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An integrative analysis of phylogenetic relationships among newts of the genus *Triturus* (family Salamandridae), using comparative biochemistry, cytogenetics and reproductive interactions

Herbert C. Macgregor[1], Stanley K. Sessions[2,4] and J. W. Arntzen[3]

[1]*Department of Zoology, University of Leicester, LE1 7RH, England*;
[2]*Developmental Biology Centre, University of California, Irvine, CA 92717, USA*;
[3]*Institute of Taxonomic Zoology, University of Amsterdam, Mauritskade 57, P.O. Box 4766, 1009 AT, Amsterdam, The Netherlands.*

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Abstract

All the currently available data with regard to morphology, palaeontology, biochemical genetics, reproductive interactions and behaviour have been collated and analysed with combinations of phenetic and numerical phylogenetic methods, and integrated into a consensus evolutionary tree for European newts of the genus *Triturus*.

A range of cytogenetic data are presented including genome sizes (amount of DNA per haploid chromosome set), Giemsa C-banded karyotypes for mitotic chromosomes, maps of lampbrush chromosomes and locations of nucleolus organisers (genes for ribosomal RNA). We have examined these data in search of evolutionary trends within the genus.

The degree to which cytogenetic data accord with the consensus tree, and the general usefulness of cytogenetic data for the reconstruction of evolutionary history are discussed.

A revised scheme for the taxonomy of *Triturus* is presented in which the genus is subdivided into two subgenera, *Triturus* and *Palaeotriton* is suggested. Subgenus *Triturus* should include all of the large bodied newts belonging to the *T. cristatus* species group as well as *T. alpestris* and *T. vittatus*. All other species are included in subgenus *Palaeotriton*.

The elevation of the former subspecies of *T. cristatus* to full species status is recommended mainly on the basis of information from studies of mitochrondrial DNA.

[4] Current address: Department of Biology, Hartwick College, Oneonta, NY 13820, USA.

Introduction

Newts of the genus *Triturus* (family Salamandridae) are all pond dwellers, widely distributed in Europe and adjacent parts of Asia (Figs 1, 2 and 3). The genus *Triturus* is a monophyletic group characterised by a complex courtship behaviour with a total absence of amplexus, and by a higher degree of sexual dimorphism than in any other amphibian genus (Halliday, 1977). They are characterised by a biphasic life history, involving aquatic larvae and metamorphosis. The genus is old and osteologically distinctive (Bolkay, 1928). Fossils of *T. marmoratus* have been recovered from Pliocene layers in Spain (Sanchiz, 1977; cf. Sanchiz and Szyndlar, 1984) and fossils of *T. cristatus* have been found in the Pliocene of Poland and Czechoslovakia (Hodrova, 1985; Sanchiz and Mlynarski, 1979). A *T. marmoratus*-like fossil has been reported from the Middle Miocene of France (Estes and Hoffstetter, 1976; Estes, 1981), and a *T. montandoni*-like fossil was found in the Upper Pliocene in Poland (Sanchiz and Mlynarski, 1979). Gonzalez and Sanchiz (1986) have reported on fossils of *Triturus* obtained from the lowermost Oligocene.

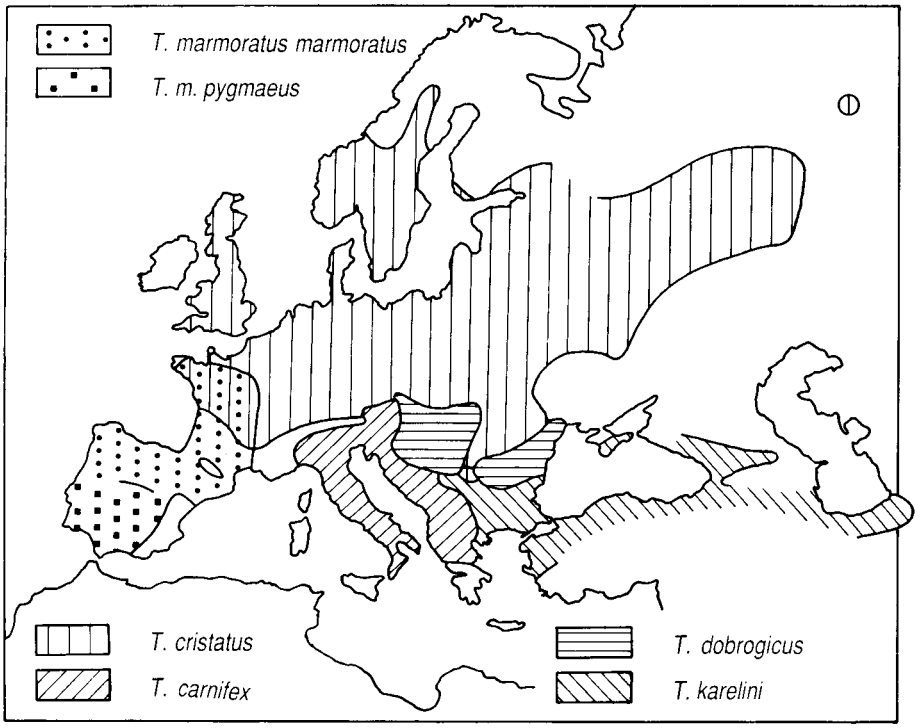


Fig. 1. Distribution map for *Triturus carnifex*, *T. cristatus*, *T. dobrogicus*, *T. karelini* and *T. marmoratus* after Thorn (1968), modified after Schmidtler (1976), Bannikov et al., (1977), Terhivuo (1981), Salvador (1985), Dorda and Esteban (1986), Zuiderwijk (1986), Wallis and Arntzen (1989) and J. W. Arntzen (unpublished data).

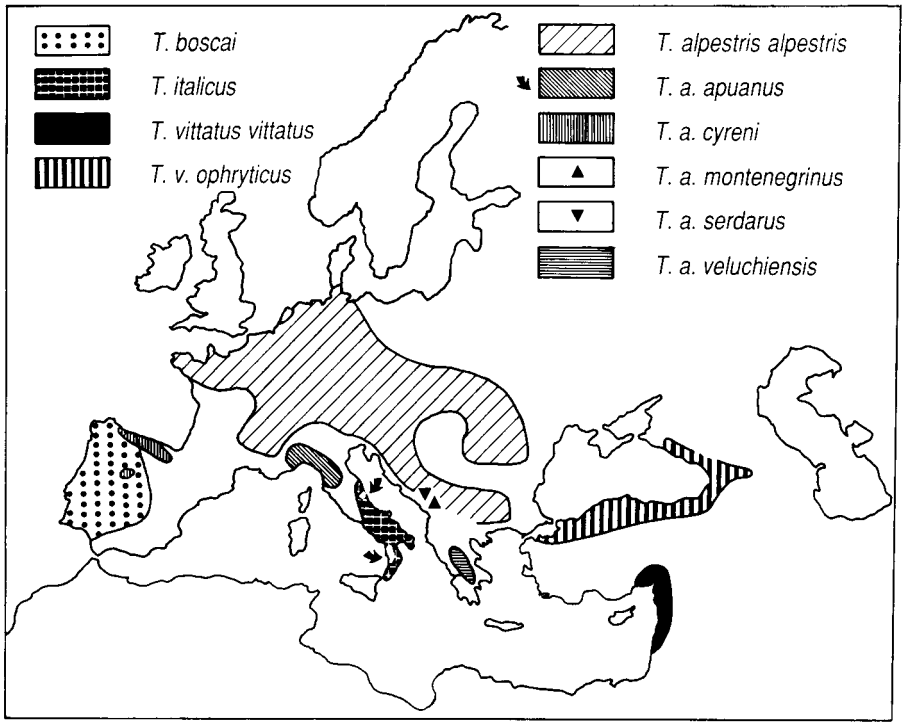


Fig. 2. Distribution map for *Triturus alpestris*, *T. boscai*, *T. italicus* and *T. vittatus* after Thorn (1968), modified after Berger et al. (1969), Dubois (1983), Salvador (1985), Arano and Arntzen (1987), Breuil and Parent (1987), Grossenbacher (1987), C. Giacoma (personal communication) and A. Zuiderwijk (personal communication).

Although *Triturus* species have long been used in comparative biological studies, uncertainties still exist concerning phylogenetic relationships within the genus. Nine species and a number of subspecies were recognized by Thorn (1968), but more recently, twelve species have been recognized (Frost, 1985) on the basis of karyological and hybridization studies by Bucci-Innocenti et al. (1983a) supported by an analysis of mitochondrial DNA (Wallis and Arntzen, 1989). Three subgenera have been recognized on osteological grounds (Bolkay, 1928; Thorn, 1968): one of large newts (*Neotriton*) including *T. carnifex*, *T. cristatus*, *T. dobrogicus*, *T. karelini* and *T. marmoratus*; one of small newts (*Palaeotriton*) including *T. boscai*, *T. helveticus*, *T. italicus*, *T. montandoni*, *T. vulgaris* and also *T. vittatus*; and a taxon (*Mesotriton*) including only *T. alpestris*.

Recently, several independent, comparative biochemical studies have focused on the genus *Triturus* (Rafinski and Arntzen, 1987; Busack et al., 1988; M. Frelow, H. Macgregor and D. Wake, unpublished). In terms of major patterns of phylogenetic relationships, these studies are in broad agreement with each other as well as with Bolkay's (1928) morphological analysis, that at least two major species groups are

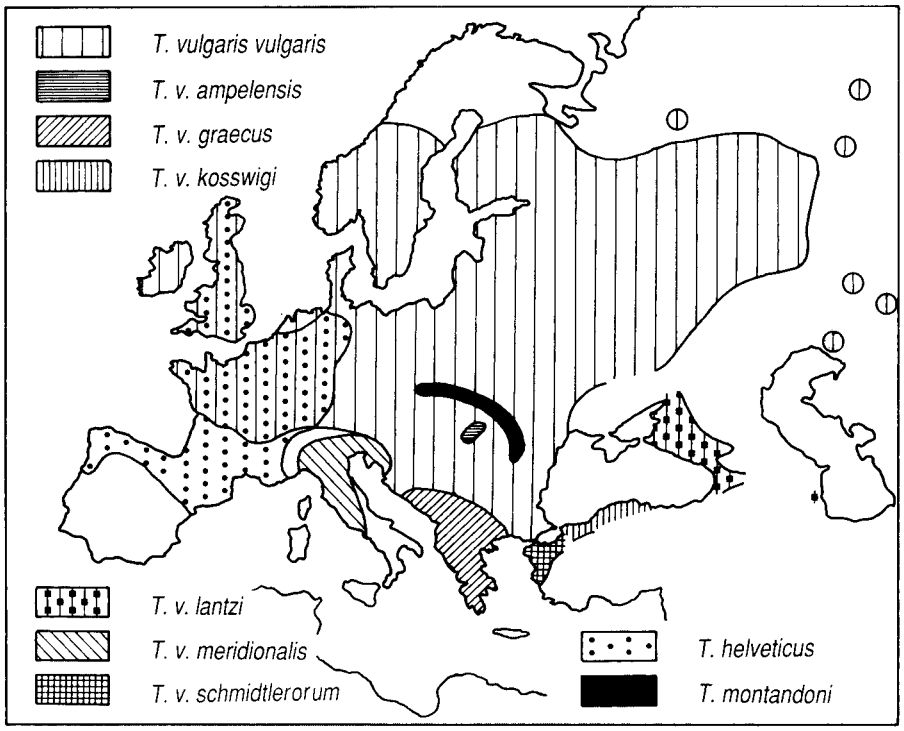


Fig. 3. Distribution map for *Triturus helveticus*, *T. montandoni* and *T. vulgaris* after Thorn (1968), modified after Bruno (1973), Bannikov et al. (1977), Dolmen (1982), Schmidtler and Schmidtler (1983), Raxworthy (1988), Regalado (1985) and A. Zuiderwijk (personal communication).

recognised; a *vulgaris* species group including *T. helveticus*, *T. montandoni*, and *T. vulgaris*, and a *cristatus* species group including *T. carnifex*, *T. cristatus T. dobrogicus*, *T. karelini* and *T. marmoratus*. These results appear also to be in agreement with available data on cytogenetics and interspecific reproductive interactions (Bucci-Innocenti et al., 1983a). In our view, problematic species whose relationships to other species in the genus are not clearly resolved by biochemical analyses include *T. alpestris*, *T. vittatus*, *T. boscai* and *T. italicus* as well as certain species within the *T. cristatus* species group. Recently published behavioural evidence (Arntzen and Sparreboom, 1987, 1989; Raxworthy, 1989) places *T. vittatus* alongside the large newts within the subgenus *Triturus*. Likewise, available evidence (Busack et al., 1988, Arntzen and Sparreboom, 1987, 1989; Frelow et al., unpublished) places *T. alpestris* in the subgenus *Triturus*, although the data here are less conclusive. In accordance with Bolkay (1928) *T. boscai* and *T. italicus* should be placed together with species belonging to the *vulgaris* group. All of these newts make up the subgenus *Palaeotriton*. A full list of recognised species and subspecies and the nomenclatorial designations used in the present study are given in Table 1.

Table 1. Taxa recognised within the genus *Triturus*.

subgenus	*Triturus*		
	cristatus species group		
		cristatus superspecies	
			T. carnifex
			T. cristatus
			T. dobrogicus
			T. karelini
		T. marmoratus	*marmoratus*
		" "	*pygmaeus*
	T. vittatus	*vittatus*	
	" "	*ophryticus*	
	T. alpestris	*alpestris*	
	" "	*apuanus*	
	" "	*cyreni*	
	" "	*montenegrinus*	
	" "	*serdarus*	
	" "	*veluchiensis*	
subgenus	*Palaeotriton*		
	T. boscai		
	T. italicus		
	vulgaris species group		
		T. helveticus	
		T. montandoni	
		T. vulgaris	*vulgaris*
		" "	*ampelensis*
		" "	*graecus*
		" "	*kosswigi*
		" "	*lantzi*
		" "	*meridionalis*
		" "	*schmidtlerorum*

It is the purpose of this paper to review the information available on protein biochemistry and interspecific reproductive interactions, and to propose a consensus phylogenetic tree based on data derived from these and other approaches. We will then present a range of cytogenetic data and examine the extent to which our consensus tree helps us to understand mechanisms, directions and rates of genome and chromosome evolution.

Phylogeny

Protein electrophoresis

The tree of descent Bolkay (1928) proposed for *Triturus* has recently been tested in two studies of the biochemical systematics of the genus (Rafinski and Arntzen, 1987; Frelow, Macgregor and Wake, unpublished). Rafinski and Arntzen (1987)

examined the electrophoretic properties of nineteen proteins from representatives of nine species. Frelow et al. looked at twenty-two proteins from seventeen populations of seven species.

Dendrograms were constructed from both data sets using the same phenetic and phylogenetic methods as applied by Rafinski and Arntzen (1987). We employed Nei's (1972) genetic distance (D) in the unweighted pair-grouping method using arithmetic averages (UPGMA, Sneath and Sokal, 1973). The dendrograms obtained from both data sets are very similar, and point to the existence of two main groups within the genus *Triturus* (Figs. 4 and 5). On the basis of these electrophoretic data the position of *T. alpestris* in relation to the main groups remains unresolved. The *cristatus* species group and a group of small newts including *T. boscai*, *T. helveticus* and *T. vulgaris* are both differentiated at a level of $D = 0.70 - 0.90$. On the basis of the data of Frelow et al. (Table 2), *T. alpestris* clusters with the group of large-bodied newts at a level of $D = 0.85$, while in the study of Rafinski and Arntzen (1987) this species is separated from all others at a level of $D = 1.5$.

Two main objections to the method of hierarchical clustering employed in the above are its phenetic nature and the implicit assumption that evolutionary rates are uniform. Both these possible disadvantages are circumvented by the Wagner algorithm. Unrooted Wagner trees were produced using the TreeTools computer program algorithm (Ellis, 1985). In this program the enzyme data are examined on a locus by locus basis. For each locus a transformation network was produced in which OTU's were connected by (single locus) Rogers' genetic distance (D_R, Rogers, 1972). The advantages of this precise method of coding are described in a recent publication by Swofford and Berlocher (1987).

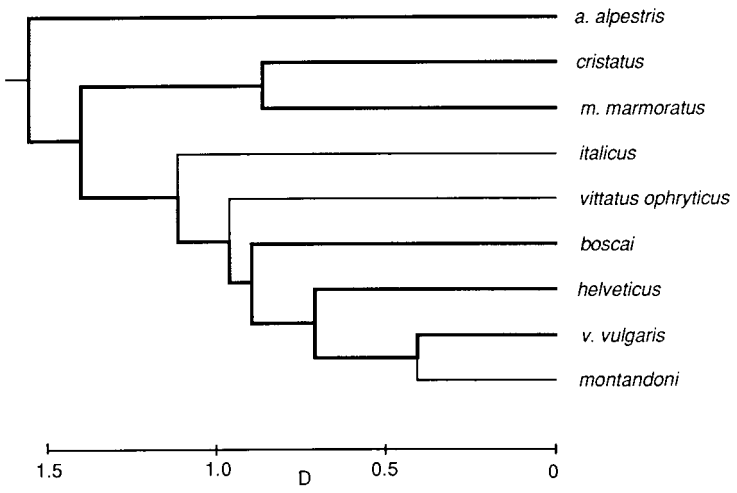


Fig. 4. UPGMA-dendrogram for 9 species of the genus *Triturus* based on Nei's genetic distance (D) measured over 19 loci (Rafinski and Arntzen, 1987). The cophenetic correlation coefficient is 0.82. Thick lines connect the six (sub)-species that were also studied by Frelow et al., (see figure 5).

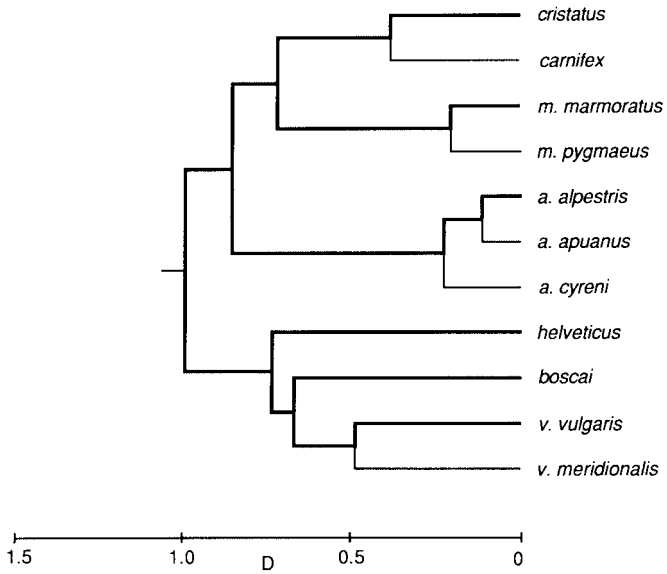


Fig. 5. UPGMA-dendrogram for 7 species (11 forms) of the genus *Triturus* based on Nei's genetic distance (D) measured over 22 loci (data from M. Frelow, H. C. Macgregor and D. B. Wake, unpublished). The cophenetic correlation coefficient is 0.80. Thick lines connect the six (sub)-species that were also studied by Rafinski and Arntzen (1987), (see figure 4).

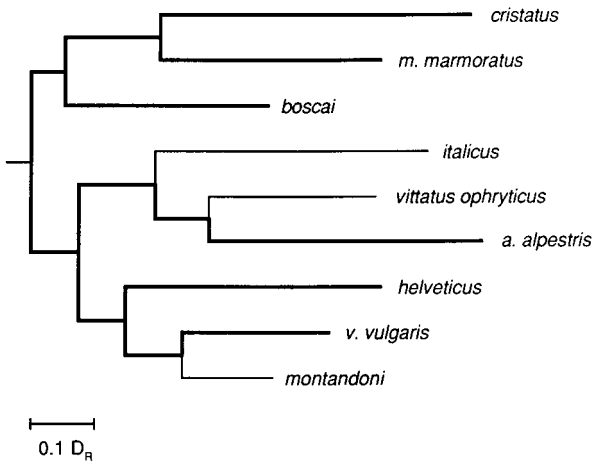


Fig. 6. Minimum length Wagner tree for 9 species of the genus *Triturus* based on allozymic transformation networks with Rogers' genetic distance (D_R) measured over 19 loci (data from Rafinski and Arntzen, 1987). The undirected tree is rooted in such a way as to minimise the total variance of evolutionary rates. Its cophenetic correlation coefficient is 0.92. Thick lines connect the six (sub)-species that were also studied by Frelow et al., (see figure 7).

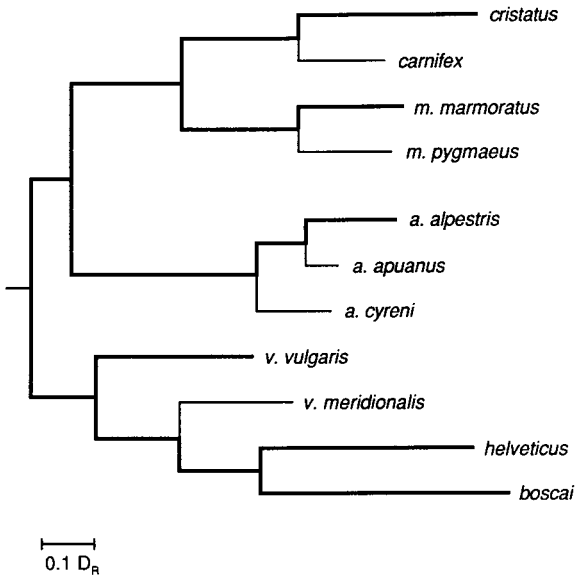


Fig. 7. Minimum length Wagner tree for 7 species (11 forms) of the genus *Triturus* based on allozymic transformation networks with Rogers' genetic distance (D_R) measured over 22 loci (data from M. Frelow, H. C. Macgregor and D. B. Wake, unpublished). The undirected tree is rooted in such a way as to minimise the total variance of evolutionary rates. Its cophenetic correlation coefficient is 0.88. Thick lines connect the six (sub)-species that were also studied by Rafinski and Arntzen (1987), (see figure 6).

For an evolutionary interpretation, the undirected trees have to be rooted. The best method is outgroup rooting, but since no outgroup was included in either study, we rooted the undirected trees by minimising the variance in evolutionary rates among phyletic lines (Farris, 1972), although we realise that with this method of rooting the assumption of uniform rates of evolutionary change is reintroduced. The most parsimonious solution was selected out of a total of 20,000 iteractions for each data set (Figs. 6 and 7).

The results of both UPGMA and Wagner analyses of the data of Frelow et al. point to the existence of two groups within *Triturus*. In both analyses *T. alpestris* is joined with the *cristatus* species group. The Wagner tree constructed from data provided by Rafinski and Arntzen (1987) is broadly in agreement with these findings, but *T. alpestris* (and *T. vittatus*) is grouped with the small-bodied newts, while *T. boscai* is joined with the *cristatus* species group.

Nei's measure of genetic differentiation has been used to estimate divergence times because of its relation to immunological distance and rates of nucleotide substitution for which divergence times have been calibrated. If we accept the calibration of Maxson and Maxson (1979), made for lungless salamanders of the family *Plethodontidae*, in which one unit of D is equivalent to approximately fourteen million years of isolation, we can obtain some rough estimates of time of divergence and compare them with what is known from the fossil record. The

Table 2. Nei's genetic distance (to the right of diagonal) and Rogers' genetic distance (below diagonal) for all pairs of *Triturus* (sub)-species studied by M. Frelow, H. C. Macgregor and D. B. Wake (unpublished). On the diagonal in boldface type, sample-size and number of populations studied (in brackets). A list of the localities that were sampled, a description of electrophoretic conditions used and the electromorph frequencies scored for the 22 protein loci that were studied, is available from the senior author (HCM). A table of single-locus genetic distances is available from JWA.

	1	2	3	4	5	6	7	8	9	10	11
1. *T. a. alpestris*	**5(1)**	0.11	0.26	1.26	0.92	0.80	0.87	0.70	0.80	0.86	0.81
2. *T. a. apuanus*	0.13	**5(1)**	0.20	1.27	0.95	0.80	0.85	0.91	0.79	1.07	0.89
3. *T. a. cyreni*	0.25	0.18	**5(1)**	1.26	0.96	0.79	0.84	0.96	0.79	0.85	0.72
4. *T. boscai*	0.71	0.72	0.71	**5(1)**	1.02	1.08	0.72	1.30	1.18	0.71	0.65
5. *T. cristatus*	0.61	0.62	0.62	0.64	**18(2)**	0.38	1.51	0.84	0.82	1.02	1.32
6. *T. carnifex*	0.57	0.58	0.56	0.65	0.36	**35(4)**	1.00	0.62	0.58	0.98	0.85
7. *T. helveticus*	0.59	0.58	0.58	0.53	0.76	0.62	**10(2)**	1.04	0.94	0.85	0.64
8. *T. m. marmoratus*	0.52	0.61	0.63	0.72	0.59	0.49	0.65	**5(1)**	0.19	0.71	0.75
9. *T. m. pygmaeus*	0.55	0.55	0.55	0.69	0.57	0.47	0.62	0.21	**6(1)**	0.78	0.52
10. *T. v. vulgaris*	0.59	0.67	0.59	0.53	0.64	0.63	0.58	0.51	0.57	**8(2)**	0.49
11. *T. v. meridionalis*	0.57	0.59	0.52	0.49	0.73	0.58	0.50	0.55	0.43	0.42	**5(1)**

values, as indicated in Table 2 and Figs. 4 and 5, suggest that the main radiation of the genus dates back to the Miocene. This is in good accordance with the fossil record (see introduction) and with the results of immunological studies (Busack et al., 1988).

Reproductive interactions

Reproductive interactions have long been considered central to taxonomic issues, particularly with regard to evaluating species status. Since 1903 over 40 different inter- and intraspecific hybridizations within the genus *Triturus* have been carried out and/or described by at least nineteen different investigators. Consequently, a large body of data exists concerning reproductive interactions within the genus.

From the standpoint of natural hybridisation in the wild, there is known to be extensive hybridisation leading to wide zones of intergradation between subspecies of *T. vulgaris* (Schmidtler and Schmidtler, 1983). *T. vulgaris* hybridises with *T. montandoni* in the foothills of the Carpathians (Fuhn, 1963; Hofmann, 1908) and exceptionally it hybridises with *T. helveticus* (Griffiths et al., 1987). *T. cristatus* and *T. marmoratus* regularly hybridise (Vallée, 1959; Arntzen, 1986a,b). It is noteworthy that all natural hybridisation is within recognised species groups.

A list of all reported hybridisations together with their consequences is shown in Table 3. The list that we present may not be entirely complete, but we hope it may nevertheless prove useful as a basis for reference and as another source of evidence relating to taxonomic relationships within the genus.

A number of general conclusions can be drawn from these data. The majority of cases of hybridization between subgenera could only be obtained by artificial fertilisation, from which we may conclude that there are signficant pre-zygotic isolating factors separating the species involved. However, courtship behaviour followed by natural fertilisation was accomplished in several *Triturus* × *Palaeotriton* crosses. Bataillon (1927) does not say whether his crosses between *T. helveticus* (*Palaeotriton*) and *T. marmoratus* (*Triturus*) were the result of natural or artificial fertilisation, but the inference is that they were a consequence of natural fertilisation. If this inference is correct, then these are the only reported hybridisations between subgenera that are natural and led to the production of adult newts.

From the standpoint of developmental capacity of intra- and inter-subgeneric hybrids it is clear that most combinations, except those involving *T. vittatus*, will produce adult newts, but that in all cases there is more or less failure of gonad formation and meiosis and the adult hybrids are certainly incapable of producing F_2 or F_2 backcross generations.

Crosses between genera, involving either North American newts, other salamandrids or plethodontids failed completely, although G. Mancino and his colleagues (personal communication) have reported fertilisation and cleavage and some subsequent development of embryos.

Table 3. Reproductive interactions among species of the genus *Triturus* and between *Triturus* and other salamandrid and non-salamandrid urodele genera. Abbreviations are as follows: aa = *T. alpestris alpestris*, ap = *T. a. apuanus*, b = *T. boscai*, cr = *T. cristatus*, cx = *T. carnifex*, d = *T. dobrogicus*, h = *T. helveticus*, it = *T. italicus*, k = *T. karelini*, ma = *T. marmoratus*, mo = *T. montandoni*, vt = *T. vittatus*, vm = *T. vulgaris meridionalis*, vv = *T. v. vulgaris*, Nv = *Notophthalmus viridescens*, St = *Salamandrina terdigitata*, Hi = *Hydromantes italicus* (Family Plethodontidae).

Interaction	A	B	C	D	E	F	G	H	I	J	K	L	References
aa × ap		+				+		+			+	+	16
aa × b	+				+								12, 34, 35
aa × cr	+				+								16, 17, 24, 25
aa × h	+							+					1, 24, 25
aa × ma		+						+					1
aa × vv	+					+	+	+		+			16, 25, 35
ap × b	+	+		+		+		+		+			16, 20
ap × vm	+					+	+	+		+			16, 20
ap × vt	+	+				+		+		+			16
b × h	+							+					25
b × it	+					+							5
b × vm	+					+							5
cr × cx	+	+				+		+		+		+	6, 7, 30
cr × h	+							+					25
cr × k	+	+				+		+		+		+	6, 7, 30
cr × ma	+	+	+			+	+	+		+		+	13, 14, 19, 33, 34, 35
cr × vm	+				+		+	+		+			16, 25
cr × vt	+			+									16
cr × vv	+							+					35
cx × k	+	+				+		+		+		+	6, 7, 30
cx × ma	+	+				+		+		+		+	14, 15, 23, 39, 35
cx × vm	+				+		+	+		+			4, 20, 21, 22
cx × vt	+			+									16
d × ma	+	+				+	+	+		+		+	16
d × vt	+			+									16
h × ma		+						+					1
h × vm		+				+		+		+		+	3, 18, 27
h × vv		+	+			+		+		+		+	8, 9, 11, 16, 26, 27 35, 36
Hi × vm	+			+									16
it × vv	+	+				+		+		+			2, 17, 28, 35
k × ma	+	+				+	+	+		+		+	14
k × vt	+			+									16
ma × vm	+				+		+	+		+			16
ma × vt	+			+									16
mo × vm		+				+		+			+	+	16
mo × vv			+			+		+			+	+	10, 26, 34, 35
Nv × vm	+		+										16
St × vm	+		+										16
vm × vt	+	+				+		+					5, 16, 35

Table 3 (*continued*)

Pre-zygotic		
	A	Successful in vitro fertilization
	B	Successful courtship leading to mating in the laboratory
	C	Successful courtship leading to mating in the wild
Post-zygotic development		
	D	Eggs fertilised, but no cleavage
	E	Eggs fertilised, but low percentage of cleavage or cleavage abnormal; those eggs that do not cleave normally give normal development through to hatching
	F	Eggs fertilised, normal development to hatching
	G	Larvae showing polyploidy
	H	Adult newts produced
Meiosis and reproductive potential		
	I	Gonad formation with normal gonial mitoses, but no meiosis
	J	Some meiosis but low yield of functional gametes
	K	Meiosis normal, leading to functional eggs and sperm
	L	Adult hybrids fertile and able to produce F_2 or F_2 backcross generations.

Key to References in Table 3

1. Bataillon (1927)
2. Benazzi (1954)
3. Benazzi (1957)
4. Benazzi and Lepori (1949)
5. Bucci-Innocenti et al. (1983a)
6. Callan (1982)
7. Callan and Spurway (1951)
8. Feldmann (1981)
9. Freytag (1950)
10. Fuhn et al. (1975)
11. Griffiths et al. (1987)
12. Lantz (1934)
13. Lantz (1947)
14. Lantz and Callan (1954)
15. Macgregor and Horner (1980)
16. G. Mancino (personal communication)
17. Mancino (1961)
18. Mancino and Scali (1964)
19. Mancino et al. (1973)
20. Mancino et al. (1976)
21. Mancino et al. (1978)
22. Mancino et al. (1979a)
23. Mancino et al. (1979b)
24. Pariser (1935)
25. Pariser (1936)
26. J. Rafinski (personal communication).
27. Scali and Mancino (1968)
28. Schreitmüller (1910)
29. Sims et al. (1984)
30. Spurway and Callan (1950)
31. Spurway and Callan (1960)
32. White (1946)
33. Wolterstorff (1903)
34. Wolterstorff (1904)
35. Wolterstorff (1925)
36. Wolterstorff and Freytag (1951)

Phenetic analysis of reproductive interactions

Our approach to understanding the taxonomic significance of the data on reproductive interactions with the genus *Triturus* has been as follows. First we have divided the features of hybridisations into three categories, pre-zygotic, post-zygotic development, and meiosis and reproductive potential (see Table 3). We have done this because, clearly, without fertilisation there will be no development, without development there will be no adults and without adults there is no reproductive potential. Secondly, we have allocated an all-or-nothing, + or −, score to each

Table 4. Half-matrix of 'hybridization scores' for species of *Triturus*, indicating relative levels of successful reproductive interaction (from Table 3; see also Fig. 8).

Species	1	2	3	4	5	6	7	8	9	10	11	12
1. *T. alpestris*												
2. *T. boscai*	6											
3. *T. carnifex*	–	–										
4. *T. cristatus*	2	–	6									
5. *T. dobrogicus*	–	–	–	–								
6. *T. helveticus*	2	2	–	2	–							
7. *T. italicus*	–	2	–	–	–	–						
8. *T. karelini*	–	–	6	6	–	–	–					
9. *T. marmoratus*	2	–	6	8	7	2	–	7				
10. *T. montandoni*	–	–	–	–	–	–	–	–	–			
11. *T. vittatus*	5	–	2	2	2	–	–	2	2	–		
12. *T. vulgaris*	5	2	5	5	–	6	5	–	5	5	4	

feature. We have then used the total plus score for each hybridisation to construct the similarity matrix shown in Table 4. The matrix was then converted into a dendrogram (Fig. 8) using the UPGMA method.

We recognise that our analysis is somewhat limited by lack of data, as can be seen from the gaps in both Tables 3 and 4. Many of these gaps, however, are truly significant. The cross *T. alpestris* × *T. cristatus*, for example, required artificial fertilisation and failed to produce adult newts, consequently there are no entries under F-L in Table 3. The cross *T. cristatus* × *T. marmoratus* receives the highest score since it leads naturally to adult newts capable of producing backcross generations; so there are altogether eight entries in Table 3. Likewise, although some of the gaps in Table 4 signify that crosses that might have been attempted have not been and therefore no data are available, many other gaps are significant in the sense that geographical separation of species (see Figs. 1–3) precludes natural hybridisation.

Notwithstanding the incomplete database, the dendrogram generated by our analysis is remarkably similar to those generated by comparative morphological and biochemical studies, and this result goes some way towards vindicating our approach and suggests that reproductive compatibility, via artificial and/or natural hybridisation, is a good indicator of the evolutionary relationships in this group of urodeles.

As in other analyses, two relatively tight clusters of species are resolved (Fig. 8); the *vulgaris* species group (plus *T. italicus*) and the *cristatus* species group. Within these groups the branching order is different from that of other analyses with a trend towards the grouping of species in which hybridisation has been more extensively investigated: *T. cristatus* – *T. marmoratus* and *T. helveticus* – *T. vulgaris*. The remaining species *T. alpestris*, *T. boscai* and *T. vittatus* occupy positions outside the two major species groups.

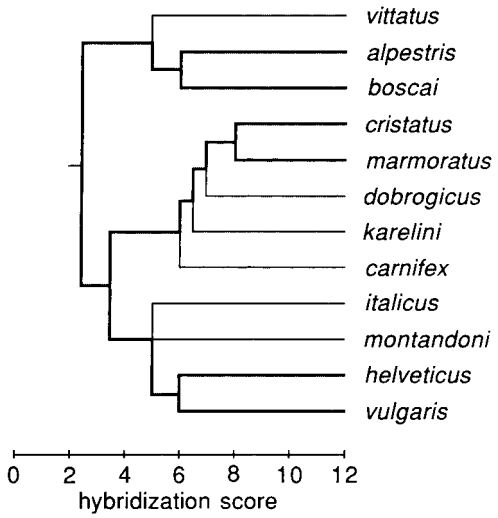


Fig. 8. UPGMA-dendrogram constructed from the table and similarity matrix of reproductive interactions presented in Tables 3 and 4.

Other approaches

Other approaches to *Triturus* taxonomy have recently been explored. Wallis (1987) and Wallis and Arntzen (1989) have examined mitochondrial (mt) DNA insertion polymorphism and variation in the genus. All *Triturus* species so far examined possess mtDNA circles of 16300–16500 base pairs (bp), which is fairly typical of vertebrate mt genomes. Numerous within-species nucleotide-substitution polymorphisms have been found but intra-specific nucleotide-divergence values are quite low when compared to some natural populations of vertebrates. On the other hand, numerous substantial insertions (40–140 bp) have been found in mt genomes from *T. cristatus* and related species in the *cristatus* group. One individual had a 8500 bp insertion sequence. All of these insertion polymorphisms map to the largely non-coding control region of the mt genome, and in the case of the larger insertions they are simply tandem duplications of a part of the mt molecule. In *Triturus*, mtDNA length variation would seem to be a useful parameter for studies of fine-scale population structure.

Perhaps most importantly, the former subspecies of *T. cristatus* have been shown by studies across contact zones to be effectively genetically isolated (Wallis and Arntzen, 1989; Arntzen and Wallis, in prep.). Analysis of their mtDNA has shown them to be deeply divergent to a degree that strongly supports their elevation to specific status (Wallis & Arntzen, 1989). Of these four species a phylogenetic analysis of mtDNA data indicates *carnifex* and *cristatus* to be the most closely related pair.

T. Burke (unpublished observations) has carried out a preliminary survey of the risosomal DNA of *Triturus* by looking at the restriction fragments produced by

each of five enzymes from DNA of nine species. His analysis so far is not as satisfactory as a phylogenetic analysis of restriction maps, but it nonetheless already shows a distinct clustering of those species that are known to be most closely related on the basis of other kinds of data, and it strongly supports the division of the genus into the two designated subgenera. Against this background, we consider that analysis of cloned ribosomal DNAs would certainly be a powerful approach in sorting out the phylogeny of the older lineages in this genus.

Arntzen and Sparreboom (1987, 1989) present a catalogue of courtship behaviour of *Triturus* newts and analyse these data in a phylogenetic context, simultaneously with data from protein electrophoresis. Their synthetic approach is particularly effective as the older as well as the younger evolutionary lineages within the genus are described by the behavioural and biochemical characters, respectively.

Construction of a consensus evolutionary tree

The data that we have presented and discussed so far uphold a single unified hypothesis of evolutionary descent of *Triturus* species. However, the alternative evolutionary trees that we have shown in Figs. 4–8 are not identical. Differences exist to the extent that a strict consensus representation of the trees is not informative. To overcome this problem we have constructed a composite consensus tree for all *Triturus* species, starting with a framework consisting of the six species that feature in the studies of biochemical genetics and reproductive interactions. These six species, *T. alpestris*, *T. boscai*, *T. cristatus*, *T. helveticus*, *T. marmoratus* and *T. vulgaris*, are connected by thick lines in Figs. 4–8. In constructing the consensus tree we counted the number of internal nodes, including the root, separating species over the tree. The sums of these values over the five trees for all 15 pairwise combinations were represented in a half matrix, from which the consensus tree was constructed by applying the UPGMA-method. The consensus tree is represented by thick lines in Fig. 9. However, the ability for species to hybridise may be plesiomorphous (a shared primitive condition). Hybridisation potential does correctly link species only on the assumption that it is time dependent and inversely correlated with the degree of divergence. Although this assumption seems sensible and correct, we repeated the construction of the framework consensus tree deleting the hybridisation data. This approach gave an identical result. Moreover, the consensus tree is in line with independently derived conclusions based on albumin immunological distances (Busack et al., 1988), which bolsters our confidence in the combined approach presented above. *T. cristatus* is grouped with *T. marmoratus* (as in all constituent trees) which group in turn clusters with *T. alpestris*. This configuration is supported by most of the electrophoretic analyses. *T. helveticus* is grouped with *T. vulgaris* (cf. Figs. 4, 6 and 8) to which in turn *T. boscai* is added. *T. boscai* is not assigned to any species group by Busack et al. (1988) and the only direct support for this latter configuration comes from the UPGMA-analysis of the protein electrophoretic data of Rafinski

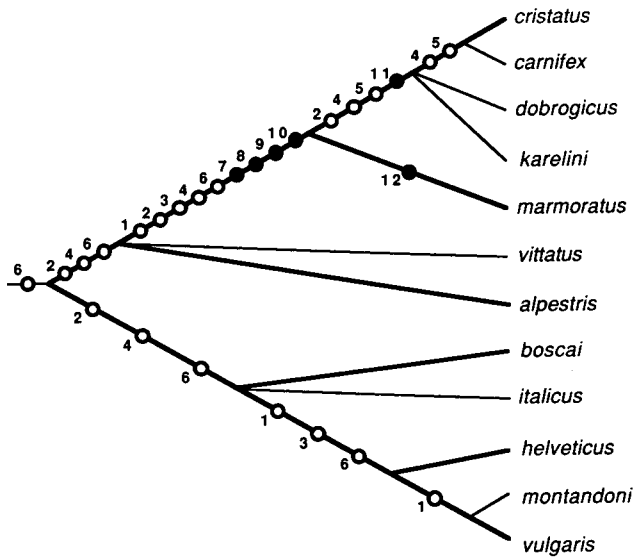


Fig. 9. Composite consensus evolutionary tree of the genus *Triturus*. The tree contains three unresolved trichotomies (details see text). Studies with supportive data are indicated on the tree; solid dots refer to cytogenetic characters: (1) protein electrophoretic data by Rafinski and Arntzen (1987) and (2) Table 2 of this paper, (3) reproductive isolation (reviewed in the present paper), (4) immunological data (Busack et al., 1988), (5) mitochondrial DNA sequencing data (Wallis and Arntzen, 1989), (6) data from courtship behaviour (Arntzen and Sparreboom, 1987, 1989), (7) chromosome 1 syndrome (Sims et al., 1984, Sessions et al., 1988, (8) C-value (reduction in *cristatus* species group), (9) decrease in heterochromatin count, (10) decrease in C-band number, (11) decrease in intrakaryotypic variation in chromosome shape, and (12) additional modifications of the chromosome 1 developmental arrest syndrome (Sessions et al., 1988).

and Arntzen (1987) and the analysis of newt courtship behaviour (Arntzen and Sparreboom, 1989).

To this framework, the species *T. carnifex*, *T. dobrogicus* and *T. karelini* were added (Fig. 9) on the basis of analyses of mitochondrial DNA (Wallis and Arntzen, 1989), that we consider to provide strong and more convincing data than the analysis of reproductive interactions. *T. montandoni* and *T. vulgaris* we consider sister species on the basis of their high genetic similarity (Rafinski and Arntzen, 1987) and high hybridization potential in the field (Fuhn, 1963; Hofmann, 1908). Unfortunately, the cytogenetic aspects of this natural hybridization have not been studied.

Conflicting data mainly concern the relative positions of *T. dobrogicus*, *T. vittatus* and *T. italicus*. These species are therefore attached to our composite evolutionary tree by a trichotomous branching structure. The remaining minor areas of data conflict are widely scattered over the tree (see Fig. 9, cf. Figs. 4–8 and cited papers).

Cytogenetics

Three main sets of cytogenetic characteristics will be considered here: genome size (expressed as C-value, the total amount of nuclear DNA in a haploid nucleus); mitotic chromosome structure, including chromosome morphology, number and position of C-bands, amount of cytologically visible heterochromatin, and gene loci; and finally, oocyte lampbrush chromosomes.

Genome size

All species of *Triturus* have relatively large genomes, and so are in the forefront of arguments concerned with excess, selfish, or junk DNA and its signficance, if any, in relation to morphology, development or evolution (Horner and Macgregor, 1983; Sessions and Larson, 1987). Several authors have reported C-values for various species of *Triturus* and certain other salamandrids (Callan, 1972; Olmo, 1973, 1974, 1983; Olmo and Morescalchi, 1975; see Table 5). All values have been

Table 5. Published genome sizes (expressed as C-values, or haploid amount of DNA) of various salamandrid species. References: 1) Mancino et al (1977); 2) Olmo (1983); 3) Sessions (unpublished); 4) this paper (from Table 2).

Species	Mean	References
Euproctus montanus	23	1
Notophthalmus viridescens	35	2
Paramesotriton hongkongensis	34	2
Salamandra atra	33	2
Salamandra salamandra	33	1
Salamandrina terdigitata	21	1
Taricha granulosa	29	2, 3
T. rivularis	30	2, 3
T. sierrae	32	3
T. torosa	29	2, 3
Triturus alpestris alpestris	24	4
T. a. apuanus	24	2
T. boscai	24	4
T. carnifex	22	2, 4
T. cristatus	21	2, 4
T. dobrogicus	22	1, 4
T. helveticus	23	1, 4
T. italicus	22	1
T. karelini	21	4
T. marmoratus marmoratus	21	1, 4
T. montandoni	26	4
T. vittatus ophryticus	28	4
T. vulgaris meridionalis	24	2
T. v. vulgaris	29	4
Tylototriton verrucosus	25	2

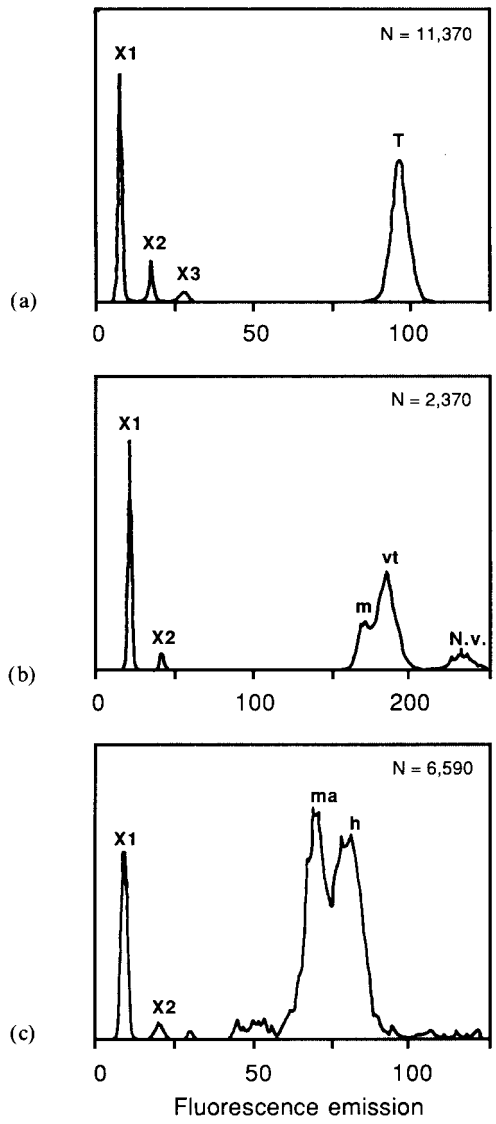


Fig. 10a. Flow cytophotometric scan of a mixed preparation of nuclei from *Xenopus laevis* and *T. v. vulgaris*. X1, single *Xenopus* nuclei; X2, adhering pairs of *Xenopus* nuclei; X3, adhering triplets of *Xenopus* nuclei; T, *Triturus* nuclei, N, total number of nuclei measured.

Fig. 10b. Flow cytophotometric scan of a mixed preparation of nuclei from *X. laevis* (X1, X2), *T. montandoni* (m), *T. vittatus* (vt) and *Notophthalmus viridescens* (N.v.).

Fig. 10c. Flow cytophotometric scan of a mixed preparation of nuclei from *X. laevis* (X1, X2), *T. marmoratus* (ma) and *T. helveticus* (h).

obtained from Feulgen-stained erythrocyte nuclei using a microspectrophotometer. No one author has measured C-values of all species of *Triturus* and only one author has reported C-values for representatives of all three subgenera (Olmo, 1983). The C-values of some species have never been measured.

We have used a flow cytometer to measure C-values of eleven of the twelve species of *Triturus* (Fig. 10). Nuclear DNA amount was measured using erythrocyte nuclei that were isolated with 0.01 M citric acid with 0.05% Nonidet P40 (Horner and Macgregor, 1983), resuspended in ethanol, and stained with propidium iodide or DAPI in phosphate buffer. Problems encountered using this technique included the standard chosen as well as the fluorochrome used. Using erythrocyte nuclei from *Xenopus laevis* as our standard, most of our estimated C-values were extraordinarily high compared to those obtained by other workers (Table 6) and also showed large differences between two sets of measurements. These problems may reflect the nearly ten-fold difference between the genome sizes of tests and standard, and they disappeared when we used urodele standards to calculate C-values (Table 6).

Our observations concerning C-value variation among species of *Triturus* are very similar to those reported by other workers (Tables 5 and 6). The major feature that we see in C-value variation within the genus is a distinction between the 'large'-bodied newts of the *cristatus* species group with relatively small genomes, and all the remaining species which have relatively large genomes. Species belonging

Table 6. C-values of species of *Triturus* based on flow cytometric measurements of RBC nuclei. C-values were calculated from the ratio of cytometric values of test specimens to those of standard specimens of 'known' C-values. All specimens, except *T. montandoni*, *T. vittatus*, and *Notophthalmus viridescens*, were measured on the same day using *T. cristatus* as the standard (published C-value = 22.0; Olmo, 1983). *Triturus montandoni*, *T. vittatus*, and *N. viridescens* were measured at a later date, and standardised to *N. viridescens* (published C-value = 35.0; Olmo, 1983).

Species	No. of nuclei $\times 10^{-3}$	Cytometric units $\pm$ SD	Ratio test/standard	Estimated C-value*
T. carnifex	9.0	87.0 $\pm$ 3.70	1.00	22.0 (33)
T. cristatus	10.3	85.0 $\pm$ 3.20	0.98	21.6 (32)
T. dobrogicus	16.7	83.0 $\pm$ 3.20	0.95	20.9 (31)
T. karelini	26.3	81.0 $\pm$ 3.00	0.93	20.5 (30)
T. marmoratus	3.9	83.0 $\pm$ 4.70	0.95	20.9 (31)
T. vittatus	6.9	72.0 $\pm$ 2.50	0.79	27.7 (20)
T. alpestris	11.3	93.0 $\pm$ 4.40	1.07	23.5 (35)
T. boscai	13.3	93.0 $\pm$ 2.60	1.07	23.5 (35)
T. helveticus	11.4	98.0 $\pm$ 3.50	1.13	24.9 (37)
T. montandoni	5.4	69.0 $\pm$ 2.90	0.76	26.6 (27)
T. vulgaris	2.9	114.0 $\pm$ 4.13	1.31	28.8 (43)
N. viridescens	1.9	91.0 $\pm$ 2.40	1.00	35.0 (34)

* Values in parentheses are those obtained when *Xenopus* nuclei were used as the standard (see text).

to the *cristatus* group have closely similar genome sizes in the range of 21–22 picograms, whereas other species have a wider range of genome size, from 22–29 picograms (mean = 25 ± 2.6 pg; Table 5).

The significance of smaller C-values in the *cristatus* species group is undoubtedly worthy of further exploration. Smaller genomes generally indicate smaller cells and shorter cell-cycle times (Horner and Macgregor, 1983), and cell size and cycle time are clearly important in relation to rates of development and growth (Sessions and Larson, 1987). It would therefore seem appropriate to determine relative growth rates and cell-cycle times for species that are representative of the large-bodied and small bodied newts. A valuable assessment of the evolutionary significance of changes in genome size might then be possible, taking account of all that is known of the natural history and geographical distributions of the species concerned. The only specific comment that we wish to make here by way of an example relates to *T. cristatus*, a species with one of the lowest C-values of the genus. *T. cristatus* extends far into the northernmost parts of Europe, where growing seasons are short and mean annual temperatures are low, and it is almost always found with at least one of the small-bodied species of *Triturus* in the same ponds. Under such conditions, rapid somatic and reproductive growth may be an advantage, particularly for an animal that must grow to a relatively large adult size. This matter has been discussed in more detail by Macgregor and Sessions (1986a,b).

Mitotic chromosome structure

Of all the 17 idiograms of karyotypes presented here (Figs. 11–13), 10 were constructed from preparations made in H. C. Macgregor's laboratory employing the BaOH/Giemsa method of C-staining. Four others were constructed from preparations made by Dr. Pilar Herrero and Dr. Michael Schmid employing the same technique. The remaining three, *T. vulgaris meridionalis*, *T. montandoni* and *T. italicus* were constructed from preparations made by Dr. M. Ragghianti and her colleagues employing the NaOH/Giemsa method of C-banding. We recognise that the Giemsa C-band patterns produced in these latter three species may not be strictly comparable with those of the other 14 on account of the different C-banding techniques that were used. However, in our experience, the differences between NaOH- and BaOH-induced C-banding are usually small, and such as they may be, we have judged that they are unlikely to affect seriously our analyses or conclusions.

The karyotypes of all species of *Triturus* contain twelve pairs of biarmed (metacentric or submetacentric) chromosomes, and the gradation in length from chromosome 1 to 12 is more or less the same for all species (Figs. 11–13). Phylogenetic analysis of individual chromosomal variation in *Triturus* is seriously hampered by our inability to identify homologous chromosomes between species. The only chromosomes that we can identify as homologues between species of *Triturus* are the sex chromosomes and possibly those that carry conserved NOR loci (see below). All species of *Triturus* appear to be characterised by X/Y sex

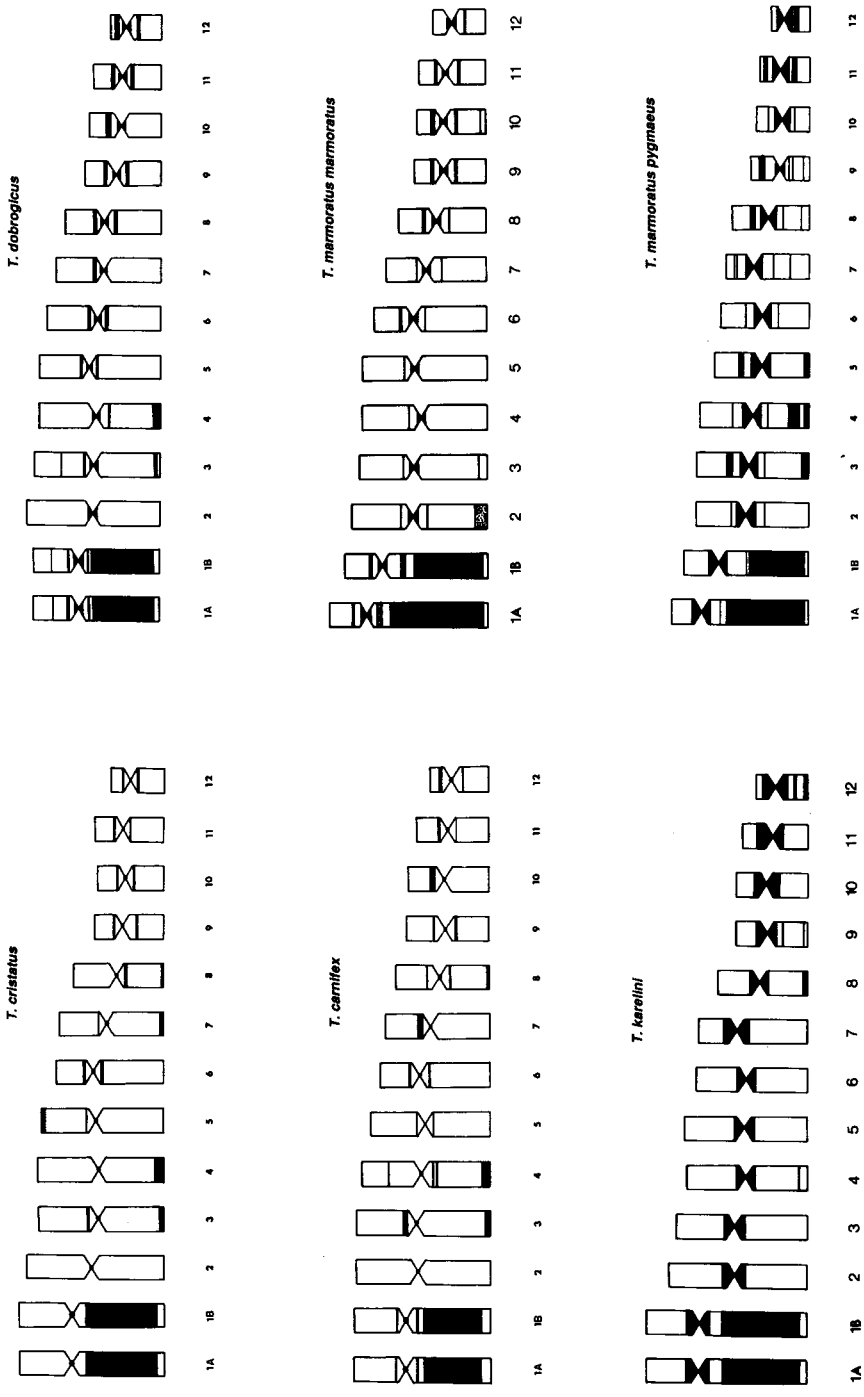


Fig. 11. Idiograms showing the Giemsa C-band patterns of chromosomes from newts belonging to the *cristatus* species group. In each case, the longest chromosome (number 1) is represented twice since it is heteromorphic and almost entirely heterochromatic in its long arm in all animals of these species. The idiogram for *T. m. pygmaeus* was constructed from data provided by Dr. P. Herrero.

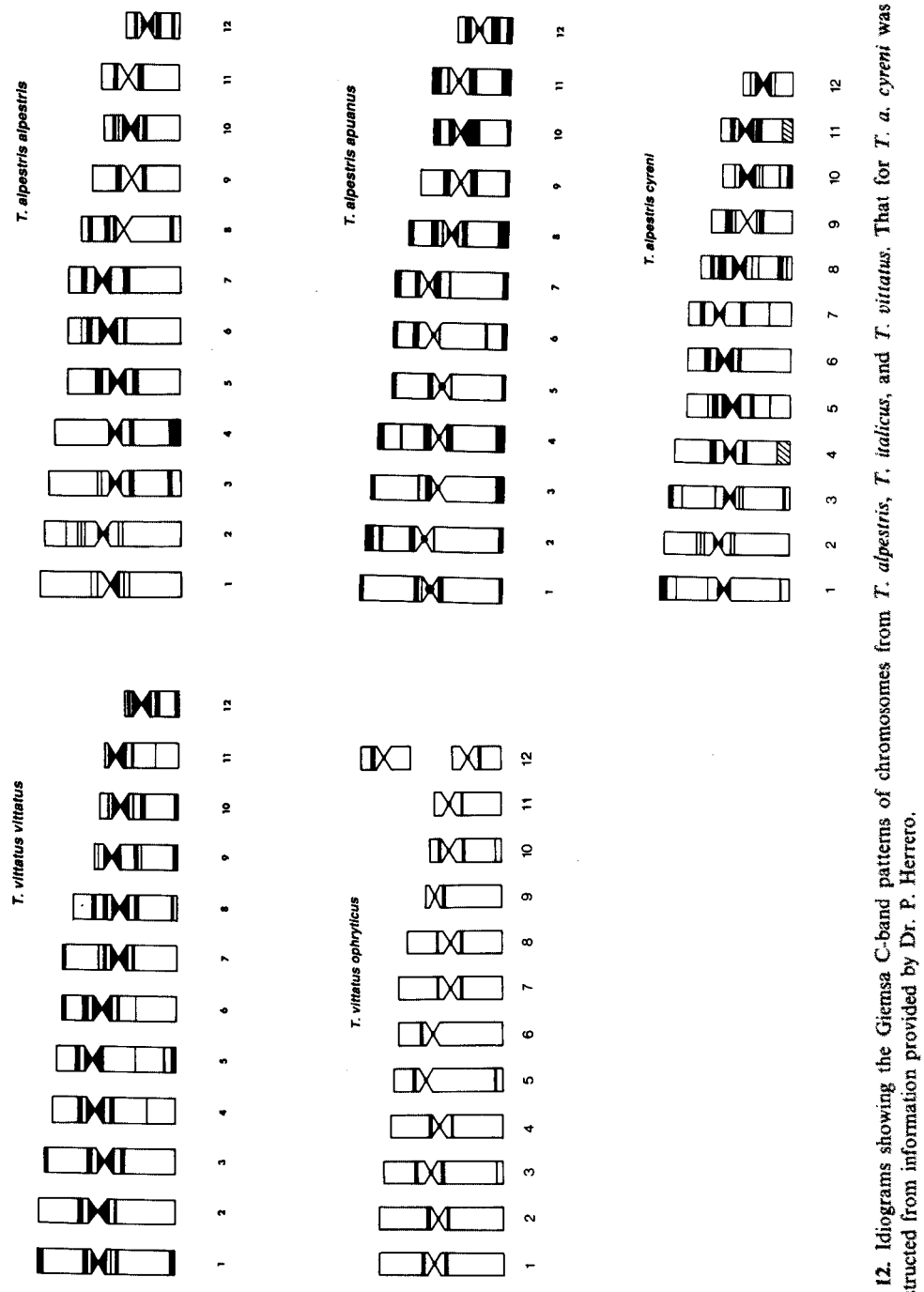


Fig. 12. Idiograms showing the Giemsa C-band patterns of chromosomes from *T. alpestris*, *T. italicus*, and *T. vittatus*. That for *T. a. cyreni* was constructed from information provided by Dr. P. Herrero.

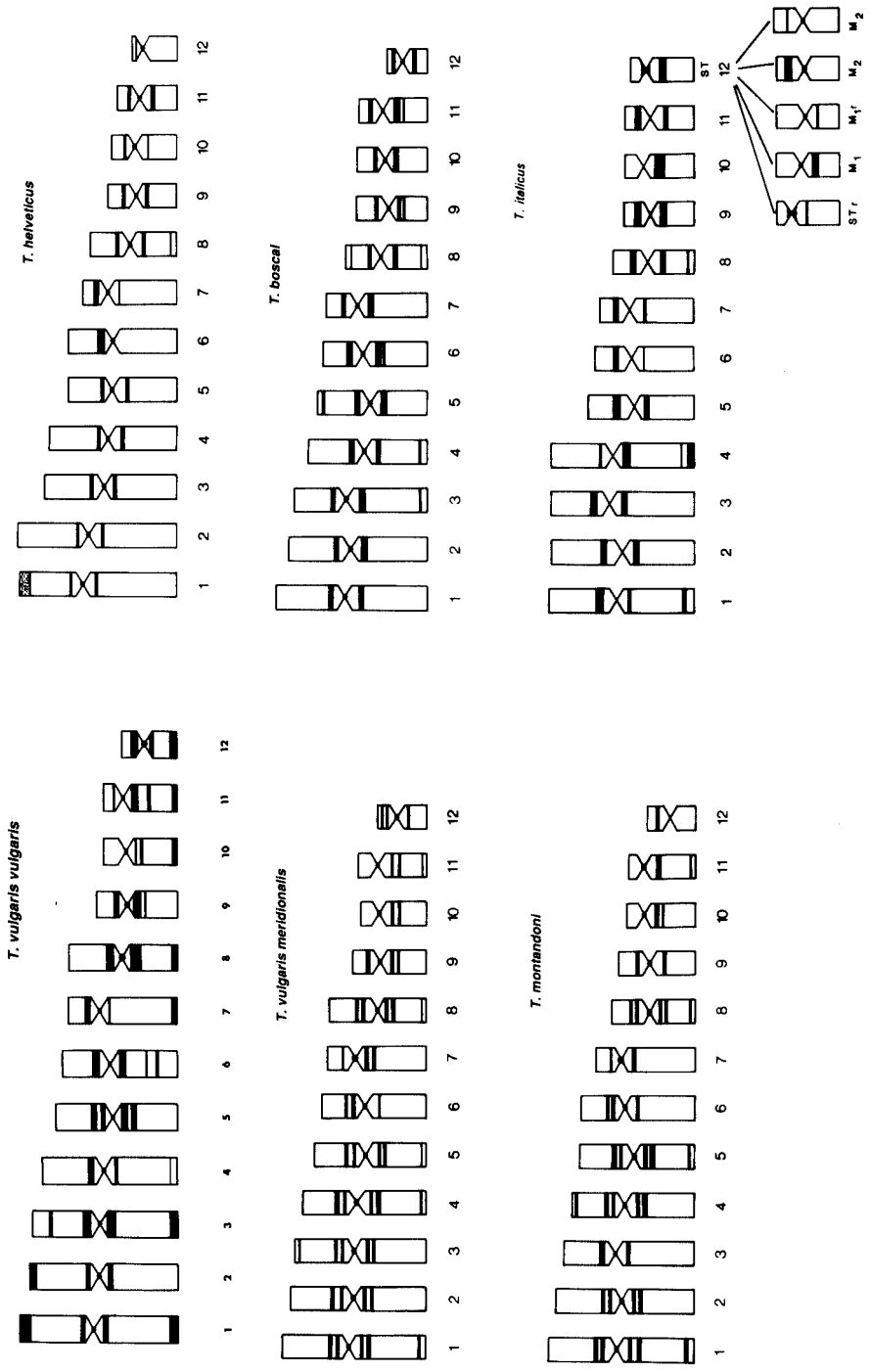


Fig. 13. Idiograms showing the Giemsa C-band patterns of chromosomes from newts belonging to the sub-genus *Palaeotriton*. That for *T. italicus* was constructed from data in Ragghianti et al. (1980).

determination, but the karyotypic position of the sex chromosome pair differs between species. The sex pair is chromosome 4 in the *cristatus* group and in *T. alpestris* and chromosome 5 in all other species (Schmid et al., 1979). The Y chromosome is distinguished from the X chromosome by telocentric heterochromatin in all species except *T. helveticus* (Table 7). The absence of Y-chromosome heterochromatin in *T. helveticus* probably reflects the large evolutionary reduction in heterochromatin that has occurred in this species (see below). Even in *T. helveticus*, however, the sex-chromosome pair is identifiable during meiosis by the presence of differential segments of the sex bivalent that lack chiasmata (Schmid et al., 1979).

The most obvious karotypic difference between species of *Triturus* concerns the peculiar chromosome-1 heteromorphism present in the *cristatus* species group but absent in all other species (Figs. 11–13, Table 7). It has been established that the chromosome-1 heteromorphism is involved in a balanced lethal genetic system in which homomorphic embryos undergo developmental arrest at late tailbud stages in all species of their group (Macgregor and Horner, 1980). This balanced lethal chromosome-1 heteromorphism may be considered uniquely derived, since it is not found in any other salamander species. It is a clearly synapomorphic character that provides the strongest available evidence that the species of *T. carnifex*, *T. cristatus*, *T. dobrogicus*, *T. karelini*, and *T. marmoratus* together constitute a monophyletic

Table 7. Cytogenetic parameters of *Triturus* species. CB = number of C-bands; HTC = amount of heterochromatin expressed as % of karyotype length; cHTC = % of total HTC that is centromeric; yHTC = visible heterochromatin on Y chromosome; HTA = heterochromatic long arms of chromosome 1 involved in a balanced lethal heteromorphism.

Species	CB	HTC	cHTC	yHTC	HTA
T. carnifex	34	12	27	+	+
T. cristatus	31	11	32	+	+
T. dobrogicus	34	12	42	+	+
T. karelini	20	23	89	+	+
T. marmoratus marmoratus	37	12	43	+	+
T. m. pygmaeus	42	31	62	+	+
T. alpestris alpestris	46	20	54	+	–
T. a. apuanus	63	29	24	+	–
T. a. cyreni	60	26	50	+	–
T. vittatus ophryticus	34	9	40	+?	–
T. v. vittatus	54	29	64	+?	–
T. boscai	44	16	28	+?	–
T. helveticus	36	11	40	–	–
T. italicus	28	11	6	+	–
T. montandoni	49	16	28	+?	–
T. vulgaris meridionalis	58	16	30	+	–
T. v. vulgaris	54	23	20	+	–

lineage (Sims et al., 1984; Sessions et al., 1988). Evidence for evolutionary modification of the chromosome-1 situation within the *cristatus* species group is provided by *T. marmoratus*, in which the heteromorphic region has apparently increased in size and is associated with embryonic arrest at an earlier developmental stage relative to other species of the *cristatus* group (Sessions et al., 1988). It will be important in future work to try to identify the chromosomal homologue to chromosome 1 in the karyotypes of other *Triturus* species. It is possible that this homologue may actually be a small chromosome which has increased in size due to growth of the C-band positive heteromorphic region (Sessions et al., 1988).

Although we are limited by our inability to identify most homologous chromosomes between species, nevertheless distinct evolutionary patterns are seen in terms of general chromosome shape, or centromere position. Newts belonging to the *cristatus* species group have more symmetrical chromosomes than do the other species. Chromosome symmetry is often expressed by 'centromere index' (CI) (Levan et al., 1964) which is calculated by dividing the length of the short arm by the total chromosome length. We have quantified interspecific differences in chromosome shape by calculating a 'mean centromere index' (MCI) for the entire karyotype of each species (Table 8) by dividing the sum of centromere indexes by the number of chromosomes within each karyotype. In addition, a measure of diversity in chromosome shape within a given karyotype is provided by the standard deviations of the MCI's (expressed as % of the mean centromere index, Table 8).

Using these values, a distinction is seen between species with relatively high MCIs (mean $= 0.42 \pm 0.01$), and low intrakaryotypic variation in chromosome shape (% SD, mean $= 14.0 \pm 3.3$), all of the *cristatus* species group, and species with

Table 8. Variation in chromosome shape in species of *Triturus* expressed as 'mean centromere index' (MCI), calculated by dividing the sum of the centromere indices of the chromosomes in each karyotype by the number of chromosomes (centromere index = length of short arm/total chromosome length). '%SD' is the standard deviation expressed as percent of the MCI.

Species	MCI	%SD
T. carnifex	0.43	10
T. cristatus	0.42	12
T. dobrogicus	0.43	13
T. karelini	0.43	13
T. marmoratus marmoratus	0.40	17
T. m. pygmaeus	0.42	19
T. alpestris	0.41	16
T. vittatus	0.37	32
T. boscai	0.39	21
T. italicus	0.39	20
T. helveticus	0.38	29
T. montandoni	0.38	29
T. vulgaris	0.40	23

relatively low MCIs and high intrakaryotypic variation in chromosome shape, (0.39 ± 0.01; 24.2 ± 5.9 respectively) in all other species. No clear patterns are apparent within each of these two groups with respect to MCIs, but we do see a pattern in terms of our measure of intrakaryotypic variation in chromosome shape. Outwith the *cristatus* species group, which shows low values, this measure resolves *T. vittatus*, with the highest value, from *T. helveticus* and *T. montandoni* with somewhat lower values, *T. boscai*, *T. italicus* and *T. vulgaris*, with still lower values, and *T. alpestris* with the lowest value of all. Within the *cristatus* species group, *T. marmoratus marmoratus* and *T. m. pygmaeus* stand out with relatively high levels of variation, accounted for by the heteromorphic chromosome 1 which is strongly asymmetrical in these species. *T. carnifex*, *T. cristatus*, *T. dobrogicus* and *T. karelini* all have substantially lower levels of intrakaryotypic variation.

Although we have not analysed these karyotypic patterns statistically, they suggest that the *cristatus* species group shows less diversity in chromosome shape than the other species. Since chromosomal shape is influenced by rearrangements (i.e. those that shift centromere position), the differences that we have identified may reflect different rates of fixation of chromosomal rearrangements which may in turn reflect differences in population structure. Regardless of their biological significance, however, our results show a concordance with other comparative analyses as represented by our consensus tree (Fig. 9) in the sense that they distinguish the *cristatus* species group (including *T. marmoratus* and *T. dobrogicus*) from other species, so bolstering our confidence that our manner of expressing chromosomal morphology does have some phylogenetic significance.

C-bands and heterochromatin

The distributions of Giemsa stained C-bands among species of *Triturus* are illustrated in Figs. 11–13. From these idiograms it is apparent that members of the *cristatus* species group have C-bands that are primarily centromeric or pericentric, but show a wide variation from one species or subspecies to another with respect to the overall amount of C-band material (Table 7). Other newts have C-bands that are primarily pericentric, interstitial, or telomeric. If we consider the total number of C-bands (Table 7), including a single block of centromeric heterochromatin as one C-band but excluding altogether the large mass of heterochromatin that occupies most of the long arm of chromosome 1 in the *cristatus* group, and if we calculate the proportion of C-band heterochromatin that is strictly centromeric (Table 7), then one firm conclusion is possible: members of the *cristatus* species group have significantly fewer C-bands (as tested by ANOVA and multiple range test) than all other species. With regard to arrangements of C-band heterochromatin within the entire genus it is clear that the fewer C-bands present the greater the proportion of C-band heterochromatin that is located at the centromeres, *T. karelini* being the extreme example of this phenomenon (Table 7).

We have attempted a numerical cladistic analysis employing presence or absence on each chromosome arm of C-bands in particular regions (centromeric, pericen-

tric, interstitial, telomeric) and classifying C-bands into 'very large' (occupying more than 1% of the total idiogram length), 'very small' (barely visible cytologically and represented on idiograms as a single thin line) and 'intermediate' (all other C-bands). The result of this analysis was taxonomically nonsensical, probably because of our inability to identify homologous chromosomes and/or C-bands. Homology of the C-bands depends on information concerning the precise sequence of the highly repeated DNA that is concentrated in each band. Such information is available in only a very few cases (see Macgregor and Sessions, 1986a,b). Furthermore, we know nothing of the rates of change or movement around the karyotype of the highly repetitive sequences that form the basis of C-band heterochromatin.

The biological significance of C-band heterochromatin depends on a clearer understanding of its changeability at the molecular level. Just as with overall genome size, heterochromatin may have an influence at the level of organismal growth and development through its effects upon cell-cycle time, the replicative chronologies of individual chromosomes, the timing of the chromosome separation at mitosis, the distribution of genetic crossovers and the degree of structural and operational linkage between 'functional' genes. Some aspects of this problem have been reviewed by Macgregor and Sessions (1968a,b), but it has to be said that for the moment we simply do not know why C-band heterochromatin is there nor why it behaves as it does.

Gene loci

Several specific gene loci have been identified by in-situ hybridisation and other techniques in several *Triturus* species as well as various other salamandrids (Table 9). Most of this information concerns nucleolus-organizing regions (NORs) and NOR-like sequences, but information is also available for 5S-ribosomal genes and histone genes in a few species. These sites appear to vary widely in position from species to species (Table 9). Nevertheless, it is possible to discern patterns suggesting directions and modes of evolutionary change.

Two main hypotheses have been presented for the mode of evolutionary change in chromosomal position of these genes. The 'repatterning' hypothesis (Mancino et al., 1977) postulates that variation in chromosomal sites of gene loci, C-bands and other cytogenetic markers reflects extensive remodelling of chromosome structure during evolution. A prediction based on this hypothesis is that evolutionary changes in the positions of cytogenetic markers within a taxon should be slow, unique and virtually irreversible. An alternative hypothesis, the 'homosequentiality' hypothesis (Macgregor and Sherwood, 1979), postulates that differences in the locations of cytologically visible cytogenetic markers reflect selective, localised amplification of different subsets of multigene families within the context of a basically stable pattern of chromosome structure. This hypothesis predicts that evolutionary changes in cytologically visible chromosomal sites should often be rapid, and reversals and parallelisms should be common.

Table 9. Chromosomal loci of ribosomal and histone genes in salamandrid species. Main NORs refers to NORs that occur as diploid pairs, while 'other sites' occur as variants between individuals, usually absent on homologous chromosomes.

Species	Chromosome number				
	Main NORs	Other sites	5SrRNA	Histones	References
Euproctus montanus	9	2, 7	–	–	5
E. platycephalus	11	–	–	–	5
Notophthalmus viridescens	3	–	1, 2, 6, 7	2, 6	7, 8, 9, 17
Pleurodeles poireti	3, 11	–	–	–	10
P. waltli	3, 11	–	–	–	10
Salamandra salamandra	11	–	–	–	4
Taricha granulosa	2, 9	–	7	–	11
Triturus alpestris	9, 11	–	–	1, 2, 4	8, 13, 18
T. boscai	8, 10	–	–	–	6
T. carnifex	6, 9	–	10	5, 8	2, 8, 12, 20
T. helveticus	10/11	2/3	–	–	22
T. italicus	3	–	several	–	4
T. marmoratus	10	–	10	–	3, 14
T. montandoni	10/11	–	–	–	19
T. vittatus	9	–	–	–	21
T. vulgaris	10/11	several	10/11	–	1, 13, 15, 16

References: 1) Barsacchi et al. (1970); 2) Barsacchi Pilone et al. (1974a); 3) Barsacchi Pilone et al. (1974b); 4) Barsacchi Pilone et al., unpublished (cited in Mancino et al., 1977); 5) Bucci-Innocenti et al. (1978); 6) Bucci-Innocenti et al. (1983b); 7) Gall, (1954); 8) Gall et al. (1981); 9) Hutchison and Pardue (1975); 10) Lacroix (1968); 11) Leon (1976); 12) Mancino et al. (1972); 13) Mancino et al. (1977); 14) Nardi et al. (1972); 15) Nardi et al. (1974); 16) Nardi et al. (1977); 17) Pukkila (1975); 18) Ragghianti et al. (1972); 19) Ragghianti et al. (1978); 20) Rudak and Callan (1976); 21) Sims (1984); 22) Sessions, unpublished.

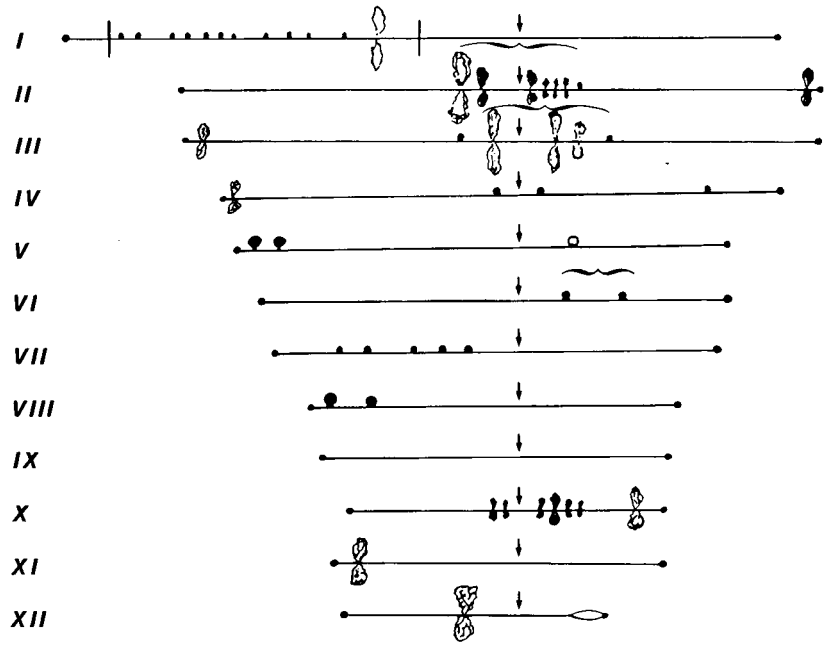
As mentioned before, most of the available information concerns NOR positions, and it is on these that we will focus our attention. NORs have been visualised by both in-situ hybridisation using molecular probes and differential silver staining. Since molecular in-situ hybridisation will label sites with sequence homology to the probe regardless of whether those sites represent functional NORs, we will consider only NOR sites that occur as diploid pairs and/or can be verified as functional NORs by silver staining or localisation of nucleoli. Four such NOR sites are seen in species of salamandrids outside the genus *Triturus*: chromosomes 2, 3, 9 and 11 (Table 9). Since there is uncertainty as to chromosome homology based on size, we will refer to these as: 2/3, 8/9, and 10/11. Defined in this way, it is apparent that variation in NOR location among species of *Triturus* involves these same three chromosomes (with one exception: *T. carnifex* with an NOR on chromosome 6; Table 9). Among these sites we can recognise 'conserved' sites that are found in a wide range of species, and 'novel' sites that are more restricted in distribution.

As in other groups of urodeles (Sessions and Kezer, 1987), salamandrids may have more than one diploid set of NORs, and *Triturus* species have either one or two sets of NORs showing no particular taxonomic pattern in this respect (Table 9). The most conserved NOR is located near the telomere of the long arms of chromosome 10/11. This NOR site is seen in two species of the subgenus *Triturus*, four species of *Palaeotriton*, and is also found in species of three other salamandrid genera (Table 9). This site appears to be the main ancestral NOR in *Triturus*. The next most conserved site is located near the telomere of the one arm of the metacentric chromosome 8/9 in one species of *Palaeotriton* and two species of *Triturus* (Table 9) and in the North American salamandrid *Taricha granulosa*. *T. alpestris* has an NOR on chromosome 8/9 but located interstitially on the long arms. A more 'rare' NOR site is located near the end of the short arm of chromosome 2/3, found in *Notophthalmus* and *Pleurodeles*, and in only one species of *Triturus* (*T. italicus*). Another rare site is an interstitial NOR on the long arm of chromosome 10/11 seen in *T. boscai* and in *Salamandra salamandra*. *T. carnifex* appears to have a unique NOR site near the telomeres of the long arms of chromosome 6.

The overall pattern of variation in NOR sites among species of *Triturus* relative to other salamandrids, suggests that evolutionary changes have mainly involved the selective appearance, disappearance, and reappearance of a few conserved sites, and therefore supports the homosequentiality rather than the repatterning hypothesis. Most significantly in the context of this paper, the transitory nature of NORs renders them less useful as characters for phylogenetic studies, since their evolution appears to be characterised by extensive homoplasy.

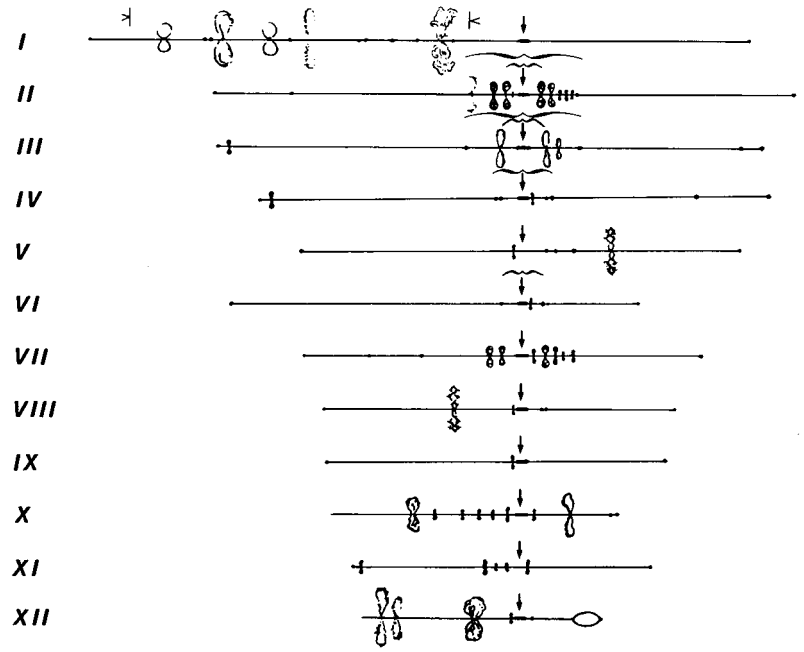
Lampbrush chromosomes

The lampbrush chromosome maps (Figs. 14–22) that we present are reproduced from those published by Barsacchi et al. (1970); Callan and Lloyd (1960; 1975); Mancino and Barsacchi (1965; 1966; 1969); Mancino et al. (1972); Nardi et al. (1972). Every species and subspecies of *Triturus* that has been examined is distinguishable on the basis of lampbrush chromosome morphology. All members of the *cristatus* species group are closely similar with respect to both the forms and distributions of most of the distinctive features of their lampbrush chromosomes. Indeed, each species of this group can be identified on the basis of lampbrush chromosome markers alone (Callan and Lloyd, 1960). For example, *T. dobrogicus* is characterised by highly distinctive lampbrush telomeres, *T. karelini* is characterised by uniquely conspicuous lampbrush centromeres, and *T. cristatus* can be identified unequivocally on the basis of a single pair of loops, the giant granular loops on its chromosome 12 (Callan and Lloyd, 1960). The lampbrush map for *T. alpestris* is quite distinct from that of any other species in so far as the majority of the loops and other structures with distinctive morphologies are clustered around the middle regions of the chromosomes (Fig. 19). The lampbrush maps for the three species of *Palaeotriton* that have been examined are broadly similar to each other



Triturus dobrogicus

Fig. 14.



Triturus karelini

Fig. 15.

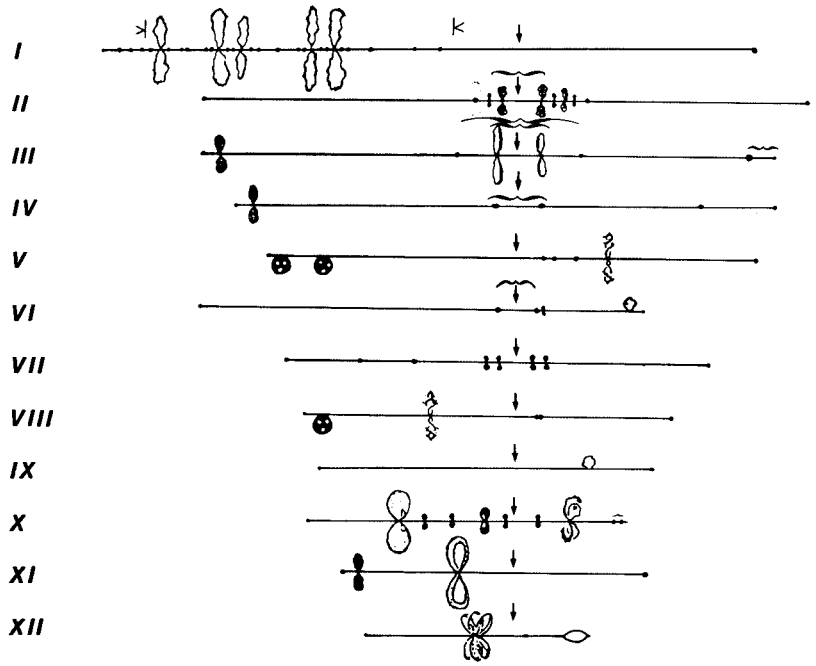


Fig. 16. *Triturus carnifex*

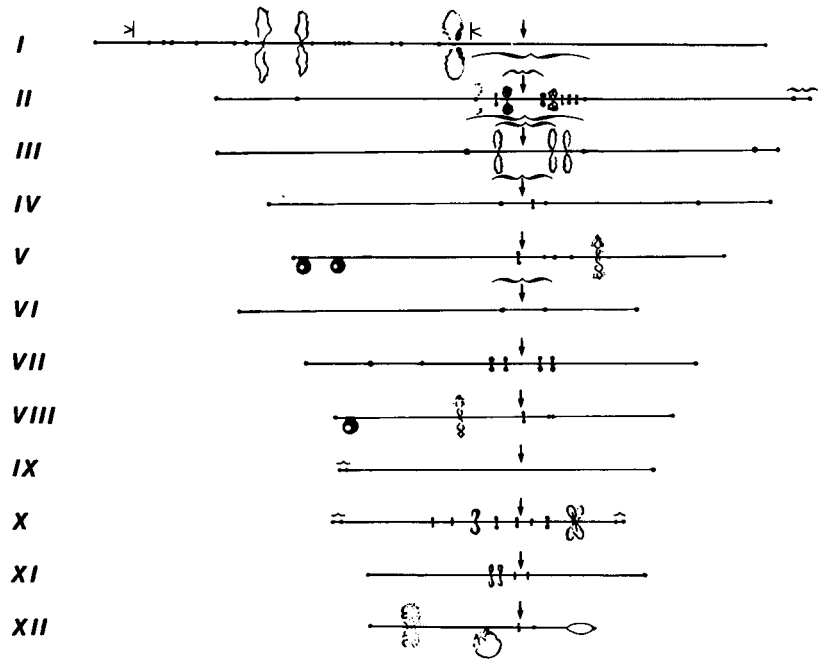


Fig. 17. *Triturus cristatus*

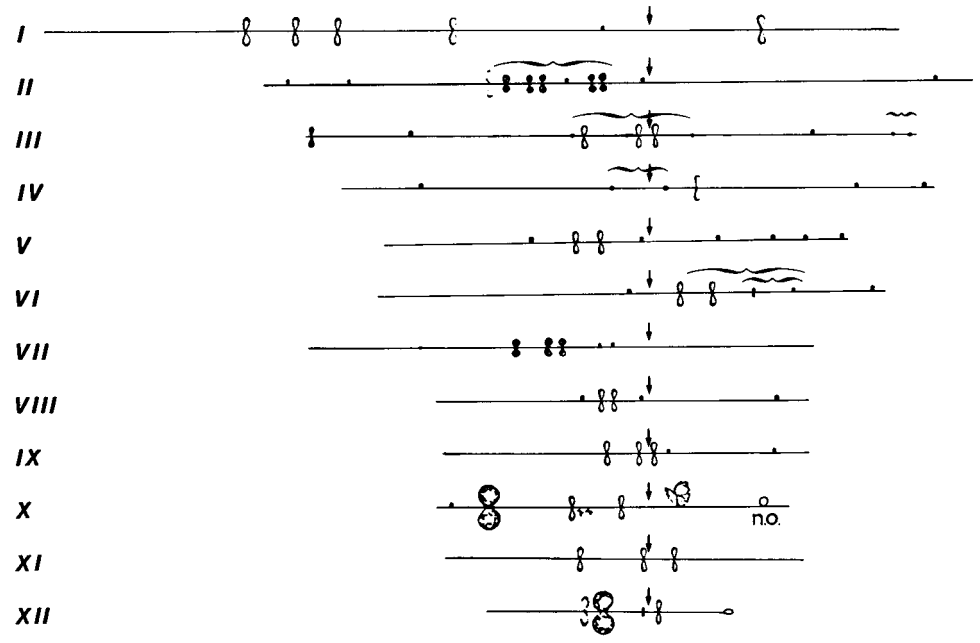

I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
n.o.
Triturus marmoratus

Fig. 18.

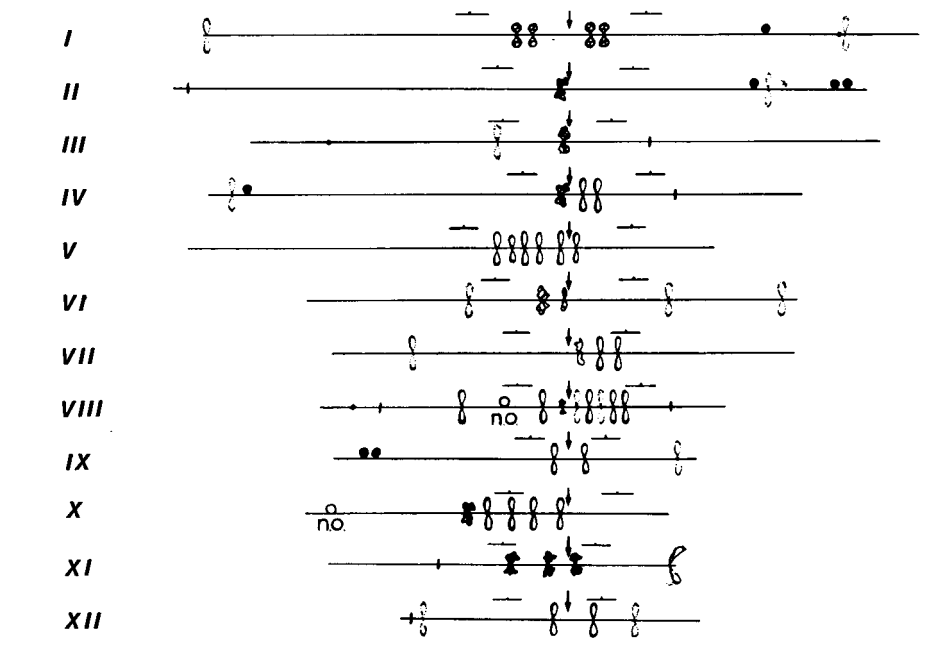

I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
n.o.
n.o.
Triturus alpestris apuanus

Fig. 19.

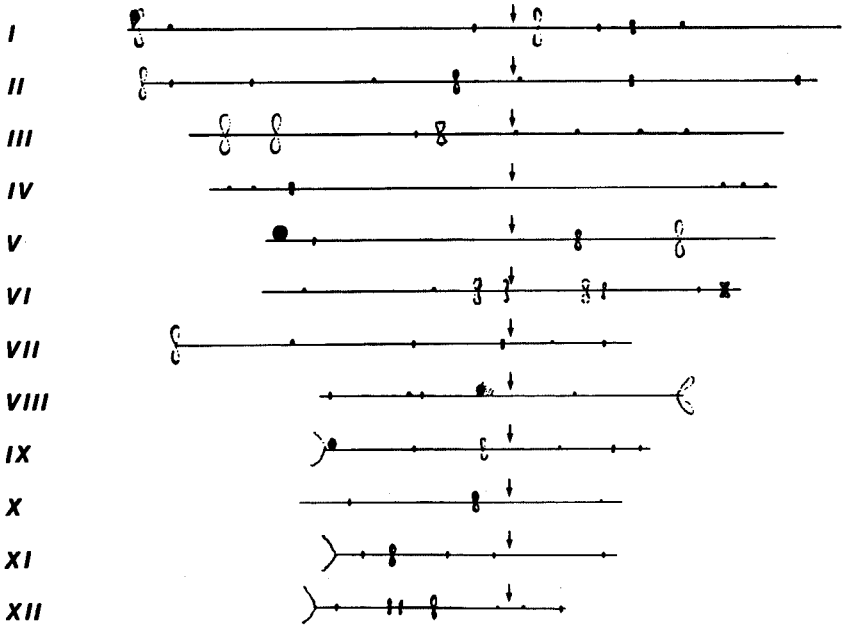

I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
Triturus helveticus

Fig. 20.

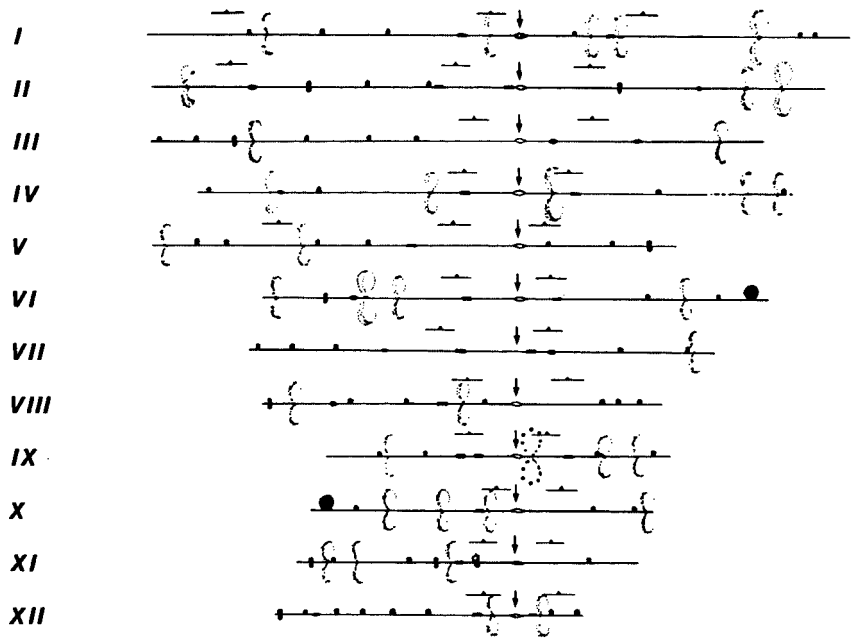

I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
Triturus italicus

Fig. 2..

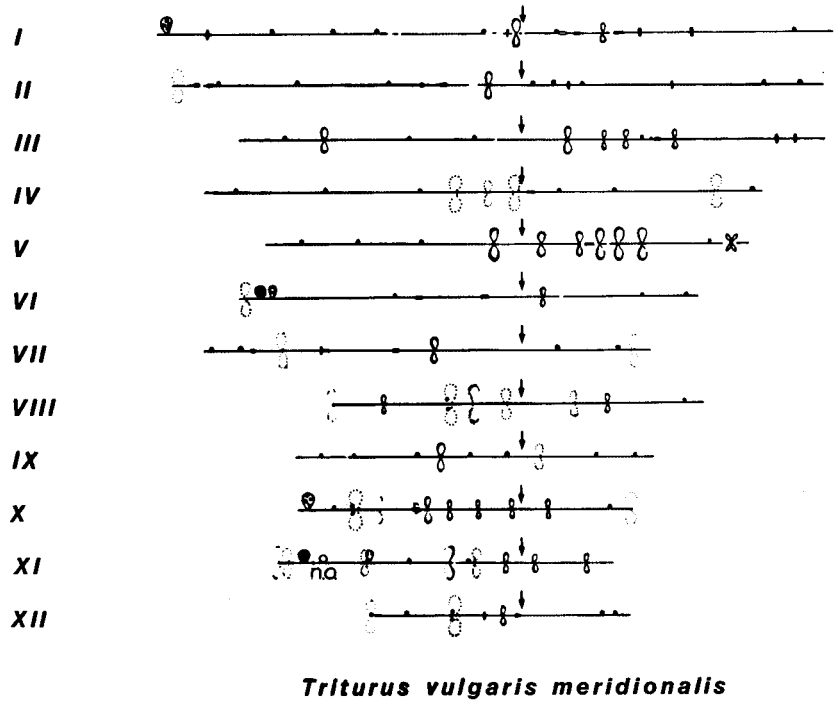


Fig. 22.

Figs. 14–22. Working maps of lampbrush chromosomes from species representative of each subgenus. These maps were originally produced by the following persons: 14, 15, 16, 17, Callan and Lloyd (1960), 18, Nardi et al., (1972), 19, Mancino et al., (1972), 20, Mancino and Barsacchi (1969), 21, Mancino and Barsacchi (1966), 22, Barsacchi et al., (1970). In each map the centromeres are indicated by vertical arrows. The lampbrush centromere positions have not been directly determined for *T. marmoratus* (Fig. 18), *T. helveticus* (Fig. 21), and *T. v. meridionalis* (Fig. 22). It is therefore not possible to be certain that all the chromosomes in each of these maps are arranged in the correct left-right orientation.

and quite distinct from those of either of the *cristatus* species group of *T. alpestris* (Figs. 14–22). The objects that form the basis of the maps are widely dispersed over the chromosome sets, and few of them are as conspicuous as some of the major markers used for identification of chromosomes from the *cristatus* group.

We have not been able to detect a clear correlation between the positions of lampbrush marker loops and the positions of C-bands on mitotic chromosomes. However, one striking correlation is clear: the very large centromeric C-bands of *T. karelini* correspond to the bars of loopless heterochromatin that flank each of the centromeres on the lampbrush chromosomes of this species. The highly repetitive DNA sequences that form the basis of this heterochromatin have been described by Baldwin and Macgregor (1985). Lampbrush chromosomes are therefore useful for taxonomy and phylogeny from three standpoints. First, as is the case with all other approaches, they show the *cristatus* species group to be distinct from all other

species. Second, they show *T. alpestris* as being quite different from the small-bodied newts, and third, they provide us with an exceptionally high-resolution means of species identification.

Analysis of cytogenetic parameters in an evolutionary context

We have analysed evolutionary patterns of C-value, number of C-bands and amount of heterochromatin (HTC) independently by partitioning changes in each parameter along the branches of our consensus tree (Fig. 23 and Table 10). Changes were partitioned along the branches of the tree using a procedure that minimises the number of hypothesised changes that are required on each branch

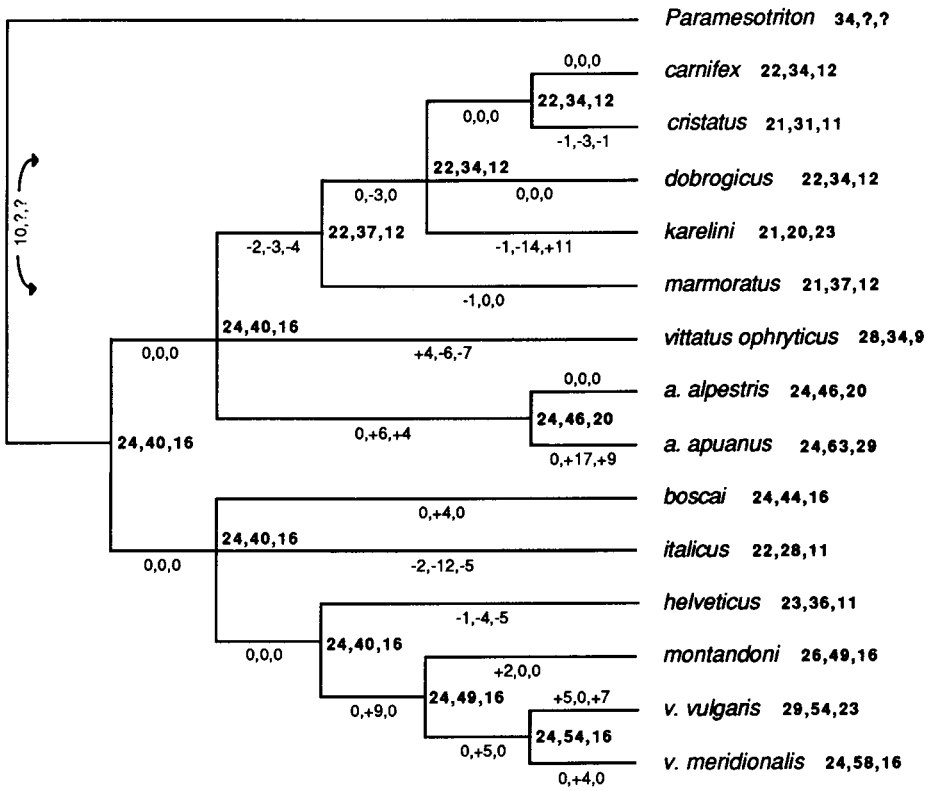


Fig. 23. Evolutionary tree showing evolutionary changes in C-value, number of C-bands, and amount of heterochromatin. Changes are partitioned along the branches of the tree using a procedure that minimises the number of hypothetical changes that are required on each branch (lineage) of the tree, without any change in the branching pattern itself, to generate the observed patterns of variation (see Farris, 1972). The branching structure of the tree used here is based on the consensus tree (Fig. 9). Character states are in boldface, evolutionary transitions in normal type.

Table 10. Phylogenetic changes in C-value, number of C-bands and amount of heterochromatin (HTC) in 19 lineages of newts of the genus *Triturus*. Changes are expressed as increases (+) or decreases (−) in percent of ancestral values inferred from the phylogenetic analysis present in Fig. 20. Ages of lineages (in millions of years) estimated from the comparative biochemical data of Rafinski and Arntzen (1987), Busack et al., (1988) and Frelow et al. (unpublished).

Lineage	Percent Change			
	C-value	C-bands	HTC	Est. Age (my)
cristatus species group	−8	−8	−25	14–28
cristatus superspecies	0	−8	0	7–12
T. marmoratus	−5	0	0	< 12
T. carnifex	0	0	0	1–3
T. cristatus	−5	−9	−8	1–3
T. dobrogicus	0	0	0	1–3
T. karelini	−5	−41	+92	< 2
T. alpestris	0	+15	+25	10–28
T. alpestris alpestris	0	0	0	1–2
T. a. apuanus	0	+37	+45	1–2
T. vittatus	+17	−15	−44	11–15
vulgaris species group	0	0	0	11–18
T. boscai	0	+10	0	10–17
T. helveticus	−4	−10	−31	7–13
T. italicus	−8	−30	−31	14–18
T. montandoni	+8	0	0	4–8
T. vulgaris	0	+10	0	4–8
T. vulgaris meridionalis	0	+7	0	7
T. v. vulgaris	+21	0	+44	7

(lineage) of the tree, without any change in the branching pattern itself, to generate the observed patterns of variation (see Farris, 1972). The analysis includes fourteen species and subspecies of newts of the genus *Triturus* and the salamandrid genus *Paramesotriton* as a comparator for genome size.

Our analysis suggests that the genus *Triturus* has experienced relatively modest evolutionary changes in genome size. Only nine of a total of twenty-three lineages show evolutionary changes in C-values, and most of these are decreases (Table 10). None of the decreases are strikingly large (i.e. at least 10% of the inferred ancestral values: Fig. 23). The only substantial changes are two large increases, one in the lineage leading to *T. vittatus* and the other in the lineage leading to *T. v. vulgaris* (Table 10).

Thirteen of the twenty-three lineages show evolutionary changes in the number of cytologically visible C-bands, of which nine are large: five increases and four decreases. The two largest changes in the number of C-bands are the increase in the lineage leading to *T. a. apuanus* and the decrease in the lineage leading to *T. karelini*. The lineage leading to *T. italicus* shows a decrease almost as large as that of the *T. karelini* lineage. Other lineages with large decreases in number of C-bands

are *T. helveticus* and *T. vittatus*. Lineages showing large increases in number of C-bands are *T. alpestris*, *T. boscai*, *T. vulgaris*, and the lineage leading to *T. montandoni* and *T. vulgaris*.

Nine lineages show evolutionary changes in the cytologically visible amount of heterochromatin (HTC, expressed as a percent of the total karyotype length), eight of which involve 10% or more of inferred ancestral values, with equal numbers of increases and decreases (Fig. 23 and Table 10). One lineage, *T. karelini*, stands out in showing a very large increase over the ancestral value. This increase seems to be due mainly to an accumulation of centromeric HTC (Table 10). Relatively large increases in HTC are also seen in lineages leading to *T. alpestris* and *T. a. apuanus*, and *T. v. vulgaris*. Large decreases in HTC are seen in lineages leading to *T. helveticus*, *T. italicus*, *T. vittatus* and to the *cristatus* species group.

Seven lineages show no change in any of the three cytological parameters. Where more than one change occurred, the changes tend to be in the same direction (Fig. 23). For example, five out of six lineages experiencing changes in both C-values and C-band number show decreases in both parameters; five out of seven lineages showing changes in both C-value and HTC show changes in the same direction (one in which they both increased, and four in which they both decreased); seven out of eight lineages show changes in the same direction in both C-band number and amount of heterochromatin (two cases of increase and five cases of decrease). Six lineages show changes in all three parameters, and four of these were all decreases. Three of those lineages lead to species or subspecies (*T. carnifex*, *T. dobrogicus*, and *T. a. alpestris*). The other three lineages lead to the subgenera *Triturus* and *Palaeotriton* and to the *vulgaris* species group.

Two lineages stand out as exceptions to most of the correlations analysed above: *T. karelini* and *T. vittatus*. *T. karelini* is unusual in showing a large decrease in C-band number associated with a large increase in heterochromatin amount. *T. vittatus* stands alone in the genus as the only lineage in which a large increase in genome size is accompanied by a large decrease in both the number of C-bands and the amount of heterochromatin.

The *cristatus* species group is characterised by a pattern of change in these cytological parameters which is quite different from other species of *Triturus*: C-band number, heterochromatin amount, and genome size have all tended to decrease, whereas in the other newts increases in C-value or number of C-bands have occurred with or without changes in other parameters in either direction. In *Palaeotriton* however, decreases in heterochromatin amount are always associated with large decreases in C-band number, and the single increase in heterochromatin amount is associated with a large increase in genome size (*T. v. vulgaris*). These results suggest that changes in genome size, C-band number, and heterochromatin amount are evolutionary correlates of each other.

Four lineages that stand out in showing the largest amount of total change in the cytological parameters are *T. karelini*, *T. vittatus*, *T. italicus* and *T. v. vulgaris*. The change in *T. karelini* is accounted for by growth of centromeric heterochromatin. Changes in two of the other lineages are more interesting because they coincide with morphological peculiarities at the organismal level. *T. vittatus* has the largest body

size outside the *cristatus* group and shows a large increase in genome size with large decreases in C-bands and heterochromatin. *T. italicus*, on the other hand, has the smallest body size in the genus and shows a decrease in genome size associated with very large decreases in C-bands and heterochromatin. The biological significance of this relationship between cytological and organismal characteristics remains to be assessed.

Approximate rates of change in cytogenetic characters among lineages of *Triturus* can be estimated on the basis of the ages of the taxa concerned. *T. karelini* is thought to be up to 2 or 3 my old (Busack et al., 1988; Wallis and Arntzen, 1989). According to the molecular-clock calibrations mentioned above, *T. a. apuanus* would be about 1.5 my old and *T. v vulgaris* would be about 7 my old (Frelow et al., unpublished; see Table 2). The lineages that have experienced the highest rates of change are *T. karelini* (decrease in C-bands and increase in HTC), *T. alpestris apuanus* (increase in C-bands and HTC), and *T. v. vulgaris* (increase in C-value and in HTC).

Discussion

In this paper we have tried to integrate a range of genetic and cytogenetic information on *Triturus*. The results from each widely different approach are always broadly in agreement, and sometimes sharply so, concerning the overall pattern of evolutionary relationships in the genus. All analyses agree in discerning two well-defined clusters of closely related species: the *cristatus* species group consisting of *T. carnifex*, *T. cristatus T. dobrogicus T. karelini*, and *T. marmoratus*, and the *vulgaris* species group consisting of *T. helveticus*, *T. montandoni*, *T. vulgaris*. The relationships of four remaining species are less clear. Two of these, *T. boscai* and *T. italicus*, appear to be closely related to the *vulgaris* group, while the remaining two, *T. alpestris* and *T. vittatus*, appear to be most closely related to the *cristatus* group. Accordingly, we recommend the recognition of two subgenera instead of the three proposed by Bolkay (1928). To be sure, the *cristatus* species group is distinct on account of the large sizes and osteology of the animals, as Bolkay pointed out, and it is also chromosomally distinct on account of the heteromorphism for chromosome 1. However, in consideration of all the evidence now available, these characters alone do not justify the designation of the group as a subgenus in its own right and apart from *T. alpestris* and *T. vittatus*. One thing that would seem to be clear from our studies, with particular regard to the *T. cristatus* group, is that there is undoubtedly a 'market' for cytogenetic data in phylogenetic analysis, provided that appropriate outgroups are included. A most interesting development in this field is the recent application by Ragghianti et al. (1988) of monoclonal antibodies to the mapping of specific sites on chromosomes of Amphibia. We hope, and indeed anticipate, that this approach will prove extremely valuable in identifying individual chromosomes and establishing homologies between chromosomes of related species.

Problematic species in all analyses are *T. boscai* and *T. italicus*, which may probably be correctly assigned to subgenus *Palaeotriton*, and *T. alpestris* and *T.*

vittatus for which presently available evidence favours inclusion in the subgenus *Triturus*. *T. vittatus*, the only non-European species, is by far the least understood of the *Triturus* species. The animal is known for the aggressive behaviour between rival males. A similar, but less fierce behaviour has been reported for *T. cristatus* and *T. marmoratus* (Zuiderwijk and Sparreboom, 1986). Although aggression is difficult to describe and quantify in newts, and similar behaviour is almost certainly present in other salamandrid species, it may nonetheless be a good synapormorphy that justifies the placing of *T. vittatus* alongside the *T. cristatus* species group. This matter has been discussed in some detail by Arntzen and Sparreboom (1989) and by Raxworthy (1989). The species *T. alpestris T. boscai*, *T. italicus*, and *T. vittatus* apparently reflect deep differentiation of ancient lineages, and more work is needed to resolve more confidently their phylogenetic positions within the genus.

Since 1983, Bucci-Innocenti et al., (1983a) have generally been followed in elevating *T. carnifex*, *T. cristatus*, *T. dobrogicus* and *T. karelini* to full species status. This view is supported by the data of Wallis and Arntzen (1989) who showed through their studies of mitochondrial DNA that transition zones between the species are narrow and (cytoplasmic) gene flow probably is absent or very limited. We consider that the data we have analysed in this paper provide further support for the assignment of full species status to each of the members of the original subspecies of *T. cristatus*.

We consider that *Triturus* is in many senses an ideal group of animals for evolutionary studies. Every accessible aspect of its biology has proved to be workable from the standpoint of obtaining objective taxonomic and evolutionary information; and to judge from the limited information currently available on highly repetitive DNA from *Triturus* (see Macgregor and Sessions, 1986a,b) such difficulties as we have had with the chromosomes will probably be resolved by a further concerted investigation at the molecular level. Some indication of the usefulness of repetitive DNA sequence comparisons in taxonomic studies of Amphibia was given by the studies of Mizuno and Macgregor (1974) with regard to the salamander genus *Plethodon*. *Triturus* is likely to be even more promising in this respect since, unlike *Plethodon*, so much of its repetitive DNA seems to have been organised into the large cytologically identifiable blocks that are manifest as C-bands.

Finally we wish to comment on species protection and conservation. For *Triturus* in Europe the overall pattern is now clear: the most variation, species sub-division and subspeciation is found in southern regions. There is naturally a case for habitat protection in all parts, but in our view, southern Europe should have the highest priority in this regard. The large-bodied species are the most vulnerable on account of their particular habitat requirements. Species that live in geographically monotonous areas are especially vulnerable because in such areas environmental changes are likely to be on a large scale. *T. dobrogicus*, for example, is probably under considerable threat because of extensive modernisation of agriculture and drainage of the Danube marshes. Very little is known about *T. vittatus* in southern U.S.S.R, Turkey and Israel. It should also be bourne in mind that although the aquatic habitat is popularly seen as the most characteristic one for newts, it being

the place where they reproduce, all the species spend by far the greater part of their lives on land. Therefore the importance of the terrestrial habitat and specifically of hiding sites and shelter in the vicinities of ponds should not be overlooked. This matter has been discussed by Schoorl and Zuiderwijk (1981) on the basis of a comparative study showing the importance of the terrestrial habitat. With regard to trading in *Triturus*, all European species have been regularly advertised for sale to the public, although recently introduced strict legislation is now taking effect. The clear trend has been that the larger the species range, the more commonly is it traded. A notable exception is *T. marmoratus*, usually seen as the most attractive species by persons who keep newts as 'pets'. It seems that trade in marbled newts has not yet entirely ceased, notwithstanding the fact that they are fully protected by law in all the countries where they occur. An excellent report on the subject has been published by van Koolwijk (1987).

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Corresponding Editor: P. A. Alberch.