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Mitochondrial DNA insertion polymorphism and germ line heteroplasmy in the *Triturus cristatus* complex

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Restriction enzyme analysis of mitochondrial (mt) DNA isolated from oocytes of 185 individuals of the *T. cristatus* complex collected from 10 European countries has demonstrated that large length variation (>40 bp) is a common feature of the group. Insertion polymorphism was found both within and among populations, and in all cases maps to the control region of the molecule. In addition, 2 individuals from Pisa, (Italy) were each found to be heteroplasmic for 2 large insertions comprising tandem repeats of 1100 bp of the control region. Large-scale length variation has been described in a few other lower vertebrates, but some of the insertion variants within populations described here are of unprecedented size (up to 8500 bp). This is in dramatic contrast to mammalian mtDNA in which size variation is largely restricted to small (< 15 bp) insertions and deletions.

INTRODUCTION

Metazoan mitochondrial (mt) DNA has proved of great interest to evolutionary geneticists for several reasons. The supercoiled closed circular genome exhibits extreme economy of organisation, packing genes for 2 rRNAs, 22 tRNAs and 13 mRNAs into 15·7–19·5 kbp (Brown, 1985). The control region includes the D-loop, a triplex structure of essentially non-coding DNA containing a hybridised H-strand fragment representing replication initiation. It is flanked upstream by the gene for $tRNA^{Pro}$, and downstream by a non-coding region followed by the $tRNA^{Phe}$ and 12S rRNA genes. The size of the maternally inherited mt genome varies little across related taxa, and the gene map is highly conserved. It has been widely shown that mammalian (and possibly all chordate) mtDNA evolves some 5–10× faster than nuclear DNA in terms of the rate of base substitution (Miyata *et al.*, 1982; Cann *et al.*, 1984; Brown, 1985), making it a highly informative molecule for the analysis of population structure and closely related taxa (Avise *et al.*, 1979, 1983; Saunders *et al.*, 1986). Although evolution of the mt genome proceeds primarily by base substitution, with transition substitutions hugely outnumbering transversions (Brown, 1985; Hasegawa *et al.*, 1985), there is growing evidence that insertion/deletion polymorphism occurs in natural populations (see Discussion). It is also becoming clear that not all individuals are homoplasmic, *i.e.* homogeneous for the same mt genotype throughout all body tissues; results from some species of animals now show that organisms may contain and transmit heterogeneous populations of mitochondria (*e.g.*, Solignac *et al.*, 1984). These findings have relied largely upon insertion polymorphism to permit unambiguous demonstration of heteroplasmy, as it is difficult to distinguish heterogeneity for presence and absence of restriction enzyme site(s) from possible partial digestion of mtDNA.

This paper documents several insertion polymorphisms occurring in populations of the great crested newt, *Triturus cristatus*. Some of these insertions are particularly large, in one case a reiteration of over 50 per cent of the entire mt genome, resulting in the largest recorded animal mt genome. Two instances of heteroplasmy are described that result from 2- and 3-fold reiterations of 1100 bp of DNA in the control region of the molecule. This paper also includes a simple method for the isolation and restriction enzyme analysis of amphibian mtDNA.

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MATERIALS AND METHODS

Newts

Triturus cristatus is found throughout Europe with the exception of south-west France and the Iberian peninsula. Four races/subspecies are currently recognised (Thorn, 1968): *cristatus*, the more northerly and most widely distributed form; *carnifex*, found south of the Italian Alps in Italy and east to the Dinaric Alps; *dobrogicus*, the slender Danube valley form found from Vienna to the Black Sea; *karelinii*, the larger south-east European form found from the Balkans to the Caucasus. While each of these subspecies is typified by certain coloration, pattern and body form, morphological variation among populations is marked. In Yugoslavia in particular, the *carnifex / karelinii* transition is not clear cut (Kalezić and Stevanović, 1980; pers. obs.). However, the disparity of the four subspecies for chromosome (*e.g.* Bucci-Innocenti *et al.*, 1983; Macgregor and Sessions, 1986) and isozyme (Kalezić and Hedgecock, 1980; Frelow *et al.*, submitted; Rafiński and Arntzen, submitted) markers has led some authors to ascribe specific status. Some of the population samples from Yugoslavia and Greece are not clearly classifiable, and are referred to as "*carnifex*".

Newts were captured from ponds, ditches and streams by repeated sweeping with hand-held nets, and transported at 4°C. They were kept in aquaria and fed with chopped liver, earthworms and tubifex until required. Newts were immobilised in MS222 (ethyl m-aminobenzoate) solution for 10 min, rinsed, photographed and measured before dissection.

Mt DNA isolation

A procedure simplified from Lansman *et al.*, (1981) was developed to isolate amphibian oocyte mtDNA. Dissected ovaries were homogenised in 5 ml MSTC (210 mM mannitol, 70 mM sucrose, 50 mM tris-HCl *p*H 7·5, 3 mM $CaCl_2$) with 6–8 strokes of a Dounce homogeniser. 0·5 M EDTA *p*H 7·5 was added to 10 mM, and nuclei and debris were removed by spinning down at 800*g* for 2× 10 min. Mitochondria were then pelleted at 20,000*g* for 20 min, resuspended in 15 ml MSTE (MST, 10 mM EDTA), repelleted, resuspended in 2·3 ml STE (100 mM NaCl, 50 mM tris-HCl, 10 mM EDTA, *p*H 7·5) and lysed by the addition of 100 μl 20 per cent SDS. After adding 250 μg of RNAse A and 250 units of RNAse Tl to the lysate, it was incubated at 37°C for 30 min. 5 units of proteinase *K* were added and incubation continued for up to 2 hours. An equal volume of phenol/chloroform (100 g phenol, 100 ml $CHCl_3$, 0·1 g 8-hydroxyquinoline, 4 ml isoamyl alcohol; stored under 10 mM tris-HCl *p*H 7·5 at 4°C) was added to the lysate, and mixed by pipette. After centrifuging at 2000*g*, the extraction process was repeated twice, and residual mtDNA was back-extracted from the phenol phases with 3 ml ddH_2O. The pooled supernatants were dialysed against TE (4 mM tris-HCl, 0·5 mM EDTA; *p*H 8) overnight, then with fresh TE for 2 × 1 h. MtDNA was precipitated by adding 2 M sodium acetate to 0·2 M, mixing with 2 vol. of 100 per cent ethanol, cooling to −80°C, and spinning at 20,000*g* for 30 min. The pellet was resuspended in 3 × 100 μl ddH_2O and stored at −20°C. Amphibian oocytes are a particularly good source of mtDNA (Dawid and Blackler, 1972), this procedure yielding 1–3 μg mtDNA per gram of tissue.

Digestion with restriction endonucleases

Digestion of mtDNA was carried out under salt and temperature conditions appropriate to each enzyme as described by the manufacturer. Digests of 10–30 μl were done in 1·5 ml microfuge tubes, using 4 mM spermidine trihydrochloride and large excesses of restriction enzyme to enhance complete digestion. Multiple digests were done concurrently if salt conditions were compatible, or sequentially followed by levelling of salt concentrations before electrophoresis. The following restriction enzymes (recognition sites) were used: *Bgl*II (A/GATCT), *Cla*I (AT/CGAT), *Eco*RI (G/AATTC), *Eco*RV (GAT/ATC), *Hin*dIII (A/AGCTT), *Pst*I (CTGCA/G), *Pvu*II (CAG/CTG), *Sst*II (CCGC/GG), *Stu*I (AGG/CCT), *Xba*I (T/CTAGA), and *Xho*I (C/TCGAG). These were variously supplied by Amersham International, Bethesda Research Labs (BRL), New England Bio-Labs and Pharmacia. Reactions were stopped after at least 2 h by adding 1/10 volume of loading buffer (30 per cent Ficoll, 0·5 per cent sarcosyl, 0·1 per cent bromophenol blue, 0·1 M EDTA *p*H 8).

Agarose gel electrophoresis

Fragments were sized in horizontal gels of 0·45 per cent to 2 per cent agarose, depending upon fragment size. A continuous tris-borate buffer (89 mM tris, 73 mM boric acid, 2·5 mM EDTA; *p*H 8·2) was used with 0·7 μg/ml ethidium bromide added to the gel and anodal buffer.

20 cm × 20 cm gels with 2 rows of 33 wells were run at 4–5 V/cm for 2–4 h, and photographed over UV light using type 55 positive-negative Polaroid film. A variety of preheated λ digests and a 1 kbp ladder (BRL) were used to provide fragments of known sizes and to enable graphical sizing of mtDNA fragments. This versatile marker permits accurate sizing of fragments; perhaps ±1 per cent given optimal running conditions.

Restriction mapping

All of the enzymes used in this study recognise hexanucleotide sequences, and thus produce small numbers of fragments which expedites mapping. To avoid ambiguities, most of the possible pairwise combinations (double digests) were used.

RESULTS

Restriction fragment analysis

Using enzymes that linearise the mtDNA circle by cleaving once, the normal size of the *Triturus* mt genome was estimated to be 16·3 to 16·4 kbp. Linearised molecules were intermediate to the 15,721 bp *Bam*HI/*Eco*RI and 16,841 bp *Bam*HI λ fragments. *Xenopus* mtDNA linearised with *Pst*I was intermediate in size to the 17,053 bp *Kpn*I and 19,397 bp *Sma*I λ fragments.

The 185 mtDNA samples were each analysed using all 11 of the restriction enzymes. The samples displayed a variety of length variants visible in several fragments. Table 1 gives details of length variation for every population analysed, using a *Hin*dIII fragment delimited by two conserved sites across all taxa. Smaller fragments produced by other enzymes were used to size insertions within populations more accurately, but restriction site variation prevented comparison of these fragments across taxa. The values given for the smaller length variations should only be taken as approximations.

Two population samples of *cristatus* displayed insertion polymorphism; 3 newts from Mayenne site 2C9 had a 140 bp insertion (fig. 1) and 5 from Limanowa had a 45 bp insertion. For *carnifex*, newts supplied by "Xenopus Ltd." (probably from Milano) had mt genomes 140 bp larger than those from Acerra, Pisa, Firenze and Kramplje. One of these possessed a 100 bp insertion in addition to this difference, and 2 newts from Pisa were heteroplasmic for insertions of 2250 and 3350 bp (fig. 2). Populations of "*carnifex*" from eastern Yugoslavia south of Beograd possessed larger mt genomes

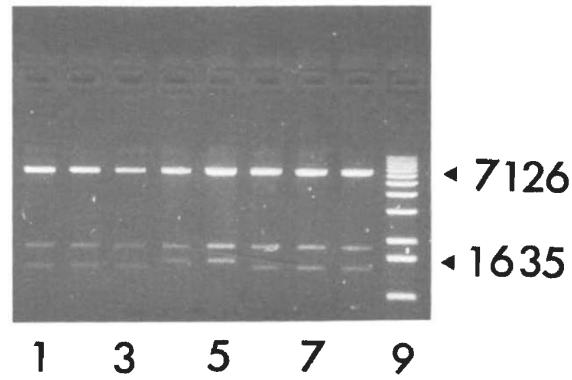

Figure 1 MtDNA of *T. c. cristatus* from Mayenne (France) digested with *Pvu*II and run on a 0·9 per cent agarose gel. Lanes 1–3, normal profile; 4–5, 140 bp insertion; 6–8, normal; 9, 1 kbp ladder marker (marker bands: 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1635, 1018, 516/506, 394, 344, 298, 220, 200, 154 and 142 bp).

than neighbouring western Yugoslav and Italian populations of *carnifex*, and showed considerable differentiation for restriction site variation (unpubl. data). Two newts from Trešnja possessed insertion polymorphisms of 100 and 140 bp in addition to the interpopulation difference, and three newts from Guberevac had 70 bp larger genomes (fig. 3). Some of the newts from Trešnja and Guberevac had slightly fuzzy control region bands which might indicate heteroplasmy for small continuously distributed insertions. Newts from Ano Kaliniki (just inside the northern border of Greece) were more like Italian *carnifex* populations for mtDNA restriction fragments profiles, and shared the *Hin*dIII fragment typical of those populations. MtDNA from one newt, however, contained extra bands for all digests revealing an insertion of unprecedented size, about 8500 bp, producing a mt genome of nearly 25 kbp (fig. 4). This is the largest recorded metazoan mt genome; Kessler and Avise (1985) mention large interspecific size differences in *Hyla*. The size of the *Hin*dIII fragment in *dobrogicus* was smaller than in the other subspecies, with no size variation observed.

Thus there is evidence for mtDNA length variation (1) among subspecies (2) among populations within subspecies (3) among individuals within populations (4) within individuals.

Restriction fragment mapping

The locations of insertions were determined by mapping representative *cristatus* and *carnifex* mt genomes (fig. 5); map positions of the *Stu*I sites have not been determined. Maps were aligned with

Table 1 MtDNA insertion polymorphism in *Triturus cristatus* sub-species as shown by the variable sized *Hin*dIII fragment

Taxon	Country	Population	*N*	Size
cristatus	England	nr. Peterborough	1	4000
		nr. Maidstone	2	4000
	France	St. Lô	1	4000
		Mayenne 314	11	4000
		Mayenne 431	15	4000
		Mayenne 2K5	4	4000
		Mayenne 2C9	7	4000
		Mayenne 2C9	3	4140
	Austria	Ottenstein	1	4000
	Poland	nr. Limanowa	5	4000
		nr. Limanowa	5	4045
	Romania	Tîrgoviste	3	4000
		Videle, nr. Bucharest	4	4000
		Sinaia	6	4000
		Cîmpeni, nr. Cluj	5	4000
		Vîrfuri, nr. Arad	3	4000
carnifex	Italy	"Xenopus"	10	4000
		"Xenopus"	1	4100
		Acerra, nr. Naples	2	3860
		Pisa	2	3860
		Pisa	2	6100+7200
		Firenze	5	3860
	Yugoslavia	Kramplje, nr. Ljubljana	6	3860
"*carnifex*"	Yugoslavia	Debrc, nr. Šabac	2	4000
		Trešnja, nr. Beograd	4	4000*
		Trešnja, nr. Beograd	1	4100*
		Trešnja, nr. Beograd	1	4140*
		Guberevac, nr. Kraljevo	1	4000
		Guberevac, nr. Kraljevo	3	4070*
		Grčak, nr. Kraljevo	4	4000
		Lučane, nr. Vranje	12	4000
	Greece	Ano Kaliniki, nr. Florina	7	3860
		Ano Kaliniki, nr. Florina	1	4950**
dobrogicus	Austria	Haidhof, nr. Baden	3	3860
		Kr.-Meiseldorf, nr. Horn	9	3860
	Yugoslavia	Podgorač, nr. Našice	1	3860
		Županja, nr. Vinkovci	4	3860
		Vršani, nr. Brčko	5	3860
		Glušci, nr. Šabac	1	3860
		Debrc	8	3860
		Beograd	6	3860
		Trešnja	3	3860
	Hungary	Öcsöd, nr. Szolnok	1	3860
	Romania	Zimnicea	1	3860
		Virfuri	1	3860
karelinii	Turkey	nr. Istanbul	2	4000

* fuzzy bands (see text)
** the ~8·5 kbp insertion in this sample included two *Hin*dIII sites, producing a double 3540 bp band also (see text)

the mammalian map using the two highly conserved *Sst*II sites, one occurring in each rRNA gene. These two sites delimit fragments of 1672 bp in *Homo* (Anderson *et al.*, 1981), 1688 bp in *Bos* (Anderson *et al.*, 1982), 1690 bp in *Mus* (Bibb *et al.*, 1981), 1700 bp in *Xenopus* (Cordonnier *et al.*, 1982) and 1750 bp in *Salmo* (Berg and Ferris, 1984). These correspond to a 1625 bp fragment in *Triturus*. The *Xenopus* mt genome is co-linear with the mammalian genome (Champagne *et al.*, 1984) and most probably with the caudate genome also; indeed, it seems likely that all vertebrate mt genomes are co-linear (Araya *et al.*, 1984). Orientation of the newt genomes against *Xenopus* was

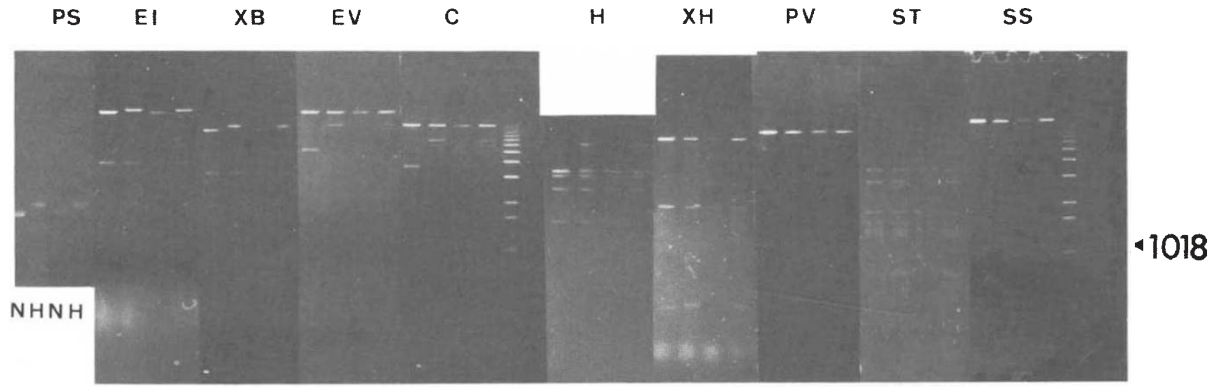

Figure 2 Composite of mtDNA of 4 *T. c. carnifex* individuals from Pisa (Italy) digested with a range of restriction enzymes and run on 0·8–0·9 per cent agarose gels. Each block of 4 comprises 2 normal (N) and 2 insertion heteroplasmic (H) individuals. Blocks are aligned by origin position; run times vary among blocks. Restriction enzymes used are (left to right): *Pst*I, *Eco*RI, *Xba*I, *Eco*RV, *Cla*I, *Hin*dIII, *Xho*I, *Pvu*II, *Stu*I and *Sst*II (see text for explanation). The 1 kpb marker (see fig. 1) is included for *Cla*I and *Sst*II.

determined by the conserved *Cla*I, *Hin*dIII and *Xba*I sites. Mt genome sizes of 17·4 kbp (Cordonnier *et al.*, 1982) and 17·7 kbp (Brown, 1983; Carr, 1983) have been reported for *Xenopus*. This is some 6–8 per cent larger than *Triturus*, a fact largely accounted for by the longer control region.

The *cristatus* insertions were visible in the *Eco*RV 3470, *Hin*dIII 4000 and *Pvu*II 1450 fragments containing the control region (fig. 1), and in the *Stu*I 3700 fragment.

All *carnifex* length variation was detectable in *Eco*RV 3330, *Hin*dIII 3860 and *Pvu*II 2080 fragments containing the control region, and in the *Stu*I 1710 fragment. The large insertions in the two newts from Pisa were visible in every digest (fig. 2). The large fragment was enlarged in the case of *Pst*I ~16,300, *Eco*RI ~13,600 and *Xba*I ~12,300 fragments. This was also the case for *Eco*RV 3330, *Cla*I 3700 and *Hin*dIII 3860, only here the two insertion length variants were clearly resolved. For *Xho*I, *Pvu*II, *Stu*I and *Sst*II, intense bands of 1100 bp were observed in addition to the usual profile. Hence it may be concluded that the insertions of 2250 and 3350 bp are tandem repeats of 1100 bp, each containing the *Xho*I, *Pvu*II and *Sst*II sites in the region of the origin of H-strand replication (fig. 5), and a *Stu*I site. One newt contained approximately equal proportions of the the 2 insertion variants in its oocyte mtDNA; in the other the 3350 bp insertion clearly predominated.

In "*carnifex*", length variation was evident in *Hin*dIII 4000, *Pvu*II 3300 and *Stu*I 3460 fragments (fig. 3), once again implicating the control region as the site of insertion. The newt from Ano Kaliniki with a particularly large insertion had large fragments *Bgl*II ~16,300, *Xho*I ~16,300 and *Eco*RI ~13,600 of greatly increased size (fig. 4). For *Pst*I, *Pvu*II, *Cla*I and *Eco*RV, there was a new fragment of ~8500 bp in addition to the normal profile. The other four enzymes gave more complex patterns each involving a single new fragment and duplicated normal fragments: *Sst*II gave a new ~6700 fragment and a double 1625 fragment, *Xba*I a new 4600 fragment and a double 4000 fragment, *Hin*dIII a new 4950 fragment and a double 3540 fragment, and *Stu*I a new 1440 fragment and double 3370, 1780 and 1710 fragments. Thus this insertion is a single duplication of the sequence of sites: *Pvu*II-*Eco*RV-*Hin*dIII-*Sst*II-*Cla*I-*Xba*I-*Sst*II-*Hin*dIII-*Pst*I-*Xba*I, including the 12S, 16S, N1 and N2 genes (fig. 5; this population does not have the *Xho*I and *Sst*II sites nearest the origin of H-strand replication, or the *Hin*dIII and *Pvu*II sites in the COI and COII genes).

None of the newts displaying intrapopulation insertion polymorphism, were in any way morphologically distinct from other members of that population.

DISCUSSION

In an extensive study of 112 humans, Cann and Wilson (1983) described 14 length variants ranging from −6 to +14 bp occurring in 9 regions of the mtDNA circle. Horai and Matsunaga (1986) examined 116 Japanese and found at least 4 variants of −7 to +14 bp, plus a possible 80 bp insertion. Additionally, every individual possessed a 60 bp insertion relative to the Cambridge sequence (Anderson *et al.*, 1981). In a study of 899 bp of the D-loop regions in 7 humans, Aquadro

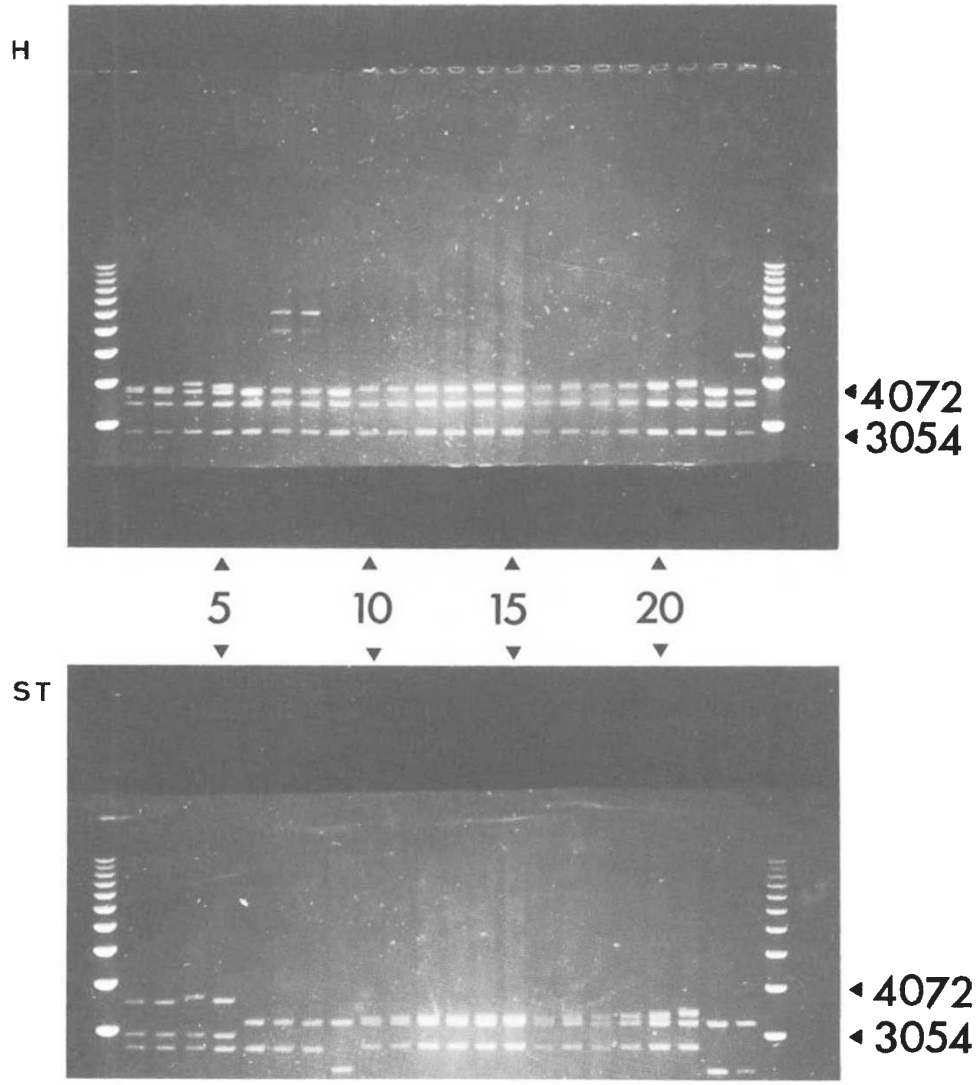

Figure 3 MtDNA of *T. cristatus subspp.* individuals digested with *Hin*dIII and *Stu*I, and run on 0·5 per cent agarose gels to show mobility differences due to variation in length of the control region fragments (see table 1). The 1 kbp marker (see fig. 1) is in the end lanes; other lanes are as follows, giving the size of the *Hin*dIII large fragment: *cristatus*: 2, 4000 bp Mayenne; 3, 4000 bp Limanowa; 4, 4140 bp Mayenne; 5, 4045 bp Limanowa. *carnifex*: 6, 3860 bp Pisa; 7–8, 6100+7200 bp Pisa; 9, 3860 bp Kramplje. "*carnifex*": 10, 4000 bp Lučane; 11, 4000 bp Grčak; 12–15, 4000 bp Trešnja; 16, 4000 bp Guberevac; 17–19, 4070 bp Guberevac; 20, 4100 bp Trešnja; 21, 4140 bp Trešnja; 22, 3860 bp Ano Kalinki; 23, 4950 bp Ano Kalinki. Fragments much smaller than 3000 bp have run off the gels.

and Greenberg (1983) and Greenberg *et al.* (1983) describe 5 small length variations amounting to nearly 9 per cent of the total variation observed. Brown and DesRosiers (1983) describe length variation in homopolymer runs of up to 4 bp between $tRNA^{Cys}$ and $tRNA^{Tyr}$ near the origin of L-strand replication in *Rattus*, and similar findings are reported in the D-loops of cows (Hauswirth *et al.*, 1984) and humans (Hauswirth and Clayton, 1985). A study of 208 individuals representing 8 species of *Mus* revelaed only one insertion variant: 12 bp (in or near the D-loop) in a single mouse (Ferris *et al.*, 1983). Surveys of 87 *Geomys pinetus* (Avise *et al.*, 1979) and 135 *Peromyscus maniculatus* (Lansman *et al.*, 1983), and a bird study (Kessler and Avise, 1985) have revealed no length variation polymorphism, although small variations could have gone undetected.

These findings contrast with those from insects, which possess a hypervariable A+T rich region overlapping the origin of replication (Clary and Wolstenholme, 1984) containing insertions of

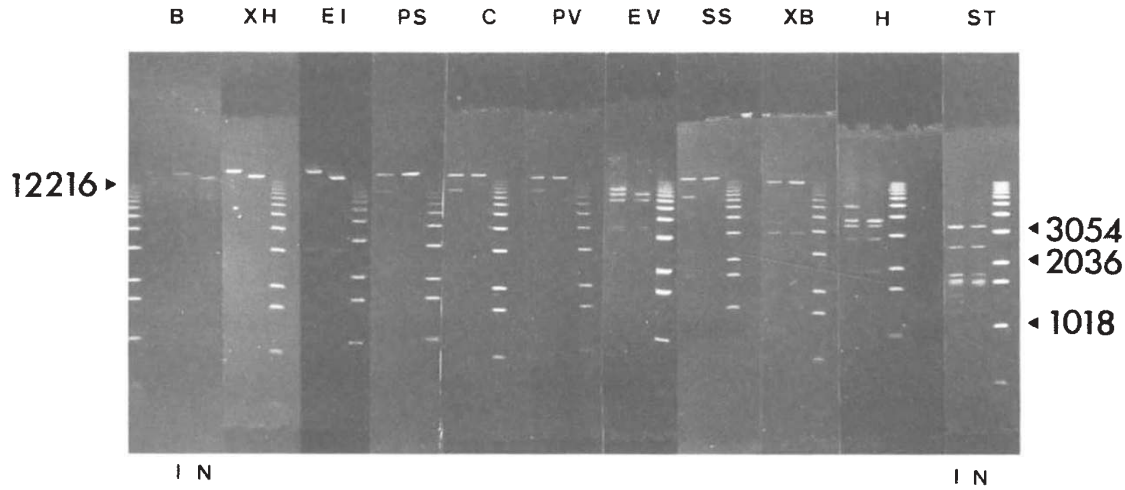

Figure 4 Composite of mtDNA of 2 *T. c.* "*carnifex*" individuals from Ano Kaliniki (Greece) digested with a range of restriction enzymes. Each strip comprises the profile of the individual containing the ~8·5 kbp insertion (I), the normal profile (N), and the 1 kbp ladder marker (see fig. 1). Restriction enzymes used are (left to right): *Bgl*II, *Xho*I, *Eco*RI, *Pst*I, *Cla*I, *Pvu*II, *Eco*RV, *Sst*II, *Xba*I, *Hin*dIII and *Stu*I (see text for explanation).

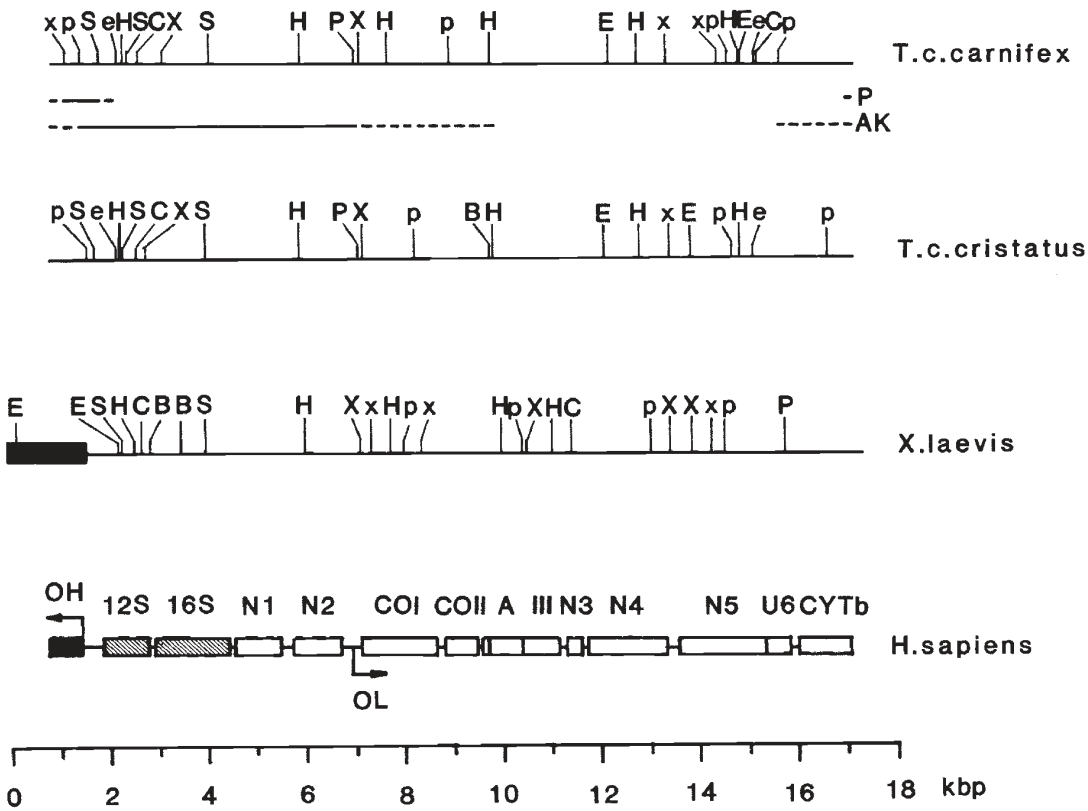

Figure 5 Restriction maps for three amphibian mtDNAs aligned against the human gene map (Anderson *et al.*, 1981). Shaded boxes represent the D-loop, hatched boxes rRNA genes, and empty boxes polypeptide coding genes. These are interspersed with tRNA genes, the origin of light-strand replication, and a few non-coding bases. The large duplicated region in newts from Pisa and Ano Kaliniki are represented by solid and dashed lines, indicating definite and possible extents of the duplications respectively. Enzyme sites are indicated by letters according to the following code: *Bgl*II, B; *Cla*I, C; *Eco*RI, E; *Eco*RV, e; *Hin*dIII, H; *Pst*I, P; *Pvu*II, p; *Sst*II, S; *Xba*I, X; *Xho*I, x.

several hundred base pairs within a number of species (Reilly and Thomas, 1980; Fauron and Wolstenholme, 1980; Solignac *et al.*, 1983; Harrison *et al.*, 1985). Recent findings in *Cnemidophorus* lizards (Densmore *et al.*, 1985; Mortiz and Brown, 1986), Ranid (Monnerot *et al.*, 1984) and Hylid (Bermingham *et al.*, 1986; Spolsky and Uzzell, 1986) frogs, and bowfin fish (Bermingham *et al.*, 1986), suggest that these groups also differ from mammals, not only in the abundance of large insertions, but by displaying heteroplasmy for many of these (table 2). Studies on other fish (Avise and Saunders, 1984; Gyllenstein *et al.*, 1985) and *Xenopus* (Carr, 1983) have not, however, disclosed such variation. In this paper, all the insertion polymorphism and heteroplasmy has been shown in oocyte mtDNA alone; there may prove to be higher levels if other organs were examined, but as these genomes are not transmitted they are not of direct relevance to evolution of the mt genome. Low levels of heteroplasmy (say <5 per cent) will also go undetected given the conditions of the study.

Large insertions therefore appear to be a recurrent feature of amphibian (and possibly reptilian and fish) mtDNA. This is in contrast to findings in mammals, in which even the largest length variations do not approach the size of many amphibian and insect insertions. Moreover, in the poikilothermic groups, considerable within population polymorphism is found. There are several factors that might impinge upon the prevalence of insertion polymorphism and heteroplasmy in natural populations: at the molecular level, the rate of generation of molecules carrying insertions; at the cellular level, the effective number of mt genomes per cell, the number of cell generations per germ line generation and degree of paternal leakage; at the population level, population size and migration rates. Additionally, selection can act at any level (for a theoretical treatment see, *e.g.* Birky *et al.*, 1983; Takahata, 1984). Mt genomes made unnecessarily larger by the presence of insertions may be at a replicative disadvantage compared to those containing normal sized genomes, but the small amount of work done addressing this possibility suggests little or no deterministic sorting (Solignac *et al.*, 1984). The "half-life" of 300 bp insertion heteroplasmy in a *Drosophila mauritiana* isofemale line is of the order of 60 generations (Solignac *et al.*, 1983), and complete sorting out may require as many as 500 generations (Solignac *et al.*, 1984).

The explanation for the disparity among groups with regard to prevalence of insertion polymorphism seems likely to lie in differences in molecular mechanisms governing replication and repair of the mitochondrial genome, and/or sequence difference and their specific effects, resulting in disparate mutation rates. In *Triturus cristatus*, all length variation described maps to the control region, and where it has been possible to determine the nature of insertions, they are tandem duplications. These may result from strand slippage and mismatching during replication (Densmore *et al.*, 1985); in the absence of sequence data, it is not possible to say whether or not there are shared end points. The fuzzy bands observed in some Trešnja and Guberevac samples imply that these newts were heteroplasmic for a continuous distribution of small length variations of 1–15 bp, as found in the conserved sequence blocks (CSBs) of mammals (Hauswirth *et al.*, 1984; Hauswirth and Clayton, 1985). This parallels findings in *Rana* in which fuzziness over a 400–700 bp range has been described (Monnerot *et al.*, 1984). The CSBs can be drawn into large hairpin structures, and may be related to insertion polymorphism in the region. Repeat sequences of 45 bp separated by 16 bp have

Table 2 Examples of heteroplasmy for mtDNA in animal populations showing % individuals heteroplasmic

Species	*N*	%	Insertion Heteroplasmy	Reference
Drosophila mauritiana	60*	(50)	500 bp	Solignac *et al.*, 1983
Bos taurus	several	high	1–10 bp	Hauswirth *et al.*, 1984
Rana esculenta	several	100	400–700 bp (continuous)	Monnerot *et al.*, 1984
Gryllus	100	12	300 and 600 bp	Harrison *et al.*, 1985
Cnemidophorus	92	>44	1–370 bp (continuous)	Densmore *et al.*, 1985
		7	35 bp	
Amia calva	52	8	≦700 bp	Bermingham *et al.*, 1986
Hyla cinerea	142	2	≦900 bp	
Hyla gratiosa	163	8	≦900 bp	
Triturus cristatus	185	>1	2250 and 3350 bp	this paper

* 30th generation of isofemale line.

been found within the *Xenopus* D-loop (Wong *et al.*, 1983); such features could also promote the tendency for length variation in *Triturus* and indeed Amphibia as a whole. The prevalence of length variation in this region also results from it being mainly non-coding and a comparatively large target sequence (>1 kbp).

It is interesting that in the case of *Cnemidophorus* it is the interspecific triploids that show highest frequency of insertions. In *Triturus*, one of the samples containing insertions and blurred control region bands (Trešnja) also displays evidence of intersubspecific cytoplasmic gene flow and possibly some nuclear gene flow (unpubl. data). Similarly, low levels of nuclear gene flow have been found between *T. cristatus* and *T. marmoratus* in the Mayenne department of France (Arntzen, 1986) where 2C9 *cristatus* display insertion polymorphism. It is thus tempting to speculate that presence of foreign genes may in some way "destabilise" the mt genome, whose replication is mediated by many nuclear gene products. This cannot be the whole story as some insertion polymorphism in *T. cristatus* occurs away from "subspecies" transition zones, but the effect may none the less exist.

The phenomenon of length variation has to be taken into account when analysing mtDNA restriction fragment profiles. It is important to distinguish this type of variation from site variation before making estimates of nucleotide divergence and inferring phylogeny. When considering fine-scale genetic differentiation, the problem of convergence may be lower for large scale length variation than for restriction site variation; each length variant is of a certain size and at a specific site in the mt genome, whereas restriction sites may "blink" off and on. Length variants have been useful in revealing population subdivision (Horai and Matsunaga, 1986; this paper), and have provided variation where restriction site variation is limited (Densmore *et al.*, 1985). They therefore have potential as markers in studies of the genetic structure of natural populations.

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