGACCTCCCTGCCCCCTCTAATATCTCAGTCTGATGAAACTTTG
Brachymystax lenok              ACTAGTTGACCTTCCAGCACCCTCTAATATCTCAGTCTGATGAAACTTTG
Salmo salar                     ACTAGTCGATCTCCCAGCACCATCTAACATCTCAGTTTGATGAAACTTTG
Oncorhynchus keta               ACTAGTCGACCTCCCAGCACCATCTAACATCTCAGTCTGATGAAACTTTG
Gadus morhua                    ATTAGTTGATCTCCCCGCCCCCTCCAATATCTCAGTATGATGAAATTTTG
Amphilophus alfari              ACTAGTCGACCTTCCCGCCCCCTCAAACATCTCTGTTTGATGAAACTTTG
Paraneetroplus siebold          ACTAGTTGATCTCCCCACGCCCTCAAACATCTCCATCTGATGAAATTTCG
Tomocichla tuba                 ACTAGTTGACCTTCCTACACCCTCAAACATCTCCGTTTGATGAAACTTTG
Poeciliopsis monacha            ATTAGTAGATCTCCCCGCCCCCGTTAATATCTCCGCCTGATGAAACTTCG
Saurida elongata                ACTAGTAGATCTCCCCGCCCCCTCCAATATTTCAGCCCTATGAAACTTCG
Chlorophthalmus albatrossis     CCTAGTTGACCTTCCCGCCCCCTCGAACATTTCAGCTTGATGAAACTTCG
Chlorophthalmus sp              TTTGGTTGACCTTCCTGCTCCCTCTAACATCTCAGCATGGTGAAACTTCG
Trachinocephalus myops          ACTTGTTGACCTCCCCGCACCCTCCAACATCTCTGCATGGTGGAACTTTG
Synodus ulae                    CCTAGTTGATCTCCCAGCGCCCTCAAATATCTCGGCTTGATGGAATTTTG
Synodus hoshinonis              ACTGGTTGACCTACCAGCACCATCCAACATTTCCGCCTGATGAAATTTCG
Sebastes umbrosus               CCTAGTTGACCTACCCGCCCCCTCAAATATTTCAGTGTGATGAAACTTCG
                                  * ** ** ** **  * **    ** ** **       ** ** ** *

Salvelinus alpinus              GTTCACTCTTAGGCCTATGTTTGGCCACCCAAATTCTTACCGGACTCTTC
Brachymystax lenok              GATCACTCCTAGGCTTATGTCTAGCCACCCAAATTCTCACCGGACTCTTC
Salmo salar                     GCTCACTCTTAGGCCTATGTCTAGCCACCCAAATCCTTACCGGGCTCTTC
Oncorhynchus keta               GTTCACTCCTAGGCTTATGCCTAGCCACCCAAATTCTTACCGGGCTCTTC
Gadus morhua                    GCTCTCTTCTAGGCCTTTGCTTAATTACTCAACTTCTAACAGGACTATTT
Amphilophus alfari              GCTCCCTACTAGGACTCTGCCTTGCCGCCCAAATCCTAACAGGCTTATTT
Paraneetroplus siebold          GCTCCCTACTAGGACTTTGTCTCGTCACCCAAATTTTAACGGGCCTATTT
Tomocichla tuba                 GCTCCTTATTAGGACTATGCCTCGTCGCTCAAATTTTAACAGGCCTATTC
Poeciliopsis monacha            GCTCCCTCCTAGGCCTCTGCCTTATTGCCCAAATTATAACTGGCCTATTT
Saurida elongata                GATCTCTCCTAGGCCTTTGCCTAATCACCCAGATCGTTACAGGACTCTTC
Chlorophthalmus albatrossis     GCTCCCTACTCGGGCTTTGTCTCGCTACACAAATTCTCACAGGATTATTC
Chlorophthalmus sp              GTTCCTTGCTTGGGCTATGTCTGGCCACACAAATTCTTACCGGGTTATTT
Trachinocephalus myops          GCTCTTTATTGGGCCTTTGCTTAGCGACTCAGATTCTGACTGGCCTCTTT
Synodus ulae                    GGTCCTTACTAGGCTTATGCCTGGCCACCCAAATCTTAACAGGCTTGTTC
Synodus hoshinonis              GGTCCCTCCTAGGCCTATGTCTAGCGACCCAAATCTTAACAGGCTTATTC
Sebastes umbrosus               GCTCTCTCTTAGGACTCTGCTTAATTATCCAAATCCTCACAGGACTATTT
                                * **  *  * **  * **  *      **  *  * ** **  * **
```

Fig 3.1 Aligned DNA sequences from 1137 bp of the cytochorme *b*.

```
Salvelinus alpinus              CTAGCCATACACTACACCTCCGATATTTCAACAGCTTTTTCCTCTGTGTG
Brachymystax lenok              CTAGCCATGCACTATACCTCTGACATTTCAACAGCTTTTTCCTCCGTCTG
Salmo salar                     CTAGCCATACACTACACCTCCGATATCTCAACAGCTTTTTCCTCTGTTTG
Oncorhynchus keta               CTAGCCATGCACTACACCTCCGACATTTCAACAGCTTTTTCCTCTGTCTG
Gadus morhua                    CTAGCCATACACTATACCTCAGACATCGAGACAGCCTTCTCATCCGTAGT
Amphilophus alfari              CTTGCAATACACTACACTTCCGACATCGCAACAGCCTTTTCATCCGTTGC
Paraneetroplus siebold          CTTGCAATACACTATACCTCCGACATCGCAACAGCCTTTTCATCTGTTGC
Tomocichla tuba                 CTTGCAATACACTACACTTCCGACATCGCAACAGCCTTTTCATCCGTCAC
Poeciliopsis monacha            TTAGCAATACACTACACCTCCGATATCTCTATAGCATTTTCATCCGTGGC
Saurida elongata                CTAGCTATACACTATACCTCCGATGTTGCTACTGCTTTCTCCTCCGTCGC
Chlorophthalmus albatrossis     TTGGCTATACATTACACTTCCGACATCGCGACTGCCTTCTCTTCCGTAAC
Chlorophthalmus sp              CTCGCTATGCACTACACCTCTGACATCGCAACAGCCTTCTCTTCAGTCAC
Trachinocephalus myops          CTTGCTATGCACTATACTTCTGACGTTGCCACCGCTTTTTCCTCCGTCAC
Synodus ulae                    TTAGCCATGCATTATACCTCTGACATTGCAACAGCCTTCTCATCAGTAAC
Synodus hoshinonis              CTAGCTATACATTACACTTCTGATATCGCCACAGCCTTTTCATCCGTTAC
Sebastes umbrosus               TTAGCCATACACTACACCTCTGATATTGCTACAGCTTTTTCTTCCGTTGC
                                 * ** ** ** ** ** ** **  *    *  ** ** ** ** **

Salvelinus alpinus              CCATATCTGCCGAGATGTAAGTTACGGCTGACTCATCCGGAATATCCACG
Brachymystax lenok              CCATATCTGTCGAGACGTTAGTTACGGCTGACTTATCCGAAATATCCACG
Salmo salar                     CCACATTTGCCGAGATGTTAGCTATGGCTGACTCATCCGTAACATTCACG
Oncorhynchus keta               CCACATCTGCCGAGATGTTAGCTACGGCTGACTAATTCGAAACATCCACG
Gadus morhua                    CCACATCTGTCGTGATGTAAACTACGGCTGACTAATTCGGAATATACATG
Amphilophus alfari              CCACATCTGTCGAGACGTAAATTATGGCTGACTTATCCGCAACATGCACG
Paraneetroplus siebold          CCACATCTGTCGAGATGTAAACTACGGCTGATTAATCCGCAACATACACG
Tomocichla tuba                 CCACATCTGCCGAGATGTTAACTATGGCTGACTAATCCGCAACATGCACG
Poeciliopsis monacha            ACACATTTGCCGAGACGTCAACTACGGATGACTAATCCGCAATATACATG
Saurida elongata                CCACATCTGCCGTGACGTAAACTACGGCTGAATGATCCGGAATCTACACG
Chlorophthalmus albatrossis     TCATATCTGCCGGGACGTGAACTACGGCTGGCTAATCCGAAATATGCACG
Chlorophthalmus sp              CCATATTTGCCGGGACGTAAATTATGGTTGACTGATCCGAAACATGCATG
Trachinocephalus myops          TCATATTTGCCGAGACGTCAACTACGGCTGGTTGATTCGGAATATGCATG
Synodus ulae                    ACACATCTGCCGTGACGTCAACTATGGCTGGCTCATCCGAAATATACATG
Synodus hoshinonis              TCACATTTGCCGCGACGTAAACTATGGCTGACTAATCCGAAACATACACG
Sebastes umbrosus               CCACATTTGCCGGGACGTAAATTACGGGTGATTCATCCGAAATCTTCACG
                                 ** ** ** ** ** ** *  ** ** **  * ** ** **  * ** *

Salvelinus alpinus              CTAACGGAGCATCTTTCTTCTTTATCTGTATTTATATACATATCGCCCGA
Brachymystax lenok              CTAACGGAGCATCCTTCTTCTTTATCTGTATTTATATGCACATCGCCCGA
Salmo salar                     CTAACGGAGCATCTTTCTTCTTTATCTGTATTTATATACACATCGCCCGA
Oncorhynchus keta               CTAACGGAGCATCTTTCTTTTTTATTTGTATTTATATACATATCGCCCGG
Gadus morhua                    CTAATGGTGCCTCTTTCTTTTTCATTTGTCTTTATATGCACATTGCCCGA
Amphilophus alfari              CCAACGGCGCATCCTTCTTCTTCATCTGCATCTACCTTCACATTGGTCGA
Paraneetroplus siebold          CCAACGGCGCATCCTTTTTCTTTATCTGCATTTACCTTCACATTGGCCGA
Tomocichla tuba                 CCAACGGCGCATCCTTTTTCTTTATCTGCATTTACCTCCACATCGGCCGA
Poeciliopsis monacha            CCAACGGAGCCTCCTTCTTTTTCATCTGCATTTACCTTCACATCGGACGG
Saurida elongata                CTAACGGAGCTTCCTTCTTCTTTATTTGCATTTATATTCACATTGCACGC
Chlorophthalmus albatrossis     CTAACGGAGCATCCTTCTTCTTCATTTGCATCTATATGCACATCGCACGA
Chlorophthalmus sp              CCAATGGAGCATCCTTTTTCTTTATTTGCATTTATATGCACATCGCACGA
Trachinocephalus myops          CCAACGGGGCCTCCTTCTTCTTTATCTGCATTTATATGCACATCGCCCGC
Synodus ulae                    CCAACGGTGCATCCTTTTTCTTTATTTGCATTTATATACACATTGCTCGG
Synodus hoshinonis              CCAATGGGGCTTCTTTTTTCTTTATTTGCATTTACATGCATGTCGCCCGA
Sebastes umbrosus               CCAACGGTGCATCCTTCTTCTTTGTATGCATCTATGCCCACATTGGCCGC
                                * ** ** ** ** ** ** **  * **  * **    **  * *  **
```

Fig 3.1 (continued)

```
Salvelinus alpinus              GGACTCTACTACGGGTCCTACCTATATAAAGAAACCTGAAATATTGGAGT
Brachymystax lenok              GGACTCTACTACGGATCCTATTTATATAAAGAAACTTGAAATATTGGAGT
Salmo salar                     GGACTTTATTATGGTTCCTATCTATATAAAGAAACCTGAAATATCGGAGT
Oncorhynchus keta               GGACTTTATTACGGATCCTACCTGTACAAAGAAACCTGGAATATCGGAGT
Gadus morhua                    GGTCTCTATTATGGTTCCTATCTTTTTGTAGAGACATGAAACATCGGGGT
Amphilophus alfari              GGGTTATACTACGGCTCCTACCTTTACAAAGAAACATGAAACGTTGGAGT
Paraneetroplus siebold          GGACTATACTATGGCTCCTACCTCTATAAAGAAACATGAAATGTAGGAGT
Tomocichla tuba                 GGGTTATACTACGGCTCTTACCTCTATAAAGAAACATGAAACGTCGGAGT
Poeciliopsis monacha            GGCCTATACTACGGCTCTTACCTCTACAAAGAAACATGAAACATTGGAGT
Saurida elongata                GGCTTATACTACGGGTCTTATCTTTACATGGAGACCTGAAACATCGGAGT
Chlorophthalmus albatrossis     GGGCTTTATTATGGCTCATATTTGTACAAGGAAACATGAAACGTAGGTGT
Chlorophthalmus sp              GGACTTTACTACGGCTCATACCTCTATAAAGAAACCTGAAACGTTGGCGT
Trachinocephalus myops          GGCCTTTACTATGGTTCATACCTTTATAAGGAGACCTGGAACGTAGGTGT
Synodus ulae                    GGACTGTACTATGGGTCATACCTCTACATAGAGACCTGAAATATCGGGGT
Synodus hoshinonis              GGCCTCTACTACGGCTCATACCTCTACATAGAAACCTGAAATATTGGTGT
Sebastes umbrosus               GGACTTTACTACGGCTCATACCTCTATAAAGAAACATGAAACATCGGGGT
                                **  * ** ** ** ** **  * *     ** ** ** **  * ** **

Salvelinus alpinus              AGTATTACTACTTCTAACTATAATGACTGCCTTTGTAGGCTACGTTCTTC
Brachymystax lenok              AGTACTACTACTTCTCACCATGATAACTGCCTTTGTCGGCTATGTCCTTC
Salmo salar                     TGTACTTCTACTTCTCACTATAATAACTGCCTTCGTAGGCTACGTTCTTC
Oncorhynchus keta               TGTACTTTTACTTCTCACTATAATAACTGCATTCGTGGGCTACGTCCTCC
Gadus morhua                    TGTCCTTTTCCTTTTAGTAATAATAACCTCTTTCGTAGGTTATGTCCTCC
Amphilophus alfari              TATCCTTCTCCTCCTAACCATAATAACTGCTTTCGTAGGCTATGTCCTCC
Paraneetroplus siebold          TGTCCTCCTCCTTTTAACCATAATAACCGCATTCGTAGGCTATGTCCTTC
Tomocichla tuba                 GATCCTTCTCCTCTTAACAATAATAACTGCATTCGTAGGCTACGTCCTCC
Poeciliopsis monacha            AGTACTTCTTCTACTTGTTATAATAACCGCCTTCGTCGGATACGTCCTCC
Saurida elongata                AATTCTGCTTCTTCTAGTAATAATAACTGCCTTCGTTGGTTATGTTCTCC
Chlorophthalmus albatrossis     CATCTTATTACTTCTCGTTATGATGACTGCCTTCGTCGGCTACGTTCTGC
Chlorophthalmus sp              TATCCTATTACTTCTCGTCATGATAACCGCTTTCGTAGGCTACGTCCTCC
Trachinocephalus myops          GATCCTCCTCCTTCTTGTCATGATGACCGCCTTTGTCGGCTACGTCCTAC
Synodus ulae                    TATTCTTCTTCTCCTTGTAATGATGACAGCGTTTGTAGGTTACGTACTCC
Synodus hoshinonis              CGTTCTTCTTCTACTCGTAATATTAACCGCTTTCGTCGGCTACGTTCTTC
Sebastes umbrosus               AGTTCTATTACTTCTAGTTATAATAACTGCTTTCGTCGGTTATGTGCTAC
                                  *  *  * **  *    **  * **  * ** ** ** ** ** ** *

Salvelinus alpinus              CATGAGGGCAAATATCCTTCTGAGGAGCCACTGTAATCACAAACCTCCTC
Brachymystax lenok              CATGAGGACAAATATCCTTCTGAGGAGCCACTGTAATCACCAACCTCCTA
Salmo salar                     CATGAGGACAAATATCCTTCTGAGGAGCCACTGTAATTACAAACCTCCTC
Oncorhynchus keta               CGTGAGGACAAATATCCTTCTGAGGAGCCACTGTAATTACAAACCTTCTT
Gadus morhua                    CCTGAGGACAAATATCATTCTGAGGAGCTACCGTAATTACGAATTTAATA
Amphilophus alfari              CATGAGGACAAATATCCTTCTGAGGCGCCACCGTCATCACCAACCTCCTC
Paraneetroplus siebold          CATGAGGACAAATGTCCTTCTGAGGGGCTACCGTTATTACCAACCTCCTC
Tomocichla tuba                 CATGAGGACAAATATCCTTTTGAGGTGCTACCGTCATCACCAACCTCCTC
Poeciliopsis monacha            CATGAGGACAAATATCCTTCTGAGGAGCTACCGTAATTACCAACCTCCTC
Saurida elongata                CTTGAGGACAAATATCCTTTTGGGGGGCCACAGTTATTACTAACCTATTA
Chlorophthalmus albatrossis     CCTGAGGACAAATGTCTTTCTGAGGGGCAACTGTAATTACCAACCTCTTG
Chlorophthalmus sp              CCTGAGGACAAATGTCTTTCTGAGGAGCAACCGTAATTACCAATCTTCTG
Trachinocephalus myops          CTTGAGGTCAGATGTCCTTCTGAGGCGCCACCGTCATCACCAACCTTCTT
Synodus ulae                    CTTGAGGTCAAATGTCTTTCTGAGGGGCGACTGTAATTACCAACCTTTTA
Synodus hoshinonis              CCTGAGGACAAATGTCGTTTTGAGGTGCAACCGTCATCACCAACCTCATA
Sebastes umbrosus               CCTGAGGCCAAATATCCTTTTGAGGTGCCACCGTTATCACCAACCTACTC
                                * ***** ** ** ** ** ** ** ** ** ** ** ** **  *  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              TCCGCTGTCCCTTACGTAGGAGGTGCCCTTGTACAATGAATTTGAGGCGG
Brachymystax lenok              TCCGCCGTCCCATATGTTGGAGGCGCCCTAGTACAATGAATTTGAGGAGG
Salmo salar                     TCCGCTGTCCCCTACGTAGGAGGCGCCCTTGTACAATGAATTTGAGGAGG
Oncorhynchus keta               TCCGCTGTCCCCTACGTAGGAGGCGCCCTAGTACAATGAATTTGAGGCGG
Gadus morhua                    TCTACTGTTCCTTATGTAGGTGATGCCTTAGTTCAATGGATCTGAGGAGG
Amphilophus alfari              TCCGCTATCCCGTACATCGGCAATTCCCTTGTCCAATGACTCTGAGGTGG
Paraneetroplus siebold          TCCGCAATTCCTTACATTGGCAACTCTCTAGTTCAATGGCTTTGAGGTGG
Tomocichla tuba                 TCCGCAGTCCCTTACATTGGCAACTCCCTGGTCCAATGACTTTGAGGTGG
Poeciliopsis monacha            TCCGCTGTCCCTTATATAGGAAACACCCTTGTCCAATGAATCTGAGGTGG
Saurida elongata                TCCGCCGTCCCCTACGTAGGCACCACCCTCGTCGAATGAATTTGGGGAGG
Chlorophthalmus albatrossis     TCCGCAGTCCCATACGTAGGAAATGCCCTAGTTCAATGGATCTGAGGGGG
Chlorophthalmus sp              TCCGCAGTCCCATACGTTGGGAACGCCTTAGTCCAATGAATTTGAGGGGG
Trachinocephalus myops          TCTGCCGTCCCCTACGTCGGCAACACCCTTGTACAGTGAATTTGGGGTGG
Synodus ulae                    TCTGCCGTCCCATACGTTGGTAATACCCTTGTCCAATGAATTTGAGGCGG
Synodus hoshinonis              TCCGCCGTCCCATATGTTGGTAACACCCTGGTCCAATGGATTTGAGGTGG
Sebastes umbrosus               TCTGAAGTACCCTACGTAGGTAACGCCCTTGTTCAATGAATTTGAGGTGG
                                **    * ** **  * **    *  * **  * **  * ** ** **

Salvelinus alpinus              ATTTTCTGTAGACAACGCCACCCTAACCCGATTTTTCGCCTTTCACTTCC
Brachymystax lenok              ATTCTCCGTAGACAACGCTACCCTGACACGATTTTTCGCCTTCCACTTCC
Salmo salar                     ATTTTCTGTAGACAACGCCACCCTAACACGATTTTTCGCCTTCCACTTCC
Oncorhynchus keta               GTTCTCCGTTGACAACGCCACCCTAACACGATTTTTCGCCTTTCACTTCC
Gadus morhua                    TTTCTCAGTAGATAATGCTACCCTAACTCGGTTTTTTGCATTCCATTTCT
Amphilophus alfari              CTTCTCAGTAGACAATGCCACTCTCACCCGATTCTTTGCCTTCCACTTTC
Paraneetroplus siebold          CTTTTCAGTAGACAATGCCACCCTCACCCGATTCTTTGCCTTCCACTTCC
Tomocichla tuba                 CTTTTCAGTAGATAACGCCACCCTCACCCGATTCTTTGCCTTCCACTTCC
Poeciliopsis monacha            ATTTTCAGTAGACAACGCCACCCTAACCCGCTTCTTCGCCTTCCACTTCC
Saurida elongata                ATTTTCTGTAGACAAGGCCACCCTAACCCGATTCTTCGCCTTCCACTTCC
Chlorophthalmus albatrossis     CTTCTCTGTCGACAATGCCACGCTCACCCGATTCTTTGCCTTTCACTTCC
Chlorophthalmus sp              CTTCTCAGTTGATAACGCCACACTCACCCGATTCTTTGCCTTCCACTTCC
Trachinocephalus myops          GTTCTCGGTAGATAACGCCACGCTTACTCGCTTCTTTGCCTTCCACTTTC
Synodus ulae                    GTTTTCGGTCGATAATGCCACCCTCACCCGGTTCTTTGCATTTCATTTCT
Synodus hoshinonis              ATTCTCAGTAGACAACGCCACCTTAACCCGATTTTTCGCATTTCACTTTT
Sebastes umbrosus               GTTCTCAGTAGACAATGCAACCCTTACCCGATTCTTTGCTTTCCACTTTT
                                 ** ** ** ** ** ** **  * ** ** ** ** ** ** ** **

Salvelinus alpinus              TATTCCCCTTCGTTATTGCAGCCGCCACAGTACTTCACCTTCTATTTCTG
Brachymystax lenok              TATTCCCATTTATCATCGCAGCTGCCACAGTCCTTCACCTCCTATTCCTA
Salmo salar                     TATTCCCATTCGTTATTGCAGCTGCCACAGTACTCCATCTTCTATTTTTA
Oncorhynchus keta               TATTCCCTTTCGTCATCGCAGCTGCTACAGTCCTTCACCTCCTATTCCTT
Gadus morhua                    TATTCCCCTTTGTTGTTGCTGCTTTTACAATACTCCACCTACTTTTTCTC
Amphilophus alfari              TCCTCCCATTTATCATCGCAGCCATAACAATAATTCACCTAATCTTCCTA
Paraneetroplus siebold          TCCTTCCTTTCATCATTGCAGCCATAACAATAATCCACCTAATTTTTCTC
Tomocichla tuba                 TCCTCCCGTTCATCATTGCAGCCATAACAATAATCCACCTAATCTTCCTC
Poeciliopsis monacha            TCCTTCCCTTCATCGTTGCAGCAGCAACCTTAGTCCACCTCATTTTTTTA
Saurida elongata                TATTTCCCTTTGTAATCGTCGCCGTCACAGCTATTCACCTTCTTTTCTTA
Chlorophthalmus albatrossis     TCTTCCCATTCGTCATCGCCGCTGTAACAGTCATCCACCTGTTGTTCCTT
Chlorophthalmus sp              TCTTTCCATTTGTCATTGCGGCCGTAACAGTTATCCACCTACTGTTTCTC
Trachinocephalus myops          TCTTCCCCTTCGTCATCGCTGCGGTTACCGTTATCCACCTCCTATTTCTT
Synodus ulae                    TATTTCCCTTCGTTATCGCAGCGGTTACCGTAATTCACCTCTTATTCCTC
Synodus hoshinonis              TATTTCCATTCGTTATCGCAGCCGTAACTGTAATCCACCTCTTGTTCTTA
Sebastes umbrosus               TGTTCCCCTTTGTAATTGCAGCCGCAACCATAGTCCACCTCCTTTTCCTT
                                *  * ** **  *  * *  **    **     * ** **  * **  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              CATGAAACCGGGTCCAATAACCCAGCAGGGATTAACTCCGACGCCGACAA
Brachymystax lenok              CACGAAACCGGATCCAACAACCCAGCAGGCATTAACTCCGACGCCGATAA
Salmo salar                     CATGAAACCGGGTCTAATAACCCAGCAGGCATCAACTCCGATGCCGATAA
Oncorhynchus keta               CACGAGACAGGATCTAATAACCCGGCAGGAATTAACTCCGATGCCGATAA
Gadus morhua                    CATGAAACAGGCTCAAATAATCCCACAGGAATCAATTCAAATGCAGACAA
Amphilophus alfari              CACGAAACCGGCTCAACAAACCCAGCAGGCCTAAACTCCGACACAGACAA
Paraneetroplus siebold          CACGAAACCGGTTCCACAAACCCAGCAGGCTTAAACTCCGACACAGACAA
Tomocichla tuba                 CACGAAACCGGATCCACAAACCCAGCAGGCCTAAACTCCGACACAGACAA
Poeciliopsis monacha            CACGAAACAGGCTCCAACAACCCCACTGGTCTAAACTCTGACGCAGACAA
Saurida elongata                CATGAAACCGGCTCCAACAACCCGACCGGTATTAACTCTGACGTAGACAA
Chlorophthalmus albatrossis     CACGAGACCGGCTCCAACAACCCCGCAGGGATTAACTCGGACGCGGACAA
Chlorophthalmus sp              CACGAAACCGGATCCAATAACCCCGCCGGTATTAATTCGGACGCAGATAA
Trachinocephalus myops          CATGAGACGGGCTCCAACAACCCGGCGGGGATTAACTCGGACGCAGATAA
Synodus ulae                    CACGAGACCGGCTCAAATAACCCCGCTGGGATTAACTCGGATGCGGACAA
Synodus hoshinonis              CACGAAACCGGATCTAACAACCCAGCCGGGATCAACTCAGATGCCGATAA
Sebastes umbrosus               CACCAAACAGGATCAAACAACCCCCTGGGCCTAAATTCAGACGCAGATAA
                                **  * ** ** ** *  ** **    **  * ** **  *    ** **

Salvelinus alpinus              AATCTCATTCCACCCCTACTTCTCGTACAAAGACCTCCTCGGTTTCGTAG
Brachymystax lenok              AATCTCGTTTCACCCTTACTTCTCATACAAAGACCTCCTCGGGTTCGTAG
Salmo salar                     AATCTCATTCCACCCTTACTTCTCATATAAAGACCTCCTCGGATTTGTAG
Oncorhynchus keta               AATCTCGTTTCACCCTTACTTCTCGTACAAAGACCTCCTAGGGTTCGTAG
Gadus morhua                    AATTCCATTCCACCCATATTTCACCTACAAAGACCTGCTTGGCTTTGCTG
Amphilophus alfari              AATTTCTTTCCACCCCTACTTCTCCTACAAAGACCTCCTAGGCTTTGGAA
Paraneetroplus siebold          AATCTCATTCCACCCCTACTTTTCCTACAAAGACCTATTAGGCTTTGTAA
Tomocichla tuba                 AATCTCATTCCACCCCTATTTCTCCTACAAAGACCTCCTAGGCTTTGCAA
Poeciliopsis monacha            AATCTCTTTCCACCCTTACTTCTCCTATAAAGACCTCCTAGGCTTTGCCT
Saurida elongata                AATCGCGTTCCATCCGTACTTCATCTATAAAGACCTCCTAGGTTTTGTAA
Chlorophthalmus albatrossis     AATTTCCTTCCACCCATACTTCTCCTACAAAGACCTCCTCGGCTTTATTA
Chlorophthalmus sp              AATTTCATTCCACCCATATTTTTCCTACAAGGATCTCCTTGGCTTTATTA
Trachinocephalus myops          GATCTCCTTTCATCCCTATTTCTCGTACAAGGATCTTTTGGGCTTTATTA
Synodus ulae                    AATTTCTTTTCATCCCTACTTTTCGTATAAAGATCTGCTTGGCTTCGTTA
Synodus hoshinonis              GATCTCGTTCCACCCTTATTTTTCCTATAAGGACTTGCTAGGATTTGTTA
Sebastes umbrosus               AATAAGCTTCCACCCCTACTTCTCATATAAAGACTTATTAGGATTTGCAG
                                 **    ** ** ** ** **    ** ** **  *  * ** **

Salvelinus alpinus              CTATATTGCTTGGCCTAACAACCCTAGCTCTTTTCGCACCTAACCTCCTA
Brachymystax lenok              CTATACTACTAGGCTTAACATCCCTAGCTCTCTTTGCACCTAATCTTCTA
Salmo salar                     CCATACTACTTGGCCTAACATCCTTAGCTCTATTCGCACCCAACCTCCTC
Oncorhynchus keta               CCATACTTCTTGGTCTAACATCCTTAGCCCTCTTTGCACCAAACCTCCTG
Gadus morhua                    TGATGCTTCTGGGCTTAACCGCCCTCGCCCTCTTCGCACCTAATTTACTC
Amphilophus alfari              TCCTTCTCATTGCCCTAATCGCCCTAGCCCTCTTCTCCCCCAACCTTCTA
Paraneetroplus siebold          TCCTACTAGTTGCCCTAATTGCTTTAGCCCTCTTTTCCCCCAACCTCCTA
Tomocichla tuba                 TCCTACTCATCGCCCTAATCGCCCTAGCTCTCTTTTCCCCCAACCTCCTA
Poeciliopsis monacha            TCCTGCTTACCACACTAATTGCCCTCGCCCTCTTCTCCCCCAACCTATTA
Saurida elongata                TTATACTTGCTGGCCTATCCGCTCTAGCCTTCTTTTACCCCAACCTCCTC
Chlorophthalmus albatrossis     TCATGATGGTGGCCCTTACCTCCCTCGCCTTGTTTTCACCAAACCTTCTG
Chlorophthalmus sp              TTATGATAGTAGCCCTCACCTCCCTTGCTCTATTCTCCCCAAATCTTCTA
Trachinocephalus myops          TTCTGATGATAGCCCTCACCTCCCTAGCTTTATTTTCCCCTAACGTGCTC
Synodus ulae                    TCCTAATAATAGCCCTTACTTCCCTTGCCCTTTTTTCACCCAATCTTCTA
Synodus hoshinonis              TCCTTATGATAGCTCTTACCTCTTTAGCCCTGTTCTCACCCAACCTGCTA
Sebastes umbrosus               TACTTGTCATTGCCCTTACATGTCTAGCTTTATTCTCACCCAACCTACTG
                                   *  *        *        * **  * **    ** **  *  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              GGAGACCCGGACAATTTCACACCAGCCAACCCCCTAGTTACCCCGCCACA
Brachymystax lenok              GGAGACCCAGACAATTTTACACCCGCCAACCCCCTAGTCACCCCACCTCA
Salmo salar                     GGGGACCCAGACAATTTTACACCTGCCAACCCCCTAGTTACTCCACCTCA
Oncorhynchus keta               GGGGACCCGGACAATTTTACGCCCGCCAACCCACTAGTCACCCCGCCTCA
Gadus morhua                    GGAGATCCAGATAATTTCACCCCTGCTAACCCCATCGTTACCCCACCTCA
Amphilophus alfari              GGAGACCCAGACAACTTCACCCCCGCAAACCCCTTGGTCACTCCCCCACA
Paraneetroplus siebold          GGAGACCCAGACAACTTCACCCCAGCAAACCCCCTAGTCACTCCCCCGCA
Tomocichla tuba                 GGAGACCCAGACAACTTCACTCCCGCAAACCCCCTAGTTACTCCCCCCCA
Poeciliopsis monacha            GGAGACCCAGAAAACTTCACTCCTGCTAACCCACTTGTAACACCTCCTCA
Saurida elongata                GGAGATCCTGACAACTTCACCCCCGCCAATCCCTTAGTTACTCCCCCCCA
Chlorophthalmus albatrossis     GGGGACCCAGACAACTTCACCCCAGCCAACCCCCTTGTTACTCCCCCCCA
Chlorophthalmus sp              GGAGACCCAGACAACTTCACCCCGGCGAACCCGCTCGTCACCCCACCACA
Trachinocephalus myops          GGCGACCCAGACAACTTTACGCCCGCTAACCCCCTTGTCACCCCTCCTCA
Synodus ulae                    GGCGACCCAGATAACTTCACCCCCGCCAACCCCCTGGTGACTCCGCCCCA
Synodus hoshinonis              GGCGACCCCGATAACTTTACCCCCGCTAATCCCCTAGTTAATCCTCCCCA
Sebastes umbrosus               GGAGACCCAGACAACTTCACCCCCGCCAACCCACTAGTTACTCCTCCCCA
                                ** ** ** ** ** ** ** ** ** ** **  * ** *  ** ** **

Salvelinus alpinus              CATCAAGCCCGAATGGTACTTCTTATTCGCCTATGCAATTCTCCGATCTA
Brachymystax lenok              CATCAAACCCGAATGATACTTCTTGTTTGCTTATGCAATTCTACGGTCTA
Salmo salar                     TATCAAGCCTGAATGATACTTCCTATTCGCCTACGCAATCCTACGCTCCA
Oncorhynchus keta               TATCAAACCCGAATGATATTTCCTATTCGCTTACGCAATCCTACGCTCTA
Gadus morhua                    TGTTAAGCCCGAATGATATTTCTTGTTTGCCTATGCCATCTTACGCTCTA
Amphilophus alfari              CATCAAACCAGAATGATACTTCCTATTTGCTTACGCCATTCTCCGATCTA
Paraneetroplus siebold          CATTAAACCAGAATGATACTTCTTGTTTGCTTACGCCATCCTCCGATCAA
Tomocichla tuba                 CATTAAACCAGAATGGTACTTCCTATTTGCTTACGCCATCCTCCGATCAA
Poeciliopsis monacha            CATTAAACCCGAGTGATATTTTCTCTTCGCATACGCCATTCTTCGTTCTA
Saurida elongata                CATCAAACCGGAATGGTACTTTCTCTTCGCCTACGCCATCCTCCGCTCCA
Chlorophthalmus albatrossis     CATTAAGCCCGAGTGATATTTCCTCTTCGCCTATGCCATCCTCCGTTCCA
Chlorophthalmus sp              TATCAAACCAGAATGGTATTTCCTCTTCGCTTATGCCATTCTTCGTTCGA
Trachinocephalus myops          CATCAAGCCTGAGTGGTACTTCCTATTCGCCTACGCCATTCTTCGGTCTA
Synodus ulae                    TATTAAGCCAGAATGGTATTTTCTTTTTGCTTACGCCATCCTACGATCCA
Synodus hoshinonis              TATTAAACCAGAGTGATACTTTCTCTTTGCCTACGCCATTCTTCGCTCCA
Sebastes umbrosus               CATTAAGCCAGAATGATATTTCCTGTTCGCATATGCAATTCTACGCTCCA
                                  * ** ** ** ** ** **  * ** ** ** ** **  * ** ** *

Salvelinus alpinus              TCCCCAATAAACTAGGAGGGGTACTCGCCCTTTTATTCTCAATCCTCGTC
Brachymystax lenok              TCCCCAATAAGCTAGGCGGAGTACTCGCCCTCTTGTTCTCCATCCTCGTC
Salmo salar                     TTCCTAACAAACTAGGCGGAGTACTCGCCCTCTTATTCTCGATCCTGGTC
Oncorhynchus keta               TCCCCAACAAACTTGGAGGGGTACTCGCCCTTTTATTCTCAATCCTTGTC
Gadus morhua                    TTCCAAATAAGCTAGGTGGCGTACTTGCACTCCTATTCTCGATTCTAGTC
Amphilophus alfari              TTCCCAACAAACTAGGCGGAGTCCTAGCACTCCTTTTCTCCATCCTAATC
Paraneetroplus siebold          TCCCGAACAAGCTGGGAGGGGTCCTTGCACTTCTCTTTTCCATTCTAATC
Tomocichla tuba                 TCCCCAACAAACTAGGAGGAGTCCTCGCACTTCTTTTCTCGATTCTAATC
Poeciliopsis monacha            TTCCCAACAAGCTGGGAGGGGTTCTTGCTCTTCTAGCCTCTATTCTAGTT
Saurida elongata                TCCCTAACAAACTAGGAGGAGTTCTTGCACTTCTTGCATCTATTCTTGTC
Chlorophthalmus albatrossis     TCCCAAATAAGCTGGGGGGTGTCCTTGCCCTTCTAGCCTCCATCCTAGTC
Chlorophthalmus sp              TCCCCAACAAACTAGGCGGGGTGCTAGCTCTGCTAGCGTCCATTTTAGTT
Trachinocephalus myops          TTCCCAATAAGCTCGGCGGCGTACTCGCCCTCCTTGCCTCCATCCTCGTC
Synodus ulae                    TCCCAAACAAACTTGGCGGGGTCCTGGCCCTTTTGGCCTCGATCTTAGTA
Synodus hoshinonis              TTCCAAACAAACTTGGAGGAGTTCTGGCCCTTCTTGCCTCTATTTTAGTA
Sebastes umbrosus               TCCCCAATAAACTGGGGGGAGTTTTAGCCCTCCTAGCTTCAATCCTTATC
                                * ** ** ** ** ** ** **  * ** **  *    ** **  *  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              CTCATAGTTGTCCCGATCCTCCACACCTCTAAACAGCGCGGACTAACCTT
Brachymystax lenok              CTTATGGTTGTCCCCATCCTTCACACCTCTAAACAACGAGGGCTAACCTT
Salmo salar                     CTTATAGTCGTCCCCATCCTCCATACCTCTAAACAACGAGGACTGACCTT
Oncorhynchus keta               CTTATAGTTGTCCCCATCCTGCATACATCTAAACAACGAGGACTGACCTT
Gadus morhua                    CTCATGGTTGTACCCTTTCTCCATACGTCAAAACAACGAGGTTTAACATT
Amphilophus alfari              CTCATGCTCGTGCCAATCCTCCACACCTCAAAACTCCGAGCCCTTACCTT
Paraneetroplus siebold          CTTATACTAGTGCCAATCCTCCACACCTCAAAACTCCGAGCCCTTACCTT
Tomocichla tuba                 CTCATACTCGTGCCAATCCTCCACACCTCAAAACTTCAGACTCTTACCTT
Poeciliopsis monacha            CTGATAGCTGTTCCCTTCCTTCACACTTCTAAACAACGAAGCCTCACCTT
Saurida elongata                CTTATACTAGTCCCCGTTCTCCACACGTCTAAACAACGGGGCTTAATATT
Chlorophthalmus albatrossis     CTAATGCTGGTCCCTTTCCTCCATACCTCTAAACAGCGAGGACTAACATT
Chlorophthalmus sp              TTGATACTGGTCCCTTTTCTCCATACCTCAAAACAGCGTGGACTAACATT
Trachinocephalus myops          CTCATGCTCGTTCCCATCCTCCATACTTCCAAGCAGCGGGGACTAACTTT
Synodus ulae                    CTTATGCTCGTTCCAATTCTTCACACCTCAAAGCAGCGAGGACTTACTTT
Synodus hoshinonis              CTAATGCTTGTCCCAATACTACACACCTCTAAGCAGCGAGGACTTACCTT
Sebastes umbrosus               CTAATGCTCGTACCATTTCTACACACGTCTAAACAACGAAGCCTCACTTT
                                 * **    ** **  * ** ** ** ** ** *  *      * *  **

Salvelinus alpinus              TCGACCACTAACTCAATTCTTATTCTGAACCCTGGTAGCAGACATACTAA
Brachymystax lenok              TCGACCACTAACCCAATTCTTATTCTGAACCCTAGTAGCAGACATACTTA
Salmo salar                     TCGCCCACTCACCCAATTCTTATTCTGGACCCTGGTAGCGGACATACTAA
Oncorhynchus keta               TCGACCACTAACCCAATTCTTATTTTGAGCCTTAGTAGCAGATATACTCA
Gadus morhua                    CCGCCCTCTTACCCAAATACTATTCTGAGTCCTCGTTGCAGATATACTAG
Amphilophus alfari              CCGACCAATTACTCAATTTTTATTTTGACTCCTAGTTGCAGACGTCATTA
Paraneetroplus siebold          CCGACCCCTTACTCAATTCTTATTCTGACTCCTAATTGCAGACGTCATAA
Tomocichla tuba                 CCGACCACTCACTCAATTTTTATTCTGGCTCCTAGTTGCAGACGTCGTTA
Poeciliopsis monacha            CCGTCCTCTCACCCAAATCCTCTTTTGACTTTTAATCGCAGACGTAGCAA
Saurida elongata                CCGCCCTCTTACCCAACTCCTCTTCTGAACATTCGTAGCAGACGTGGTGA
Chlorophthalmus albatrossis     CCGGCCCTTAACACAAATTATTTTCTGAACATTTGTAGCTAACGTCATCA
Chlorophthalmus sp              CCGTCCATTGACACAAATTCTCTTTTGAACCTTTGTCGCCAATGTTATTA
Trachinocephalus myops          CCGCCCTCTGACCCAAGTACTCTTCTGGGCCTTTGTAGCCGACGTCATCA
Synodus ulae                    CCGACCCCTAACACAGTTTCTATTCTGAACTTTCGTAGCAGATGTCATTA
Synodus hoshinonis              TCGACCCCTAACACAATTTCTTTTCTGGACTTTTGTGGCAGATGTCATTA
Sebastes umbrosus               CCGACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTA
                                 ** **  * ** **  *  * ** **     *  * ** *  *

Salvelinus alpinus              TCCTCACCTGAATTGGGGGCATGCCTGTAGAACACCCATTTATCATTATC
Brachymystax lenok              TCCTCACCTGAATTGGTGGCATACCCGTAGAACACCCATTTATTATCATC
Salmo salar                     TCCTTACCTGAATTGGAGGCATACCCGTGGAACACCCATTCATTATCATT
Oncorhynchus keta               TCCTCACCTGAATCGGGGGCATACCCGTAGAACACCCGTTCATTATTATC
Gadus morhua                    TTCTTACATGAATTGGAGGCGTACCTGTAGAACACCCCTTCATTATCATC
Amphilophus alfari              TTCTAACCTGAATTGGAGGAATGCCTGTCGAACATCCCTTTGTTATCATT
Paraneetroplus siebold          TTTTAACCTGAATTGGAGGAATACCCGTCGAACACCCATTTATCATCATT
Tomocichla tuba                 TTTTGACTTGAATTGGAGGCATACCCGTTGAACACCCATTTATCATTATC
Poeciliopsis monacha            TCCTTACATGAATCGGAGGTATACCTGTTGAACACCCATTTATTATTATT
Saurida elongata                TCCTCACATGAATTGGAGGCATGCCGGTTGAACACCCCTTTATTATTATC
Chlorophthalmus albatrossis     TCCTGACGTGAATCGGAGGTATGCCAGTCGAACACCCCTTTATTATCATT
Chlorophthalmus sp              TTCTAACTTGAATCGGAGGTATGCCGGTTGAACACCCCTTCATTATTATT
Trachinocephalus myops          TTCTCACCTGGATCGGAGGCATACCAGTCGAACACCCTTTTATCATCATC
Synodus ulae                    TCCTCACATGAATTGGGGGTATACCAGTGGAGCACCCTTTTATTATTATT
Synodus hoshinonis              TCCTCACCTGAATTGGAGGCATGCCAGTAGAACATCCATTTATTATTATC
Sebastes umbrosus               TTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTCATTATT
                                *  * ** ** ** ** **  * ** **    ** ** **  * ** **
```

Fig 3.1 (continued)

```
Salvelinus alpinus              GGCCAAGTTGCCTCTGTGATTTACTTCACCATCTTCCTAGTCCTCGCCCC
Brachymystax lenok              GGCCAAGTCGCCTCTGTAATTTACTTCACCATCTTCCTAATCCTCGCCCC
Salmo salar                     GGTCAAATTGCCTCTGTAATTTACTTTACTATCTTCCTAGTCCTTGCCCC
Oncorhynchus keta               GGCCAAATTGCCTCTGTAATCTACTTCACCATCTTCCTAGTTCTTTCCCC
Gadus morhua                    GGACAAGTGGCATCAGTACTATATTTCTCCCTCTTCCTAGTTTTATTCCC
Amphilophus alfari-sj           GGCCAAATTGCATCCTTCCTCTATTTCTCTATTTTCCTTATCTTACTGCC
Paraneetroplus siebold          GGCCAAATCGCATCTTTCCTCTATTTCTTCCTCTTCCTTATTTTACTGCC
Tomocichla tuba                 GGCCAAATCGCTTCCTTCCTCTACTTCTTCATATTCCTTATTTTACTGCC
Poeciliopsis monacha            GGTCAAGTAGCCTCCCTCCTCTACTTCTCCCTATTCCTAGTCCTCTCCCC
Saurida elongata                GGCCAGGTCGCTTCCTTCCTCTATTTCTTTCTGCTCTTGGTCTTAGTCCC
Chlorophthalmus albatrossis     GGTCAAGTAGCATCGTTCCTATACTTTCTTCTCCTTCTAGTACTCTCACC
Chlorophthalmus sp              GGGCAGGTAGCATCGTTTCTCTATTTCCTCCTCCTTTTAGTCCTATCGCC
Trachinocephalus myops          GGACAGGTAGCTTCCTTCCTATACTTTCTTCTTCTTCTCGTACTAGCCCC
Synodus ulae                    GGGCAAGTTGCCTCCTTCCTTTACTTCTTTCTACTGTTGCTATTGTCCCC
Synodus hoshinonis              GGACAAGTGGCCTCTTTCCTTTACTTCTTCCTACTTCTTTTGCTATCTCC
Sebastes umbrosus               GGACAAGTTGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACC
                                ** **  * ** **  *  * ** **    *  *  *  *  *   **

Salvelinus alpinus              CTTAGCCGGTTGGGCCGAAAATAAAGCCCTTGAATGA
Brachymystax lenok              CTTAGCCGGATGGGCCGAGAATAAAGCCCTCGAATGA
Salmo salar                     CCTGGCTGGCTGAGCTGAAAATAAAGCTCTTGAATGA
Oncorhynchus keta               CTTAGCCGGCTGGGCCGAAAATAAAGCCCTCCAATGA
Gadus morhua                    CCTTGCAGGAATAACTGAAAATAAGGCCCTTGAATGA
Amphilophus alfari-sj           CATCTCTGGACTAGCAGAAAATAAAATATTTGCATAA
Paraneetroplus siebold          CATTGCCGGACTAGCAGAAAATAAAATATTTGCATAA
Tomocichla tuba                 CACCGCCGGACTAGCAGAAAATAAAATATTTGCATAA
Poeciliopsis monacha            CACTGCAGCATGAGTAGAAAATAAAATCCTCGGATGA
Saurida elongata                CGCTGCAGGGTGACTAGAAAATAAAGCACTTGAATGA
Chlorophthalmus albatrossis     CTTAGCGGGCTGAGTGGAGAATAAAGCCCTTGATTGA
Chlorophthalmus sp              CCTTGCGGGATGGGTAGAGAACAAGGCCCTTAAATGA
Trachinocephalus myops          CCTCGCCGGCTGGGTTGAGAATAAGGCCCTTGAATGA
Synodus ulae                    AATTGCAGGGTGATTAGAAAATAAAGCCCTTGAATGA
Synodus hoshinonis              TATGGCAGGCTGACTGGAAAATAAAGCCCTCGAATGA
Sebastes umbrosus               ACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGA
                                   * *        **  * **    *    * *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              ATGGCCAACCTCCGAAAAACCCACCCACTCCTAAAAATTGCTAATGACGC
Brachymystax lenok              ATGGCTAACCTCCGAAAAACCCACCCCCTCCTAAAAATTGCTAATGACGC
Salmo salar                     ATGGCCAACCTCCGAAAAACTCACCCGCTCCTAAAAATTGCTAATGACGC
Oncorhynchus keta               ATGGCCAACCTCCGAAAAACCCACCCTCTCCTAAAAATCGCTAATGACGC
Gadus morhua                    ATGGCCAGCCTTCGGAAAACCCATCCAATCCTAAAAATTGCTAATAGCGC
Amphilophus alfari              ATGGCCAGCCTCCGAAAAACTCACCCCCTCCTAAAAATCGCAAATGACGC
Paraneetroplus siebold          ATGGCCAACCTCCGAAAAACCCACCCCCTCCTAAAAATCGCAAATGACGC
Tomocichla tuba                 ATGGCCAGCCTCCGAAAAACCCACCCTCTCCTAAAAATCGCAAATGACGC
Poeciliopsis monacha            ATGGCTAGCCTACGAAAATCCCACCCCCTACTAAAAATTGCAAACAACGC
Saurida elongata                ATGGCCATCCTCCGAAAGACACACCCCCTCATGAAAATTGCAAACGACGC
Chlorophthalmus albatrossis     ATGGCC---CTCCGAAAAACCCACCCTTTATTGAAAATCGCAAACGACGC
Chlorophthalmus sp              ATGGCC---CTCCGAAAAACTCATCCCTTACTGAAAATCGCAAACGACGC
Trachinocephalus myops          ATGGCAAGCCTGCGAAAAACCCACCCCCTACTAAAGATTGCAAACGGCGC
Synodus ulae                    ATGGCAAGCCTACGTAAAACCCACCCCCTCCTAAAAATCGCAAACGGTGC
Synodus hoshinonis              ATGGCAAGCCTACGTAAAACCCACCCGCTCCTAAAAATCGCAAACGGCGC
Sebastes umbrosus               ATGGCAAGTCTACGAAAGACACACCCCCTCCTCAAAATCGCAAACAATGC
                                *****    ** ** **  * ** **  *  * ** ** ** **    **

Salvelinus alpinus              ACTAGTCGACCTCCCTGCCCCCTCTAATATCTCAGTCTGATGAAACTTTG
Brachymystax lenok              ACTAGTTGACCTTCCAGCACCCTCTAATATCTCAGTCTGATGAAACTTTG
Salmo salar                     ACTAGTCGATCTCCCAGCACCATCTAACATCTCAGTTTGATGAAACTTTG
Oncorhynchus keta               ACTAGTCGACCTCCCAGCACCATCTAACATCTCAGTCTGATGAAACTTTG
Gadus morhua                    ATTAGTTGATCTCCCCGCCCCCTCCAATATCTCAGTATGATGAAATTTTG
Amphilophus alfari              ACTAGTCGACCTTCCCGCCCCCTCAAACATCTCTGTTTGATGAAACTTTG
Paraneetroplus siebold          ACTAGTTGATCTCCCCACGCCCTCAAACATCTCCATCTGATGAAATTTCG
Tomocichla tuba                 ACTAGTTGACCTTCCTACACCCTCAAACATCTCCGTTTGATGAAACTTTG
Poeciliopsis monacha            ATTAGTAGATCTCCCCGCCCCCGTTAATATCTCCGCCTGATGAAACTTCG
Saurida elongata                ACTAGTAGATCTCCCCGCCCCCTCCAATATTTCAGCCCTATGAAACTTCG
Chlorophthalmus albatrossis     CCTAGTTGACCTTCCCGCCCCCTCGAACATTTCAGCTTGATGAAACTTCG
Chlorophthalmus sp              TTTGGTTGACCTTCCTGCTCCCTCTAACATCTCAGCATGGTGAAACTTCG
Trachinocephalus myops          ACTTGTTGACCTCCCCGCACCCTCCAACATCTCTGCATGGTGGAACTTTG
Synodus ulae                    CCTAGTTGATCTCCCAGCGCCCTCAAATATCTCGGCTTGATGGAATTTTG
Synodus hoshinonis              ACTGGTTGACCTACCAGCACCATCCAACATTTCCGCCTGATGAAATTTCG
Sebastes umbrosus               CCTAGTTGACCTACCCGCCCCCTCAAATATTTCAGTGTGATGAAACTTCG
                                  * ** ** ** **  * **    ** ** **       ** ** ** *

Salvelinus alpinus              GTTCACTCTTAGGCCTATGTTTGGCCACCCAAATTCTTACCGGACTCTTC
Brachymystax lenok              GATCACTCCTAGGCTTATGTCTAGCCACCCAAATTCTCACCGGACTCTTC
Salmo salar                     GCTCACTCTTAGGCCTATGTCTAGCCACCCAAATCCTTACCGGGCTCTTC
Oncorhynchus keta               GTTCACTCCTAGGCTTATGCCTAGCCACCCAAATTCTTACCGGGCTCTTC
Gadus morhua                    GCTCTCTTCTAGGCCTTTGCTTAATTACTCAACTTCTAACAGGACTATTT
Amphilophus alfari              GCTCCCTACTAGGACTCTGCCTTGCCGCCCAAATCCTAACAGGCTTATTT
Paraneetroplus siebold          GCTCCCTACTAGGACTTTGTCTCGTCACCCAAATTTTAACGGGCCTATTT
Tomocichla tuba                 GCTCCTTATTAGGACTATGCCTCGTCGCTCAAATTTTAACAGGCCTATTC
Poeciliopsis monacha            GCTCCCTCCTAGGCCTCTGCCTTATTGCCCAAATTATAACTGGCCTATTT
Saurida elongata                GATCTCTCCTAGGCCTTTGCCTAATCACCCAGATCGTTACAGGACTCTTC
Chlorophthalmus albatrossis     GCTCCCTACTCGGGCTTTGTCTCGCTACACAAATTCTCACAGGATTATTC
Chlorophthalmus sp              GTTCCTTGCTTGGGCTATGTCTGGCCACACAAATTCTTACCGGGTTATTT
Trachinocephalus myops          GCTCTTTATTGGGCCTTTGCTTAGCGACTCAGATTCTGACTGGCCTCTTT
Synodus ulae                    GGTCCTTACTAGGCTTATGCCTGGCCACCCAAATCTTAACAGGCTTGTTC
Synodus hoshinonis              GGTCCCTCCTAGGCCTATGTCTAGCGACCCAAATCTTAACAGGCTTATTC
Sebastes umbrosus               GCTCTCTCTTAGGACTCTGCTTAATTATCCAAATCCTCACAGGACTATTT
                                * **  *  * **  * **  *      **  *  * ** **  * **
```

Fig 3.1 Aligned DNA sequences from 1137 bp of the cytochorme *b*.

```
Salvelinus alpinus              CTAGCCATACACTACACCTCCGATATTTCAACAGCTTTTTCCTCTGTGTG
Brachymystax lenok              CTAGCCATGCACTATACCTCTGACATTTCAACAGCTTTTTCCTCCGTCTG
Salmo salar                     CTAGCCATACACTACACCTCCGATATCTCAACAGCTTTTTCCTCTGTTTG
Oncorhynchus keta               CTAGCCATGCACTACACCTCCGACATTTCAACAGCTTTTTCCTCTGTCTG
Gadus morhua                    CTAGCCATACACTATACCTCAGACATCGAGACAGCCTTCTCATCCGTAGT
Amphilophus alfari              CTTGCAATACACTACACTTCCGACATCGCAACAGCCTTTTCATCCGTTGC
Paraneetroplus siebold          CTTGCAATACACTATACCTCCGACATCGCAACAGCCTTTTCATCTGTTGC
Tomocichla tuba                 CTTGCAATACACTACACTTCCGACATCGCAACAGCCTTTTCATCCGTCAC
Poeciliopsis monacha            TTAGCAATACACTACACCTCCGATATCTCTATAGCATTTTCATCCGTGGC
Saurida elongata                CTAGCTATACACTATACCTCCGATGTTGCTACTGCTTTCTCCTCCGTCGC
Chlorophthalmus albatrossis     TTGGCTATACATTACACTTCCGACATCGCGACTGCCTTCTCTTCCGTAAC
Chlorophthalmus sp              CTCGCTATGCACTACACCTCTGACATCGCAACAGCCTTCTCTTCAGTCAC
Trachinocephalus myops          CTTGCTATGCACTATACTTCTGACGTTGCCACCGCTTTTTCCTCCGTCAC
Synodus ulae                    TTAGCCATGCATTATACCTCTGACATTGCAACAGCCTTCTCATCAGTAAC
Synodus hoshinonis              CTAGCTATACATTACACTTCTGATATCGCCACAGCCTTTTCATCCGTTAC
Sebastes umbrosus               TTAGCCATACACTACACCTCTGATATTGCTACAGCTTTTTCTTCCGTTGC
                                 * ** ** ** ** ** ** **  *    *  ** ** ** ** **

Salvelinus alpinus              CCATATCTGCCGAGATGTAAGTTACGGCTGACTCATCCGGAATATCCACG
Brachymystax lenok              CCATATCTGTCGAGACGTTAGTTACGGCTGACTTATCCGAAATATCCACG
Salmo salar                     CCACATTTGCCGAGATGTTAGCTATGGCTGACTCATCCGTAACATTCACG
Oncorhynchus keta               CCACATCTGCCGAGATGTTAGCTACGGCTGACTAATTCGAAACATCCACG
Gadus morhua                    CCACATCTGTCGTGATGTAAACTACGGCTGACTAATTCGGAATATACATG
Amphilophus alfari              CCACATCTGTCGAGACGTAAATTATGGCTGACTTATCCGCAACATGCACG
Paraneetroplus siebold          CCACATCTGTCGAGATGTAAACTACGGCTGATTAATCCGCAACATACACG
Tomocichla tuba                 CCACATCTGCCGAGATGTTAACTATGGCTGACTAATCCGCAACATGCACG
Poeciliopsis monacha            ACACATTTGCCGAGACGTCAACTACGGATGACTAATCCGCAATATACATG
Saurida elongata                CCACATCTGCCGTGACGTAAACTACGGCTGAATGATCCGGAATCTACACG
Chlorophthalmus albatrossis     TCATATCTGCCGGGACGTGAACTACGGCTGGCTAATCCGAAATATGCACG
Chlorophthalmus sp              CCATATTTGCCGGGACGTAAATTATGGTTGACTGATCCGAAACATGCATG
Trachinocephalus myops          TCATATTTGCCGAGACGTCAACTACGGCTGGTTGATTCGGAATATGCATG
Synodus ulae                    ACACATCTGCCGTGACGTCAACTATGGCTGGCTCATCCGAAATATACATG
Synodus hoshinonis              TCACATTTGCCGCGACGTAAACTATGGCTGACTAATCCGAAACATACACG
Sebastes umbrosus               CCACATTTGCCGGGACGTAAATTACGGGTGATTCATCCGAAATCTTCACG
                                 ** ** ** ** ** ** *  ** ** **  * ** ** **  * ** *

Salvelinus alpinus              CTAACGGAGCATCTTTCTTCTTTATCTGTATTTATATACATATCGCCCGA
Brachymystax lenok              CTAACGGAGCATCCTTCTTCTTTATCTGTATTTATATGCACATCGCCCGA
Salmo salar                     CTAACGGAGCATCTTTCTTCTTTATCTGTATTTATATACACATCGCCCGA
Oncorhynchus keta               CTAACGGAGCATCTTTCTTTTTTATTTGTATTTATATACATATCGCCCGG
Gadus morhua                    CTAATGGTGCCTCTTTCTTTTTCATTTGTCTTTATATGCACATTGCCCGA
Amphilophus alfari              CCAACGGCGCATCCTTCTTCTTCATCTGCATCTACCTTCACATTGGTCGA
Paraneetroplus siebold          CCAACGGCGCATCCTTTTTCTTTATCTGCATTTACCTTCACATTGGCCGA
Tomocichla tuba                 CCAACGGCGCATCCTTTTTCTTTATCTGCATTTACCTCCACATCGGCCGA
Poeciliopsis monacha            CCAACGGAGCCTCCTTCTTTTTCATCTGCATTTACCTTCACATCGGACGG
Saurida elongata                CTAACGGAGCTTCCTTCTTCTTTATTTGCATTTATATTCACATTGCACGC
Chlorophthalmus albatrossis     CTAACGGAGCATCCTTCTTCTTCATTTGCATCTATATGCACATCGCACGA
Chlorophthalmus sp              CCAATGGAGCATCCTTTTTCTTTATTTGCATTTATATGCACATCGCACGA
Trachinocephalus myops          CCAACGGGGCCTCCTTCTTCTTTATCTGCATTTATATGCACATCGCCCGC
Synodus ulae                    CCAACGGTGCATCCTTTTTCTTTATTTGCATTTATATACACATTGCTCGG
Synodus hoshinonis              CCAATGGGGCTTCTTTTTTCTTTATTTGCATTTACATGCATGTCGCCCGA
Sebastes umbrosus               CCAACGGTGCATCCTTCTTCTTTGTATGCATCTATGCCCACATTGGCCGC
                                * ** ** ** ** ** ** **  * **  * **    **  * *  **
```

Fig 3.1 (continued)

```
Salvelinus alpinus              GGACTCTACTACGGGTCCTACCTATATAAAGAAACCTGAAATATTGGAGT
Brachymystax lenok              GGACTCTACTACGGATCCTATTTATATAAAGAAACTTGAAATATTGGAGT
Salmo salar                     GGACTTTATTATGGTTCCTATCTATATAAAGAAACCTGAAATATCGGAGT
Oncorhynchus keta               GGACTTTATTACGGATCCTACCTGTACAAAGAAACCTGGAATATCGGAGT
Gadus morhua                    GGTCTCTATTATGGTTCCTATCTTTTTGTAGAGACATGAAACATCGGGGT
Amphilophus alfari              GGGTTATACTACGGCTCCTACCTTTACAAAGAAACATGAAACGTTGGAGT
Paraneetroplus siebold          GGACTATACTATGGCTCCTACCTCTATAAAGAAACATGAAATGTAGGAGT
Tomocichla tuba                 GGGTTATACTACGGCTCTTACCTCTATAAAGAAACATGAAACGTCGGAGT
Poeciliopsis monacha            GGCCTATACTACGGCTCTTACCTCTACAAAGAAACATGAAACATTGGAGT
Saurida elongata                GGCTTATACTACGGGTCTTATCTTTACATGGAGACCTGAAACATCGGAGT
Chlorophthalmus albatrossis     GGGCTTTATTATGGCTCATATTTGTACAAGGAAACATGAAACGTAGGTGT
Chlorophthalmus sp              GGACTTTACTACGGCTCATACCTCTATAAAGAAACCTGAAACGTTGGCGT
Trachinocephalus myops          GGCCTTTACTATGGTTCATACCTTTATAAGGAGACCTGGAACGTAGGTGT
Synodus ulae                    GGACTGTACTATGGGTCATACCTCTACATAGAGACCTGAAATATCGGGGT
Synodus hoshinonis              GGCCTCTACTACGGCTCATACCTCTACATAGAAACCTGAAATATTGGTGT
Sebastes umbrosus               GGACTTTACTACGGCTCATACCTCTATAAAGAAACATGAAACATCGGGGT
                                **  * ** ** ** ** **  * *    ** ** ** **  * ** **

Salvelinus alpinus              AGTATTACTACTTCTAACTATAATGACTGCCTTTGTAGGCTACGTTCTTC
Brachymystax lenok              AGTACTACTACTTCTCACCATGATAACTGCCTTTGTCGGCTATGTCCTTC
Salmo salar                     TGTACTTCTACTTCTCACTATAATAACTGCCTTCGTAGGCTACGTTCTTC
Oncorhynchus keta               TGTACTTTTACTTCTCACTATAATAACTGCATTCGTGGGCTACGTCCTCC
Gadus morhua                    TGTCCTTTTCCTTTTAGTAATAATAACCTCTTTCGTAGGTTATGTCCTCC
Amphilophus alfari              TATCCTTCTCCTCCTAACCATAATAACTGCTTTCGTAGGCTATGTCCTCC
Paraneetroplus siebold          TGTCCTCCTCCTTTTAACCATAATAACCGCATTCGTAGGCTATGTCCTTC
Tomocichla tuba                 GATCCTTCTCCTCTTAACAATAATAACTGCATTCGTAGGCTACGTCCTCC
Poeciliopsis monacha            AGTACTTCTTCTACTTGTTATAATAACCGCCTTCGTCGGATACGTCCTCC
Saurida elongata                AATTCTGCTTCTTCTAGTAATAATAACTGCCTTCGTTGGTTATGTTCTCC
Chlorophthalmus albatrossis     CATCTTATTACTTCTCGTTATGATGACTGCCTTCGTCGGCTACGTTCTGC
Chlorophthalmus sp              TATCCTATTACTTCTCGTCATGATAACCGCTTTCGTAGGCTACGTCCTCC
Trachinocephalus myops          GATCCTCCTCCTTCTTGTCATGATGACCGCCTTTGTCGGCTACGTCCTAC
Synodus ulae                    TATTCTTCTTCTCCTTGTAATGATGACAGCGTTTGTAGGTTACGTACTCC
Synodus hoshinonis              CGTTCTTCTTCTACTCGTAATATTAACCGCTTTCGTCGGCTACGTTCTTC
Sebastes umbrosus               AGTTCTATTACTTCTAGTTATAATAACTGCTTTCGTCGGTTATGTGCTAC
                                  *  *  * **  *    **  * **  * ** ** ** ** ** ** *

Salvelinus alpinus              CATGAGGGCAAATATCCTTCTGAGGAGCCACTGTAATCACAAACCTCCTC
Brachymystax lenok              CATGAGGACAAATATCCTTCTGAGGAGCCACTGTAATCACCAACCTCCTA
Salmo salar                     CATGAGGACAAATATCCTTCTGAGGAGCCACTGTAATTACAAACCTCCTC
Oncorhynchus keta               CGTGAGGACAAATATCCTTCTGAGGAGCCACTGTAATTACAAACCTTCTT
Gadus morhua                    CCTGAGGACAAATATCATTCTGAGGAGCTACCGTAATTACGAATTTAATA
Amphilophus alfari              CATGAGGACAAATATCCTTCTGAGGCGCCACCGTCATCACCAACCTCCTC
Paraneetroplus siebold          CATGAGGACAAATGTCCTTCTGAGGGGCTACCGTTATTACCAACCTCCTC
Tomocichla tuba                 CATGAGGACAAATATCCTTTTGAGGTGCTACCGTCATCACCAACCTCCTC
Poeciliopsis monacha            CATGAGGACAAATATCCTTCTGAGGAGCTACCGTAATTACCAACCTCCTC
Saurida elongata                CTTGAGGACAAATATCCTTTTGGGGGGCCACAGTTATTACTAACCTATTA
Chlorophthalmus albatrossis     CCTGAGGACAAATGTCTTTCTGAGGGGCAACTGTAATTACCAACCTCTTG
Chlorophthalmus sp              CCTGAGGACAAATGTCTTTCTGAGGAGCAACCGTAATTACCAATCTTCTG
Trachinocephalus myops          CTTGAGGTCAGATGTCCTTCTGAGGCGCCACCGTCATCACCAACCTTCTT
Synodus ulae                    CTTGAGGTCAAATGTCTTTCTGAGGGGCGACTGTAATTACCAACCTTTTA
Synodus hoshinonis              CCTGAGGACAAATGTCGTTTTGAGGTGCAACCGTCATCACCAACCTCATA
Sebastes umbrosus               CCTGAGGCCAAATATCCTTTTGAGGTGCCACCGTTATCACCAACCTACTC
                                * ***** ** ** ** ** ** ** ** ** ** ** ** **  *  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              TCCGCTGTCCCTTACGTAGGAGGTGCCCTTGTACAATGAATTTGAGGCGG
Brachymystax lenok              TCCGCCGTCCCATATGTTGGAGGCGCCCTAGTACAATGAATTTGAGGAGG
Salmo salar                     TCCGCTGTCCCCTACGTAGGAGGCGCCCTTGTACAATGAATTTGAGGAGG
Oncorhynchus keta               TCCGCTGTCCCCTACGTAGGAGGCGCCCTAGTACAATGAATTTGAGGCGG
Gadus morhua                    TCTACTGTTCCTTATGTAGGTGATGCCTTAGTTCAATGGATCTGAGGAGG
Amphilophus alfari              TCCGCTATCCCGTACATCGGCAATTCCCTTGTCCAATGACTCTGAGGTGG
Paraneetroplus siebold          TCCGCAATTCCTTACATTGGCAACTCTCTAGTTCAATGGCTTTGAGGTGG
Tomocichla tuba                 TCCGCAGTCCCTTACATTGGCAACTCCCTGGTCCAATGACTTTGAGGTGG
Poeciliopsis monacha            TCCGCTGTCCCTTATATAGGAAACACCCTTGTCCAATGAATCTGAGGTGG
Saurida elongata                TCCGCCGTCCCCTACGTAGGCACCACCCTCGTCGAATGAATTTGGGGAGG
Chlorophthalmus albatrossis     TCCGCAGTCCCATACGTAGGAAATGCCCTAGTTCAATGGATCTGAGGGGG
Chlorophthalmus sp              TCCGCAGTCCCATACGTTGGGAACGCCTTAGTCCAATGAATTTGAGGGGG
Trachinocephalus myops          TCTGCCGTCCCCTACGTCGGCAACACCCTTGTACAGTGAATTTGGGGTGG
Synodus ulae                    TCTGCCGTCCCATACGTTGGTAATACCCTTGTCCAATGAATTTGAGGCGG
Synodus hoshinonis              TCCGCCGTCCCATATGTTGGTAACACCCTGGTCCAATGGATTTGAGGTGG
Sebastes umbrosus               TCTGAAGTACCCTACGTAGGTAACGCCCTTGTTCAATGAATTTGAGGTGG
                                **     * ** **  * **     *  * **  * **  * ** ** **

Salvelinus alpinus              ATTTTCTGTAGACAACGCCACCCTAACCCGATTTTTCGCCTTTCACTTCC
Brachymystax lenok              ATTCTCCGTAGACAACGCTACCCTGACACGATTTTTCGCCTTCCACTTCC
Salmo salar                     ATTTTCTGTAGACAACGCCACCCTAACACGATTTTTCGCCTTCCACTTCC
Oncorhynchus keta               GTTCTCCGTTGACAACGCCACCCTAACACGATTTTTCGCCTTTCACTTCC
Gadus morhua                    TTTCTCAGTAGATAATGCTACCCTAACTCGGTTTTTTGCATTCCATTTCT
Amphilophus alfari              CTTCTCAGTAGACAATGCCACTCTCACCCGATTCTTTGCCTTCCACTTTC
Paraneetroplus siebold          CTTTTCAGTAGACAATGCCACCCTCACCCGATTCTTTGCCTTCCACTTCC
Tomocichla tuba                 CTTTTCAGTAGATAACGCCACCCTCACCCGATTCTTTGCCTTCCACTTCC
Poeciliopsis monacha            ATTTTCAGTAGACAACGCCACCCTAACCCGCTTCTTCGCCTTCCACTTCC
Saurida elongata                ATTTTCTGTAGACAAGGCCACCCTAACCCGATTCTTCGCCTTCCACTTCC
Chlorophthalmus albatrossis     CTTCTCTGTCGACAATGCCACGCTCACCCGATTCTTTGCCTTTCACTTCC
Chlorophthalmus sp              CTTCTCAGTTGATAACGCCACACTCACCCGATTCTTTGCCTTCCACTTCC
Trachinocephalus myops          GTTCTCGGTAGATAACGCCACGCTTACTCGCTTCTTTGCCTTCCACTTTC
Synodus ulae                    GTTTTCGGTCGATAATGCCACCCTCACCCGGTTCTTTGCATTTCATTTCT
Synodus hoshinonis              ATTCTCAGTAGACAACGCCACCTTAACCCGATTTTTCGCATTTCACTTTT
Sebastes umbrosus               GTTCTCAGTAGACAATGCAACCCTTACCCGATTCTTTGCTTTCCACTTTT
                                 ** ** ** ** ** ** **  * ** ** ** ** ** ** ** ** **

Salvelinus alpinus              TATTCCCCTTCGTTATTGCAGCCGCCACAGTACTTCACCTTCTATTTCTG
Brachymystax lenok              TATTCCCATTTATCATCGCAGCTGCCACAGTCCTTCACCTCCTATTCCTA
Salmo salar                     TATTCCCATTCGTTATTGCAGCTGCCACAGTACTCCATCTTCTATTTTTA
Oncorhynchus keta               TATTCCCTTTCGTCATCGCAGCTGCTACAGTCCTTCACCTCCTATTCCTT
Gadus morhua                    TATTCCCCTTTGTTGTTGCTGCTTTTACAATACTCCACCTACTTTTTCTC
Amphilophus alfari              TCCTCCCATTTATCATCGCAGCCATAACAATAATTCACCTAATCTTCCTA
Paraneetroplus siebold          TCCTTCCTTTCATCATTGCAGCCATAACAATAATCCACCTAATTTTTCTC
Tomocichla tuba                 TCCTCCCGTTCATCATTGCAGCCATAACAATAATCCACCTAATCTTCCTC
Poeciliopsis monacha            TCCTTCCCTTCATCGTTGCAGCAGCAACCTTAGTCCACCTCATTTTTTTA
Saurida elongata                TATTTCCCTTTGTAATCGTCGCCGTCACAGCTATTCACCTTCTTTTCTTA
Chlorophthalmus albatrossis     TCTTCCCATTCGTCATCGCCGCTGTAACAGTCATCCACCTGTTGTTCCTT
Chlorophthalmus sp              TCTTTCCATTTGTCATTGCGGCCGTAACAGTTATCCACCTACTGTTTCTC
Trachinocephalus myops          TCTTCCCCTTCGTCATCGCTGCGGTTACCGTTATCCACCTCCTATTTCTT
Synodus ulae                    TATTTCCCTTCGTTATCGCAGCGGTTACCGTAATTCACCTCTTATTCCTC
Synodus hoshinonis              TATTTCCATTCGTTATCGCAGCCGTAACTGTAATCCACCTCTTGTTCTTA
Sebastes umbrosus               TGTTCCCCTTTGTAATTGCAGCCGCAACCATAGTCCACCTCCTTTTCCTT
                                *  * ** **  *  * *  **    **     * ** **  * **  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              CATGAAACCGGGTCCAATAACCCAGCAGGGATTAACTCCGACGCCGACAA
Brachymystax lenok              CACGAAACCGGATCCAACAACCCAGCAGGCATTAACTCCGACGCCGATAA
Salmo salar                     CATGAAACCGGGTCTAATAACCCAGCAGGCATCAACTCCGATGCCGATAA
Oncorhynchus keta               CACGAGACAGGATCTAATAACCCGGCAGGAATTAACTCCGATGCCGATAA
Gadus morhua                    CATGAAACAGGCTCAAATAATCCCACAGGAATCAATTCAAATGCAGACAA
Amphilophus alfari              CACGAAACCGGCTCAACAAACCCAGCAGGCCTAAACTCCGACACAGACAA
Paraneetroplus siebold          CACGAAACCGGTTCCACAAACCCAGCAGGCTTAAACTCCGACACAGACAA
Tomocichla tuba                 CACGAAACCGGATCCACAAACCCAGCAGGCCTAAACTCCGACACAGACAA
Poeciliopsis monacha            CACGAAACAGGCTCCAACAACCCCACTGGTCTAAACTCTGACGCAGACAA
Saurida elongata                CATGAAACCGGCTCCAACAACCCGACCGGTATTAACTCTGACGTAGACAA
Chlorophthalmus albatrossis     CACGAGACCGGCTCCAACAACCCCGCAGGGATTAACTCGGACGCGGACAA
Chlorophthalmus sp              CACGAAACCGGATCCAATAACCCCGCCGGTATTAATTCGGACGCAGATAA
Trachinocephalus myops          CATGAGACGGGCTCCAACAACCCGGCGGGGATTAACTCGGACGCAGATAA
Synodus ulae                    CACGAGACCGGCTCAAATAACCCCGCTGGGATTAACTCGGATGCGGACAA
Synodus hoshinonis              CACGAAACCGGATCTAACAACCCAGCCGGGATCAACTCAGATGCCGATAA
Sebastes umbrosus               CACCAAACAGGATCAAACAACCCCCTGGGCCTAAATTCAGACGCAGATAA
                                **  * ** ** ** *  ** **    **  * ** **  *    ** **

Salvelinus alpinus              AATCTCATTCCACCCCTACTTCTCGTACAAAGACCTCCTCGGTTTCGTAG
Brachymystax lenok              AATCTCGTTTCACCCTTACTTCTCATACAAAGACCTCCTCGGGTTCGTAG
Salmo salar                     AATCTCATTCCACCCTTACTTCTCATATAAAGACCTCCTCGGATTTGTAG
Oncorhynchus keta               AATCTCGTTTCACCCTTACTTCTCGTACAAAGACCTCCTAGGGTTCGTAG
Gadus morhua                    AATTCCATTCCACCCATATTTCACCTACAAAGACCTGCTTGGCTTTGCTG
Amphilophus alfari              AATTTCTTTCCACCCCTACTTCTCCTACAAAGACCTCCTAGGCTTTGGAA
Paraneetroplus siebold          AATCTCATTCCACCCCTACTTTTCCTACAAAGACCTATTAGGCTTTGTAA
Tomocichla tuba                 AATCTCATTCCACCCCTATTTCTCCTACAAAGACCTCCTAGGCTTTGCAA
Poeciliopsis monacha            AATCTCTTTCCACCCTTACTTCTCCTATAAAGACCTCCTAGGCTTTGCCT
Saurida elongata                AATCGCGTTCCATCCGTACTTCATCTATAAAGACCTCCTAGGTTTTGTAA
Chlorophthalmus albatrossis     AATTTCCTTCCACCCATACTTCTCCTACAAAGACCTCCTCGGCTTTATTA
Chlorophthalmus sp              AATTTCATTCCACCCATATTTTTCCTACAAGGATCTCCTTGGCTTTATTA
Trachinocephalus myops          GATCTCCTTTCATCCCTATTTCTCGTACAAGGATCTTTTGGGCTTTATTA
Synodus ulae                    AATTTCTTTTCATCCCTACTTTTCGTATAAAGATCTGCTTGGCTTCGTTA
Synodus hoshinonis              GATCTCGTTCCACCCTTATTTTTCCTATAAGGACTTGCTAGGATTTGTTA
Sebastes umbrosus               AATAAGCTTCCACCCCTACTTCTCATATAAAGACTTATTAGGATTTGCAG
                                 **    ** ** ** ** **    ** ** **  *  * ** **

Salvelinus alpinus              CTATATTGCTTGGCCTAACAACCCTAGCTCTTTTCGCACCTAACCTCCTA
Brachymystax lenok              CTATACTACTAGGCTTAACATCCCTAGCTCTCTTTGCACCTAATCTTCTA
Salmo salar                     CCATACTACTTGGCCTAACATCCTTAGCTCTATTCGCACCCAACCTCCTC
Oncorhynchus keta               CCATACTTCTTGGTCTAACATCCTTAGCCCTCTTTGCACCAAACCTCCTG
Gadus morhua                    TGATGCTTCTGGGCTTAACCGCCCTCGCCCTCTTCGCACCTAATTTACTC
Amphilophus alfari              TCCTTCTCATTGCCCTAATCGCCCTAGCCCTCTTCTCCCCCAACCTTCTA
Paraneetroplus siebold          TCCTACTAGTTGCCCTAATTGCTTTAGCCCTCTTTTCCCCCAACCTCCTA
Tomocichla tuba                 TCCTACTCATCGCCCTAATCGCCCTAGCTCTCTTTTCCCCCAACCTCCTA
Poeciliopsis monacha            TCCTGCTTACCACACTAATTGCCCTCGCCCTCTTCTCCCCCAACCTATTA
Saurida elongata                TTATACTTGCTGGCCTATCCGCTCTAGCCTTCTTTTACCCCAACCTCCTC
Chlorophthalmus albatrossis     TCATGATGGTGGCCCTTACCTCCCTCGCCTTGTTTTCACCAAACCTTCTG
Chlorophthalmus sp              TTATGATAGTAGCCCTCACCTCCCTTGCTCTATTCTCCCCAAATCTTCTA
Trachinocephalus myops          TTCTGATGATAGCCCTCACCTCCCTAGCTTTATTTTCCCCTAACGTGCTC
Synodus ulae                    TCCTAATAATAGCCCTTACTTCCCTTGCCCTTTTTTCACCCAATCTTCTA
Synodus hoshinonis              TCCTTATGATAGCTCTTACCTCTTTAGCCCTGTTCTCACCCAACCTGCTA
Sebastes umbrosus               TACTTGTCATTGCCCTTACATGTCTAGCTTTATTCTCACCCAACCTACTG
                                   *  *        *        * **  * **    ** **  *  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              GGAGACCCGGACAATTTCACACCAGCCAACCCCCTAGTTACCCCGCCACA
Brachymystax lenok              GGAGACCCAGACAATTTTACACCCGCCAACCCCCTAGTCACCCCACCTCA
Salmo salar                     GGGGACCCAGACAATTTTACACCTGCCAACCCCCTAGTTACTCCACCTCA
Oncorhynchus keta               GGGGACCCGGACAATTTTACGCCCGCCAACCCACTAGTCACCCCGCCTCA
Gadus morhua                    GGAGATCCAGATAATTTCACCCCTGCTAACCCCATCGTTACCCCACCTCA
Amphilophus alfari              GGAGACCCAGACAACTTCACCCCCGCAAACCCCTTGGTCACTCCCCCACA
Paraneetroplus siebold          GGAGACCCAGACAACTTCACCCCAGCAAACCCCCTAGTCACTCCCCCGCA
Tomocichla tuba                 GGAGACCCAGACAACTTCACTCCCGCAAACCCCCTAGTTACTCCCCCCCA
Poeciliopsis monacha            GGAGACCCAGAAAACTTCACTCCTGCTAACCCACTTGTAACACCTCCTCA
Saurida elongata                GGAGATCCTGACAACTTCACCCCCGCCAATCCCTTAGTTACTCCCCCCCA
Chlorophthalmus albatrossis     GGGGACCCAGACAACTTCACCCCAGCCAACCCCCTTGTTACTCCCCCCCA
Chlorophthalmus sp              GGAGACCCAGACAACTTCACCCCGGCGAACCCGCTCGTCACCCCACCACA
Trachinocephalus myops          GGCGACCCAGACAACTTTACGCCCGCTAACCCCCTTGTCACCCCTCCTCA
Synodus ulae                    GGCGACCCAGATAACTTCACCCCCGCCAACCCCCTGGTGACTCCGCCCCA
Synodus hoshinonis              GGCGACCCCGATAACTTTACCCCCGCTAATCCCCTAGTTAATCCTCCCCA
Sebastes umbrosus               GGAGACCCAGACAACTTCACCCCCGCCAACCCACTAGTTACTCCTCCCCA
                                ** ** ** ** ** ** ** ** ** ** **  * ** *  ** ** **

Salvelinus alpinus              CATCAAGCCCGAATGGTACTTCTTATTCGCCTATGCAATTCTCCGATCTA
Brachymystax lenok              CATCAAACCCGAATGATACTTCTTGTTTGCTTATGCAATTCTACGGTCTA
Salmo salar                     TATCAAGCCTGAATGATACTTCCTATTCGCCTACGCAATCCTACGCTCCA
Oncorhynchus keta               TATCAAACCCGAATGATATTTCCTATTCGCTTACGCAATCCTACGCTCTA
Gadus morhua                    TGTTAAGCCCGAATGATATTTCTTGTTTGCCTATGCCATCTTACGCTCTA
Amphilophus alfari              CATCAAACCAGAATGATACTTCCTATTTGCTTACGCCATTCTCCGATCTA
Paraneetroplus siebold          CATTAAACCAGAATGATACTTCTTGTTTGCTTACGCCATCCTCCGATCAA
Tomocichla tuba                 CATTAAACCAGAATGGTACTTCCTATTTGCTTACGCCATCCTCCGATCAA
Poeciliopsis monacha            CATTAAACCCGAGTGATATTTTCTCTTCGCATACGCCATTCTTCGTTCTA
Saurida elongata                CATCAAACCGGAATGGTACTTTCTCTTCGCCTACGCCATCCTCCGCTCCA
Chlorophthalmus albatrossis     CATTAAGCCCGAGTGATATTTCCTCTTCGCCTATGCCATCCTCCGTTCCA
Chlorophthalmus sp              TATCAAACCAGAATGGTATTTCCTCTTCGCTTATGCCATTCTTCGTTCGA
Trachinocephalus myops          CATCAAGCCTGAGTGGTACTTCCTATTCGCCTACGCCATTCTTCGGTCTA
Synodus ulae                    TATTAAGCCAGAATGGTATTTTCTTTTTGCTTACGCCATCCTACGATCCA
Synodus hoshinonis              TATTAAACCAGAGTGATACTTTCTCTTTGCCTACGCCATTCTTCGCTCCA
Sebastes umbrosus               CATTAAGCCAGAATGATATTTCCTGTTCGCATATGCAATTCTACGCTCCA
                                  * ** ** ** ** ** **  * ** ** ** ** **  * ** ** *

Salvelinus alpinus              TCCCCAATAAACTAGGAGGGGTACTCGCCCTTTTATTCTCAATCCTCGTC
Brachymystax lenok              TCCCCAATAAGCTAGGCGGAGTACTCGCCCTCTTGTTCTCCATCCTCGTC
Salmo salar                     TTCCTAACAAACTAGGCGGAGTACTCGCCCTCTTATTCTCGATCCTGGTC
Oncorhynchus keta               TCCCCAACAAACTTGGAGGGGTACTCGCCCTTTTATTCTCAATCCTTGTC
Gadus morhua                    TTCCAAATAAGCTAGGTGGCGTACTTGCACTCCTATTCTCGATTCTAGTC
Amphilophus alfari              TTCCCAACAAACTAGGCGGAGTCCTAGCACTCCTTTTCTCCATCCTAATC
Paraneetroplus siebold          TCCCGAACAAGCTGGGAGGGGTCCTTGCACTTCTCTTTTCCATTCTAATC
Tomocichla tuba                 TCCCCAACAAACTAGGAGGAGTCCTCGCACTTCTTTTCTCGATTCTAATC
Poeciliopsis monacha            TTCCCAACAAGCTGGGAGGGGTTCTTGCTCTTCTAGCCTCTATTCTAGTT
Saurida elongata                TCCCTAACAAACTAGGAGGAGTTCTTGCACTTCTTGCATCTATTCTTGTC
Chlorophthalmus albatrossis     TCCCAAATAAGCTGGGGGGTGTCCTTGCCCTTCTAGCCTCCATCCTAGTC
Chlorophthalmus sp              TCCCCAACAAACTAGGCGGGGTGCTAGCTCTGCTAGCGTCCATTTTAGTT
Trachinocephalus myops          TTCCCAATAAGCTCGGCGGCGTACTCGCCCTCCTTGCCTCCATCCTCGTC
Synodus ulae                    TCCCAAACAAACTTGGCGGGGTCCTGGCCCTTTTGGCCTCGATCTTAGTA
Synodus hoshinonis              TTCCAAACAAACTTGGAGGAGTTCTGGCCCTTCTTGCCTCTATTTTAGTA
Sebastes umbrosus               TCCCCAATAAACTGGGGGGAGTTTTAGCCCTCCTAGCTTCAATCCTTATC
                                * ** ** ** ** ** ** **  * ** **  *    ** **  *  *
```

Fig 3.1 (continued)

```
Salvelinus alpinus              CTCATAGTTGTCCCGATCCTCCACACCTCTAAACAGCGCGGACTAACCTT
Brachymystax lenok              CTTATGGTTGTCCCCATCCTTCACACCTCTAAACAACGAGGGCTAACCTT
Salmo salar                     CTTATAGTCGTCCCCATCCTCCATACCTCTAAACAACGAGGACTGACCTT
Oncorhynchus keta               CTTATAGTTGTCCCCATCCTGCATACATCTAAACAACGAGGACTGACCTT
Gadus morhua                    CTCATGGTTGTACCCTTTCTCCATACGTCAAAACAACGAGGTTTAACATT
Amphilophus alfari              CTCATGCTCGTGCCAATCCTCCACACCTCAAAACTCCGAGCCCTTACCTT
Paraneetroplus siebold          CTTATACTAGTGCCAATCCTCCACACCTCAAAACTCCGAGCCCTTACCTT
Tomocichla tuba                 CTCATACTCGTGCCAATCCTCCACACCTCAAAACTTCAGACTCTTACCTT
Poeciliopsis monacha            CTGATAGCTGTTCCCTTCCTTCACACTTCTAAACAACGAAGCCTCACCTT
Saurida elongata                CTTATACTAGTCCCCGTTCTCCACACGTCTAAACAACGGGGCTTAATATT
Chlorophthalmus albatrossis     CTAATGCTGGTCCCTTTCCTCCATACCTCTAAACAGCGAGGACTAACATT
Chlorophthalmus sp              TTGATACTGGTCCCTTTTCTCCATACCTCAAAACAGCGTGGACTAACATT
Trachinocephalus myops          CTCATGCTCGTTCCCATCCTCCATACTTCCAAGCAGCGGGGACTAACTTT
Synodus ulae                    CTTATGCTCGTTCCAATTCTTCACACCTCAAAGCAGCGAGGACTTACTTT
Synodus hoshinonis              CTAATGCTTGTCCCAATACTACACACCTCTAAGCAGCGAGGACTTACCTT
Sebastes umbrosus               CTAATGCTCGTACCATTTCTACACACGTCTAAACAACGAAGCCTCACTTT
                                 * **    ** **  * ** ** ** ** ** *  *      * *  **

Salvelinus alpinus              TCGACCACTAACTCAATTCTTATTCTGAACCCTGGTAGCAGACATACTAA
Brachymystax lenok              TCGACCACTAACCCAATTCTTATTCTGAACCCTAGTAGCAGACATACTTA
Salmo salar                     TCGCCCACTCACCCAATTCTTATTCTGGACCCTGGTAGCGGACATACTAA
Oncorhynchus keta               TCGACCACTAACCCAATTCTTATTTTGAGCCTTAGTAGCAGATATACTCA
Gadus morhua                    CCGCCCTCTTACCCAAATACTATTCTGAGTCCTCGTTGCAGATATACTAG
Amphilophus alfari              CCGACCAATTACTCAATTTTTATTTTGACTCCTAGTTGCAGACGTCATTA
Paraneetroplus siebold          CCGACCCCTTACTCAATTCTTATTCTGACTCCTAATTGCAGACGTCATAA
Tomocichla tuba                 CCGACCACTCACTCAATTTTTATTCTGGCTCCTAGTTGCAGACGTCGTTA
Poeciliopsis monacha            CCGTCCTCTCACCCAAATCCTCTTTTGACTTTTAATCGCAGACGTAGCAA
Saurida elongata                CCGCCCTCTTACCCAACTCCTCTTCTGAACATTCGTAGCAGACGTGGTGA
Chlorophthalmus albatrossis     CCGGCCCTTAACACAAATTATTTTCTGAACATTTGTAGCTAACGTCATCA
Chlorophthalmus sp              CCGTCCATTGACACAAATTCTCTTTTGAACCTTTGTCGCCAATGTTATTA
Trachinocephalus myops          CCGCCCTCTGACCCAAGTACTCTTCTGGGCCTTTGTAGCCGACGTCATCA
Synodus ulae                    CCGACCCCTAACACAGTTTCTATTCTGAACTTTCGTAGCAGATGTCATTA
Synodus hoshinonis              TCGACCCCTAACACAATTTCTTTTCTGGACTTTTGTGGCAGATGTCATTA
Sebastes umbrosus               CCGACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTA
                                 ** **  * ** **  *  * ** **     *  * **  *  *

Salvelinus alpinus              TCCTCACCTGAATTGGGGGCATGCCTGTAGAACACCCATTTATCATTATC
Brachymystax lenok              TCCTCACCTGAATTGGTGGCATACCCGTAGAACACCCATTTATTATCATC
Salmo salar                     TCCTTACCTGAATTGGAGGCATACCCGTGGAACACCCATTCATTATCATT
Oncorhynchus keta               TCCTCACCTGAATCGGGGGCATACCCGTAGAACACCCGTTCATTATTATC
Gadus morhua                    TTCTTACATGAATTGGAGGCGTACCTGTAGAACACCCCTTCATTATCATC
Amphilophus alfari              TTCTAACCTGAATTGGAGGAATGCCTGTCGAACATCCCTTTGTTATCATT
Paraneetroplus siebold          TTTTAACCTGAATTGGAGGAATACCCGTCGAACACCCATTTATCATCATT
Tomocichla tuba                 TTTTGACTTGAATTGGAGGCATACCCGTTGAACACCCATTTATCATTATC
Poeciliopsis monacha            TCCTTACATGAATCGGAGGTATACCTGTTGAACACCCATTTATTATTATT
Saurida elongata                TCCTCACATGAATTGGAGGCATGCCGGTTGAACACCCCTTTATTATTATC
Chlorophthalmus albatrossis     TCCTGACGTGAATCGGAGGTATGCCAGTCGAACACCCCTTTATTATCATT
Chlorophthalmus sp              TTCTAACTTGAATCGGAGGTATGCCGGTTGAACACCCCTTCATTATTATT
Trachinocephalus myops          TTCTCACCTGGATCGGAGGCATACCAGTCGAACACCCTTTTATCATCATC
Synodus ulae                    TCCTCACATGAATTGGGGGTATACCAGTGGAGCACCCTTTTATTATTATT
Synodus hoshinonis              TCCTCACCTGAATTGGAGGCATGCCAGTAGAACATCCATTTATTATTATC
Sebastes umbrosus               TTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTCATTATT
                                 *  * ** ** ** ** **  * ** **    ** ** **  * ** **
```

Fig 3.1 (continued)

```
Salvelinus alpinus              GGCCAAGTTGCCTCTGTGATTTACTTCACCATCTTCCTAGTCCTCGCCCC
Brachymystax lenok              GGCCAAGTCGCCTCTGTAATTTACTTCACCATCTTCCTAATCCTCGCCCC
Salmo salar                     GGTCAAATTGCCTCTGTAATTTACTTTACTATCTTCCTAGTCCTTGCCCC
Oncorhynchus keta               GGCCAAATTGCCTCTGTAATCTACTTCACCATCTTCCTAGTTCTTTCCCC
Gadus morhua                    GGACAAGTGGCATCAGTACTATATTTCTCCCTCTTCCTAGTTTTATTCCC
Amphilophus alfari-sj           GGCCAAATTGCATCCTTCCTCTATTTCTCTATTTTCCTTATCTTACTGCC
Paraneetroplus siebold          GGCCAAATCGCATCTTTCCTCTATTTCTTCCTCTTCCTTATTTTACTGCC
Tomocichla tuba                 GGCCAAATCGCTTCCTTCCTCTACTTCTTCATATTCCTTATTTTACTGCC
Poeciliopsis monacha            GGTCAAGTAGCCTCCCTCCTCTACTTCTCCCTATTCCTAGTCCTCTCCCC
Saurida elongata                GGCCAGGTCGCTTCCTTCCTCTATTTCTTTCTGCTCTTGGTCTTAGTCCC
Chlorophthalmus albatrossis     GGTCAAGTAGCATCGTTCCTATACTTTCTTCTCCTTCTAGTACTCTCACC
Chlorophthalmus sp              GGGCAGGTAGCATCGTTTCTCTATTTCCTCCTCCTTTTAGTCCTATCGCC
Trachinocephalus myops          GGACAGGTAGCTTCCTTCCTATACTTTCTTCTTCTTCTCGTACTAGCCCC
Synodus ulae                    GGGCAAGTTGCCTCCTTCCTTTACTTCTTTCTACTGTTGCTATTGTCCCC
Synodus hoshinonis              GGACAAGTGGCCTCTTTCCTTTACTTCTTCCTACTTCTTTTGCTATCTCC
Sebastes umbrosus               GGACAAGTTGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACC
                                ** **  * ** **  *  * ** **     *  *  *  *  *    **

Salvelinus alpinus              CTTAGCCGGTTGGGCCGAAAATAAAGCCCTTGAATGA
Brachymystax lenok              CTTAGCCGGATGGGCCGAGAATAAAGCCCTCGAATGA
Salmo salar                     CCTGGCTGGCTGAGCTGAAAATAAAGCTCTTGAATGA
Oncorhynchus keta               CTTAGCCGGCTGGGCCGAAAATAAAGCCCTCCAATGA
Gadus morhua                    CCTTGCAGGAATAACTGAAAATAAGGCCCTTGAATGA
Amphilophus alfari-sj           CATCTCTGGACTAGCAGAAAATAAAATATTTGCATAA
Paraneetroplus siebold          CATTGCCGGACTAGCAGAAAATAAAATATTTGCATAA
Tomocichla tuba                 CACCGCCGGACTAGCAGAAAATAAAATATTTGCATAA
Poeciliopsis monacha            CACTGCAGCATGAGTAGAAAATAAAATCCTCGGATGA
Saurida elongata                CGCTGCAGGGTGACTAGAAAATAAAGCACTTGAATGA
Chlorophthalmus albatrossis     CTTAGCGGGCTGAGTGGAGAATAAAGCCCTTGATTGA
Chlorophthalmus sp              CCTTGCGGGATGGGTAGAGAACAAGGCCCTTAAATGA
Trachinocephalus myops          CCTCGCCGGCTGGGTTGAGAATAAGGCCCTTGAATGA
Synodus ulae                    AATTGCAGGGTGATTAGAAAATAAAGCCCTTGAATGA
Synodus hoshinonis              TATGGCAGGCTGACTGGAAAATAAAGCCCTCGAATGA
Sebastes umbrosus               ACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGA
                                   * *        **  * **     *    * *
```

Fig 3.1 (continued)

used in the phylogenetic analysis were as follows: NC000861 *Salvelinus alpinus*, AF125213 *Brachymystax lenok*, U12143 *Salmo salar*, AF125212 *Oncorhynchus keta*, NC002081 *Gadus morhua*, AF009950 *Amphilophus alfari*, AF009937 *Paraneetroplus siebold*, AF009941 *Tomocichla tuba*, AF047346 *Poeciliopsis monacha*, and AF031516 *Sebastes umbrosus*. For the NJ analyses, Kimura's two-parameter distance correction (Kimura, 1980) was made by using all positions of a codon and excluding gap sites. Statistical confidence of the phylogenetic tree was assessed using bootstrapping (1000 iterations).

3.2.3 Cytogenetic analysis

In order to investigate cytogenetic features of these species of Aulopiformes, I made preparation of chromosomes in *Synodus ulae*, *Synodus hoshinonis*, and *Trachinocephalus myops* and *Chlorophthalmus albatrossis*. To increase the number of metaphase cells, animals were colchicinized by an intraperitoneal injection of colchicine solution three hours before preparation. Kidneys were then removed and minced. Kidney cell suspension was treated by hypotonic solution (75 mM KCl) for 30 min at room temperature and was fixed with methanol and acetic acid (3 :1). The fixed materials were dropped onto precleaned slides and were air-dried. Slides were stained by Giemsa solution. In metaphase cells, the nuclear organizer regions (NORs) were observed by the silver staining method (Howell and Black 1980), and the constitutive heterochoromatin was demonstrated by the

C-banding method (Sumner 1972). I observed 20 to 30 cells of metaphase for each species.

3.3 Results

3.3.1 Phylogenetic analysis

The topology of the phylogenetic tree suggested that the six Aulopiform species examined formed a monophyletic group, although the bootstrap value at the branching point between a cluster which consist of the genus of *Saurida* and the other five species was a bit low (Fig. 3.2). I also found that the two synchronous hermaphroditic species of the order Aulopiformes were grouped as a single cluster with a high bootstrap value (99%). On the other hand, six gonochoristic species were separated into four clusters. One cluster (called "cluster 1") contained *Trachinocephalus myops* and the three hermaphroditic species. The second cluster (called "cluster 2") was composed of the two gonochoristic species, *Synodus ulae* and *Synodus hoshinonis*. The third cluster (called "cluster 3") contains one gonochorisitc species, *Aulopus japonicus*. The last cluster (called "cluster 4") contains two gonochoristic species, *Saurida* sp. and *Saurida elongata*.

3.3.2 Cytogenetic analysis of sex chromosomes

It is generally believed that heteromorphic sex chromosomes of fish are

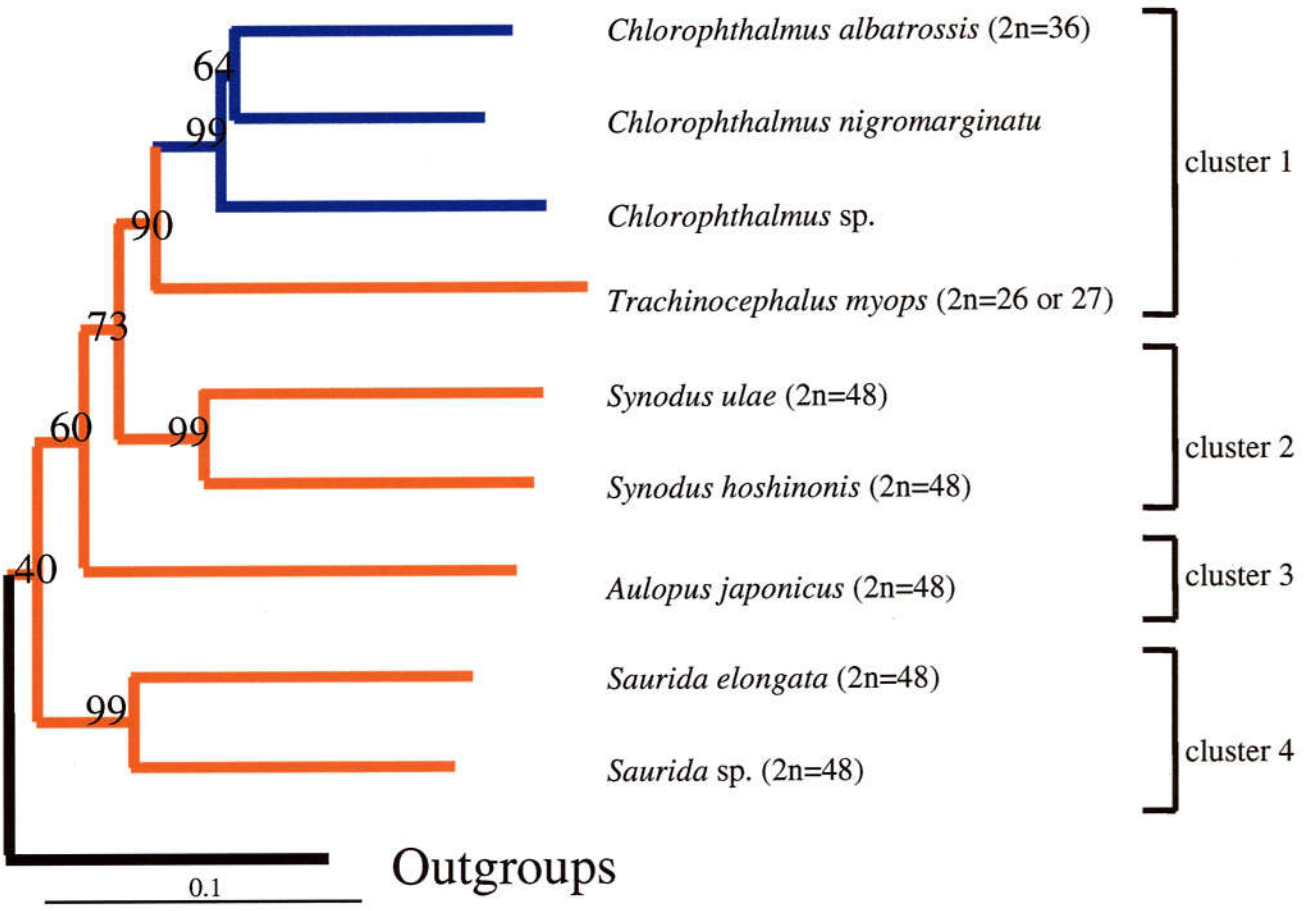


Fig. 3.2 Phylogenetic tree cytochrome *b* in the order Aulopiformes reconstructed by the neighbor-joining method.
A gray box indicated hermaphroditic species.
A white box indicated gonochoristic species.
The numbers in parantheses indicate the number of diploid chromosomes.

indistinguishable under the microscope, whereas *Saurida elongata* was suggested to have one microchromosome as a possible sex chromosome in the female (Nishikawa and Sakamoto 1978). At first, I inspected cytogenetic features in three gonochoristic species (*Synodus ulae, Synodus hoshinonis* and *Trachinocephalus myops*; in Fig. 3.2). Surprisingly, I found that heteromorphic sex chromosomes are present in all three species. Herein, I investigated these heteromorphic sex chromosomes in detail by the C-band and the silver staining methods, and I obtained clearly stained metaphases in several preparations. In fact, the microchromosome in the female of *Synodus ulae* was stained in the whole region by the C-band staining method (Fig. 3.3). Likewise, by the silver staining method, the microchromosome in the female of *Synodus hoshinonis* was clearly detected in the entire region except centromeric region (Fig. 3.4). Because these microchromosomes stained by the C-band or silver staining methods were not detected in the males of *Synodus ulae* and *Synodus hoshinonis*, I propose that these microchromosomes are heteromorphic sex chromosomes. I also found that only female individuals had heteromorphic sex chromosomes in *Trachinocephalu myops* because single acrocentric and subtelocentric chromosomes in only females were stained by the C-band staining method (Fig. 3.5). As a result, gonochoristic species were found to have generally heteromorphic sex chromosomes. On the other hand, heteromorphic chromosomes were not found in any of the hermaphroditic species (Fig. 3.6), implying that the sex chromosomes do not exist in the hermaphroditic species.

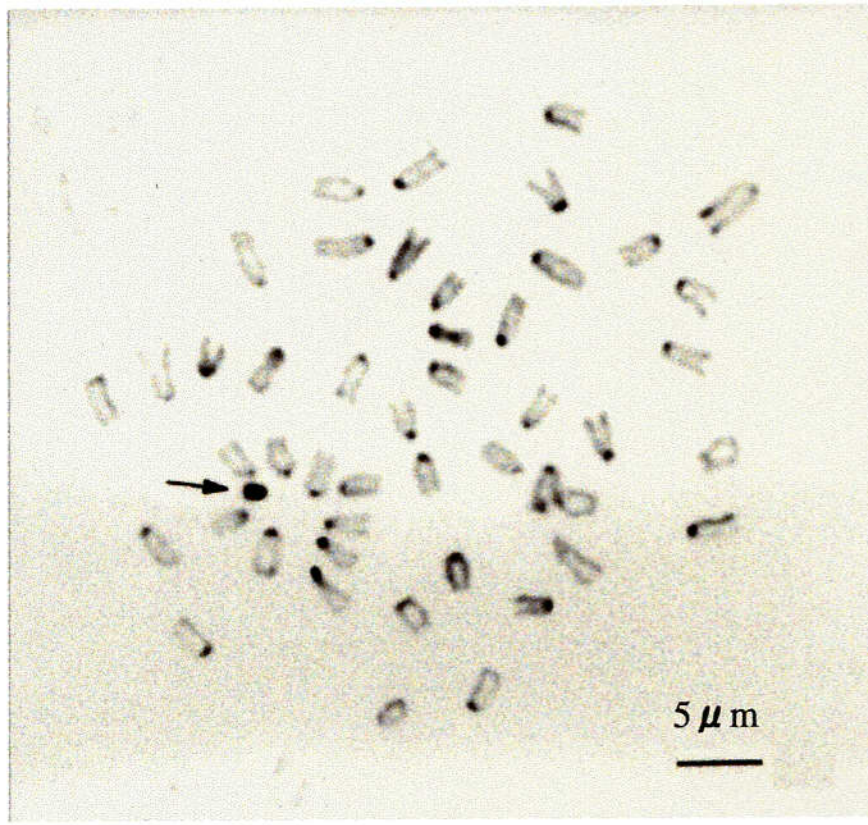


Fig. 3.3 C-band staining of metaphase chromosomes of a female in *Synodus ulae*. The arrow indicates a hetetochromatin region.

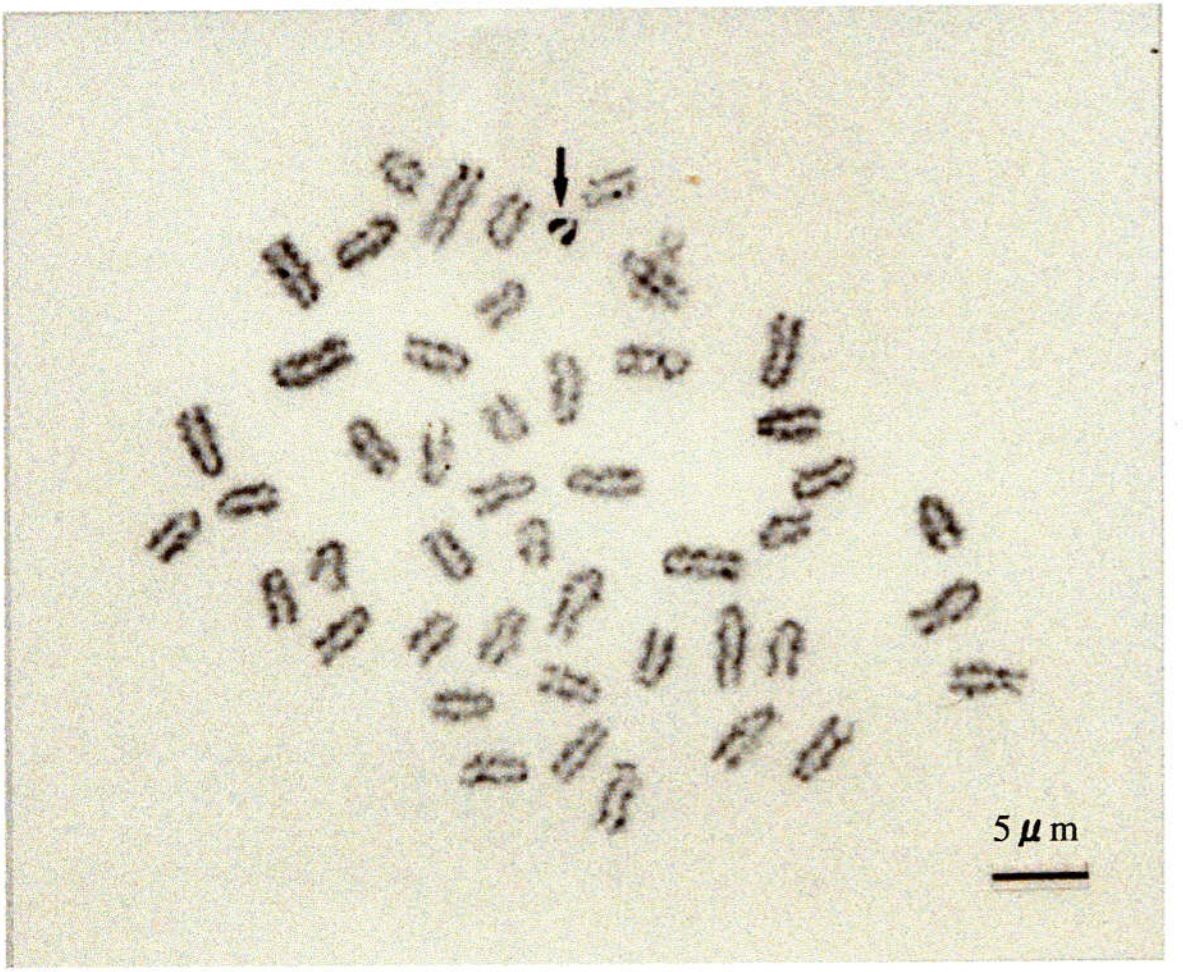


Fig. 3.4 Silver staining of metaphase chromosomes of a female in *Synodus hoshinonis*. The arrow indicates the NORs region.

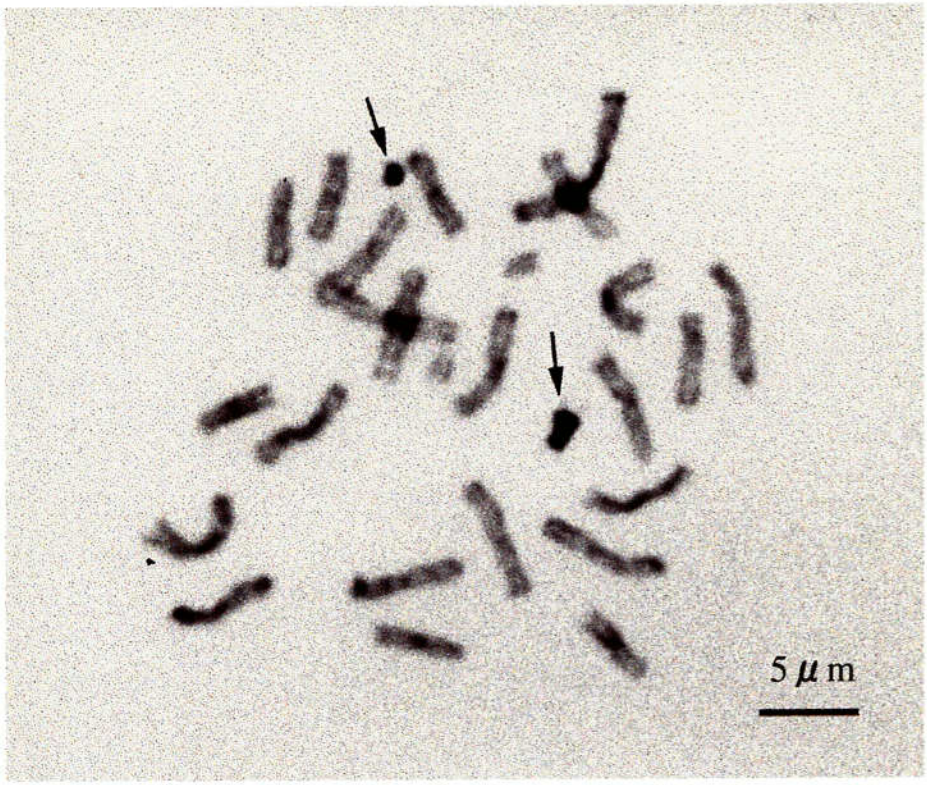


Fig. 3.5 C-band staining of metaphase chromosomes of a female in *Trachinocephalus myops*. Arrows indicate the heterochromatin region.

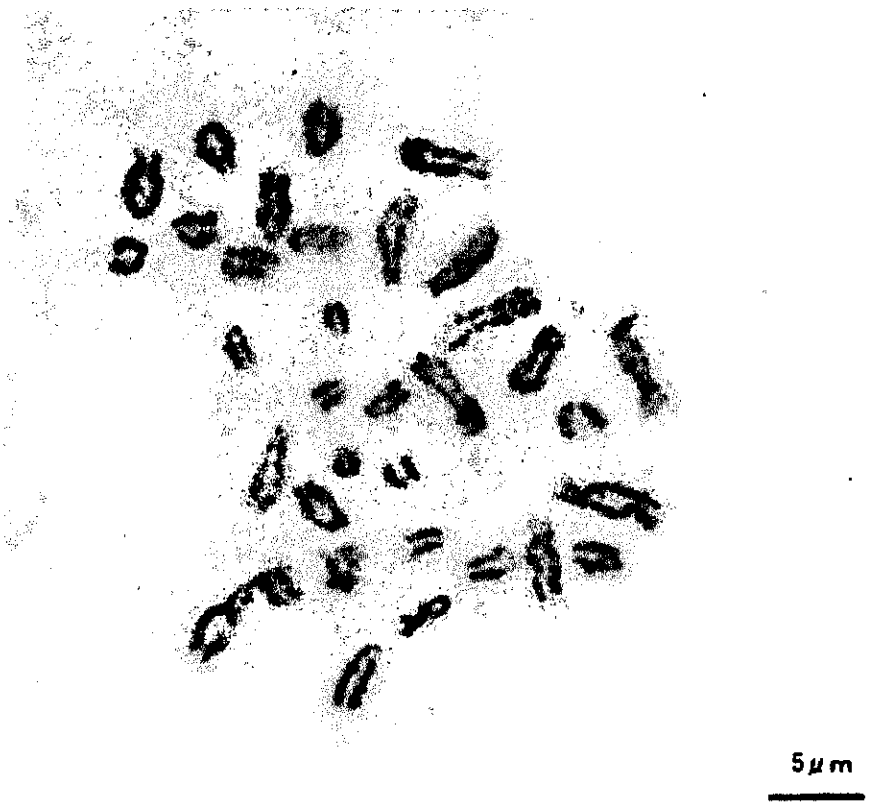


Fig. 3.6 Silver staining a metaphase chromosome in *Chlorophthalmus albatrossis.*

3.3.3 The number of diploid chromosomes

I counted the number of diploid chromosomes in three gonochoristic species and one hermaphroditic species. In both males and females, *Synodus ulae* and *Synodus hoshinonis* had 48 chromosomes (Figs. 3.3-4). For *Trachinocephalus myops*, I found a difference in the number of chromosomes between males and females: Males had 26 chromosomes whereas females had 27 chromosomes (Fig 3.5). For the hermaphroditic species (*Chlorophthalmus albatrossis*), I found 36 chromosomes (Fig. 3.6).

Because a female of *Saurida elongata* has been reported to have 48 chromosomes (Nishikawa and Sakamoto 1978), all the three species, *Synodus ulae*, *Synodus hoshinonis* and *Saurida elongata*, have the same number of chromosomes. Thus, the common ancestor of the two genera of *Saurida* and *Synodus* was considered to have 48 chromosomes. Since the numbers of chromosomes for *Trachinocephalus myops* and *Chlorophthalmus albatrossis* differ from those of the Saurida and Synodus species significantly, it is highly probable that the lineage leading to cluster 1 has been subjected to extensive chromosomal rearrangements, so that the chromosome numbers may have been reduced.

3.4 Discussion

According to Smith's proposal (1975), gonochoristic species were expected to have been derived from hermaphroditic species. However, our molecular phylogenetic analysis clearly indicated that hermaphroditic species were evolutionarily derived from gonochoristic species. Although it is difficult to estimate the time of emergence of hermaphroditic species in Aulopiformes, the phylogenetic tree obtained (Fig. 3.2) suggests they emerged immediately after other Aulopiform species had diverged from the common ancestor.

Our cytogenetic data indicates that gonochoristic species had heteromorphic sex chromosomes. According to a previous paper (Nishikawa and Sakamoto 1978), *Saurida elongata* was reported to have one microchromosome as a possible sex chromosome in a female. Those facts indicated that the microchromosomes were retained in the genus of *Saurida* and *Synodus*. Thus, it can be inferred that the common ancestor of the genus of *Saurida* and *Synodus* possessed the microchromosomes. In other words, heteromorphic sex chromosomes had been established once in the evolutionary course of the order of Aulopiformes. However, our cytogenetic analysis as well as molecular phylogenetic analysis indicated that the hermaphroditic species might have lost the heteromorphic sex chromosomes during evolution. Thus, I suggest a possibility that heteromorphic sex chromosomes can disappear from the genome even if they have appeared once in evolution.

Here, I focused on the number of chromosomes to examine the disappearance process of sex chromosomes. I pointed out that chromosome numbers of the species in cluster 1 (2n = 26 to 36) differ from clusters 2, 3 and 4 (2n = 48), indicating that large scale rearrangement of chromosomes has occurred in the lineage of cluster 1. Thus, it is quite possible that disappearance of sex chromosomes was accomplished along with the large-scale rearrangement of chromosomes.

Taking into account Ohno's hypothesis (1967) that heteromorphic sex chromosomes might have emerged from autosomes, I propose the hypothesis that heteromorphic sex chromosomes have undergone repeated events of appearance and disappearance in the evolutionary course of fish.

Chapter 4

Estimation of genome size in the order Aulopiformes

4.1 Introduction

According to Ohno (1970), genome duplication hardly occurs in mammals, bird and reptiles, which have highly differentiated sex chromosomes, because genome duplication disrupt the sex determination systems of them. On the other hand, in amphibian and fish species, which have not highly differentiated sex chromosomes, genome duplication is allowed.

Many researchers have observed various genome sizes in fish species (Ohno 1970; Hingegardner and Rosen 1972; Tiersch et al. 1989; Vadim et al. 1993; Ronchetti et al. 1995; Suzuki et al. 1995; Alexander 1998; Lamatsch et al. 2000). The large variation of genome size is due to duplication, deletion and large-scale rearrangement in chromosomes.

In Chapter 3, I mentioned that gonochoristic aulopiform species have female heteromorphic sex chromosomes. I also described that hermaphroditic species are different from gonochoristic species in the

number of diploid chromosomes. The data of the number of diploid chromosomes suggest that large-scale chromosome rearrangements have occurred in evolution. To investigate the large-scale chromosome rearrangement in detail, I estimated the genome sizes of gonochristic and hermaphroditic species, and compared one with the other and compared gonochristic species with hermaphroditic species.

4.2 Materials and methods

I have chosen two aulopiform species, *Synodus hoshinonis* (gonochoristic species) and *Chlorophthalmus albatrossis* (hermaphroditic species), for the comparative analysis. First, whole blood was taken from by a heparinized syringe and fixed by 99% ethanol. The fixed blood was then stained by propidium iodide. Fixed and stained whole blood of *Oncorhyncus mkissis* was used as control. Since the fluorescent intensity corresponds to the amount of DNA, I measured the fluorescent intensity of propidium iodide by using EPICS ALTRA flow cytometrer (Beckman Coulter). To estimate the genome size, I compared the samples from *S. hoshinonis* and *C. albatrossis*) with control (*O. mkissis*) in the fluorescent intensity of propidium iodide.

4.3 Results

Both gonochoristic and hermaphroditic species showed a half of the fluorescent intensity of the control (Fig. 4.1 and 4.2). The result indicates that there is no obvious difference between the gonochoristic and hermaphroditic species in the genome size. The genome size of gonochoristic and hermaphroditic species are a half of the *O. mkissis* of genome size.

4.4 Discussion

There are many reports about the genome size in fish species (Ohno 1970; Hingegardner and Rosen 1972; Tiersch et al. 1989; Vadim et al. 1993; Ronchetti et al. 1995; Suzuki et al. 1995; Alexander 1998; Lamatsch et al. 2000). Since the reported data of DNA contents were indicated in weight per cell (picogram/cell), I converted my data to DNA contents (pg/cell) to compare them with the reported data. It is known that *O. mkissis*, has 5.5 pg/cell in DNA contents (Pierrez and Ronot 1991). Taking into account this value (5.5 pg/cell), I could calculate the weight of DNA per cell for the two. My estimation indicates that both the gonochoristic and hermaphroditic specie have 2.75 pg/cell in DNA contents.

According to Hingerdner and Rosen (1972), two gonochoristic aulopiform species (*Synodus lucioceps* and *Synodus foetens*) have 2.4 pg/cell.

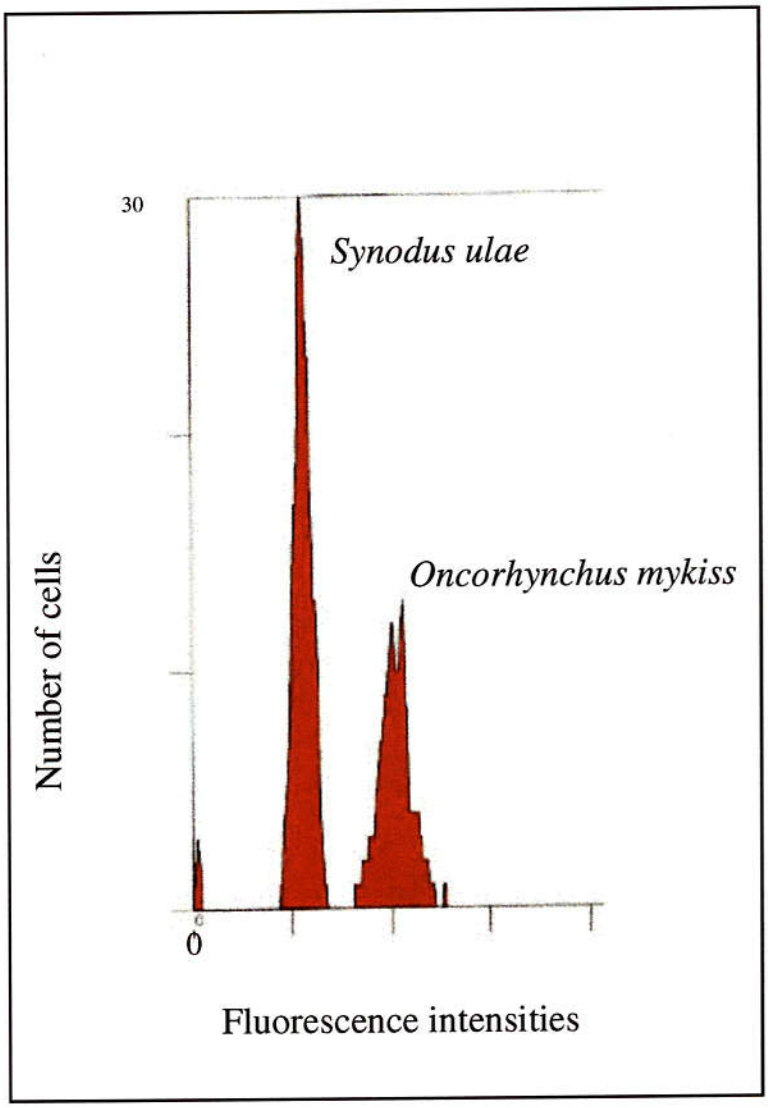


Fig. 4.1 Histogram of genome size measurement by flowcytometer in *Synodus ulae* and *Oncorhynchus mikiss* (control).

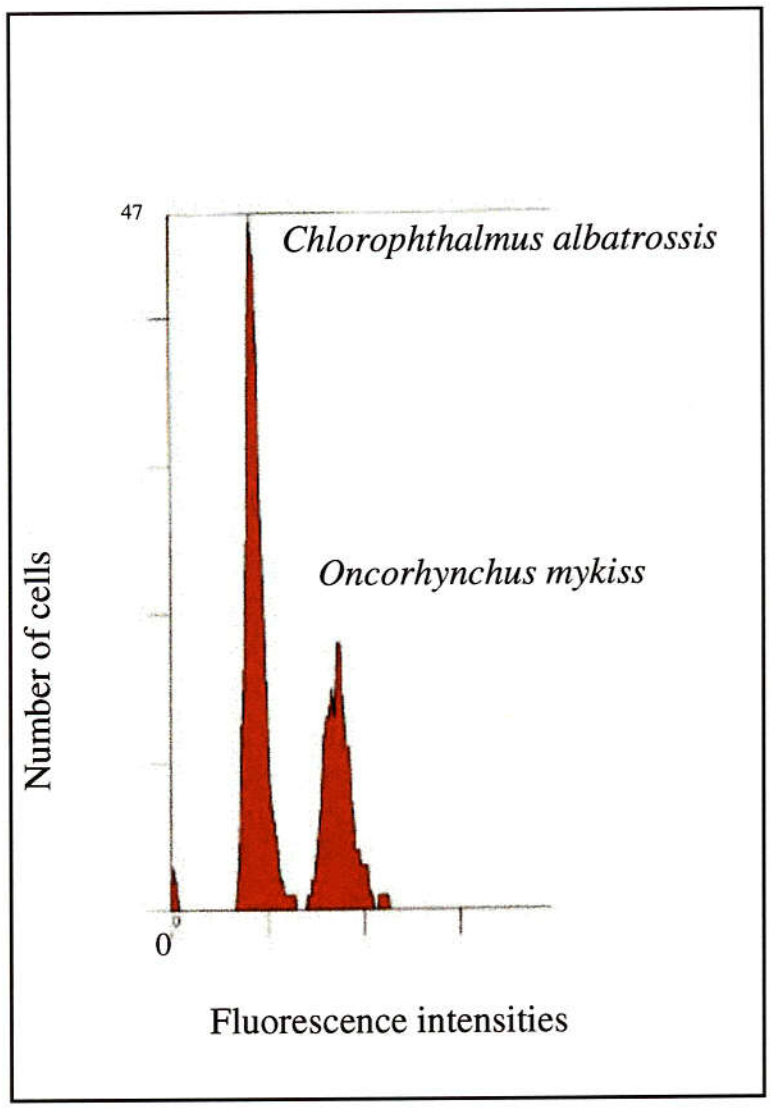


Fig. 4.2 Histogram of genome size measurement by flowcytometer in *Chlorophthalmus albatrossis* and *Oncorhynchus mikiss* (control).

This value (2.4 pg/cell) is close to my data (2.75 pg/cell). Therefore, the genome size might have been stable in aulopiform species in evolution. This indicates that the large-scale chromosomes rearrangement in the aulopiform species have occurred without large-scale changes in genome size.

Chapter 5

Identification of Z chromosomes in *Aulopus japonicus*

5.1 Introduction

Sex of mammals and birds is strongly controlled by the genetic sex determination system. Namely, the highly differentiated sex chromosomes such as XY chromosome or ZW chromosome systems are commonly observed in mammals and birds. On the other hand, sex of fish is generally not so strongly controlled by the genetic sex determination system. Thus, highly differentiated sex chromosomes are not observed in most fish species. Therefore, it is difficult to find a common rule in the occurrences of differentiated sex chromosomes in fish species. Indeed, the occurrence of sex chromosomes does not correspond to phylogenetic relationships among fish species (Ojima 1983). There is report of fish that the XY and ZW sex determination systems exist in the same lineage (Galetti Jr et al. 1981). Taking into account these reports, I have concluded that highly differentiated sex chromosomes are not conserved in the same linage for long time of fish species.

In chapter 2, I mentioned the finding of female heteromorphic sex chromosome systems in the order Aulopiformes. Since all the species studied have a female heteromorphic sex chromosome, the ZW chromosome system might be conserved among the gonochoristic species. I could clearly identify a very small chromosome as W chromosome in the gonochoristic aulopiform species. However, I could not identify the Z chromosome in gonochoristic aulopiform species studied in chapter 3, because morphological difference is not clear between Z chromosomes and autosomes.

There are reports on the presence of repetitive sequences in sex chromosome in several fish species (Galetti and Foresti 1986; Nanda et al. 1993; Moran et al. 1996; Born and Bertollo 2000). In my study, repetitive sequences have been identified on W chromosome in two aulopiforme species (*Synodus ulae* and *Synodus hoshinonis*) by using C-banding and silver staining methods. If Z chromosome also contains a characteristic repetitive sequence in the aulopiform species, we would identify Z chromosome by using it as probe for Fluorescence *in-situ* hybridization (FISH).

Aulopus japonicus also tends to inhabit shallow waters and belongs to the gonochoritic aulopiform. It has two advantageous points. Firstly, we can easily obtain many specimens in this species. Secondly, we can keep this species in an aquarium tank for a long time. Thus, to investigate a sex chromosome at the molecular level, this species would be a suitable material.

In this chapter, I will describe how I identify Z chromosome by using 5S rDNA and telomeric repetitive sequence as probe for FISH. I sequenced 5S rDNA and its adjacent non-transcripted intergenic spacers (NTS).

5.2 Materials and methods

5.2.1 Samples

Fourteen specimens of *Aulopus japonicus* (seven male and seven female individuals) were collected in the Suruga bay. All of the samples were sexed by macroscopic examination of the gonad. Six specimens (three male and three female individuals) of *Aulopus japonicus* were used for FISH analyses, and all the fourteen specimens were used for sequencing 5S rDNA and NTS.

5.2.2 DNA extraction and PCR amplification

The blood of *Aulopus japonicus* was diluted in TNES-urea buffer (Asahida et al. 1996). After keeping it for several days to weeks at room temperature, 20 ul protenase K was added and the solution was incubated about 2 hours at 60℃. This was followed by two phenol-chloroform and two chloroform extractions. After precipitation with two volumes of ethanol, the pellet was rinsed in 70% ethanol, moderately dried and dissolved in Tris-EDTA buffer. 5S rDNA sequences were amplified by using PCR for all the specimen. The primers used were 5SF and 5SR (Pendas et al. 1994). The thermal cycle profile was carried out under 10 s at 94℃, annealing for 10 s at 55℃ and extension for 20 s at 72℃.

5.2.3 Preparation of probes

The PCR products of 5S rDNA sequence were labeled by fluorescein isothiocyanate (FITC). A telomeric sequence (TTAGGG) was labeled by fluorescent cyanine dye (Cy3). To label the 5S rDNA and telomeric sequences, I conducted PCR reaction as the following cycle profile: denatureation for 10 sec at 94℃, annealing for 10 sec at 55℃ and extension for 60 sec at 72℃.

5.2.4 Chromosome slides and Fluorescence *in-situ* hybridization (FISH)

In order to perform FISH, I prepared chromosome slides. To increase the number of metaphase cells, the specimens were colchicinized by intraperitoneal injection of colchicines solution 3 hours before preparation. Kidneys were then removed and minced, and its cell suspension was treated by hypotonic solution (75 mM KCl) for 30 min at room temperature. The suspension was then fixed with methanol and acetic acid (3:1). The fixed materials were dropped onto precleaned slides and air-dried. The slides were stained by Giemsa solution.

To conduct Fluorescence *in-situ* hybridization, chromosomal DNA and the probes were denatured by 70% formaide solution at 70-85 ℃. The probes is placed on the slide and the slide were incubated at 37℃ during overnight. After washing the slides, they were counterstained by 4',

6-diamidino-2-phenyindole (DAPI). The slides were observed under the fluorescence microscope.

5.2.5 Cloning and nucleotide sequencing

The PCR products were cloned into pT7Blue vector (Novagen) and sequenced by using dye-labeled terminatiors (Applied Biosystems). I sequenced partial 5S rDNA and NTS regions in 85 clones; 33 clones derived from a male individual and 52 clones derived from a female individual. These sequence were aligned by ClustalX (Thompson et al. 1997)

5.3 Results

5.3.1 Cytogenetic features and FISH analyses

In order to study cytogenetic features of this species, I performed Giemsa staining. Then, I compared the male with the female in cytogenetic feature under the microscope. All the female individuals have a heteromorphic chromosome that is clearly distinguished from the other chromosomes. Therefore, it can be said that *Aulopus japonicus* has a female heteromorphic sex chromosome as shown in Fig 5.1.

In both the male and female individuals, 48 chromosomes were observed. The number of diploid chromosomes is similar to those of most gonochoristic aulopiform species. There is no difference between the male

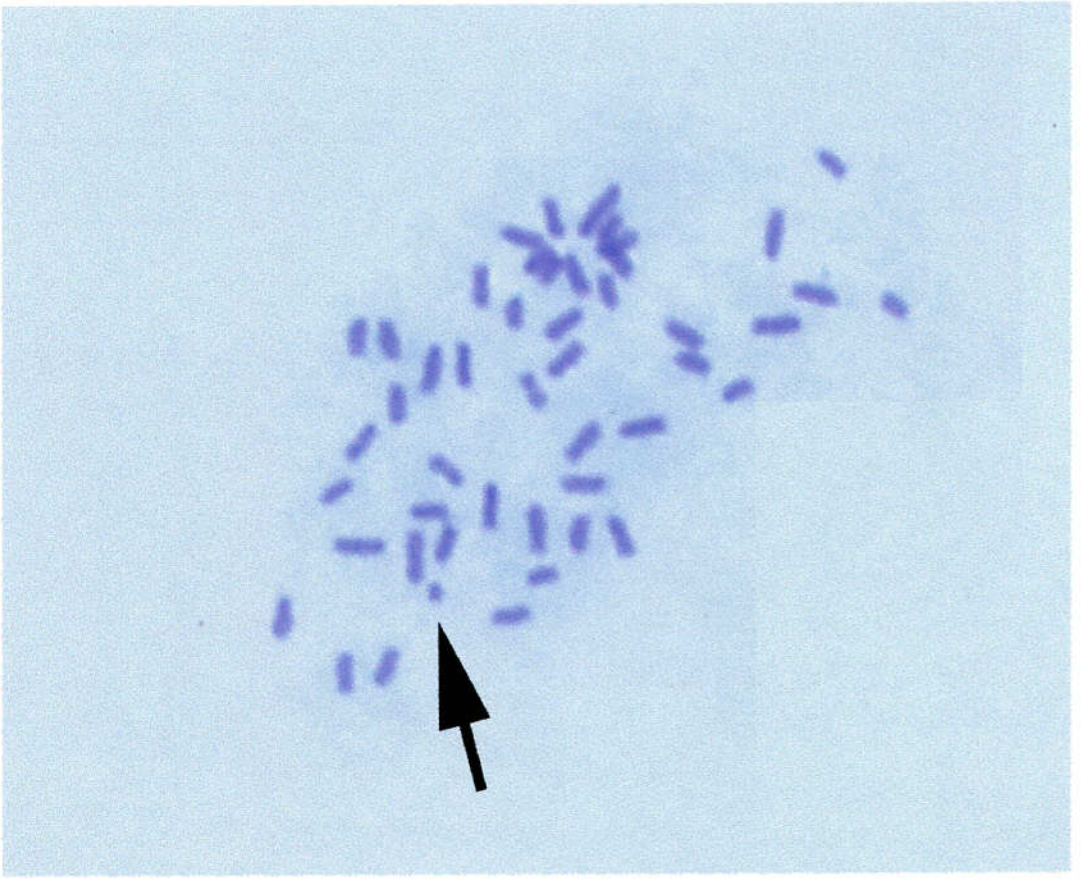


Fig. 5.1 Giemsa staining of metaphase chromosome of a female in *Aulopus japonicus*. The arrow indicates a heteromorphic sex chromosome.

and female individuals in the number of diploid chromosomes. Taking into account the female heteromorphic chromosome and the number of diploid chromosomes, I argue that *Aulopus. japonicus* has the ZW chromosome system. While I could identify W chromosome, I could not identify Z chromosome in this species by using Giemsa staining method in this species.

To identify Z chromosome of this species, I used 5S rDNA sequence and a telomeric sequence as probes for FISH analyses. I could detect both 5S rDNA (green) and telomeric sequence (pink) signals as shown in Fig 5.2. Although the signals of the telomeric sequence were detected in most chromosomes, I could not identify Z chromosome by using the telomeric probe.

However, I found a strong signal of 5S rDNA on W chromosome in the female metaphase cells. I also detected double signals of 5S rDNA on a large chromosome (Fig. 5.2). This result indicates that W chromosome has highly repetitive 5S rDNA sequences, and that the large chromosome is Z chromosome. To confirm the existence of Z chromosome, I performed FISH analyses in metaphase cells of the male individuals. In the male methaphase cells, 5S rDNA signals appeared on two large chromosomes (Fig. 5.3). This indicates that the male individual has two copies of Z chromosomes in the diploid cell.

To reveal differences between Z and W chromosomes, I compared the two chromosomes in size and structure. Even though W chromosome is much smaller than Z chromosome, W chromosome has more copies of 5S rDNA than Z chromosome (Fig 5.4). Thus, this species has highly

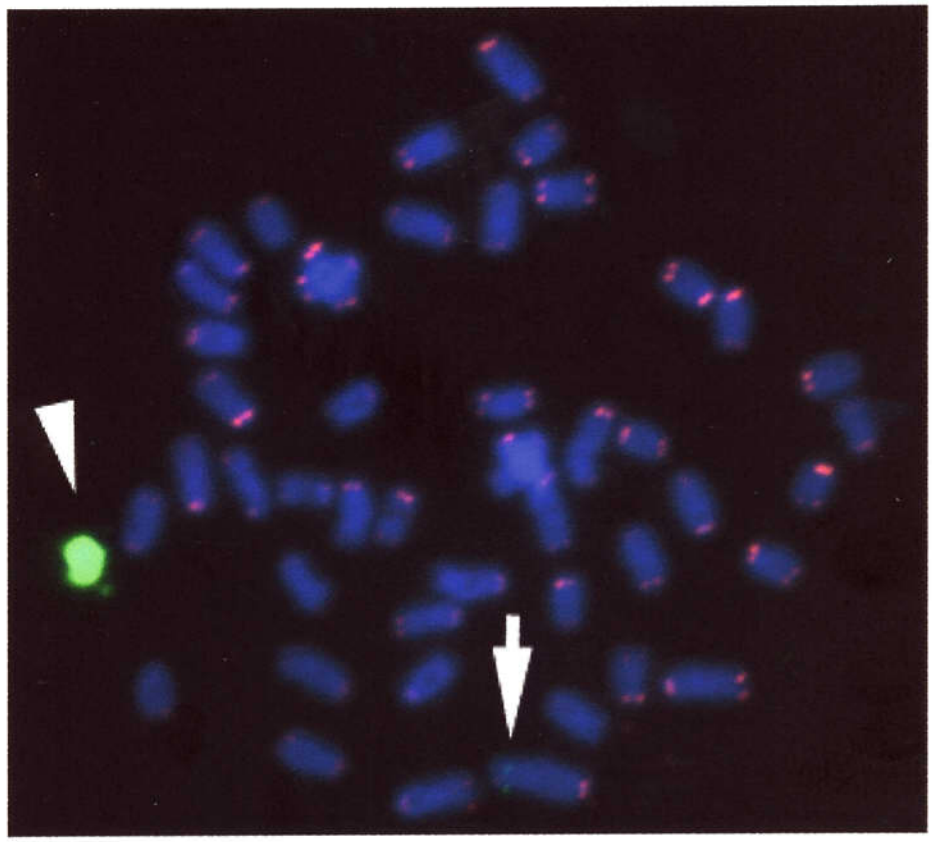


Fig. 5.2 Metaphase chromosomes of a female with 5S rDNA (green) and telomeric sequence (pink) probes in *Aulopus japonicus*. The white arrow indicated Z chromosome. The white arrowhead indicated the W chromosome.

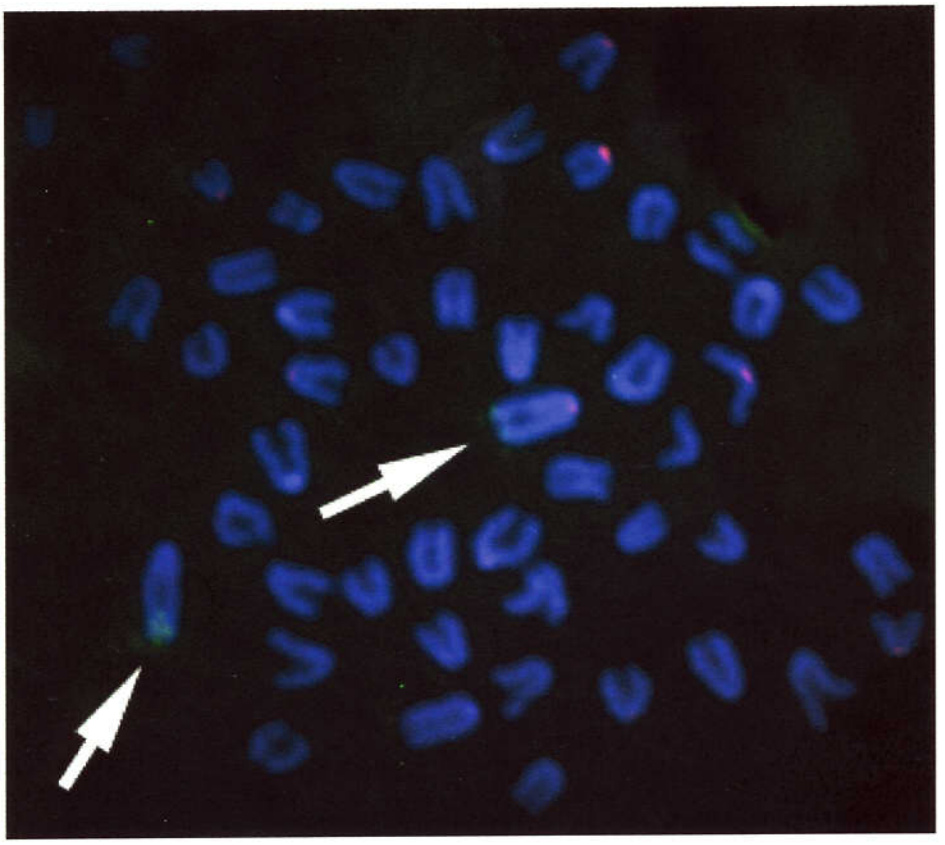


Fig. 5.3 Metaphase chromosomes of male with 5S rDNA (green) and telomeric sequence (pink) probes in *Aulopus japonicus*. White arrows indicated Z chromosomes.

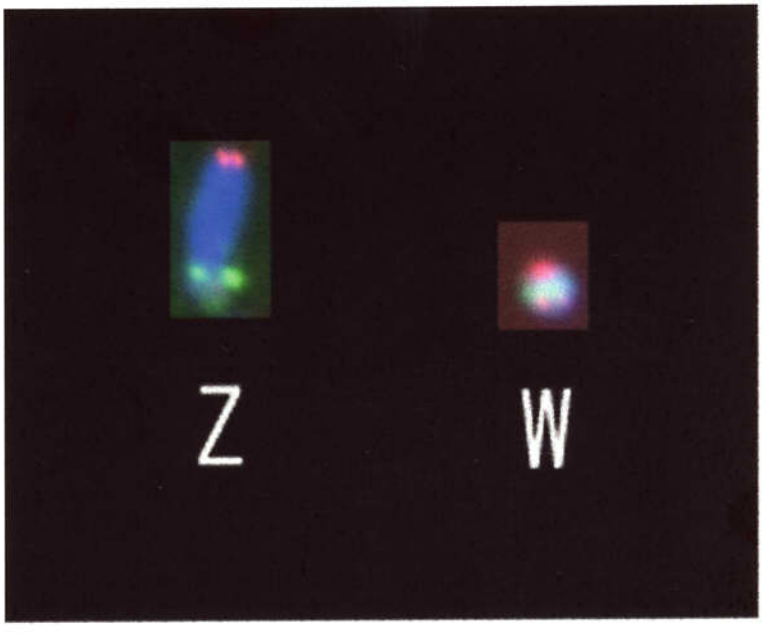


Fig. 5.4 The Z and W chromosomes of *Aulopus japonicus* with 5S rDNA (green) and telomeric sequence (pink) probes.

differentiated the Z and W sex chromosomes both in size and the number of repetitive sequences.

5.3.2 Characterization of 5S rDNA and NTS sequence

It has been demonstrated that 5S rDNA sequences are located on Z and W chromosomes in *Aulopus. japonicus.* Generally, 5S rDNA and its adjacent non-transcripted intergenic spacer (NTS) form a 5S rDNA cluster. In a trout species (*Onchorhynchus tshawytscha*), different types of NTS are observed in the same individual (Pendas et al. 1994). To elucidate whether *Aulopus japonicus* also has various NTS types or not, I conducted sequencing and PCR amplification in this species.

To investigate the variation of 5S rDNA and NTS of the species, I performed PCR amplification and sequencing. I amplified 5S rDNA sequence and NTS in the seven male and seven female individuals under the same condition. The products of PCR amplification were divided into two DNA fragments of discrete size on the agarose gel. The first band was 900 bp and the second band was approximately 1000 bp in length (Fig. 5.5). Although the male individuals differed from the female ones in efficiency of amplification of the second band, we could detect both bands in all of lanes on the gel. To categorize these fragments, I performed cloning and sequencing of 5S rDNA and NTS. I cloned 85 fragments totally. Thirty-three fragments were derived from a male individual and fifty-five fragments were derived from a female individual. All fragments consist of

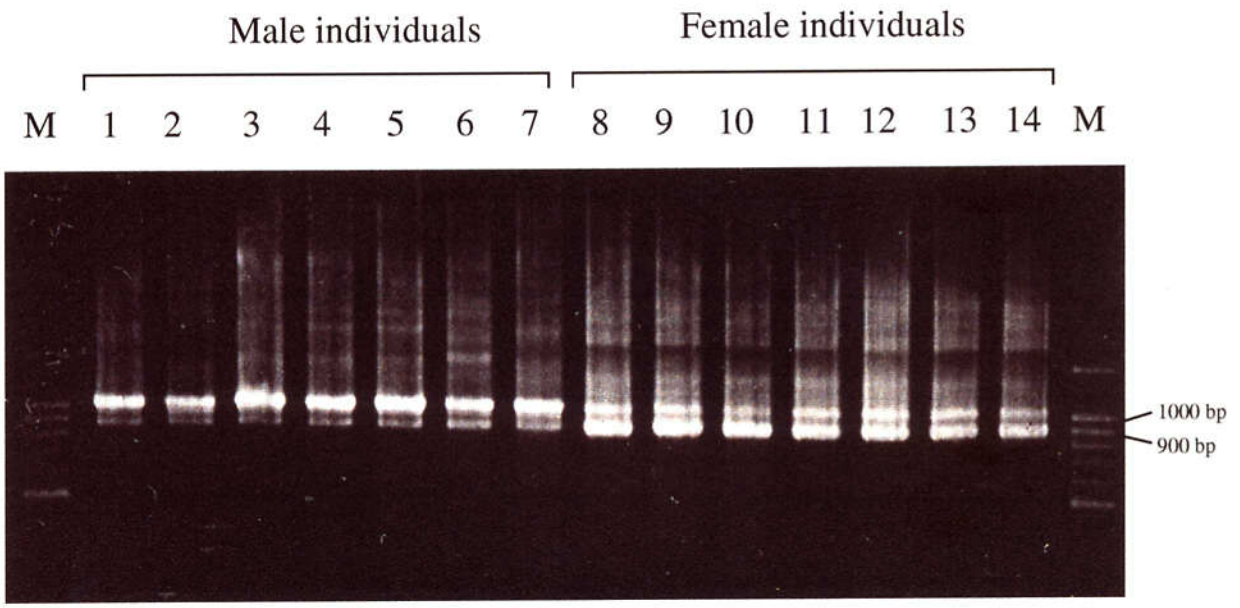


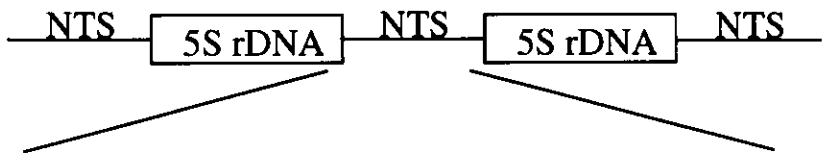
Fig. 5.5 PCR products of 5S rDNA and NTS were size-fractionated by electrophoresis through a 1.5 % agarose gel and stained with ethidium bromide.

5S rDNA and NTS regions. These fragments were clearly categorized into two NTS types. It was difficult to make alignment between first fragment (called "type A") and second fragments (called "type B") in NTS regions (Fig 5.6), because the NTS region highly diverged from each other.

To confirm the existence of both "type A" and "type B" in this species, I prepared four primers A5' (5'-GATTCCGCAGTGAGACACCA-3'), A3'1 (5'-GGATACA- CACACCATACGGTCCAG-3'), B5' (5'-GATCCACCAAATGGA GGCTACG-5') and B3'1 (5'-CACAGTGTCCCGTCGGATCGATTGG-3') from "type A" and "type B" sequences and conducted PCR amplification in seven male and seven female individuals. Using those primers, I amplified PCR products, which are approximately 700bp each (Fig 5.7 and Fig 5.8). The result of PCR amplification indicates that the male and female individuals possess "type A" and "type B" sequences.

5.4 Discussion

In chapter 3, I proposed that gonochoristic aulopiform species have the female heteromorphic sex chromosomes. I have also mentioned that *Aulopus japonicus* has a female heteromorphic sex chromosome in this chapter. Moreover, I have shown that W chromosome is smaller than Z chromosome in *Aulopus japonicus.* This result strongly suggests that the species has a highly differentiated ZW chromosome system. Because the heteromorphic sex chromosome is also found in a different family of aulopiform species, it is suggested that the ZW chromosome system has been



Type A

TGGGAATACCAGGTGCTGTAAGCGTTTTCACAGAATTTTTCACGCCAGTAGAAACAATCTATTAGA
TTCCGCAGTGAGACACCAATTTATGAATTATCTTTTCATTTATTTCTCTTTTTAAGAGTTTTGTCTG
AGCATTTGACACAAAGACTGATAGTAACTGCTGAAAATGACTTTTATCCTACTGTAATACTCTTAA
GGATGTAAGTTAAACGTTTTGTTTACATTACATTTGTACTAATGTTATCTTGACCCTCCCCGATGG
TTTTCCTTGAACCTCCCTGAGTGACTGGGGAAAACTGCATGACCCTGCCTCCACTATAAATAACC
ATATGTCTTTATATTCACACCAAATGTAGGTAGTATTGGAGCCAATGCAACAAGCTAATACCACGG
AGGGAGTGAGAGCTATCACCAAAACAAACTAATTTCTTCCGCAAAATGCAAATCCTCCTTACAAA
CCACATCATCACACAAGCTGTGTGTGCACTTCAGCAGAATATGGGTGCACGACAGCCAACCACCC
TACCACGCTAAACCAACAACATTTAATAAGCCTACAGACACGCACAAACGCACACACACACACAC
ACACACACACACAGAGAGTGAGAGAAAGATGACGATTGTAGGCCATTGGGGGAAATAAAAGACAT
TTTAAAAAATGTATTCATAGTTACATCAACCACTTGTTCAGTAAATAAGTTAGTGGTCAATAAGGA
ACAAATCTTGAAATAATTCAAACTGGACCGTATGGTGTGTGTATCCTNAAAAGGCCAATGTNGCC
CCACAAGANTCCTN

Type B

TGGGAATACCAGGTGCTGAAAGCGTTTTCATCCACTTTTTCACGACAGTCGAAACAATCTATTTAT
ACAGCAATGAACCACAAAAAACACATACTATGATCCACCAAATGGAGGCTACGAACTGAACAGCT
GTATTGGGAACTGAAGGTAGATGTTAAGAATCAAGATGGTTTTTAGAGGGAAGAATAAAAAGATA
CACGGTACATTTCATACATACACCTGTAGTCTAGAGCAGTGGTTCCCTACCTTTTTTGTCTTATGT
ACCCCTTGAGCTGTTGCCATGCCAACAGTACCCCCTCACTCATGCGTGTTGATGATTCACTTAAG
TTTGTTCTATTAGATTGATTAAACTTTTCGTATCTACACATCTGATTATAAAATAAAAGTCATTTGA
ATACGTTCCTTAAATACAAAGACATTTACACTTATTGTGAGTGAAAAAATAATATTCAGACTTACT
CTGAAGCAGTTTCAACTCCTTTTTGTCAGTCCCTTGAGCTTTAATGAGACACTTGAGCTTGTTTGT
CTTTCATAAGCTTTGATAGTTCTTTTACTAGGTTGTTCTCCACATTTAGCTTGTTCCTCTACTTCGT
TTTGATGTGAGTCAAAGCTGAGGAGGTCACTTCACACATACATCTAACATACATCCCACTAACAT
CTGTCATTTCGTCCAACTGCAAAGTGAACTGACCTGCTTGCTTCATTTTATCCACCACTTGTACAA
CAATAGTAATCGACATACCATCGATCCGACGGGACACTGTGTTGTTTGATAATGGGACTGTCTCG
AGTGCTTTGGCTGCTTTCTTGTCAATCGTATTTTCAGCTAAAACTATGGAATGAGAATGAGATCTT
CGACGATAGAATGTGTCTTTATTGATTTAGCCACAAGTAGTGACACAGCCTGTCTCCAAAAAANC
AAATNTCGNCCCCCTCAAGNNCCNC

Fig. 5.6 Organization of a 5S rDNA repeated region and the deduced nucleotide sequence of 5S rDNA and NTS sequence. Boldface type indicated 5S rDNA region.

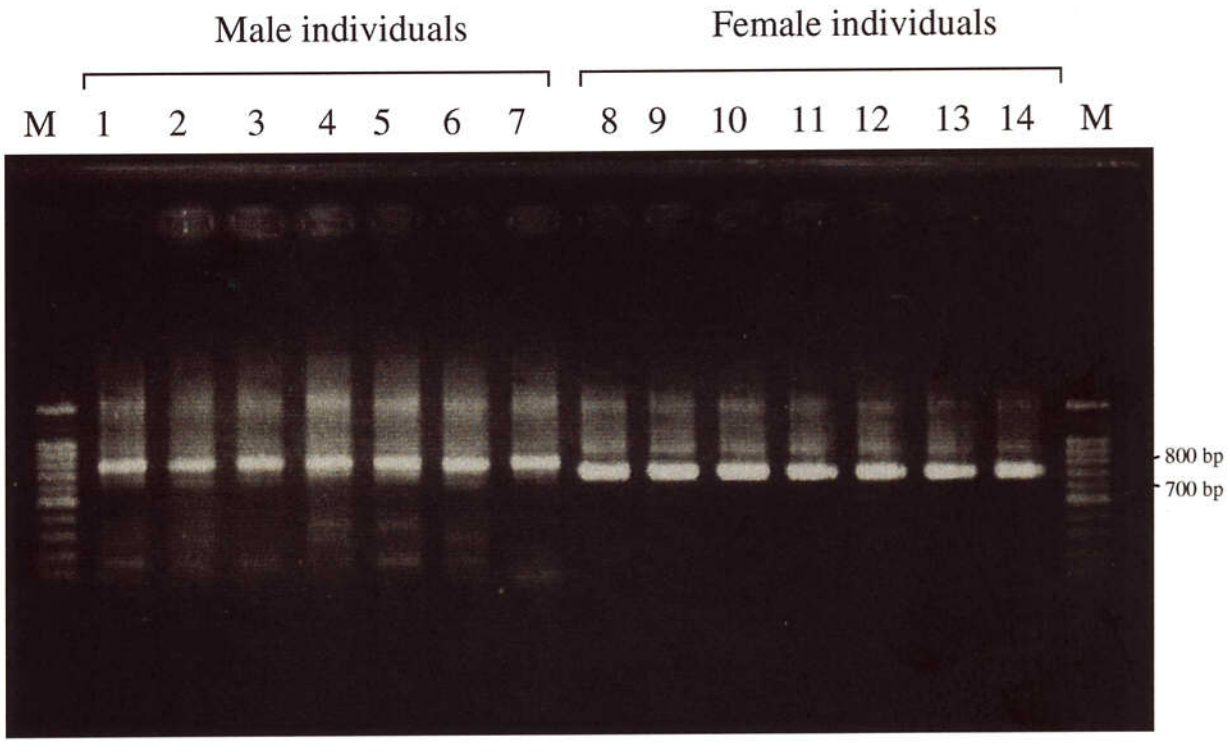


Fig. 5.7 PCR products with A5' and A3' primers were size-fractionated by electrophoresis through a 1.5 % agarose gel and stained with ethidium bromide.

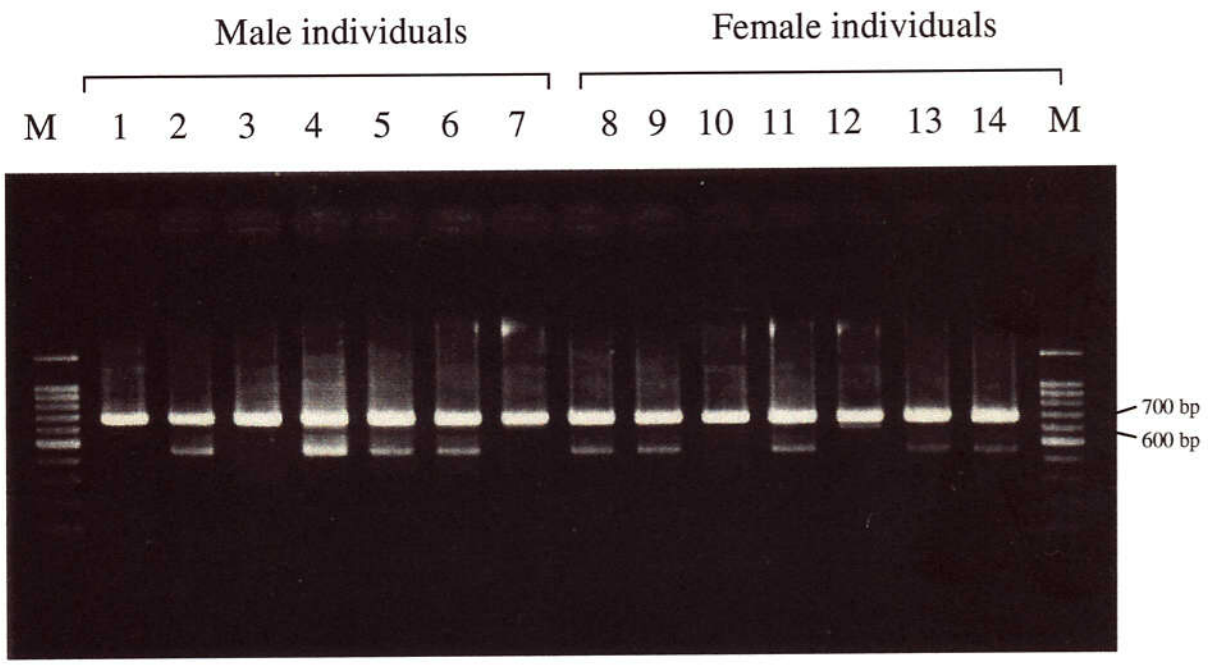


Fig. 5.8 PCR products with B5' and B3' primers were size-fractionated by electrophoresis through a 1.5 % agarose gel and stained with ethidium bromide.

widely conserved among gonochoristic aulopiform species.

To investigate the possibility of recombination between the Z and W chromosomes, I carried out FISH for Z and W chromosomes of this species. This resulted in finding of a homologous region between Z and W chromosomes. Since 5S rDNA sequences are located on the tip of the long arm of Z chromosome, this region should correspond to some region in W chromosome. Thus, the region of the W chromosome may attach to the tip of the long arm of Z chromosome during meiosis.

From an evolutionary point of view, I compared this species with other species with respect to the ZW chromosome systems. Although the ZW chromosome system was observed in other fish species, most of Z and W chromosome are not differentiated each other and not conserved in the same linage. Exceptionally, the genus *Leporinus* is known as a fish group which has differentiated Z and W chromosomes. In the comparison of sex chromosomes between the genus *Leporinus* and the order *Aulopiformes*, we can find an interesting point in the size of these sex chromosomes. In the genus *Leporinus*, Z chromosome is smaller than W chromosome in most of studied species (Koehler et al. 1997). On the contrary, in the order Aulopiformes, the W chromosome is smaller than Z chromosome. This contrasting result indicates that the two ZW sex chromosome systems have been developed independently in each linage. I also compared the size of sex chromosome between the order Aulopifomes and bird species. The result shows that Z chromosome is lager than W chromosome in both animals. Thus, it is indicated that the same mechanism might have acted

on the ZW chromosomes of aulopifome species and birds, even if their ZW chromosome systems have been independently developed.

Fossil data suggested that a common ancestor of the order Aulopiformes existed in Cretaceous (http://www.fishbase.org). Thus, I would suggest that the ZW chromosme system of the order Aulopiformes was developed more than 70 million years ago. According to a comparative study of sex chromosomes in bird species (Shetty et al. 1997), the ZW chromosome system was developed more than 80 million years ago. Thus, I proposed the hypothesis that the ZW chromosome systems of fish and birds have a common history in time scale and development.

Chapter 6

Amplification and characterization of female specific fragment in *Aulopus japonicus*

6.1 Introduction

To consider the process and mechanism of sex chromosome evolution in vertebrates, we usually compare those of mammals and birds which have well known XY and ZW chromosomes, respectively (Graves and Shetty 2001). Since the sex chromosomes might have been developed and conserved independently in mammals and birds, these sex chromosomes could be materials suitable for the investigation of sex chromosome evolution. Some comparative studies at the molecular level have been progressed in mammals (Nanda et al. 1999).

Sex-specific sequences were identified in several fish species (Nakayama et al. 1994; Reed et al. 1995; Delvin et al. 1998; Forbes et al. 1994). Detailed linkage analyses have also been performed in medaka, zebra fish and trout from which sex specific sequences can be isolated (Postlethwait et al. 2000; Naruse et al. 2000; Sakamoto et al. 2000).

However, we cannot compare sex-specific sequence of these fish with those of other vertebrates (mammals and birds), because there is no guarantee that the sequences have been conserved owing to evolutionary instability of sex chromosomes of fish. Then, the question is whether or not we can find a fish species that have conserved sex chromosomes and sex specific genes.

In chapter 3, I showed the existence of a female heteromorphic sex chromosome in the order Aulopiformes. I also demonstrated that *Aulopus japonicus* has the highly differentiated ZW chromosomes as mammals and birds have in chapter 5. Thus, the order Aulopiformes is suitable for investigating the process and mechanism of sex chromosome evolution in vertebrates. In particular, if we can identify a female-specific sequence in this order, the sequence will give us insight into the evolution of sex chromosomes.

I have successfully shown that 5S rDNA and nontrancscribed spacer (NTS) sequences are located on Z and W chromosomes in *Aulopus japonicus*. These NTS sequence are classified into two types, types A and B. The two types form two separate clusters (Fig 6.1). This is in agreement with the report which 5S rDNA and NTS alternately locate on a chromosome (Pendas et al. 1994). Although the precise locations of type A and type B clusters on Z and W chromosomes are unknown, there is the possibility that a sequence exists between type A and type B clusters. If it is the case, the intervening sequence can be amplified by PCR. Thus, I performed amplification of a female-specific sequence by using NTS sequences primer and cloned it.

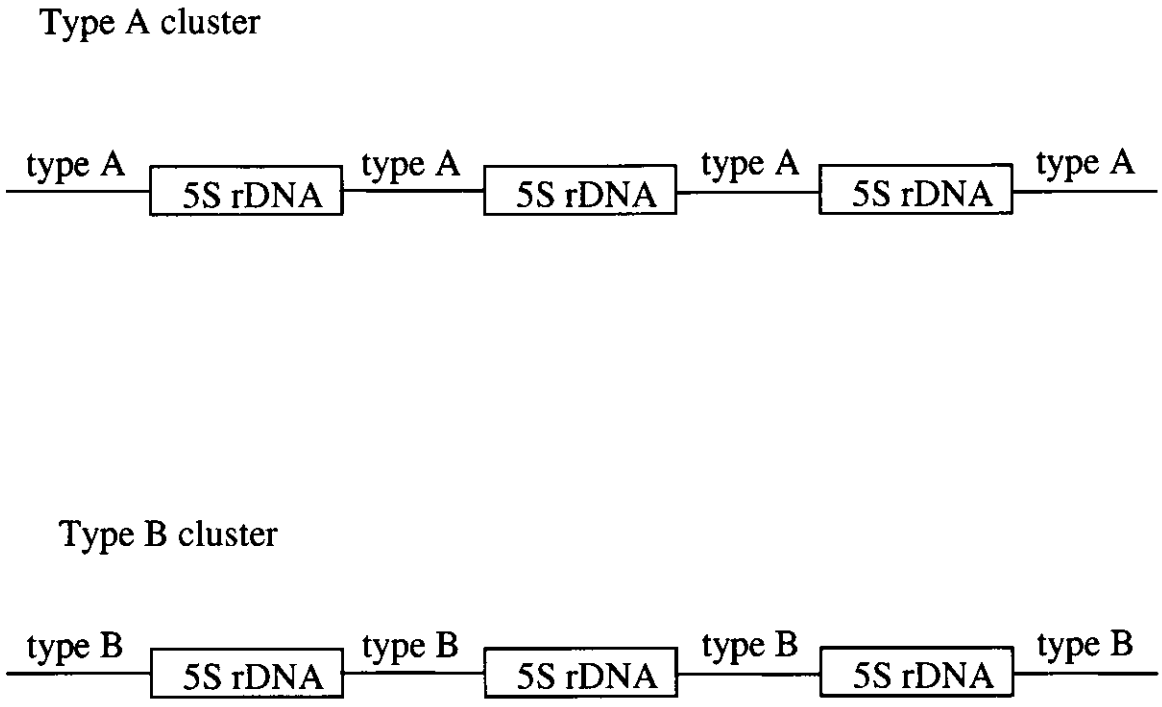


Fig. 6.1 Diagrammatic representation of two types of 5S rDNA clusters (type A cluster and type B cluster)

6.2 Materials and methods

6.2.1 Samples

14 specimens of *Aulopus japonicus* (seven males and seven females) were collected in the Suruga bay. All the samples were sexed by macroscopic examination of the gonad.

6.2.2 DNA extraction

Total blood was diluted in TNES-urea buffer (Asahida et al. 1996). After several days to weeks at room temperature, 20 ul protenase K was added and the solution was incubated about 2 h at 60℃. This was followed by two phenol-chloroform and two chloroform extractions. After precipitation with two volumes of ethanol, the pellet was rinsed in 70% ethanol, moderately dried and dissolved in Tris-EDTA buffer. These all template DNA were diluted with Tris-EDTA buffer to adjust the same concentration (100ng/μ l).

6.2.3 PCR amplification, cloning and sequencing

To amplify female-specific sequence, I prepared four primers A5' (5'-GATTCCGCAGTGAGACACCA-3'), A3'1 (5'-GGATACACACACCATA-CGGTCCAG-3'), B5' (5'-GATCCACCAAATGGAGGCTACG-5') and B3'1

(5'-CACAGTGTCCCGTCGGATCGATTGG-3'). The thermal cycle profile was as follows; denatureation for 10 second at 94 ℃, annealing for 10 second at 55℃ and extension for 4 minute at 72℃. The same PCR amplification was conducted to different concatenation of templates (10ng/μl and 100ng/μl). PCR product was cloned into pUC19 and pT7Blue vectors (Novagen). These clones were sequenced by using dye-labeled terminatiors (Applied Biosystems).

6.3 Results

I performed PCR amplification for the seven male and seven female individuals. By using A5' and B3'1 primers, I could amplify some fragments in several male and several female individuals (Fig. 6.2). But, I could not find a sex-specific fragment by these primers. By using B5' and A3'-1 primers, however, I could then amplify a large fragment that is more than 6 kb long in all the seven of female individuals (Fig. 6.3). We could detect the same band in none of the male individuals. These results indicate that a female-specific sequence was successfully amplified by using B5' and A3'-1 primes. When I changed the concentration of template DNA to check the accuracy of PCR amplification, the large fragment band appeared in all female individuals. Thus, it was confirmed that a female-specific fragment was amplified by PCR.

I then conducted cloning and sequencing to characterize the female-specific fragment. The female specific fragment was disgusted by

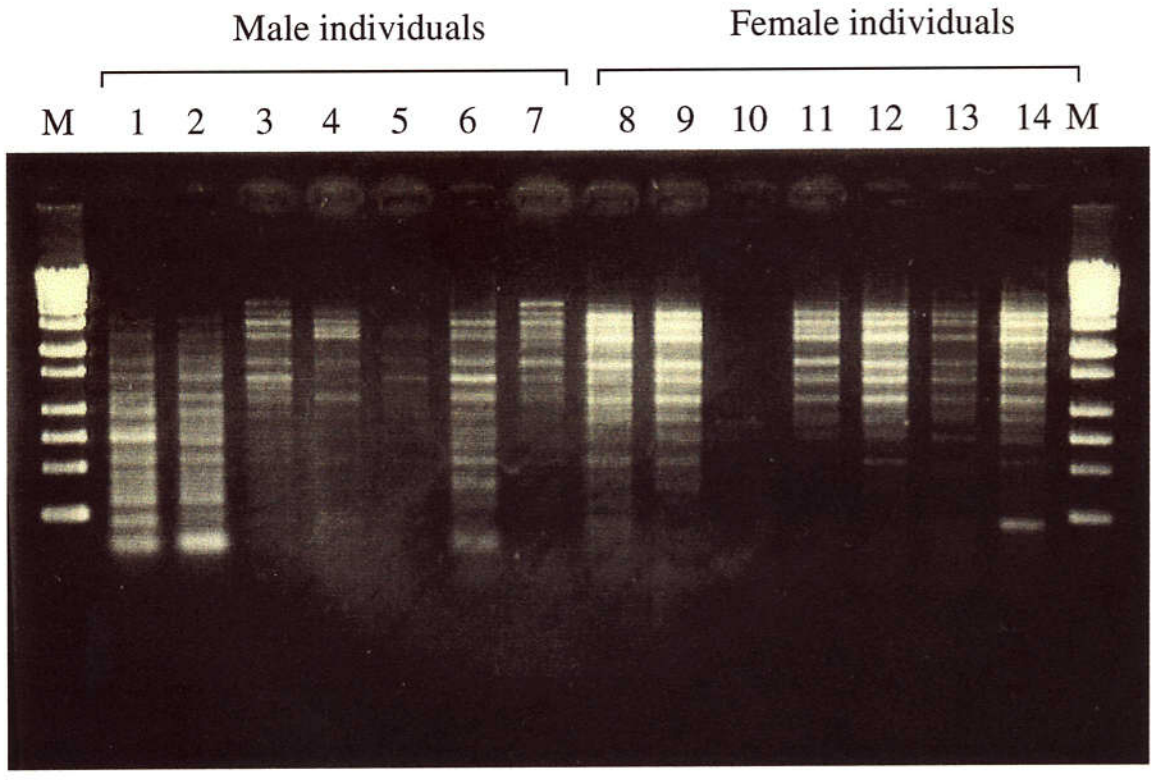


Fig. 6.2 PCR products with A5' and B3' primers were size-fractionated by electrophoresis through a 1% agarose gel and stained with ethidium bromide.

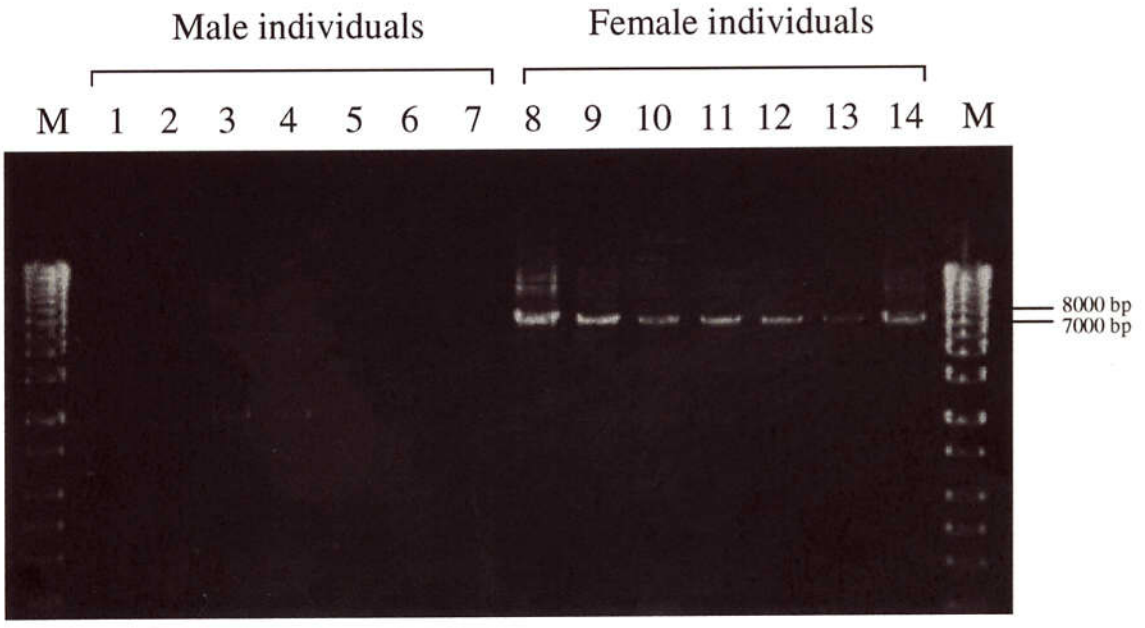


Fig. 6.3 PCR products with A3' and B5' primers were size-fractionated by electrophoresis through a 1% agarose gel and stained with ethidium bromide.

Sau HA I and cloned into the pUC19 vector. I performed sequencing of fifteen inserts from both 5' and 3' ends by using M13 and M13 reverse primers. For these sequences (30 sequences), I carried out homology search against protein sequence database (SWISS-PLOT release 40.5) by using BLASTX (Altschul et al. 1997). The result of the BLAST search shows that 12 sequences are similar to several amino acid sequences. Most sequences are related to transposable elements, and two sequences (01_1118_4G and 01_1118_4GR) are closely related to mitogen activating protein kinase (mapk) in high identity (Table 6.1). The result implies that a "mapk-like" sequence is located on W chromosome in *Aulopus japonicus*. To determine whether the "mapk-like" sequence has the complete gene structure of a mapk gene, I conducted sequencing of the clone that contains the "mapk-like" sequence (Fig. 6.4).

The resulted sequence indicates that three parts of the "mapk-like" sequence are highly similar to mapk 6 (Fig 6.5). Therefore, the "mapk-like" sequence could be functional.

6.4 Discussion

I could successfully amplify a W chromosome-specific fragment. The result of PCR amplification indicates that the fragment exists in the region which recombination has been suppressed. Thus, the fragment might have been evolved independently from Z chromosome after the establishment of the regions. I also found a "mapk-like" sequence in the fragment. It is

Table 6.1 Summaries of BLASTX results

Query sequences	Protein names of hit sequences	Accession number	Expectation value
01_1124_1br	Transposon TX1 hypothetical 149 kDa protein.	P14381	3.00E-18
01_1124_1cr	Transposon TX1 hypothetical 149 kDa protein (ORF 2).	P14381	1.00E-27
01_1124_2br	Transposon TX1 hypothetical 149 kDa protein.	P14381	5.00E-37
01_1124_2fr	Retrovirus-related POL polyprotein.	P11369	1.00E-23
01_1124_3gr	Kelch-like ECH-associated protein 1.	Q14145	9.00E-12
01_1118_4G	Mitogen-activated protein kinase 6.	Q16659	4.00E-14
01_1118_4GR	Mitogen-activated protein kinase 6.	Q16659	3.00E-27
01_1124_4ar	LINE-1 reverse transcriptase homolog.	P08547	6.00E-13
01_1124_4br	LINE-1 reverse transcriptase homolog.	P08547	2.00E-12
01_1124_4dr	LINE-1 reverse transcriptase homolog.	P08547	8.00E-08
01_1124_4hr	Mitogen-activated protein kinase 6.	Q16659	4.00E-27
01_1124_5cr	Minor tail protein M.	P03737	1.00E-15
01_1124_5dr	Host specificity protein J.	P03749	2.00E-21

TCAGGAAACAGCTATGAACCATGATTACGCCAAGCTCTAATACGACTCACTATAGGGAAAGCTT
GCATGCCTGCAGGTCGACTCTAGAGGATCTACTAGTCATATGGATTCCACCAAATGGAGGCTAC
GAGCACACGCGCACCATCCTCCCTCCATATGCAAAACACAAGCTTGTCTCACAGCCACACGGAA
ACAAATGCATGCAAAAAGGGGAAAAGCATCCTCACTTGATGTCATGTATACATTTTGTCCACAC
AGCCATAGCCGAAGTCATATTTGCTCCTTTTGTCTGGCACTTGTTTTTTTTCTTTCTGTGAACT
CATTTCCCTTGTTCCCTCCTTTCTCTCTCTCTCTCTCAGCCCTTGATTTCCTGGAGAAGATCCT
GACCTTTAACCCCATGGACCGTCTGACGGCGGAGGAAGCCCTGGCCCACCCCTACATGGCGGAC
TACTCCTTTCCCCTGGACGAGCCCATCTCTCTGCACCCCTTCCACATCGAGGACGAGGTGGACG
ACATCCTGCTCATGGACCAGAGCCACAGCCACACCTGGGACAGGTACACACACACACACACACA
CACACACACACACACACACACACTCTGGCGGGACGGAGTATAGGGACGGGTGTTTGCTTTGTTC
TGGTTTAGTACAGGGCTACGGGGGAGATGGGTGCATATGTTACATAGAGCTGGTGTGAGGGCCA
AAACTCTTGGATCTACAAATGGATAACCTGTTCAAGAATAAAAAACAACGGGCTTATTTACAGA
TCCACTCCCATGTATTTTTCACTTTAGTGCGCGAACGACATGCCAGTTTAAGCGAGGGGGTGTG
TACACATTAACTCAAGAGGGTCTGGTGAACAAGGCACAGAGTGATGACCCACTCTGTGTAAATT
CATTAAATCACCCATTTAAATCATATGCTTAAATAAAAAAATAAAATAAGTTCTTAAAAAGTAA
GATTGGGGTTTTGAATGGGTTTCGTGGTTCTTAAGAGTCATGAATCTTGCCCACAGCATAGAGA
AGTACATAAAGTTTCAAGTCAACTAAAAAAATTGGAGGTACGAGTTTTCGCCTGACAGCAGTGA
TGGGTATTACTTACTTACAACTTCCTGTGTGTCCGGTAATTTAGGTGGCCTTATTCACACATTT
GCAAATGTTTGTGTATTGGAGTCTTAACTCCGATCTTTTATTACAGGTACCACGACAGCCAGCT
GTCCGAGGCTGACTGGCACCTGsACAGCAACCACGACTCCGACGAGGTGCAGGTCGACCCGCGG
GCTCTCTCTGTTGTCACCGACGAGGAGGAGGTCCAGGTGGGTTTGAACACACTTGCGCCCACGC
ATAACCCCTTATTTAACTCATTCTACATGTTACAGCAAAGACATAATTTATTATCTAGGTTTCG
TACTCAAAATAAGGAAATTTAAAAACAAGTGAATTTAAGTTTTTTTTTGGGCATCCATCTTAAT
GGTTCTAATATCTGTAATATCTCCCAGGTGGACCCTCGTAAGTACGCTGATGGCGAACGGGAGA
AATTCCTGGACGAGCCGTCCTTCGACTACTCCTCTCTGTTCCCGCCGGAGCGCTCCTGGGAGGA
GGAGGACCACCACGAGAACAAGTACTGTGACCTGGAGTGCAGCCACACCTGCAACTACAAGGCC
GTGTCGCCCTCGTACCTCGACAACCTCATCTGGAGGGACAGCGAGGTCAACCACTACTACGAAC
CCAAGCTCATCATCGATCTCTCCAACTGCAAGGAGCAGCAGAGCAAGGACAAGGCCGACCGCAA
GGCCAAGAGCAAGTGTGAGAAGAACGGGCTGGTGAAGGCCCAGATAGCCCTGCAGGAGGCCGAG
AAGACCCAGGGGCCCGTGGAGAAGGACAGGGAGCAGGAGAAGCACCAGACACTGCCCCAGAGCC
AACAGAGCCCAAGCTTCGACTTTGACTCCTTCATTGCCGACACCATCAAACTGAGCCTGCAGCC
AGAGCCCTGCCCAGAGGTGGCCGTGCTGAGCGAGGTGGGCCTCCTCAACGAGCTCAACTCCTCC
GTTTCTCAGCTGGAGGCCCCTCGGTCGGGCTCCATGTCTAAATCCATCAGCCAGGAGAAGGAGG
AGAAGTGCCTGGTCAATCTGGCCCAGTTGGGGGGAGGAGGAGGAGTGCTGGGAGGAGTTGGTGG
GGATGTTGTCAGAGCTGCACACCCTTGGGAGAGCTTTGGTGGCGGAGAGAGAGTAGGGGAGAAT
GGCTGTTTGATAGACGAGGCATGCTGGGACATTGGTAAAGAGGACCAGCTCCAGAAGGAGAGCA
CTTACACCAGCTACCTGGCCCGTATGGTGTGTGTATCCAGGAGGGCCTTTCCAAGGTAAATCCC
CTCCT

Fig. 6.4 The "mapk-like" sequence in *Aulopus japonicus.*

A

```
MK06_RAT_(P27704)          356   RYHDCQFSEHDWPIHNNFDIDEVQLDPRALSDVTDEEEVQV  396
MK06_MOUSE_(Q61532)        356   RYHDCQFSEHDWPIHNNFDIDEVQLDPRALSDVTDEEEVQV  396
MK06_HUMAN_(Q16659)        356   RYHDCQFSEHDWPVHNNFDIDEVQLDPRALSDVTDEEEVQV  396
The "mapk-like"           1197   RYHDSQLSEADWHLXSNHDSDEVQVDPRALSVVTDEEEVQV 1319
                                 ****.*:** ** : .*.* ****:****** *********
```

B

```
MK06_RAT_(P27704)          395   QVDPRKYLDGDREKYLEDPAFDTS--YSAEPCWQYPDHHENKYCDLECSHTCNYKTRSPS
MK06_MOUSE_(Q61532)        395   QVDPRKYLDGDREKYLEDPAFDTS--YSAEPCWQYPDHHENKYCDLECSHTCNYKTRSSP
MK06_HUMAN_(Q16659)        395   QVDPRKYLDGDREKYLEDPAFDTN--YSTEPCWQYSDHHENKYCDLECSHTCNYKTRSSS
The "mapk-like"           1497   QVDPRKYADGEREKFLDEPSFDYSSLFPPERSWEEEDHHENKYCDLECSHTCNYKAVSPS
                                 ******* **:***:*::*:** .   :..* .*:  ******************: *..

MK06_RAT_(P27704)                YLDNLVWRESEVNHYYEPKLIIDLSNWKE  486
MK06_MOUSE_(Q61532)              YLDNLVWRESEVNHYYEPKLIIDLSNWKE  486
MK06_HUMAN_(Q16659)              YLDNLVWRESEVNHYYEPKLIIDLSNWKE  486
The "mapk-like                   YLDNLIWRDSEVNHYYEPKLIIDLSNCKE 1710
                                 *****:**:****************** **
```

C

```
MK06_RAT_(P27704)          289   ALDFLEQILTFSPMDRLTAEEALSHPYMSIYSFPTDEPISSHPFHIEDEVDDILLMDETH
MK06_MOUSE_(Q61532)        289   ALDFLEQILTFSPMDRLTAEEALSHPYMSIYSFPTDEPISSHPFHIEDEVDDILLMDETH
MK06_HUMAN_(Q16659)        289   ALDFLEQILTFSPMDRLTAEEALSHPYMSIYSFPMDEPISSHPFHIEDEVDDILLMDETH
The "mapk-like"            359   ALDFLEKILTFNPMDRLTAEEALAHPYMADYSFPLDEPISLHPFHIEDEVDDILLMDQSH
                                 ******:****.***********:****: **** ***** ****************::*

MK06_RAT_(P27704)                SHIYNWERY 357
MK06_MOUSE_(Q61532)              SHIYNWERY 357
MK06_HUMAN_(Q16659)              SHIYNWERY 357
The "mapk-like"                  SH--TWDRY 559
                                 **  .*:**
```

Fig. 6.5 Sequence comparisons of rat, mouse, human Mapk 6 and the "mapk-like" sequence. Three parts of the "mapk-like" sequence (A: 1197bp to 1319bp), (B: 1497bp to 1710bp) and (C: 359bp to 559bp) correspond to Mapk 6 partial regions in amino acid sequence.

known that a mapk gene responds to chemical and physical stresses, thereby controlling cell survival and adaptation (Chang and Karin 2001). However, we have not found any evidence that mapk works as the sex-determining gene in vertebrates. Therefore, it is interesting to show for the first time that the "mapk-like" sequence I found is a candidate of the sex-determining gene.

As the origin of the "mapk-like" sequence, I can consider two cases. The first case is that the "mapk-like" sequence moved into the W chromosome by a transposable element. The female-specific fragment is known to contain transposable elements. The second case is that a common ancestor of aulopiform species had an ancestral sequence of the "mapk-like" sequence.

If the second case is correct, it is highly possible that the "mapk-like" sequence has been conserved in most gonochoristic aulopiform species. In mammals, it is known that Sry is located on the Y chromosome, and that Sry was derived from Sox 3 located on X chromosome in evolution (Katoh and Miyata 1999). Take into accounts of the evolutionally relationship between Sry and Sox3 in mammals, I propose that the "mapk-like" sequence was derived from a mapk like sequence located on Z chromosomes.

Chapter 7

Conclusion

It has been generally known that fish has not highly differentiated sex chromosomes that are distinguished from the other autosomes under the microscope. Differentiated sex chromosomes exceptionally observed have not been conserved in evolution. My cytogenetic and phylogenetic data indicate for the first time that highly differentiated sex chromosomes have been conserved in the same linage of the order Aulopiformes.

According to Ohno (1970), the whole genome duplication hardly took place in mammals, birds and reptiles, because of the well-established chromosomal sex determining mechanism. The whole genome duplicaton in those vertebrates would confuse their sex determining mechanisms. In amphibians and fish, on the other hand, genome duplication is allowed because most fish and amphibians do not have well-established chromosomal sex determining mechanism. In chapter 3, I proposed that the genome size has been stable in the order Aulopiformes in evolution. Therefore, it can be stated that the existence of highly differentiated sex chromosomes prevents the whole genome duplication in vertebrates. However, there are tetraploid mammals reported (Gallardo et al. 1999), suggesting that at least existence of sex chromosome may have affected evolution of genome size.

In this study, I demonstrated that W chromosome of *Aulopus japonicus* contains a lot of repetitive 5S rDNA clusters. This indicates that the W chromosome has few genes. On the other hand, the Z chromosome is larger than the W chromosome and has only a small number of repetitive 5S rDNA cluster, indicating that the Z chromosomes has more genes than the W chromosome. I thus conclude that the sex chromosomes of *Aulopus japonicus* are similar to those of mammals and birds in organization, the number of genes and that of repetitive elements. Even if sex chromosomes have evolved independently in each linage, the common mechanism might have acted in sex chromosome differentiation among the fish species, mammals and birds.

I could successfully amplify the W chromosome-specific fragment of *Aulopus japonicus*. The fragment contains a "mapk-like" sequence. In chapter 3, I suggested that the W chromosome might have disappeared in evolution. The "mapk-like" sequence will give us a clue to investigate whether the W chromosome actually disappeared or was incorporated into another chromosome in evolution.

As an extension of this study, male-driven evolution is an interesting issue. Male-driven evolution of mammals and birds having XY and ZW sex chromosomes, respectively, has been studied (Shimmin et al. 1993; Chang et al. 1995; Agunik et al. 1997; Kahn and Quinn 1999). If we can identify a mapk-like sequence on the Z chromosome, we would compare the Z chromosome with the W chromosome of aulopiform species in view of molecular evolution. The comparison will give us implication as to whether

male-driven evolution is a common rule or not in vertebrates. Moreover, if we could compare mapk-like sequence between gonochoristic and hermaphroditic species, we would also be able to understand sexuality from the viewpoint of molecular evolution.

References

Agunik A. I., Bishop C. E., Lerner J. I., Agulnik S. I and Solovyev V. V. (1997) Analysis of mutation rates in the SMCY/SMCX genes shows that mammalian evolution is male driven. Mammalian Genome 8: 134-138

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Alexander E. V. (1998). Genome size and GC-percent in vertebrates as determined by flow sytometry: the triangular relationship. Cytometry 31: 100-109

Asahida, H., Kobayashi, T., Saitoh, K., and Nakayama, I. 1996. Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentration of Urea. Fisheries Science 62 (5), 727-730.

Baroiller J. F., Guiguen Y. and Fostier A. (1999) Endocrine and environmental aspects of sex differentiation in fish. Cell Mol Life Sci 55:910-931

Born G. G. and Bertollo L. A. (2000) An XX/XY sex chromosome system in a fish species, Hoplias malabaricus, with a polymorphic NOR-bearing X chromosome. Chromosome Res; 8(2): 111-8.

Chang B. Hung-Hunn and Li Wen-Hsiung (1995) Estimating the intensity of male-driven in rodents by using X-linked and Y-linked *Ube 1* genes and pseudogenes. J Mol Evol 40: 70-77

Chang L. and Karin M. (2001) Mammalian MAP kinase signaling cascades. Nature, vol 410: 37-40

Chen T.R (1969) Karyological heterogamety of deep-sea fishes. Postilla 130, 1-29.

Chen T.R. and Ebeling A.W (1974) Cytotaxonomy of Californian Myctophoid fishes. Copeia, 1974: 839-848

Delvin R. H., Stone G. W. and Smailus D. E. (1998) Extensive direct-tandem organization of a long repeat DNA sequence on the Y chromosome of Chinook salmon (*Oncorhynchus tshawytscha*). J Mol Evol 46: 277-287

Filho, O. M., Bertollo L. A. C., and Galetti Jr. P. M. (1980) Evidences for a multiple sex chromosome systems with female heterogamety in *Apareiodon affinis* (Pisces. Parodontidae). Caryologia 33: 38-91.

Forbes S. H., Knudsen K. L., North T. W., and Allendorf F. W. (1994) One of two growth hormone genes in coho salmon in sex linked. Proc. Natl. Acad. Sci. USA 91: 1628-1631

Galetti, Jr P. M., Foresti F., Bertollo L. A. C. and Filho O. M. (1981) Heteromorphic sex chromosomes in three species of the genus Leporinus (Pisces. Anostomidae). Cytogenet. Cell Genet. 29: 138-142

Galetti, Jr,. P. M. and Foresti F. (1986) Evolution of the ZZ/ZW system in Leporinus (Pisces, Anostomidae). Cytogenet. Cell Genet. 43: 43-46.

Gallardo M. H., Bickham J. W., Honeycutt R. L., Ojeda R. A and Kohler N (1999) Discovery of tetraploidy in a mammal Nature 401: 341

Graves J. A. M. and Shetty S. (2001) Sex from W to Z: evolution of vertebrate sex chromosomes and sex determining genes. J Exp Zool 290: 449-462

Howell W. M. and Black, D. A. 1979. Location of the nucleous organizer regions on the sex chromosomes of the banded killifish, Fundulus diaphanus. Copeia 544-546.

Kahn N. W. and Quin T. W (1999) Male-driven evolution among Eoaves? A test of the replicatice division hypothesis in a heterogametic femal (ZW) system. J Mol Evol 49:750-759

Katoh K. and Miyata T. (1999) A heuristic approach of maximum likelihood method for inferring phylogenetic tree and an application to the mammalian SOX-3 origin of the testis-determining gene SRY.
FEBS Lett. 463(1-2):129-32.

Kimura M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol.Evol 16(2):111-120 .

Koehler M. R., Dehm D., Guttenbach M., Nanda I., Haaf T., Molina W. F., Galetti P. M., Jr, Schmid M. (1997) Cytogenetics of the genus *Leporinus* (Pisces, Anostomidae). 1. Karyotype analysis, heterochromatin distribution and sex chromosomes. Chromosome Res.: 5(1): 12-22.

Lamatsch D.K., Steinlein C. and Schmid M. (2000) Noninvasive determination of genome size and ploidy level in fishes by flow cytometry: detection of triploid *Poechila Formosa*. Cyttometry 39:91-95

Marin I. and Baker B. S. (1998) The evolutionary dynamics of sex determination.
Science 25;281(5385):1990-4

Mead G. W. (1960) Hermaphroditism in archibentic and pelagic fishes of the order Iniomi. Deep-Sea Res. 11: 569-596

Moran P, Martinez JL, Garcia-Vazquez E, Pendas AM. (1996) Sex chromosome linkage of 5S rDNA in rainbow trout (Oncorhynchus mykiss). Cytogenet Cell Genet; 75(2-3): 145-50.

Morita Y., Hasegawa K., Nishigaki R., Shimada A., Wada H., Kusakabe T., Suzuki N., Kinoshita M., Kanamori A., Terado T., Kimura H., Nonaka M. and Shima A. (2000) A detailed likage map of medaka, Oryzias latipes: comparative genomics and genome evolution. Genetics 154: 1773-1784

Nakamura A. and Kuwamura T. (1987) "Sex change in fishes" Tokai universtiy press

Nakayama I., Foresti F., Tewari R., Schartl M and Chourrout D. (1994) Sex chromosome polymorphism and heterogametic males revealed by two cloned DNA probes in the ZW/ZZ fish *Leporinus elongatus*. Chromosoma 103: 31-39

Naruse K., Fukamachi S., Mitani H., Kondo M., Matsuoka T., Kondo S., Hanamura N., Reed K. M., Bohlander S. K. and Phillips R. B. (1995) Microdissection of the Y chromosome and fluorescence in situ hybridization analysis of the sex chromosomes of lake trout, *Salvelinus namaychush*. Chromosome Res 3: 221-226

Nanda I., Schartl M., Epplen J. T, Feichtinger W, and Schmid M. (1993) Primitive sex chromosomes in poeciliid fishes harbor simple repetitive DNA sequences. J Exp Zool. 265(3): 301-8.

Nanda I, Shan Zhihong, Schartl M., Burt D. W., Hoehler M., Nothwang H., Grutzner F., Paton I. R., Windsor D., Dunn I., Engel W., Staeheli R., Mizuno S., Haaf T. and Schmid M. (1999) 300 million years of conserved synteny between chicken Z and human chromosome 9. Nature genetics 21:258-259

Nelson, J.S., (1994) Fishes of the World. Third ed,. Wiley, New York.

Nishikawa S and Sakamoto K. (1978) Comparative studies on the chromosomes in Japanese fishes. Ⅳ. Somatic chromosomes of two lizardfishes. J. Shimon. Univ. Fish. 27: 117-13

Ohno S. 1974. "Animal cytogenetics" vol. 4, Chordata 1. Gebrunder Borntraeger, Berlin.

Ohno S. (1967) Sex chromosomes and Sex Linked Genes. Springer, Berlin.

Ojima Y (1983) Fish Cytogenetics. In: Sharma, AK, Sharma A, eds. Shromosomes in Evolution of Eukaryotic Groups Vol.1. Boca Raton FL: CRC Press, pp. 11-145.

Ota K., Kobayashi T, Ueno K., Gojobori T. (2000) Evolution of heteromorphic sex chromosomes in the order Aulopiformes
Gene 23; 259(1-2): 25-30

Pendas A. M., Moran P., Freije J. P. and Garcia-Vazquez E. (1994) Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenet Cell Genet 67: 31-36

Pierrez J and Ronot X. (1991) Use of diploid and triploid trout erythrocytes as internal standards in flow cytometry. Cytometry 12: 275-278

Postlethwait J. H., Woods I. G., Ngo-Hazelett P., Yan Y. L., Kelly P. D., Chu F., Huang H., Hill-Force A., and Talbot W. S. (2000) Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Res 10(12):1890-902

Ronchetti E., Salvadori S., and Deiana A. M. (1995) Genome size and AT content in Anguilliformes. Eur. J. Histochem 39: 259-265

Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425

Sakamoto T., Danzmann R. G., Gharbi K., Howard P., Ozaki A., Khoo S. K., Woram R. A., Okamoto N., Ferguson M. M., Holm Lars-Erik, Guyomard R and Hoyheim B. (2000) A microsatellite likege map of rainbow trout (*Oncorhynchus mkiss*) Characterized by large sex-specific differences in recombination rates. Genetics 155: 1331-1345

Shetty S, Griffin DK, Graves JA.(1997)
Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. Chromosome Res. 7(4): 289-95.

Shimmin L. C., Chang B. Hung-Junn, and Li Wen-Hsiung (1993) Male-driven evolution of DNA sequences. Nature 362: 745-747

Smith, C. L. (1975) "The evolution of hermaphroditism in fishes" In: R. Reinboth (ed.) Intersexuality in the Animal Kingdom. pp. 295-310, Springer Verlag, Berlin.

Sumner, A. T. (1972) A simple technique for demonstrating centrometric heterochromatin. Exp. Cell Res. 75: 304-306

Suzuki A., Taki Y., Mochizuki M., and Hirata J. (1995) Chromosomal speciation in Eurasian and Japanese Cyprinidae (Pisces, Cypriniformes). Cytobios 83: 171-186

Thompsom J.D., Gibon T.J., Plewniak F, Jeanmougin, F. and Higgins D.G., (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876-4882.

Tiersch T. R., Chandler R. W, Kallman K. D and Wachtel S. S. (1989) Estimation of nuclear DNA content by flow cytometry in fishes of the genus *Xiphophorus*. Comp. Biochem. Physiol. Vol. 94B, No. 3: 465-468

Uyeno T and Miller R. R. (1971) Multiple sex chromosomes in Mexican cyprinodontid fish. Nature 281: 452-453

Vadim J. B., Andrei I. P. and Boris F. G. (1993) DNA content in Eurasian sturgeon species determined by flow cytometry. Cytometry 14: 377-383.