Cold Fusion: Massive Karyotype Evolution in the Antarctic Bullhead Notothenia coriiceps

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ABSTRACT

Half of all vertebrate species share a series of chromosome fusions that preceded the teleost genome duplication (TGD), but we do not understand the causative evolutionary mechanisms. The “Robertsonian-translocation hypothesis” suggests a regular fusion of each ancestral acro- or telocentric chromosome to just one other by centromere fusions, thus halving the karyotype. An alternative “genome-stirring hypothesis” posits haphazard and repeated fusions, inversions, and reciprocal and nonreciprocal translocations. To study large-scale karyotype reduction, we investigated the decrease of chromosome numbers in Antarctic notothenioid fish. Most notothenioids have 24 haploid chromosomes, but bullhead notothen (Notothenia coriiceps) has 11. To understand mechanisms, we made a RAD-tag meiotic map with ~10,000 polymorphic markers. Comparative genomics aligned about a thousand orthologs of platyfish and stickleback genes along bullhead chromosomes. Results revealed that 9 of 11 bullhead chromosomes arose by fusion of just two ancestral chromosomes and two others by fusion of three ancestral chromosomes. All markers from each ancestral chromosome remained contiguous, implying no inversions across fusion borders. Karyotype comparisons support a history of: (1) Robertsonian fusions of 22 ancestral chromosomes in pairs to yield 11 fused plus two small unfused chromosomes, like N. angustata; (2) fusion of one of the remaining two ancestral chromosomes to a preexisting fused pair, giving 12 chromosomes like N. rossii; and (3) fusion of the remaining ancestral chromosome to another fused pair, giving 11 chromosomes in N. coriiceps. These results raise the question of what selective forces promoted the systematic fusion of chromosomes in pairs and the suppression of pericentric inversions in this lineage, and provide a model for chromosome fusions in stem teleosts.

KEYWORDS

Notothenioid Antarctica Robertsonian translocation teleost genome duplication karyotype reduction

Compared to placental mammals, teleost fish have remarkably similar chromosome numbers: 58% of 580 teleost species studied have 24 or 25 haploid chromosomes (Naruse et al. 2004). The distribution shows a small secondary peak at ~50 chromosomes (25 of 580 species, 0.4%) (Naruse et al. 2004), which mostly involves species like salmonids and carps, whose lineages experienced a relatively recent genome duplication event (Ohno et al. 1967; Allendorf and Thorgaard 1984). Placental mammals, on the other hand, have wildly variable karyotypes with four small peaks at 19 (4.7% of mammals), 21 (8.5%), 22 (8.1%), and 24 (8.8%) haploid chromosomes, with the remaining 70% distributed between 3 and > 50 chromosomes (Naruse et al. 2004). Even within a single order, different placental mammals can have dramatically different karyotypes; for example, Cervidae (deer) vary between 3 and 40 chromosomes in the haploid set and Rodentia (rodents) range between 5 and 51 (Schertman 2012). Teleosts with a sequenced genome generally preserve chromosome numbers similar to the ancestral karyotype: zebrafish (Danio rerio) with 25 chromosomes (Amores and Postlethwait 1999), medaka (Oryzias latipes) and platyfish (Xiphophorus maculatus) both with 24 (Ocalewicz 2004; Kasahara et al. 2007), cod
(Gadus morhua) with 23 (Ghigliotti et al. 2012), fugu pufferfish (Takifugu rubripes) having 22 (Miya et al. 1995), and both stickle- back (Gasterosteus aculeatus) and green pufferfish (Tetraodon nigroviridis) with 21 haploid chromosomes (Grutzner et al. 1999; Upton et al. 2011). Karyotypic stability in teleosts vs. variability in mammals is even more notable given that teleosts have been diverging for more than twice as long as placental mammals, ~230 vs. ~65 MY (Hurley et al. 2007; Wible et al. 2007; Santini et al. 2009). These observations pose questions concerning evolutionary mechanisms that appear to have constrained karyotypic evolution in teleosts or to have permitted variable karyotypes in mammals.

Karyotypic stability among teleosts is even more surprising given the TGD, which occurred ~226–316 MYA (Amores et al. 1998; Taylor et al. 2003; Jaillon et al. 2004; Hurley et al. 2007; Santini et al. 2009). The TGD initially doubled the chromosome number, and the return to functional diploidy involved a substantial number of chromosomal rearrangements after the TGD (Nakatani et al. 2007). The TGD occurred after the divergence of the teleost lineage from the Holostean lineage, which includes spotted gar (Lepisosteus oculatus) and bowfin (Amia calva), ~350 MYA (Hoegg et al. 2004; Amores et al. 2011). Surviving holosteans have slowly evolving genomes (Braasch et al. 2016) and, remarkably, many entire gar and chicken chromosomes have orthologous gene content and even chromosome size (Braasch et al. 2016), suggesting that few interchromosomal exchanges occurred in these lineages since gar and chicken last shared a common ancestor ~450 MYA. Because the nonteleost fish spotted gar has 29 chromosomes in its haploid set (Braasch et al. 2016), extensive chromosome fusion events must have occurred in the teleost lineage to achieve the 25 haploid chromosomes found in most of today’s teleosts despite the TGD. Comparative genomic analyses showed that pairwise chromosome fusions occurred in the teleost lineage before the TGD (Braasch et al. 2016). Understanding the mechanisms of such massive fusion events would help us to understand genome evolution for half of all vertebrates.

Although teleost genomes retained extraordinarily stable chromosome numbers with few interchromosomal exchanges (translocations) after the chromosomal fusion events that preceded the TGD, comparative genomic analyses have shown that teleosts experienced frequent intrachromosomal rearrangements (inversions and transpositions) (Postlethwait et al. 1998; Naruse et al. 2004). For example, platyfish and medaka karyotypes remained remarkably similar with few translocations between chromosomes but with numerous transpositions and inversions since their lineages diverged ~120 MYA (Amores et al. 2011, 2014). An example is linkage group (LG) 9 of the platyfish, which, although sharing reciprocally exclusive conserved synteny with LG4 of medaka, experienced >30 inversions and transpositions with respect to the latter (Amores et al. 2014). These observations pose a problem: what forces permitted intrachromosomal rearrangements but restricted interchromosomal exchanges in teleosts? Solutions could come from exploring situations that counter these rules, situations in which translocations are many but perhaps inversions might be few. To probe these issues, we investigated a case of extensive chromosome number reduction in Antarctic nototheniid fishes.

Within teleosts, a single subgroup of percomorph fish, the notothenioids, currently dominates the subzero (~1.9°C) water of the Southern Ocean (Eastman 2005). After the separation of Antarctica, South America, and Australia ~35 MYA (Near et al. 2015), the Drake Passage opened and the Circumpolar Currents formed, thereby isolating the Southern Ocean and allowing it to gradually chill to its current frigid condition ~10–14 MYA (Kempton 1977). During this prolonged cooling period, many taxa became locally extinct, liberating numerous ecological niches into which notothenioids could radiate (Eastman 1993; Eastman and McCane 2000). As notothenioids conquered Antarctic waters, they evolved key innovations, including the origin of antifreeze glycoproteins (AFGs) (Devries 1988; Chen et al. 1997; Cheng and Chen 1999; Cheng and Detrich 2007) and a greatly altered inducible heat shock response (Hofmann et al. 2000; Place and Hofmann 2005; Huth and Place 2013; Buckley et al. 2004; Buckley and Somero 2009; Detrich et al. 2012).

Like most teleosts, Antarctic notothenioids generally have 24 chromosome pairs (see Figure 1). Even icefish (Channichthys, Figure 1) retain this ancestral karyotype despite their highly derived features: delayed and reduced bone and scale mineralization, substantial deposition of lipids, the loss of myoglobin and/or hemoglobin genes, the diffusion of oxygen directly through scaleless skin, decreased oxygen demand associated with changes in the density and morphology of mitochondria, and increased relative heart size and pumping volume (Koch 2005; Albertson et al. 2010; Friedrich and Hagen 1994; Hagen et al. 2000; Hemmingsen 1991; Egginton et al. 2002; Sidell et al. 1997; O’Brien et al. 2000).

However, several notothenioid lineages retained ancestral morphologies but evolved karyotypes with a dramatically reduced chromosome number (Figure 1), including Lepidonotothen nudifrons (1n = 14), Trematomus loembergii (1n = 14), and the Bathydracon marri and Rakovitza glacialis lineages (1n = 19 and 18, respectively) (Tomaszkiewicz et al. 2011; Morescalchi et al. 1992b; Near and Cheng 2008; Ozouf-Costaz et al. 1991). However, the most dramatic karyotype evolution among notothenioid fish involves the lineage of the genus Notothenia. Fish in the monophyletic group Notothenia angustata, N. microlepidota, and N. magellanica (Dettai et al. 2012) have 13 chromosomes as a haploid set. N. rossii has 12, and N. coriiceps has the further reduced set of 11 haploid chromosomes (Priodrina and Neyelov 1984; Doussau de Bazignan and Ozouf-Costaz 1985; Van et al. 1987; Ozouf-Costaz and Doussau de Bazignan 1987; Ozouf-Costaz et al. 1991; Pisano et al. 2003b) (Figure 1). These observations raise the question: what evolutionary processes resulted in the N. coriiceps genome possessing fewer than half the number of chromosomes that is found in most other notothenioids and indeed, most other teleost fish?

N. coriiceps could have evolved a greatly reduced karyotype by any of several mechanisms. Under a simple “Robertsonian-translocation hypothesis,” each of the 24 ancestral haploid acro- or telocentric chromosomes fused with just one other chromosome at their centromeres (Robertsonian translocations) (Robertson 1916) to produce 12 chromosomal pairings but evolved karyotypes with a dramatically reduced chromosome number (Figure 1), including Lepidonotothen nudifrons (1n = 14), Trematomus loembergii (1n = 14), and the Bathydracon marri and Rakovitza glacialis lineages (1n = 19 and 18, respectively) (Tomaszkiewicz et al. 2011; Morescalchi et al. 1992b; Near and Cheng 2008; Ozouf-Costaz et al. 1991). However, the most dramatic karyotype evolution among notothenioid fish involves the lineage of the genus Notothenia. Fish in the monophyletic group Notothenia angustata, N. microlepidota, and N. magellanica (Dettai et al. 2012) have 13 chromosomes as a haploid set. N. rossii has 12, and N. coriiceps has the further reduced set of 11 haploid chromosomes (Priodrina and Neyelov 1984; Doussau de Bazignan and Ozouf-Costaz 1985; Van et al. 1987; Ozouf-Costaz and Doussau de Bazignan 1987; Ozouf-Costaz et al. 1991; Pisano et al. 2003b) (Figure 1). These observations raise the question: what evolutionary processes resulted in the N. coriiceps genome possessing fewer than half the number of chromosomes that is found in most other notothenioids and indeed, most other teleost fish?
bullhead chromosome consisting of segments from several ancestral chromosomes.

To distinguish these two hypotheses, we made sex-specific meiotic maps for N. coriiceps using sequence-based markers produced by RAD-seq (Baird et al. 2008; Miller et al. 2007a,b; Amores et al. 2011) and identified ancestral chromosomes by comparative genomics with outgroups. For comparative genomics, we identified coding sequences on RAD-tag markers mapped on the N. coriiceps map to stickleback and platyfish genome sequences (Schartl et al. 2013; Amores et al. 2014; Jones et al. 2012). Because notothenioids and sticklebacks both occupy Near’s clade XIII of spiny-ray fishes, DNA sequences of notothenioids and stickleback should be more similar than DNA sequences of notothenioids and platyfish, which occupies clade VII (Near et al. 2012b). Nevertheless, the karyotype of stickleback with 21 chromosomes is more derived than platyfish with 24. The Robertsonian fusion hypothesis predicts that ancestral chromosomes would mostly be fused one-to-one. In contrast, the genome stirring hypothesis predicts a disorganized, rather haphazard arrangement of parts of ancestral chromosomes. Results showed that 22 of the 24 chromosomes present in the ancestral Notothenioid (as inferred from comparative genomics with stickleback and platyfish) fused in pairs, and then the remaining two unfused chromosomes joined two previously merged chromosomes to form today’s N. coriiceps karyotype of 11 haploid chromosomes. In contrast to the dramatic, extensive, and concerted evolution of Robertsonian translocations experienced in the N. coriiceps lineage, the current karyotype shows no inversions across centromeres since the fertilization event were 2200 bp over the samples of muscle, liver, fin, and spleen were stored in 100% EtOH at −20°C until further use. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN). The University of Oregon IACUC approved all protocols (13-27RR).

Creation of RAD-tag libraries
Genomic DNA was purified from the male and female parents and 244 of their progeny. RAD-tag libraries were created as described (Amores et al. 2011). DNA from each fish was digested with high-fidelity SfiI (New England Biolabs) restriction enzyme overnight and each sample was separately barcoded with P1 and P2 adaptor ligations overnight, using 96 different five-nucleotide barcodes. Fifty nanograms of pooled, size-selected DNA was amplified by PCR for 12 cycles and the PCR product was gel purified by excising a 200–500 bp fraction. Libraries were sequenced on an Illumina GAII or HiSeq2000 to obtain 100-nucleotide single-end reads.

Marker genotyping
Sequenced reads from the Illumina runs were sorted by barcode, allowing up to one mismatched nucleotide in the barcode sequence. Reads containing uncalled bases and those that had an average phred quality score that fell below 10 over 15% of the read length were discarded. Retained reads were genotyped using the software Stacks (Catchen et al. 2011). Progeny with < 800,000 reads were excluded.

MATERIALS AND METHODS

Animals
Adult N. coriiceps were collected by bottom trawls or baited traps deployed from the ARSV _Laurence M. Gould_ southwest of Low Island [Antarctic Specially Protected Area (ASPA) 152, Western Bransfield Strait; latitudes 63°15′S–63°30′S, longitudes 62°00′W–62°45′W, bounded on northeast by Low Island] or west of Brabant Island (ASPA 153, Eastern Dallmann Bay; latitudes 63°53′S–64°20′S and longitudes 62°16′W–62°45′W, bounded on the east by the shoreline of Brabant Island) in the Palmer Archipelago in May 2008. Fish were transported alive to Palmer Station, Antarctica, where they were maintained in seawater aquaria at ~1°C until they were about a month old, in midembyo stages [see Postlethwait et al. (2016)], and then were stored in 100% EtOH at ~−20°C. Eggs from a single female and sperm from a single male were stripped, gametes were mixed, and progeny from this single in vitro fertilization event were maintained at ~−1°C until they were about a month old, in midembyo stages [see Postlethwait et al. (2016)], and then were stored in 100% EtOH at ~−20°C. The male and female parents were killed by MS-222 overdose and samples of muscle, liver, fin, and spleen were stored in 100% EtOH at ~−20°C until further use. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN). The University of Oregon IACUC approved all protocols (13-27RR).
from Stacks analysis. The parameters for Stacks provided to denovo_map.pl were: a minimum of five reads for a stack (-m 5), up to four differences when merging stacks into loci (-M 4), and up to two fixed differences when merging loci from the parents into the catalog (-n 2).

Stacks exported data into JoinMap 4.1 (Van Ooijen 2006) for linkage analysis.

Map construction

Of the 244 F1 individual progeny sequenced, 52 markers of type ab × cd (segregating 1:1:1:1) were heterozygous in both parents with two male-specific alleles and two female-specific alleles; markers of type ef × eg (segregating 1:1:1:1) were heterozygous in both parents with one male-specific allele, one female-specific allele, and one shared allele; markers of type hk × hk (segregating 1:2:1) were heterozygous in both parents with two shared alleles; markers of type lm × ll (segregating 1:1) were heterozygous in the female parent and homozygous in the male parent; and markers of type nn × np (segregating 1:1) were homozygous in the female parent and heterozygous in the male parent. LG, linkage group; Gac, conserved markers for stickleback; Xma, conserved markers for platyfish.

| LG | Female Map cM | Male Map cM | Female Markers | Male Markers | Total Markers | ab × cd | ef × eg | hk × hk | lm × ll | nn × np | Synteny to Gac | Synteny to Xma |
|----|---------------|-------------|----------------|--------------|---------------|--------|--------|--------|--------|--------|---------------|---------------|
| 1  | 104.3         | 106.4       | 457            | 468          | 896           | 34     | 112    | 114    | 310    | 326    | 176           | 136           |
| 2  | 100           | 103.3       | 487            | 503          | 976           | 18     | 100    | 106    | 367    | 385    | 167           | 122           |
| 3  | 92.5          | 107.6       | 454            | 443          | 862           | 27     | 106    | 100    | 319    | 310    | 197           | 128           |
| 4  | 111.5         | 96.2        | 441            | 487          | 894           | 35     | 118    | 110    | 296    | 335    | 184           | 120           |
| 5  | 101.3         | 91.1        | 408            | 439          | 841           | 23     | 91     | 107    | 294    | 326    | 199           | 138           |
| 6  | 105           | 102.2       | 415            | 456          | 853           | 28     | 99     | 108    | 288    | 330    | 178           | 124           |
| 7  | 101.7         | 104.5       | 421            | 438          | 831           | 23     | 95     | 89     | 303    | 321    | 165           | 125           |
| 8  | 105.5         | 86.9        | 446            | 406          | 801           | 21     | 116    | 84     | 309    | 271    | 149           | 102           |
| 9  | 113.5         | 96.2        | 384            | 396          | 764           | 17     | 95     | 97     | 272    | 283    | 197           | 131           |
| 10 | 105.1         | 108.5       | 367            | 405          | 740           | 15     | 95     | 81     | 253    | 296    | 147           | 101           |
| 11 | 95.6          | 98          | 346            | 359          | 680           | 15     | 78     | 68     | 253    | 266    | 126           | 93            |
| Total| 1136         | 1100.9      | 4626           | 4800         | 9138          | 256    | 1105   | 1064   | 3264   | 3449   | 1885          | 1320          |

Table 1 Total genetic length in centimorgans (cM) and total number of markers for each linkage group

Markers of type ab × cd (segregating 1:1:1:1) were heterozygous in both parents with two male-specific alleles and two female-specific alleles; markers of type ef × eg (segregating 1:1:1:1) were heterozygous in both parents with one male-specific allele, one female-specific allele, and one shared allele; markers of type hk × hk (segregating 1:2:1) were heterozygous in both parents with two shared alleles; markers of type lm × ll (segregating 1:1) were heterozygous in the female parent and homozygous in the male parent; and markers of type nn × np (segregating 1:1) were homozygous in the female parent and heterozygous in the male parent. LG, linkage group; Gac, conserved markers for stickleback; Xma, conserved markers for platyfish.

Analysis of conserved syntenies

The sequence from each RAD marker was used as a search query against the platyfish (Amores et al. 2014) and stickleback (Jones et al. 2012) genome databases using an e-value cutoff of 1e−12 and a minimum sequence alignment overlap of 75% of the RAD-tag length. Markers with sequence conservation to multiple locations were eliminated from the analysis.

Genome size and coverage

Genome length was estimated using Method-4 of Chakravarti et al. (1991) in which each LG length is adjusted by the factor m+1/m−1, where m is the number of markers on the LG, and by the method described in (Fishman et al. 2001 #70), in which twice the value of marker spacing, s, is added to the length of each LG. Total map coverage was calculated as the ratio between observed and estimated genome length.

Data availability

RAD-tag sequences of all mapped individuals are available online at the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra; accession number SRP047484).
RESULTS

Making the map
Sequencing produced 15.3 million sequences for both the male and female parents of the map cross and an average of 2.2 million sequences for individual progeny. Stacks software yielded a total of 74,508 RAD-tags, of which 60,349 were present in at least 10 progeny individuals. We identified SNPs segregating in 14,396 RAD-tags, and of those, 11,808 were present in at least 150 progeny fish. (Supplemental Material Table S1.) Of the 11,808 mapped RAD-tag markers present in at least 150 of 192 progeny, 9138 markers mapped to the male map, or to the female map, or to both (Table 1), at the minimum required LOD of 25. The dataset included 1,654,303 total genotypes, 96,038 (5.5%) missing genotypes, and 1853 (0.1%) manually corrected genotypes. JoinMap 4.1 assigned markers to 11 LGs, which equals the number of cytogenetically described chromosomes (Phan et al. 1987; Ozouf-Costaz et al. 1991; Tomaszkiewicz et al. 2011). The total number of markers belonging to each LG ranged from a high of 976 in N. coriiceps LG2 (Nco2) to a low of 680 markers in Nco11. (Figure S1.) Map lengths were similar for both sexes: 1136 cM for the female map and 1100.9 cM for the male map; the combined consensus map was 1300 cM long. LG sizes ranged between 92.5 and 113.5 cM for the female map and between 86.9 and 108.5 cM for the male map. The estimated genome length based on genetic map data showed that the RAD-tag map covers from 99.1 to 99.4% of the total genome length (Chakravarti et al. 1991; Fishman et al. 2001). Because the bullhead notothen genome is ~637 Mb (Shin 2014), the genome has a density of ~14.3 mapped markers per Mb.

Sex-specific recombination rates
Although the sex-specific linkage maps were similar in total length, the two maps differed substantially in the distribution of recombination events along the chromosomes. In the female map, recombination was more frequent near the centromeres, but in the male map, the recombination rate was higher near the telomeres; Figure 2 shows an example for LG1 (Nco1, N. coriiceps LG 1). Centromere position was estimated from cytogenetic data (Ozouf-Costaz et al. 1991; Phan et al. 1987; Tomaszkiewicz et al. 2011) as the block of markers with greatly reduced recombination near the middle of LGs for these metacentric chromosomes.

Segregation distortion
Most markers showed expected Mendelian segregation ratios. However, the segregation of some markers, fell outside expected Mendelian segregation ratios, a phenomenon that can occur if gametes or embryos have viability defects due to linked loci. For example, Nco11 showed little segregation distortion across most of the chromosome (Figure 3, A and B). In contrast, 464 markers (4.7%) showed significant segregation distortion (P < 0.01), nearly all (426/464) of which localized in two large sex-specific blocks. One block of segregation distortion included 244 markers distributed between 39.1 and 60.7 cM in the Nco1 male map (Figure 3, C and D), and the other large block of segregation distortion included 182 markers located between 39.8 and 65.7 cM in the Nco4 female map (Figure 3, E and F). For example, lm × ll markers in the female LG4 map showed 72 homozygous individuals and 120 heterozygous individuals, a ratio of 0.75: 1.25 [or, for markers with the opposite polarity, 120 homozygotes to 72 heterozygotes (a ratio of 1.25: 0.75)] rather than the expected 1: 1 (96:96) segregation ratio. Some ab × cd markers in the same region of LG4 showed a segregation ratio of 1.33; 1.17: 0.73: 0.77 (64 ac genotypes: 56 ad: 35 bc: 37 bd) rather than the expected 1: 1: 1: 1 ratio (48: 48: 48: 48 individual genotypes). When scoring female and male alleles for such markers, the segregation of the female ab alleles was distorted (120:72) and that of the male bc alleles was normal (99:93), in agreement with the pattern of the surrounding male and female markers in the region. Three additional small blocks of significant segregation distortion were also identified: (1) one block...

Figure 2 Male and female linkage maps for N. coriiceps chromosomes Nco1. The position of the centromere (Cen, gray box) is estimated based on published karyotype information and greatly reduced recombination rates. Connecting lines indicate the position of markers present in both the male and female chromosomes. Markers with sequence conservation to stickleback (Gac) or platyfish (Xma) sequences are color coded based on chromosome origin within these two species. For clarity, some loci do not list all polymorphic RAD-tags that mapped to the indicated position. Note sex-specific recombination rates across the chromosome. RAD, restriction site-associated DNA.
involved two widely separated markers and was barely statistically significant at position 70.5 in the male Nco11 map (Figure 3A); (2) another block involved four markers, two in each of two widely separated regions on Nco3; (3) and the final block involved 32 markers at position 52.2–56.9 cM in the female Nco5 map (Figure S2).

**Analysis of conserved syntenies**

Comparative genomic analyses are necessary to test whether the Robertsonian translocation hypothesis or the genome stirring hypothesis better predict the mechanism giving rise to the greatly reduced bullhead notothen karyotype. To obtain comparative data, we identified conserved sequences in our mapped RAD-tag sequences by BLAST comparisons of the *N. coriiceps* RAD-tag sequences to the stickleback and platyfish reference genome sequences. Fortunately, the restriction enzyme we used for making RAD-tags cuts preferentially at exon–intron borders, which makes protein-coding sequences appear more frequently than by chance (McCluskey and Postlethwait 2015). Among the 9138 mapped markers, 1320 (14.4%) had significant sequence similarity to sequences in the platyfish genome (Schartl et al. 2013) and 1885 (20.6%) had significant similarity to sequences in the genome of stickleback (Jones et al. 2012), to which bullhead notothen is more closely related than it is to platyfish (Near et al. 2012a,b). A total of 81 RAD-tags with conserved sequence were located on unmapped platyfish scaffolds, and 197 RAD-tags with conserved sequence were located on unmapped stickleback scaffolds. A display of markers with sequence homology along the genetic map allowed us to conduct comparative genomic analyses.

Although platyfish is an outgroup to the stickleback + notothenioid clade (Near et al. 2012), the platyfish haploid karyotype of 24 chromosome pairs containing 48 chromosome arms (NF = 48) reflects more closely the ancestral condition than the stickleback karyotype with 21 chromosome pairs (NF = 58) (see summary Figure 6) due to chromosome rearrangements in the stickleback lineage (Urton et al. 2011). Bullhead vs. platyfish and stickleback comparisons showed that 9 of the 11 bullhead chromosomes each have homology to two platyfish or stickleback chromosomes. Figure 2, for example, shows male and female maps for Nco1 with conserved markers for platyfish LG5 (Xma5) and stickleback LG IX (GacIX) on the top arm and platyfish Xma9 and stickleback GacVIII on the lower arm [for nomenclature, consider that the example marker called 44742Nco1Xma5GacIX means marker number 44742 on bullhead Nco1, which has sequence similarity to a locus on platyfish LG5 and stickleback LG IX].
**DISCUSSION**

Here, we report the first dense genetic linkage map for any species of Antarctic fish. The 11 bullhead LGs correspond to the number of chromosomes detected cytogenetically (Phan et al. 1987; Ozouf-Costaz et al. 1991; Tomaszkiewicz et al. 2011). We conclude that the linkage map spans the entire bullhead genome.

Analysis of chromosome numbers and arm numbers, coupled with the bullhead genetic map, shows the pattern of karyotype evolution in notothenioids and their relatives. Basally diverging notothenioids, like *Cottoperca gobio* and *Bovichtus variegatus*, have a diploid number of 48 and a chromosome arm number of 48 (Figure 1 and references therein), indicating that all ancestral chromosomes were likely acrocentric or telocentric. The next most recently branching lineage, represented by *Pseudaphritis urvillii*, still has 2n = 48, but has NF = 52 rather than NF = 48, indicating that two pericentric inversions brought the centromere toward the middle of the chromosome for two ancestral chromosomes, a condition shared by all of the Cryonotothenioidea (Figure 1 and references therein). Within the Harpagiferidae–Artedidraconidae–Bathydraconidae–Channichthyidae clade, chromosome counts and arm numbers are mostly rather stable, although some lineages experienced a few chromosome fusions and pericentric inversions (Figure 1).

Within the Nototheniidae, some lineages show chromosome fusions, fissions, and pericentric inversions, especially in the *Trematomus* genus, for example *T. loemnbergii* with a diploid set of 28 or 30 chromosomes having 52 chromosome arms and *T. nicolai* with a diploid set of 57 or 58 chromosomes and 83–84 chromosome arms (Figure 1). However, the most dramatic karyotype evolution is in the *Notothenia–Paranothenia* clade, the mechanisms of which our comparative genetic map elucidates.

**A model for karyotype reduction in the Notothenia–Paranothenia clade**

Comparative genomic analyses showed that 9 of the 11 bullhead notothen chromosomes originated by fusion of two ancestral chromosomes, two bullhead chromosomes arose from the fusion of three ancestral chromosomes, and that no chromosome inversions or transpositions were detected across the centromeres (Figure 6). These comparative genomic data rule out the genome-stirring hypothesis for the reduction of the bullhead karyotype compared to outgroups (Figure 1) and are what would be expected from the hypothesis of an organized series of mostly one-to-one Robertsonian translocations.
Coupling our bullhead map data with published chromosome numbers for notothenioids and their nearest outgroups (Figure 1) provides a model to explain the massive reduction of chromosome number in the *Notothenia* species group (Figure 7). First, the data suggest that the last common ancestor of the *Notothenia* species group had 24 chromosomes in its haploid set (Figure 7, step 1) because the nearest
outgroup, the Nototeniidae including Dissostichus mawsoni, L. squamifrons, and T. lepidorhinus, has mainly 24 haploid chromosomes, as do most fish in the sister lineage, which includes Harpagifer antarcticus and icefish (Channichthyidae) like Chionodraco rastrosipinosus and C. aceratus (Figure 1). Second, a pericentric inversion in one of the smallest chromosomes created a small metacentric chromosome (the smallest, blue chromosome in Figure 7B). This small metacentric chromosome is still present in N. angustata, N. magellanica, and N. rossii (Doussau de Bazignan and Ozouf-Costaz 1985; Pisano et al. 2003b). Third, 22 ancestral chromosomes in the haploid karyotype fused together one-to-one, reducing the chromosome number from 24 pairs of acrocentric chromosomes to 11 large metacentric chromosomes and two small unfused chromosomes, giving a haploid karyotype with 13 chromosomes. N. angustata and N. magellanica possess such a karyotype today (Figure 1 and Figure 7 step 3). Because the lineage of N. coriceps + N. rossii diverged from N. angustata + P. magellanica ~8 MYA (Near et al. 2012), this massive one-to-one chromosome fusion event is either older than 8 MY or, less parsimoniously, happened more recently but twice, and independently in the N. angustata + N. magellanica lineage. Fourth, after the divergence of the N. coriceps + N. rossii clade from the N. angustata + N. magellanica clade, one of the small, previously unfused chromosomes became fused to one of the existing, already-fused metacentric chromosomes, creating the current Nco2 or Nco4 chromosomes. N. rossii shows a karyotype today that would fit this model (Figure 7, step 4). In a final step, the last small, previously unfused metacentric chromosome joined another existing metacentric chromosome to create the current Nco2 or Nco4 in N. coriceps (Figure 7, Step 5). An alternative, less parsimonious possibility not ruled out by our data on bullhead notothen is that some of the predicted chromosome fusions in the model originated independently in some of the four species in the Nototenia clade. Experiments similar to the ones we report here for bullhead notothen are required to test the hypothesis of evolution to similar chromosome numbers but independent chromosome fusion events.

**Inversions**

Because all conserved syntenic markers were contiguous with no intermixing of markers belonging to one of the individual ancestral chromosomes with markers from the other ancestral chromosome across their position of contact in each fused bullhead chromosome, we conclude that no pericentric inversions occurred in the bullhead lineage after the chromosomal fusion events that led to the reduction in
chromosome number. This is notable because the chromosome fusions do not appear to be recent; they are shared by other members of the Notothenia clade, including N. magellanica (n = 13), N. angustata, (n = 13), and N. rossii (n = 12). It is still possible that pericentric inversions occurred but that they are small and masked by reduced recombination rates near centromeres. We know that other groups of Antarctic fish did experience recent pericentric inversions because, for example, Pagetopsis macropterus has a diploid number of 47 or 48, similar to the ancestral state, but an arm number of 58, rather than the ancestral values of 2n = 48 and NF = 52 as in several other icefish, including Chaenocephalus aceratus. Although putative paracentric inversions appear in some chromosome arms, without a closer outgroup, it is not possible to determine if the rearrangements happened before or after the chromosome fusions.

Recombination rates
Uneven recombination rates along chromosomes, like the situation discovered here for bullhead chromosomes, occur in other species too. In zebrafish and spotted gar for example, recombination rates are higher at the telomeres in the male map and higher near the centromeres in the female map (Howe et al. 2013; Amores et al. 2011). In contrast to bullhead notothen, where most chromosomes are similar in size, in zebrafish the female map is more than twice the length of the male map (2.74: 1.0) (Singer et al. 2002).

Segregation distortion
Segregation distortion in the male map on Nco1 and in the female map on Nco4 is unlikely to be due to genotyping errors because many adjacent markers show the same distortion; thus, this situation is likely to be biological in origin. Segregation distortion could be explained by interactions between male and female alleles: if one of the two alleles at a locus in the male parent was a partial stage-specific dominant embryonic lethal in the context of the female allele (this would be the < 1: 1: 1: 1 case). In other cases, segregation distortion could result from epistatic interactions: if in the male, both alleles at locus “a” functioned well in the genomic context of male alleles at locus “b” but the male allele at locus “a” was deleterious in the context of heterozygosity for the female allele at locus “b.” Or if the male were a heterozygote for a paracentric inversion, then after homologous pairing in meiosis, a recombination event within the deletion would result in monocentric and dicentric chromosomes, which would lead to aneuploid gametes, embryo lethality, and segregation distortion. Segregation distortion might also be expected for sex chromosomes, but our results showed distortion on different chromosomes in the female and male, making the sex chromosome explanation less likely. In addition, cytogenetic analyses of bullhead failed to detect any sex chromosomes (Phan et al. 1987; Ozouf-Costaz et al. 1991; Tomaszkiewicz et al. 2011).
Conclusions

The systemic and massive chromosome fusion events that occurred a few million years after the divergence of the Nototthenia clade from the Dissostichus + Lepidontothen + Trematomus clade provide a model to explain how teelots could have evolved fewer chromosomes than their last common ancestor with their Holostean sister group (amia + gars), despite chromosome doubling associated with the TGD. Analyses of conserved synteny of spotted gar (L. oculatus, Loc), chicken (G. gallus, Gga), and medaka fish (O. latipes, Ola) indicate that chromosome fusions occurred in the teelot lineage before the TGD (Braasch et al. 2016). For example, the two orthologous chromosome pairs Loc13/Gga14 and Loc15/Gga27 together show conserved synteny to both Ola19 and Ola8, which are duplicate (paralogous) chromosomes derived from the TGD (Braasch et al. 2016). This pattern would appear if, after the divergence of gar and teelot lineages, the teelot orthologs of Loc13 and Loc15 fused together, followed by the duplication of the fused chromosome in the TGD event to produce the homeologous (paralogous) medaka chromosomes Ola19 and Ola8. The organization of the gar genome shows that the fusions envisaged to have preceded the TGD (Nakatani et al. 2007) occurred in the few million years after the divergence of gar and teelot lineages but before the TGD. While our comparative genomic analysis of the bullhead map shows that rapid concerted, regular chromosome fusions can and do occur, they leave open several questions, including: what forces would methodically cause chromosomes to fuse regularly one-to-one in a lineage; did the one-to-one fusion of 22 chromosomes to 11 happen at all once or over a protracted period of several million years since the divergence of the Nototthenia lineage from its sister group; and what caused the remaining two unfused chromosomes in the last common ancestor of N. rossii and N. coriceps to fail to fuse to each other but instead join already fused chromosomes? A possible answer might be that some molecular feature of chromosome structure makes it more difficult to fuse a metacentric chromosome with another chromosome than to fuse two acrocentric or telocentric chromosomes.

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