

Maintenance of species boundaries despite rampant hybridization between three species of reef fishes (Hexagrammidae): implications for the role of selection

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Hybridization and introgression are antagonistic to the process of speciation. If hybrids are viable and backcross, introgression will slow or prevent the evolution of reproductive isolation between populations. If species hybridize upon secondary contact, introgression will ultimately erode species boundaries. If hybridization is rampant and gene flow is high, the proportion of hybrids will approach values expected with random mating, and introgression will overcome species boundaries, homogenizing populations. Alternatively, if species are genetically distinct, the proportion of hybrids would be significantly less than expected with random mating, gene flow would be interrupted by selection against hybrids, and inviability or sterility of F_1 hybrids or backcrosses would be expected. Here, we investigate a system with characteristics expected by both of these opposing scenarios. Hybridization occurs between three species of reef fishes in the genus *Hexagrammos* at unexpected high frequencies in a zone of distributional overlap. Backcrossed individuals are detected, indicating F_1 hybrids are viable and capable of reproducing. Yet, these species are genetically distinct at multiple loci. To study this apparent paradox, we estimate the relative proportions of hybrids, patterns of symmetry, inviability, and cytonuclear disequilibria using one mitochondrial and two nuclear markers. We invoke selection against hybrids, at various life history stages, in the maintenance of species boundaries in this system. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, 91, 135–147.

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INTRODUCTION

There is substantial evidence supporting the evolution of reproductive isolation in allopatry across taxa (Rice and Hostert, 1993; Palumbi, 1994). However, the absence of strict allopatry results in various levels of gene flow, which is antagonistic to the process of speciation. Therefore, in partial or complete sympatry, it

is important to understand how populations can diverge into separate species despite the homogenizing effects of genetic exchange (Avice, 1994; Porter and Johnson, 2002). Indeed, hybridization, gene flow, and introgression are integral to the concept of species, and hybrid zones have been increasingly recognized as fertile grounds for evolutionary studies concerning models of speciation and maintenance of species boundaries (Hewitt, 1988; Anderson and Thompson, 2002).

Populations exhibiting hybridization generally progress towards one of two opposing scenarios. First, if

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hybridization is rampant and gene flow is high, the proportion of hybrids will approach values expected under random mating (i.e. no reproductive isolation). In this scenario, introgression will eventually overcome species boundaries and homogenize populations, as has been observed in Darwin's ground finches of the genus *Geospiza* (Freeland and Boag, 1999). Alternatively, if species are genetically distinct, the proportion of hybrids will be significantly less than expected under a random mating model, gene flow will be interrupted by selection against hybrids, and inviability or sterility of F_1 hybrids or backcrosses is expected. Furthermore, when hybridization occurs, reproductive isolation is expected to increase with genetic distance (Coyne and Orr, 1989). Therefore, closer relatives are expected to have greater reproductive compatibility relative to more distantly related species pairs. Here, we describe patterns of hybridization in a system that does not conform to these theoretical expectations.

The genus *Hexagrammos* (greenling) includes six species of temperate reef fishes that are endemic to the North Pacific. Hybridization occurs between three genetically distinct species that exhibit fixed differences at multiple molecular loci (Crow, Kanamoto and Bernardi, 2004). We found surprising trends in the observed proportions of hybrids, patterns of hybridization, and sex ratio of F_1 hybrids. Only two of six potential classes of F_1 hybrids are observed in nature, and these crosses are not between closest relatives. Finally, viable backcrossed individuals are observed, indicating potential for introgression between all three species. Therefore, we invoke selection against hybrids or early generation backcrosses, as the only plausible explanation for the maintenance of species boundaries in this system.

BACKGROUND ON *HEXAGRAMMOS* SPECIES

Hybrids between three *Hexagrammos* species have previously been described from a zone of distributional overlap in Peter the Great Bay, Russia, based on the occurrence of morphological intermediates (Balanov and Antonenko, 1999; Balanov *et al.*, 2001). The ranges of the two southern species, *Hexagrammos otakii* (Jordan and Starks, 1895) and *Hexagrammos agrammus* (Temminck and Schlegel, 1843), occur in sympatry off Japan and the Korean peninsula and overlap slightly with the ranges of three northern species, including *Hexagrammos octogrammus* (Pallas, 1814), between latitudes 40°N and 44°N (Fig. 1). The hybrids described in Peter the Great Bay are between the northern species *H. octogrammus* and the southern species *H. agrammus* (Balanov and Antonenko, 1999) or *H. otakii* (Balanov *et al.*, 2001). Interspecific mating between all three species has been described based on underwater observations off northern Japan

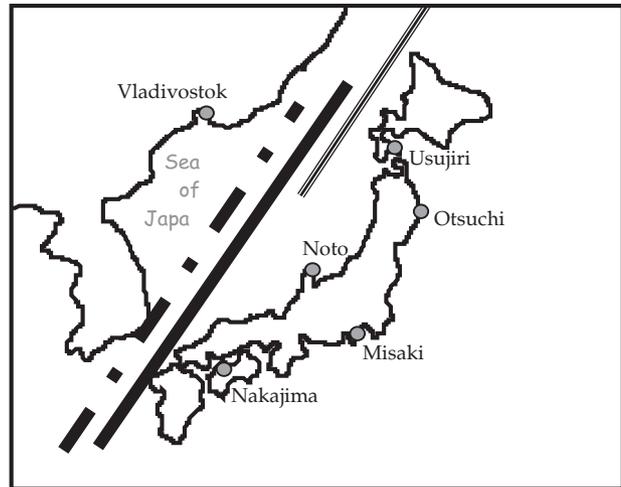


Figure 1. Cartoon of three *Hexagrammos* species distributions in the Northwestern Pacific and Sea of Japan. Bars indicate approximate latitudinal ranges for *Hexagrammos agrammus* (dashed), *Hexagrammos otakii* (solid), and *Hexagrammos octogrammus* (striped). Dots indicate locations where *Hexagrammos* fishes have been sampled. Hybrids have been sampled off Vladivostok, Russia; Otsuchi and Usujiri, Japan.

(Munehara, Kanamoto & Miura, 2000), but adult hybrids have not previously been described from that region. In both of these geographical localities of distributional overlap, nothing is known about the relative abundance of hybrids, or the role of hybridization in the evolution of these species.

Hexagrammos males guard benthic nests with several clutches of eggs deposited from multiple females (Crow, Powers & Bernardi, 1997). Therefore, hybridization occurs as a result of non-assortative mating behaviour, not from random dispersal of gametes produced by broadcast spawning. Females are multiple spawners (Kurita, Sano & Shimizu, 1995; Crow *et al.*, 1997; Antonenko, 1999) and each species exhibits a protracted spawning season associated with water temperature that varies with season and latitude. The peak spawning periods for the two southern sympatric species are the most divergent in time, but they do intersect. Reproductive season is approximately from mid September through October for *H. agrammus*, and from October to mid November for *H. otakii*. By contrast, the reproductive season of *H. octogrammus* occurs approximately from late September through early November, overlapping considerably with the reproductive seasons of the two southern species.

SYMPATRIC AND ALLOPATRIC SPECIATION

The importance of sympatric speciation has been viewed with skepticism because the homogenizing

effect of gene flow is antagonistic to the evolution of reproductive isolation (Via, 2001). However, sympatric speciation can occur when divergent selection acts on traits that directly, or pleiotropically, contribute to prezygotic reproductive isolation (Kondrashov and Mina, 1986; Rice and Salt, 1990), such as disparity in breeding season (as observed in sympatrically distributed *Hexagrammos* species). It is also plausible if linkage disequilibrium occurs between divergently selected loci and loci affecting mate choice because this could overcome 'selection-recombination antagonism' (Felsenstein, 1981). Therefore, sympatric speciation initially occurs when selection acts on relatively few loci resulting in prezygotic reproductive isolation. Accordingly, Coyne and Orr (1989, 1997) observed, with remarkable consistency, that prezygotic isolation evolves faster than postzygotic isolation in sympatry, relative to allopatry.

Allopatric speciation is caused by genetic drift or selection that results in disruption of coadapted gene complexes in hybrids. These Dobzhansky–Muller incompatibilities explain the intrinsic, or developmentally-mediated, loss of fitness in hybrids, but not ecologically-mediated selection against intermediate phenotypes (Rice and Hostert, 1993; Blier, Dufresne and Burton, 2001), and their role is widely accepted in the evolution of postzygotic isolation between allopatric species (Lijtmaer, Mahler and Tubaro, 2003; Turelli and Orr, 2000; Porter and Johnson, 2002).

Our objectives in this study are: (1) to determine the role of hybridization in processes of speciation by comparing observed proportions of hybrids with expected values under a random mating model, at one site in an area of distributional overlap-off Usujiri, Japan; (2) to evaluate the evidence for introgression between the three hybridizing *Hexagrammos* species; and (3) to infer patterns in mating system and potential fitness costs associated with hybridization using a combination of nuclear and mitochondrial molecular markers.

MATERIAL AND METHODS

SAMPLE COLLECTION

In 1998, 144 *Hexagrammos* fishes were sampled from five sites in Japan: Misaki Marine Biological Station (University of Tokyo), Noto Marine Laboratory (Kanazawa University), Nakajima Marine Biological Station (Ehime University), Otsuchi Marine Research Center (University of Tokyo), and Usujiri Marine Biological Station (UMBS) (Hokkaido University), by trap, spear, and hook and line (Fig. 1). These samples were used as a general survey of the distribution patterns of *Hexagrammos* hybrids.

In 2000, *Hexagrammos* fishes ($N = 229$) were sampled near UMBS, Hokkaido, Japan. Of these, five samples were collected randomly ($N = 76$) using trap,

trammel net, and hook and line, and an additional 153 individuals were obtained opportunistically from concurrent research projects by trap, spear, trammel net, and hook and line. Thus, a total of 373 individuals were surveyed.

DIAGNOSTIC MORPHOLOGICAL CHARACTERS, STANDARD LENGTH, AND SEX RATIOS

Species or hybrid assignments for individuals used in this study were based on genotyping at three molecular loci. However, the *Hexagrammos* species considered in this study exhibit external character sets that are diagnostic for each species including the number of lateral lines, supra-orbital cirri, and caudal fin morphology (Fig. 2). We mapped these character sets for every individual to evaluate the range of intermediate morphology associated with hybrids, and to determine whether hybrids exhibit diagnostic external characters. *Hexagrammos agrammus* (hereafter referred to as *Hag*) exhibit one lateral line, two pairs of supra-orbital cirri, and a round caudal fin (Fig. 2A). *Hexagrammos octogrammus* (hereafter referred to as *Hoc*) exhibit five paired lateral lines (Fig. 2B, numbered 1–5), one pair of supra-orbital cirri, and a round caudal fin. *Hexagrammos otakii* (hereafter referred to as *Hot*) exhibit five paired lateral lines two pairs of supra-orbital cirri, and a square caudal fin (Fig. 2C). These species-specific sets of characters are hereafter referred to as 'classic' morphology. Differences in standard lengths among pure species and hybrids were evaluated by analysis of variance (ANOVA). All data were tested for normality and were log-transformed if necessary. Differences in sex ratios were assessed by comparing observed numbers of males and females, for each species (*Hag*, *Hoc*, and *Hot*), with expected (equal) proportions by chi-square analysis.

GENETIC DETERMINATION OF PURE SPECIES, HYBRID, OR BACKCROSS STATUS: DNA EXTRACTION, POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION, AND RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPS)

Fin clips were collected and air-dried on paper for DNA extraction. Tissues were digested overnight at 55 °C in 500 µL salt extraction buffer (400 mM NaCl, 10 mM Tris, 2 mM EDTA, 1% SDS, 20 µg mL⁻¹ proteinase K). DNA was purified by standard phenol : chloroform extraction and isopropanol precipitation (Sambrook, Maniatis & Fritsch, 1989).

Fixed differences in pure species were determined from sequences of six loci (*cyt b*, *16S*, *CaM*, *S7RP*, *Ldh*, and *ck*) from ten to 17 individuals each of *Hoc*, *Hot*, and *Hag* in a previous phylogenetic study (Crow *et al.*, 2004). These individuals exhibited 'classic' morpholog-

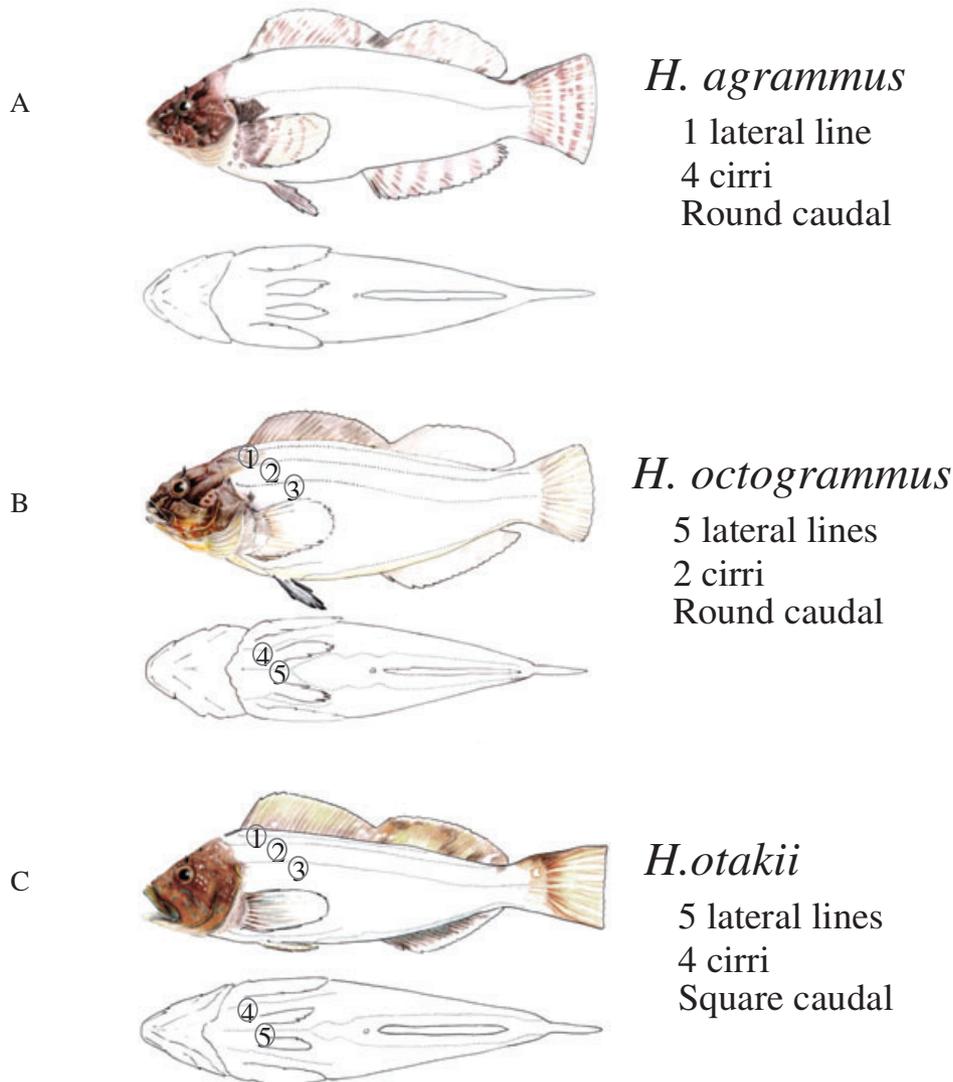


Figure 2. Typical or 'classic' morphology of pure *Hexagrammos* species exhibiting hybridization. Sets of character states including number of lateral lines, number of cirri, and caudal fin morphology are diagnostic for pure species. Note that *Hexagrammos agrammus* does not exhibit the multiple lateral lines characteristic of this genus.

ical features and were sampled from various locations throughout that species' range. Three loci were optimized for genetic determination of hybrids using RFLP analysis from two nuclear loci (*S7RP* and *CaM*) and one mitochondrial locus (*16S*). Selected loci were PCR amplified for RFLP analysis using primers: (1) the first intron of the S7 ribosomal protein: (*S7RP*) EX1F and (*S7RP*) EX2R (Chow and Hazama, 1998); (2) the fourth intron of the calmodulin gene (*CaM*): CALMex4F and CALMex5R (Chow, 1998); and (3) *16S* ribosomal subunit 16SAR and 16SBR (Kocher *et al.*, 1989). Each 50 μ L reaction contained 10–100 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1.5 units of Taq DNA Polymerase (Perkin-

Elmer), 0.25 mM dNTPS, and 0.3 μ M each primer. The amplification cycling profiles were: (1–2) 30 s at 94 °C, 1 min at 60 °C, and 2 min at 72 °C (\times 35 cycles); (3) 45 s at 94 °C, 45 s at 48 °C, and 1 min at 72 °C (\times 35 cycles).

The *CaM* and *S7RP* loci exhibit fixed differences between *Hexagrammos* species corresponding to unique restriction sites for each *Hexagrammos* species and all possible combinations of *F*₁ hybrid parental ancestry. The restriction enzymes *Bsm*I and *Bssh*II (New England BioLabs, Inc.) were used to restrict *Hot* and *Hag CaM* PCR products, respectively. The restriction enzyme *Bbs*I (New England BioLabs, Inc.) was used to restrict *Hoc S7RP* PCR products and the

restriction enzyme *Eco*NI (New England BioLabs, Inc.) was used to restrict the *16S* mtDNA locus. PCR products were run (simultaneously with products from both restriction reactions for *CaM*) on 1% agarose gels and visualized using ethidium bromide.

Every individual sampled in 2000 for the hybridization study ($N = 229$) was genotyped at all three diagnostic loci (*16S*, *S7RP*, and *CaM*) for assignment of pure species or hybrid status. For the 109 general survey samples taken in 1998, the maternal ancestor of all individuals was inferred by sequencing or RFLP of the *16S* ribosomal subunit mtDNA locus, and individuals exhibiting morphological variations were sequenced at two nuclear loci (*S7RP* and *CaM*) to determine hybrid status.

All F_1 hybrids will be detected when heterozygous at both nuclear loci. The inclusion of a second nuclear marker allows detection of backcrossed individuals to determine if F_1 hybrids are capable of reproducing, and if introgression could occur. The estimated number of backcrosses in this study is potentially underestimated because with only two nuclear loci they can incorrectly be assigned as pure species or F_1 hybrids. However, the probability of erroneously assigning backcrosses is equivalent across categories (in this case, three pure species and two hybrid classes) and therefore is relatively low in any one category (Boecklen and Howard, 1997). Because mtDNA is maternally inherited in fishes, the *16S* mtDNA locus enabled us to elucidate the maternal ancestor of individuals derived from hybrid matings, and to infer parentage of hybrid individuals and patterns such as asymmetric hybridization. Departure from random mating was determined by chi-square analysis using proportions of pure species from random samples at the study site to estimate expected proportions. Cytoneuclear disequilibria statistics (Asmussen, Arnold & Avise, 1987; Avise, 2001) were used to describe associations between genotypic frequencies at autosomal loci and haploid cytoplasmic genes.

RESULTS

PRELIMINARY RESULTS FROM GENERAL SURVEY

The presence of *Hexagrammos* hybrids was verified from samples taken in 1998. The distribution of hybrids was estimated from 109 *Hexagrammos* individuals sampled off five Marine Research Stations throughout Japan (Fig. 1). Twenty-one individuals exhibiting morphological variations were verified as F_1 hybrids or backcrosses. F_1 hybrids were of two types: (1) between *H. octogrammos* and *H. agrammus* ($N = 4$, 3.67%) and (2) *H. octogrammos* and *H. otakii* ($N = 9$, 8.26%). The notation used hereafter for F_1 hybrids will denote the maternal ancestor first (e.g.

Hoc is the maternal ancestor of *Hoc/Hot* hybrids). Hybrids were not observed at Misaki Marine Biological Station, Noto Marine Laboratory, or Nakajima Marine Biological Station (Fig. 1). *Hoc/Hag* hybrids were collected at Otsuchi and Usujiri ($N = 2$ each). All *Hoc/Hot* hybrids were collected at Usujiri ($N = 9$). There were eight backcrossed individuals, composing 7.34% of the 109 individuals. Of the eight backcrossed individuals, six were sampled from Otsuchi and two from Usujiri. Thus, UMBS, where most hybrids occurred and the ranges of *Hot*, *Hag*, and *Hoc* overlap, was selected as the focus for a more detailed hybridization study. From these preliminary data, two interesting patterns emerged regarding F_1 hybrids: all are female and *H. octogrammos* is the maternal ancestor in all cases.

HYBRIDIZATION STUDY AT UMBS: PROPORTIONS OF PURE SPECIES AND HYBRIDS

Species or hybrid assignments for all individuals sampled in summer 2000 at UMBS were based on genotypic data from one mtDNA locus and two nuclear loci for estimating relative proportions of pure species and hybrids occurring at this site. Of the 76 randomly sampled *Hexagrammos* individuals collected near UMBS, 31% were hybrids or backcrosses with 6.58% F_1 *Hoc/Hag*, 19.74% F_1 *Hoc/Hot*, and 5.26% hybrid-backcrosses (Table 1). There was no significant difference in the frequencies of pure species and hybrids between the 76 randomly sampled individuals and the additional 153 opportunistically sampled individuals ($\chi^2 = 8.078$, d.f. = 5, $P = 0.152$). Therefore, these data were combined ($N = 229$) yielding the following observed proportions: 16.59% *Hag*, 42.36% *Hoc*, 18.34% *Hot*, 5.24% *Hoc/Hag*, 11.79% *Hoc/Hot*, and 5.68% backcrosses (Table 1).

EXTERNAL MORPHOLOGICAL CHARACTERS ASSOCIATED WITH HYBRIDS AND BACKCROSSES

Individuals genetically characterized as *Hoc/Hag* hybrids exhibited morphological traits that were intermediate between parental species. *Hoc/Hag* hybrids were consistently characterized by missing fourth, and absent or abbreviated first, second, and fifth lateral lines. These hybrid females exhibited round caudal morphology and were equally likely to exhibit two, three or four cirri.

Hoc/Hot hybrids exhibited considerable variation in external morphology, but so did the parental species *Hoc*. These hybrid females were more likely to exhibit reverse branching of the fourth lateral line compared to parental species (four-fold relative to *Hoc*, and 1.5-fold relative to *Hot*), and no branching of the fourth lateral line (common in *Hot* but not in *Hoc*). All 27

Table 1. Number of individuals, sex, and relative proportions of *Hexagrammos* pure species and hybrids sampled in 2000 at Usujiri Marine Biological Station

Species	Male	Female	Not sexed	Total	Proportions
<i>Hag</i>	21 (5)	15 (4)	2 (2)	38 (11)	16.6 (14.5)
<i>Hoc</i>	32 (3)	41 (1)	24 (23)	97 (27)	42.4 (35.5)
<i>Hot</i>	18 (6)	17 (5)	7 (3)	42 (14)	18.3 (18.4)
<i>Hoc/Hag</i>		11 (4)	1 (1)	12 (5)	5.2 (6.6)
<i>Hoc/Hot</i>		20 (11)	7 (4)	27 (15)	11.8 (19.7)
<i>Hag/Hoc</i>				0 (0)	0 (0)
<i>Hag/Hot</i>				0 (0)	0 (0)
<i>Hot/Hoc</i>				0 (0)	0 (0)
<i>Hot/Hag</i>				0 (0)	0 (0)
Backcross	2 (0)	11 (4)	0 (0)	13 (4)	5.7 (5.3)

Data are all samples combined ($N = 229$), with data in parenthesis indicating a subset of the first ($N = 76$) taken from randomly collected samples. Hybrid classes are denoted with maternal ancestor first. Relative proportions of *Hexagrammos* pure species and hybrids are shown in the last column. For clarity, zeros (0) are omitted from the first three columns.

Hoc/Hot hybrids exhibited one pair of cirri and round tail (like *Hoc*) with one exception in each category.

There were significant differences in mean standard lengths among pure species and hybrids (Fig. 4; one-way ANOVA, fixed factors, $F = 44.44$, d.f. = 4,92, $P < 0.001$). This comparison was made between five categories (three pure species and two hybrid classes). Backcrosses and males were not included in this analysis because backcrosses were of mixed heritage and there were no male F_1 hybrids. Because parental species *Hot* is significantly larger in standard length compared to *Hoc* and *Hag*, we found that standard length is a diagnostic feature for adult *Hoc/Hot* F_1 hybrids.

Thirteen backcrossed individuals were detected indicating (1) that F_1 females are viable and capable of reproducing, and (2) potential for introgression. The backcrossed individuals could be grouped into four apparent types including: (1) *Hag* maternal ancestor with *Hoc* ancestry; (2) *Hoc* maternal ancestor with *Hoc/Hot* ancestry; (3) *Hot* maternal ancestor with *Hoc/Hot* ancestry; and (4) *Hot* maternal ancestor with *Hag* ancestry (Table 2). Backcrossed individuals did exhibit morphological variation but there were no external morphological trends that significantly distinguished them from pure species. Of the 13 hybrid-backcrossed individuals, 11 were female and two were male.

ASYMMETRICAL HYBRIDIZATION AND INVIABILITY OF MALE HYBRIDS

Hexagrammos octogrammus was the maternal ancestor of all 39 F_1 hybrids sampled at UMBS in 2000. This pattern is consistent either with asymmetric hybridization (i.e. sex-based directionality in interspecific matings), or differential survivorship of hybrid prog-

eny from random interspecific matings. In addition, all F_1 hybrids sampled (that could be sexed) were female, yet sex ratios of parental species did not differ significantly from equal (χ^2 , d.f. = 1, *Hag* $P = 0.3173$, $N = 36$; *Hoc* $P = 0.2922$, $N = 73$; *Hot* $P = 0.8658$, $N = 35$). Of the 229 *Hexagrammos* fishes sampled in 2000 at UMBS, 73 males were genetically scored and none were found to be F_1 hybrids (73 were female pure species, 31 were female F_1 hybrids, and 41 were not sexed, Table 1). Patterns of asymmetric hybridization and inviability of males were consistent in samples taken in 1998 and 2000.

DEPARTURES FROM RANDOM MATING

Assortative mating, random mating, and rampant hybridization

We compared observed vs. expected proportions of hybrids to determine the relative importance of hybridization vs. assortative mating. We constructed a Punnett square to estimate expected proportions of pure species and hybrids under random mating with the assumptions that: (1) genotypic frequencies are representative of the study area and (2) sex ratios of pure species are equal (as shown above). Overall, the observed pattern of pure species and hybrid frequencies was significantly different from random (Fig. 3). This result is driven by the differences in observed frequencies of pure species being greater than expected with random mating, indicating assortative mating is dominant, and the fact that four of the six classes of hybrids did not occur, indicating unidirectional mating or selection against these hybrid classes. However, the two hybrid classes that did occur were approximately equal to or greater than what would be expected by random mating. Surprisingly, these data implicate

Table 2. Genetic and morphological characterization of backcrossed individuals

ID	Nuclear DNA enzymes cut			Sex	Cirri (N)	Tail	Lateral line morphology	
	mtDNA	<i>Hot</i> and <i>Hag</i>	<i>Hoc</i>					
	<i>16S</i>	<i>CaM</i>	<i>S-7</i>					
J LL	1	<i>Hag</i>	<i>Hoc</i>	<i>Hoc</i>	Female	2	R	Like <i>Hoc/Hag</i> with No fourth
J	2	<i>Hoc</i>	<i>Hot/Hoc Hyb</i>	<i>Hoc</i>	Female	2	R	Like <i>Hot</i> but two cirri R tail
J	3	<i>Hoc</i>	<i>Hoc</i>	<i>Hoc/? Hyb</i>	Female	2	R	Fourth LL not branched or extended
J	4	<i>Hoc</i>	<i>Hoc</i>	<i>Hoc/? Hyb</i>	Female	2	R	Like <i>Hot</i> but fourth LL not branched
J	5	<i>Hoc</i>	<i>Hot/Hoc Hyb</i>	<i>Hoc</i>	Female	2	R	Like <i>Hot</i> but two cirri, R tail
J	6	<i>Hot</i>	<i>Hot/Hoc Hyb</i>	<i>Hoc</i>	Female	2	R	Fifth broken, fourth LL not extended
J	7	<i>Hot</i>	<i>Hot/Hoc Hyb</i>	Not <i>Hoc</i>	Female	2	R	Fourth LL not extended, Right not branched
J	8	<i>Hot</i>	<i>Hot</i>	<i>Hoc/? Hyb</i>	Female	2	S	Like <i>Hot</i> , fifth not joined and fourth reverse branch
J	9	<i>Hot</i>	<i>Hot</i>	<i>Hoc/? Hyb</i>	Female	4	S	Like <i>Hot</i>
J	10	<i>Hot</i>	<i>Hot</i>	<i>Hoc/? Hyb</i>	Female	4		Like <i>Hot</i> but fifth LL abbreviated and not joined
J	11	<i>Hot</i>	<i>Hot</i>	<i>Hoc/? Hyb</i>	Male	4	S	Like <i>Hot</i> but fourth LL broken and reverse branched
J	12	<i>Hot</i>	<i>Hag</i>	Not <i>Hoc</i>	Male	4	R	Like <i>Hag</i>
J	13	<i>Hot</i>	<i>Hag</i>	Not <i>Hoc</i>	Female	4	R	Like <i>Hag</i>

Genotypes are inferred from restriction fragment length polymorphism data of three loci. Morphological characteristics include lateral line (LL) configuration, number of supraorbital cirri, and caudal fin morphology. J refers to sampling location in Japan.

R, round; S, square.

rampant hybridization for some species pairs, and a high potential for introgression and incomplete reproductive isolation. These results are unexpected because introgression is antagonistic to maintenance of species boundaries, yet these three species are genetically distinct. An important clue is the low frequency of backcrosses compared to the expected value (estimated using only the proportions of female F_1 hybrids that occur in nature). The expected proportion of backcrosses is 43.48%, although only 5.26% were observed, indicating that gene flow is significantly interrupted given the observed F_1 hybrid proportions.

Cytonuclear disequilibria

There was a nonrandom association between maternally inherited mtDNA haplotypes and biparentally inherited nuclear genotypes. Whereas the expected frequencies of pure species and hybrids under a random mating scenario were estimated from frequencies of individuals, the cytonuclear disequilibria statistics were based on frequencies of genotypes (Table 1). The cytonuclear disequilibrium statistic (D), introduced by Clark (1984) and Asmussen *et al.* (1987) is used to infer cytonuclear signatures describing reproductive behaviours associated with various hybridizing systems including patterns of introgression and sex-

based directionality to interspecific matings (Avice, 2001). The theory was developed for the conventional system of hybridization between two species. This system is unique in that three species are involved in hybridization; however, the statistics for pairwise comparisons were highly significant for hybridizing species pairs (Table 3). The cytonuclear signature indicates sex-based directionality to interspecific matings and introgression between species.

DISCUSSION

Species within the genus *Hexagrammos* are genetically distinct, yet hybridization occurs between three species resulting in six potential classes of hybrids. Adults from four classes of hybrid crosses are not observed, including hybrids between sister taxa *Hot* and *Hag*. Accordingly, these species exhibit the greatest disparity in breeding season. Surprisingly, the two classes of hybrids that are observed exhibit unexpected high frequencies relative to observed proportions of pure species. These hybrids, resulting from matings between *Hoc* females and *Hot* or *Hag* males, are progeny of species pairs that are not closest relatives. The fact that *Hoc* is the maternal ancestor of all F_1 hybrids sampled has resulted in significant pat-

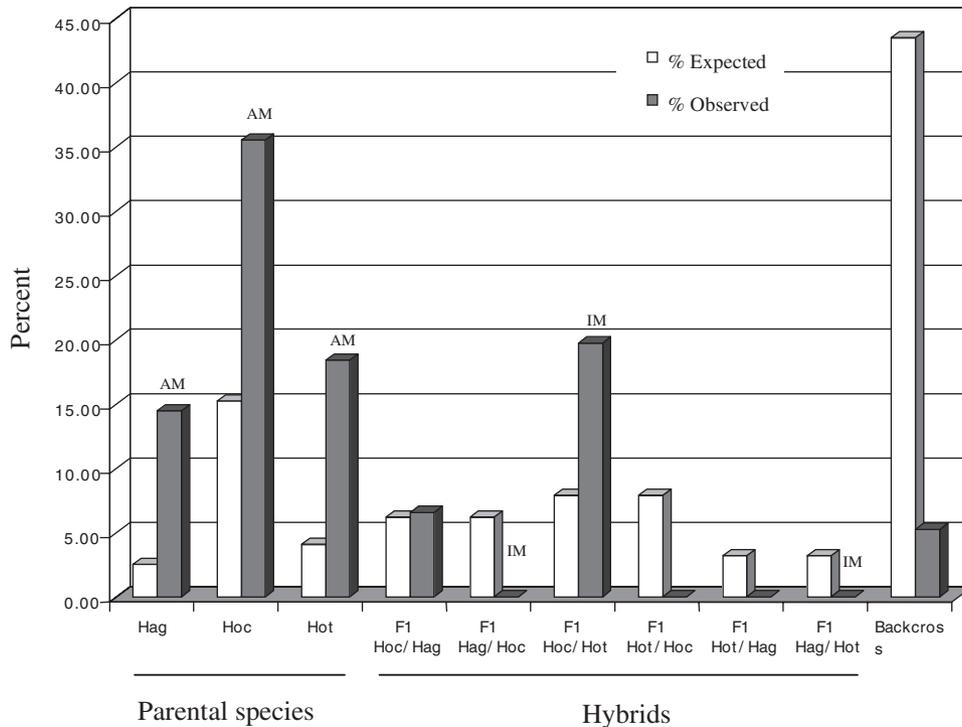


Figure 3. Observed species and hybrid composition vs. expected under random mating. Expected frequencies were calculated with a punnett square using observed frequencies of pure species and hybrids from random samples ($N = 76$; Table 1) and assuming equal sex ratios. The overall distribution of pure species and hybrids is significantly different from random mating (χ^2 , d.f. = 1 $P < 0.001$). However, the observed frequency of *Hoc/Hag* hybrids is approximately equal to expected proportions with random mating, and the frequency of *Hoc/Hot* hybrids is greater. AM, assortative mating; IM, interspecific mating observed in nature (Munehara *et al.*, 2000).

terns of cytonuclear disequilibria (Table 3) and asymmetric hybridization. This pattern of asymmetry could be driven by behaviour (i.e. only *Hoc* females and *Hag* and *Hot* males hybridize) or by negative selection against other classes of hybrids. Adult backcrossed individuals are observed, indicating potential for gene flow and introgression. However, selection against hybrids is implicated in this system for several reasons. First, there is direct evidence for interspecific mating between all three species, yet four classes of hybrids are not observed in nature as adults. Second, all F_1 hybrids are female, yet sex ratios are equal in pure species; therefore, males are presumed inviable and selection is implicated as imposing a fitness cost of 50% or greater in those crosses. Finally, species boundaries are maintained in the presence of rampant hybridization and viable backcrosses. Without selection against hybrids or backcrosses, introgression and erosion of species boundaries would be expected. Accordingly, the number of backcrossed individuals is far less than expected, given the proportions of the two F_1 hybrid females that do occur.

PHYLOGENETIC RELATIONSHIPS AND PATTERNS OF HYBRIDIZATION IN *HEXAGRAMMOS* SPECIES

Hybridization within the genus *Hexagrammos* is unique because it involves three species. Both morphological (Shinohara, 1994) and molecular data (Crow *et al.*, 2004) indicate that *H. agrammus* and *H. otakii* are closest relatives with a sequence divergence of 1.78% (*cyt b* and *16S*) and 100% bootstrap replicate support. These sister taxa occur in sympatry throughout their range, yet hybrids between them are not observed. The morphological phylogenetic hypothesis indicates the sister clade of *Hot* and *Hag* includes *H. octogrammus* (and *H. lagocephalus*; Shinohara, 1994), whereas the molecular data indicate *H. octogrammus* is the singular sister taxon of this clade with sequence divergences of 3.02% and 2.43%, respectively (Crow *et al.*, 2004). Interestingly, *Hexagrammos* hybrids that are observed are not progeny resulting from hybridization between closest relatives that co-occur, but rather between these and their allopatric congener *H. octogrammus*.

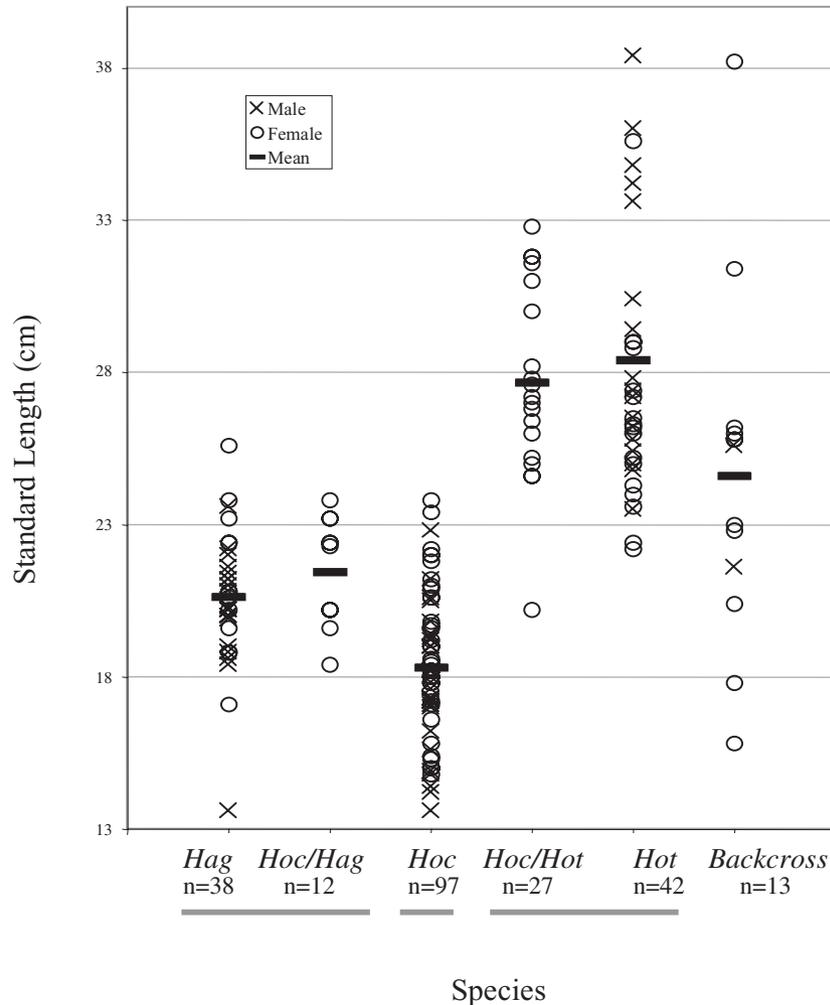


Figure 4. Standard lengths and sex of 229 *Hexagrammos* fishes sampled from Usujiri Marine Biological station in 2000. Significant differences and groups are indicated by horizontal bars ($F = 44.44$, d.f. = 4,92, $P < 0.001$).

PURE SPECIES AND HYBRIDS ARE GENETICALLY AND MORPHOLOGICALLY DISTINCT

We found that all individuals exhibiting ‘classic’ morphology (from 229 individuals sampled in Japan 2000 including 28 *Hag*, 23 *Hoc*, and 24 *Hot*) were genetically scored as pure species. Furthermore, all individuals assigned as hybrids or backcrosses exhibited intermediate morphology. However, nine individuals exhibiting atypical morphological features were diagnosed as pure species, either indicating there is morphological plasticity (but not genetic heterogeneity at the loci examined) or, more likely, these individuals are backcrosses, and introgression is slightly greater than estimated.

Although assignments were made based on genetic data alone, there were diagnostic morphological features for both observed classes of hybrids. All F_1 *Hoc/Hag* hybrids are identifiable by a configuration of lat-

eral lines and number of cirri that is intermediate to parental species (e.g. missing fourth lateral line). Alternatively, F_1 *Hoc/Hot* hybrids can be diagnosed by standard length relative to the parental species *Hoc*. All individuals resembling *Hoc*, yet larger than 25 cm SL, were found to be an F_1 *Hoc/Hot* hybrid or backcrossed individual.

BACKCROSSES AND INTROGRESSION

Both the proportions of backcrosses observed and their genetic make up implicate fitness costs associated with backcrosses. The proportion of observed backcrosses is 8.26-fold less than expected considering the proportions of F_1 hybrid females. Even if we assume that individuals exhibiting intermediate morphology but scored as pure species are backcrosses, the observed frequency is still far less than

Table 3. Number of cytonuclear genotypes observed for hybrid population of *Hexagrammos* fishes ($N = 229$) at Usujiri Marine Fisheries Station, Japan 2000

mtDNA Haplotype	Nuclear genotype <i>CaM</i>							
	<i>Hag/Hag</i>	<i>Hoc/Hag</i>	<i>Hoc/Hoc</i>	<i>Hoc/Hot</i>	<i>Hot/Hot</i>	<i>Hot/Hag</i>	<i>Hag/Hag</i>	
<i>Hag</i>	38	0	1	0	0	0	38	
<i>Hoc</i>	0	12	99	29	0	0	0	
<i>Hot</i>	2	0	0	2	46	0	2	
<i>Hag</i>	38	0	1	0	0	0	38	

Cytonuclear disequilibria												
	<i>Hoc/Hag</i>				<i>Hoc/Hot</i>				<i>Hot/Hag</i>			
	D_1	D_2	D_3	D	D_1	D_2	D_3	D	D_1	D_2	D_3	D
With <i>CaM</i>	0.187	-0.021	-0.167	0.177*	0.153	0.037	-0.190	0.172*	0.236	0.000	-0.236	0.236
With <i>S7RP</i>	0.187	-0.021	-0.167	0.177*	0.153	0.020	-0.174	0.163*	0.237	0.000	-0.237	0.237

*Statistical significance.

Data shown are number of individuals in each haplotype (16S mtDNA) by genotype (*CaM*) class. Cytonuclear disequilibria statistics were calculated for each pairwise comparison between three hybridizing species: *Hexagrammos agrammos* (*Hag*), *Hexagrammos octogrammus* (*Hoc*), and *Hexagrammos otakii* (*Hot*). Consistent patterns of cytonuclear disequilibria were observed in both nuclear loci (*CaM* and *S7RP*).

expected. Another interesting aspect of the cytonuclear data for the observed backcrosses is their unexpected genetic make up (Table 2). The cytonuclear signature exhibited by *Hexagrammos* hybrids indicates potential introgression between all species and implicates sex-based directionality in interspecific matings or hybrid survivorship. Because F_1 hybrids are female and *Hoc* is their maternal ancestor, the genetic architecture in this system is expected to be profoundly affected, in that autosomal genes will move between all parental species but mitochondrial genes will move in one direction only, from *Hoc* to *Hot* or *Hag* via *Hoc* female-mediated introgression. Therefore, backcrossed individuals would be expected to exhibit *Hoc* as their maternal ancestor. Yet nine of 13 backcrossed individuals exhibit *Hag* or *Hot* as their maternal ancestors (Table 2). One explanation is that these individuals are backcrosses from *Hot/Hag* hybrids, but this is unlikely because the probability of detecting F_1 hybrids is greater than detecting backcrosses, and F_1 *Hot/Hag* hybrids were not observed. A more plausible explanation is that these individuals are advanced backcrosses between male offspring from an F_1 backcross and females of another species. This pattern suggests that first-generation backcrosses, with *Hoc* maternal ancestry, have reduced fitness, which would be consistent with interruption of gene flow, and the low number of backcrosses observed.

A final consideration is that F_2 hybrids are not possible because all F_1 hybrids are female.

MATING SYSTEM AND/OR SELECTION

There is clear evidence for assortative mating in pure species, and observed frequencies are significantly greater than would be expected with random mating (Fig. 3). However, rampant asymmetrical hybridization occurs in nature with hybrid frequencies equal to (*Hoc/Hag*) or exceeding (*Hoc/Hot*) values expected with random mating. The observed pattern of asymmetry could be explained by behaviour, natural selection, or a combination of both. For example, the significant pattern of cytonuclear disequilibrium could only be caused by features of the mating system or selection against hybrids such as negative epistatic interactions between mitochondrial cytotypes and nuclear genomic backgrounds (Asmussen *et al.*, 1987). First, prezygotic behavioural factors could be associated with asymmetrical hybridization expressed as preferences in mate choice such as sex-based directionality in interspecific matings. In this system, males and *Hoc* females are likely to be nondiscriminating. However, *Hot* females are likely to be discriminating because they have not been observed to mate heterospecifically (Munehara, Kanamoto and Miura, 2000), nor have F_1 hybrids with *Hot* maternal ancestry been detected. Alternatively, hybrid matings could

occur randomly but negative selection could eliminate progeny from some crosses. The absence of *Hexagrammos* hybrids from at least two hybrid classes where interspecific mating has been directly observed at the same site (Munehara *et al.*, 2000) implicates negative selection in the development of these hybrids (Fig. 3). When the frequency of hybrids is significantly less than expected under a closed system model, strong selection against hybrids has been invoked (Bert and Arnold, 1995; Helbig *et al.* 2001). One study on hybridization in *Mercenaria* clams inferred a complex pattern of selection acting on hybrids based on deviations from expected frequencies under a random mating model (Bert and Arnold, 1995). The authors inferred endogenous, negative selection when observed frequencies were less than expected frequencies, and exogenous, positive selection when observed frequencies exceeded expected frequencies.

In this system, both mate choice and selection against hybrids contribute to the maintenance of species boundaries. *Hot* females likely mate assortatively, but not males, *Hag* females, or *Hoc* females. Selection against hybrids is implicated in the maintenance of species boundaries following three lines of evidence. First, adult hybrids from several potential crosses are not observed despite direct observations of interspecific mating. Second, males are inviable in the F_1 generation, resulting in a minimum fitness cost of 50%. Finally, rampant hybridization would result in complete introgression and collapse of species boundaries unless interrupted by selection.

Of the six potential hybrid classes between these three species, only two are observed in nature as adults, even though several classes of interspecific mating have been observed by underwater video surveillance (Munehara *et al.*, 2000). The absence of F_1 hybrids between *Hot* and *Hag* is likely influenced by reproductive character displacement in the timing of reproduction for these species. There is no evidence that *Hot* females mate interspecifically but *Hot* males do. Interspecific mating with *Hag* females was observed underwater, and *Hoc/Hot* hybrids were the most abundant class of hybrids-with a frequency two-fold greater than expected with random mating. Although adult hybrids between *Hot* and *Hag* do not occur, hybridization with *Hoc* could mediate introgression between all three species. Indeed, evidence from backcrossed individuals indicates *Hot* and *Hag* mixed ancestry, but the overall proportions of backcrosses is low. Heterospecific mating was observed between *Hoc* females and *Hot* males, but also between *Hag* females and both *Hoc* and *Hot* males. Munehara *et al.* (2000) did not include observations of *Hag* male nests. However, the present study provides evidence that *Hag* males do mate interspecifically because *Hoc/Hag* hybrids were common as adults. To summarize, there

is direct evidence for four of the six types of interspecific matings, but adult progeny are observed for only two of those crosses. This suggests that selection against hybrids in at least two classes is contributing to differential survivorship.

Of the *Hexagrammos* hybrids that occur as adults, males are inviable in the F_1 generations, resulting in fitness costs associated with hybridization that are 50% or greater. The inviability of males is suggestive of Haldane's rule, which predicts that reduced fitness among F_1 hybrids tends to occur in the heterogametic sex (Haldane, 1922), is common in organisms exhibiting hybridization, and is indicative of ongoing speciation processes (Coyne and Orr, 1989). Karyotypes of four *Hexagrammos* species do not indicate obvious sex chromosomes (Matsumiya, Sakamoto & Nishikawa, 1980). However, these data suggest that sex is likely to be genetically determined in this group of fishes.

Clearly, there is potential for gene flow and introgression between hybridizing *Hexagrammos* species. Population genetic theory predicts that, in a neutrally evolving system, only one migrant per generation would eventually homogenize populations, regardless of population size. One of the most interesting aspects of this system is that species boundaries are being maintained despite hybrid frequencies equal to or greater than expected with random mating (i.e. rampant hybridization). This suggests that introgression is being interrupted by negative selection. Indeed, we found that the proportion of backcrosses was significantly less than expected given the observed proportions of F_1 hybrid females, and the genetic composition of backcrosses exhibited unexpected maternal ancestry suggesting that first-generation backcrosses, with *Hoc* maternal ancestry, exhibit reduced fitness. Therefore, selection must be enforcing fitness costs against hybrids at later life history stages.

MECHANISMS OF SPECIATION

Implicating models of speciation depends on a comparison of prezygotic vs. postzygotic reproductive isolation, and intrinsic (genetic incompatibilities) vs. extrinsic (ecological) negative selection. In the case of sympatric *Hexagrammos* species, divergent natural selection is implied in the evolution of reproductive season disparity, which is consistent with sympatric speciation. These sister taxa are most closely related, yet appear to exhibit the greatest degree of reproductive isolation because adult hybrids between these species are not observed. This is consistent with prezygotic isolation evolving faster in sympatry relative to allopatric species pairs. However, interspecific mating was observed between *Hag* females and *Hot* males; therefore, we have argued that intrinsic genetic

incompatibilities also may play a role in the reproductive isolation of these species.

In the case of allopatric species pairs, genetic incompatibilities are expected to cause developmentally-mediated loss of fitness in hybrids. We have argued that may be the case because *Hag/Hoc* hybrids do not occur as adults, even though interspecific mating has been observed and, more importantly, because males are inviable in the adult hybrids that do occur. However, the relative proportion of adult F_1 hybrids between allopatric species pairs *Hoc/Hag* and *Hoc/Hot* was quite unexpected because it predicts complete introgression between these species. Therefore, we invoke negative selection at various life history stages because introgression appears to be minimal.

CONCLUSIONS AND SIGNIFICANCE

Although the species status of fishes in the genus *Hexagrammos* is supported, reproductive isolation between *Hot*, *Hag* and their allopatric relative *Hoc* is not complete. Here, we reveal a complex system in nature with striking patterns and interesting characteristics. First, this system is unique because introgression could occur between three species via hybridization from species pairs that are not closest relatives. Second, patterns of hybridization in this system exhibit characteristics of antagonistic evolutionary processes. Proportions of two hybrid classes are equal to or exceed values expected with random mating, and we observe viable adult backcrosses indicating potential for gene flow and introgression. However, *Hexagrammos* species are genetically and morphologically distinct. How could species boundaries be maintained in the presence of introgression via hybridization, at the levels observed? The absence of some classes of hybrids despite observed mating in nature, the inviability of male hybrids, and low levels of introgression imply gene flow is interrupted by strong selection against hybrids at various life history stages. Further studies should be conducted to determine whether differential survivorship occurs between all pairwise crosses of hybrid matings in this genus. By parsing intrinsic vs. extrinsic negative selection, and prezygotic vs. postzygotic reproductive isolation, the relative importance of fitness costs associated with hybridization can be evaluated to implicate potential mechanisms of speciation. Because this complex system represents a rare opportunity to address the genetic architecture of reproductive isolation in nature, the significance of this research extends beyond the natural history of the genus *Hexagrammos*.

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