Isolation and Characterization of Salmonid Telomeric and Centromeric Satellite DNA Sequences

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Abstract

Satellite DNA clones with a 37 bp repeat unit were obtained from *Bgl*II-digested genomic DNA of masu salmon (*Oncorhynchus masou*) and chum salmon (*O. keta*). Fluorescence *in situ* hybridization (FISH) analysis with the isolated clones as a probe showed that these repetitive sequences were localized in the telomeric regions of chromosomes in both species. Southern and dot blot analyses suggested conservation of homologous sequences with similar repeat unit in other salmonids including the species of the genus *Oncorhynchus* and *Salvelinus*, but lack or scarcity of such sequences in the genus *Hucho* and *Salmo*. Similarly, polymerase chain reaction (PCR)-based cloning of satellite DNA referring to a reported Rainbow trout (*O. mykiss*) centromeric sequence was successful for the *Oncorhynchus*, *Salvelinus* and *Hucho* species. The obtained satellite DNA clones were localized with FISH in the centromeric regions of chromosomes of the species from these three genera. Although PCR cloning of the centromeric satellite DNA was failed in the *Salmo* species due to some base changes in the priming sites, dot blot hybridization analysis suggested conservation of homologous satellite DNA in the genus *Salmo* as in the other three genera. In the neighbor-joining tree of cloned centromeric satellite DNA sequences, the genus *Oncorhynchus* and *Salvelinus* formed adjacent clades, and the clade of the *genus Hucho* included the reported centromeric sequence of the genus *Salmo*. Conservation pattern and molecular phylogeny of the telomeric and centromeric satellite DNA sequences isolated herein support a close phylogenetic relationship between the genus *Oncorhynchus* and *Salvelinus* and between the *Salmo* and *Hucho*.

Introduction

Eukaryotic genome contains a substantial fraction of tandemly repeated DNA sequences termed satellite DNA, which often form heterochromatin (Bickmore and Craig, 1997). While some satellite DNA locates on specific chromosome regions such as centromere and telomere, others are specific to certain chromosomes such as sex chromosomes or B chromosomes (Mestriner *et al.*, 2000; Phillips, 2001; Stein, Phillips & Devlin, 2001; Ziegler *et al.*, 2003). Since fish chromosomes are generally small and numerous, it is difficult to distinguish each homologous chromosome pair morphologically (Fujiwara and Abe, 2002). Chromosome-specific or chromosome region-specific satellite DNA, if any, may therefore become a useful cytogenetic marker in fish. In fact, chromosome-specific satellite DNA has been successfully used to detect homeologous chromosomes (Mantovani, Abel & Mestriner, 2004; Ziegler *et al.*, 2003) and chromosome rearrangement (Mestriner *et al.*, 2000) between closely related fish species.

The family Salmonidae comprises three subfamilies, Coregoninae, Thymallinae, and Salmoninae (Crespi and Fulton, 2004). The subfamily Salmoninae contains five genera *Brachymystax*, *Hucho*, *Salvelinus*, *Salmo* and *Oncorhynchus*. Phylogenetic relationship of Salmoninae has been studied by using morphological data (Stearly and Smith, 1993) and several nuclear and mitochondrial DNA markers (Shedlock *et al.*, 1992; Murata *et al.*, 1993; Oohara, Sawano & Okazaki, 1997; Oakley and Phillips, 1999). However, the evolutionary relationships among salmonids are still uncertain, especially the relationship between the genus *Salvelinus*, *Salmo* and *Oncorhynchus* (Crespi and Fulton, 2004). To make the salmonine phylogenetic relationships clearer, further analysis using other DNA or chromosomal markers is needed to determine the

relationships at genus-level.

This study aims to isolate and characterize repetitive satellite DNA sequences localizing at telomeric and centromeric regions, to utilize them as a tool for analyzing karyotypic evolution in salmonine fishes. Both chromosome regions are essential to maintain chromosome structure and play a key role in eukaryotic mitotic and meiotic chromosome segregation (Bickmore and Craig, 1997). Therefore, it is highly conceivable that satellite DNA sequences besides authentic centromere protein box (CENP box) and alphoid sequences of centromere and $(TTAGGG)_n$ of telomere in vertebrate should also have evolutionary significance among related fish species or groups.

Materials and methods

Fish samples

Genomic DNA was extracted from whole blood according to standard protocols (Sambrook *et al.*, 1989) from 12 salmonine species including the Japanese huchen *Hucho perryi*, Japanese char *Salvelinus leucomaenis leucomaenis*, Japanese char "Iwana" *S. leucomaenis japonicus*, Brook trout *S. fontinalis*, Dolly varden *S. malma*, Atlantic salmon *Salmo salar*, Brown trout *S. trutta*, Rainbow trout *Oncorhynchus mykiss*, Masu salmon *O. masou*, Coho salmon *O. kisutch*, Sockeye salmon *O. nerka* and Chum salmon *O. keta*. Adult fishes were obtained from the Nanae Freshwater Station, Field Science Center for Northern Biosphere, Hokkaido University. Only homed chum salmon adult fishes were obtained from the Oshima Salmon Breeding Association. Ten to 12-day-old embryos of some of these salmonines were obtained for chromosome preparations from artificial fertilization between parental fishes obtained from the Nanae Freshwater Station.

Cloning of satellite DNA in *O. masou* and *O. keta*

About 10 μg genomic DNA of *O. masou* and *O. keta* was digested with 14 restriction enzymes (RE; *Alu*I, *Apa*I, *Bgl*II, *Bam*HI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hind*III, *Hpa*I, *Pst*I, *Sau*3AI, *Sma*I, *Xho*I) for screening of tandemly-arrayed satellite DNA bands. RE-digested DNA band was extracted from agarose gel using QIA quick gel extraction system (Qiagen), and inserted into the vector pUC18 *Bam*HI site (Nippon gene) using Ligation-Convenience Kit (Nippon gene). The recombinant plasmids were transformed into JM109 competent cells (Nippon gene) following the standard protocols (Sambrook, Fritsch & Maniatis, 1989). Positive clones were obtained by blue-white selection, and high copy number clones in the genome were selected by dot blot analysis.

Nucleotide sequence analysis and homology search

DNA sequences were analyzed by the ABI PRISM 3130xl capillary autosequencer after sequencing reaction using Big Dye terminators v1.1 cycle sequencing kit (Applied Biosystems) following the manufacturer's instructions, with M13 forward -21 (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGACC-3') sequence primers. Both strands were read more than twice for accurate analysis. Sequence alignment was performed using the CLUSTAL W program (Thompson, Higgins & Gibson, 1994). The DNA Data Bank of Japan (DDBJ) was screened for sequence homology search using the BLAST algorithm.

Chromosome preparations and fluorescence *in situ* hybridization (FISH)

Mitotic chromosome preparations were obtained from early embryos by the method described in Inokuchi *et al.* (1994). Fluorescence *in situ* hybridization (FISH) was performed according to Fujiwara *et al.* (1998) with a modification. Chromosome slides were hardened at 65ºC for 3 hours and then denatured at 70ºC for 1 min in 70%

formamide in 2x SSC, and dehydrated in 70% and 100% ethanol at 4ºC. The DNA probes were labeled with biotin-16-dUTP by nick translation kit (Roche). After hybridization, the slides were washed in 50% formamide for 20 min at 37ºC, and in 2x SSC and 4x SSC for several minutes at room temperature. Detection of the probe was conducted with avidin-FITC conjugate. Two rounds of signal amplification were conducted using a biotinylated anti-avidin antibody. Finally chromosome preparations were counterstained with propidium iodide. The slides were observed under Eclipse E800 (Nikon), and the images were captured with black and white CCD camera Pixera Penguin 150CL-CU (Pixcera). FISH images were processed using Penguin Mate Ver.1.0.8. application program for RGB pseudocolor imaging (Pixcera) on a computer.

Southern blot analysis

Southern blot analysis was conducted according to the standard protocols (Sambrook, Fritsch & Maniatis, 1989). Genomic DNA was digested with *Bgl* Ⅱ, transferred onto nylon membrane Hybond N+ (Amersham), and baked at 80ºC for 2 hours. Labelling of probe, hybridization at 42ºC for overnight, and detection of signals were performed using the aforementioned AlkPhos Direct labelling and detection system with CDP-star (Amarsham) according to the manufacture's instruction. Hybridization signals were observed by the lumino image analyser LAS-1000 mini (Fujifilm).

Dot blot analysis

One μg genomic DNA of each species was blotted onto membrane Hybond N+ (Amersham), denatured with 1 M NaOH, and baked at 80ºC for 2 hours. Labelling of probe and hybridization (at 45ºC for overnight) was conducted as described above.

Polymerase Chain Reaction (PCR)-based cloning of centromeric satellite DNA

Because the telomeric sequence is likely conserved in salmonid fishes as

described below and so does the centromeric sequence, we decided to isolate centromeric sequences based on the previous findings (Reed, Dorschner & Phillips, 1997). The PCR primer sequences were designed from the *O. mykiss* centromeric repetitive DNA, Om-D3-7 (Reed, Dorschner & Phillips, 1997; Acc.No.AF021227), using GENETIX ver.6 (Genetix). The two primers, Om-D3-7F (5'-TCCAGAGTGCATCAAACATGA-3') and Om-D3-7R (5'-CAGAAATAACGCCTTTTCCA-3'), were used for PCR. The PCR mixture contained 100 ng of template DNA, 0.5 μl of each primer, 0.2 unit of *Taq* DNA polymerase (Sigma), 5 μl of 10x PCR buffer, 1.5 mM of $MgCl_2$, and 0.2 mM each of dNTPs in 50 μl solution. The reaction using a PROGRAM TEMP CONTROL SYSTEM PC-320 (ASTEC) was performed with the following cycling profile: pre-cycling denaturation at 95ºC for 3 min, and then 30 cycles of denaturation at 95ºC for 30 sec, annealing at 60ºC for 30 sec, extension at 72ºC for 30 sec, and post-cycling extension at 72ºC for 5 min. The PCR products were checked with 3 % agarose gel electrophoresis.

Fresh PCR products were cloned with TOPO TA cloning kit for Sequencing (Invitrogen), following the manufacturer's instruction. Phylogenetic analysis was conducted according to the neighbor-joining (NJ) method using Kimura's two-parameter distance (Kimura, 1980) with PHYLIP ver.3.63 (Felsenstein, 1989). Confidence for the NJ tree topology was estimated by bootstrapping with 1000 replication of plausible trees (Felsenstein, 1985).

Results

Isolation and characterization of satellite DNA in *O. masou* and *O. keta*

With screening of satellite DNA bands after digestion with several RE, *Bgl*II-digested genomic DNA of *O. masou* showed tandemly-arrayed satellite DNA bands, ranging from about 70bp to 230bp, in agarose gel electrophoresis (Figure 1A lane a). The satellite DNA, approximately 70 bp in size, was cloned. By dot blot analysis, 7 clones (Om9, Om54, Om74, Om78, Om82, Om92 and Om98) were estimated to have high copy number clones in the *O. masou* genome, and selected for further analysis (data not shown). The nucleotide sequence of each clone was shown in Figure 2A. On FISH analysis, the isolated satellite DNA clones located on telomeric regions of the most chromosomes, but not all (Figure 1B left). In some metacentric chromosomes, telomeric FISH signals were located on one of chromosome arms.

In *Bgl*II-digested genomic DNA of *O. keta*, tandemly-arrayed satellite DNA bands also were found in agarose gel electrophoresis (Figure 1A lane b). The satellite DNA was also cloned as *O. masou*. By dot blot analysis, 7 clones (Ok20, Ok22, Ok35, Ok54, Ok79, Ok94, Ok112) were estimated to have high copy numbers in the *O. keta* genome (data not shown). Sequences of each clone of *O. keta* satellite DNAs were shown in Figure 2B. FISH analysis indicated that these clones also located on telomeric regions of most chromosomes of *O. keta*, same as *O. masou* (Figure 1B right).

These isolated clones from *O. maosu* and *O. keta* showed high homology with the arctic char *Salvelinus alpinus* DNA repeat region (Accession number : Acc.No.L01078), lake trout *S. namaycush* clone SnAluI-16 (Acc.No.U27090) and rainbow trout *Oncorhynchus mykiss* clone Om-A42a repetitive DNA (Acc.No.AF021228).

Since homology search showed the same results with *O. masou*, multiple sequence alignment was conducted with the consensus sequences of five species, *O. masou* and *O. keta* satellite clones obtained here, *S. alpinus* DNA repeat region

(Acc.No.L01078), lake trout *S. namaycush* clone SnAluI-16 (Acc.No.U27090), and *O. mykiss* clone (Acc.No.AF021228), by CLUSTAL W (Figure 2C). In this alignment, these repetitive sequences of five species were almost same, and they shared the same one repeat unit. The length of this repeat unit was 37 bp and it was composed of 66.4% of AT content on average.

In Southern blot analysis probed with the Om9 clone, tandemly-arrayed signals were detected in the species of the genus *Oncorhynchus* and *Salvelinus*, but not detected in those of the genus *Salmo* and *Hucho* fishes (Figure 1C). Because of no signals in *Salmo* and *Hucho* species, dot blot analysis was further conducted to confirm the lack of this sequence. In *Hucho perryi* genome, weak signal was observed by dot blot analysis, but not in *Salmo* species (Figure 1D).

PCR-based cloning of centromeric satellite DNAs in salmonid species

PCR amplification of centromeric sequences were conducted with genomic DNA of 12 species (*H. perryi*, *S. fontinalis*, *S. leucomaenis leucomaenis*, *S. leucomaenis japonicus*, *S. malma*, *S. salar*, *S. trutta*, *O. mykiss*, *O. masou*, *O. kisutch*, *O. nerka*, *O. keta*). In the *Salvelinus*, *Oncorhynchus* and *Hucho* species, PCR product with target size was co-amplified with tandemly-arrayed products, but in the genus *Salmo*, PCR products were not obtained.

To confirm the PCR product with target size contained really centromeric sequence, PCR products of *O. mykiss* were cloned and sequenced. Three clones (Omy25, Omy28 and Omy29) were isolated, and all of them showed almost the same sequence as the reported *O. mykiss* centromeric sequence of Om-D3-7 (Reed, Dorschner & Phillips, 1997) (data not shown). In *O. masou*, *O. keta*, *S. leucomaenis japonicus*, *S. fontinalis, S. malma* and *H. perryi*, the PCR products were successfully cloned and

sequenced as shown in Table 1.

Because of no PCR products in the genus *Salmo*, dot blot analysis was conducted. The signal was detected in all examined species including two of the genus *Salmo* (data not shown).

Blast search indicated that the sequences of each of the above isolated clones had high homology with the *O. mykiss* centromeric sequence Om-D3-7, but in *H. perryi* clones except for Hp83 showed a greater homology with the reported *S. salar Hpa*I centromeric sequence (Acc.No.AY703447); the similarity was 87% for Hpe35, 89% for Hpe38, 87% for Hpe50, 87% for Hpe53, 86% for Hpe69, and 87% for Hpe81, respectively. Moreover, most isolated clones have high homology partially with the reported *S. namaycush* clones (Acc.No.U27092, U27086, U27094, and U27095), among which one (U27092) had longer sequence than the isolated clones but the other three were short (data not shown).

The NJ consensus tree of isolated clones is shown in Figure 3. In the NJ tree, cloned centromeric sequences were clustered by genus but not necessarily by species, although the bootstrap values were low. The NJ consensus tree indicated that the genus *Salvelinus* was separated into major two groups (Salvelinus-A and Salvelinus-B) and the genus *Oncorhynchus* also was clustered into two groups (Oncorhyncus-A and Oncorhynchus-B). On the tree, A and B were tentatively designated by the distance from the clade of *Hucho*, i.e. A for proximate clade and B for distant clade. Because no PCR products were obtained from the genomic DNA of *S. salar*, a part of the reported *Hpa* I sequence of this species (AY703447), the portion from 31 bp to 219 bp, was used in the NJ phylogenetic analysis. This *S. salar* centromeric sequence was clustered in the same group with *H. perryi,* as expected (Figure 3). Alignment between cloned

sequences of *O. perryi* and the centromeric sequence of *S. salar* disclosed the sequence difference in primer regions (data not shown), which explains why amplification was failed in the genus *Salmo*. Also, the portion from 399 bp to 591 bp of the reported sequences of *S. namaycush* (U27092) showing high homology with isolated clones was included in the NJ analysis, even though the sequences of primer regions were different from the Om-D3-7 sequence (data not shown). The partial U27092 sequence was clustered in the Salvelinus-A group (Figure 3).

FISH analysis was conducted with isolated clones as probes (Hpe38, Hpe50, Hpe83, Sle1, Sle9, Sfo2, Sfo24, Sfo42, Smala19, Smhok81, Smhok83, Oma2, Oma8, Oma10, Oma21, Oma14, Oke35 and Oke49) but not all the results are shown here (Figure 4). All analyzed clones were localized at centromeric regions of the chromosomes of *H. perryi, S. leucomaenis japonicus, S. fontinalis, S. malma, O. masou,* and *O. keta*, although FISH signals were considerably weak with clones Hpe35 and Oma8 in *H. perryi* and *O. masou*, respectively (data not shown). We could not identify the signal differences between Oncorhynchus-A and -B clones, or Salvelinus-A and -B clones (data not shown). The *O. masou* Om14 showed specifically centromeric signals on one pair of chromosomes (Figure 5a). To investigate the specific localization of Om14 in other salmonid fishes, interspecific FISH with this clone was conducted on the chromosomes of *H. perryi*, *S. fontinalis* and *O. keta*. In all three species, however, the Om14 did not show chromosome-specific localization of FISH signals.

Discussion

In this study, both the telomeric and centromeric sequences in salmonine fishes were analyzed. Telomeric satellite DNA sequences isolated from *Bgl*II-digested

genomic DNA in *O. masou* and *O. keta* were found to have an identical 37 bp repeat unit between two species. In addition, a multiple sequence alignment with the sequences obtained and those retrieved from a DNA databank showed that the same repeat unit was conserved in *Salvelinus alpinus* (Hartley and Davidson, 1994), *S. namaycush* (Reed and Phillips, 1995), and *O. mykiss* (Reed, Dorschner & Phillips, 1997). The telomeric sequences isolated in this study were localized with FISH in most, but not all, chromosomes of *O. masou* and *O. keta* (Figure 1B). Furthermore, in some metacentric chromosomes, telomeric FISH signals was located on one of chromosome arms, and the authenticated $(TTAGGG)_n$ repeats were not identified in the isolated clones (Figures 2). These results are obviously different from the previous FISH observations with $(TTAGGG)_n$ repeats in several salmonid fishes (Phillips and Reed, 1996). It is thus conceivable that the isolated sequences could actually be subtelomeric ones, probably having a role of chromosome structure maintenance in association with the $(TTAGGG)_n$ sequence.

The functional centromeres are generally featured by CENP box or alpha satellite DNA (Phillips and Reed, 1996). The centromere sequences isolated herein by PCR-based cloning did not show any homology with such functional centromeric sequences by BLAST search, and not located at the centromeric region of all chromosomes in the examined species. This situation is different from the previous observation with a centromeric repetitive DNA family having a sequence motif common to vertebrate centromeric satellite DNA isolated from *Sparus aurata* genome, which localized in the centromere of all chromosomes (Garrido-Ramos *et al.*, 1995). Therefore, the centromeric sequences isolated in this study may not be involved in the functional mitotic machinery of centromere.

It is noteworthy that the clone Om14 sequence was localized in the centromeric region of only one chromosome pair of *O. masou*, whereas this sequence was mapped to the centromeric regions of several different chromosome pairs in *H. perryi*, *S. fontinalis* and *O. keta* (Figure 5). It is unknown at present as to whether the Om14 sequence actually clusters on a single chromosome pair or extensively localizes at low copy number in other chromosomes of *O. masou*. The observed difference in the distribution pattern of this sequence among the examined species could be related to chromosome rearrangement in the process of karyotypic evolution. In addition, this sequence may become a useful chromosome marker, especially when preparing a cytogenetic map of *O. masou*.

Tetraploid origin of salmonid fish, resulting from ancestral genome duplication (Ohno, Wolf & Atkin, 1967), has been widely accepted. This tetraploidization has been postulated to cause complex patterns of salmonid chromosome evolution (Hartley, 1987). Since salmonid fishes have the almost same chromosome arm number close to 100, salmonid chromosome evolution has been hypothesized to occur by Robertsonian rearrangement (Hartley, 1987; Phillips and Ráb, 2001). It is thus necessary to establish phylogenetic relationships of salmonid fish to consider their chromosome evolution. Conflicts about relationships at genus-level in Salmoninae are as to whether the genus *Oncorhynchus* and *Salmo* are sister group or not. Based on the morphological data, these two genera are thought to be sister taxa (Stearley and Smith, 1993), which also is supported by a molecular phylogenetic data of interspersed nucleotide elements (SINEs) (Murata *et al.*, 1996). However, recent molecular phylogenetic analyses using growth hormone gene (GH) intron (Oakley and Phillips, 1999), microsatellite DNA (Angers and Bernatchez, 1997), and vitellogenin gene (Buisine, Trichet & Wolff, 2002) suggest that

the genus *Oncorhynchus* is close to the genus *Salvelinus*, but not to the genus *Salmo*.

Conservation of isolated telomeric sequence family with a 37 bp repeat unit in all the *Oncorhynchus* and *Salvelinus* species examined herein and those reported in the literature, but absence in the genus *Salmo* or very few copy number in the genus *Hucho*, seem compatible with the above mentioned molecular phylogenetic findings of salmonid fish using nuclear DNA markers. Additionally, the topology of the NJ consensus tree made with centromeric sequences cloned herein showed that the genus *Oncrohynchus* is more close to the genus *Salvelinus* than the genus *Salmo* as a whole (Figure 3), although the clusters of Salvelinus-A and –B are separated by Oncorhynchus-A. Considering that *H. perryi* is the most primitive species among those examined herein (Phillips, Oakley & Davis, 1995), it is conceivable that the sequence group A of *Salvelinus* and *Oncorhynchus* is more primitive than the group B. Inclusion of the reported single *Salmo* centromeric sequence in the *Hucho* cluster also seems favor a notion that the genus *Hucho* and *Salmo* are closely related. Thus, both the centromeric and telomeric sequences isolated herein suggest a close phylogenetic relationship between the *Salmo* and *Hucho* rather than between the *Oncorhynchus* and *Salmo*. However, the present molecular findings are not compatible with Hartley's proposal of salmonine groups based on the karyotype data (1987).

Molecular evolution of centromeric and telomeric sequences isolated in this study is unknown at present. It is conceivable that their evolutionary process is not necessarily the same each other. Probable conservation of the isolated telomeric sequences was suggested only by the dot blot analysis in the present study. Thus, molecular phylogenetic analysis using sequence data of the telomeric DNA from the examined species is needed to confirm the evolutionary scenario of salmonine fish.

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Figure legends

Figure 1. (A) Electrophoresis of genomic DNA digested with *Bgl*Ⅱ restriction enzyme. Lane (a) is *O. masou* genomic DNA digested with *Bgl*Ⅱ and Lane (b) is *O. keta* genomic DNA digested with *Bgl*Ⅱ. Lane (M) is DNA size marker. Arrow head indicates satellite DNA bands cloned for analysis. (B) left: FISH analysis of the satellite clone Om9 on *O. masou* chromosomes (2n=66). right; FISH analysis of the satellite clone Ok35 on *O. keta* chromosomes (2n=74). White bars indicate scale of 10 ,,m. (C) Southern blot analysis of the clone Om9. Each lane contains 10 mg of genomic DNA digested with *Bgl*Ⅱ restriction enzyme. DNA samples of each lane are (a) *O. masou*, (b) *O. keta*, (c) *O. mykiss*, (d) *S. trutta*, (e) *S. salar*, (f) *S. malma*, (g) *S. leucomaenis leucomaenis*, (h) *S. leucomaenis japonicus*, (i) *S. fontinalis*, (j) *H. perryi*. The size marker is illustrated in left side of this picture and the size is given in bp. (D) Dot blot analysis. Each blot contains 1 μg of genomic DNA. DNA samples of each dot are (a) *H. perryi*, (b) *S. fontinalis*, (c) *S. leucomaenis japonicus*, (d) *S. leucomaenis leucomaenis*, (e) *S. malma*, (f) *S. salar*, (g) *S. trutta*, (h) *O. mykiss*, (i) *O. masou*, (j) *O. kisutch* ,(k) *O. nerka*, (l) *O. keta*, (m) Om78 PCR products amplified with M13 forward -21 and reverse primers as positive control.

Figure 2. (A) Aligned sequences of seven satellite clones of *O. masou,* (B) Aligned sequences of seven satellite clones of *O. keta*. Consensus sequence is shown on the top. Dots mean same bases and dashes mean gaps. (C) Alignment of telomeric sequences of two examined species and three reported sequences. The last 10bp sequence of *O. keta* is not shown in this figure. L01078 is repeated sequence of *S. alpinus* and AF021228 is

that of *O. mykiss* Om-A42a. U27090 is the partial sequence of *S. namaycush* clone SnAlu Ⅰ-16. Dashes mean gaps and asterisks indicate same bases. Underlines indicate repeat units.

Figure 3. Neighbor-joining consensus tree rooted with Hpe69 with nodal values for bootstrap support over 50% of the 1000 replicated trees. Centromeric sequences of *O. mykiss* (the partial Om-D3-7 sequence ranging from 12 bp to 204 bp), *S. namaycush* (the partial U27092 sequence from 399 bp to 591 bp) and *S. salar* (the partial AY703447 sequence from 31 bp to 219 bp) are included in the analysis.

Figure 4. Fluorescence *in situ* hybridization of isolated clones on chromosomes of *O. masou* (2n=66) (a), *O. keta* (2n=74)(b), *H. perryi* (2n=62) (c), *S. leucomaenis japonicus* (2n=84) (d), *S. fontinalis* (2n=84) (e), and *S. malma* (2n=82)(f), respectively, probed with (a)Oma2, (b)Oke49, (c)Hpe50, (d)Sle9, (e)Sfo42, and (f)Smhok81. White bars indicate scale of 10 μm.

Figure 5. Fluorescence *in situ* hybridization on chromosomes of *O. masou* (a), *H. perryi* (b), *O. keta* (c), and *S. fontinalis* (d) respectively, using the Oma14 clone of *O. masou*. White bars indicate scale of 10 μm.

colour figure

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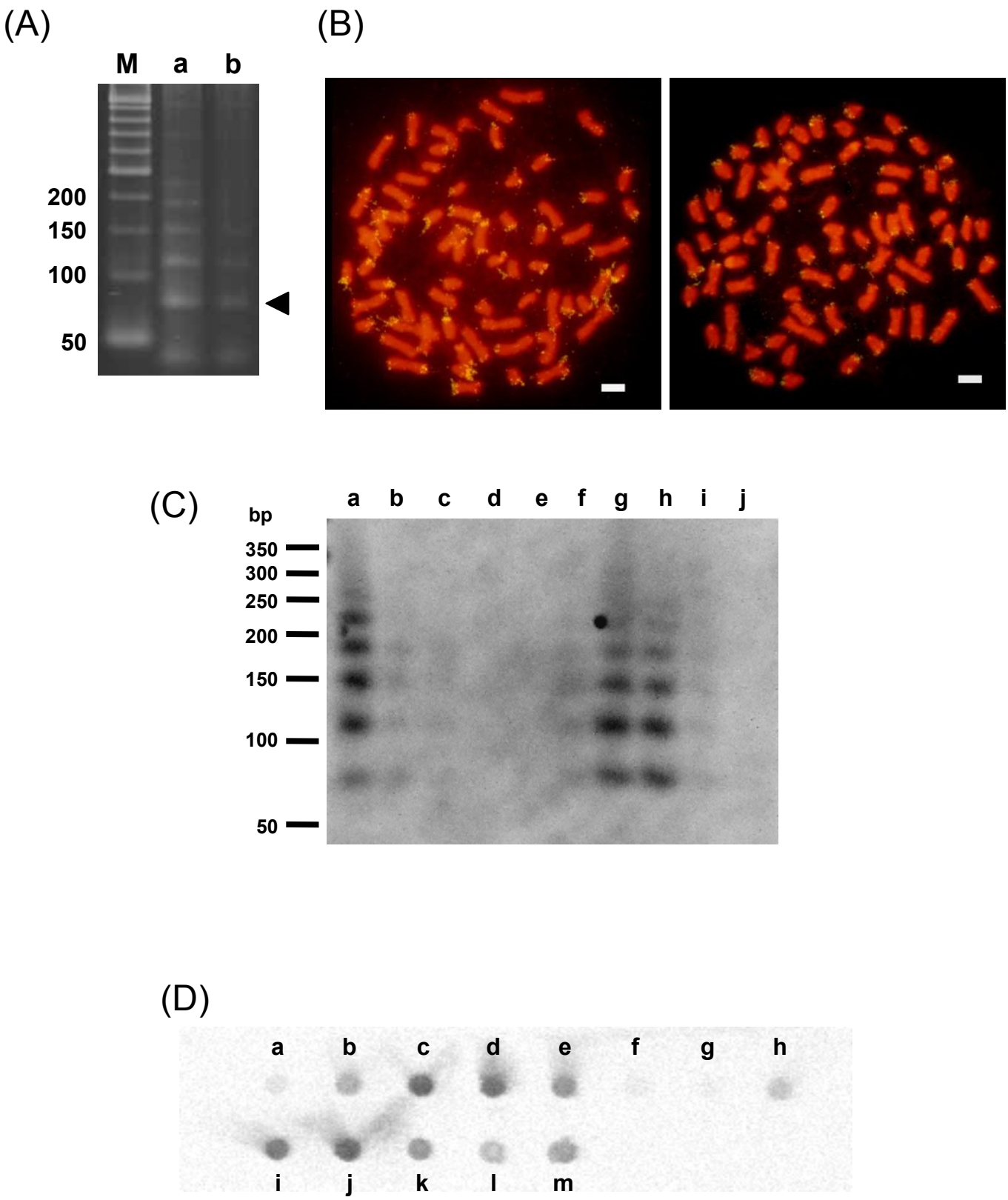

Figure 1 Saito et al.

(A)

```
Consensus 1: TAGCTGGTCT GTCATAATAT AATAGAGACA GTAGATCTAG CTGGTCTGTC ATAATATAAT AGAGACAGTA ARWCWRKMTG GTCTGTCATA GTA  :93

Om9       1: .......... ....G..... .......... ...T.....C .......... .......... ..........                          :70
Om54      1: .......... ........A. C .C........ ......G... .......... .......... .......TGG . GA. AGTA             :78
Om74      1: ..C....... ..........A .......... ......T... .......... .......... .......... . AT. TAGCTG GTCTGTCATA GTA  :93
Om78      1: .T........ ......T... .......... ......A... .......... .......... ..........                          :70
Om82      1: .......... .......... .......... ...C...... .......... .......... ..........                          :70
Om92      1: .......... .......... .......... ...T.....C .......... ..TT....G. ..........                          :70
Om98      1: .......... .......... C......... .......G.. .......... .......... ........C.                          :70
```

(B)

```
Consensus   1: GATCTAGCTG GTCTGTCATA ATATAATAGA GACAGTAGAT CTAGCTGGTC TGTCATAATA TAATAGAGAC AGTAGTTCTA GAAGATCATG GCTGACTGGC GGATCTAGCT GCTCTATGCA GACTGGCAGT  :130

Ok20        1: .......... .......... ...C...... C......... .G........ .......... ......C..G .A....                                                                :76
Ok22        1: .......... .......... .......... .....A.... TG........ ........T.. .......... ......                                                              :76
Ok35        1: .......... .......... .......... .......T.. .......... .......... .......... ......                                                              :76
Ok54        1: .......... .......... .......... .......T.. .......... .......... .......... .....A.... .......... .......... .......... .......... ..........  :130
Ok79        1: .......... .......... ...C...... .........C .......... .....A.... C......... ......                                                              :76
Ok94        1: .......... .......... .......... .......... A.......... .......... .......... ......                                                              :76
Ok112       1: .......... .......... .......... .......... A.T....... .......... .......... T.....                                                              :76
```

(C)

```
O. masou    1   ----TAGCTG GTCTGTCATA ATATAATAGA GACAGTAGAT CTAGCTGGTC TGTCATAATA  56
O. keta     1   GATCTAGCTG GTCTGTCATA ATATAATAGA GACAGTAGAT CTAGCTGGTC TGTCATAATA  60
L01078      1   GATCTAGCTG GTCTGTCATA ATATAATAGA GACAGTATAT CTAGCTGGTC TGTCATAATA  60
U27090    361   TCTTGAGCTG GTCTGTCATA ATATAATAGT GGCAGTAGAT CTAGTTGGTC TGTCATTATA 420
AF021228    1   --------CTG GTCTGTCATA ATATAATAGA GACAGTATAT CTCGCTGGTC TGTCATAATA  53
                      *** ********** *********  * ***** ** ** * ***** ****** ***

O. masou   57   TAATAGAGAC AGTAARWCWR KMTGGTC-TG TCATAGTA                          93
O. keta    61   TAATAGAGAC AGTAGTTCTA GAAGATCATG GCTGACTGGC GGATCTAGCT GCTCTATGCA 120
L01078     61   TAATAGAGAC AGTAGATC                                               78
U27090    421   TAATAGAGAC AGTAGATCTA G                                          441
AF021228   54   TAATAGAGAC AGTAGATCTA G                                           74
                ********** ****    *
```

Figure 2 Saito et al.

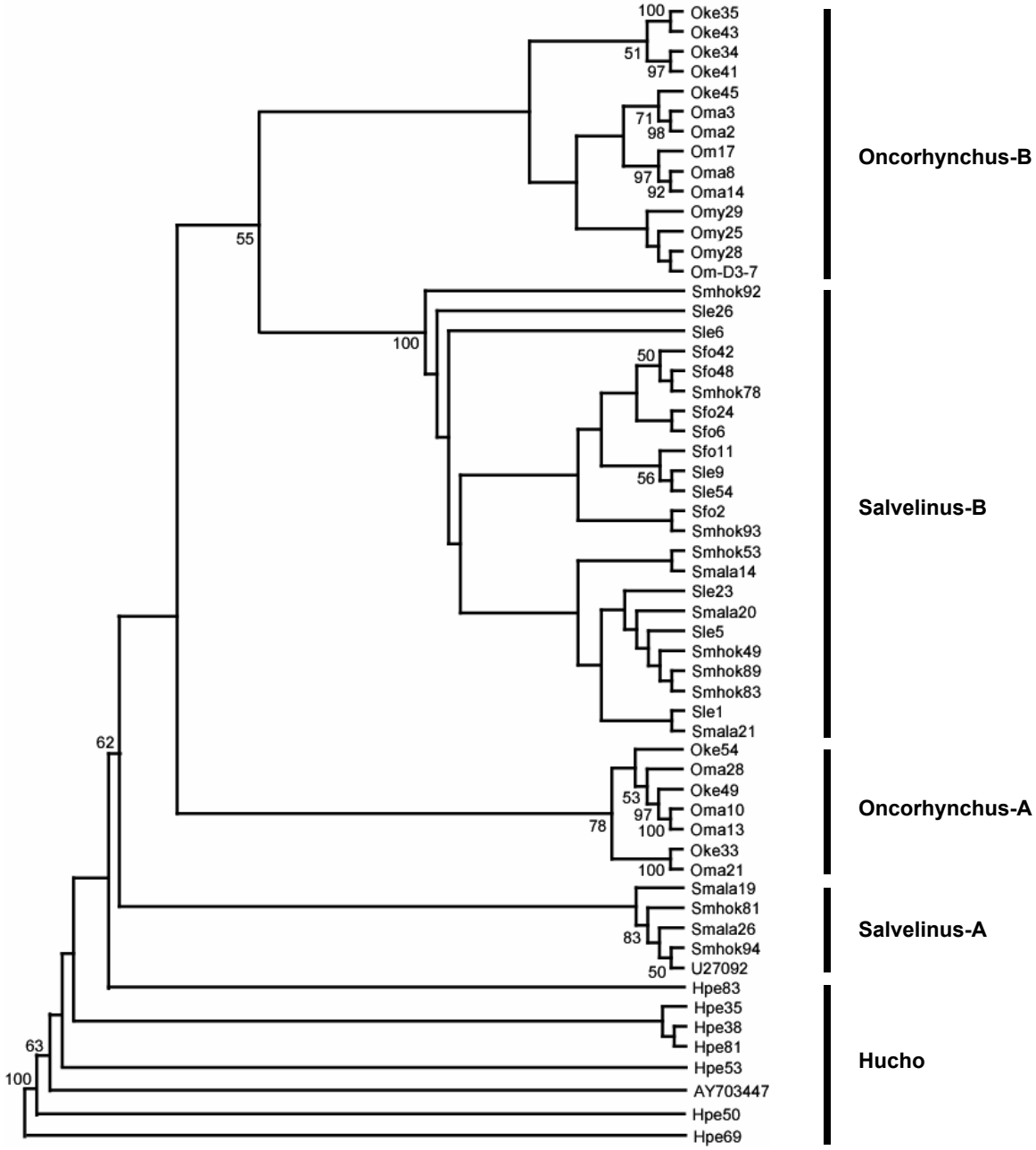

Figure 3 Saito et al.

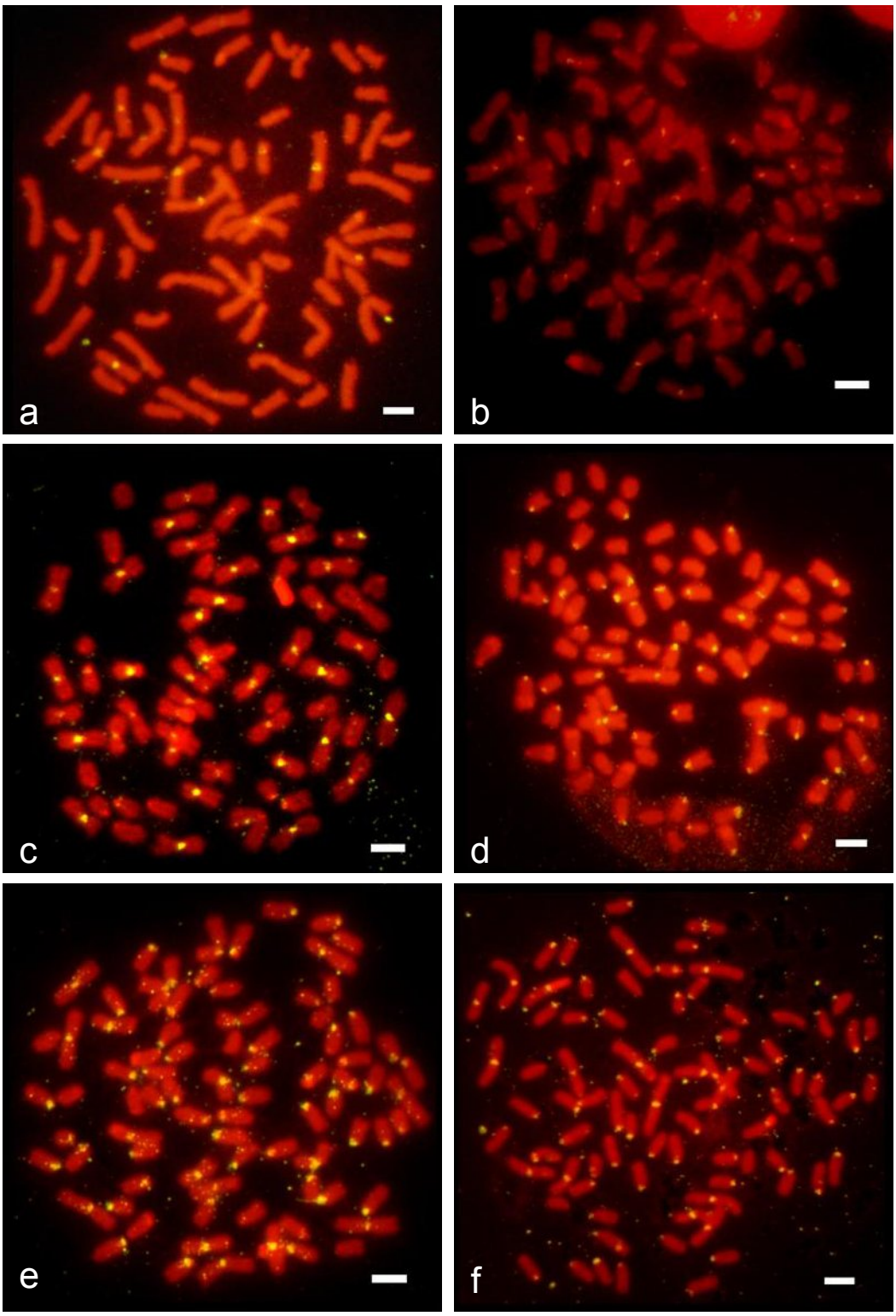

Figure 4 Saito et al.

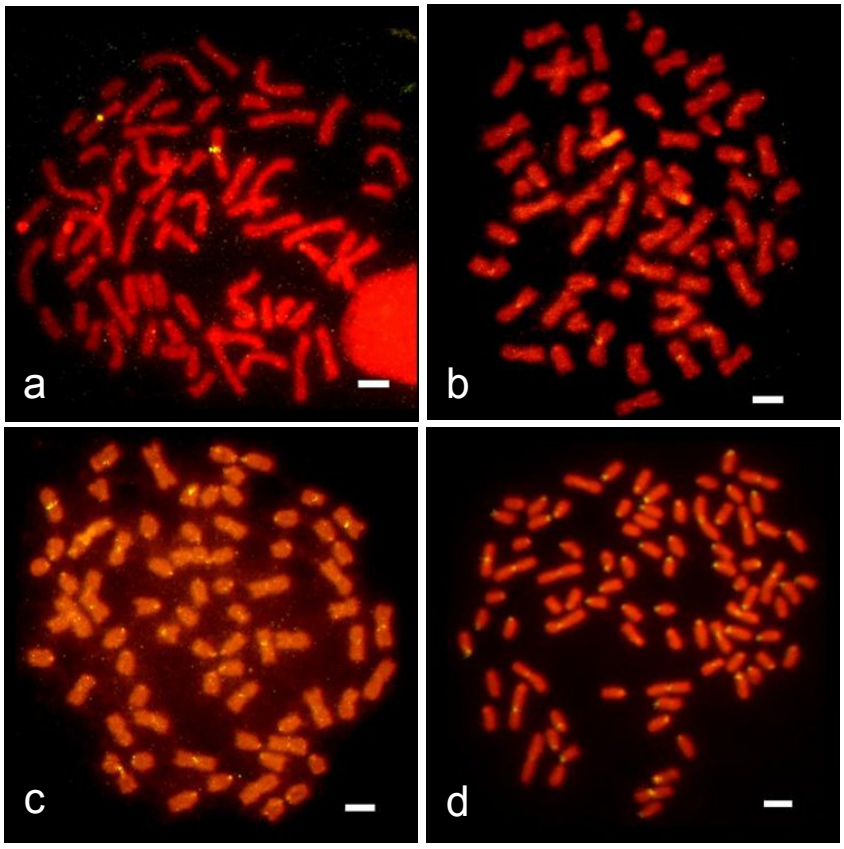

Figure 5 Saito et al.

Table 1. Summary of centromeric satellite DNA clones isolated by PCR-based cloning in this study

species	Abbreviation	clone names
Hucho perryi	Hpe	Hpe35,Hpe38,Hpe50,Hpe53,Hpe69,Hpe81,Hpe83
Salvelinus leucomaenis japonicus	Sle	Sle1,Sle5,Sle6,Sle9,Sle23,Sle26,Sle54
S. fontinalis	Sfo	Sfo2,Sfo6,Sfo11,Sfo24,Sfo42,Sfo48
S. malma (Alaska)	Smala	Smala14,Smala19,Smala20,Smala21,Smala26
S. malma (Hokkaido)	Smhok	Smhok49,Smhok53,Smhok78,Smhok81,Smhok83,Smhok89,Smhok92,Smhok93,Smhok94
Oncorhynchus mykiss	Omy	Omy25,Omy28,Omy29
O. masou	Oma	Oma2,Oma3,Oma8,Oma10,Oma13,Oma14,Oma17,Oma21,Oma28
O. keta	Oke	Oke33,Oke34,Oke35,Oke41,Oke43,Oke45,Oke49,Oke54