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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 life span of 9–11 weeks for the shortest-lived strain. Natural populations of *N. furzeri* display differences in life span, aging biomarkers, behavior, and color, which make *N. furzeri* a unique vertebrate system for studying the genetic basis of these traits. We mapped regions of the genome involved in sex determination and tail color by genotyping microsatellite markers in the F_2 progeny of a cross between a short-lived, yellow-tailed strain and a long-lived, red-tailed strain of *N. furzeri*. 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. The microsatellite-based linkage map we developed for *N. furzeri* will also facilitate analysis of the genetic architecture of traits that characterize this group of vertebrates, including short life span and adaptation to extreme environmental conditions.

THE Nothobranchius fish species are present in eastern and southeastern Africa, where they populate ephemeral water pools that often undergo complete desiccation during the dry season (Terzibasi *et al.* 2008; Reichard *et al.* 2009; 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 (Wourms 1972; Inglima *et al.* 1981; Genade *et al.* 2005).

Nothobranchius furzeri is the shortest-lived species of the Nothobranchius genus, with an intergeneration time of 40 days, a median life span of 9–11 weeks, and a maximum life span of 12–15 weeks for the shortest-lived strain GRZ (Valdesalici and Cellerino 2003; Genade *et al.* 2005; Valenzano *et al.* 2006; Terzibasi *et al.* 2008, 2009; Hartmann *et al.* 2009). 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 life span 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* (Terzibasi *et al.* 2008; Reichard *et al.* 2009). Similar color polymorphism among males is also observed in other species of Nothobranchius (Wildekamp 2009) and in other fish species, including guppies and

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cichlids (Hughes *et al.* 1999; Brooks and Endler 2001; 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 discernible 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 (Volff 2005; Marshall Graves 2008). For example, medaka, platyfish, guppy, and sticklebacks all have recently evolved genetic sex-determining systems (Volff and Schartl 2002; Peichel *et al.* 2004; Shapiro *et al.* 2009; Tripathi *et al.* 2009a), 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 for 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; Walter *et al.* 2004; Tripathi *et al.* 2009b), and of 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 intraspecific 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 life span.

MATERIALS AND METHODS

Fish housing and husbandry: Fish were grown at 25° in a centralized filtration water system at a density of two fish per gallon tank. Fish were fed freshly hatched Artemia brine shrimp until 3 weeks of age and then dried Chironomid bloodworms two 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 $CaCl_2$, 0.02 mm $NaHCO_3$, pH 7.3) (Rembold *et al.* 2006). Fry were placed in 0.2-gallon tanks at the density of five 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 F_1 generation (54 fish), nine independent "families" (eight tanks with a spawning pair in each tank and one tank with three males and four females spawning together) were formed, and fertilized eggs were collected. A total of 413 F_2 individuals were produced. An independent, reciprocal cross was established between one male MZM-0403 and one female GRZ (cross 2). Two F_1 individuals from cross 2 produced a total of 34 F_2 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 F_2 adult males and all 23 F_1 males from cross 1 by visual inspection, immediately preceding submersion in ethanol. *N. furzeri* male fish are extremely colorful, and thus male color at death was clearly visible. F_1 and F_2 fish with yellow tails overlaid with red spots were scored as "yellow."

Sex phenotyping: Sex was assessed at death on the basis of 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 *Rsa*I. Fragments (1000–1650 bp) were cloned into the pCR-Blunt-TOPO vector (Invitrogen) and transformed into chemically competent *Escherichia coli*. Transformants were selected for the presence of CA/GT microsatellites using a [^{32}P]dCTP end-labeled $(CA)_{15}$ hybridization probe (Elim Biopharmeceuticals) 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° and that amplify fragments between 150 and 400 bp 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 5540 sequences composing 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 that met the following criteria were identified: (i) one microsatellite per sequence, (ii) at least 20 repeat units for dinucleotide repeats, (iii) at least 100 bp of microsatellite flanking sequence, and (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: A total of 244 pairs of primers were used to amplify microsatellites from the grandparents and 246 F_2 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 F_2 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 four terminal markers of each LG and to the singletons. PCRs were performed in 5 μl in 384-well plates with 0.2 units Taq DNA polymerase, 1× 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° for 5 min, followed by 30 cycles of 30 sec at 94°, 45 sec at 56°, and 45 sec at 72° and 8 cycles of 30 sec at 94°, 45 sec at 53°, and 45 sec at 72°. 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° 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 (F_1) genotypes were tractable for only 96 of 246 F_2 individuals. Raw data are available for download (supporting information, File S1).

The map length was computed by adding $2 \times s$ to each LG length in centimorgans, where s is the average intermaker 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 centimorgans 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 F_2 red-tailed fish ($n = 61$) of cross 1, genotyped for all 148 microsatellites. Only red-tailed F_2 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: $\mathrm{LOD} = M \times \mathrm{LOD10}(m) + (N - M) \times \mathrm{LOD10}(1 - m) - N \times \mathrm{LOD10}(0.5)$, 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 at which all red individuals have both MZM-0403 alleles, $m \times 100$ corresponds to the distance in centimorgans 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 cosegregated with tail color as query against the medaka genome (October 2005 MEDAKA1 assembly) at Ensembl (http://www.ensembl.org/Oryzias_latipes/Info/Index). The microsatellites flanking regions having a P value $<10^{-5}$, and 100% sequence identity over >22 bp was considered a significant hit. 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 subcloned in the pCR 2.1-TOPO cloning vector (Invitrogen).

Identification and genotyping of *mc1r* sequence polymorphism: The *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 F_2 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).

Mapping sex: The genotypes at 148 microsatellites of 239 F_2 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, F_2 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 the male *vs.* female sex-determining region was determined by scoring recombination events in two specific F_1 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 the sex-specific allele distribution in the offspring of these two F_1 families (22 F_2 individuals for family 3 and 46 F_2 individuals for family 7—68 F_2 individuals total) at the sex-linked markers that were heterozygous for both F_1 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"), by using a $(CA)_{15}$ probe because CA micro-

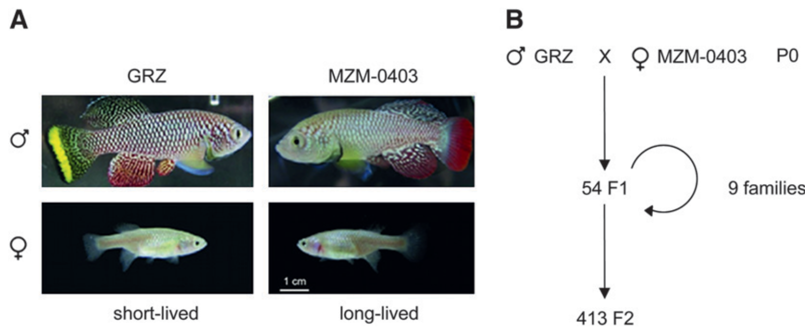

FIGURE 1.—Cross between two strains of *N. furzeri* that differ in color and life span (A) Color phenotypes of GRZ and MZM-0403. (B) A yellow-tailed, short-lived male GRZ and a red-tailed, long-lived female MZM-0403 were the founders of cross 1.

satellite 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 F_1 progeny, which were used to form nine families (Figure 1B). A total of 413 F_2 progeny from these F_1 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 (Table S1). 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, which is consistent with this strain being recently derived from the wild and propagated in captivity for no more than seven generations (Terzibasi *et al.* 2008; Hartmann *et al.* 2009).

To build the linkage map, we genotyped 246 F_2 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 >50% of the individuals. The resulting *N. furzeri* linkage map consists of 25 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 intermarker distance of 5.3 cM. Considering 25 cM as the maximum intermarker 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 ($25 \times 10 + 25 \times 6 = 400$ 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 F_1 and F_2 generations for a "yellow" *vs.* a "red" caudal fin. All F_1 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 F_1 male fish with advancing age (data not shown). In the 203 males of the F_2 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 ($\chi^2_1 = 2.76$, $P = 0.09$), 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 the 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 F_2 generation inherited both alleles from the red MZM-0403 grandparent. These results indicate that the yellow/red tail-color trait is determined mainly 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 the sequenced fish species most closely related

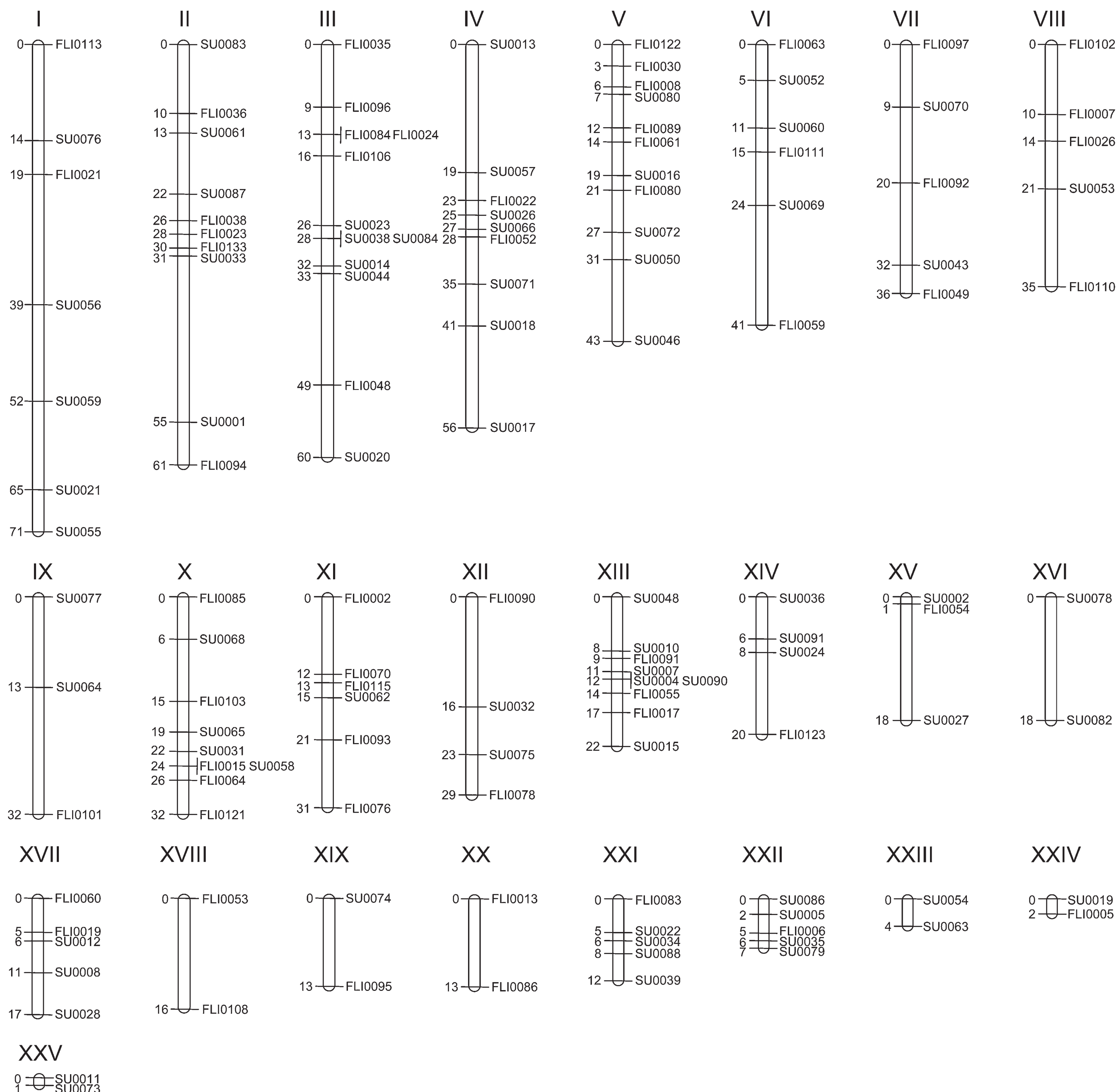

FIGURE 2.—Microsatellite-based genetic linkage map of *N. furzeri*. Each linkage group is designated by a Roman numeral and ordered on the basis of genetic length. The map distance in centimorgans is reported on the left side of each linkage group. Microsatellite loci identified at Stanford University were labeled as 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*.

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 colinearity 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 the following: melanocortin 1 receptor (*Mc1r*) and its ligand (*Asip*) (Rees 2003;

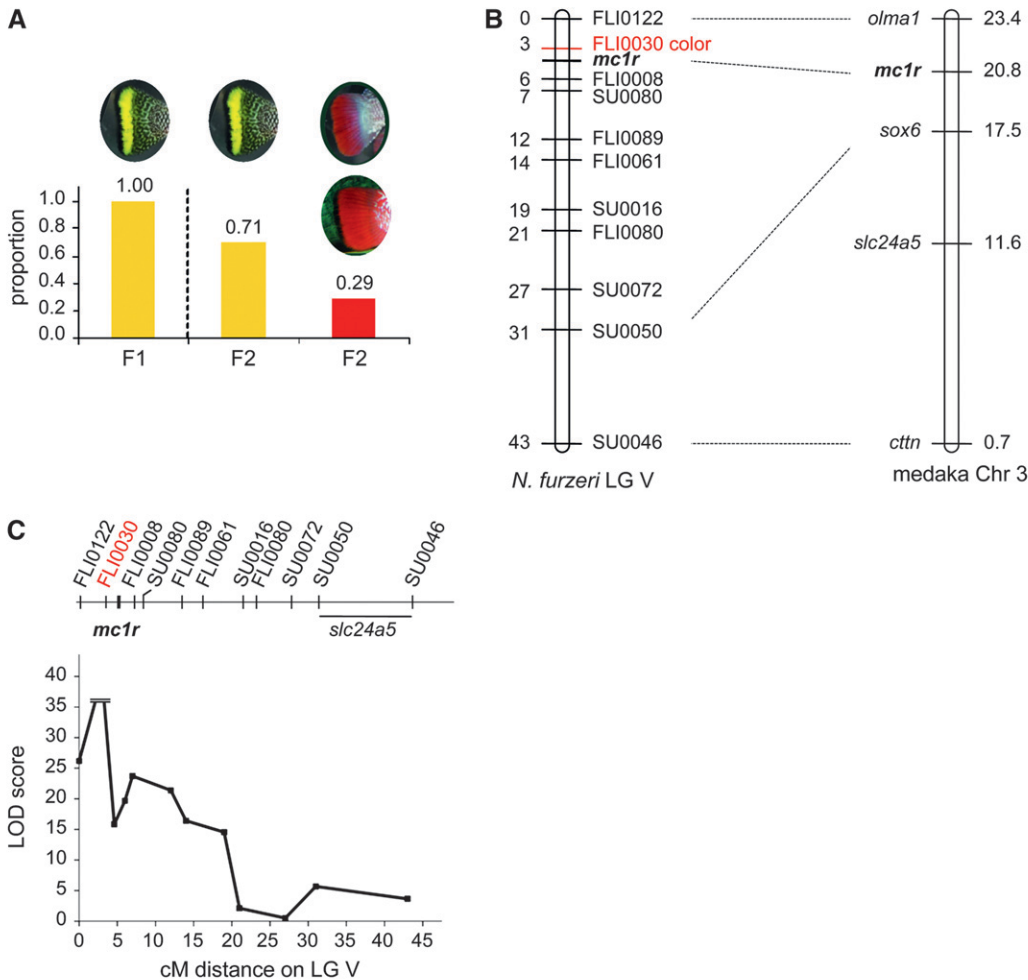

FIGURE 3.—Genetics of the tail color in *N. furzeri* males. (A) Transmission of the color trait in the F_1 and F_2 generations. A χ^2 test shows that the ratio is close to 3:1. Yellow tails always have black vertical bars. Red tails can be with (bottom) or without (top) black vertical bars. (B) LG V contains the color locus, which corresponds to marker NfuFLI0030 (FLI0030). The expected map location of *N. furzeri mc1r* is indicated in bold. The distance in centimorgans is reported on the left side of LG V. *mc1r* is represented between NfuFLI0030 and NfuFLI0008 and closer to NfuFLI0030 than to NfuFLI0008 because there is stronger linkage among *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 centimorgans 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 megabases 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 two short horizontal lines. *slc24a5* was not directly mapped and therefore is represented by a horizontal line between marker NfuSU0050 (SU0050) and NfuSU0046 (SU0046).

SHRIVER *et al.* 2003; HOEKSTRA 2006; SULEM *et al.* 2008; LE PAPE *et al.* 2009), 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* (SULEM *et al.* 2007; HAN *et al.* 2008), *TPCN2* (BONILLA *et al.* 2005; SULEM *et al.* 2008), *Sly* (ALIZADEH *et al.* 2009), *oca2* (SHRIVER *et al.* 2003; PROTAS and PATEL 2008), 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 nonsynonymous 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 three 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 F_2 fish allowed us to place *mc1r* on LG V, the LG

TABLE 1

Genotype frequencies of microsatellite markers on the color locus containing LG V

Markers	Genotype		
	MZM-0403	GRZ	GRZ/MZM-0403
FLI0122	46 (97)	0 (0)	1 (2)
FLI0030[a]	48 (97)	0 (0)	0 (0)
mc1r	26 (85)	0 (0)	1 (3)
FLI0008	35 (93)	0 (0)	0 (0)
SU0080	50 (93)	1 (2)	3 (6)
FLI0089	41 (91)	0 (0)	3 (7)
FLI0061	40 (85)	1 (2)	6 (13)
SU0016	41 (73)	1 (2)	13 (23)
FLI0080	9 (64)	1 (7)	4 (29)
SU0072	5 (36)	1 (7)	8 (57)
SU0050	28 (56)	3 (6)	19 (38)
SU0046	25 (42)	3 (5)	31 (53)

Genotype frequencies for 11 markers and for *mc1r* on LG V in 61 F_2 red-tailed males. The number of red individuals is presented. Genotype frequencies are presented as percentages in parentheses. Only complete genotypes with both alleles present were reported; therefore, percentage values do not add up to 100.

[a] Marker corresponding to the LOD peak.

that contains the color locus (Figure 3B). However, there was one recombinant red-tailed F_2 fish with a GC genotype, as well as three recombinant F_2 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 F_2 individuals whereas the latter was calculated only in red F_2 males. The distance between *mc1r* and the color locus indicates that the 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 F_1 and F_2 generations. The F_1 and F_2 offspring displayed an even sex ratio (F_1: $\chi^2_1 = 0.184$, $P = 0.668$; F_2: $\chi^2_1 = 1.64$, $P = 0.2$) (Figure 4A), consistent with a genetic sex determination system. We sorted the genotypes by sex and searched for markers that show alleles that are present predominantly in males or in females. We identified nine microsatellite markers on LG XIII that were linked with sex. Interestingly, five of these makers (NfuSU0004, NfuSU0007, NfuSU0010, NfuSU0090, and NfuFLI0091) show a significant male-specific allelic bias in that most of the F_2 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 whether the male-specific allelic bias is shared by different *N. furzeri* strains, we genotyped the sex-linked markers in the F_2 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 whether recombination is suppressed at the sex-linked markers in *N. furzeri*, we independently calculated male and female meiotic recombination frequencies in 68 F_2 offspring from two F_1 families of our first cross (cross 1). We performed this analysis in F_2 individuals instead of F_1 individuals because the P0 MZM-0403 female was homozygous at all sex-linked loci. F_1 females were heterozygous at the sex-linked loci, except at marker NfuSU0004, which was therefore excluded from the analysis. In F_2 progeny from each of the two F_1 families, there was no recombination event for markers NfuSU0090, NfuSU0007, NfuSU0010, and NfuFLI0091 in males, whereas females displayed a total of six recombination events, which 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 nine 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 $P = 1.5 \times 10^{-7}$), although there is no 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 chromo-

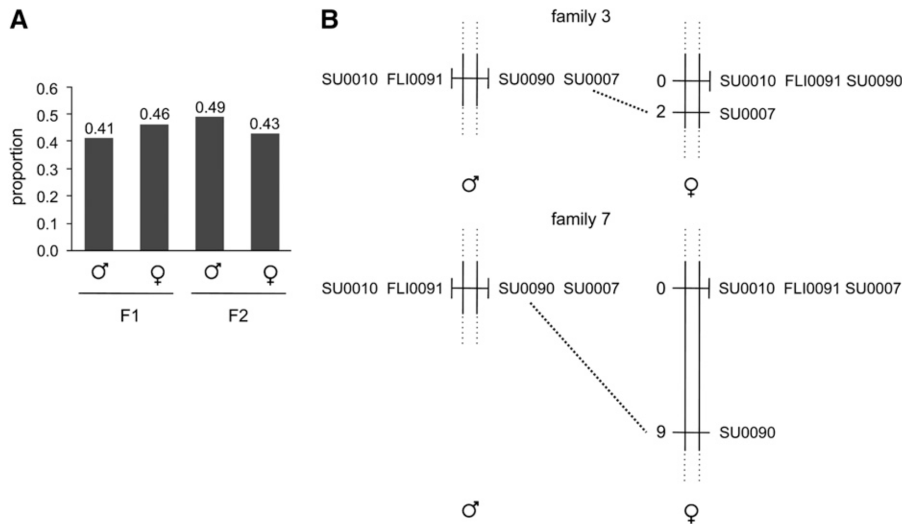

FIGURE 4.—Sex determination in *N. furzeri* (A) The proportion of males and females in the F_1 (23 males and 26 females) and F_2 (203 males and 178 females) generations. The χ^2 test shows that the ratio is 1:1 (F_1 χ^2_1 = 0.184, P = 0.668; F_2 χ^2_1 =1.64, P = 0.2). The individuals that could not be phenotyped as males or females (undetermined) are not reported; therefore the proportion of males and females does not add up to 1. (B) Recombination scores for males and females at LG XIII for two F_1 families (family 3 = 22 individuals; family 7 = 46 individuals). In both families, F_2 males do not show recombination at the four sex-linked markers NfuSU0010 (SU0010), NfuFLI0091 (FLI0091), NfuSU0007 (SU0007), and NfuSU0090 (SU0090). In contrast, F_2 females show recombination at markers NfuSU0007 (SU0007) and NfuSU0090 (SU0090). Note that F_2 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 displays only the markers on LG XIII that were heterozygous in the F_1 parents of family 3 and family 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.

some in another species of medaka (*Oryzias latipes*) (Matsuda *et al.* 2002; Kasahara *et al.* 2007) 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 of these species.

TABLE 2

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

Markers	m1 allele (%) ♂	m1 allele (%) ♀	m2 allele (%) ♂	m2 allele (%) ♀	f allele (%) ♂	f allele (%) ♀
Cross 1						
SU0048	0	0	77	27	23	73
SU0010	50	0	23	24	27	76
FLI0091	48	0	23	26	29	74
SU0007	51	0	22	28	27	72
SU0004	50	0	0	0	50	100
SU0090	49	0	21	26	30	74
FLI0055	0	0	80	28	20	72
FLI0017	0	0	70	21	30	79
SU0015	0	0	65	31	35	69
Cross 2						
SU0010	50	0	15	25	35	75
FLI0091	53	0	27	25	20	75
SU0007	50	0	30	18	20	82
SU0090	0	0	67	25	33	75

Genotype frequencies of sex-linked markers on LG XIII in 160 males and 86 females (cross 1) and in 20 males and 14 females (cross 2). m1 and m2 are alleles derived from the male grandparent. The m1 allele is exclusively present in F_2 males. The f allele is derived from the female grandparent. Cross 1: GRZ male crossed with MZM-0403 female. Cross 2: MZM-0403 male crossed with GRZ female.

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 (Ohtsuka *et al.* 1999; Naruse *et al.* 2000). 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/oxg__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.* 2008, 2009).

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* (Rees 2003; Hoekstra 2006). The Mc1r protein is a G-protein-coupled re-

ceptor 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 (Valverde *et al.* 1995; Mundy 2009) 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 *A. 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 *de novo* (Parichy 2003; Braasch *et al.* 2007; Protas and Patel 2008).

The red and yellow grandparents of our cross have a nonsynonymous variation in the coding sequence of *mc1r*, leading to a change in amino acid in the Mc1r protein from an aspartic acid in the red strain to a histidine in the yellow strain. However, this nonsynonymous 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 *N. furzeri*. Fine mapping will reveal whether color determination in *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 Nothobranchius, coloration is specific to males and is likely shaped by female mate selection. For example, in *Nothobranchius guentheri*, females prefer conspecific males on the basis of 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 *et al.* 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 *N. furzeri*, it will be interesting to test whether natural populations of *N. furzeri*, as well as different species of the genus Nothobranchius, harbor polymorphisms in this region.

Sex determination in *N. furzeri*: Sex can be determined by mechanisms that are genetic, environmental, or a combination of both (Volff 2005; Marshall Graves 2008). 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 in 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 nonrecombining region, similar to that in medaka, guppies, platyfish, and sticklebacks (Kondo *et al.* 2001; Matsuda *et al.* 2002; Nanda *et al.* 2002; Volff and Schartl 2002; Peichel *et al.* 2004; Schultheis *et al.* 2006; Ross and Peichel 2008; Shapiro *et al.* 2009; Tripathi *et al.* 2009a). 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. Synteny 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 the *O. javanicus* sex chromosome (chromosome 16), raising the interesting question of whether the sex-determining regions in these two different species were 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, 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.

Life span, color, and sex determination in *N. furzeri*: A major advantage of developing *N. furzeri* as a model system is its short life span and the presence of natural populations with differences in mean and maximal life span. Our cross did not allow us to map quantitative trait loci (QTL) for longevity because a large number of F_2 fish died prematurely because of 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 life span 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 life span.

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

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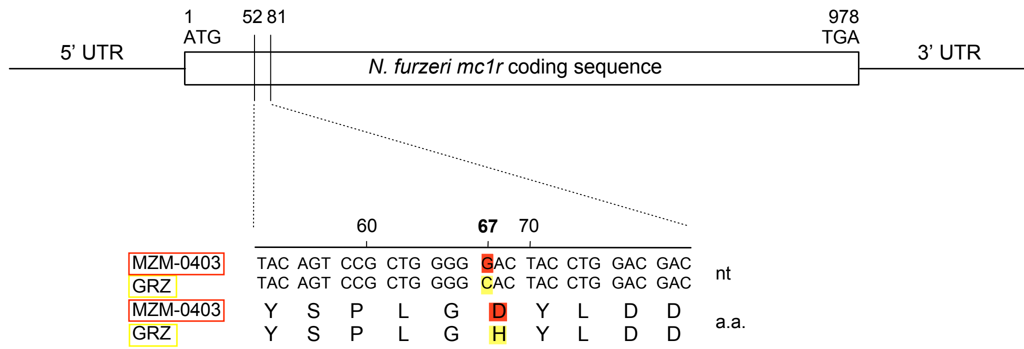

FIGURE S1.—*N. furzeri mc1r* sequence polymorphism and resulting amino acid change in Mc1r protein in MZM-0403 and GRZ. UTR: untranslated region.

TABLE S1

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

ID	Unit repeat	Forward primer	Reverse primer	size range (bp)
NfuSU0001	CA	GGGTGTATGGCAGCAAGATT	TGCACTGCAAATCTGAAGGA	211-247
NfuSU0002	CA	CACAAGCAAGGTGAAGTCCA	GCGACACTCAAGCCTCCTAC	250-258
NfuSU0003	CA	CTCCTTTGTCTGGCTTCTGG	TGAAGCGGTTTGAAGTTTTG	250-267
NfuSU0004	GAA	AATGAAAAGCATCACTGCACA	TTTTGTGAAGTGCCTCGTGA	195-207
NfuSU0005	CA	AAGGTTCAACCACTCCATCG	AGGACAGAAGGCTGCATGTT	208-295
NfuSU0006	CA	CTGACGTGCTGCGTCTCTAC	GGCATTTTGCCACACATAAA	186-215
NfuSU0007	CA	GGCTAAGCCTTGCTGACAGA	CAGGGAGCTGAAAACCTCAG	166-214
NfuSU0008	CA	GCAAACTCGCTAGCTTGGAT	CACCGGCATGATCAGTGTTA	293-301
NfuSU0009	CA	TTGGACATGTGGACGTGACT	GAAACCTTCAGCATTTGAGCA	217-262
NfuSU0010	CA	CGCAGTCTGATCAAATCGTGT	TGTTTGAAGGTTCACATTCATTATC	220-272
NfuSU0011	CA	AGGAATCAGGCTCAGAGCAA	AGCTGAGGATGGTGTTTTGG	215-257
NfuSU0012	CA	CCCACAGCGGATTAACAGAG	GTTATTGACCAAGGCGAGGA	211-267
NfuSU0013	CA	GGGGAGTGCTGTAGGACAAC	ACCTGCATTTGCCAGGTTA	606-655
NfuSU0014	CA	CATCAATGATCCCTGGCTCT	GATGAAGGCTTTCCGTTCAC	214-222
NfuSU0015	CA	CTGAAGGGACCTCAGCACTC	CGAGTGAAACTGCTCACCTCT	214-245
NfuSU0016	CA	CATGGCTAAACCGTGATGAA	GAAGGACGCCAGCTATGAAG	209-240
NfuSU0017	CA	CCTCTTCTCCCAACGAACAC	TGCATCAGCTTCATTTCTGG	240-261
NfuSU0018	CA	GTGGTTTTGGCTGTTCTCGT	GAAGAGGAACAAAGGAGGGTTT	274-338
NfuSU0019	CA	TTCAAGCAAATGCAGGACAG	GCAGGCATGCAATCTTACAG	173-224
NfuSU0020	CA	GCCTTCTCCTGTGCACTTTC	GCTTCTCCTCTTTTGCGAGA	571-642
NfuSU0021	CA	GGGTTAGCACATGGTCGAGT	CAACGACTTTGGAGTTGCATT	181-210
NfuSU0022	CA	AACACAGCTCTCGTAAGGAGGTA	TTCAGACTTGTCTTACTACCATGTTT	198-238
NfuSU0023	CA	TGCATTAAAACGCGTTAATCTT	CGTTTTCTCCTGTCTTCTGTCC	203-245
NfuSU0024	CA	TTCAGCAGCTGTTCACCATC	ATGAATTGCATGTTCCCACA	172-226
NfuSU0025	CA	TGAATGCCACCTTCTCTCCT	CGTCCTGAGCAGAGTTTGAA	183-214
NfuSU0026	CA	GCCAGTAATCGAAAGGTTGC	AGCTCCTTTCAGCGTCAGAG	261-309
NfuSU0027	CA	TCCAGCTGAATCGGTAATGA	AAACTCGAGGGTGCAATCTG	164-226
NfuSU0028	CA	GCCAGGAAGCAATAAAACCA	CAGAACTGATAGCAACGTGAGAA	174-194
NfuSU0031	CA	CTGAACAAGCTCCCCAATGT	CATGTTTTATGCCCTGCATTT	121-219
NfuSU0032	CA	GCCCCACACATCACTTTTCT	CCAGAGCCACAAAGACACAA	226-268
NfuSU0033	CA	ATTAGGACCGGGTTTTGGTC	CCTTTTTCCTCTTGCTTTCG	211-235
NfuSU0034	CA	CATTCCTTTGGATGCCATTT	CAGGTGCAGAGCTGTCTGAG	310-320
NfuSU0035	CA	GCTGCGGTTATTCCTCTGTC	ATGAAACTGGCCACACCAAT	240-264
NfuSU0036	CA-GA	TGCTTTGTGTGAGCATGTGA	CTGGACAGCACTGGGAAGTT	259-298
NfuSU0038	CA	TCTCCGTGGTCAAGTCACAG	TCCCAAGCAGATCTGGAAAG	118-171
NfuSU0039	CA	ATGGCATTTTTCATGGAAGC	CAGATGGGTCAACAGGCTTT	202-210

NfuSU0040	CA	TAAGCACAATCCGCCTCTTT	GCCCCTTCAACTCATGTCAC	171-203
NfuSU0042	CA	CACACAGAAATGAGGGCAAA	GCTGGTATTGCAACAGGACA	222-229
NfuSU0043	CA	GATGGCACACACACACAACA	GCCTGAGTCTGGTTTGCATT	218-226
NfuSU0044	CA	TGTGGCAGCTTTAATGAGGA	AACCCAGCTCTGACACTTGTT	608-651
NfuSU0045	CA	TGTCTGGATGTGGATGGAGA	TACTGGACATGCTGGTCTGG	208-216
NfuSU0046	CA	GCCAAACTTAAATAATAAAACTAGGG	GTCATCACGTTCACGCATTT	210-226
NfuSU0048	CA	TTCTGTTGGTTGCAGAGACG	AGATCCACCTGTGCCGTTAC	441-444
NfuSU0049	CA	CTGGACAAAGTGCCAATCAC	CTCCCACAGTCCCAAAACAT	196-197
NfuSU0050	CA	CCAGAATGAACAATACTCAGATCAA	GCAGCTTAGTTTAATGATATCACAATG	252-295
NfuSU0052	CA	GGGCCAAATTCAAACACATT	AGGGGGCTTGTTGATTTTGT	226-236
NfuSU0053	CA	GCCGGCTGAAATTACACCTA	TGACAGCAAAACCAGGCTAA	256-264
NfuSU0054	CA	CAAGACTTTACAGTGTGTGCTTTTG	GTCGGACATTACCCCTGCTA	157-174
NfuSU0055	CA	GCAGTCATAAGCAGTCTTTTGG	TCTTTCCCCCAAAATTCAAA	172-192
NfuSU0056	CA	GCAAGGCCTGATGTTGATCT	TATGTCAGCAACCCTGGTAGG	160-188
NfuSU0057	CA	GCAGGATCGCTCATTACCAT	TCCAAGGAAGCGTATTTTCA	337-383
NfuSU0058	CA	CTTTTCCCCTCCTTCCTCTG	ACTAGCGGCTTGTTTCTCCA	208-255
NfuSU0059	CA	GCTGCAACGCAATAGTTTCA	AAAGGCAGAGTTGGTCGAAA	220-230
NfuSU0060	CA	CTAGCCACTCCCCTGGTTTA	CCGTCACGATGTGCTGATAC	216-248
NfuSU0061	CA	TGAAGGGAAAGCAGGTGACT	CCTCAGGTCTGGCATTCATT	242-255
NfuSU0062	CA	GGGCTTTTAATTCCCCTCAT	TTCAACCAATTACATCAGTTTGTG	248-258
NfuSU0063	CA	TGTCTCCAGATGCAGAGGAA	TGCATCATTAGACAGCATAACG	253-263
NfuSU0064	CA	CACACTGATGAATCGCATCC	CAGCCCAAAATAACCCTCAA	242-257
NfuSU0065	CA	ATAACCTAGGCCAGGGAGGA	TGTCATGTGCAGACACATCC	176-187
NfuSU0066	CA	GCCAGTAATCGAAAGGTTGC	AGTTTGTCCCAGGAAGCGTA	447-495
NfuSU0067	A	CATGTCCAGCCTCAGAGTGA	AGGATACGGACCCTCGAAGA	208-211
NfuSU0068	CA	TCACTGGGGATGGAGAAGAC	GCTGCTAAATTCCTGCATCA	224-261
NfuSU0069	A	AGTGGGGAAAGGGGTAACAG	GCAAAACAGAAATATATGAAAAACCA	252-254
NfuSU0070	CA	TTGTCCCCTACCAACGCTAA	AAACCGTGCGATTTGCTTTA	380-419
NfuSU0071	CA	CATAAACCCCACGCTGAGTC	CAGAGAAACTTTTGCTGCACAC	254-258
NfuSU0072	CA	CTGGTTCAGAGTCCGAGAGC	CATGGAGAATGCAGAGTTTCC	210-237
NfuSU0073	CA	GCCACTGAATTCCTCAGCTC	CCAGCAAAGGCTAGCTTGAA	242-274
NfuSU0074	CA	AAATGTGACGCCAAACCTTC	CCAACATAGCATCACGGTTG	189-194
NfuSU0075	TAA	TTGCTACAAGGCAACAGCTC	AAGCAAATATGATTTACCTACAAGAAA	265-388
NfuSU0076	CA	CAGCAAGAAAGTTAATTCTGACGA	AGGCGCTTTGCAATACAGTT	202-220
NfuSU0077	CA	GTTCGGGTGAGAGAGCAGAC	CAGTTATAGCTCCGCCCATT	193-269
NfuSU0078	CA	GCAGCAATAAGGAATCATCCA	TGGTGATGACCGATCACAGT	188-201
NfuSU0079	CA	TTGCTTCCAAATCCTAAATCC	CACATTGATGTGACGTTTGTGA	296-306
NfuSU0080	CA	ATCCTTTCCTCCCTCTCTGC	AAAGCAGACCTTGGTGTCACT	175-239
NfuSU0082	CA	ACAAATGCACAGCAGCTCAC	CCATTTGTTGCCTGAACTGA	258-262
NfuSU0083	CA	GGATATGTCACGTGGGGAAC	CCGTGGTGCTGAAACAAGAT	221-230
NfuSU0084	CA	AACAGCATCACACCAACCAA	GTCAGCATCGACAGCAGTGT	245-257

NfuSU0085	A	TGTTCAAGGCATCTTGTGGA	GGTTCAAGGGTCACACAGGT	210-241
NfuSU0086	CA	GGAAACTTTACACATCCAGGTC	GCTACTGGAGACGAAAATCTGAA	195-239
NfuSU0087	CCT	TGATGAGTCTTGCCATTTCG	CCCTAAAGGAATGATTGTCTCG	170-213
NfuSU0088	CA	GGCTCTGCAATTACACTTGG	GCAAGTGGGTTTTAACAGTGC	193-201
NfuSU0089	CA	TTTATCCGTTTAATTATCCACTAGGA	CCAGCAAATTGCATCAAGTC	232-240
NfuFLI0002	CA	GTAACCATGGACACATTTCTTG	GACATCCTTGATGCTCAATGAA	358-366
NfuFLI0005	CA	CACCAACTGTGGAGTAGTGC	CCCAACATGAACAAAGACGC	183-212
NfuFLI0006	CA	GCAAGCAGCACCCTTTATTTC	GAAAGTAGGGGTCCCACACA	299-312
NfuFLI0007	CA	GATGGCGTGTCATTCACTAC	CCTCCAACATGGTCAGCTCT	187-235
NfuFLI0008	TA	CATGTGCTCCCTTTCATTCAA	CTGGAAATGAGCTCTGCAAC	232-266
NfuFLI0013	CA	TCACCCACTTACCACACCAA	ATGGATGCCATCAAGGAGAG	238-253
NfuFLI0015	CA	TTGGTCACACCTCTGGTTTC	CCCAAAGCCAGCTATTAGTCC	243-274
NfuFLI0017	CA	CCGGTCTCAGGAGTATCTAC	GGAGGTGAAACCCCCATAAA	261-303
NfuFLI0019	CA	CCAAAAGAGGTGGGAGCATA	GAGCCAAATCCTCAAGCTCA	214-260
NfuFLI0021	CA	CTGGAGCACTGACAATAAAGC	AAAGTGCAGCTGTCCCTGAT	276-303
NfuFLI0022	ATT	AGTGACCTTGGGTGTTCTG	GCTAGGCGGTTAAAGTGTC	248-239
NfuFLI0023	CA	GGTTAGGGTTAGGGTGAATC	GTACAGCACCTGCCTTAGTC	321-395
NfuFLI0024	CA	CTCCAGCAATACAGACCTTG	GTGCAGAAAGTTGTGAAACAC	250-255
NfuFLI0026	AC	AGCATCCCACTGTGTGTGTA	GCGCGCAGTTCTTCATCT	188-218
NfuFLI0029	CA	GTTTGAAAACCCACAATGCAC	CGCACATCTGATCACTCTCA	238-259
NfuFLI0030	CA	CAGAAGCTAAAGGCCAGACG	GGGAAACAATAGGGAACCAC	174-205
NfuFLI0032	CA	AAGTCGGATCAGAGCCAAGA	CTATCAGTGGTCTCAGTC	196-143
NfuFLI0033	AATC	GACTGACTCACAGGGTCAC	CGAATGACCACAGTCACCAA	229-231
NfuFLI0035	CA	CCTTTCCCCTGAACACGTAA	GTGGACCAACATACATGCG	228-265
NfuFLI0036	CA	CCCAGCTTGGAGTCTTTCAG	CAGTTCAAAGCAGTGAGAGG	343-345
NfuFLI0038	CA	CAGTAGGAGGGAGAAGCAG	CTTTGTCAGCTTGCTCTAGG	245-330
NfuFLI0043	GA	TCCACTGCAGCAGAGCTAAG	AGACTCCAGCCCCAGGTTT	200-243
NfuFLI0045	GT	TCTAGCCTCTCAGTGTGGAC	GACTCAAAGAGGCGAATCAG	388-430
NfuFLI0048	AAT	GAGTTTGTTCATAGGAAAGC	GTGTTAGTGGTGGTCAGAGG	309-312
NfuFLI0049	GT	GCAGTCAAGGTGTGAAAGAG	TGCAGTCACATGGAGGTAG	319-343
NfuFLI0052	TTC	CTACATCAGCAATCCACCTG	GGGCTATGCTTGTCTTTCAG	347-410
NfuFLI0053	CA	GCTAACAGTAATGGGGCTTC	GAAGTTGAGTCTGCCATCG	287-315
NfuFLI0054	CA	CTGTACTGAGACACTAACTG	AGGTCACAGGATGCTAACTG	248-256
NfuFLI0055	CA	GATGAACCACAGAAGACCAG	CACACCAGGGGTCTGAATAC	226-244
NfuFLI0059	GT	GGGGTTTTCAGAGGTAGAAC	CAGAGCGACTCACTTTTGAC	550-595
NfuFLI0060	GT	CGTTCTACCAGACAACTGACC	GCTACTGAGAGCAATCCAAG	406-436
NfuFLI0061	AAG	CTTCTGTCCCATCAGCAG	GCAAGATTTGGTTGCATTCA	447-474
NfuFLI0063	GT	CTTCCATCCAGACCAGAAG	TGTTTGGAATGGCCATGAC	428-440
NfuFLI0064	CA	CTCCTCTCCCTAAGCATACC	GTCAGTGTTGTGCCATTGTC	352-360
NfuFLI0070	CA	CACATGGGGTGACTGATG	TAAAGCCCGTCATGTGTC	317-360

NfuFLI0076	CA	CCATACCAAACTCTGTGGAG	GCTCTTTGAGTTCCACACC	468-487
NfuFLI0078	TGC	AGAGTGTGACCAAAGCTCAG	TCTGCACCTGAGTCCTATTC	282-333
NfuFLI0080	TG	ATAGGAGGAGAGCAAGAACG	GTCAGTCAGACTCACATATC	280-337
NfuFLI0082	CA	GTGTTGGCTCTCTTGTGTG	GTGATGCAGGCTAAATGC	318-395
NfuFLI0083	CA	GACTCCAGTAGCTGCCTATC	ATCCTGGAGGTCGTGGTAAC	466-495
NfuFLI0084	TAA	CTCTGATTGGTGGAAGACAG	GCAGTGTGTGAATGTGAGAG	352-354
NfuFLI0085	GT	CTAACCTACGGGCTCTTTTC	GGTTTGTCCGAGGTTTTG	498-515
NfuFLI0086	CA	GAACAGAGTCATTCGACACG	AGTGTAACAAGGGCAGTGTG	452-528
NfuFLI0087	TA	CTCCCCTTTGAGATCATACC	TGGGGAGATTATGTGTGC	386-421
NfuFLI0089	TA	GAACCAAAATCCCACCTC	CAAGACATCCCAGACCATC	280-287
NfuFLI0090	GT	TGACGCATTGGCAAGTAG	AGCAGATTGGGGAGTTTG	298-305
NfuFLI0091	CA	ACGCTGACTCTACCCAGTC	CTGCCTGCTACTGACAATG	355-373
NfuFLI0092	TC	TGTCATCCAGAGTTCCTGAC	GGCTGCACCAAAATGTAG	295-305
NfuFLI0093	TTA	CAAGGCTGAAAACCGAAC	CCCAAAAGTGTTGCTCAG	407-412
NfuFLI0094	TA	TCCCCCTGATAGAAAGTAGC	GGGCAGGTGTGAGTTATGTC	208-216
NfuFLI0095	GT	AGTGAAGCCTCTGCTAATCC	GAGAGGAGTCGTGTTTACCC	493-500
NfuFLI0096	TC	ATGTAGCCGTGGGTTAGTG	AAGCAGGAGGGATGAAAC	302-339
NfuFLI0097	TTTC	TCAGAGCAGGATAACCAGTG	CCCCCACTAACCTACAACG	360-404
NfuFLI0101	CA	TAAGGACCTGGCTTACACAC	CAAGTAGCCAGGGTCAATC	260-312
NfuFLI0102	CA	CAGGTTGCATCAAGTCACTG	TTTTTACTCTCTTGGACATCG	380-412
NfuFLI0103	GT	AGTCACCCCCAAATCAAC	TTGTCGGTCATTATCACTTG	404-437
NfuFLI0107	GT	AGTGACCAGACGCTTGAGAC	GACCGCAGCTAAAGTAGCAC	407-462
NfuFLI0108	CA	CTGTGATTAAGCTCGGACTC	CAACCATACCCGACTTGG	279-298
NfuFLI0109	TTC	TTCAGGCTTCCTGTTCACTC	GACCCCAGAAACTTCAGATG	455-465
NfuFLI0110	GT	ATCTGAGCTGGAGTGTGATG	TGCAACTATCTGCCTCACAG	195-207
NfuFLI0111	TC	TCTCTTAGACTCGCCTCTGC	ACAGCTCTACCCTCAACTCG	376-457
NfuFLI0113	AATC	TGAGTGAGTTTGGTCTGTCG	ACGTGCTCCCTTCATGTG	413-285
NfuFLI0115	GT	GAGCCAGTGATGTGCTGAG	GTGTTGTCCATGAAGACGTG	403-449
NfuFLI0121	TA	CAGCACTCCACTTATCTACGC	ACAGCAGGTAGCATCATCTC	393-457
NfuFLI0122	CA	TGCTACCCGTATGCTACAAG	CTGCCATCTGCTGTTTGTC	310-364
NfuFLI0123	CA	GTGTGGACCAGCAATATGTC	ACTGATGGAGCACTTTCACC	254-274
NfuFLI0125	TG	TCGGTGTAGCTCTAACAAGC	ACTTGCATTGGAGACTCGTG	334-361
NfuFLI0127	GT	GACACACGGGGATCAAATAC	CCTGTTTGCAGAAACGAGTC	557-616
NfuFLI0128	GT	ACCAGTTCCAACCAGTATGC	AGCTGGAGCACAGATAGAGG	378-418
NfuFLI0133	GT	TAAGAGCCTGTGTGAACCTC	GACTCCTTGCTGCATTTG	564-590

FILE S1

Linkage map input data file for JoinMap3.0

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