Coexistence of Y, W, and Z sex chromosomes in *Xenopus tropicalis*

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Homomorphic sex chromosomes and rapid turnover of sex-determining genes can complicate establishing the sex chromosome system operating in a given species. This difficulty exists in *Xenopus tropicalis*, an anuran quickly becoming a relevant model for genetic, genomic, biochemical, and ecotoxicological research. Despite the recent interest attracted by this species, little is known about its sex chromosome system. Direct evidence that females are the heterogametic sex, as in the related species *Xenopus laevis*, has yet to be presented. Furthermore, *X. laevis*' sex-determining gene, DM-W, does not exist in *X. tropicalis*, and the sex chromosomes in the two species are not homologous. Here we identify *X. tropicalis*' sex chromosome system by integrating data from (*i*) breeding sex-reversed individuals, (*ii*) gynogenesis, (*iii*) triploids, and (*iv*) crosses among several strains. Our results indicate that at least three different types of sex chromosomes exist: Y, W, and Z, observed in YZ, YW, and ZZ males and in ZW and WW females. Because some combinations of parental sex chromosomes produce unisex offspring and other distorted sex ratios, understanding the sex-determination systems in *X. tropicalis* is critical for developing this flexible animal model for genetics and ecotoxicology.

sex chromosomes | sex determination | sex reversal | gynogenesis | *Xenopus tropicalis*

Sex chromosomes are unique karyotype features present only in species with genetic sex determination. They originate from autosomes when a gene/locus assumes the sex-determining role either by acquisition of a sex-specific trigger or by gene dosage effects on gonadal differentiation (1). Two main sex chromosome systems can be found in vertebrates with genetic sex determination: XX/XY (in which males are heterogametic) and ZZ/ZW (with heterogametic females). Nevertheless, sex chromosomes are extraordinarily variable, and other combinations also can be found, ranging from female heterogamety with 00/0W or ZZ/Z0 sex chromosomes (2, 3) to male heterogamety with XX/X0 sex chromosomes (3). Furthermore, complex XY and ZW systems are possible when fusions, fissions, and translocations between sex chromosomes and autosomes take place (e.g., X_1X_2Y, XY_1Y_2, or Z_1Z_2W) (for a review see ref. 1).

Among vertebrates, amphibians, reptiles, and fish show remarkable variation in sex-determining mechanisms and sex chromosome systems, not only between closely related species but even within species. Taxa have been described with environmental sex determination and cryptic sex chromosomes in reptiles (4), multiple sex chromosomes and polyfactorial sex determination in amphibians and fishes (5–7), and frequent transitions between ZZ/ZW and XX/XY sex chromosome systems in related species (8–11), with the extreme example of *Rana rugosa*, in which both sex chromosome systems are differentiating independently in different populations (12).

It has been proposed that sex chromosomes differentiate when a sex-determining region accumulates sexually antagonistic alleles, favoring suppression of recombination (13). Nonrecombining regions of sex-specific chromosomes (Y and W) degenerate progressively, leading eventually to cytogenetically distinct heteromorphic sex chromosomes (14, 15). In the most extreme cases, the Y or W chromosome is lost entirely, resulting in the X0 and Z0 systems (3, 16, 17). Nevertheless, not all sex chromosomes are morphologically distinct. This lack of differentiation does not always indicate a recent origin of the sex chromosomes, as is the case in ratite birds and boid snakes (18, 19). Two hypotheses have been proposed to explain the lack of differentiation between some pairs of sex chromosomes: (*i*) occasional recombination between them, as could happen in sex-reversed individuals (20), and (*ii*) frequent turnover of sex chromosomes because of rapid changes of sex-determining genes, preventing sex chromosome differentiation (21).

Amphibians present genetic sex determination (GSD), with temperature affecting sex differentiation in some species of the genera *Pleurodeles, Hynobius, Bufo,* and *Rana* and artificial polyploids of the genus *Xenopus* (11, 22, 23). Despite ubiquitous GSD, the sex chromosome pair has been identified in only about 4% of analyzed species (24), not because of the lack of cytological studies [more than 25% of known Anura taxa have been karyotyped (25, 26)], but because amphibian sex chromosomes are usually homomorphic (25).

The diploid pipid frog *Xenopus tropicalis* has become an important model for developmental biology, genetics, evolutionary genomics, and ecotoxicology, but little is known about its sex chromosome system or mechanism of sex determination. All species from the genus *Xenopus* have homomorphic sex chromosomes (27), including *Xenopus laevis* (28). However, breeding sex-reversed animals shows that females are the heterogametic sex in *X. laevis* (29–31). Thus, estradiol treatment during development results in sex-reversed genetically male, phenotypically female animals, which produce all-male offspring when mated to normal untreated males. Conversely, sex-reversed females obtained by exposure to grafts of

Significance

As in most amphibians, sex chromosomes of the model species *Xenopus tropicalis* are homomorphic, complicating identification of the heterogametic sex. Using genetic approaches, we have proved the existence of three types of sex chromosomes (Y, W, and Z), defining three kinds of males (YZ, YW, and ZZ) and two kinds of females (ZW and WW). The existence of both male and female heterogametic individuals in one species is an extremely rare situation in nature, because some sex chromosome combinations produce offspring with sex ratios different from 1:1. Thus, parental sex chromosomes must be taken into account when *X. tropicalis* is used in multigeneration genetic studies or in ecotoxicological assays of endocrine disruptors with gender effects.

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testicular tissue produce offspring with a female:male sex ratio of 3:1 (30). Among the female offspring from these sex-reversed females, one third were predicted to be WW "superfemales," confirmed by crosses to normal males resulting in all-female clutches (31).

The only sex-determining gene identified in amphibians to date is the *DM-W* gene from *X. laevis* (32). This gene is located on the W chromosome of this species (32, 33), confirming the female heterogamety previously demonstrated by sex-reversal experiments. However, *DM-W* is present in only some *Xenopus* species and not in *X. tropicalis* or other amphibians (34).

Genetic markers linked to the *X. tropicalis* sex-determining locus were identified in an amplified fragment-length polymorphism study (35). These markers largely place to sequence scaffolds that genetically map to the short arm of linkage group 7 (chromosome 7) (36, 37).

Although it has been proposed that *X. tropicalis*' sex chromosome system is similar to that of *X. laevis* (33, 35, 36), experimental support for this hypothesis is limited. Here we provide the first (to our knowledge) description of the sex chromosome system of *X. tropicalis*, integrating data from (*i*) breeding sex-reversed individuals, (*ii*) gynogenesis (both gynogenetic diploids and completely homozygous double haploids), (*iii*) triploids, and (*iv*) crosses among several laboratory strains. Our analysis shows that both males and females in *X. tropicalis* can be either homogametic or heterogametic. At least three different types of sex chromosomes exist: Y, W, and Z, observed in YZ, YW, and ZZ males and ZW and WW females. Because some parental combinations can lead to unisex offspring or other distorted sex ratios, understanding the sex determination systems in *X. tropicalis* is critical for the development of this flexible model system.

Results

In *X. laevis* and closely related species, the heterogametic sex is thought to be female, i.e., ZW (34). To determine whether *X. tropicalis* uses a ZZ/ZW or XX/XY sex chromosome system or variants thereof, we analyzed offspring from sex-reversed animals, gynogenesis, triploids, and conventional crosses.

Chemical Sex Reversal and Breeding. The heterogametic sex can be identified by analyzing the sex ratio in the offspring of sex-reversed individuals crossed with normal animals.

Male-to-female sex reversal was induced in *golden* and (*N*/*IC* F1)*IC* tadpoles by exposing developing larvae to synthetic estrogen (ethynylestradiol or β-estradiol 3-benzoate, respectively). Seventy-eight of eighty estrogen-treated tadpoles were phenotypically female at maturity (Table 1). High estrogen doses (640 ng/L ethynylestradiol or 50 μg/L β-estradiol) resulted in a large proportion of sterile animals with ovaries but few or no detectable oocytes.

Sex-reversed genetic males among phenotypic females were identified using sex-linked markers (Fig. 1*A*) (35). Mating these animals to untreated related males produced 100% male offspring [one clutch from *golden* and two from (*N*/*IC* F1)*IC* sex-reversed males, with 100 phenotypically normal male offspring each], demonstrating that sex-reversed males were not heterogametic (XY), because ♀ (sex-reversed) XY × ♂ XY would result in both male and female offspring. These data support a ZZ/ZW sex determination system in which males are homogametic: ♀ (sex-reversed) ZZ × ♂ ZZ = 100% ♂ ZZ.

Female-to-male sex reversal was obtained using the aromatase inhibitor fadrozole on *golden* strain tadpoles; all fadrozole-exposed tadpoles exhibited male characteristics. Treated animals that genotyped as sex-reversed females were mated with control female siblings, producing offspring with 3:1 female:male sex ratios. These results are possible only if sex-reversed females are ZW and are consistent with a ZZ/ZW sex chromosome system but not a XX/XY system: ♀ ZW × ♂ (sex-reversed) ZW = ♀ ZW + ♀ ZW + ♀ WW + ♂ ZZ. To confirm that one third of the female offspring were WW superfemales, females were genotyped for sex-linked markers (35), and those lacking Z-linked alleles in this family (i.e., WW) were mated with control males. Consistent with a maternal WW genotype, the offspring were all female, indicating that these matings were ♀ WW × ♂ ZZ = ♀ ZW. For a pedigree of the sex-reversal experiment, see Figs. S1 and S2.

Table 1. Sex ratios observed in clutches after different treatments with synthetic estrogens

Strain	Estrogen	Dose, μg/L	No. females	No. males
golden	EE2	0.640	24*	0
(*N*/*ICF1*)*IC*	β-Estradiol	10	40	2†
(*N*/*ICF1*)*IC*	β-Estradiol	50	14‡	0
Total			78	2

EE2, ethynylestradiol.
*Females identified in three spawns (n = 8 each).
†All males with small testes.
‡Some females were sterile.

Sex of Gynogenenetic Offspring. Production of viable diploid animals derived solely from the maternal genome, or gynogenesis, provides an independent method for analyzing sex-determination systems. Two different methods can be used to rescue viable diploid embryos from nonviable haploids (produced by fertilization with UV-irradiated sperm) (Fig. 2*A*): (*i*) suppressing polar body extrusion by early cold shock (ECS), and (*ii*) suppressing first cleavage following DNA replication by late cold shock (LCS). When maternal loci are heterozygous, LCS results in isogenic double-haploid embryos homozygous at all loci in 50:50 allelic ratios. For the sex-determining locus, an XX maternal genome will produce all-female offspring, whereas ZW females should produce 50% ZZ male and 50% WW superfemale LCS offspring. In ECS, the retained second polar body contains the sister chromatid products of meiotic recombination, so gynogenetic offspring can be homozygous or heterozygous in ratios that reflect the distance of the locus from the centromere (38).

For this experiment two hybrid females [(*N*/*IC*)F1] were bred with a F3 male (female *Cam4*/*N* × male [female *Cam4*/*N* × (*Cam4*/*N*/*PacBio*)F1) F2]) (see pedigree in Fig. S2). We used hybrid animals (*N*/*IC* females and *Cam4*/*N*/*PacBio* males) to obtain many two- and three-allele polymorphisms for distinguishing the origin of offspring. Because the paternal genome can occasionally "leak" through the UV treatment, gynogenetic origin was confirmed using a simple sequence repeat (SSR) polymorphism that was homozygous in the father and distinct from the two maternal alleles at that locus (Fig. 2 *B* and *C*). In this way, nine nongynogenetic diploid (N maternal + N paternal) leaker individuals were identified in LCS (three individuals) and ECS (six individuals) experiments, and three triploids (2N maternal + N paternal) arose in ECS experiments.

We also genotyped multiple centromeric and distal maternal polymorphisms (see markers listed in Table 2) to distinguish true LCS-derived double haploids from gynogenetic diploids formed by spontaneous polar body failure. Gynogenetic diploids (either from ECS or spontaneous polar body failure) are homozygous near centromeres, but the probability of heterozygosity rises with distance from the centromere. On the other hand, double haploids formed by LCS are homozygous at all positions (38). We observed only one gynogenetic diploid formed by spontaneous polar body failure (labeled as GD in Fig. 2*C*) among LCS-derived double haploids.

The sex of postmetamorphic gynogenetic offspring was determined by inspecting dissected gonads (Fig. 1 *B* and *C* and Table 3). The presence of males among gynogenetic offspring produced by LCS and ECS is incompatible with a maternal XX sex chromosome constitution but supports female heterogametic ZW sex chromosomes. As predicted for a heterozygous sex-determining locus, in LCS double haploids roughly equal numbers of both sexes (nine males, 14 females; P value = 0.2024) were observed. To check the ZW hypothesis, one LCS female (predicted to be a WW superfemale)

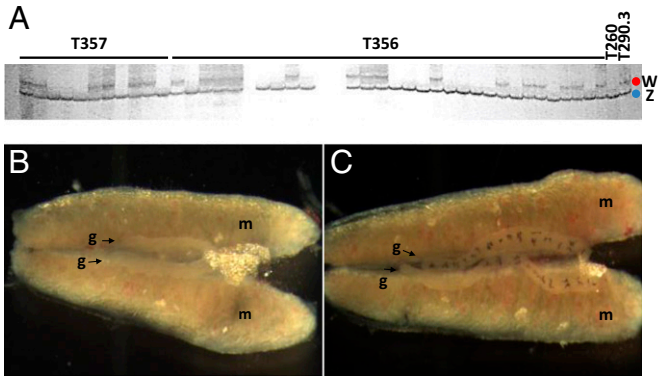

Fig. 1. Genotypic and phenotypic sex of *X. tropicalis* samples. (*A*) Genotypic sex of estrogen-treated animals (T356 and T357) was determined using several sex-linked SSLP markers with informative polymorphisms in their progenitors. The father of clutches T356 and T357 (T260) is homozygous for the SSLP marker shown in *A*; the mother (T290.3) is heterozygous, with the longer allele (red dot) located on the W chromosome and the shorter allele (blue dot) located on the Z chromosome (data obtained from grandparents). When this marker is used, ZW individuals have two bands, and ZZ individuals have only one. Sex-reversed males are identified as phenotypic females with a ZZ genotypic constitution for several informative sex-linked markers. (*B* and *C*) *X. tropicalis* gonads (g) attached to mesonephroi (m) from male (*B*) and female (*C*) tadpoles at stage NF 60. When the gonad has differentiated morphologically, the sex of tadpoles can be established by direct observation of the gonad under the stereoscope after dissection.

was bred with a *Cam4*/*N* male. This cross produced all-female offspring (n = 43 individuals), consistent with ♀ WW × ♂ ZZ = all ♀ ZW. These data confirm a ZW sex chromosome constitution for the females used as mothers in gynogenesis experiments.

The proportion of males (~7%) observed among the ECS gynogenotes (14 males in 196 individuals) is consistent with the sex-determining locus located near the end of chromosome 7. Because of its distal position, recombination is common between this locus and the centromere, and gynogenesis is expected to produce a high frequency of heterozygosity (ZW females) and lower, but roughly equal, numbers of ZZ males and WW superfemales with zero or even numbers of intervening recombination events.

The gene-centromere distance of the sex-determining locus can be determined from the sex ratio of ECS-derived gynogenetic diploid offspring by half-tetrad analysis (39, 40). Using the Kosambi mapping function, 0.43 [= frequency of recombination = 1 – (2 × frequency of males (ZZ)], gives a distance of 65 cM from the locus to the centromere, indicating the locus and centromere are not linked.

Sex of Triploid and Diploid Siblings. The presence of ZW females and ZZ males in *X. tropicalis* could result either from a dominant female-determining W chromosome or from a gene dosage effect from two Z chromosomes. To distinguish between these possibilities, we generated triploid individuals by ECS polar body suppression on normal (non–UV-treated sperm) fertilized eggs and diploid controls, using genetic backgrounds (hybrid *N*/*IC* females and *Cam4*/*N*/*PacBio* outbred males) similar to those in the gynogenesis experiment. Ploidy was confirmed using several three- or four-allele polymorphisms (two maternal and one or two distinct paternal) (Fig. 3) from several linkage groups; triploids bear all three or four alleles at a given locus. If a ZW system is operating at the distal sex-determining locus, the Z dosage hypothesis predicts >90% male triploids (ECS ratios plus additional sperm Z allele: ~7% ZZZ supermale, ~86% ZZW male, ~7% ZWW female). Alternatively, if the W-linked sex-determining allele behaves as a simple dominant, then ZZW individuals will be female, and triploids would be >90% female.

Unexpectedly, the male:female sex ratio in diploid controls was 3:1 (χ^2; P = 0.1853) rather than 1:1 (χ^2; P < 0.0001), and the ratio in the two sets of triploid offspring did not differ significantly from 1:1 (χ^2; P = 0,265) (Table 4). This result is inconsistent with the simple ZZ/ZW sex chromosome system inferred from gynogenesis and sex-reversal data, supporting neither a dominant W allele nor Z dosage. Because the gynogenesis data unambiguously show that the females used in these experiments are ZW, one possible explanation for these results is that the *Cam4*/*N*/*PacBio* outbred hybrid males used have a dominant male-determining Y allele (see the pedigree of the animals used in these experiments in Fig. S3). Triploid and diploid sex ratios thus can be explained if the father was YZ:

$$\begin{aligned}\text{Diploid cross: } \male\ \text{YZ} \times \female\ \text{ZW} &= (2n)\\ &= \male\ \text{YZ} + \male\ \text{YW} + \male\ \text{ZZ}\\ &\quad + \female\ \text{ZW}(\text{sex ratio } 3\male{:}1\female)\\ \text{Triploids: } \male\ \text{YZ} \times \female\ \text{ZW} &= (3n)\\ &= \sim 43\%\male\ \text{YZW} + \sim 43\%\female\ \text{ZZW}\\ &\quad + \sim 10.5\%(\male\ \text{YZZ} + \male\ \text{ZZZ}\\ &\quad + \male\ \text{YWW}) + \sim 3.5\%\ \female\ \text{ZWW}\\ &\quad (\text{sex ratio} \sim 1\male{:}1\female).\end{aligned}$$

These data also suggest that dosage of Z and W chromosomes has no effect on the sex determination triggered by W- or Y-linked sex-determining alleles in triploid *X. tropicalis*, because ZZW tadpoles are female and YWW tadpoles are male.

These results provide evidence that different sex chromosome systems exist in some strains or stocks of *X. tropicalis*. In the experiment above, the dominant male-determining allele (Y chromosome) can likely be traced to the *PacBio* stock (see pedigree in Fig. S3*A*). When the triploid experiment was repeated using progenitors of a different strain (*IC*) (see pedigree in Fig. S3*B*), the sex ratio in diploid controls was not different from 1:1, but 33 female and three male triploid offspring were observed. The deviation from the 1:1 sex ratio described in triploids (8% males) is equivalent to that observed in ECS experiments (7% males) and is a consequence of the distal position of the sex-determining locus. For this cross, the results are consistent with a ZZ/ZW sex chromosome system, with no effect of Z dosage but with a W-linked dominant sex-determining allele.

Inter- and Intrastrain Pairings Confirm Multiple Sex Chromosome Systems. Sex-reversal and gynogenesis experiments using several strains provide strong evidence for a ZZ/ZW sex chromosome system, whereas crosses to generate triploids and diploids siblings suggest the presence of Y chromosomes in some *X. tropicalis* strains but not in others. Conventional crosses provide further evidence for Y, W, and Z sex chromosomes in several *X. tropicalis* laboratory strains.

A *TGA* strain male heterozygous for a dominant sex-linked marker [v4.0 scaffold 605_116800-117215 (35)] was crossed to an outbred N^{NB1} female lacking the marker. Resulting offspring consisted of females possessing the marker (n = 14) and males lacking it (n = 13), indicating a dominant paternally-inherited male sex-determining allele, characteristic of an XX/XY sex chromosome system in which the male is the heterogametic sex.

In crossing these hybrid *N*/*TGA* F1 animals produced offspring with ~1:1 sex ratios, with the sex-linked marker absent or present in both males and females (spawns P30, P31, and P32). These results differ from those reported in ref. 35 using the *golden* strain and with those described above using sex-reversed *golden* and (*N*/*IC* F1)*IC* individuals, in which sex determination operated under maternal control in a ZZ/ZW sex chromosome system.

To characterize the sex chromosome system in these frogs further, hybrid *N*/*TGA* animals were crossed to *golden* animals from sex-reversal studies that previously had been characterized as ZZ males, WW females, ZZ females, and ZW females (summarized in Fig. 4). When hybrid *N*/*TGA* females were mated with *golden* ZZ

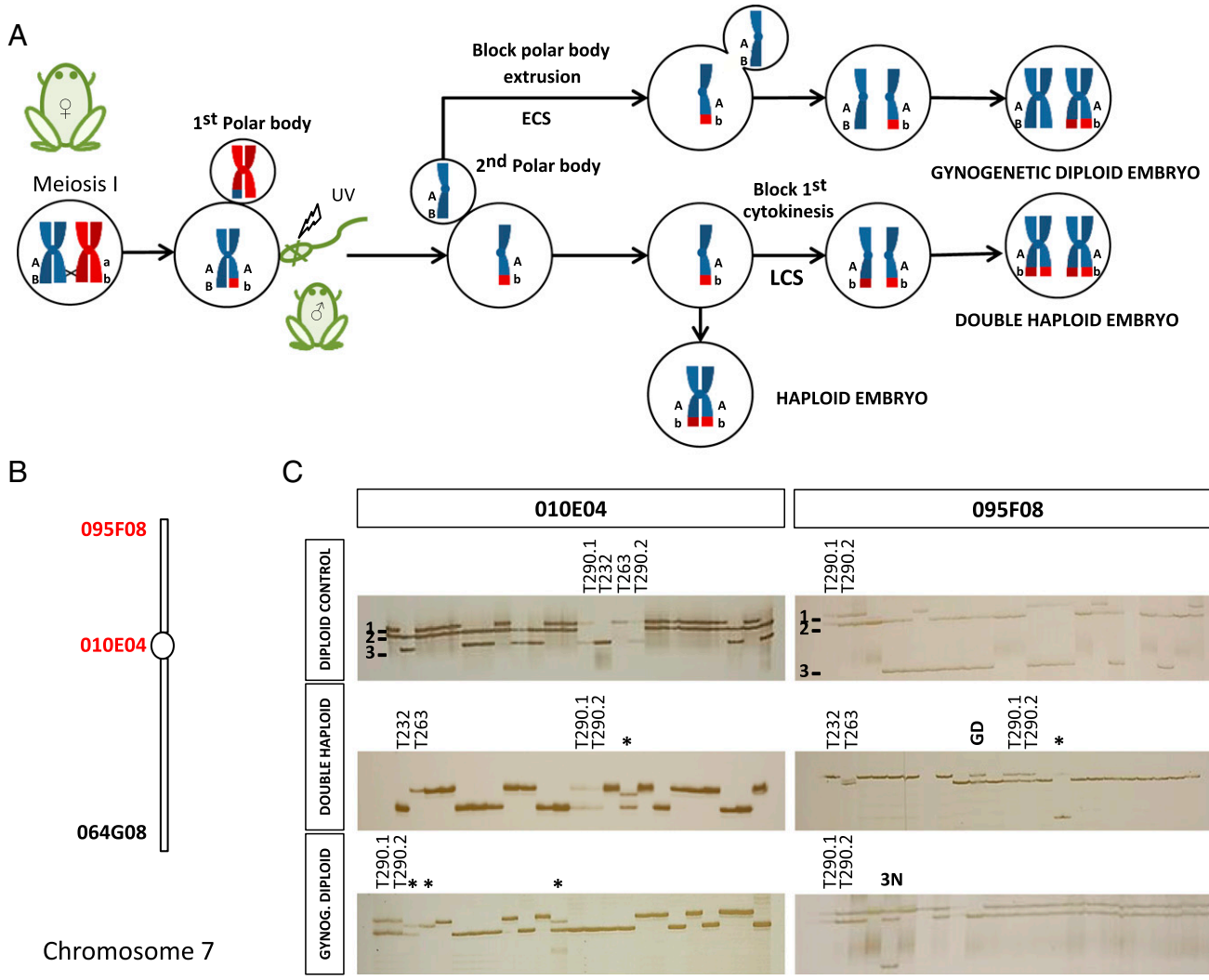

Fig. 2. Gynogenetic animals: production, confirmation of absence of paternal DNA, and verification of double haploids and gynogenetic diploids obtained by LCS and ECS, respectively. (*A*) To obtain gynogenetic offspring, the paternal contribution is blocked by UV-irradiation of sperm. Haploid (nonviable) embryos are rescued to viable diploidy either by suppressing second polar body formation with ECS to produce gynogenetic diploid embryos (heterozygous at many distal loci) or by blocking first cytokinesis using LCS and producing completely homozygous double haploids. (*B*) Chromosomal position of the two markers shown in *C* (red) on *X. tropicalis* chromosome 7 according to Wells et al. (36). (*C*) Genotypes for markers 010E04 (centromeric) and 095F08 (distal) in nongynogenetic diploid controls, double haploids, and gynogenetic diploids. A three-allele system for marker 010E04 allows the identification of the paternal contribution because the father is homozygous for allele 2, not present in the mothers (T290.1 and T290.2), heterozygous for alleles 1 and 3. Both triploid and nongynogenetic leaker individuals (labeled as 3n and *, respectively) were observed among true double haploids and gynogenetic diploids. The polymorphism for marker 095F08 (together with all markers included in Table 2) distinguishes true isogenic double haploids from gynogenetic diploids (GD) arising from spontaneous polar body failure. Double-haploid individuals are homozygous for all markers analyzed, whereas gynogenetic diploids are homozygous for centromeric markers, with a high probability of heterozygosity at distal loci.

males, all-female offspring were produced; in seven spawns (P0, P6, P7, P8, P9, P10, and P11) there were >300 female offspring with only one anomalous male. This result is possible only if hybrid *N/TGA* females are WW. On the other hand, hybrid *N/TGA* females mated with *TGA* males (spawns P27, P28, and P29), outbred N males (spawns P77 and P78), or other hybrids (spawns P30, P31, and P32) produced ~1:1 male:female ratios in offspring. Assuming these hybrid *N/TGA* females are WW, the males used in these crosses cannot be ZZ but must be YZ or YW (Fig. 4 and Table S2).

The presence of Y chromosomes was tested further by mating hybrid *N/TGA* males with females of known sex chromosome constitution. In contrast to the results obtained from sex-reversal studies using *golden* strain animals, in which offspring of WW superfemales and normal males produced all-female offspring, crossing a male *N/TGA* hybrid to a *golden* WW superfemale resulted in both male and female offspring (P79). Conversely, when a male *N/TGA* hybrid was crossed with a sex-reversed *golden* ZZ female (P80), only three offspring survived to metamorphosis, but two of these were female, again a result that is incompatible with a ZZ genotype in the male *N/TGA* hybrid. These results can be explained only if hybrid *N/TGA* males are YW so that mating to WW superfemales or sex-reversed ZZ females can produce both sexes.

Finally, three spawns between an *N/TGA* hybrid male and a *golden* ZW female produced mixed broods of male and female offspring (P33, P34, and P35). The ZW *golden* females used in these crosses bear the dominant sex-linked marker [v4.0 scaffold 605_116800-117215 (35)] on their W chromosome (passing the marker to daughters when bred to ZZ males lacking the marker). Because male *N/TGA* hybrid did not possess the marker, it was possible to establish if the marker remained linked to the female phenotype. The marker was inherited similarly to the *N/TGA*

Table 2. Markers used to confirm gynogenetic origin of individuals

Chromosome	Marker name*	Position*	Distance to centromeric marker, cM†
1	013H11	64.20	0
	033F11	11.67	52.53
2	054A08	4.71	2.27
	020G03	4.18	1.74
3	060F12	0.89	46.3
4	039A03	40.98	3.56
5	115C04	27.19	2.38
	053D07	6.23	23.34
6	003G06	41.18	0.66
7	095F08	0	69.11
	010E04	69.11	0
8	045E03	12.09	0.23
	024E12	12.14	0.18
9	023G03	61.11	0
10	096E03	46.64	—

Genotyping gynogenetic samples for centromeric and distal SSR markers differentiates LCS-derived double haploids (homozygous at all loci) from gynogenetic diploids formed by polar body failure (homozygous near centromeres but usually heterozygous at distal loci) (see Fig. 2*A*).
*Marker name and chromosomal position, in cM, according to Wells, et al. (36).
†Distances (cM) to centromeric markers described in Khokha, et al. (38). See Table S1 for more details.

hybrid-hybrid crosses: some females and males possessed the marker, and some did not. If the *N*/*TGA* hybrid male were YW, female offspring possessing the sex-linked marker would be expected to have a WW genotype, and females lacking the marker would have a ZW genotype. This prediction was confirmed by mating one female of each type to a known ZZ *golden* male. Offspring from the female with the marker consisted of all females (P90; $n = 46$), whereas the female without the marker had both male and female offspring (P01; $n = 10$ males and $n = 17$ females).

Taken together, these results provide direct evidence of the presence of Y chromosomes in *N*/*TGA* male hybrids, because male individuals are produced when the *N*/*TGA* male hybrids are bred to WW females. Thus, the presence of males among the offspring of crosses that involve WW females can be explained only if a dominant, paternally-inherited Y-linked allele is present in the father. Furthermore, interstrain pairings also show that WW superfemales can be obtained without gynogenesis or hormonal sex reversal by breeding YW males to ZW (or WW) females.

The results shown in Fig. 4 predict three possible sex chromosome constitutions for *X. tropicalis* males (YW, YZ, and ZZ) and two for females (ZW and WW) that, accordingly, will result in six different types of progenies with 0:1, 3:1, and 1:1 male:female sex ratios (Table 5).

All-female clutches (predicted when ZZ males breed with WW females) were observed in cross T369 [LCS WW superfemale (*N*/*IC*) × ZZ male (Cam4/N)]; in seven crosses between *N*/*TGA* hybrid females and ZZ *golden* males (P0, P6, P7, P8, P9, P10, and P11; Fig. 4); and in one cross between a supposed WW hybrid female [*golden*/(*N*/*TGA*)F1] and a ZZ *golden* male (P90; Fig. 4). Also, the presence of WW females among outbred N females was proved when one female (N^{NB1}) was crossed to a *golden* ZZ male; the resulting all-female spawn (P92: $n = 22$) indicated that this N^{NB1} female was WW.

We also identified spawns with 3:1 male:female sex ratios (predicted when YZ males breed with ZW females) (Table S3). By these criteria, males T139 (*Cam4*/*N*/*PacBio*), M.Po1, and M.Po2 (N^{EXR}) are YZ, whereas females T290.7, T290.6, T290.7 (*N*/*IC*), and F.Po3 (N^{EXR}) must be ZW. This identification was confirmed by the inheritance of parental sex-linked markers (Fig. S4).

Sex ratios that do not differ significantly from 1:1 can be obtained from four possible combinations of parental sex chromosomes: ZZ × ZW, YZ × WW, YW × ZW, and YW × WW. Analysis of sex-linked markers is needed to establish progenitor sex chromosome constitutions.

Clutches from ZZ and ZW pairs are easy to identify as long as the ZW female is heterozygous for a sex-linked marker and the ZZ male does not have an identical genotype. In this situation, paternal alleles can be found in both sexes, whereas male offspring inherit the maternal allele not shared by female offspring.

The remaining crosses that produce 1:1 sex ratios (YZ × WW, YW × ZW, and YW × WW) are characterized by the presence of a Y chromosome in the male progenitor. In this case the analysis of sex-linked sequence polymorphisms is not sufficient to establish the sex chromosome constitution of progenitors and offspring. Thus, although it is possible to identify Y-linked sequence polymorphisms, it is not possible to establish if the other paternal sex chromosome is Z or W or whether the female parent is ZW or WW. Establishing the sex chromosome constitution in these cases requires additional crosses to individuals with known sex chromosome constitution. This strategy was used to determine the sex chromosome constitution of males M.N2 (N^{NB3}) and M.Po1 (N^{EXR}) [bred with ZW females T290.3 (cross 28) and T290.7 (cross 29), respectively]. In the first case the male:female sex ratio was 1:1 (cross 28; 12 males and 14 females; $n = 26$), whereas in the second case it was 3:1 (cross 29; 103 males and 37 females, $n = 140$). From these data it can be deduced that M.N2 is YW and M.Po1 is YZ. The sex chromosome constitution of females can be established in the same way by breeding to known ZZ males; WW females will produce all-female offspring, but the sex ratio of ZW females will be 1:1 (see Fig. 4).

Discussion

Sex Chromosomes in *X. tropicalis*. Although the sex chromosome system in *X. tropicalis* has been proposed to be ZZ/ZW (33, 35, 36), as in *X. laevis*, our analysis shows that it is more complex. Using sex-reversed individuals, gynogenesis, triploids, and conventional crosses, we detect at least three different sex chromosomes (Y, W, and Z), with three types of males (YZ, YW, and ZZ) and two types of females (ZW and WW). Pairing these male and female sex chromosome constitutions can result in six different classes of spawns with 0:1, 3:1, and 1:1 male:female sex ratios (Table 5).

Previously published experimental support for a ZZ/ZW system in *X. tropicalis* is limited to an early linkage map (41). The frequency of polymorphisms in the maternal genome of this map cross was greater than in the paternal genome, as is consistent with female heterogamety. However, a simpler explanation is that this preliminary linkage analysis was based on a single outbred (more polymorphic) female crossed to an inbred (less polymorphic) male. Another method for obtaining sex-linked polymorphisms, restriction site-associated DNA-tag analysis, failed to identify sex-specific heterozygosity in the *X. tropicalis* genome, as is consistent with a large pseudoautosomal region in sex chromosomes (37).

Table 3. Sex of gynogenetic offspring (double haploids and gynogenetic diploids) of two hybrid females [(female *N* × male *IC*) F1]

Genotype	Sex	Total	$P^{\dagger}$	$P^{\ddagger}$
Double haploid (LCS)	Male	9		
	Female	14	0.2024	0.2971
Gynogenetic diploid (ECS)	Male	14		
	Female	182	<0.0001	<0.0001

H_0, 1:1 sex ratio.
†Binomial distribution.
‡χ^2 distribution.

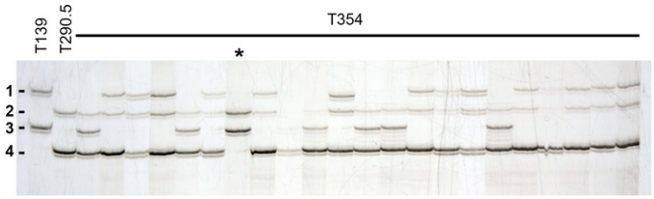

Fig. 3. Genotyping of triploid offspring. Triploid offspring are genotyped using polymorphic markers when both progenitors are heterozygous for different pairs of alleles (results for marker 033F11 are shown; see Table S1 for details). This type of marker allows identification of paternal (T139, heterozygous for alleles 1 and 3) and maternal (T290.5, heterozygous for alleles 2 and 4) contributions. Most triploid individuals (T354) will have three different alleles (one paternal and both maternal alleles), although triploids with two copies of one of the maternal alleles are also possible (*). The use of several markers differentiates diploids from triploids with two copies of the same allele.

We have detected males with a Y chromosome (either YZ or YW) in the strains *TGA*, *golden* and N^{NB1}, N^{NB3}, and N^{EXR} and in the *PacBio* stock; and Z chromosomes and ZZ males have been confirmed in the *golden*, N^{NB2}, N^{EXR}, and *IC* strains. The presence of both types of males in two commonly used laboratory stocks (*golden* and N^{EXR}) suggests that distorted sex ratios will not be limited to interstrain crosses and may be an important consideration for researchers. Y chromosomes have not yet been detected in the *IC* strain, but the genotypes analyzed derive from a single inbred (F8–F11) lineage, and Y alleles could yet be found in outbred *IC* stocks or other inbred lines. In the same way, ZZ males were not identified in *PacBio* stock or in the *TGA* strain, but a small number of animals were analyzed in these cases.

It is unlikely that variation in *X. tropicalis* sex chromosomes reflects a situation similar to that recently described in *Danio rerio* (42) where some laboratory strains acquired new mechanisms of sex determination after original sex chromosomes were lost in some strains during domestication. The longer lifespan and generation time of *X. tropicalis* means that most stocks that have not been intentionally inbred are unlikely to have experienced multigeneration bottlenecks. Also, some stocks, such as *PacBio*, are composed of wild-caught and presumably outbred animals of diverse geographical origins. Furthermore, analysis of previously described sex-linked markers (35) used in our work demonstrates that the Y, Z, and W alleles are all linked to the same genetic region (see Fig. S4) located at the distal tip of chromosome 7p (assembly v7.1).

Our data do not address how these three sex chromosomes may be organized in natural populations of *X. tropicalis*. The animals analyzed come from laboratory stocks whose geographical origins are not always known (e.g., *PacBio*) or whose pedigrees may be incomplete and could include founders from multiple collection sites. Several possibilities exist for the sex chromosome systems in natural populations of this species. The existence of more than two types of sex chromosomes in the same species has been described in fish, amphibians, and even mammals (6, 7, 43–47) and can occur in several forms. In some fish species, the existence of two or more nonhomologous sex pairs allows coexistence of more than two types of sex chromosomes. This situation has been described in *Oreochromis aureus* and *Oreochromis mosambicus* (7, 43) but may not be applicable to *X. tropicalis* because the inheritance of maternal and paternal alleles of sex-linked markers always correlates with the sexual phenotype (except for a reduced number of recombinants between these markers and the sex-determining locus).

The presence of three sex chromosomes in laboratory stocks of *X. tropicalis* could reflect geographical variation in sex chromosomes in wild populations. Such geographical variation has been described in *R. rugosa*, in which it is possible to find several XX/XY and ZZ/ZW sex chromosome systems in separate geographical regions, so only one type of sex chromosome system exists in each population (48). A similar situation could be possible in *X. tropicalis* if geographical isolation favored splitting sex chromosome systems when the change of heterogamety took place. If so, sex chromosomes in *X. tropicalis* are likely to have a recent origin because gynogenetic WW females are viable and fertile, indicating that deleterious mutations have not yet accumulated on the W chromosome of this species. In *R. rugosa*, however, WW females with W chromosomes from the same population die before metamorphosis (49, 50).

The other possibility is that the sex chromosome system in *X. tropicalis* resembles those observed in some fish species, where more than two types of sex chromosomes exist because of the presence of several sex-determining alleles in the same or closely-linked loci. An example is *Xiphophorus maculatus*, a species with three sex chromosomes (W, Y, and X), in which females can be XX, XW, or YW, and males are YY or XY (43, 44). In addition to *X. maculatus*, several rodent species show three sex chromosomes with different masculinizing/feminizing capacity. Some *Akodon* species bear an unusual Y chromosome (known as "Y*") that has lost its masculinizing capacity, producing XY* females (45). On the other hand, in the species *Myopus schisticolor*, *Dicrostonyx groenlandicus*, and *Dicrostonyx torquatus*, a special X chromosome has been described (X*) that is able to suppress the male-determining effect of the Y chromosome so the individuals with this chromosome, including X*Y animals, develop as females (46, 47).

The presence of Y chromosomes in strains with different geographic origins (Ivory Coast, Cameroon, Sierra Leone, and Nigeria; see Table 6 for details about the origin of the laboratory strains used) scattered along the distribution area of *X. tropicalis* would support this possibility. Nevertheless, the sex chromosome system in natural populations of *X. tropicalis* is unknown, and both models are possible. ZZ/ZW and XX/XY populations could be isolated, or the three sex chromosomes could coexist in the same population because WW individuals are viable. In the first scenario, sampling animals from different populations could have introduced the Y chromosome into *X. tropicalis* laboratory strains with a ZZ/ZW system. On the other hand, in the second scenario, it would be of great interest to analyze the frequency of each sex chromosome in different populations and check how they are maintained in nature.

The Sex-Determining Locus in *X. tropicalis*. The sex-determination factor appears to be located on the short arm of *X. tropicalis* chromosome 7 (35, 36). The position of the sex-determining locus can affect the sex ratio in gynogenetic diploids and triploids if female is the heterogametic sex. Thus, if the sex-determining locus is not close to the centromere, and recombination has not been suppressed in the heterogametic sex, recombination will increase the frequency of ZW females in the offspring (51).

The high frequency of females (93%) among *X. tropicalis* diploid gynogenotes is caused by recombination events between

Table 4. Sex ratios from triploid and diploid siblings obtained from the cross depicted in Fig. S3*A*

EXPERIMENT (ploidy)	Cross	Male	Female	$P(\chi^2)^{\dagger}$	$P(\chi^2)^{\ddagger}$
Triploid* (3N)	T354	101	83	0.1845	<0.0001
	T355	27	21	0.3864	0.0027
Total		128	104	0.1151	<0.0001
Diploid[§] (3N)	T352	165	47	<0.0001	0.3414
	T353	148	42	<0.0001	0.3568
Total		313	89	<0.0001	0.1853

*Triploid offspring: ECS + untreated sperm.
[†]H_0: sex ratio 1:1.
[‡]H_0: sex ratio 3:1.
[§]Diploid offspring: no ECS + untreated sperm.

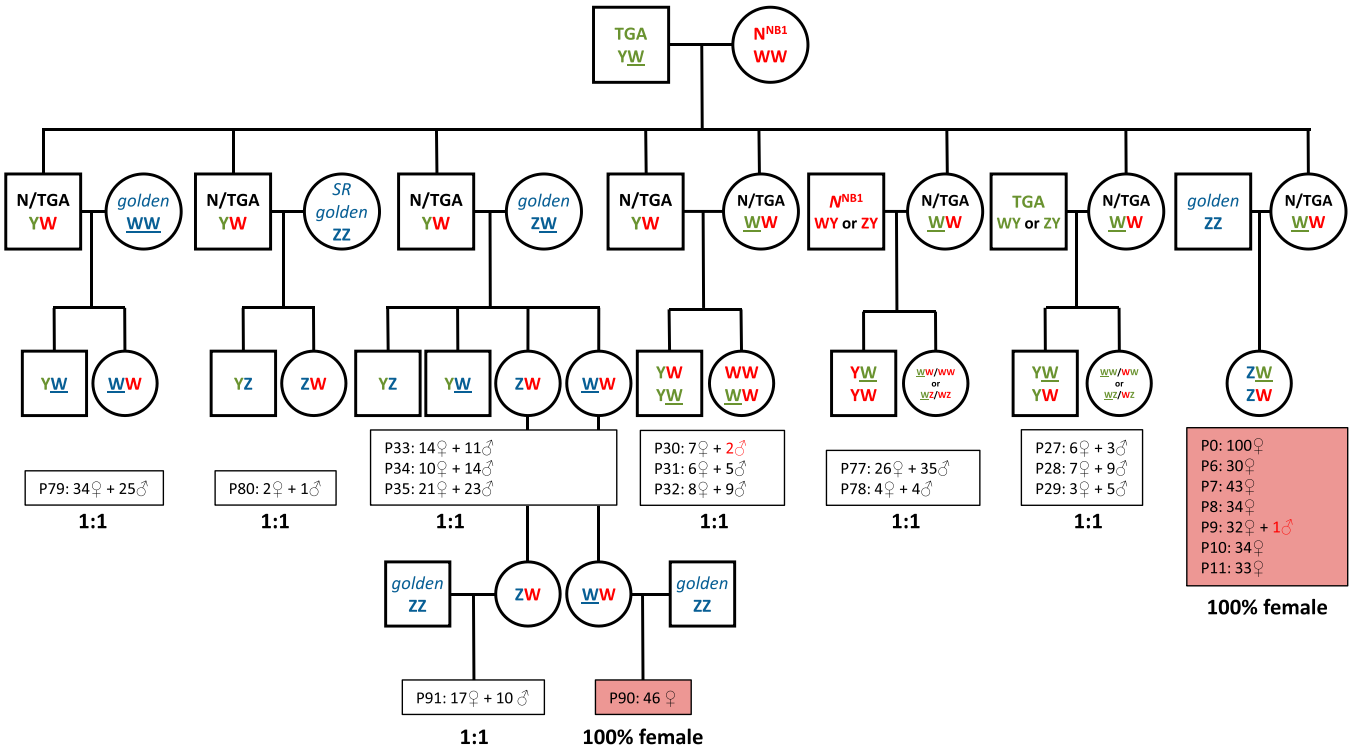

Fig. 4. Sex ratios and sex-linked marker inheritance in interstrain pairings prove the existence of Y, W, and Z sex chromosomes. The pedigree chart of the interstrain pairings performed shows the sex of the offspring obtained in different spawns (P0 to P91). All-female offspring were observed in two types of crosses (labeled in red), both involving *golden* ZZ males (obtained from breeding sex-reversed ZZ females with ZZ males). These offspring are possible only if the mothers are WW. Without sex reversal or gynogenesis, WW females can be produced only if males with a W chromosome (YW) are bred with ZW or WW females. Because all hybrid *N/TGA* females analyzed were WW, the sex chromosome constitution of the progenitors used to produce these hybrids was YW (*TGA* male) and WW (N^{NB1} female). Other *TGA* and N^{NB1} males used in this pedigree have a Y chromosome, although the data do not distinguish YZ from YW. The proposed sex chromosome constitution for all the animals in the pedigree chart is depicted together with a color code that indicates the strain or provenance (green: *TGA*; red: Nigerian; blue: *golden*). SR indicates a sex-reversed individual obtained by ethynylestradiol exposure (see pedigree chart in Fig. S1).

the sex-determining locus and the centromere in the prophase of the first meiotic division, as observed in other taxa (51–53), and confirms that the sex-determining locus in this species is far from the centromere. Because gynogenetic diploids are completely maternally derived and are analogous to half a tetrad, they can be used to establish the genetic distance of any locus to the centromere (38–40). In this case, using the Kosambi mapping function, the genetic distance between the centromere and the sex-determining locus can be established as 65 cM, indicating it is located in a distal position and is not linked to the centromere.

The sex-determining gene in *X. laevis*, *DM-W*, is not present in the genome of *X. tropicalis* (34), whose mechanism for allocating sex remains unknown. A single genetic locus with at least three alleles seems the simplest explanation, but two or more tightly linked genes are also possible. A hypothesis based on gene dosage, as proposed for *X. maculatus* (44), does not seem to be supported by our data, because diploid *X. tropicalis* individuals with two Z chromosomes are male, but triploid ZZW individuals are females; likewise WW individuals are females but triploid YWW individuals are males. A different situation is observed in *Buergeria buergeri*, an amphibian with heteromorphic sex chromosomes, in which ZZZ individuals are males, ZWW individuals are females, and those with a ZZW sex chromosome constitution can be either males or females, with a 1:1 sex ratio (52). These differences suggest that distinct molecular mechanisms operate in these species. For example, the dominance relationship for sex chromosomes in *X. maculatus* can be established as W>Y>X (YW individuals are females) whereas in *X. tropicalis* Y>W>Z (YW individuals are male).

Table 5. Three sex chromosomes explain observed sex ratios and sex-linked marker inheritance in *X. tropicalis*

Maternal genotype	Paternal genotype	Offspring genotypes	Offspring sex ratio	Clutches
ZW♀	ZZ♂	ZW♀, ZZ♂	1:1 ♀:♂	T350, T358, T396, T397, P91
ZW♀	YZ♂	ZW♀, YW♂, YZ♂, ZZ♂	1:3 ♀:♂	T352, T353, X10, X20, X24, X29, P93
ZW♀	YW♂	WW♀, ZW♀, YW♂, YZ♂	1:1 ♀:♂	X28, P33, P34, P35
WW♀	ZZ♂	ZW♀	All♀	T369, P0, P6, P7, P8, P9, P10 P11, P90, P92
WW♀	YZ♂	ZW♀, YW♂	1:1 ♀:♂	P27, P28, P29, P77, P78
WW♀	YW♂	WW♀, YW♂	1:1 ♀:♂	P30, P31, P32, P79, P27, P28, P29, P77, P78

Two female genotypes (ZW and WW) and three male genotypes (ZZ, YZ, and YW) can be paired in six ways. Four pairings will produce 1:1 male:female sex ratios with the sex chromosome constitution indicated in each case. It also is possible to obtain all-female offspring or 3:1 male:female sex ratios. For each of the six possible pairings, the clutches that support each cross have been indicated. The underlined clutches P27, P28, P29, P77, and P78 were produced by WW females bred to males that could be YZ or YW. The genotype of these males could be established by breeding their female offspring (obtained when bred to WW females) with ZZ males. All-female offspring are produced by daughters of YW males, whereas 1:1 male:female offspring are produced by daughters of YZ males.

Table 6. Strains and origin of *X. tropicalis* surveyed in this work

Strain/stock	Geographical origin	Breeding	Source
IC	Ivory Coast	Inbred (F8–F11)	NIMR (L.B.Z.)
N	Nigeria	Inbred (F10)	NIMR (L.B.Z.)
N^{NB1}	Nigeria	Outbred	Nasco Biology
N^{NB2}	Nigeria	Outbred	CABD (J. L. Gómez-Skarmeta), from Nasco Biology
N^{NB3}	Nigeria	Outbred	IDIBELL (A. Llobet), from Nasco Biology
N^{EXR}	Nigeria	Outbred	IDIBELL (A. Llobet), from EXRC (University of Portsmouth)
golden	Unknown*	Outbred	UC (Richard Harland)
TGA	Ivory Coast/Cameroon/Sierra Leone	Outbred	UC (Richard Harland)
Cam4/N	Unknown	Outbred	NIMR (L.B.Z.)
PacBio	Unknown (several geographic locations)	Not applicable	Pacific Biological Supply

CABD, Centro Andaluz de Biologia Desarrollo, Seville, Spain; EXRC, European *Xenopus* Research Centre, University of Portsmouth, Portsmouth, United Kingdom; IDIBELL, Bellvitge Biomedical Research Institute, Barcelona, Spain; NIMR, National Institute for Medical Research, London; UC, University of California, Berkeley, CA.

*Phylogenetic analysis of mitochondrial DNA suggests these animals originated in Nigeria (37).

The sex ratio of triploid offspring artificially obtained in several amphibian taxa depends on the species analyzed and ranges from mainly all-female to all-male clutches (54–59). A higher frequency of females among triploid offspring has been described in *Notophthalmus viridescens* (54) and *Ambystoma mexicanum* (55). In *Bombina orientalis*, *Pelophylax nigromaculatus*, and *Pelophylax porosus brevipoda* (56, 57) the sex ratios of triploid offspring are close to 1:1, whereas all-male offspring or male-biased sex ratios were found in *Hyla japonica* and *R. rugosa* (Hiroshima) (58, 59). Sex ratios of triploid offspring can be explained by female or male heterogamety and the relative position of the sex-determining locus relative to the centromere. However, other considerations, such as multiple sex chromosomes, sex-linked differences in recombination rates, or possible homozygous lethal effects of sex-specific chromosomes (Y or W), should be taken into account.

The sex of triploid interspecies hybrids also reveals some differences among *Xenopus* species. When endoreduplicated oocytes from hybrid females (*X. laevis* × *Xenopus gilli*) are fertilized with the sperm of several species from the genus *Xenopus* (*X. laevis*, *X. gilli*, *Xenopus borealis*, and *Xenopus muelleri*), the sex ratios in triploid offspring depend on the sperm donor species and, sometimes, on temperature (23). *X. borealis* and *X. muelleri* are likely to use a different sex-determination mechanism than *X. laevis* because the *DM-W* gene does not appear to be present in these species (34). If the sex chromosomes in *X. borealis* and *X. muelleri* are not homologous to the sex chromosome pair in *X. laevis*, hybrids will have two nonhomologous sex chromosome pairs, complicating the interpretation of data from triploids (*X. laevis*/*X. gilli*/*X. borealis* or *X. laevis*/*X. gilli*/*X. muelleri*). The situation of triploids and tetraploids with *X. laevis* + *X. gilli* genomes is different, because these species are closely related. The *DM-W* gene may be present in both, and they hybridize in nature (60). The results obtained by Kobel and Pasquier (23) point to a Z dosage effect over the feminizing effect of the W chromosome in these species, a situation not observed in *X. tropicalis*. Further experiments with triploid and tetraploid clutches from these two species are necessary to characterize the effect of sex chromosome dosage on the sex-determination mechanism of each species. On the other hand, the effect of temperature on these hybrids is of great interest and deserves further research to elucidate its mechanism and whether it occurs only in hybrids.

Mapping the sex-determining locus in *X. tropicalis* and identifying candidate genes is hindered by the quality of available genome assemblies for the short arm of chromosome 7, where the sex-determining region lies. Long-range contiguity in the v7.1 chromosome-scale genome assembly is based on synteny with other vertebrate genomes and is known to be imperfect in this region. Comparison between sequences from laser-microdissected chromosome 7p arms and the v7.1 assembly reveals large regions assembled in 7p belonging to other chromosomes and regions assigned to chromosomes 1, 3, and 4 that are physically located on chromosome 7p (61).

Evolution of the Sex-Determining Locus. The existence of different sex-determining mechanisms in related species or even in populations of the same species points to a rapid evolutionary change in the control of sexual development in fish, reptiles, and amphibians, a trend quite different from what is observed in birds and mammals (62).

The swift change described in the sex chromosome systems in amphibians is probably the result of a quick turnover in sex-determining genes. Identification and mapping of sex-linked genes in several species indicate that a conserved sex-linked linkage group does not exist in class Amphibia (24, 63), with variability observed even within species (64). Thus, the gene *Got1*, closely sex-linked in *B. buergeri* (65), is autosomal in *R. rugosa*. On the other hand, three genes mapped on the sex chromosomes of *R. rugosa* (*AR*, *SF1*/*Ad4BP*, and *Sox3*) are not located on the sex chromosomes of *X. tropicalis* or *X. laevis* (33). Furthermore, only one sex-determining gene has been identified in amphibians, the *DM-W* gene in *X. laevis*, and it is present in only some species of the genus *Xenopus* (34). These data indicate that almost any chromosomal pair can be the sex pair in this group. Apart from the plasticity of the sex-determining pathway of this vertebrate group, the high frequency of transitions between sex chromosome systems in amphibians may have been facilitated if differentiation between sex chromosomes is minimal, as demonstrated by the long pseudoautosomal region found in *X. tropicalis* (37).

Concluding Remarks. *X. tropicalis* is quickly becoming a relevant amphibian model species because of the notable advantages that this species offers for research (66, 67). Nevertheless, it is surprising that the existence of three sex chromosomes has not been revealed before. This lack of information may have been caused, in part, by the difficulty of studying natural populations. Also, *X. tropicalis* research focuses on early development, with less analysis after spawns mature, so skewed sex ratios may go unnoticed. All-female clutches in laboratory colonies also may have been erroneously attributed to unintentional endocrine disruption, e.g., from plastic residues in husbandry containers. Finally, most laboratory animals are provided by a small number of breeders, and the allele frequency at the sex-determining locus may be different in different batches.

Our demonstration of this multiple sex chromosome system has critical implications for the use of *X. tropicalis* in ecotoxicology studies. Stocks and strains with defined sex-determination alleles will be essential for interpreting sex phenotypes resulting from larval endocrine disruptor treatments. Strains that produce unisex offspring (for example known sex-reversed ZZ females crossed to

ZZ males or WW females crossed to ZZ males) greatly increase the sensitivity and statistical significance of assays detecting feminizing or masculinizing agents.

Materials and Methods

Strains. Sex-determination systems were surveyed in a range of available laboratory strains and commercial stocks of *X. tropicalis* (Table 6). *Golden* and *TGA* strains were the kind gift of Richard Harland (University of California, Berkeley, CA). Inbred *IC* and *N* strains were obtained from Robert Grainger (University of Virginia, Charlottesville, VA) and were inbred further to F11 and F10, respectively. Outbred *N* strain animals, initially purchased from Nasco Biology or from the European *Xenopus* Resource Centre, were the kind gift of José Luis Gómez-Skarmeta (Centro Andaluz de Biología del Desarrollo, Seville, Spain) and Artur Llobet (IDIBELL, Barcelona). *PacBio* animals were purchased from Pacific Biological Supply. In hybrids nomenclature, parental strain names are separated by a slash, with the female parent listed first and the male second. Thus, (*N*/*IC* F1) *IC* is the offspring of an (*N* × *IC*)F1 female backcrossed to an *IC* male.

Animal Husbandry. All animal protocols were approved by the ethics committee for research on animals of the institution where the protocols were performed. The care and treatment of animals used in this research was conducted in accordance with policies on animal care provided by regulations of the European Union and the United States.

All frogs and tadpoles were housed in flow-through tanks (except during sex-reversal treatment) using filtered, UV-treated water according to previously described protocols on *X. tropicalis* husbandry (ref. 68, faculty.virginia.edu/xtropicalis/, and tropicalis.berkeley.edu/home/). Ovulation and mating were induced by two injections of human chorionic gonadotropin (Sigma-Aldrich) as described previously (38, 69). Animals were killed by immersion in 2 g/L bicarbonate-buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich). Phenotypic sex of tadpoles and juvenile animals was determined by dissection and morphological examination of the gonads (Fig. 1 *B* and *C*). Tadpole development was staged according to Nieuwkoop and Faber (NF stages) (70).

Chemical Induction of Sex Reversal. Male-to-female sex reversal of tadpoles was induced by aqueous exposure of larvae to the synthetic estrogen ethynylestradiol (640 ng/L) (Sigma-Aldrich) or to β-estradiol 3-benzoate (10 and 50 μg/L) (Sigma-Aldrich). Female-to-male sex reversal was effected using 100 μg/L of the pharmaceutical aromatase inhibitor fadrozole (Novartis Pharma AG) (69). Tadpoles were treated with hormone or inhibitor from 2 d after fertilization (about NF 35) until completion of metamorphosis (NF 66) and then were raised to reproductive maturity. Sex-reversed individuals were identified using sex-linked polymorphisms either from the genetic map (36) or as described in Olmstead et al. (35).

Gynogenesis. Viable embryos derived solely from the maternal genome were produced in two ways (Fig. 2*A*). Gynogenetic diploids or gynogenotes were obtained using ECS to suppress polar body formation in haploid embryos, rescuing them to viable diploidy (71). Briefly, females were induced to ovulate, and eggs were expressed manually into 1× Marc's Modified Ringers (MMR) to inhibit premature activation. Testes were dissected from a freshly killed male and were crushed in L15 medium (PAA) + 10% (vol/vol) calf serum (PAA). The dispersed sperm suspension was irradiated with UV (50–70,000 mJ) and incubated with eggs for 3–5 min following removal of the 1× MMR buffer. Eggs then were flooded with 0.05× MMR, cold-shocked in an ice bath for 7.5 min about 5 min after flooding, and returned to ∼22 °C for further development. After 2–4 h, cleaving embryos were sorted from unfertilized eggs.

Alternatively, isogenic double-haploid embryos were produced by suppression of the first cleavage following DNA duplication in haploid embryos, using a similar LCS for 5 min ∼48 min after flooding (71) (note that the time to the appearance of the first cleavage furrow varies with temperature and egg batch). LCS embryos that showed delayed cleavage were selected for raising.

For each gynogenesis experiment, control and haploid embryos were also produced.

Triploid *X. tropicalis*. Triploid *X. tropicalis* were obtained by ECS treatment 5 min after fertilization as above, except that nonirradiated sperm were used, leading to retention of the second polar body in addition to the diploid zygote nucleus.

Genotyping. Although sex-linked markers have been identified in *X. tropicalis* (35), polymorphisms vary among individuals and must be defined for each cross. Here we have used a dominant marker located on scaffold 605 at positions 116800–117215 in the *X. tropicalis* genome (ver. 4.0) (72) and revealed with the primers 5′-GCCCAAGCAATATAAGGGCTTGTT-3′ forward and 5′-TGTCCTGCCCTATTGCTCCCGTAA-3′ reverse together with positive control primers (forward: 5′-TATTCCTGCTTTGCCCGGCG-3′ and reverse: 5′-GGTGTGGTCATCAGAGCATCCAAT-3′). We also used previously described simple sequence-length polymorphisms (SSLPs) (35) located on scaffolds 1778 and 6092 in the *X. tropicalis* genome (positions 6,156–6,312 and 2,392–2,601, respectively, in assembly v4.0) (72). These markers are located on chromosome 7p and are sex-linked (35, 37).

The gynogenetic origin of individuals was confirmed by the absence of paternal alleles for the SSLP markers 010E04 and 063F04 (36), with different alleles in male and female progenitors. LCS-derived double haploids and gynogenetic diploids formed by polar body failure were identified by genotyping with the centromeric and distal SSLP markers listed in Table 2 and Table S1 (36). *X. tropicalis* genome assemblies used to assign these markers to scaffolds/chromosomes are v4.1, composed entirely of sequence-based scaffolds (72), v7.1 (for a comparison with v4.1, see refs. 73 and 61), and v8.0 (from the Rokhsar and Harland groups at the University of California, Berkeley, CA), all available through Xenbase.

Tissue samples from toes or muscle were digested with 200 μg/mL Proteinase K (Sigma Aldrich) in lysis buffer [50 mM Tris·HCl (pH 8.8), 1 mM EDTA, 0.5% Tween-20] and were used directly in PCR. Genotyping reactions were run in duplicate and separated on 40% acrylamide (19:1) (Merck) or 3% (wt/vol) agarose gels.

Gynogenetic Mapping. To estimate the distance between the sex-determining locus and the centromere, the recombination frequency was calculated as half the frequency of heterozygous gynogenotes [heterozygous ZW females = 1 − (2 × number of males (ZZ)] (39). The Kosambi mapping function was used to convert the recombination frequency to map distance in cM (74).

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