Mapping Loci Associated with Tail Color and Sex Determination in the Short-Lived Fish *Nothobranchius furzeri*

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ABSTRACT

The African fish *Nothobranchius furzeri* is the shortest-lived vertebrate species that can reproduce in captivity, with a median lifespan of 9-11 weeks for the shortest-lived strain. There exist several natural populations of *N. furzeri* that display differences in lifespan, aging biomarkers, behavior, and color, which make *N. furzeri* a unique vertebrate system for studying the genetic basis of these traits. Here we report a first generation microsatellite-based linkage map for *N. furzeri* that allowed us to identify loci linked with tail color and sex. *N. furzeri* linkage map was generated by genotyping the F2 progeny of a cross between a short-lived, yellow-tailed strain and a long-lived, red-tailed strain. The map contains 25 linkage groups and has a total length of 1012 cM. We identified one region linked with the yellow/red tail color that maps close to *melanocortin 1 receptor* (*mc1r*), a gene involved in pigmentation in several vertebrate species. Analysis of the segregation of sex-linked markers revealed that *N. furzeri* has a genetic sex determination system with males as the heterogametic sex, and markedly reduced recombination in the male sex-determining region. Our results demonstrate that both naturally-evolved pigmentation differences and sex determination in *N. furzeri* are controlled by simple genetic mechanisms and set the stage for the molecular genetic dissection of factors underlying such traits. *N. furzeri* linkage map will also help analyze the genetic architecture of traits that characterize this group of vertebrates, including short lifespan and adaptation to extreme environmental conditions.
INTRODUCTION

The *Nothobranchius* fish species are present in Eastern and South-Eastern Africa, where they populate ephemeral water pools that often undergo complete desiccation during the dry season (REICHARD 2009; TERZIBASI et al. 2008; WILDEKAMP 2009). *Nothobranchius* species tend to live in extreme habitats and have evolved unique adaptations to harsh environmental conditions, including extremely short life cycles, resistance to a wide range of temperatures and water salinity, embryonic development that does not require the presence of water, and a developmental diapause that allows embryos to survive for months in dry conditions (GENADE et al. 2005; INGLIMA et al. 1981; WOURMS 1972).

*N. furzeri* is the shortest-lived species of the *Nothobranchius* genus, with an inter-generation time of 40 days, a median lifespan of 9-11 weeks, and a maximum lifespan of 12-15 weeks for the shortest-lived strain GRZ (GENADE et al. 2005; HARTMANN et al. 2009; TERZIBASI et al. 2009; TERZIBASI et al. 2008; VALDESALICI and CELLERINO 2003; VALENZANO et al. 2006). Natural populations of *N. furzeri* can vary substantially in phenotypic traits. For example, *N. furzeri* strains derived from Zimbabwe and Northern Mozambique (e.g. GRZ) exhibit a shorter lifespan than strains derived from more humid areas in Southern Mozambique (e.g. MZM-0403) under controlled conditions (TERZIBASI et al. 2008). The extremely short life cycle of *N. furzeri* and the presence of natural populations with phenotypic variations make this species a promising model system for studying aging and adult-specific traits, including color and behavior.
The color pattern of the adult male tail differs among *N. furzeri* strains. GRZ males show a yellow submarginal band and a black marginal band (yellow morph) whereas MZM-0403 males display a broad red band (red morph) in the caudal fin (Figure 1A). This dichromatism is present in natural populations of *N. furzeri* (REICHARD 2009; TERZIBASI et al. 2008). Similar color polymorphism among males is also observed in other species of *Nothobranchius* (WILDEKAMP 2009), and in other fish species, including guppies and cichlids (BROOKS and ENDLER 2001; HUGHES et al. 1999; MAAN et al. 2004). Differences in male color morphs within the same species are associated with sexual preference by females, different recognition by predators depending on the habitat, and differential susceptibility to pathogens (PRICE et al. 2008), which could all influence the evolution of this trait. Despite the widespread variation in *Nothobranchius* coloration, the genetic basis of this trait is unknown.

Genetic information on *N. furzeri* is still limited. The *N. furzeri* genome is 1.6-1.9 Gb in size and is characterized by a high repeat content (45%) (REICHWALD et al. 2009). *N. furzeri* has 19 chromosomes, but no morphologically discernable sex chromosomes (REICHWALD et al. 2009). The sex determination system in *N. furzeri* has not been characterized yet. Sex can be determined either genetically or environmentally in fish (MARSHALL GRAVES 2008; VOLFF 2005). For example, medaka, platyfish, guppy and sticklebacks all have recently evolved genetic sex-determining systems (PEICHEL et al. 2004; SHAPIRO et al. 2009; TRIPATHI et al. 2009a; VOLFF and SCHARTL 2002), whereas zebrafish do not
have a clear genetic basis of sex determination (VON HOFSTEN and OLSSON 2005).

Genetic studies in *N. furzeri* would greatly benefit from the building of a linkage map in this species of fish. However, to date there is no linkage map available for *N. furzeri* or any *Nothobranchius* species, although linkage maps have been generated for fish of the same order, e.g. *Poecilia reticulata* (guppy) and *Xiphophorus maculatus* (platyfish) (KHOO et al. 2003; TRIPATHI et al. 2009b; WALTER et al. 2004), and the same superorder, e.g. *Oryzias latipes* (medaka) (WADA et al. 1995).

Here, we report a microsatellite-based linkage map for *N. furzeri* using a genetic cross between the short-lived yellow-tailed GRZ strain and the long-lived red-tailed MZM-0403 strain. This *N. furzeri* linkage map allowed us to map the male-specific tail color trait on linkage group (LG) V. Synteny analysis revealed that LG V has homology to a region of the medaka genome that contains the *melanocortin 1 receptor (mc1r)* gene, which is known to play a key role in vertebrate pigmentation. We identified a sequence polymorphism in *mc1r* between the two strains of *N. furzeri*, allowing us to map *mc1r* on LG V. This analysis revealed that *mc1r* is located in close proximity to the color locus, but that the sequence polymorphism is probably not causative for the color difference. We also found that sex is genetically determined in *N. furzeri*, with males as the heterogametic sex. The sex-determining region is located on LG XIII and is characterized by male-specific suppression of recombination. Our
findings will be pivotal for the identification of the genetic determinants of color in
*N. furzeri*, and for expanding our knowledge about sex-determination
mechanisms in vertebrates. Due to the array of intra-specific phenotypic
differences displayed by the various populations of *N. furzeri*, this linkage map
will also be a key tool for mapping phenotypic variation in this short-lived
vertebrate species, including differences in lifespan.
MATERIALS AND METHODS

Fish housing and husbandry:

Fish were grown at 25 °C in a centralized filtration water system at a density of 2 fish per gallon tank. Fish were fed freshly hatched *Artemia* brine shrimp until 3 weeks of age and then dried *Chironomid* bloodworms 2 times per day every day. Adults spawned freely in the system. Tanks were inspected daily and freshly laid embryos were collected and stored in dry peat moss until they were ready to hatch, as indicated by spontaneous twitching inside the eggshell. Once ready to hatch, embryos were immersed in Yamamoto embryo solution (17 mM NaCl, 2.7 mM KCl, 2.5 mM CaCl₂, 0.02 mM NaHCO₃ pH 7.3) (REMBOLD et al. 2006). Fry were placed in 0.2-gallon tanks at the density of 5 fry per tank.

Cross between two strains of *N. furzeri*:

One male from the GRZ strain was crossed with one female from the MZM-0403 strain (cross #1). From the F1 generation (54 fish), 9 independent ‘families’ (8 tanks with a spawning pair in each tank and one tank with 3 males and 4 females spawning together) were formed, and fertilized eggs were collected. A total of 413 F2 individuals were produced. An independent, reciprocal cross was established between one male MZM-0403 and one female GRZ (cross #2). Two F1 individuals from cross #2 produced a total of 34 F2 individuals.
Color phenotyping:

Fish were removed from tanks at death, rinsed in tap water and stored in 100% ethanol. Tail color (yellow vs. red) was scored in all 203 F2 adult males and all 23 F1 males from cross #1 by visual inspection, immediately preceding submersion in ethanol. *N. furzeri* male fish are extremely colorful, thus male color at death was clearly visible. F1 and F2 fish with yellow tails overlain with red spots were scored as 'yellow'.

Sex phenotyping:

Sex was assessed at death based on the presence or absence of the typical male tail coloration. Fish that had not yet reached sexual maturity at death were scored as “undetermined”.

Microsatellite identification by hybridization:

Genomic DNA was isolated from a 7-week old MZM-0403 male and digested with *RsaI*. 1000-1650bp fragments were cloned into the pCR®-Blunt-TOPO® vector (Invitrogen) and transformed into chemically competent *E. coli*. Transformants were selected for the presence of CA/GT microsatellites using a $^{32}$P-dCTP end-labeled (CA)$_{15}$ hybridization probe (Elim Biopharmeceuticals, Inc.) following a described method (PEICHEL et al. 2001). Of the 773 positive clones analyzed, 318 contained microsatellites and 311 met the following criteria: (i) at least 15 repeat units, and (ii) at least 100 bp of sequence flanking the microsatellite. To amplify these microsatellite repeats by PCR, primers that have melting temperatures between 57-63 °C and that amplify fragments between
150-400bp were designed using Primer3 (http://frodo.wi.mit.edu/). A 5' M13 sequence (5'-TGTAAAACGACGGCCAGT-3') was added to all forward primers to enable fluorescent labeling of fragments during PCR (SCHUELKE 2000).

**Microsatellite identification by whole genome sample sequencing:**

Whole genome sample sequencing of a male specimen of the GRZ strain resulted in 5,540 sequences comprising a total of 5.4 Mb (REICHWALD et al. 2009). Through *in silico* analyses using Sputnik (http://cbi.labri.fr/outils/Pise/sputnik.html), 289 microsatellites were identified which met the following criteria: *(i)* one microsatellite per sequence, *(ii)* at least 20 repeat units for dinucleotide repeats, *(iii)* at least 100 bp of microsatellite flanking sequence, *(iv)* perfect repeats. Primers were designed using the GAP4 module of the Staden Sequence Analysis Package, as described (REICHWALD et al. 2009). One hundred and thirty nine microsatellites were experimentally validated by PCR and subsequent genotyping using ABI3730xl analyzers and GeneMapper software v4 (Applied Biosystems).

**Genotyping:**

244 pairs of primers were used to amplify microsatellites from the grandparents and 246 F2 offspring (160 males and 86 females) of cross #1. One hundred and fifty two microsatellite markers were informative for the grandparents, 148 of which were used to genotype F2 individuals. Of the 246 individuals genotyped to build the map, 234 were genotyped at all the markers whereas 12 were genotyped only at the 121 markers corresponding to the 4
terminal markers of each LG and to the singletons. PCRs were performed in 5 μl in 384-well plates with 0.2U Taq DNA polymerase, 1X PCR buffer (50 mM KCl, 100 mM Tris HCl pH 9.0, 0.1% Triton-X), 0.5 ng/μl DNA, 0.25 mM dNTP (Invitrogen), 800 nM FAM56-labeled M13 primer, 10-20 nM M13-forward primer, and 400-800 nM reverse primer. Samples were heated to 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 45 s at 56 °C and 45 s at 72 °C and 8 cycles of 30 s at 94 °C, 45 s at 53 °C and 45 s at 72 °C. Amplicons were denatured by incubation in denaturation solution (1:1.15 Hi-Di™ Formamide (Applied Biosystems) and 1:300 GeneScan™-500 LIZ™ Size Standard (Applied Biosystems)) at 95 °C for 5 min, and electrophoresed on an ABI 3730 capillary sequencer. Chromatographs were analyzed manually using PeakScanner™ software v1.0 (Applied Biosystems).

**Linkage map generation and map length calculation:**

Genotypes were scored according to JoinMap 3.0 (VAN OOIJEN and VOORRIPS 2001). The Kosambi mapping function was used to convert recombination frequencies (REC = 0.4) to centimorgans (cM). The map was also calculated using Haldane's function to account for double crossing-overs, which gave similar results. The assignment of markers to LG was carried out with a LOD score threshold of 4 and a maximum linkage distance of 25 cM. The calculation of phase cannot be exploited in this particular case, since parental (F1) genotypes were tractable for only 96 out of 246 F2 individuals. Raw data are available for download (Supplemental data).
The map length was computed by adding $2s$ to each LG length in cM, where $s$ is the average inter-maker distance, to account for chromosome ends, as described (TRIPATHI et al. 2009b). This measure was averaged with the measure obtained by multiplying each LG’s length in cM by $(m+1)/(m-1)$, where $m$ is the number of markers in each LG, as described (TRIPATHI et al. 2009b).

**Mapping color:**

Color mapping was computed manually by scoring the recombination events in all the F2 red-tailed fish ($n = 61$) of cross #1, genotyped for all 148 microsatellites. Only red-tailed F2 fish were used to map color because the red phenotype is more reliable than the yellow phenotype, as yellow-tailed fish can develop red spots with advancing age (see below). These genotypes were then further included in the whole-genome map calculation. For each microsatellite marker, the presence of two, one, or none of the two alleles inherited from the red tailed MZM-0403 grandparent was scored. The LOD score for each marker was calculated according to the following formula: $\text{LOD} = M \times \text{LOD10}(m) + (N-M) \times \text{LOD10}(1-m) - N \times \text{LOD10}(0.5)$, where $M$ is the number of recombination events, $m$ is the fraction of recombination events over all the alleles ($m = M/N$), $N$ is twice the number of genotyped individuals (corresponding to the total number of alleles genotyped), and LOD10 is the 10-base logarithm. Considering the color marker position as the position where all red individuals have both MZM-0403 alleles, $m \times 100$ corresponds to the distance in cM of each marker from the color-gene. This analysis was performed over all the markers in the map.
**Synteny analysis:**

BLASTn searches were performed using the flanking regions from all 11 microsatellites that co-segregated with tail color as query against the medaka genome (Oct. 2005 MEDAKA1 assembly) at Ensembl (http://www.ensembl.org/Oryzias_latipes/Info/Index). The microsatellites flanking-regions having a p value lower than $10^{-5}$ and 100% sequence identity over more than 22 bp were considered significant hits. Search sensitivity was set to “no optimization”.

**Cloning of mc1r:**

Total RNA was isolated from caudal fins of three male adult individuals (two GRZ and one MZM-0403) using Trizol (Invitrogen). cDNA was generated using MMLV reverse transcriptase (Clontech), according to the manufacturer’s protocol. A 681 bp fragment of *N. furzeri mc1r* was generated by PCR using primers derived from conserved mc1r regions in medaka, stickleback, *Takifugu* and *Tetraodon* (forward primer: 5' GAA CCG CAA CCT GCA CTC 3'; reverse primer: 5' GGG TCG ATG AGC GAG TTA CA 3'). A 1402 bp DNA fragment containing the mc1r open reading frame and 5' and 3' untranslated regions was amplified by RACE PCR (Clontech) and sub-cloned in the pCR 2.1-TOPO cloning vector (Invitrogen).

**Identification and genotyping of mc1r sequence polymorphism:**

mc1r 1402 bp region was amplified from the grandparents of cross #1 (male GRZ and female MZM-0403), cloned and entirely sequenced. A sequence
polymorphism between GRZ and MZM-0403 was identified at nucleotide 67 of the mc1r coding sequence. This polymorphism was genotyped in 61 F2 fish from cross #1. For sequencing, a 312 bp region flanking nucleotide 67 of mc1r was amplified by PCR using the following primers (forward primer: 5’ GTG GAC CCC TGC TTT AAT GA 3’; reverse primer: 5’ TAG TAC ATG GGC GAG TGC AG 3’). The PCR products were purified using a PCR purification kit (Qiagen) and sequenced using Molecular Cloning Laboratories (http://www.mclab.com). Sequences were analyzed using Sequencher 4.7 (Gene Codes Corporation).

**Mapping sex:**

The genotypes at 148 microsatellites of 239 F2 individuals from cross #1 were sorted by sex (female, male, unknown) to isolate the markers carrying a significant sex-biased allele distribution. To confirm these results, F2 individuals from cross #2 (34 individuals) were also genotyped at 9 sex-linked microsatellites identified in cross #1 and sorted by sex (female, male, unknown). The presence of potentially suppressed recombination in male vs. female sex-determining region was determined by scoring recombination events in two specific F1 families of cross #1 (family 3 and family 7), in which the two parental pairs were both heterozygous at all the sex-linked loci. The sex-specific recombination events were determined by assessing sex-specific allele distribution in the offspring of these two F1 families (22 F2 individuals for family 3 and 46 F2 individuals for family 7, 68 F2 individuals total) at the sex-linked markers that were heterozygous for both F1 parents and that had more than two alleles.
RESULTS

Microsatellite-based linkage map for *N. furzeri*:

To develop genetic markers for linkage mapping in *N. furzeri*, we identified microsatellites using two strategies of large-scale genomic library screening and sequencing. In the first approach, we screened a genomic library from MZM-0403, a wild-derived strain of *N. furzeri* with a broad red marginal band in the caudal fin (‘red morph’), using a (CA)$_{15}$ probe, as CA microsatellite repeats are frequent in fish genomes. We sequenced 773 positive clones and identified 318 clones containing microsatellites, 105 of which gave rise to a PCR product. In the second approach, we performed whole genome sample sequencing of GRZ, which has a black marginal band and a yellow submarginal band in the caudal fin (‘yellow morph’) (REICHWALD *et al.* 2009). We identified 289 clones containing microsatellites by *in silico* analysis using Sputnik (http://cbi.labri.fr/outils/Pise/sputnik.html), 139 of which were experimentally validated.

To generate a linkage map, we set up a cross between one long-lived red-tailed MZM-0403 female and one short-lived yellow-tailed GRZ male (Figure 1A). We obtained 54 F1 progeny, which were used to form nine families (Figure 1B). Four hundred and thirteen F2 progeny from these F1 families developed into adulthood (Figure 1B). We genotyped 244 microsatellite markers in the grandparents and found that 152 (62%) were polymorphic and thus informative for building a genetic map (Supplemental Table 1). All microsatellite markers were homozygous in the GRZ male grandparent, except those linked with sex
(see below), confirming that GRZ is an inbred strain (Reichwald et al. 2009).

Forty one percent of the microsatellite markers (63 of 152) were heterozygous in the MZM-0403 female grandparent, consistent with this strain being recently derived from the wild and propagated in captivity for no more than seven generations (Hartmann et al. 2009; Terzibasi et al. 2008).

To build the linkage map, we genotyped 246 F2 fish at 148 of the 152 informative microsatellite markers (see Materials and Methods). Significant linkage was found for 138 (93 %) microsatellite markers and 10 markers were singletons. Six of the 138 linked markers were excluded from the map calculation because they gave incomplete genotypes in more than 50% of the individuals. The resulting *N. furzeri* linkage map consists of 25 linkage groups (LGs), with 2-12 markers per LG (Figure 2). Since *N. furzeri* has only 19 chromosomes (Reichwald et al. 2009), we anticipate that some of the linkage groups will coalesce when additional markers are included. The total calculated map length is 1012 cM, with an average inter-marker distance of 5.3 cM. Considering 25 cM as the maximum inter-marker distance, 10 singletons, and 6 more LGs than chromosomes, we estimate that up to 400 cM could still not be accounted for in this map (25*10 + 25*6 = 400 cM), corresponding to 28% of the *N. furzeri* genome (400/(400+1012)). Thus, we have generated a first-generation microsatellite-based linkage map for *N. furzeri* that can be used to map phenotypic variation between the populations of this species.
Mapping tail color on LG V in *N. furzeri*:

A conspicuous difference between the GRZ and MZM-0403 strains of *N. furzeri* is the color and pattern of the caudal fin in males (Figure 1A, Figure 3A). To examine the genetic basis of this dichromatism, we scored all males in the F1 and F2 generations for a ‘yellow’ versus a ‘red’ caudal fin. All F1 males display a yellow morph, indicating that yellow is dominant over red (Figure 3A). We also observed the progressive appearance of red spots in the caudal fin of adult F1 male fish with advancing age (data not shown). In the 203 males of the F2 generation, the yellow/red tail-color trait segregated as 142 ‘yellow’ (yellow and yellow with red spots) and 61 ‘red’ (Figure 3A). The ratio between the two color morphs is close to 3:1 (χ² = 2.76, p = 0.09), which suggests Mendelian transmission.

To map the yellow/red tail-color trait, we genotyped all the red males of our cross (61 fish) at all 148 markers. We found a strong linkage signal on LG V (Figure 3B, Table 1). The peak LOD score corresponds to marker NfuFLI0030 marker (Figure 3C, Table 1). At the current map resolution, the NfuFLI0030 marker is indistinguishable from the color locus, i.e. all red males in the F2 generation inherited both alleles from the red MZM-0403 grandparent. These results indicate that the yellow/red tail-color trait is mainly determined by one locus, although we cannot exclude that multiple linked loci contribute to this color trait or that other minor unlinked loci may influence the trait.
We sought to identify candidate genes that underlie the tail-color trait in *N. furzeri*. Synteny with other species can be used to infer the position of genes on a linkage map of a species without a sequenced genome (GROSS et al. 2008). We performed a BLASTn search in the medaka genome with the known sequences flanking the 11 microsatellite markers of LG V as queries, since medaka is a sequenced fish species most closely related to *N. furzeri* (REICHWALD et al. 2009). Markers NfuFLI0122, NfuSU0050 and NfuSU0046 displayed significant sequence homology with, respectively, *olma1* (cardiac muscle actin, orthologous to Human *ACTC1*, p<10^{-17}), *sox6* (sry box containing gene 6, p<10^{-13}), and *cttn* (cortactin, p<10^{-11}) on medaka chromosome 3 (Figure 3B). In addition, NfuFLI0122, NfuSU0050 and NfuSU0046 are in the same order on *N. furzeri* LG V as their respective counterparts on medaka chromosome 3 (Figure 3B). Together, these results suggest that there is synteny and co-linearity between *N. furzeri* LG V and medaka chromosome 3.

We next asked whether genes known to mediate skin/hair pigmentation in other vertebrate species are present in the region that is syntenic to *N. furzeri* LG V on medaka chromosome 3 (Figure 3B). The genes that we specifically examined were: melanocortin 1 receptor (*Mc1r*) and its ligand (*Asip*) (HOEKSTRA 2006; LE PAPE et al. 2009; REES 2003; SHRIVER et al. 2003; SULEM et al. 2008), the *Kit* receptor and its ligand (*Kitl*) (GEISSLER et al. 1988; MILLER et al. 2007; SULEM et al. 2007), *CBD103* (CANDILLE et al. 2007), *MATP/SLC45A2* (NORTON et al. 2007), *SLC24A4* (SULEM et al. 2007), *SLC24A5* (LAMASON et al. 2005), *IRF4* (HAN et al. 2008; SULEM et al. 2007), *TPCN2* (BONILLA et al. 2005; SULEM et al. 2007).
2008), Sly (ALIZADEH et al. 2009), oca2 (PROTAS and PATEL 2008; SHRIVER et al. 2003), and TYR (SHRIVER et al. 2003; SULEM et al. 2007). Of all these genes, only mc1r and slc24a5 were located on the medaka chromosome syntenic to N. furzeri LG V. Conversely, the annotated genes on medaka chromosome 3 do not include any other genes known to be associated with color determination, although this does not rule out their existence. In medaka, mc1r is located 2.6 Mb from olma1, the marker that is syntenic with NfuFLI0122, whereas slc24a5 is located outside of the medaka region that is syntenic to the region linked with color in N. furzeri (Figure 3B). Together, these results suggest that mc1r is a better functional candidate for tail color than slc24a5 in N. furzeri.

To determine if mc1r is the gene underlying male tail color, we cloned N. furzeri mc1r cDNA and mapped mc1r on the linkage map. Sequence comparison of GRZ and MZM-0403, the strains used in our cross, revealed a single nucleotide polymorphism at position 67 of the mc1r coding sequence (C in GRZ and G in MZM-0403) (Figure S1, GenBank GQ463613). This variation is a non-synonymous substitution that results in a change from histidine (GRZ) to aspartic acid (MZM-0403) at amino acid 23 in the N-terminal region of the Mc1r protein (Figure S1). Sequencing 3 additional specimens of each strain confirmed that MZM-0403 individuals were homozygous for G, and GRZ individuals were homozygous for C at position 67 of the mc1r coding sequence. The segregation of this mc1r polymorphism in 61 F2 fish allowed us to place mc1r on LG V, the LG that contains the color locus (Figure 3B). However, there was one recombinant red-tailed F2 fish with a GC genotype, as well as three recombinant
F2 yellow-tailed fish with a GG genotype at position 67 (data not shown) and these genotypes were independently confirmed. These results indicate that *mc1r* is closely linked to the color locus, but 1.7 cM away from it. The marker to which *mc1r* is most closely linked is NfuFLI0030 (Figure 3B). The distances between *mc1r* and NfuFLI0030 (2 cM) and between *mc1r* and the color locus (1.7 cM) are not the same, because the former was calculated in both male and female F2 individuals whereas the latter was calculated only in red F2 males. The distance between *mc1r* and the color locus indicates that Mc1r amino acid difference is unlikely to be causal for the yellow/red color determination in *N. furzeri*, although we cannot rule out the involvement of cis-acting elements for the *mc1r* gene in the determination of color. An alternative possibility is that another gene in close proximity to *mc1r* is involved in color determination in *N. furzeri*.

**A sex determination system on linkage group XIII in *N. furzeri***:

Sex is another phenotype that differs between the two grandparents of our cross. To establish the sex determination system in *N. furzeri*, we counted the number of males and females in the F1 and F2 generation. The F1 and F2 offspring displayed an even sex ratio (F1: $\chi^2 = 0.184$, p = 0.668; F2: $\chi^2 = 1.64$, p = 0.2) (Figure 4A), consistent with a genetic sex determination system. We sorted the genotypes by sex and searched for markers that show alleles that are predominantly present in males or females. We identified 9 microsatellite markers on LG XIII that were linked with sex. Interestingly, 5 of these makers (NfuSU0004, NfuSU0007, NfuSU0010, NfuSU0090 and NfuFLI0091) show a significant male-specific allelic bias in that most of the F2 males carry a
combination of a fixed, male-specific allele and a non-fixed, non sexually-biased allele (Table 2). Importantly, the male-biased markers are the only ones for which the male grandparent (GRZ) is heterozygous. These results strongly suggest that male is the heterogametic sex in *N. furzeri* and that the sex determination system is XY/XX. To analyze if the male-specific allelic bias is shared by different *N. furzeri* strains, we genotyped the sex-linked markers in the F2 progeny (20 males and 14 females) of a reciprocal cross of a male MZM-0403 and a female GRZ (cross #2), and found a male-allelic bias at the same loci (Table 2). Together, these results indicate the presence in *N. furzeri* of a conserved haplotype for the male sex chromosome with marked sex-linkage disequilibrium.

Chromosomal regions carrying a sex-determining locus are characterized by suppressed meiotic recombination (Charlesworth 2004; Marshall Graves 2008). To test if recombination is suppressed at the sex-linked markers in *N. furzeri*, we independently calculated male and female meiotic recombination frequencies in 68 F2 offspring from two F1 families of our first cross (cross #1). We performed this analysis in F2 individuals instead of F1 individuals because the P0 MZM-0403 female was homozygous at all sex-linked loci. F1 females were heterozygous at the sex-linked loci, except at marker NfuSU0004, which was therefore excluded from the analysis. In F2 progeny from each of the two F1 families, there was no recombination event for markers NfuSU0090, NfuSU0007, NfuSU0010, and NfuFLI0091 in males, whereas females displayed a total of 6 recombination events, that account for a map distance of 2 cM for family 3, and 9 cM for family 7 at markers NfuSU0007 and NfuSU0090 respectively (Figure 4B).
This result indicates that recombination is largely suppressed in males in the sex-linked region of LG XIII, and further supports the conclusion that male is the heterogametic sex in *N. furzeri*.

To determine if the sex-linked markers in *N. furzeri* have similarities with sex-determining regions in other fish species, we searched for synteny between the 9 microsatellite markers on LG XIII on *N. furzeri* and medaka chromosomes. Marker NfuSU0015 on LG XIII shows significant homology with a region on medaka chromosome 16 (BLASTn p = 1.5x10^-7), although there isn’t any annotated gene in this region. Interestingly, chromosome 16 corresponds to the sex chromosome in one medaka species (*Oryzias javanicus*) (*TAKEHANA et al.* 2008), raising the interesting possibility that sex-determination in *N. furzeri* and *O. javanicus* might have evolved from a common system. However, *N. furzeri* LG XIII is not syntenic with the sex-determining chromosome in another species of medaka (*Oryzias latipes*) (*KASAHARA et al.* 2007; *MATSUDA et al.* 2002) and with the sex-determining LGs in two stickleback species (*PEICHEL et al.* 2004; *ROSS and PEICHEL* 2008; *SHAPIRO et al.* 2009), suggesting that the sex-determination system of *N. furzeri* probably arose independently from that of these species.
DISCUSSION

Linkage map in *N. furzeri*:

Our first generation microsatellite-based linkage map consists of 25 LGs. The number of LGs is higher than the number of chromosomes visible in a metaphase spread (19) (REICHWALD et al. 2009). First generation linkage maps usually contain more LGs than chromosomes and these LGs tend to collapse if more markers are added (NARUSE et al. 2000; OHTSUKA et al. 1999). Thus, it is likely that the current LG number will eventually collapse to 19 if additional markers/meioses are added. Our analyses also show that the phylogenetic relationship between *N. furzeri* and medaka allows the use of sequence similarity and synteny to predict the location of specific genes on the *N. furzeri* map. The synteny between medaka and *N. furzeri* is likely to be high, given that medaka and the more distant stickleback genomes already exhibit remarkable synteny (http://oxgrid.angis.org.au/ensembl_grids/oxt__gacu_all__vs__olat_all__500.html). A high level of synteny will be particularly useful in identifying candidate genes underlying specific traits, as was recently described for the cave fish *Astyanax mexicanus* (GROSS et al. 2009; GROSS et al. 2008).

Color determination in *N. furzeri*:

Our linkage map allowed us to identify a locus linked with male yellow/red tail color on LG V in *N. furzeri*. Synteny analysis between *N. furzeri* and medaka revealed a potential candidate gene for color, *mc1r* (HOEKSTRA 2006; REES 2003). The Mc1r protein is a G protein-coupled receptor for the Agouti ligand Asip. In mice, mutations in either *Mc1r* or *Asip* result in changes in the pattern of
melanogenesis and in coat color (JACKSON 1993), and affect the relative amounts of eumelanin and pheomelanin in mammalian melanocytes (ANDERSSON 2003). In humans, \(MC1R\) mutations are associated with red hair and fair skin (MUNDY 2009; VALVERDE et al. 1995), and with the presence of freckles and skin sensitivity to sun (SULEM et al. 2007). However, while \(mc1r\) is important for the brown pigmentation in different populations of the cave fish Astyanax mexicanus (GROSS et al. 2009), whether \(mc1r\) plays any role in yellow/red pigmentation in fish is unknown. In fact, fish pigmentation is fundamentally different from that of mammals, because it depends on several cell types, including xantophores and erythrophores, which can synthesize yellow pigments \textit{de novo} (BRAASCH et al. 2007; PARICHY 2003; PROTAS and PATEL 2008).

The red and yellow grandparents of our cross have a non-synonymous variation in the coding sequence of \(mc1r\), leading to a change in amino acid in Mc1r protein from an aspartic acid in the red strain to a histidine in the yellow strain. However, this non-synonymous change in Mc1r does not map exactly at the color-locus, but 1.7 cM from it, likely ruling out its direct functional implication in male coloration. This amino acid change is in the extracellular N-terminal domain of Mc1r, a region poorly conserved between different teleost species and not essential for ligand binding (SELZ et al. 2007). This region does not contain any of the known Mc1r mutations that have previously been found to be causative for color differences in other species (SELZ et al. 2007). Thus, at this point, we consider unlikely an implication of \(mc1r\) in fish yellow/red color determination, although we cannot exclude a potential involvement of \(mc1r\) via...
cis-regulation. It is more likely that another gene located in the same region is responsible for color determination in \textit{N. furzeri}. Fine mapping will reveal if color determination in \textit{N. furzeri} is mediated by mc1r or by another gene.

Mapping the color locus will provide important cues for the evolution of a trait under strong sexual selection. In the genus \textit{Nothobranchius} coloration is specific to males and is likely shaped by female mate selection. For example, in \textit{N. guentheri}, females prefer conspecific males based on color (HAAS 1976). More intensely colored males are preferred over less intensely colored ones (HAAS 1976). However, a bright coloration also renders males more conspicuous to predators and likely comes with a survival cost, in line with the observation that in the wild, the sex ratio for this species is biased toward females, which are not colored (HAAS 1976; REICHARD 2009). The balance between the fitness advantage due to mate preference and the fitness costs due to predation may underlie the rate of evolution of male coloration in this species. When we identify a gene or regulatory region underlying the difference in color in \textit{N. furzeri}, it will be interesting to test if natural populations of \textit{N. furzeri}, as well as different species of the genus \textit{Nothobranchius}, harbor polymorphisms in this region.

\textbf{Sex determination in \textit{N. furzeri}:}

Sex can be determined by mechanisms that are genetic, environmental, or a combination of both (MARSHALL GRAVES 2008; VOLFF 2005). Environmental factors that control sex determination in fish species include water temperature, density, and social interactions. Genetic control of sex determination is governed
by the presence of sex chromosomes (visible sex chromosomes or heteromorphic chromosomes) that can be present either in males (XY) or females (ZW). We found that *N. furzeri* has a genetic sex-determination system, with males as the heterogametic sex, indicative of an XY/XX system. The male sex-determining region in *N. furzeri* harbors a non-recombining region (NRY), similar to medaka, guppies, platyfish, and sticklebacks (KONDO et al. 2001; MATSUDA et al. 2002; NANDA et al. 2002; PEICHEL et al. 2004; ROSS and PEICHEL 2008; SCHULTHEIS et al. 2006; SHAPIRO et al. 2009; TRIPATHI et al. 2009a; VOLFF and SCHARTL 2002). In line with sticklebacks and medaka (KONDO et al. 2001; PEICHEL et al. 2004; SHAPIRO et al. 2009), the sex linkage group in *N. furzeri* does show major differences in recombination rates when computed independently for males and females, and *N. furzeri* males consistently share a sex haplotype.

Syntenic analysis between *N. furzeri* sex-linked markers and the known medaka and stickleback sex-chromosomes revealed that the sex-linked LG in *N. furzeri* (LG XIII) is syntenic with *O. javanicus* sex chromosome (chromosome 16), raising the interesting question of whether the sex-determining regions in these two different species derived from the same ancestral chromosome or arose independently from one another.

**Color and sex determination in *N. furzeri***:

Genes underlying sexually attractive traits, such as bright coloration, are often located on sex-chromosomes (LINDHOLM and BREDEN 2002). In guppies, many of the male color traits (dorsal fin black, central blue white spot, anterior orange spot, etc.) map to the sex chromosome, although other color traits map to
autosomes (LINDHOLM and BREDEN 2002; TRIPATHI et al. 2008; TRIPATHI et al. 2009b). Similarly, in platyfish (Xiphophorus maculatus), a number of color traits (iris color, body colors, fin color) map to sex chromosomes, while others (black comets on the fin) map to autosomes (BASOLO 2006). Having color and sex linked may help maintain sexual dimorphism and provide an evolutionary advantage. In N. furzeri, male color does not map to the LG containing the sex-determining region, at least in our first-generation linkage map. This could be due to the incompleteness of the map – it is conceivable that LG V and LG XIII would merge if additional markers/specimens are used. Alternatively, color and sex-determining regions may segregate independently in N. furzeri, perhaps because the evolutionary advantage of linking male tail color and sex has not emerged yet in this species.

**Lifespan, color, and sex determination in N. furzeri:**

A major advantage of developing *N. furzeri* as a model system is its short lifespan and the presence of natural populations with differences in mean and maximal lifespan. Our cross did not allow us to map QTL for longevity because a large number of F2 fish died prematurely, due to the unexpected presence of the parasite *Glugea sp.* in the fish housing system (data not shown). Nevertheless, preliminary data suggest that there is no simple link between the yellow/red color and longevity or between lifespan and sex (data not shown). Additional crosses performed in controlled environmental conditions will be needed for the mapping of QTL for longevity in *N. furzeri.*
Concluding remarks:

Our study reports the generation of the first linkage microsatellite-based map for the short-lived fish *N. furzeri*. This genetic tool allowed us to identify a single locus linked with color determination in males, as well as to reveal the sex-determination system of this species. This map will also be of key importance in determining the genetic architecture of other traits that characterize this unique group of organisms, particularly differences in lifespan.
ACKNOWLEDGEMENTS

We thank David Kingsley and Greg Barsh for intellectual input on the project. We thank Steve Arnott from the Kingsley lab for guidance for linkage mapping analysis. We thank Craig Miller, Katie Peichel, and Michael Shapiro for helpful suggestions throughout this project. We thank Sabrina Fullhart for her help with fish husbandry and fish pictures. We thank Tom Hofmann for expert technical assistance. We are grateful to the Kingsley lab for the use of the ABI sequencer. We thank members of the Brunet lab, as well as Steve Arnott, Greg Barsh, David Kingsley, Craig Miller, Katie Peichel, Dmitri Petrov, and Michael Shapiro for critically reading the manuscript. We thank Martin Reichard for communication on his findings on N. furzeri color in the wild. This work was supported by an R21 grant from NIH/NIA (AG030464) (A.B.), a fellowship from the Stanford Center on Longevity (D.R.V.), and by a PAKT grant from the Leibniz-Gemeinschaft, Germany (C.E. and M.P.).
TABLE LEGENDS

TABLE 1. Genotype frequencies of microsatellite markers on the color locus-containing LG V

Genotype frequencies for 11 markers and for mc1r on LG V in 61 F2 red-tailed males. Highlighted in red is the marker corresponding to the LOD peak. The number of red individuals is presented. Genotype frequencies are in parentheses. Only complete genotypes with both alleles present were reported, therefore % values do not add up to 100.

TABLE 2. Genotype frequencies of sex-linked markers on the sex-determining region on LG XIII

Genotype frequencies of sex-linked markers on LG XIII in 160 males and 86 females (cross #1) and 20 males and 14 females (cross #2). m1 and m2: alleles derived from the male grand-parent. The m1 allele is exclusively present in F2 males. f: allele derived from the female grandparent. Cross #1: GRZ male crossed with MZM-0403 female. Cross #2: MZM-0403 male crossed with GRZ female.
FIGURE LEGENDS

FIGURE 1. Cross between two strains of *N. furzeri* that differ in color and lifespan

(A) Color phenotypes of GRZ and MZM-0403. (B) A yellow-tailed short-lived male GRZ and a red-tailed long-lived female MZM-0403 were the founders of cross #1.

FIGURE 2. Microsatellite-based genetic linkage map of *N. furzeri*

Each linkage group is designated by a Roman number and ordered based on genetic length. The map distance in centimorgans (cM) is reported on the left side of each linkage group. Microsatellite loci identified at Stanford University were labeled SU followed by a four-digit number based on their order of identification. Microsatellite loci identified at the Fritz Lipmann Institute were labeled as FLI followed by a four-digit number. In the text, these markers are termed NfuSU or NfuFLI followed by a four-digit number, with Nfu standing for *N. furzeri*.

FIGURE 3. Genetics of the tail color in *N. furzeri* males

(A) Transmission of the color trait in the F1 and F2 generations. Chi-square test shows that the ratio is close to 3:1. Yellow tails always have black vertical bars. Red tails can be with (lower panel) or without (upper panel) black vertical bars.

(B) LG V contains the color locus, which corresponds to marker NfuFLI0030 (FLI0030). The expected map location of *N. furzeri* mc1r is indicated in red. The distance in cM is reported on the left side of LG V. *mc1r* is represented in
between NfuFLI0030 and NfuFLI0008 and closer to NfuFLI0030 than to NfuFLI0008 because there is stronger linkage between mc1r, NfuFLI0030, and NfuFLI0122 than between mc1r and NfuFLI0008 and because mc1r is 4 cM from NfuFLI0122 and 2 cM from NfuFLI0030. Note that the distances in cM between NfuFLI0122, NfuFLI0030, mc1r, and NfuFLI0008 do not add up because the distances between mc1r and each of these three microsatellite markers were calculated independently. Dashed lines indicate the synteny of microsatellite markers on N. furzeri LG V and annotated genes on medaka chromosome 3. The distance in Mb is reported on the right side of medaka chromosome 3. (C) LOD score plot of the color locus region. The LOD score is undetermined at marker NfuFLI0030 (FLI0030) due to lack of recombination between this marker and the color locus. Therefore the peak at NfuFLI0030 is represented by //. slc24a5 was not directly mapped and therefore is represented by a horizontal line between marker NfuSU0050 (SU0050) and NfuSU0046 (SU0046).

FIGURE 4. Sex determination in N. furzeri

(A) The proportion of males and females in the F1 (23 males and 26 females) and F2 (203 males and 178 females) generations. Chi-square test shows that the ratio is 1:1 (F1 $\chi^2_1 = 0.184$, p= 0.668; F2 $\chi^2_1 = 1.64$, p=0.2). The individuals that could not be phenotyped as males or females (undetermined) are not reported, therefore the proportion of males and females do not add up to 1. (B) Recombination scores for males and females at LG XIII for two F1 families (family 3 = 22 individuals; family 7 = 46 individuals). In both families, F2 males do not show recombination at the four sex-linked markers NfuSU0010 (SU0010),
NfuFLI0091 (FLI0091), NfuSU0007 (SU0007), NfuSU0090 (SU0090). In contrast, F2 females show recombination at markers NfuSU0007 (SU0007) and NfuSU0090 (SU0090). Note that F2 females show recombination at different markers in family 3 and family 7, which is likely due to the low number of individuals that were genotyped. This figure only displays the markers on LG XIII that were heterozygous in the F1 parents of family 3 and 7 and therefore allowed the analysis of male vs. female meioses. The partial representation of LG XIII is depicted by dotted lines. The map distance in centimorgans is reported on the left of the LGs.

SUPPLEMENTAL TABLE LEGEND

TABLE S1
Informative 152 microsatellite markers used to generate *N. furzeri* linkage map.

SUPPLEMENTAL FIGURES LEGEND

FIGURE S1
*N. furzeri mc1r* sequence polymorphism and resulting amino acid change in Mc1r protein in MZM-0403 and GRZ. UTR: untranslated region.

SUPPLEMENTAL DATA
Linkage map input data file for JoinMap3.0.
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Telomeres shorten while Tert expression increases during ageing of the


Table 1

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Figure 1

A

GRZ  MZM-0403

♂
♀

short-lived  long-lived

B

♂ GRZ  X  ♀ MZM-0403  P0

54 F1  9 families

413 F2
Figure 2
Figure 3

A

![Proportion graph](image)

B

![Genetic map](image)

C

![LOD score graph](image)
Figure 4

A

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B

Family 3

SU0010 FLI0091 SU0090 SU0007

Family 7

SU0010 FLI0091 SU0090 SU0007

SU0007

SU0090

SU0007

SU0090

SU0010 FLI0091 SU0090