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## Sympatric speciation in a genus of marine reef fishes

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### Abstract

Sympatric speciation has been contentious since its inception, yet is increasingly recognized as important based on accumulating theoretical and empirical support. Here, we present a compelling case of sympatric speciation in a taxon of marine reef fishes using a comparative and mechanistic approach. *Hexagrammos otakii* and *H. agrammus* occur in sympatry throughout their ranges. Molecular sequence data from six loci, with complete sampling of the genus, support monophyly of these sister species. Although hybridization occurs frequently with an allopatric congener in an area of slight distributional overlap, we found no  $F_1$  hybrids between the focal sympatric taxa throughout their coextensive ranges. We present genetic evidence for complete reproductive isolation based on SNP analysis of 382 individuals indicating fixed polymorphisms, with no shared haplotypes or genotypes, between sympatric species. To address questions of speciation, we take a mechanistic approach and directly compare aspects of reproductive isolation between allopatric and sympatric taxa both in nature and in the laboratory. We conclude that the buildup of reproductive isolation is strikingly different in sympatric vs. allopatric taxa, consistent with theoretical predictions. Lab reared hybrids from allopatric species crosses exhibit severe fitness effects in the  $F_1$  or backcross generation. No intrinsic fitness effects are observed in  $F_1$  hybrids from sympatric species pairs, however these treatments exhibited reduced fertilization success and complete pre-mating isolation is implied in nature because  $F_1$  hybrid adults do not occur. Our study addresses limitations of previous studies and supports new criteria for inferring sympatric speciation.

**Keywords:** allopatric, genomic conflict, reproductive isolation, speciation, sympatric

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### Introduction

Sympatric speciation is the evolution of reproductive isolation in the absence of geographic barriers. This topic has been hotly debated throughout the history of evolutionary biology, but it has gained increasing support from empirical work and theoretical models predicting its plausibility in a variety of natural conditions [Dieckmann & Doebeli 1999; Kirkpatrick & Ravigne 2002; Coyne & Orr 2004; Gavrillets 2004; Doebeli *et al.* 2005; Barluenga *et al.* 2006; Savolainen *et al.* 2006; Schlieuwen *et al.* 2006; reviewed in Via (2001)]. However, empirical studies in natural settings are still scarce

(Coyne & Orr 2004; Bolnick & Fitzpatrick 2007)—often occurring in restricted environments and based largely on a limited number of taxa. With a theoretical framework in place, the focus of the sympatric speciation debate has now shifted from establishing its plausibility to determining how commonly and in what contexts, it occurs in nature (Bolnick & Fitzpatrick 2007).

Coyne & Orr (2004) outline four criteria to infer sympatric speciation: a sympatric distribution, a monophyletic sister species relationship, complete reproductive isolation and an ecological setting where allopatric speciation is unlikely, such as an isolated island habitat or a crater lake of recent origin. However, considering only those taxa stringently consistent with the fourth criterion may be limiting our understanding of the importance of sympatric speciation in a variety of

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natural settings. Marine settings may well be the antithesis of isolated and recently emerged environments, yet together form the largest habitat on earth. Divergence with gene flow is likely to dominate marine systems due to high dispersal potential and large migration rates. Therefore sympatric speciation may play a larger role in this environment compared to terrestrial habitats with more potential for vicariance. Although, empirical evidence supports surprisingly high levels of population structure in a variety of marine taxa despite high connectivity and dispersal potential (Jones *et al.* 1999; Swearer *et al.* 1999), therefore allopatric divergence is still considered important.

It has been argued that allopatric speciation is the null model against which sympatric speciation must be weighed (Futuyma & Mayer 1980; Coyne & Orr 2004). However, others have argued that this approach is too conservative because of sampling bias (i.e. limited taxa investigated) and insufficient or inconclusive data that fail to reject the null hypothesis, leading to a high probability of a Type II error (Berlocher 1998; Mallet 2005; Bolnick & Fitzpatrick 2007). As such, several authors have called for a shift from geographic inference to a more mechanistic approach to differentiating speciation processes (Via 2001; Bolnick & Fitzpatrick 2007; Fitzpatrick *et al.* 2008; Flitzpatrick *et al.* 2009). Under this approach, theoretical predictions based on models of speciation processes may be used to evaluate sympatric and allopatric speciation on an equal basis, allowing broader consideration of the circumstances in which sympatric speciation may occur.

Theoretical expectations for sympatric vs. allopatric speciation differ with respect to both the mechanism and rate of divergence driving speciation and predict differences in the build up of pre-zygotic and post-zygotic processes (Turelli *et al.* 2001). Allopatric speciation is caused by genetic drift or selection that results in disruption of co-adapted gene complexes in hybrids (Dobzhansky 1936; Muller 1942). These Dobzhansky-Muller incompatibilities are due to differences in expression patterns and dominance for co-adapted gene complexes exhibiting epistasis. Dobzhansky-Muller incompatibilities explain intrinsic or developmentally mediated, loss of fitness in hybrids (Rice & Hostert 1993; Blier *et al.* 2001; Turelli *et al.* 2001) and their role is widely accepted in the evolution of post-zygotic isolation between allopatric species [Turelli & Orr 2000; Porter & Johnson 2002; reviewed in Lijtmaer *et al.* (2003)]. In sympatry, Coyne & Orr (1989, 1997) observed, with remarkable consistency, that pre-zygotic isolation evolves faster than post-zygotic isolation relative to allopatry. In sympatric speciation, intrinsic post-zygotic isolation is not expected to evolve initially, rather extrinsic ecological factors are associated with

the build up of reproductive isolation (Coyne & Orr 2004). Another key finding regarding sympatric speciation is that it can readily occur when selection acts on reproductive isolation as a correlated character via pleiotropy (Kondrashov & Mina 1986; Rice & Salt 1988, 1990) or if linkage disequilibrium occurs between divergently selected loci and loci affecting mate choice (Felsenstein 1981; Gavrillets 2003). In other words, sympatric speciation can readily occur when divergent selection acts on characters that directly or indirectly affect assortative mating (Gavrillets 2003), such as divergence in mating season/flowering time or ecological/habitat specialization when mating occurs locally in the adaptive habitat. Empirical work has shown that sympatric species generally experience multiple selective forces or multifarious natural selection, on resource use, habitat choice, mate choice and life history traits [reviewed in Via (2001)].

Here, we present evidence for sympatric speciation in a complex marine system with features that, remarkably, allow us to make a direct comparison between sympatric and allopatric speciation processes among species in the same genus, thereby controlling for phylogenetic effects. We focus on three species of marine fishes (genus *Hexagrammos*) that occur in the North-western Pacific. The southern species, *H. agrammus* and *H. otakii* are completely sympatric, with coextensive geographic ranges. These ranges overlap slightly with their northern, mostly allopatric congener, *H. octogrammus* (Fig. 1). Hybridization has been reported within this area of overlap, based on genetics and the occurrence of morphological intermediates (Balanov & Antonenko 1999; Balanov *et al.* 2001; Crow *et al.* 2007). In one area,

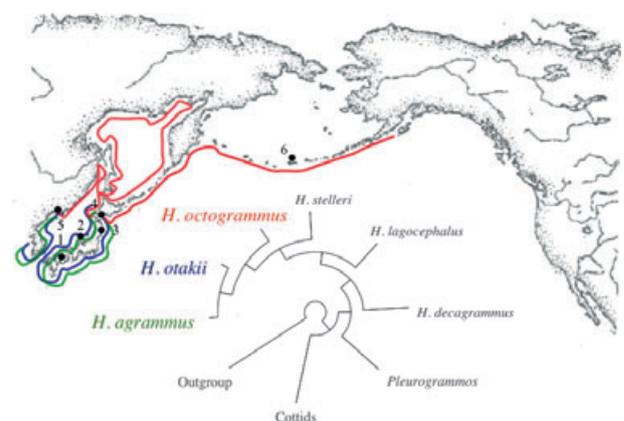


Fig. 1 Geographic distributions of three *Hexagrammos* species and Hexagrammid phylogeny [adapted from Crow *et al.* (2004)]. Dots indicate sampling locations at Nakajima Marine Biological Station<sup>1</sup>, Noto Marine Laboratory<sup>2</sup>, Otsuchi Marine Research Center<sup>3</sup>, Usujiri Marine Biological Station<sup>4</sup>, Peter the Great Bay, Russia<sup>5</sup>, and Kachemak Bay, Alaska<sup>6</sup>.

hybridization occurs at surprisingly high frequencies (Crow *et al.* 2007). These adult hybrids occurring in nature are progeny from allopatric species pairs only and hybrids between the two sympatric species *H. agrammus* and *H. otakii* were not observed. Hybridization is asymmetric, as *H. octogrammus*, the mostly allopatric northern species, is the maternal ancestor of all F<sub>1</sub> hybrids sampled. For example, naturally occurring hybrids are genetically intermediate between *H. octogrammus* and *H. agrammus*, or *H. octogrammus* and *H. otakii*, hereafter referred to as *Hoc/Hag* or *Hoc/Hot* respectively, with hybrid notation indicating the maternal ancestor first. Finally, all F<sub>1</sub> hybrids sampled are female (Crow *et al.* 2007), likely resulting from Haldane's (1922).

This system offers a unique opportunity to study processes of speciation using a mechanistic approach because the focal sympatric species pair and allopatric species pairs can still be artificially crossed in the laboratory, producing offspring that exhibit a range of phenotypes. Therefore, we were able to perform genetic crosses between all species pairs to directly compare aspects of pre-zygotic and post-zygotic reproductive isolation in both sympatric and allopatric species pairs. We invoke allopatric speciation as the null only because it is most conservative with which to test our specific hypotheses, while acknowledging it may be too conservative for some studies of sympatric speciation. We demonstrate that the sympatric species *H. otakii* and *H. agrammus* meet three of the four criteria for sympatric speciation as outlined by Coyne & Orr (2004), and make no assumption about the historical geographic distributions of modern sympatric species *H. otakii* and *H. agrammus*. Rather, we directly test the hypothesis that there is a difference in the build up of reproductive isolation between species evolved in

sympatry vs. allopatry, as manifest by pre-mating isolation or gamete incompatibility (pre-zygotic RI) and/or intrinsic negative fitness effects (post-zygotic RI). Using this comparative approach, we find that patterns of hybrid fitness and genomic conflict are strikingly different between sympatric species *H. otakii* and *H. agrammus*, relative to allopatric pair crosses among taxa in the same phylogenetic, geographic and ecological system. We conclude that there is no evidence supporting allopatric speciation for the focal sympatrically distributed species, based both on comparison of speciation processes and fit to theoretical predictions about the mechanisms of speciation. Finally, we provide circumstantial evidence further supporting sympatric speciation by describing a suite of divergent morphological and ecological characters that simultaneously affect reproductive isolation via pleiotropy-consistent with theoretical expectations and likely resulting from divergent natural selection.

## Materials and methods

### Sampling

A total of 484 individuals of *Hexagrammos* species (*H. otakii*, *H. agrammus*, *H. octogrammus* or their hybrid intermediates) were sampled from various sites within their respective ranges (Fig. 1) including four sites in Japan (Nakajima Marine Biological Station, Ehime University; Noto Marine Laboratory, Kanazawa University; Otsuchi Marine Research Center, University of Tokyo; and Usujiri Marine Biological Station, Hokkaido University), three sites in Russia within Peter the Great Bay (Vityas Bay; Vostok Bay, and Amursky Bay, Institute of Marine Biology, Russian Academy of Sciences in

**Table 1** Sampling locations and year for 484 *Hexagrammos* species

	Sample location			1998	2000	1998	2000	2002	Total
	Naka	Noto	Ots	Usu	Usu	PGB	PGB	Kach	
<i>H. agrammus</i>	10	11	24	8	<b>38</b>				91
<i>H. otakii</i>			10	4	<b>42</b>		3		59
<i>H. octogrammus</i>			1	19	<b>97</b>		<b>112</b>	15	244
F <sub>1</sub> Hybrid <i>Hoc/Hot</i>				9	<b>27</b>	1	2		39
F <sub>1</sub> Hybrid <i>Hoc/Hag</i>			2	2	<b>12</b>	5	3		24
Backcross, mixed ancestry, not F <sub>1</sub>			6	2	<b>13</b>		6		27
									484
Genotypes verified by SNP analysis			8	13	<b>229</b>	6	<b>126</b>		<b>382</b>

Sample genotypes verified by SNP analysis shown in bold. Code for sample location: Naka, Nakajima Marine Biological Station, Japan; Noto, Noto Marine Laboratory, Japan; Ots, Otsuchi Marine Research Center, Japan; Usu, Usujiri Marine Biological Station, Japan; PGB, Peter the Great Bay, Russia; Kach, Kachemak Bay, Alaska.

Vladivostok), and one site in Alaska (Kachemak Bay) by trap, trammel net, spear or hook and line between 1998 and 2002 (Table 1). Muscle or fin tissue was collected and preserved in ethanol for standard DNA extraction (described in Crow *et al.* 2004, 2007).

#### *Monophyly of sympatrically distributed sister species H. otakii and H. agrammus*

A molecular phylogeny of the family Hexagrammidae was constructed based on six loci including two mitochondrial genes—cytochrome b (*cytb*) and 16S ribosomal subunit (16S), and four nuclear introns from the following loci: S7 ribosomal protein (*S7RP*), Calmodulin (*CaM*), creatine kinase (*ck*) and lactate dehydrogenase gene (*Ldh*). Sampling, DNA extraction and PCR conditions, sequence analysis and GenBank Accession numbers are described in Crow *et al.* (2004). Three to 12 individuals of *H. agrammus*, *H. otakii* and *H. octogrammus* (from various locations throughout their respective ranges) were sequenced at all six loci (Fig. 1, Table 1). Monophyly of *H. agrammus* and *H. otakii* and the phylogenetic relationships within the genus, with complete taxon sampling, were assessed using the combined data set of all six loci. In addition, timing of divergence between *H. octogrammus*, *H. agrammus* and *H. otakii* was estimated based on molecular divergence. Sequence divergence of the mitochondrial cytochrome b was used, with a generally accepted rate of 1.5–2.5% per million years for fishes (McCune & Lovejoy 1998). Sequence divergence was estimated with an optimal substitution model for our data, as determined by Modeltest 3.7 [Posada & Crandall (1998), a TrN+I+G model was obtained].

#### *Evidence for complete reproductive isolation*

Sequences indicated fixed differences between species at all six genetic loci. In addition to sequencing several individuals of each species sampled throughout their respective ranges, fixation of single nucleotide polymorphisms (SNPs) was verified by restriction mapping of an additional 382 individuals (Table 1). We used this assay to genetically identify hybrid intermediates and to verify that there were no shared haplotypes or genotypes between *H. agrammus* and *H. otakii*. To accomplish this goal, we designed the restriction mapping assay based on SNPs that are diagnostic for *H. agrammus*, *H. otakii* and *H. octogrammus* (Table 1) from three loci. The 16S, *CaM* and *S7RP* loci exhibit fixed differences between *Hexagrammos* species corresponding to unique restriction sites for each species and all possible combinations of F<sub>1</sub> hybrid parental ancestry. The restriction enzyme *EcoNI* (New England BioLabs, Inc.) was used to restrict PCR products of the 16S mtDNA locus of

*H. octogrammus* in two sites, one site for *H. otakii* and did not restrict 16S product of *H. agrammus*. The restriction enzymes *BsmI* and *BssHII* (New England BioLabs, Inc.) were used to restrict *CaM* PCR products of *H. otakii* and *H. agrammus* respectively and the restriction enzyme *BbsI* (New England BioLabs, Inc.) was used to restrict *H. octogrammus* *S7RP* PCR products (Table 2). PCR products were run (simultaneously with products from both restriction reactions for *CaM*) on 1% agarose gels and visualized using ethidium bromide. Every individual that exhibited intermediate morphological characters and every individual sampled in 2000 for a concurrent hybridization study was genotyped at three diagnostic loci for assignment of pure species or hybrid status ( $n = 382$ , Table 1). Using this approach we were able to identify F<sub>1</sub> hybrids and individuals of pure species ancestry. All F<sub>1</sub> hybrids were detected via heterozygosity at two nuclear loci and because mtDNA is maternally inherited in fishes, the 16S mtDNA locus enabled us to elucidate the maternal ancestor of individuals derived from hybrid matings to infer parentage and patterns of asymmetric hybridization. In addition we were able to identify individuals of backcrossed descent—albeit an underestimate based on only two nuclear markers (as discussed in Crow *et al.* 2004), as those individuals that were not consistently heterozygous at both nuclear loci.

In addition to genetic markers that distinguish *H. agrammus*, *H. otakii* and *H. octogrammus*, these species are morphologically distinct in a number of characters including variation the number of lateral lines, supra-orbital cirri and caudal fin morphology (Fig. 2). For example *H. agrammus* exhibits one lateral line, whereas *H. otakii* and *H. octogrammus* exhibit five. However the latter two are differentiated by differences in number of cirri and caudal morphology (Fig. 2, Crow *et al.* 2007). Further, we compared differences in standard length from mature adults sampled in Japan 2000 of *H. otakii* ( $n = 35$ ) and *H. agrammus* ( $n = 33$ ) via two tailed, Student's *t*-test. We mapped SL, lateral line configuration, number of orbital cirri and caudal fin morphology for every individual sampled ( $n = 484$ ) to identify individuals with intermediate morphologies as potential hybrids.

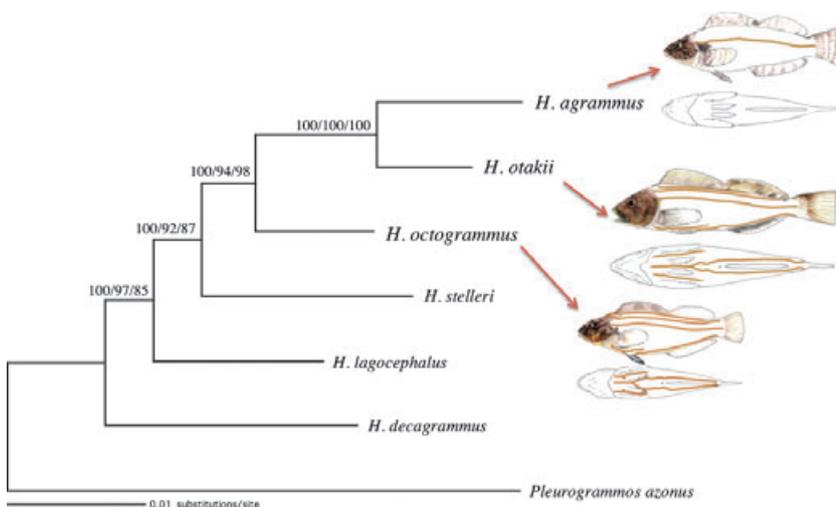
#### *Comparison of reproductive isolation patterns and processes in sympatry vs. allopatry*

In this study, we directly compare speciation processes between sympatric and allopatric taxa by comparing fitness costs associated with interspecific mating and patterns of genomic incompatibilities between all pairwise crosses of *H. agrammus*, *H. otakii* and *H. octogrammus*. Specifically, we compared pre-zygotic and post-zygotic

**Table 2** Twenty-seven single nucleotide polymorphisms that distinguish three *Hexagrammos* species

Locus	GenBank accession	SNP alignment position identity	Polymorphism Hag/Hot/Hoc	Enzyme and recognition sequence
16S (46497 bp)	AY583111 ( <i>Hag</i> )	97	A/G/G	
	AY583112 ( <i>Hot</i> )	<b>201</b>	—/C/T	*EcoNI cuts <i>Hoc</i> CCTAATAAAGG
	AY583113 ( <i>Hoc</i> )	<b>207</b>	<b>G/G/A</b>	*EcoNI cuts <i>Hoc</i> CCTAATAA <u>AGG</u>
		264	C/T/T	
		291	T/C/T	
CaM (352 bp)	AY583169 ( <i>Hag</i> )	48	T/T/G	
	AY583170 ( <i>Hot</i> )	51	A/G/A	
	AY583171 ( <i>Hoc</i> )	53	C/C/T	
		105	T/A/T	
		148	A/G/A	
		149	C/C/T	
		156	G/G/A	
		193	T/T/-	
		205	G/G/A	
		220	C/C/A	
		248	T/C/T	
		250	C/C/A	
		<b>261</b>	<b>T/C/T</b>	*BSMI cuts <i>Hot</i> GCATTC
		271	T/C/T	
	<b>274</b>	<b>G/A/A</b>	*BSSHII cuts <i>Hag</i> GCGCGC	
	<b>279</b>	<b>C/C/T</b>	*BSSHII cuts <i>Hag</i> GCGCG <u>C</u>	
	301	G/C/C		
57RP (490 bp)	AY583188 ( <i>Hag</i> )	46	T/T/A	
	AY583189 ( <i>Hot</i> )	83	C/T/T	
	AY583190 ( <i>Hoc</i> )	143	A/A/G	
		220	G/G/A	
		242	G/G/T	
		243	T/T/G	
		247	A/A/C	
		<b>248</b>	<b>C/C/G</b>	*BbsI cuts <i>Hoc</i> <u>GTCTTC</u>
		445	C/C/T	

Sample genotypes verified by SNP analysis shown in bold.



**Fig. 2** Phylogram indicating genetic relationships of fishes in the genus *Hexagrammos* based on six molecular markers including two mtDNA loci and four nuclear introns. Statistical support at nodes is expressed as BPP/MP/NJ. *Pleurogrammus azonus* is used as the outgroup. Morphological character sets that are diagnostic for species include differences in the number of lateral lines, supra-orbital cirri and caudal fin morphology.

fitness components affecting fertility and viability including fertilization success, embryonic development, hatching success and larval survivorship from pure

crosses, reciprocal hybrid crosses and introgressive (i.e. F<sub>1</sub> hybrid backcross) crosses. F<sub>2</sub> crosses are not possible because male F<sub>1</sub> hybrids do not occur.

### Fertilization and development

Laboratory crosses were performed to compare fertilization success, development and survivorship all from possible combinations of crosses ( $n = 13$ , Table 3). Egg incubation and larval rearing occurred in a 'common garden' setting with shared, recirculating sea water for 30 days after hatching. Each treatment cross was replicated three times. Each parental individual was genotyped to verify morphological species assignment and, as a result, some replicates were lost from some treatments (summarized in Table 1) due to incorrect assignment (e.g. a backcrossed individual was mistaken for a pure species before genetic characterization). Gametes were extracted from gravid adults and fertilized *in vitro*. Fertilization was indicated by cleavage 1–2 h later and fertilization success was roughly estimated by counting the proportion of eggs fertilized in a randomly chosen microscopic field of view. Starting size of egg masses was comparable in each replicate (i.e. eggs approximately formed a single layer on the bottom of a Petri dish) and contained approximately 500–750 eggs. Fertilized clutches were transferred to 10 L buckets fitted with a 100  $\mu$  mesh screen to allow water flow. Filtered seawater was passed through ultraviolet sterilization (OmniZap UV Sterilizer, 25 Watt UV, 17 inches long, 3 inches diameter) before flowing into a temperature controlled holding tank with a crushed coral biological filter. Water temperature was set to mimic natural spawning conditions (between 14 and 17 °C). Water was then pumped from this source into buckets containing clutches, by passing through a bubbler for oxygenation. To maintain a constant temperature environment, treatment buckets were immersed in circulating water within each water table. Because larval fish

tend to swim into the current, swim more in high light environments and expend copious amounts of energy compensating for turbulence (Duray *et al.* 1997), water tables were maintained in a darkened room and every effort was made to standardize water flow into each bucket and reduce turbulence. Clutches were incubated for 20–23 days until hatching. Larval development was characterized as normal or abnormal at hatching. Hatching success was characterized as good (most hatched) or impaired (few hatched).

### Larval survivorship

After hatching, 121 individuals were placed in each treatment bucket to control for density dependent effects. Beginning 1 day after hatching, larvae were fed rotifers (*Brachionus* sp.) three times daily and brine shrimp (*Artemia* sp.) once per day beginning on day 7. Rotifers were enriched with nutrients ('Aquaran-Special Fish Egg Powder Plus' nutrient enrichment; BASF Japan Ltd, Tokyo, Japan) for at least 1 h before being fed to larvae. Brine shrimp were enriched with nutrients for less than 1 h because they tended to exhibit reduced tolerance to the nutrient treatment. The density of rotifers and brine shrimp presented to larvae at each feeding were approximately 6–10 per mL and 2–4 per mL respectively. Food densities remained constant regardless of the number of surviving larvae. Surviving larvae were counted each day and dead larvae removed, until no larvae remained or 30 days had passed. Survivorship curves were log transformed for comparison of mortality rates. The effect of treatment cross on larval survivorship slopes was assessed by ANOVA. Treatments were then combined into two categories—pure (within) and hybrid (between) crosses—and further compared by a general contrast (one degree of freedom) test for differences in slope. The variability in survivorship among treatments was evaluated by comparing treatment coefficients of variation (CV) of slopes using ANOVA. The care and handling of all vertebrate animals used in this study was in compliance with the Institutional Animal Care and Use Committee (IACUC) and NIH policies.

**Table 3** All pairwise combinations of pure, hybrid and backcross treatments in larval survivorship experiments

Specific crosses	F1 hybrid crosses	Backcrosses
Hag × Hag (4)	Hoc × Hag* (2) Hag × Hoc (3)	Hoc/Hag × Hoc (2) Hoc/Hag × Hag (3)
Hoc × Hoc (3)	Hoc × Hot* (3) Hot × Hoc (3)	Hoc/Hot × Hoc (2) Hoc/Hot × Hot (3)
Hot × Hot (3)	Hot × Hag (3) Hag × Hot (1)	

Number of successful replicates is shown in parentheses.

\*Hybrids that occur in nature as adults.

### Results

#### *Monophyly of sympatrically distributed sister species H. otakii and H. agrammos*

For inferring the monophyly of sister taxa *H. otakii* and *H. agrammos* and the phylogenetic relationships of all members of the genus *Hexagrammos*, three to 12 individuals of each species, exhibiting diagnostic morphology and sampled from various locations throughout their ranges, were sequenced at six loci including two mito-

chondrial genes and four nuclear introns [as described in Crow *et al.* (2004)]. For each locus, few or no differences (i.e. zero to four polymorphisms) were found within species. However, fixed differences between species were identified at each locus corresponding to 31 SNP loci (Table 2). Of the 2013 characters analysed, 204 were variable with 69 parsimony informative sites. Monophyly of sister taxa *H. agrammus* and *H. otakii* was inferred by all analyses with high levels of support (Fig. 2). Because there were no significant differences in tree topologies for the genus *Hexagrammos* from individual loci, data sets were combined to obtain a single most-parsimonious tree, which was identical to the neighbour-joining tree and to the maximum-likelihood tree (tree length = 260 steps, Fig. 2). The data indicate that *H. agrammus* and *H. otakii* are indeed closest relatives with a sequence divergence of 1.78% and 100% statistical support (based on all six loci combined, indels removed). *H. octogrammus* is the sister taxon of this clade with sequence divergences of 3.02% and 2.43% from *H. agrammus* and *H. otakii* respectively. *H. stelleri* is the next closest relative followed by *H. lagocephalus*, with *H. decagrammus* as the most ancestral within the genus (Fig. 2). The genus *Pleurogrammus* forms a monophyletic sister clade of the genus *Hexagrammos* (Crow *et al.* 2004). There were no shared genotypes or haplotypes between sympatric species *H. agrammus* and *H. otakii*, therefore we found no evidence to question their species status. The monophyly of *H. agrammus* and *H. otakii* is further supported by a previous phylogenetic study based on meristics and comparative osteology (Shinohara 1994).

#### Evidence for complete reproductive isolation

*Hexagrammos agrammus* and *H. otakii* are morphologically distinct. In addition to diagnostic character sets including number and configuration of lateral lines, supraorbital cirri, and caudal fin morphology, they exhibit significant differences in standard length (SL,  $P = 1.694 \times 10^{-11}$ ,  $t = 8.0127$ ,  $df = 71$ , two-tailed, equal variances, from sexually mature adults sampled in Japan 2000), where mean SL for *H. otakii* was 28.28 cm ( $n = 38$ ) and 20.63 cm for *H. agrammus* ( $n = 35$ ). Because *H. otakii* is significantly larger in standard length than *H. octogrammus* and *H. agrammus*, we found that standard length was an additional diagnostic feature for adult *Hoc/Hot* F<sub>1</sub> hybrids. For example, all individuals with round caudal morphology, but standard length greater than 24 cm, was later verified to be a hybrid between *H. octogrammus* and *H. otakii* (Crow *et al.* 2007). We mapped diagnostic morphological character sets for every individual sampled ( $n = 484$ ) to identify individuals with intermediate morphologies as potential hybrids. While there were no individuals with intermediate morphology

sampled in the southern sites where the sympatric species *H. agrammus* and *H. otakii* co-occur (i.e. in Nakajima or Noto), several individuals with intermediate morphology were sampled in other locations (Table 1).

We designed a genetic assay to establish patterns of reproductive isolation between these *Hexagrammos* species based on restriction mapping of single-nucleotide polymorphisms (SNPs) that were diagnostic for species and all combinations of F<sub>1</sub> hybrids. There were five, 17, and nine SNPs between species in the *16S*, *CaM* and *S7RP* loci respectively (Table 2). Fixation of six SNPs was further confirmed by restriction mapping of an additional 382 individuals of *H. agrammus*, *H. otakii* and *H. octogrammus* (or hybrid verification of morphological intermediates). We genotyped all morphological intermediates sampled from any site and year, and all individuals sampled in year 2000 at two sites where hybrids have previously been observed (Usujiri and Peter the Great Bay,  $n = 355$ ) and not a single individual was found to be an F<sub>1</sub> hybrid between *H. otakii* and *H. agrammus*.

While morphological intermediates were not observed at Noto Marine Laboratory, or Nakajima Marine Biological Station (Fig. 1), 90 individuals exhibiting morphological variations were genetically verified as F<sub>1</sub> hybrids or backcrosses from the northern sites where the distribution of the sympatric species overlaps slightly with *H. octogrammus*, at Otsuchi, Usujiri, and within Peter the Great Bay [described in Crow *et al.* (2007)]. F<sub>1</sub> hybrids were of two types only: (i) between a female *H. octogrammus* and male *H. agrammus* ( $n = 24$ ) or (ii) a female *H. octogrammus* and male *H. otakii* ( $n = 39$ , Table 2). From all *Hexagrammos* fishes sampled ( $n = 484$ ) across sites and sampling dates, no F<sub>1</sub> hybrids between *H. otakii* and *H. agrammus* were observed (Table 1).

#### Comparison of reproductive isolation patterns and processes in sympatry vs. allopatry

We performed genetic crosses between all possible species pairs (Table 3) to directly compare aspects of pre-zygotic and post-zygotic reproductive isolation (RI) between species pairs distributed in sympatry and allopatry. Aspects of pre-zygotic RI included pre-mating isolation inferred from frequencies of hybrids that occur in nature and fertilization success from genetic crosses. For post-zygotic RI we looked at fitness components affecting viability such as embryonic development, hatching success and larval survivorship from pure crosses, hybrid crosses and backcrosses. Using a comparative approach, we found that patterns of hybrid fitness and genomic conflict were strikingly different in larvae from the sympatric pair *H. otakii* and *H. agrammus*, relative to allopatric pair crosses in this system.

*Fertilization, development, hatching and survivorship for pure crosses*

All replicates of pure species crosses were consistent and therefore established a standard for normal fertilization success, development, hatching success and larval survivorship. For example all replicates of pure species crosses exhibited >90% fertilization success,

normal larval development, and uniformly high hatching success ( $n = 10$ , Tables 3 and 4, Figs 3 and 4). Survivorship curves were steep in pure species crosses and hybrid crosses producing viable fry had similar results. Surprisingly, differences in post-zygotic RI were only manifest as developmental abnormalities occurring before hatching and did not include differences in survivorship.

**Table 4** Summary of fitness components characterized at various stages in larval development in pure, hybrid and backcross treatments between three species of *Hexagrammos* fishes. Notation for hybrid crosses indicate maternal ancestor first. Comments in red indicate negative fitness costs associated with hybridization relative to parental crosses

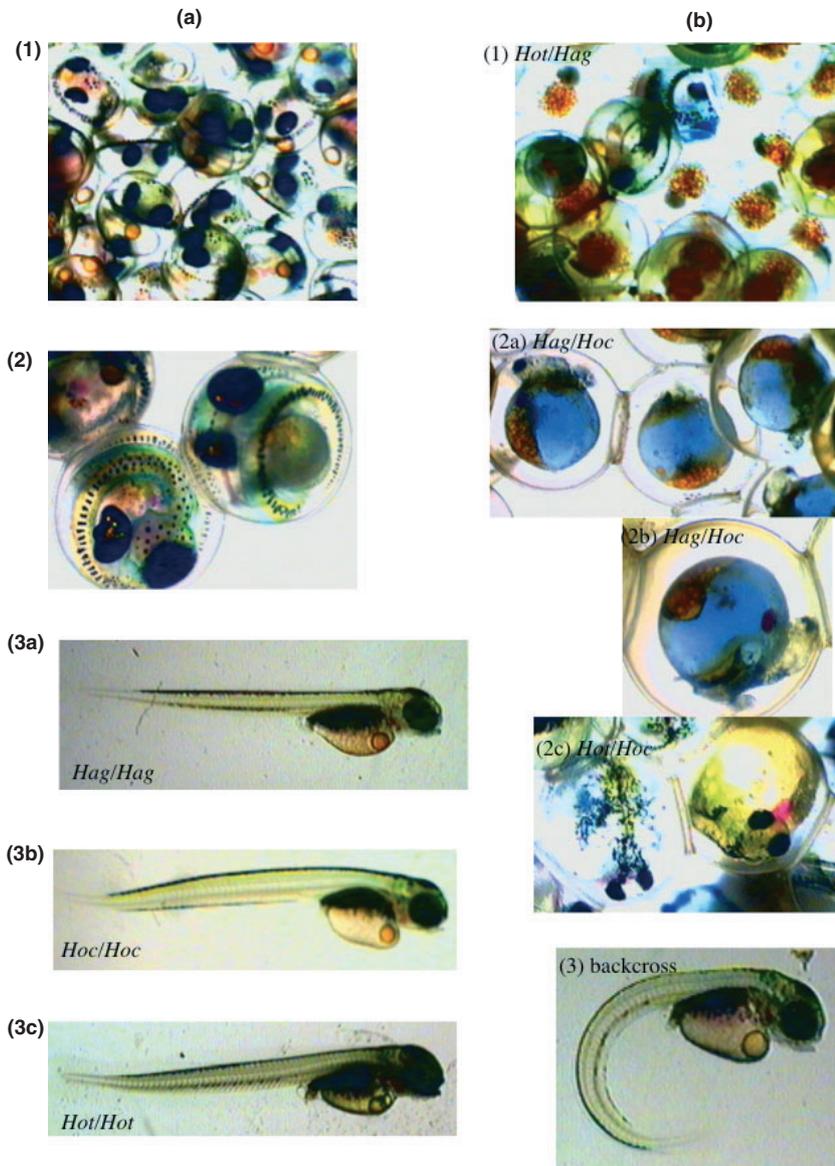
Cross	<i>n</i>	Mating in nature	Fertilization success	Larval development	Hatching	Larval survivorship	Adults in nature	GSI adults	backcrosses
<b>Pure</b>									
<i>Hag</i> × <i>Hag</i>	4	Yes	Good	Normal	Good	Normal	Yes		
<i>Hoc</i> × <i>Hoc</i>	3	Yes	Good	Normal	Good	Normal	Yes		
<i>Hot</i> × <i>Hot</i>	3	Yes	Good	Normal	Good	Normal	Yes		
<b>Hybrid</b>									
<i>Hoc/Hot</i>	3	Yes	Good	Normal	Good	Normal	Yes	reduced	not ok
			Good	Normal	Good	Normal	Obs >> exp		
			Good	Normal	Good	Normal			
<i>Hot/Hoc</i>	3	No	Good	Abnormal	None	N/A	No	N/A	
			Reduced	Abnormal	None	N/A			
			Poor	Abnormal	None	N/A			
<i>Hoc/Hag</i>	2	Yes	Good	Normal	Good	Normal	Yes	reduced	ok
			Good	Severely retarded	Impaired	Normal	Obs = exp		
					(121 hatched)				
<i>Hag/Hoc</i>	3	Yes	Good	Normal	Good	Normal	No	N/A	
			Good	Severely abnormal	None	N/A			
			Good	Severely abnormal	None	N/A			
<i>Hag/Hot</i>	1	Yes	Reduced	Normal	Good	Normal	No	N/A	
<i>Hot/Hag</i>	3	No	Reduced	Normal	good	Normal	No	N/A	
			Reduced	Normal	good	Normal			
			None	N/A	N/A	N/A			
<b>Backcross</b>									
<i>Hoc/Hot-Hoc</i>	5	N/A	Good	Severely retarded	Impaired	N/A	Probably	N/A	
					(six hatched)				
<i>Hoc/Hot-Hoc</i>			Reduced	No data	No data	Steep slope			
<i>Hoc/Hot-Hot</i>			Poor	Retarded	Impaired	Normal			
					(35 hatched)				
<i>Hoc/Hot-Hot</i>			Good	Mostly normal	Some deformed	Normal			
<i>Hoc/Hot-Hot</i>			Good	Normal	Good	Normal			
<i>Hoc/Hag-Hoc</i>	5	N/A	Poor	Severely retarded	Impaired	Steep slope	Probably	N/A	
					(124 hatched)				
<i>Hoc/Hag-Hoc</i>			Good	Normal	Good	Normal			
<i>Hoc/Hag-Hag</i>			Good	Normal	Good	Normal			
<i>Hoc/Hag-Hag</i>			Good	Normal	Good	Normal			
<i>Hoc/Hag-Hag</i>			Good	Normal	Good	Normal			

Fertilization success: good = 90–100%, reduced = 50–89%, poor = 6–49%, none = 0–5%

Larval development: normal = uniform development, retarded = arrested development, abnormal = gross abnormalities

Hatching: good = most hatched, impaired = few hatched, some deformed or none = self explanatory

Larval survivorship: normal = no significant difference from pure species crosses



**Fig. 3** Normal development of larvae from pure crosses exhibited, in column A (1) good fertilization success, (2) uniform development, and (3a-c) normal hatching and survivorship. Abnormal development of larvae from representative hybrid crosses exhibited terminal phase fitness effects, in column B: (1) poor fertilization success in one replicate cross between sympatric species; (2) developmental fitness effects in larvae from allopatric species crosses including (2a) arrested development and failure to develop posteriorly, (2b) deformity with disencephaly, (2c) retarded development; and (3) developmental fitness effects in larvae from backcross treatments included deformities such as sway backs (100% mortality after hatching). Note that some replicates from hybrid crosses exhibited no fitness effects and were indistinguishable from pure species crosses, as illustrated in 3a-c.

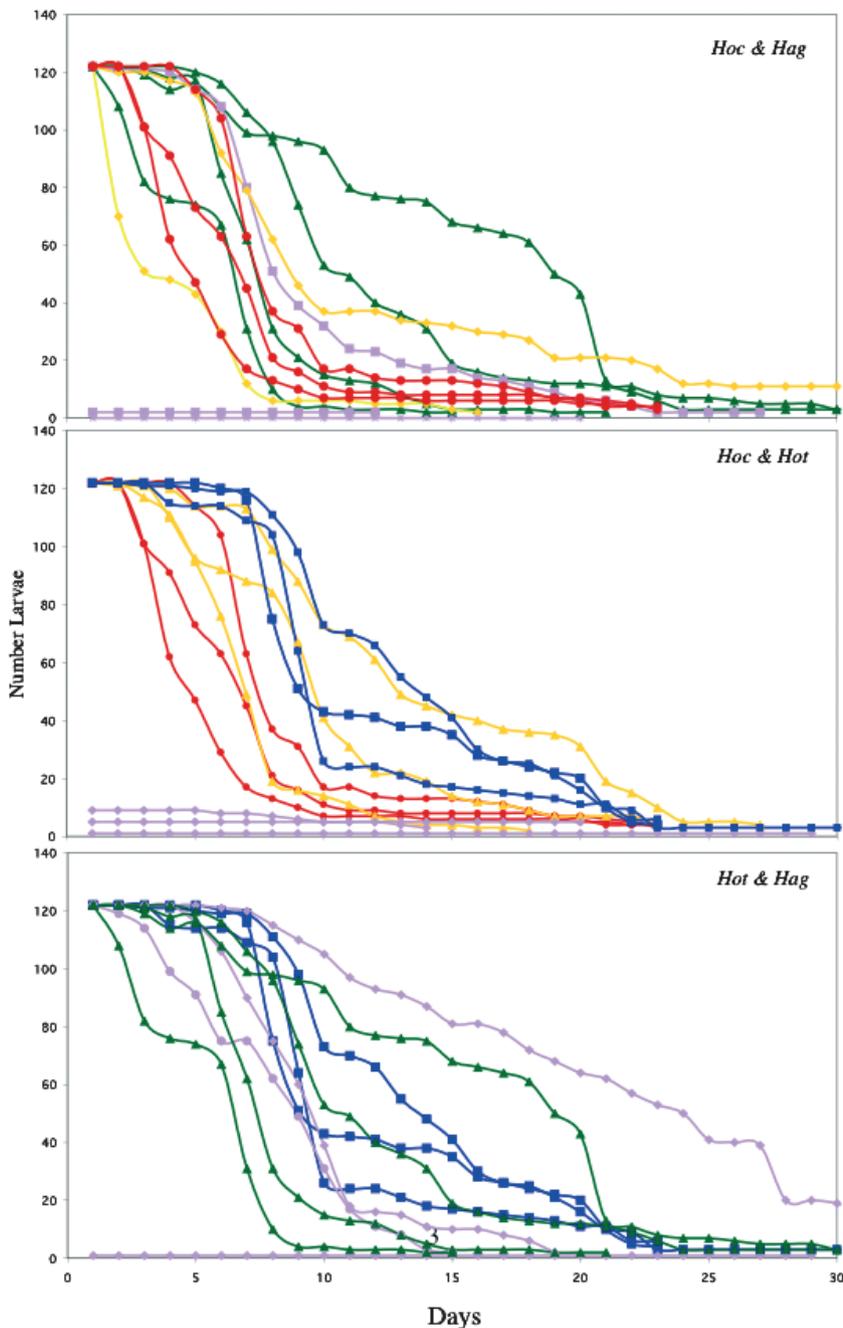
*Patterns of reproductive isolation for sympatric species crosses: fertilization, development, hatching and survivorship*

Crosses between sympatric sister taxa *H. otakii* and *H. agrammus* exhibited reduced fertilization success in all replicates and zero fertilization success in one replicate (Table 4, Figs 3 and 4). In replicates where fertilization did occur, larvae exhibited normal development, good hatching success and normal larval survivorship in both reciprocal cross treatments and there was no significant difference from pure species crosses (Table 4, Figs 3 and 4; see comparison of survivorship between pure species and all hybrid crosses below). Unfortunately, while proportions of fertilized eggs were counted, data were recorded nominally for some

replicates and therefore quantitative interpretation of these results is not possible.

*Patterns of reproductive isolation for allopatric species crosses and backcrosses*

Crosses between allopatric species exhibited severe fitness effects in one or more of the life history stages examined. All replicates of the hybrid cross treatment between *H. otakii* (female) and *H. octogrammus* (male), exhibited complete loss of fitness during embryonic development and two replicates exhibited some fitness effects at the life history stage of fertilization (Table 4, Figs 3 and 4). Severe developmental effects in these replicates included arrested development and failure to development posterior to the trunk region (Fig. 3). All



**Fig. 4** Comparison of survivorship between replicates of pure and hybrid crosses. X-axis is number of days and Y-axis is number of larvae surviving. Pure *H. octogrammus* crosses shown in red, pure *H. agrammus* crosses shown in green and pure *H. otakii* crosses shown in blue. Each graph illustrates two parental pure crosses and the reciprocal hybrid crosses between them. Hybrid crosses that occur in nature are shown in yellow (e.g. *Hoc/Hag*, top; and *Hoc/Hot*, middle) and those that do not occur in nature are shown in lavender (e.g. *Hag/Hoc*, top; and *Hot/Hoc*, middle). Crosses between allopatric species pairs are shown in the top two graphs and crosses between sympatric species are shown in the bottom graph. Replicates with zero survivorship at day 1 exhibited complete loss of fitness at developmental stages before hatching.

replicates of this treatment failed to produce any viable fry and therefore larval survivorship was not relevant (Table 4).

Alternatively, *H. octogrammus* (female)  $\times$  *H. otakii* (male) larvae exhibited no fitness effects in the  $F_1$  generation (Table 4, Figs 3 and 4), but severe negative effects occurred in the backcross generation at one or more life history stages in four of the five replicates (Table 4). Examples of negative fitness effects in the backcross generation included severe physical deformities such as curved spines and misplaced organs.

Crosses between the allopatric species pair *H. agrammus* and *H. octogrammus* exhibited severe fitness effects in one or two replicates of each reciprocal cross treatment in the  $F_1$  generation (Table 4). Severe developmental effects in these replicates included failure of posterior development at the post-cephalic region or dissociation of the cephalic region (Fig. 3). However, these treatments were not completely asymmetric (as was observed in crosses between the other allopatric species pair *H. octogrammus* and *H. otakii*) and each reciprocal cross treatment had one replicate exhibiting normal fer-

tilization, development and hatching with no significant differences in survivorship relative to pure species crosses. When backcrossed, fitness effects were less pronounced and occurred in only one of five replicates.

While there was clear evidence for selection against most hybrid treatments relative to pure crosses at the levels of fertilization and development, for those replicates that did produce viable larvae, there were only slightly significant differences in the slopes (ANOVA,  $F = 2.485$ ,  $df = 8$ ,  $P = 0.055$ ) of larval survivorship (log transformed data). When backcrossed replicates were removed and remaining data were pooled into two categories of pure and hybrid crosses, there was no significant difference in larval survivorship ( $P = 0.034$ , pooled variance  $t = 0.26$ ,  $df = 19$ ). Therefore, instead of differences in mortality rates, selection against hybrids was manifest in an 'all or nothing' fashion and was incurred at developmental stages before hatching.

## Discussion

### *Divergence in sympatry*

In this study we infer sympatric speciation between two species of marine reef fishes—*H. otakii* and *H. agrammus*, based several lines of evidence. First, we provide evidence for three of the four criteria necessary to infer sympatric speciation: a sympatric distribution, a monophyletic sister species relationship (with complete sampling of the genus) and complete reproductive isolation as indicated by fixed differences between species at multiple loci (with large sample sizes taken from various locations throughout their ranges). We argue that the fourth criterion of 'occurring in an isolated habitat where allopatric speciation is unlikely' is restrictive because, ultimately, we want to know how common sympatric speciation is and in what types of settings it occurs in nature. To compensate for the lack of an ecological setting rendering allopatric speciation unlikely, we take a mechanistic approach, evaluating theoretical predictions about the consequences of sympatric vs. allopatric speciation. By directly comparing aspects of RI between allopatric and sympatric taxa, both in nature and the laboratory, we found evidence for intrinsic post-zygotic RI (i.e. reduced hybrid fitness) in two allopatric species pairs, conforming to theoretical expectations for allopatric speciation. We then compared patterns of post-zygotic RI for two of the same taxa distributed in sympatry and found no evidence for post-zygotic RI, even though reproductive isolation is essentially complete based on absence of shared genotypes/haplotypes and absence of hybrids in nature. In short, the sympatric species pair exhibits complete pre-zygotic RI and no evidence for post-zygotic RI at the

life history stages examined, whereas two allopatric species pairs exhibit severe fitness effects resulting in significant post-zygotic RI at one or more of the life history stages examined and little pre-zygotic RI (at least with respect to mate choice by *Hoc* females). The balance of evidence firmly establishes both the plausibility of sympatric speciation between *H. otakii* and *H. agrammus* and a clear difference in patterns of divergence and reproductive isolation relative to allopatric species pairs. Based on evidence supporting established criteria for inferring sympatric speciation and the contrast in speciation patterns in sympatric vs. allopatric distributed species, we conclude that allopatric speciation with secondary contact is not the most likely explanation for the divergence between the sympatric taxa *H. otakii* and *H. agrammus*.

In further support of this finding, we provide several lines of circumstantial evidence supporting sympatric speciation that conforms to theoretical expectations. First, we find that the build up of pre-zygotic RI has occurred faster than post-zygotic RI in the sympatric taxa and, second, that pre-zygotic RI has evolved faster in sympatry vs. allopatry in a direct comparison that controls for lineage specific effects—both classic hallmarks of sympatric speciation (Coyne & Orr 1989, 1997). Second, sympatric species exhibit character displacement in ecological traits that directly affect reproductive isolation—depth distribution of nests, nest site substrate and timing of reproduction; and traits that could affect mate choice—divergent body size, nuptial colouration, lateral line configuration and caudal fin morphology (Table 5). Character displacement in these seven traits, with potential for direct and pleiotropic effects on assortative mating is consistent with theoretical expectations for sympatric speciation. For example, the simplest situation favouring sympatric speciation occurs when both assortative mating and disruptive selection are affected by the same phenotypic character (Rice 1984). This model is most feasible when mate choice involves ecologically important traits such as habitat preference (Rice 1984), timing of reproduction or sexually selected traits such as body size (Nagel & Schluter 1998; Jones *et al.* 2003) or nuptial displays.

*Hexagrammos* fishes have an interesting reproductive natural history that includes male parental care, sexual selection, high dispersal potential and hybridization, all of which influence the evolution and distribution of the genus. Males defend benthic nests with several clutches of eggs and therefore exhibit nest site fidelity and clearly defined mating grounds. As a result, when hybridization occurs it is due to non-assortative mating, not the union of randomly dispersed gametes produced by broadcast spawning. Nests typically contain several clutches of eggs at various stages of development

**Table 5** Summary of divergently selected characters in morphology and ecological traits associated with reproductive isolation

	<i>H. octogrammus</i>	<i>H. agrammus</i>	<i>H. otakii</i>	Source
Reproduction				
Spawning season	October	September–October	Late October–November	Figure S1 and Sekigawa (2003)
Depth distribution of nests (m)	0.5–3.0	0.5–3.5	6.5–19.0	Kimura & Munehara (2010)
Nest substrate	Algal turf	Algal turf	Rock outcrops	Kimura & Munehara (2010)
Morphology				
Average SL (cm)	18.1 ( $\pm$ , $n = 38$ )	20.5 (+, $n = 97$ )	28.2 (+, $n = 42$ )	Crow <i>et al.</i> (2007)
Lateral line number	5	1	5	Illustrated in Crow <i>et al.</i> (2007)
Caudal fin	Round	Round	Square	Illustrated in Crow <i>et al.</i> (2007)
Nuptial colouration	Pelvic anal fin black	Pelvic anal fin black	Body bright yellow	Munehara <i>et al.</i> (2000)

(Crow *et al.* 1997; Munehara *et al.* 2000; Kimura *et al.* 2007). Females are multiple spawners (Kurita *et al.* 1995; Crow *et al.* 1997; Antonenko 1999) and clutches within a nest are contributed from multiple females (Crow *et al.* 1997; Kimura *et al.* 2007). Each species exhibits a protracted spawning season associated with water temperature that varies with season and latitude.

The focal sympatric taxa exhibit a suite of divergently selected characters that have direct effects on reproductive isolation, including timing of reproduction and depth distribution of nests. The peak spawning period for the two southern sympatric species are the most divergent in time with reproductive season occurring approximately from mid September through October for *H. agrammus* and from late October to November for *H. otakii*. In 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 (Table 5). Gonadosomal Indices (GSI) were measured for all *Hexagrammos* females sampled at Usujiri Marine Biological Station in fall 2000 as gonad weight/body weight (with gut removed). These data were plotted by sampling date and are consistent with divergence in spawning time between sympatric species (Fig. S1, Supporting Information). However, these samples were not taken regularly throughout the reproductive period due to limited availability of boats and we do not propose that these data fully characterize maximum GSI or complete spawning period. Further, *H. agrammus* and *H. otakii* exhibit character displacement in depth distribution and substrate type of nests with *H. otakii* being distributed significantly deeper and significant differences in nest associated algal turf and substrate (Kimura & Munehara 2010). Yet, the reproductive period does overlap and the ecological distribution of each species is well within cruising range (*sensu* Mayr 1947) of the others because females move freely through the available reproductive habitat to select mates creating opportunity for interspecific mating. This

is supported by direct observation of mating behaviour between *H. agrammus* females and *H. otakii* males by underwater video surveillance (Munehara *et al.* 2000). The character displacement in depth distribution and timing of reproduction are likely under disruptive natural selection and contribute to assortative mating.

*Hexagrammos agrammus* and *H. otakii* also exhibit character displacement in traits likely to be associated with sexual selection including nuptial displays and body size. They are morphologically distinct as *H. otakii* exhibits five paired lateral lines, two pairs of supra-orbital cirri and a square caudal fin and *H. agrammus* exhibits only one lateral line, two pairs of supra-orbital cirri and a round caudal fin (Fig. 2). *H. otakii* exhibit larger body sizes that are significantly different from *H. agrammus* and *H. octogrammus* in standard length (Crow *et al.* 2007). They exhibit divergence in patterns of sexual dimorphism as well. *H. agrammus* and *H. octogrammus* exhibit a blotchy pattern that becomes much more pronounced and darker during the reproductive season, whereas *H. otakii* males exhibit a nuptial transformation from a light blotchy pattern of white, blueish-grey and gold, to an extreme solid yellow-gold colouration during the spawning season. Differences in body size and nuptial displays may provide visual cues that are likely to be associated with sexual selection and assortative mating.

Another, more complex, scenario that could contribute to sympatric speciation is that of pleiotropic effects on characters affecting behavioural reproductive isolation and selection against hybridization such as gamete incompatibility, or linkage disequilibrium between loci responsible for these traits. We found evidence for both behavioural reproductive isolation and gamete incompatibility between these species. First, crosses between *H. otakii* and *H. agrammus* ( $n = 4$ ) were the only treatments to exhibit zero percent fertilization in one replicate and reduced fertilization success in all remaining replicates. Second, adult hybrids do not occur in nature even though experimental crosses produced viable

larvae indicating pre-zygotic isolation has evolved faster than post-zygotic reproductive isolation. Although linkage disequilibrium between these traits was not tested directly, taken together these data suggest multiple opportunities for sympatric speciation to have occurred and is consistent with the observation that sympatric speciation is more likely to occur under multifarious natural selection on multiple traits [reviewed in Via (2001)].

Multifarious selection on traits associated with assortative mating may be much more common in nature than is typically characterized in studies of hybridization. Multiple modes of selection have likely contributed to the evolution of sexual selection resulting in speciation between these taxa. For example, males appear to be non-discriminatory, but sexual selection is implied in females. First, the fact that hybrids between *H. otakii* and *H. agrammus* exhibit the least severe fitness costs yet hybrids are not sampled in nature implies that females are mating assortatively. Second, *H. otakii* and *H. agrammus* females do not appear to mate with allopatric *H. octogrammus* males or if they do, their progeny are met with severe fitness costs. Therefore, females of *H. otakii* and *H. agrammus* are under multifarious and simultaneous selective pressures to mate assortatively because if they mate with sympatric males they experience reduced fertilization success and if they mate with allopatric males they experience near complete loss of fitness. In contrast, *H. octogrammus* females appear to be the least discriminate, because the genetic signatures of hybrids demonstrates that they mate readily with males of all three species in the area of distributional overlap, and indeed fitness costs associated with interspecific mating by *H. octogrammus* females are less severe and do not occur until the next generation. This explains the apparent latency in the evolution of assortative mating in *H. octogrammus* females and suggests potential for reinforcement between allopatric species pairs.

#### *Divergence in allopatry*

Allopatric speciation is caused by genetic drift or selection resulting in developmentally mediated, loss of fitness in hybrids [Dobzhansky 1936; Muller 1942; Rice & Hostert 1993; Turelli & Orr 2000; Blier *et al.* 2001; Porter & Johnson 2002; reviewed in Lijtmaer *et al.* (2003)]. We found interesting trends in genomic interactions evident from patterns of post-zygotic RI in larvae produced from reciprocal hybrid crosses between allopatric species *H. octogrammus* and both *H. otakii* and *H. agrammus*. Genome-wide incompatibilities between these allopatric species suggest that divergence has been built up in the absence of gene flow. For example, *Hot/Hoc* F<sub>1</sub> hybrids did the poorest overall and all replicates ( $n = 3$ ) exhib-

ited abnormal development and near complete loss of fitness (Table 1). In contrast, F<sub>1</sub> hybrids from the reciprocal cross (*Hoc/Hot*) fared well overall. In fact, this was the only F<sub>1</sub> hybrid treatment that did not incur fitness costs in any of the three replicates. The former suggests that there is a significant negative genomic interaction between *H. otakii* mitochondrial genes on the *H. octogrammus* nuclear genetic background, while the latter suggests that the nuclear genomes of these two species are compatible. While cytonuclear incompatibilities could also be caused by negative interactions between maternally provided oocyte cytoplasm and the hybrid's nuclear genes, these features of the cytoplasm would be encoded by the nuclear genes of the maternal parent, still resulting in genomic conflict. Regardless of the source of cytonuclear incompatibility, the only alternative explanation for the observed prevalence of *Hoc/Hot* F<sub>1</sub> hybrids occurring in nature is asymmetrical hybridization. However, these results provide clear evidence for intrinsic negative selection on hybridization between *H. otakii* females and *H. octogrammus* males.

Even though *Hoc/Hot* F<sub>1</sub> hybrids occurred in nature in surprisingly high frequencies [i.e. two to three times more frequently than would be predicted by random mating alone, Crow *et al.* (2007)] there was evidence for selection against these hybrids. First, all *Hoc/Hot* F<sub>1</sub> hybrids sampled in nature were female, implying male inviability and a significant fitness cost. More importantly, when backcrossed, *Hoc/Hot* F<sub>1</sub> hybrids exhibited significant loss of fitness in four of five replicates. Therefore, negative selection is implied for *Hoc/Hot* hybrids at later life history stages.

In crosses between *H. octogrammus* and *H. agrammus*, some replicates between both *Hoc/Hag* and *Hag/Hoc* crosses exhibited severely abnormal development and near complete loss in fitness. In contrast, one replicate in each class fared well and exhibited no differences from pure crosses at the life history stages characterized (Table 4). This could be explained by differences in expression patterns and dominance for genes exhibiting epistasis. These negative epistatic interactions between loci or Dobzhansky-Muller incompatibilities, may be adaptive in their normal genetic background, but result in loss of fitness when brought together in hybrids (Turelli & Orr 2000; Orr & Turelli 2001), suggesting divergence in allopatry. Of these two hybrid classes, only *Hoc/Hag* F<sub>1</sub> hybrids were found in nature as adults, therefore either negative selection from further cytonuclear incompatibilities acts on *Hag/Hoc* hybrids at life stages beyond 30 days of larval survivorship or *H. agrammus* females mate assortatively where *H. octogrammus* females do not. Again, of the *Hoc/Hag* F<sub>1</sub> hybrid adults sampled in nature, all were female, implying sex-linked incompatibilities as well.

*Could sympatric species H. otakii and H. agrammus have diverged in allopatry?*

If we make the conservative assumption that allopatric speciation is the null model against which sympatric speciation must be tested (Futuyma & Mayer 1980; Coyne & Orr 2004; Bolnick & Fitzpatrick 2007) then we must consider the possibility that *Hexagrammos otakii* diverged from *H. agrammus* in allopatry and character displacement occurred during secondary contact. It is crucial to note that under this scenario we would still expect to see some degree of post-zygotic reproductive isolation or intrinsic negative fitness effects, built up during allopatric speciation. We saw no such evidence in lab-crossed hybrids between *H. otakii* and *H. agrammus* during the life history stages tested. In fact, these larvae did not exhibit any of the intrinsic post-zygotic fitness costs that would be expected under allopatric speciation yet were apparent in two allopatric species crosses involving these same taxa (e.g. *Hoc/Hag* and *Hoc/Hot* hybrids). Alternatively, sympatric species exhibited greater pre-zygotic reproductive isolation than allopatric species pairs, as evidenced by the complete lack of naturally occurring hybrids, reduced fertilization success and absence of shared genotypes. The allopatric species pairs have not acquired the same degree of pre-zygotic reproductive isolation as the focal sympatric species, even though they have been divergent for a longer period of time. Indeed, divergence in allopatry would not predict pre-zygotic isolation to evolve faster, rather mutations would fix randomly or under selection, causing incompatibilities resulting in reduced hybrid fitness, as was observed in allopatric species crosses. In summary, we found no evidence for post-zygotic RI (i.e. reduced fitness of hybrids) between sympatric species, which is in stark contrast to patterns of post-zygotic RI between two examples of allopatric species pairs among the same taxa. Therefore we find no evidence to support species divergence in allopatry with character displacement or reinforcement occurring after secondary contact.

*Frequencies of hybrids can be explained by fitness costs incurred at various life history stages*

The observed frequencies of hybrids in this system can be explained by intrinsic negative selection resulting from a buildup of genomic incompatibilities in hybrids from allopatric species pairs and pre-mating isolation, including reduced gamete compatibility, between sympatric species pair *H. otakii* and *H. agrammus*. Hybridization between allopatric species pairs could not be explained by mating behaviour alone, because if fitness costs in hybrids were not manifest, complete introgression

between all three species would be expected via frequent hybridization with *H. octogrammus*. Because these species exhibit fixed differences, our *a priori* hypothesis was that negative selection against all hybrid classes would be manifest.

Indeed strong selection against hybrids was manifest in all crosses between allopatric species pairs, at one or more of the life stages evaluated. However, there is an apparent latency in selection against hybrids in the two classes occurring abundantly in nature, explaining the unexpected high frequencies of those classes of hybrids and indicating ongoing speciation processes in these taxa. For example, the frequency of *Hoc/Hot* hybrids exceeded the expected proportion under a random mating model (Crow *et al.* 2007) and this was the only hybrid class with no apparent loss of fitness in the F<sub>1</sub> generation. However, males of this hybrid class are inviable and there is evidence for strong negative selection when backcrossed. This is consistent with the observation that these species are discrete and exhibit fixed differences, with low levels of introgression. The reciprocal class of hybrids, *Hot/Hoc*, exhibited near complete loss in fitness in all replicate crosses and accordingly, these hybrids were not observed in nature.

Larvae from both reciprocal crosses between *H. octogrammus* and *H. agrammus* exhibited strong negative selection in some replicates, but one replicate in each treatment exhibited no fitness effects in the F<sub>1</sub> generation. This explains the fact that *Hoc/Hot* F<sub>1</sub> hybrids occur almost twice as frequently in nature than *Hoc/Hag* F<sub>1</sub> hybrids. However, *Hoc/Hag* F<sub>1</sub> hybrids were observed in nature while the reciprocal *Hag/Hoc* hybrids were not. This difference might be attributed to the inference that *H. octogrammus* females are less discriminating than *H. agrammus* and *H. otakii* in mate selection.

*Proposed mechanism for sympatric speciation*

The evolutionary relationships of *Hexagrammos* fishes are well characterized based on genetics and morphology. We estimate that the common ancestor of *H. otakii* and *H. agrammus* diverged from *H. octogrammus* approximately 2.2–3.6 million years ago, based on their *cytb* sequence divergence (5.42%), and assuming an average 1.5–2.5% sequence divergence per million years (inferred from a generally accepted molecular clock for *cytb* in fishes). Based on life history traits shared by *H. octogrammus* and *H. agrammus*, this ancestor likely was distributed in shallow rocky reefs with nests in abundant algal turf. Morphological traits including round caudal fin, smaller body size and disruptive colour patterns in nuptial displays likely were present in the common ancestor as adaptations to this complex environment for increased maneuverability and crypsis.

A protracted spawning season likely occurred in fall, possibly associated with water temperature cooling. Approximately 1.2–2.0 million years ago, *H. otakii* diverged from *H. agrammus* and expanded into deeper rocky reef environments. Nest sites of *H. otakii* occur in significantly deeper water and are associated with different benthic substrate types and less algal turf than *H. agrammus* and *H. octogrammus*. In this environment, guarding males and nest sites are more visible and therefore nest guarding tactics likely shifted from crypsis and hiding to more aggressive tactics involving chasing. Therefore, increased body size, conspicuous nuptial colouration and square caudal morphology may have been selectively advantageous, as they would confer increased advertising and thrusting capabilities.

This type of niche specialization has been described in other examples of ecological speciation as a result of divergent selection on ecological and morphological traits. One example involves two Midas cichlids that diverged in sympatry in Lake Apoyo, Nicaragua, with an ancestral benthic form and sympatric sister taxon with a more elongated body shape that is associated with the limnetic environment (Barluenga *et al.* 2006). Another example is the repeated evolution of marine sticklebacks invading fresh water habitats in which case a benthic form with armour reduction has repeatedly diverged from an ancestral limnetic form with pelvic spines and a high number of armoured body plates (Schluter & Rambaut 1996; Rundle *et al.* 2000). Ecologically based extrinsic reproductive isolation is unique to ecological speciation (Rundle & Nosil 2005) although this pattern is not specific to divergence in sympatry, parapatry or allopatry (Schluter 1998). While our study does provide evidence for character displacement in ecological traits directly associated with RI, it does not test for *extrinsic* RI associated with niche specialization, but it would be interesting to know if hybrids between the sympatric taxa *H. otakii* and *H. agrammus* exhibit reduced fitness in either parental habitat at later life history stages. Regardless, we provide evidence that intrinsic genetic incompatibilities readily evolve in allopatry, yet are not apparent between the same taxa that occur in sympatry at the life history stages examined.

#### Significance and summary

How does this system compare with others? Previous studies have been criticized because of the exclusive use of mitochondrial DNA, small sample sizes or insufficient taxon sampling [outlined in Barluenga *et al.* (2006); Schlieven *et al.* (2006)]. Our study addresses these limitations and more than satisfies theoretical conditions under which sympatric speciation is

plausible. We infer sympatric speciation from multiple lines of evidence including three established criteria for sympatric speciation. The only caveat is that the focal sympatric species do not occur in an isolated habitat of recent origin where allopatric speciation is unlikely. Rather they occur in one of the most common habitats on earth, a marine setting where high dispersal potential may not be conducive to allopatric speciation, but in no way precludes it. To compensate for the lack of an ecological setting rendering allopatric speciation unlikely, we directly compare aspects of RI between allopatric and sympatric taxa, both in nature and the laboratory, to infer speciation processes. Theoretical expectations for allopatric speciation predict the build up of post-zygotic RI (i.e. reduced hybrid fitness). As a control, we were able to test for post-zygotic RI in two allopatric distributed species pairs and indeed found evidence conforming to this expectation at various life history stages. We then compared patterns of post-zygotic RI in two of the same taxa, which are monophyletic sister taxa and share a sympatric distribution across their entire range and found no such evidence for post-zygotic RI. Based on this contrast, we reject the null hypothesis of allopatric speciation with secondary contact to explain the divergence between the sympatric taxa. In addition, we found further evidence supporting sympatric speciation conforming to theoretical expectations including evidence for the build up of pre-zygotic RI occurring faster in sympatry vs. allopatry and faster than post-zygotic RI; and character displacement in traits affecting reproductive isolation. We argue that divergent natural selection on characters affecting both niche partitioning and mate choice simultaneously via pleiotropy more than satisfies the plausibility of sympatric speciation. These characters imply adaptation to a deeper, colder habitat and niche specialization by *H. otakii*. Taken together, the evidence supports a compelling case of sympatric speciation in a genus of marine reef fishes. By studying natural hybridization between three related species we were able to use a novel comparative approach to infer multiple selective pressures associated with speciation and reveal a complex system of reproductive isolation in nature. We agree that mechanistic processes should be emphasized in speciation studies, and note that our understanding of the relative importance of sympatric speciation is incomplete, because natural systems have not yet been adequately described.

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### Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Gonadosomal Indices (GSI) of *H. agrammus* and *H. otakii* females sampled during September–November 2000 at Usujiri Marine Biological Station. GSI values greater than five indicate reproductive maturity. Green and dark blue dots indicate data from this study and solid lines indicate trends. Light blue dots indicate GSI for *H. otakii* taken in 1999 (Sekigawa *et al.* 2003). No samples were collected between September 22 and October 15, but calculated GSIs support reported spawning season and character displacement in spawning season.

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