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Electrophoretic patterns of hemoglobin in different *Xenopus* species, subspecies and interspecies hybrids[1]

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Summary. Hemoglobins of 12 diploid and polyploid species, 6 subspecies and 15 different inter-species hybrids of the genus *Xenopus* were compared by electrophoresis on polyacrylamide gels or by isoelectrofocusing. Multiple hemoglobin bands were detected in all taxa. - Each species can be identified by its specific hemoglobin pattern, in spite of the intraspecific polymorphism observed within 5 of the 12 species. In contrast to the observed interspecific variation, the hemoglobin pattern of the six subspecies of *Xenopus laevis* is almost invariable and does not allow an unequivocal identification of these taxa. - Hemoglobin patterns of all interspecies hybrids represent the sum of those of their parental species. In spite of this codominant expression of all parental hemoglobins, no hybrid molecules containing globins of both parents can be detected.

Introduction

The different species and subspecies of the African clawed toad, genus *Xenopus,* represent from an evolutionary point of view a most interesting group of lower vertebrates. *Xenopus tropicalis,* considered to be the most ancient extant species of the genus[2], has a mitotic chromosome number of $2n = 20$[24]. *X. epitropicalis,* a newly described species[7], has a mitotic chromosome number of $2n \mp 40$ and is, therefore, tetraploid and, moreover, closely related to *X. tropicalis* with respect to its morphology and karyotype[28]. Numerous species, amongst them *X. laevis,* which is

widely used in embryological and molecular genetic analyses, have a chromosome number of 2n = 36[25]. The 3 species *X. vestitus*, *X. wittei* and *X. amieti* (2n = 72) are tetraploid and *X. ruwenzoriensis* (2n = 108) represents a hexaploid species with respect to *X. laevis*[12,26,27,29]. However, since the group of species with 36 chromosomes must already be considered as being tetraploid with respect to *X. tropicalis*[2], the highly polyploid species would be octoploid (2n = 72) and dodecaploid (2n = 108), respectively. Recent analyses of the genome of *X. laevis* have revealed that it contains 2 genes for serum albumin[34], 2 pairs of loci for vitellogenin[30], and 2 genes for some of the ribosomal proteins[3]. Finally, Jeffreys et al.[11] have shown that the closely linked α- and β-globin genes of *X. tropicalis* are duplicated in the genome of *X. laevis*. This duplication represents the first evidence at the gene level of the tetraploid origin of *X. laevis*.

In spite of the heterogeneity of the genus at the chromosome level, the different species and subspecies are very similar morphologically[35]. From a taxonomic point of view it is, therefore, essential to have suitable molecular markers available to distinguish any taxon of the genus. Recently, Vonwyl and Fischberg[37] and Mann et al.[14] have shown that identification of most of the taxa is possible by comparing the electrophoretic patterns of their LDH and of their sperm-specific nuclear proteins, respectively. These markers, however, have the disadvantage that the animals have to be sacrificed for analysis. For this reason we started to investigate the electrophoretic pattern of the hemoglobins (Hb) of the different *Xenopus* taxa, since Hb can be easily obtained without killing the animals. An earlier comparison of the Hb of different *Xenopus* species and subspecies has been undertaken, but did not allow an identification of most of the species analyzed[17].

In addition to the numerous *Xenopus* taxa several inter-species hybrids are available in our laboratory[18,35]. Since the polyploid species of the genus *Xenopus* are thought to be of allopolyploid origin[18,26-28], it would be interesting to know how the parental Hb's are expressed in the different inter-species hybrids.

The results presented here show that each *Xenopus* species can be identified by its unique Hb pattern, whereas the 6 different subspecies of *X. laevis* show almost the same Hb types. Moreover, all parental Hb types are codominantly expressed in inter-species hybrids but no chimeric Hb molecules can be detected.

Materials and methods. Animals. Adult animals of the genus *Xenopus* were used in all experiments. Different species and subspecies as well as their geographical origin are listed in the table. The F_1 hybrids of different parental species were produced in our laboratory either by natural or artificial fertilization[35]. The hybrid combinations and sample size are also indicated in the table.

Hb preparation. Blood was collected either by heart puncture of anesthetized animals which were subsequently killed, or from the femoral vein of animals immobilized with 0.1% (w/v) MS 222 (Sandoz, Basel), using a heparinized capillary pipette. In the latter case the animals recovered about 30 min after they had been transferred back to normal water. Blood cells

Origin of the analyzed species and subspecies of the genus *Xenopus* and combinations of interspecies hybrids examined

Species, subspecies	Abbreviation	Chromosome number 2n	Origin		Sample size	Hybrid combinations ♀ × ♂		Sample size
X. tropicalis (Gray)	T	20	Adiopodoume	Ivory Coast	2			
X. epitropicalis[7]	E	40	Kinshasa	Zaïre	3			
X. muelleri (Peters)	M1	36	Ifakara	Tanzania	3	M1	LV2; C	3; 3
	M2			Malawi	2			
X. species nova VI[a]	SPNVI	36[b]		Ghana	5			
X. borealis Parker	B1	36	Samburu Range	Kenya	1			
	B2		Marsabit	Kenya	2			
	B3		Maralal	Kenya	2			
	B4		Kiambu	Kenya	3			
	B5		Nairobi	Kenya	3	B5	M1; C	1; 3
X. clivii Peracca	C	36		Ethiopia	3	C	M1; B5	3; 3
X. fraseri Boulenger	F	36	Foulassi	Cameroon	4			
X. species nova III	SPNIII	36[b]		Ethiopia	2			
X. laevis	L							
X. l. laevis (Daudin)	LL	36	Fish Hoek	South Africa	3	LL	C	3
X. l. sudanensis Perret	LS	36[b]	Galim	Cameroon	1			
X. l. subspecies nova I[a]	LSSPNI	36[b]		Malawi	1			
X. l. petersi Bocage	LP	36		Simbabwe	2	LP	F	3
X. l. victorianus Ahl	LV1	36	Kitanga	Uganda	1	LV1	B4	3
	LV2		Shama	Ruanda	2	LV2	M1	3
X. l. bunyoniensis Loveridge	LB	36[b]	Luhonda	Ruanda	5			
X. vestitus Laurent	V	72	Mutanda	Uganda	6	V	LP; F; W	1; 3; 3
X. amieti[12]	A	72	Galim	Cameroon	2			
X. wittei[23]	W	72	Echuya	Uganda	4	W	V	3
X. ruwenzoriensis[8]	R	108	Semliki Valley	Uganda	4	R	LB	3

[a] Recognized as new taxa by one of us (M.F.); [b] J. Tymowska, personal communication.

were washed 3 times by centrifugation at 2000 × g for 5 min, resuspended in 5 vol. of 50% cold amphibian Ringer solution (6.6 g NaCl, 0.15 g KCl, 0.15 g $CaCl_2$ in 1 l distilled water adjusted to pH 7.8 with $NaHCO_3$) and frozen at −20 °C. The erythrocytes could be stored over several weeks without alteration of the electrophoretic properties of their Hb. Red cell lysis, partial Hb purification and determination of Hb concentration were performed as described by Moss and Ingram[16]. Hb samples of 6 mg/ml in 50% Ringer solution were converted to cyanmetHb by addition of 0.2 vol. of 2% $K_3Fe(CN)_6$, 0.5% KCN, 0.1% $NaHCO_3$. These samples could not be stored for more than 12 h without alteration of their electrophoretic properties. 10–20 µl of a solution containing 6 µl of cyanmetHb, 25 µl of 50% Ringer, 20 µl of 0.1% bromphenol blue in 40% sucrose and 2 µl of 2-mercaptoethanol was incubated at room temperature for 30 min prior to electrophoresis.

Polyacrylamide gel electrophoresis. The polyacrylamide gel system of Davis[4] was used either in cylindrical or in slab gels. After electrophoresis, gels were stained for total protein with amido black or with Coomassie Blue G250 (Serva) and destained in 7% acetic acid. Hb specific staining was performed with benzidine as described by Moss and Ingram[16].

Isoelectric focusing. Electrofocusing was performed on ready-made thin-layer LKB Ampholine polyacrylamide gel plates with a pH range of 3.5–9.5. (LKB-1804, LKB, Sweden), using the LKB 2117 Multiphor apparatus. 10–15 µl of a solution containing 10 µl cyanmetHb (5 mg/ml), 40 µl of 50% Ringer and 3 µl of 2-mercaptoethanol was incubated for 30 min at room temperature prior to focusing. After focusing and determination of the pH gradient, gels were fixed, stained with Coomassie blue, destained, dehydrated in a glycerol solution and dried according to the producer's instructions (LKB, Sweden).

Results.

1. *Hb patterns in different species.* The Hb patterns of the following species were compared: *X. tropicalis* (2n = 20); *X. epitropicalis* (2n = 40); *X. muelleri, X. species nova VI, X. borealis, X. clivii, X. fraseri, X. sp. n. III* (2n = 36); *X. vestitus, X. amieti, X. wittei* (2n = 72) and *X. ruwenzoriensis* (2n = 108). 1–3 major and some minor Hb bands can be detected for each species. *X. tropicalis* and *X. epitropicalis*, 2 closely related species[28], differ strikingly from one another by their Hb pattern (T and E in figs 1 and 2). The pattern of *X. tropicalis* is characterized by 3 weak bands in the most cathodal region of the gel, while its major Hb and the minor bands migrate to a position similar to those of most bands of the other species. In *X. epitropicalis* Hb migrates as a diffuse large band that cannot be resolved into distinct bands, even under intensified reducing conditions. *X. muelleri, X. sp. n. VI* and *X. borealis*, 3 other closely related species[18,19,25], exhibit great similarities in the major bands of their Hb patterns (M, SPN VI, and B in fig. 2). Yet, 2 of the 3 species display polymorphic Hb phenotypes (SPN VI and B1–B5 in fig. 2). *X. clivii* shows a pattern with 2 major and 5 minor bands (C in figs 1 and 2). *X. fraseri* has 1 Hb phenotype with 2 equally stained major bands and a 2nd one with 2 additional minor bands (F in figs 1 and 2). The Hb pattern of *X. sp. n. III* contains 2 major and 1 minor bands (SPN III in figs 1 and 2).

The Hb pattern of the 4 highly polyploid species with a chromosome number of either 72 or 108 are shown in figures 1 and 2. A marked species-specific pattern can be observed for each taxon. Polymorphic Hb patterns can be seen in *X. vestitus* and *X. wittei*. This polymorphism concerns either some quantitative differences between the common bands (*X. vestitus*, V in fig. 2) or the appearance of additional bands in some individuals as compared to others (*X. wittei*, W in fig. 2).

2. *Hb patterns in different subspecies.* The patterns of 6 subspecies of *X. laevis*, all with 2n = 36 chromosomes, were compared: *X. l. laevis, X. l. sudanensis, X. l. subspecies nova I, X. l. petersi, X. l. victorianus*, and *X. l. bunyoniensis*. A unique Hb pattern consisting of 2 major and 1 minor bands, resembling the pattern observed in *X. species nova III*, was found in all subspecies except in *X. l. bunyoniensis*. In this subspe-

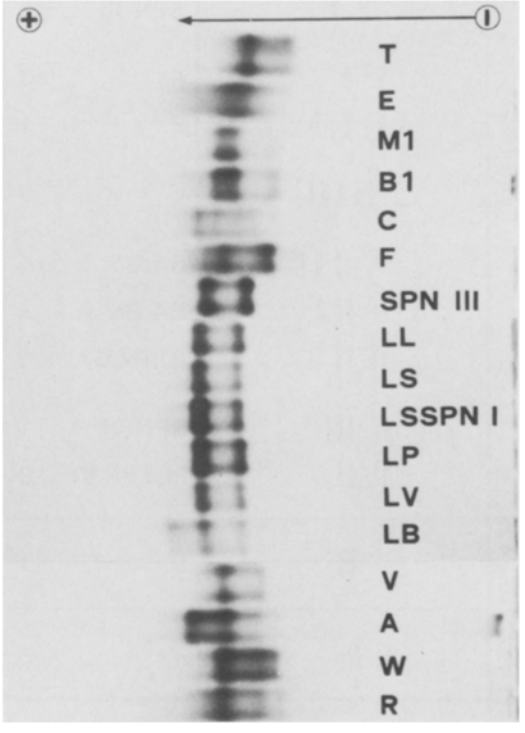

Figure 1. Individual Hb phenotypes of 17 *Xenopus* species and subspecies. Hb of all taxa was run on a 5.5% acrylamide slab gel. Abbreviations are the same as in the table.

cies an alternative pattern with 3 major bands was observed in some individuals (L and LB in fig. 2).

3. *Hb patterns in inter-species hybrids.* 15 inter-species *Xenopus* hybrids were analyzed (table). The results reveal that Hb patterns of all hybrid combinations represent the qualitative sum of the patterns of parental species without any evidence for the existence of hybrid molecules. Figures 3A and 3B show the pherograms of 4 representatives of the 15 analyzed hybrid combinations. A striking similarity can be observed between the pattern of the reciprocal hybrids of *X. muelleri* (M1) and *X. clivii* (C), named M1 × C and C × M1, and of *X. vestitus* (V) and *X. wittei* (W), named V × W and W × V. Furthermore these patterns are identical to those resulting from a mixture of Hb of the parental species (M1 + C and V + W), thus revealing a lack of hybrid molecules. In several hybrid combinations quantitative differences were observed between some electrophoretic bands of reciprocal hybrids (fig. 3B). These differences cannot be seen in the electrofocusing patterns (fig. 3D). To test whether

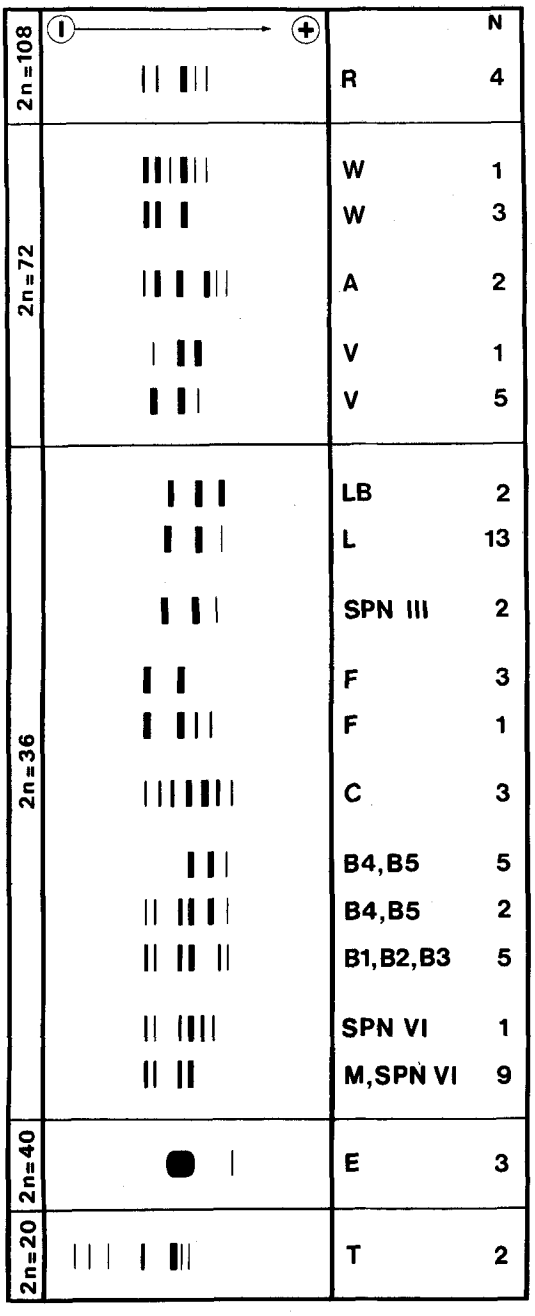

Figure 2. Graphic representation of the Hb patterns of the 18 *Xenopus* species and subspecies observed in 5.5% acrylamide slab and tube gels. Species abbreviations see table. 2n = diploid chromosome number; N = frequencies of the observed phenotypes.

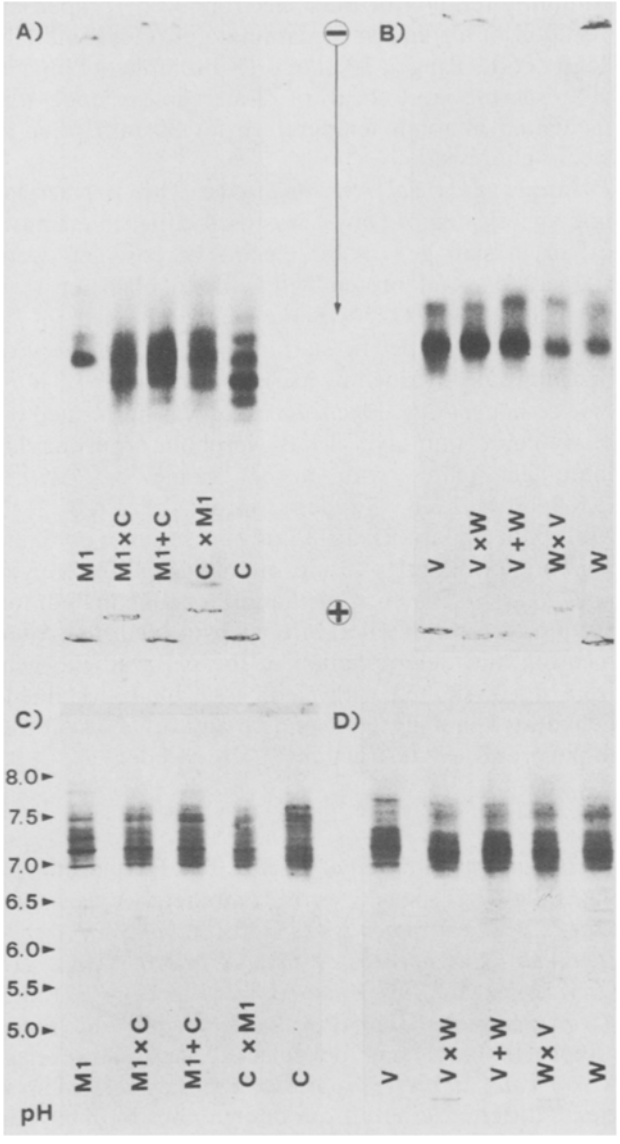

Figure 3. Comparison of the Hb patterns of reciprocal inter-species hybrids with those of their parental species.
A) and C): Ml and C Hb of *X. muelleri* and *X. clivii,* respectively. M1 + C mixed Hb of the two species. M1 × C and C × Ml Hb of their reciprocal hybrids.
B) and D): V and W Hb of *X. vestitus* and *X. wittei,* respectively. V + W mixed Hb of the two species. V × W and W × V Hb of their reciprocal hybrids.
A) and B) show electrophoretic patterns in 5.5% acrylamide tube gels. C) and D) represent the patterns after isoelectrofocusing.

the lack of chimeric Hb bands is due to limits in the resolving power of polyacrylamide gel electrophoresis, we separated the hybrids and their parental Hb by electrofocusing (figs 3C and 3D). The major Hb bands of all *Xenopus* species and inter-species hybrids are resolved within a pH range of 7.0–7.5. The isoelectrofocusing patterns of Hb of reciprocal hybrids (M1 × C and C × M1; V × W and W × V) and those of the mixture of Hb of the parental species (M1 + C; V + W) were identical and confirm the results obtained by gel electrophoresis. It must be noted that no correspondence can be established between the Hb bands obtained by electrophoresis and those obtained by isoelectrofocusing.

Discussion. The present study demonstrates that each species of the genus *Xenopus* can be identified by its specific Hb pattern and that each inter-species hybrid has a Hb pattern representing the sum of those of both parents.

The diversity of the Hb pattern corresponds to that reported for LDH isozymes[37] and for sperm nuclear proteins[14] among *Xenopus* species. Nevertheless, groups of closely related species have been defined with respect to their morphology[12,35], and to some cytogenetic characters, namely their karyotype[25,27], their nuclear DNA content[22], or the degree of bivalent formation during meiosis in inter-species hybrids[13,18]. How does the electrophoretic Hb pattern compare with these characters?

The diploid species, *X. tropicalis* (2n = 20), and the tetraploid one, *X. epitropicalis* (2n = 40), are closely related with respect to their morphology (unpublished results) and to their karyotypes[28]. By contrast, their Hb patterns and also their LDH isozyme patterns[36] are very different.

Among the different species and subspecies with 2n = 36 chromosomes, *X. muelleri* and *X. borealis* are closely related to each other with respect to their morphology[35], to their cytogenetic characters[18,22,25], to the antigenicity of the heavy chains of their immunoglobulins[10], as well as to their sperm nuclear proteins[14]. The similarity of their Hb patterns reported here also supports this close relationship. It seems that *X. sp. n. VI* is, with regard to its Hb, systematically very close to the 2 species mentioned above.

Cytogenetic analyses of *X. clivii* revealed a quite significant taxonomic distance between this species and other *Xenopus* taxa[18,22]. However, Tymowska[25] and Mann et al.[14] believe that *X. clivii, X. muelleri* and *X. borealis* may be related with regard to the localization of their nucleolar organizer regions and the resemblance of their sperm nuclear proteins respectively. However, the Hb pattern of *X. clivii* is completely different from those of any other species, confirming the rather large systematic distance between *X. clivii* and the rest of the genus.

X. fraseri differs from all other *Xenopus* species possessing less than 72 chromosomes with respect to its cytogenetic and biochemical characters[14,18,25,37]. The specific Hb pattern of *X. fraseri* will be discussed in relation to the highly polyploid species (see below).

The 6 subspecies of *X. laevis* can hardly be distinguished cytogenetically[18,19,22,25]. The similarity of their sperm nuclear proteins[14] and of their Hb patterns confirms the poor divergence of these subspecies.

X. species nova III is a newly discovered species that has not hitherto been described. Its phylogenetic relationship with other taxa of the genus is largely unknown. However, similarities between this species and the subspecies of *X. laevis* with respect to their sperm nuclear proteins[14] and to their Hb pattern indicate a close relationship between these taxa.

Amongst the 4 highly polyploid species, *X. vestitus* and *X. wittei* (2n = 72) are cytogenetically closely related to each other[13,22,27]. A close relationship between these 2 species is also supported by the resemblance of their sperm nuclear proteins[14]. The Hb patterns of these 2 species show, however, slight differences in the migration of all major bands.

X. amieti (2n = 72) and *X. ruwenzoriensis* (2n = 108) are very similar morphologically and both resemble *X. fraseri* (2n = 36)[12]. They also possess similar sperm nuclear proteins[14]. Yet the Hb patterns of these 3 species differ from each other and do not suggest a close relationship between them. These differences are not necessarily astonishing, as species with such different chromosome numbers most certainly possessed, at least initially, unequal numbers of globin genes and some of them may have diverged substantially during evolution.

The comparison of the Hb patterns in the genus *Xenopus* supports only a few of the phylogenetic relationships indicated by morphological and cytogenetic analysis of the different taxa. This observation confirms the opinion that morphological and molecular characters seem to evolve at different rates in anuran amphibians[33].

The Hb patterns of all inter-species hybrids are additive, i.e. they represent the qualitative sum of the parental patterns. It is obvious that the globin genes of both parents of a hybrid are expressed. Codominant Hb expression in adult inter-species hybrids has also been observed in the genus *Rana*[6,20,21] and in the genus *Bufo*[9]. Codominant gene expression in adults of several *Xenopus* hybrid combinations has also been reported for non-identified proteins[5] as well as for several isozymes[31,32,38]. In contrast to the electrophoretic pattern of dimeric or tetrameric isozymes the Hb pherograms of *Xenopus* hybrids contain no detectable chimeric molecules. Such molecules, containing globins of both parents, are supposed to exist in vivo but seem to be unstable during electrophoresis[15]. This

phenomenon may be one of the reasons why the Hb patterns of the highly polyploid *Xenopus* species (2n = 72 and 2n = 108), all supposed to be of allopolyploid origin, are not much more complex than those of most of the originally tetraploid species (2n = 36, see fig. 2). Alternatively, the absence of a highly complex pattern could be due to partial diploidization in the highly polyploid species. The mechanism seems to be responsible for the presence of only 2 functional nucleolar organizer regions per genome[25-29].

In order to get a better insight into the molecular variation and evolution of the Hb in the genus *Xenopus* a comparative electrophoretic study of the globins of the *Xenopus* species and subspecies is in progress.

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