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Title

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

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

Running Title: Two-way backcrossing reduces extinction risk

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Author contributions: SS is interested in the evolutionary ecology of hemiclinal reproduction, and carried out the karyological analysis and msDNA analysis. SM assisted the collection of materials and the karyological observation. KA contributed the genetic analysis. HM led this study, and carried out msDNA analysis. All authors discussed and approved the manuscript.

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Abstract

Unisexual vertebrates (*i.e.*, those produced through clonal or hemiclonal reproduction) are typically incapable of purging deleterious mutations, and, as a result, are considered short-lived in evolutionary terms. In hemiclonal reproduction (hybridogenesis), one parental genome is eliminated during oogenesis, producing haploid eggs containing the genome of a single parent. Hemiclonal hybrids are usually produced by backcrossing hemiclonal hybrids with males of the paternal species. When hemiclonal hybrids from a genus of greenlings (*Hexagrammos*) are crossed with males of the maternal species, the progeny are phenotypically similar to the maternal species and produce recombinant gametes by regular meiosis. The present study was conducted to determine if the hemiclonal genome is returned to the gene pool of the maternal species in the wild. Using a specific cytogenetic marker to discriminate between such progeny and the maternal species, we observed that *Hexagrammos* hybrids mated with maternal and paternal ancestors at the same frequency. This two-way backcrossing in which clonal genomes are returned to the gene pool where they can undergo recombination plays an important role in increasing the genetic variability of the hemiclonal genome and reducing the extinction risk. In this way, hybrid lineages may have survived longer than predicted through occasional recombinant generation.

Keywords: Hybridogenesis, hemiclonal reproduction, marine fish, unisex, karyology, host switch

Introduction

Hybridization between two distantly related species is typically associated with the disruption of normal meiosis due to pairing incompatibilities between homologous chromosomes (Dawley, 1989). Some vertebrates have escaped this constraint by switching the reproduction mode from bisexual to unisexual, employing mechanisms such as clonal reproduction (parthenogenesis and gynogenesis) or hemiclonal reproduction (hybridogenesis) (Dawley 1989; Vrijenhoek *et al.*, 1989; Lampert and Scharl, 2008). Since the Amazon molly, *Poecilia formosa* was first described in 1932 (Hubbs and Hubbs, 1932), unisexual species (biotypes) have been confirmed in more than 50 naturally occurring fishes, amphibians, and reptiles. In most of these taxa, reproduction is either clonal or hemiclonal and without recombination (Avis, 2015; Warren *et al.*, 2018). In clonal reproduction, females produce unreduced diploid eggs, which require sperm to trigger embryogenesis, and sometimes “paternal leakage” occurs. In hemiclonal reproduction, both the maternal and paternal haploid genomes are expressed in somatic cells following fertilization. But during oogenesis, one parental genome, usually the paternal one, is eliminated from germ cells, producing haploid eggs that carry only the other parental genome (Schultz, 1961; Cimino, 1972; Tunner and Heppich-Tunner, 1991). In this way, one entire parental genome is displaced at every generation change. Hemiclonal reproduction is less common than clonal reproduction and has

been reported in only six taxa to date (Lamatsch and Stöck, 2009): freshwater fishes, such as *Poeciliopsis* hybrids (Miller and Schultz, 1959; Schultz, 1969), *Squalius alburnoides* (*Rutilus alburnoides*) (Carmona *et al.*, 1997), and *Hypseleotris* (Schmidt *et al.*, 2011); the edible frog *Pelophylax esculenta* (*Rana esculenta*) (Ogielska, 2009); the marine fish genus *Hexagrammos* (Kimura-Kawaguchi *et al.*, 2014); and the stick insect genus *Bacillus* (Tinti and Scali, 1992).

Unisexual reproduction eliminates the cost of producing males. As a result, organisms that reproduce unisexually can reproduce twice as fast as organisms that reproduce sexually (Maynard Smith, 1978; 1992). 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. This is because recombination can greatly increase genetic diversity. Genetic diversity in unisexual populations arises chiefly through mutations, and over time, deleterious mutations can accumulate in clonal genomes through the process known as Muller's ratchet (Muller, 1964; Kondrashov, 1988; Otto and Lenormand, 2002; Burt and Trivers, 2006). The accumulation of mutations is thought to lead to the extinction of unisexual lineages within 10,000-100,000 generations (Lynch and Gabriel, 1990; Lynch *et al.*, 1993; Loewe and Lamatsch, 2008). However, in *P. formosa*, which has a generation time of 3–4 months, gynogenetic hybrids have existed for more than approximately 500,000 generations (Loewe and Lamatsch, 2008). Also, *P. monaca-lucida* hemiclinal hybrids have

existed far longer than 100,000 generations (Quattro *et al.*, 1992). In *P. formosa* and unisexual salamanders (*Ambystoma jeffersonianum-laterale* complex), it is thought that genetic diversity was brought by introgression deriving from paternal-genome fragments (Bogart *et al.*, 2009, Avise 2015, Warren *et al.*, 2018). If hybrids have enough genetic diversity when they start unisexual reproduction, they may survive longer without recombination (Warren *et al.*, 2018). However, the accumulation of deleterious mutations can lead to the extinction of unisexual lineages because such one-way genetic introgression does not remove the mutations. The reason why hybrids have existed so long despite apparently accumulating mutation remains elusive because it is difficult to obtain empirical evidence for extended longevity in clonal or hemiclinal lineages.

The genus *Hexagrammos* comprises six species of greenlings that are common fish in coastal waters of the North Pacific Ocean (Rutenberg, 1970). One boreal species, the masked greenling *H. octogrammus* (hereafter *Hoc*), and two temperate species, the fat greenling *H. otakii* (*Hot*) and the spotty belly greenling *H. agrammus* (*Hag*), have sympatric distributions in coastal areas of southern Hokkaido (Japan) and Primorsky Krai (Russia) (Rutenberg, 1970; Crow *et al.*, 2010). In addition, two unisexual hybrids occur in these areas, and it has been clarified using amplified fragment length polymorphism (AFLP) markers, microsatellites, flow cytometry and morphological analysis that they reproduce hemiclonally (Kimura-Kawaguchi *et al.*, 2014). The natural hemiclinal hybrids that produce haploid eggs containing only the *H. octogrammus* genome (maternal ancestor) generate hemiclinal

offspring by fertilization with haploid sperm of *H. agrammus* or *H. otakii* (paternal species) (Fig. 1A; Kimura-Kawaguchi *et al.*, 2014). The *Hoc** genome of the hemiclinal hybrids has been differentiated somewhat from the *Hoc* genome of the pure species because the F₁-hybrids arising from crossbreeding between *Hoc* and *Hag* (or *Hot*) generate recombinant gametes (eggs and spermatozoa) through regular meiosis (Fig. 1B; Kimura-Kawaguchi *et al.*, 2014). The two hemiclinal hybrids are denoted as *Hoc*/Hag* and *Hoc*/Hot* (“*” indicates that the genetic factors required for inducing hemiclinal reproduction are present), whereas artificial F₁ hybrids are denoted as *Hoc*×*Hag* or *Hoc*×*Hot* (A×B indicates A: female species × B: male species artificial crossing, and A/B indicates hemiclinal hybrids).

Experiments using artificial crosses have shown that the offspring of maternal backcrosses between *Hoc*/Hag* hybrids and *Hoc* (hereafter BC-*Hoc*) also produce recombinant gametes (Suzuki *et al.*, 2017). BC-*Hoc* has a *Hoc** genome inherited from the hemiclinal hybrid and a normal *Hoc* genome inherited from the *Hoc* male (Fig. 1C). However, BC-*Hoc* has the same morphological phenotype as normal *Hoc*, so BC-*Hoc* cannot be discriminated from normal *Hoc* based on morphology alone. In a previous karyological study, we found that the *Hoc** genome has 2-3 large metacentric chromosomes formed by centric-fusion of two chromosomes, or Robertsonian translocation, that are not present in the contemporary *Hoc* genome (Suzuki *et al.*, 2017). Therefore, BC-*Hoc* has these large metacentric chromosomes, which are transmitted hemiclinally from the *Hoc*/Hag* hybrid (Suzuki *et al.*, 2017). Such a marked difference between karyotypes is a useful cytogenetic marker for identifying

ancestral *Hoc** and contemporary *Hoc* genomes. Some offspring of BC-*Hoc* having a *Hoc** genome (*Hoc**/*Hoc*) should be carriers of hemiclinal reproduction. If carriers are found in the natural environment, it can be considered that the hemiclinal genome will return to the *Hoc* gene pool. When carriers are crossed with *Hag* males breaking the established reproductive isolation (Crow *et al.*, 2007, 2010; Kimura and Munehara, 2011), carriers have the potential to produce hemiclinal hybrids. Thus, BC-*Hoc* individuals play an important role in increasing the genetic diversity of the hemiclinal lineage by transmitting the hemiclinal genome to the gene pool of the *Hoc* population.

In this study, we sampled egg masses from both *Hoc* and *Hag* breeding territories to test for evidence of back-crossing between hemiclinal hybrids and both of their parental species. By testing eggs from *Hag* territories, we were able to determine whether the egg masses were spawned by *Hag*, *Hoc* (*Hoc**) or *Hoc**/*Hag* individuals. By testing eggs from *Hoc* territories, we were able to test for evidence of hemiclinal hybrids backcrossing with their maternal species.

Materials and Methods

Tissue and egg collection

Males of *Hoc* and *Hag* maintain breeding territories in which they guard egg masses laid by multiple females (Kimura and Munehara, 2011). We collected egg masses that had been spawned in breeding territories of *Hoc* and *Hag*, and used cytogenetic markers or genetic

markers to identify egg masses that were spawned by *Hoc**/*Hag* hybrids. All fish- and eggs were collected by scuba diving off the coast of Usujiri (41°57'N; 140°58'E) in southern Hokkaido in September and October of 2015 and 2016. The territories are typically located in shallow areas, with the preference for the extent of seaweed coverage differing slightly between *Hoc* and *Hag* (Kimura and Munehara, 2011). Species identification was performed based on the number of lateral lines and number of pairs of flaps on the head according to Shinohara (1994) and Nakabo (2013). To clarify parentage and whether the mothers of egg masses in the male's territory were hemiclinal hybrids or pure species, the tip of the right pectoral fin was cut off with a pair of scissors after a territorial male was caught using a hand net. After the fin clips were collected, all fish were released near their territories. Using a pair of scissors or tweezers, approximately 50 eggs were collected from each egg mass (equivalent to 0.5-1% of all eggs in the egg mass). To avoid admixture of egg samples, eggs were collected only from egg masses that were not attached to any other egg masses. A total of 285 egg masses were collected; in 2015, 77 egg masses were collected from 14 *Hag* territories and 56 were collected from 20 *Hoc* territories, in 2016, 96 egg masses were collected from 16 *Hag* territories and 56 were collected from 28 *Hoc* territories. 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 the hatched larvae were preserved in 99% ethanol at -20°C until genetic analysis. Fish and

embryos were treated in accordance with the Regulations of Animal Experimentation at Hokkaido University (26-1).

Identification of mitochondrial DNA haplotypes

Since the maternal species of the hemiclinal hybrids is *Hoc* (Crow *et al.*, 2007, 2010; Kimura-Kawaguchi *et al.*, 2014), mitochondrial DNA (mtDNA) analysis was used to detect the *Hoc* haplotypes in egg masses 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 the 12S and 16S rRNA (12-16S rRNA) gene regions. AmpliTaq 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.

Detection of hemiclinal hybrids by microsatellite DNA

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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. Alleles not found in the territorial male were assumed to derive from a female (or sneaker *Hag* males). When all offspring in an egg mass shared only a single maternal allele at all the loci investigated, the egg mass was considered to have inherited a hemiclinal genome. If any 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 normal individuals, not hemiclinal hybrids. When egg masses are partially fertilized by sneaker males, the offspring likely possess alleles not found in the territorial male (Munehara *et al.*, 2016). In such cases, the size range of each allele was applied to determine whether the allele size is seen in *Hag* or *Hoc* populations. Eight offspring were randomly selected from each egg mass, and their genotypes were compared with the territorial *Hag* male that guarded them.

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) (Megl  cz *et al.*, 2010). A 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 12 individuals of *Hot*. Finally, based on the low Hardy-Weinberg equilibrium deviation probabilities and sufficiently high heterozygosity within each species, four (*Hexoc* 2, *Hexoc* 9-2, *Otakii* 5, *Otakii* 6) of 22 loci were selected for analysis (Table 1 and Table 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 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.).

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 hemiclinal hybrids (Suzuki *et al.*, 2017). Consequently, the identity of the parent fish, *i.e.*, whether they are *Hoc**/*Hag* or *Hoc*, can be determined by chromosome analysis.

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Chromosome preparation followed Suzuki *et al.* (2017). The karyotypes were determined using chromosomes 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 metacentric (*m*), submetacentric (*sm*), subtelocentric (*st*), or telocentric (*t*). 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.

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 during 2015 and 2016, respectively, from *Hag* territories, 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 belonged to *Hoc* (Table S1).

For these 11 egg masses, the offspring were genotyped using seven microsatellite loci, and the genotypes were compared to those of the territorial males. In nine of these egg masses, the offspring shared a single allele from the mother on every locus, but shared either one of two paternal alleles (Table 3). Therefore, these egg masses were considered to have inherited the *Hoc** genome by hemiclinal reproduction. 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 (Table 3). 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 a tetra-nucleotide motif, offspring shared an allele known from the *Hoc* population. Therefore, in these two egg masses, the alleles shared among offspring were also inherited by hemiclonal reproduction. These results show that the 11 egg masses with the *Hoc* haplotype were spawned by hemiclonal hybrids (*Hoc**/*Hag*) and that no egg masses were spawned by *Hoc* individuals in *Hag* territories.

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 $2sm + 10st + 36t$, 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 4). Three of the karyotypes were identical to those observed in BC-*Hoc* in a previous study (Suzuki *et al.*, 2017), and the remaining two karyotypes (type 4 and type 5) were observed for the first time in the present study. The type 4 karyotype comprised $1(1)m + 2 sm + 8 st + 36 t$ (numerals in parentheses indicate the number of large metacentric chromosomes), 47 chromosomes and 50 arms (Fig.

2A). Type 5 comprised $2(2)m + 2 sm + 6 st + 36 t$, 48 chromosomes and 50 arms, including two micro-chromosomes (Fig. 2B). The karyotypes of F₁ hybrids (crossing between *Hoc* and *Hag*) were intermediate between the two parental species, and large metacentric chromosomes were not observed (Suzuki *et al.*, 2017). Thus, the type 4 and type 5 karyotypes were also categorized as the BC-*Hoc* karyotype, suggesting that the hemiclinal hybrids mated with *Hoc* males. Finally, seven (6.3%) of the 112 egg masses were spawned by hemiclinal hybrids (*Hoc**/*Hag*), and no egg masses were spawned by *Hag* individuals in *Hoc* territories.

Discussion

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

The two *Hexagrammos* hybrids reported by Kimura-Kawaguchi *et al.*, (2014) were the first evidence of a hemiclinal reproduction system in marine organisms. Of these hybrids, the hemiclinal 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). It is well known that hemiclinal hybrids between closely related species occur frequently in contiguous habitats (Barton and Hewitt, 1985; Arnold, 1997; Buggs, 2007). Although the three *Hexagrammos* species have established a firm system of reproductive isolation, and no evidence of genetic introgression has been detected to date (Crow *et al.*, 2007, 2010; Kimura and Munehara, 2011), egg masses showing the *Hoc*

haplotype have occasionally been found in *Hag* territories (Kimura *et al.*, 2007). The present study revealed that all of the egg masses showing the *Hoc* haplotype were spawned by hemiclonal hybrids (*Hoc*/Hag*), and not *Hoc* individuals. In addition, using cytogenetic markers, we could discriminate egg masses spawned by the hemiclonal hybrids in *Hoc* territories from those spawned by *Hoc* individuals. In hemiclonal reproduction, the entire paternal genome is replaced at every generation change. Indeed, once a hemiclonal hybrid (*Hoc*/Hag*) crossbreeds with *Hot*, another type of hemiclonal hybrid (*Hoc*/Hot*) can arise through “host switching” (Fig. 1D) (Munehara *et al.*, 2016). Genealogical analysis of *Hoc* and two hemiclonal hybrids has shown that anomalous crossbreeding between a hemiclonal hybrid (*Hoc*/Hag*) and *Hot* has occurred only once in the past (Munehara *et al.*, 2016). Conversely, in the present study, hemiclonal hybrids (*Hoc*/Hag*) mated normally with *Hoc* at the same frequency as they mated with *Hag*, which is a typical backcross pattern. These results suggest that BC-*Hoc* individuals play an important role in increasing the genetic diversity of the hemiclonal lineage.

The hybrid offspring produced from crosses between the hemiclonal hybrids and males of the maternal species are diploid and bear two sets of the maternal species’ genome. In the *Poeciliopsis* hemiclonal system, none of the offspring artificially produced from such a cross are hemiclonal reproduction; all are fully fertile males and females that produce recombinant gametes (Vrijenhoek, 1993). Similarly, in the hemiclonal system of water frogs, offspring produced by artificial backcrossing with the maternal ancestor produce recombinant gametes

(Holsbeek and Jooris, 2010). The results of those laboratory experiments are identical to the results obtained in studies of the *Hexagrammos* hemiclinal system (Kimura-Kawaguchi *et al.*, 2014; Suzuki *et al.*, 2017). 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 hemiclinal hybrids (*Hoc**/*Hag*) and both *Hag* and *Hoc* could be considered 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 the extinction risk. The present study is the first to demonstrate that two-way backcrossing occurs consistently in natural environments. The discovery of this mating pattern can provide a rational hypothesis for why hemiclone lineages have survived longer than predicted through “occasional recombinant generation”.

Longevity of hemiclinal lineages

Since hemiclinal hybrids do not exchange genetic material through recombination, they accumulate deleterious mutations in their hemiclinal genomes, and genetic diversity is limited (Lynch and Gabriel, 1990; Lynch *et al.*, 1993). These characteristics increase the extinction risk of hemiclinal lineages (Muller, 1964; Lynch and Gabriel, 1990; Burt and Trivers, 2006). There are several considerations concerning the mechanisms employed by unisexual organisms to generate genetic variability, including partial incorporation of paternal chromosomes and increasing ploidy (Schartl *et al.*, 1995; Lampert and Schartl,

2008). However, compared with such incidental phenomena, recombination produces higher genetic variability through the rearrangement of homologous chromosomes and crossing over during meiosis. In triploid green toads (*Bufo baturae*), females auto-reduplicate one maternal genome before meiosis and transmit one clonal and one recombined genome to the offspring (pre-equalizing hybrid meiosis) (Stöck *et al.*, 2012). In diploid vertebrates, since the genomes including “hybridogenetic factors” are markedly altered by recombination during conspecific breeding, the resulting hemiclone lineages have a lower risk of going extinct. In this way, many of the deleterious mutations that accumulate over time are removed from the hemiclone genome. If the hemiclone hybrids compete with the maternal species, despite being able to reproduce twice as fast as sexual reproduction, the hybrid lineages are likely to be short-lived on an evolutionary timescale.

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* (a 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 thought 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 10,000 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 100,000-1,000,000 years ago. This implies that the *Hoc**/*Hag* hybrid lineage is older than theoretically expected. Thus, the hemiclonal (hybridogenetic) system may extend the longevity of hybrid lineages by recombinant generation.

Regeneration of hemiclonal lineages through two-way backcrossing

The occurrence of hemiclonal reproduction can be explained by the "balance hypothesis" (Wetherington *et al.* 1987; Moritz *et al.* 1989). Wetherington *et al.*, (1987) suggested that "establishment of a new unisexual-hybrid lineage may be limited to a narrow window, in which the combining genomes are sufficiently dissimilar to disrupt gametogenesis and yet similar enough to coordinate development of a stable phenotype". However, because *Hexagrammos* F1-hybrids generated normally recombinant gametes (eggs and spermatozoa) through meiosis, hemiclonal reproduction might not always be caused by the extent of the genomic dissimilarity between species (Kimura-Kawaguchi *et al.*, 2014; Suzuki *et al.*, 2017). In addition, it was suggested that genetic factors capable of inducing hemiclonal reproduction exist in the hemiclonal genome (Kimura-Kawaguchi *et al.*, 2014). However, these factors do not function under high genetic compatibilities between homeologous chromosomes, and BC-*Hoc* individuals bearing two sets of *Hoc* genomes produce recombinant gametes (Suzuki *et al.*, 2017). Indeed, the large metacentric chromosomes found in BC-*Hoc* that are inherited from hemiclonal hybrids fissure to form two separate chromosomes during meiosis (Suzuki *et al.*, 2017). These previous studies show that BC-*Hoc* fulfilled the role of a normal diploid

Hoc, and that there were no karyological differences between two sets of *Hoc* genomes in offspring from a crossing between BC-*Hoc* and *Hoc*. In addition to these results, the present study showed the hemiclone genome is returned to the ‘gene pool’ of the *Hoc* population in the wild. After BC-*Hoc* reproduces in the wild, if carriers hybridize with *Hag*, a new hemiclone lineage would arise (Fig. 3). 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 hemiclone hybrids (Munehara *et al.*, 2016). Even if the genetic factors responsible for inducing hemiclone reproduction are encoded by multiple genes, new hemiclone lineage will still arise, albeit more rarely. The hypothesis suggests that BC-*Hoc* 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 hemiclone reproduction will be renewed through recombination at the time of generation changes.

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Fig. 1. The specimen lineages of Hexagrammos hybrids referred in the present study. (A) Typical mating patterns of natural hybrids, (A-1) paternal backcross between hemiclinal hybrids (Hoc^*/Hag) and males of the paternal species (Hag), (A-2) paternal backcross between hemiclinal hybrids (Hoc^*/Hot) and males of the paternal species (Hot). (B) Artificial F-1 hybrids between Hoc and Hag . (C) Maternal backcross between hemiclinal hybrids (Hoc^*/Hag) and males of the maternal species (Hoc). (D) Host-switching of hemiclinal hybrids (Hoc^*/Hag) from Hag to Hot , that is, the origin of another hemiclinal hybrids (Hoc^*/Hot). All abbreviated species signs were denoted in the text. Blue, orange and green colors of fish shapes indicate genomes of *H. octogrammus*, *H. agrammus* and *H. otakii*, respectively.

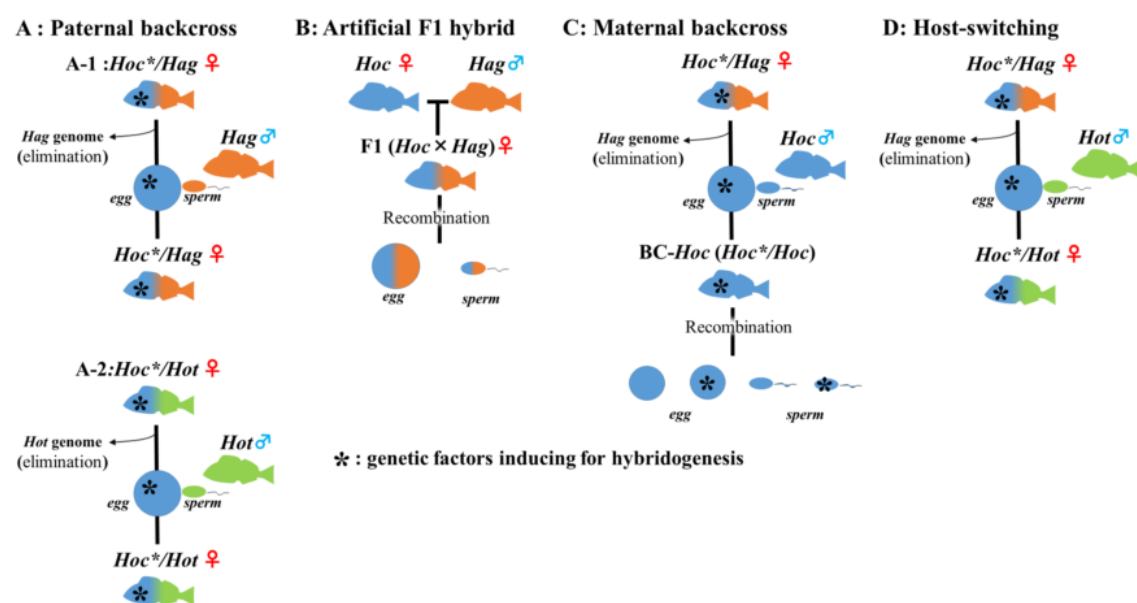


Fig. 2. Mitotic metaphase chromosome spreads and karyotypes of maternal backcrosses. (A) BC-Hoc type 4 showed $1(1)m + 2\text{ sm} + 8\text{ st} + 36\text{ t}$, (B) BC-Hoc type 5 comprised $2(2)m + 2\text{ sm} + 6\text{ st} + 36\text{ t}$ and two micro-chromosomes. m: Metacentric, sm: submetacentric,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

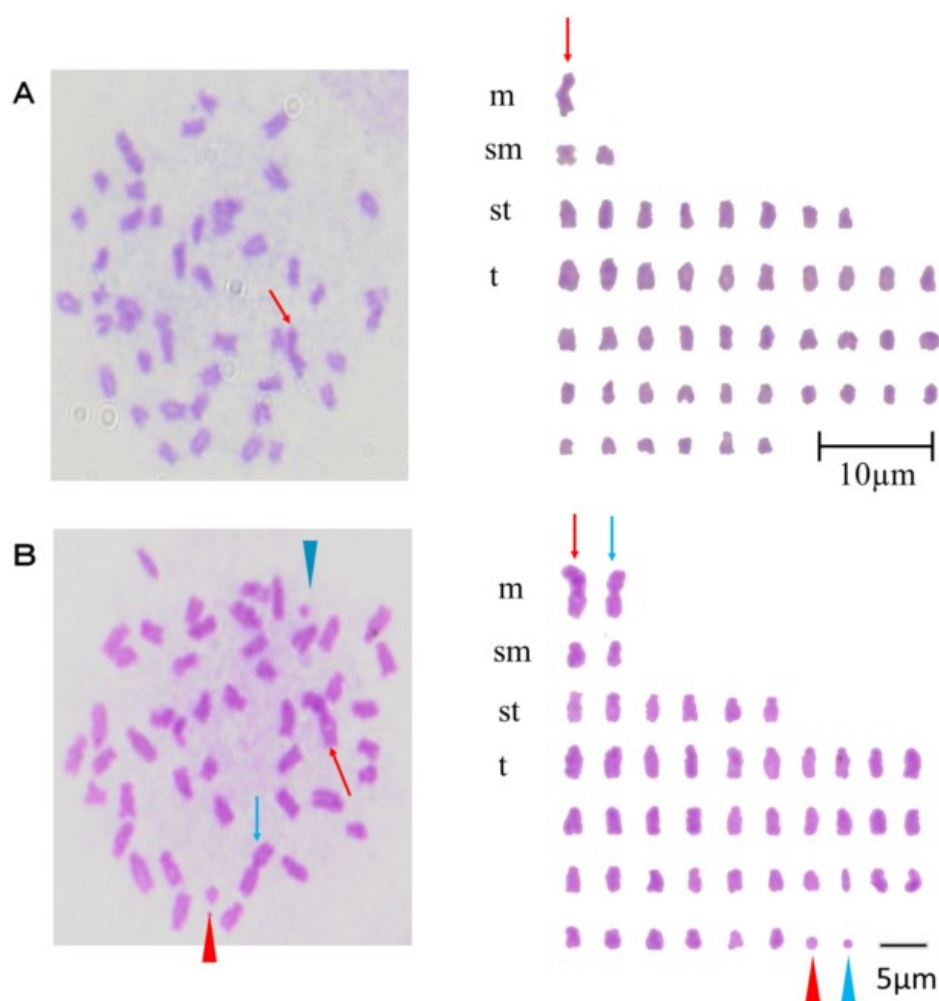


Fig. 3. Hypothesis for renewing hemiclinal lineages that accumulated deleterious mutations through the recombinant generation (BC-Hoc). When a natural hemiclinal hybrid (Hoc^*/Hoc) was crossbred with a male of the maternal ancestor (Hoc), their offspring, BC-Hoc (Hoc^*/Hoc), would produce recombinant gametes. The hemiclinal genome of the hybrid would be assimilated into Hoc population, and some deleterious mutations in the genome would be taken off by recombination while mating among Hoc . When carrier males mate with normal Hoc females, the mtDNA haplotype of carriers would be replaced. When one of the carriers mates with a Hag male, a new hemiclinal lineage will arise.

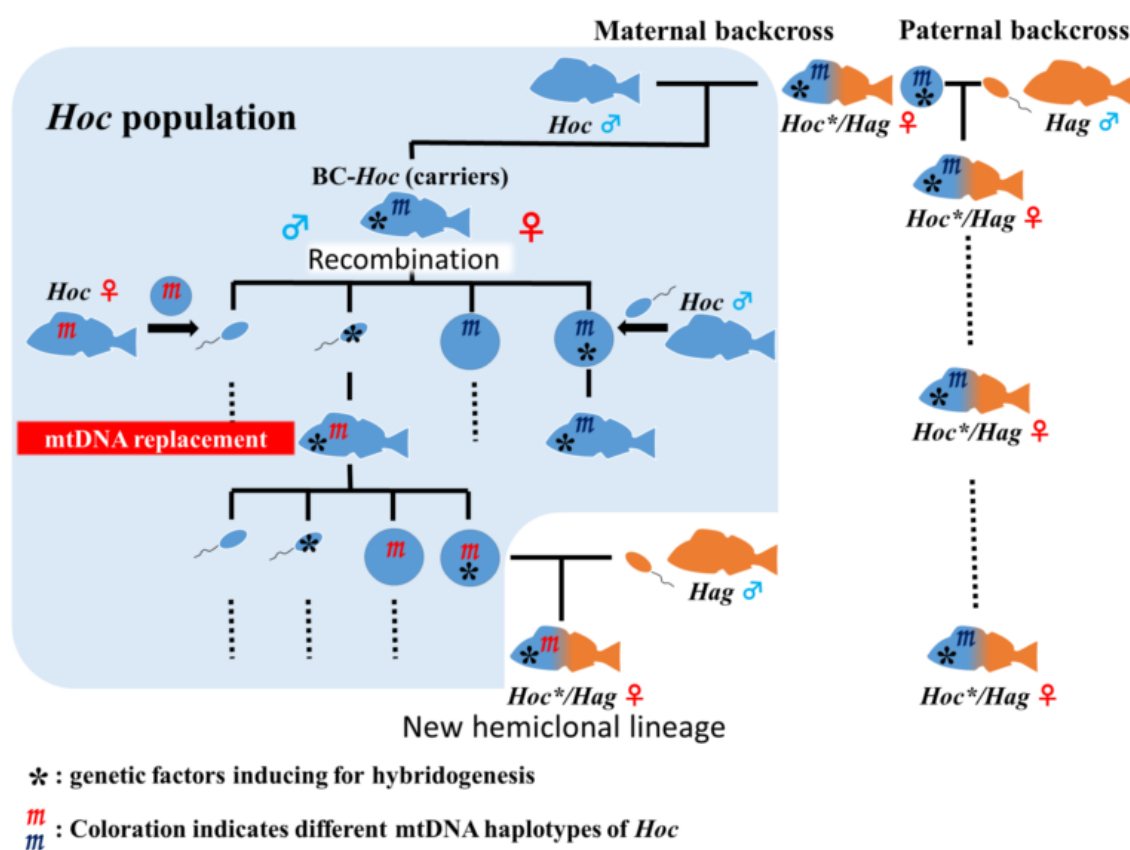


Table 1. Sequences of the four PCR primers used in this study.

Sequences of the remaining three primers (*Hexoc* 6, 14, 21) used in this study are given in the previous studies (Kimura-Kawaguchi *et al.*, 2014, Munehara *et al.*, 2016).

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

Table 2. Summary of four polymorphic microsatellite loci for the two parental species.

Ho and *He*, correspondence was observed between the expected and observed heterozygosities. The remaining three microsatellite loci are described in the previous studies (Kimura-Kawaguchi *et al.*, 2014, Munehara *et al.*, 2016).

Species	Locus	Size range (bp)	No of allele	Ho	He	Chi-squared test
<i>H. octogrammus</i> (N=30)	<i>Otakii 6</i>	130-174	12	0.900	0.859	1.000
	<i>Hexoc 2</i>	88-106	10	0.793	0.852	0.035
	<i>Otakii5</i>	127-142	6	0.800	0.788	0.942
	<i>Hexoc 9-2</i>	116-184	18	0.931	0.927	0.663
<i>H. agrammus</i> (N=40)	<i>Otakii 6</i>	100-156	15	0.875	0.908	0.439
	<i>Hexoc 2</i>	78-94	6	0.525	0.482	1.000
	<i>Otakii5</i>	140-158	7	0.825	0.753	0.989
	<i>Hexoc 9-2</i>	122-150	8	0.790	0.812	0.995

Table 3. Genotypes of offspring from egg masses deposited in *Hag* territories showing the *Hoc* mtDNA haplotype. Yellow and green cells indicate alleles that were inherited hemiclonally and alleles inherited from sneaker males, respectively.

2015		Genotypes of microsatellite DNA													
Clutch NO.	Individual NO.	<i>Hexoc</i> 6		<i>Hexoc</i> 14		<i>Hexoc</i> 21		<i>Hexoc</i> 2		<i>Hexoc</i> 9-2		<i>Otakii</i> 5		<i>Otakii</i> 6	
1-5	Territorial male	11 2	12 2	86	88	11 8	11 8	78	88	13 0	13 0	14 6	15 2	10 4	11 6
	Offspring 1	11 2	11 2	88	12 4	11 8	15 2	78	96	13 0	15 2	13 6	15 2	11 6	13 8
	Offspring 2	11 2	12 2	88	12 4	11 8	15 2	88	96	13 0	15 2	13 6	15 2	10 4	13 8
	Offspring 3	11 2	11 2	86	12 4	11 8	15 2	88	96	13 0	15 2	13 6	14 6	11 6	13 8
	Offspring 4	11 2	11 2	88	12 4	11 8	15 2	88	96	13 0	15 2	13 6	15 2	10 4	13 8

	Offspring 5	11 2	12 2	88	12 4	11 8	15 2	N/ A	N/ A	13 0	15 2	13 6	15 2	10 4	13 8
	Offspring 6	11 2	12 2	88	12 4	11 8	15 2	78	96	13 0	15 2	13 6	15 2	10 4	13 8
	Offspring 7	11 2	11 2	86	12 4	11 8	15 2	88	96	13 0	15 2	13 6	14 6	10 4	13 8
	Offspring 8	11 2	12 2	88	12 4	11 8	15 2	78	96	13 0	15 2	13 6	15 2	11 6	13 8
2-2	Territorial male	11 8	12 4	92	92	10 2	13 2	84	88	13 0	13 0	14 0	15 2	13 6	14 8
	Offspring 1	11 8	12 0	92	12 4	10 2	15 2	84	96	13 0	15 2	13 6	14 0	13 8	14 8
	Offspring 2	11 8	12 0	92	12 4	10 2	15 2	88	96	13 0	15 2	13 6	15 2	13 6	13 8
	Offspring 3	11 8	12 0	92	12 4	10 2	15 2	88	96	13 0	15 2	13 6	14 0	13 6	13 8
	Offspring 4	12	12	92	12	13	15	84	96	13	15	13	15	13	13

		0	4		4	2	2			0	2	6	2	6	8
	Offspring 5	11 8	12 0	92	12 4	10 2	15 2	88	96	13 0	15 2	13 6	15 2	13 6	13 8
	Offspring 6	12 0	12 4	92	12 4	13 2	15 2	88	96	13 0	15 2	13 6	14 0	13 6	13 8
	Offspring 7	12 0	12 4	92	12 4	13 2	15 2	88	96	13 0	15 2	13 6	14 0	13 8	14 8
	Offspring 8	12 0	12 4	92	12 4	13 2	15 2	84	96	13 0	15 2	13 6	14 0	13 8	14 8
2-6	Territorial male	11 8	12 4	92	92	10 2	13 2	84	88	13 0	13 0	14 0	15 2	13 6	14 8
	Offspring 1	12 0	12 4	92	12 6	13 2	15 2	88	96	13 0	15 2	13 6	15 2	13 8	14 8
	Offspring 2	12 0	12 4	90	12 6	11 4	15 2	88	96	12 6	15 2	13 6	14 0	13 8	14 4
	Offspring 3	12 0	12 4	92	12 6	10 2	15 2	84	96	13 0	15 2	13 6	15 2	13 6	13 8

	Offspring 4	12 0	12 8	90	12 6	10 2	15 2	88	96	12 6	15 2	13 6	14 0	13 8	14 4
	Offspring 5	12 0	12 4	92	12 6	10 2	15 2	84	96	13 0	15 2	13 6	15 2	13 6	13 8
	Offspring 6	12 0	12 4	88	12 6	11 4	15 2	88	96	12 6	15 2	13 6	15 2	12 8	13 8
	Offspring 7	12 0	12 4	90	12 6	11 4	15 2	88	96	14 6	15 2	13 6	14 0	12 8	13 8
	Offspring 8	11 8	12 0	N/ A	N/ A	10 2	15 2	88	96	13 0	15 2	13 6	15 2	13 6	13 8
3-4	Territorial male	12 4	12 6	88	86	11 4	12 0	88	90	13 0	14 2	14 3	14 6	10 4	11 6
	Offspring 1	12 0	12 6	86	12 6	12 0	15 2	90	96	13 0	15 2	13 6	14 3	11 6	13 8
	Offspring 2	12 0	12 6	88	12 6	12 0	15 2	90	96	13 0	15 2	13 6	14 6	11 6	13 8
	Offspring 3	12	12	86	12	11	15	90	96	14	15	13	14	11	13

		0	4		6	4	2			2	2	6	3	6	8
	Offspring 4	12 0	12 4	86	12 6	11 4	15 2	88	96	14 2	15 2	13 6	14 3	10 4	13 8
	Offspring 5	12 0	12 6	88	12 6	12 0	15 2	90	96	14 2	15 2	13 6	14 6	10 4	13 8
	Offspring 6	12 0	12 4	86	12 6	11 4	15 2	88	96	14 2	15 2	13 6	14 3	11 6	13 8
	Offspring 7	12 0	12 4	86	12 6	11 4	15 2	90	96	14 2	15 2	13 6	14 3	10 4	13 8
	Offspring 8	12 0	12 6	88	12 6	12 0	15 2	88	96	14 2	15 2	13 6	14 6	10 4	13 8
5-1	Territorial male	11 4	12 2	86	92	11 2	11 2	78	90	13 0	15 0	14 3	14 9	12 8	12 8
	Offspring 1	11 4	12 0	92	12 6	11 2	15 2	90	96	15 0	15 2	13 6	14 3	12 8	13 8
	Offspring 2	12 0	12 2	86	12 6	11 2	15 2	78	96	15 0	15 2	13 6	14 9	12 8	13 8

	Offspring 3	11 4	12 0	92	12 6	11 2	15 2	78	96	15 0	15 2	13 6	14 3	12 8	13 8
	Offspring 4	11 4	12 0	86	12 6	N/ A	N/ A	90	96	13 0	15 2	13 6	14 9	12 8	13 8
	Offspring 5	11 4	12 0	92	12 6	11 2	15 2	78	96	15 0	15 2	13 6	14 3	12 8	13 8
	Offspring 6	12 0	12 2	92	12 6	11 2	15 2	78	96	13 0	15 2	13 6	14 3	12 8	13 8
	Offspring 7	11 4	12 0	86	12 6	11 2	15 2	90	96	15 0	15 2	13 6	14 9	12 8	13 8
	Offspring 8	11 4	12 0	86	12 6	11 2	15 2	90	96	15 0	15 2	13 6	14 9	12 8	13 8
9-1	Territorial male	11 8	12 0	86	86	10 4	10 6	88	88	13 0	14 6	14 0	14 6	10 4	11 6
	Offspring 1	12 0	12 2	86	12 4	10 6	15 2	88	96	13 0	15 2	13 6	14 0	10 4	13 8
	Offspring 2	12	12	86	12	10	15	88	96	13	15	13	14	10	13

		0	2		4	6	2			0	2	6	6	4	8
	Offspring 3	11 8	12 2	86	12 4	10 4	15 2	88	96	14 6	15 2	13 6	14 0	11 6	13 8
	Offspring 4	11 8	12 2	86	12 4	10 6	15 2	88	96	13 0	15 2	13 6	14 0	11 6	13 8
	Offspring 5	12 0	12 2	86	12 4	10 6	15 2	88	96	13 0	15 2	13 6	14 6	11 6	13 8
	Offspring 6	11 8	12 2	86	12 4	10 4	15 2	88	96	14 6	15 2	13 6	14 6	N/ A	N/ A
	Offspring 7	12 0	12 2	86	12 4	10 6	15 2	88	96	13 0	15 2	N/ A	N/ A	10 4	13 8
	Offspring 8	12 0	12 2	86	12 4	10 6	15 2	88	96	13 0	15 2	13 6	14 0	10 4	13 8
9-4	Territorial male	11 8	12 0	86	86	10 4	10 6	88	88	13 0	14 6	14 0	14 6	10 4	11 6
	Offspring 1	10 6	12 6	92	11 8	12 8	17 0	88	94	15 0	16 8	13 3	14 3	13 2	15 8

	Offspring 2	10 6	12 6	92	11 8	12 8	17 0	88	94	15 0	16 8	13 3	14 3	13 2	15 8
	Offspring 3	10 6	12 6	88	11 8	13 0	17 0	88	94	15 0	16 8	13 3	14 0	12 8	15 8
	Offspring 4	10 6	12 6	88	11 8	12 8	17 0	88	94	15 0	16 8	13 3	14 0	13 2	15 8
	Offspring 5	10 6	12 6	92	11 8	13 0	17 0	88	94	15 0	16 8	13 3	14 0	13 2	15 8
	Offspring 6	10 6	12 6	92	11 8	12 8	17 0	88	94	15 0	16 8	13 3	14 3	12 8	15 8
	Offspring 7	10 6	12 6	88	11 8	12 8	17 0	88	94	15 0	16 8	13 3	14 3	13 2	15 8
	Offspring 8	10 6	12 6	92	11 8	13 0	17 0	88	94	15 0	16 8	13 3	14 3	12 8	15 8
2016		Genotypes of microsatellite DNA													
Clutch NO.	Individual NO.	<i>Hexoc 6</i>	<i>Hexoc 14</i>	<i>Hexoc 21</i>	<i>Hexoc 2</i>	<i>Hexoc 9-2</i>	<i>Otakii 5</i>	<i>Otakii 6</i>							

6-4	Territorial male	12 2	12 4	86	90	10 8	11 8	88	88	12 8	15 2	14 6	15 2	12 0	15 2
	Offspring 1	12 0	12 2	86	12 4	10 8	15 2	88	96	15 2	15 2	13 8	15 2	12 0	13 8
	Offspring 2	12 0	12 4	86	12 4	11 8	15 2	88	96	12 8	15 2	13 8	15 2	13 8	15 2
	Offspring 3	12 0	12 4	90	12 4	11 8	15 2	88	96	12 8	15 2	13 8	14 6	12 0	13 8
	Offspring 4	12 0	12 2	86	12 4	11 8	15 2	88	96	12 8	15 2	13 8	15 2	13 8	15 2
	Offspring 5	12 0	12 2	86	12 4	10 8	15 2	88	96	12 8	15 2	13 8	15 2	13 8	15 2
	Offspring 6	12 0	12 4	86	12 4	11 8	15 2	88	96	15 2	15 2	13 8	15 2	12 0	13 8
	Offspring 7	12 0	12 4	90	12 4	11 8	15 2	88	96	12 8	15 2	13 8	14 6	12 0	13 8
	Offspring 8	12	12	90	12	11	15	88	96	12	15	13	14	12	13

		0	4		4	8	2			8	2	8	6	0	8
6-5	Territorial male	12 2	12 4	86	90	10 8	11 8	88	88	12 8	15 2	14 6	15 2	12 0	15 2
	Offspring 1	12 0	12 4	90	12 4	11 8	15 2	88	96	12 8	15 2	13 8	14 6	13 8	15 2
	Offspring 2	12 0	12 4	90	12 4	11 8	15 2	88	96	15 2	15 2	13 8	14 6	13 8	15 2
	Offspring 3	12 0	12 2	86	12 4	10 8	15 2	88	96	12 8	15 2	13 8	14 6	12 0	13 8
	Offspring 4	12 0	12 2	86	12 4	10 8	15 2	88	96	15 2	15 2	13 8	15 2	12 0	13 8
	Offspring 5	12 0	12 4	90	12 4	11 8	15 2	88	96	12 8	15 2	13 8	14 6	12 0	13 8
	Offspring 6	12 0	12 2	90	12 4	10 8	15 2	88	96	12 8	15 2	13 8	14 6	12 0	13 8
	Offspring 7	12 0	12 2	86	12 4	10 8	15 2	88	96	15 2	15 2	13 8	15 2	13 8	15 2

	Offspring 8	12 0	12 4	90	12 4	11 8	15 2	88	96	15 2	15 2	13 8	14 6	12 0	13 8
16-6	Territorial male	12 4	12 6	86	88	10 6	13 2	88	88	12 6	12 6	14 0	14 3	10 4	13 6
	Offspring 1	12 0	12 6	88	12 4	13 2	15 2	88	96	12 6	14 8	13 8	14 3	10 4	13 8
	Offspring 2	12 0	12 4	88	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 3	10 4	13 8
	Offspring 3	12 0	12 6	86	12 4	13 2	15 2	88	96	12 6	14 8	13 8	14 0	13 6	13 8
	Offspring 4	12 0	12 6	88	12 4	13 2	15 2	88	96	12 6	14 8	13 8	14 3	10 4	13 8
	Offspring 5	12 0	12 4	86	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 3	13 6	13 8
	Offspring 6	12 0	12 4	88	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 3	13 6	13 8
	Offspring 7	12	12	88	12	13	15	88	96	12	14	13	14	13	13

		0	6		4	2	2			6	8	8	3	6	8
	Offspring 8	12 0	12 6	86	12 4	13 2	15 2	88	96	12 6	14 8	13 8	14 0	10 4	13 8
16-7	Territorial male	12 4	12 6	86	88	10 6	13 2	88	88	12 6	12 6	14 0	14 3	10 4	13 6
	Offspring 1	12 0	12 4	86	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 0	13 6	13 8
	Offspring 2	12 0	12 4	86	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 0	10 4	13 8
	Offspring 3	12 0	12 6	88	12 4	13 2	15 2	88	96	12 6	14 8	13 8	14 3	10 4	13 8
	Offspring 4	12 0	12 4	88	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 3	10 4	13 8
	Offspring 5	12 0	12 4	88	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 3	10 4	13 8
	Offspring 6	12 0	12 4	88	12 4	10 6	15 2	88	96	12 6	14 8	13 8	14 3	13 6	13 8

	Offspring 7	12 0	12 6	88	12 4	13 2	15 2	88	96	12 6	14 8	13 8	14 3	10 4	13 8
	Offspring 8	12 0	12 6	86	12 4	13 2	15 2	88	96	12 6	14 8	13 8	14 0	10 4	13 8

Table 4. Five different karyotypes observed in egg masses from *Hoc* territories.

Typical type	Number of egg mass	Karyotype				Centric fusion	micro chromosome	FN	2n
		m	sm	st	t				
<i>H. octogrammus</i>	105	0	2	10	36	0	0	50	48
BC- <i>Hoc</i> (Type 1)	2	2	2	6	36	2	0	50	46
BC- <i>Hoc</i> (Type 2)	1	3	2	6	34	3	0	50	45
BC- <i>Hoc</i> (Type 3)	1	3	2	6	34	3	2	50	47
BC- <i>Hoc</i> (Type 4)	2	1	2	8	36	1	0	50	47
BC- <i>Hoc</i> (Type 5)	1	2	2	6	36	2	2	50	48