Ancient Constraint Sow the Seed of Vertebrate; Evolutionary Insight for the Segmental Development of the Vertebrate Pharyngeal Arch

January 2014

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A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Science

(Doctoral Program in Biological Sciences)

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Abstract

On the origin of the vertebrate head, the developmental relationship between the somite and the pharyngeal arch has been controversial issue for long time. I revealed the indispensable function of medaka *pax1* for the pharyngeal segmentation. Unexpectedly, *pax1* knockdown shed light on the primary segmentation of the mesoderm. This notion allowed me to consider the unveiled relationship between somite and pharyngeal arch. In addition to the results from medaka, my observation of the amphioxus and lamprey development of the pharyngeal segmentation suggests the evolutionary conserved segmental mechanism based on the primary rhythm of somite.

On the other hand, in the course of the sequence analysis of lamprey mtDNA, I found the dynamic nature of the repetitive sequences. Although the initial motivation was to find genetic marker for identifying lamprey species, my results rather contribute to understand the novel aspects of the molecular evolution in vertebrate.

Finally, I discus the evolutionary scenario of the vertebrate head from the aspect of the developmental constraints. The ancestor of vertebrates probably acquired the pharyngeal arch by using somite pattern, while it also evolved the anterior arches where the constraints were relaxed.

General Introduction

Looking out over the various morphologies of bilaterian animals, in their body, the repetitive structures are often recognized along anteroposterior axis (Minelli and Fusco, 2004). This segmental body plan is adopted broadly across major phyla, for example, Arthropoda, Annelida and Chordata (Minelli and Fusco, 2004), which represent three major branches of bilaterians; Ecdysozoa, Lophotrochozoa and Deuterostome, respectively (Halanych et al., 1995: Aguinaldo et al., 1997; Sarrazin et al., 2012). Although the issue whether the last common ancestor of bilateria called urbilateria possess the segmental body plan is deeply rooted (Kimmel, 1996; Balavoine and Adoutte, 2003), recent studies have pointed out the crucial common grounds among developmental mechanism of bilaterian segmentation and supported the idea of the segmented urbilateria (Martin and Kimelman, 2009; Dray et al., 2010; Steinmetz et al., 2011). Basically, the primary reiteration of embryos is brought about by sequential segregation of mesodermal units called somite (Martin and Kimelman, 2009). In vertebrate, the somites arise from a posterior zone of active cell proliferation (Maroto et al., 2012). In short germ insects except for *Drosophila*, the most anterior segments are also segregated sequentially from an embryonic posterior zone of cell

proliferation (Davis and Patel, 2002). Additionally, the body segments of annelid are also brought about by the mesodermal cells from the posterior growth zone (de Rosa et al., 2005). Finally, the genes related to the cyclic segregation of somite are similar among these animals, for example, *hairy* cognates in vertebrate and insect (Muller et al., 1996), and *hedgehog* in arthropod and annelid (Dray et al., 2010).

On the ontogeny and phylogeny of bilaterian animals, the segmental body plan has played a critical role for generating diverged morphology. The establishment of the repetitive somites gives organism the redundancy for the usage of each somite and results in the development and evolution of the segment-specific organs (Lemons and McGinnis, 2006). In addition, the combinatorial usages of segments also contribute the highly sophisticated structure such as insect head (Lemons and McGinnis, 2006). These aspects of the segmental body plan can be regarded as one of the most important basis of the animal morphological evolution. On the other hand, as well as such notable contributions of the segmentation for the morphological evolution, another aspect of the segmental body plan, which seems to restrict the morphological diversity, should be pointed out. Because the primary reiteration generated by somitogenesis sets down the fundamental pattern of the development, the developmental patterns of organogenesis after somitogenesis are sometimes forced to adhere the primary pattern of the

somitogenesis. The typical example is the arthropod body. Within the arthropods, the exoskeletons, appendages, nervous system, kidneys, muscles and body cavity are distributed segmentally, and these segmental patterns are imposed by the primary segmentation of somite (Deutsch, 2004). In vertebrate, although it doesn't exhibit segmental appearance so much as arthropod, the axial skeletons, innervation patterns and the streams of neural crest cells follow the primary reiteration by somitogenesis (Rickmann et al., 1985; Bernhardt and Schachner, 2000). These secondarily imposed reiterative patterns are applied to the concept of developmental constraints (Wagner, 1994; Kuratani, 2003). Concerning the evolution of the developmental mechanisms, the developmental constraints restrict or bias the direction of the evolution because of the causality among developmental mechanisms. Namely, the conserved body plan of segmentation among bilaterian animals can be interpreted as the broad constraints for the developmental and evolutionary patterns of the subsequent organogenesis (Knoll and Carroll, 1999).

Despite of the conserved ancient developmental constraint brought about by somite, in vertebrate, another segmentation is recognized. In the developing trunk, the primary segmental pattern come about by somites is called somitomerism (de Beer, 1937), on the other hand, another segmental pattern in vertebrate is called

branchiomerism because it is generated by the reiterated pharyngeal arches (branchial arches) of vertebrate embryos (Romer, 1995). The pharyngeal arch is the metameric structure in the embryonic vertebrate head. Through the development, the pharyngeal arch contributes to various organs called pharyngeal derivatives such as jaw, auditory ossicles, gill skeletons, thymus, parathyroid and urtimobranchial body (Graham et al., 2005). Historically, branchiomerism has been thought to be corresponding to somitomerism because the vertebrate head is seen a serial array of mesodermal segments by researchers called segmentalists (Goodrich, 1930). The segmentalists have postulated the presence of the head somites, and thought that the primary segmental pattern inheres in the head somite called somitomere, not in any other tissue (Goodrich, 1930). The important rationales of the segmentalists for the presence of somitomere are the head cavities of shark embryo and anterior somites of amphioxus (Goodrich, 1930; Holland, 2000; Kuratani, 2003). However, recent studies have revealed the histological and genetic differences of the head cavities from trunk somite (Adachi and Kuratani, 2012; Adachi et al., 2012). Furthermore, while amphioxus develops somites in the most anterior region, the developmental mechanism of the anterior somites is different from the posterior segmentation (Bertrand et al., 2011). These studies support the idea that loss of the anterior somitomerism contributes to

the vertebrate head by relaxing the developmental constraint by segmental pattern of mesoderm.

During the segmental development of the pharyngeal arch, the pharyngeal endoderm plays a crucial role for the separation of each arch (Graham et al., 2005). The pharyngeal endoderm develops the repetitive outpocketings called pharyngeal pouch. Unlike somitomerism, because the segmental pattern is thought to inhere not in the mesoderm but in the endoderm itself, branchiomerism has the primary segmental pattern generated by the pharyngeal endoderm (Kuratani, 2003; Graham et al., 2005). On the other hand, the developmental relationships between endoderm and mesoderm also have been reported in vertebrate species (Piotrowski et al., 2003; Crump et al., 2004; Zhang et al., 2006). Therefore, in order to describe the evolutionary process of the vertebrate pharyngeal arch and the vertebrate head, it is necessary to understand the mechanism of the pharyngeal arch segmentation. Fortunately, the pharyngeal segmentation is not an endemic feature of vertebrate. Because the pharyngeal gill slits in the non-vertebrate deuterostomes are homologous to the vertebrate pharyngeal pouch, the comparative developmental researches are informative to understand the origin of the vertebrate pharyngeal arch (Peters et al., 1995; Holland et al., 1995; Ogasawara et al., 1999; Ogasawara et al., 2000; Gillis et al., 2012).

In addition to the importance of the non-vertebrate deuterostomes, a living jawless-vertebrate lamprey is also informative to infer the early state of the pharyngeal arch (Ogasawara et al., 2000; Shimeld and Donoghue, 2012). The study of lamprey development is motivated principally by the phylogenetic position of these organisms (Shimeld and Donoghue, 2012). Lamprey provides a window into understanding the developmental processes present in early vertebrates and, hence, a key to understanding what has changed during the evolution of novel structures. In fact, previous studies in lamprey have advanced understandings of several vertebrate features including jaws, fins and neural crest cells (Shimeld and Donoghue, 2012). In spite of such notable importance of lamprey developmental studies, lampreys have never been taken through a complete life cycle in captivity, and developmental studies are based on wild-caught specimens. Gravid adults can be collected and held for some time in cool fresh water, before strip-spawning and in vitro fertilization (Nikitina et al., 2009). However, because of their relatively simple morphology, identification of species is often difficult. In particular, their larvae called ammocoete are amazingly similar each other, and the different multi-species share a habitat in Japan. Hence, the reliable molecular marker is desired (Yamazaki and Goto, 1998).

In the present thesis, firstly, I focused on Japanese lampreys to obtain the

genetic markers for the species identification. The initial motivation was material augment of lamprey species for later developmental study while, sequence analysis of the Japanese lamprey species revealed rather interesting aspect of the molecular evolution of their mitogenome. In the first chapter, I describe the dynamic evolution of the repetitive sequence in the mitochondrial DNA of Japanese lampreys, genus *Lethenteron*.

Secondarily, I challenged unveiling the developmental mechanism and the evolutionary pathway of the vertebrate pharyngeal arch in the second chapter. I focused on the endodermal segmentation and on the expression and function of *pax1* gene in medaka embryo. The experiments revealed the indispensable function of medaka *pax1* for proper pouch segmentation while, surprisingly, the mesodermal cells in pharynx seemed to be segmented without pouch segmentation. Examing the results from medaka, lamprey and amphioxus together, I tried to elucidate the evolutionary scenario of the pharyngeal arch and vertebrate head.

Chapter 1

Repetitive Sequences in the Lamprey Mitochondrial DNA Control Region and Speciation of *Lethenteron*

1. 1. Introduction

Sequence variation in mitochondrial DNA (mtDNA) has been widely used for molecular phylogenetic studies. Within mtDNA, the control region has the highest evolutionary rate and serves as a molecular marker for examining relatively recent events, such as among populations (Avise et al., 1987; Avise, 1991). The control region shows sequence variation in nucleotide substitutions, indels, and the numbers of tandem repeat sequences. Numerous studies have documented copy number variation within populations, and sometimes in single individuals (Ludwig et al., 2000; Hoarau et al., 2002; Mjelle et al., 2008).

Most of the copy number variation is thought to arise from slipped-strand mispairing during mtDNA replication (Buroker et al., 1990; Broughton and Dowling, 1994; Mundy and Helbig, 2004). This strand slippage is facilitated by the secondary structure of the repeat sequence. In addition to slipped-strand mispairing,

recombination accounts for some of the sequence variation among repeats (Hoarau et al., 2002; Mjelle et al., 2008).

Lamprey mtDNA contains two repeat regions within the control regions, NC1 and NC2 (Lee and Kocher, 1995). Recently, White and Martin (2009) analyzed the copy number variation of the least brook lamprey, *Lampetra aepyptera*, and reported that the copy number variation in NC1 is due to slipped-strand mispairing.

Besides their phylogenetic position as a basal group of vertebrates, lampreys are an interesting biological group in terms of speciation. All lamprey species breed in fresh water, where they spend several years as suspension or detritus feeders (Hardisty, 1971). This stage is known as the ammocoetes larval stage. After metamorphosis, while some species parasitize fish and other animals, other species do not feed after metamorphosis and breed within several months. These non-parasitic species are believed to have evolved several times from ancestral parasitic species (Zanandrea, 1959; Hardisty, 1971; Hubbs, 1971). Variation in the lamprey life history may be one of the factors inducing speciation in lampreys.

The present study focused on four *Lethenteron* species around Japan: *Lethenteron japonicum*, *Lethenteron kessleri*, and two cryptic species of *L.* sp. N and *L.* sp. S (Yamazaki and Goto, 1998; Yamazaki et al., 2006). Of these, *L. japonicum* is

parasitic species, which grow in the sea after metamorphosis, while *L. kessleri* and the two cryptic species of *L.* sp. N and *L.* sp. S have an entirely freshwater life cycle and non- parasitic. Molecular phylogenetic studies indicated that *L.* sp. S diverged from the most basal node, and *L.* sp. N, *L. japonicum*, and *L. kessleri* form a monophyletic clade (Yamazaki et al., 2006). Although several fixed nucleotide polymorphisms in the cytochrome oxidase subunit I gene (*COI*) have been identified in *L.* sp N. and *L.* sp. S, no fixed nucleotide polymorphisms distinguish *L. japonicum* from *L. kessleri* (Yamazaki et al., 2006). To date, only one fixed allele, of malate dehydrogenase 3 (MDH3), has been recognized as a distinct molecular characteristic distinguishing *L. japonicum* and *L. kessleri* (Yamazaki and Goto, 1998).

Because *L. japonicum* is parasitic and *L. kessleri* is non-parasitic, these two species are readily distinguished as adults. However, the ammocoetes larvae of these species are quite difficult to distinguish, based on morphology. Additionally, some individuals of *L. japonicum* have been reported to remain in fresh water (Yamazaki et al., 1998). Thus, genetic markers that distinguish these species are desirable.

In this study, I characterized the molecular structure of the mitochondrial control regions of the Japanese lamprey species, seeking molecular markers to identify *Lethenteron* species. I report the highly variable nature of the NC2 repeats (Lee and

Kocher, 1995) in *Lethenteron*. A detailed analysis of the sequence of the repeat arrays allowed me to trace some of the evolutionary history of the generation of variable repeats. However, I could not recover a phylogenetic signal that distinguished *L. japonicum* and *L. kessleri*. These two species likely diverged too recently to detect genetic fixed genetic loci in mtDNA.

1. 2. Materials and Methods

1. 2. 1. Amplification and Sequencing of the mtDNA Control Region and *SoxD* Intron

The sampling localities and numbers of individuals of four species of *Lethenteron* examined are summarized in Table 1–1. In the present study, I regard *L.* sp. S as a species of *Lethenteron* following previous studies, however, as described in Yamazaki et al. (2006), the classification of the genera *Lethenteron* and *Lampetra* should be re-examined in the future studies. DNA was extracted from body tissues using a DNeasy Kit (QIAGEN). The primers used to amplify the NC2 region of mtDNA control region and *SoxD* are shown in Table 1–2. These amplified approximately 700 bp of the mtDNA control region and 400 bp of the *SoxD* intron. PCR conditions as follows; initial denaturing at 95°C for 3 minites, 35 cycles of 95°C for 1 minite, 65°C for 1minite and 72°C for 1minite. For the PCR, PrimeSTAR GXL polymerase (Takara) was used. The nucleotide sequences were determined directly from the PCR fragments after treatment with ExoSAP (GE Healthcare) by using ABI PRISM 377 or 310.

1. 2. 2. Sequencing the Complete mtDNA of *L.* sp. S

The complete mtDNA of specimen Ss3 was sequenced. First, the mtDNA was amplified in six fragments, using the six primer pairs (Table 1–2). Fragments generated were subsequently sequenced by primer walking.

1. 2. 3. Sequence Analyses

The sequences were compared using the Genetyx software (Genetyx). A network analysis of nucleotide substitutions was performed using TCS 1.21 (Clement et al., 2000). The secondary structure of the DNA was analyzed using MFOLD (Zuker, 2003).

1. 3. Results

1. 3. 1. Characterization of the mtDNA Control Region

To examine the genetic structure of *Lethenteron*, I analyzed the sequence variation in NC2 between $tRNA^{Glu}$ and *cytB* (Lee and Kocher, 1995). In this region, repeat sequences have already been reported for *Petromyzon marinus* (Lee and Kocher, 1995) and *Lampetra fluviatilis* (Delarbre et al., 2000), and I found repeat sequences in the four Japanese species (Fig. 1–1). I sequenced the repeat region from 41 specimens of the four species and found that the repeats were highly variable in terms of both copy number and the nucleotide sequences of the repeat arrays (Fig. 1– 2A–C). The copy number and sequences were variable even within local populations, in contrast to a report that the NC1 repeat of *Lampetra aepyptera* in the United States lacked polymorphisms within local populations (White and Martin, 2009). I did not detect any heteroplasmy, and PCR amplification always resulted in a single DNA band, which was sequenced directly. Detailed analyses of the repeat sequences reveal highly dynamic evolution of the repeat sequences within populations, as described below.

In order to examine the dynamic nature of the repeats, I classified the repeat

sequences based on network analysis of the repeat sequence (Fig. 1–2A, B). Because the network analysis indicated that A3 and B2 formed nodes of the network (Fig. 1–2B), I classified the repeat sequences into two types based on the 22nd position of the repeat: T in the A-type and C in the B-type. A3 and B2 were also observed in *L. fluviatilis* (Delarbre et al., 2000) and *P. marinus* (Lee and Kocher, 1995), respectively. The rest of the repeat sequence types were derived via distinct substitutions or indels. All of the repeat sequences possessed multiple T sequences at their 3′-end, and this number was quite variable. I identified the sequences of the repeat arrays in the form type-number of repeats. For example, A3–5 has the A3-type sequence with five Ts at the 3′-end (Fig. 1–2A). The published sequence of *P. marinus* mtDNA is B2–7, while those of *L. fluviatilis* are A4-6 and A3–5, 8, and 7 (Fig. 1–2C).

1. 3. 2. Characterization of the Novel Repeat Sequences that Emerged from tRNA Genes

I found another type of the repeat sequence in some individuals of *L.* sp. S from Senju, between $tRNA^{Thr}$ and $tRNA^{Glu}$ (tRNA-Thr/Glu repeat: Fig. 1–3). The novel repeat unit: tRNA-Thr/Glu repeat originated at the 3′-end of the $tRNA^{Thr}$ and the complementary sequence of the 3′-end of the $tRNA^{Glu}$ (Fig. 1–3B). The unit was

repeated up to five times in *L.* sp. S. Because the repetitive sequence originated from the 3′-end of $tRNA^{Thr}$ and $tRNA^{Glu}$, I examined whether these tRNA genes were functional. I examined the entire sequence of mtDNA for *L.* sp. S (GenBank/EMBL/DDBJ Acc. No. AB565771).

1. 3. 3. Analysis of the Secondary Structure of the DNA

In order to consider the mechanism of repeats by slipped-strand mispairing, the secondary structures of the A3–5 in the NC2 repeat, the two tendem repeats of A3–5 in the NC2 repeat and the 3′ half of $tRNA^{Thr}$ and the 3′ half of the complementary strand of $tRNA^{Glu}$ were examined. Within each sequence, stem-loop structures were expected (Fig. 1–4).

1. 3. 4. Sequence Analysis of *SoxD* Intron

I compared the nucleotide sequences in the rest of the control region between *L. japonicum* and *L. kessleri*. Although there were some single nucleotide polymorphisms in the sequences, none was fixed in either species. Searching for genetic markers, I also compared the approximately 400 bp sequence of the *SoxD* intron (Ohtani et al., 2008). Although there were two deletions, including one from a CA microsatellite, no fixed

indel or substitution was observed in either species (Fig. 1–5).

1. 4. Discussion

1. 4. 1. Repeat Dynamics within and among Species

Unexpectedly, I found the distinct variable repeats in the NC2 region. As shown in Fig. 1–2C, the number of repeats is not fixed within species, with six or seven repeats in *L. japonicum*, three to eight repeats in *L.* sp. N, and four or five repeats in *L.* sp. S. Although some individuals within populations possess the same copy number of repeat arrays, the internal architecture of the repeat arrays was quite different. Among the 41 individuals, only two groups shared identical repeat architecture (js5/ji1/ji2 and Nk5/Nk8). Some individuals showed traces of recent events that led to the copy number variation. One example was js4 (*L. japonicum* from Shiribeshi), in which two arrays of the repeat (B2–6 and A1–5) were duplicated in the third to sixth repeats (Fig. 1–2C). This pattern of repeats supports the occurrence of slipped-strand mispairing after the two repeat units unfolded, and subsequently two arrays were inserted in the repeats.

Additionally, nk3 shows evidence of slipped-strand mispairing. It has two arrays of the A9-type sequence at the 3′-end of the repeat. Because A9 required two substitutions from the ancestral A3, these were not likely to have emerged simultaneously in the third and fourth repeats, but more likely arose due to

slipped-strand mispairing. Similarly, the two arrays of A5 at the 5′-end of Ns2 also likely arose via slipped-strand mispairing.

These two cases indicated that slipped-strand mispairing has inserted either one or two copies of the repeat array. Insertion of a single copy is due to folding of a single array of the repeat, while insertion of two copies is due to the folding of the two arrays. MFOLD analyses of the possible DNA secondary structures (Zuker, 2003) indicated that the repeat sequence folds stably both as a single array and as two arrays (Fig. 1–4).

These observations suggest that the copy number of the repeat is highly variable, and turnover of the repeats is rapid. This, in turn, indicates that the same copy number does not guarantee the same evolutionary history. Thus, I believe that copy number variation is not suitable as a genetic marker in *Lethenteron* species.

Regarding the high turnover rate, the case of jm2 is also worth noting. The repeat in jm2 has distinct sequences in its second to fourth arrays, which involve insertion of CTTTTT in the repeat (C1 and C2 in Fig. 1–2D). The sequence of the third repeat lost 5′ nucleotide stretch of AATTGT (C3 in Fig. 1–2D). This replacement of the repeat sequence occurred without changing the number of repeats; jm1 retained the typical architecture of the arrays of *L. japonicum* (four B-type arrays and three A-type

arrays). If slipped-strand mispairing accounts for the replacement of the repeat arrays, it should accompany the increase in copy number. Although I cannot exclude the possibility that the loss of the original B2-type of the repeat arrays occurred after the slippage event, I may need to consider other mechanisms of the gene conversion, such as intermolecular recombination (Hoarau et al., 2002; Mjelle et al., 2008). Recent studies documented instances of mtDNA recombination (Hoarau et al., 2002; Mjelle et al., 2008), and some experimental evidence has been reported in other taxonomic groups (Lunt and Hyman, 1997; Ladoukakis and Zouros, 2001).

In this regard, the case of the Kamo population of *L.* sp. S also requires special attention. Two individuals, sk1 and sk2, possessed distinct repeat sequences (Fig. 1–2E). The D1 sequence might have emerged via substitution and duplication of the internal sequence (Fig. 1–2E) and D3 might have emerged via further insertion and deletion of the sequence (Fig. 1–2F). Note that all of the repeat arrays were replaced by the D-type, and no A-type repeat arrays were left.

I also found another type of the repeats between $tRNA^{Thr}$ and $tRNA^{Glu}$ of the Ss individuals (Fig. 1–3). The unit was repeated up to five times in *L.* sp. S. This expansion of the repeat is best explained by slipped-strand mispairing, facilitated by the secondary structure of the tRNA stems (Fig. 1–4). Additionally, the 3′-abutting

sequence of the $tRNA^{Thr}$ anticodon and complementary sequence of $tRNA^{Glu}$ possess 13 bp of identical sequence (double-ended arrows in Fig. 1–4B), which also facilitates the folding of the replication strand and subsequent slippage. The nucleotide sequence of the 13 bp nucleotide sequence was identical in *P. marinus* (Fig. 1–6; Lee and Kocher, 1995), although the repeat was not detected in the individual for which the full-length mtDNA was analyzed.

In the full-length sequence of the mtDNA of *L* sp. S, the gene arrangement was identical to that in *P. marinus* and *L. fluviatilis*. There are several nucleotide sequences that need to be translated using $tRNA^{Thr}$ and $tRNA^{Glu}$, and no additional tRNA genes were found in the mitochondrial genome. Thus, I concluded that the two tRNA genes are likely functional.

1. 4. 2. Molecular Markers for *L. japonicum* and *L. kessleri*

My primary motivation for analyzing the control region of the *Lethenteron* species was to search for molecular markers suitable for species identification. However, present results suggest that the copy number is too variable for species identification, and I did not find reliable characteristics that distinguished *L. japonicum* from *L. kessleri*. Yamazaki and Goto (1998) found that only the MDH3 locus

showed fixed allele differences in *L. japonicum* and *L. kessleri*. No allele was fixed in the remaining 26 allozymes tested and the COI sequence did not distinguish the two species (Yamazaki et al., 2006). No fixed differences were observed in the *SoxD* intron in the two species. This suggests that these two species diverged quite recently (Yamazaki and Goto, 1998; Yamazaki et al., 2006). Adult *L. japonicum* and *L. kessleri* are generally easy to distinguish because they differ markedly in size. Although *L. japonicum* continues to grow in the sea after metamorphosis, and *L. kessleri* stays in fresh water and does not grow after metamorphosis, Yamazaki et al. (1998) reported that some individuals of *L. japonicum* do stay in fresh water after metamorphosis. Additionally, it is difficult to distinguish the ammocoetes larvae of these species. Thus, for species identification, I need to find a reliable molecular marker. These two species may show fixed genetic markers in a restricted chromosome region around MDH3 locus (Yamazaki and Goto, 1998).

In the present study, I found quite a dynamic nature of the repeat sequences of the *Lethenteron* mtDNA control region. Most of the 41 individuals examined show unique architectures of repeat arrays in their mtDNA control region. The detail analyses of the repeat arrays provided evidences that most of the variations can be due to slipped-strand mispairing. Some cases, such as the repeat arrays found in specimens

sk1, sk2, and jm2, might be better explained by gene conversion, due to intermolecular recombination. Although my primary purpose to find the molecular markers to distinguish between *L. japonicum* and *L. kessleri* was not achieved, the highly dynamic nature of the repeat sequences in *Lethenteron* mtDNA control region provided a unique system to analyze the details of the molecular evolution of repeat sequences. In addition, the present study further strengthened the idea that these two species diverged quite recently. This rather encouraged me to seek for the evolutionary transition from the parasitic lifestyle of *L. japonicus* to non-parasitic lifestyle of *L. kessleri*. Investigations on the genomic sequences around the MDH3 locus may give me clues to understand the evolutionary transition.

General Discussion

Molecular Evolution of Lamprey mtDNA and Biological Contributions

Current researches in the field of evolutionary developmental biology are flourishing in a wide sphere. High quality embryology by using 3D reconstruction on a computer sheds light on the deeply rooted issue on the early evolution of the vertebrate (Oisi et al., 2013). Dramatic innovation of the imaging technologies allows researches to observe signal molecules such as RA (Shimozono et al., 2013). Experimental evolution and population genetics also make a breakthrough by means of next generation sequencers, and researchers can detect numerous transitions of heredity traits occurring without morphological alternation (Barrick and Lenski, 2013). Because of this situation, what is strongly bringing out researches' interests is the evolution in the field (Garfield et al., 2013). To describe the current evolution in the field, it is necessary to reveal the evolutionary history of the target genetic architectures.

I described the dynamic evolution of repeat sequences of the lamprey mtDNA. Although the initial motivation of the research was to find the reliable molecular markers for species identification, I successfully described the short-term molecular evolution in the actual field. Lamprey is intriguing animal from various biological

aspects; the important phylogenetic position to understand early evolution of vertebrate (Shimeld and Donoghue, 2012), the architecture of speciation (Yamazaki et al., 2006), lifestyle polymorphism (Yamazaki et al., 1998) and the genetic rearrangement in somatic cells (Smith et al., 2012). I believed that my research contributed to reveal one of the interesting aspects of the biology of lamprey. Recently, whole genome sequence of *Petromyzon marinus* was reported, it must accelerate more innovation of lamprey research (Smith et al., 2013).

In the light of the molecular biology, one could argue that my description of the repeat evolution revealed the molecular nature of lamprey mtDNA in the vital system. Currently, such accumulation of apparently senseless mutations attract rising attention because novel biological meanings are found after another in the sequences annotated rather senseless once or unknown function. For example, ENCORD project on human genome suggested that most of whole genome of human is transcribed (Kavanagh et al., 2013). Additionally, in mouse development, sequence length of introns in *Hes7* gene, which is a clock of somitegenesis of the vertebrate, regulates the pace of oscillation of the gene and the segmental rhythm of somite (Harima et al., 2013). I don't intend to find novel function on the lamprey repetitive sequence now, while it may be a lesson telling researchers importance of honest and detailed observation at a

level of one base.

Finally, scarcity of Japanese lamprey species should be argued. As mentioned above repeatedly, needless to say, lamprey is quite valuable animal in wide-range biology (Shimeld and Donoghue, 2012). However, environmental condition surrounding Japanese lampreys is getting worse, in fact, lamprey species in Japan are listed as endangered species. In order to prevent Japanese lamprey resources from depletion and species extinctions, we have to consider seriously on the environmental issues. Present research revealed a part of genetic architecture of lamprey species, and I hope my findings will contribute to conservations of lampreys, even if only a little.

On the Origin of the Vertebrate Head

Numerous researchers have been attracted to the sophisticated morphologies of the vertebrate head and its complexities. On the evolution of the vertebrate head, a major issue is whereabouts of the primary developmental pattern of the head and its evolutionary origin (Kuratani, 2003). In this study, I found the evidence supporting the presence of the mesodermal segmentation in the pharyngeal arch, at least posterior than PP3. I have already mentioned the presence of the mechanical boundary between PP2 and PP3. Taking advantaging these notions, I would like to discuss the

evolutionary process of the vertebrate head.

In the light of segmetntalists, the anterior segmentation of amphioxus somite is crucial evolutionary support as homolog of the somitomeres in the vertebrate head (Holland, 2000). However, resent study on amphioxus development has revealed the somatic boundary defined by whether FGF-sensitive or insensitive (Bertrand et al., 2011). According to that previous research, the boundary is identified between the third and fourth somite. Interestingly, according to my observation of amphioxus larvae, this boundary seems to be congruent with the anterior boundary defined by whether the somite bears a gill slit or no gill slit. Additionally, our developmental study on medaka pointed out the different regions of the pharyngeal pouch, namely *pax1*-independent development of PP1 and PP2 and *pax1*-dependent development of the more posterior pouches. As mentioned in chapter 2, this boundary is consistent with the boundary defined by RA dependency because RA-deficient zebrafish, mouse and quail show the lack of the third and more posterior pouches (Quinlan et al., 2002; Mark et al., 2004; Kopinke et al., 2006). On the other hand, all gill slits of amphioxus are RA-sensitive.

In sum, PP3 and more posterior pouches of the vertebrate may be homologous to the amphioxus gill slit, while the anterior two are likely to have been evolved during

vertebrate evolution. My observation of lamprey pharyngeal pouch and somite is also consistent with this idea because PP1 and PP2 developed below the unsegmented paraxial mesoderm (S0) but PP3 and PP4 were formed beneath the first and second somite respectively. The evolutionary scenario loomed out of these clues is; the evolution of the anterior pouch, firstly, the most anterior mesoderm has lost the segmentation, secondly, the unsegmented region has been expanded and finally, new signal center, which induces the anterior pouch morphogenesis, may have been acquired, for example, Fgfs from mesoderm and neural ectoderm (Crump et al., 2004). The morphological variation in the anterior region of the vertebrate head is evidently abundant such as brain case, jaws and hyoid skeletons (Santagati and Rijli, 2003). The stem vertebrate may have acquired the new pouches, PP1 and PP2, where the somitomeric constraints have been relaxed (Bertrand et al., 2011). It is possible that this event promoted flexible usage of the anterior germ layers including neural crest cells.

Although this scenario is based on the developmental mechanism of the pharyngeal segmentation in chordate, another scenario, which is based on the conserved expression patterns among deuterostome animals, can be argued. These two will elucidate the same goal as far as the former places is based on the framework that

the evolution of the developmental mechanism can be occurred on the ready established developmental mechanism because conserved gene expression patterns should have a role for the ancestral developmental process. For considering the latter scenario, recent studies in hemichordate development are quite informative (Lowe et al., 2003; Gillis et al., 2012; Pani et al., 2012). In order to elucidate the grounded scenario for the evolution of the vertebrate head, knowledge on the developmental mechanism in the primitive animals are still missing. Fortunately, the pharyngeal segmentation is well conserved among deuterostomia, and progresses of the developmental studies on hemichordate and amphioxus are surprising (Benito-Gutierrez et al., 2013). In the near future, foreshadowing sprinkled in each developmental process will be recovered. Even in my bumbleheaded research, I could reveal the crucial clues to correlate branchiomerism with somitomerism.

During the evolution of the vertebrate head, the initial constraint of urbilateria body plan brought about by primitive somitogeneis may be a 'seed' with great potential for the variable morphological evolution. Today, 'flowers' of the vertebrate is blooming with notable complex head structures, in which the anterior constraint is relaxed partially. The flower allows us to discuss, to hear and to give more than a passing thought to a long way of our evolutionary process.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Professor Hiroshi Wada (University of Tsukuba) for providing me with the great supports in his laboratory. I really appreciate Professor Katsuo Furukubo–Tokunaga, Associate Professors Yasunori Sasakura and Masanao Honda (University of Tsukuba) for their critical reading of this thesis. I received generous supports from Professor Yosuke Takahama and Dr. Norimasa Iwanami (University of Tokushima) for providing me with medaka *tbx1* MO. I am grateful to Associate Professor Yuji Yamazaki (University of Toyama) for providing me the lamprey samples (*L. kessleri*). I thank Assistant Professor Shinichi Yokobori (Tokyo University of Pharmacy and Life Science) for his critical advice for sequence analysis very much. I am indebted to Associate Professor Masaru Matsuda (Utsunomiya University) for his teaching me microinjection technique. I appreciate Mr. Tadashige Kishi for providing me with *L. japonicum* samples. I also appreciate Drs. Hector Escriva and Stephanie Bertrand (Université de Paris) for providing me with *B. lanceolatum* larvae and their kind supports in Banyuls sur Mer. Special thanks are due to the members of Wada's laboratory for insightful discussion.

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Tables

Table 1–1
Collection sites and number of specimens examined in the study of lamprey sequence analysis.

Taxon	Collected site	Number of specomens examined	Individual recognition	Acc. nos. for NC2	Acc. nos. for *SoxD* intron
Lethenteron japonicum	Shiribeshitoshibetsu R. (Hokkaido, Japan)	5	js1~js5	AB567688–92	AB565490–92 (js1–3)
	Ishikari R. (Hokkaido, Japan)	3	ji1, ji2, ji3	AB567693–95	
	Mogami R. (Yamagata, Japan)	2	jm1, jm2	AB567696–97	
L. kessleri	Irtysh R. (Upper Ob, Kazakhstan)	5	k1~k5	AB567698–702	AB565493–94 (k1–2)
L. sp. N	Shiribeshitoshibetsu R. (Hokkaido, Japan)	5	Ns1~Ns5	AB567703–07	
	Kamo R. (Upper Shogawa, Toyama, Japan)	12	Nk1~Nk12	AB567708–16, AB567729–31	
L. sp. S	Senju R. (Upper Onga, Fukuoka, Japan)	5	Ss1~Ss5	AB567734–38	
	Kamo R. (Upper Shogawa, Toyama, Japan)	2	Sk1, Sk2	AB567732–33	

Table 1–2
Primers used in this study of lamprey sequence analysis.

	Primer name	Sequence
For NC2	NC2–F	5'-GCTGCCGAATACACAAAAACAACCATCAT-3'
	NC2–R	5'-TTGGCATGGAGGTTTCGTATAAGCCATCC-3'
For *SoxD* intron	SoxD–F	5'-GACGAGAGGCGGAAAATCCTTCAAGCTT-3'
	SoxD–R	5'-GAGGCTTGTACTTGTAGTCGGGATACTTC-3'
For complete mtDNA of *L.* sp. S	Lsmt1F	5'-CTAGTAGATCTTCCTTCTCCTGCTAATAT-3'
	Lsmt1R	5'-GTTCCAGTGTAGGGTTAACGGTTATTAGTT-3'
	Lsmt2F	5'-CCCTATAACCACTATTAAGTAATCCTATAT-3'
	Lsmt2R	5'-GATCTTGTGCAATTTGAATAAGGAGAGTAA-3'
	Lsmt3F	5'-GCTCAAAGTGCAGGCTCTGCCACACTTCTT-3'
	Lsmt3R	5'-GGAGGGTAGCTAATCAGCTAAAAACTTTAA-3'
	Lsmt4F	5'-GGGATTATCTCTCACGTAGTTGCTTATTAT-3'
	Lsmt4R	5'-GTGATGGCCTAGAAAGGTGCCTTCTCGAA-3'
	Lsmt5F	5'-GTCTTTATTCTGCTTTTAACCCTCTACCTT-3'
	Lsmt5R	5'-CATGTTTAATGAAAAGAGAGCGGTTAAAGT-3'
	Lsmt6F	5'-CCTCTAATATCATTCTGATGACTTATAATA-3'
	Lsmt6R	5'-CCAGTGCTTTATATTTAAGCTATCAAAGCT-3'

Figures

Fig. 1–1 Fig. 1. Nucleotide sequence of the non-coding region 2 (NC2) of the mtDNA control region of *Lethenteron japonicum*, specimen js1. In this specimen, six NC2 repeats are observed.

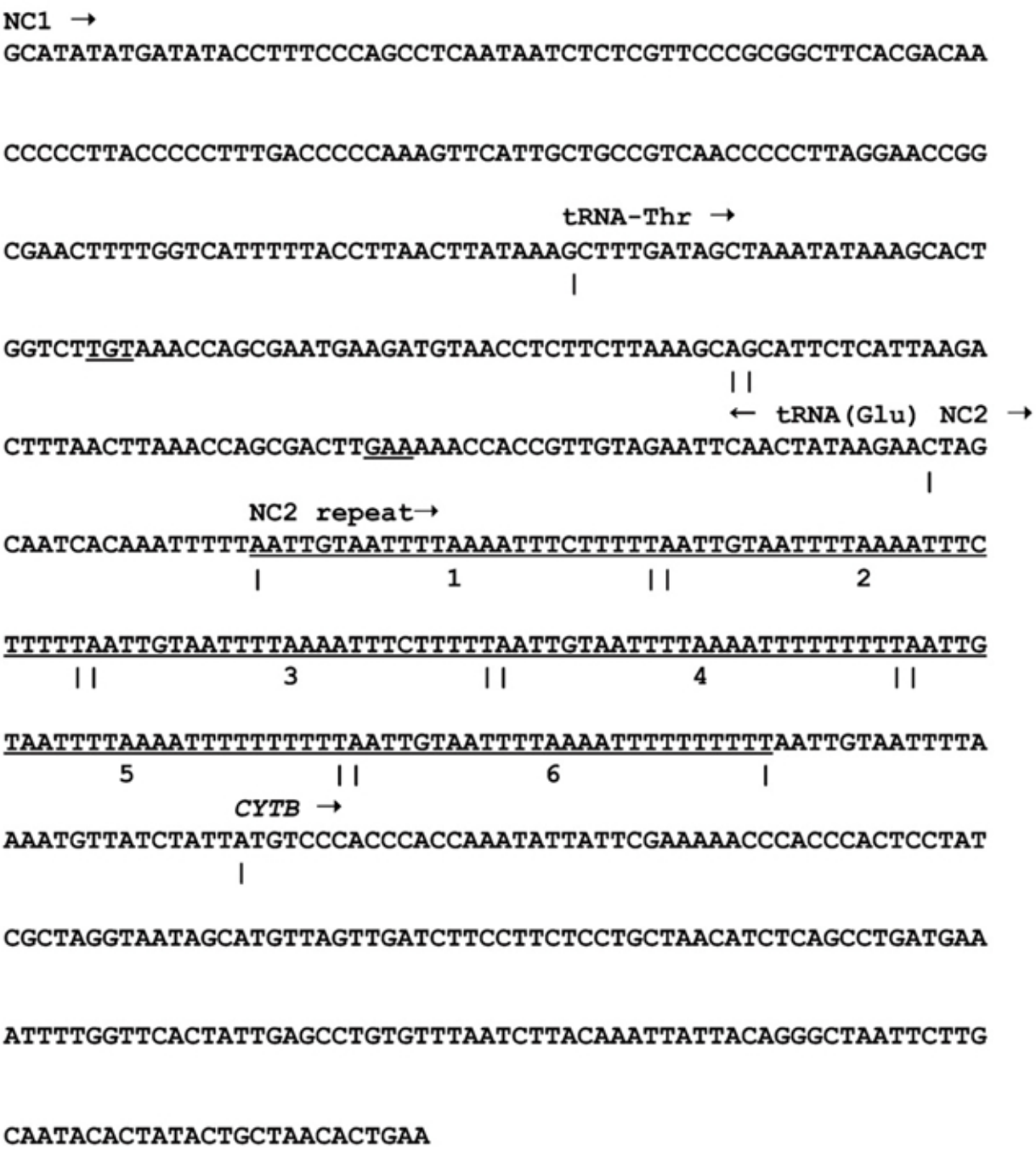

NC1 →
tRNA-Thr →
← tRNA(Glu) NC2 →
NC2 repeat→
1
2
3
4
5
6
CYTB →

Fig. 1–2. Sequence types and repeat architecture of the NC2 repeats. (A) Alignment of repeat sequences. (B) Network analysis of the repeat sequences. Numbers on the nodes indicate the nucleotide sites of the repeat in which substitutions occur between the sequences. (C) Schematic illustration of architecture of the repeat sequences in NC2 for each specimen. (D) Schematic illustration of a putative evolutionary history of the C-type array of repeats. Alignment of the C-type repeat arrays. Evolutionary scheme for the C-type. The C-type of repeat array probably originated from the B2-type via 3′ extension of the CTTTTT sequence. (E, F) Schematic illustration of a putative evolutionary history of the D-type array of repeats. Type D1 arose from type A3 via duplication of the 3′ part of the repeat (E). Subsequently, D2 and D3 were derived from the D1 sequence (F).

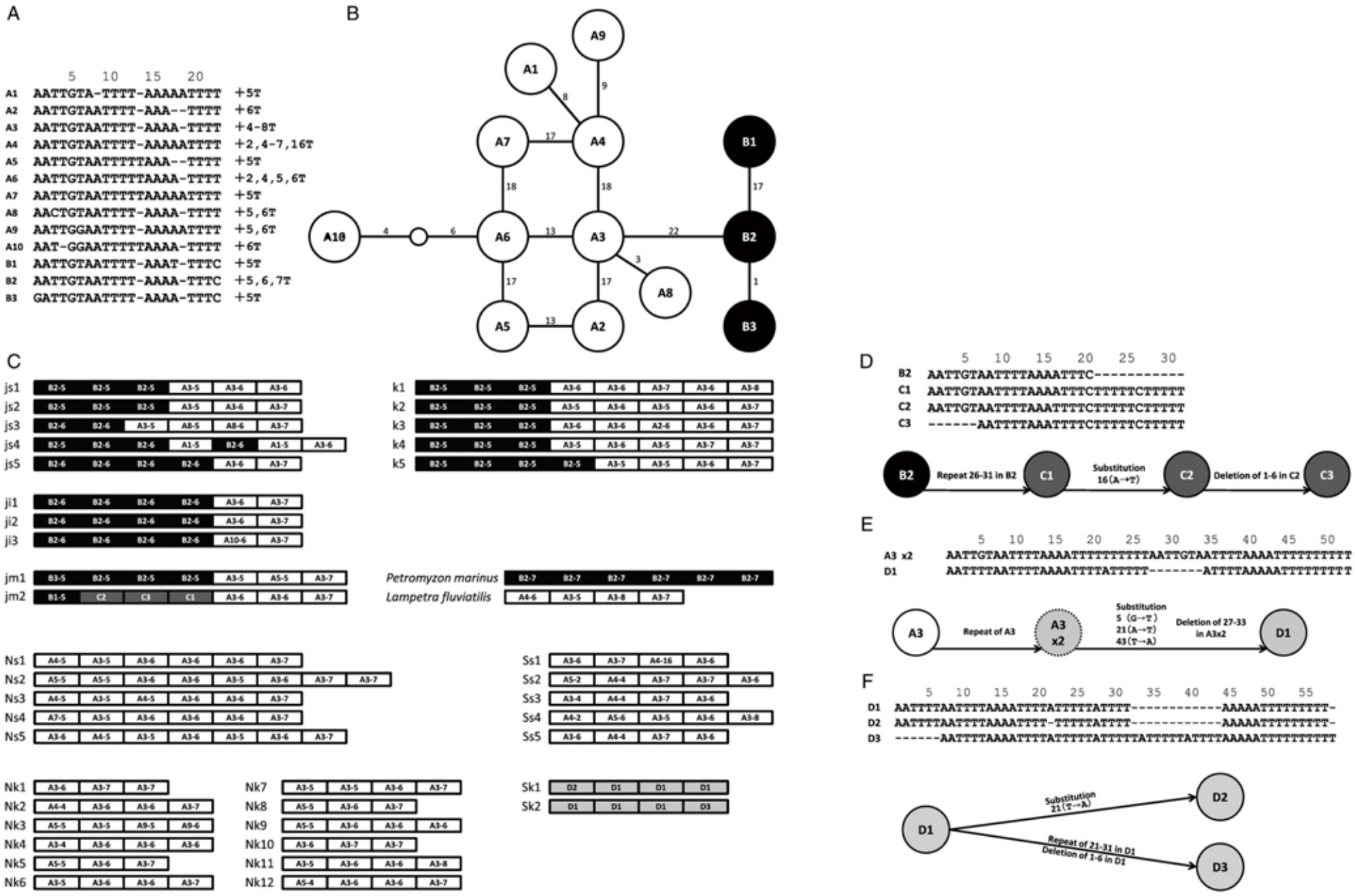
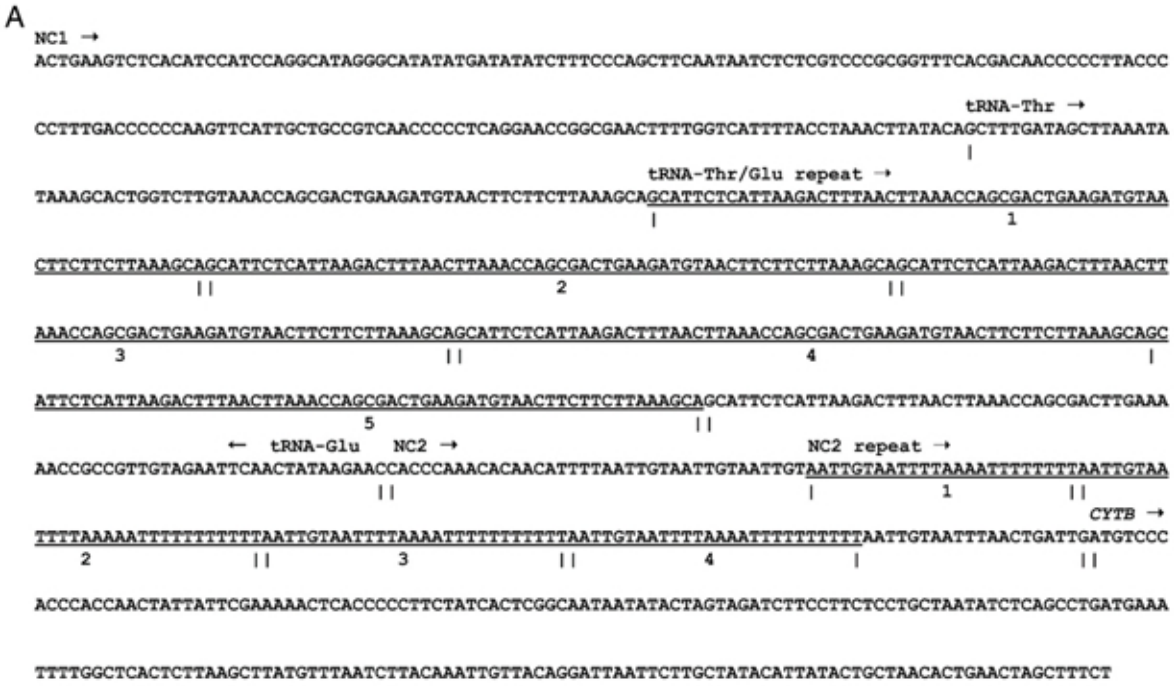
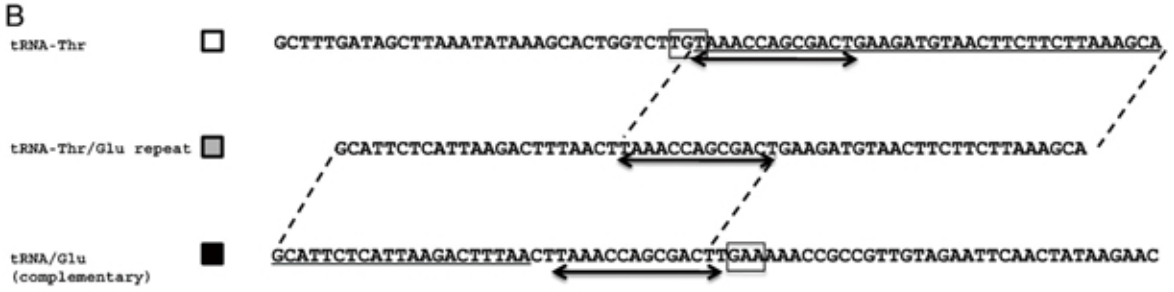

A
5 10 15 20
A1 AATTGTA-TTTT-AAAAATTTT +5T
A2 AATTGTAATTTT-AAA--TTTT +6T
A3 AATTGTAATTTT-AAAA-TTTT +4-8T
A4 AATTGTAATTTT-AAAAATTTT +2,4-7,16T
A5 AATTGTAATTTTTAAA--TTTT +5T
A6 AATTGTAATTTTTAAAA-TTTT +2,4,5,6T
A7 AATTGTAATTTTTAAAAATTTT +5T
A8 AACTGTAATTTT-AAAA-TTTT +5,6T
A9 AATTGGAATTTT-AAAAATTTT +5,6T
A10 AAT-GGAATTTTTAAAA-TTTT +6T
B1 AATTGTAATTTT-AAAT-TTTC +5T
B2 AATTGTAATTTT-AAAA-TTTC +5,6,7T
B3 GATTGTAATTTT-AAAA-TTTC +5T
B
A9
A1
A7
A4
B1
A10
A6
A3
B2
A8
A5
A2
B3
C
js1 B2-5 B2-5 B2-5 A3-5 A3-6 A3-6
js2 B2-5 B2-5 B2-5 A3-5 A3-6 A3-7
js3 B2-6 B2-6 A3-5 A8-5 A8-6 A3-7
js4 B2-5 B2-6 B2-6 A1-5 B2-6 A1-5 A3-6
js5 B2-6 B2-6 B2-6 B2-6 A3-6 A3-7
ji1 B2-6 B2-6 B2-6 B2-6 A3-6 A3-7
ji2 B2-6 B2-6 B2-6 B2-6 A3-6 A3-7
ji3 B2-6 B2-6 B2-6 B2-6 A10-6 A3-7
jm1 B3-5 B2-5 B2-5 B2-5 A3-5 A5-5 A3-7
jm2 B1-5 C2 C3 C1 A3-6 A3-6 A3-7
k1 B2-5 B2-5 B2-5 A3-6 A3-6 A3-7 A3-6 A3-8
k2 B2-5 B2-5 B2-5 A3-5 A3-6 A3-5 A3-6 A3-7
k3 B2-5 B2-5 B2-5 A3-6 A3-6 A2-6 A3-6 A3-7
k4 B2-5 B2-5 B2-5 A3-5 A3-6 A3-5 A3-7 A3-7
k5 B2-5 B2-5 B2-5 B2-5 A3-5 A3-5 A3-6 A3-7
Petromyzon marinus B2-7 B2-7 B2-7 B2-7 B2-7 B2-7
Lampetra fluviatilis A4-6 A3-5 A3-8 A3-7
Ns1 A4-5 A3-5 A3-6 A3-6 A3-6 A3-7
Ns2 A5-5 A5-5 A3-6 A3-6 A3-5 A3-6 A3-7 A3-7
Ns3 A4-5 A3-5 A4-5 A3-6 A3-6 A3-7
Ns4 A7-5 A3-5 A3-6 A3-6 A3-6 A3-7
Ns5 A3-6 A4-5 A3-5 A3-6 A3-5 A3-6 A3-7
Ss1 A3-6 A3-7 A4-16 A3-6
Ss2 A5-2 A4-4 A3-7 A3-7 A3-6
Ss3 A3-4 A4-4 A3-7 A3-6
Ss4 A4-2 A5-6 A3-5 A3-6 A3-8
Ss5 A3-6 A4-4 A3-7 A3-6
Nk1 A3-6 A3-7 A3-7
Nk2 A4-4 A3-6 A3-6 A3-7
Nk3 A5-5 A3-5 A9-5 A9-6
Nk4 A3-4 A3-6 A3-6 A3-6
Nk5 A5-5 A3-6 A3-7
Nk6 A3-5 A3-6 A3-6 A3-7
Nk7 A3-5 A3-5 A3-6 A3-7
Nk8 A5-5 A3-6 A3-7
Nk9 A5-5 A3-6 A3-6 A3-6
Nk10 A3-6 A3-7 A3-7
Nk11 A3-5 A3-6 A3-6 A3-8
Nk12 A5-4 A3-6 A3-6 A3-7
Sk1 D2 D1 D1 D1
Sk2 D1 D1 D1 D3
D
5 10 15 20 25 30
B2 AATTGTAATTTTAAAATTTC----------
C1 AATTGTAATTTTAAAATTTCTTTTTCTTTTT
C2 AATTGTAATTTTAAATTTTCTTTTTCTTTTT
C3 ------AATTTTAAATTTTCTTTTTCTTTTT
B2
Repeat 26-31 in B2
C1
Substitution 16(A→T)
C2
Deletion of 1-6 in C2
C3
E
5 10 15 20 25 30 35 40 45 50
A3 x2 AATTGTAATTTTAAAATTTTTTTTTAATTGTAATTTTAAAATTTTTTTTTT
D1 AATTTTAATTTTAAAATTTTATTTTT-------ATTTTAAAAATTTTTTTTT
A3
Repeat of A3
A3 x2
Substitution 5 (G→T) 21(A→T) 43(T→A)
Deletion of 27-33 in A3x2
D1
F
5 10 15 20 25 30 35 40 45 50 55
D1 AATTTTAATTTTAAAATTTTATTTTTATTTT----------AAAAATTTTTTTTT-
D2 AATTTTAATTTTAAAATTTT-TTTTTATTTT----------AAAAATTTTTTTTT-
D3 ------AATTTTAAAATTTTATTTTTATTTTTATTTTTATTTTAAAAATTTTTTTTT
D1
Substitution 21(T→A)
D2
Repeat of 21-31 in D1 Deletion of 1-6 in D1
D3

Fig. 1–3. Sequence of the novel repeat originating from *tRNA^{Thr}* and *tRNA^{Glu}*. (A) Nucleotide sequence of the 3′ half of the mtDNA control region of L. sp. S (Ss3). This specimen possesses five arrays of the tRNA-Thr/Glu repeat. (B) Sequence comparison of *tRNA^{Thr}*, *tRNA^{Glu}*, and the repeat sequence; 13 bp of identical nucleotide sequences are shown by the double-ended arrows. These sequences in *tRNA^{Thr}* and *tRNA^{Glu}* may have facilitated the slipped-strand mispairing (see text for details). The tRNA anticodons are boxed. (C) Variation in the number of the repeats in five specimens of L. sp. S. White, gray, and black boxes indicate the sequence of *tRNA^{Thr}* and *tRNA^{Glu}* (complementary) and tRNA-Thr/Glu repeat as shown in (B).


A
NC1 →
ACTGAAGTCTCACATCCATCCAGGCATAGGGCATATATGATATATCTTTCCCAGCTTCAATAATCTCTCGTCCCGCGGTTTCACGACAACCCCCTTACCC
tRNA-Thr →
CCTTTGACCCCCCAAGTTCATTGCTGCCGTCAACCCCCTCAGGAACCGGCGAACTTTTGGTCATTTTACCTAAACTTATACAGCTTTGATAGCTTAAATA
tRNA-Thr/Glu repeat →
TAAAGCACTGGTCTTGTAAACCAGCGACTGAAGATGTAACTTCTTCTTAAAGCAGCATTCTCATTAAGACTTTAACTTAAACCAGCGACTGAAGATGTAA
1
CTTCTTCTTAAAGCAGCATTCTCATTAAGACTTTAACTTAAACCAGCGACTGAAGATGTAACTTCTTCTTAAAGCAGCATTCTCATTAAGACTTTAACTT
2
AAACCAGCGACTGAAGATGTAACTTCTTCTTAAAGCAGCATTCTCATTAAGACTTTAACTTAAACCAGCGACTGAAGATGTAACTTCTTCTTAAAGCAGC
3
4
ATTCTCATTAAGACTTTAACTTAAACCAGCGACTGAAGATGTAACTTCTTCTTAAAGCAGCATTCTCATTAAGACTTTAACTTAAACCAGCGACTTGAAA
5
← tRNA-Glu NC2 →
NC2 repeat →
AACCGCCGTTGTAGAATTCAACTATAAGAACCACCCAAACACAACATTTTAATTGTAATTGTAATTGTAATTGTAATTTTAAAATTTTTTTTAATTGTAA
1
CYTB →
TTTTAAAAATTTTTTTTTTTAATTGTAATTTTAAAATTTTTTTTTTTAATTGTAATTTTAAAATTTTTTTTTTAATTGTAATTTAACTGATTGATGTCCC
2
3
4
ACCCACCAACTATTATTCGAAAAACTCACCCCCTTCTATCACTCGGCAATAATATACTAGTAGATCTTCCTTCTCCTGCTAATATCTCAGCCTGATGAAA
TTTTGGCTCACTCTTAAGCTTATGTTTAATCTTACAAATTGTTACAGGATTAATTCTTGCTATACATTATACTGCTAACACTGAACTAGCTTTCT

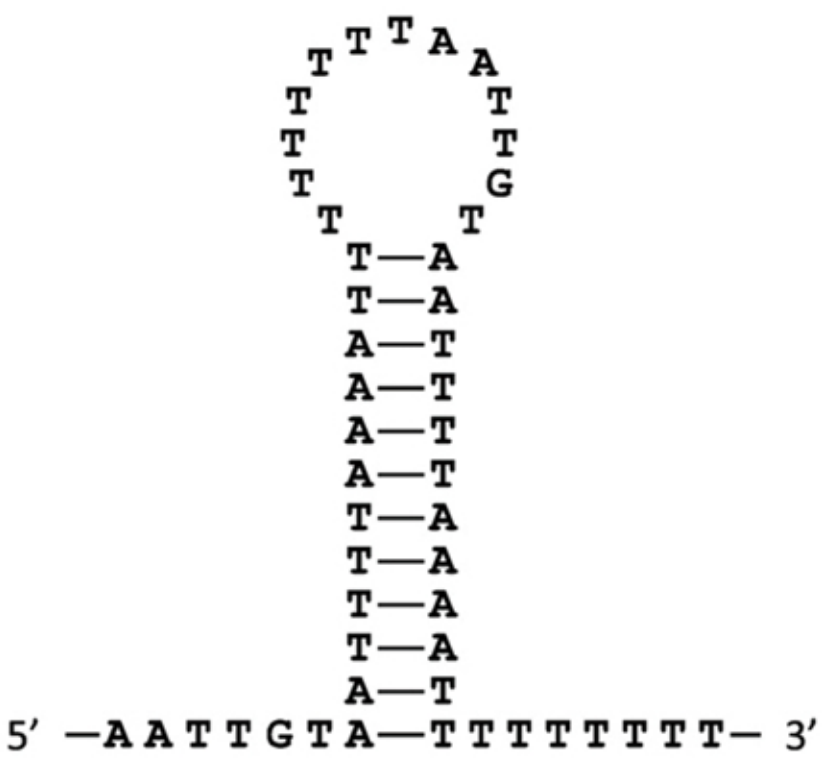

B
tRNA-Thr
GCTTTGATAGCTTAAATATAAAGCACTGGTCTTGTAAACCAGCGACTGAAGATGTAACTTCTTCTTAAAGCA
tRNA-Thr/Glu repeat
GCATTCTCATTAAGACTTTAACTTAAACCAGCGACTGAAGATGTAACTTCTTCTTAAAGCA
tRNA/Glu
(complementary)
GCATTCTCATTAAGACTTTAACTTAAACCAGCGACTTGAAAAACCGCCGTTGTAGAATTCAACTATAAGAAC


C
Ss1
Ss2
Ss3
Ss4
Ss5

Fig. 1–4. Possible secondary structure of the NC2 repeat. (A) The secondary structure of the NC2 repeat of A3–5. The estimated free energy was -2.27 kcal at 120 mM Na^+, 2 mM $Mg2^+$, and 20 °C. (B) The secondary structure of the two tandem repeats of A3–5. The estimated free energy was -9.79 kcal at 120 mM Na^+, 2mM $Mg2^+$, and 20 °C. (C) Possible secondary structure of the sequence of the 3′ half of $tRNA^{Thr}$ and the 3′ half of the complementary strand of $tRNA^{Glu}$, which mediate the generation of the repeats. The estimated free energy was -6.13 kcal at 120 mM Na^+, 2 mM $Mg2^+$, and 20 °C.

A

B



C

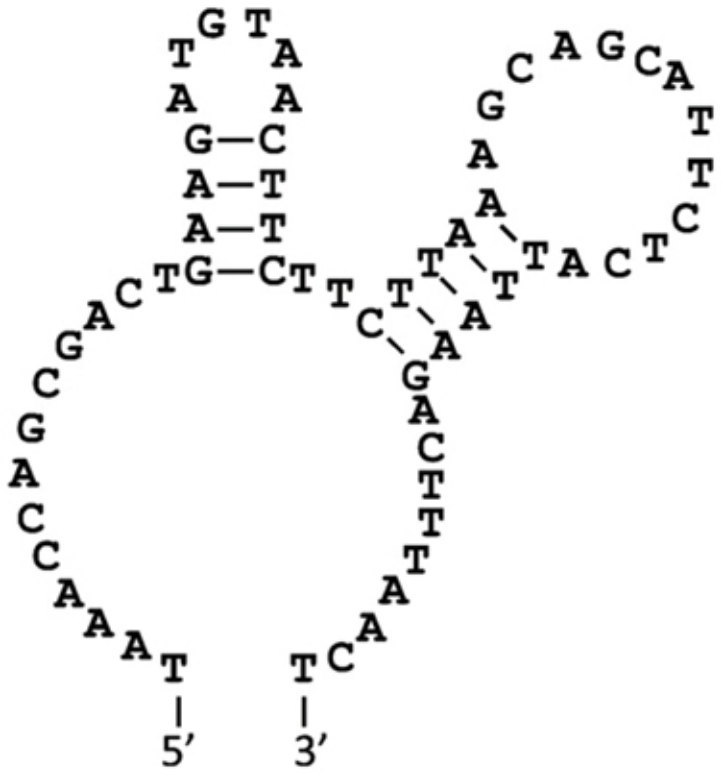


Fig. 1–5. Alignment of the *SoxD* intron sequence. Although there are some indels, no fixed substitution was observed between *L. japonicum* and *L. kessleri*.

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          10        20        30        40        50        60        70        80        90       100
js1 GGACACACACACACACACACAAAGATCCCCCGTAGAGCCTCAATGCACTTTTTGGGGCAAGTGCGTTAGTGAGCACCGATGAAGGCCGGCACGGCACACG
js2 ....................................................................................................
js3 ....................................................................................................
k1  ..................--..................................................................................
k2  ....................................................................................................

         110       120       130       140       150       160       170       180       190       200
js1 AGAGGGAGGTTGACCAGCACCATACCCGGCCGCCTTTGTCTCCCTCGTGACAATGTTTGCACATCGCACGAGGTACTCATTTAAGGCTGAGTCGACCTAG
js2 ....................................................................................................
js3 ....................................................................................................
k1  ....................................................................................................
k2  ....................................................................................................

         210       220       230       240       250       260       270       280       290       300
js1 GGGAGTCTAGACACACACGCGTGAGTCTAGAAACACACGCGTGTGAGCCTACACACGTGCGTGTGCAGACATTTTGTACTCGTATATATTATCCGAACAT
js2 ....................................................................................................
js3 ....................................................................................................
k1  ....................................................................................................
k2  ....................................................................................................

         310       320       330       340       350       360       370       380       390       400
js1 ATTTAAAGAAACTGTTTATAAAATAATAGTTAGCGTCATTGCGGAGAGTATATGTAACAAAATAGATAAGCAAACAATACTCATGTATGCATATGTCCCT
js2 ...........................................................................-........................
js3 ....................................................................................................
k1  ....................................................................................................
k2  ....................................................................................................
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Fig. 1–6. Alignment of the *tRNA*^{Thr} and *tRNA*^{Glu} in *Lethenteron* and *P. marinus*. The 13 stretches of the identical nucleotide sequence are conserved in *P. marinus*, although the repeat sequence was not reported.

tRNA^{Thr}, tRNA^{Glu}