HERPETOLOGICAL JOURNAL, Vol. 8, pp. 13-17 (1998)

MITOCHONDRIAL RDNA PHYLOGENY IN *XENOPUS*

H. R. KOBEL, B. BARANDUN AND CH. H. THIÉBAUD*

*Laboratoire de Génétique and * Département de Zoologie et de Biologie Animale, 154 Route de Malagnou, CH-1224 Chêne-Bougeries/Genève, Switzerland*

Based on morphological, biochemical and karyological characters, the genus *Xenopus* can be divided into two main groups (subgenera), *Silurana* and *Xenopus*, and the latter into five subgroups. The relationships among these five subgroups are less clear. Since all except one species are allopolyploid (tetra-, octo- and dodecaploid), they are, by definition, not monophyletic. In principle, sequence data would permit unravelling of these complex relationships, provided that all duplicated genes were conserved. However, that is not the case: redundant genetic information tends to become lost, interrupting phylogenetic lines of descent of the genes. Since the mitochondrial genome is inherited in a purely matrilinear manner, problems linked to polyploidy are seemingly avoided. However, this character is not monophyletic either. At least at the start of an allopolyploid speciation, mitochondria of both parental species can be present though one or the other type eventually becomes extinct. Which one is conserved is probably random. Nevertheless, it may be interesting to compare the phylogeny of mitochondria to species trees based on nuclear characters. We sequenced about 600 bp of mitochondrial 12s and 16s rRNA genes of the diploid *X. tropicalis*, of most tetraploid species, and of the octoploid *X. wittei*. Trees obtained with Neighbor Joining, Maximum Likelihood and Maximum Parsimony methods essentially confirm the *tropicalis, laevis* and *muelleri* groups and subgroups, whereas the *fraseri* subgroup is less well defined. Mitochondria of *X. clivii* and *X. largeni*, members of the *muelleri* and the *laevis* subgroup respectively, show only a low bootstrap score when connected to any subgroup, thus forming a polytomy of several species. Divergence of the same sequence between *Rana catesbeiana* and *R. temporaria*, for which immunological and zoogeographic considerations suggest a possible age of roughly 30-40 Ma, was used for tentative calibration of the *Xenopus* mitochondrial tree. This calibration is necessary for comparison with other phylogenetic data on this genus.

INTRODUCTION

Polyploid *Xenopus* are thought to have arisen through interspecific hybridization. The main evidence for this is that natural and laboratory-made hybrid females spontaneously produce endoreduplicated eggs in variable proportions. Backcrosses with both parental species may result in tetraploid animals (tetraploid with respect to the ploidy of the parental species). Both females and males occur in this F 3 generation, because the dominant female determining genetic mechanism of sex determination is abolished in favor of an environmental mode of sex determination in such experimental allopolyploids (Kobel, 1996).

Although *Xenopus* species can be assigned to several groups and subgroups (Fig. 1) as defined by morphological, karyological, biochemical and parasitological characteristics (reviewed by Kobel *et al.*, 1996; Tymowska, 1991; Graf, 1996; Tinsley, 1996), relationships between these groups remain obscure. In a few cases, hypothetical parental species or parental subgroups can be ascribed to higher polyploids (Fig. 1). However, for the three subgroups at the tetraploid level (4X=36), no diploid species survived that could be studied as potential parental species, though a number of such species certainly did occur at one time.

Theoretically, sequences of duplicated genes that are present in polyploids permit one to look back at dichotomies of extinct diploid parental species. Thus, sequence divergence between 17 duplicated genes of *X. l. laevis* suggests an age of 27-35 Ma of its parental species as measured by the 80 Ma old divergence of the homologous genes between man and rodents (Hughes & Hughes, 1993). In an earlier comparison of globin cDNA sequences (Knöchel *et al.*, 1986) within and between the diploid *X. tropicalis* and the tetraploid *X. borealis* and *X. l. laevis*, diploid and tetraploid species showed a very distant relationship. Whereas the diploid ancestors of the tetraploids diverged possibly 50 Ma ago, dichotomy of the two tetraploid species is thought to have occurred more recently (15-20 Ma) at the tetraploid level. However, the data probably better fit a model comprising three diploid ancestral species and two separate allopolyploidization events as origin of the two tetraploids. Hence, sequence data are not apt to distinguish speciation events between the different ploidy levels nor do they contain information on the allopolyploidization events themselves. Nevertheless, sequence data are valuable in providing insight into the speciation of the ancestral species of allopolyploids. Polyploidy also means redundancy of genetic information which is prone to loss of duplicated genes, thereby interrupting phylogenetic lines in a gene tree. It has been estimated that 25-50% of duplicated genes have been silenced in the tetraploid *X. l. laevis* (Graf & Kobel, 1991; Hughes & Hughes, 1993). The situation is worse in higher polyploids because their parental species might have already lost some duplicated genes

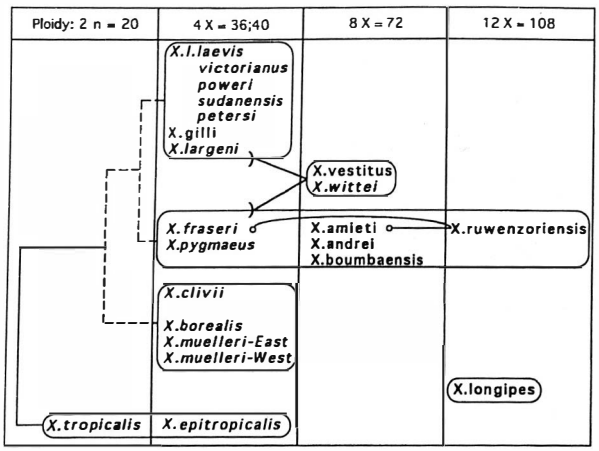

FIG. 1. Composition of the genus *Xenopus*. The species are arranged in groups of related taxa (Kobel *et al.*, 1996), and with respect to ploidy (Tymowska, 1991). The two main branches can be regarded as subgenera *(Xenopus*, *n*=9; *Silurana,* comprising *X. tropicalis and X. epitropicalis*, *n*=10). The tentative connections at the diploid level between tetraploids indicate that these groups descend from extinct diploid species of the subgenus *Xenopus*. The three subgroups might have originated from different hybrid combinations between the postulated diploid species. Species in italics were included in this study.

before the next round of allopolyploidization occurred (Kobel & Du Pasquier, 1986). Also, tetraploids of the various subgroups seem to have silenced genes in an independent manner, each species conserving its own collection of duplicated genes.

Sequences from the mitochondrial genome, which is inherited in a purely matrilinear manner, would seem to avoid problems linked to polyploidy. However, this character also is not monophyletic. At least at the start of an allopolyploidization, mitochondria of both parental species can be present, though one or the other type eventually will be lost. Which one is conserved is probably random. Consequently, different mitochondrial trees may fit equally well to a particular "true" species tree (Fig. 2).

A first attempt to unravel relationships between polyploid *Xenopus* species using mitochondrial genomes (Carr *et al.*, 1987), resulted in a number of contrasting trees of similar parsimony. The study revealed rather pronounced differences between restriction maps of the species, which prompted us to sequence parts of the mitochondrial 12s and 16s rRNA genes from 15 *Xenopus* taxa. Although mitochondrial gene trees, as stated above, do not necessarily parallel allopolyploid species trees, additional information of this kind contributes to an understanding of the complex reticulate evolution of this genus.

MATERIALS AND METHODS

ANIMALS

For the 15 species and subspecies of *Xenopus* analysed, see Fig. 1, names in italics. All animals were laboratory bred at the University of Geneva. Eggs of a wild caught *Rana temporaria* were also included in order to have, together with the published sequence of *R. catesbeiana* (Nagae *et al.*, 1988) a pair of *Rana* species for comparison and calibration.

CLONING

The mitochondrial genome of most *Xenopus* species contains only two Sac II restriction sites, delimiting a fragment of about 1700 bp that comprises parts of the 12s and 16s rRNA genes (Carr *et al.*, 1987). Mitochondria were isolated from eggs by differential centrifugation, and their DNA purified by proteinase K treatment and ethanol precipitation. After digestion with Sac II or Ksp I nucleases, fragments were inserted into Bluescript II KS vector at its Sac II site. Transformed DH 5a *E. coli* were selected and their vectors used to transform JM 105 strains in order to induce ssDNA for sequencing by the dideoxy method. All ma-

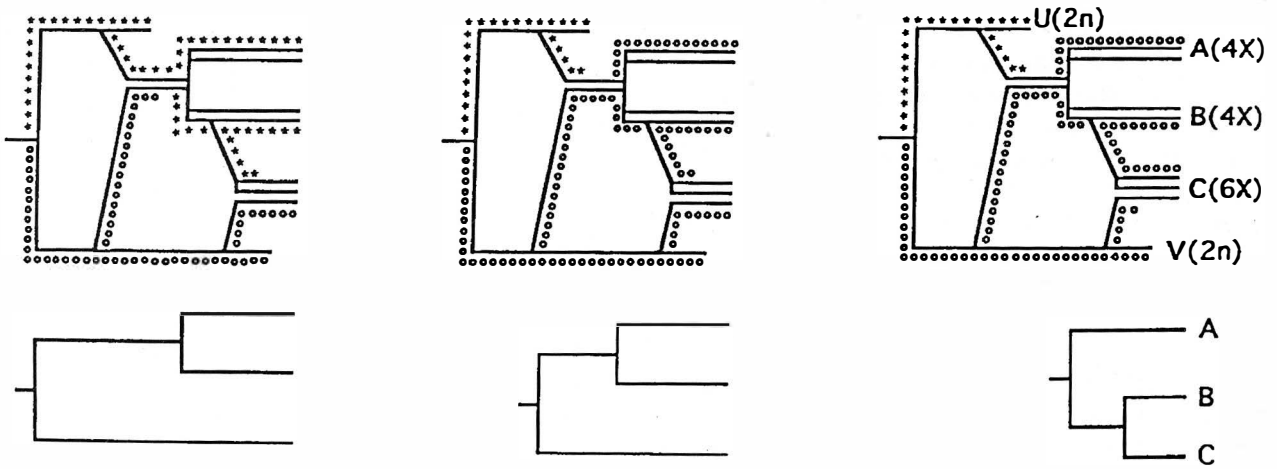

FIG. 2. Model trees demonstrating the limited parallelism between mitochondrial trees and the "true" species trees in reticulate evolution. Several contrasting mitochondrial trees may fit to the same species tree, because of the random loss of parental mitochondrial types. Upper row: — identical nuclear trees; ***and ooo mitochondrial lines. Second row : corresponding mitochondrial trees as inferred from sequence data; diploid species omitted.

nipulations were done following Sambrook *et al.* (1989).

SEQUENCE ANALYSIS

Sequences were aligned by hand, consulting secondary structure models for rRNAs (Dams *et al.*, 1988; Gutell & Fox, 1988; Springer & Douzery, 1996) and different alignments proposed by the Clustal V program (Higgins *et al.*, 1992). Trees were constructed by Neighbor Joining (NJ) (Saitou & Nei, 1987), Maximum Likelihood (ML) (Felsenstein, 1993) and Maximum Parsimony (MP) (Swofford, 1993), using the GDE 2. 2 software (Larson *et al.*, 1993). Robustness of the NJ and MP trees was estimated with 1000 bootstrap replications (Felsenstein, 1985).

RESULTS AND DISCUSSION

ALIGNMENT, VARIABLE SITES

About 600 bp of rDNA were sequenced; 12s : 2319-2618, 16s : 3742-4041 (bp numbering of Roe *et al.* 1985). Five bp (3907, 3976, 3997, 4044, 4028) could not be found in any species; on the other hand, two bp (2551+, 4035+) not listed there, were present in all species.

For most parts of the sequences, alignment is straightforward by introducing gaps where necessary. The consensus length, including *Rana* species, is 598 bp + 16 indels. Some species have shorter sequences, e.g. *X. wittei* 592 bp, *R. temporaria* 572 bp. However, for a few short stretches, especially the loops connecting stem 17 with 18 (Springer & Douzery, 1996), alignment remains ambiguous. These loops (18 bp and gaps) were therefore deleted from the analysis. Among the remaining 596 bp and gaps, 235 are variable sites (12s : 106, incl. 54 positions to accommodate for *Rana*; 16s : 129, incl. 48). Distribution of variable sites appears not to be random, e. g. more than 70% of the variable sites of the 12s fragment are found in the 5' half. Transitions are up to 3. 5x more frequent than transversions.

The secondary structure model for mammalian 12s (Springer & Douzery, 1996) is not applicable exactly to *Xenopus;* some helices contain more, others fewer potential stem nucleotides than the mammalian model shows. A number of compensatory replacements have occurred between *Xenopus* species as well as with respect to mammalian helices.

TOPOLOGY OF TREES

NJ (Fig. 3) and ML gave essentially the same mitochondrial rDNA trees. The subgenera *Silurana* and *Xenopus* are well separated. Within the latter, the *muelleri* subgroup represents the sister group to the remainding species, of which the *laevis* subspecies form a defined entity. However, neither *X. clivii* (*muelleri* subgroup) nor *X. largeni (laevis* subgroup) are placed with their respective subgroup (Fig. 1). Instead, these two species, together with the three species of the *fraseri* subgroup form an ill-defined cluster with very short common branches. Omitting the two species

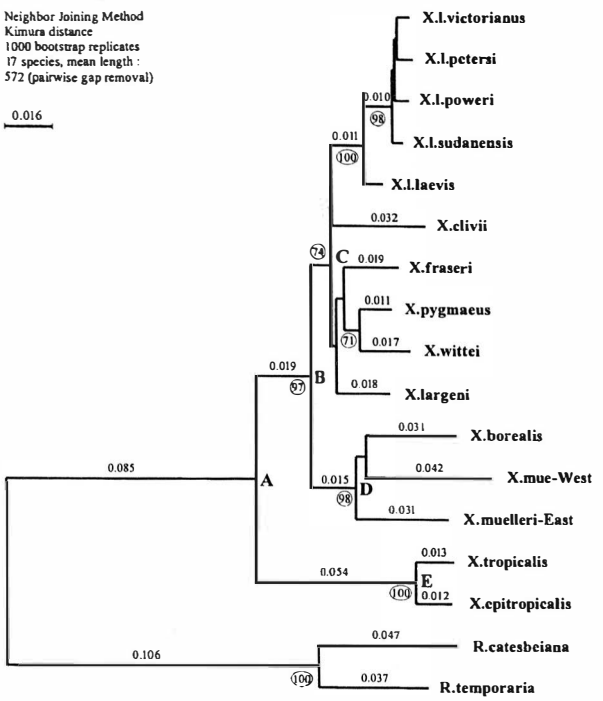

FIG. 3. Neighbor Joining tree with branch lengths in nucleotide replacements per site and bootstrap scores (circled, values above 70 only).

gives better support for the relationship [*muelleri* subgroup *((fraseri* subgroup) (*laevis* subgroup))]. MP, applying a bootstrap 50% majority-rule, places the five species together with the *laevis* branch as a polytomy, with the *muelleri* subgroup as sister group.

Within the *muelleri* subgroup, *X. muelleri*-East and *X. muelleri*-West show an amount of sequence divergence such that both have to be regarded as separate species, confirming evidence from other traits such as biochemical characters and parasite fauna (Tinsley, pers. comm.). *X. borealis* appears more closely related to *X. muelleri*-West, but their common branch is short, pointing to a trichotomy, i. e. a more or less simultaneous speciation. That *X. clivii* is not attached to this branch of the tree, is not a contradiction to the species grouping of Fig. 1. The four species could still have emerged from the same allopolyploidization event, *X. clivii* having conserved its mitochondrial type from the one parental species while the remaining species of this subgroup conserved the mitochondrial type of the other parent. The same argument holds also for *X. largeni*. This is a matter of interpretation since, as stated above, a mitochondrial gene tree shows only half of the parental relationships of allopolyploids.

The clade of the *laevis* subgroup comprises five taxa that are regarded as subspecies with *X. l. laevis* as the basal subspecies having diverged earlier than the other four. Therefore, it seems appropriate to upgrade the rank of this taxon to the species level. The remainder are so similar in their rDNA sequences that subspecies status seems adequate in spite of the distinctness in mating calls (Vigny, 1979) and genetic make-up (Graf, 1989). This contradictory situation demands further investigation.

How can the topology of these mitochondrial rDNA trees be interpreted? Since the sequence data contain no information about the ploidy level on which the various dichotomies occurred, one can only speculate. While there is no doubt that nodes A and E (Fig. 3) occurred at a diploid level, dichotomy B could have occurred either at a diploid or tetraploid level. In the former case, the two post-B branches would thus represent two parental species among an unknown number of diploid species, such as must be postulated to account for the duplicated nuclear genes of tetraploids. Sequences of duplicated genes of *X. l. laevis* (see below) possibly coalesce at a point which coincides with node B of the mitochondrial tree, enhancing the view that the two branches represent a diploid level indeed. Nodes C and D, on the other hand, are polytomies rather than dichotomies, a natural outcome if allopolyploidization triggered a burst of speciation. This is easily conceivable since allopolyploidy assembles adaptations of two parental species and creates genetic redundancy with which evolution may experiment. The emergence of polyploid *Xenopus* then likely took place somewhere between nodes B and C, D. How many polyploidization events occurred cannot be known. Also, more diploid species might have been implicated in allopolyploidization than appear in the tree, but for which mitochondria were lost.

Branch Lengths, Calibration

Branch lengths vary between species and between subgroups; *tropicalis* and *muelleri* groups together with *Rana* have 15% longer branches than *fraseri* and *laevis* subgroups.

Measured by a mammalian standard, sequence divergences in *Xenopus* are rather low, as is the case in some other non-mammalian vertebrates (Avise *et al.*, 1992; Martin *et al.*, 1992). In order to propose a more appropriate calibration, we collected similar data also for *Rana temporaria*. Comparison with the published sequence of *R. catesbeiana* (Nagae *et al.*, 1988) gives a dichotomy for which external evidence exists to determine age. First, it has been proposed (Duellman & Trueb, 1986) that *Rana* was transported by the drifting Indian subcontinent from Africa to Asia and arrived there about 34 Ma ago, from which the ancestors of the two species migrated in opposite directions to Europe and to North America. Second, immunological distances (Post & Uzzell, 1981; Uzzell, 1982) suggest an age of 33-43 Ma for the separation between these two branches. This gives an estimate of the substitution rate for these rDNA fragments of roughly 0.12% per Ma. Considering that this figure is based on only two species, this calibration cannot be more than tentative and has to be taken cautiously.

Using this value for *Rana*, separation between the two anuran families goes back about 130 Ma, which is an estimate that agrees with other evidence. Fossils of Pipidae are known from 120 Ma (Baz, 1996). Since these already show adaptations to an aquatic life style, the age of the family must be older. Mitochondrial rDNA sequences of extant *Xenopus* species coalesce at 48 Ma, contrasting with values of immunological distance (33 Ma; Bisbee *et al.*, 1977) and globins (110 Ma; Knöchel *et al.*, 1986). However, as demonstrated in Fig. 2, mitochondrial gene trees do not necessarily show the deepest nodes of species trees. In this figure, diploid species were omitted from the mitochondrial trees for the sake of simplicity. By including species V, for example in the third tree, it can be seen that the resulting tree does not show a coalescence point as deeply rooted as that of the duplicated nuclear genes of the polyploid species. Nevertheless, it seems unlikely that the *Silurana* line is directly implicated in the polyploidy of the subgenus *Xenopus*.

Node B (Fig. 3) has an age of 30 Ma, which is very close to the age (27-35 Ma) of 17 duplicated nuclear genes of the tetraploid *X. l. laevis* (Hughes & Hughes, 1993). The divergence between duplicated genes in an allotetraploid specifies the dichotomy that led to the two diploid parental species of that allotetraploid. Hence, the coincidence in the ages of mitochondria and nuclear genes strongly suggest that mitochondrial node B represents a dichotomy at the diploid level also. The first polyploid *Xenopus* species then would have originated posterior to node B. Nodes C and D represent polytomies rather than dichotomies. As discussed above, such bursts of speciation are not unexpected consequences of allopolyploidizations.

The youngest diploid node (10 Ma; node E) lies in the subgenus *Silurana*. If *X. epitropicalis* is also an allotetraploid, as differences within its chromosome quartets suggest (Tymowska, 1991), the dichotomy at node E then indicates the existence of a second diploid species. A relatively recent origin of *X. epitropicalis* can also be inferred from the presence of duplicates of almost all genes analysed so far in this species (unpublished; L. Du Pasquier, pers. comm.). This second species has not been found though not many localities have been sampled in the Congo rainforest. However, since only a single diploid species is known in the entire genus, one has to envisage the possible capability of allopolyploids to outcompete their diploid ancestors, and that the second diploid *tropicalis*-like species may be extinct.

CONCLUSION

The relatively short rDNA fragments yielded a surprising amount of information. Although mitochondrial gene trees do not necessarily parallel species trees in the case of allopolyploid (reticulate) speciation, the trees (NJ, ML, MP) confirm postulated subdivisions in the subgenus *Xenopus*. However, the similarity of the mitochondria of the *fraseri* and *laevis* subgroups indicates they may be more closely related to each other than was previously thought. Two other features in the trees are of special interest. Node B, by using a *Rana* mitochondrial calibration, appears to coincide with the coalescence point of duplicated genes of the tetraploid *X. l. laevis*. One may infer therefore that this deeper part

of the tree still represents a diploid level. On the contrary, the polytomies that follow could reflect bursts of speciation caused by allopolyploidization events.

ACKNOWLEDGEMENTS

We are indebted to MM J. Pawlowsky, J. Montaya and C. DeVarga for discussion and computer assistance, to M A. Solaro for animal care, as well as to the reviewers for stylistic improvements.

REFERENCES

Avise, J. C., Bowen, B. W., Lamb, T., Meylan, A. B. & Bermingham, E. (1992). Mitochondrial DNA evolution at a Turtle's pace: Evidence for low genetic variability and reduced microevolutionary rate in Testudines. *Mol. Biol. Evol.* **9**, 457-473.

Baéz, A. M. (1996). The fossil record of the Pipidae. In *The biology of Xenopus*, 391-401. Tinsley, R. C. & Kobel, H. R. (Eds). Oxford : Oxford University Press.

Bisbee, C. A., Baker, M. A., Wilson, A. C., Hadii-Azimi, J & Fischberg, M. (1977). Albumin phylogeny for clawed frogs (*Xenopus*). *Science* **195**, 785-787.

Carr, S. M., Brothers, A. J. & Wilson, A. (1987). Evolutionary inferences from restriction maps of mitochondrial DNA from 9 taxa of *Xenopus* frogs. *Evolution* **41**, 176-188.

Dams, E., Hendricks, L., Van de Peer, Y., Neefs, J-M., Smits, G., Van den Bempt, J. & De Wachter, R. (1988). Compilation of small ribosomal RNA sequences. *Nuc. Acids Res.* **16**, Supplement: r87-rl73.

Duellman, W. E. & Trueb, L. (1986). *Biology of Amphibians*. New York : McGraw-Hill.

Felsenstein, J. (1985). Confidence limits on phylogenies : An approach using the bootstrap. *Evolution* **39**, 783-791.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package, version 3. 5c.) Seattle: Dept. Genetics, University of Washington.

Graf, J-D. (1989). Genetic mapping in *Xenopus laevis*: Eight linkage groups established. *Genetics* **123**, 389-398.

Graf, J-D. & Kobel, H. R. (1991). Genetics of *Xenopus laevis*. In *Methods in Cell Biology*, vol. 36, 19-34. Kay, B. K. & Peng, H. B. (Eds). New York: Academic Press.

Graf, J-D. (1996). Molecular approaches to the phylogeny of *Xenopus*. In *The biology of Xenopus*, 379-389. Tinsley, R. C. & Kobel, H. R. (Eds.). Oxford: Oxford University Press.

Gutell, R. R. & Fox, G. E. (1988). A compilation of large subunit RNA sequences presented in a structural format. *Nuc. Acids Res.* **16**, Supplement: r175-r269.

Higgins, D. G., Bleasby, A. J. & Fuchs, R. (1992). CLUSTAL V: Improved software for multiple sequence alignment. *CABIOS* **8**, 189-191.

Hughes, M. K. & Hughes, E. (1993). Evolution of duplicate genes in a tetraploid animal, *Xenopus laevis*. *Mol. Biol. Evol.* **10**, 1360-1369.

Knöchel, W., Karge, E., Basner, A. & Meyerhof, W. (1986). Globin evolution in the genus *Xenopus*: Comparative analysis of cDNAs coding for adult globin polypeptides of *X. borealis* and *X. tropicalis*. *J. Molec. Evol.* **23**, 211-223.

Kobel, H. R. (1996). Allopolyploid speciation. In *The biology of Xenopus*, 391-401. Tinsley, R. C. & Kobel, H. R. (Eds). Oxford : Oxford University Press.

Kobel, H. R. & Du Pasquier, L. (1986). Genetics of polyploid *Xenopus*. *Trends Genet.* **2**, 310-315.

Kobel, H. R., Loumont, C. & Tinsley, R. C. (1996). The extant species. In *The biology of Xenopus*, 9-33. Tinsley, R. C. & Kobel, H. R. (Eds). Oxford : Oxford University Press.

Larson, N., Olsen, G. J., Maidak, B. L., McCaughey, M. L., Overbeek, R., Macke, T. J., Marsh, T. L. & Woese, C. R. (1993). The ribosomal database project. *Nuc. Acids Res.* **21**, 3021-3023.

Martin, A. P., Naylor, G. J. P. & Palumbi, S. R. (1992). Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. *Nature* **357**, 153-155.

Nagae, Y., Fujii, H., Yoneyama, Y., Goto, Y. & Okazaki, T. (1988). Nucleotide sequences of the *Rana catesbeiana* mitochondrial small (12s) and large (16s) ribosomal RNA genes. *Nuc. Acids Res.* **16**, 10363.

Post, T. & Uzzell, T. (1981). The relationship of *Rana sylvatica* and the mono-phyly of the *Rana boylii* group. *Syst. Zool.* **30**, 170-180.

Roe, B. A., Ma, D-P.,Wilson, R. K. & Wong, J. F-H. (1985). The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *J. Biol. Chem.* **260**, 759-9774.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning*, 2nd ed., New York : Cold Spring Harbor Lab. Press.

Saitou, N. & Nei, M. (1987). The neighbor joining method : a new method for constructing phylogenetic trees. *Molec. Biol. Evol.* **4**, 406-425.

Springer, M. S. & Douzery, E. (1996). Secondary structure and patterns of evolution among mammalian mitochondrial 12s RNA molecules. *J. Mol. Evol.* **43**, 357-373.

Swofford, D. L. (1993). PAUP: Phylogenetic Analysis Using Parsimony, version 3.1.1.Illinois Natural History Survey, Champaign, II.

Tinsley, R. C. (1996). Evolutionary inferences from host and parasite co-speciation. In *The biology of Xenopus*, 403-420. Tinsley, R. C. & Kobel, H. R. (Eds). Oxford : Oxford University Press.

Tymowska, J. (1991). Polyploidy and cytogenetic variation in frogs of the genus *Xenopus*. In *Amphibian cytogenetics and evolution*, 259-297. Green, D. M. & Sessions, S. K. (Eds). San Diego : Academic Press.

Uzzell, T. (1982). Immunological relationship of western Palearctic water frogs (Salientia : Ranidae). *Amphibia-Reptilia* **3**, 135-143.

Vigny, C. (1979). The mating calls of 12 species and subspecies of the genus *Xenopus* (Amphibia : Anura). *J. Zool.*, Lond. **188**, 103-122.

Accepted: 1.7.97