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Common Spontaneous Sex-Reversed XX males of the Medaka *Oryzias latipes*

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ABSTRACT

In the medaka, a duplicated version of the *dmrt1* gene, *dmrt1bY*, has been identified as a candidate for the master male sex-determining gene on the Y chromosome. By screening several strains of Northern and Southern medaka we identified a considerable number of males with normal phenotype and uncompromised fertility, but lacking *dmrt1bY*. The frequency of such males was >10% in some strains and zero in others. Analysis for the presence of other Y-linked markers by FISH analysis, PCR, and phenotype indicated that their genotype is XX. Crossing such males with XX females led to a strong female bias in the offspring and also to a reappearance of XX males in the following generations. This indicated that the candidate male sex-determining gene *dmrt1bY* may not be necessary for male development in every case, but that its function can be taken over by so far unidentified autosomal modifiers.

GENETIC mechanisms that determine the development of the male and female sex have developed independently many times during evolution. This is reflected by such diverse sex-determining mechanisms as the autosome-to-one-sex-chromosome ratio and the female or male heterogamety. In some studied cases, even in established sex-determining systems, new sex chromosomes can appear due to mutation and invade the population (Charlesworth 1991; Charlesworth and Charlesworth 2000).

Fish display a wide spectrum of genetic sex-determining mechanisms (for reviews see Baroiller *et al.* 1999; Devlin and Nagahama 2002). One extreme, yet very thoroughly analyzed situation, is found in the swordtails and platyfish of the genus Xiphophorus. Here, in closely related species diverse modes of sex determination like polygenic sex determination, XX/XY and WZ/ZZ systems are operating. In most populations of the Southern platyfish *Xiphophorus maculatus*, even three sex chromosomes, X, Y, and W, coexist. Exceptionally so-called atypical sex determination was observed. This was explained by the action of rare alleles of autosomal modifiers that can influence or override the sex chromosome genes (Kallman 1984). The sex-determining region has been mapped in these fish (Morizot *et al.* 1991; Gutbrod and Schartl 1999) but the sex-determining gene(s) is unknown so far.

Another classic fish model for studying genetic sex determination is the medaka *Oryzias latipes*. In this organism crossing over between X and Y chromosomes was shown for the first time in vertebrates (Aida 1921). In line with the early theories of genetic sex determination (Bridges 1922, 1925; Winge 1934) the medaka was described as having a polygenic sex-determining system of multiple male- and female-determining factors spread over different chromosomes and as being polymorphic in a given population, however, with a strong epistasis of those factors that concentrate on the X and Y chromosome (Aida 1936). This view was later given up (see Yamamoto 1975). Today it is generally accepted that the medaka has a firm genetic female XX/male XY sex-determination system, with a master sex-determining gene on the Y chromosome that initiates the determination of the bipotential embryonic gonad similar to the action of *Sry* in mammals. However, unlike in mammals and many other groups of animals, treatment with sex steroids at the sensitive period of gonadal development can result in all types of functional sex reversals (Yamamoto 1975). A single report was made on spontaneous atypical sex determination (Aida 1936). Of 5000 males analyzed, 7 were detected in one laboratory strain, which, due to a linked marker, were diagnosed to be XX. In the absence of a detailed genetic map and of knowledge about the sex-determining gene, this finding remained enigmatic.

Recently, a first candidate for the male sex-determining gene from a fish species was cloned in the medaka (Matsuda *et al.* 2002; Nanda *et al.* 2002). The male sex-determining candidate gene of medaka is a duplicated version of the autosomally located *dmrt1a* gene. It is designated *dmrt1bY*. The high similarity even in introns between *dmrt1a* and *1bY*, in conjunction with molecular phylogenetic analysis, indicates a recent origin of *dmrt1bY* from its autosomal counterpart (Kondo *et al.* 2003). *dmrt1bY* is expressed during male embryonic development, preceding the differentiation of the gonad. In adults it is expressed exclusively in the Sertoli cells of the testis. The expression pattern is not altered by sex-

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reverting steroid treatment (Nanda *et al.* 2002). The strongest evidence for *dmrt1bY* as in fact being the master regulatory gene for male development comes from the finding that naturally occurring mutations in this gene lead to XY male-to-female sex reversals (Matsuda *et al.* 2002). This leads to the conclusion that *dmrt1bY* is necessary for male development.

Conceptual translation of *dmrt1bY* reveals a predicted protein with all conserved residues of a DM domain containing transcription factor. Not much is known about the biochemical and biological function of vertebrate *dmrt* genes in general and *dmrt1* in particular. There is genetic evidence—in line with the expression pattern—that *dmrt1* plays an important role as a downstream sex-determination/sex-differentiation gene in male development of some reptiles, birds, and mammals (Raymond *et al.* 1999, 2000; Smith *et al.* 1999; De Grandi *et al.* 2000; Kettlewell *et al.* 2000; Moniot *et al.* 2000). How *dmrt1bY*, as a duplicate of a downstream sex-determination/sex-differentiation gene, could assume a function at the top of the sex-determination cascade and how it could act molecularly in medaka as a master male sex-determining gene is totally unclear at the moment.

Two lines of evidence indicate that sex determination in medaka is at an early stage of evolution and has not reached a similar stability as in other organisms like birds and mammals. First, both X and Y chromosomes are homomorphic, indicating that the molecular differentiation process of the sex chromosomes leading to recombinational isolation over large parts has not progressed to a stage where it becomes visible. Sex chromosomal crossovers occur over almost the entire length of the corresponding linkage groups. In fact, the Y-chromosome-specific region appears to be very small, estimated to be only a few hundred kilobases in length. Second, the genotypic sex can be easily reverted by hormone treatment. As another facet of the instability of the genetic sex-determination system we report here that in several medaka strains, fish with an XX chromosome constitution spontaneously become fully fertile males. The frequency of such XX males in some strains can be as high as >10%. The results indicate the presence of autosomal modifiers for sex determination and that under certain conditions *dmrt1bY* is dispensable for genetic sex determination in medaka.

MATERIALS AND METHODS

Fish: All fish used in this study were taken from closed colony breeding stocks and are derived either from the highly inbred medaka lines HNI, HB32C, i-3, HdrR (Hyodo-Taguchi and Sakaizumi 1993), Kaga, SOK (A. Shimada and A. Shima, unpublished results), and Quart (Wada *et al.* 1998) or from the noninbred Carbio and Wü strain. SOK is derived from Korea; HNI and Kaga are derived from the northern population of Japan; and HB32C, i-3, HdrR, and Quart are derived from the southern population of Japan. The Carbio strain was established during 1986–1988 from fish obtained from Carolina Biological Supplies. Carbio fish are derived from the southern population of *O. latipes* and are homozygous for the *b′* (variegated) allele at the *B* pigmentation locus. Wü is a wild-type-pigmented hybrid strain of HNI and HB32C, the Y chromosome being derived from the Southern population of Japanese medaka. Both strains have been bred as closed colonies since their establishment.

All fish were raised and maintained under the same standard conditions (at 27°, with a light cycle of 14 hr light and 10 hr dark). Determination of sex ratios in the Carbio and i-3 strain was done from fish kept in large population tanks. The entire egg clutches from all females that had spawned were collected over several days and pooled and the hatched larvae were raised together until sexual maturation.

Sexing of fish: The phenotypic sex was first determined from secondary sex characters (shape of dorsal and anal fins, spines on male anal fin rays) and confirmed by functional egg or sperm production or gonad histology. No sterile fish with immature or mature gonads were detected in this study. The genotypic sex was diagnosed from the presence or absence of the *dmrt1bY* gene by PCR from fin clip DNA (Altschmied *et al.* 1997) using allele-specific primers such as DMT1k (5′ CAA CTT TGT CCA AAC TCT GA 3′) and DMT1l (5′ AAC TAA TTC ATC CCC ATT CC 3′) at an annealing temperature of 56°. Eventually a series of other *dmrt1bY*-specific primers were used. This information is available upon request. Furthermore, *casp6*, which is a polymorphic marker that differentiates the X and Y chromosome in fish of the AA2 strain derived from the Southern population of medaka (Kondo *et al.* 2001), was employed for genotyping. The primers used in this previous study amplified also *casp6* from the HB32C strain. Primers intf2 (5′ TAG CAC TTT CAC ATT TCC AAG C 3′) and c6R (5′ CGT CTC TCG ATG AGA ATA GAA ACC 3′) at an annealing temperature of 60° were used.

Southern blot analysis: DNA from individual fish was obtained from pooled organs as described (Schartl *et al.* 1995). Five micrograms of genomic DNA was digested with restriction enzymes, separated on 0.8% agarose gels, and blotted onto nylon membranes (Hybond N+, Amersham Buchler). Membranes were hybridized under conditions of moderate stringency (hybridization in 35% formamide, 0.1% Na-pyrophosphate, 50 mm Tris-HCl, pH 7.5, 5× SSC, 1% sodium dodecyl sulfate (SDS), 5× Denhardt's, 100 μg/ml calf thymus DNA at 42°, washing in 1× SSC/1% SDS at 60°) with the *OlaDmrt1a* probe (4-kb *Eco*RI fragment from cosmid 73K2481).

FISH analysis: For fluorescence *in situ* hybridization (FISH), mitotic chromosome preparations were made directly from pooled spleen, gills, and cephalic kidney cells after exposing the fish several hours to a 0.02% solution of colchicine. Prior to hybridization, slides were subjected to pepsin (0.01%) and formaldehyde (1%) treatment and denaturation in 70% formamide in 2× SSC (pH 7.0) at 70° following the standard procedure.

Two different bacterial artificial chromosome (BAC) clones, one spanning the Y-chromosome-specific region (BAC 15H17) and one containing a flanking maker (BAC 98J17, *SL1*), were separately labeled by nick-translation with biotin-16-dUTP or digoxigenin-11-UTP (Roche Molecular Biochemicals, Mannheim, Germany). Labeled DNA at a concentration of 10 ng/μl was coprecipitated with 150 ng/μl calf thymus DNA and 100 ng/μl sonicated medaka genomic DNA and redissolved in 50% formamide, 10% dextran sulfate, and 2× SSC. After 10 min denaturation at 75° and reannealing at 37° for 30 min, 20 μl of probe mixture was applied to a denatured slide and sealed under a coverslip. Following overnight incubation at 37°, the slides were washed at 45° in 50% formamide, 2× SSC for 15 min and for an additional two times of 5 min each, with 1× SSC at 60°.

TABLE 1

Sex ratios in medaka i-3 and Carbio strains

Brood	Females	Males	
		i-3 strain	
1	62	61	
2	25	23	
3	43	42	
4	47	49	
5	44	38	
6	33	36	
Total	254	249	(chi square = 0.05)
		Carbio strain	
1	18	25	
2	38	32	
3	24	15	
4	23	16	
5	21	15	
6	18	15	
7	42	46	
8	27	26	
Total	211	190	(chi square = 1.1)

The locations of the hybridization sites were detected with rhodamine-conjugated avidin (Vector, Burlingame, CA) and antidigoxigenin (monoclonal)-conjugated fluorescein (Sigma, St. Louis) followed by further signal enhancement of biotinylated probe using biotinylated antiavidin and rhodamine-conjugated avidin. Likewise, the sheep anti-mouse FITC conjugate was used to enhance the signal of the digoxygenated probe. Chromosomes and cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI). Slides were mounted with antifade medium and the hybridization signal was visualized on a Zeiss epifluorescence microscope equipped with a computer-controlled thermoelectronically cooled charged-coupled device camera. Digitized images of the FITC, rhodamine, and DAPI signals of metaphase spread were captured separately and merged using the Easy FISH 1.0 software (Applied Spectral Imaging). At least 20 metaphase plates for both probes were simultaneously examined to evaluate the hybridization pattern.

RESULTS

With a candidate for the male sex-determining gene and linked molecular markers now available we wanted to reinvestigate the classical finding of Aida (1936) of the rare, exceptional XX males. The sex ratio in two representative medaka strains, Carbio and i-3, was determined by the unambiguous phenotypic secondary sex characters and male and female gamete production. No significant deviation from the expected 1:1 ratio was found (Table 1).

When males of various strains were PCR genotyped for the medaka male sex-determining gene, we found a strikingly large number of animals that did not show the expected amplification product (Figure 1a). The lack of *dmrt1bY* was confirmed by Southern blot analysis.

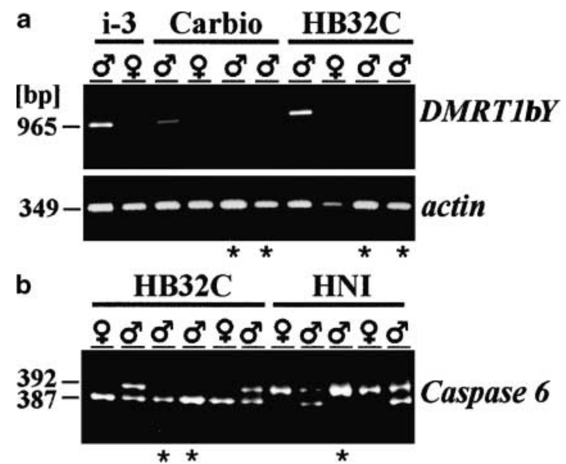

Figure 1.—PCR genotyping of medaka males and females. (a) Presence of the *dmrt1bY* PCR product in normal males of the i-3, Carbio, and HB32C strains. No PCR product was amplified from females and the aberrant males (*). For control, an aliquot of the same DNA was used for *actin* PCR. (b) Hemizygosity of normal males of the HB32C and HNI strains for *caspase 6*. Females and the aberrant males (*) show only the X chromosomal PCR product.

All the aberrant males showed a restriction fragment pattern like females (Figure 2). Such males were then genotyped for the linked sex chromosomal marker *casp6*, which is located outside the Y-chromosome-specific fragment in the pseudoautosomal region. The genetic distance between *casp6* and *dmrt1bY* is ∼1 cM (Nanda *et al.* 2002). Primers that give a 392-bp product from the Y chromosomal allele and a 387-bp product from the X chromosomal allele of Southern medaka and a 387-bp product from the Y chromosomal allele and a 392-bp product from the X chromosomal allele of Northern medaka were used (Kondo *et al.* 2001). All aberrant males had only the X chromosomal alleles (Figure 1b). In the HdrR strain a dominant color marker (orange body coloration, *R*) on the Y chromosome was also absent from the *dmrt1bY*-negative males.

FISH analysis was done on metaphase chromosomes

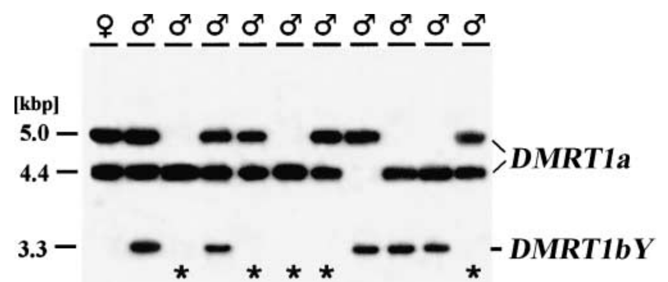

Figure 2.—Southern blot analysis of *Eco*RI-digested DNA from female, normal male, and aberrant (*dmrt1bY* negative) males (*) from the Carbio strain, hybridized with the *OlaDmrt1a* probe. This probe detects the autosomal *dmrt1a* (two alleles present in Carbio) as well as the Y chromosomal *dmrt1bY* under conditions of moderate stringency (see Nanda *et al.* 2002).

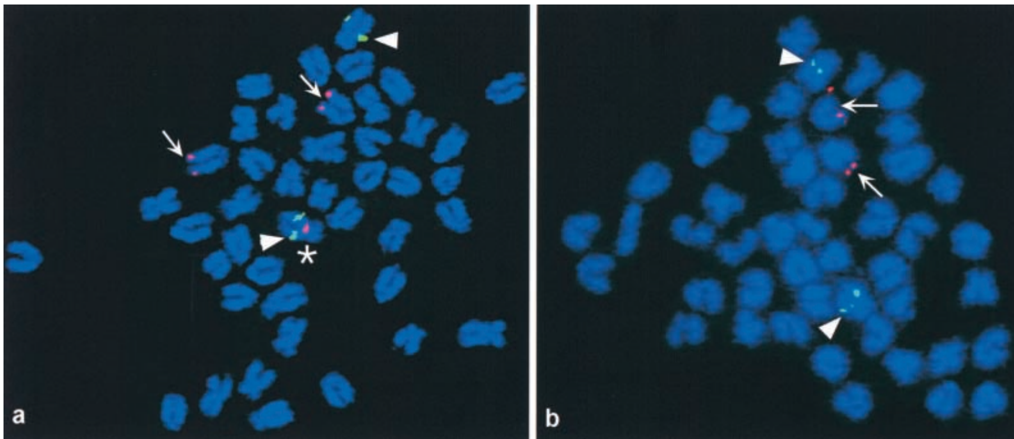

FIGURE 3.—FISH pattern of Y-specific (BAC 15H17) and sex-chromosome-specific (BAC 98J17) probes on XY (a) and XX (b) male metaphase chromosomes. Note the presence of three hybridization signals (red) for the BAC 15H17 in XY males as compared to two spots in XX males. The two relatively weak signals (arrows) represent the autosomal *dmrt1a* locus (linkage group 9), which can be seen in both XY and XX males. The additional prominent signal (*) is specific to the Y chromosome (a) that is absent in the XX males (b). BAC 98J17 (green signal) containing the marker locus *SL1* detects both sex chromosomes (arrowheads).

of such males using BAC 15H17 as probe. This BAC contains only sequences from the Y-specific region, including *dmrt1bY*, and hybridizes only to the Y chromosome in normal males (XY, Figure 3a), but not to the X. In addition, it shows a weak cross-hybridization with the telomeric region of linkage group 9, which is the location of the autosomal *dmrt* gene cluster and some other sequences that were coduplicated during the event that created the Y-specific fragment. To identify both sex chromosomes visually, a marker that gives hybridization signals on the long arm of both the X and the Y chromosome was used (Figure 3a). Contrary to XY males, in all the analyzed metaphases of XX males no specific FISH signal was detectable with the Y-specific BAC on either one of the sex chromosomes (Figure 3b), indicating the absence of most of the Y-specific region in these males. However, the Y-specific probe still cross-hybridized with the autosomal *dmrt1a* locus on linkage group 9 (Figure 3b). Repeated FISH experiments on XX males were carried out, which consistently corroborated the finding of the Southern hybridization experiment.

To investigate the frequency of the occurrence of XX males, we tested a total of eight strains for the presence of XX males by diagnostic PCRs. The frequency of XX males was highly variable (Table 2). In the i-3 strain, not a single XX male was among 81 tested males. Also, in the Kaga strain, no XX male was found. A low frequency of XX males was found in Quart and HNI (3 and 4%, respectively). In other strains, XX males were more frequent, for instance, 12% in Carbio. In the HdrR strain, we found 8 XX males, which were initially identified in the population tanks by the lack of the R phenotype and were confirmed by PCR for the absence of *dmrt1bY*.

To further test the XX chromosome constitution of the aberrant males, several of them were mated to single females (Table 3). In every case there was a strong bias toward female offspring, ranging from 100 to 89%. When some of the rare F_1 males were crossed again to single females, the strong female bias was seen again except for one case. HB32C male 4-6 had 28 female and 36 male offspring. Three of his sons were tested by crossing to i-3 females and produced all female offspring. One F_1 male (Carbio 1-1) was tested in an outcross with 20 of his sisters. The female-to-male ratio here was 4:1. From all crosses several offspring males were PCR tested for the absence of *dmrt1bY*.

DISCUSSION

Using the candidate male sex-determining gene *dmrt1bY* as a marker, we unexpectedly detected a high number of functional males with uncompromised fertility that did not have this gene.

A possible explanation for the absence of *dmrt1bY* could be that this gene is not located at the male sex-determination locus, but that it is only a linked marker. The males lacking *dmrt1bY* (and the closely linked Y chromosomal allele of *casp6*) would then be recombinants due to XY crossovers in the pseudoautosomal region. In such a case, a similar proportion of females with *dmrt1bY* should be present. However, in 304 females not a single individual was found with *dmrt1bY*.

The crossings of the *dmrt1bY*-lacking males confirmed their sex chromosomal constitution to be indeed XX. In most cases a strong female-biased offspring was obtained. The reoccurrence of XX males in the following generations can be taken as evidence that the sex reversal in the parental male may not be due to some unidentified environmental effect. The seven XX males earlier reported in medaka were explained by a lowering of the female determining potency of the X chromosome and thus polygenic autosomal male determinants becoming epistatic (AIDA 1936). Both XX males described in the guppy *Poecilia reticulata* (WINGE 1930) and the two XX males of the platyfish *X. maculatus* (ÖKTAY 1959; KALLMAN 1984) were all explained by the action of

TABLE 2

Frequency of males lacking *dmrt1bY* in different strains of medaka

Strain	Gender/genotype			
	Female (*dmrt1bY*−)	Male (*dmrt1bY*+)	Female (*dmrt1bY*+)	Male (*dmrt1bY*−) (%)
HB32C	16	11	0	2 (18)
Carbio	178	268	0	32 (12)
Quart	7	26	0	1 (3)
i-3	57	81	0	0
Wü	14	17	0	2 (12)
HNI	12	25	0	1 (4)
Kaga	15	20	0	0
SOK	5	12	0	2 (17)
Total	304	460	0	40

autosomal modifier genes. These autosomal modifiers were proposed to override the sex chromosomal genes; however, it was impossible to decide whether they suppress a female-determining locus on the X chromosome or act as male inducers.

In the medaka, it has been shown that *dmrt1bY* is the only functional gene in a Y-chromosomal-specific segment. This segment is absent from the X chromosome, and outside this region both sex chromosomes are homologous (Nanda *et al.* 2002). It appears reasonable to assume that the autosomal modifiers have a male-determining activity analogous to *dmrt1bY*. The crossing data do not support a simple monogenic dominant or recessive trait for such autosomal modifiers. The hypothesis that they are polygenic receives some support from the fact that in most crosses (*e.g.*, Carbio males 1 and 2) the number of male offspring was lower than expected and in one (HB32C male 4-6) was much higher than expected, reflecting differences in the genotype of the females used for the cross. The autosomal

TABLE 3

Progeny test of *dmrt1bY* negative males

P male	*P* female	F_1 females	F_1 males	F_1 total	Female bias (%)
Quart					
m15	Quart	44	3	47	97
HB32C					
m 11	HB32C	47	0	47	100
m 6	HB32C	47	6[a]	53	89
m 0 (m 6)[a]	HB32C	5	0	5	100
m 1 (m 6)[a]	HB32C	54	3	57	95
m 2 (m 6)[a]	HB32C	37	3	40	93
m 3 (m 6)[a]	HB32C	117	8	125	94
m 4 (m 6)[a]	HB32C	28	36[b]	64	42
m 1 (m 4/ m 6)[b]	i-3	15	0	15	100
m 5 (m 4/ m 6)[b]	i-3	16	0	16	100
m 7 (m 4/ m 6)[b]	i-3	14	0	14	100
HdrR					
m 13	HdrR	15	1	16	94
m 17	HdrR	19	1	20	95
m 18	HdrR	9	0	9	100
m 30	HdrR	21	1	22	95
Carbio					
m 1	Carbio	142	1[c]	143	99
m 2	Carbio	47	0	47	100
m 1(m 1)[c]	Carbio	159	44	203	78

[a] F_1 males from HB32C male 6.
[b] F_2 males from F_1 male 4.
[c] F_1 male from Carbio male 1, outbreeding with 20 female siblings.

modifiers may be polymorphic and strain specific and even absent or present at low frequency in certain strains, such as in i-3. This situation would explain why the number of XX males dropped to zero when males from the HB32C male 4-6 offspring were outcrossed to i-3 females.

Contrary to all earlier reports, which described XX males as an extremely rare phenomenon, our data indicate that in medaka they are very common. This now offers the possibility to identify the linkage groups carrying the autosomal modifiers after repeated backcrossing and to map and identify the genes. The high frequency of XX males may also justify reevaluating Aida's theory of a polygenic sex-determination system with epistasis of sex chromosomal genes in medaka.

So far, three mutant Y chromosomes have been found in the medaka, all of which were found in XY sex-reversed females. One mutant Y (designated Y−) lacks most or the entire male-specific region, including *dmrt1bY*; one (YwAwr) has a frameshift that leads to a premature termination of the *dmrt1bY* protein; and the third (YwSrn) has an intact *dmrt1bY* coding region, although a so far unknown mutation suppresses *dmrt1bY* expression in the embryo. This led to the conclusion that *dmrt1bY* is required for normal testicular differentiation (Matsuda *et al.* 2002).

The frequent appearance of XX medaka males makes a more differentiated view necessary. The fact that through hormonal treatment even fully fertile XX males can be obtained (Yamamoto 1955) already indicated that the Y chromosome does not contain genes required for correct differentiation of the testes and for male fertility. The appearance of sexually uncompromised spontaneous XX males clearly shows that *dmrt1bY* is not obligatory for male sex determination under physiological conditions. This does not question the role of the *dmrt1bY* gene as a sex-determining gene and as the master regulator of male sexual development in most cases, but it indicates that *dmrt1bY* function can become dispensable. Obviously one or several autosomal genes can induce male sexual development as well. Such autosomal modifiers have been identified through crossing analyses also in the platyfish *X. maculatus* (Kallman 1984). However, in this fish *dmrt1bY* is not present (Veith *et al.* 2003). Thus, even if the autosomal modifiers were homologous, they probably substitute for a different molecular event.

The Y chromosome of medaka is still at an early stage of evolution and in fact may be the youngest naturally occurring sex chromosome known so far. It is, however, unclear whether the fact that autosomal modifiers can override the XY system (whose molecular correlate is the presence and expression of *dmrt1bY*) is a reflection of a situation in which the molecular processes bringing about the male sex-determination system are not firmly established and robust. Several cases in which new sex chromosomes that arose by mutation from the normal chromosome complement invaded a population and took over the function of the previous ones have been described. In the vole *Ellobius lutescens*, for instance, such a mechanism has led to the elimination of *Sry* (Just *et al.* 1995), which is the male sex-determining gene in the overwhelming majority of mammals. The autosomal modifiers of *dmrt1bY* function in medaka may represent such newly emerging sex-determining genes.

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