Karyotype Differentiation between Two Stickleback Species (Gasterosteidae)

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Key Words

Chromosome evolution • Fish sex chromosome • Fluorescence in situ hybridization • Karyotype differentiation • Stickleback

Abstract

The stickleback family (Gasterosteidae) of fish is less than 40 million years old, yet stickleback species have diverged in both diploid chromosome number (2n) and morphology. We used comparative fluorescence in situ hybridization (FISH) on 2 stickleback species, *Gasterosteus aculeatus* (2n = 42) and *Apeltes quadracus* (2n = 46), to ascertain the types of chromosome rearrangements that differentiate these species. The *A. quadracus* karyotype contains more acrocentric and telocentric chromosomes than the *G. aculeatus* karyotype. By using bacterial artificial chromosome probes from *G. aculeatus* in our FISH screen, we found that 6 pericentric inversions and 2 chromosome fusions/fissions are responsible for the greater number of acrocentric and telocentric chromosomes in *A. quadracus*. While most populations of *G. aculeatus* have an XX/XY sex chromosome system, *A. quadracus* has a ZZ/ZW sex chromosome system, as previously reported. However, we discovered that a population of *A. quadracus* from Connecticut lacks heteromorphic sex chromosomes, providing evidence for unexpected sex chromosome diversity in this species.

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Stickleback fishes are small teleosts of the family Gasterosteidae, which comprises 6 species that inhabit temperate freshwater and marine environments across the northern hemisphere [Wootton, 1976]. Previous studies on chromosome number and morphology revealed diverse karyotypes among stickleback species [Chen and Reisman, 1970; Ocalewicz et al., 2011] as well as within a single species [Kitano et al., 2009]. Stickleback species also have different sex chromosome systems. Both three-spine sticklebacks (*Gasterosteus aculeatus*) and ninespine sticklebacks (*Pungitius pungitius*) have XX/XY (male heteromorphic) sex chromosome systems, but comparative fluorescence in situ hybridization (FISH) mapping and genetic linkage analyses indicate that these sex chromosome systems arose independently [Peichel et al., 2004; Ocalewicz et al., 2008; Ross and Peichel, 2008; Ross et al., 2009; Shapiro et al., 2009]. A third species, the fourspine stickleback (*Apeltes quadracus*), has a ZZ/ZW (female heteromorphic) sex chromosome system [Chen and Reisman, 1970; Ross et al., 2009]. In addition, the black-spotted stickleback (*G. wheatlandi*) and *G. aculeatus* populations from the Sea of Japan each have independently evolved X₁X₁X₂X₂/X₁X₂Y sex chromosome systems [Kitano et al., 2009; Ross et al., 2009].

The phylogenetic relationships between species in the stickleback family have previously been disputed [Matern, 2004]. However, a recent comprehensive phylogeny has resolved the evolutionary relationship among the sticklebacks [Kawahara et al., 2009]. When all available karyotype data for sticklebacks are mapped onto this

Fig. 1. A phylogeny of the family Gasterosteidae, incorporating major findings from this study and other reports. The phylogeny is based on Kawahara et al. [2009], though branch lengths are arbitrary. Listed are the common name and scientific name for each stickleback species, as well as the diploid number, the heteromorphic sex chromosome system (if known), and the chromosome on which the sex determination gene is found (if known). Chromosomes in all stickleback species are numbered in reference to *G. aculeatus*.

	Species	2n	System	Chr	References
Gasterosteidae	Fourspine <i>A. quadracus</i>	46	ZW	–	Chen and Reisman, 1970; Ross et al., 2009; this study
	Fifteenspine <i>S. spinachia</i>	–	–	–	–
	Brook <i>C. inconstans</i>	46	–	–	Ross et al., 2009
	Ninespine <i>P. pungitius</i>	42	XY	12	Ocalewicz et al., 2008; Shapiro et al., 2009; Ross et al., 2009; Ocalewicz et al., 2011
	Black-spotted <i>G. wheatlandi</i>	42	X ₁ X ₂ Y male 2n = 41	12, 19	Ross et al., 2009
	Threespine <i>G. aculeatus</i>	42	X ₁ X ₂ Y male 2n = 41	9, 19	Kitano et al., 2009
	Threespine <i>G. aculeatus</i>	42	XY	19	Peichel et al., 2004; Ross and Peichel, 2008; Ocalewicz et al., 2011; this study

phylogeny, it appears that sticklebacks have diverged more rapidly in karyotype than previously appreciated, since even closely related stickleback species have different diploid chromosome numbers, karyotypes, and sex chromosome systems (fig. 1).

To explore the rapid evolution of stickleback karyotypes in more detail, we designed a comprehensive FISH survey to identify the major genomic rearrangements that have occurred in the karyotypes of 2 stickleback species since they diverged from a common ancestor. We chose 2 species with different diploid chromosome numbers and divergent karyotypes, the threespine stickleback (*G. aculeatus*) and the fourspine stickleback (*A. quadracus*) [Chen and Reisman, 1970; Ross et al., 2009]. In addition, while *G. aculeatus* has an XX/XY sex chromosome system found on chromosome 19 [Peichel et al., 2004; Ross and Peichel, 2008], the ancestral chromosome pair that evolved into the *A. quadracus* ZZ/ZW sex chromosome pair is not yet known. Thus, we designed our survey to uncover the genomic rearrangements that generated the diverse karyotypes of these species and to reveal the identity of the *A. quadracus* ZZ/ZW sex chromosome pair.

Material and Methods

Fish Collection and Breeding

We collected male and female *A. quadracus* from the West River Memorial Park in New Haven, Connecticut, in May 2009. These males and females were brought into the laboratory and allowed to breed naturally in 110-liter aquarium tanks in July and

August 2009. We grew progeny from these matings together in 474-liter aquarium tanks until adulthood. Tissue from both wild-caught *A. quadracus* individuals and their progeny was used to prepare metaphase chromosome spreads (see below). In April and June 2010, we collected male *G. aculeatus* from 2 locations in Lake Washington: Union Bay in Seattle, Washington and the Mercer Slough Nature Park in Bellevue, Washington (Washington permit 10-049). We housed these fish in 110-liter aquarium tanks. Metaphase chromosome spreads were prepared using tissue from wild-caught *G. aculeatus* individuals (see below). For both species, we kept fish in summer lighting conditions (16 h light, 8 h dark) at approximately 16°C in 0.35% saltwater (3.5 g/l Instant Ocean salt (Aquarium Systems, Mentor, Ohio, USA); 0.4 ml/l NaHCO₃). We fed the fish with live brine shrimp nauplii twice daily. All procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (protocol 1575).

Selection of FISH Probes

We used *G. aculeatus* bacterial artificial chromosome (BAC) clones from the CHORI-215 library [Kingsley et al., 2004] as probes for FISH (table 1). To identify BAC probes for each end of each *G. aculeatus* chromosome pair, we extracted 200 kb of genomic sequence from the end of each *G. aculeatus* chromosome assembly (BROAD S1 assembly, February 2006, available at http://www.ensembl.org/Gasterosteus_aculeatus/index.html) and used this sequence in a BLAST search of the publicly available BAC end sequences [Kingsley and Peichel, 2007]. If both the T7 and SP6 end sequence reads of the BAC clones aligned within the 200-kb genomic sequence we selected, we used those paired end sequence reads from that BAC in a BLAT search against the *G. aculeatus* genome. If the paired end sequence reads of the BAC clone aligned to the desired chromosome assembly in opposing orientation, with expected separation based on the average size of a CHORI-215 BAC insert [Kingsley et al., 2004], we then used the BAC clone as a FISH probe for that *G. aculeatus* chromosome (table 1).

Table 1. BAC clones used as FISH probes in this study

Chro- mo- some	Length, bp	BAC	Color	Location, bp
1	28,185,914	CH215-01F04	purple	808,402–984,867
		CH215-36H04	green	26,520,970–26,685,556
2	23,295,652	CH215-14A06	purple	1,256,893–1,362,297
		CH215-51B03	green	22,208,609–22,374,157
3	16,798,506	CH215-22E01	purple	904,453–1,069,729
		CH215-18O23	green	14,811,263–14,979,917
4	32,632,948	CH215-37J16	purple	1,324,641–1,484,145
		CH215-03K06	green	31,667,549–31,830,249
5	12,251,397	CH215-10F03	purple	906,711–1,099,727
		CH215-15A11	green	11,086,429–11,245,223
6	17,083,675	CH215-39G07	purple	1,009,878–1,175,833
		CH215-30J20	green	16,083,765–16,163,690
7	27,937,443	CH215-24C20	purple	1,118,953–1,279,901
		CH215-50F19	green	26,759,364–26,929,269
8	19,368,704	CH215-18K21	purple	1,728,015–1,897,447
		CH215-50P04	green	18,234,424–18,411,738
9	20,249,479	CH215-09O14	purple	1,205,121–1,367,430
		CH215-35K20	green	18,760,858–18,914,793
10	15,657,440	CH215-32N20	purple	939,436–1,124,528
		CH215-13K03	green	14,608,763–14,740,402
11	16,706,052	CH215-22E24	purple	1,328,616–1,481,096
		CH215-32F20	green	15,594,645–15,722,431
12	18,401,067	CH215-35E14	purple	951,837–1,050,566
		CH215-19P17	green	17,405,092–17,588,100
13	20,083,130	CH215-29N20	purple	1,278,281–1,129,621
		CH215-41J19	green	18,303,573–18,452,590
14	15,246,461	CH215-50I22	purple	1,523,325–1,641,391
		CH215-04P19	green	14,054,345–14,228,449
15	16,198,764	CH215-42G09	purple	1,136,990–1,288,015
		CH215-47G02	green	14,910,768–15,091,535
16	18,115,788	CH215-25D03	purple	5,417,553–5,595,117
		CH215-59O23	green	15,244,265–15,412,230
17	14,603,141	CH215-03L14	purple	1,687,087–1,798,208
		CH215-25N10	green	13,556,132–13,679,389
18	16,282,716	CH215-60K06	purple	2,420,846–2,594,158
		CH215-58L04	green	14,897,105–15,061,421
19	20,240,660	CH215-23N18	purple	849,540–1,016,623
		CH215-16P13	green	4,756,773–4,923,988
20	19,732,071	CH215-32D17	purple	16,730,161–16,890,598
		CH215-09B01	green	18,782,835–18,919,085
21	11,717,487	CH215-11F14	purple	982,376–1,039,352
		CH215-34H24	green	10,642,915–10,791,824

For each *G. aculeatus* chromosome, the total length in the *G. aculeatus* genome assembly (version BROAD S1, February 2006, http://www.ensembl.org/Gasterosteus_aculeatus/index.html) is given. The pseudocolor for each BAC clone is indicated, as are the positions of the ends of each BAC clone in the respective chromosome assembly.

For 18 of 21 *G. aculeatus* chromosomes, the selected BAC clones aligned to a region within 2.6 Mb of the predicted ends of that chromosome assembly and hybridized to the ends of chromosomes in *G. aculeatus* male metaphase spreads. For 3 chromosomes, 1 BAC clone aligned to a genomic sequence within 2.6 Mb of the predicted ends of that chromosome assembly, but did not hybridize to the end of a chromosome pair in *G. aculeatus* male metaphase spreads. In those 3 cases, we screened additional 200-kb regions of those chromosome assemblies until we identified BAC clones that met the above selection criteria and hybridized to the ends of a chromosome pair in male *G. aculeatus* metaphase spreads. For the chromosomes involved (chromosomes 16, 19 and 20), errors in the *G. aculeatus* genome assembly, as previously reported for chromosome 19 [Ross and Peichel, 2008], or population-specific chromosome rearrangements could account for these observations.

Cytogenetic Analysis

We prepared metaphase spreads as described previously [Ross and Peichel, 2008] with the following modifications. We performed intraperitoneal injections of 5 μ l (*A. quadracus*) or 10 μ l (*G. aculeatus*) of 1% colchicine in phosphate-buffered saline into adult fish and incubated them for 12–14 h in an aquarium. After euthanizing the fish in 0.025% tricaine methanesulfonate (MS-222), we determined the sex of individual fish by examination of the gonads. We combined spleen tissue from several individuals to make metaphase spreads of males and females from both species (table 2).

We conducted FISH as previously described [Ross and Peichel, 2008], with the following modifications. We extracted and purified BAC DNA using an AutoGen 740 automated system (AutoGen, Holliston, Mass., USA) and quantified BAC DNA by gel electrophoresis. Using the Vysis nick translation kit (Abbott Labs, North Chicago, Ill., USA), we labeled 1 μ g of each BAC clone with either ChromaTide Alexa Fluor 488-5-dUTP or 568-5-dUTP (Invitrogen, Carlsbad, Calif., USA). Two hundred nanograms of the labeled clones were ethanol precipitated with 10 μ g salmon sperm DNA and 10 μ g genomic DNA from the species used in the experiment. We allowed hybridization to proceed for 1–2 nights at 37°C. We viewed washed slides using the 100 $\times$ objective on a Nikon Eclipse 80i microscope (Nikon, Shinjuku, Tokyo, Japan) with an automated filter turret using Chroma filters 31000v2 (DAPI), 41001 (FITC), and 41004 (Texas Red) (Chroma, Bellows Falls, Vt., USA). We captured images using a Photometrics Cool-snap ES2 camera (Photometrics, Tucson, Ariz., USA) and used NIS Elements imaging software (BR 3.00, SP7, Hotfix8, Build 548, Nikon, Shinjuku, Tokyo, Japan) to pseudocolor those images grey (DAPI), green (Alexa 488) and purple (Alexa 568).

Chromosome Morphology

For *G. aculeatus* and *A. quadracus*, we used images from FISH experiments (figs. 2, 3; data not shown) to assess the morphology of each chromosome. We classified chromosomes according to this average arm length ratio (long arm:short arm, L:S). Chromosomes were classified as metacentric (1.0–1.7), submetacentric (1.7–3.0), acrocentric (3.0–7.0), or telocentric (7.0 or greater), following Levan et al. [1964] and Schrader et al. [1997] (table 3). The only exception to this method is *G. aculeatus* chromosome 19, which is a heteromorphic XX/XY sex chromosome pair [Peichel et al., 2004; Ross and Peichel, 2008]. Thus, we measured the arm

Table 2. Number of individual samples and pools analyzed by FISH in *A. quadracus* and *G. aculeatus* and karyotyped in *A. quadracus*

	FISH analyses			Karyotypic analyses		
	total pools analyzed	individuals/pool (range)	metaphases analyzed/chromosome (range)	total pools analyzed	individuals/pool (range)	metaphases analyzed
<i>A. quadracus</i> ♀	8	12 (9–17)	5 (3–10)	3	11 (7–15)	6
<i>A. quadracus</i> ♂	7	8 (7–11)	3 (3–5)	2	8 (6–11)	5
<i>G. aculeatus</i> ♂	5	21 (15–36)	3 (3–4)	N/A	N/A	N/A

N/A = Not applicable.

Table 3. Chromosome morphology and evidence for chromosome rearrangements differentiating the chromosomes of *G. aculeatus* and *A. quadracus*

<i>G. aculeatus</i> chromosome	Mean arm ratio (L:S)	Chromosome morphology	<i>A. quadracus</i> chromosome	Mean arm ratio (L:S)	Chromosome morphology	Proposed rearrangement
1	1.90	submetacentric	1	3.33	acrocentric	pericentric inversion
2	5.28	acrocentric	2	4.30	acrocentric	
3	4.67	acrocentric	3	5.10	acrocentric	pericentric inversion
4	2.15	submetacentric	4a	3.39	acrocentric	fission/fusion
			4b	5.04	acrocentric	
5	2.13	submetacentric	5	1.98	submetacentric	paracentric inversion
6	4.38	acrocentric	6	5.92	acrocentric	paracentric inversion
7	1.23	metacentric	7a	4.88	acrocentric	fission/fusion
			7b	6.52	acrocentric	
8	9.17	telocentric	8	1.32	metacentric	pericentric inversion
9	6.04	acrocentric	9	3.41	acrocentric	
10	13.68	telocentric	10	3.48	acrocentric	
11	5.37	acrocentric	11	11.65	telocentric	
12	5.51	acrocentric	12	3.77	acrocentric	
13	5.74	acrocentric	13	3.08	acrocentric	
14	1.49	metacentric	14	1.36	metacentric	
15	9.97	telocentric	15	3.06	acrocentric	
16	9.73	telocentric	16	8.12	telocentric	
17	1.42	metacentric	17	3.23	acrocentric	pericentric inversion
18	6.19	acrocentric	18	3.52	acrocentric	
19 (X)	2.67	submetacentric	19	2.44	submetacentric	
20	9.88	telocentric	20	1.36	metacentric	pericentric inversion
21	1.39	metacentric	21	3.36	acrocentric	pericentric inversion

lengths of the single X chromosome in male *G. aculeatus*, but did not measure the Y chromosome (fig. 3; data not shown). For all FISH experiments and karyotype analyses, we examined multiple metaphase spreads from multiple pools of individual fish (table 2). Based on the chromosome morphology, relative centromere position, and BAC probe hybridization positions for each chromosome in our FISH screen, we determined the types of chromosome rearrangements that could explain the observed differences between the *G. aculeatus* and *A. quadracus* karyotypes (table 3).

We also calculated the fundamental number (major chromosome arm number; NF) for both sexes of *G. aculeatus* and *A. quadracus* [Matthey, 1949; Klinkhardt, 1998]. For calculating NF, we followed White [1978] and Klinkhardt [1998] and considered that metacentric and submetacentric chromosomes each have 2 major chromosome arms, while acrocentric and telocentric chromosomes each have 1 major chromosome arm. We inferred NF and other karyotype features for *G. aculeatus* females (table 4) based on the absence of the metacentric Y and the presence of a second submetacentric X chromosome [Ross and Peichel, 2008].

Fig. 2. Major chromosomal rearrangements between *G. aculeatus* and *A. quadracus*. Two-color FISH images are shown for the 10 *G. aculeatus* chromosomes that show visible evidence for chromosome rearrangement. Pairs of homologs from individual metaphase spreads of *G. aculeatus* males and *A. quadracus* females are shown. White arrowheads indicate the position of the centromere.

Fig. 3. No major differences in chromosome morphology or probe hybridization between 11 *G. aculeatus* and *A. quadracus* chromosome pairs. Two-color FISH images are shown for both homologs of each chromosome pair. White arrowheads indicate the position of the centromere. One FISH probe for chromosome 19 does not hybridize to the metacentric Y chromosome, most likely due to a large deletion on the Y chromosome in *G. aculeatus* [Ross and Peichel, 2008].

Fig. 4. DAPI-stained karyotypes of an *A. quadracus* female (upper panel) and an *A. quadracus* male (lower panel) from the West River in New Haven, Connecticut.

Results

FISH Screen Identifies the Chromosome Morphology of Each G. aculeatus Chromosome Pair

The *G. aculeatus* male karyotype consists of 42 chromosomes: 8 autosomes are metacentric, 6 are submetacentric, 16 are acrocentric, 10 are telocentric, and there is 1 submetacentric X and 1 metacentric Y (table 4) [Ross and Peichel, 2008]. The relatively high NF (58) for *G. aculeatus* compared to its diploid number ($2n = 42$) reflects a relatively large number of metacentric and submetacentric chromosomes in the karyotype. The selected BAC clones (table 1) hybridize by FISH to the ends of each chromosome (figs. 2, 3). By combining FISH with measurements of chromosome arm length, we were able to assign each *G. aculeatus* chromosome pair to a morphological class (table 3).

FISH Screen Identifies the Major Differences between the G. aculeatus and A. quadracus Karyotypes

We used the same BAC clones selected for the *G. aculeatus* male FISH analysis in a similar survey of the chromosomes in *A. quadracus* females and males (figs. 2, 3). The *A. quadracus* karyotype consists of 46 chromosomes, of which 6 chromosomes are metacentric, 4 are submetacentric, 32 are acrocentric, and 4 are telocentric (fig. 4; table 4). The dominance of acrocentric and telocentric

chromosomes in the *A. quadracus* karyotype (78%) is reflected in a lower ratio of NF to diploid chromosome number; NF:2n is 56:46 for *A. quadracus*, while it is 58:42 for *G. aculeatus* (table 4).

Two of the larger chromosome pairs in the *G. aculeatus* karyotype, chromosomes 4 (submetacentric) and 7 (metacentric), each correspond to 2 pairs of relatively small acrocentric chromosomes in *A. quadracus* (fig. 2; table 3). Eight chromosome pairs appear to have undergone inversions in the time since *G. aculeatus* and *A. quadracus* diverged; 6 are pericentric inversions, which encompass the centromere, and 2 are paracentric inversions, which do not encompass the centromere (fig. 2; table 3). Five of these pericentric inversions have resulted in visibly different morphology for the orthologous *G. aculeatus* and *A. quadracus* chromosomes. Probes from 11 *G. aculeatus* chromosome pairs show identical hybridization patterns and morphologies in both *G. aculeatus* and *A. quadracus* (fig. 3).

A. quadracus Males and Females from Connecticut Do Not Have Heteromorphic Sex Chromosomes

A heteromorphic ZZ/ZW sex chromosome pair has been described in *A. quadracus* populations from Maine [Chen and Reisman, 1970] and Massachusetts [Ross et al., 2009]. However, we found no evidence for a heteromorphic sex chromosome pair in *A. quadracus* male or fe-

Table 4. Major features of the karyotypes of *G. aculeatus* and *A. quadracus*

Species	Sex	2n	Chromosomes				NF
			metacentric	submetacentric	acrocentric	telocentric	
<i>G. aculeatus</i>	female	42	8	6+2X	16	10	58
	male	42	8+Y	6+X	16	10	58
<i>A. quadracus</i>	female	46	6	4	32	4	56
	male	46	6	4	32	4	56

2n = Diploid number; NF = fundamental number.

male progeny from specimens collected in the West River in New Haven, Connecticut (fig. 4). In addition, hybridization patterns were identical in metaphase spreads from male and female *A. quadracus* for all FISH probes used in this study (data not shown).

Discussion

Diversity of the A. quadracus Sex Chromosomes

We undertook a comparative FISH study to identify the major genomic rearrangements that have occurred in lineages separating 2 stickleback species, *A. quadracus* and *G. aculeatus*. These species are intriguing subjects for such a study, not only because of reported differences in karyotype, but also due to the divergence of sex chromosome systems [Chen and Reisman, 1970; Ross et al., 2009]. In *G. aculeatus*, chromosomes 19 are an XX/XY sex chromosome pair [Peichel et al., 2004; Ross and Peichel, 2008]. Two previous studies have shown that *A. quadracus* populations from Maine and Massachusetts have a ZZ/ZW sex chromosome pair [Chen and Reisman, 1970; Ross et al., 2009]. In both studies, the W chromosome is described as a large acrocentric chromosome, while the Z chromosome is also acrocentric, but has a smaller long arm than the W chromosome. Genetic markers from chromosome 19 are not linked to the sex determination locus in an *A. quadracus* family from Massachusetts [Ross et al., 2009], and no genetic markers linked to a sex determination locus have been found in *A. quadracus*.

In the present study of *A. quadracus* from Connecticut, we find that neither females nor males have a heteromorphic chromosome pair indicative of either a ZZ/ZW or XX/XY sex chromosome system. Two possible explanations could account for the differences in sex chromosomes between the Connecticut population of *A.*

quadracus and the Maine and Massachusetts populations. First, *A. quadracus* from Connecticut could have a cryptic (homomorphic) ZZ/ZW sex chromosome pair that is homologous to the ZZ/ZW pair in the Massachusetts and Maine populations. The larger size of the W chromosome relative to the Z chromosome in those populations [Chen and Reisman, 1970; Ross et al., 2009] could be due to an increase in the amount of heterochromatin in the W chromosome relative to the Z chromosome following the cessation of recombination with the Z chromosome. If negligible amounts of heterochromatin were found in the *A. quadracus* W chromosome of the Connecticut population, then the Z and W chromosomes in this population would not be morphologically distinguishable. Such a population-specific difference in the amount of heterochromatin or repetitive DNA sequences present on the sex chromosomes has been reported in the guppy *Poecilia reticulata* [Hornaday et al., 1994], the platyfish *Xiphophorus maculatus* [Nanda et al., 2000], and the glass knifefish *Eigenmannia virescens* [Almeida-Toledo et al., 2001]. Differences in the amount of heterochromatin on the sex chromosomes have also been reported between closely related species of salmonids [Moran et al., 1996] and South American catfishes [Andreata et al., 1992].

Second, *A. quadracus* from Connecticut could have a different sex determination mechanism than the Massachusetts and Maine ZZ/ZW sex chromosome system. This mechanism could be genetic, environmental, or a combination of genetic and environmental signals. Extreme variation in the mechanism of sex determination within a single species is not without precedent. In the Atlantic silverside, *Menidia menidia*, both environmental and genetic sex determination mechanisms are present and vary by latitude [Conover and Heins, 1987]. Although we currently have little data on the ecology or

population structure of *A. quadracus*, it will be interesting to determine whether variation in sex determination mechanisms is associated with any ecological or geographical factors in this species.

Even though the *A. quadracus* population in this study does not have a heteromorphic ZZ/ZW sex chromosome system, our comparative FISH study could help to identify the ZZ/ZW sex chromosome system in *A. quadracus*. The ZZ/ZW pair in both Massachusetts and Maine populations of *A. quadracus* is one of the larger chromosome pairs in the karyotype [Chen and Reisman, 1970; Ross et al., 2009]. In addition, the karyotypes of the Connecticut and Massachusetts *A. quadracus* are similar, differing only in the presence of the large W chromosome in *A. quadracus* females from the Massachusetts population [Ross et al., 2009]. Thus, the heteromorphic ZZ/ZW pair in the Massachusetts *A. quadracus* karyotype could correspond to one of the larger chromosome pairs in the Connecticut *A. quadracus* karyotype. Our FISH experiments showed that chromosomes 1, 2, 9, 12, 13, 19, and 20 were among the larger chromosome pairs in their respective metaphase spreads. Two of these (chromosomes 12 and 19) have previously been ruled out as the Massachusetts *A. quadracus* ZZ/ZW pair [Ross et al., 2009]; however, the other large chromosomes remain targets for further studies to identify the ZZ/ZW sex chromosome pair in *A. quadracus*.

Population differences in sex chromosomes and sex determination mechanisms are also found in other species of fish. Studies have documented sex chromosome diversity within species of poeciliid fish [Nanda et al., 2000; Volff and Schartl, 2001; Schultheis et al., 2009] and within several species of neotropical fish, including *E. virescens* [Almeida-Toledo et al., 2002; Henning et al., 2011], *Erythrinus erythrinus* [Bertollo et al., 2004; Cioffi et al., 2010], and *Hoplias malabaricus* [Bertollo et al., 2000; Cioffi and Bertollo, 2010]. Sex chromosome systems also differ among populations of other stickleback species. *G. aculeatus* from the Sea of Japan have an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system, which likely arose from the ancestral XX/XY sex chromosome system found in other *G. aculeatus* populations [Kitano et al., 2009]. Black-spotted sticklebacks (*G. wheatlandi*) from a population in Maine have an XX/XY sex chromosome system [Chen and Reisman, 1970], while at least 1 *G. wheatlandi* population in Massachusetts has an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system [Ross et al., 2009]. Future studies should reveal whether most *A. quadracus* populations have ZZ/ZW sex chromosomes, or if this species has a diverse array of sex determination mechanisms.

Mechanisms Responsible for the Differences in Karyotype between G. aculeatus and A. quadracus

Although *G. aculeatus* and *A. quadracus* diverged approximately 40 million years ago, our comparative FISH study has uncovered genomic rearrangements encompassing nearly half of the 21 *G. aculeatus* chromosome pairs. These rearrangements account for the 2 major karyotypic differences between these species: *A. quadracus* has a higher diploid number, coupled with a lower number of metacentric and submetacentric chromosomes, when compared to *G. aculeatus* (table 4).

The larger diploid number (46) in *A. quadracus* is explained by our finding that 2 *G. aculeatus* chromosome pairs (4 and 7) correspond to 4 *A. quadracus* chromosome pairs. Examination of other stickleback species does not reveal whether the *G. aculeatus* or *A. quadracus* diploid number is more similar to the ancestral state (fig. 1). The closest extant relative of *G. aculeatus* also has a diploid chromosome number of 42, as does a more distant relative, the ninespine stickleback, *P. pungitius* [Ocalewicz et al., 2008, 2011; Ross et al., 2009; Shapiro et al., 2009]. However, many differences in chromosome morphology exist between the *Gasterosteus* species and *P. pungitius* [Ocalewicz et al., 2008, 2011; Ross et al., 2009]. A recent report shows that *P. pungitius* has a higher NF:2n than *G. aculeatus* [Ocalewicz et al., 2011], indicating a higher number of metacentric and submetacentric chromosome pairs in *P. pungitius*. However, it is not known if the chromosome pairs that differ in morphology in *G. aculeatus* and *P. pungitius* are identical to the chromosome pairs that differ in *G. aculeatus* and *A. quadracus* in this study. The closest extant relative of *P. pungitius*, the brook stickleback (*Culaea inconstans*), has the same diploid number as *A. quadracus*, but these 2 species also differ in karyotype [Chen and Reisman, 1970; Ross et al., 2009]. No published reports document the diploid chromosome number and chromosome morphology of the fiftenspine stickleback (*Spinachia spinachia*), which is the closest extant relative of *A. quadracus* [Kawahara et al., 2009]. Thus, we lack comprehensive karyotype data from a sufficient number of stickleback species and populations to know whether the karyotype of *G. aculeatus* or *A. quadracus* is the more ancestral for this family.

Though the ancestral stickleback karyotype is unknown, our FISH screen has clarified some of the mechanisms that have shaped the *A. quadracus* and *G. aculeatus* karyotypes since they diverged. While metacentric and submetacentric chromosomes make up less than one quarter of the *A. quadracus* karyotype, they comprise

more than one third of the *G. aculeatus* karyotype (table 4). One factor that contributes to this difference is the separation of *G. aculeatus* chromosomes 4 and 7 in the *A. quadracus* karyotype (fig. 2; table 3). The differential state of these chromosomes in *G. aculeatus* and *A. quadracus*, and the fact that they are both acrocentric in *A. quadracus*, suggest that Robertsonian fusions and/or centric fissions have played roles in the evolution of stickleback karyotypes. These fusions may leave signatures in the form of interstitial telomeric sequences (ITSs) in the species with the derived chromosome state. A recent study of a *G. aculeatus* population from Poland failed to detect any ITSs by FISH; however, it is possible that ITSs were eliminated or remain in a copy number that is below the detection threshold for FISH [Ocalewicz et al., 2011]. Thus, the ancestral state of chromosomes 4 and 7 in the stickleback family is still unknown, and proper determination of the ancestral state will clarify whether fusions or fissions are responsible for these differences in karyotype.

We also uncovered evidence for inversions involving at least 8 chromosomes, based on differences in relative centromere position and/or BAC probe hybridization positions between *G. aculeatus* and *A. quadracus*. Six of these inversion events encompass the centromere, and 5 of those inversions alter the gross chromosome morphology between *G. aculeatus* and *A. quadracus*. In particular, 3 of these 5 inversions are on chromosomes that are metacentric or submetacentric in *G. aculeatus* and acrocentric or telocentric in *A. quadracus* (table 3), which is one factor that accounts for the lower NF in this species compared to *G. aculeatus* (table 4). Future studies of chromosome morphology and relative probe hybridization positions in other stickleback species could further illuminate the role of pericentric inversions in the evolution of karyotypes across this family.

Conclusions

Our FISH survey has identified the major chromosome rearrangements that have shaped the *G. aculeatus* and *A. quadracus* karyotypes since they diverged approximately 35 million years ago. The *A. quadracus* karyotype contains a large number of acrocentric and telocentric chromosomes that can be explained by several pericentric inversions and Robertsonian fusion or centric fission events. Importantly, we discovered unexpected diversity among *A. quadracus* sex chromosomes. Male and female individuals from Connecticut do not harbor heteromorphic (distinguishable) sex chromosomes, in contrast to other *A. quadracus* populations in New England. Future studies are required to uncover the true diversity of sex chromosomes and sex determination mechanisms in this species and to learn how the diverse array of sex chromosome systems evolved in the stickleback family.

Acknowledgements

We thank Thomas Near for providing our wild-caught *A. quadracus* samples, the Broad Institute and Stanford Human Genome Center for allowing us to use BAC end sequences and the initial stickleback genome sequence assembly prior to publication, Joseph Ross for providing both protocols and training for stickleback cytogenetics and FISH, Kohta Yoshida for helpful discussions on chromosome evolution in fishes, and Anna Greenwood for her unique insights, discussions, and advice as this project progressed. We thank Barbara Trask, Joseph Ross, and Anna Greenwood for helpful comments and suggestions regarding this manuscript. Finally, we thank Stephen Uy, who translated key passages from Robert Matthey's *Les Chromosomes des Vertébrés*. Our work was funded by National Institutes of Health grants P50 HG002568 (C.L.P.), R01 GM089733 (C.L.P.), and T32 HG00035 (J.R.U.).

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