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The fate of duplicated immunity genes in the dodecaploid *Xenopus ruwenzoriensis*

Louis Du Pasquier[1], Melanie Wilson[2], Benedicte Sammut[3]

[1]University of Basel, Department of Zoology and Evolutionary Biology University of Basel Vesalgasse 1 CH-4051 Basel Switzerland, [2]University of Mississippi Medical Center, Department of Microbiology University of Mississippi Medical Center2500 North State StreetJackson, MS 39216-4505, [3] Washington University School of medicine in St-Louis Department of bone and mineral diseases 660 South Euclid Avenue, Campus Box 8301 St. Louis, MO 63110

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1. ABSTRACT

The relatively recent dodecaploid *Xenopus (X.) ruwenzoriensis* (108 chromosomes) permits to catch the phenomenon of gene silencing or modification in the act. This is interesting for genes related to the immune system, many of which can be selected as a consequence of their utility or eliminated as a consequence of their cost. Among receptor genes, a trend toward diploidization is seen: neither T cell receptor, Immunoglobulin, or Major Histocompatibility Complex Class I and II and linked loci are present on all paralogs. The fate of linked Class I and Class II loci can be independent from one another. Six Class II beta sequences can be detected in heterozygous *X. laevis* and *X. ruwenzoriensis* but they are distributed on two (disomic) loci in *X. laevis* versus 6 (polysomic) loci in *X. ruwenzoriensis*. In the same two species 2 Class I sequences can be detected in heterozygous *X. laevis* versus 4 in *X. ruwenzoriensis*. One interpretation is that natural selection acts more on the number of genes than on the mode of inheritance (polysomic versus disomic).

2. INTRODUCTION

In 1970 Susumu Ohno proposed that the genome of vertebrates was the result of rounds of whole genome duplications that occurred during phylogeny. This bold proposition was originally formulated without any precise time points or strong molecular biology support. It is now well supported and has been refined based on data from analyses of many genome sequencing projects (1, 2, 3). The genome of vertebrates appears indeed to consist in sets of paralogous regions, which suggests that two rounds of duplication took place in the ancestors of the present gnathostomes. In modern vertebrates, it is rare to see the four straight sets of paralogs conserved because the duplicated genes had different fates. Some genes are kept, others are silenced or deleted, others are later duplicated once more, and the overall picture is far from clear. The fate of duplicated genes remains a question not easy to address since there are practically no models that would allow catching the phenomenon of gene silencing or conversion in the act. Still, series of polyploid species exist,

and among them perhaps the most well known is a series of clawed toads (South African frog) species that have recently undergone polyploidization, which allows for investigation of gene stability following diplo-tetra-octo- and dodecaploidization within the same genus or closely related ones (*Xenopus.*and *Silurana*).

As immunologists we want to ask specific question relative to the fate of duplicated immune genes. Why is there in general only one Major Histocompatibility Complex (MHC) in gnathostome vertebrates? What would happen if all T Cell Receptors (TCR) and immunoglobulin (Ig) genes were kept after polyploidization? What about allelic exclusion in polyploid animals? To date, several genes, including recombination-activating genes (RAG), Ig, MHC linked Class I and II, low molecular weight proteasome (LMP), transport associated proteins (TAP), have been followed in at least some of the polyploid *Xenopus* species, with frequent evidence for reduction to disomic inheritance (4). However, the dodecaploid *X. ruwenzoriensis* species, being among the most recent, are the ones that offer the best possibilities to observe the process of gene modification at an intermediary stage. The purpose of this chapter is to review the published data on RAG, Ig, TCR, and MHC linked genes in polyploid species and to present new material concerning the immune genes of *X. ruwenzoriensis*. Altogether we shall see that the number of genes that can be maintained matters more than the preservation or not of a mode of polysomic inheritance.

3. THE QUESTIONS

In vertebrates an increase in genome size could a priori create problems. Polyploid cells are usually bigger than the original diploid cells even if some cell size reduction occurs after the polyploidization event (5). Because *Xenopus* dodecaploid individuals are usually smaller than tetraploid species they will have fewer cells altogether. In the case of the immune system where lymphocyte clone size clearly is important for functionality and where each cell by virtue of allelic exclusion expresses only one receptor (Ig or TCR), this could lead to under expression of some receptor genes and to limited, perhaps too limited, cell numbers for the various specificities. Therefore, there may have been selective pressure to reduce the number of receptor genes during the evolution of the newly formed polyploids. In contrast, benefits may derived from the increase in genetic material that stems from polyploidization, which could lead to diverse gene families. In summary one can anticipate certain contradictory forces affecting the different immune genes in these species. In the case of antibody genes the question is "will all variable germline elements be expressed in a species having three times more genes but 40 times less cells?" as the situation is when comparing *X. laevis* (4n) and *X. ruwenzoriensis* (12n)" Perhaps the cell numbers in *X. ruwenzoriensis* lymphocyte clones will be too small to allow a given specificity to be efficient. For MHC Class I and II genes, one can also envision problems following duplications. First the necessity to become tolerant to one's self will create selection problems during the establishment of the immune repertoire in an allopolyploid species. For example, since polyploidization involves hybridization between different species, which have their own diverging MHC and TCR variable gene sequences. Therefore T cell education, especially when cell numbers are limited, could result in holes in the repertoire, i.e. many anti-self clones would be deleted during the phase of negative selection. In addition, even in the case of MHC where polymorphism is the rule and where many loci products are co-expressed on the cell surface, another potential problem could occur. In dodecaploid animals, assuming that all genes after duplication are conserved, cells could co-express in principle 12 original alleles for each component of the MHC Class I alpha and MHC Class II alpha and beta loci. Each of these allelic forms would then be endowed with a specific peptide binding capacity. Thus, it is possible that each form would be expressed on the cell surface at such a low density that a threshold of functionality could not be reached.

4. THE MODEL

4.1. The *Xenopus* polyploid species

The relationships among all of the *Xenopus* species, including their chromosome numbers, are presented in the chapter by Ben Evans in this special issue. The range from 2n to 12n is unique among vertebrates and especially interesting since the polyploidization events that generated the various genomes occurred over a relatively short period of time, approximately 60 million years. By opposition the vertebrates' specific 2R occurred probably much earlier: 400 to 450 million years ago (6). Therefore *Xenopus* with several relatively recent polyploidy species like the two dodecaploid ones might offer precisely the possibility to catch the phenomenon of gene silencing in the act, before the stabilization of the genomes. Another advantage of the *Xenopus* model is that laboratory generated polyploid individuals can be produced by species hybridization, providing a time 0 control. Such animals could in principle allow picking possible instant silencing (equivalent of Lyonisation) events (7). Moreover *Xenopus* is a large and convenient lab animal it also offers the possibility to study the expression of the concerned genes, of which we shall offer a couple of examples that are involved in humoral and cellular immune responses of these frogs.

4.2. *Xenopus ruwenzoriensis*

This species, in addition to the dodecaploid *X. longipes*, is a relatively recent polyploidy species that resulted from a set of allopolyploidizations (see chapter1). In other words its 108 chromosome represent a variation of the number 36 present in the *X. laevis* -like series. However, while two 12n species have been reported but only *X. ruwenzoriensis* has been studied somehow in detail. From earlier functional studies *X. ruwenzoriensis* appeared to have a larger antibody repertoire than the 4n *X. laevis* or the 2n *Silurana tropicalis* (4). On the other end *X. ruwenzoriensis* had kept several of its MHC genes under polysomic inheritance. Molecular analysis in the recent years have clarified or explained the molecular genetics behind this expression and functional data.

5. ANSWERS? GENES RELATED TO IMMUNITY IN A DODECAPLOID *XENOPUS*

The immunity genes presented in this review are all part of the adaptive arm of the *Xenopus* immune system that is characterized by the somatic generation of antigen receptors and their functioning in the context of antigen presentation by MHC encoded Class I and II molecule. The somatic rearrangement of Ig and TCR is mediated by the RAG-1 and RAG-2 enzyme, and the evolutionary fate of RAG 1 has been studied in X. *ruwenzoriensis* (8, 10).

5.1. RAG-1

RAG-1 and RAG-2 are the enzymes mediating the somatic rearrangement of T and B cell receptor genes. These genes have been cloned in *X. laevis* (9).From an immunological view point the molecular evolution of duplicated paralogous RAG-1 genes in many species of *Xenopus* could therefore be explored in order examine the fate of paralogs of this gene at the DNA level in terms of recombination, positive selection, and gene degeneration or deletion. Two major lineages of RAG-1 genes shave been identified in *Xenopus*– alpha and beta – and as expected only one copy of RAG-1, was found in the diploid *S. tropicalis* genome.

The next level of genomic complexity are the members of the tetraploid group where 2n =36 (or 40 in the *Silurana* lineage). The members of the *X. laevis*-like 4n typically have 2 RAG-1 loci, although in some species one paralog has degenerated. Moreover two differently sized RAG-1 mRNA transcripts, potentially derived from different paralogs, are indeed expressed in the thymus of *X. laevis* (9), and both may be functional at the protein level. Thus these non-degenerated RAG genes are candidates for redundancy, subfunctionalization, or novel function. Likewise, some octoploid *Xenopus* species exhibit evidence of pseudogenization of RAG1 paralogs. Altogether in the 2 dodecaploid species where up to 6 paralogs were expected, the number of non-degenerate paralogs suggested silencing of up to four in *X. longipes* and up to three in *X. ruwenzoriensis*. The RAG gene in *X. ruwenzoriensis* exists indeed as 4 copies (two pairs) corresponding to two major lineages alpha and beta that most likely correspond to some of the constituting haplotypes of the species, which retained their specific characteristic, i.e. in this case they did not homogenize each other by gene conversion following duplications. The alpha and beta lineages are always maintained regardless of the species and their ploidy number. The pair that disappeared must have been deleted or modified by introduction of stop codons and frameshift mutations as seen in other cases in some paralogs (8). Most interestingly (10) this degeneration occurred in paralogs inherited from only one of the diploid progenitor species. Other deletions have been reported for MHC, Ig and genes other than RAG (see below) and it would be interesting (even if difficult because of recombination) to see if those genes also disappeared from the same paralog out of which the RAG genes were deleted. Perhaps some of the constituting genomes in a polyploidy species are less compatible with one another and will be more prone to deletion in general than the other. It would also be interesting to monitor the species supposed to have participated in the speciation events for transposable elements. Such elements that could show strong species specific associations (M. Wilson L. Du Pasquier unpublished) could perhaps participate in selective elimination or displacements of genes as was suggested to explain certain aspects of the Ig heavy chain locus structure and function in fish (11).

From a functional point of view is there a selective advantage to keep two or more RAG loci? Is the presence of two messages in *X. laevis* an example of subfunctionalization? Is one RAG function not restricted to lymphocytes? After all a sea urchin, *Strongylocentrotus purpuratus*, form of RAG does not seem to be lymphocyte specific (12) and RAG1 message has been identified in the brain of the urodele amphibian *Pleurodeles* (13).

5.2. Immunoglobulin (Ig) heavy chain genes

X. ruwenzoriensis produces a visibly larger antibody heterogeneity in response to haptens such as DNP (14). One explanation could be that the number of functional Ig genes in this dodecaploid (12n) species is higher than in species of a lesser ploidy, especially relative to tetraploid (4n) species because polysomic inheritance of Ig genes has been maintained. So how many Immunoglobulin genes are still present in *X. ruwenzoriensis*?

5.2.1. Number of Ig loci

X. laevis and *gilli*, both tetraploid species, have a single Ig locus (15, 16) indicating that pseudogenization has occurred in a second locus that is predicted in a tetraploid species. In contrast *X. ruwenzoriensis* genome seems to be constituted of an assembly of genomes of such species. Therefore it is reasonable to assume that *X. ruwenzoriensis* could have 3 times the number of Ig loci as that of 4n species. In addition the relatively rare occurrence of multivalents at meiosis (17) lets anticipate a pairing of chromosome according to what happens in normal meiosis hence gamete composition used in Figures 3 and 5 and in tables 1 and 2 In a disomic tetraploid genome with no pseudogenization, one expects two loci, each with two alleles, to segregate independently. In a disomic dodecaploid genome with no pseudogenization, one expects six loci, each with two alleles, to segregate independently. This expectation in mind, one can examine the actual number of segregating alleles in *X. laevis* (a tetraploid) and *X. ruwenzoriensis* (a dodecaploid).

Southern blotting with IgM constant region and J region probes of haploid progenies of *X. ruwenzoriensis* females and regular families always show the deletion of one pair of Ig heavy (H) chain gene alleles, i.e. 2 pairs are always present not 6 as expected if the three expected loci were maintained (Figure 2). The presence of 2 Ig loci was also confirmed by *in situ* hybridization on chromosomes, albeit for Variable region genes of the VH1 family only (Figure 2 and ref 18). The polysomic inheritance of IgH chain loci in *X. ruwenzoriensis* may have to do with the relative young age of the species. Perhaps a diverse response is an advantage counterbalancing other forces that

Figure 1. Four species of clawed toads. *Silurana (Xenopus) tropicalis* (2n = 20 chromosomes), *Xenopus laevis* (2n = 36 chromosomes, *Xenopus wittei* (2n = 72) and *Xenopus ruwenzoriensis* (2n=108)

tend to reduce gene numbers in an organism after polyploidization. Such a counterbalance would result in longer time periods before reduction of the gene numbers. Evidence for this occurring comes from examining VH diversity patterns. Briefly, when VH gene banding patterns are compared among the different polyploid *X.*species, *X. ruwenzoriensis* always exhibited on the average a higher number of VH genes. However the number of bands was not directly proportional to the ploidy. *X. ruwenzoriensis* 12n never had twice as many VH genes as *X. laevis* 4n.

5.2.2. Mode of inheritance

Another way to examine the inheritance is *in situ* hybridization on chromosomes for all VH as in Figure 3, which clearly shows reduction of VH1 to disomy in 4n *X. laevis* and the loss of one pair of genes in *X. ruwenzoriensis* (18). However the technique can be technically challenging and it was developed after inheritance data had already been acquired by Mendelian segregation studies. Figure 3 represents some of such studies using haploid X. *ruwenzoriensis* tadpoles where the number of bands is reduced to one half as compared to a heterozygous wild animal, which simplifies the analysis (16). The Figure clearly shows the presence of 2 Ig heavy chain C mu loci in each haploid set of *X. ruwenzoriensis* chromosomes. For the VH5 family the gene number is surprisingly similar in 4n and 12n animals. With Hind III or EcoRI digestions 8-9 bands are detected on blots from heterozygous individuals, whether from *X. laevis*, (*X. laevis X. gilli*) LG7 hybrids (which are F1 hybrids), or *X. ruwenzoriensis* species. We are aware that the screening of VH numbers by southern may underestimate and be inaccurate. However orders of magnitude differences in estimating VH numbers were excluded when comparing southern blot estimation with actual sequences from the animal (16). Therefore the fact that *X. ruwenzoriensis* also contains 8-9 VH5 members (i.e. VH5 specific hybridizing bands to be precise), like LG7, reflects selection pressure exerted on the overall number of genes rather than on the mode of inheritance and it is clearly polysomic.

The other VH families were studied in *X. ruwenzoriensis* and analyzed for complexity and heritability by southern blotting. In terms of the putative deletions or conservations of genes, one could expect in the F1 from parents heterozygous at all loci a pattern of inheritance that follows the laws of monohybridism (1pair of alleles maintained, 4 Classes of segregants) dihybridism (two pairs of alleles maintained, 16 Classes of segregants) or trihybridism (3 pairs of alleles maintained, 64 Classes of segregants). In terms of the restriction enzymes and probes used, the patterns of segregation estimated by restriction fragment length polymorphism gave rise to different numbers of Classes, from 9 to 13, which is compatible with dihybridism (2 loci maintained, one deleted). Unfortunately without *in situ* hybridization, it is impossible to decide whether all the relatively complex patterns (some examples in Table 1) are due to dihybridism with high levels of recombination or to some trihybridism. Trihybridism would imply the presence of some orphan VH genes on chromosomes that have lost the C genes. Only VH specific *in situ* hybridization would resolve this issue. Most probably these orphan genes could not easily be used since they would involve rearrangement across their own chromosome. Their presence could be the result of the progressive disruption of the Ig heavy chain locus on one set of paralogs. The amount of recombination differed from one family to the other (Table 1). For example VH, 8, 11 did not show any recombination in the progenies whereas 1, 2, 3 (known to form a group of interspersed members (15,19) showed 12% ,VH 9 16%, 4, 6, and 7 25% and finally VH5 30% (table 1 and data not shown). If these

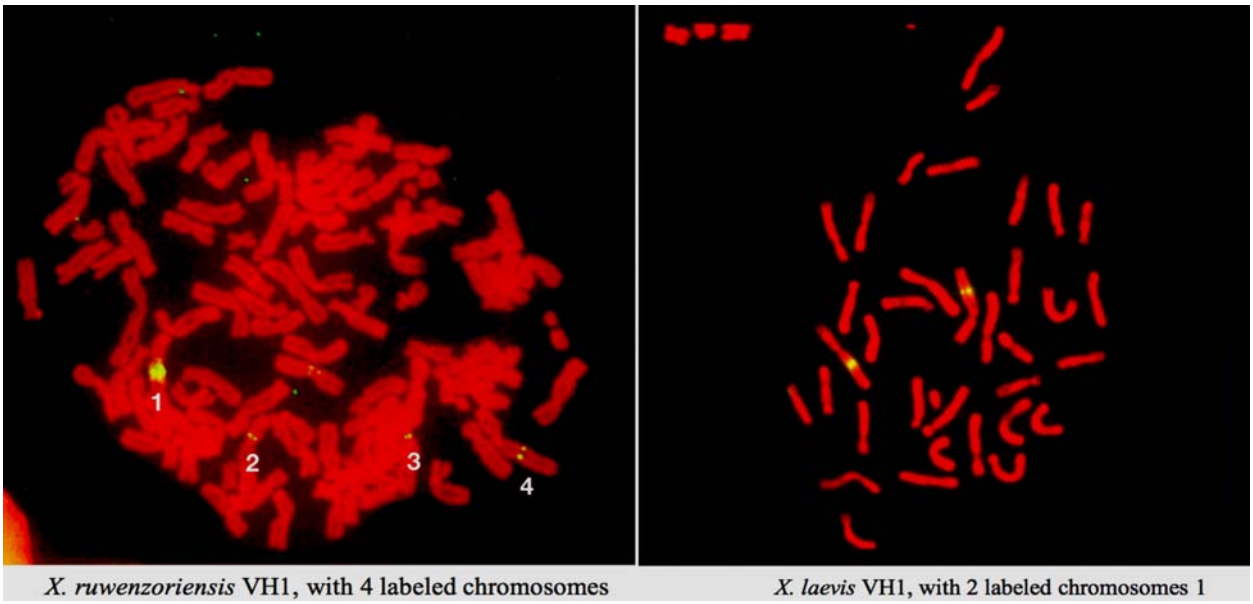

Figure 2. *Xenopus ruwenzoriensis* metaphase (left) stained with immunoglobulin VH1 probe. Compare with *Xenopus laevis* (right).

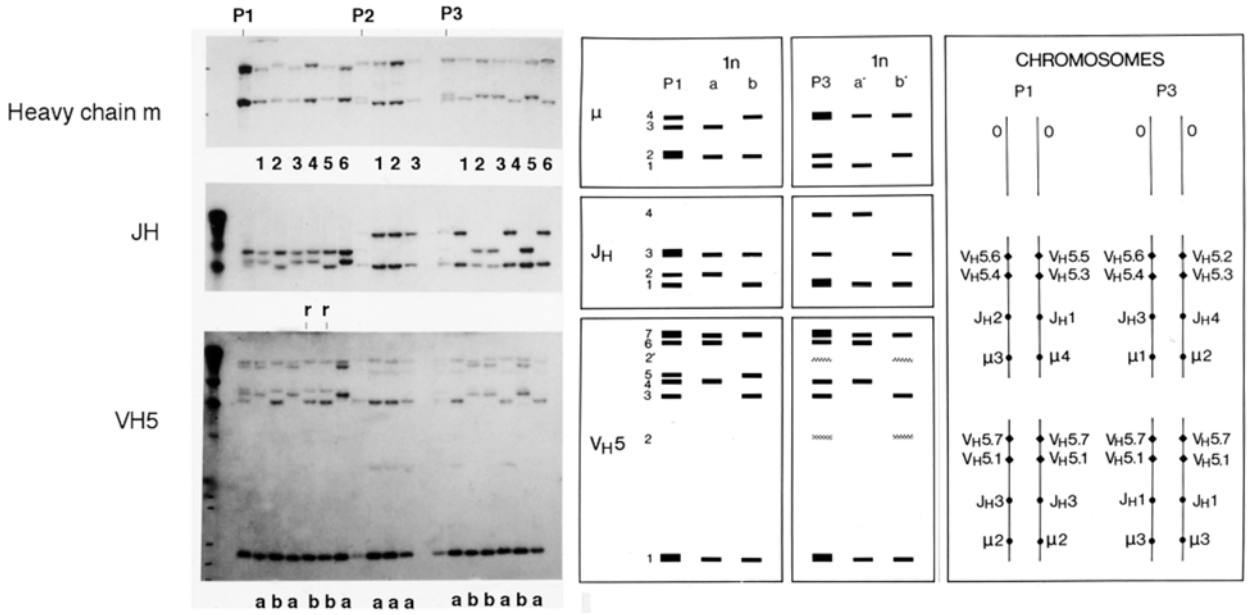

Figure 3. Immunoglobulin JH, Cmu, VH5 segments segregation in haploid progenies of *X. ruwenzoriensis*. Left: southern blot analysis three parental females P 1, 2, 3 were used. "r" indicates recombinants. (VH5 was the family with the highest percentage of recombination with respect to Cmu (see text). Numbers 1,2, 3 etc. correspond to sibs from each parent. Right: interpretation of the results for p1 and p3. The figures on the chromosomes correspond to the classification of the band on the right part of the panel. a b, a'b' *ruwenzoriensis* 1n (each contains 54 chromosomes) contents. Due to inbreeding in our colony and to the fact that our original *X. ruwenzoriensis* came all from the same narrow spot near Bundibugyo in Ouganda, the two animals used for segregation of haploid tadpoles were both homozygous at one of the pair of Ig carrying chromosome . Due to recombination the locus could be homozygous in some places and heterozygous in other as shown on figure for the relatively simple VH 5 family (r arrows). Southern blots have been monitored by densitometry to monitor homozygosity or heterozygosity at the constant region gene locus. The thick bands correspond to homozygosity.

variations were only due to recombination between the 2 complete conserved Ig heavy chain loci, they would give us an idea of the relative position of the different VH families relative to the C mu gene.

Table 1. Examples of VH gene segregation in a 24 sibs family of *X. ruwenzoriensis*. Southern blot analysis of RFLP data probed with VH specific probes

VH8	**Aa**	**Ab**	**Ba**	**Bc**
Ca	1, 7, 13, 21	4, 18	5, 10, 12	2, 15, 22,
Cd	9, 11, 16	17, 24	14	
Dc	8	20	3, 6	23
Dd	19			
VH 11	**Aa**	**Ab**	**Ba**	**Bb**
Ca	1, 4, 9, 11, 16, 24	18	5, 10	15, 22
Cb	17	13, 21	14	12
Da	8	20	3, 6	23
Db	19			
VH 1, 2, 3	**Aa**	**Ab**	**Ba**	**Bb**
Ca	1, 7, 13, 16	4	2	5, 10, 12,
Cb	9, 11			15, 22
Da	19, 20, ***23***		3, 6, ***14***, ***17***	
Db	8			
VH9	**Aa**	**Ab**	**Ba**	**Bb**
Ca	1, *5*, 7	*10*, 13, 21, *12*	2, 5, 22	
Cb	9, 11,16	24		
Da	8	20, *23*	3, 6	
Db	19			
Vh7	**Aa**	**Ab**	**Ba**	**Bb**
Ca	1, 5, 10, 12, 13, 21	4, 14, 17, 24	2, 18, 15, 22	
Cb	9, 11, 16, 20			
Da	8		23	
Db	19		3, 6	
6 /24				
C mu, J	**A**		**B**	
C	1, 4,7, 9, 11, 13, 16, 17, 18, 21, 24		2, 5, 10, 12, 15, 14, 22	
D	8, 19, 20		3, 6, 23	

If one accepts that the classical law according to which the percentage of recombination grows with the distance to the V mu gene, then theVH8 pattern is consistent with a polymorphic (RFLP) group of genes showing a segregation pattern consistent with dihybridism but no recombination. [2] For VH1, 2, 3 the pattern is compatible with this group of genes being relatively close to C mu with moderate recombination (14, 17, 23). For C and J the polymorphism did not permit to distinguish the 4 categories of gametes expected from the dihybridism. Hence, the presence of only 4 classes of segregants is found with these probes. See figure 3 for the distribution of the C, J and VH5 genes on chromosomes. The original linkage groups (i.e. those of J and mu) have been attributed a color, that allows to follow the recombination (italics) for each VH family.

Table 2. Distribution of class II determinants in progenies (54 chromosomes) of (*ruwenzoriensis*) x (*laevis*) hybrids

Parent *ruwenzoriensis* family A female 108 chr				Parent *laevis* family B male homozygous 36 chr	
1n *ruw.*	1n *ruw.*	Gametes *ruw.* Following independent sgregation	Constitution of hybrids 54 chr: 4 classes	1n *laevis*	1n *laevis*
4	4	412 415 435 432	f412 f415 f435 f432	f	f
1	3				
2	5				
Parent *ruwenzoriensis* family B male				Parent *laevis* family B female heterozygous	
1n *ruw*	1n *ruw*	Gametes *ruw.* Following independent sgregation	Constitution of hybrids: 54 chr: 16 classes	1n *laevis*	1n *laevis*
1	2	135 136 145 146 235 236 245 246	f135 f136 f145 f146 f235 f236 f245 f246 g135 g136 g145 g146 g235 g236 g245 g246	f	g
3	4				
6	5				

Explanation of gametes' constitution: - Family A: Numbers (1to 6) for *ruwenzoriensis* correspond to the various loci and their alleles with the same code as in figure 5 i.e. position of the respective bands on Southern blots; f and g are *laevis* MHC haplotypes. The f haplotype contains 3 class II beta loci (visible on figure 5 center: ff lane). The fact that MLR is under the control of one genetic region only in *laevis* was demonstrated in the ref (7). The dissection of this single region in class II beta loci DAB. DCB and DBB was published earlier (30) and the reduction to disomic inheritance of the MHC in *laevis* was demonstrated further in ref (18) by *in situ* hybridization. This family was also used in figure 5. - Family B : The family B was used in the published MLR experiment (7). Of the 16 possible classes, only 10 were found due to the small size of the progeny. However finding 10 MLR classes in the family B is incompatible with the presence of only 4 Class II beta paralogs like was found for Class Ia paralogs. This demonstrates the differential silencing (deletion) within the Major histocompatibility complex.

In summary, the average number of VH genes is higher in *X. ruwenzoriensis* than in S. *tropicalis* or *X. laevis* although the number is not strictly proportional to the number of conserved Ig loci. Polysomy is maintained in the VH genes based on the segregation pattern of VH genes in families. Functionally, more antibodies spectrotypes were detected in *X. ruwenzoriensis* (14) than in 2n or 4n species, suggesting that the Ig V region/cell number paradox mentioned earlier has been somehow not strong enough to hamper the immune capacity of the smallish *X. ruwenzoriensis*.

5.3. Allelic exclusion

Each B lymphocyte expresses a single species of antibody with a unique specificity via a process termed allelic exclusion. Two models have been proposed:

The regulated model of allelic exclusion proposes that Ig Light (L) chain V gene assembly must proceed on one chromosome at a time and that protein products from a functional Ig L rearrangement (one that encodes an Ig L chain that associates with the preexisting Ig H chain to make surface Ig) mediates allelic exclusion through feedback inhibition of further Ig L rearrangement (20).

The stochastic model, proposes that inefficient V (D) J rearrangement mediates allelic exclusion (21). V (D) J rearrangement would proceed simultaneously on both HC or LC alleles, but both would rarely be expressed in an individual B cell because the generation of functional joins occurs infrequently. However, such a model, in the absence of feedback, predicts no regulation; so both alleles should ultimately be rearranged in mature B cells.

In *X. ruwenzoriensis* that kept two heavy chain loci instead of one, we studied the expression of species-specific surface IgM in *X. ruwenzoriensis* or (*X. ruwenzoriensis)* x (*X. laevis)* hybrids. *Xenopus ruwenzoriensis* is a small species with relatively small number of large lymphocytes and a large number of genes. Allelic exclusion is indeed observed in *X. ruwenzoriensis*, lymphocytes do not produce all of the immunoglobulins encoded by the Ig genes present in its genome (22). There is an apparent lack of cells producing multiple Ig in polyploid hybrids having more than one pair of functional Ig genes. This suggests first that the selection pressure to make a lymphocyte synthesize only one antibody is high, and that allelic exclusion already existed in the ancestors of amphibians which appeared 300 million years ago. Second, if allelic exclusion follows the stochastic model, the frequency of multiple successful rearrangements has to be very low, resulting in a huge waste of lymphocyte precursors. Given selective pressure to rapidly develop an immune system in tadpoles, which have a very small number of lymphocytes (less than 10^5 at the onset of immunological competence (23), we argue that this latter explanation is less likely.

5.4. TCR

At the present, even though only preliminary Southern blot data are available from a limited number of species, there appears to be a strong pressure against the presence of more than one TCR beta locus. In *X. ruwenzoriensis* where more than one MHC (see below) and more than one Ig H loci persist. Southern blots data show the presence of only one TCR beta locus, which is consistent with the presence of one locus and two alleles. A similar result was also obtained with *X. longipes* (24).

5.5. MHC and MHC linked genes

Acute allograft rejection (a Class I mediated phenomenon) and the mixed lymphocyte reaction (MLR, a Class II mediated phenomenon) are under disomic inheritance in the few species of *Xenopus* tested, whether with 2n (S. *tropicalis*) 4n (*X. laevis*, *X. gilli*) or 8n chromosomes (*X. vestitus, X. amieti*). Therefore in most polyploid species one or more of the MHC genes must have been silenced. However In *X. ruwenzoriensis* 12n MLR is under polysomic inheritance. The size of the families obtained in *X. ruwenzoriensis* were always rather small and the segregation pattern did not allow us to determine whether all MHC genes were maintained or whether some had been silenced. Fortunately MLR in *laevis-ruwenzoriensis* hybrids, *in situ* hybridization southern blot and sequencing in *ruwenzoriensis* gave the final answer as regards the situation of MHC genes in this species (18, 25).

5.5.1. Class I genes

In *X. ruwenzoriensis* 4 Class Ia (Classical) haplotypes have been recognized (instead of 2 in *X. laevis*) in segregation analysis using southern blot and Class I alpha1 domain probes. Also several alleles have been sequenced in *X. ruwenzoriensis* that suggests a situation similar to RAG. However the Class Ia genes of *ruwenzoriensis* have been homogenized during evolution and all sequences recovered so far bear a new *X. ruwenzoriensis* specific marker: one deletion (25). So even though the locus is under polysomic inheritance, its diversity resembles more that of 4 alleles of the same product rather than the product of 2 different highly divergent lineages like in the case of LMP or TAP loci. As a consequence, for Class I alpha2/alpha3 domain all *X. ruwenzoriensis* alleles form a monophyletic cluster nested within a larger clade of *X. laevis* and *X. gilli* However *X. ruwenzoriensis* alpha1 domains are intermingled together with *X. laevis* sequences (26).

The fate of non-classical Class Ib has been monitored by *in situ* hybridization in S. *tropicalis*, *X. laevis*, and *X. ruwenzoriensis*, and the situation is similar to that of the classical Class Ia. Four loci are maintained in the dodecaploid *X. ruwenzoriensis*. At the time these studies were performed, Class Ib were the only Class I genes to be studied by *in situ* hybridization because Class Ib genes promote a strong signal because they exist under at least 10 20 copies per haploid genome. No single copy gene signal could be obtained in *Xenopus* by *in situ* hybridizations. Since then, however, single copy gene hybridization has been obtained by others, and more precise data could certainly be obtained now (27). Although not physically linked to Class Ia and to Class II the Class Ib genes are located on the same chromosome but far from one another (28 and Figure 4)

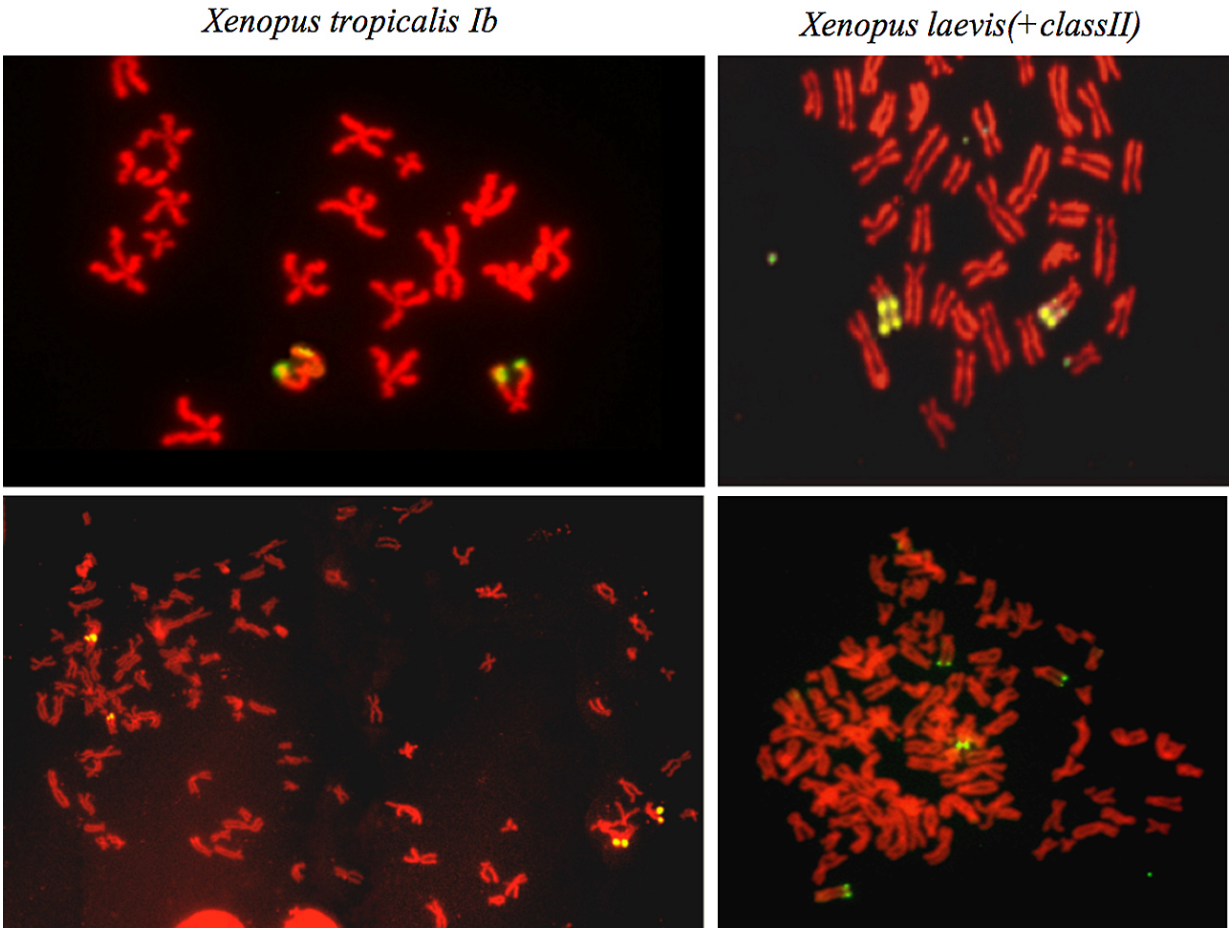

Figure 4. *In situ* hybridization with MHC Class Ib probes and double stain with Class II beta.

5.5.2. Class II genes

5.5.2.1. Segregation studies

In *X. ruwenzoriensis* the segregation of Class II genes has been followed exclusively on Southern blots by restriction fragment length polymorphism in family studies. An explanation of the distribution of Class II genes in *X. ruwenzoriensis*, *laevis* and their hybrids used in the experiments reported below is given in table 2. Functionally the segregation pattern of the Class II mediated Mixed Leukocyte Reaction (MLR) determinants in *Xenopus* families was consistent with at least ten MLR identical Classes in a progeny of a cross *laevis X. ruwenzoriensis* (ref. 7 family B).

This is compatible with some Class II MLR determinants being present on each of the 6 constituting MHC haplotypes of the original species, which is in contrast to the 4 observed for Class Ib (Figure 4). The functional test is crucial, since it adds the notion of functionality to that of the conservation of a gene segment that can be obtained by PCR or seen on a Southern blot.

This polysomic (i.e., presence of more than 2 alleles) inheritance suggested by MLR has been confirmed by southern blot hybridization using Class II beta specific probes (Figure 5). Family studies of (ff) x (*X. ruwenzoriensis)* progenies (family A) with Southern blotting clearly show that the 6 Class II band segregate independently from one another (5 are visible on the Figure because the parent was homozygous at one of the loci (see also table 2), i.e. the duplicated Class II bands are not linked like they are on the *X. laevis* haplotype each of which contains three loci. On the contrary the three different bands identified by domain specific hybridization and that correspond to the different DAB, DCB, DBB *X. laevis* Class II beta loci sequences (29) appear in the homozygous *X. laevis* ff strain. The 3 Class II beta loci all segregate together, and *in situ* hybridization proved that those loci are located in tandem on the single MHC chromosome pair present in the tetraploid *X. laevis* (Figure 4b).

5.5.2.2. Sequence data

When the *X. ruwenzoriensis* Class II beta genes (Figure 6) were compared to the published *X. laevis* sequences both species had different Class II beta loci fall into two main categories (Figure 7; 29): One branch with one locus DB, and one branch with two loci that duplicated later, DA/DC.

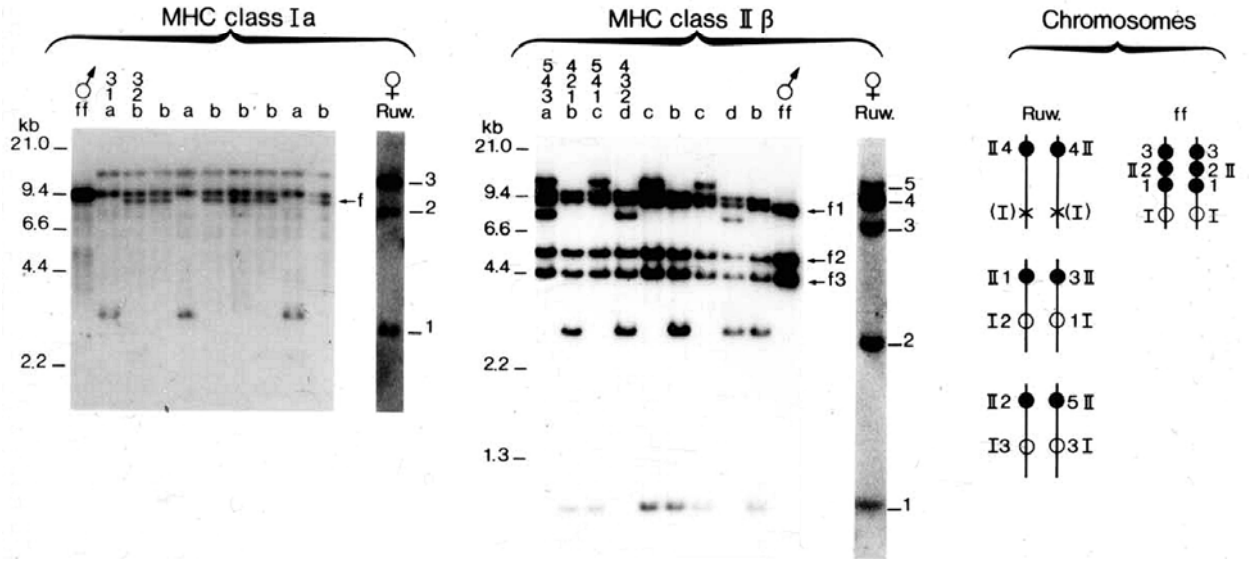

Figure 5. Segregation of Class Ia (left) and Class II beta (center) *ruwenzoriensis* genes in (*ff*) X (*ruwenzoriensis*) hybrids with chromosome attribution (right) fitting with the segregations patterns. Bands were given number in function of their molecular size starting from bottom to top. Due to the type of cross all individual possess the *f* haplotype only polymorphic *ruwenzoriensis* haplotypes segregate. *Ruwenzoriensis* parent was homozygous at one of the Class Ia loci (band 3, I3 in the chromosome diagram) and at one of the Class II beta loci (band 4, II4 in the chromosome diagram). See also table 2.

In *X. ruwenzoriensis* two major branches in the tree of Class II beta sequences are observed, like in *laevis*. One is easily recognizable as a homolog of DB the other one that is made of sequences tat are neither DA nor DC and where the bootstrap values are not significant. The final number of Class II beta genes recovered from a single heterozygous individual is 2 DB and 4 neither DA nor DC which represent 6 different sequences in heterozygous individuals, the same number as in a fully heterozygous *X. laevis*, but with the difference that the six genes are not in two groups of 3 loci but are present as one locus on each of the 6 paralogous chromosomes due to polyploidy. The comparison of Class II beta sequences (Figure 6) suggests an independent evolution of the *laevis* and *ruwenzoriensis* loci except for the DBB locus for which good *ruwenzoriensis* homologue exist. The main feature derived from the tree is that the A/C duplication within the second branch occurred after the speciation of the *laevis* and *ruwenzoriensis*. Everything looks as if the number of Class II beta "non-DB" loci was under a selective pressure to allow a certain amount of duplication but not too many. When comparing Class II gene number in other species the picture is similar Class II loci are often more duplicated than the Class I. A number close to 6 is perhaps an optimal one (see (30)). Similar to Class I the mode of inheritance apparently did not play an important role in the selection processes: polysomic or disomic could be used. The differences between the beta chain genes reside mainly in the beta 1 domain whereas the beta 2 sequence is highly conserved between *X. laevis* and *X. ruwenzoriensis*. A model compatible with the situation encountered is presented in Figure 8.

5.5.3. MHC linked LMP7genes

LMP7 is an (MHC)-encoded catalytic subunit of the 20S immunoproteasome, which is responsible for the production of antigenic peptides that are presented by the MHC Class I molecules. Two highly diverged allelic lineages of LMP7 characterized by eight diagnostic amino acid residues, termed LMP7A and LMP7B, have been identified previously in *X. laevis*. Of fourteen *Xenopus* and *Silurana* species, ten had both LMP7A and LMP7B, and the other 4 (*tropicalis, mulleri, borealis, amieti*) had only LMP7A. *Xenopus ruwenzoriensis* specimens are among those that can have the two LMP7A and LMP7B genes. Since *X. ruwenzoriensis* is believed to be derived from ancestors most closely related to *X. clivii* and *X. fraseri*, which diverged from *X. laevis* about 30 MYA, the primary structure of the LMP7A and LMP7B allelic lineages seems to have been conserved for at least 30 MY. Based on the high level of sequence divergence (31), and the apparent absence of balancing selection Hughes (32) concluded that this high degree of divergence between LMP7A and LMP7B alleles can only be explained by a recent introgression of one allele into *X. laevis* by hybridization with a distantly related species. However the observation in polyploid *Xenopus* rules out this explanation, since at least 11 species, some of which diverged more than 50 MYA, shared these two lineages (33).

5.5.4. TAPs

Two distinct lineages of Class Ia and LMP7 loci were previously identified, thus suggesting co-evolution among "Class I region" genes. This *Xenopus* MHC "Class I region" lies between Class II and Class III genes. There are two remarkably divergent distinct alleles at both the TAP1

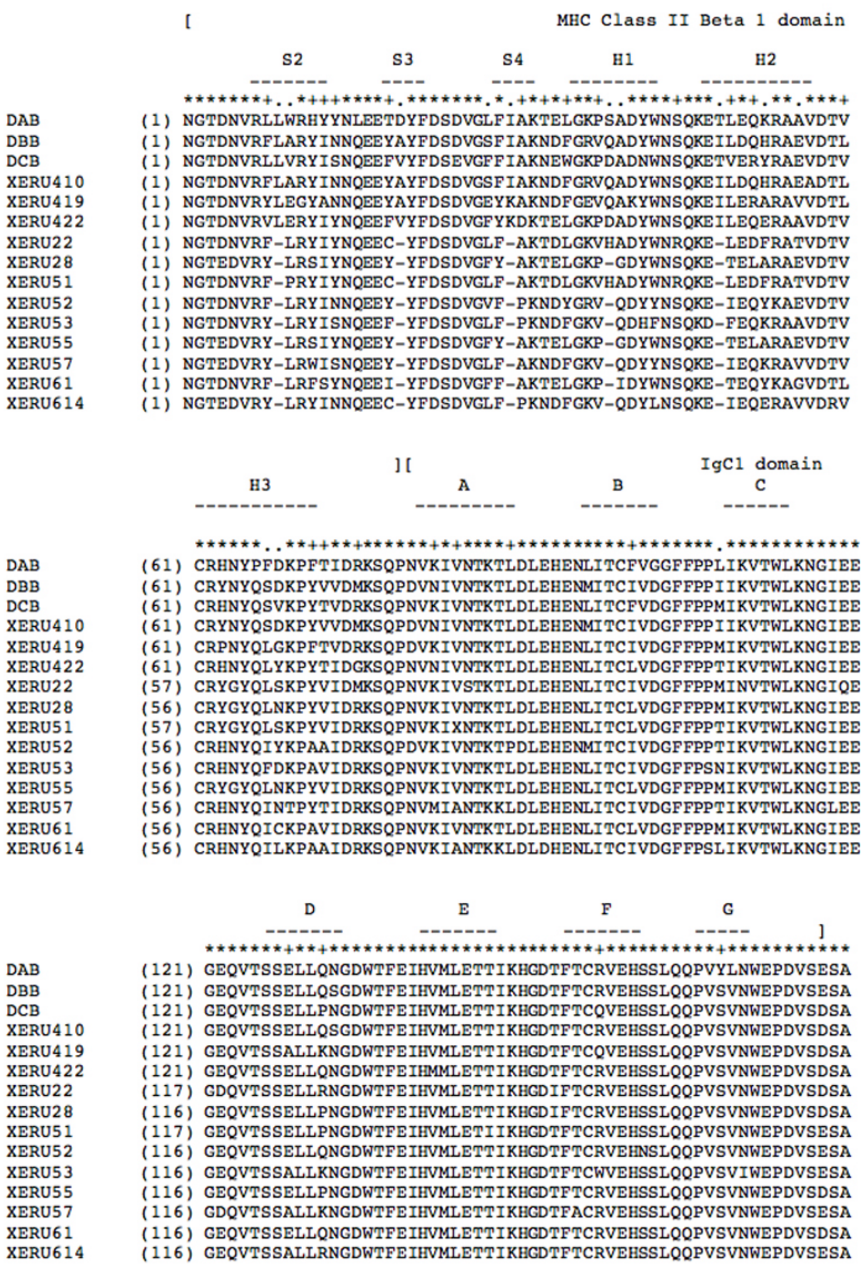

Figure 6. Alignment of *Xenopus laevis* and *ruwenzoriensis* Class II beta amino acid sequences. Predictions of domains, helices and strands have been superimposed. DAB, DBB, DCB sequences are from *X.laevis* , XERU, from *X. ruwenzoriensis.*

and TAP2 loci. They apparently diverged from each other 60-80 million years ago. The two lineages are present in different frequencies in *X. laevis* and *X. tropicalis*. Like the LMP7 lineages, the TAP lineages were detected in most *Xenopus* species that diverged from a common ancestor 50 MYA ago (34) suggesting that the "Class I region" biallelic lineages are under balancing selection. Similarly to LMP, two lineages of TAP1 and 2 were detected in the same panel of animals. These lineages seem to match those of LMP7 (35). The two lineages are found at the same frequency the number of hybridizing band did not increase with ploidy, except in *ruwenzoriensis*. However *X. longipes*, which like *X. ruwenzoriensis* is dodecaploid, show as many bands as *laevis*. Unfortunately, however, the sample size of *X. longipes* was limited to one individual.

5.5.5. CTX

That the deletion of MHC linked loci does not affect all loci at once is shown by the pattern of inheritance of the MHC loosely linked gene CTX that has been retained in all species and is a faithful indicator of ploidy. The maintenance of all copies of a gene in polyploid species especially in dodecaploid forms such as X *ruwenzoriensis* is a rare event. Among genes expressed by

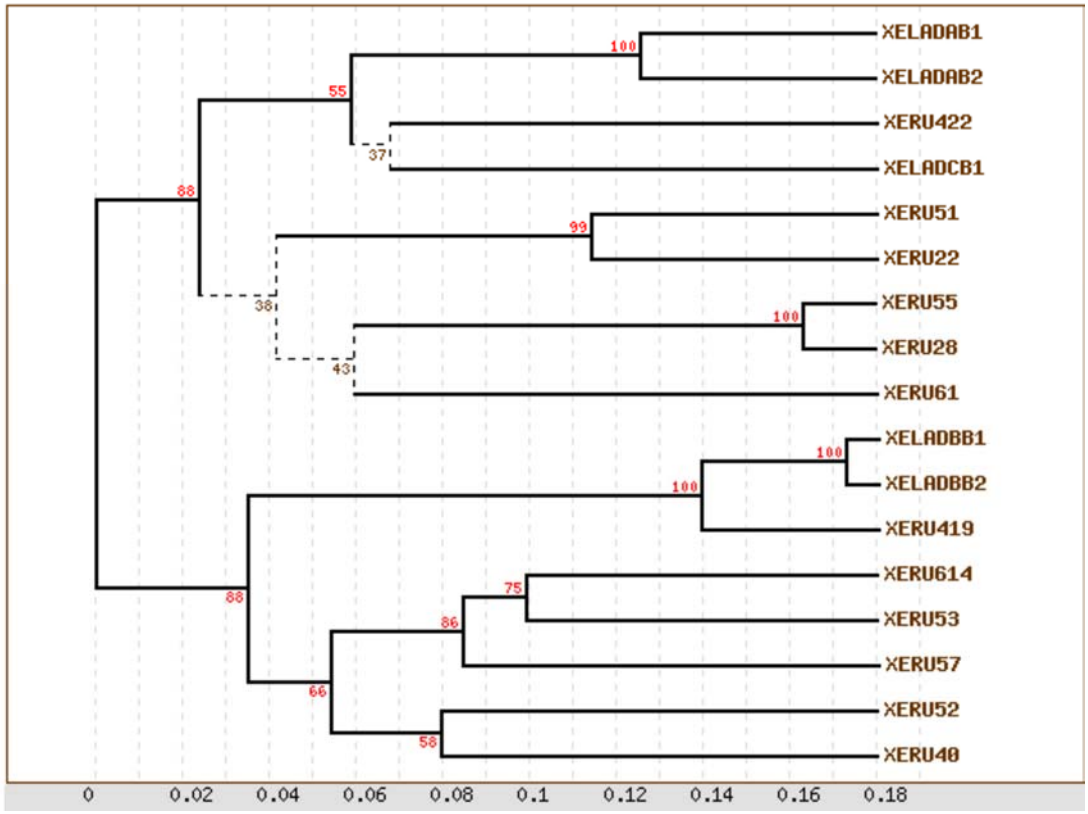

Figure 7. Phylogenetic relationships between *X. laevis* and *X. ruwenzoriensis* Class II beta chain loci DNA sequences (same origin as in the alignment of Fig 6). All sequences start in with O are from *X. laevis* DAB, DBB and DCB products (alleles) sequences starting witrh Xeru are from *Xenopus ruwenzoriensis*. Xeru 53, 614, 57, 410, 419 are homologous to DBB *laevis* sequences, whereas XeRU 22, 51, 28, 55, 422, 61 cannot be firmly reattached to either DAB or DCB lineages. Clustal alignment and neighbour joining tree were drawn with the genebee cluster algorithm found at http//:www.genebee.msu.su:genebee.html.

lymphocytes the only one that we encountered so far was that of the thymus cortical marker CTX whose genes are present on all chromosomes in *ruwenzoriensis* as suggested by southern blot analysis. However we do not know if all copies are functional (36) and confirmation by *in situ* chromosome hybridization has not been obtained. The fact that the can be expression of CTX in the gut and on the surface of thymocytes in tetraploid suggests this possibility. CTX is a family whose multiple members in mammals and birds have different tissue distributions and their genes are indeed located on paralogous chromosomes (chromosomes 3,11,21,1 in human (37). *S. tropicalis* has one CTX locus, *X. laevis* two, *X. ruwenzoriensis* 12, expression studies in these species should be quite interesting in term of potential subfunctionalization.

6. CONCLUSIONS

In the polyploid species *Xenopus ruwenzoriensis* the number of different gene sequences related to adaptive immunity was less than or equal to the number predicted by the ploidy. Deletions were noticed in several cases, in that sense that the relevant genes could not be identified any longer, by hybridization for instance. Deletions take time and can affect the different loci independently of one another.

6.1. Deletions take time

That deletion(s) takes time is indicated by the co-expression of all MHC linked genes in lab made polyploids upon which time has not had an opportunity to let selections happen. There was no Lyonisation for any the immunity genes examined. All histocompatibility antigens and the Class II determinants necessary for MLR and acute graft rejection were co expressed in those individuals (7). The antibody diversity of lab made polyploid was also larger than that of diploid (14).

A comparison among dodecaploid generated at different times during evolution would therefore be interesting in this context, unfortunately too little is known about the genesis of the 12 species to use them as model yet. However there exist already visible differences between *X. longipes* and *X. ruwenzoriensis*. If one trusts low stringency Southern blot hybridization with domain specific probes e.g. TCR, then *X. longipes* has distinctly fewer genes than *X. ruwenzoriensis*. This is also observed for MHC I and II and limited data obtained in those two species indeed suggest differences in the state of genome reduction. Again *in situ* hybridization could be developed further, provided live specimens could be kept and cell proliferation assays developed to get chromosomes. Also, all of the studies presented here are very preliminary and

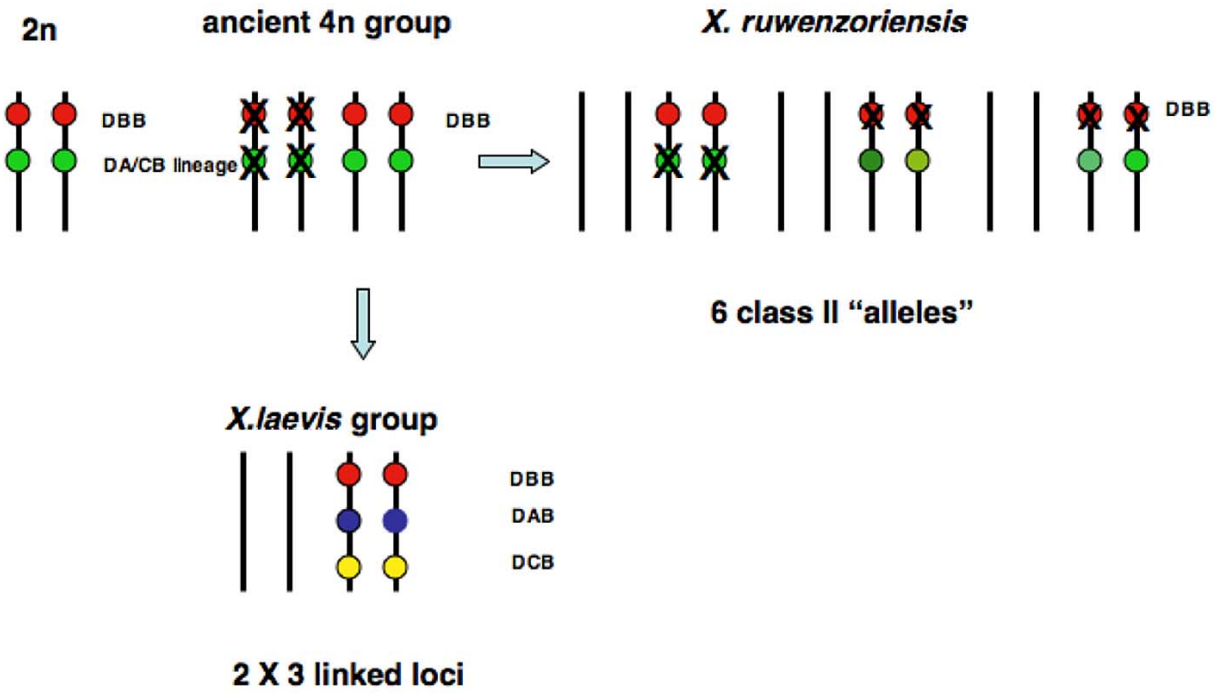

Figure 8. Model of duplications to explain the Class II beta situation in *X. ruwenzoriensis* and *laevis.*

should be complemented by polymorphism studies in populations. The fact that *X. ruwenzoriensis* is encountered in a single environment, that of the Semliki forest in Uganda when the other 12 n *X. longipes* is from lake Oku in the Cameroons could in principle allow interesting comparisons between different conditions of selection and their effects on two similar genotypes. A new field collection would probably be very rewarding.

6.2. Differential deletions

The Ig, TCR, MHC and linked loci have been submitted to differential gene silencing or deletion. The dodecaploid species *ruwenzoriensis* contains 12 MHC-linked CTX genes but only 4 Class Ia, 4 clusters of Class Ib and 6 Class II genes. Moreover the comparison of gene numbers across all the species of *Xenopus* suggests that the number of Class II beta loci was under strong selective pressure to maintain a specific number of gene copies at each locus. This number is also reached in *X. laevis* but following late tandem duplications. *X. ruwenzoriensis* turns out to be a key species because it shows intermediary stages in the history of different duplicated loci; neither complete reduction to disomic inheritance nor complete conservation of all the duplicated loci. It also shows that different immunity related loci have had different histories and what we observe now is probably the result of a temporary and perhaps fragile balance between exploiting diversity and dealing with the risks engendered by this diversity. The interesting issue of subfunctionalization (see addendum) of duplicated genes has to be envisaged for the genes that remain multiple in the polyploid species such as RAG-1 and CT*X.* Situations similar to those described for some enzymes (4) could occur also among immunity related genes. For the receptor genes showing multiple specificities, whether TCR, Ig or MHC Class I or II, as a rule the number of genes that is acceptable seems to be a factor more important than the modes of inheritance as illustrated by the case of Class II beta. Numbers may be in fact so important that two closely linked genes like Class II and Ia have had different fates following polyploidization. In order to further characterize the significance of the mode of inheritance of immune-related loci, much work remains to be done. Are two times three loci of a diploid a situation identical to 6 x 1 locus of a hexaploid in term of potential for evolution? To understand this aspect more alleles and loci should be studied in both types of *Xenopus*.

One could anticipate that the silencing and gene conversion often observed may work in the same direction in dealing with the problem linked to the greater number of genes in polyploid species. Bothe would reduce the number of epitopes. The reduction in number of epitopes is probably more important than the number of genes themselves and two times the same gene probably is different from two different genes. A consequence is that autopolyploidy may generate completely different conditions of selection with the same number of genes as those obtained by allopolyploidization. To what extent is the original genetic multiplicity and diversity due to the inheritance from different genomes maintained or transformed? Certainly multiple mechanisms are involved (38). Depending on which species participated in the allopolyploidization event, some haplotypes could come from species submitted to different environments and offer to the polyploid hybrid a pool of sequences with multiple advantages for the new species. For immunity related genes, is the observation made for RAG general, i.e. the preferential deletion within some of the subgenomes? One

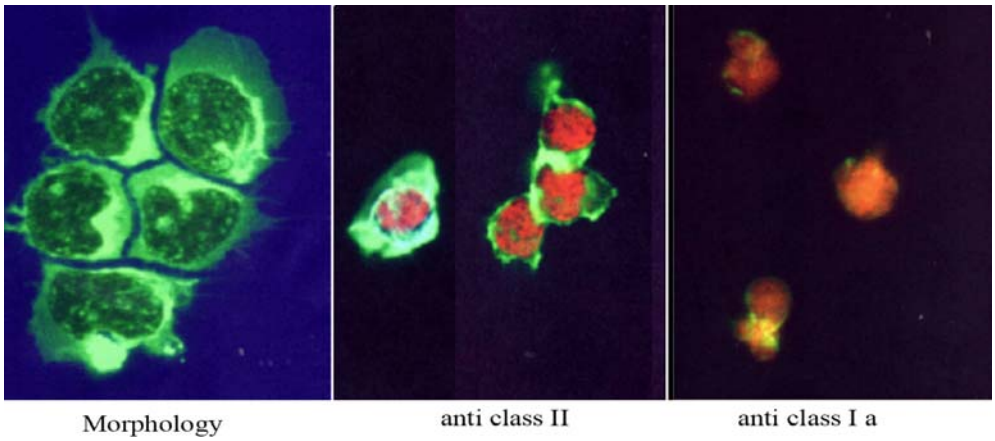

Figure 9. Morphology and immuno-staining of TOUF cells grown *in vitro*

environment might favor maintenance of diversity, another the selection of a given set of receptors. As an example of these possible fluctuations MHC Class I genes whether Classical or non-Classical apparently tend to get homogenized which probably reduces the functional heterogeneity. This might not be a general feature of Class I evolution since in another order of amphibians, the urodeles, a vast heterogeneity of Class I Classical-like genes are present (39). Interestingly the animals of this order have generally poor adaptive responses (40).

The independent fates of various immunity related genes that delicate balance which has to be reached between conservation and deletion, is perhaps one of the striking features that emerge from this review. However there is no rule that would apply to all MHC or all antigen specific receptor genes. Each immunity related gene (and probably each gene) is submitted to its own set of constraints and pressures and if, sometimes, analogous results are observed it might be due to very different reasons and mechanisms.

7. ADDENDUM

7.1. A case of subfunction? Over expression of a MHC Class II locus, DAB, in a *Xenopus* tumor of hemopoietic origin

Sub functionalization has been mentioned as one possible outcome of the fate of duplicated genes. *Xenopus* offers perhaps one such case.

In September 2000 a tumor of hemopoietic origin was detected in a male resulting from the cross of an outbred female with a male ff. Several lenticular nodules under the skin of the thigh were noticed and the spleen was enlarged spleen (1 cm in diameter) and all white. It looked very different from the thymic tumor reported earlier. The single cell suspension that was obtained yielded cells with the morphology of leukocytes. In the absence of a histocompatible host for transfer they were put in culture. What grew out and was called TOUF had the following phenotype: CTX -, CD8 -, Ig -,Class Ia +, Class II +++ (Figure 9).

The main characteristic of this cell line was the great intensity for class II staining, never seen before. Also, *in vitro*, the cells behaved in a way never seen before: they would remain at distance from one another and did not form a pellet even though they were not adherent.

The cells grew very slowly (5 generations in 6 days) compared to the other lymphoid tumors of *Xenopus* and never procured large number of cells. Transfer into bottle was impossible. Yet enough cells were harvested to make a cDNA library and the MHC class II expression was checked and all the three loci alleles were found expressed at the message level. Given the great difficulty encountered in the various MHC projects to obtain DAB messages from mixtures of lymphocytes populations, the above mentioned overexpression suggests a differential regulation of a specific class II in this cell lineage and perhaps a special subfunction for DAB one out of three *Xenopus* Class II beta loci.

Karyotype showed some euploid cells with 36 chromosome but also several cases of aneuploidy 35, 37 and 41 chromosomes.

The location of nodules in various place sin the body suggested that metastasis had already taken place when the tumor was noticed, but we have no formal proof that this was truly a tumor except the clonality of the class II products (6 sequences from the six loci ofwhich the 3 expected ff sequences from the parental). The homogeneity in phenotype and the possibility to derive cell lines *in vitro* can also be taken an argument in favor of the tumor nature of the TOUF cell line.

The over expression of class II message, especially DAB could be due to the deregulation occurring in a tumorous tissue. It could also be due to the fact that this was a tumor of a special cell type rarely encountered in normal tissues and that did not have the feature of a T or B cell. The nucleus morphology of the nucleus was reniform (Figure 1). They could correspond to monocytes.

Earlier report based on northern blots with 3'UTs of the three loci of *Xenopus laevis* concluded to a large expression of all loci in the hemopoietic tissue (ref 30). This was not confirmed by later cDNA analysis of spleen messages where almost exclusively DBB and DCB were detected (B. Sammut unpublished). In the earlier screening some confusion might have been caused by the sharing of some repetitive elements in the 3'UT regions of the probes used for screening northern blots. The discovery of a cell type like TOUF suggests a possible subfunctionilization of one duplicate of the beta locus: DAB. The duplication that generated DAB may not have to do with polyploidization, but similar evolutionary fate may have affected MHC genes duplicated in the various polyploidy species of *Xenopus.*

8. AKNOWLEDGMENTS

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Abbreviations: C (mu): constant region gene of immunoglobulin (of the mu isotype) , CTX: cortical thymocyte marker of Xenopus, D: diversity segment of immunoglobulin genes , H: heavy chain of Immunoglobulin, Ig: Immunoglobulin, J: joining segment of immunoglobulin gene, L: light chain of immunoglobulin, LG: (Xenopus laevis) x (Xenopus gilli) hybrid, LMP: low molecular weight proteasome , MHC: major histocompatibility complex , MLR: mixed lymphocyte reaction, RAG: recombination-activating genes , TAP: transport associated proteins, TCR: T-cell receptor, 2R: two rounds of duplication, V: variable immunoglobulin gene segment , VH: variable heavy chain gene

Key Words: Polyploidy, Major Histocompatibility Complex, Class II, Immunoglobulins, Amphibians, Chromosomes, Review

Send correspondence to: Louis Du Pasquier, University of Basel, Department of Zoology and Evolutionary Biology University of Basel Vesalgasse 1 CH-4051 Basel Switzerland, Tel: 0041612670367, Fax: 0041612670362, E-mail: dupasquier@dial.eunet.ch

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