Doctoral Thesis

Evolutionary ecological study on lineage longevity of *Hexagrammos* hemiclonal hybrids: two-way backcrossing, recombination through occasional generation changes and synthesis of renewed hemiclone

（アイナメ属半クローン雑種の系統寿命に関する進化生態学的研究：二方向の戻し交配、間欠的組換え世代の出現と半クローン世代の更新）

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General introduction

Hybridization has been mentioned in the context of evolutionary diversification (Seehausen 2004). Hybridization is an important source of genetic variation, functional innovation, adaptation to new habitats, and also causes breakdown or reinforcement of isolating barriers (Anderson and Stebbins 1954, Stebbins 1959; Lewontin and Birch 1966, Anderson 1968). It can lead to even new species or new ecotypes (Coyne and Orr 2004, Mallet 2007). On the other hands, hybridization between two distantly related species is typically associated with the disruption of normal meiosis due to pairing incompatibilities between homoeologous chromosomes (Dawley 1989). Some vertebrates however, have escaped from this constraint by altering their reproduction from sexual mode to unisexual mode, such as clonal reproduction (parthenogenesis and gynogenesis) or by employing hemiclonal reproduction (hybridogenesis) (Dawley 1989, Vrijenhoek 1989, Lampert and Schartl 2008). In 1932, the Amazon molly, *Poecilia formosa*, was the first unisexual vertebrate to be described (Hubbs and Hubbs 1932). After that, unisexual species were confirmed in 50 or more naturally occurring fish, amphibian and reptile, and most of them reproduced clonally (Avise 2015, Warren *et al.*, 2018). Unlike gynogenesis and parthenogenesis which involve clonal reproduction without any contribution of the paternal genome, in hybridogenesis, the sperm genome is used to produce somatic cells, but it is eliminated from germ cells during oogenesis (Schultz 1961, Cimino 1972, Tunner and Heppich-Tunner 1991). In this way, the entire paternal genome is displaced by incorporation of the other sperm genome at every generation. Hemiclonal reproduction is less widespread than clonal reproduction and to date has only been reported in six taxa, freshwater fishes, *Poeciliopsis* hybrids (Miller and Schultz 1959; Schultz 1969), *Tropidophoxinellus alburnoides* (Carmona *et al.*, 1997), and *Hypseleotris* complex (Schmidt *et al.*, 2011); the stick insect *Bacillus*

hybrids (Tinti and Scali 1992), the frog *Pelophylax esculentus* (*Rana esculentus*) (Ogielska 2009), and the marine fish *Hexagrammos* hybrids (Kimura-Kawaguchi *et al.*, 2014).

Based on karyological evidences, Cimino (1972) was the first to propose that the paternal genome of *Poeciliopsis* hybrids is eliminated before the onset of meiosis as chromosome fragments. In *Pelophylax esculentus* (*Rana esculentus*), it was shown paternal genome has been eliminated in gonial cells as Nucleus-like body (NLB) containing eliminated chromosome (Tunner and Heppich-Tunner 1991, Ogielska 1994, 2009, Chmielewska *et al.*, 2018). For *Bacillus* hybrids it was hypothesized that the elimination of a genome takes place during the formation of polar bodies formation, i.e. at meiotic metaphases I and II (Tinti and Scali 1992). Those mechanisms of genome elimination are associated with genome-recognition of each homoeologous chromosome between the karyotypes of two different species during gametogenesis (Roeder 1997). Like this, when the genetic divergence between the parental genomes has to be sufficiently large to affect meiosis in hybrids but not so large that it causes a significant decrease in hybrid viability, clonal or hemiclonal animals could be produced between the parental species. This hypothesis has been named "balance hypothesis" (Moritz *et al.*, 1989). In addition to this genomic heterogeneity, the existence of genetic factors that induce hybridogenesis has been verified by artificial crossing experiments among *Poeciliopsis* hybrids and parental species. *Poeciliopsis* hemiclonal hybrids that originated between females of *P. monacha* and males of *P. lucida* were hemiclonally reproduced by mating with males of *P. lucida* (Schultz 1969). However most of F1 embryos of *P. monacha* that were artificially fertilized by spermatozoa from *P. lucida* did not retain sufficient yolk to develop until the larvae could feed independently, only 7% of such mating produce viable offspring (Schultz 1973, Leslie and Vrijenhoek 1978). The findings of these

studies suggested the presence of specific differences in the genetic elements between the clonal or hemiclonal genomes of natural hybrids and the genomes of the maternal species (Leslie and Vrijenhoek 1978). This means that at least two genetic problems must be solved in order to occur clonal or hemiclonal reproduction: the genomic heterogeneity (genetic affinity) between the parental species and getting the genetic elements.

The most important difference between sexual reproduction and unisexual reproduction, that is, while individuals of sexual reproduction lose half of their genome by recombination and meiosis, gynogens and hybridogens pass on their entire maternal diploid and haploid genomes, respectively, to their descendants, and are absolved from the cost of producing males (Maynard Smith 1978, 1992). Unisexual reproduction is therefore considered to have two-fold reproductive advantage. Consequently, such system is considered to increase twice as fast as sexual reproduction, and once a unisexual lineage appears, it is considered to be at an advantage when colonizing new habitats or when competing with sexual reproduction in the mother species (Vrijenhoek 1978, Avise 2008, Lehtonen *et al.*, 2013). However, despite this advantage, most eukaryotes have retained sexual reproduction and recombination as a reproductive strategy, and only a small number of taxa have developed unisexual modes of reproduction.

The primary reason for retaining sexual reproduction as a reproductive strategy is because genetic diversity does not increase in the absence of recombination, and because deleterious mutations accumulate within clonal genomes. The theoretical disadvantages of clonal reproduction have been examined extensively. Essentially, the genetic heterogeneity of a genotype is generated through a combination of recombination during gametogenesis and the subsequent union of recombinant zygotes, and additionally mutation may contribute partially for genetic variation.

The concomitant increase in genetic variability is advantageous for adaptation to environmental perturbations and for increasing resistance to parasites (Bengtsson 2009, Neiman and Koskella 2009). Compared to the relative absence of mechanisms that increase genetic heterogeneity in unisexual reproduction, the advantage of these mechanisms in sexually reproducing organisms has been referred to as The Red Queen hypothesis (Van Valen 1977, Bell 1982, Barton and Charlesworth 1998). In reproduction without recombination, the descendants inherit an entire genome that has gradually accumulated deleterious mutations with every generation. This condition is referred to as Muller's ratchet (Muller 1964, Kondrashov 1988, Otto and Lenormand 2002, Burt and Trivers 2006). These defects are thought to lead to extinction of unisexual lineages within at most 10^4-10^5 years due to a combination of natural selection such as genetic drift and Muller's ratchet (Lynch and Gabriel 1990, Lynch *et al.*, 1993, Loewe and Lamatsch 2008). However, in *P. formosa* which have a generation time of 3–4 months, gynogenetic hybrids have existed for more than approximately 500,000 generations (Loewe and Lamatsch 2008, Warren *et al.*, 2018). Also *P. monaca-lucida,* hybridogentic hybrids have lasted far exceeding the theoretical estimates of 100,000 generations (Quattro *et al.*, 1992). Despite the theoretical aspects of clonal reproduction having been examined extensively, such apparent contradictions have remained elusive by difficulty to obtain empirical evidences.

The genus *Hexagrammos* contains six species that are very common fish in the coastal waters of the North Pacific Ocean (Rutenberg 1970). Of these species, a boreal species, the Masked greenling *H. octogrammus* (hereafter *Hoc*), and two temperate species, the Fat greenling *H. otakii* (*Hot*) and the Spottybelly greenling *H. agrammus* (*Hag*), have a sympatric distribution in the coastal areas of southern Hokkaido and Primorsky Krai of Russia (Rutenberg 1970, Crow *et al.*, 2010). The

hybrid zone inhabited by these three *Hexagrammos* species contains two unisexual hybrids which reproduced hemiclonally (Kimura-Kawaguchi *et al.*, 2014). Hybrids that produced haploid eggs containing only the *Hoc* genome (maternal ancestor) generated hemiclonal offspring by fertilization with haploid sperm of *Hag* or *Hot* (paternal species) (Kimura-Kawaguchi *et al.*, 2014). Existence of certain genetic factors that induce hybridogenesis was demonstrated by mating experiments of *Hexagrammos* hybrids (Kimura-Kawaguchi *et al.*, 2014), as had been assumed from *Poeciliopsis* hybrids. Therefore, the hemiclonal *Hoc** genome of the natural hybrids was differentiated from *Hoc* genome of the pure species.

The two natural hybrids were denoted as *Hoc*/Hag* and *Hoc*/Hot* ("*" indicates that the genetic factors required for inducing hybridogenesis are present). It is well known that natural hybrids between closely related species occur frequently in contiguous habitats (Barton and Hewitt 1985, Buggs 2007). However, the three *Hexagrammos* species have established a firm reproductive isolation system (Kimura and Munehara 2011) with no evidence of genetic introgression detected to date (Crow *et al.*, 2007, 2010). Consequently, natural hybridization among the three *Hexagrammos* species is considered to occur only rarely at present. By reproducing hemiclonally, the entire paternal genome is replaced at every generation change. Indeed, a natural hybrid between *Hoc** and *Hot* (*Hoc*/Hot*) has been shown to arise by anomalous hybridization, or host switching, between the other natural hybrid (*Hoc*/Hag*) and the pure species (*Hot*), and not from interspecific hybridization between *Hoc* and *Hot* (Munehara *et al.*, 2016). Like this, host switching is considered to have been an important event in the evolution of *Hexagrammos* hybrids. Therefore, it is appropriate prospect that the natural hybrids (*Hoc*/Hag*) often backcross with *Hoc* also, because the natural hybrids consist of each genome set from *Hoc** and *Hag*. The offspring from the backcross (abbreviated as BC-*Hoc*) has a *Hoc**

genome inherited from the hemiclonal hybrid, and a normal *Hoc* genome inherited from the *Hoc* male. BC-*Hoc* has the same morphological phenotype as normal *Hoc*, so BC-*Hoc* cannot be discriminated from normal *Hoc* based on morphology alone. Genotyping with microsatellite DNA revealed that BC-*Hoc* hybrids produced recombinant gametes. This situation needs novel genetic markers for advance of this study.

In this thesis, I aimed to clarify the mechanisms to obtain how to get longevity in *Hexagrammos* hemiclonal hybrids. In Chapter 1, I compared the karyotypes of three *Hexagrammos* species and several hybrids to determine the reproductive mode employed by BC-*Hoc* hybrids and the mode of inheritance of the hemiclonal genome, in order to clarify the genetic differences between natural hybrids and artificial hybrids. As a result, novel genetic marker to discriminate BC-*Hoc* and normal *Hoc* was developed. In Chapter 2, I searched for the hybrid eggs deposited in *Hoc* and *Hag* breeding territories using karyological and genetic makers. In chapter 3, accumulation of deleterious mutations in the natural hybrid genome was ascertained from fertility of gametes that sub-mating offspring from maternal backcross produced. In chapter 4, I evidenced a long term longevity strategy of unisexual hemiclonal hybrids through repetitive artificial crossing experiments.

Fish and embryos were treated in accordance with the Regulations of Animal Experimentation at Hokkaido University.

Chapter 1
Karyological evidence of hybridogenesis in Greenlings (Teleostei: Hexagrammidae)

Changing of reproduction system from recombinant to (hemi) clonal is often caused by dependence of ploidy (Arai 2009). In *Misgurnus angullicaudatus*, the diploid loach produces clonal diploid gametes, but triploid loach, which has arisen from a clone diploid egg taking in a haploid sperm, produces haploid gametes of one parental genome transmitted without recombination (Morishima *et al.*, 2008). In this reproduction system of triploid called meiotic hybridogenesis, homogeneous genomes with high affinity are paired for meiosis, and a heterogeneous haploid genome is eliminated (Morishima *et al.*, 2008, Arai 2009). There are also reports of aneuploids in genus *Poeciliopsis* hybrids having various genetic modalities including hemiclonal reproduction (Vrijenhoeck 1993). Not only ploidy level but also change of karyotype loaded with abnormal chromosomes may get new capability or cause certain gene illness in connection with reproduction system. In wolf fish, *Hoplias malabaricus,* sex chromosome differs among populations, so in some population sex chromosome unites with another chromosome (Cioffi *et al.*, 2011a, 2011b). Therefore, a comparison of karyotypes among the natural hybrids and artificially made F1-hybrids may detect any significant differences on genetic elements. The karyotypes of *H. otakii* and *H. agrammus* have been studied by Nishikawa and Sakamoto (1982) and Matsumiya *et al.*, (1980), and chromosome number of *H. octogrammus* has been observed by Makino (1937). In this chapter, I conducted a comparative study of karyotype morphology among three *Hexagrammid* species, the natural hybrids and artificially made some kinds of hybrids including F1-hybrids.

Materials and Methods

Collection of parental fish

For artificial fertilization, the three *Hexagrammos* species (*H. octogrammus; Hoc, H. agrammus; Hag*, and *H. otakii; Hot*) and the natural hybrids were caught using traps and/or fishing rods in the shore of Usujiri Fisheries Station (N41° 57', E140° 58') of the Field Science Center for Northern Biosphere, Hokkaido University in southern Hokkaido, Japan from 2011 to 2013. Species identification was performed based on the number of lateral lines, number of pairs of flaps on the head, and caudal fin shape according to Shinohara (1994) and Nakabo (2013). Morphologically intermediate individuals of these three characters were separated as hybrids. The natural hybrids between *Hoc* and *Hag* (*Hoc*/Hag*) were identified by the number of lateral lines and flap pairs: lack of the forth lateral line and one flap on the posterior region of the head. The natural hybrids between *Hoc* and *Hot* (*Hoc*/Hot*) were identified by the number of flap pairs and the shape of the caudal fin: lack of a flap pair on the posterior region of the head and caudal fins with a rounded margin. Hybrid identification based on these morphological characteristics was genetically verified by Kimura-Kawaguchi *et al.* (2014).

Before artificial fertilization, the captured live fishes were kept in 500 L tanks containing concrete blocks as hiding places. Fresh seawater was pumped into the tank to ensure that the water temperature was the same as that in the natural environment. Fishes were fed daily with Japanese anchovy, krill and artificial pellets (Otohime, Nishinmarubeni Co., Japan).

Chromosome spreads were prepared from embryos of *Hoc, Hag*, *Hot*, the two natural hybrids (**Hoc/Hag* and **Hoc/Hot*), artificial F_1-hybrids (*Hoc* × *Hag* and *Hoc* × *Hot*), and several backcrosses, as described below. The generalized format used to represent the hybrid crosses in this study was as follows: A × B and A/B, where A is

the female species, B is the male species, and "/" and "×" indicate natural hybrids and artificial crossing, respectively. F_1-hybridization between species is depicted here as *Hoc* × *Hag* and *Hoc* × *Hot*. Different symbols are used to represent these hybrids, because the natural hybrids reproduce hemiclonally by backcrossing with paternal species, whereas F_1-hybrids resulting from hybridization between species produced recombinant gametes (Kimura-Kawaguchi *et al.*, 2014). In such a situation, paternal backcrosses of natural hybrids are described as (*Hoc*/Hag*) × *Hag* and (*Hoc*/Hot*) × *Hot*, and they thoroughly correspond with the natural hybrids *Hoc*/Hag* and *Hoc*/Hot*, respectively. In the same way, maternal backcrosses of natural hybrids are referred to as BC-*Hoc* 1: (*Hoc*/Hag*) × *Hoc* and BC-*Hoc* 2: (*Hoc*/Hot*) × *Hoc*. Both the artificial hybrids comprise a *Hoc** genome and a *Hoc* genome. When they matured at 2 years, it was found that both sexes of BC-Hoc were produced, which meant that both BC-*Hoc* 1 × *Hoc* and *Hoc* × BC-*Hoc* 1 could be crossed in order to observe the resulting karyotypes and to investigate their respective reproductive modes. The lineages of specimens used for observation of a chromosome were indicated in Fig. 1-1.

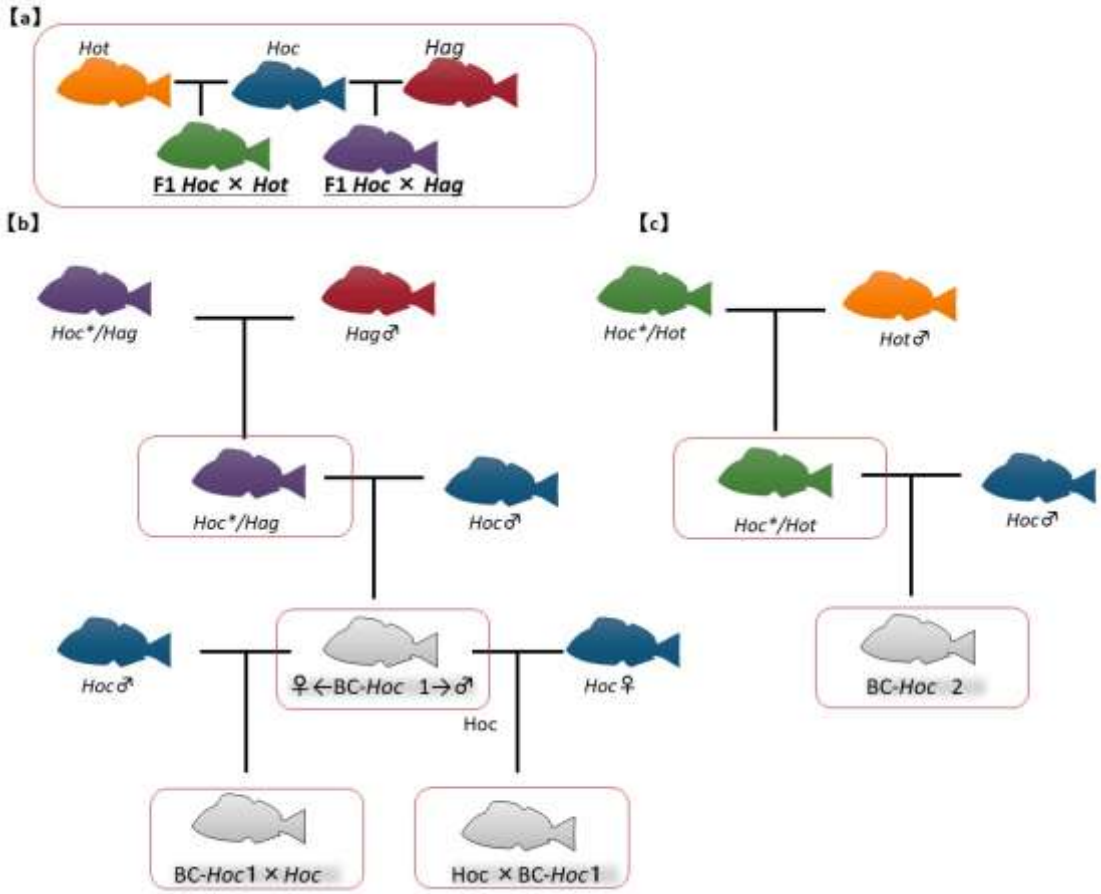

Fig. 1-1. The lineages of specimens used in this study. (a) Artificial F_1-hybrids, (b) *Hoc*/Hag* hybrid lineage, (c) *Hoc*/Hot* hybrid lineage. All abbreviated species signs were referred to text. Red square indicate species that observed in this chapter.

Artificial fertilization

Ovulation was confirmed by applying gentle pressure to the abdomen. When the females ovulated, the eggs were evenly divided into separate petri dishes. Milt was collected with a hematocrit tube. The amount and time of handling of the fish was kept to a minimum to ameliorate suffering and distress, and collecting eggs and spermatozoa were conducted with anesthesia (MS-222). Sperm motility was checked by adding seawater under a microscope (× 40). After checking, sperm with high motile activity were used for artificial fertilization. The eggs were fertilized with a sufficient quantity of semen (5-10 μl) and fertilization was conducted using a standard dry method. After thinly spreading the eggs, spermatozoa were added with gentle mixing using a spatula. The fertilized eggs were then incubated at room temperature for several minutes to complete fertilization, followed by incubation at 11-15°C for 3 to 7 days until the eyes appeared in separate cages (Fig. 1-2). For identification purposes, all of the parental fish used for artificial fertilization were identified using internal electric tags or external plastic tags.

Fig. 1-2. The incubation tank (A) and cages (B) that raises an egg mass separately.

This system constantly send fresh sea water to eggs.

Collection of egg masses from natural spawning grounds

Most of the embryos were prepared by artificial fertilization, but some were collected from natural breeding sites. Eggs of *Hoc*/Hot* natural hybrids were collected from *Hot* spawning grounds on the western side of Usujiri fishing port by SCUBA from October to December, 2011 to 2013. The eggs of natural hybrids deposited in *Hot* territories were identified based on the color of the oil droplets in the yolk using the method of Kimura *et al.*, (2007). *Hag* egg masses were collected in the coastal waters of Sado Island (N38° 04', E138° 14') using SCUBA in December 2011. These egg masses were also incubated at 15°C until the eyed embryo stage.

Chromosome preparations and analyses

Karyotypes of *Hot* and *Hag* have been studied by Matsumiya *et al.* (1980) and Nishikawa and Sakamoto (1982), and the modal chromosome number of *Hoc* has been estimated by Makino (1937). In the present study, including the pure species, a total of 43 egg batches were prepared for chromosome analysis. The number of egg batches from each species (strains) were 5 *Hot*, 5 *Hag*, 6 *Hoc*, 3 *Hoc* × *Hag*, 3 *Hoc* × *Hot*, 5 *Hoc*/Hag*, 5 *Hoc* × *Hot*, 3 BC-*Hoc*1, 3 BC-*Hoc*2, 2 BC-*Hoc*1 × *Hoc* and 3 *Hoc* × BC-*Hoc*1. Yolk was aseptically removed from eyed embryos in 0.9% NaCl solution with tweezers under a microscope (SZX, Olympus Co., Japan). The resulting embryos were then kept in a 0.9% NaCl solution containing 0.0025% colchicine for 1.5 to 2.5 h, before being placed into a 0.075 M KCl hypotonic solution for 30-60 min at room temperature and then fixed with a Carnoy solution (methanol:acetic acid=3:1) in microtubes overnight. After resuspending the cells in an aqueous solution using a micropipette, chromosome spreads were obtained by dripping the aqueous solution on a glass microscope slide from a height of 30 cm. The chromosome spreads were then stained with 4% Giemsa in phosphate buffer (pH=6.8) for 30 min.

Chromosomes were observed under a microscope (BX51, Olympus Co.). The karyotypes were determined by chromosomes that were reconstructed from Giemsa-stained metaphase plates using a microscope equipped with a digital camera (VB-7010, Keyence Co., Japan). According to Levan *et al.,* (1964), chromosomes were classified as *m* = metacentric; *sm* = submetacentric; *st* = subtelocentric and *t* = telocentric. The fundamental number (NF) was calculated by assigning 2 arms for *m* and *sm* and 1 arm for *st* and *t*. A pair of homologous chromosomes was determined by the relative length of the short and long arms of each chromosome.

Results

Karyotypes of three *Hexagrammos* species

Among 62 metaphases from six batches of *H. octogrammus* (*Hoc*), the modal chromosome number was 2n=48 (Table 1). The karyotype was determined as 2*sm* + 10*st* + 36*t,* and the NF was 50 arms (Fig. 1-3a).

Among nine metaphases from five batches of *H. agrammus* (*Hag*), the modal chromosome number was 2n=48. The karyotype was 8*m* + 26*sm* + 14*st,* and NF was 82 arms.

Among 22 metaphases from five batches of *H. otakii* (*Hot*), the modal chromosome number was 2n=48. The karyotype was 6*m* + 12*sm* + 22*st* + 8*t,* and the NF was 66 arms.

Karyotypes of artificial F_1-hybrids

Among 120 metaphases from three batches of *H. octogrammus* × *H. agrammus* (*Hoc* × *Hag*), the modal chromosome number was 2n=48. The karyotype was 4*m* + 14*sm* + 12*st* + 18*t,* and the NF was 66 arms (Fig. 1-3b).

Among 121 metaphases from three batches of *H. octogrammus* × *H. otakii* (*Hoc* × *Hot*), the modal chromosome number was 2n=48. The karyotype was 3*m* + 7*sm* + 16*st* + 22*t*, and the NF was 58 arms (Fig. 1-3c). These results showed that the karyotypes of both F_1-hybrids were intermediate between two parental species.

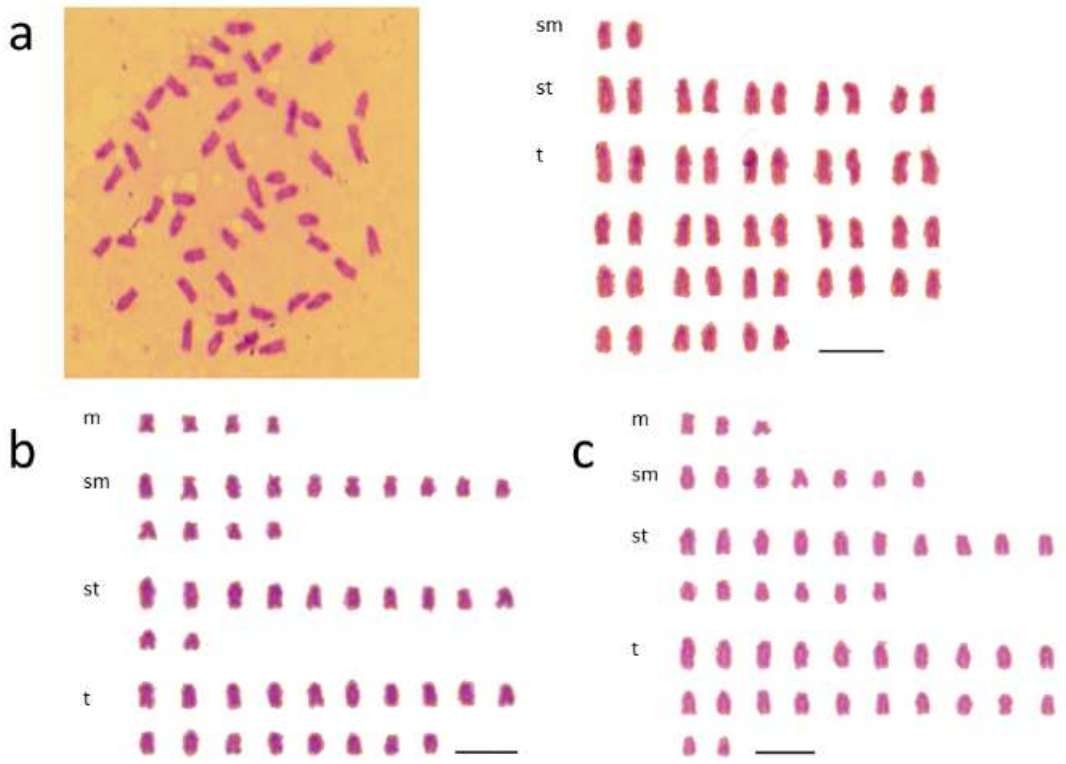

Fig. 1-3. Mitotic metaphase chromosome spread and karyotypes. (a) *H. octogrammus*, (b) F_1-hybrid (*Hoc* × *Hag*), (c) F_1-hybrid (*Hoc* × *Hot*).

Table 1-1. Karyological observations of the three Hexagrammos species, and the natural hybrids and the artificial hybrids.
Yellow cells indicate the most frequent chromosome numbers of each species and hybrid.

Species	Observed clutch	Observed cells	Individuals	observed chromosome							Type	Karyotype				Centric fusion	Micro chromosomes	NF	2n
				～43	44	45	46	47	48	49		*m*	*sm*	*st*	*t*				
H. octogrammus	6	62	10		2	2	4	8	45	1		0	2	10	36			50	48
H. agrammus	5	9	5				2	1	6			8	26	14	0			82	48
H. otakii	5	22	30				4	4	14			6	12	22	8			66	48
Hoc×*Hag*	3	120	13	2	1	3	2	10	99	3		4	14	12	18			66	48
Hoc×*Hot*	3	121	12	3	1	6	7	12	86	6		3	7	16	22			58	48
Hoc/Hag			7		8	13	53	7	7		Type 1	6	14	8	18	2		66	46
	5	140	5	9	3	27	2				Type 2	7	14	8	16	3		66	45
			1	1				10			Type 3	7	14	8	16	3	2	66	47
Hoc/Hot			10		7	13	30	8	1		Type 1	5	7	12	22	2		58	46
	5	151	6	20	9	45	2			2	Type 2	6	7	12	20	3		58	45
			2			2	8	4			Type 3	6	7	12	20	3	1	58	46
			7	2	2	4	42				Type 1	2	2	6	36	2		50	46
	3	90	3	6	1	25					Type 2	3	2	6	34	3		50	45
BC-*Hoc*1: (*Hoc*/*Hag*)×*Hoc*			1		1	3	1	6			Type 3	3	2	6	34	3	2	50	47
			7	1	6	41	1	1			Type 1	3	2	6	34	3		50	45
BC-*Hoc*2: (*Hoc*/*Hot*)×*Hoc*	3	101	3	3	44	4					Type 2	4	2	6	32	4	1	50	45
BC-*Hoc*1×*Hoc*	2	80	8		1			8	69	2		0	2	8	38			50	48
Hoc×BC-*Hoc*1	3	59	6			1	7	5	45	1		0	2	8	38			50	48

Karyotypes of the natural hybrids

Among 140 metaphases from five batches of *H. octogrammus* /*H. agrammus* (*Hoc*/Hag*), two or three large metacentric chromosomes were observed in all of the cells (Table 1). These large chromosomes were inferred to comprise two chromosomes that had become joined, and which are mentioned in the discussion. In addition, two microchromosomes were observed in some cells. The modal chromosome numbers in these hybrids were 2n=45-47 and the karyotypes were divided into three types as follows:

(Here and below, the numerals in parenthesis represent the number of large-size metacentric chromosomes).

Type 1: 6(2)*m* + 14*sm* + 8*st* + 18*t,* 46 chromosomes and 66 arms (Fig. 1-4a).

Type 2: 7(3)*m* + 14*sm* + 8*st* + 16*t,* 45 chromosomes and 66 arms (Fig. 1-4b).

Type 3: 7(3)*m* + 14*sm* + 8*st* + 16*t,* 47 chromosomes including 2 microchromosomes and 66 arms (Fig. 1-4c).

In *Hoc*/Hot* crosses, among 151 metaphases from five batches, two or three large metacentric chromosomes and one microchromosome were observed in all of the cells. The modal chromosome numbers were 2n=45 or 46, and the karyotypes were divided into three types.

Type 1: 5(2)*m* +7 *sm* + 12*st* + 22*t*, 46 chromosomes and 58 arms (Fig. 1-4d).

Type 2: 6(3)*m* + 7*sm* + 12*st* + 20*t,* 45 chromosomes and 58 arms (Fig. 1-4e).

Type3:6(3)*m* + 7*sm* + 12*st* + 20*t,* 46 chromosomes including one microchromosome and 58 arms (Fig. 1-4f).

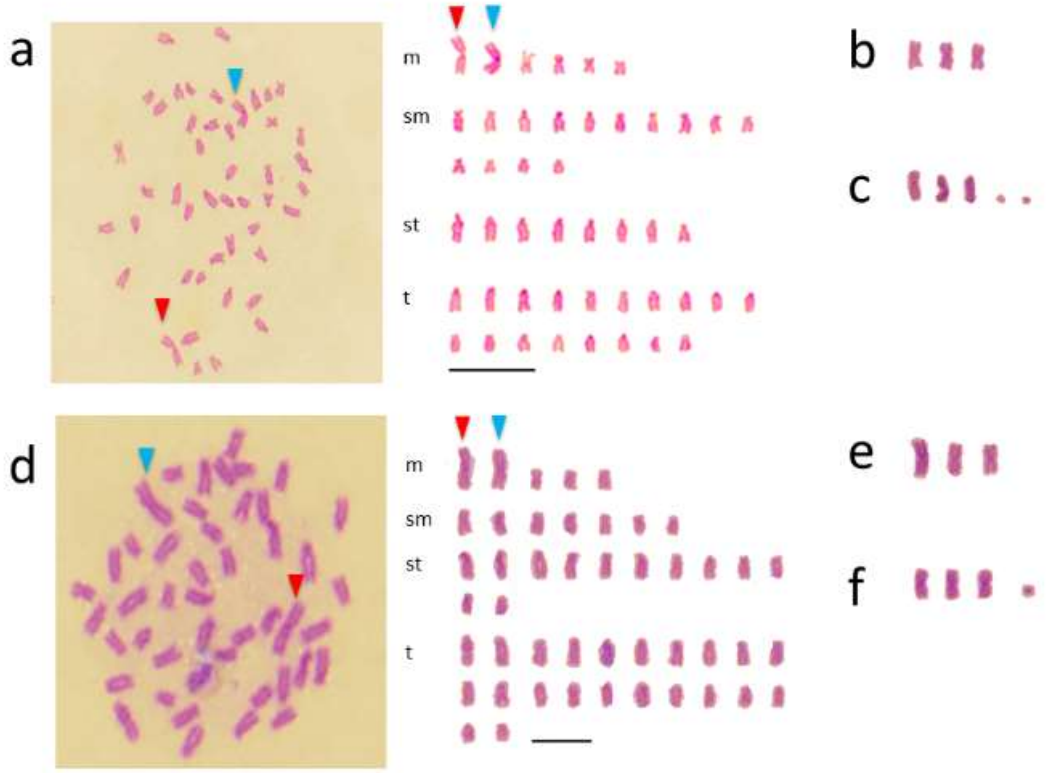

Fig. 1-4. Mitotic metaphase chromosome spreads and karyotypes of natural hybrids. (a) *Hoc*/Hag* type 1, (b) large metacentric chromosomes of *Hoc*/Hag* type 2, (c) large metacentric chromosomes and microchromosome of *Hoc*/Hag* type 3; (d) *Hoc*/Hot* type 1, (e) large metacentric chromosomes of *Hoc*/Hot* type 2, (f) large metacentric chromosomes and microchromosome of *Hoc*/Hot* type 3. Arrow heads showed large metacentric chromosomes.

Karyotypes of maternal backcross; natural hybrid × *H. octogrammus* (BC-*Hoc*)

Among 90 metaphases from three batches of BC-*Hoc*1 (*Hoc**/*Hag*×*Hoc*), two or three large metacentric chromosomes and two microchromosomes were also observed. The modal chromosome number was 2n=45-47, and the karyotypes were divided into three types.

Type 1: 2(2)*m* + 2*sm* + 6*st* + 36*t,* 46 chromosomes and 50 arms (Fig. 1-5a).

Type 2: 3(3)*m* + 2*sm* + 6*st* + 34*t,* 45 chromosomes and 50 arms (Fig. 1-5b).

Type 3: 3(3)*m* + 2*sm* + 6*st* + 34*t,* 47 chromosomes including two microchromosomes and 50 arms (Fig. 1-5c).

Among 101 metaphases from three batches of BC-*Hoc*2 (*Hoc**/*Hot* × *Hoc*), three or four large metacentric chromosomes and one microchromosome were observed in all the cells. The modal chromosome number was 2n=45, and the karyotypes were divided into 2 types.

Type 1: 3(3)*m* + 2*sm* + 6*st* + 34*t,* 45 chromosomes and 55 arms (Fig. 1-5d).

Type 2: 4(4)*m* + 2*sm* + 6*st* +32*t,* 45 chromosomes including one microchromosome and 50 arms (Fig. 1-5e).

These results showed that, of the *Hoc* genomes, although the genome compositions of both BC-Hoc1 and BC-Hoc2 are diploid, the karyotypes differed slightly from normal *Hoc*.

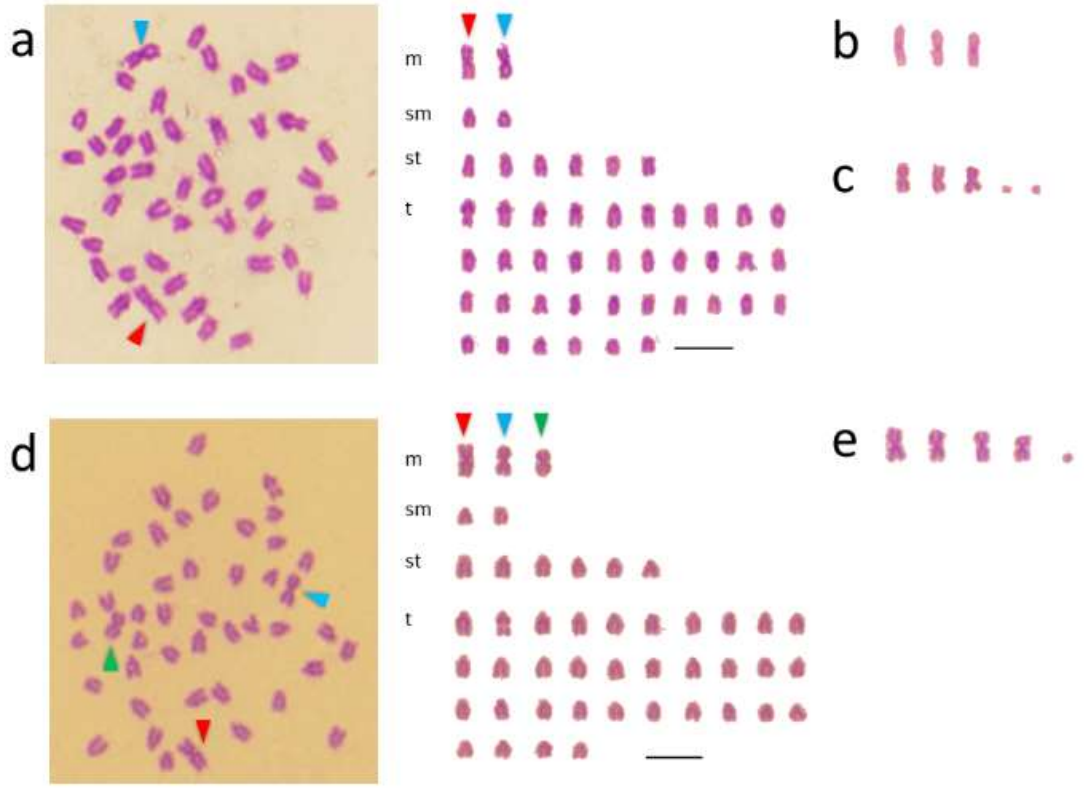

Fig. 1-5. Mitotic metaphase chromosome spreads and karyotypes of maternal backcross. (a) BC-*Hoc*1 ((*Hoc**/*Hag*) × *Hoc*) type 1, (b) large metacentric chromosomes of BC-Hoc1 ((*Hoc**/*Hag*) × *Hoc*) type 2, (c)) large metacentric chromosomes and microchromosome of BC-*Hoc*1 ((*Hoc**/*Hag*) × *Hoc*) type 3; (d) BC-*Hoc*2 ((*Hoc**/*Hot*) × *Hoc*) type 1, (e) large metacentric chromosomes and microchromosome of BC-*Hoc*2 ((*Hoc**/*Hot*) × *Hoc*) type 2. Arrow heads showed large metacentric chromosomes.

Karyotype of BC-*Hoc*1 × *Hoc*

Among 80 metaphases from two batches of BC-*Hoc*1 × *Hoc*, none of the large metacentric chromosomes or microchromosomes were observed in any of the cells. The modal chromosome number was 2n=48, the karyotype was 2*sm* + 8*st* + 38*t*, and the NF was 50 arms (Fig. 1-6a).

Karyotype of *Hoc* ×BC-*Hoc*1

Among 59 metaphases from three batches of *Hoc* × BC-*Hoc*1, none of the large metacentric chromosomes or microchromosomes were observed in any of the cells. The modal chromosome number was 2n=48, the karyotype was 2*sm* + 8*st* + 38*t,* and the NF was 50 arms (Fig. 1-6b). BC-*Hoc*1 × *Hoc* and *Hoc* × BC-*Hoc*1 exhibited the same karyological morphology. The genomic composition of these hybrids was a diploid *Hoc* genome and a *Hoc** genome, which was the same as BC-*Hoc*; however, the karyotype of BC-*Hoc*1 × *Hoc* and *Hoc* × BC-*Hoc*1 differed from both BC-*Hoc* and the pure *Hoc* species.

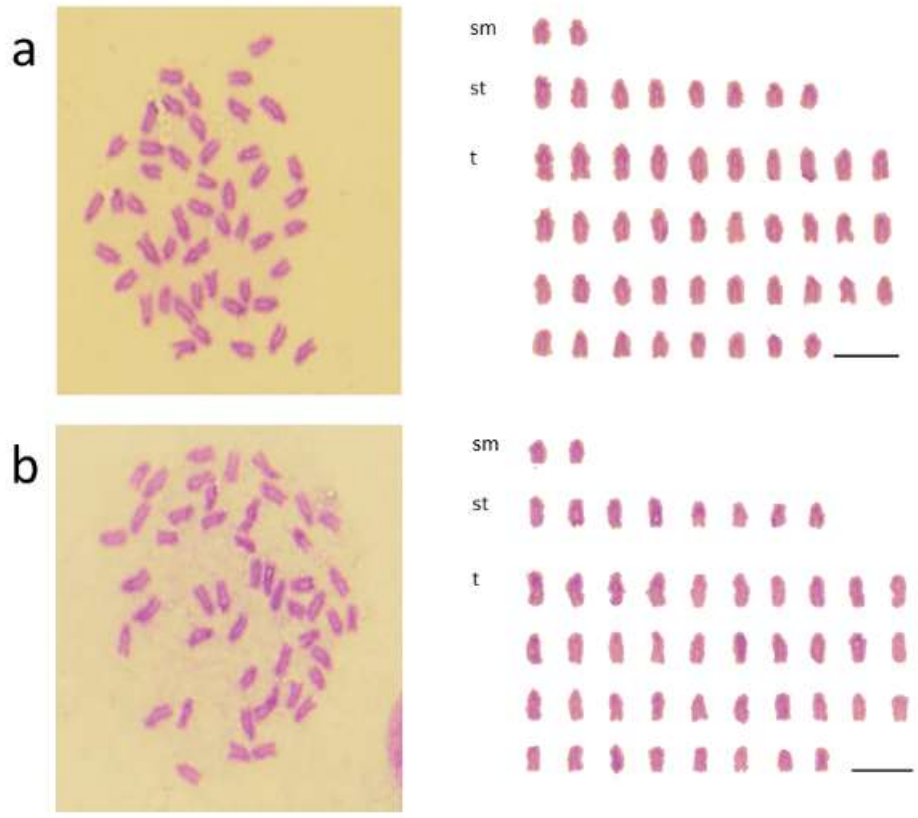

Fig. 1-6. Mitotic metaphase chromosome spreads and karyotypes. (a) BC-*Hoc*1 × *Hoc*; (b) *Hoc* × BC-*Hoc*1

Discussion

Karyotypes of three *Hexagrammos* species and artificially produced F_1-hybrids

The results obtained for the pure species in the present study corroborated those of previous studies (Makino 1937, Matsumiya *et al.*, 1980, Nishikawa and Sakamoto 1982). The karyotypes of the three *Hexagrammos* species were observed to have diversified, especially that of *Hoc*, which was comparatively larger than the other two species. For example, while *Hot* and *Hag* have 6 and 8 metacentric chromosomes that are not found in *Hoc.* The *Hoc* has 36 telocentric chromosomes while *Hot* has only 8 and *Hag* has none. *Hag* and *Hot* have also been shown to be genetically closer to each other than either is to *Hoc* (Shinohara 1994, Crow *et al.*, 2004), and flow cytometry analysis has shown that *Hoc* has 1.5-times as much genetic material as *Hag* and *Hot* (Kimura-Kawaguchi *et al.*, 2014). The more marked karyological differences between *Hoc* and the other two species observed in this study therefore correspond to these previous observations of genetic differences among these three species.

Robertsonian translocation in the natural hybrids

Natural hybrids (*Hoc**/*Hot* and *Hoc**/*Hag*) hemiclonally produce haploid eggs with a maternally derived genome, whereas artificial F_1-hybrids (*Hoc* × *Hag* and *Hoc* × *Hot*) produce recombinant gametes by meiosis (Kimura-Kawaguchi *et al.*, 2014). Although the karyotypes of both F_1-hybrids were intermediate between the two parental species, the karyotypes of both natural hybrids differed from those of each F_1-hybrid and comprised large metacentric chromosomes. BC-*Hoc*, which have *Hoc** and *Hoc* genomes, exhibited karyotypes that comprised two to three large metacentric chromosomes. The large metacentric chromosomes appear to have been transmitted from the *Hoc** genome of the natural hybrid. Since karyotypes of *Hoc* and *Hag* were $0m + 2sm + 10st + 36t$ and $8m + 26sm + 14st + 0t$, respectively, the numerical mean was $4m + 14sm + 12st + 18t$, which corresponded to the karyotype of the F_1-hybrid, *Hoc* × *Hag*. If the two united chromosomes were formerly subtelocentric, then $2st$ would be counted as $1m$. Type 1 of the hemiclonal hybrid *Hoc**/*Hag*, which has two large metacentric chromosomes, was $6(2)m + 14sm + 8st + 18t$ and was applied to this conversion. In the case of Type 2, if one of the three united chromosomes was a previously submetacentric or telocentric chromosome, then $2t$ would be counted as $1m$. Type 2 of *Hoc**/*Hag* was $7(3)m + 14sm + 8st + 16t$, which was applied to this conversion, as were the karyotypes of another hemiclonal hybrid (*Hoc**/*Hot*).

The large metacentric chromosomes were concluded to have united based on the finding that the two chromosomes fused near the centromere. This type of large metacentric chromosome is considered to correspond to “centric-fusion” and this is referred to as a Robertsonian translocation (Lajus 2007). Robertsonian translocation in fish was described as a polymorphic condition in the Reticulated dascyllus, *Dascyllus reticulates* (Ojima and Kashiwagi, 1981). In *Hexagrammos*, the

differences in the karyotypes of natural hybrids and artificial F_1-hybrids are considered to reflect the different reproductive modes. In previous studies on hybridogenesis and gynogenesis, Robertsonian translocation has not been reported based on karyological observations (Cimino 1973, Zaleśna *et al.*, 2011).

Fission of Robertsonian translocation

The *Hoc** genome is identical to the haploid karyotype of the *Hag* genome subtracted from the *Hoc*/Hag* karyotype of natural hybrids obtained through hemiclonal reproduction. Type 1 and 2 karyotypes of *Hoc*/Hag* are calculated as $2(2)m + 1sm + 1st + 18t$ and $3(3)m + 1sm + 1st + 16t$, respectively. The karyotypes of BC-*Hoc* 2, which were produced by adding hemiclonal eggs and haploid sperm from *Hoc*, can be represented as $2(2)m + 2sm + 6st + 36t$ and $3(3)m + 2sm + 6st + 34t$. The former is identical to the Type 1 karyotype of BC-*Hoc* 2, while the latter is somewhat similar to the Type 3 karyotype of BC-*Hoc* 2. Specifically, the slight difference between the latter and the Type 3 karyotype of BC-*Hoc* 2 is the presence of a large metacentric chromosome consisting of $2st$. Interestingly, while the large metacentric chromosomes were inherited by the somatic cells of BC-*Hoc*, they disappeared in BC-Hoc 1 × *Hoc* and *Hoc* × BC-*Hoc* 1 crosses. These findings imply that the large chromosomes had been fissured, probably in germ line cells.

Based on these results, the following process of fission in these large metacentric chromosomes is proposed. All of the chromosomes, including the large chromosomes, are bivalent before meiosis. When the homologous chromosomes are paired at the first meiotic division, the large metacentric chromosomes should combine with the two homologous chromosomes. The tetrad should be composed of two pairs of homologous chromosomes. After random segregation and recombination of genomes from the female and the male, the large chromosomes fissure into two different chromosomes during the second meiotic division (Fig.1-7). In this way, all of the chromosomes of the *Hoc** genome is homologous to those of the *Hoc* genome, and BC-*Hoc* produces recombinant gametes by undergoing meiosis.

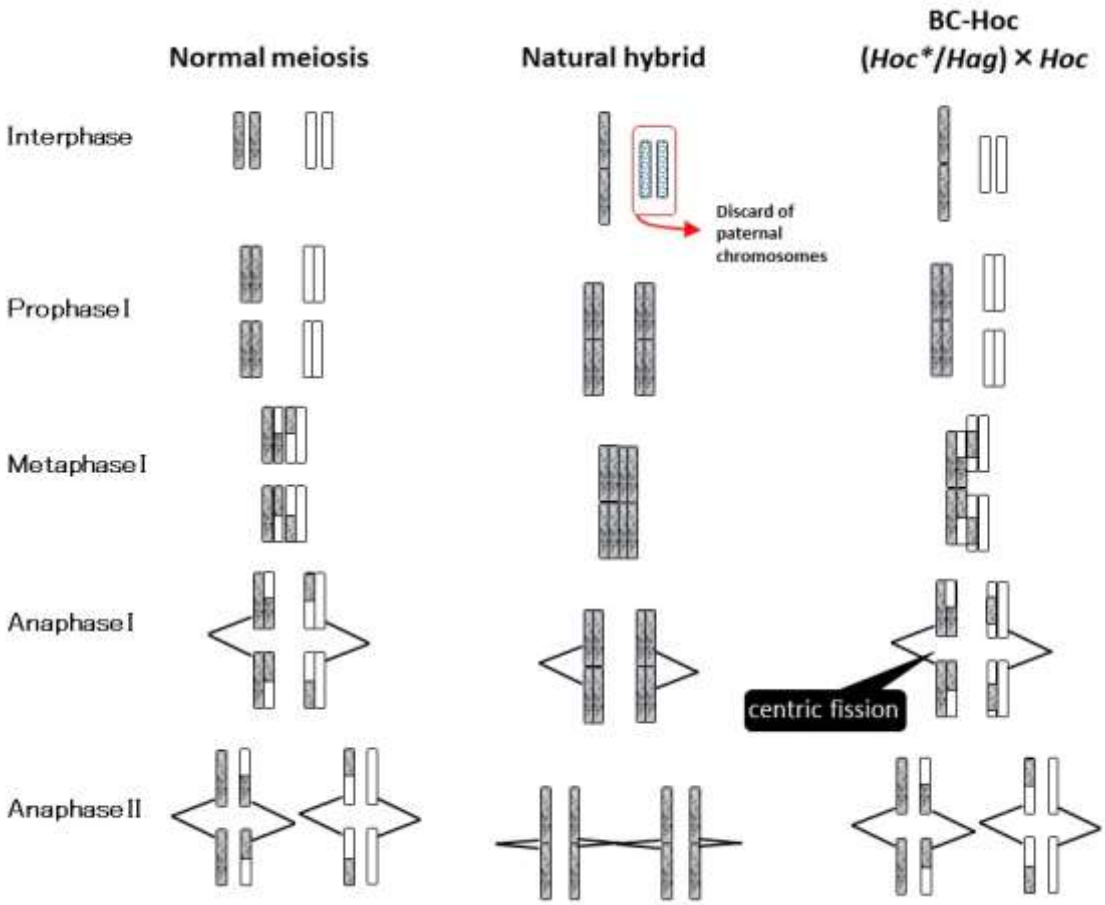

Fig 1-7. Mechanism of disappearance of metacentric chromosomes during gametogenesis in BC-*Hoc*. During gametogenesis of the natural hybrid, paternal genome is eliminated, and haploid maternal genome is transmitted to gametes after duplication. In BC-*Hoc*, fused metacentric choromosomes divide into two homologous telocentric ones, and meiosis is normally conducted.

Derivation of microchromosomes

In addition the large metacentric chromosomes, one or two microchromosomes were also observed in natural hybrids, but not in the pure species. Microchromosomes were also found in the gynogenetic fish, *P. formosa* (Schartl *et al.*, 1997, Nanda *et al.*, 2007). Since microchromosomes diversify in pigment cells, these excess chromosomes are considered to be one way of producing genetic divergence in a gynogenetic strain (Ojima 1983, Warren *et al.*, 2018). In *P. formosa*, the microchromosomes are acquired from males due to gene leakage attributed to a failure in the normal sperm-exclusion mechanism (Schartl *et al.*, 1997, Nanda *et al.*, 2007). Microchromosomes found in *Hexagrammos* hybrids likely comprise the short arms of submetacentric or subtelocentric chromosomes that were torn off when the two chromosomes formed a large metacentric chromosome. In intraspecific crosses, all homologous chromosomes typically pair during meiosis; however, in hybrids, it can be difficult for some chromosomes to pair precisely due to slight differences between homoeologous chromosomes. Unlike the gynogenetic fish, Poecilia formosa, the microchromosomes in *Hexagrammos* hybrids may be transmitted from the hybrid genome.

Chapter 2
Unisexual hybrids break through an evolutionary dead end by two-way backcrossing

Experiments using artificial crosses showed that the offspring of backcrosses between natural hybrids and *Hoc* produce recombinant gametes (Chapter 1). BC-*Hoc* comprises both a *Hoc** genome inherited from the hemiclonal hybrid, and a normal *Hoc* genome inherited from the *Hoc* male. BC-*Hoc* has the same morphological phenotype as normal *Hoc*, so BC-*Hoc* cannot be discriminated from normal *Hoc* based on the morphology. BC-*Hoc* is thus the pivot generation that transmits the hemiclonal genome from the natural hybrids to gene-pool of *Hoc* population. Consequently, the ability to detect BC-*Hoc* is therefore indispensable to clarifying the evolutionary mechanisms escaping Muller's ratchet or red queen hypothesis associated with the hemiclonal lineage.

In Chapter 1, I found that the *Hoc** genome has several large metacentric chromosomes that are not present in the contemporary *Hoc* genome, and that BC-*Hoc* has these large metacentric chromosomes which are transmitted hemiclonally from *Hoc*/Hag*. Such a marked difference between karyotypes is a useful cytogenetic marker for identifying ancestral *Hoc** and contemporary *Hoc* genomes. Males of the *Hexagrammos* species commonly establish breeding territories to guard egg masses that have been spawned by a multiple number of females (Kimura and Munehara 2011). We collected egg masses that had been spawned in breeding territories of the parental species, *Hoc* and *Hag,* and used cytogenetic markers to identify egg masses that were spawned by *Hoc*/Hag* (BC-*Hoc*).

Materials and Methods

Tissue and egg collection

All fish and egg collection activities were conducted by scuba diving off the coast of Usujiri (N 41'57; E 140'58) in southern Hokkaido, Japan, in September and October of 2015 and 2016. Males of *Hoc* and *Hag* maintain a breeding territory in which they guard the egg masses laid by the females. The territories are typically located in shallow areas, with the preference for the extent of seaweed coverage differing slightly between species (Kimura and Munehara 2011). Species identification of *Hoc* and *Hag* was based on Chapter 1. To clarify parentage and whether the mothers of egg masses in the male's territory were hemiclonal hybrids or pure species, the tip of the right pectoral fin was cut off with a pair of scissors after the territorial males were caught using hand nets. After fin clips were collected, all of the fish were released near their territories. Using a pair of scissors or tweezers, approximately 50 eggs in each egg mass (equivalent to 0.5-1% of an entire egg mass) were then collected from each egg mass. To avoid admixture of egg samples, eggs were only collected from egg masses that were not attached to any other egg masses. The fin samples were preserved in 99% ethanol and stored at -20°C until genetic analysis in the laboratory. Eggs collected from *Hoc* territories were incubated at 15°C until the eyed embryo stage when they were fixed with Carnoy fixative for karyological observation of the chromosomes. Eggs from *Hag* territories were incubated at 15°C until hatching, and then hatched larvae were preserved in 99% ethanol at -20°C until genetic analysis.

Identification of mitochondrial DNA haplotypes

Since the maternal species of the natural hybrids is *Hoc* (Kimura-Kawaguchi *et al.*, 2014; Crow *et al.*, 2007, 2010), mitochondrial DNA (mtDNA) analysis was used to detect the *Hoc* haplotypes in egg masses that were spawned in *Hag* territories.

Total genomic DNA was extracted using a Quick Gene DNA tissue kit S (Fujifilm, Japan) according to the manufacturer's instructions and stored in a refrigerator at 4°C until use. The mtDNA haplotype analysis was performed using the method of Kimura *et al.*, (2007), with multiplex amplified product length polymorphism (APLP) analysis of 12S and 16S rRNA (12-16S rRNA) gene regions. Ampli*Taq* Gold DNA polymerase (Applied Biosystems, CA) was used to assure high annealing stringency of species-specific primers to complementary diagnostic sites. The PCR products were separated by electrophoresis on 1.5% agarose gels for 30 min at 100 V and visualized under UV light following staining with SYBR Green II (Takara Bio Inc., Japan). In the 12-16S rRNA region, fragments of 446 bp and 1177 bp in length were identified as belonging to the *Hag* and *Hoc* haplotypes (*Hoc* or *Hoc**/*Hag*), respectively. A total of 77 and 96 egg masses from 14 and 16 territories were analyzed in 2015 and 2016, respectively.

Detection of hemiclonal hybrids by microsatellite DNA

Both *Hoc* and *Hoc*/Hag* have the *Hoc* mtDNA haplotype. To ascertain whether the egg masses showing the *Hoc* haplotype were spawned by *Hoc* or *Hoc*/Hag* individuals, the inheritance pattern of the maternal alleles was determined from parentage analysis using microsatellite DNA markers. Seven microsatellite loci were analyzed, three of which had been used in previous studies (Kimura-Kawaguchi *et al.*, 2014, Munehara *et al.*, 2016) while the other four were newly developed in the present study using a Roche 454 next-generation sequencing system (Roche Holding AG, Switzerland). A total of 269,197 reads from *Hoc* and 314,191 reads from *Hot* were obtained, and the unique sequence contigs containing microsatellite motifs were identified using the QDD software package (Ver. 3.1) (Meglécz *et al.*, 2010). Total of 199 *Hoc* loci and 253 *Hot* loci were selected for analysis. After considering the length of the PCR products (100-400 bp) and microsatellite motifs, 69 loci were screened for polymorphisms using template DNA derived from 12 individuals of *Hoc* and *Hot*. Finally, based on the low Hardy-Weinberg equilibrium deviation probabilities and sufficiently high heterozygosities within each species, four (*Hexoc* 2, *Hexoc* 9-2, *Otakii* 5, *Otakii* 6) out of 22 loci were selected for the analysis. The characteristics of the newly designed microsatellite loci are shown in Table 2-1 and Table 2-2.

The PCR mixes contained 5 μl of EmeraldAmp PCR Master Mix (Takara Bio Inc.), 0.2 μl of each primer, 1 μl of template DNA (50–100 ng/μl) and 3.6 μl of water to give a final volume of 10 μl. PCR reactions were conducted using a PCR thermal cycler (Takara Bio Inc.), with an initial denaturation step of 60 s at 94°C, followed by 25–30 cycles of 30 s at 94°C, 30 s at the annealing temperatures shown in Table 2-1, and 60 s at 72°C. Genotyping was performed by electrophoresis on 6–10% polyacrylamide gels stained with SYBR Green II (Takara Bio Inc.).

Eight offspring were randomly selected from each egg mass, and their genotype was compared with the territorial *Hag* male that guarded them. Alleles not found in the territorial male were supposed to allot to a female (or sneaker *Hag* males). When all of the offspring in an egg mass shared a common maternal allele, the egg mass was considered to be hemiclonal. Even if only one offspring in an egg mass inherited a maternal allele that differed from the other offspring, the egg mass was considered to contain regular recombinant eggs from wildtype individuals. When the egg masses were partially fertilized by sneaker males, the offspring likely possessed alleles not found in the territorial male (Munehara *et al.*, 2016). In such cases, the size range of each allele was compared to the allele sizes typical of the *Hag* population.

Table. 2-1 PCR primer sequences used in this study.

Locus (accession no.)	Motif	Annealing temp.	Cycle	Sequence 5'------3' (Upper, Forward; Lower, reverse)
Hexoc 2	AC	60	25	GAAGGTAGACTTGGTCCAGGTTGAT
(LC341210)				GTCCTCTTGTGTTCCATGTCCC
Hexoc 9-2	TCTG	59	27	AAACAATTTCCCCTTGCAGGA
(LC341211)				GAATTTCAAGATTGCTTTGTGATCC
Otakii 5	GAG	60	27	TCTCCTAGCGGTTGTGAGTGTAAA
(LC341208)				TGATCAGACTCATCCTCTTCCTCA
Otakii 6	TATC	60	26	TTCTGATTACAACACAAACAAGGG
(LC341209)				GCATTGATGTAGCAGCAAACAACT

Table 2-2. Summary of polymorphic microsatellite loci for the two parental species. For Ho and He, correspondence was observed between the expected and observed heterozygosities.

Species	Locus	Size range (bp)	No of allele	Ho	He	Chi-squared test
H. octogrammus (N=*30*)	*Otakii 6*	130-174	12	0.9	0.859	1
	Hexoc 2	88-106	10	0.793	0.852	0.035
	Otakii5	127-142	6	0.8	0.788	0.942
	Hexoc 9-2	116-184	18	0.931	0.927	0.663
H. agrammus (N=*40*)	*Otakii 6*	100-156	15	0.875	0.908	0.439
	Hexoc 2	78-94	6	0.525	0.482	1
	Otakii5	140-158	7	0.825	0.753	0.989
	Hexoc 9-2	122-150	8	0.79	0.812	0.995

Karyological observations of chromosomes

For egg masses from *Hoc* territories, although the genome composition of BC-*Hoc* (i.e., offspring derived from mating *Hoc*/Hag* and *Hoc*) individuals was identical to that of *Hoc* individuals, BC-*Hoc* individuals inherit hemiclonally certain large metacentric chromosomes and micro-chromosomes from the natural hybrids (Chapter 1). Consequently, the identity of the parent fish, i.e., whether they are *Hoc*/Hag* or *Hoc*, can be determined by chromosome analysis.

Chromosome preparation followed by chapter 1. The karyotypes were determined using chromosomes that were reconstructed from Giemsa-stained metaphase plates using a microscope (BX51, Olympus Co., Japan) equipped with a digital camera (VB-7010, Keyence Co., Japan). According to Levan *et al.* (1964), chromosomes were classified as *m* = metacentric, *sm* = submetacentric, *st* = subtelocentric and *t* = telocentric. The fundamental number (FN) was calculated by assigning 2 arms for *m* and *sm*, and 1 arm for *st* and *t*. A pair of homologous chromosomes was defined based on the relative length of the short and long arms of each chromosome. Karyological observations were performed on a total of 56 egg masses from 20 and 28 territories in 2015 and 2016, respectively.

Results

Maternal species identification of egg masses in *Hag* territories

In 70 (90.9%) of the 77 egg masses and 92 (95.8%) of the 96 egg masses collected from *Hag* territories during 2015 and 2016, APLP analysis showed that the mtDNA haplotypes were identical to the *Hag* type. In the remaining seven (9.1%) and four (4.2%) egg masses, the mtDNA haplotypes were identified as belonging to *Hoc* (Table 2-3).

For these 11 egg masses, the offspring in each egg mass were genotyped using seven microsatellite loci and the genotypes were compared to those of the territorial males. Of all the microsatellite DNA loci examined, the offspring of nine of these 11 egg masses shared only one maternal allele. In the remaining two egg masses, the offspring shared one allele, but some of the offspring shared no alleles with the territorial male, implying that a sneaker male fertilized some of the eggs. Of the seven microsatellite DNA loci, three of the shared alleles were out of the size range of alleles found in the *Hag* population. In two of the remaining four microsatellite DNA loci, with tetra-nucleotide motif, insertion (or deletion) of two bases was observed in *Hag*; i.e., the shared alleles were inherited from *Hoc*, but not from *Hag* (Fig. 2-1). Therefore, in both egg masses, the alleles shared among offspring in an egg mass were hemiclonally inherited from *Hoc*/Hag*. These results showed that the 11 egg masses with the *Hoc* haplotype were spawned by the natural hybrids, *Hoc*/Hag*, and that no egg masses were spawned by *Hoc* individuals in *Hag* territories (Table 2-4).

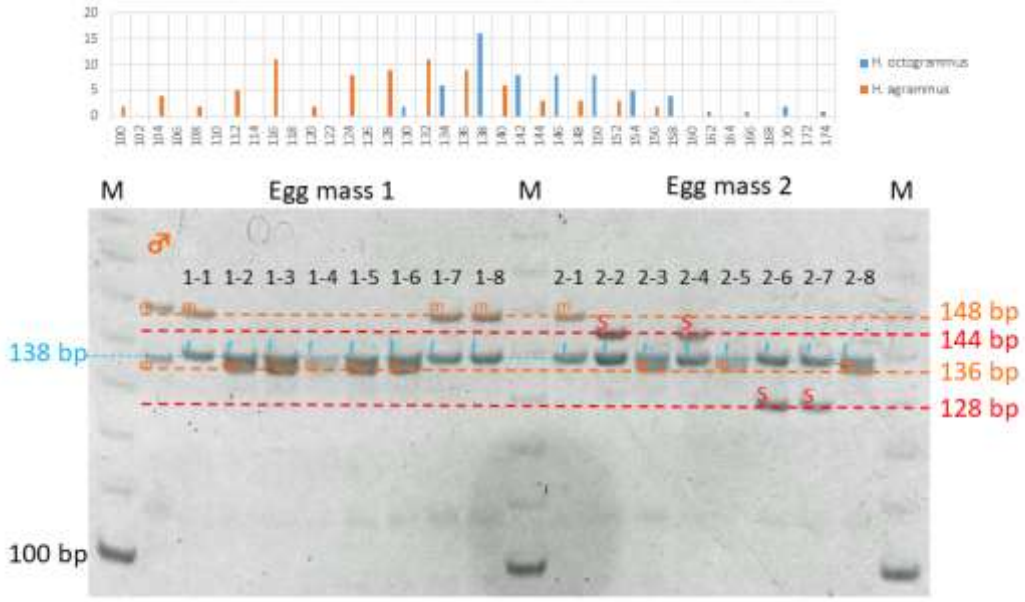

Fig. 2-1. Hemiclonal inheritance demonstrated using microsatellite DNA. Histogram showing allele frequencies of *Otakii* 6 with tetra-nucleotide motif (top), and polyacrylamide gel electrophoresis to visualize the alleles (bottom). All of the offspring from egg mass 1 inherited either a 136-bp or a 148-bp allele from the territorial male, and a 138-bp was inherited hemiclonally from the maternal species (*Hoc**/*Hag*). The 128-bp and 144-bp alleles were inherited from sneaker males of *Hag*, judging from insertion (or deletion) of 2 bases in *Hag* alleles. M: 10 bp ladder marker, m (yellow): territorial male alleles, f (sky blue): maternal alleles, s (red): sneaker male alleles.

Table 2-3 APLP analysis to detect the Hoc haplotypes in egg masses that were spawned in Hag territories. Yellow cells showed that the mtDNA haplotypes were identical to the Hoc type.

2015		Genotypes of mt DNA (12-16s rRNA)														
Breeding territory NO.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Breeding male		446	446	446	446	446	446	446	446	446	446	446	446	446	446	
Egg mass NO.	1	446	446	446	446	1177	446	446	446	1177	446	446	446	446	446	
	2	446	1177	446		446	446	446	446	446	446	446	446	446	446	
	3	446	446	446		446	446	446	446	446	446	446	446	446		
	4	446	446	1177		446	446		446	1177	446	446	446	446		
	5	1177	446			446	446		446		446	446	446	446		
	6		1177				446		446			446	446			
	7		446				446		446			446	446			
	8		446						446			446				
	9								446							
	10								446							
	11								446							
	12								446							
	13								446							
A number og egg masses		5	8	4	1	5	7	3	13	4	5	8	7	5	2	77
															Hybrid egg ratio	9.09

2016		Genotypes of mt DNA (12-16s rRNA)																	
Breeding territory NO.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Breeding male		446	446	446	446	446	446	446	446	446	446	446	446	446	446	446	446	446	
Egg mass NO.	1	446	446	446	446	446	446	446	446	446	446	446	446		446	446	446	446	
	2	446	446	446	446	446	446	446	446	446	446	446	446		446	446	446	446	
	3	446	446	446	446	446	446	446	446	446	446	446	446		446	446	446		
	4			446		446	1177	446	446	446	446	446	446		446	446	446		
	5					446	1177	446	446	446		446	446		446	446	446		
	6						446	446	446	446		446			446	446	1177		
	7							446	446	446		446				446	1177		
	8							446	446			446					446		
	9							446	446								446		
	10							446	446								446		
	11								446								446		
	12																446		
A number og egg masses		3	3	4	3	5	6	10	11	7	4	8	5	0	6	7	12	2	90
																	Hybrid egg ratio(		4.17

Table 2-4. Genotypes of offspring from egg mass deposited in *Hag* territories showing *Hoc* haplotype of mtDNA. Yellow cells and the green cells were shown the alleles inhertied hemiclonally and the alleles inherited from sneaker, respectively.

2015 Clutch NO.		Genotypes of microsatellite DNA													
	Individual NO.	Hexoc 6		Hexoc 14		Hexoc 21		Hexoc 2		Hexoc 9-2		Otakii 5		Otakii 6	
1-6	Territorial male	112	122	86	88	118	118	78	88	130	130	146	152	104	116
	Offspring 1	112	112	88	124	118	152	78	96	130	152	136	152	116	138
	Offspring 2	112	122	88	124	118	152	88	96	130	152	136	152	104	138
	Offspring 3	112	112	86	124	118	152	88	96	130	152	136	146	116	138
	Offspring 4	112	112	88	124	118	152	88	96	130	152	136	152	104	138
	Offspring 5	112	122	88	124	118	152	N/A	N/A	130	152	136	152	104	138
	Offspring 6	112	122	88	124	118	152	78	96	130	152	136	152	104	138
	Offspring 7	112	112	86	124	118	152	88	96	130	152	136	146	104	138
	Offspring 8	112	122	88	124	118	152	78	96	130	152	136	152	116	138
2-2	Territorial male	118	124	92	92	102	132	84	88	130	130	140	152	136	148
	Offspring 1	118	120	92	124	102	152	84	96	130	152	136	140	138	148
	Offspring 2	118	120	92	124	102	152	88	96	130	152	136	152	136	138
	Offspring 3	118	120	92	124	102	152	88	96	130	152	136	140	136	138
	Offspring 4	120	124	92	124	132	152	84	96	130	152	136	152	136	138
	Offspring 5	118	120	92	124	102	152	88	96	130	152	136	152	136	138
	Offspring 6	120	124	92	124	132	152	88	96	130	152	136	140	136	138
	Offspring 7	120	124	92	124	132	152	88	96	130	152	136	140	138	148
	Offspring 8	120	124	92	124	132	152	84	96	130	152	136	140	138	148
2-6	Territorial male	118	124	92	92	102	132	84	88	130	130	140	152	136	148
	Offspring 1	120	124	92	126	132	152	88	96	130	152	136	152	138	148
	Offspring 2	120	124	90	126	114	152	88	96	126	152	136	140	138	144
	Offspring 3	120	124	92	126	102	152	84	96	130	152	136	152	136	138
	Offspring 4	120	128	90	126	102	152	88	96	126	152	136	140	138	144
	Offspring 5	120	124	92	126	102	152	84	96	130	152	136	152	136	138
	Offspring 6	120	124	88	126	114	152	88	96	126	152	136	152	128	138
	Offspring 7	120	124	90	126	114	152	88	96	146	152	136	140	128	138
	Offspring 8	118	120	N/A	N/A	102	152	88	96	130	152	136	152	136	138
3-4	Territorial male	124	126	88	86	114	120	88	90	130	142	143	146	104	116
	Offspring 1	120	126	86	126	120	152	90	96	130	152	136	143	116	138
	Offspring 2	120	126	88	126	120	152	90	96	130	152	136	146	116	138
	Offspring 3	120	124	86	126	114	152	90	96	142	152	136	143	116	138
	Offspring 4	120	124	86	126	114	152	88	96	142	152	136	143	104	138
	Offspring 5	120	126	88	126	120	152	90	96	142	152	136	146	104	138
	Offspring 6	120	124	86	126	114	152	88	96	142	152	136	143	116	138
	Offspring 7	120	124	86	126	114	152	90	96	142	152	136	143	104	138
	Offspring 8	120	126	88	126	120	152	88	96	142	152	136	146	104	138
5-1	Territorial male	114	122	86	92	112	112	78	90	130	150	143	149	128	128
	Offspring 1	114	120	92	126	112	152	90	96	150	152	136	143	128	138
	Offspring 2	120	122	86	126	112	152	78	96	150	152	136	149	128	138
	Offspring 3	114	120	92	126	112	152	78	96	150	152	136	143	128	138
	Offspring 4	114	120	86	126	N/A	N/A	90	96	130	152	136	149	128	138
	Offspring 5	114	120	92	126	112	152	78	96	150	152	136	143	128	138
	Offspring 6	120	122	92	126	112	152	78	96	130	152	136	143	128	138
	Offspring 7	114	120	86	126	112	152	90	96	150	152	136	149	128	138
	Offspring 8	114	120	86	126	112	152	90	96	150	152	136	149	128	138
9-1	Territorial male	118	120	86	86	104	106	88	88	130	146	140	146	104	116
	Offspring 1	120	122	86	124	106	152	88	96	130	152	136	140	104	138
	Offspring 2	120	122	86	124	106	152	88	96	130	152	136	146	104	138
	Offspring 3	118	122	86	124	104	152	88	96	146	152	136	140	116	138
	Offspring 4	118	122	86	124	106	152	88	96	130	152	136	140	116	138
	Offspring 5	120	122	86	124	106	152	88	96	130	152	136	146	116	138
	Offspring 6	118	122	86	124	104	152	88	96	146	152	136	146	N/A	N/A
	Offspring 7	120	122	86	124	106	152	88	96	130	152	N/A	N/A	104	138
	Offspring 8	120	122	86	124	106	152	88	96	130	152	136	140	104	138
9-4	Territorial male	118	120	86	86	104	106	88	88	130	146	140	146	104	116
	Offspring 1	106	126	92	118	128	170	88	94	150	168	133	143	132	158
	Offspring 2	106	126	92	118	128	170	88	94	150	168	133	143	132	158
	Offspring 3	106	126	88	118	130	170	88	94	150	168	133	140	128	158
	Offspring 4	106	126	88	118	128	170	88	94	150	168	133	140	132	158
	Offspring 5	106	126	92	118	130	170	88	94	150	168	133	140	132	158
	Offspring 6	106	126	92	118	128	170	88	94	150	168	133	143	128	158
	Offspring 7	106	126	88	118	128	170	88	94	150	168	133	143	132	158
	Offspring 8	106	126	92	118	130	170	88	94	150	168	133	143	128	158

2016 Clutch NO.	Individual NO.	Genotypes of microsatellite DNA													
		Hexoc 6		Hexoc 14		Hexoc 21		Hexoc 2		Hexoc 9-2		Otakii 5		Otakii 6	
6-4	Territorial male	122	124	86	90	108	118	88	88	128	152	146	152	120	152
	Offspring 1	120	122	86	124	108	152	88	96	152	152	138	152	120	138
	Offspring 2	120	124	86	124	118	152	88	96	128	152	138	152	138	152
	Offspring 3	120	124	90	124	118	152	88	96	128	152	138	146	120	138
	Offspring 4	120	122	86	124	118	152	88	96	128	152	138	152	138	152
	Offspring 5	120	122	86	124	108	152	88	96	128	152	138	152	138	152
	Offspring 6	120	124	86	124	118	152	88	96	152	152	138	152	120	138
	Offspring 7	120	124	90	124	118	152	88	96	128	152	138	146	120	138
	Offspring 8	120	124	90	124	118	152	88	96	128	152	138	146	120	138
6-5	Territorial male	122	124	86	90	108	118	88	88	128	152	146	152	120	152
	Offspring 1	120	124	90	124	118	152	88	96	128	152	138	146	138	152
	Offspring 2	120	124	90	124	118	152	88	96	152	152	138	146	138	152
	Offspring 3	120	122	86	124	108	152	88	96	128	152	138	146	120	138
	Offspring 4	120	122	86	124	108	152	88	96	152	152	138	152	120	138
	Offspring 5	120	124	90	124	118	152	88	96	128	152	138	146	120	138
	Offspring 6	120	122	90	124	108	152	88	96	128	152	138	146	120	138
	Offspring 7	120	122	86	124	108	152	88	96	152	152	138	152	138	152
	Offspring 8	120	124	90	124	118	152	88	96	152	152	138	146	120	138
16-6	Territorial male	124	126	86	88	106	132	88	88	126	126	140	143	104	136
	Offspring 1	120	126	88	124	132	152	88	96	126	148	138	143	104	138
	Offspring 2	120	124	88	124	106	152	88	96	126	148	138	143	104	138
	Offspring 3	120	126	86	124	132	152	88	96	126	148	138	140	136	138
	Offspring 4	120	126	88	124	132	152	88	96	126	148	138	143	104	138
	Offspring 5	120	124	86	124	106	152	88	96	126	148	138	143	136	138
	Offspring 6	120	124	88	124	106	152	88	96	126	148	138	143	136	138
	Offspring 7	120	126	88	124	132	152	88	96	126	148	138	143	136	138
	Offspring 8	120	126	86	124	132	152	88	96	126	148	138	140	104	138
16-7	Territorial male	124	126	86	88	106	132	88	88	126	126	140	143	104	136
	Offspring 1	120	124	86	124	106	152	88	96	126	148	138	140	136	138
	Offspring 2	120	124	86	124	106	152	88	96	126	148	138	140	104	138
	Offspring 3	120	126	88	124	132	152	88	96	126	148	138	143	104	138
	Offspring 4	120	124	88	124	106	152	88	96	126	148	138	143	104	138
	Offspring 5	120	124	88	124	106	152	88	96	126	148	138	143	104	138
	Offspring 6	120	124	88	124	106	152	88	96	126	148	138	143	136	138
	Offspring 7	120	126	88	124	132	152	88	96	126	148	138	143	104	138
	Offspring 8	120	126	86	124	132	152	88	96	126	148	138	140	104	138

Identification of maternal species in *Hoc* territories using cytogenetic markers

Most of the karyotypes of the fertilized eggs in the egg masses collected from *Hoc* territories were identical to the typical *Hoc* karyotype, which comprised 2*sm* + 10*st* + 36*t*, 48 chromosomes and 50 arms. However, 1–3 atypical chromosomes were observed from three (5.4%) of the 56 egg masses in 2015 and four (7.1%) of the 56 egg masses in 2016. The karyotypes with atypical chromosomes were divided into five types based on the number of large metacentric chromosomes and micro-chromosomes (Table 2-5). Three of the karyotypes were identical to those observed in BC-*Hoc* in Chapter 1, and the remaining two karyotypes (type 4 and type 5) were observed for the first time in the present study. The type 4 karyotype was composed of 1(1)*m* + 2 *sm* + 8 *st* + 36 *t* (the numerals in parenthesis represent the number of large metacentric chromosomes), 47 chromosomes and 50 arms (Fig. 2-2A). The type 5 was composed of 2(2)*m* + 2 *sm* + 6 *st* + 36 *t,* 48 chromosomes and 50 arms, including two micro-chromosomes (Fig. 2-2B). Both types were categorized as the BC-*Hoc* karyotype, suggesting that the natural hybrids mated with *Hoc*. Thus, seven (6.3%) of the 112 egg masses were spawned by natural hybrids (*Hoc**/*Hag*), and no egg masses were spawned by *Hag* individuals in *Hoc* territories.

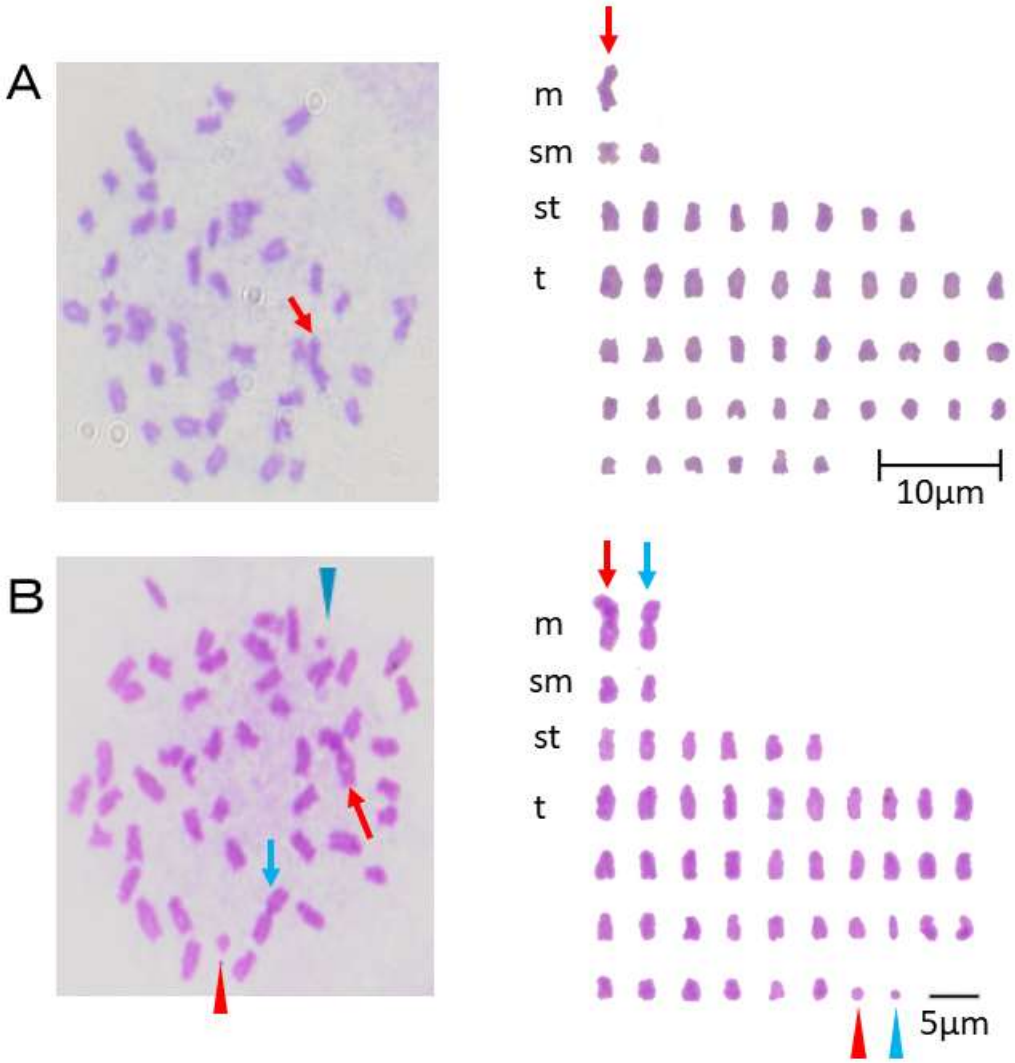

Fig. 2-2. Mitotic metaphase chromosome spreads and karyotypes of maternal backcrosses. (A) BC-*Hoc* ((*Hod*/*Hag*) × *Hoc*) type 4 showed 1(1)*m* + 2 *sm* + 8 *st* + 36 *t*, (B) BC-*Hoc* ((*Hod*/*Hag*) × *Hoc*) type 5 comprised 2(2)*m* + 2 *sm* + 6 *st* + 36 *t* and two micro-chromosomes. m: Metacentric, sm: submetacentric, st: subtelocentric, t: telocentric chromosomes, arrows: large-size metacentric chromosomes, arrowheads: micro-chromosomes.

Table 2-5. Five different karyotypes (Type 1 to 5) were observed in egg masses from *Hoc* territories.

Typical type	Observed eggmsses	Karyotype				Centric fusion	Micro chromosome	FN	2n
		m	sm	st	t				
H. octogrammus	105	0	2	10	36	0	0	50	48
BC-*Hoc*(Type 1)	2	2	2	6	36	2	0	50	46
BC-*Hoc*(Type 2)	1	3	2	6	34	3	0	50	45
BC-*Hoc*(Type 3)	1	3	2	6	34	3	2	50	47
BC-*Hoc*(Type 4)	2	1	2	8	36	1	0	50	47
BC-*Hoc*(Type 5)	1	2	2	6	36	2	2	50	48

Discussion

First observation of two-way backcrossing in hemiclonal hybrids in the natural environment

The two reported *Hexagrammos* hybrids constitute the first evidence of a hybridogenetic system in marine organisms (Kimura-Kawaguchi *et al.*, 2014). Of these hybrids, the natural hybrid (*Hoc**/*Hag*) and the ancestral species are distributed in shallow areas, but the utilization of seaweed as a spawning substrate differs slightly between *Hoc* and *Hag* (Kimura and Munehara 2011). Although reproductive isolation between *Hoc* and *Hag* has been demonstrated (Crow *et al.*, 2007, Kimura-Kawaguchi *et al.*, 2014), egg masses showing the *Hoc* haplotype have occasionally been found in *Hag* territories (Kimura *et al.*, 2007). The present study clarified that all of the egg masses showing the *Hoc* haplotype were spawned by the natural hybrids (*Hoc**/*Hag*), and not *Hoc* individuals. In addition, using cytogenetic markers, egg masses that had been spawned by the natural hybrids in *Hoc* territories could be discriminated from the egg masses of *Hoc* individuals. In hybridogenesis, the entire paternal genome is replaced in one generation. Indeed, once a natural hybrid (*Hoc**/*Hag*) crossbred with *Hot*, another type of natural hemiclonal hybrid (*Hoc**/*Hot*) arose by such "host switching" (Munehara *et al.*, 2016). Genealogical analysis of *Hoc* and two natural hybrids showed that anomalous crossbreeding between a natural hybrid (*Hoc**/*Hag*) and *Hot* had only occurred on one occation in the past (Munehara *et al.*, 2016). Conversely, natural hybrids (*Hoc**/*Hag*) have mated normally with *Hoc* at the same frequency as they have mated with *Hag*, which is a typical backcross pattern. The discovery of this mating pattern using a cytogenetic marker to discriminate between BC-*Hoc* and diploid *Hoc* individuals is surprising.

The hybrid offspring produced from crosses between the hybridogens and males of

the maternal species are diploid and bear two sets of the maternal species' genome. In the *Poeciliopsis* hybridogenetic system, none of the offspring artificially produced from such a cross are hybridogenetic; all of the offspring are fully fertile males and females that produce recombinant gametes (Vrijenhoek 1993). Similarly, in the hybridogenetic system of water frogs, offspring produced by artificial backcrossing with the maternal ancestor produced recombinant gametes (Holsbeek and Jooris 2010). The results of those experiments are identical to the results obtained in studies on the *Hexagrammos* hybridogenetic system (Kimura-Kawaguchi *et al.*, 2014, Chapter 1). However, this mating pattern had not yet been verified in the natural environment because genetic markers capable of discriminating between BC-*Hoc* and diploid *Hoc* individuals had not yet been developed. The typical mating pattern observed between the natural hybrids (*Hoc**/*Hag*) and both *Hag* and *Hoc* could be considered to be an example of destabilizing hybridization by two-way backcrossing rather than host switching. The destabilizing hybridization hypothesis was first proposed by Lynch (1984) as a mechanism for reducing extinction risk. The present study is the first to demonstrate that two-way backcrossing occurs consistently in natural environments.

Return of hemiclonal genomes to the *Hoc* gene pool through two-way backcrossing

The hybrids bear the genomes of both parents. In some clonal and hemiclonal systems, the ancestral maternal species is typically extinct and has been replaced by a unisexual hybrid that can multiply twice as fast as sexually reproducing organisms (Maynard Smith 1992, Vrijenhoek and Paker 2009). For example, in the *Hypseleotris* species complex, the maternal species competing with the hybridogens became extinct (Schmidt *et al.*, 2011). When the maternal species coexisted with the hybridogens, two-way backcrossing with the hybridogens must have occurred. *Hexagrammos* hybrids coexist with the maternal species in the hybrid zone (Kimura-Kawaguchi *et al.*, 2014), and when the natural hybrids crossbreed with males of the maternal ancestor, the offspring (BC-*Hoc* = *Hoc*/Hoc*) produce recombinant gametes (Chapter 1). Although the genomic heterogeneity (genetic affinity) between the two parental species plays an important role in facilitating hybridogenesis (Moritz *et al.*, 1989), it cannot alone induce hybridogenesis (Kimura-Kawaguchi *et al.*, 2014, Chapter 1). In addition, it was suggested that genetic factors capable of inducing hybridogenesis exist in the hemiclonal genome (Kimura-Kawaguchi *et al.*, 2014) however, these factors do not function under high genetic compatibilities between homeologous chromosomes such as *Hoc* and *Hoc** (Chapter 1). Therefore, BC-*Hoc* individuals bearing two sets of *Hoc* genomes produce recombinant gametes. Indeed, the large metacentric chromosomes found in BC-*Hoc* that are inherited from natural hybrids fissure to form two separate chromosomes during meiosis (Chapter 1). The findings of this study showed that BC-*Hoc* fulfill the role of a normal diploid *Hoc*, and that there are no karyological differences between two sets of *Hoc* genomes in offspring from a crossing between BC-*Hoc* and *Hoc*. In this way, the hemiclonal genome is returned to the 'gene pool' of the *Hoc* population.

Diversification of hemiclonal lineages

If all of the genetic factors that induce hybridogenesis are contained within a single major gene, then half of the BC-*Hoc* individuals would be carriers of hybridogenesis; such essential genetic factors would include characters such as discarding of the paternal genome. If BC-*Hoc* females are crossed with *Hag* males, breaking the established reproductive isolation, then these carriers will produce hybridogens. Some of BC-*Hoc* offspring have a *Hoc** nuclear genome that undergoes recombination once and has the same mtDNA haplotype as the grandmother (the natural hybrid: *Hoc**/*Hag*) (Fig. 2-3 A). However, once the mtDNA has been replaced over the course of several generation changes among conspecific individuals (*Hoc*), if hybridogenesis carriers hybridize with *Hag*, a new hemiclone lineage would arise (Fig. 2-3 B). This hypothesis is supported by the existence of several hybrid lineages within the polymorphic genealogical tree analyzed from 2,498 base pairs of mtDNA for *Hoc* and natural hybrids (Munehara *et al.*, 2016). Even if the genetic factors responsible for inducing hybridogenesis are encoded by multiple genes, new hybridogens will still arise, albeit more rarely. The hypothesis suggests that two-way backcrossing played an important role in the genetic diversification of hemiclone lineages. In addition, genomes that contained accumulated deleterious mutations and the genetic factors that induce hybridogenesis will be renewed through recombination at the time of generation changes.

High levels of mtDNA diversity were reported in *P. monacha-lucida* (Quattro *et al.*, 1991), which was confirmed to produce viable offspring after artificial backcrossing with males of *P. monacha* (Beukeboom and Vrijenhoek 1998). In *P. monacha-lucida* case also, while maternal species coexisted with hybridogens, two-way backcrossing by hybridogens occurred, which over time, resulted in hybridogenesis carriers hybridizing with the paternal species. The high levels of mtDNA diversity observed

in hybridogenetic systems probably indicates that a series of such events occurred occasionally.

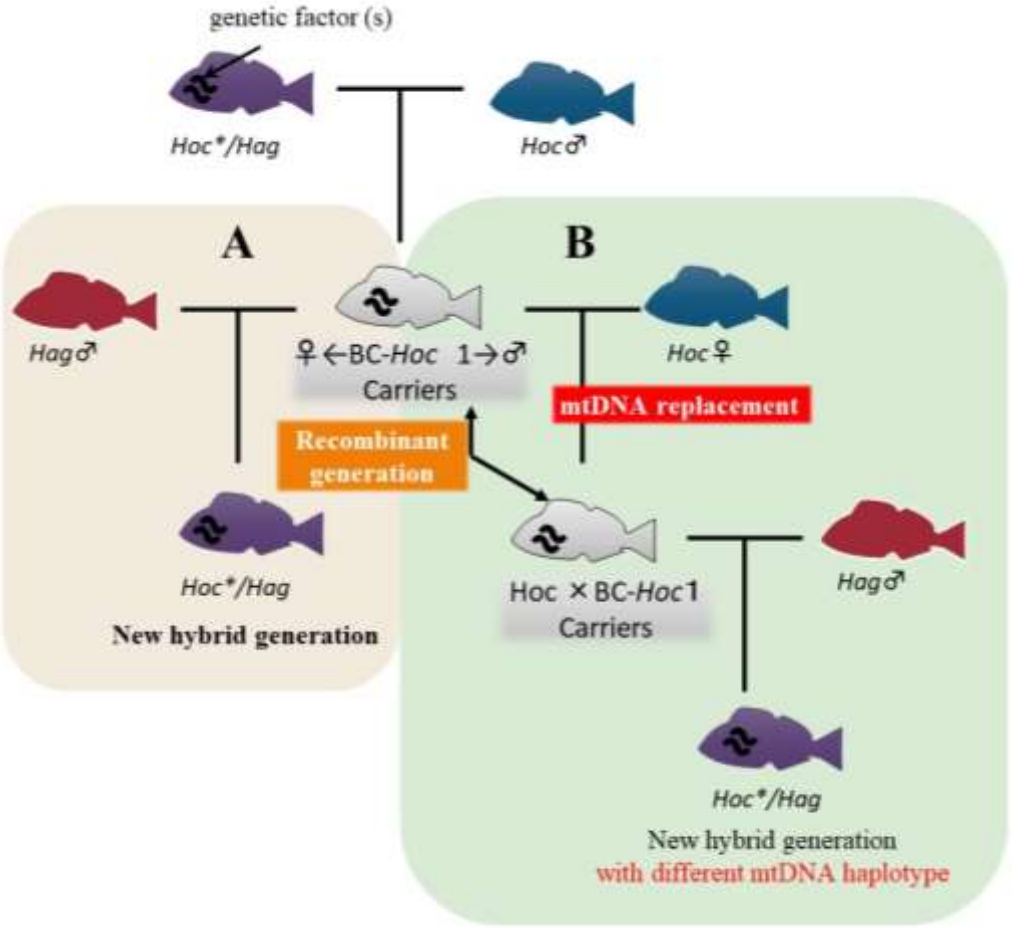

Fig. 2-3. Role of BC-*Hoc* in facilitating the establishment of new hybridogenetic lineages. When a *Hoc*/Hag* hybrid mates with a male of the maternal species, the backcrossed offspring become *Hoc* (BC-*Hoc*: carriers). If all of the genetic factors required for inducing hybridogenesis are included in a single gene, then half of the resulting offspring will be hybridogen carriers. (A) If the female carriers mate with *Hag*, then the offspring may produce a new hybridogenetic lineage. (B) If fertile male carriers mate with normal *Hoc* females, then the resulting progeny will inherit the mtDNA haplotype of the normal *Hoc.* Carriers are considered to have altered the mtDNA haplotype. When a carrier mates with a *Hag* male, a new hybridogenetic lineage also will arise.

Longevity of hemiclonal lineages

There has been extensive discussion of whether the actual lineage longevities of most unisexual organisms exceed theoretical expectations (Maynard Smith 1992, Schartl *et al.*, 1995). By modelling natural selection and deleterious mutation loads, Lynch & Gabriel (1990) proposed that clonal lineages are unlikely to survive more than 10^4 to 10^5 generations. In *Hexagrammos* species, Crow *et al.* (2010) proposed that the common ancestor of *H. otakii* and *H. agrammus* (temperate species) diverged allopatrically from *H. octogrammus* (boreal species) approximately 2.2–3.6 million years ago, and that *H. otakii* and *H. agrammus* diverged sympatrically approximately 1.2–2.0 million years ago. Secondary contact between the temperate species and boreal species then occurred after sympatric speciation (Shinohara 1994, Brykov and Podlesnykh 2001). *Hoc**/*Hot* hybrids are considered to have arisen by host switching between *Hoc**/*Hag* and *Hot* approximately 20,000–30,000 years ago (assuming a generation period of 2 years, approximately 10^4 generations have passed) (Munehara *et al.*, 2016). Together, these results suggest that the first *Hoc**/*Hag* hybrid likely arose after secondary contact, which occurred before host switching approximately 10^5-10^6 years ago. This implies that the *Hoc**/*Hag* hybrid lineage is older than theoretically expected.

Recombination of hemiclone lineages

Since hybridogens do not exchange genetic material through recombination, they accumulate deleterious mutations in their hemiclonal genomes (Lynch and Gabriel 1990, Lyunch *et al.*, 1993). Typically, hybridogenetic lineages lose their resistance to parasites and their ability to adapt to abrupt environmental changes (Red Queen hypothesis); further, deleterious mutants tend to accumulate in the hemiclone genome (Muller's ratchet). These characteristics tend to contribute towards increasing the extinction risk of hybridogenetic lineages (Muller 1964; Burt and Trivers 2006; Lynch and Gabriel 1990). There are several considerations concerning the mechanisms employed by unisexual organisms to generate genetic variability, including partial incorporation of paternal chromosomes and increasing ploidy (Lampert and Schartl 2008, Schartl *et al.*, 1995). However, compared with such incidental phenomena, recombination produces much more genetic variability through the rearrangement of homologous chromosomes and crossing over during meiosis. Since the genomes including hybridogenetic factors are markedly altered by recombination during conspecific breeding, the resulting hemiclone lineages are less at risk from extinction. In this way, many of the deleterious mutations that have accumulated over time are removed from the hemiclonal genome. If the hybridogens outcompete the maternal species, then, despite having a two-fold reproductive advantage (cost of recombination; Felsenstein, 1974), the hybrid lineages are likely to be short-lived on an evolutionary timescale. Thus, the hybridogenetic system extends the longevity of hybrid lineages by recombination during occasional generation changes.

Chapter 3
Assessment of deleterious mutation loads in the natural hybrid genome

Karyotypic variation has been reported in many fishes (Arai 2011). In the order Characiformes, the modal diploid number is 2n=54 chromosomes with few differences among species (Claudio *et al.*, 2007). In *Dascyllus reticulatus*, polymorphisms have been reported, including 4 typical karyotypes due to Robertsonian translocation (Ojima and Kashiwagi, 1981). In *Hoplias malabaricus,* sex chromosomes differ among populations (Cioffi *et al.*, 2011a,b). In the case of humans, it is known that a translocation can cause various genetic diseases (Roux *et al.*, 2005). For example, the Robertson translocation caused by imbalanced division between the 14th and the 21st chromosomes during meiosis causes stillbirth or Down syndrome (Roux *et al.*, 2005).

Polyploidy of hybrids in fish results in sterility and is widely used in aquaculture to improve characteristics such as growth (Arai, 1997). However, some hybrids can produce unusual gametes that are fertile. For Japanese loach, which is the product of at least two cryptic species, female triploids produce haploid gametes by meiotic hybridogenesis (Morishima *et al.*, 2008). In this type of gametogenesis, which was first reported by Alves *et al.* (1998), the bivalents segregate to produce haploid gametes, whereas unpaired chromosomes are excluded. During meiosis, the chromosomes of the cryptic species sharing greater homology often shows preferential pairing, while the chromosomes of the other species remain unpaired (Morishima *et al.*, 2008).

Thus, structural changes in chromosomes affect the reproduction ability of individuals, and hybrids with atypical karyotypes have potential of producing irregular gametes. Although BC-*Hoc* differs from the normal *Hoc* in karyotype, these

gametes show fertility (Chapter 1). In this chapter, the ploidy level of BC-*Hoc* somatic cells and milt will be examined using a flow cytometer, and these hybrids will be compared with pure species and F_1-hybrids.

Materials and Methods

Parental fish collection

For use in artificial fertilization, two *Hexagrammos* species (*H. octogrammus, Hoc; H. agrammus, Hag*) and natural hybrids were caught using traps and/or by fishing rod in the vicinity of the Usujiri Fisheries Station (N41° 57', E140° 58'), the Field Science Center for Northern Biosphere, Hokkaido University in southern Hokkaido, Japan from 2011 to 2013. Collection and rearing of parental fishes was as described in Chapter 1. For individual identification, all parental fish used for artificial fertilization were identified by internal electronic tags or external plastic tags.

Estimation of deleterious extent of hemiclonal genomes

First, BC-*Hoc* individuals were produced by backcrossing *Hoc*♀/*Hag* hybrids with *Hoc* males in 2011. When BC-*Hoc* matured after 2 years, sibling mating (sib-mating) individuals were produced. BC-*Hoc* offspring, which are abbreviated as F_2-BC-*Hoc* (BC-*Hoc* × BC-*Hoc*), included both females and males and possessed some homologous alleles from the hemiclonal genome. BC-*Hoc* × *Hag* offspring were also produced by a cross between a BC-*Hoc* female and a *Hag* male, which had been kept in the Usujiri fisheries station since 2010 using artificial fertilization methods described in Chapter 1. The lineages used this chapter are indicated in Fig. 3-1.

To estimate the accumulated mutation load in the hemiclonal genome, the fertility of gametes produced by F_2-BC-*Hoc* was tested by the following procedures. For F_2-BC-*Hoc* females, fertilization, development and hatching of F_2-BC-*Hoc* eggs fertilized by sperm of pure species (*Hoc, Hag* or *Hot*) were examined. For F_2-BC-*Hoc* males, eggs of natural hybrids fertilized by these sperm were examined in the same manner. In addition, the ploidy of milt was determined by flow cytometry (CyFlow; Partec, Germany) after morphological observation by optical microscope (Olympus, BX51).

Fertilization was indicated by cleavage occurring 1–2 h from artificial insemination and fertilization rates were roughly estimated by counting eggs in a randomly chosen microscopic field.

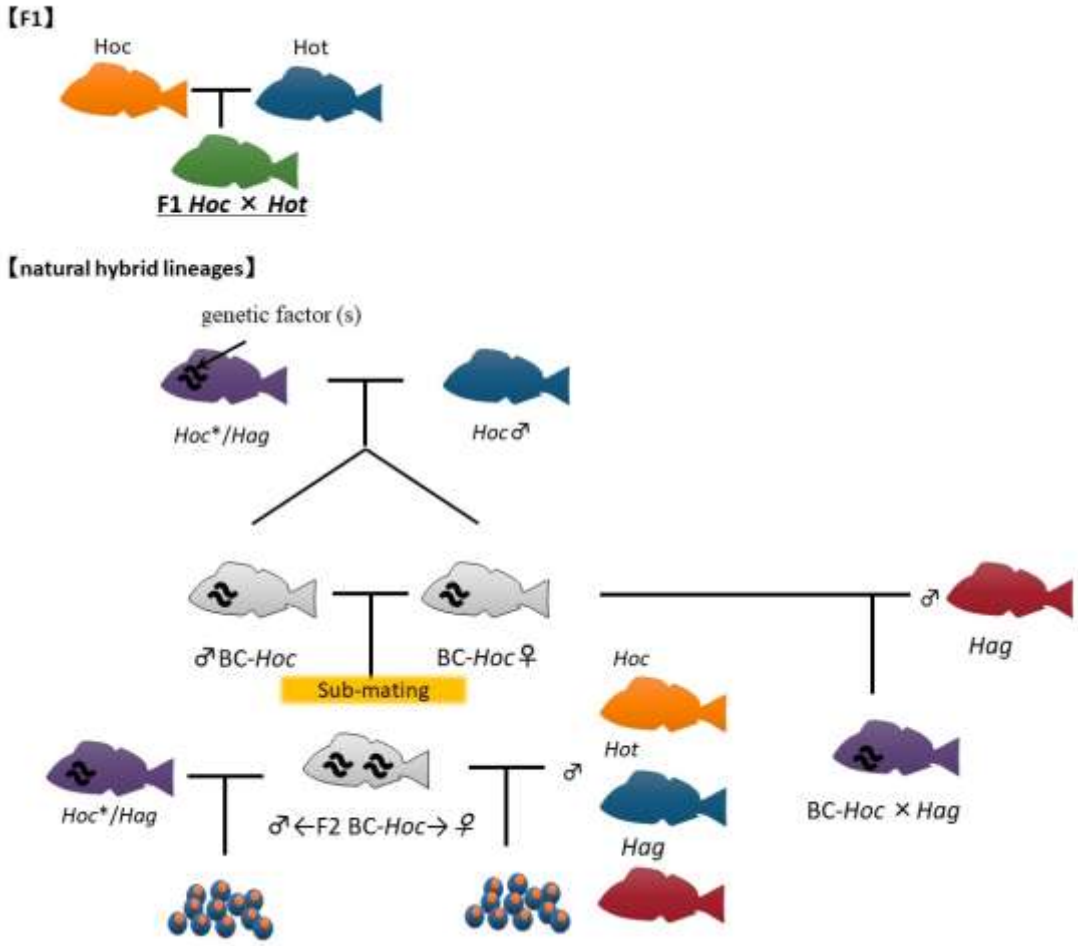

Fig. 3-1. Lineage of specimens in this chapter. Lineage and species abbreviations are defined in the text.

Ploidy determination

For not only F_2-BC-*Hoc* × *Hoc* (n=20) but also three *Hexagrammos* species (*n*=1, each species), BC-*Hoc* (*n*=2) and F1-hybrid (*Hoc*/*Hag*, *n*=2), relative DNA content of milt and somatic cells of the pectoral fin were measured by flow cytometry (CyFlow; Partec, Germany). Somatic cells of *Hot* were used as the standard control. Relative DNA content of target cells was estimated from the channel number of the peaks with the value of the standard set at 200. For setting the DNA value of normal diploid (2n) cells as 2C based on the standard, haploid (1n), triploid (3n) and tetraploid (4n) cells were designated as 1C, 3C and 4C, respectively. Preparation of samples followed Zhang and Arai (1996a).

Results

Ploidy determination in fin and milt

Spermatozoa and tissue cells of *Hot* and *Hag* males were considered to have the standard 2C DNA content (Fig. 3-2 A, C), and DNA content of *Hoc* was determined to be approximately 2.6C (Fig 3-2 E). Most milt from diploid males of *Hot* and *Hag* had 1C DNA and *Hoc* had 1.3C DNA (Fig. 3-2 B, D, F). DNA content of both *Hag* and *Hot* was about 70% relative to that of *Hoc*, and this result was the same as a previous study (Kimura-Kawaguchi *et al.*, 2014).

Somatic cell content of BC-*Hoc* and F_2-BC-*Hoc* was the same as that of *Hoc* (Fig. 3-3 A, C). In BC-*Hoc*, the major peak of milt had 1.3C DNA content (Fig. 3-3 B). F_2-BC-*Hoc* individuals also showed the same value of DNA content as *Hoc* and BC-*Hoc* (Fig 3-3 D).

Somatic cell content of F_1-hybrids and BC-*Hoc* × *Hag* takes an intermediate value between *Hoc* and *Hag* (Fig 3-4 A, C, E). For milt content, 1 of 2 F_1-hybrid males showed a major peak at approximately 1.2C DNA content, with minor peaks at about 2.3C DNA content (Fig. 3-4 B). Other F_1-hybrids showed a major peak at 2.3C DNA content and two minor peaks at approximately 1.2C and 4.6C DNA content (Fig. 3-4-D). BC-*Hoc* × *Hag* male also had a major peak at approximately 1.2C DNA content with two minor peaks at about 2.3C and 4.6C DNA content (Fig. 3-4-F). These results suggest that males of F_1-hybrids and BC-*Hoc* ×*Hag* fail to produce normal haploid spermatozoa during spermatogenesis. In particular, since 4.6C sperm appears more frequently, it seems that meiosis has stopped after the genome duplication.

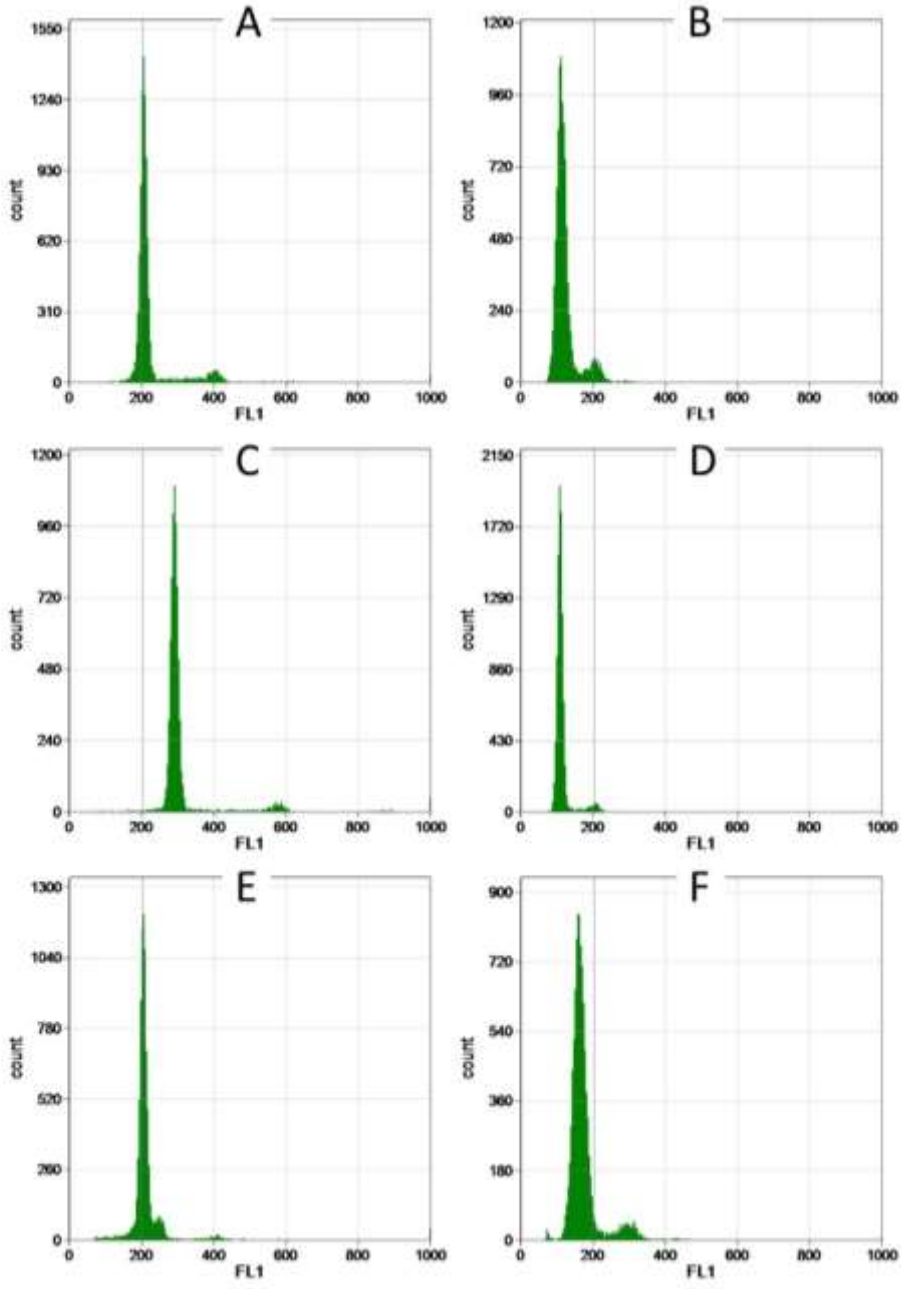

Fig. 3-2. Flow cytometry histograms of somatic cell (left) and milt (right) samples collected from *Hot* (A, B), *Hag* (C, D), and *Hoc* males (E, F). Flow cytometry of a *H. otakii* somatic cell was used as the standard (200). *Hoc* DNA count is higher than that for *Hot* and *Hag*. Y-axis denotes cell number and X-axis denotes channel number.

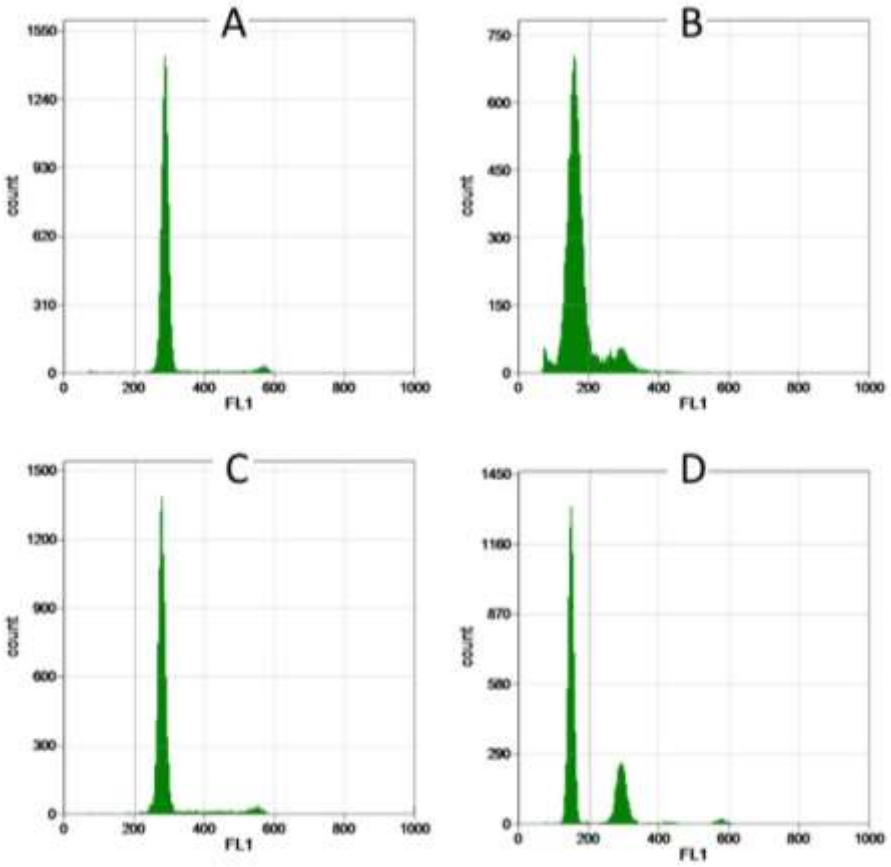

Fig. 3-3. Flow cytometry histograms of somatic cell (left) and sperm (right) samples collected from BC-*Hoc* (A, B) and F2-BC-Hoc (C, D). Flow cytometry of *H. otakii* somatic cell (Fig. 3-1A) was taken as the standard (200). There is almost no difference in the comparison with normal *Hoc.* Y-axis denotes cell number and X-axis denotes channel number.

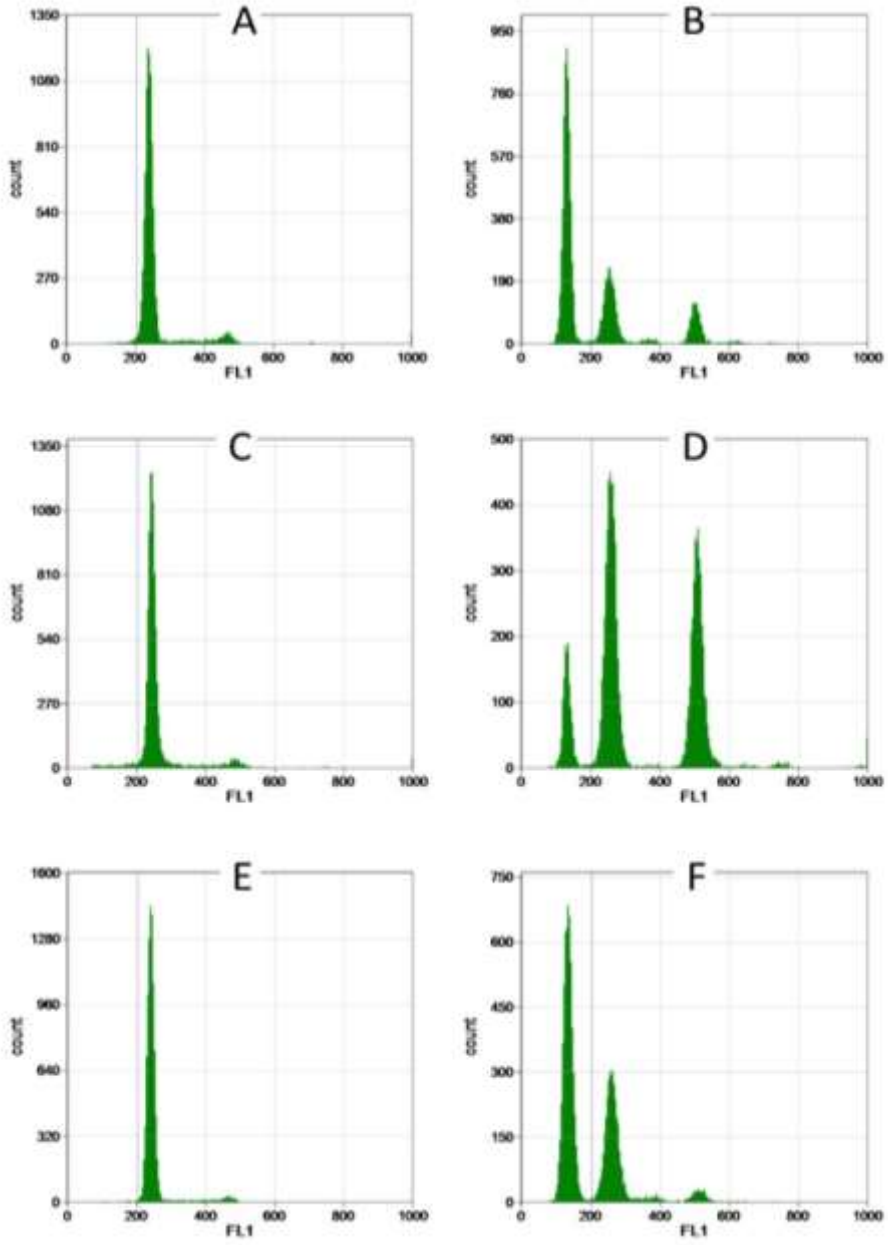

Fig 3-4. Flow cytometry histograms of somatic cell (left) and sperm (right) samples collected from F1-1 (A, B), F1-2 (C, D) and BC-*Hoc* ×*Hag* (E, F). Flow cytometry of *H. otakii* somatic cell (Fig. 3-1A) was taken as the standard (200). DNA counts of somatic cells for all individuals were midway between those of *Hoc* and *Hag*. However, these hybrids showed triple peaks at 1.6C, 2.3C and 4.6C DNA content. Y-axis denotes cell number and X-axis denotes channel number.

State of fertilized eggs and embryos

Seven out of 12 egg clutches from females of F_2-BC-*Hoc* showed fertilization of 90% or more, and the remaining clutches were also 80% or higher. Although these results were almost identical as the experimental results of Crow *et al.* (2010), most embryos had explicit abnormalities, such as vertebrae distortion, and in 5 clutches, none survived to hatching (Fig. 3-5 A B C). In 7 of the clutches only a few larvae hatched from each (Fig. 3-5 D) (Table 3-1).

In 5 out of 12 clutches fertilized by sperm from F_2-BC-*Hoc* males, fertilization rates were 90% or more, and the remaining ones showed fertilization rates under 90%, with the lowest fertilization rate of 56% (Table 3-1). However, some embryos in 3 clutches showed development over several days, and died before hatching.

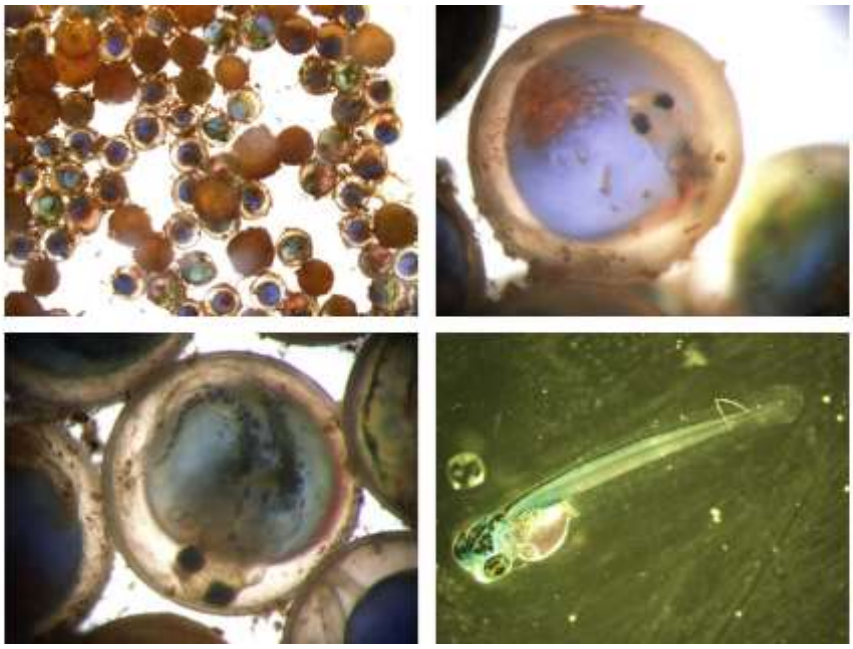

Fig. 3-5. Abnormal development in F_2-BC-*Hoc* × *Hoc.* A: egg masses showed abnormalities during the embryonic stage, B: arrested development and failure to develop posteriorly, C: distortion in the vertebrae, D: one of few hatched larvae.

Table 3-1 Fertilization success, development to embryonic stage and hatching rate in F2-BC-Hoc female × male (upper) and natural hybrids ×F2-BC-Hoc male (Lower).

Mother (F2 BC-*Hoc*)	Father	Fertilization success (%)	Embryonic stage (%)	Hatch
231731	*Hag* 158502	97.6	46.4	3 larvae
231731	*Hot*	94.0	51.2	3 larvae
C-569	*Hoc* 878103	80.0	14.8	0
C-569	158502	90.1	54.5	20 larvae
C-568	158502	85.5	26.2	some larvae
231764	158502	87.8	65.0	18 larvae
231764	878103	92.0	58.2	29 larvae
231727	158502	92.5	16.7	0
231727	878103	90.0	16.3	0
373646	158502	92.1	70.0	0
379103	158502	94.0	0.0	0
379103	158502	89.3	0.0	0

Mother	Father (F2 BC-*Hoc*)	Fertilization success (%)	Embryonic stage (%)	Hatch
Hoc/*Hot* 138345	948371	72.7	0	0
138345	948340	61.0	0	0
138345	948352	56.3	0	0
138345	948408	68.1	0	0
Hoc/*Hag* 948422	948397	87.0	43.5	0
948422	948421	94.5	60	0
948422	948432	91.1	45.9	0
948345	948371	no data	51.4	no deta
948399	948352	68.2	0	0
881483	948423	93.4	0	0
948401	948378	98.4	0	0
948433	948378	93.2	0	0

Discussion

Genetic characteristics of BC-*Hoc*

From the findings of Chapter 1, there are no differences in chromosome number among the three species—*Hag, Hot* and *Hoc* (2n = 48), but BC-*Hoc* has some karyotypes showing Robertsonian translocation as well as microchromosomes (2n = 45-48). Unbalanced chromosomal division often causes abnormal gametogenesis (Roux *et al.*, 2005). In males of triploid Chinese loach (diploid female × tetraploid male), most individuals have spermatozoa that shows multiple peaks on DNA flow cytometry (Li *et al.*, 2011, 2016). In the fertilization experiments using gametes of triploids and diploids, offspring were abnormal (Zhang and Arai, 1999; Li *et al.*, 2016). Although BC-*Hoc* has some different karyotypes, DNA content appears as a normal diploid, identical to normal *Hoc*. Furthermore, BC-*Hoc* individuals generated normal gametes through recombination with fissions of the Robertsonian translocation (Chapter 1). Thus, BC-*Hoc* can show the same behavior in the *Hoc* population as normal *Hoc*.

Abnormal spermatogenesis of F1 and BC-*Hoc* × *Hag*

Most species do not hybridize due to the effectiveness of isolating mechanisms, which may be classified as prezygotic or postzygotic (Michalowski, 1964). Even if hybridization occurs, the hybrids in most cases are sterile or have low fertility caused by disturbances during meiosis (Ogielska, 2009). In hemiclonal water frog (*Ph. esculentus*), the development and differentiation of testes are delayed (Schmidt, 1993; Ogielska and Bartmańska, 1999; Pruvost *et al.*, 2013). Even if offspring of hybrids mature, spermatozoa are scant or tubules are abnormal (Ogielska, 2009). In *Hexagrammos* hybrids, fertility of F_1-hybrids is suggested to be incomplete and spermatozoa are abnormal (unpublished). DNA content in somatic cells of F_1-hybrids between *Hoc* females and *Hag* males showed a value intermediate of the two parental species, and this result was same as BC - *Hoc* × *Hag*. However, both F_1-hybrids and BC-*Hoc* × *Hag* produce normal haploid spermatozoa with minor peaks also appearing at approximately 2.3C (diploidy) and 4.6C (tetraploidy) DNA content. In the interspecific hybrid male loach, abnormal spermatozoa is suggested to be produced by meiotic arrest after replication of chromosomes, which elevates the DNA content from 2C (diploidy) to 4C (tetraploidy) (Li *et al.*, 2016). Since tetraploid peaks are also observed in F_1-hybrids, cytokinesis is considered to occur after genome duplication is stopped. Therefore, in F_1-hybrids lacking genetic factors that induce hybridogenesis, gametogenesis was assumed to be suspended due to low compatibility of homologous chromosomes.

Detection of accumulated deleterious mutation loads in the hemiclonal genome

The absence of meiosis causes the accumulation of deleterious mutations with every generation (Muller's ratchet; Muller, 1964; Kondrashov, 1988; Otto and Lenormand, 2002; Burt and Trivers, 2006). In *Poeciliopsis* hemiclonal hybrids, experimental observations suggested that hemiclonal genomes might be degenerated by mutation loads (Vrijenhoek, 1984). Backcrosses of *P. monacha-lucida*×*monacha* males with *P. monacha-lucida* females produced very few offspring (Vrijenhoeck, 1984). In hemiclonal water frog, most offspring produced by inbreeding of *Ph. esculentus* within a population also produced nonviable embryos and few viable juveniles (*Ph. ridibundus*) (Vorburger, 2001a, b; Guex *et al.*, 2002; Christiansen *et al.*, 2005, 2010; Pruvost *et al.*, 2013). However, crossings of *Ph. esculentus* individuals between different populations produced normal tadpoles, most of which survived and successfully completed metamorphosis (Vorburger, 2001a). These results suggest inviability is caused by homozygosity for recessive deleterious mutations at particular gene loci or caused by mutation loads accumulated in non-recombining genomes through Muller's ratchet (Guex *et al.*, 2002).

In this study, sib-mating of BC-*Hoc* should produce homozygous alleles at many loci in the offspring (F_2-BC-*Hoc*). Although F_2-BC-*Hoc* individuals showed the same DNA content value as *Hoc* and BC-*Hoc*, eggs fertilized with sperm of F_2-BC-*Hoc* died before hatching. In F_2-BC-*Hoc* females, all produced eggs that showed abnormalities in the embryonic stage. These results suggest that individuals that can not adequately eliminate deleterious mutations have reduced fitness. Lynch and Gabriel (1990) showed that clonal lineages are unlikely to survive more than 10000 to 100000 generations, which is consistent with existing data on parthenogenetic animals. Assuming that the molecular clock of mtDNA in genus *Hexagrammos* is 1.5–2.5%

per million years (Crow *et al.*, 2004; Meyer *et al.*, 1990), host switching likely first occurred 17,000–27,000 years ago, and assuming a generation period of 2 years, about 10000 generations have passed (Munehara *et al.*, 2016). Therefore, fitness of offspring may have decreased due to accumulation of deleterious mutations over this timeframe. On the other hand, in *P. monacha-lucida*, even if the homozygous condition is deleterious, mutations in clonal genomes may be masked by heterozygous condition produced by contribution of the mate (Leslie and Vrijenhoek, 1978). In *Hexagrammos* hybrids, deleterious mutations were accumulating in a hemiclonal genome, and gene transfer from other populations or other recombination is important to increase fitness of offspring and to avoid homozygosity..

Chapter 4
Renewal of hemiclone genome

As discussed in Chapter 2, longevity of hybrid lineages in the hybridogenetic system could be extended by recombination during occasional generation changes. BC-*Hoc* is the pivot generation that transmits the hemiclonal genome from natural hybrids to the *Hoc* gene pool. Through conspecific mating within the *Hoc* gene pool, the *Hoc** genome set, including the genetic factors required for inducing hybridogenesis, would be recombined. After several generations, the cross of a *Hoc* female hybridogen carrier with a *Hag* male would break the established reproductive isolation, and the carrier would produce a hybridogen. This hypothesis is supported by the existence of several hybrid lineages within the polymorphic genealogical tree produced from the analysis of 2,498 base pairs of mtDNA for *Hoc* and natural hybrids (Munehara *et al.*, 2016). From the findings of Chapter 1 and 3, males of BC-*Hoc* are suggested to produce normal sperm, and their offspring are viable. If a fertile male BC-*Hoc* mates with a normal *Hoc* female, then the resulting progeny will inherit the mtDNA haplotype of normal *Hoc*.

In this chapter, renewal of the hemiclone genome was achieved by artificial crossbreeding through several generations with initiation from a natural hemiclonal hybrid.

Materials and Methods

Artificial fertilization

For artificial fertilization, two *Hexagrammos* species (*Hoc* and *Hag*) and their natural hybrid (*Hoc*/Hag*) were caught by trap and/or fishing rod in the vicinity of the Usujiri Fisheries Station (N41° 57', E140° 58') of the Field Science Center for Northern Biosphere, Hokkaido University in southern Hokkaido, Japan from 2010 to 2016. Parental species were identified following Shinohara (1994) and Nakabo (2013), as described in Chapter 1. Artificial fertilization methods were conducted according to Crow *et al.* (2010) and Chapter 1.

Gametes from *Hoc*/Hag* hybrids were extracted from ovulated adults and fertilized with *Hoc* sperm. Offspring from maternal backcrosses between the natural hybrids (*Hoc*/Hag*) and *Hoc* are referred to as BC-*Hoc*. Eggs of *Hoc* females were artificially inseminated with sperm of BC-*Hoc*. Then, when *Hoc* × BC-*Hoc* matured, their eggs were inseminated with the sperm of *Hag* males. Finally, (*Hoc* × BC-*Hoc*) × *Hag* were crossed with *Hag* or *Hoc* males, and 22 of (*Hoc* × BC-*Hoc*) × *Hag* females matured, and 45 egg masses were obtained. The sperm used for artificial fertilization was obtained from 3 *Hag* or 4 *Hoc* males. To clarify parentage and whether the mothers used in these experiments were hemiclonal hybrids or not, the tip of the right pectoral fin was cut off with a pair of scissors after fertilization. The fin samples were preserved in 99% ethanol at -20°C until genetic analysis was performed in the laboratory. Fertilization of eggs was judged by reaching first cleavage at 1–2 h after insemination, and fertilization success of each clutch was estimated by the ratio of fertilized eggs among 100 randomly chosen eggs. The clutches were incubated for 5–7 days at 11–15°C until the embryonic stage. The percentage of eggs in the embryonic stage was evaluated by the same methods as the fertilization rate. At 20–23 days after fertilization, hatched larvae were preserved in 99% ethanol and stored at -20°C

until genetic analysis in the laboratory. Hatching success of each clutch was judged as being “Normal” (most larva of clutch hatched) or “Bad” (only a few larvae hatched from the clutch hatched). The lineages of specimens used in this chapter are shown in Fig. 4-1.

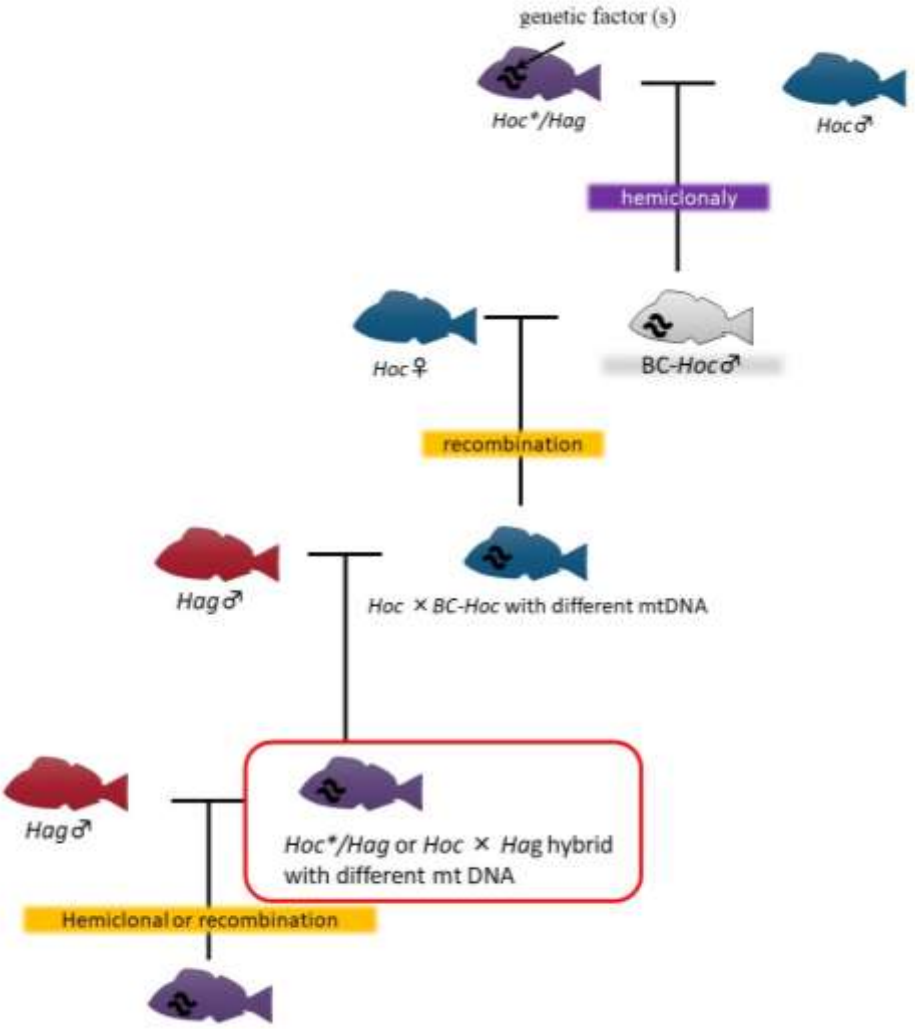

Fig 4-1.Lineage of specimens in this chapter. The renewal lineage enclosed in the red square is used to ascertain hemiclonal reproduction in experiments in this chapter. In this lineage, the male of the maternal backcrossing (BC - *Hoc*) is used to replace the mtDNA of the original hemiclonal hybrid.

Detection of hemiclonal hybrids

To ascertain whether (*Hoc* × BC-*Hoc*) × *Hag*) offspring are produced hemiclonally or by recombination, inheritance patterns of maternal alleles were determined through parentage analysis using microsatellite DNA markers. To determine the reproductive mode of (*Hoc* × BC-*Hoc*) × *Hag* hybrids through backcrossing, 7 clutches from 5 (*Hoc* × BC-*Hoc*) × *Hag* individuals were genotyped using microsatellite loci. Seven offspring from each clutch produced by (*Hoc* × BC-*Hoc*) × *Hag*) hybrids and paternal individuals were genotyped at 7 loci at which the hybrid exhibited heterogeneity. When both maternal alleles were identified in a clutch, the hybrid was regarded as being capable of producing recombinant gametes. When all offspring in a clutch shared one or the other of the maternal alleles, the clutch was considered to be hemiclonal. In order to confirm that the lineage reproduces hemiclonally, microsatellite analysis was additionally conducted at three loci.

Total genomic DNA was extracted using a Quick Gene DNA tissue kit S (Fujifilm, Japan) according to manufacturer's instructions and stored in a refrigerator at 4°C until use. Seven loci of the microsatellite DNA were amplified using the primer sets of Kimura-Kawaguchi *et al.* (2014), Munehara *et al.* (2016) and Chapter 2: *Hexoc* 6, *Hexoc* 14, *Hexoc* 21, *Hexoc* 2, *Hexoc* 9-2, *Otakii* 5, and *Otakii* 6. Polymerase chain reaction (PCR) mixes contained 5 μl of EmeraldAmp PCR Master Mix (Takara Bio Inc.), 0.2 μl of each primer, 1 μl of template DNA (50–100 ng/μl) and water (3.6 μl) to reach the reaction volume of 10 μl. Reactions were incubated in a PCR Thermal Cycler (Takara Bio Inc.) with an initial denaturation step of 60 s at 94°C, followed by 25–30 cycles of 30 s at 94°C, 30 s at the annealing temperature (Chapter 2, Table 2-1) and 60 s at 72°C. The genotypes were verified by separating the PCR products on 6%–10% polyacrylamide gels stained with SYBR Green II (Takara Bio Inc.).

Chromosome observation

Karyological observations were performed on a total of 8 clutches from 5 individuals of (*Hoc* × BC-*Hoc*) × *Hag*. Chromosomes were prepared as described in Chapter 1 and observed under a microscope (BX51, Olympus Co.). Karyotypes were determined from chromosomes reconstructed from Giemsa-stained metaphase plates using a microscope equipped with a digital camera (VB-7010, Keyence Co., Japan). Following Levan *et al.* (1964), chromosomes were classified as *m* = metacentric; *sm* = submetacentric; *st* = subtelocentric and *t* = telocentric. The fundamental number (NF) was calculated by assigning 2 arms for *m* and *sm* and 1 arm for *st* and *t*. A pair of homologous chromosomes was determined by the relative length of the short and long arms of each chromosome.

Results

Fertilization rates

Among 45 egg masses, fertilization rate ranged from 4% to 98%. In 29 of 45 (64%) clutches, all eggs died before reaching the embryonic stage (Fig. 4-2 A). In the remaining 16 clutches (16%), some eggs developed to the stage of forming embryos (Table 4-1). In 7 of these 16 clutches, development of all eggs was arrested at the stage of extension of the posterior trunk before hatching (Fig. 4-2 B C). In the other 9 (20%) clutches, viable larvae hatched, but two or more larvae showed critical deformities, with the major deformity being curvature of the spine (Fig. 4-2 D).

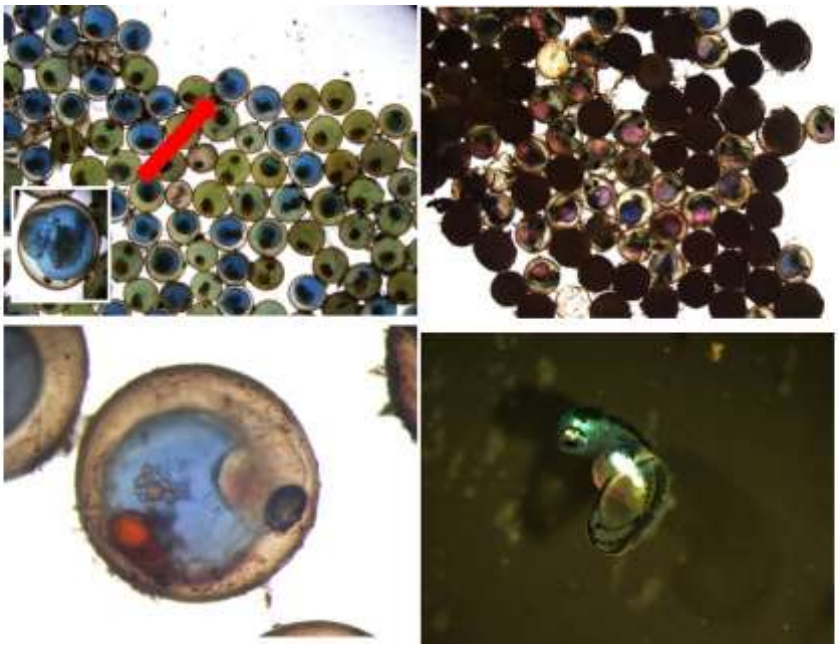

Fig. 4-2. Abnormal development of larvae in this experiment. (A), Dead eggs before first cleavage, (B) and (C), eggs arrested development at the stage of extension of the posterior trunk before hatching, (D), distortion in the vertebrae.

Table 4-1 Fertilization success, development of embryonic stage and hatching rate by artificial fertilization experiments between (*Hoc* ♀×BC-*Hoc* ♂) ×*Hag* and males. In hatching rate, ○ indicates that hatching was sufficient, ▲ indicates few hatched individuals or that most individuals were abnormal. Yellow highlight indicates lineage in which the reproduction pattern could be confirmed.

Mothers	Fathers	Fertilization success (%)	embryonic stage (%)	Hatch
(*Hoc* ♀×BC-*Hoc* ♂) *Hag* 395761	*Hag* 399097	71.63120567		dead
386200	*Hoc* 401014	59.71731449		dead
385772	*Hoc* 401014	76.20967742		dead
386462	*Hoc* 401014	69.44444444		dead
397365	*Hag* 396495	52.81690141		dead
386368	*Hoc* 398691	94.20289855		dead
387228	*Hoc* 398691	72.04724409		dead
369678	*Hag* 396495	70.12448133		dead
386450	*Hoc* 398691	90.10238908		dead
395172	*Hag* 396495	91.5407855		dead
385759	*Hoc* 398691	98.3935743	73.790323	○
386200	*Hag* 397250	82.96529968		dead
386462	*Hag* 397250	4.761904762		dead
385857	*Hag* 397250	60.17699115		dead
386269	*Hag* 397250	93.40277778	5.6994819	dead
398850	*Hoc* 398691	73.66412214		dead
385812	*Hoc* 398691	83.92282958		dead
397340	*Hoc* 398691	81.31147541	1.8450185	dead
395761	*Hoc* 398691	97.61904762	54.761905	▲
397365	*Hoc* 398691	55.44871795		dead
387228	*Hoc* 398691	75.29880478		dead
385759	*Hag* 397250	94.77351916	5.7613169	dead
398850	*Hoc* 398691	93.06122449	18.061674	▲
825551	*Hoc* 398691	90.66666667	3.4602076	dead
386901	*Hag* 397250	96.94656489	80.694981	○
385812	*Hag* 397250	87.61904762		dead
386368	*Hag* 397250	84.58781362		dead
397365	*Hoc* 398691	89.80392157		dead
387228	*Hag* 397250	92.5093633		dead
825536	*Hag* 397250	95.56313993	7.1917808	dead
385857	*Hoc* 398691	74.84662577		dead
396678	*Hoc* 398691	84.98023715		dead
386269	*Hag* 397250	97.25490196	61.780105	○
825594	*Hag* 397250	68.75	57.249071	○
385772	*Hag* 397250	71.32616487		dead
825571	*Hoc* 825600	83.92156863		dead
825536	*Hoc* 825592	90.2173913	17.721519	dead
386269	*Hoc* 825592	88.32116788	26.923077	○
386901	*Hag* 396495	95.63636364	58.245614	○
398850	*Hoc* 825592	82	7.860262	▲
396194	*Hag* 396495	85.61151079		dead
395761	*Hag* 396495	68.29268293		dead
387228	*Hoc* 825592	87.09677419		dead
386269	*Hoc* 825592	92.67399267	49.003984	dead
825594	*Hoc* 825592	90.38461538		dead

Reproduction mode of (*Hoc* × BC-*Hoc*) × *Hag*

Six clutches from 5 (*Hoc* × BC-*Hoc*) × *Hag* individuals were genotyped for 7 microsatellite loci. For two individuals of (*Hoc* × BC-*Hoc*) × *Hag* hybrids, all of the offspring shared one of the two maternal alleles in all the 7 microsatellite loci (Table 4-2), suggesting that haploid gametes were produced hemiclonally. In the remaining three individuals, both maternal alleles for all microsatellite loci tested were found in each clutch, suggesting a recombinant mode of producing gametes (Table 4-2). These results demonstrate that hemiclonal reproduction was caused by some genetic bases, and the lineage was regenerated via carrier individuals (even if the carriers were males).

Table 4-2 Genotypes of offspring from ((*Hoc*×BC-*Hoc*) *Hag*) by artificial fertilization experiments. Yellow highlight indicates alleles showing hemiclonal maternal inheritance.

		Genotypes of microsatellite DNA													
Clutch NO.	Individual NO.	*Hoc6*		*Hoc14*		*Hoc21*		*Otakii5*		*Otakii6*		*Hexoc2*		*Hexoc9-2*	
♀2 (386269)	Female	124	104	128	92	168	128	152	133	182	134	104	86	144	120
	Offspring 1	110	104	128	88	168	122	152	133	182	120	104	88	144	130
	Offspring 2	110	104	128	88	168	122	152	133	182	116	104	88	144	130
	Offspring 3	124	104	128	88	168	116	152	133	182	116	104	88	144	134
	Offspring 4	110	104	128	88	168	122	152	133	182	116	104	86	144	130
	Offspring 5	124	104	128	86	168	116	152	133	182	120	104	88	144	134
	Offspring 6	110	104	128	88	168	122	152	133	182	116	104	88	144	134
	Offspring 7	124	104	128	88	168	116	152	133	182	116	104	88	144	134
	Male-*Hag* (397250)	124	110	88	86	122	116	152	152	120	116	88	86	134	130
♀8=♀2 (386269)	Female	124	104	128	92	168	128	152	133	182	134	102	86	144	120
	Offspring 1	160	104	128	124	168	150	136	133	182	158	102	94	144	130
	Offspring 2	160	104	128	124	168	150	136	133	182	158	102	96	180	144
	Offspring 3	160	104	128	124	168	150	136	133	182	158	102	94	180	144
	Offspring 4	160	104	128	128	168	150	136	133	182	158	102	94	180	144
	Offspring 5	106	104	128	124	168	168	133	130	182	158	102	96	144	130
	Offspring 6	160	104	128	128	168	150	133	130	182	158	102	94	144	130
	Offspring 7	106	104	128	128	168	150	133	130	182	158	102	94	144	130
	Male-*Hoc* (825592)	160	106	128	124	168	150	136	130	158	158	96	94	180	130
♀9 (398850)	Female	138	118	132	88	148	100	155	130	146	116	98	88	128	120
	Offspring 1	138	106	132	124	168	148	130	130	158	146	98	96	180	120
	Offspring 2	160	138	132	124	148	148	130	130	158	146	98	96	130	120
	Offspring 3	160	138	132	124	148	148	130	130	158	146	98	96	130	120
	Offspring 4	138	106	132	128	168	148	136	130	158	146	98	94	180	120
	Offspring 5	138	106	132	124	168	148	130	130	158	146	98	96	180	120
	Offspring 6	138	106	132	128	168	148	136	130	158	146	98	94	130	120
	Offspring 7	160	138	132	128	148	148	136	130	158	146	98	96	180	120
	Male-*Hoc* (825592)	160	106	128	124	168	148	136	130	158	158	96	94	180	130
♀1 (825594)	Female	126	104			166	126							148	130
	Offspring 1	110	104			166	122							134	130
	Offspring 2	110	104			166	122							148	134
	Offspring 3	110	104			166	122							148	134
	Offspring 4	126	110			126	122							130	130
	Offspring 5	126	124			126	116							148	134
	Offspring 6	126	124			166	116							148	134
	Offspring 7	110	104			126	122							134	130
	Male-*Hag* (397250)	124	110			122	116							134	130
♀3 (386901)	Female		126			166	126							182	150
	Offspring 1	126				166	148							150	130
	Offspring 2	126				148	126							150	130
	Offspring 3	126				148	126							182	130
	Offspring 4	126	110			140	126							182	130
	Offspring 5	122				166	148							150	130
	Offspring 6	122	110			166	140							182	130
	Offspring 7	122				166	148							150	130
	Male-*Hoc* (398691)					148	140							130	130
♀6 (385759)	Female	118	104			164	100							146	124
	Offspring 1	110	104			164	122							146	134
	Offspring 2	110	104			164	122							134	124
	Offspring 3	118	110			122	100							146	130
	Offspring 4	110	104			164	122							146	134
	Offspring 5	110	104			164	122							134	124
	Offspring 6	124	118			116	100							130	124
	Offspring 7	118	110			122	100							146	134
	Male-*Hag* (397250)	124	110			122	116							134	130

Karyotype of ((*Hoc*×BC-*Hoc*)×*Hag*)×*Hag*

Among 55 metaphases from 8 clutches of (*Hoc*×BC-*Hoc*)×*Hag*)×*Hag*, the modal chromosome number of *Hexagrammos* species was 2n=48. In ((*Hoc*×BC-*Hoc*)×*Hag*)×*Hag* hemiclonal lineages, the karyotype was 4m + 14sm + 11st + 19t and the NF was 66 arms (Fig 4-3). From the findings of Chapter 1, the karyotype of *Hag* was 8*m* + 26*sm* + 14*st* (NF = 82 arms), and the karyotype of *Hoc* × BC-*Hoc* was 2*sm* + 8*st* + 38*t* (NF = 50). These results showed that the lineages were intermediate between *Hoc*×BC-*Hoc* and *Hag*. No large metacentric chromosomes were observed in either hemiclonal reproduction or recombination modes. This finding suggests that large metacentric chromosomes were not reformed by regeneration of a hemiclone.

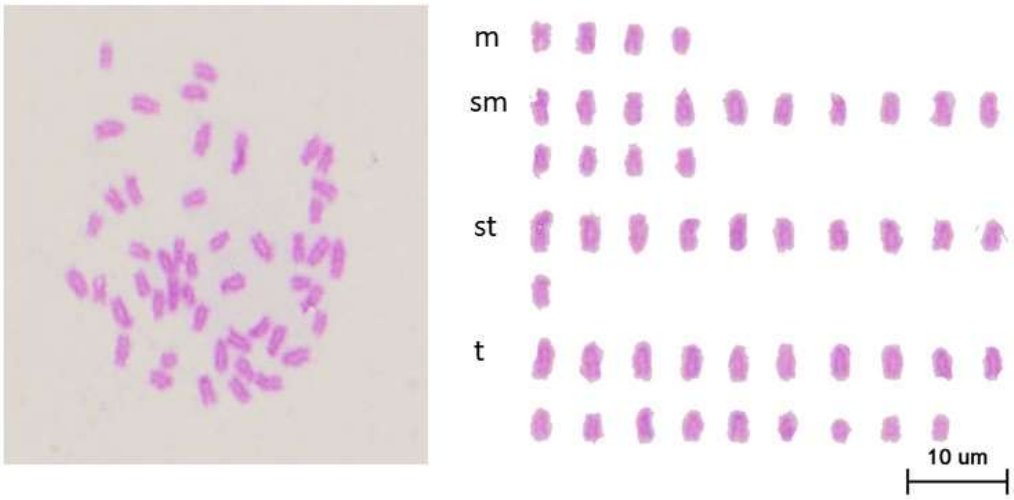

Fig. 4-3. Mitotic metaphase chromosome spreads and karyotypes of ((*Hoc*×BC-*Hoc*)×*Hag*)×*Hag* hemiclonal lineages. The karyotype was 4m + 14sm + 11st + 19t and were intermediate between Hoc×BC-*Hoc* and *Hag*.

Discussions

Genetic factors for hybridogenesis

Hemiclonal organisms accumulate deleterious mutations due to reproduction without recombination or exchange of genetic material (Muller, 1964). The accumulation of deleterious mutations makes it difficult for clonal populations or species taxa to survive over a long time (Lynch and Gabriel, 1990), and hemiclonal organisms are thought to have weak resistance to perturbations in the environment or attacks by parasites (the Red Queen theory; Van Valen, 1977). In *Hexagrammos* hybrids, hemiclonal genomes could be recombined in the *Hoc* gene pool. Although the genetic affinity between parental species plays an important role in facilitating hybridogenesis, it is not considered to be entirely sufficient for inducing hybridogenesis (Kimura-Kawaguchi *et al.*, 2014; Munehara *et al.*, 2016, chapter 2). Chapter 2 suggests that BC-*Hoc* can eliminate deleterious mutations by producing recombinant gametes in the natural environment. In this chapter, production of a renewal hemiclone through maternal backcrossing from the natural hybrid was confirmed. Two of the 5 (*Hoc*×BC-*Hoc*)×*Hag* individuals hemiclonally produced gametes but the remaining 3 individuals produced recombinant gametes. This suggests that hemiclonal reproduction occurs by not only genetic compatibility but certain genetic factors. In this cross pattern ((*Hoc*×BC-*Hoc*)×*Hag*), if all of the genetic factors inducing hybridogenesis are contained within a single major gene, then a quarter of the (*Hoc*×BC-*Hoc*)×*Hag* individuals would be carriers of hybridogenesis. In addition, because most (*Hoc* ×BC-*Hoc*) ×*Hag* failed to produce viable larva, the genetic factors responsible for hybridogenesis must be located separately on at least two loci. This result contradicts the balance hypothesis proposed by Moritz *et al.* (1989). Thus, the hybridogenetic system can eliminate deleterious mutations by recombination during occasional generation changes and extend the longevity of

hybrid lineages.

Occasional generation changes may increase the diversity of mtDNA, and *P. monacha-lucida* has been reported to have high levels of mtDNA diversity (Quattro *et al.*, 1991). In this hybrid, genealogical analyses with allozyme and mitochondrial DNA showed that hybridogenetic females arose more than once (Quattro *et al.*, 1991; Burt and Trivers, 2006). In *Hexagrammos* hybrids, genealogical analysis using mtDNA revealed that *Hoc**/*Hot* hybrids formed a cluster with *Hoc**/*Hag* that did not contain any *Hoc* individuals (Munehara *et al.*, 2016). On the other hand, some *Hoc**/*Hag* hybrids shared alleles with *Hoc*. When fertile BC-*Hoc* males mated with normal *Hoc* females, progeny inherited the normal *Hoc* mtDNA haplotype (Fig. 4-1). Consequently, a *Hoc* individual possessing genetic factors capable of inducing hybridogenesis (carrier) is considered to have altered the mtDNA haplotype (Fig. 4-1). Moreover, when a carrier mates with a *Hag* male, a new hemiclone lineage will arise (Fig. 2-3 B in Chapter 2, Fig. 4-1). Even if the mutation facilitating hybridogenesis occurred only once, hybridogens showing different haplotypes can share the mutation. This hypothesis is in agreement with the genealogical relationship of *Hoc* and two natural hybrids (Munehara *et al.*, 2016).

General Discussion

Karyotype evolution in Hexagrammid fishes

The hexagrammid comprising 5 genera and 12 species are widely distributed in the North Pacific and in the adjacent Bering Sea, Sea of Okhotsk and Sea of Japan (Gorbunova, 1970; Rutenberg, 1970). The karyotypes of *H. otakii, H. agrammus, H. lagocephalus* (2m+6sm+28st+12t), *H. stelleri* (4m+12sm+12st+20t) and *Pleurogrammus azonus* (18m+8sm+12st+10t) were previously investigated (Yu *et al.*, 1992, Nishikawa and Sakamoto, 1982; Matsumiya *et al.*, 1980; and this study). *H. octogrammus,* was reported to have only chromosome number (FN) by Makino (1937), and the karyotype was clarified for the first time in this study. Crow *et al.* (2004) investigated the phylogenetic relationships of the Hexagrammidae using two mtDNA and four nuclear loci. Applying karyotype diversity to this molecular phylogenetic tree, *P. azonus*, which has 18 metacentric chromosomes, first diverged from the ancestral species, and then *H. lagocephalus* (2m+6sm+28st+12t) arose. With speciation of *Hexagrammos* to *H. stelleri, H. octogrammus, H. otakii* and *H. agrammus*, the (sub)metacentric chromosome number increased and the (sub)terocentric chromosome number decreased. In Perciformes, karyotypes that are composed of 48 single-arm type (telocentric) chromosomes are thought to be the primitive state (Ohno *et al.*, 1968; Mabuchi *et al.*, 2002). In Labridae, which has a decrease in FN due to fusion of chromosomes, the karyotypes (2n=48, FN=48) of genus *Thalassoma* are thought to be the most primitive karyotype among 50 species investigated (Ojima and Kashiwagi, 1979, 1980; Alvarez *et al.*, 1986; Mabuchi *et al.*, 2002). Excluding *H. octogrammus*, which has many terocentric chromosomes, this evolutionary pattern of karyotype can be applied to *Hexagrammos,* suggesting that metacentric chromosomes increased during the speciation of *Hexagrammos* spp.

In Chapter 1, inheritance of *Hoc* chromosomes from hybrids was observed to

potentially increase the large metacentric chromosomes with Robertsonian translocation through generational change. Robertsonian translocation was a useful cytogenetic marker for identifying ancestral *Hoc** that fissioned in meiosis during gametogenesis of BC-*Hoc*, which was produced by a cross between the natural hybrid and *Hoc*. Thus, the karyotype of *Hoc* shows a terocentric chromosome that occupies a large part and may have originated from repeated hybridization through two-way backcrossing.

Return to gene pool and coexistence with parent species

Secondary contact between the closely related temperate species and boreal species occurred after sympatric speciation (Shinohara, 1994; Brykov and Podlesnykh, 2001). This 'Hybrid zone' is defined as a geographic area where individuals from genetically distinct groups meet and mate, resulting in the production of offspring of mixed ancestry (Harrison, 1993; Arnold *et al.*, 1999). *Hexagrammos* hybrids produced viable offspring by hemiclonal reproduction without introgressive hybridization between the genomes of parental species, and those hybrids coexist with parental species (Kimura-Kawaguchi *et al.*, 2014). Unisexual reproduction modes such as clonal or hemiclonal are considered to have a two-fold reproductive advantage (Maynard Smith, 1978; 1992). Such species are considered to increase twice as fast as those utilizing sexual reproduction, and once a unisexual lineage appears, it is considered to be at an advantage when colonizing new habitats or competing with sexual reproduction in the mother species (Vrijenhoek, 1978; Avise, 2008; Lehtonen *et al.*, 2013). Consequently, there are some lineages in which only hemiclonal reproduction remains and the parent species have disappeared (Schmidt *et al.*, 2011).

In Chapter 2, producing gametes in the natural environment was shown have

the potential to reverse translocation in BC-*Hoc*. As a result, the karyotype BC - *Hoc* × *Hoc* (or *Hoc* ×BC-*Hoc*) contained many telocentric chromosomes, as with karyotype of *Hoc* (Chapter 1). Since the large metacentric chromosome markers disappeared, it is almost impossible to distinguish between *Hoc* and offspring of BC-*Hoc*. These results suggest that BC - *Hoc* and its offspring are genetically introgressive into the *Hoc* population. In addition to these results, natural hybrids (*Hoc**/*Hag*) have mated normally with *Hoc* at the same frequency as they have mated with *Hag* (Chapter 2). That is, half of the hybrid genome has been returned to the *Hoc* population and the two-fold advantage of hemiclonal hybrids is reduced. Thus, BC-*Hoc* (carriers of hybridogenesis and the first recombination generation) plays an important role in coexisting with the parental species.

Longevity of hemiclone lineage

Asexual lineages lose their resistance to parasites and their ability to adapt to abrupt environmental changes (Red Queen hypothesis), and deleterious mutations should be accumulated in the hemiclone genome (Muller's ratchet). These defects increase the extinction risk of the hybridogenetic lineage (Lynch and Gabriel, 1990; Lynch *et al.*, 1993; Vrijenhoek *et al.*, 1993), and asexual lineages are thus often considered to be "evolutionary dead ends," "blind alleys," and "no-hopers" (Wetherington *et al.*, 1987). By modelling natural selection and deleterious mutation loads, Lynch and Gabriel (1990) predicted that clonal lineages are unlikely to survive beyond 10^4 to 10^5 generations. However, *P. formosa*, a gynogenetic hybrid has continued for more than approximately 500,000 generations, far exceeding the prediction (Warren *et al.*, 2018), and *Poeciliopsis* hybridogenetic lineages are thought to have continued for more than 100,000 generations (Quattro *et al.*, 1992). In *Hexagrammos* species, Crow *et al.* (2010) proposed that the common ancestor of *H.*

otakii and *H. agrammus* (temperate species) diverged allopatrically from *H. octogrammus* (boreal species) approximately 2.2–3.6 million years ago and that *H. otakii* and *H. agrammus* diverged sympatrically approximately 1.2–2.0 million years ago. Secondary contact between the temperate species and boreal species then occurred after sympatric speciation (Shinohara, 1994; Brykov and Podlesnykh, 2001). *Hoc**/*Hot* hybrids are considered to have arisen by host switching between *Hoc**/*Hag* and *Hot* approximately 20,000–30,000 years ago (assuming a generation period of 2 years, approximately 10^4 generations have passed) (Munehara *et al.*, 2016). These findings suggest the first *Hoc**/*Hag* hybrid also likely arose after secondary contact that occurred before host switching, approximately 10^5–10^6 years ago, which exceeds theoretical expectation (Munehara *et al.*, 2016). In recent years, Warren *et al.* (2018) reported that micro DNA insertion produces nuclear genetic diversity and longevity of clonal lineages. Along with this mechanism, hybridogenetic systems may evolve another way to increase genetic divergence because of changes in the paternal genome at every generation change.

The mating patterns of natural hybrids (*Hoc**/*Hag*) backcrossing both parental species with almost equal frequency could be considered to be the typical example of two-way backcrossing rather than a destabilizing hybridization. The destabilizing hybridization hypothesis was first proposed by Lynch (1984) as a possible mechanism for reducing extinction risk. Maternal backcross (BC-*Hoc*) involves the normal diploid organisms (*Hoc*) returning hemiclonal genomes to the recombinant generation (Chapter 2). In Chapter 4, (*Hoc*×BC-*Hoc*)×*Hag* was shown to produce gametes hemiclonally as evidenced by regeneration of the hemiclonal lineages. mtDNA of the initial hybridogens was replaced and the nuclear genome was recombined in the *Hoc* gene pool during *Hoc*×BC-*Hoc* generation. These findings indicate that renewal of the hemiclonal lineage arises after several recombinations

of genome sets and reduction of accumulated deleterious mutation loads.

In other hemiclonal lineages, studies on genetic diversity or longevity of hybrids have been conducted in *Ph. esculentus*, which uses sexual reproduction. Most of the offspring from matings between two *Ph. esculentus* individuals in a population are nonviable, but some viable *Pe. ridibundus* individuals are produced from hybrid mating (Vorburger, 2001a, b; Guex *et al.*, 2002; Christiansen *et al.*, 2005, 2010; Pruvost *et al.*, 2013). The inviability of offspring from natural matings between two *Ph. esculent*a is suggested to be caused by fixed deleterious mutations in the *P. ridibundus* genome (Vorburger, 2001 a, b). Viable *Pe. ridibundus* females resulting from the cross between *Ph. esculentus* produced recombinant gametes as normal *Pe. ridibundus*, and they may reduce the load of deleterious mutations in the *Pe. ridibundus* genome (Holsbeek and Jooris, 2010). Because *Pe. ridibunda* is usually absent in mixed populations of *Ph. lessonae* and *Ph. esculenta* (Schmidt, 1993), recombination of the hemiclonal genome only occurs in matings between *Ph. esculentus*. Thus, though this mechanism, mating between hemiclonal hybrids beneficially increases the long-term fitness of individuals in a hybridogenetic population in *Ph. esculentus* (Som and Reyer, 2006; Christiansen and Reyer, 2009). The appearance of males and females makes it possible to increase genetic diversity, and the mechanism is different from that in *Hexagrammos* hybrids.

Aphid species reproduce asexually throughout the spring and summer months but switch to sexual forms to produce overwintering eggs only in the autumn (Loxdale and Lushai, 2003). This life cycle aids in resetting chromosome telomeres, the physical ends of linear eukaryotic chromosomes, as well as to increase genetic diversity and eliminate deleterious mutations within the population (Loxdale and Lushai, 2003). If the function of telomeres is lost, DNA will be damaged and the cell will die; that is, individuals with short telomeres have short longevity (Fagagna *et*

al., 2003). Therefore, recombination in (hemi)clonal lineages is important not only to extend lineage longevity but also to maintain individual longevity. Even unisexual organisms gain genetic diversity by incorporating a recombination generation into the life cycle.

Features of hybridogenetic system of *Hexagrammos* hybrids

Hexagrammos hybrids have two significant benefits when compared to other hemiclonal system. One is that *Hexagrammo*s hybrids still coexist with both parental species. To clarify the population structure of hybridogens demographically, populations including both parental species must be analyzed. *Hexagrammos* hybrids have originated from hybridization between boreal species and temperate species, which inhabit geographically different oceans with contact zones. The larger contact zones reduce the extinction risk of the hemiclone lineage, and these conditions are more likely to produce new lineages than other hybridogenetic systems of restricted habitats, such as lake and rivers. The second is that *Hoc** hybrid can be discriminated from *Hoc* diploid by genetic maker. Hemiclonal hybrids have genomes that thoroughly correspond with those of natural hybrids containing *Hoc** and *Hag* (or *Hot*) genomes. BC-*Hoc* hybrids produce recombinant gametes, although these gametes comprise *Hoc** and *Hoc* genomes. These relationships suggest that the paternal genome is not eliminated when it meets a conspecific genome. In *Hexagrammos* hybrids, although the genetic affinity between parental species plays an important role in facilitating hybridogenesis, it is not considered to be entirely sufficient for inducing hybridogenesis. Consequently, the case described in this study does not corroborate the balance hypothesis proposed by Moritz *et al.* (1989).

Unisexual organisms of hybridization origin should be described by genomic

heterogeneity between parents, called “Balance hypothesis” (Moritz, 1989) or “Heterosis hypothesis” (Wetherington, 1987). In addition to genomic heterogeneity, the presence of genetic factors inducing hybridogenesis have been verified first by artificial crossing experiments among *Hexagrammos* hybrids and species (Kimura-Kawaguchi *et al.*, 2014). Hemiclonal reproduction is a spontaneous ultimate “gene drive” that is a promising way to sterilize harmful insects through “genome editing” (Caplan *et al.*, 2015; Lunshof, 2015). Unisexual hybrids are valuable materials for exploring the evolution of sex, even if these are not taxonomically described.

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