Genetic Mapping in *Xenopus laevis*: Eight Linkage Groups Established

Jean-Daniel Graf

Station de Zoologie Expérimentale, Université de Genève, 1224 Chêne-Bougeries, Switzerland

Manuscript received April 17, 1989
Accepted for publication June 26, 1989

ABSTRACT

Inheritance of alleles at 29 electrophoretically detected protein loci and one pigment locus (albinism) was analyzed in *Xenopus laevis* by backcrossing multiply heterozygous individuals generated by inter-subspecies hybridization. Pairwise linkage tests revealed eight classical linkage groups. These groups have been provisionally numbered from 1 to 8 in an arbitrarily chosen order. Linkage group 1 includes *ALB-2* (albumin), *ADH-1* (alcohol dehydrogenase), *NP* (nucleoside phosphorylase), and a^p (periodic albinism). Linkage group 2 contains *ALB-1* and *ADH-2*, and probably is homeologous to group 1. Linkage group 3 comprises *PEP-B* (peptidase B), *MPI-1* (mannosephosphate isomerase), *SORD* (sorbitol dehydrogenase), and *mIDH-2* (mitochondrial isocitrate dehydrogenase). Linkage group 4 contains *GPI-1* (glucosephosphate isomerase) and *EST-4* (esterase 4). Linkage group 5 contains *GPI-2* and *PEP-D* (peptidase D). Linkage group 6 comprises *ACP-3* (acid phosphatase), *sME* (cytosolic malic enzyme), and *GLO-2* (glyoxalase). Linkage group 7 consists of *sSOD-1* (cytosolic superoxide dismutase), *GPD-2* (glycerol-3-phosphate dehydrogenase), *mME* (mitochondrial malic enzyme), and the sex determining locus. Linkage group 8 includes *FH* (fumarate hydratase) and *TRF* (transferrin). Recombination frequencies between linked loci showed differences related to the genomic constitution (parental subspecies) and to the sex of the heterozygous parent. Independent assortment was observed between the duplicate *ALB* loci. This is true for the duplicate *ADH*, *GLO*, and *MPI* loci as well, supporting the view that these genes have been duplicated as part of a genome duplication that occurred in the evolutionary history of *X. laevis*. Comparative analysis of genetic maps reveals a possible conservation of several linkages from the Xenopus genome to the human genome.

XENOPUS laevis is widely used as an experimental animal in developmental and molecular biology, and constitutes a model system for the study of vertebrate embryogenesis. It is therefore surprising that so little effort has been devoted to the task of establishing a genetic map of this organism. Of the nearly 50 developmental mutations described so far, six have been mapped with respect to the centromere (THIÉBAUD, COLOMBELLI and MÜLLER 1984; REINSCHMIDT *et al.* 1985). In addition, allelic variants of several protein genes have been identified (VONWYL and FISCHBERG 1980; WOLFF and KOBEL 1985; BÜRKI 1987) and the sex-determining locus has been localized with respect to the centromere (COLOMBELLI, THIÉBAUD AND MÜLLER 1984) and to a linked enzyme locus (GRAF 1989).

Comparative studies of karyotypes (TYMOWSKA and FISCHBERG 1982) and DNA content (THIÉBAUD and FISCHBERG 1977) indicate that the various *Xenopus* species constitute a polyploid series in the proportions 2:4:8:12. DNA contents correspond closely, whereas chromosome numbers are multiples of either $x = 9$ or $x = 10$, so that the above series reads in chromosome numbers as 20:40 and 36:72:108 (KOBEL and DU PASQUIER 1986). This variation in basic number reflects chromosomal rearrangements that took place during the evolutionary history of the genus Xenopus. The only diploid species known so far is *Xenopus tropicalis* ($2n = 20$). *X. laevis* (36 chromosomes) is tetraploid with respect to DNA content but must be regarded as a functional diploid in view of the exclusive occurrence of bivalents at meiosis (MÜLLER 1974; J. TYMOWSKA, personal communication). *X. laevis* is therefore referred to as "tetraploid-derived." Molecular studies of gene structure and chromosomal arrangement have focused so far on *X. tropicalis* and *X. laevis*. For instance, the α- and β-globin genes are closely linked and form a single gene cluster in *X. tropicalis*, whereas *X. laevis* has two similar clusters, reflecting the genome duplication that occurred in its evolutionary history (JEFFREYS *et al.* 1980; HOSBACH, WYLER and WEBER 1983). In contrast, segregation analysis of the four vitellogenin genes in *X. laevis* indicated that three of them (A_1, A_2, and B_1) belong to the same classical linkage group, whereas the fourth (B_2) segregates independently (SCHUBIGER and WAHLI 1986). Since *X. tropicalis* has three vitellogenin genes (*i.e.*, two closely related type-A and a single type-B gene), the chromosomal arrangement found in *X. laevis* supports an evolutionary scenario combining gene duplication, genome duplication, and gene elimination (SCHUBIGER and WAHLI 1986).

This genetic complexity of tetraploid-derived *X.*

The publication costs of this article were partly defrayed by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

TABLE 1

Origin of backcross progenies used for segregation analysis

Cross	Female parent	Male parent
1	[*X. l. victorianus* × *X. l. laevis* a^p] hybrid	*X. l. laevis* a^p
2	[*X. l.* subspecies M × *X. l. laevis* a^p] hybrid	*X. l. laevis* a^p
4	*X. l. victorianus*	[*X. l. victorianus* × *X. l. laevis* a^p] hybrid
5	[*X. l. poweri* × *X. l. laevis* a^p] hybrid	*X. l. laevis* a^p

laevis makes it an interesting object for the study of genome organization and chromosomal evolution. It is noteworthy that the karyotype of *X. laevis* presents a diploid-like arrangement, i.e. morphologically distinct pairs of chromosomes (TYMOWSKA 1977), indicating that structural changes have affected the homeologous chromosomes. Evidently, the genetic mapping of duplicate loci and duplicate linkage groups would provide a wealth of information on the evolution of genome architecture in polyploid Xenopus in general.

The present study is based on analyses of linkage relationships among 30 genetic loci in four backcross families derived from parents heterozygous at multiple loci. These heterozygous individuals were obtained by hybridizing different subspecies of *X. laevis* that had different alleles for many of the loci examined. It had been established previously that these hybrids are fertile and show normal pairing of homologs at meiosis (MÜLLER 1977).

MATERIALS AND METHODS

Animals: A series of crosses were made between laboratory-bred animals of the following subspecies: *Xenopus laevis laevis* Daudin (L) homozygous for a^p (periodic albino mutant; HOPERSKAYA 1975), *X. laevis victorianus* Ahl (V), *X. laevis* new subspecies I (M) originating from southern Malawi, and *X. laevis poweri* Hewitt (P). Ovulation was artificially induced and fertilization was done *in vitro* following the procedure described in GURDON (1967). First generation hybrids between V and L, M and L, or P and L, were backcrossed to *X. laevis laevis* homozygous for a^p or to the other parental subspecies (Table 1). Samples of backcross progenies were reared through metamorphosis. Subadult frogs were killed and dissected. Blood was drawn from the heart into a pipette that had been washed with a 3.2% solution of tri-sodium citrate and centrifuged to separate the blood cells and plasma. Samples of liver, skeletal muscle, kidney and duodenum were removed and stored at −70° until analyzed.

Electrophoresis: Plasma proteins were separated on 7.5% nondenaturing polyacrylamide gels (PAGE) following the technique described in GRAF and FISCHBERG (1986). The bands corresponding to transferrin were identified using a partial purification procedure given in ROGERS (1973). For isozyme analysis, tissue samples were homogenized on ice using a ratio of 1 g tissue to 3 ml extraction buffer (5 mM $MgCl_2$, 25 mM KCl, 25 mM tris-HCl pH 7.4, with 0.01% 2-mercaptoethanol added just before use). The homogenates were centrifuged at 12,000 × *g* for 5 min. The supernatants were submitted to horizontal starch gel electrophoresis for 15 hr at 4°. Five different buffer systems were used. AC, amine-citrate; electrode: 0.04 M citric acid, adjusted to pH 6.0 with *N*-(3-aminopropyl)-morpholine, gel: 1/20 dilution of electrode buffer; DT, discontinuous tris-citrate; electrode: 0.30 M H_3BO_3, 0.06 M NaOH; gel: 0.076 M tris, 0.005 M citric acid; TC, continuous tris-citrate; electrode: 0.135 M tris, 0.045 M citric acid; gel: 1/15 dilution of electrode buffer; TG, Tris-glycine; electrode: 0.053 M tris, 0.147 M glycine; gel: 1/4 dilution of electrode buffer; TP, Tris-phosphate; electrode: 0.1 M tris, 0.1 M NaH_2PO_4; gel: 1/20 dilution of electrode buffer. Gels were made using 60 g starch (University of Languedoc, Montpellier, France) and 500 ml gel buffer; 10 µl 2-mercaptoethanol were added before degassing.

Enzymes (Table 2) were stained following the procedures described in HARRIS and HOPKINSON (1976). For ACP, the substrate was 4-methylumbelliferyl phosphate. For EST, a mixture of α-naphthyl acetate and α-naphthyl propionate was used. The substrates for peptidases were Leu-Gly-Gly for PEP-B and Phe-Pro for PEP-D.

Nomenclature: Many enzymes occurred as multiple isozymes. When mitochondrial and supernatant forms occurred (BÜRKI 1987; GRAF 1989), they were distinguished by the prefix "m" or "s" as, for example, mME and sME. In other cases, isozymes were numbered so that the most anodal form was designated "1," *e.g.*, GPI-1 and GPI-2. Isozymes of LDH and CK were named according to previously established nomenclature (WOLFF and KOBEL 1982; BÜRKI 1985). Allelic variants of each locus were assigned small letters, so that the most anodal allozyme was designated by "a."

Data analysis: Phenotypic ratios in each backcross family were examined for agreement with Mendelian segregation ratios and independent assortment of loci. Contingency chi-square statistics were used to test for the independent assortment of pairs of loci. Estimates of recombination fractions and their standard errors were calculated according to SERRA (1965).

RESULTS

Electrophoretic phenotypes of protein loci: Most of the 21 proteins analyzed in this study occurred as multiple isozymes showing various levels of tissue specificity. Evidence for the genetic control of multiple isozymes by separate loci was provided by the heritable variation in one form independently of the others.

Acid phosphatase occurred as three isozymes (ACP-1, ACP-2, ACP-3) in kidney and liver homogenates. Only *ACP-3* was found to be polymorphic, and heterozygotes had two-band ACP-3 phenotypes. Gels stained for aconitase revealed three isozymes in kidney. The most anodal band was specific to the cytosolic fraction (sACO), whereas the two others, *i.e.*, mACO-1, mACO-2, were mitochondrial isozymes (BÜRKI 1987). Heterozygotes for *mACO-1* had two-band phenotypes. Serum albumin consisted of one major band (ALB-1) and one minor band (ALB-2), as illustrated in GRAF and FISCHBERG (1986). Alcohol dehydrogenase occurred as two isozymes in liver ho-

TABLE 2

List of polymorphic proteins used for linkage analysis

Enzyme or blood protein	Abbreviation	EC No.	Buffer[a]	Tissue used
Acid phosphatase	ACP	3.1.3.3	TP	Kidney
Aconitase	ACO	4.2.1.3	TG	Kidney, muscle
Albumin	ALB		PAGE	Plasma
Alcohol dehydrogenase	ADH	1.1.1.1	DT	Liver
Creatine kinase	CK	2.7.3.2	AC	Kidney, muscle
Esterase	EST	3.1.1.1	TG, DT	Liver
Fumarate hydratase	FH	4.2.1.2	TG	Liver, muscle
Glucosephosphate isomerase	GPI	5.3.1.9	TG, AC	Kidney, liver, muscle
Glycerol-3-phosphate dehydrogenase	GPD	1.1.1.8	AC	Muscle
Glyoxalase I	GLO	4.4.1.5	TG	Liver, muscle
Isocitrate dehydrogenase	IDH	1.1.1.42	TP	Kidney, muscle
Lactate dehydrogenase	LDH	1.1.1.27	TP	Kidney, liver
Malic enzyme	ME	1.1.1.40	AC	Kidney, muscle
Mannosephosphate isomerase	MPI	5.3.1.8	TG	Muscle
Nucleoside phosphorylase	NP	2.4.2.1	TG	Kidney, liver
Peptidase B	PEP-B	3.4.3.1	DT	Liver
Peptidase D	PEP-D	3.4.3.1	AC	Kidney, duodenum
6-Phosphogluconate dehydrogenase	PGD	1.1.1.44	AC	Kidney, muscle
Sorbitol dehydrogenase	SORD	1.1.1.14	TP, DT	Kidney, liver
Superoxide dismutase	SOD	1.15.1.1	TC, DT	Liver
Transferrin	TRF		PAGE	Plasma

[a] The buffer systems used for electrophoresis are described in text.

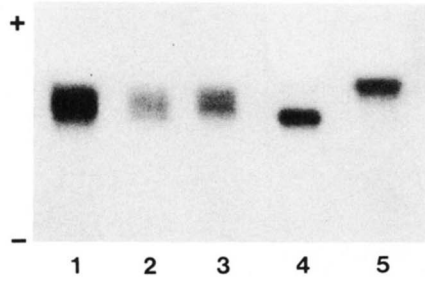

FIGURE 1.—Electrophoretic patterns of fumarate hydratase. The *FH* genotypes are: (1)–(3) *a/b*; (4) *b/b*; (5) *a/a*. Liver homogenates were run in a starch gel using a tris-glycine (TG) buffer system.

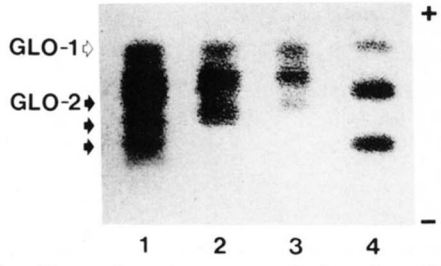

FIGURE 2.—Electrophoretic patterns of glyoxalase. The *GLO-1* genotypes are: (1)–(4) *a/a*. The *GLO-2* genotypes are: (1) *c/e*; (2) *c/d*; (3) *c/c*; (4) *e/e*. Liver homogenates were run in a starch gel using a tris-glycine (TG) buffer system.

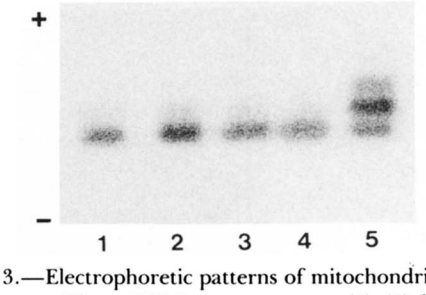

FIGURE 3.—Electrophoretic patterns of mitochondrial isocitrate dehydrogenase. The *mIDH-2* genotypes are: (1)–(4) *b/b*; (5) *a/b*. Kidney homogenates were run in a starch gel using a tris-phosphate (TP) buffer system.

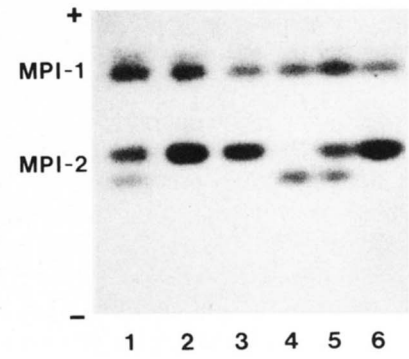

FIGURE 4.—Electrophoretic patterns of mannosephosphate isomerase. The *MPI-1* genotypes are: (1)–(6) *a/a*. The *MPI-2* genotypes are: (1), (5) *b/c*; (2), (3), (6) *b/b*; (4) *c/c*. Muscle homogenates were run in a starch gel using a tris-glycine (TG) buffer system.

mogenates, as already reported by WESOLOWSKI and LYERLA (1983). The isozyme with higher staining intensity and higher anodal mobility was designated ADH-1. Although heterozygotes for either *ADH-1* or *ADH-2* showed three-band phenotypes, attesting to the dimeric structure of these isozymes, no heterodimer could be observed between ADH-1 and ADH-2. Creatine kinase occurred as four isozymes with well-defined tissue specificity. The most anodal band (CK-C) was the major isozyme in brain, stomach, and kidney, whereas CK-A-1 and CK-A-2′ were the major isozymes in skeletal muscle and heart muscle. The fourth isozyme, corresponding to the least anodal

TABLE 3

Single-locus segregation at each of 30 genetic loci in crosses 1, 2, 4 and 5

Locus	Cross	Parental genotypes		Offspring	χ^2
		Female	Male		
albinism a^p	1	$a^p/+$	a^p/a^p	52 a^p/a^p; 40 $a^p/+$	1.56
	2	$a^p/+$	a^p/a^p	32 a^p/a^p; 28 $a^p/+$	0.27
	5	$a^p/+$	a^p/a^p	47 a^p/a^p; 58 $a^p/+$	1.15
ACP-3	4	*a/a*	*a/b*	41 *a/a*; 35 *a/b*	0.47
	5	*a/b*	*b/b*	58 *b/b*; 46 *a/b*	1.38
mACO-1	5	*a/b*	*a/a*	44 *a/a*; 40 *a/b*	1.19
ALB-1	1	*b/d*	*a/b*	15 *a/b*; 26 *b/b*; 27 *a/d*; 24 *b/d*	3.91 (df = 3)
	2	*b/c*	*b/b*	28 *b/b*; 32 *b/c*	0.27
	4	*d/d*	*b/d*	41 *d/d*; 36 *b/d*	0.32
ALB-2	1	*a/c*	*a/a*	50 *a/a*; 42 *a/c*	0.70
	2	*a/b*	*a/a*	31 *a/a*; 29 *a/b*	0.07
	5	*a/b*	*a/a*	54 *a/a*; 49 *a/b*	0.24
ADH-1	1	*a/b*	*a/a*	50 *a/a*; 42 *a/b*	0.70
	2	*a/c*	*a/a*	31 *a/a*; 29 *a/c*	0.07
	4	*b/b*	*a/b*	33 *b/b*; 45 *a/b*	1.85
	5	*a/b*	*a/a*	54 *a/a*; 51 *a/b*	0.09
ADH-2	4	*b/b*	*a/b*	39 *b/b*; 34 *a/b*	0.34
	5	*a/b*	*a/a*	20 *a/a*; 34 *a/b*	0.25
CK-C	2	*a/b*	*a/a*	25 *a/a*; 35 *a/b*	1.67
	4	*b/b*	*a/b*	31 *b/b*; 27 *a/b*	0.28
CK-A-2	5	*a/b*	*a/a*	65 *a/a*; 40 *a/b*	5.95*
EST-1	1	*a/b*	*b/b*	45 *b/b*; 46 *a/b*	0.01
EST-4	5	*a/b*	*b/b*	50 *b/b*; 45 *a/b*	0.26
FH	1	*a/b*	*a/a*	57 *a/a*; 35 *a/b*	5.26*
	4	*b/b*	*a/b*	37 *b/b*; 41 *a/b*	0.20
	5	*a/b*	*a/a*	53 *a/a*; 52 *a/b*	0.01
GPI-1	2	*a/b*	*b/b*	21 *b/b*; 39 *a/b*	5.40*
	5	*b/c*	*b/b*	51 *b/b*; 54 *b/c*	0.09
GPI-2	1	*a/b*	*a/a*	47 *a/a*; 45 *a/b*	0.04
	4	*a/a*	*a/b*	36 *a/a*; 42 *a/b*	0.46
GPD-2	4	*a/a*	*a/b*	48 *a/a*; 30 *a/b*	4.15*
GLO-1	5	*a/b*	*a/a*	59 *a/a*; 45 *a/b*	1.88
GLO-2	1	*c/e*	*c/c*	42 *c/c*; 50 *c/e*	0.70
	2	*b/c*	*c/c*	31 *c/c*; 29 *b/c*	0.07
	5	*a/c*	*c/c*	56 *c/c*; 49 *a/c*	0.47

* Significant deviation from the expected Mendelian ratio, at $P < 0.05$ level with df = 1.
** Significant at $P < 0.01$ level with df = 1.

band in stomach and eye homogenates, probably is a mitochondrial CK (WOLFF and KOBEL 1985). The genetic control of the muscle-specific CK isozymes is unusual in that the CK-A-2′ isozyme is a heterodimer consisting of a CK-A-1 subunit and a second subunit (CK-A-2) that apparently does not form any detectable homodimer (BÜRKI 1985; ROBERT and KOBEL 1988). Therefore, genotypes at the *CK-A-2* locus can only be deduced from the simultaneous examination of the CK-A-1 and CK-A-2′ isozymes. Gels stained for esterase activity revealed four isozymes designated EST-1, EST-2, EST-3 and EST-4 by order of decreasing anodal mobility. Fumarate hydratase (FH) occurred as one band in liver and muscle homogenates (Figure 1). Glucosephosphate isomerase occurred as three isozymes, *i.e.*, two homodimers and one heterodimer, in most tissues. GPI-1 was the major isozyme in skeletal muscle and heart muscle, whereas GPI-2 was predominant in liver and kidney. Zymograms of glycerol-3-phosphate dehydrogenase from skeletal muscle had three bands, presumably controlled by two separate loci. Only GPD-2, *i.e.*, the least anodal form in *X. laevis laevis*, showed scorable genetic variation. Glyoxalase I occurred as three isozymes, controlled by two separate loci (GLO-1 and GLO-2) in liver and kidney (Figure 2), whereas heart and skeletal muscle contained almost exclusively the GLO-2 isozyme. Zymograms of isocitrate dehydrogenase from heart or skeletal muscle had four bands; the most anodal one was the cytosolic form (sIDH), and the other three bands were mitochondrial isozymes (BÜRKI 1987) controlled by two loci, *i.e.*, *mIDH-1* and *mIDH-2*. In kidney homogenates, only mIDH-2 and sIDH were detectable (Figure 3). Gels stained for lactate dehydrogenase showed complex band patterns resulting from the expression of at least three separate loci, *i.e.*, *LDH-A*, *LDH-B*, and *LDH-C* (WOLFF and KOBEL 1982). Malic enzyme occurred as two isozymes, *i.e.*, one cytosolic

Locus	Cross	Parental genotypes		Offspring	χ^2
		Female	Male		
mIDH-2	1	*a/b*	*b/b*	33 *b/b*; 47 *a/b*	2.45
	4	*a/a*	*a/b*	22 *a/a*; 36 *a/b*	3.38
LDH-C	1	*a/b*	*b/b*	50 *b/b*; 41 *a/b*	0.89
	2	*a/b*	*b/b*	34 *b/b*; 26 *a/b*	1.07
	4	*a/a*	*a/b*	36 *a/a*; 42 *a/b*	0.46
	5	*a/b*	*a/a*	52 *a/a*; 48 *a/b*	0.16
sME	1	*a/c*	*c/c*	44 *c/c*; 48 *a/c*	0.17
	2	*a/c*	*c/c*	22 *c/c*; 38 *a/c*	4.27*
	4	*a/a*	*a/c*	41 *a/a*; 37 *a/c*	0.20
	5	*a/c*	*c/c*	57 *c/c*; 48 *a/c*	0.77
mME	1	*a/b*	*a/a*	53 *a/a*; 39 *a/b*	2.13
	2	*a/b*	*a/a*	24 *a/a*; 36 *a/b*	2.40
	4	*b/b*	*a/b*	36 *b/b*; 42 *a/b*	0.46
	5	*a/c*	*a/a*	43 *a/a*; 52 *a/c*	0.85
MPI-1	1	*a/b*	*a/a*	39 *a/a*; 53 *a/b*	2.13
	2	*a/c*	*a/a*	27 *a/a*; 33 *a/c*	0.60
	5	*a/c*	*a/a*	42 *a/a*; 63 *a/c*	4.20*
MPI-2	1	*a/b*	*b/b*	48 *b/b*; 44 *a/b*	0.17
	4	*a/a*	*a/b*	38 *a/a*; 40 *a/b*	0.05
	5	*b/c*	*b/b*	48 *b/b*; 57 *b/c*	0.77
NP	1	*a/b*	*a/a*	51 *a/a*; 41 *a/b*	1.08
PEP-B	2	*a/b*	*a/a*	28 *a/a*; 32 *a/b*	0.27
PEP-D	1	*a/b*	*a/a*	48 *a/a*; 44 *a/b*	0.17
	4	*b/b*	*a/b*	31 *b/b*; 47 *a/b*	3.28
PGD-2	5	*a/b*	*b/b*	54 *b/b*; 51 *a/b*	0.09
SORD	1	*b/c*	*b/b*	38 *b/b*; 54 *b/c*	2.78
	4	*c/c*	*b/c*	26 *c/c*; 52 *b/c*	8.67**
	5	*a/b*	*b/b*	43 *b/b*; 62 *a/b*	3.44
sSOD-1	1	*a/b*	*a/a*	43 *a/a*; 49 *a/b*	0.39
	4	*b/b*	*a/b*	50 *b/b*; 28 *a/b*	7.38**
	5	*a/b*	*a/a*	58 *a/a*; 47 *a/b*	1.15
TRF	1	*c/d*	*c/c*	51 *c/c*; 41 *c/d*	1.08
	2	*c/d*	*c/c*	23 *c/c*; 37 *c/d*	3.26
	4	*a/d*	*c/d*	20 *a/c*; 16 *a/d*; 23 *c/d*; 18 *d/d*	1.39 (df = 3)
	5	*b/c*	*c/c*	53 *c/c*; 48 *b/c*	0.25

(sME) and one mitochondrial (mME), in most tissues (BÜRKI 1987; GRAF 1989). The mitochondrial ME usually was the most anodal of the two. Mannose-phosphate isomerase occurred as two isozymes, controlled by two separate loci, *i.e.*, MPI-1 and MPI-2, in heart and skeletal muscle (Figure 4). Zymograms of nucleoside phosphorylase (NP) from liver and kidney had one band in homozygotes and four bands in heterozygotes. Gels stained for peptidase B (PEP-B) and peptidase D (PEP-D) showed one band in homozygotes and three in heterozygotes. 6-Phosphogluconate dehydrogenase occurred as three isozymes controlled by two loci, *i.e.*, *PGD-1* and *PGD-2*; the PGD-2 isozyme appeared to be more active than PGD-1 in all tissues tested. Zymograms of sorbitol dehydrogenase (SORD) from liver and kidney had a single band in homozygotes and five bands in heterozygotes. Superoxide dismutase occurred as four bands; the three most anodal bands were cytosolic isozymes controlled by two loci, *i.e.*, *sSOD-1* and *sSOD-2*, whereas the fourth band was the mitochondrial isozyme (BÜRKI 1987). Transferrin (TRF) occurred as one band in homozygotes and two bands in heterozygotes.

Inheritance of electrophoretic variants: For each cross and each polymorphic locus, the segregation of parental alleles was analyzed and tested for conformity to expected Mendelian ratios for disomic inheritance. Most crosses were of the test cross type, and therefore were expected to yield homozygotes and heterozygotes in a proportion of 1:1. In some cases, both parents were heterozygous but had different alleles, yielding four genotypic classes with an expected ratio of 1:1:1:1.

The segregation data presented in Table 3 show an overall agreement with Mendelian ratios. In a few cases, however, the observed numbers diverge significantly from the expected. There is an apparent deficiency of heterozygotes for CK-A-2 in cross 5 ($P < 0.025$), for FH in cross 1 ($P < 0.025$), for GPD-2 in cross 4 ($P < 0.05$), and for SOD-1 in cross 4 ($P <$

TABLE 4

List of loci used for pairwise linkage tests in crosses 1, 2, 4 and 5

Cross	Sex	Characteristics of informative parent: Genomic constitution[a]	Characteristics of informative parent: Heterozygous loci
1	f	VL	*a^p, ALB-1, ALB-2, ADH-1, EST-1, FH, GPI-2, GLO-2, mIDH-2, LDH-C, sME, mME, MPI-1, MPI-2, NP, PEP-D, SORD, sSOD-1, TRF.*
2	f	ML	*a^p, ALB-1, ALB-2, ADH-1, CK-C, GPI-1, GLO-2, LDH-C, sME, mME, MPI-1, PEP-B, TRF.*
4	m	VL	*ACP-3, ALB-1, ADH-1, ADH-2, CK-C, FH, GPI-2, GLO-2, GPD-2, mIDH-2, LDH-C, sME, mME, MPI-2, PEP-D, SORD, sSOD-1, TRF.*
5	f	PL	*a^p, ACP-3, mACO-1, ALB-2, ADH-1, ADH-2, CK-C, CK-A-2, EST-4, FH, GLO-1, GLO-2, GPI-1, LDH-C, sME, mME, MPI-1, MPI-2, PGD-2, SORD, sSOD-1, TRF.*

[a] Genomic constitution is indicated by the abbreviations of the two parental subspecies, female listed first; L, laevis; M, subspecies I; P, poweri; V, victorianus.

0.01). In contrast, there is an apparent excess of heterozygotes for GPI-1 in cross 2 ($P < 0.025$), for sME in cross 2 ($P < 0.05$), for MPI-1 in cross 5 ($P < 0.05$), and for SORD in cross 4 ($P < 0.01$). In sum, eight of 70 tests made for single-locus segregation showed significant departure from the expected Mendelian ratio. This proportion can be considered relatively low, since three to four departures at a significance level of 0.05 would be expected by chance alone. Therefore, it may be assumed that meiosis in the four Xenopus hybrids tested proceeded normally.

Linkage analysis: In the four crosses analyzed, pairwise linkage tests of 13 to 22 loci were performed (Table 4). In most pairwise comparisons, four genotypes were found among the offspring. Since the linkage phase (coupling or repulsion) in the heterozygous parent was known, the genotypes in the progeny could be identified unambiguously as parental or recombinant. The offspring with either parental genotype were grouped in a single "parental" class, and those with either recombinant genotype were grouped in a "recombinant" class. Contingency table analyses were performed based on numbers expected for independent assortment. For evidence of linkage, the significance level of $P < 0.01$ was required.

Of 361 pairs of loci tested for linkage, 20 showed significant deviation from independent assortment in at least one of the crosses examined. The contingency table analyses of these 20 pairs of loci for all crosses in which they were tested are presented in Table 5. In addition, the linkage analyses for four pairs of duplicated loci are presented in Table 6.

There is an overall agreement between the four crosses concerning the status of linkage or independence of most pairs of loci. Nevertheless, two exceptions have to be pointed out. First, *mME* and *SORD* showed joint segregation in cross 5 (recombinant fraction 0.37) but independent assortment in crosses 1 and 4 (Table 5). Since the evidence for linkage of these two loci is tenuous, *mME* and *SORD* are conservatively considered unlinked. Secondly, *ACP-3* and *GLO-2* showed joint segregation in cross 4 (recombinant fraction 0.30) and independent assortment in cross 5. In this case, linkage is strongly supported by unambiguous data indicating that *ACP-3* and *GLO-2* are both linked to *sME* (Table 5).

From the data presented in Table 5, eight linkage groups were defined, comprising 23 genetic loci (Figure 5). It is worth noting that large discrepancies occur among the various crosses with respect to recombination frequencies between linked loci. These differences appear to be associated with the sex of the heterozygous parent and with its genomic constitution (parental subspecies).

For convenience, the linkage groups have been numbered from 1 to 8 in an arbitrarily chosen order. Linkage group 1 includes four loci, albumin-2, alcohol dehydrogenase-1, nucleoside phosphorylase, and periodic albinism. The recombination frequencies were *ALB-2*, 0%, *ADH-1*, 3%, *NP*, 5%, *a^p* in a VL female (hybrid of *X. laevis victorianus* and *X. laevis laevis*), and *ALB-2*, 5%, *ADH-1*, 26%, *a^p* in a PL female (hybrid of *X. laevis poweri* and *X. laevis laevis*). Linkage group 2 includes *ALB-1* and *ADH-2*, and probably is homeologous to linkage group 1.

Linkage group 3 comprises four loci, peptidase B, mannosephosphate isomerase-1, sorbitol dehydrogenase, and mitochondrial isocitrate dehydrogenase-2. The position of *PEP-B* with respect to *SORD* and *mIDH-2* has not been determined. The recombination frequencies were: *MPI-1*, 1%, *SORD*, 11%, *mIDH-2* in a VL female, and *SORD*, 2%, *mIDH-2* in a VL male.

Linkage group 4 includes glucosephosphate isomerase-1 (*GPI-1*) and esterase-4 (*EST-4*), with recombination frequency 2% in a PL female. Linkage group 5 contains glucosephosphate isomerase-2 (*GPI-2*) and peptidase D (*PEP-D*); their recombination frequency was 1% in a VL female and 6.4% in a VL male.

Linkage group 6 comprises three loci, acid phosphatase-3, cytosolic malic enzyme, and glyoxalase-2, with recombination frequencies *ACP-3*, 22%, *sME*, 10%, *GLO-2* in a VL male, and *ACP-3*, 34%, *sME*, 28%, *GLO-2* in a PL female.

Linkage group 7 contains four loci, cytosolic superoxide dismutase-1, glycerol-3-phosphate dehydrogenase-2, mitochondrial malic enzyme, and sex (data in GRAF 1989). The recombination frequencies were

TABLE 5

Classical linkages detected in pairwise comparisons between loci tested in this study

Locus pair	Cross	Offspring		χ^2	rf [a]	SE (rf)
		Parental	Recombinant			
a^p ALB-2	1	84	8	62.78**	0.09	0.03
	2	53	7	35.27**	0.12	0.04
	4	76	27	23.31**	0.26	0.04
a^p ADH-1	1	84	8	62.78**	0.09	0.03
	2	53	7	35.27**	0.12	0.04
	5	78	27	24.77**	0.26	0.04
a^p NP	1	87	5	73.09**	0.05	0.02
ACP-3 GLO-2	4	50	21	11.84**	0.30	0.05
	5	58	46	1.38		
ALB-1 ADH-2	4	72	1	69.05**	0.01	0.01
ALB-2 ADH-1	1	92	0	92.00**	0.00	0.00
	2	60	0	60.00**	0.00	0.00
	5	98	5	83.97**	0.05	0.02
ALB-2 NP	1	89	3	80.39**	0.03	0.02
ADH-1 NP	1	89	3	80.39**	0.03	0.02
EST-4 GPI-1	5	93	2	87.17**	0.02	0.01
FH TRF	1	64	28	14.08**	0.30	0.05
	4	71	7	52.51**	0.09	0.03
	5	73	28	20.05**	0.28	0.04
GPI-2 PEP-D	1	91	1	88.04**	0.01	0.01
	4	73	5	59.27**	0.06	0.03
GPD-2 mME	4	60	18	22.61**	0.23	0.05
GPD-2 sSOD-1	4	60	18	22.61**	0.23	0.05
GLO-2 sME	1	74	18	34.08**	0.20	0.04
	2	43	17	11.26**	0.28	0.06
	4	66	7	47.69**	0.10	0.03
	5	76	29	21.04**	0.28	0.04
mIDH-2 MPI-1	1	70	10	45.00**	0.12	0.04
mIDH-2 SORD	1	71	9	48.05**	0.11	0.04
	4	57	1	54.07**	0.02	0.02
mME SORD	1	43	49	0.33		
	4	46	32	2.51		
	5	66	39	6.94**	0.37	0.05
MPI-1 PEP-B	2	55	5	41.67**	0.08	0.04
MPI-1 SORD	1	91	1	88.04**	0.01	0.01
	5	104	1	101.04**	0.01	0.01

[a] Recombinant fraction: recombinant/total.
** Significant at $P < 0.01$ level with df = 1.

TABLE 6

Linkage analysis of duplicate loci

Locus pair	Cross	Offspring		χ^2
		Parental	Recombinant	
ALB-1 ALB-2	1	43	49	0.39
	2	27	33	0.60
ADH-1 ADH-2	4	42	31	1.66
	5	34	30	0.25
GLO-1 GLO-2	5	53	51	0.04
MPI-1 MPI-2	1	51	41	1.09
	5	47	58	1.15

sSOD-1, 23%, *GPD-2*, 23%, *mME* in a VL male. The mean recombination frequency between *mME* and *sex* is 6%, based on segregation analysis in three females, *i.e.*, VL, ML and PL (GRAF 1989). The position of the sex-determining locus with respect to *sSOD-1* and *GPD-2* has not been established.

Linkage group 8 includes fumarate hydratase (*FH*) and transferrin (*TRF*); their recombination frequency varied from 9% in a VL male to 30% in a VL female.

The linkage groups here established cannot for the time being be associated with individual chromosomes.

It is worth noting that linkage group 4 has not been tested for independent assortment with linkage group 5. Similarly, some of the eight unassigned genetic loci (mACO-1, CK-C, CK-A-2, EST-1, GLO-1, LDH-C, MPI-2, PGD-2) were not tested in all possible combinations (Table 3).

DISCUSSION

The inheritance results presented in this paper indicate that subspecies hybrids can be used successfully

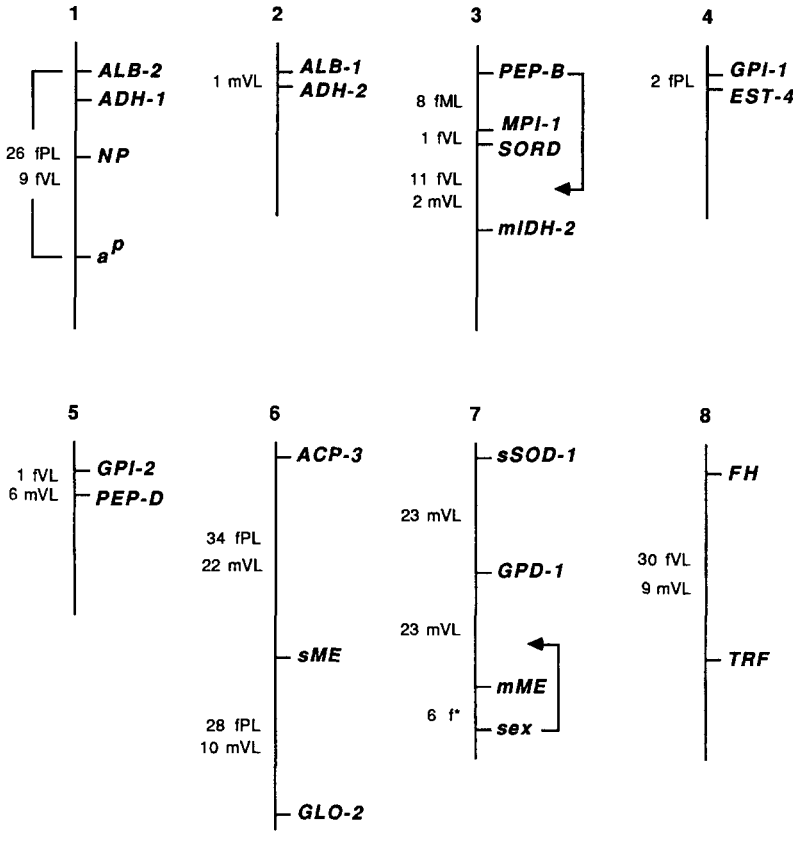

FIGURE 5.—Linkage groups in hybrids between various subspecies of *X. laevis*. The groups have been numbered from 1 to 8 in an arbitrarily chosen order. Arrows indicate the two possible positions of a locus in a group, when the data were insufficient to determine its exact position. Estimated recombination frequencies (in percent) are indicated on the left side of the linkage groups. When different crosses yielded different recombination frequencies, the highest and lowest estimates are given, followed by the sex and subspecies constitution of the hybrid in which they occurred. Subspecies abbreviations are as follows: L, laevis; M, subspecies I; P, poweri; V, victorianus. (*) indicates an average value based on three crosses (data in GRAF 1989).

for linkage analysis of the *X. laevis* genome. The major advantage of using hybrid backcrosses resides in the fact that hybrids are heterozygous at multiple loci, with the consequence that every single cross allows a maximum number of pairwise linkage tests to be performed.

In most cases, the loci showing linkage association in one hybrid combination were found to be linked in other hybrids where they could be tested. However, estimates of recombination frequencies between linked loci showed differences related to the genomic constitution (parental subspecies) and to the sex of the heterozygous parent. For instance, the map distance between *ALB-2* and a^p was considerably larger in a PL female ($rf = 0.26$) than in a VL female ($rf = 0.09$). Nevertheless, the same females displayed similar recombination frequencies for the locus pairs *FH/TRF* or *MPI-1/SORD*. When a VL male is compared to a VL female, recombination estimates are greater in the female for three pairs of loci (*FH/TRF*; *GLO-2/sME*; *mIDH-2/SORD*) and greater in the male for one pair (*GPI-2/PEP-D*). These results indicate that the probability of recombination, at least in certain segments of the genome, is affected by sex and other genetic differences. Recombination appears to be less frequent generally in males than in females, although sex differences are not uniform throughout the genome, and might even occur in opposite directions at different locations in the genome. Definite evidence for reduced recombination in males has been found in other vertebrate species, *e.g.*, in salmonid fish (JOHNSON, WRIGHT and MAY 1987) and in man (BARKER *et al.* 1987). In their detailed study of human chromosome 7, BARKER *et al.* (1987) observed that the male/female difference in recombination rate was not uniform throughout the chromosome but was concentrated in a few intervals. It has been proposed that, when sex differences occur, recombination usually is less frequent in the heterogametic sex (reviewed in DUNN and BENNETT 1967). The preliminary results obtained with *X. laevis*, a species in which the female is heterogametic (reviewed in GRAF 1989), are not consistent with this hypothesis.

There is compelling evidence, from data on karyotype and DNA content (THIÉBAUD and FISCHBERG 1977), that *X. laevis* arose by tetraploidization. Moreover, the exclusive occurrence of bivalents at meiosis (MÜLLER 1974) supports an allotetraploid origin. Molecular studies of globins (HOSBACH, WYLER and WEBER 1983) and α-actin (STUTZ and SPOHR 1986) have shown that the genome of *X. laevis* contains twice as many genes encoding these proteins as the genome of the diploid *X. tropicalis*. Similarly, the present study provides genetic evidence for the expression of duplicate loci coding for serum albumin (ALB), alcohol dehydrogenase (ADH), glucosephosphate isomerase (GPI), glyoxalase (GLO) and mannosephosphate isomerase (MPI). Four duplicate pairs (*ALB-1/ALB-2*; *ADH-1/ADH-2*; *GLO-1/GLO-2*; *MPI-1/MPI-2*) were tested for joint segregation each in at least one back-

cross family, but none of these loci was found to be linked with its duplicate. However, *ALB-1* showed close linkage with *ADH-2*, as did *ALB-2* with *ADH-1*, suggesting that the entire *ALB - ADH* linkage group has been duplicated. These results (*i.e.* independent assortment of duplicate loci and duplicate linkage groups) strongly support the view that the greater complexity of the *X. laevis* genome (as opposed to *X. tropicalis*) was produced by genome duplication and not by tandem duplications.

The supposed homology of many enzyme loci throughout the vertebrates allows to compare the genetic maps of closely related as well as distantly related species. For instance, the linkage map of *X. laevis* (Figure 5) can be compared with those of other amphibian species, *i.e.*, *Rana pipiens*, *Rana berlandieri* (WRIGHT *et al.* 1983) and *Rana nigromaculata* (NISHIOKA, OHTANI and SUMIDA 1987), as well as with the very detailed human gene map (MCKUSICK 1987).

Xenopus linkage groups 1 and 2 (Figure 5): *ALB* and *ADH* are also linked in *R. berlandieri* and *R. nigromaculata*, and this group includes an albino locus in *R. nigromaculata* (*NP* not mapped in *Rana*); similarly, *ALB* and *ADH* are syntenic on human chromosome *4*, whereas *NP* is located on chromosome *14*. Linkage group 3 (Figure 5): *PEP-B* and *MPI* are also linked in *R. pipiens* and *R. nigromaculata* (*SORD* and *mIDH* not mapped in Rana); *MPI*, *SORD* and *mIDH* are syntenic on human chromosome *15*, whereas *PEP-B* is located on chromosome *12*. Linkage groups 4 and 5 (Figure 5): *GPI* and *PEP-D* are also linked in *R. berlandieri*, and syntenic on human chromosome *19*. Linkage group 6 (Figure 5): *GLO* and *ACP* are linked in *R. pipiens* (*sME* not mapped in this species); *GLO* and *sME* are syntenic on human chromosome *6*. Linkage group 7 (Figure 5): *sSOD* is also sex-linked in *Rana pipiens*: in addition, *GPD*, "*ME-A*" and "*SOD-B*" are linked in *R. nigromaculata* but it is unclear whether "*ME-A*" and "*SOD-B*" designate the cytosolic or mitochondrial form of these enzymes. In the human gene map, *GPD*, *mME*, and *sSOD* are located on three different chromosomes. Linkage group 8 (Figure 5): *FH* and *TRF* have not been mapped in *Rana*; they are not syntenic in the human genome.

In sum, the loci that were tested in both Xenopus and Rana showed similar linkage relationships in these two taxa, suggesting that an important proportion of linkage groups have been conserved between these distantly related anuran genera. More surprising is the apparent conservation of linkages between the Xenopus and the human gene maps. Of eight linkage groups identified in Xenopus, five showed some similarity with human syntenic groups. It certainly would be premature to make a statement about the significance of this apparent conservation. The elaboration of a more detailed genetic map of *X. laevis* should provide new insights as to which of the observed similarities truly reflect persistent ancestral gene arrangements. It is noteworthy that the loci for peptidase D (PEP-D) and glucosephosphate isomerase (GPI) were found to be classically linked in all vertebrate taxa where they were tested, *e.g.*, salmonid fish (JOHNSON, WRIGHT and MAY 1987), *R. pipiens* (WRIGHT *et al.* 1983), *X. laevis* (this article), mouse (WOMACK 1987), and man (MUKUSICK 1987), suggesting that this linkage relationship has been conserved for more than 300 million years. This example supports the view that some linkage groups might effectively be stable over very long periods of evolution.

I am grateful to H. GLOOR for critically reading the manuscript. I thank BRUNELLA COLOMBELLI, HANS RUDOLF KOBEL and CHARLES THIÉBAUD for stimulating discussions and encouragement. PATRICE BAATARD, MONIQUE FORNALLAZ and ANDRÉ SOLARO provided excellent technical assistance. This work was supported by the Swiss National Science Foundation, grant 3.596-0.87.

LITERATURE CITED

BARKER, D., P. GREEN, R. KNOWLTON, J. SCHUMM, E. LANDER, A. OLIPHANT, H. WILLARD, G. AKOTS, V. BROWN, T. GRAVIUS, C. HELMS, C. NELSON, C. PARKER, K. REDICKER, M. RISING, D. WATT, B. WEIFFENBACH AND H. DONIS-KELLER, 1987 Genetic linkage map of human chromosome 7 with 63 DNA markers. Proc. Natl. Acad. Sci. USA **84:** 8006–8010.

BÜRKI, E., 1985 The expression of creatine kinase isozymes in *Xenopus tropicalis*, *Xenopus laevis laevis*, and their viable hybrid. Biochem. Genet. **23:** 73–88.

BÜRKI, E., 1987 Comparative analysis of electrophoretic protein phenotypes in the genus *Xenopus* (Anura: Pipidae). PhD thesis No. 2244, University of Geneva, Switzerland.

COLOMBELLI, B., C. H. THIÉBAUD and W. P. MÜLLER, 1984 Production of WW superfemales by diploid gynogenesis in *Xenopus laevis*. Mol. Gen. Genet. **194:** 57–59.

DUNN, L. C., and D. BENNETT, 1967 Sex differences in recombination of linked genes in animals. Genet. Res. **9:** 211–220.

GRAF, J.-D., 1989 Sex linkage of malic enzyme in *Xenopus laevis*. Experientia **45:** 194–196.

GRAF, J.-D., and M. FISCHBERG, 1986 Albumin evolution in polyploid species of the genus *Xenopus*. Biochem. Genet. **24:** 821–837.

GURDON, J. B., 1967 African clawed toads, pp. 75–84 in *Methods in Developmental Biology*, edited by N. H. WILT and N. K. WESSELS. Crowell Company, New York.

HARRIS, H., and D. A. HOPKINSON, 1976 *Handbook of Enzyme Electrophoresis in Human Genetics*. North Holland, Amsterdam.

HOPERSKAYA, O. A., 1975 The development of animals homozygous for a mutation causing periodic albinism (a^p) in *Xenopus laevis*. J. Embryol. Exp. Morph. **34:** 253–264.

HOSBACH, H. A., T. WYLER and R. WEBER, 1983 The *Xenopus laevis* globin gene family: chromosomal arrangement and gene structure. Cell **32:** 45–53.

JEFFREYS, A. J., V. WILSON, D. WOOD and J. P. SIMONS, 1980 Linkage of adult α- and β-globin genes in *X. laevis* and gene duplication by tetraploidization. Cell **21:** 555–564.

JOHNSON, K. R., J. E. WRIGHT, JR., and B. MAY, 1987 Linkage relationships reflecting ancestral tetraploidy in salmonid fish. Genetics **116:** 579–591.

KOBEL, H. R., and L. DU PASQUIER, 1986 Genetics of polyploid *Xenopus*. Trends Genet. **2:** 310–315.

MCKUSICK, V. A., 1987 The human gene map, pp. 534–597 in *Genetic Maps 1987*, edited by S. J. O'BRIEN. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MÜLLER, W. P., 1974 The lampbrush chromosomes of *Xenopus laevis* (Daudin). Chromosoma **47:** 283–296.

MÜLLER, W. P., 1977 Diplotene chromosomes of *Xenopus* hybrid oocytes. Chromosoma **59:** 273–282.

NISHIOKA, M., H. OHTANI and M. SUMIDA, 1987 Chromosomes and the sites of five albino gene loci in the *Rana nigromaculata* group. Sci. Rep. Lab. Amphibian Biol. Hiroshima Univ. **9:** 1–52.

REINSCHMIDT, D., J. FRIEDMAN, J. HAUTH, E. RATNER, M. COHEN, M. MILLER, D. KROTOSKI and R. TOMPKINS, 1985 Gene-centromere mapping in *Xenopus laevis*. J. Hered. **76:** 345–347.

ROBERT, J., and H. R. KOBEL, 1988 Purification and characterization of cytoplasmic creatine kinase isozymes of *Xenopus laevis*. Biochem. Genet. **26:** 543–555.

ROGERS, J. S., 1973 Protein polymorphism, genic heterozygosity and divergence in the toads *Bufo cognatus* and *B. speciosus*. Copeia **1973:** 322–330.

SCHUBIGER, J.-L., and W. WAHLI, 1986 Linkage arrangement in the vitellogenin gene family of *Xenopus laevis* as revealed by gene segregation analysis. Nucleic Acids Res. **14:** 8723–8734.

SERRA, J. A., 1965 *Modern Genetics*. Academic Press, London.

STUTZ, F., and G. SPOHR, 1986 Isolation and characterization of sarcomeric actin genes expressed in *Xenopus laevis* embryos. J. Mol. Biol. **187:** 349–361.

THIÉBAUD, C. H., and M. FISCHBERG, 1977 DNA content in the genus *Xenopus*. Chromosoma **59:** 253–257.

THIÉBAUD, C. H., B. COLOMBELLI and W. P. MÜLLER, 1984 Diploid gynogenesis in *Xenopus laevis* and the localization with respect to the centromere of the gene for periodic albinism a^p. J. Embryol. Exp. Morphol. **83:** 33–42.

TYMOWSKA, J., 1977 A comparative study of the karyotypes of eight *Xenopus* species and subspecies possessing a 36-chromosome complement. Cytogenet. Cell Genet. **18:** 165–181.

TYMOWSKA, J., and M. FISCHBERG, 1982 A comparison of karyotype, constitutive heterochromatin, and nucleolar organizer regions of the new tetraploid species *Xenopus epitropicalis* Fischberg and Picard with those of *Xenopus tropicalis* Gray (Anura, Pipidae). Cytogenet. Cell Genet. **34:** 149–157.

VONWYL, E., and M. FISCHBERG, 1980 Lactate dehydrogenase isozymes in the genus *Xenopus*: species-specific patterns. J. Exp. Zool. **211:** 281–290.

WESOLOWSKI, M. H., and T. A. LYERLA, 1983 Alcohol dehydrogenase isozymes in the clawed frog, *Xenopus laevis*. Biochem. Genet. **21:** 1003–1017.

WOLFF, J., and H. R. KOBEL, 1982 Lactate dehydrogenase of *Xenopus laevis laevis* and *Xenopus borealis* depends on a multiple gene system. J. Exp. Zool. **223:** 203–210.

WOLFF, J., and H. R. KOBEL, 1985 Creatine kinase isozymes in pipid frogs: Their genetic bases, gene expressional differences, and evolutionary implications. *J. Exp. Zool.* **234:** 471–480.

WOMACK, J. E., 1987 Comparative gene mapping: A valuable new tool for mammalian developmental studies. Dev. Genet. **8:** 281–293.

WRIGHT, D. A., C. M. RICHARDS, J. S. FROST, A. M. CAMOZZI and B. J. KUNZ, 1983 Genetic mapping in amphibians. Isozymes Curr. Top. Biol. Med. Res. **10:** 287–311.

Communicating editor: S. L. ALLEN