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ORIGINAL ARTICLE

Centromere inactivation on a neo-Y fusion chromosome in threespine stickleback fish

Jennifer N. Cech · Catherine L. Peichel

Abstract Having one and only one centromere per chromosome is essential for proper chromosome segregation during both mitosis and meiosis. Chromosomes containing two centromeres are known as dicentric and often mis-segregate during cell division, resulting in aneuploidy or chromosome breakage. Dicentric chromosome can be stabilized by centromere inactivation, a process which reestablishes monocentric chromosomes. However, little is known about this process in naturally occurring dicentric chromosomes. Using a combination of fluorescence in situ hybridization (FISH) and immunofluorescence combined with FISH (IF-FISH) on metaphase chromosome spreads, we demonstrate that centromere inactivation has evolved on a neo-Y chromosome fusion in the Japan Sea threespine stickleback fish (*Gasterosteus nipponicus*). We found that the centromere derived from the ancestral Y chromosome has been inactivated. Our data further suggest that there have been genetic changes to this centromere in the two million years since the formation of the neo-Y chromosome, but it remains unclear whether these genetic changes are a cause or consequence of centromere inactivation.

J. N. Cech
Divisions of Basic Sciences and Human Biology, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave North, Mailstop C2-023, Seattle, WA 98109, USA

J. N. Cech
Graduate Program in Molecular and Cellular Biology, University of Washington, Seattle, WA 98195, USA

Present Address:
C. L. Peichel (✉)
Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, 3012 Bern, Switzerland
e-mail: cpeichel@fhcrc.org
e-mail: catherine.peichel@iee.unibe.ch

Abbreviations

BAC	Bacterial artificial chromosome
CENP-A	Centromere protein A
ChIP-seq	Chromatin immunoprecipitation sequencing
Chr	Chromosome
DAPI	4′,6′-Diamidino-2-phenylindole
FISH	Fluorescence in situ hybridization
GacCEN	Threespine stickleback (*Gasterosteus aculeatus*) centromeric repeat sequence
IF-FISH	Immunofluorescence combined with FISH
IP	Immunoprecipitation
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline Tween-20

Introduction

The centromere of a chromosome was first described by Walther Flemming as the primary constriction observed on condensed chromosomes during both mitosis and meiosis (Flemming 1880). This primary constriction is the region of the chromosome where microtubules attach, and it is necessary for the segregation of chromosomes during cell division. Many organisms contain "regional" centromeres, which are found at a single location on each chromosome and comprised of kilobases to megabases of DNA. The sequence of these regional centromeres is often repetitive and AT rich, but the specific sequence varies dramatically among species (Henikoff et al. 2001; Alkan et al. 2011; Melters et al. 2013), and can even vary among centromeres within a species (Nagaki et al. 2004; Piras et al. 2010; Tek et al. 2010; Shang et al. 2010; Gong et al. 2012). Centromeres do share a common epigenetic characteristic, which is the presence of a histone variant called centromere protein A (CENP-A) that replaces histone H3 in centromeric nucleosomes (Palmer et al. 1987, 1989, 1991; Sullivan et al. 1994a). The presence of CENP-A is considered the hallmark of an active centromere (Warburton et al. 1997; Heun et al. 2006; Marshall et al. 2008; Allshire and Karpen 2008; Barnhart et al. 2011; Mendiburo et al. 2011; Sekulic and Black 2012; Fukagawa and Earnshaw 2014; McKinley and Cheeseman 2016).

While it is essential that chromosomes have a centromere, in organisms with regional centromeres, it is also essential that every chromosome has one and only one active centromere. A normal monocentric chromosome only has a single centromere that can bind microtubules and form a bipolar spindle, allowing normal chromosome segregation. However, a dicentric chromosome has two centromeres that can both bind microtubules, leading to instability during cell division. Barbara McClintock first described this instability and termed it the breakage-fusion-bridge cycle (McClintock 1939, 1941). If the microtubules from spindle poles on the opposing sides of the cell bind to the two centromeres, the chromosome will be pulled in opposite directions. This leads to merotelic kinetochore attachments where the dicentric chromosome lags in the middle during anaphase as it is being pulled to opposite poles. The physical tension on the chromosome can lead to breakage of the chromosome. When the broken ends are replicated during the next S-phase of the cell cycle, the broken ends can anneal back to each other, recreating a dicentric. Not only does the breakage-fusion-bridge cycle start again, but the breakage and reannealing leads to gene loss and gene gain. This is evident in cancer cells where dicentrics are often observed, leading to cells with "increased genetic heterogeneity" (Gisselsson et al. 2000, 2001).

Thus, dicentric chromosomes created by chromosome fusions are predicted to be very unstable. However, chromosome fusion (and fission) events are common during evolution as evidenced by the extensive variation in chromosome number among species. For example, in multicellular eukaryotes, chromosome number ranges from a diploid number of two in the Jack jumper ant (Crosland and Crozier 1986) to 1260 in the Adders tongue fern (van der Burg 2004). In eutherian mammals, the diploid chromosome number ranges from 6 in the Indian muntjac to 102 in the viscacha rat (Gallardo et al. 1999); this diversity results from many independent fusion and fission events (Ferguson-Smith and Trifonov 2007). Even closely related species can vary in chromosome number; for example, the Chinese muntjac has a diploid chromosome number of 46, while the Indian muntjac has a diploid number of 6 in females or 7 in males (Shi et al. 1980; Yang et al. 1995; Wang and Lan 2000). It is speculated that a series of chromosome fusion events between the telocentric chromosomes present in the Chinese muntjac created the much larger Indian muntjac chromosomes (Wang and Lan 2000). Chromosome fusions have also occurred in the primate lineage; human chromosome 2 is a fusion between chimp chromosomes 2a and 2b (Lejeune et al. 1973; IJdo et al. 1991; Avarello et al. 1992). Similar chromosome fusion and fission events are common in many taxa and can account for much of the diversity of karyotype number observed today. Many of these chromosome fusion events must have created dicentric chromosomes and therefore been accompanied by mechanisms to stabilize the chromosome fusions.

To date, however, relatively little is known about the mechanisms involved in the stabilization of dicentric chromosomes during evolution in natural populations. Most of our current understanding of how dicentrics are stabilized comes from studies of artificially induced or engineered dicentric chromosomes in yeast (Pobiega and Marcand 2010; Sato et al. 2012), fruit flies (Agudo et al. 2000), and human cell lines (Higgins et al. 1999, 2005; Stimpson et al. 2010, 2012), as well

as a few examples of dicentric chromosomes found in plants (Sears and Câmara 1952; Han et al. 2006; Zhang et al. 2010; Gao et al. 2011; Koo et al. 2011; Liu et al. 2015) and human patients (Therman et al. 1989; Maraschio et al. 1990; Fisher et al. 1997; Page and Shaffer 1998; Sullivan and Willard 1998; Lange et al. 2009; Stimpson et al. 2012). In some engineered systems, the dicentric chromosome can simply break in order to reestablish monocentric chromosomes (Pobiega and Marcand 2010; Sato et al. 2012). In other engineered or patient-derived dicentrics, chromosome fusions can be stably inherited because the two centromeres on a fusion chromosome are close enough to each other to act as a single centromere (Koshland et al. 1987; Page and Shaffer 1998; Sullivan and Willard 1998; Higgins et al. 1999; Lange et al. 2009). However, distance between the centromeres does not fully explain the stable maintenance of dicentric chromosomes, and in many cases, there is inactivation of one of the centromeres to reestablish functionally monocentric chromosomes (Stimpson et al. 2012). Centromere inactivation can either occur by genetic or epigenetic inactivation of one centromere. In genetic inactivation, deleterious mutation of the DNA sequence of one centromere prevents it from binding CENP-A. In epigenetic inactivation, the DNA of the inactivated centromere is still present, but CENP-A is not. In either case, the functional consequence of the loss of CENP-A is that the centromere does not recruit kinetochore components and therefore does not attach to microtubules during cell division. Although both genetic and epigenetic inactivation have been observed in the systems studied to date (Stimpson et al. 2012), almost nothing is known about the relative contribution of these mechanisms to dicentric stabilization during evolution.

The stickleback family (Gasterosteidae) provides a unique opportunity to study the mechanisms that stabilize natural dicentric chromosomes during evolution because there have been several chromosome fusion events that have occurred within the past 35 million years (Kitano et al. 2009; Ross et al. 2009). Previous work has identified a chromosome fusion involving the ancestral metacentric Y chromosome present in the Pacific Ocean threespine stickleback (*Gasterosteus aculeatus*). This Y chromosome has fused to the acrocentric chromosome 9 in males of the Japan Sea threespine stickleback (*Gasterosteus nipponicus*) species within the past two million years, creating an X_1X_2Y sex chromosome system in which the ancestral

X is X_1, the unfused chromosome 9 is X_2, and the fused chromosome is called the neo-Y (Kitano et al. 2009) (Fig. S1). Because this fusion involved the metacentric Y and an acrocentric chromosome, the neo-Y should have been dicentric upon formation. Preliminary data had suggested that there was no loss of a chromosome arm to bring the two centromeres closer on the Japan Sea neo-Y chromosome or rebreakage of the fusion chromosome. These data suggested that centromere inactivation has occurred on the Japan Sea neo-Y chromosome.

In this study, we aimed to first determine if centromere inactivation has occurred on the neo-Y, and to then test whether inactivation is due to a genetic or epigenetic mechanism. Both the genetic and epigenetic mechanisms of centromere inactivation ultimately result in only one functional centromere, as defined by the presence of CENP-A. If centromere inactivation has occurred, we expected to see only one region on the neo-Y with CENP-A staining. If no centromere inactivation has occurred, we expected to see two regions of CENP-A staining on the neo-Y. Using CENP-A antibody staining, we determined that the Japan Sea neo-Y has only one active centromere, as defined by the presence of CENP-A. In an extension of our previous study (Cech and Peichel 2015), we then performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) to determine the centromere sequence in the Japan Sea species. We then used fluorescence in situ hybridization (FISH) to demonstrate that there have likely been genetic changes to the ancestral Y centromere on the Japan Sea neo-Y.

Materials and methods

Fish use and care

Two species of lab-reared threespine stickleback fish were used in this study: Pacific Ocean (*G. aculeatus*) and Japan Sea (*G. nipponicus*). Lab-reared fish were derived from wild-caught fish collected in Akkeshi on Hokkaido Island, Japan (Kitano et al. 2007, 2009). Fish were kept in 3.5 % saltwater (3.5 g/l Instant Ocean salt (Spectrum Brands, USA); 0.4 ml/l sodium bicarbonate) in 29-gal aquarium tanks, at 16 °C and 16 h light/8 h dark. Fish were fed once daily with live *Artemia* nauplii and once daily with frozen *Mysis* shrimp. All institutional and national guidelines for the care and use of

laboratory animals were followed, and all procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (protocol 1575).

CENP-A immunoprecipitation followed by high-throughput sequencing

Chromatin immunoprecipitation (IP) with the stickleback-specific CENP-A antibody (Cech and Peichel 2015) was performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology, USA); 0.1 g of spleen and liver tissue was taken from three Japan Sea females (one single replicate and one pooled replicate of two individuals) and four Japan Sea male sticklebacks (two pooled replicates of two individuals each). The chromatin immunoprecipitation was performed at the same time and following the same protocol as for the two Pacific Ocean female samples described in Cech and Peichel (2015). To identify the Japan Sea consensus centromere sequence, analysis was performed using the same methods as in Cech and Peichel (2015). All raw fastq sequence files are available in the NCBI Sequence Read Archive (Pacific Ocean female data: SRP063504; Japan Sea male and female data: SRP081499).

FISH on metaphase spreads and interphase nuclei

Lab-reared Pacific Ocean and Japan Sea fish were injected with 10 µl of 1 % colchicine in phosphate-buffered saline (PBS) for 14–16 h to arrest metaphase cells. Metaphase and interphase cells were obtained following the same protocol described in Ross and Peichel (2008), with the exception that metaphase slides were dried at 50 °C, not 37 °C. The threespine stickleback centromeric repeat sequence (GacCEN) probe was made as described in Cech and Peichel (2015). Bacterial artificial chromosome (BAC) probes listed in Table 1 were prepared following the protocol in Urton et al. (2011) and labeled with either Alexa-488 or Alexa-568.

Metaphase slides were first washed for 5 min with phosphate-buffered saline Tween-20 (PBST), fixed for 10 min in 4 % paraformaldehyde in PBS, then washed again in PBST for 5 min. Subsequent FISH hybridization with only the GacCEN probe or only BAC probes was performed as described in Cech and Peichel (2015). For metaphase slides hybridized with combined GacCEN and BAC probes, 10 µl of the GacCEN probe

was first lyophilized at 50 °C for 4 h. The dried GacCEN probe was then resuspended with either 10 µl of a single BAC probe in hybridization buffer or 20 µl of equal parts of two BAC probes in hybridization buffer.

Telomere staining

Telomere staining was performed according to the manufacturer's instructions using the DAKO telomere PNA FISH kit/FITC no. K5325 (Agilent Technologies, USA).

Immunofluorescence-FISH (IF-FISH)

For IF-FISH, 15 Pacific Ocean or Japan Sea embryos at 48 h postfertilization were dounced in 10 ml 0.56 % potassium chloride using a glass dounce. The remainder of the IF-FISH protocol follows Cech and Peichel (2015).

Microscopy

Images were taken using a Nikon Eclipse 80i microscope (Nikon, Japan) with an automated filter turret (Chroma filters 31000v2 (DAPI), 41001 (FITC), and 41004 (Texas Red); Chroma, USA) using the ×100 objective. Following Cech and Peichel (2015), images were pseudocolored using NIS Elements imaging software (BR 3.00, SP7, Hotfix8, Build 548, Nikon, Japan).

Results

No loss of chromosome arms on the Japan Sea neo-Y fusion chromosome

To determine if the neo-Y fusion was a complete fusion between both chromosomes, or if large portions of each or one of the respective p-arms was lost, we performed two types of FISH experiments. First, we conducted hybridization with BAC probes homologous to the ends of chromosome 9 (44L12) and the Y chromosome (101E8) that are near the fusion site, as well as the end of the Y chromosome (188J19) opposite to the fusion site (Table 1). These probes all hybridize to the neo-Y on Japan Sea male metaphase chromosomes, demonstrating that loss of an entire chromosome arm has not occurred on the neo-Y fusion chromosome (Fig. 1).

Table 1 List of BACs used in this study

Chr	BAC	Library	Genomic position (bp)	Cytogenetic position[a]
9	44L12	CHORI-213	497,589–729,621	Distal end of short arm
9	35K20	CHORI-215	18,760,858–18,914,793	Distal end of long arm
X	188J19	CHORI-213	2,384,200–2,563,816	Distal end of short arm
Y	188J19	CHORI-213	Unknown	Distal end of short arm
X	180J08	CHORI-213	3,377,138–unknown	Proximal end of short arm
Y	180J08	CHORI-213	Unknown	Proximal end of long arm
X	171H24	CHORI-213	5,465,262–5,643,381	Proximal end of long arm
Y	171H24	CHORI-213	Unknown	Proximal end of short arm
X	101E08	CHORI-213	11,446,792–11,651,387	Middle of long arm
Y	101E08	CHORI-213	Unknown	Distal end of long arm
Y	91G03	CHORI-215	Unknown	Distal end of long arm

[a] Position is relative to the centromere; proximal is closer to the centromere and distal is further from the centromere. The genomic positions of the BACs on chromosome 9 and the X chromosome were determined by BLASTing the publicly available BAC end sequences (Kingsley and Peichel 2007) to the updated threespine stickleback genome assembly (Glazer et al. 2015). The genomic positions of BACs on the Y chromosome are unknown because a complete genome assembly of the Y chromosome is not available

Hybridization of these same BAC probes on Pacific Ocean male metaphase chromosomes further demonstrates that there have not been gross rearrangements between the Japan Sea and Pacific Ocean sticklebacks on either chromosome 9 or the Y chromosome (Fig. S2).

Second, to test if there was truly no loss of any genetic material, we also used a FISH probe to the 6-bp telomere sequence (TTAGGG) found in all vertebrates (Meyne et al. 1989). There is no internal telomere signal on the neo-Y chromosome, indicating that some loss of genetic material encompassing at least the telomeres has occurred on the neo-Y chromosome (Fig. S3). Interestingly, telomere loss can induce chromosome fusions (Gisselsson et al. 2001; Maser and DePinho 2002; Bailey 2006; Pobiega and Marcand 2010; Murnane 2010; Stimpson et al. 2010). However, we do not know whether loss of telomeres is a cause or consequence of the neo-Y fusion. Taken together, these data do suggest that the neo-Y chromosome resulted from a fusion between two nearly complete monocentric chromosomes, creating a dicentric neo-Y chromosome. The neo-Y chromosome is present in all Japan Sea males examined to date, and we have no evidence for dicentric chromosome segregation-associated aneuploidy in the Japan Sea males (i.e., no sex ratio bias in the offspring of Japan Sea males; data not shown). Furthermore, there is evidence of only one constriction on the neo-Y in metaphase chromosome spreads (Fig. S3). Thus, we hypothesized that the neo-Y

chromosome has been stabilized by centromere inactivation via either genetic or epigenetic inactivation.

Evidence for centromere inactivation on the Japan Sea neo-Y chromosome

Using CENP-A antibody staining in combination with FISH, we determined that only one region of CENP-A staining is found on the neo-Y in multiple independent metaphase spreads from Japan Sea males (Fig. 2). The CENP-A staining was flanked by BAC probes present at the end of the Y near the fusion breakpoint and at the distal end of chromosome 9, indicating that the active centromere is on chromosome 9 (Fig. 2). The CENP-A staining on the neo-Y is in a similar location to the CENP-A staining found on the unfused chromosome 9 (X_2), further suggesting that the active centromere on the neo-Y is retained from the ancestral acrocentric chromosome 9 (Fig. 2f).

To confirm that the ancestral Y chromosome centromere was active before the fusion that created the neo-Y occurred, we performed CENP-A staining in combination with FISH using a BAC probe (91G03; Table 1) that specifically labels the Y chromosome on Pacific Ocean male chromosomes (Fig. S4). We found normal CENP-A staining on the Pacific Ocean Y centromere (Fig. S5). This is evidence that the ancestral Y chromosome had an active centromere and that the Y
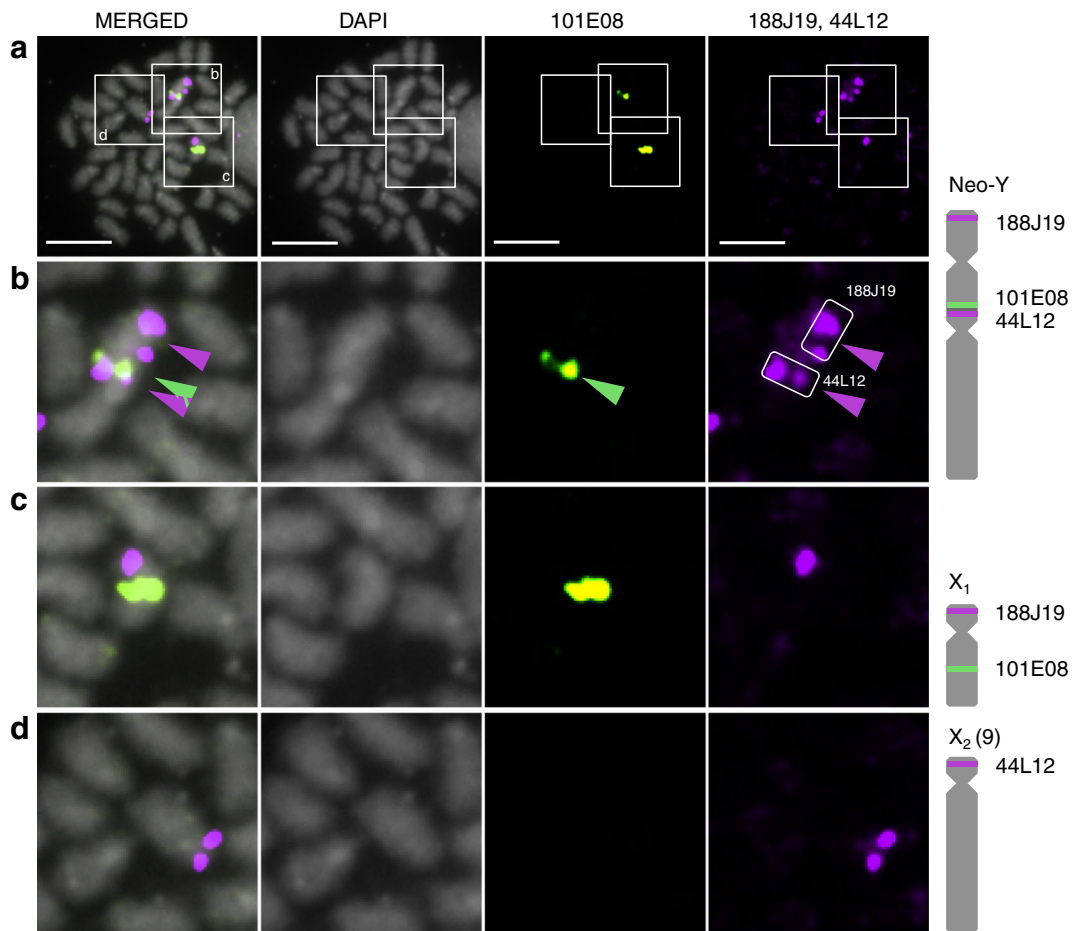
Fig. 1 The Japan Sea neo-Y is a fusion between two complete chromosomes. FISH with the ancestral X and Y chromosome BACs 188J19 (*purple*) and 101E08 (*green*) and the chromosome 9 BAC 44L12 (*purple*) on a Japan Sea male metaphase spread shows **a** three chromosomes with BAC hybridization each highlighted by *square boxes*. Higher magnification of the boxed regions in **a** shows the neo-Y fusion (**b**), the X_1 chromosome, which is derived from the ancestral X chromosome (**c**) and the X_2 chromosome, which is derived from the ancestral chromosome 9 (**d**). *Scale bar*, 5 μm

chromosome centromere was inactivated after the fusion to chromosome 9 in Japan Sea males.

Evidence for genetic inactivation on the Japan Sea neo-Y chromosome

Because we found evidence for only a single active centromere on the Japan Sea neo-Y chromosome, we sought to determine whether there has been genetic inactivation (i.e., deletion or alteration) of the ancestral Y chromosome centromere. We had previously identified the centromere sequence (GacCEN) in the Pacific Ocean threespine stickleback (Cech and Peichel 2015). Although this centromere sequence appears to be present on all autosomes, including chromosome 9 and the

X chromosome, we observed very weak hybridization of the centromere sequence to the Y chromosome in the ancestral Pacific Ocean population (Cech and Peichel 2015). It was unclear whether the centromere on the Y chromosome has a completely different sequence, a highly divergent sequence, or a reduced number of repeats. Here, we further explored these possibilities in order to analyze the fate of the ancestral Y chromosome centromere on the Japan Sea neo-Y. We performed additional FISH experiments on Pacific Ocean males with the centromere probe and confirmed that centromere hybridization on the ancestral Y is weak and variable. Over 11 independent FISH experiments, we counted 29 metaphase spreads with positive GacCEN staining and 21 with negative GacCEN staining
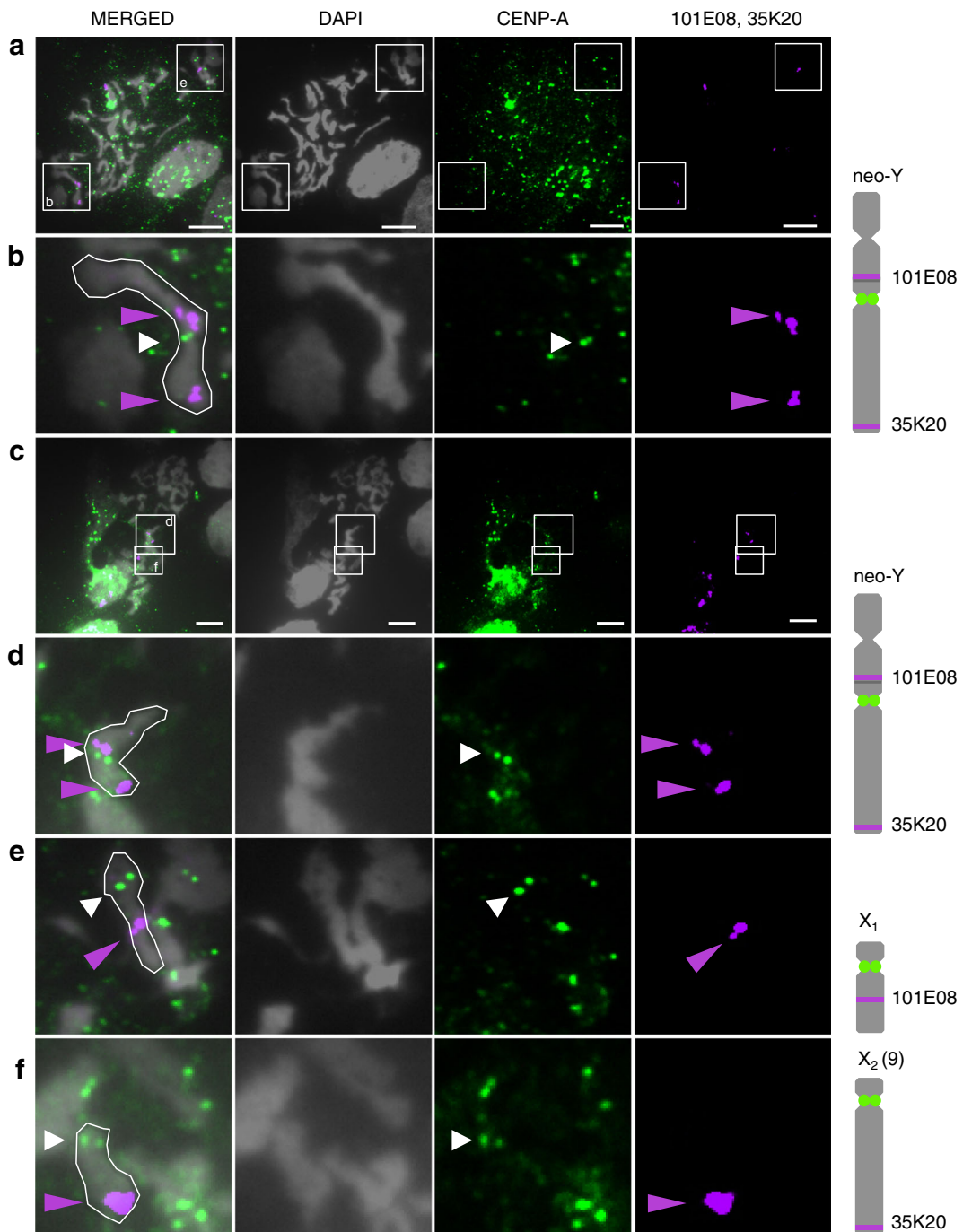
Fig. 2 The CENP-A antibody only localizes to one region on the Japan Sea neo-Y chromosome. **a**, **c** Two independent metaphase spreads from Japan Sea embryos stained with the CENPA antibody (*green*) as well as the X and Y chromosome BAC 101E08 (*purple*) and the chromosome 9 BAC 35K20 (*purple*). **b**, **d** Higher magnifications of the boxed regions in **a** and **c**, highlighting the neo-Y chromosome with the two regions of BAC staining (*purple arrowheads*) flanking two distinct CENP-A puncta (*white arrowhead*). The CENP-A staining is located on the chromosome 9-derived part of the neo-Y chromosome. **e** A higher magnification of the boxed region in **a**, highlighting the ancestral X_1 chromosome; 101E08 hybridizes to the middle of the long arm of X_1 (*purple arrowhead*), and there is one region of two CENP-A puncta (*white arrowhead*). **f** A higher magnification of the boxed region in **c**, highlighting the unfused chromosome 9 (X_2); 35K20 hybridizes to the end of the long arm of X_2 (*purple arrowhead*), and there is one region of two CENP-A puncta (*white arrowhead*). *Scale bar*, 5 μm

(Table 2). This variability is demonstrated by both positive and negative staining on metaphase spreads from the same male (Fig. 3). These data suggest that the Y centromere is likely not a completely different sequence but instead might represent a divergent repeat sequence and/or a reduced number of the same repeat sequence.

To determine whether the centromere sequence in the Japan Sea species is the same as in the Pacific Ocean species, we also performed CENP-A ChIP-seq in Japan Sea males and females at the same time we performed the Pacific Ocean CENP-A ChIP-seq (Cech and Peichel 2015). We had previously demonstrated that the Pacific Ocean and Japan Sea CENP-A genes only differ by a single amino acid (Cech and Peichel 2015), which is not in the region of the protein used for the antibody design (Fig. S6). Following previously described methods (Cech and Peichel 2015), we first identified the 500 most abundant sequence clusters in each IP sample and then analyzed the clusters with a fold enrichment of greater than one in the IP relative to the input (Table 3). Using these enriched sequence clusters, we identified a consensus CENP-A-associated putative centromere sequence in both Japan Sea males and females. As we found previously, most of the sequence clusters enriched in the IP relative to the input aligned to this consensus sequence (Table 3). The enriched sequence clusters that did not align to the consensus sequence did not align to each other and were among the clusters with the lowest enrichment in each sample (Table 3). Thus, these experiments identified one major CENP-A-associated repeat in the Japan Sea males and females; this Japan Sea putative centromere sequence is 98.7 % similar to the Pacific Ocean GacCEN and is also 186 bp and 61.2 % AT rich (Fig. S7). Similar to the Pacific Ocean population, the Japan Sea GacCEN probe shows hybridization to the constriction on metaphase spreads

Table 2 GacCEN staining on Y centromere from Pacific Ocean Y and Japan Sea neo-Y

|	Pacific Ocean Y	Japan Sea neo-Y
GacCEN positive staining	29	0
GacCEN negative staining	21	26
Number of males	5	6
Number of experiments	11	4

The number of metaphase spreads with GacCEN positive and negative staining, the number of males used to generate the metaphase spreads, and the number of independent FISH experiments are indicated

(Fig. S8) as well as colocalization with CENP-A (Fig. S9).

We performed FISH using the GacCEN probe in combination with BAC probes on Japan Sea metaphase spreads to determine whether it is present on the neo-Y. There is strong hybridization of the GacCEN probe to the ancestral chromosome 9 centromere, providing evidence that this centromere has not been deleted on the neo-Y (Fig. 4). Although we also observed strong GacCEN staining on both X chromosomes in Japan Sea females (Fig. S10), there was no hybridization of the GacCEN probe to the ancestral Y centromere on the neo-Y (Fig. 4). In contrast to the variable hybridization of the GacCEN probe to the Y chromosome in Pacific Ocean males (Fig. 3), the lack of GacCEN hybridization to the Y chromosome was consistently observed on 26 different metaphase spreads from six independent Japan Sea males over four different experiments (Table 2). Because we do not know the specific sequence of the Y centromere, we cannot determine whether the lack of staining to the Y centromere on the neo-Y is due to deletion of the centromeric DNA on the neo-Y or further divergence of the centromeric DNA on the neo-Y. However, these data do suggest that there has been a genetic alteration to the ancestral Y chromosome centromere on the Japan Sea neo-Y.

To further assess whether there has been a deletion of the ancestral Y centromere on the neo-Y, we examined the hybridization pattern of BAC probes flanking the ancestral Y centromere on the neo-Y. Previous work had identified two BAC probes (171H24 and 180J08; Table 1) flanking the centromeric constriction on both the X and Y chromosomes in the Pacific Ocean species (Ross and Peichel 2008). Note that the order of these probes is different between the X and Y chromosomes due to the presence of several inversions on the Y chromosome relative to the X (Ross and Peichel 2008). There is no loss of hybridization with either probe on the Japan Sea neo-Y (Fig. 5), indicating that these large regions flanking the centromere are still present. However, when compared to hybridization on the Japan Sea X, these probes do appear to be closer together on the neo-Y (Fig. 5). Additionally, hybridization with these two probes and the GacCEN probe shows that there is also a slight distance between these two probes on the ancestral Pacific Ocean Y chromosome, along with weak internal centromere staining (Fig. 6b). By contrast, there is no internal GacCEN signal between these two BACs on the Japan Sea

Fig. 3 GacCEN staining is weak and variable on the ancestral Pacific Ocean male Y chromosome centromere. FISH with the GacCEN probe (*green*) and Y chromosome BAC 91G03 (*purple*) on four different metaphase spreads from the same Pacific Ocean male. Two metaphase spreads show GacCEN staining on the Y chromosome (**a**–**d**), while two metaphase spreads lack GacCEN staining on the Y chromosome (**e**–**h**). **b**, **d** Higher magnification of the boxed regions in **a** and **c** highlighting Y chromosomes with weak GacCEN staining (*green arrowhead*), and **f**, **h** higher magnification of the of the boxed regions in **e** and **g** highlighting Y chromosomes with no GacCEN staining. *Scale bar*, 5 μm
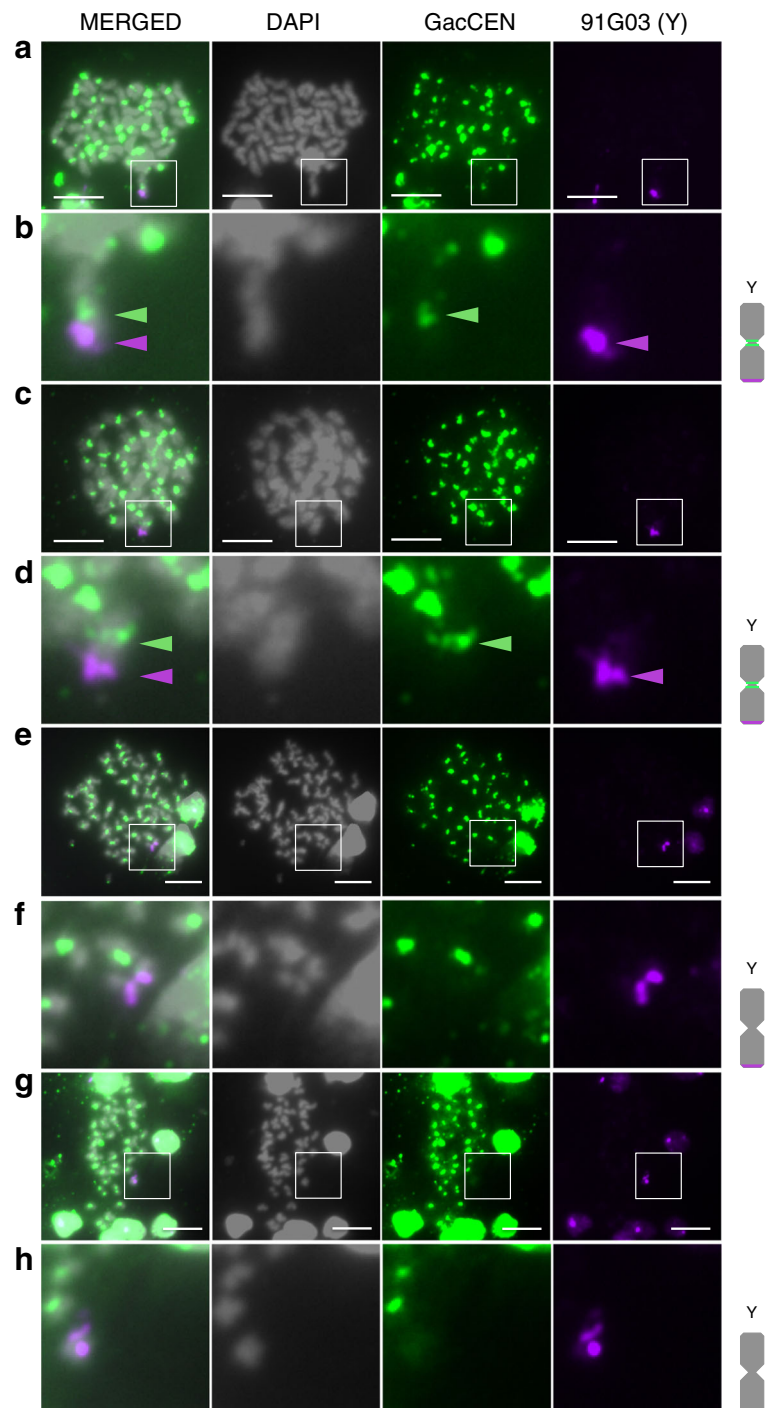
neo-Y (Fig. 6e), although there is a clear separation between these BACs and internal GacCEN signal on both X chromosomes in Japan Sea females (Fig. S11). These data suggest, but do not definitively prove, that there has been a deletion of the ancestral Y chromosome

centromere on the Japan Sea neo-Y. However, the absence of GacCEN hybridization strongly supports a model in which there has been genetic inactivation of the ancestral Y centromere on the Japan Sea neo-Y. Still, it is important to note that we cannot determine whether

Table 3 Summary of CENP-A ChIP-seq data in two Japan Sea male replicates and two Japan Sea female replicates

	Japan Sea male 1	Japan Sea male 2	Japan Sea female 1	Japan Sea female 2
Number of enriched sequence clusters in 500 most abundant	228	230	175	170
Number of enriched sequence clusters with GacCEN repeat	220	196	166	97
Average fold enrichment of IP/input	10.44	10.19	13.58	3.00
Range of fold enrichment across all sequence clusters	1.00–39.29	1.00–41.62	1.03–49.36	1.00–7.24
Range of fold enrichment for clusters without GacCEN	1.00–1.17	1.00–3.72	1.03–2.09	1.00–1.29
Total IP reads	34,418,733	28,294,604	23,676,014	32,155,558
Total input reads	32,317,012	26,307,950	24,794,940	38,968,765

genetic changes to this centromere led to its inactivation or whether genetic changes occurred after an initial phase of centromere inactivation that was mediated by an epigenetic mechanism.

Discussion

Overall, we have shown that the Japan Sea neo-Y chromosome fusion has been stabilized by centromere inactivation. The ancestral Y centromere has been inactivated as evidenced by loss of CENP-A staining, and the active neo-Y centromere is derived from chromosome 9. While we currently cannot conclusively determine whether the ancestral Y centromere sequence has been deleted or has changed in some other way on the neo-Y, our experiments suggest there have been genetic changes to the Y centromere on the Japan Sea

neo-Y. Our evidence for a partial deletion or loss of centromeric DNA is consistent with genetic deletions observed in engineered dicentrics in yeast and humans (Stimpson et al. 2010; Sato et al. 2012) and in human patients (Maraschio et al. 1990; Fisher et al. 1997). However, further experiments are required to identify the specific mechanism of genetic inactivation of the Y centromere on the neo-Y. These experiments are hindered because there is currently not a Y chromosome sequence assembly in threespine stickleback, as is the case in most organisms. However, we are currently working to obtain an assembled sequence of the Pacific Ocean threespine stickleback Y chromosome, which will allow us to test for genetic deletion by identifying the unique sequences at the centromere boundaries. Combining this assembly with CENP-A ChIP-seq experiments in Pacific Ocean males will also allow us to determine whether there are Y chromosome-
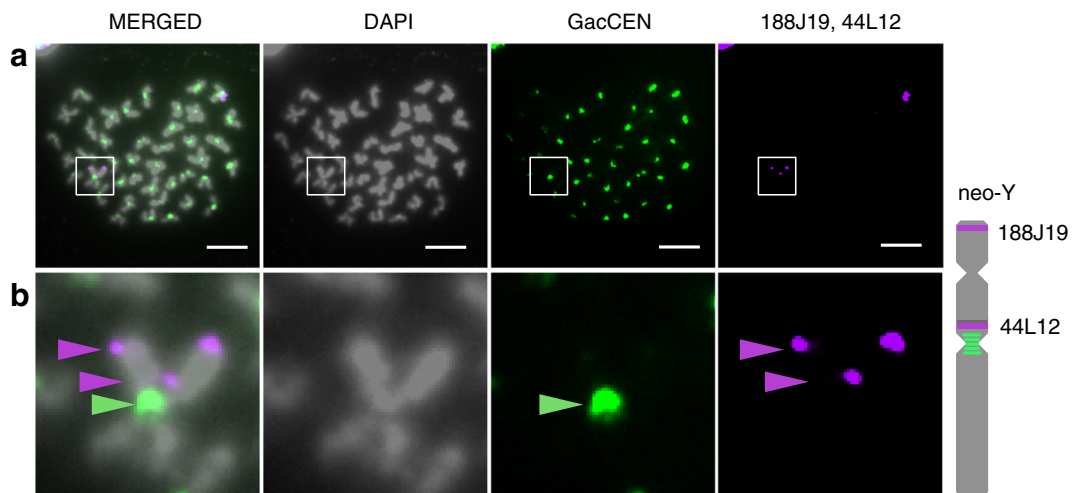

Fig. 4 GacCEN hybridizes to the centromere of chromosome 9 on the Japan Sea neo-Y chromosome. FISH with the GacCEN probe (*green*), Y chromosome BAC probe 188J19 (*purple*), and a chromosome 9 BAC probe 44L12 (*purple*) on a metaphase spread from a Japan Sea male is shown in **a**. **b** A magnification of the neo-Y chromosome from the boxed region in **a**. The GacCEN probe only localizes to the region of the ancestral chromosome 9 centromere on the neo-Y. *Scale bar*, 5 μm
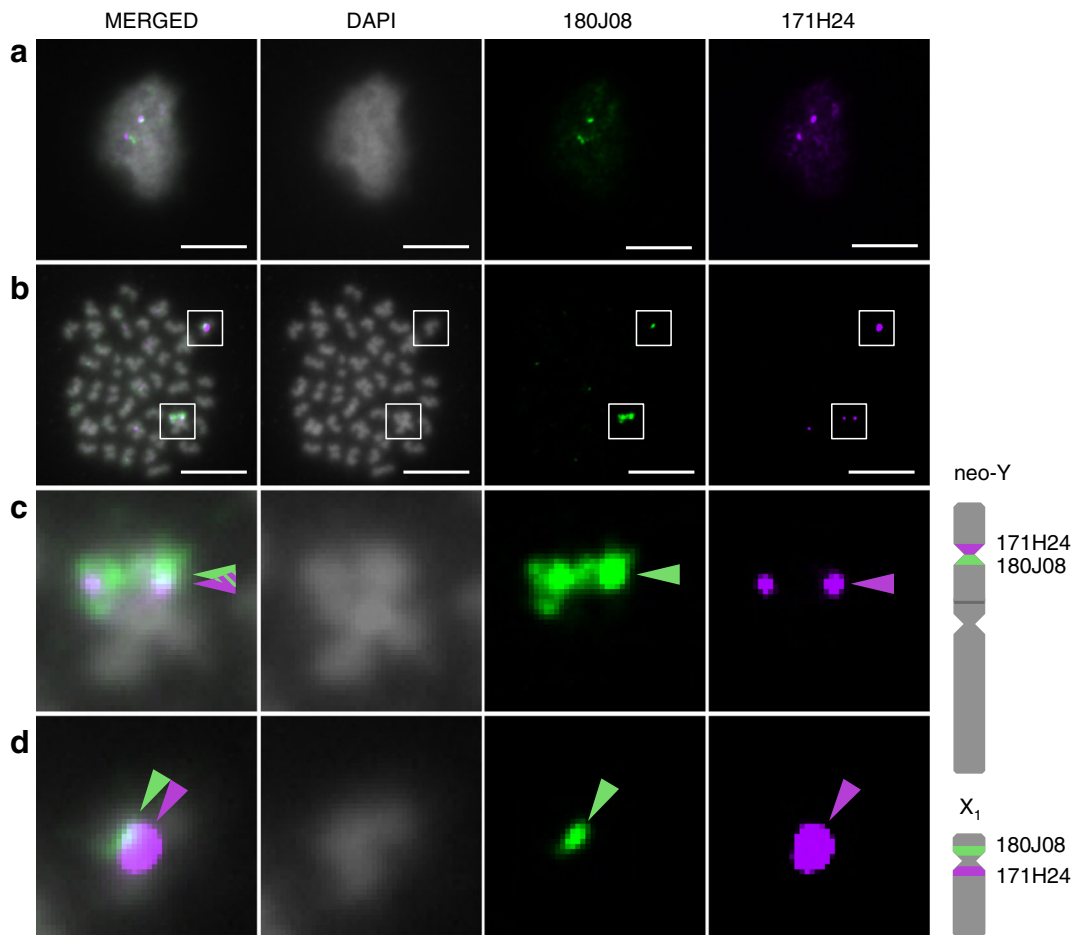
Fig. 5 Mapping the regions flanking the ancestral Y centromere on the neo-Y fusion. FISH with the X and Y BACs 180J08 (*green*) and 171H24 (*purple*) on Japan Sea male interphase nuclei (**a**) and metaphase spreads (**b**). **c**, **d** Higher magnifications of the boxed regions in **b**. Both probes are present on the neo-Y (**c**) and the ancestral X_1 (**d**), although the probes appear to be closer together on the neo-Y (**c**) as compared to the ancestral X_1 (**d**). *Scale bar*, 5 μm

specific centromeric repeats in the ancestral Pacific Ocean sticklebacks and whether there have been genetic changes to these repeats on the Japan Sea neo-Y.

It would also be interesting to determine whether additional epigenetic changes have occurred on the inactive centromere. For example, inactive centromeres in wheat show different patterns of histone methylation than those found on active centromeres (Zhang et al. 2010), while the DNA at inactivated centromeres in maize is hypermethylated (Koo et al. 2011). Currently, nothing is known about histone modifications or DNA methylation at either active or inactive centromeres in threespine sticklebacks.

Interestingly, the ancestral Y chromosome has independently fused to the acrocentric chromosome 12 in males of the blackspotted stickleback (*Gasterosteus wheatlandi*) species within the past 15 million years, creating an independent neo-Y chromosome (Ross et al. 2009). From an evolutionary perspective, it is intriguing that of the seven known species of stickleback, two have undergone fixation of a Y-autosome fusion. It will be particularly interesting to test whether the Y chromosome centromere is also inactivated on the blackspotted neo-Y because previous studies in plants and humans have suggested that there may be differences in centromere size or strength that lead to the preferential inactivation of one centromere on dicentric chromosomes (Sullivan et al. 1994b; Han et al. 2009; Zhang et al. 2010; Liu et al. 2015). Although we do not know if the ancestral Y chromosome centromere in the threespine stickleback is smaller or weaker, we do know that it
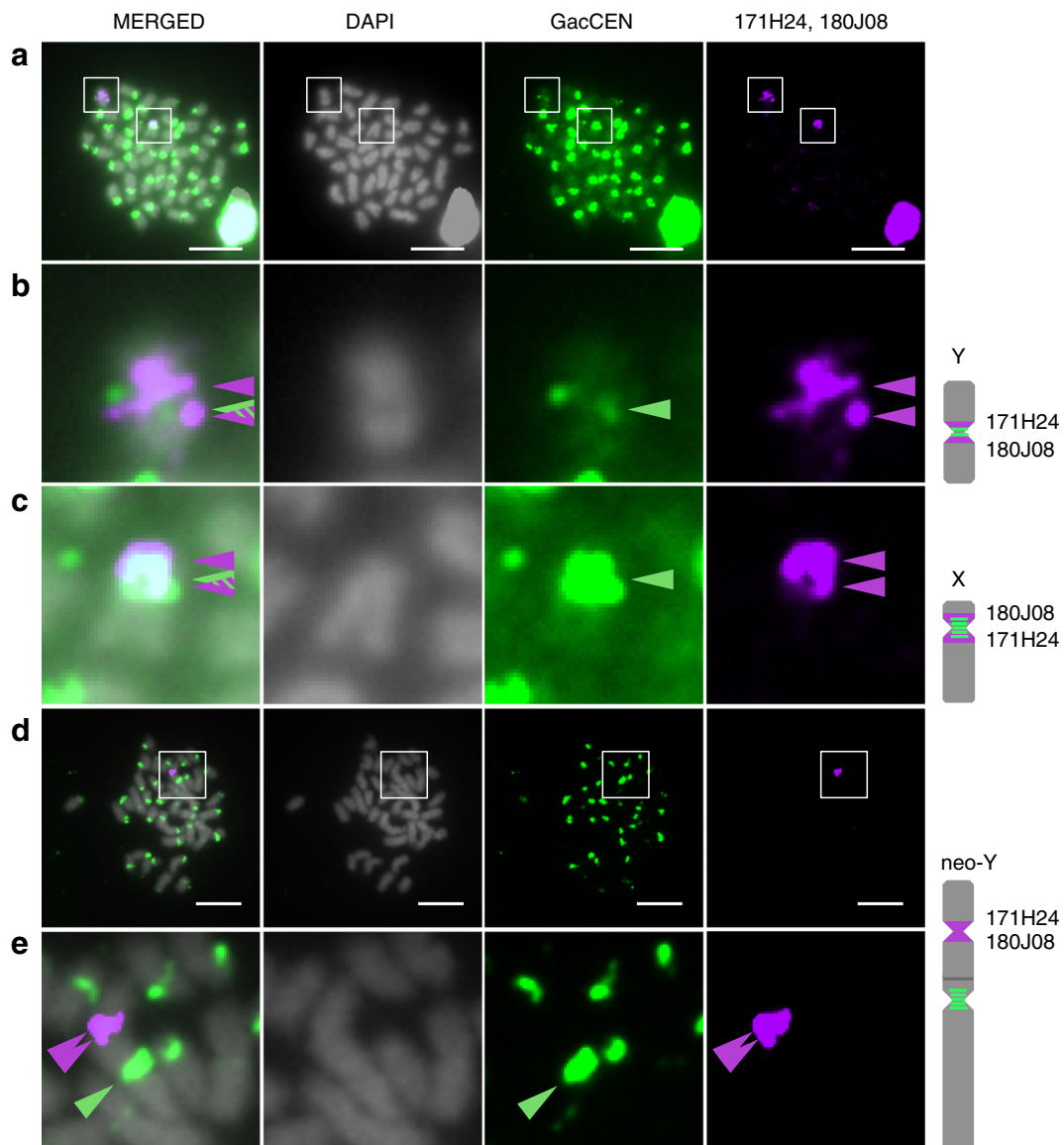
Fig. 6 GacCEN and centromere flanking regions on the Pacific Ocean X and Y and Japan Sea neo-Y chromosomes. FISH with the X and Y BACs 180J08 and 171H24 (*purple*) and GacCEN (*green*) on a metaphase spread from a Pacific Ocean male is shown in **a** and a metaphase spread from a Japan Sea male in **d**. **b**, **c** Magnifications of the boxed regions in **a** showing two distinct regions of staining (*purple arrowheads*) flanking weak GacCEN staining (*green arrowhead*) on the unfused Y (**b**) and two distinct regions of staining (*purple arrowheads*) flanking strong GacCEN staining (*green arrowhead*) on the X (**c**). **e** A magnification of the boxed regions in **d** showing that the two centromere flanking probes (*purple arrowheads*) are very close together on the neo-Y with no GacCEN staining in between. The strong GacCEN staining (*green arrowhead*) is from the chromosome 9 centromere on the neo-Y. *Scale bar*, 5 μm

differs from the centromeres on the rest of the chromosomes (Cech and Peichel 2015), as is also true in humans and mice (Wolfe et al. 1985; Pertile et al. 2009; Miga et al. 2014). Taken together, these data suggest the intriguing possibility that Y chromosomes might be better able to tolerate fusions than other chromosomes. Interestingly, Y-autosome fusions are more common that X-autosome fusions in fish and squamate reptiles (Pennell et al. 2015). However, the contribution of centromere strength or centromere inactivation to the establishment and fixation of chromosome fusions during evolution remains to be explored.

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