SOME ASPECTS OF THE CYTOLOGY OF EUROPEAN NEWTS
(GENUS TRITURUS)

by

SIMON H. SIMS

A thesis submitted for the degree of
Doctor of Philosophy of the
University of Leicester

August, 1984
Thesis
15.11.1984
STATEMENT

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled "Some Aspects of the Cytology of European Newts (Genus Triturus)" is based on work conducted by the author in the Department of Zoology, of the University of Leicester, mainly during the period between October 1981 and June 1984.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by reference. None of the work has been submitted for another degree in this or any other University.

ACKNOWLEDGEMENTS

I would like to thank all the people in this laboratory for their help, support and friendship during this work. In particular my thanks go to Dr. Alma Swan and Jaquie Boswell.

For excellent technical assistance, my thanks go to Ms. Heather Horner and especially Mrs. Lesley Barnett whose never ending and unquestioning help is more than greatly appreciated.

I would like to thank Dr. Michael Schmid for allowing me to visit and learn so much at the Institute for Human Genetics, University of Würzburg, W. Germany.

I would like to thank all the people in the Department of Zoology who have helped me in any way throughout my studies.

I cannot thank my family enough for their love and support which is always there.

Finally, I warmly thank Professor Herbert Macgregor for all his help, guidance, encouragement, and support throughout my work. I also thank him for providing me with excellent research facilities.

This work was carried out whilst in receipt of a research grant from the Medical Research Council, which is gratefully acknowledged.
1.1 General Introduction 1

1.1.1 European newts (Triturus) 1
1.1.2 The 'cristatus' species group 8
1.1.3 The 'vulgaris' and 'alpestris' species groups 17

1.2 Chromosome Banding and Mechanisms of Action 23

1.2.1 C-banding 24
1.2.2 Fluorescence banding 27
1.2.3 DAPI staining 29
1.2.4 Mithramycin staining 30
1.2.5 Mechanisms of counterstain enhancement of banding 31

2. The Cytology of Triturus cristatus and T. marmoratus with Particular Reference to Chromosome 1

2.1 Introduction 33

2.2 Methods and Materials 38

2.2.1 Animals 38
2.2.2 Preparation of animals and tissues 39
2.2.3 Mitotic chromosome preparations 40
2.2.4 Giemsa C-banding 41
2.2.5 Conventional staining with orcein 42
2.2.6 Fluorescence staining 43

2.2.6.1 DAPI/actinomycin D staining 43
2.2.6.2 Mithramycin/distamycin A staining 45
2.2.6.3 Counterstaining with methyl green 47
2.2.6.4 Sequential staining 48
2.2.7 Histology and cytology of the testis

2.2.7.1 Fixation and embedding

2.2.7.2 Sectioning and staining

2.2.8 Microscopy and photography

2.2.9 Newt embryo chromosome preparations

2.3 Results

2.3.1 Karyology

2.3.2 C-banding of the karyotypes

2.3.3 C-banding of chromosome 1

2.3.4 Chromosome 1 in meiosis

2.3.5 F1 hybrid embryos

2.3.6 Fluorochrome staining

2.3.7 Fluorochrome banding of chromosome 1

2.3.8 Banding of centromeric heterochromatin

2.3.9 General fluorochrome banding

of the karyotypes

2.3.10 Chromosomal rearrangements

2.4 Discussion

2.4.1 General karyotypic features

2.4.2 Chromosomes 1

2.4.3 The evolution of the heteromorphism

of chromosome 1

3. The Sex Chromosomes of Triturus

3.1 Introduction

3.2 Results

3.2.1 Mitotic sex chromosomes

3.2.2 Meiotic sex bivalents
4. **Triturus** Interspecific Cytology

4.1 Introduction 149

4.2 Results 154

4.2.1 General karyotypic features 154

4.2.2 Distribution of C-heterochromatin 155

4.2.2.1 Centromeric and pericentric heterochromatin 155

4.2.2.2 Terminal, subterminal and interstitial heterochromatin 157

4.2.3 Cytology of an F1 hybrid between **T. h. helveticus** and **T. v. vulgaris** 160

4.2.4 Chromosome polymorphisms 161

4.3 Discussion 163

Summary 183

References 185
1.1 GENERAL INTRODUCTION

Urodeles comprise eight families with about 350 species. In the Amphibia as a whole, there appears to be a trend in karyological evolution from a karyotype with a fairly large chromosome number with numerous microchromosomes and telocentrics to one with fewer chromosomes and only large metacentrics and submetacentrics (Morescalchi, 1970, 1971, 1975). The order Urodela has families that exemplify this trend, from the primitive Hynobiidae and Cryptobranchidae to the more evolved families such as the Salamandridae.

The Salamandridae contain the Palearctic newts with 2n=24 (N.F. = nombre fondamentale = 48) and the Nearctic newts with 2n=22 (N.F. = 44). The Palearctic newts include Triturus and Pleurodeles, and the Nearctic newts include Taricha and Notophthalmus. The karyotypes of these newts are very similar, all chromosomes being large metacentrics or submetacentrics. Acrocentric and telocentric chromosomes are absent, indicating that these groups are highly evolved.

1.1.1 EUROPEAN NEWTS (Triturus)

The genus Triturus is recognised as having nine good species, with numerous subspecies or races. The species have for a long time been organised into three distinct groups (Lantz, 1947) (Table 1). The groups were compiled according to general morphological data and hybrid experiments. Since this early grouping, there have been many studies on Triturus, demanding a reappraisal of the taxonomic
relationships within the genus. Weighting of certain taxonomic measures can greatly influence ideas on relationships. A review mainly focussed on different cytological aspects within Triturus is intended to shed light on certain relationships without suggesting that they are definitive.

The mitotic karyotypes of all the Triturus species, and for that matter all of the Palearctic salamandrids, appear remarkably well conserved in general morphology (Herrero, 1982a, b; Mancino et al., 1977; Morescalchi, 1973, 1975). Centromere indices and relative lengths appear to correspond well within the group, with a few notable exceptions.

Despite the gross similarity in karyotypes, the species are not truly homosequential. This is confirmed by detailed studies of lampbrush chromosome marker loops and Giemsa C-banding patterns. Conspicuous lampbrush 'marker' loops have been mapped or at least noted in some subspecies from all of the nine Triturus species (Barsacchi et al., 1970; Bucci-Innocenti et al., 1983b; Callan and Lloyd, 1960, 1975; Mancino and Barsacchi, 1966, 1969; Mancino et al., 1972b; Nardi et al., 1972a; Ragghianti et al., 1972, 1978). The centromeric positions of lampbrush chromosomes are not

* - Derived from the term 'homosequential complex', which was introduced by Carson et al. (1967) to describe speciation without chromosomal rearrangement in Hawaiian Drosophila.
always readily identifiable, but conspicuous loops/structures and their positions have been noted. The differences in morphology and/or position of marker loops is not easily related between different taxa. For example, subspecific differences in the presence or absence of a particular loop cannot solely be attributed to allelic differences between the taxa. The discrepancies may reflect some other phenomenon such as changes in amount of DNA or differences in transcriptional activity.

Giemsa C-banding patterns show the distribution of constitutive heterochromatin throughout the chromosome complement. C-bands are generally consistent and reliable chromosome markers and any change in position of a band, between individuals or races etc., may reflect some form of chromosomal rearrangement.

Can data from lampbrush chromosomes and mitotic C-bands be correlated to help reinforce any evidence of cytological change? Lampbrush loops with a particular morphology suggest the transcription or accumulation of a particular gene product(s). To link a marker loop with a block of heterochromatin (C-band) would be of questionable value. This association would, however, help to determine the interspecific cytological derivation of a particular gene or gene product and this may be of some phylogenetic use, i.e. does a particular lampbrush loop/C-band always occur on a particular chromosome or has it changed position many times in different lineages. To date, there are two lampbrush
features that have been positively associated with C-bands on mitotic chromosomes in *Triturus* (Mancino et al., 1972a). These are the nucleolus organiser region (NOR) and sphere sites. Spheres are objects which have been found associated with amphibian lampbrush chromosomes at consistent sites and they also occur free in the nucleoplasm of the germinal vesicle. Spheres are products of unknown genic origin, although sphere sites have been found to be closely associated with the histone gene cluster in *Notophthalmus viridescens* (Gall et al., 1981). The NOR site can occasionally be detected in 'lampbrush' chromosome preparations by the presence of a nucleolus or by nucleic acid in situ hybridisation with rDNA or rRNA (Hennen et al., 1975; Mancino et al., 1972a; Morgan et al., 1980). The NOR in 'mitotic' chromosomes is characterised by a secondary constriction in conventionally stained karyotypes. This region also stains positively with the silver staining technique (Nardi et al., 1978; Ragghianti et al., 1977; Schmid, 1983) and the C-banding technique (Schmid, 1978a, b), and it can be localised by in situ hybridisation with rDNA or rRNA (Barsacchi-Pilone et al., 1974a,b; Batistoni et al., 1978; Hennen et al., 1975; Morgan et al., 1980; Nardi et al., 1977). Mancino et al. (1972a) have correlated sphere sites with C-bands on mitotic chromosomes, the significance of which is unknown. The sites of these markers varies considerably between species. Why is there such variation? Have these sites been moved during the course of evolution from an original ancestral *Triturus* position? If this is so, then considerable chromosome rearrangement has occurred,
along with multiplication of some sites (NORs and sphere sites, in this case) in some species. NORs may occur in different places in the karyotype because of reintegration of the amplified rDNA in the developing oocyte (Schmid, 1980c). Macgregor and Sherwood (1979) have proposed a hypothesis to explain the variable position of NORs in certain plethodontid salamanders, which have highly conserved karyotypes of varying C-value. These authors suggest and provide evidence for the existence of a basic set of potential ribosomal sites in the chromosome set. The genes at these sites may amplify up to form clusters that are large enough to be easily detectable by in situ hybridisation or they may decline to an almost imperceptible level. Consequently, all species may possess the same potential ribosomal sites, yet only certain sites are detectable in any one species or population. In this respect, it is interesting to note that Nardi et al. (1978) have found several ribosomal sites in *T. vulgaris meridionalis* by *in situ* hybridisation of 18S+28S rRNA to mitotic chromosomes. Not all of these ribosomal sites are detectable in any one individual and the sites are inherited in a Mendelian fashion. The actual situation in *Triturus* is probably not as clear-cut as that for the plethodontid salamanders. As will be shown, there is considerable evidence for chromosomal rearrangement within *Triturus*. However, chromosomal rearrangement alone can not explain the existence of variable numbers of particular chromosomal sites. One can only speculate as to the effects that the positions of markers have on speciation in *Triturus*. Their
relocation, multiplication and/or deletion during evolution may be consequential rather than causal in speciation.

Each of the *Triturus* species studied so far show quite distinctive C-banding patterns of the mitotic karyotype (Bucci-Innocenti et al., 1983b; Herrero, 1982b; Mancino et al., 1977; Nardi et al., 1973; Ragghianti et al., 1978; Schmid et al., 1979). The C-banding patterns reveal extensive chromosome repatterning during speciation, or at least considerable cytological differentiation. C-banding facilitates easy recognition of individual chromosomes which is important for analysing interspecific variation and for hybrid spermatogenesis. Unfortunately, most studies involving hybrid meiosis were performed on conventionally stained chromosome preparations. Although a wealth of information has been obtained from these studies, precise details of which chromosomes were involved in meiotic associations were not readily discernible.

Many different cytological phenomena have been used in assessing the taxonomic affinities between newt groups. Before discussing differences between newt taxa, it is best to have some knowledge of these phenomena for a better understanding of the genetic and taxonomic relationships. Most of the valuable information derives from meiotic work on both parental species and hybrid individuals. The following are considered as useful taxonomic measures:
1. **Chiasma frequencies and chiasma position in parental species and F hybrids.**

Much of the work in this area was performed on conventionally stained chromosome preparations (e.g. Callan and Lloyd, 1960; Lantz and Callan, 1954; Spurway and Callan, 1960). Chiasma frequency and position tend to vary between the parental species and differ from the F hybrids. Recent studies on C-banded preparations allow identification of the chromosomes involved in an association (Mancino et al., 1977, 1978).

2. **Meiotic pairing and the incidence of univalents and multivalents.**

The degree of pairing is usually lower in the F hybrid compared to that in the parental forms. The extent of pairing may be reflected by the number of univalents present in hybrid meiosis. The number of univalents present in hybrids has been correlated with taxonomic distance between the parental forms (Müller, 1977). The incidence of multivalents in a diploid hybrid individual are usually indicative of translocation differences between the parental chromosome sets.

3. **Fertility of the F and backcross hybrids.**

Genetic distance can be assessed by the degree of reduction of fertility of hybrid individuals. The further
apart the parental forms, the earlier spermatogenesis is blocked in the hybrid.

Essentially, the degree of taxonomic relationship between parental groups is inversely proportional to the magnitude of meiotic irregularities in the hybrid.

1.1.2 THE 'cristatus' SPECIES GROUP.

The cristatus group comprise Triturus cristatus, with its four subspecies or races, and T. marmoratus with its two subspecies/races. Very little is known about the subspecies, T. m. pygmaeus. All discussions here will concern the nominate subspecies, T. m. marmoratus.

T. cristatus is distributed over most of Europe, including Britain, and eastwards into Asia but excepting southern France and the Iberian peninsula. T. marmoratus is found over the Iberian peninsula and western France. The distribution range of T. c. cristatus is sympatric with T. marmoratus in central and western France. These present ranges are thought to be the result of expansion from refuges of the last Pleistocene ice age, 90 000 - 10 000 years B.P.. T. cristatus would have expanded from refuges in central Scandinavia and T. marmoratus from refuges in southern to central France (Zuiderwijk, 1980). These species are known to produce natural hybrids within the zone of sympatry, thus, reproductive isolation is not entirely
Much of the cytological work of this group has been performed on laboratory and experimentally produced inter- and intraspecific hybrids.

Consider, first, the meiotic properties of the specific forms. Meiotic pairing (synapsis) in newts begins at the telomeres and progresses backwards towards the centromeres forming a characteristic "bouquet" arrangement. Chiasmata distribution is known to differ between the sexes in Triturus species, as may the mean chiasma frequencies (Callan and Perry, 1977; Watson and Callan, 1963).

T. marmoratus has a chiasma frequency ranging from 23.5-25.0 per spermatocyte nucleus (Lantz and Callan, 1954). The ranges of spermatocyte chiasma frequencies for T. cristatus differ between the subspecies:

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. c. carnifex</td>
<td>30.7-32.1</td>
<td>31.0</td>
</tr>
<tr>
<td>T. c. cristatus</td>
<td>36.5-38.5</td>
<td>35.2</td>
</tr>
<tr>
<td>T. c. karelinii</td>
<td>39.5-42.2</td>
<td></td>
</tr>
</tbody>
</table>

* Spurway and Callan (1950)
** White (1946)
*** Wickbom (1945)

No data are available for T. c. danubialis (= dobrogicus).

In all the cristatus subspecies, chiasma distribution in
male meiosis is unrestricted, except in those chromosome arms where only one chiasma occurs where it tends to be terminal. This contrasts with female meiosis, where the chiasma frequency is between 23 and 25 for all subspecies, and chiasma localisation tends to be procentric (Watson and Callan, 1963). It is, perhaps, worth noting here that terminal associations in female meiosis do not always equate with chiasmata. The distribution of chiasmata is independent or unrelated to the sequential character of synapsis because in both sexes synapsis progresses from the tips of the chromosomes and is complete (Callan and Perry, 1977).

Attention has not been paid to this matter in T. marmoratus, but it would seem that chiasmata are probably unlocalised tending to be terminal or subterminal in spermatocytes (Lantz and Callan, 1954) and largely procentrically localised in oocytes (deduced from Nardi et al., 1972a).

Spermatogenic studies with conventionally stained preparations reveal the presence of chiasmata, but do not allow accurate identification of the chromosomes involved. Meiotic data from T. cristatus x T. marmoratus 1 F hybrids seem to show a considerable degree of variation within and between individuals. However, there are some general trends.

T. marmoratus will readily form hybrids with all of the subspecies of cristatus in captivity. All F hybrids of crosses between T. cristatus and T. marmoratus show male sterility. Males fail to produce normal numbers of functional gametes, whereas hybrid oogenesis tends to be
more normal (Lantz, 1947; Spurway, 1953). Lantz (1947) notes that in *cristatus* x *marmoratus* crosses, many embryos die before hatching, as would be expected from the 50% mortality of all offspring from the *cristatus* group (Macgregor and Horner, 1980). Also, any young which do survive metamorphosis, may elicit arrested development at sexual maturity. However, the occasional hybrid may grow larger and live longer than either parental form. The incidence of hybrid male sterility is in accordance with Haldane's rule (1922) which postulates that the male, in this case, should be the heterogametic sex. Male heterogamety has been proved to be the situation for *Triturus* by various means, such as chromosome banding and H-Y antigen studies (Engel and Schmid, 1981; Schmid et al., 1979).

Most studies on hybrid meiosis have involved spermatogenesis. In all *cristatus* x *marmoratus* hybrids, the chiasma frequency is lower than in either of the parental forms. Chiasma localisation is terminal or subterminal. The reduced chiasma frequency may be the result of either asynapsis or a later desynapsis (White, 1973). General opinion tends to favour asynapsis. Lantz and Callan (1954), and Spurway (1953) noted multivalent formation in only the *T. marmoratus* x *T. c. karelinii* hybrids. Mancino et al. (1979), however, demonstrated multivalent associations in *T. marmoratus* x *T. c. carnifex* hybrids.

The presence of inversions is believed to prevent chromosome pairing (i.e. result in asynapsis) and the presence of
multivalents is evidence for translocational differences between the parental chromosome sets. The inability to demonstrate multivalents, however, is not evidence against the presence of translocations, as failure of pairing may prevent their manifestation, depending on genetic relatedness of the parental forms.

Summary of chiasma frequencies in male meiosis of hybrids:

i. \( T.m \times T.c.\text{carnifex} \) (F) \( 5.8-10.8 \)

ii. \( T.m \times T.c.\text{karelinii} \) (F) \( 5.84 \)

iii. \( (T.m \times T.c.\text{karelinii}) \times T.m \) (BACKCROSS) \( 7.5-21.3 \)

iv. \( ([T.m \times T.c.k] \times T.m) \times ([T.m \times T.c.k] \times T.m) \) \( 24.9 \)

v. \( ((T.m \times T.c.k.) \times T.m) \times T.c.k. \) (DOUBLE BACKCROSS) \( 10.27-11.00 \)

\( T.m = T.\text{marmoratus} \). \( T.c.k = T.\text{c. karelinii} \).

( i - Lantz and Callan, 1954; Spurway, 1953. ii - White, 1946. iii - Lantz and Callan, 1954. iv - Spurway, 1953. v - White, 1946.).

Some of the backcross newts (iii) show multivalents. 'iv' is an inbred backcross hybrid resulting from a brother-sister mating of 'backcross' hybrids. This individual showed a normal marmoratus chiasma frequency and phenotype, and was presumed to be of an all marmoratus chromosome constitution. Most of the F hybrids show a great mortality up to or just after metamorphosis. Any newts surviving this period show hybrid vigour. In the F and backcross generations, many
larval anomalies are seen along with high mortality at metamorphosis.

In the marmoratus x cristatus hybrids, there is extensive spermatid degeneration, although, some mature sperm are found in some individuals. Many unbalanced spermatocytes occur at anaphase I (which is often delayed and may never take place) with many unpaired chromosomes (Lantz and Callan, 1954). These authors have noted that spermatid degeneration is usually after anaphase II and sometimes after anaphase I.

As previously mentioned, multivalents signify translocations. The presence of a tri- or quadrivalent indicates that a single translocation has taken place, a quinque- or sexivalent signifies two translocations. Reciprocal translocations result in 'closed' multivalent associations. The absence of closed multivalents does not mean that reciprocal translocations are not present. It may be that enough preparations have not been analysed or unequal chromosome lengths have been exchanged.

In interracial hybrids within cristatus, a similar story appears, but the meiotic anomalies are not as extensive as in the interspecific hybrids. The chiasma frequencies are quite variable in male meiosis of the F hybrids:

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Chiasma Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>karelinii x cristatus</td>
<td>21.2</td>
</tr>
<tr>
<td>carnifex x karelinii</td>
<td>15.1 - 21.2</td>
</tr>
</tbody>
</table>
Chiasmata are generally terminal. There is some failure of pairing, although just under half of the meioses have all of the chromosomes paired (Callan and Spurway, 1951; Spurway and Callan, 1950). Only hybrids involving *karelinii* showed multivalents. Because of the failure of pairing, it was suggested that of the three races analysed, all differed from each other by inversions. However, *karelinii* differs from *cristatus* and *carnifex* by at least two translocations. Spermatid degeneration varies considerably between and even within hybrid individuals. All males showed at least some sperm formation and some showed no degeneration at all. Backcross animals showed considerable mortality at and before metamorphosis (Callan and Spurway, 1951).

The overall view from these studies is that the different subspecies/races of *T. cristatus* differ from each other by 1-2 translocations and that *T. marmoratus* differs from *T. cristatus* by up to four translocations involving possibly six chromosomes.

Interspecific hybrids involving *T. cristatus* and the more distantly related species, *T. vulgaris*, show extensive meiotic anomalies with spermatid degeneration occurring early - at metaphase/anaphase I (Benazzi and Lepori, 1949). The early spermatid degeneration is the result of extensive
pairing failure giving rise to many univalents.

The following table summarises relationships based on meiotic anomalies in individuals from hybrid crosses:

Spermatid degeneration:

<table>
<thead>
<tr>
<th>Cross</th>
<th>Degeneration</th>
<th>Relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cristatus</em> x <em>vulgaris</em></td>
<td>Early - amongst early spermatogonia.</td>
<td>Distant</td>
</tr>
<tr>
<td><em>marmoratus</em> x <em>cristatus</em></td>
<td>Late - anomalies at first meiotic metaphase. Usually degeneration after second meiotic division.</td>
<td>Close</td>
</tr>
<tr>
<td><em>cristatus</em> subspecific crosses</td>
<td>Late, but only partial - entirely post-meiotic, all hybrids show some degree of sperm production.</td>
<td>Very close</td>
</tr>
</tbody>
</table>

The introduction of chromosome banding techniques has allowed more detailed analysis of hybrid spermatogenesis, although, these studies are far from complete. Mancino et al. (1978) showed that hybridisation between *T. c. carnifex* and *T. vulgaris meridionalis* results in a low percentage of egg cleavage, a shift in sex ratio favouring females, and very few first meiotic metaphases in the male hybrids. Many univalents were evident, signifying some failure of pairing. Most associations were terminal, occasionally forming trivalents. However, banding revealed that associations could be between chromosomes of both parental sets and between those of just one parent (inter- and intragenomal
associations, respectively). Many associations appeared to be non-homologous. Very few sperm were actually formed, those present being anomalous. The magnitude of these meiotic irregularities confirm the distant relationship between *cristatus* and *vulgaris*.

Mancino et al. (1979) also found inter- and intraspecific associations, believed to be true chiasmata, between *T. marmoratus* and *T. c. carnifex*. On this basis, it appears that some of the associations seen in previous (uniformly stained) studies may not have been truly intergenomal. The number of intragenomal associations was, however, small in comparison with the *cristatus x vulgaris* hybrids. All of the associations were terminal. The inter and intragenomal terminal associations could be explained by a hypothesis suggested by White (1973). White believed that some of the terminal chiasmata are not evidence of translocation heterozygosity. He considered that numerous translocations had occurred involving minute terminal regions between non-homologous chromosomes, giving rise to many similar chromosome ends within a chromosome set. This would result in duplication-deletion karyotypes that are tolerated because, the translocated regions are small. These translocations are only detected when complete synapsis is inhibited e.g. heterozygosity in hybrid individuals. So, heteromorphic bivalents can form (as in *cristatus x vulgaris*) due to homology of minute terminal regions, resulting in true chiasmatic association between non-homologous chromosomes.
With certainty, the *cristatus* group can be considered apart from the other *Triturus* species, especially when other cytological features are examined. *T. cristatus* and *T. marmoratus* are the only two species, in this genus, to show a heteromorphism for the largest chromosome (chromosome I) with respect to a long heterochromatic tract in the long arm (Callan and Lloyd, 1960; Macgregor, 1979, 1982; Macgregor and Andrews, 1977; Mancino et al., 1977; Morgan, 1978).

1.1.3 THE 'vulgaris' AND 'alpestris' SPECIES GROUPS.

The two remaining species groups are the 'vulgaris' group and the 'alpestris' group (Lantz, 1947). The alpestris group consists of just this one species and its subspecies, whereas the vulgaris group consists of the remaining six *Triturus* species (Lantz, 1947). The taxonomic affinities between and within these groups are in doubt.

*T. vulgaris* is found over most of Europe, as is *T. cristatus*. *T. h. helveticus* is found in northern France, Germany and Britain, and is sympatric with the nominate subspecies of *vulgaris* for most of its range. In the zone of sympathy, *T. helveticus* and *T. vulgaris* do not hybridise, even though both are commonly found in the same breeding ponds. The two species are believed not to hybridise due to behavioural differences in courtship (Halliday, 1977). This effectively isolates the two species prezygotically. *T. helveticus* is, however, known to breed readily with allopatric subspecies.
of *vulgaris* in the laboratory (Lantz, 1947). The Pyrenees are thought to have been a major geographical barrier in the isolation of these species, as with *T. cristatus* and *T. marmoratus*. The last ice-ages of the Pleistocene are considered to have been important in the differentiation of these two species (Spurway and Callan, 1960).

Experimental hybrids have been made between *T. vulgaris meridionalis* and *T. helveticus helveticus* (Spurway and Callan, 1960). Both parental species have similar chiasma frequencies:

<table>
<thead>
<tr>
<th>Species</th>
<th>Chiasma Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. vulgaris meridionalis</em></td>
<td>22.2 - 24.4 (*)</td>
</tr>
<tr>
<td><em>T. helveticus helveticus</em></td>
<td>22.2 - 24.3 (*), 21.5 - 22.4 (**)</td>
</tr>
</tbody>
</table>

* - Spurway and Callan, 1960  
** - Watson and Callan, 1963

The chiasma are proterminally localised in both species, although more so in *T. h. helveticus* (Spurway and Callan, 1960; Watson and Callan, 1963). In *T. h. helveticus*, the chiasma frequency of oocytes is about 25.0, where chiasmata are unrestricted in distribution (Callan and Perry, 1977; Watson and Callan, 1963). It appears, therefore, that in *T. helveticus* responsibility for recombination is taken by the female, where recombination can occur in all parts of the chromosomes. Gene exchange in males occurs only distally. This situation contrasts with *cristatus* and *marmoratus* where
unlocalised chiasma distribution is found in the male, females having essentially procentric chiasmata.

In the vulgaris x helveticus F hybrid, the mean chiasma frequency per nucleus was 7.6, which is much reduced from either of the parental frequencies (about 23). Most chiasmata were terminal with over half of the cells showing one or more multivalents. Callan and Spurway deduced from the multivalents present that the two species differ from each other by no more than 3 translocations, of which at least one is reciprocal as judged from the presence of closed multivalents.

Spermatocyte degeneration is almost entirely post-meiotic with about half of the spermatid nuclei failing to form mature sperm. Hybrids were either inviable or showed hybrid vigour. Although, there is high infant mortality, those surviving show vigour, growing to a much larger size than the parental species, but they are sterile.

Spurway and Callan (1960) conclude that translocations are associated with newt speciation. They are more likely to become established in small isolated populations at the periphery of the geographical range. Probably only one inseminated female is necessary to establish this population, giving rise to fixation of a translocation in the homozygous state. This will effectively prevent later reintegration with the main species population. It is unlikely that these translocations are selectively
advantageous, but arise and become established by chance.

*T. montandoni* is found in the Carpathian and Tatra mountains. Its range is completely sympatric with *T. vulgaris vulgaris*. Spontaneous natural hybrids are known to occur between these species. Viable $F_1$ and $F_2$ hybrids have been experimentally produced (Ragghianti et al., 1978). *T. montandoni* is considered to be the most closely related species to *T. vulgaris*, for several reasons. Many karyotypic features are similar, such as certain C-bands, and number and position of sphere sites and NORs (Ragghianti et al., 1978). Cytologically, *T. montandoni* is considerably different from *T. helveticus* and *T. italiensis*. Experimental hybrids have been produced between *T. montandoni* and *T. vulgaris meridionalis* (note - not the sympatric subspecies). In this cross, there was 95% cleavage, all of which survived hatching. However, only 11% cleavage was observed with a *T. vulgaris* x *T. helveticus* cross (Benazzi and Lepori, 1949) and even lower with *T. vulgaris* x *T. italiensis*. Thus, there is strong evidence for a very close relationship between *T. vulgaris* and *T. montandoni*.

*T. italiensis* is found in southern Italy only. Its range is partially sympatric with *T. vulgaris meridionalis*, which is found in northern and central Italy. The C-banding patterns and other cytological features, such as centromeric lampbrush bars in *T. italiensis* and the different positions of sphere sites on the lampbrush chromosomes, differentiate these two species well. There is no record of the occurrence
of natural hybrids.

The range of *T. boscai* is limited to the central and western areas of the Iberian peninsula. *T. alpestris* has a fairly extensive range from western Russia to northern and eastern France and from southern Denmark to north Italy and central Greece. Lantz (1947) states that *T. boscai* hybridises freely with *T. alpestris*. In fact, *T. boscai* is sympatric for much of its range with *T. alpestris*. However, Bucci-Innocenti et al. (1983b) have failed to produce viable embryos from experimental hybridisations with this pair of species. They believe that effective post-zygotic barriers are in operation.

*T. alpestris* is sympatric with *T. boscai* on the west of its range, and allopatric with *T. vittatus* on the east of its range (*T. vittatus* being found in the Caucasus, northern and southern parts of Asia Minor, Syria, Lebanon and Israel). *T. vittatus* readily mates with *T. alpestris* in captivity resulting in 66% cleavage (i.e. fertilisation and resultant cleavage) and considerable survival to hatching. *T. vittatus* will not, however, mate readily with *boscai,* *vulgaris* or *cristatus* (Bucci-Innocenti et al., 1983b).

A complex and perhaps confusing network of relatedness has been built up. Bucci-Innocenti et al. (1983b) believe that *Triturus* should be divided into two species groups, as opposed to the three of Lantz (1947). These would be the *cristatus* and *vulgaris* groups. This would appear to be a
sensible division, if only to differentiate the *cristatus* group from the rest of the *Triturus* species. However, this system does not sufficiently emphasise the complex affinities of the remaining species, particularly within the proposed *vulgaris* group. *T. alpestris* readily forms hybrids with *T. vittatus* in captivity, is supposed to form natural hybrids (although no reliable records are available) with *T. boscai*, and produces anomalous experimental hybrids with *T. vulgaris*. These data indicate a distant relationship of *alpestris* to *vulgaris*, but should not exclude the former from a grouping together with the other *Triturus* species.

A general view of relationships in *Triturus* is presented here, although, the picture is far from complete and greatly needs further hybridological and cytological study. There is a serious need for a more complete study of banding of *Triturus* chromosomes. The intention of this study is to characterise fully the chromosomes of *Triturus* using various chromosome banding techniques. Detailed banding studies help in the understanding of some of the relationships between different species and determine whether and to what extent the chromosomes have diverged during the course of evolution. The first part of this study is concerned with an unusual chromosomal situation found only in *T. cristatus* and *T. marmoratus*, which results in the death of half of all the offspring produced.
1.2 CHROMOSOME BANDING AND MECHANISMS OF ACTION

Banding techniques have been developed to differentiate regions along chromosome arms on the basis of differential staining. Some banding techniques can reveal multiple banding patterns along the length of the chromosome in certain vertebrate classes (mammals, birds and reptiles). The multiple banding techniques include G-banding (usually a protease or hot saline treatment followed by Giemsa staining), R-banding (hot phosphate buffer treatment and Giemsa staining) and Q-banding (using quinacrine dyes). The multiple banding methods cannot be used with amphibian chromosomes. It is suggested that the high degree of condensation of amphibian metaphase chromosomes compared to other vertebrate classes, helps to obscure the resolution of light and dark bands produced by multiple banding techniques (Schmid, 1978a,b). Recently, G-banding patterns have been produced for part of the *Xenopus laevis* chromosome complement (Sekiya and Nakagawa, 1983). However, the reproducibility of this technique is questionable. There are several reliable and reproducible techniques that will differentially stain amphibian chromosomes. These include C-banding, silver staining and several fluorescence banding techniques.

What do the bands, produced by these techniques, signify and how are they produced?
1.2.1 C-BANDING

C-banding is a well established technique used to stain differentially chromosomal locations of constitutive heterochromatin, using the Giemsa stain. C-banding was first discovered by Pardue and Gall (1970) when using their technique for nucleic acid in situ hybridisation. They found that after processing their slides, heterochromatin of the centromeres of mouse chromosomes stained intensely with Giemsa. These regions also correspond to the localisation of their satellite DNA probe. The hybridisation method has since been adapted for C-banding, the most commonly used methods being those of Arrighi and Hsu (1971, 1974) and Sumner (1972).

The mechanisms of C-banding are not fully understood. However, certain factors are known to be important. The chromosome structure has to be altered in some way to affect the staining of euchromatic and heterochromatic regions. The most commonly used C-banding methods involve the treatment of preparations with alkali followed by hot saline and Giemsa staining. The original concept of the mechanism of banding was that the DNA was denatured and allowed to renature in the hot saline. Heterochromatic regions, known to be composed of highly repeated DNA, were believed to renature more quickly than euchromatic regions, and therefore, take up stain more intensely. Other lines of evidence indicate that the actual situation is not as simple as this. Giemsa is a non-base specific DNA stain that forms
a magenta compound with the chromosomal DNA. The magenta compound consists of two molecules of methylene blue and one molecule of eosin Y, which binds to chromosomal DNA at two sites by hydrogen bonding (Sumner and Evans, 1973). It is the spatial organisation of the binding sites in chromatin which is believed to affect the intensity of staining. The technique is known selectively to remove more DNA from euchromatin than from heterochromatin (Comings et al., 1973). Furthermore, treatment of chromosomes with DNase will also extract more DNA from euchromatic regions, resulting in C-banding (Alfi et al., 1973). These factors do tend to suggest that the DNA in heterochromatic regions is more resistant to extraction, possibly as a consequence of greater chromatin condensation. Certain chromosomal proteins are also known to be extracted from isolated nuclei and chromosomes during the C-banding procedure, yet, those remaining probably help prevent chromatin loss from heterochromatin (Burkholder and Duczek, 1980, 1982). Electron microscope studies of banded chromosomes also suggest that the chromatin of C-band regions remains highly condensed and resistant to extraction (Burkholder, 1975). So, tightness of binding of proteins to DNA in C-bands may influence the degree of chromatin extraction during the C-banding procedure. C-banding, along with G- and Q-banding, is known to oxidise the protein sulphydryl (SH) groups to disulphide (SS) cross-links. However, banding does not affect the distribution of SH or SS groups along the chromosome, both being uniformly distributed (Sumner, 1984). It was concluded that chromosome banding cannot be connected
with either the distribution or the oxidation state of sulphur in chromosomal protein. Clearly, C-banding is more than just selective staining of fast renaturing, highly repeated DNA in heterochromatin, as originally thought. Only non-histone chromosomal proteins can be involved in banding as histone proteins are extracted during fixation, the usual fixative being composed of acetic acid and methanol (Sumner et al., 1973). DNA-protein interactions are obviously important in the production of C-bands, but the exact nature of these remains to be elucidated.

Numerous properties have been ascribed to constitutive heterochromatin, although, not every heterochromatic block shows all of the same properties:

(i) It is in a condensed state throughout the cell-cycle.
(ii) It is late replicating in S-phase of the cell-cycle.
(iii) It is associated with highly repeated DNAs and therefore, shows genetic inertness and is generally not transcribed.
(iv) Meiotic crossing-over is rare in constitutive heterochromatin, although, it may facilitate chromosome pairing and promote interchromosomal associations.
(v) It can show quantitative intraspecific variation with no apparent phenotypic effect.
(vi) Rearrangements involving constitutive heterochromatin are rare in meiotic cells, but are
C-bands can vary in their intensity of staining, suggesting an element of heterogeneity in heterochromatin. That is to say, it is not a universal property of heterochromatin to either stain or not stain, there can be various degrees of staining and presumably various types of heterochromatin. This type of heterogeneity can be seen, for example, in the heterochromatic metaphase Y-chromosome of *Drosophila melanogaster* (Hsu, 1971), or in the heterochromatic Y-chromosome of *Gastrotheca riobambae* (Schmid et al., 1983a, b), or in the heterochromatic, heteromorphic region of chromosome 1 in *Triturus cristatus* (Morgan, 1978). These examples show variations in staining intensity within large blocks of heterochromatin. Smaller discrete blocks of heterochromatin can also vary in staining intensity, as can be seen by examining C-banded karyotypes from different species of Anura (Schmid, 1978a, b). The variations in staining intensity may reflect chromatin packing order differences and/or interspersion of small blocks of euchromatin, which probably affect DNA accessibility for dye binding and/or differential DNA-non-histone protein interactions.

1.2.2 FLUORESCENCE BANDING

Constitutive heterochromatin can also be localised by the use of nucleotide base-specific fluorochromes. Certain fluorescent dyes show base specific binding and fluorescence. These DNA affinity labels can be used to
complement C-banding and further characterise heterochromatin in terms of base composition.

Some of the most commonly used dyes for banding are quinacrine, quinacrine mustard and their derivatives. The quinacrine dyes have the properties of fluorescing brightly when bound to AT-rich DNA, and actually show quenched fluorescence when bound to GC-rich DNA.

The dyes used in this study were the AT specific fluorochrome, 4'-6-diamidino-2-phenylindole (DAPI), and the GC specific fluorochrome, mithramycin. The base specificity of these fluorochromes with chromosomal DNA has been firmly qualified (Lin et al., 1977; Schweizer, 1976a).

Banding with AT specific dyes is complemented by the reverse banding patterns achieved with GC specific dyes (Schnedl et al., 1977). Banding patterns with fluorochromes alone, however, are generally of a poor resolution. Consequently, fluorochrome banding techniques have been improved by the use of contrasting agents. Non-fluorescent base specific dyes are used as counterstains in conjunction with the fluorochrome (the primary stain). Contrast enhancement has been usefully demonstrated on chromosomes from diverse sources such as human and various other mammalian species, anurans and plants (Jorgenson et al., 1978; Schmid, 1980a; Schweizer 1976a, b, 1981).
1.2.3 DAPI STAINING

The Trypanocidal antibiotic, DAPI, is closely related to Hoechst 33258, sharing similar staining properties and a partly similar structure. DAPI binds preferentially with AT rich DNA, the fluorescent intensity increasing with increasing molar content of AT base pairs (Lin et al., 1977). A strong dye-DNA interaction occurs with AT-rich, helical DNA in the major groove, without intercalation (Comings, 1975; Müller and Gautier, 1975; Lin et al., 1977). Chromatin protein involvement is believed to play a minor role, in relation to the dye-DNA interaction, in chromosomal fluorescent studies with DAPI (Lin et al., 1977). It is therefore safe to assume that with DAPI any strong fluorescence of a certain chromosomal region is a consequence of its enriched content of AT base pairs.

DAPI can be counterstained with a non-fluorescent GC specific compound to enhance contrast between heterochromatic and euchromatic chromosomal regions (Schweizer, 1976a, b). The GC specific counterstain used is actinomycin D. Actinomycin D binds to helical DNA by intercalation, with an absolute specificity for guanine, where one GC base pair is required for dye-DNA binding (Müller and Crothers, 1975). Actinomycin D will not fluoresce with UV excitation at the wavelengths required for DAPI fluorescence, however, the counterstaining does cause an overall reduction in fluorescence intensity (Schweizer, 1976a, b).
1.2.4 MITHRAMYCIN STAINING

Mithramycin and closely related chromomycin A are antibiotic dyes which exhibit a secondary fluorescence when bound to DNA. These dyes effectively bind to helical DNA with a strong specificity for guanine (Behr et al., 1969). The dye-DNA interaction is significantly enhanced by the presence of divalent cations, such as Mg$^{2+}$ (Ward et al., 1965). Binding of these dyes is probably mediated by hydrophobic interactions of the essentially lipophylic sugar side chains to the minor groove of DNA, where about one molecule of dye occupies 3-4 nucleotide base pairs (Behr et al., 1969). These dyes probably do not intercalate since they do not cause unwinding of helical DNA (Waring, 1970).

Mithramycin can be counterstained with an AT specific dye to enhance chromosome banding of GC rich regions (Schmid, 1980a). Distamycin A serves the purpose of counterstain here, because it is an optically inactive oligopeptide antibiotic. It binds with a high specificity to AT-rich, helical DNA, by hydrogen bonding, possibly further stabilised by van der Waals forces in the minor groove, without intercalation (Zimmer, 1975).

Methyl green can also be used as a counterstain with mithramycin (Schweizer, 1976a). Methyl green has a high affinity for helical DNA with a strong AT specificity (Müller and Gautier, 1975).
1.2.5 MECHANISMS OF COUNTERSTAIN ENHANCEMENT OF BANDING

Numerous combinations of dye pairs can be used for fluorescent banding. Depending on the dye pair used, there are two mechanisms by which banding contrast can be enhanced.

One mechanism is by transfer of electronic excitation energy from the fluorochrome (donor) to the counterstain (acceptor). This mechanism primarily requires there to be spectral overlap between the donor fluorescence and acceptor absorption, and depends on the linear spatial separation of the two bound dyes (Sahar and Latt, 1978, 1980).

The second mechanism is one of direct binding competition, such that the counterstain excludes binding of the primary stain (Jorgenson et al., 1978). One or to some extent both mechanisms may be operating to reduce the fluorescence of the primary stain, where both dyes are bound in close proximity.

In the present study, contrast enhancement is probably mediated by energy transfer for the 'methyl green/mithramycin' and 'actinomycin D/DAPI' dye pairs, and by direct binding competition for the 'distamycin A/mithramycin' dye pair (interpreted by substitution of related dyes in data from Sahar and Latt, 1980).
SUMMARY OF DYE-DNA INTERACTIONS

AT specific dyes

1. DAPI : AT fluorescence
2. Distamycin A : non-fluorescent
3. Methyl green : non-fluorescent
4. Actinomycin D/DAPI : AT fluorescence with good contrast

GC specific dyes

1. Mithramycin : GC fluorescence
2. Actinomycin D : non-fluorescent
3. Distamycin A/mithramycin : GC fluorescence with good contrast
4. Methyl green/mithramycin : GC fluorescence with good contrast
2. THE CYTOLOGY OF TRITURUS CRISTATUS AND T. MARMORATUS
WITH PARTICULAR REFERENCE TO CHROMOSOME 1.

2.1 INTRODUCTION

Both Triturus cristatus and T. marmoratus have been found to have an unusual and perhaps unique chromosomal situation involving the largest chromosome of the set, chromosome 1. In 1960, Callan and Lloyd detailed and mapped the main features of the lampbrush chromosomes of the subspecies of T. cristatus. They found that lampbrush bivalent 1 is heteromorphic for a region which encompasses most of the long arm, from near the telomere up to just below the centromere. The heteromorphism is in relation to the number, morphology and position of distinctive marker loops between the two half-bivalents. The heteromorphic region or heteromorphic arm (HTA) was also defined as never forming chiasmata during diplotene of oogenesis.

The HTA was at first interpreted as being involved in sex determination (Callan and Lloyd, 1960), T. cristatus having a ZW/ZZ sex determining system. However, evidence from $F_1$ hybrid data, between cristatus and marmoratus suggested that the male was the heterogametic sex (Lantz, 1947). $F_1$ hybrid males, being sterile, are usually heterogametic, according to "Haldane's rule" (1922).

A similar heteromorphic region has been shown in lampbrush bivalent 1 of T. marmoratus (Nardi et al., 1972a; Mancino
and Nardi, 1971). Chromosome 1 is more submetacentric in marmoratus than in cristatus and the heteromorphic region in the former does not extend as far up towards the centromere. The achiasmate region in marmoratus, however, extends beyond the centromere and into the short arm. In marmoratus too, this chromosome was considered to be involved in sex determination (Mancino and Nardi, 1971; Nardi et al., 1972a).

The heteromorphic region appears deeply stained after Giemsa C-banding in both males and females (Mancino et al., 1973; Rudak and Callan, 1976; Schmid and Krone, 1976). Little attention was paid to this fact by the latter authors, however, Morgan (1978) realized that the HTAs were present and achiasmate in both sexes and dispelled their association with sex determination. The present study has revealed the sex chromosomes in males of both species, indicating an XY/XX sex determining system. In fact, the heteromorphism of chromosome 1 need not have anything to do with sex determination in these newts.

The HTAs have since been the subject of much research and many interesting features have been elucidated. Chromosome 1, being the largest of the set, is more submetacentric than any of the larger chromosomes of the set. It also differs from all other Triturus species, where the largest chromosome is always metacentric. The long arm of chromosome 1 shows a difference in length between the two homologues (Callan and Lloyd, 1960; Nardi et al., 1972a), a difference
which is more marked in *T. marmeratus* than in *T. cristatus*. This tends to suggest a differential growth of the long arms which could be envisaged as a differential accumulation of heterochromatin within the isolated heteromorphic regions. The growth of the long arm could also account for the submetacentric appearance of the chromosome, the original form probably being more metacentric. In the same vein, chromosome 1 may have originally been a smaller chromosome in the set, later being 'promoted' to the largest by expansion of the HTA. Whatever the case, the origins of chromosome 1 can only be speculated upon.

Further work revealed more interesting features of chromosome 1. Macgregor and Andrews (1977) found that middle repetitive (m.r.) DNA (Cot 0.2 - 50) from *T. c. carnifex* was distributed more or less evenly throughout the chromosome set, using nucleic acid in situ hybridisation. The m.r. probe labelled all chromosomal regions evenly in mitotic preparations. However, hybridisation of the probe to RNA transcripts of lampbrush chromosomes showed that several loops in the HTA labelled heavily after a short period of exposure. Few loops labelled elsewhere in the lampbrush chromosome set. It appeared that a disproportionate number of loops were transcribing m.r. DNA in the HTA. The fast and heavy labelling nature of these loops suggested that they consisted of tandemly repeated sequences complementary to sequences in the probe. The distribution of labelled loops in the heteromorphic region varied between both half-bivalents, between preparations and between individuals,
even for chromosomes from like-sized oocytes (and presumably, therefore, at the same stage of the transcriptional programme in oogenesis). The variation between half-bivalents is to be expected. However, the other variations in the distribution of labelled loops along the chromosome arms can partly be attributed to the long length of the chromosomes and their differential stretching during isolation from the germinal vesicle. No significant underlying trends in the pattern of labelled loop distribution could be determined for any one individual or any one size class of oocyte.

Further studies by Varley et al. (1980a, b), Macgregor (1979) and Macgregor et al. (1981) have shown that the heteromorphic region is disproportionately rich in highly repeated (h.r.) DNA. Three classes of h.r.DNA have been isolated from total DNA from T. c. carnifex by renaturation kinetics, caesium salt centrifugation and cloning of restriction endonuclease fragments. In all cases, in situ hybridisation to lampbrush chromosome RNA transcripts showed that the h.r.DNAs were being preferentially transcribed by certain loops in the heteromorphic region. A few other loops throughout the rest of the chromosome set were also seen to transcribe h.r.DNA. The degree of labelling of loops in the HTA showed that sequences in the h.r.DNA probe were either tandemly repeated in some loops or interspersed with other sequences in other loops. The distribution pattern of labelled loops in the HTA shows a degree of variation between individuals (Macgregor, personal communication).
Morgan (1978) suggested that several 'forms' of each HTA may be present in any one population. This suggestion was based on the fact that positions of marker loops in the heteromorphic region vary from individual to individual (Callan and Lloyd, 1960) and C-banding patterns of the HTAs show inconsistencies.

Finally, and perhaps most importantly, the heteromorphism is always observed in both sexes. This begs the question as to what happens to individuals that inevitably have the constitution of being homomorphic for either homologue of chromosome 1? Macgregor and Horner (1980) found that any embryo homomorphic for chromosome 1 showed arrested development at tailbud stage and eventually died. This effectively means that 50% of all offspring produced die before even hatching.

One objective of this study was to investigate further the heteromorphism of chromosome 1 at the cytological level, using various chromosome banding techniques. Also, the distribution of C-band heterochromatin was assessed within the cristatus species group. It was found that the organisation of chromosomal regions has not changed as much as originally expected (see section 1.1.2).
2.2 METHODS AND MATERIALS

2.2.1 Animals

Triturus cristatus cristatus (Laurenti 1768). Specimens were kindly collected by Dr. T. Halliday in Oxfordshire, England.

T. c. carnifex (Laurenti 1768). Specimens were collected in northern Italy and supplied by Xenopus Ltd. Biological Suppliers, Redhill, Surrey, England.

T. c. karelinii (Strauch 1870). Specimens were collected in Turkey and supplied by Xenopus Ltd.

T. marmoratus marmoratus (Latreille 1800). Specimens were collected in north eastern Spain and were also supplied by Xenopus Ltd.

T. alpestris apuanus (Bonaparte 1839). Specimens were kindly given by Dr. G. Barsacchi, collected in Pisa, Italy.

T. boscai (Lataste 1879). Specimens were collected in Portugal and supplied by Xenopus Ltd.

T. helveticus helveticus (Razoumowsky 1789). Specimens were collected by this laboratory from Mayene, France. Some specimens were collected in Oxfordshire and kindly supplied by Ms. J. Roberts.

T. vittatus ophryticus (Gray 1835). Specimens were collected in Israel and supplied by Xenopus Ltd.

T. vulgaris vulgaris (L. 1758). Specimens were collected in Oxfordshire and kindly supplied by Ms. J. Roberts.

All animals were kept in a tank room at 13 degrees C under
a 12 hours dark, 12 hours light regime. The animals were well fed on a diet of Tubifex worms and liver.

2.2.2 Preparation of Animals and Tissues

Either the testes or gut epithelium were used as a source of dividing cells for chromosome preparations. All preparations were made by the 'squash' technique (see next section). Experimental animals were taken from the tank room (at 13 degrees C) and kept at room temperature (23-25 degrees C). The rise in temperature is thought to induce a higher mitotic index in the testes and encourage meiotic divisions in males of the right condition. Meiotic divisions are normally only found in males just after the breeding season, in September for T. cristatus and T. marmoratus. A high mitotic index was induced in the gut by feeding a newt with as many earthworms as it would take for at least 4 days (after being brought to room temperature). The abrasive soil material in earthworm gut may help to stimulate epithelial turnover in the newt's intestine.

After this intensive feeding period, a newt would be given an intraperitoneal injection of 0.2% colchicine (Sigma, C-9754), in 0.65% NaCl, at a dose of 0.05 ml per gram body weight. Newts were killed 48 hours post-injection by anaesthetising in MS222 (Sigma, E-1626), followed by cervical severage. The intestine and testes were removed and placed immediately in amphibian hypotonic solution, 0.05M KCl. The intestine was slit open along its length and
thoroughly washed in the KCl solution to remove all traces of mucus and food debris. The intestine was given a total time of 30-35 min, and the testes 40-45 min in the hypotonic KCl solution. Tissues were then fixed in freshly prepared, ice-cold '3:1' (ethanol:glacial acetic acid). The fixative was changed twice in the first two hours and thereafter only changed whenever preparations were to be made. Tissues were left overnight before any chromosome preparations were made. Tissues can be stored indefinitely in the 3:1 fixative at -20 degrees C.

2.2.3 Mitotic Chromosome Preparations

Fixed tissue fragments of about 1 mm were used for squash preparations. Each fragment was dissociated in a drop of 45% acetic acid on a subbed slide. Any large pieces of connective tissue or other debris were removed. The preparation was squashed under a siliconised coverslip, between the fold of a piece of filter paper. Preparations were air dried directly after removal of the coverslip by the dry-ice method of Conger and Fairchild (1953). It was found that the chromosome morphology was well preserved by this procedure. If after removal of the coverslip by dry-ice, slides were placed immediately in 95% ethanol for 10 min before air drying, then the chromosomes appeared slightly 'fuzzy'. Immersion in 95% alcohol is apparently too drastic a dehydration step and the chromatin is dissociated to some degree.
2.2.4 Giemsa C-Banding

Chromosome preparations were 'aged' in an incubator at 65 degrees C for 12-18 hours. The slides were then processed as follows:

(1) Incubation in freshly filtered 0.07M barium hydroxide \([\text{Ba(OH)}_2]\) at 37 degrees C for 10 min. \(\text{Ba(OH)}_2\) is a saturated solution at a molarity of 0.07M. However, the solubility of \(\text{Ba(OH)}_2\) increases with increasing temperature. So, the solution was pre-warmed in a 37 degrees C water bath for 10-15 min before filtration.

(2) A brief rinse in 0.1N HCl for 5 sec at room temperature, to remove the barium carbonate film picked up from the surface of the \(\text{Ba(OH)}_2\) solution.

(3) 2-3 rinses, 10 sec each, in distilled water at room temperature.

(4) Incubation in 2xSSC (0.3M sodium chloride, 0.03M sodium citrate. pH 7.0) at 65 degrees C for 75-90 min.

(5) A brief rinse in distilled water followed by air drying or staining.

(6) Staining for 20 min in 100 ml Sørensen
phosphate buffer (5 ml 0.067M KH$_2$PO$_4$, 5ml 0.067M Na$_2$HPO$_4$, 90 ml distilled water, pH 6.8) plus 3 ml Eastman Giemsa (C.I. 15510; Eastman Kodak Ltd., New York, C 8685) in a Coplin jar.

(7) Thorough washing in running tap water, with a final rinse in distilled water, followed by air drying.

(8) Preparations need not be mounted, although, some were made permanent by clearing in xylene for 5 min and mounting in Xam (B.D.H. Chemicals Ltd.).

2.2.5 Conventional Staining with Orcein

Some preparations were stained with orcein (C.I. 1242; Sigma, 0-3626). This stain is particularly useful for staining meiotic preparations, revealing details of chromosome structure and morphology which are normally lost after the C-banding technique.

Slides need not be aged for orcein staining. 3-4 drops of aceto-orcein (1% orcein in 45% acetic acid) were placed on chromosome preparations and overlaid with a coverslip. After staining for 20 min the coverslip was removed and the preparation was washed in 90% ethanol for 30 sec, followed by air drying. Some preparations were cleared in xylene and mounted in Xam.
2.2.6 Fluorescence Staining

Chromosome preparations do not have to be aged for any of the fluorochrome staining techniques. The fluorochrome (primary stain) is normally used in conjunction with a non-fluorescent dye (counterstain) which acts to enhance the fluorochrome banding. Preparations may be stained first with the counterstain followed by the primary stain or vice-versa. As a matter of course, in this study, the counterstain was always used first.

2.2.6.1 DAPI/Actinomycin D Staining

Solutions:

McIlvaine's citric acid - Na HPO buffer
2  4
Solution A : 0.1M citric acid.
Solution B : 0.2M Na HPO .
2  4

For pH 7.0, 18.5 ml of solution 'A' was mixed with 81.5 ml of solution 'B'.
For pH 7.5, 7.5 ml of solution 'A' was mixed with 92.5 ml of solution 'B'.

Actinomycin D (Sigma, A-4262), GC-specific counterstain.
Stock solution: 2 mg/10 ml in McIlvaine's buffer, pH 7.0.
Working solution: 0.1 mg/ml diluted from stock with buffer solution.
When preparing the stock solution, actinomycin D can first be dissolved in 1-2 drops of methanol. The stock solution can be kept for several weeks at 0-4 degrees C, in light-free conditions.

DAPI [4',6-diamidino-2-phenylindole] (Boehringer Mannheim), AT-specific primary stain.

Stock solution: 5 ug/ml in McIlvaine's buffer, pH 7.0.

Working solution: 0.5 ug/ml diluted from stock with buffer solution.

DAPI is particularly insoluble in aqueous media and must first be dissolved in 1-2 drops DMSO (dimethyl sulfoxide) or methanol, the former being the most effective. The stock solution can be kept for several weeks in dark conditions at 0-4 degrees C.

Procedure:

(1) 3-4 drops of the actinomycin D solution were placed on each preparation and overlaid with a coverslip. Slides were stained for 15 min in the dark, at room temperature.

(2) Slides were rinsed in buffer, pH 7.0, for 30 sec and air dried.
3-4 drops of the DAPI solution were then placed on the preparations and overlaid with a coverslip. The slides were stained for 15 min in the dark.

Slides were rinsed in buffer at pH 7.0 for 10 sec followed by a rinse in buffer at pH 7.5 for 10 sec. After rinsing slides were air dried and mounted in saturated saccharose, blotting any excess mountant. Fluorescence was found to be more stable if preparations were kept for 2 days at 0-4 degrees C in the dark, before viewing under ultra violet light (UV).

2.2.6.2 Mithramycin/Distamycin A Staining

Solutions:

McIlvaine's buffer, pH 7.0 only, see DAPI/actinomycin D staining.

Distamycin A (Sigma, D-6135), AT-specific counterstain.

Working solution: 4 mg/ml in McIlvaine's buffer, pH 7.0.

Distamycin A is unstable in solution and is best used within an hour of preparation. The compound itself must be kept at -20 degrees C in the dark.
Mithramycin (Sigma, M-4136), GC-specific primary dye.

Stock solution: 2.5 mg/5 ml in buffer, pH 7.0, as bought.

Working solution: 0.1 mg/ml with 10mM magnesium chloride, diluted from the stock solution with McIlvaine's buffer, pH 7.0.

Mithramycin can be stored for several weeks at 0-4 degrees C, in the dark.

Procedure:

(1) 3-4 drops of freshly prepared distamycin A solution were placed on the preparations and overlaid with a coverslip. Slides were stained for 15 min, in the dark.

(2) After staining, slides were rinsed for 15 sec in buffer, pH 7.0, and air dried.

(3) 3-4 drops of the mithramycin solution were then placed on the slides and overlaid with a coverslip. Slides were stained for 15 min in the dark.

(4) Slides were rinsed for 15 sec in buffer at pH 7.0. After air drying, slides were mounted in saturated saccharose, blotting away any excess
mountant. Before viewing with UV, slides were stored at 0-4 degrees C for 2 days (or at least overnight) to allow the stains to stabilise and reduce fading of the fluorescence.

2.2.6.3 Counterstaining with Methyl Green

As an alternative to the counterstain distamycin A, the AT-specific dye methyl green (C.I. 20315; G.T.Gurr Ltd.) was also used.

Working solution: 0.1% methyl green in McIlvaine's buffer, pH 7.0.

Before this solution could be prepared, the methyl green dye had to be extracted with chloroform to remove traces of crystal violet. Chloroform was filtered through the dye using a Buchner funnel. Filtration continued until the chloroform passed through as a clear solution or very pale pink/blue.

Chromosome preparations were stained with the methyl green solution for 20-25 min (3-4 drops under a coverslip). After staining, preparations were rinsed in buffer, pH 7.0, for 30 sec. After air drying the slides could then be stained with mithramycin.
2.2.6.4 Sequential staining

After staining for either AT- or GC-specific fluorescence, it was possible to destain the preparations in order to restain again with the alternative dye pair. Slides were processed as follows:

(1) Coverslip was removed and the saccharose mountant was washed off with distilled water for 10 min.

(2) Slides were dehydrated through an increasing alcohol series - 50%, 70%, 90% and 100% ethanol, 2 min in each.

(3) Slides were then destained in either pyridine or '3:1' (ethanol:acetic acid) for two days, the latter being most effective. Slides were passed through two changes of 100% ethanol, 2 min each, before air drying and restaining with the appropriate procedure.

The destaining removes most of the bound dyes, but a residual fluorescence usually remains which does not greatly affect the subsequent staining.

2.2.7 Histology and Cytology of the Testes

The testes were prepared for wax embedding and sectioning
using a cytological fixative. This allows the general tissue organisation to be determined whilst preserving chromatin structure for cytological observation of spermatogenesis.

2.2.7.1 Fixation and Embedding

Fixative:

San Felice's Fluid

Solution A: 64 ml 1% chromium tetroxide,

4 ml glacial acetic acid.

Solution B: formalin (4% paraformaldehyde, aqueous solution).

17 ml solution 'A' was added to 8 ml solution 'B', just before use.

Procedure:

(1) Excised tissues were fixed for 24 hours in freshly prepared San Felice's fluid.

(2) The fixative was washed out of the testes with running tap water for 8-12 hours.

(3) Tissues were then dehydrated through an alcohol series and cleared in xylene as follows:

- 70% ethanol 1 hour,
- 90% ethanol 1 hour,
- 100% ethanol 30 min,
- 100% ethanol 30 min,
- xylene 30 min.
(4) Tissues were vacuum embedded in Paraplast wax at 55 degrees C. Tissues were passed through four changes of molten Paraplast before embedding, each change lasting 20 min.

2.2.7.2 Sectioning and Staining

15 µm and 20 µm sections were cut on an "LKB 2218 Historange" microtome. Sections were stained with either crystal violet or Mayer's haematoxylin and eosin, after removal of wax in xylene.

Crystal violet staining

Crystal violet (C.I. 22529; G.T.Gurr Ltd.) was prepared as a 1% aqueous solution. The iodine solution was prepared by dissolving 1 g iodine in 100 ml 2% aqueous potassium iodide solution.

(1) Sections were brought to water (100% ethanol, 90% ethanol, 70% ethanol, distilled water, 2 min in each).

(2) Sections were stained in the crystal violet solution for 15 min, followed by a rinse in distilled water.

(3) Sections were transferred to the iodine solution for 1 min, followed by a rinse in
(4) Sections were dehydrated through fresh 70%, 90% and 100% ethanol, 10 sec in each. This was followed by a further change of 100% ethanol for 1 min.

(5) Sections were cleared in xylene for 2 min and mounted in Xam.

Only chromatin and chromosomes are stained by this technique, appearing deep blue/purple.

Mayer's Haematoxylin and Eosin Staining

Mayer's haematoxylin was prepared as follows: 0.25 g haematoxylin (C.I. 75290; G.T.Gurr Ltd.) was dissolved in 250 ml distilled water. Then, 0.05 g sodium iodate, 12.50 g aluminium ammonium sulphate, 0.25 g citric acid and 12.50 g chloral hydrate were added, one at a time, ensuring that each substance had dissolved before adding the next. Eosin (C.I. 45386; G.T.Gurr Ltd.) was prepared as a 1% solution in 90% ethanol.

(1) Sections were brought to water and stained in Mayer's haematoxylin for 7-10 min. Sections were then washed and "blued" in running tap water.

(2) Sections were taken through an alcohol series
to 90% ethanol and stained in eosin for 15-30 sec, followed by a thorough wash in 90% ethanol for 20 sec.

(3) Sections were dehydrated through two 1 min changes of 100% ethanol, cleared in xylene for two min and mounted in Xam.

With this procedure, chromatin stains deep blue and cytoplasmic material stains pink.

2.2.8 Microscopy and Photography

Preparations for bright field observation were viewed under a Zeiss Universal microscope, fitted with planapochromat objectives. Photographs were taken with Kodak Panatomic X (ASA 32), EFKE KB14 (ASA 20) or Agfa Gevaert Copex Pan AHU Tri-13 (ASA 25) film.

Fluorescence preparations were examined on either a Zeiss Photomicroscope or a Zeiss Universal microscope equipped with a Zeiss III RS epi-fluorescence condenser. The source of UV being an HBO/50W high pressure mercury lamp. The epi-fluorescence condenser was fitted with Zeiss filter set 48 77 02 for DAPI staining, and filter set 48 77 09 for mithramycin staining. Photographs were taken using a range of exposure times (up to 30 sec) with Ilford Pan F film (ASA 50).
Chromosome measurements: chromosome drawings were made from highly enlarged negative projections. Measurements were made using a graphics tablet of an Apple II microcomputer.

2.2.9 Newt Embryo Chromosome Preparations

There is a regular 50% arrest of embryonic development at tailbud stage in *T. cristatus* and *T. marmoratus* (Macgregor and Horner, 1980). In order to examine the chromosomes of arresting embryos, it was important to be able to recognise which embryos were showing arresting development before mitotic divisions had ceased altogether. Embryos arrest between stages 28 and 34 of embryogenesis, but show signs of slower development at around stage 26 (Horner and Macgregor, 1984). Embryos were examined from three different mating pairs: a *T. c. carnifex* pair, a *T. marmoratus* pair and a hybrid mating between *T. c. carnifex* (female) and *T. marmoratus* (male).

The development of fertilised eggs was monitored over an 8 day period. Arresting embryos showed stunted development after day 6 (at 25 degrees C) and complete arrest by day 8. About half of the embryos continued normal development beyond this period. Arresting embryos were dissected out of their jelly capsules between days 6 and 7, and were placed in a 0.2% solution of colchicine in filtered pond water, for 2-3 hours. They were then fixed in ice-cold '3:1' and stored at -20 degrees C.
Embryos were fixed for at least 24 hours before making chromosome preparations. Yolk sacs were removed from the fixed embryo before squash preparations were made from separate head and tail regions. It was found generally that head regions possessed more mitoses than tail regions. Chromosome preparations were aged and C-banded as in section 2.2.4.
THE CYTOLOGY OF TRITURUS CRISTATUS AND T. MARMORATUS WITH
PARTICULAR REFERENCE TO CHROMOSOME 1.

2.3 RESULTS

2.3.1 Karyology

_Triturus cristatus_ and _T. marmoratus_ possess 12 pairs of chromosomes which are numbered according to size, chromosome 1 being the largest. The chromosomes can also be arranged into three groups on the basis of size. The first group consists of the largest chromosomes (nos. 1-4) which are metacentric, with the exception of the submetacentric chromosome 1. The second group of medium-sized chromosomes (nos. 5-8) are submetacentric and metacentric. The third group contains the smallest chromosomes (nos. 9-12) which again are metacentric to submetacentric. Tables 2 and 3 gives the relative lengths (R.L.) and centromere indices (C.I.) for three of the subspecies of _T. cristatus_ and for _T. marmoratus_. Relative length is computed as: (length of the chromosome x 100)/(length of the haploid genome). Centromere index is: length of the short arm/length of the entire chromosome.

For _Triturus_, it is usual for all of the large group chromosomes to be metacentric, yet, both _T. cristatus_ and _T. marmoratus_ have a considerably submetacentric chromosome 1. With respect to chromosome 1, one of the most obvious differences between the two species is that of C.I.,
marmoratus being more submetacentric than cristatus. Also, in marmoratus, the homologues of chromosome 1 differ markedly in R.L. and C.I., a difference which is not as apparent in the mitotic chromosomes 1 of cristatus. The length differences in marmoratus are due entirely to the long arm. In cristatus, the long arm of one homologue is noticably longer, but the difference is only convincingly resolvable in the very long lampbrush chromosomes of diplotene oocytes (Callan and Lloyd, 1960). Mitotic chromosomes are about 50 times more contracted than lampbrush chromosomes. So any size difference in cristatus chromosomes 1 appears insignificant at metaphase. The longer homologue of each species has been called chromosome 1A and the shorter, chromosome 1B (Macgregor and Horner, 1980). The identification of the longer homologue in cristatus and its correspondence with the longer homologue in marmoratus was determined using C-banding patterns of chromosomes in hybrid embryos (see section 2.3.5).

With the exception of chromosomes 1, the two species appear, superficially, to be very similar with respect to C.I.. However, there are some disparities in R.L.. If the chromosomes of all subspecies are ordered according to similarities in C.I., then they are in agreement with previous orderings (Callan and Lloyd, 1960; Herrero, 1982b). The differences in R.L. are accounted for by differential degrees of contraction and overstretching during experimental manipulation.
In conventionally stained preparations using orcein, one would expect to see a secondary constriction at the sites of the nucleolus organiser regions (NORs). NORs are found on chromosome 9 and occasionally 6 in *cristatus* subspecies, and on chromosome 10 in *marmoratus*. However, no secondary constrictions could be resolved (Fig. 1). The chromosome preparations were made from animals which had been kept at room temperature for several days before being killed. Secondary constrictions can be induced with cold-shock treatment to the animal. Larvae kept at 4 degrees C for 4 days before sacrifice show numerous secondary constrictions throughout the chromosome complement. The number and position of cold induced secondary constrictions varies between chromosomal spreads, but always coincide with sites of constitutive heterochromatin (Fig. 2). The results are in agreement with studies by Rudak and Callan (1976), who found cold-induced constrictions to be Giemsa C-positive in *T. cristatus*.

2.3.2 C-Banding of the Karyotypes

After C-banding, euchromatin remains weakly, but uniformly stained. Regions of constitutive heterochromatin are intensely stained with Giemsa. All attempts, in the present study, to differentiate euchromatin by standard G-banding (hot 2xSSC or trypsin treatments), R-banding (hot buffered saline) and Q-banding (quinacline staining) techniques, failed to show any linear differentiation along euchromatic chromosomal regions.
Constitutive heterochromatin, as shown by C-banding, is distributed in positions that are characteristic for each chromosome and, therefore, allow identification of all twelve chromosome pairs. The banding patterns are characteristic in each species and even between subspecies. The main distribution of heterochromatin is at the centromeres and at pericentric regions on one or both sides of the centromere. C-bands can also be seen at some terminal, subterminal and interstitial sites (Figs. 3-10).

In _T. c. cristatus_ and _T. c. carniex_, the centromeric heterochromatin appears as a small spot (Figs. 3, 4 and 7-10). In _marmoratus_, half of the chromosomes possess very large centromeric blocks of heterochromatin which often obscure the primary constriction. This is a useful feature for distinguishing _marmoratus_ chromosomes (Fig. 6). In _T. c. karelinii_, the centromeres are even more striking in their content of heterochromatin. All chromosomes, except chromosome 7, have immense blocks of centromeric heterochromatin which often obscure the constriction (Fig. 5). The centromeric C-staining extends into the arms of the chromosome, away from the centromere. _T. c. karelinii_ is the only subspecies of _cristatus_ to show these massive blocks of heterochromatin. _T. c. karelinii_ is also the only _cristatus_ subspecies to show distinctive centromeres in lampbrush chromosomes, appearing as dense, loopless, chromomeric aggregates, referred to as 'centromere bars' (Callan and Lloyd, 1960). Another feature of _karelinii_ which makes it
unique amongst the *Triturus* species examined in this study, can be seen in the interphase nuclei (Fig. 16). These nuclei possess several, very large chromocentres which are aggregated clumps of centromeric heterochromatin (Baldwin, Macgregor and Sims, unpublished observations). This situation is similar to the fused blocks of centromeric heterochromatin that have been described for certain species of plethodontid salamanders (Kezer and Macgregor, 1971; Macgregor et al., 1973). Meiotic material was not present in the animals studied, so it is not known if the centromeres fuse in male meiotic prophase nuclei.

Not all C-bands stain with equal intensity. As a rule, all heterochromatic blocks stain more intensely than euchromatin. In *cristatus* and *marmoratus*, some bands may be relatively large and yet, may not be strongly stained. This gives an impression of the heterochromatin being 'diffuse', as in the terminal band of the long arm of chromosome 5. Some blocks of heterochromatin are very small and cannot always be resolved on fully contracted metaphase chromosomes. More elongated prometaphase chromosomes show the smaller bands more clearly. However, the longer prometaphase chromosomes are less likely to separate well in spreads, and generally exhibit many overlapping regions - a problem which is troublesome enough with fully contracted urodele chromosomes!

Some C-bands may consistently show size heteromorphisms between homologous pairs from any one individual. This
phenomenon is common and may vary between individuals. Some examples of C-band polymorphisms are shown for T. c. carnifex in Figs. 7-10. A striking C-band heteromorphism is seen between the terminal blocks of heterochromatin in the long arms of chromosome 4 in males only. In all cristatus subspecies, one homologue usually has a single small block of heterochromatin in the terminal position. The other homologue has two blocks separated by a small region of euchromatin. In marmoratus, the difference is more prominent in that one homologue has two heterochromatic blocks, whereas the other has three. The heteromorphism is confined to males. Both homologues in females from cristatus and marmoratus possess one or two bands, respectively. Males are therefore the heterogametic sex and an XY/XX sex determining mechanism is in operation. The situation is detailed in section 3.2.

2.3.3 C-Banding of Chromosome 1

The most obvious feature of the C-banded karyotypes is the long heterochromatic tract in the long arm of chromosome 1. The heterochromatic tract constitutes most of the long arm, being flanked by small blocks of euchromatin at the terminal and procentric regions, and it corresponds to the heteromorphic region of lampbrush chromosomes 1 (Callan and Lloyd, 1960; Nardi et al., 1972a). The heteromorphic region constitutes about half of the entire chromosome (Table 4). T. marmoratus shows the greatest size difference of heteromorphic regions between chromosomes 1A and 1B. It is
the difference in size of the heteromorphic region which accounts for the difference in R.L. and C.I. of these two chromosomes.

The heterochromatin of the heteromorphic region does not stain uniformly. Each homologue has its own distinctive C-banding pattern within the heteromorphic arm (HTA) by which it can be identified. Chromosome preparations were made from several tissue sources (testes, intestinal epithelium, spleen, limb blastema and tail-tip blastema) and it was found that the banding patterns were constant for all tissues. Figures 11-14 show the banding patterns for chromosomes 1A and 1B in marmoratus and the cristatus subspecies. These banding patterns are always found in all individuals from a given population. However, in T. c. carnifex, two other forms of chromosome 1A have been found within a population. These 1A variants occur at a low frequency. All three forms of chromosome 1A are similar, but there are subtle differences. One variant has a very intensely staining proximal segment (Macgregor and Horner, 1980; Macgregor et al., 1983). Another has a strongly staining distal segment which actually extends to the tip of the long arm, with no terminal block of euchromatin (Fig. 15).

In marmoratus, the heteromorphic region does not extend as far up towards the centromere as it does in the cristatus subspecies. Chromosomes 1A and 1B show an additional identifying feature in the pericentric C-band of the long
arm. The weakly staining pericentric band is adjacent to the centromere in chromosome 1A, but is nearer the heteromorphic region in chromosome 1B. The difference in position of this band probably signifies a small paracentric inversion heterozygosity within the region between the centromere and the heteromorphic region. Evidence from lampbrush chromosomes supports this view, in that a small cluster of distinctive loops appears in a different position each half-bivalent within the proposed inverted segment (Sims et al., 1984).

C-banded interphase nuclei show many heterochromatic spots corresponding to C-bands of the metaphase chromosomes (Fig.16). T. c. cristatus and T. c. carnifex nuclei appear very similar with many small heterochromatic dots. T. c. karelinii has the same small dots and, in addition, possesses several large chromocentres. T. marmoratus has a combination of small and relatively large heterochromatic dots in its interphase nuclei. The size of the larger dots reflects the size of the large centromeric heterochromatin on some of the chromosomes. The large dots do not fuse to form chromocentres of the size seen in karelinii. The heteromorphic arms (HTAs) are not seen in interphase nuclei in any obvious form. However, as cells enter prophase, the chromatin of the HTAs contracts and the heteromorphic regions can be seen as long 'beaded' strands of heterochromatin (Fig. 17). In early prophase nuclei, heterochromatic dots can be seen to come together, forming the long heterochromatic tracts of the HTAs. By mid-
prophase, the HTAs are obvious. This suggests that the HTAs are not entirely heterochromatic, but consist of an array of heterochromatic 'beads' linked with short stretches of euchromatin. Only the heavily staining 'beads' persist throughout the cell-cycle in a condensed state.

2.3.4 Chromosome 1 in Meiosis

Meiosis in newts is shown in Figs. 18 and 19 by conventional staining with orcein. Leptotene is quite usual, slight condensation of chromatin being evident. Zygote is represented by chromatin being more condensed after pairing of homologues. Pairing proceeds from the tips of the chromosomes, at the nuclear envelope, towards the centromeres. The chromosomes are arranged in a polarised fashion, with centromeres at one pole of the nucleus and the distal tips projecting towards the other pole. This conformation gives the typical 'bouquet' arrangement. In some zygotene/pachytene nuclei stained with orcein one can see some chromosome strands which have not paired (Fig. 18). In C-banded preparations, these unpaired strands are obviously heterochromatic and correspond to the heteromorphic region of chromosome 1. At diplotene/diakinesis, chiasmata are obvious and the chromatids involved in a cross-over are often apparent. By first meiotic metaphase, the chromosomes have contracted considerably more than they would have done at mitotic metaphase. Meiotic metaphase II chromosomes are rarely seen, as this division follows and proceeds very quickly after the first meiotic division.
However, when present, second meiotic metaphase chromosomes are even more contracted and result in an 'X' conformation (Fig. 19c). Kezer (1970) and Kezer and Macgregor (1971) described the 'diffuse' stage of meiosis in salamanders as coming between pachytene and diplotene. This stage is believed to represent a decondensation of the chromosome structure, similar to the lampbrush stage of diplotene oocytes. Although the diffuse stage has been described for many animals, there is no evidence, to date, that any major transcriptional activity occurs during this stage. The diffuse stage is difficult to identify in squash preparations as it resembles leptotene. Sectioned material can be used to identify the diffuse stage as meiosis progresses in waves throughout certain regions of the testes. Figs. 20 and 21 show a longitudinally sectioned testis of *T. c. carnifex*, relating the structural organisation of the organ. Testes are found in a dorsal position, on both sides of the midline, around the middle to lower region of the body cavity. Each testis may be composed of 1-3 lobes or pairs of lobes (depending on the seasonal condition of the testis). Meiosis and spermatogenesis proceed in a diagonal wave through the each lobe, from a dorsal anterior position to ventral posterior. Each lobe is subdivided into lobules which contain numerous follicles. Follicles are roughly spherical sacs or vesicles enclosing numerous germ cells. The cells within a follicle are more or less synchronised in their stage of spermatogenesis. The occurrence of meiotic stages in a testis is seasonal and usually occurs soon after the breeding season. Meiotic stages are often found in *T.*
cristatus and *T. marmoratus* during September. Spermatogonia undergoing mitotic divisions normally occupy the ventral anterior end of the testis. Further down the testis, in a dorsal posterior direction, spermatocytes in meiosis should be evident. All year round, at the dorsal posterior end of the testis, the follicles are composed of elongating spermatids and/or mature sperm bundles.

Unfortunately, very few meiotic cells were seen in the testes sectioned for this study, so the existence of cells passing through the diffuse stage cannot be confirmed for *Triturus*.

C-banded squash preparations show the heterochromatic dots in leptotene nuclei to be mainly concentrated at one pole. These dots are likely to be the material that goes to make up the centromeric and pericentric C-bands of the chromosomes. The arrangement of the chromosomes probably remains unaltered after the preceding mitotic division, only a decondensation of euchromatin having taken place (Fig. 22a). This would leave the chromosomes in a suitable conformation for subsequent synapsis during zygotene: centromeres at one pole with arms extending towards the other pole. The HTAs are not clearly distinguishable in leptotene nuclei. However, by zygotene, the chromatin has condensed sufficiently for the heteromorphic regions to be visible as long strongly staining threads. In Fig. 22e and f, the HTAs can be seen as separate strands of chromatin which remain asynaptic throughout zygotene and pachytene.
Figure 23a shows clearly that the euchromatic tip of chromosome 1 has synapsed, but the heteromorphic region has remained unpaired. Chiasmata are often seen in the euchromatic distal tip of the HTAs.

The failure to pair prevents chiasma formation within the heteromorphic region. Chiasmata are, therefore, never seen in this region in diplotene to metaphase I bivalents (Fig. 24). Chiasmata most often form in three domains of bivalent 1 in male meiosis of *T. c. carnifex*: the tip of the short arm, near the centromere of the short and long arm, and at the euchromatic tip of the long arm. In *T. c. cristatus*, a chiasma may occur in any position along the short arm, near the centromere of the long arm and at the euchromatic tip of the long arm. Meiotic divisions were not observed in *T. c. karelinii* (no males were available in suitable condition). The chiasma frequency for chromosome 1 is $2.18 \pm 0.67$ (n=20) in *T. c. cristatus* and $2.15 \pm 0.67$ (n=20) in *T. c. carnifex*. The chiasma frequency of chromosome 1 in *T. marmoratus* is lower, being $1.51 \pm 0.51$ (n=20). In *marmoratus*, chiasmata are restricted to the distal tip of the short arm and the euchromatic tip of the long arm. It appears that the achiasmate region in *marmoratus* is not confined to the heteromorphic region, but extends as far up the chromosome as the centromere and may even extend into the short arm. On the rare occasion, chromosomes 1 in *marmoratus* first metaphase can be seen as univalents (Fig. 25). Normal segregation of the univalents cannot be determined.
The distribution of chiasmata in bivalents from *T. marmoratus* (Fig. 25) tend to be terminal, but can be intercalary. In *T. c. cristatus* and *T. c. carnifex* chiasmata do not appear to be localised (Figs. 26 and 27). Chiasmata are seen in terminal, interstitial and procentric positions. Many metaphase I chromosome spreads in *T. c. cristatus* are composed of essentially closed rings with terminal chiasmata. However, this cannot be taken as evidence for terminalisation of chiasmata as many spreads show a high number of interstitial chiasmata. The incidence of position of chiasmata for a particular chromosome is random and varies from spread to spread. In spreads where a chromosome has two widely separated chiasmata, making the bivalent adopt a loop-like conformation, there is a high tendency for bivalent interlocking (Fig. 23b). Interlocking involves the overlapping of half-bivalents, often giving rise to complex 'chain' conformations. Interlocking does not necessarily involve only circular bivalents. Rod shaped bivalents, with only one chiasma, can be found within the loop of a circular bivalent.

White (1946) found interlocking bivalents in *T. c. cristatus*, but not in *T. c. carnifex*. Results here show interlocking in both subspecies and in *marmoratus*, but with a higher incidence in *T. c. cristatus*. Under normal conditions, the significance, if any, of interlocking is not known. It probably does not hinder chiasma formation and does not affect the normal segregation of bivalents at anaphase I.
2.3.5 Hybrid Embryos

Macgregor and Horner (1980) established that 50% of all offspring from *T. cristatus* and from *T. marmoratus* show arrested development at tail-bud stage of embryogenesis and subsequent death. The arrested development of the embryos was related to their chromosomal constitution. Arresting embryos were homomorphic for chromosome 1, having either two chromosomes 1A or two chromosomes 1B. Individuals heteromorphic for chromosome 1 developed normally. It was decided to re-examine this situation using embryos from a hybrid mating between *T. marmoratus* (male) and *T. c. carnifex* (female). The mating was experimentally induced using injections of gonadotrophin. At the same time, arresting embryos were examined from normal matings involving *T. c. carnifex* parents and *T. marmoratus* parents. Embryos presumed to be arresting were used for chromosomal analysis before actual arrest, so as to ensure the presence of mitotic divisions. Figures 28-32 show C-banded chromosomes taken from arresting embryos. Unfortunately, the morphology of chromosomes from embryos is usually of poor quality due to difficulties in administering colchicine and the presence of large amounts of yolk platelets. Chromosomes 1 from arresting embryos were clearly seen to be homomorphic, those shown in Figs. 28 and 29 being two chromosomes 1A. In a normal marmoratus mating, one can see two chromosomes 1B in one arresting embryo, easily distinguished because of the position of the pericentric band of the long arm and the
banding within the heteromorphic region (Fig. 30).

Examination of F hybrid embryos showed that about 40% (46 out of 116 embryos) were arresting. Chromosome preparations were successfully made from some of the embryos and their C-banding patterns are shown in Figs. 31 and 32. In each of the preparations made, a 1A chromosome from carnifex was found together with a 1A chromosome from marmoratus. It was on this basis that each homologue of chromosome 1 was correlated with its equivalent homologue in both species. It was thus possible to correlate the longer chromosome of marmoratus (1A) with the corresponding 1A chromosome from carnifex.

It is interesting to note that in the hybrid mating, the chromosome 1A from carnifex happened to be one of the variant forms. This chromosome has essentially the same banding as the typical form of 1A, but the heterochromatin of the heteromorphic region extends to the distal tip. Also, the distal third of the heteromorphic region is more intensely stained than the rest of the HTA. This variant has only been seen in one other individual from the same population.

2.3.6 Fluorochrome Staining

To gain more information about the composition of heterochromatin, the chromosomes were stained with base specific fluorescent dyes in conjunction with a non-fluorescent
counterstain. With both dyes used here, DAPI (AT specific) and mithramycin (GC specific), the dye alone fluoresces brightly along all chromosomes and differential fluorescence is not easily distinguished. Differential staining is enhanced when non-fluorescent dyes, of the opposite base specificity are used with the fluorochromes. The resultant fluorescence is greatly reduced in intensity, although contrast between a heterochromatic band and euchromatin is improved considerably. The counterstains act by either quenching the fluorescence of a fluorochrome bound nearby, or they compete with the fluorochrome for binding sites. The mechanism of action depends on which counterstain is being used.

2.3.7 Fluorochrome Banding of Chromosome 1

Figures 33 and 34 show chromosomes 1 stained to show either regions of high AT composition (actinomycin D/DAPI) or GC-richness (distamycin A/mithramycin). For both types of dye pair, euchromatin stains uniformly. With actinomycin D/DAPI, any region fluorescing brighter than euchromatin is considered to be AT-rich. Any region fluorescing more weakly than euchromatin is GC-rich. The opposite is true for distamycin A/mithramycin and methyl green/mithramycin dye pairs, where strong fluorescence indicates GC-richness, and dull or absence of fluorescence shows AT-richness. The centromeric heterochromatin of marmoratus chromosomes 1 has a GC bias in composition. The centromeric heterochromatin of all the cristatus subspecies shows a weaker fluorescence
than euchromatin with actinomycin D/DAPI. However, with mithramycin dye-pairs, the centromeres show either an equal or a reduced fluorescence compared to euchromatin. The centromeric heterochromatin probably has a base pair composition similar to that of euchromatin, but there is some confusion about the exact nature of centromeric heterochromatin (see next section).

In all subspecies, the pericentric heterochromatin is AT-rich in chromosome 1. T. c. karelinii only has pericentric heterochromatin in the short arm, whereas it is present in both arms in all other subspecies. In T. marmoratus, the pericentric heterochromatin is located at different distances from the centromere in the long arm of chromosome 1A and 1B. As with C-banding, the position can be used to identify either of the chromosomes 1.

In all subspecies, the heteromorphic regions have the same characteristics of base pair composition. The heteromorphic regions have an overall slight bias towards GC-richness. With mithramycin staining, the heteromorphic regions show a slightly stronger fluorescence than euchromatin. In T. c. cristatus and T. c. carnifex, one homologue has a distally located, bright GC-rich band in the heteromorphic region. This band is not evident in all individuals of T. c. carnifex. Differential mithramycin staining of the heteromorphic region is not sufficient to allow identification of chromosomes 1A and 1B separately, in T. marmoratus and T. c. karelinii. Staining with DAPI shows the
heteromorphic regions to have an overall dull fluorescence, indicating GC-richness. Along the length of the heteromorphic regions there are localisations of AT-richness seen as bright speckles rather than discrete bands. The distribution of AT-rich speckles differs between chromosomes 1A and 1B, and between the subspecies. The speckles are difficult to map as they are small and their relative positions vary with the degree of chromosome contraction. Speckles may be clustered at certain positions and can be used as an identifying feature. For example in T. c. carnifex, one heteromorphic region has a pair of prominent, distally located speckle clusters.

The complementarity of DAPI and mithramycin with regard to staining AT- or GC-rich chromosomal regions is best demonstrated by the sequential staining of a preparation. The preparation can be stained with one dye pair, destained and then restained with another dye pair. The effect is most strikingly seen at the site of the NORs, which are characteristically GC-rich. The NORs fluoresce brightly with distamycin A/mithramycin but, lose all of their fluorescence when destained and restained with actinomycin D/DAPI (Fig. 35).

2.3.8 Banding of Centromeric Heterochromatin

All centromeres show a positive reaction with Giemsa C-band ing. The amount of centromeric heterochromatin is characteristic for a particular chromosome. The amount may
be small as in some chromosomes of *cristatus* and *carnifex* or may be extremely large as in *karelinii*. There are four lines of evidence to suggest that centromeric heterochromatin, as revealed by banding techniques, may have different properties from other sites of constitutive heterochromatin.

1. Phase-contrast micrographs of *T. c. karelinii* chromosomes show the classical chromosome morphology with a distinct centromeric constriction (Figs. 36 and 37). When the chromosomes are C-banded, the constriction may be obscured. Certain *marmoratus* chromosomes appear to have a large Giemsa-staining body over the centromeric constriction. Occasionally, the centromere may appear as an elongated, deeply staining constriction. In *karelinii*, the phenomenon is more pronounced. The centromeres often appear as a large deeply staining mass. The primary constriction is not always completely obscured, but is often hidden.

2. Fluorochrome staining shows pericentric heterochromatin to be AT-rich (using actinomycin D/DAPI). Centromeric heterochromatin fluoresces less intensely than euchromatin, suggesting GC-richness. This situation is clearly seen with the large centromeric regions of *karelinii* chromosomes. However, when preparations are stained with mithramycin dye-pairs, centromeric regions do not fluoresce brightly, as expected. The centric regions show slightly reduced fluorescence compared to euchromatin, suggesting AT-richness. Obviously, there are conflicting results here, although some *marmoratus* centromeres do actually show a bright fluorescence with mithramycin (compare Figs. 33 and 34).
(3) Chromosomes from T. c. carnifex were taken through the C-banding procedure, but were stained with quinacrine instead of Giemsa. Quinacrine fluoresces brightly at AT-rich chromosomal regions. The relatively small centromeric heterochromatin of all chromosomes in carnifex showed a bright fluorescence, indicating AT-richness. However, contrary to previous findings, the heteromorphic region also fluoresced more brightly than euchromatin. The results indicated that the heteromorphic region was AT-rich rather than being generally slightly GC-rich (Fig. 39).

(4) Actinomycin D/quinacrine staining of T. c. carnifex chromosomes shows the heteromorphic region to be generally GC-rich, as expected, with an overall lower fluorescence than euchromatin. However, some chromosomes showed brightly fluorescing centromeric heterochromatin, suggesting AT-richness (Fig. 40).

How can the above results be explained? T. marmoratus and T. c. karelinii clearly possess a centric constriction as seen in phase-contrast micrographs of fixed, unstained preparations. Feulgen staining shows the centromeric constriction to contain DNA. Highly repeated DNA sequences are present, in large number at the centromeres of karelinii chromosomes (Baldwin and Macgregor, unpublished observations). The highly repeated sequences would account for the strong C-staining at the centromeres. However, Giemsa surely cannot bind to DNA alone as originally suggested by Comings and Avelino (1975) and Comings et al. (1973). Silver
staining of nuclei and chromosomes is used to highlight the interphase nucleoli and chromosomal nucleolus organizer regions. Silver staining can also stain a number of other cytological structures such as centromeric dots (Buys and Osinga, 1980). Recently, it was found that both Giemsa and silver nitrate stain the same set of acidic phosphoproteins from nucleolar isolates (Buys and Osinga, 1984). At least one of these proteins is associated with DNA. Thus, one has to take into account at least phosphoproteins as well as DNA in Giemsa staining. Much of the centromeric staining after C-banding could be the result of a dye-DNA reaction. Whatever the case, much of the Giemsa staining material may be dissociated slightly after the C-banding technique to give a large staining mass that obscures the centromeric constriction. A similar situation has been observed in human chromosomes where the structure of the stalks and satellites of acrocentric chromosomes is 'loosened' by C-banding (Craig-Holmes and Shaw, 1971).

When stained with fluorochromes, *karelinii* centromeric constrictions are not obscured. This further lends support to the idea that C-banding disrupts the centromeric structure. Both AT-specific and GC-specific staining show less fluorescence than euchromatin. It is possible that a high concentration of centromeric proteins inhibits dye binding, preventing its base composition from being determined accurately. The proteins which may be preventing dye-binding at the centromeres, certainly have no inhibitory effect on the adjacent pericentric heterochromatin which
flouresces brightly with DAPI. Neutral caesium chloride gradients of *karelinii* total DNA show no satellite peaks away from the main band (Baldwin, unpublished observations). So it seems likely that the centromeric heterochromatin has no strong bias in base pair composition. The reduced fluorescence at *karelinii* centromeres may just reflect a proteinaceous inhibition of dye-binding and/or fluorescence.

Chromosome regions fluorescing brightly after quinacrine staining (or derivatives) are known to reflect a high AT base pair content (Ellison and Barr, 1972; Weisblum and de Haseth, 1972). Regions showing quenched quinacrine fluorescence are GC-rich. But, the relationship between base pair content and fluorescence is not so clear-cut. The centromeric heterochromatin of mice, *Mus musculus*, chromosomes is very faintly fluorescent after quinacrine staining (Rowley and Bodmer, 1971). Yet, the centromeric heterochromatin contains an AT-rich satellite DNA (Pardue and Gall, 1970) and would be expected to fluoresce brightly. Weisblum (1973) found that the degree of quinacrine fluorescence was not directly related to the AT:GC base pair ratio of the DNA, but to the interspersion of GC base pairs within DNA. Thus, an AT-rich satellite with a regular interspersion of an occasional GC-base pair would result in a reduced fluorescence. Interpretation of studies with quinacrine must be treated with caution.

Why should centromeres of C-banded *carnifex* chromosomes fluoresce brightly with quinacrine? One explanation could be
that GC-base pairs may be preferentially removed from chromatin during the C-banding procedure. This may leave sufficiently long stretches of AT base pairs to show enhanced quinacrine fluorescence. C-banding has been shown to involve depurination and beta-elimination (Holmquist, 1979). Depurinated euchromatic DNA may solubilise faster than heterochromatic DNA, resulting in a greater loss of DNA from euchromatin. The differential loss of DNA is manifested in a stronger staining of heterochromatin than euchromatin. DNA-protein interactions may be involved in the differential DNA solubilisation rates between euchromatin and heterochromatin, rather than different degrees of chromatin condensation (Burkholder and Weaver, 1977; Holmquist, 1979). In light of the results obtained with *Triturus* chromosomes, it seems unlikely that depurination (adenine and guanine) of centromeric heterochromatin could account for bright quinacrine fluorescence. Quinacrine may be binding nonspecifically to chromatin proteins at the centromeres. Quinacrine and quinacrine mustard bind to DNA by intercalation (Bontemps and Fredericq, 1974; Lerman, 1964; Selander, 1973). Thus, a more likely explanation for the strong centromeric fluorescence may be that the fragmentation of DNA by the C-banding procedure allows a greater degree of dye intercalation.

Staining of chromosomes with actinomycin D/quinacrine also reveals some AT-rich, brightly fluorescing centromeres. With this dye pair, the GC-specific actinomycin D may be sheltering the "quenching" effect of GC base pairs on
quinacrine fluorescence. It is only the centromeric staining that differs from actinomycin D/DAPI fluorescence. The heteromorphic region of chromosome 1 stains exactly the same with actinomycin D/quinacrine as it does with actinomycin D/DAPI. The inconsistent results with quinacrine were the major reasons why it was not used extensively in this study. The use of DAPI and related dyes more accurately reflect the base composition of chromosomal regions studied (for a review see Schweizer, 1981).

Finally, a comment must be made on the observation of "centromeric dot" staining. Many currently used C-banding procedures utilise barium hydroxide in the alkali denaturation step. Barium hydroxide is not as strong a reagent as sodium hydroxide, and its action is much more controllable. To achieve C-banding with sodium hydroxide an incubation period of less than 30 seconds at room temperature in a 0.07M solution is required. If chromosome preparations are exposed to 0.07M NaOH at 20 degrees C, for 3 minutes, then normal C-banding is lost (Fig. 38). The resultant chromosomes appear 'empty', although contour delineation is maintained. Interestingly, a dot appears over each chromatid at the site of the centromere on all chromosomes. The size of all centromeric dots is approximately the same and does not reflect the size of the centromeric C-heterochromatin. Some dots appear at additional sites at interstitial and subterminal positions. Similar centromeric dot staining has been produced on human chromosomes (Cd-staining), by treating with a hot balanced
salt solution (Eiberg, 1974). UV-light photolysis of Hoechst 33258 stained Allium chromosomes, not substituted with BrdU, also causes subsequent Giemsa staining of like-sized pairs of dots at the centromeres of all chromosomes and secondary constrictions (Sato and Sato, 1982). Sato and Sato (1982) believe the centromeric dots to be the kinetochores or at least contain them. Chromatin is extracted during UV photolysis, leaving contour delineated chromosomes. Contour delineation of chromosomes, without staining centromeric dots, has been achieved by various chemical and photo-oxidative treatments, which involve DNA loss (Drets and Navello, 1980; Drets et al., 1978). The centromeric staining produced in the present study probably involves extensive chromatin loss, as the chromosomes stain weakly even after prolonged Giemsa staining. The chromatin loss may be due to excessive DNA fragmentation and solubilisation, brought about by the long incubation in NaOH (Holmquist, 1979). The centromeric dots may represent a core-structure of DNA and protein which is particularly resistant to extraction. Interphase nuclei also possess between 1 and 4 small staining bodies, of variable size, which are most probably the nuleoli (Fig. 38). Nucleoli are not normally revealed by C-banding procedures. The present method may be extracting most of the chromatin, leaving the proteinaceous 'shell' of the nucleoli.

2.3.9 General Fluorochrome Banding of the Karyotypes

Few heterochromatic regions are GC-rich. The most prominent
GC-rich bands are those of the nucleolus organiser region (NOR). In situ hybridisation of rDNA or rRNA to chromosomes shows the NOR to be on chromosome 9 in *T. c. carnifex* and on chromosome 10 in *T. marmoratus* (Barsacchi-Pilone et al., 1974a, b; Hennen et al., 1975). In *T. c. carnifex*, some individuals have ribosomal genes on chromosome 6 (Hennen et al., 1975). The sites of the NORs have not been located in the other cristatus subspecies. The following summarises the major mithramycin-positive (GC-rich) chromosomal regions, including the NORs. Chromosomes 1 are not included in this summary.

*T. c. cristatus*: The NOR is evident in a subterminal position on the long arm of chromosome 9. Often the NOR is only detectable on one homologue. The heterozygosity is also seen with C-banding, where only one homologue of chromosome 9 shows a subterminal C-band. C-banding of chromosome 12 shows a 'double' pericentric band in the long arm. One of these bands fluoresces brightly with mithramycin. Few other very small GC-rich bands are apparent (Fig. 41).

*T. c. carnifex*: The NOR is evident as a GC-rich band in an interstitial position on the short arm of chromosome 9. The NOR may differ in size between the two homologues. Some individuals show a GC-rich band in a subterminal position on the short arm of chromosome 6. By comparison with in situ hybridisation studies, this region is probably an NOR (Barsacchi-Pilone et al., 1974a; Hennen et al., 1975). Several other C-bands may be GC-rich within the chromosome set. These regions are usually present on only one homologue of a pair and the sites vary between individuals (Fig. 44).
**T. c. karelinii**: The NOR is again on chromosome 9. However, two potential sites may show bright fluorescence with mithramycin dye-pairs. One type of chromosome 9 has been noted with GC-rich bands in a subterminal and pericentric position in the long arm. Another type of chromosome 9 has a GC-rich band only at a subterminal position in the long arm. No other prominent GC-rich regions were observed (Fig. 45).

**T. marmoratus**: The NOR is in a subterminal position on the long arm of chromosome 10. The size of the NOR often varies between the homologues. Some of the larger blocks of centromeric heterochromatin may show a slight GC-richness (Fig. 47).

The majority of the remaining sites of constitutive heterochromatin are AT-rich, most notably the pericentric regions (Figs. 42, 43, 46 and 48). In all the subspecies examined the pericentric heterochromatin of all chromosomes is AT-rich. In *T. c. karelinii* the pericentric heterochromatin appears as clusters of AT-rich speckles. Actinomycin D/DAPI staining shows the pericentric regions far more clearly than in C-banded preparations, where the heterochromatin appears 'diffuse' or indistinguishable from centric heterochromatin. In *T. c. cristatus*, *T. c. carnifex* and *T. marmoratus* the pericentric heterochromatin may be visible as a discrete AT-rich band spanning each chromatid. At the microscope, changing the plane of focus shows that the band follows a spiral conformation around the chromatid. The clarity of the 'spiralisation' depends on the degree of chromosome contraction.
There are two major sites of C-heterochromatin which show no bias in their base pair composition. The bands are in a subterminal position in the long arms of chromosomes 5 and 8 in all of the subspecies. The bands show euchromatic levels of fluorescence with both DAPI and mithramycin dye-pairs, but show distinct C-banding. The two sites of heterochromatin correspond to the positions of sphere loci in lampbrush chromosomes. *T. c. cristatus* and *T. c. carnifex* consistently show subterminally located spheres in the long arms of lampbrush chromosomes 5 and 8. In *T. c. karelinii*, spheres are not reliable landmarks, but when present they are on chromosomes 5 and 8 in the same positions as the other *cristatus* subspecies. Spheres have never been observed on *marmoratus* lampbrush chromosomes. The presence of C-heterochromatin at these regions in mitotic chromosomes of all subspecies suggests that these sites have been conserved within the 'cristatus' species group. Interestingly, Gall et al. (1981) found that sphere sites are associated with histone gene loci in *Notophthalmus viridescens*. Using the same histone gene probe for in situ hybridisation (a 9kb genomic histone gene cluster of *N. viridescens* cloned into a lambda phage vector), the authors found labelling in a subterminal position of the long arms of mitotic chromosomes 5 and 8 in *T. c. carnifex*. The same was true for the corresponding mitotic sites of sphere loci in *T. alpestris alpestris*. So, although spheres may or may not be present in lampbrush chromosomes, the mitotic C-bands at these sites reflect the presence of histone gene clusters or of
lampbrush sphere loci. The fluorochrome staining shows that the heterochromatin associated with these sites has an approximately equal ratio of AT:GC base pairs as euchromatin.

Minor, small C-bands are not always evident with fluorochrome staining. The ability to see some of the smaller fluorescing regions often depends on the plane of focus.

2.3.10 Chromosomal Rearrangements

Chromosome rearrangements in the higher Urodela are considered to be a relatively rare event. However, a very unusual situation was found in one male *marmoratus*. C-staining of mitoses from the intestinal epithelium revealed a wide range of chromosome rearrangements. Most of the rearrangements involved chromosome 1B, only one was found to involve chromosome 1A. Figure 49 shows examples of the types of rearrangement found. Figures 49c and d show what at first glance was taken to be an isochromosome of the long arms of chromosome 1B. Closer observation reveals this to be a translocation of most of the long arm of a chromosome 1B to the short arm of another 1B chromosome. The origin of the situation is difficult to explain unless one assumes that irregular segregation of mitotic chromosomes had occurred earlier in that cell lineage. Two chromosomes 1B might then come to occupy the same nucleus, along with the remainder of the complement. One chromosome 1B could then translocate to
the other, resulting in a chromosome with two long arms of chromosome 1B and a small centric fragment which may be lost. Alternatively, the lineage may have had a normal complement but, some time following an S-phase, one sister chromatid of a 1B chromosome may have broken and reattached to the short arm of the other sister chromatid. The end result would be the 'bi-long armed' chromosome 1B and a small roughly metacentric chromosome which would probably be lost.

Metaphase spreads from the testes of this same animal showed only normal chromosome complements. So, rather importantly, the rearrangements would not have been passed on through the germ-line. The intestine and testes were the only tissues prepared for chromosome analysis, so no other tissues could be examined for rearrangements. Nevertheless, this is probably the first known case of a somatically derived mosaicism in the Amphibia.

A chromosomal rearrangement was also found in one T. c. cristatus male. The rearrangement involved a pericentric inversion in one homologue of chromosome 6. Chromosome 6 is normally a submetacentric chromosome (C.I. = 0.338). However, the inverted homologue is more metacentric, with a C.I. of 0.439 (Fig. 50). The metacentric homologue has been seen in mitoses from both the intestinal epithelium and the testes. It is therefore likely that the rearrangement was inherited from one of the parents. From banding of mitotic chromosomes, the size of the inverted segment can be
estimated. It seems likely to consist of a small portion of the long arm including the pericentric band, extending into the short arm but not including the pericentric band of the short arm. Much of the short and long arms remain unaffected, a factor that is important when considering chromosome pairing in meiosis.

Fortunately, meiotic divisions were found in the testes of this animal. All metaphase I chromosome sets appeared normal, with no evidence of univalents, and therefore no indication of gross asynapsis of the chromosomes 6. Due to the nature of synapsis, the tips of the chromosomes pair first, synapsis proceeding away from the distal ends and towards the centromeres. The inversion, being quite small, leaves much of the short arm and most of the long arm free to pair with the typical form of chromosome 6. Bivalents 6 appear normal in that they possess a chiasma in each bivalent arm (Figs. 51 and 52). However, the positions of the chiasmata never coincide with the region of the inversion. It cannot be determined from zygotene nuclei whether the inverted region ever pairs. It may be that this region does synapse, but pairing collapses at a later stage. The problem could be resolved by examination of synaptonemal complexes with silver staining (Counce and Meyer, 1973; Moses et al., 1975, 1982).

The regular pairing of most of the inverted chromosome 6 means that the rearrangement can be passed on through the germ-line to future generations. The rearrangement would
presumably remain stable in a population, with no speciation value. Chromosome polymorphisms have been described for the smallest chromosome in *T. italicus*, in several populations over southern Italy (Bucci-Innocenti et al., 1983a).
THE CYTOLOGY OF TRITURUS CRISTATUS AND T. MARMORATUS WITH PARTICULAR REFERENCE TO CHROMOSOME 1.

2.4 DISCUSSION

2.4.1 General Karyotypic Features

Although the karyotypes of T. marmoratus and of all subspecies of T. cristatus appear superficially different on the basis of C-banding, the differences are largely quantitative. For many of the C-bands, the positions in the karyotypes have been conserved, inter- and intraspecific differences being largely in size of the C-bands concerned (Fig. 53).

C-banding allows identification of a particular chromosome which can then be compared between species and subspecies. The centromeric positions of most chromosomes appear to have been conserved through speciation and subspecific differentiation. The relative size of a chromosome may have changed, affecting its position in the karyotype. For example, the submetacentric medium-sized chromosome is ordered as number 6 in cristatus and carnifex, but as number 7 in karelinii and marmoratus. The disparity may be an artifactual consequence of preparation or more probably size expansion/contraction through changes in the amount of highly and middle repetitive DNA sequences.

Examination of the cristatus subspecies shows that the
karyotypes of *cristatus* and *carnifex* are virtually identical with regard to centromere index and position and size of most C-bands. The major differences between the karyotypes is the banding of the heteromorphic region on chromosome 1 and the positions of the NORs on chromosome 9. The corresponding chromosomes in the karyotype of *karelinii* have the same relative centromere positions as in *cristatus* and *carnifex*, but there are obvious differences in the amounts of heterochromatin. In *karelinii*, the heterochromatin of the centromeres is massive on all chromosomes except chromosome 7. Just why this chromosome has escaped expansion of the centromeric heterochromatin is not known. Pericentric C-bands are present in *karelinii* at all the same sites as in *cristatus* and *carnifex*, although all of them stain more weakly in *karelinii*. The pericentric C-band of the long arm of chromosomes 1 is not evident in *karelinii*. The pericentric C-bands appear as 'diffuse', grey staining regions, whereas centromeric heterochromatin is much more strongly stained. With actinomycin D/DAPI staining, the *karelinii* pericentric heterochromatin is clearly visible as clusters of brightly fluorescing AT-rich speckles. The same is true for the other sites of AT-rich heterochromatin. Each AT-rich speckle has a homogeneous composition and is probably tightly condensed. It seems as if each of the DAPI-positive speckles consist of short tracts of AT-rich heterochromatin separated by short stretches of euchromatin. The interspersion of euchromatin within the pericentric heterochromatin would account for the diffuse nature of the C-banding. There may be a 'dilution' effect by euchromatin resulting in grey
C-bands. Heterochromatinisation has involved different sequences in pericentric regions compared to the centromeres. This is seen by the strong AT-bias of pericentric heterochromatin.

T. marmoratus shows all the major sites of C-bands as the cristatus subspecies. The differences are largely quantitative in terms of corresponding C-bands. One of the important differences between marmoratus and the cristatus subspecies is the NOR bearing chromosome. On the basis of relative length, this chromosome is numbered as 10 in marmoratus and 9 in the cristatus subspecies. It is likely that these chromosomes are derived from the same ancestral chromosome, but during speciation have changed slightly in length. The position of the NOR within the chromosome is different for all subspecies, the changes probably brought about by inversions. However, Mancino et al. (1979) believe the NOR bearing chromosomes do not pair in F hybrid spermatogenesis between T. marmoratus and T. c. carnifex. They locate the NOR on meiotic bivalents by the 'absence' of a C-staining region. In the present study, the NORs always stain positively with Giemsa C-banding. The situation should be re-examined using either mithramycin staining or in situ hybridisation of rRNA/rDNA to locate the NORs. It is my strong feeling that the NOR bearing chromosomes of marmoratus and cristatus subspecies do pair during F hybrid meiosis.

Overall, there is conservation of the relative positions of
most of the major C-bands and centromeric indices. This does not mean that chromosomal rearrangements have not occurred, the positions of NORs being an important example. But, whatever rearrangements have taken place, they have not drastically disrupted the general morphology of the karyotype as a whole.

What is clear is that subspecific differentiation can involve larger changes in amounts of heterochromatin than speciation. Heterochromatin probably plays a minor role in speciation, but cannot be excluded from having some effect on the fitness of an individual as some rodent species can show clinal populational variations in the amount of heterochromatin in the karyotype (Bradshaw and Hsu, 1972; Pathak et al., 1973). One important property assigned to heterochromatin is its ability to affect recombination frequency and distribution. John and Miklos (1979) describe how, in the grasshopper (Atractomorpha), some chromosomes may be polymorphic for the amount of telomeric heterochromatin. The heterozygous presence of the telomeric heterochromatin causes chiasma distribution to be moved away from the telomere in that bivalent. Thus, heterochromatin polymorphisms can affect the relative frequency of gene combinations. These authors also review how, in some dipteran species, the presence of an extra block of heterochromatin on one chromosome can affect recombination in other chromosomes. These sorts of changes of recombination and gene frequencies may be particularly important in newt populations, where habitats can be quite
diverse. The lifestyle of newts is such that new pond habitats may be colonised by relatively few individuals. New populations may expand giving rise to a 'founder effect'. The only significant cytological correlation that can be made with the large amounts of centromeric heterochromatin in karelinii is the chiasma frequency. T. c. karelinii has a higher chiasma frequency in male meiosis than the other cristatus subspecies and T. marmoratus (see section 1.1.2, p10). Of course, there is no way of knowing whether the high chiasma frequency is a direct consequence of large amounts of centromeric heterochromatin. But, karelinii is potentially capable of producing gametes with a different range of genetic variation compared to other species/subspecies with lower chiasma frequencies. This may confer a greater adaptive value on individuals colonising new habitats.

2.4.2 Chromosomes 1

Each of the cristatus subspecies and T. marmoratus have different and distinctive C-banding patterns within the heteromorphic regions of chromosomes 1. Linear differentiation of C-staining along the heteromorphic regions suggests a heterogeneity in composition of the heterochromatin. Differential staining of heterochromatin is by no means restricted to Triturus. The best examples of linear differential C-staining are found in heterochromatin of sex chromosomes. Mengden (1981) demonstrated variation in C-staining intensity in the W chromosomes of numerous species
of snake and the Hispaniolan parrot (*Amazona ventralis*). Other examples include the Y chromosomes of man (Jalal et al., 1974), *Drosophila melanogaster* (Hsu, 1971) and some hamster species (Vistorin et al., 1976, 1977). Schmid (1978a, b) has noted consistent differences in staining intensities of heterochromatic blocks in many anuran species.

What factors affect and are involved in heterochromatin heterogeneity? Schmid (1978b) observed that the demonstration of weakly staining (grey or diffuse) heterochromatin is dependent on the duration of the alkali denaturation step of the C-banding technique. The alkali denaturation step results in DNA loss from chromatin (Comings et al., 1973) and also facilitates further loss of DNA in the subsequent hot saline step (Comings et al., 1973; Holmquist, 1979). Chromatin condensation (Burkholder, 1975) and DNA-protein interactions (Burkholder and Weaver, 1977) affect the preferential loss of material from euchromatin rather than heterochromatin, during C-banding. The amount of highly repeated DNA sequences also has an effect on C-staining. Highly repeated satellite DNA sequences are absent from the mouse Y chromosome, yet it stains as grey C-heterochromatin (Chen and Ruddle, 1971; Pardue and Gall, 1970). Mengden (1981) noted that the W chromosome of the snake, *Pseudechis porphyriacus*, often shows close sister chromatid apposition at regions which stain strongly with C-banding. The association is akin to the formation of chromocentres from the fusion of non-homologous centric
heterochromatin in *Drosophila*, *T. c. karelinii* and some mammalian species. The heterochromatin of the fusions is known to contain similar or the same highly repeated satellite DNA sequences.

To summarise, darkly staining C-heterochromatin is usually homogeneous in composition of highly repeated DNA sequences. This type of heterochromatin may be in a very condensed state and/or has strong DNA-protein binding. Less intensely staining C-heterochromatin does not possess sequences at a very high repetition frequency or else the highly repeated sequences are more dispersed in euchromatin. The consequences of the reduced repetition frequency and therefore heterogeneous sequence composition is that 'grey' heterochromatin is more prone to extraction during the C-banding procedure and does not stain as intensely with Giemsa.

As regards chromosome 1 in *T. cristatus*, it is known that highly repeated sequences are preferentially located within the heteromorphic region and centric regions (Macgregor, 1979; Varley et al., 1980a, b). However, the heteromorphic region is not completely composed of highly repeated DNA. In situ hybridisation using middle repetitive DNA shows a fairly even, but patchy distribution over the entire chromosome set. The heteromorphic region possesses no more or no less middle repetitive DNA than any other chromosomal region (Macgregor, 1978; Macgregor and Andrews, 1977). It seems likely that the differential C-staining of the
heteromorphic region is largely dependent on the pattern of interspersion of highly repeated sequences. Darker staining regions probably contain a higher local concentration of highly repeated DNA sequences.

The heteromorphic region has probably followed the same general line of evolution in all subspecies despite the differences in C-banding patterns. It is likely that the evolution has involved expansion or depletion of the same or similar sequences in all subspecies. This can be deduced from fluorochrome staining which shows the heteromorphic region to have a slight overall bias to GC-richness with small localised regions of AT-richness, in all subspecies examined. The consequence of this may be better understood if one looks at the possible ways in which the heteromorphic region and the associated developmental mortality originally arose and evolved. The following section suggests some possibilities for the evolution of chromosome 1 in *T. cristatus* and *T. marmoratus*.

2.4.3 The Evolution of the Heteromorphism of Chromosome 1

The origins of the unusual heteromorphism of chromosome 1 and its existence being essential for normal development, presents a problem. Any explanation remains highly speculative.

The independent derivation of the situation in the two species (*cristatus* and *marmoratus*) seems highly improbable.
and the evidence from this study is firmly against the idea.

First, we need to consider why both chromosomes 1A and 1B are essential for normal development. There are two possibilities. The most likely explanation being that recessive lethal mutations have accumulated at certain loci in the heteromorphic region. At least two recessive lethal mutations, one on each chromosome at different loci, are needed to account for the inviability of the homozygotes. Alternatively, an essential gene complex(es) may have been split between the two heteromorphic regions, requiring both chromosomes 1A and 1B to be present for all gene products to be represented.

The chromosome 1 situation bears some similarities with the degeneration of Y chromosomes in XY/XX sex determining systems. However, there is the additional complication of the fact that both homologues of chromosome 1 resemble the Y sex chromosome. The evolution of Y chromosomes will be discussed and later related to the situation in newts. The following discussion summarises the possible mechanisms leading to degeneration of the Y chromosome in XY/XX systems.

The initial state of the sex chromosomes was considered to be similar to that of an autosomal pair with both homologues having the same structural morphology and the same complement of genes (Muller, 1914; Ohno, 1967). Part or all of the Y chromosome does not cross-over with the X and is
called the differential segment. Muller (1914) suggested that the differential segment, always being heterozygous and free from recombination, will accumulate deleterious or lethal recessive mutations which are never expressed as they never become homozygous. The loss of function from the Y loci is not subject to selection and is sheltered by the X loci. However, Fisher (1935) pointed out that both X and Y should be subject to the same rate of mutation to deleterious alleles. Therefore there should be selection against Y deleterious mutations when an individual also has a deleterious allelic mutation on the X chromosome. But, the similarity of mutation rates for X and Y is dependent on an infinite population size. In a finite population, it has been shown that random drift will lead to a higher rate of chance fixation of deleterious recessive mutants at a Y locus, despite the selective disadvantage (Nei, 1970). The population size has to be lower than 10,000 for this to occur. Charlesworth (1978) argued that the small population size reflected only the deme and not the species size. Therefore, the increased fixation of Y mutations would not be occurring in the species as a whole, unless the species size was less than 10,000 (which is not the case for most animals). Also, the deleterious mutations have to have very mild effects to become fixed by chance, as recessive lethals generally show a reduction in viability of the heterozygote. So, much of the preceding discussion does not account for the degeneration of the Y chromosome.

Consider an alternative theory proposed by Muller (1964),
coined "Muller's ratchet" by Felsenstein (1974) and developed by Charlesworth (1978). In a finite population, there will be Y chromosomes with no deleterious mutations, some with one, two, etc. Y chromosomes with the same number of mutations need not have them at the same loci. Assume that the effect of each deleterious mutation is small and that the fitness differences between Y chromosomes with no mutations and those with one is also small (the same applies between other classes of Y chromosomes with 2 and 3 mutations, 3 and 4, etc.). Therefore in a population with a high rate of mutation to deleterious alleles relative to population size, there is a likelihood of Y chromosomes with the lowest number of mutations (i.e. no mutations) to be lost by chance. With no recombination and the chances of back mutation being extremely small, the class with no mutations cannot be regenerated. Muller's ratchet has therefore moved up a notch and the class of Y chromosomes with no mutations has been lost. Chromosomes with one mutation now become the lowest class and too will tend to be lost. So, the process continues leading to many non-functional genes and degeneration of the Y chromosome.

Perhaps the most analogous situation to chromosome 1 in newts, is that of the sex chromosomes of bryophytes which show haploid dioecy. Bryophytes have haploid and diploid phases in their life cycle, the haploid state being the dominant phase. The sexes are haploid, females carrying an X chromosome, males a Y. The diploid zygote is always heterozygous. There is no recombination between the X and Y.
The similarities with the newt situation are clear, newts are always heterozygous for chromosome 1 with no recombination in the heteromorphic region. As can be envisaged with chromosome 1, bryophytes can accumulate detrimental mutations on one chromosome, provided that the other compensates for it. Both chromosomes can accumulate mutations in a reciprocal fashion, degeneration not exceeding 50% on each chromosome (Bull, 1978a). Loss of genes by mutation would probably require small population size and complete recessivity of the mutation (Nei, 1970).

In bryophytes, genes are sheltered on either chromosome only in the diploid (Bull, 1978a), which is the situation found in the newts. Bull predicts that neither sex chromosome in the bryophytes should show any major degeneration. Change in chromosome size between the sex chromosomes can occur by aneuploid additions. Aneuploid additions comprise portions of the genome which are non-essential as they are represented elsewhere. The additions can be of heterochromatin or euchromatin. Euchromatic additions can degenerate by heterochromatinisation (as in Y chromosomes in diploid dioecy) because the genes are present elsewhere in the genome. So, in bryophytes, any difference in size of the sex chromosomes can be accounted for by additions to the larger chromosome. Also, heterochromatin, if present, should be found on either both chromosomes or just on part(s) of the larger one. This situation best explains the growth of chromosome 1 rather than its degeneration. Chromosome 1 in cristatus and marmoratus is much larger than the largest chromosome of any of the other Triturus species (without a
heteromorphic region). The heteromorphic region seems to consist of blocks of heterochromatin joined by stretches of euchromatin. Therefore, one can envisage growth by heterochromatinisation of non-essential portions, including mutated loci, and possibly aneuploid additions to the heteromorphic region. Muller's ratchet can operate in both heteromorphic regions but only to a limited degree. An increase in mutations can occur in both heteromorphic regions, provided that they are reciprocal at a locus, both regions sheltering each other. Degeneration by Muller's ratchet could only reach about 50% in both heteromorphic regions.

The above discussion assumes that the heterozygous nature of chromosome 1 was the starting point. As to how the heterozygosity initially arose is a more complicated problem. One of the first steps in the evolution of the heteromorphic region was probably suppression of crossing-over. Suppression of crossing-over by heterochromatin (John and Miklos, 1979) would seem unlikely as the heteromorphic region has probably become heterochromatic as a consequence "Muller's ratchet" which requires recombination suppression as a starting point. Therefore, it would seem more probable that crossing-over was suppressed by a structural rearrangement. The rearrangement would have been a large paracentric inversion within the long arm of chromosome 1. This would effectively suppress crossing-over as inversion loops do not occur in newts (Hartley and Callan, 1977), or at least not within large inversions (Mancino et al., 1970). The
inversion could easily be maintained in a population. However, we need a situation where individuals heterozygous for the inversion are at a selective advantage over those homozygous for the normal or the inverted chromosome 1. Wright (1941) suggested a means by which translocations could be homozygously fixed, leading to speciation. He proposed that a translocation could have occurred in a peripheral population, the size of which must have been very small. In such a population, translocation heterozygotes have no intrinsic advantage over homozygotes, and the homozygotes become fixed by chance. The colonising population could then become genetically adapted to its new environment. The new population would be preserved from any influx from the central population because of the relative infertility of the translocation heterozygotes. Natural selection would act against the heterozygotes which would form aneuploid gametes as a result of disjunctional disturbances at meiosis. The new population, therefore, becomes isolated from the central population. Spurway (1953) and Spurway and Callan (1960) envisaged this situation in colonising newt populations which possibly consist of offspring from just one inseminated female. The female or her mate must have been derived from a zygote of which one gamete had the new translocation (or which had occurred a few generations before). Homozygotes for the translocation would accidently become fixed and selection would lead to a greater fitness in the new environment. The population would remain distinct from the original population because of selection against heterozygotes in the 'hybrids'. Similarly,
a situation can be envisaged for the origin of the chromosome 1 heteromorphism but not without problems. All offspring from an inseminated female (invading a new environment) must contain the inversion in one chromosome 1. This alone will only generate a polymorphic population of individuals heteromorphic for the inversion or homomorphic for either the normal or inverted chromosome. If however, one of the original pioneer chromosomes 1 possessed a recessive lethal mutation, then a situation similar to XY/XX sex chromosome systems would build up. Homozygotes for the mutation would be lost, leaving heterozygotes (equivalent to XY) and homozygotes without the mutation (=XX). The present day permanent heterozygosity of chromosome 1 would probably not establish because of the sheltering effect of the non-mutated chromosome which is free to recombine in the homozygotes. Homozygotes lacking the mutation would have to be at a selective disadvantage in the new environment to encourage the heterozygous situation and promote the likelihood of accumulation of more lethal recessive mutations in both chromosomes by Muller's ratchet. Dobzhansky and Pavlovsky (1955) described a population of Drosophila tropicalis which mainly comprised heterozygotes for a certain inversion in the left arm of the second chromosome. Homozygotes die between egg and adult stages, resulting in approximately 50% mortality of all offspring. The heterozygotes for the inversion show such a great fitness that the population thrives. Other populations of the same species show a reduced frequency or absence of the inversion. Accordingly, one can picture a situation in the
newts where heterotic heterozygotes for the inversion have a greater adaptive value than the homozygotes in the new environment. An initial high incidence of heterozygotes encourages the chances of accumulation of mutations in the heteromorphic region, which should eventually make the heterozygosity requisite for development. It is worth noting here that Felsenstein (1974) did simulations of Muller's ratchet and suggested that the ratchet effect is more visible in smaller populations. There should be as much relative effect at one population size as at another, but the rate of its operation should be inversely proportional to the population size. Therefore, a population of 50 should incorporate as many unfavourable mutations in 50 generations as a population size of 100 would in 100 generations. It seems likely that the original newt population, being very small, would rapidly accumulate mutations. The mutations may be able to drift to fixation provided that homozygotes are at a strong selective disadvantage.

If the pioneer offspring had originally possessed two recessive lethal mutations, at different loci, one on each chromosome 1, then there would be no problem. But the chances of this happening would be extremely small.

The situation, so far, can be summarised as follows:

(1) An inversion in the long arm of chromosome 1 suppressed crossing-over in the structural heterozygotes.

(2) The inversion heterozygosity was fixed in a small colonising population, aided by the accumulation of
recessive lethals in the region of the inversion, in both homologues (the heteromorphic region), giving a balanced lethal system.

(3) Heterochromatinisation established the limits of the heteromorphic region, as a consequence of and accompanying accumulation of mutations.

Recessive lethal mutations can occur at random loci in any chromosome 1, in the population. However, selection will maintain reciprocal mutations at essential gene loci, such that homologues will always be heterozygous for a mutation at a particular locus. With time, all pairs of chromosomes 1 in a population will possess the same "mirrored" patterns of mutations, establishing a chromosome 1A and 1B. Any further speciation of this line should preserve the same forms of chromosomes 1A and 1B, without any changes in the patterns of mutations at essential gene loci. This is exactly what we see in *cristatus* and *marmoratus*. F hybrids require a 1A and a 1B chromosome for development, irrespective of which chromosome came from which parent. *T. marmoratus* and *T. cristatus* have retained the same genic constitution of the chromosomes 1, although the chromosomes have diverged in structure and heterochromatin content. Heterochromatinisation of the heteromorphic region is not so tightly controlled as the pattern of mutations. Heterochromatin can build up in all 'non-essential' portions of the heteromorphic region. How is heterochromatinisation mediated? Of prime consideration would be the accumulation of repetitive sequences. Smith (1973) suggested that unequal
sister chromatid exchange could result in the generation of repeated sequences, and could homogenise and fix families of tandemly repeated sequences. Highly repeated sequences are known to be scattered throughout the heteromorphic region (Macgregor, 1979; Varley et al., 1980a, b). Unequal sister chromatid exchange could account for all the heterochromatinisation seen in the heteromorphic region. Unequal crossover does not always lead to growth of a chromosomal region, as one sister chromatid will contain duplications, and the other deletions. This effect should average out, resulting in no net growth, if unequal exchanges occur at random. However, growth could conceivably occur by duplications, as any deletion in one chromatid of a homologue carrying essential sequences is selected against. Any deletion of a functional sequence may not be sheltered by the other homologue.

Genes could become inactivated or mutated by the insertion of DNA elements within regulatory or coding regions (Green, 1980; Gehring and Paro, 1980). This possibility was used to explain Y degeneration in Drosophila miranda which possesses multiple sex chromosomes (Steinemann, 1982). D. miranda is monosomic for an autosome referred to as the X chromosome. Its homologue is fused to the Y in males, forming a neo-Y chromosome. Steinemann hybridised a cRNA probe from unfractionated DNA to the chromosomes. The cRNA was believed to be representative of highly repeated sequences. The probe labelled heavily all over the neo-Y, but very little label was seen over the X. It seems that the neo-Y is
accumulating repetitive sequences that are not present to any great degree on the former homologue, \( X^2 \). The coupling of an \( X \) chromosome to the \( Y \) allows degeneration, in the "Mullerian" sense, and repetitive sequences can invade all regions of the neo-\( Y \). A similar invasion of certain repetitive sequences throughout the heteromorphic region of chromosome 1 could be envisaged, accounting for the distribution of certain highly repeated sequences (Varley et al., 1980a, b).

Spurway (1953) considers that during the Pliocene and Pleistocene, newts lived in colonies crossing many ecological barriers. At each barrier, colonising newts had the potential for speciation. The ancestral stock of newts, heteromorphic for chromosome 1, may have been split up into several populations, possibly by the ice-sheets of the Pleistocene. The new newt colonies genetically adapted to their new environments. The functional genic content of their chromosomes 1 should have been under great selective pressure to remain unaltered, although differential accumulation of heterochromatin could occur provided that it did not inactivate gene function. This may help to account for the different C-banding patterns of the present day species and subspecies.

Why does \( T. \) marmoratus have much more submetacentric chromosomes 1 than any of the \( cristatus \) subspecies? The difference is not due solely to the growth of the long arm, as can be seen in F hybrids where the short arm of
marmoratus is much shorter than its cristatus counterpart. The change in centromere position was probably caused by a pericentric inversion in one of the chromosomes 1 (Fig. 54). The other homologue may have acquired the new arrangement by a cross-over in the stretch of homologous euchromatin above the heteromorphic region and below the inversion. In this way, both chromosomes 1A and 1B acquire the marmoratus conformation. The change in centromere position accompanied but, probably did not cause speciation of the present day cristatus and marmoratus newts. A further rearrangement took place on the marmoratus chromosome 1B, taking the form of a small paracentric inversion in the long arm between the centromere and the heteromorphic region. The rearrangement shifted the pericentric C-band from near the centromere to a position close to the heteromorphic region. The effect of the paracentric rearrangement was to suppress crossing-over in the entire long arm, with the exception of the euchromatic tip (Nardi et al., 1972a; present study). Further evidence for the latter rearrangement comes from studies of lampbrush chromosomes, where the two chromosomes 1 differ with respect to the positions of a cluster of conspicuous loops (Sims et al., 1984).

As a final point, it is worth giving some indication of the age of the chromosome 1 heteromorphism. Fossil records of T. cristatus date back to about 8 million years ago, and those of T. marmoratus to about 15 million years ago (Estes and Hoffstetter, 1976; Sanchiz and Mlynarski, 1979). The lethality associated with the heteromorphism was certainly
established before the divergence of the two species, making the chromosome 1 heteromorphism a very old phenomenon, possibly dating back 20 million years.
3. THE SEX CHROMOSOMES OF TRITURUS.

3.1 INTRODUCTION

In many dioecious animals, sex is determined by the two factor mechanism of male or female heterogamety. In male heterogamety, a diploid sex chromosome constitution of XX results in a female, and XY a male. A different notation is used for female heterogamety, where ZZ individuals are male and ZW are female. In all higher vertebrates (mammals and birds), the heterogametic sex is determined by the presence of heteromorphic sex chromosomes. In the lower vertebrates (fish, amphibia and reptiles), on the other hand, there are numerous examples of cytologically indistinguishable, homomorphic sex chromosomes. In these systems, heterogamety can be determined by any of three commonly used means:

(i) Sex-reversal experiments. The phenotypic sex can be reversed from the genotypic sex by treating young stages with either temperature extreme or with hormones (estradiol). Sex reversal can also be achieved by the transplantation of embryonic gonadal ectoderm. Here, transplanting a testis primordium into a female embryo can induce the ovary primordium to develop and function as a testis. Mating of sex-reversed females (phenotypic males) with normal females, or vice-versa, can reveal heterogamety by analysis of sex ratios in the offspring. For example, if in a system with female heterogamety, a sex-reversed male (female phenotype, male genotype giving Z gametes only) was
mated with a normal male (Z gametes only), then the resultant offspring would be all males (ZZ). If male heterogamety was operating, then XY would be crossed with XY giving offspring of the constitution XX, XY and YY in a 1:3 ratio of females to males (provided that YY is viable). By this reasoning, female heterogamety has been determined for Ambystoma mexicanum (grafting - Humphrey, 1942, 1945), Pleurodeles waltlii (heat treatment - Dournon et al., 1984), and Xenopus laevis (estradiol treatment - Gallien, 1953), all of which have structurally homomorphic sex chromosomes.

(ii) Sex-linked marker genes. Segregation of a visible sex-linked marker can be used to determine the heterogametic sex: for example, sex-specific colour genes in some fish (reviewed by Ohno, 1967; 1976).

(iii) Presence of H-Y antigen. Immunological studies in vertebrates have revealed the presence of a cell surface antigen specific to the heterogametic sex (Wachtel and Ohno, 1979). The antigen is commonly referred to as the H-Y antigen as it was originally found in mice and other mammals with male heterogamety (Eichwald and Silmser, 1955; Wachtel et al., 1974). Females of ZW/ZZ systems are also H-Y (or more correctly, H-W) positive (Zaborski et al., 1979). Cross-reaction experiments have shown the H-Y antigen to be well conserved in vertebrates, and can be used as a tool for determining heterogamety in species with homomorphic sex chromosomes.
It is now widely accepted that the original state of the sex chromosomes was that of a homomorphic pair, as heterogamety can be distinguished in the absence of any chromosomal heteromorphism. Evolution of the homomorphic pair tends to progress towards extreme heteromorphism. Cases of extreme X-Y or Z-W heteromorphism show several characteristic features. The X (Z) chromosome generally resembles that of an autosome in terms of gene content and staining properties. The Y (W) chromosome, on the other hand, is often much smaller and is largely heterochromatic. The Y (W) is not totally degenerate, but lacks most if not all X-linked genes. The physical differences between X and Y, and the linkage of genes with the sex factors, are maintained by the suppression of recombination between the two chromosomes. The regions of the X and Y which do not recombine are known as the "differential" segments, as opposed to the "common" or "homologous" segments which can freely recombine and, therefore, show no sex-linkage.

How do homomorphic sex chromosomes evolve into a highly differentiated heteromorphic state? There seems to be no doubt that isolation of opposing sex factors, on part or parts of the originally homologous sex chromosomes, is essential for any differentiation to occur. Ohno (1967) pointed out that if sex was determined by a pair of alleles at a single locus, then there would be no need for isolation between the X and the Y. Free recombination between a dominant male-determining gene on the Y, with a recessive female-determining gene on the X, would neither upset the
sex determining mechanism nor disturb the 1:1 sex ratio. Isolation of a long segment on the sex chromosomes is more meaningful in that it will allow a number of opposing sex factors to accumulate, and will facilitate differentiation of the homologous elements. Discussions of sex chromosome differentiation revolve around two major hypotheses accounting for isolation of opposing sex factors and the evolution of heteromorphism:

(1) Model involving structural rearrangements. Ohno (1967) emphasised the importance of structural rearrangements in helping to suppress crossing-over. A structural rearrangement, such as a pericentric inversion, need not itself suppress recombination, but would help reinforce any isolating mechanism already operating to keep sex factors opposed. A pericentric inversion would mark the initial step in differentiation of the homologous X and Y, which would not differ in size but in centromere position. Further heteromorphic differentiation would follow, such as heterochromatinisation of the Y. Structural rearrangements could cause recombination suppression, but initial isolation may also be related to the gonadal environment affecting chiasma frequency between X (Z) and Y(W) (Yamamoto, 1961), or to sex-specific gene effects like suppressor genes in the Y (W) (Bowen, 1965; Darlington, 1958).

(2) Model of accumulation of sex-specific satellite DNA. Recombination between the sex chromosomes may be suppressed by heterochromatinisation of the Y (W) through accumulation
of sex-specific satellite DNA sequences (Singh et al., 1976, 1980; Ray-Chaudhuri et al., 1971). This hypothesis suggests that the first cytological differentiation of the sex chromosomes is the appearance of heterochromatin. Satellite sequences arise on the Y (W) by a saltatory replication event and spread through the chromosome by numerous small rearrangements. Structural differentiation, by pericentric inversions, would be secondary to the accumulation of sex-specific satellite DNA. A failing of this hypothesis is that cross-over suppression is invoked by the differential accumulation of heterochromatin. Heterochromatin may indeed reduce recombination (John and Miklos, 1979), but for highly repeated sequences to accumulate preferentially on the Y (W) recombination must have already been suppressed in the region of accumulation.

Both models fail to explain adequately the initial isolation of sex factors, although, pericentric inversion could suppress crossing-over in some cases. Nevertheless, both hypotheses indicate different routes to the same end: heteromorphic sex chromosomes, with a heterochromatic, largely genetically inert Y (W).

The lower vertebrates are littered with examples of sex chromosomes at all stages of evolutionary differentiation. The higher vertebrates have attained an advanced state of sex chromosome evolution. The lower vertebrates, therefore, provide essential clues to the mechanism of sex chromosome differentiation. Both XY/XX and ZW/ZZ systems are found in
all lower vertebrate orders, suggesting a polyphyletic origin of sex chromosomes within these orders. The following summary relates examples supporting either chromosomal rearrangement or heterochromatinisation as the initial event in differentiation of homologous sex elements. Ambiguous situations are also mentioned.

Evidence for pericentric inversion model.

One race of *Phyllodactylus marmoratus* (Gekkonidae) shows a heteromorphism of Z and W chromosomes in centromere position. The Z and W are equal in length and no heterochromatinisation of the W is evident. G-banding reveals that a pericentric inversion has taken place in the W chromosome (King and Rofe, 1976).

The Z and W chromosomes of *Acrantophis dumereli* (Boidae) are of similar size but, have different centromere positions. G-banding suggests that a pericentric inversion has taken place in the W chromosome (Mengden and Stock, 1980).

Studies with conventionally stained karyotypes have shown various stages of sex chromosome evolution in the snake family, Colubridae. Some species have a sex heteromorphism for centromere position only e.g. *Drymarchon conais cauperi*. Pericentric inversion was determined as the origin of the differentiation of sex chromosomes in the Colubridae (Becak et al., 1964; Becak and Becak, 1969). However, banding studies were not carried out at this time to determine any
degree of heterochromatinisation.

The sex chromosomes of some species populations show a high propensity for structural rearrangement. Rearrangements have been shown to be the primary cause of differentiation of the sex chromosomes in the plethodontid salamander *Aneides ferreus* (Kezer and Sessions, 1979) and in the gekkonid lizard, *Gehyra purpurascens* (Moritz, 1984) without any changes or accumulation of C-band heterochromatin. However, there is no evidence to suggest that the rearrangements confer the initial isolation of the sex factors.

**Evidence for sex-specific satellite DNA model.**

Ray-Chaudhuri et al. (1971) invoked heterochromatinisation as the first step towards heteromorphic sex chromosomes in their study of snakes. The elapsid snake *Naja naja naja*, has structurally homomorphic sex chromosomes yet, the W is heterochromatic and forms a W-chromatin body in interphase nuclei. Subsequent work by Singh et al. (1976) involved isolating a satellite DNA from females of a colubrid snake *Elaphe radiata*. The satellite (sat III) was referred to as being sex-specific as it was found in a much greater quantity in females than in males. Another satellite (sat IV) was found exclusively in female DNA, but in too small a quantity to purify. Sat III cRNA was hybridised to chromosomes of snake species showing different stages of sex chromosome evolution.
Members of the primitive boid family, *Python reticulatus* and *Xenopeltis unicolor*, have morphologically identical sex chromosomes, with no evidence of heterochromatinisation from C-banding. Sat III cRNA showed no specific labelling to any chromosome in these snakes. However, the sex chromosomes of *Ptyas mucosus* (colubrid snake) are morphologically homomorphic but, the W shows slight C-staining. In this case, sat III cRNA was found to preferentially label the W chromosome. *Natrix piscator* (Colubridae), *Bungarus caeruleus* and *B. fasciatus* (Elapidae) show highly differentiated, morphologically heteromorphic sex chromosomes, with a C-positive W chromosome. Labelling of sat III cRNA was exclusively on the W chromosomes. Taken together, Singh et al. (1976, 1980) suggest that the evidence points to accumulation of sex-specific highly repeated sequences on the original W chromosome by saltatory replication. Spread of the sequences throughout the W chromosome by many minor structural rearrangements was believed to facilitate further isolation of the Z and W. Structural rearrangements and degeneration would then follow, being secondary to satellite DNA accumulation.

Other cases have been found in support of the sex satellite DNA hypothesis. Some structurally heteromorphic sex chromosomes are known in the Amphibia. C-banding has distinguished heteromorphisms in otherwise structurally homomorphic pairs. The Indian frog, *Rana tigrina*, has homomorphic sex chromosomes but, C-banding shows that the W is almost completely heterochromatic (Chakrabarti et al.,
1983). *R. tigrina* therefore, supports the spread of heterochromation as the initial differentiating factor of sex chromosomes. Schmid et al. (1979) have C-banded the structurally homomorphic sex chromosomes of some European newts, *Triturus a. alpestris*. *T. h. helveticus* and *T. v. vulgaris*. *T. alpestris* and *T. vulgaris* show a heteromorphism for a terminal C-band on the long arm of a metacentric pair, in males only. *T. helveticus* shows no terminal C-banding in either of its sex chromosomes. In male meiosis, the X-Y bivalent of all three species has a much reduced chiasma frequency in the long arm. Schmid et al. (1979) take the results to support the view that heterochromatinisation of the tip of the long arm of the Y, in *T. alpestris* and *T. vulgaris*, represents the beginning of sex chromosome differentiation in *Triturus*. The general occurrence of chiasmata in interstitial regions in male meiosis of urodeles, was inferred to facilitate telomeric C-band heteromorphisms. A cytologically undetectable structural change in *T. h. helveticus*, with no Y-associated C-heterochromatin, was suggested to account for the reduction in chiasma frequency of the long arm. Schmid and colleagues also suggested that sex specific satellite DNA sequences may already be present, but undetectable in the Y chromosome. As these sequences accumulate, they may further aid recombination suppression and result in heterochromatinisation.

In further support of the satellite DNA model, Ray-Chaudhuri et al. (1971), and Schempp and Schmid (1981) have found
morphologically homomorphic sex chromosomes to differ by asynchronies in DNA replication. The X and Y chromosomes of Rana esculenta can only be differentiated by a short late replicating segment in the long arm of the Y (Schempp and Schmid, 1981). Ray-Chaudhuri et al. (1971) noted late replication in the W chromosome of some snake species. These findings were interpreted as the differential accumulation of sex-specific sequences in the late replicating segment of the Y (W). The late replicating segment would hinder pairing and crossing-over at zygotene, therefore isolating this region of the sex chromosomes. Late replication would, thus, mark the early stages of heterochromatinisation. However, the asynchronous replication was detected in 'mitotic' chromosomes and may not be applicable to meiotic chromosomes, where there is no evidence for late replication affecting pairing or recombination. Heterochromatin with highly repeated DNA sequences mostly shows the property of late replication. Also, heterochromatin is known to show a reduced crossing-over frequency (John and Miklos, 1979). Until highly repeated sequences or C-staining are positively identified with a late replicating segment, no correlation should be made between asynchronous replication and meiotic isolation.

Ambiguous evidence.

There are examples from which it is difficult to determine whether structural changes or heterochromatinisation are primary differentiation events in sex chromosome evolution.
The colubrid snake *Coluber fasciolus* has Z and W chromosomes of similar size, differing by a pericentric inversion in the W. The W chromosome has also undergone heterochromatinisation. Ray-Chaudhuri et al. (1971) suggested that the inversion followed heterochromatinisation, since other unrelated snakes with morphologically homomorphic sex pairs show only heterochromatinisation of the W. Obviously, there is no way of knowing which event came first. Either pericentric inversion or heterochromatinisation could have differentiated the Z and W, but isolation may have preceded and therefore facilitated the occurrence of these events.

The closely related ranid frogs *Pyxicephalus adspersus* and *Tomopterna delalandii* (=*Pyxicephalus delalandii*) show sex chromosomes at different stages of evolution. *T. delalandii* has sex chromosomes of similar size, differing slightly in centromere position. The W chromosome shows some evidence of heterochromatinisation by C-staining. *Pyxicephalus adspersus*, on the other hand, shows highly heteromorphic sex chromosomes with a much reduced size and a considerable amount of heterochromatin in the W chromosome. Schmid (1980b) described the evolutionary transition to have involved initial accumulation of heterochromatin followed by secondary structural changes. The evidence could also favour the reverse sequence of events.

The X and Y chromosomes of the new world teiid lizard, *Cnemidophorus tigris*, are of equal length but, differ by a
small pericentric inversion of the Y (Bull, 1978b; Cole et al., 1969). The medial segments of the X and Y, in the region of the inversion, do not cross-over in male meiosis. This example is interesting in that the cross-over free region of the Y chromosome shows less C-band heterochromatin than the X (Bull, 1978b). It appears that 'deheterochromatinisation' has followed (or preceded) pericentric inversion in the Y chromosome. Alternatively, some degree of heterochromatinisation has taken place in the X chromosome of this species.

The marsupial frog, Gastrotheca riobambae, is unusual in that heterochromatinisation of the Y chromosome has been accompanied by an increase in size. The Y chromosome is about 1.5 times longer than the X, and is almost completely heterochromatic (Schmid et al., 1983a, b). Unequal mitotic sister chromatid exchanges can result in heterochromatin changes or polymorphisms (Kurnit, 1979). Unequal exchanges can account for the generation of highly repeated sequences and duplication or deletion of blocks of heterochromatin. Alternatively, the greater size of the Y could be attributed to heterochromatinisation of an aneuploid autosomal translocation(s) to the Y.

What conclusions can be drawn from the presented evidence, on sex chromosome differentiation?

Probably the most overriding conclusion is that there is no one mechanism of sex chromosome differentiation. However,
one must bear in mind that certain rules, or more precisely 'trends', can be followed. All mechanisms rely on isolation of opposing sex factors as the starting point. The build-up of sex-specific satellite DNA sequences cannot be causal in the initial isolation of X (Z) and Y (W) segments. The presence of satellite sequences on the Y would help in suppression of pairing or at least cross-over, but in order to accumulate preferentially on the Y, the differential segments would have to be already isolated. Any recombination between the differential segments would pass the 'Y-specific' sequences and sex-factors to the X and render the segments homologous.

How can the differential segments be isolated?

(a) Sex-linkage of colour genes is disrupted in sex-reversed males of the cyprinodont fish *Oryzias latipes*. Recombination between X and Y is increased five-fold when in an ovarian environment (Yamamoto, 1961). Isolation here is not so much a property of the X and Y themselves, but is dependent on the gonadal environment housing the sex chromosomes.

(b) Interracial crosses of the brine shrimp, *Artemia salina*, have shown that different lines of W chromosomes exhibit different frequencies of crossing-over with the Z. The differences in effective isolation between the Z and W may be due to either inversions, deletions, or suppressor genes in the W chromosome (Bowen, 1965).

(c) Structural rearrangements, be they cytologically obvious pericentric or cryptic paracentric inversions may, in some
cases, effectively isolate part of the sex chromosomes. Some rearrangements may be deleterious as any cross-over between an inverted segment may give rise to duplications and deletions, resulting in aneuploid gametes (White, 1973), and would be under the scrutiny of natural selection.

(d) Chiasma localisation may effectively keep differential segments isolated while sex factors are accumulating in an opposed fashion. Any change in the Y to isolate permanently the X and Y would be of selective advantage in dioecious animals, fully establishing the pair on the road to differentiation. For example, chiasma may show terminal localisation in the potentially heterogametic sex. If sex factors were medially located, then they would be kept separate at meiosis. Separation of the medial region of the homologous pair would also allow highly repeated sequences to establish in this region on the Y (W), which would further facilitate isolation and mark the first stages of differentiation. Alternatively, a pericentric inversion may occur while sex factors are isolated by chiasma localisation, further reinforcing the separation.

By virtue of sex factor isolation, differentiation can proceed via structural rearrangement and heterochromatinisation, in any order. Both reinforce the isolation. Loss of gene function from the Y by mutation and heterochromatinisation make it prone to degeneration, in most cases of highly evolved sex chromosomes (e.g. mammalian Y and bird W). Degeneration, probably by unequal sister chromatid exchange deletion is the general trend, but not always the rule.
The functional significance of sex-specific heterochromatin is not fully understood. One would expect saltatory events to be to some degree random in the Y or W, i.e. 'amplification' of no one particular sequence in all cases. Singh et al. (1980) isolated another sex-specific minor satellite (BKm) from the banded krait snake, Bungarus fasciatus. BKm cRNA was shown to hybridise preferentially to the W chromosome of a variety of snakes with differentiated sex chromosomes. BKm sequences were shown to be conserved throughout the different snake families. Furthermore, components of the BKm satellite DNA were found to be conserved throughout eukaryotes, as cRNA hybridised to total DNA of bird, mouse, man and Drosophila (Singh et al., 1981). This suggested a functional importance of certain BKm sequences in sex determination. However, the conserved BKm sequences were not shown to be restricted in location to the sex chromosomes in these cases. Nevertheless, a functional significance was advocated as restricting BKm sequence divergence, on the basis of evolutionary persistence (Singh et al., 1981). On the contrary, Tone et al. (1982) arrive at a different conclusion from studies of W-specific repetitive DNA in Gallus domesticus. The authors show that the common finding of a heterochromatic W chromosome in birds is not paralleled by a largely homologous repetitive DNA component. The repetitive DNA family may be considerably variable in sequence composition. It was suggested that size, organisation and numbers of a repeating component were more important than sequence itself, in heterochromatinisation.
and therefore, maintenance of a sex-determining system. Further studies by Tone et al. (1984) indicate that there may be two types of repetitive sequences associated with highly evolved sex chromosomes. One being sequences that are relatively enriched on the sex chromosome, but found over a wide range of different taxa in vertebrates and invertebrates (e.g. Bkm sequences). The second type of sequences are highly concentrated on the sex chromosome and restricted to a limited taxonomic group. Such sequences have been isolated by restriction endonuclease digestion of female domestic fowl DNA (Tone et al., 1982, 1984). The restriction fragments also show evidence of extensive methylation in the female genome (Tone et al., 1984).

The present study aims to extend the work of Schmid et al. (1979), on the sex chromosomes of Triturus. Several species were examined in order to confirm a monophyletic origin of sex-determination in Triturus, and to help clarify the mode of differentiation of the sex chromosomes.
3.2 RESULTS

Mitotic sex chromosomes were examined in the following species/subspecies:

T. alpestris apuanus *
T. boscai
T. cristatus cristatus *
T. c. carinifex *
T. c. karelinii
T. helveticus helveticus
T. marmoratus marmoratus *
T. vittatus ophryticus
T. vulgaris vulgaris *

Meiotic sex bivalents were observed in individuals from the subspecies marked with an asterisk. Unfortunately, T. italicus and T. montandoni were not available for this study.

The sex chromosomes of Triturus are large to medium-sized elements, being chromosomes number 4 or 5 in the set. T. vittatus ophryticus appears to have submetacentric sex chromosomes, whereas all other species have a metacentric pair. Sex heteromorphisms, where seen, only involve the long arm. The heteromorphism is one of differential heterochromatinisation between the X and the Y chromosomes. The degree of heterochromatinisation varies between subspecies, in some cases involving the X as well as the Y.
3.2.1 Mitotic Sex Chromosomes.

The short arms of the sex chromosomes are considered to be entirely homologous, as judged by C-banding and studies of chiasma distribution in meiosis (see next sub-section). The short arms are largely euchromatic with few sites of heterochromatin (Fig. 55). The pericentric heterochromatin may vary between subspecies, between the X and the Y, and between the long and the short arms. Leaving aside the pericentric heterochromatin, the following are descriptions of the short and long arms:

**Short arms.**

1. *T. v. vulgaris* (chromosome 4). The short arm is entirely euchromatic, although occasionally, the distal tip may show very slight heterochromatinisation. The telomeric C-heterochromatin stains only slightly more strongly than euchromatin, appearing as a 'diffuse' grey tip.

2. *T. h. helveticus* (chromosome 5). The sex chromosomes are near metacentric elements. The only site of heterochromatin is a diffuse, grey terminal to subterminal C-band. A very small grey C-band may be seen near the pericentric heterochromatin.

3. *T. boscari* (chromosome 4). The short arms bear diffuse terminal C-heterochromatin. The diffuse heterochromatin does not stain as intensely as pericentric C-bands. Again, a small but distinct, grey intercalary C-band can be seen in a position near the pericentric heterochromatin.
(4) *T. alpestris apuanus* (chromosome 4). Diffuse, grey, C-staining telomeres are seen in the short arms. A more compact, though lightly staining C-band can be seen in an intercalary position. The intercalary band is mid-way along the chromosome arm.

(5) *T. marmoratus*, *T. cristatus cristatus*, *T. c. carnifex* and *T. c. karelinii* (chromosomes 4). All of these subspecies show the same pattern of C-staining in the short arm. The only site of heterochromatin is a small, distinct, grey C-band in a median position along the short arm. There is no evidence of diffuse heterochromatin at the telomeres.

**Long arms.**

The long arms show different degrees of heterochromatinisation, which fall into three general classes (A, B and C) based on the pattern of C-banding of the tips of the X and Y chromosomes. The three classes represent an evolutionary progression from minimal to 'substantial' heterochromatinisation.

(A) *T. h. helveticus*. The long arms were originally described as having no C-heterochromatin in either the X or Y (Schmid et al., 1979). The present study shows that in some individuals there is slight heterochromatinisation of the telomeres in both X and Y. The heterochromatin is diffuse, grey, C-staining material (as in the short arms), which is not always easily resolvable. The visualisation of lightly staining heterochromatin is largely dependent on the C-banding technique and the condensation of the chromosomes. When visible, the terminal C-heterochromatin is seen to be
present in roughly equal amounts on the X and Y.

(B) This class represents the first stage of differentiation of the long arms of the sex chromosomes. The class can be subdivided into two groups depending on the amount of Y-heterochromatin present, (i) one block and (ii) two blocks of heterochromatin.

(i) *T. v. vulgaris*. The Y chromosome has a deeply staining terminal block of heterochromatin. The X, on the other hand, shows either no terminal heterochromatin or just faint, diffuse heterochromatin. The rest of the long arms of the X and Y are euchromatic.

*T. boscai*. Like *T. vulgaris*, this species bears a terminal block of C-heterochromatin on the Y chromosome. The X chromosome shows no evidence of heterochromatinisation at the telomere. However, both the X and the Y have a small, distinct, grey C-band in a median position along the long arm. Thus, the only difference between these chromosomes is the heteromorphism for the terminal C-band.

(ii) *T. alpestris apuanus*. The Y chromosome has a large deeply staining mass of heterochromatin at the telomere. More elongated chromosomes show the terminal heterochromatin to be made up of two adjacent blocks. The X chromosome bears only diffuse grey heterochromatin at its tip. The euchromatin of the rest of the long arm bears one or two small lightly staining bands in intercalary positions. The positions of these bands on the X and Y could not be determined accurately due to
different degrees of chromosome contraction.

(C) This class represents the more evolutionarily advanced stage in that it shows the greatest degree of heterochromatinisation. In all cases here, the X chromosome has distinct strongly staining heterochromatin in a terminal or subterminal position. This class can be further subdivided into two groups based on the degree of heterochromatinisation of the X and the Y.

(i) *T. cristatus cristatus*, *T. c. carnifex* and *T. c. karelinii*. These subspecies usually show one terminal heterochromatic block on the X and two on the Y. Some individuals of *cristatus* and *carnifex* may possess two small terminal blocks on the X chromosome. The 'Y-heterochromatin' appears as a long tract of C-staining material. However, variation in staining intensity within the tract gives the impression of two adjacent blocks. This is apparent in *T. c. karelinii* where the extreme limits of the heterochromatic tract stain more intensely than the middle region. The heterochromatic block of the X chromosome is terminal in *T. c. cristatus* and *T. c. carnifex*, but is slightly subterminal in *T. c karelinii* where the extreme tip is euchromatic. A very fine, grey C-band is often discernible about a third of the way along the long arm, from the centromere, in all of the subspecies. The band is present on both the X and the Y.

(ii) *T. marmoratus* has a substantial tract of heterochromatin at the tips of both the X and Y
chromosomes. The Y-heterochromatin appears as three distinct blocks. The most terminal two blocks are stained very intensely. The proximal block is not as large, slightly less intensely stained and is separated from the other two blocks by euchromatin. The X chromosome has two terminal C-bands, smaller than those on the Y. The distal band stains more intensely than the proximal band. The bands are separated by a short stretch of euchromatin. As with the cristatus subspecies, there is a small grey C-band about a third of the way along both the X and the Y, from the centromere.

Measurements of the long arms of the sex chromosomes show no statistically significant difference between the X and the Y. Measurements suffer from variation due to differential chromosome contraction, stretching during preparation and margin of error of the method of measurement. Thus, it cannot be accurately determined whether heterochromatinisation of the Y has involved an increase in length of the long arm. The heterochromatinisation may be the result of (a) transition of existing euchromatin into heterochromatin, (b) addition of 'supernumary heterochromatin', or (c) expansion or duplication of heterochromatin after an initial euchromatin/heterochromatin transition event. Both (b) and (c) would be expected to increase the length of the long arm.

The terminal heterochromatin of the X and Y chromosomes in
T. marmoratus and T. cristatus is distinctly Giemsa C-positive; although slight differences in staining intensities suggest a heterogeneous composition of the heterochromatin. Fluorochrome staining does not highlight the sex chromosome heterochromatin as much as C-banding. For the most part, the Y-heterochromatin fluoresces only slightly less intensely than euchromatin with actinomycin D/DAPI. With mithramycin dye pairs, the Y-heterochromatin fluoresces slightly more brightly than euchromatin. The difference is often only resolvable down the microscope, as the fluorescence fades quickly. The fluorochrome staining shows that much of the Y-heterochromatin has a slight bias towards GC-richness. However, actinomycin D/DAPI staining reveals an additional AT-rich component within both the X and the Y-heterochromatin.

Heterochromatic blocks can be seen in C-banded and fluorochrome stained somatic interphase nuclei. A range of different sized blocks are clearly visible. In some cases, larger blocks represent fusions of smaller blocks. However, in no case can any C-heterochromatin be positively identified as the Y-heterochromatin.

T. vittatus ophryticus must be considered separately as there is, at present, no strong evidence that the C-band heteromorphism is in fact sex-related. Chromosomes 5 in the two males showed a terminal heteromorphism of C-banding in the long arm. However, neither meiotic material nor female tissues were available to confirm that the heteromorphism is
sex-specific and associated with a reduced chiasma frequency. The heteromorphism may just be indicative of a chromosomal polymorphism. If the heteromorphism does represent male heterogamety, then it is unusual in that these sex chromosomes are the only pair in *Triturus* that are distinctly submetacentric. One homologue (putative Y) has a deeply staining terminal block separated from a smaller subterminal block by a short stretch of euchromatin. The other homologue (putative X) possesses only a small subterminal C-band, with a diffuse heterochromatic telomere. Interestingly, the interstitial euchromatin of the long arm possesses several small C-bands along its length, differing in staining intensity and position between the two homologues, which suggest a lack of structural homology.

3.2.2 Meiotic Sex Bivalents.

The positions of chiasmata in a chromosome set is not constant for all species of *Triturus*. Chiasma distribution also varies between the sexes. Table 5 summarises the chiasma distributions for meiotic bivalents in *Triturus*.

Meiotic material was found in some of the subspecies, enabling recombination of the sex bivalents to be observed. In all the subspecies examined, there were no obvious signs of asynapsis of a euchromatic segment at zygotene. The bouquet arrangement of zygotene/pachytene nuclei may easily
obscure any failure in pairing of the sex bivalents, as many
synapsed chromosome arms overlap. Asynapsis in heterochroma-
tic regions is more readily identifiable because of the
stronger C-staining, as in the case of the heteromorphic
region of chromosome 1 in *T. cristatus* (section 2.3.4). From
the present study, it cannot be positively said that part or
parts of the sex bivalents do not undergo meiotic pairing.

As a general rule, *T. alpestris*, *T. cristatus* and *T.
marmoratus* show no localisation of chiasmata in male
meiosis (Table 5). Chiasmata can be seen in distal,
procentric and interstitial chromosomal regions (Figs. 24,
25, 26 & 56). Male meiosis of *T. vulgaris* is characterised
by distal localisation of chiasmata (Fig. 57). These factors
should be taken into account when examining chiasma
distribution in the sex bivalents. Typical sex bivalent
conformations at diplotene/metaphase I are shown in Fig.
55. The sex bivalents are easily identified within a set
because of the C-band heteromorphism of the long arms.

*T. alpestris apuanus*. One, or more usually two chiasmata are
seen in the short arm of the sex bivalent, in any position.
At least one chiasma frequently occurs between the long
arms. Chiasma formation can extend well into the long arms,
happening in any position from the procentric region to the
subterminal region. No chiasmata or associations were seen
between the heteromorphic tips of the long arms.

*T. marmoratus, T. c. cristatus* and *T. c. carnifex*. One or
two chiasmata may be seen between the short arms of the sex
bivalent. The chiasmata are most often in terminal or near-centric position, occasionally interstitial, which is typical for the larger bivalents of these subspecies (Watson and Callan, 1963; present study). The heteromorphic long arms may show one or no associations. The association, if present, is always between the terminal heterochromatin of the X and the Y half-bivalents. It is difficult to determine the exact nature of the association. The occurrence of chiasmata between heterochromatin has not been recorded (John, 1976). If the association were to be a true chiasma between euchromatin, then the cross-over would have to be proximal to the heterochromatic blocks of the X and Y. In T. marmoratus, a chiasma could occur between the euchromatin which separates the proximal C-band from the distal band(s) on the X and Y. The chiasma may even be proximal to all of the C-bands. In either case, metaphase I bivalents should show the subterminal association clearly, despite the great degree of chromatin contraction. Many associations do, in fact, appear to be terminal or sometimes 'lateral'. The lateral heterochromatic associations may indicate a subterminal chiasma. There is no strong evidence for terminalisation of chiasmata in newts (Spurway and Callan, 1960), so it is unlikely that terminal associations are subterminal chiasma that have slipped to the end of the bivalent arm. If subterminal chiasmata do occur, then one would expect to find metaphase II sex chromosomes with different amounts of terminal heterochromatin between the two sister chromatids of the long arm. Very few metaphase II chromosomes were actually observed, probably because the
second meiotic division occurs rapidly after the first (Callan and Taylor, 1968). Of those seen, the separated half-bivalents showed no quantitative difference between terminal heterochromatin of the sister chromatids. However, in one anaphase I spread, where half-bivalents have just separated, the sex bivalents have one chromatid with a large block of telomeric heterochromatin and one chromatid with a small block (Fig. 58). Clearly a subterminal chiasma has formed. So, interestingly, there are two types of association between the long arms of the sex chromosomes. One being terminal associations which are probably the result of attraction between heterochromatic segments. The second association is that of a chiasmate exchange most probably between the euchromatin just below the terminal heterochromatin. There is no evidence for crossing-over in the majority of the length of euchromatin of the long arm.

*T. vulgaris.* Terminal localisation of chiasmata is characteristic of male meiosis, in this species (Callan and Perry, 1977; present study). Only one chiasma was ever seen in the sex bivalents. The chiasma was always in a distal position on the short arm. The long arm, exhibiting the C-band heteromorphism, was always achiasmate.

To summarise, chiasmata occur at typical positions for a particular subspecies and at a typical frequency in the short arm of the sex bivalents. However, there is a tendency for part or, in some cases, all of the euchromatin of the long arm to remain achiasmate, in male meiosis.
3.2.3 Satellite DNA.

Some attempt was made to demonstrate a sex-specific satellite component from T. a. alpestris total DNA, which may be correlated with the Y-heterochromatin. T. a. alpestris was used as it shows telomeric heterochromatin in the Y chromosome only, and therefore any specific satellite associated with this chromosome would be present in males only. Total DNA from males and females were run on a neutral caesium chloride gradient. No sex-specific satellite peaks were evident. Fluorochrome staining of the chromosomes suggests that most of the Y-heterochromatin is only slightly more GC-rich than euchromatin. Thus, if a male specific satellite were present, then it may be cryptic, hidden in the main band peak. Digestion of total DNA with several restriction endonucleases also showed no sex differences.
3.3 DISCUSSION

The C-band heteromorphisms combined with a degree of consistent recombination suppression in male individuals cytologically confirm an XY/XX sex-determining system in *Triturus*. Male heterogamety has been demonstrated in *T. vulgaris* where male cells are positive for the H-Y antigen (Engel and Schmid, 1981). *T. italicus* and *T. montandoni* were not used in the present study. However, in *T. italicus* a heteromorphism for a terminal C-band in the long arm of chromosome 2 has been noted in males only (Nardi et al., 1973; Mancino et al., 1977). In spermatogenesis, the long arm of bivalent 2 remains achiasmatic (Mancino et al., 1977). No sex related C-band heteromorphism has been found in *T. montandoni* (Ragghianti et al., 1978). Hybridological and cytological studies show that *T. montandoni* is closely allied to, and completely sympatric with *T. vulgaris* (Ragghianti et al., 1978; Spurway, 1953). It would seem probable that *T. montandoni* shares male heterogamety with the rest of *Triturus*. So, all of *Triturus* may have the same type of sex determining system; but is there any evidence for a monophyletic origin of the sex chromosomes?

In all species the sex chromosomes are a large to mediumsized, metacentric pair, with the exception of *T. vittatus*, where the potential sex pair are submetacentric. Discussion of *vittatus* sex chromosomes should be treated with
reservation until stronger evidence confirms that chromosomes are the sex elements. There certainly seems to be evidence for a common origin of the sex chromosomes within the "cristatus" group. *T. marmoratus* and the *cristatus* subspecies all possess similar sex chromosomes in that all the short arms bear a small C-band in a median position, and all long arms have a similar type of heterochromatic heteromorphism (class C - see results). *T. alpestris apuanus* also has this type of short arm, with a C-band mid-way along its length. *T. helveticus* and *T. boscai* have a small interstitial C-band near the pericentric region of the short arms. *T. vulgaris* has no demonstrable interstitial, short arm C-bands.

The trend towards increasing heterochromatinisation of the tips of the long arms together with a degree of recombination suppression suggests that all *Triturus* sex chromosomes are evolving in the same way. It is not certain that the same ancestral chromosome pair is involved in all species, though it would seem likely.

If all the present day sex chromosomes are derived from the same ancestral pair, then in the ancestral *Triturus* line, the sex factors would have had to be at least isolated in a chromosomal region in the long arm of the X and Y. Subsequent speciation would have carried the same isolated region into all the present day species. Most of the *Triturus* species were probably distinct about 20 million years ago, based on fossil evidence (Hecht and Hoffstetter,
1962). Therefore, the initial separation of the sex factors would have been very ancient.

The model of Singh et al. (1976) and Ray-Chaudhuri et al. (1971) proposes that heterochromatinisation through the accumulation of sex specific satellite sequences was the first stage of sex chromosome differentiation. The model of Ohno (1967) stresses the importance of pericentric inversions, the rearrangement either causing isolation of sex factors or maintaining isolation once established. The situation in Triturus appears to follow the 'heterochromatinisation' model. There is a trend from sex chromosomes with no or minimal heterochromatinisation to those with significant accumulation of Y-heterochromatin. The situation is complicated by the tendency for the X to accumulate heterochromatin as well as the Y. The model of Singh et al. (1976) suggests that the build-up of satellite sequences specific to the Y (or W) will suppress crossing-over, therefore isolating the sex bivalents (or parts of them). Many minor structural inversions are proposed to spread the satellite DNA throughout the Y chromosome, thereby reinforcing meiotic isolation of the differential segments. However, unless the sex bivalents were already isolated (at least in the region of the differential segments) then, it is difficult to foresee an accumulation of satellite sequences unique to the Y chromosome. Free recombination would prevent sex factors from building-up in an opposed fashion and any accumulation of satellite DNA would be passed to both X and Y.
The original isolation of the differential segments may have been a paracentric inversion which would not be detected by general cytological methods. High resolution studies of lampbrush chromosomes can be used to reveal evidence of inversions (Hartley and Callan, 1977). The antiquity of the sex determining system has probably resulted in morphological and transcriptional divergence of the differential segments, thereby obscuring any possible evidence of a structural change at the lampbrush level. For example, a small differential segment has been found in the otherwise homologous Z and W lampbrush chromosomes of *Pleurodeles poireti* and *P. waltlii* (Lacroix, 1968a, b, 1970). However, the differential segments showed no homologies in chromomeric pattern or marker loop distribution and therefore provided no clues as to possible structural rearrangements.

Isolation enabling accumulation of "Y-DNA/heterochromatin" may be a consequence of chiasma distribution. Certainly, the terminal localisation of chiasmata in male meiosis of *T. vulgaris* and *T. helveticus* (Spurway and Callan, 1960; Watson and Callan, 1963) would keep the long arms of the sex chromosomes largely isolated. The isolation in male meiosis would favour the opposition of sex factors, sex-linkage of genes with opposing effects in the two sexes (Fisher, 1931), and the build-up of Y-heterochromatin. But chiasmata are not localised in *T. alpestris*, *T. cristatus* and *T. marmoratus*. So, speciation in *Triturus* has been accompanied by different mechanisms of chiasma distribution. If sex factor isolation
did occur in the ancestral *Triturus* line, then terminal chiasma localisation would have helped in the process. One cannot exclude other genetic mechanisms in suppressing recombination, as originally pointed out by Darlington (1937).

What mechanisms are involved in the accumulation of heterochromatin? The association of satellite DNA with constitutive heterochromatin is well established correlation (Pardue and Gall, 1970; Yunis and Yasmineh, 1971). Several models have been proposed for the evolution of satellite DNA. Satellite DNA is composed of sequences that may have long-range and/or short-range periodicities. The mechanisms involved in producing long-range repeats and short-range (or simple sequence) repeats may be the same. Britten and Kohne (1968) propose that certain sequences are replicated many times and are integrated into the genome. Their general model suggests no mechanisms to explain the sequence replication and it only accounts for an increase in a particular sequence (or sequence family). Models accounting for expansion of a sequence include the "rolling circle" model (Hourcade et al., 1973), where a sequence is excised from the genome, extrachromosomally replicated and finally reintegrated at a certain point in the genome. This model could account for long-range repetitions of a sequence, but it lacks evidence and only accounts for an increase in repeat number. C-band heterochromatin and satellite DNA have often been shown to vary. The variation includes deletion as well as expansion of heterochromatin.
Most models accounting for an increase or decrease of satellite sequences revolve around a mechanism of unequal cross-over, originally developed by Smith (1973). An unequal sister chromatid exchange (SCE) will result in one chromatid having lost a particular sequence while the other will have duplicated the sequence. Repeated unequal exchanges will give rise to multiplication and homogenisation of a family of repeated sequences. The very nature of an unequal exchange can account for both expansion and deletion of satellite sequences. For an unequal exchange to have any evolutionary significance, they must occur in the germ-line. Recombination rarely occurs in heterochromatin (John, 1976), making unequal mitotic rather than meiotic exchanges the most likely candidates for the model. Most of the evidence favours the unequal mitotic exchange model. For example, magnification of rDNA in Drosophila can be attributed to unequal mitotic cross-overs (Tartof, 1973), and heterogeneity in yeast rDNA is believed to result from unequal cross-overs in mitosis (Petes and Botstein, 1977). Unequal SCEs could generate short-range and long-range repeats, depending on the size of the exchange. Keyl (1965a, b) proposed the 'replicative loop' model for the evolution of highly repeated DNA. The model was proposed to explain a geometric expansion of DNA in homologous chromomeres in different subspecies of Chironomus thummi. When a replication loop forms in a chromomere, the daughter strands do not separate but break and rejoin end-to-end before replication is complete. This model has been developed to explain tandem repetitions, deletions and palindromic
sequences, depending on which chromatids break and rejoin (Kurnit, 1979; Maio et al., 1977). Maio et al. (1977) modified Keyl's hypothesis to account for the fact that certain long range repeats in some mammals are either even-numbered or geometric multiples of nucleosome length sequences (Brown et al., 1979; Maio et al., 1977; Musich et al., 1977a, b). The suggestion was that unequal cross-over involves exchanges of multiples of nucleosome length, possibly mediated by certain endonuclease recognition sites, during the replication process. The model is attractive because large blocks of repetitive sequences may be involved, allowing rapid expansion of an array with fewer cross-over events than are required by the random exchange model of Smith (1973, 1976).

Large scale unequal cross-over events are often needed to explain C-band polymorphisms (Craig-Holmes and Shaw, 1971). C-heterochromatin changes can be induced by the use of the alkylating agent, mitomycin C, which simulates gross unequal heterochromatic exchange (Hoehn and Martin, 1972, 1973).

Having established that heterochromatin can accumulate, relatively quickly, how can it affect the evolution of the sex chromosomes? Two major effects are important here, (i) the effects of heterochromatin on recombination and (ii) the effect of heterochromatinisation on Y-degeneration.

The extent of pairing homology cannot be assessed from the zygotene nuclei of the newts in the present study. The
degree of homology can be inferred from positions of chiasmata, assuming no terminalisation or minimal slippage. What factors affect pairing? In *Drosophila*, the chromosomes of male meiosis undergo pairing, although there is no recombination and no formation of a synaptonemal complex (Rasmussen, 1973). Yamamoto (1979) convincingly demonstrated that euchromatic homology is essential for autosomal pairing. Deletions and additions of heterochromatin have no effect on pairing. The sex chromosomes of *Drosophila* may have no euchromatic homology, and in this case, heterochromatic pairing is dependent on the presence of certain "pairing sites" rather than homology per se (Yamamoto and Miklos, 1977). Pairing of the sex chromosomes need not involve heterochromatin, as seen in the end-to-end associations of mammalian X and Y chromosomes. The synaptonemal complex of mouse and human sex chromosomes does not involve the heterochromatic regions of the X (Moses et al., 1975, 1982). There is no doubt that position and amount of heterochromatin can influence recombination within and between chromosomes (for review see John and Miklos, 1979). Gross deletions of heterochromatin from *Drosophila* X chromosomes may not affect pairing and normal segregation, but does drastically reduce recombination (Yamamoto and Miklos, 1978).

In *Triturus*, the telomeres pair first in the bouquet arrangement. Although heterochromatin may not be important for pairing, a heteromorphism for distal heterochromatin would obviously disrupt the process. But, how severely would
the heteromorphism affect recombination in that chromosome? 

*T. vulgaris* and *T. alpestris* have similar sex chromosome heteromorphisms. In *vulgaris*, chiasmata are normally terminally located. Prevention from forming a chiasma in the distal region of the long arm would effectively prevent chiasmate association in the entire arm. *T. alpestris*, on the other hand, shows unrestricted chiasma distribution. The heteromorphism only prevents chiasma formation in the distal tip of the chromosome arm. This suggests that the differential segment may not be very large. It must be remembered that a small chromosome segment in newts constitutes a lot of DNA. For example, just half of the long arm of the X and Y chromosomes comprises about 2.5% of the genome length. This is approximately equivalent to 0.6 pg DNA of the haploid genome.

In *T. marmoratus* and the *cristatus* subspecies, crossing-over is suppressed in most of the long arm. These subspecies are potentially capable of unrestricted chiasma distribution, but the evidence indicates that recombination may only occur in a subterminal region just below the terminal heterochromatin. This essentially means that the X and the Y differ only by the distal tips. Therefore the sex factors must be located within the terminal heterochromatin. The distal location of the sex factors in a relatively small chromosomal segment probably applies to all *Triturus* species. This would certainly explain why the distal tip of the sex chromosomes in all species is always free from crossing-over. The general nature of chiasmata distribution in
each species dictates the form that the sex-bivalent adopts. For example, in helveticus and vulgaris, the long arm is entirely achiasmate as the normal distal location of a chiasma is prevented by the terminal differential segment. Similarly, in alpestris, chiasma are normally unlocalised, the presence of the terminal differential segments preventing crossing-over in the distal portion only of the long arm. In marmoratus and the cristatus subspecies, the situation is slightly different. Chiasmata are normally unlocalised. Therefore one would expect crossing-over to occur at least in the interstitial region of the long arm. Associations only ever occur distally, despite the fact that most, if not all, of the euchromatic stretch of the long arm is homologous between the X and the Y. This can be said as a subterminal chiasma renders the X and the Y different only by the distal heterochromatin. The distal heterochromatin in the cristatus species group may be influencing cross-over suppression in most of the long arm. Cross-over suppression can occur in the absence of heterochromatin, as in helveticus. But whatever mechanism prevents recombination between part(s) of the long arms also allows heterochromatin to accumulate on the Y. The presence of heterochromatin may locally reinforce chiasma suppression. As heterochromatin further accumulates, it may exert more of an influence on recombination, suppressing crossing-over in the entire long arm (as in marmoratus and the cristatus subspecies). Thus, heterochromatin may develop as a consequence of recombination suppression, but beyond a certain point it may exert its own influence on recombination. It is doubtful that all
terminal associations are chiasmate, in the *cristatus* group. Many may be the consequence of heterochromatic attraction, which may itself facilitate chiasma formation subterminally.

The only clear example of lack of homology in the long arm comes from *T. vittatus*. The long arms of chromosomes 4 show a different distribution of small intercalary C-bands between the two homologues. It is unfortunate that this is the one example with the least confirmatory evidence from other sources for chromosomes 4 being the sex pair. The different distribution of intercalary C-bands could have arisen from minor paracentric inversions, which would be expected to prevent crossing-over in the long arm. One must exercise caution in applying structural rearrangements to all *Triturus* sex chromosomes as all other species show only homologies of intercalary C-bands in the X and Y. *T. alpestris apuanus* particularly emphasises homology with chiasma forming well into the long arm. *T. vittatus* may just be an exception where the putative sex chromosomes are prone to rearrangements (note - chromosomes 4 are also submetacentric). In addition, the differential segments probably only comprise a relatively small chromosomal region.

The X and Y chromosomes are probably still largely homologous in terms of gene content. Provided that recombination persists in the long arms of *T. alpestris*, one would not expect extensive 'degeneration' of the Y. In *marmoratus* and *cristatus*, the X and Y terminal C-bands may have arisen from the expansion of homologous sequences
rather than a meiotic exchange from the Y to the X. The consistent size difference between the X- and Y-heterochromatin is evidence against X-Y chiasmate exchange. Also, *T. c. karelinii* shows a subterminal C-band in the long arm of the X, which is likely to have arisen by a sequence replication event rather than exchange of heterochromatin from the Y. SCEs are known to take place in heterochromatin as shown by the occurrence of a dicentric ring chromosome from an originally monocentric, heterochromatic ring Y chromosome in *Drosophila* (Yamamoto and Miklos, 1978). Also, BrdU substitution studies show the existence of SCEs in heterochromatin (Holmquist and Comings, 1975; Natarajan and Klasterska, 1975). Although, in some cases SCEs may be more frequent at heterochromatin/euchromatin boundaries (Bostock and Christie, 1976). Clearly, there is the potential for unequal exchanges to account for the independent growth of X and Y heterochromatin.

Evidence for similarities between X and Y heterochromatin come from fluorochrome staining. X and Y C-bands are heterogeneous in Giemsa staining, but both chromosomes possess an AT-rich and slightly GC-rich components. Heterochromatin associations at meiosis have been reported for the AT-rich centromeres of the acrocentric chromosomes in pigs (Schwarzacher et al., 1984) and for the GC-rich centromeres of cattle (Mayr et al., 1979). Schwarzacher et al. (1984) suggest that the associations are dependent on close proximity and similar composition of the centromeric heterochromatin. The sex chromosomes of *Triturus* may pair
completely or at least are brought into close proximity, during zygotene, to facilitate the X-Y association. Mayfield and Ellinson (1975) suggest that heterochromatic associations are mediated by certain proteins which bind to specific DNA sequences and to protein molecules of their own kind, accounting for interchromosomal associations of homologous regions. In this connection, a protein binding to a specific satellite sequence has been found in Drosophila melanogaster (Hsieh and Brutlag, 1979). So, the situation in the cristatus group may be that the X and Y heterochromatin associate due to similar sequence composition and the close proximity resulting from meiotic pairing. Interestingly, snake sex-specific BKm satellite sequences have been hybridised to total DNA from T. cristatus carnifex. Results show that BKm sequences do hybridise to the newt DNA, and fractionally more so in males than females (Jones and Singh, 1982). If BKm sequences were hybridising to the DNA from the X and Y C-bands, then this would confirm the similar composition of the sex chromosomes' heterochromatin.

The Y-heterochromatin probably harbours the Y-sex factors. Y-heterochromatin will tend to accumulate as a consequence of recombination suppression and the sheltering effect of the X chromosome. As Y-heterochromatin builds-up, it may exert its own effects on chromosome behaviour which, more often than not, will tend to reinforce X-Y heteromorphism and degeneration of the Y.
4. TRITURUS INTERSPECIFIC CYTOLOGY.

4.1 INTRODUCTION

In section 1.1, it was outlined that general karyotype morphology, in terms of chromosome number, lengths and arm lengths, has been conserved within the Salamandridae. In Triturus, the work of Callan, Lantz and Spurway concluded that despite similarities in the overall 'shape' of a karyotype, specific and subspecific differentiation had proceeded via, or at least been accompanied by, several translocations and inversions. Mancino and co-workers, using C-banding and in situ hybridisation of certain repeated genes, consider that 'chromosome repatterning' is important in differentiation of species and subspecies. Their work has shown that within Triturus, the amount and distribution of C-heterochromatin and the distribution of certain genes has undergone extensive 'repatterning' during evolution.

However, from the present study on the cristatus subspecies and T. marmoratus, it can be seen that the overall morphology of the karyotype and most of the sites of constitutive heterochromatin have been conserved. There are some changes in sites of C-heterochromatin and gene loci which can be explained by the occurrence of inversions. Specific and subspecific differentiation may be accompanied by changes in the amount of C-heterochromatin at certain sites. Some sites may be subject to expansion or diminution within a population, but most of the quantitative
differences between the species/subspecies are consistent and characteristic for that group.

Within the *cristatus* species group, at least, there appears to be no firm evidence from chromosome banding for either translocational differences or extensive chromosome repatterning. What has led previous investigators to the conclusion of widespread reorganisation, or repatterning, in *Triturus*?

The work of Callan, Lantz and Spurway on hybrid male meiosis in *Triturus* used conventional staining where results showed that there is a reduction in chiasma frequency from parental values. Chiasmata tend to be terminal and multivalent associations may be present. The hybrids were between species and subspecies, and the degree of meiotic anomalies tended to reflect the expected relationships between the parental forms based on other criteria (morphological and hybridological). The extent of pairing failure and multivalent formation was quite variable within an individual and between individuals of the same mating. Some of the chiasmate associations were between non-homologous chromosomes of the parental sets. White (1961, 1973) explained this situation in *Triturus* by proposing that many, if not all, of the telomeric regions in newt chromosomes are homologous, the homologies being due to a series of translocations of small segments between the telomeres of the chromosomes. Callan (personal communication) believes that this may be the case as he has found no evidence for
translocational differences when comparing the distribution patterns of lampbrush loops of the *cristatus* subspecies. It seems that in hybrid meiosis, pairing is disrupted and non-homologous chromosome telomeres are more likely to associate forming chiasmata. The situation is complicated by the fact that C-banding studies show that chiasmate association can occur between non-homologous chromosomes of the same parental genome and occasionally in intercalary positions in F\textsuperscript{1} hybrids between *T. marmoratus* and *T. c. carnifex* (Mancino et al., 1979).

In addition, all studies have involved male hybrids which are largely or completely sterile. Callan (unpublished observations) found that female hybrids between, *cristatus* subspecies, are largely fertile. Female hybrid meiosis is characterised by a reduction in chiasma frequency compared to the parental frequencies, with the occasional univalent formation. However, chiasmata usually form in procentric or interstitial regions. Thus, synapsis in female hybrids probably proceeds normally and homology between parental chromosomes extends further than at the telomeres only. Obviously, the 'laws' governing homology and synapsis in hybrid meiosis vary with the gonadal environment. Classical genetics defines synapsis and crossing-over as occurring between homologous segments, although this may not be the case in male newt hybrids. A similar situation is seen in the inter-racial hybrids of the polytypic grasshopper species, *Caledia captiva*, where 'anomalous' chiasmata are seen in male meiosis. Shaw and Wilkinson (1978) interpret
the anomalous associations in grasshopper hybrids as purely non-homologous and consider them to "have arisen as a consequence of hybridity following the breakdown of an unknown mechanism which under normal circumstances precludes the association of non-homologues". It seems that hybrid meiotic processes are upset by a physiological imbalance in the heterogametic sex, as predicted by Haldane's rule (1922). At present, it is not possible to comment beyond this. Nevertheless, the extent of meiotic anomalies between different parental forms still reflects the degree of relatedness. For example, meiotic breakdown is greater in T. vulgaris x T. cristatus hybrids than in hybrids between the cristatus subspecies (see section 1.1.2).

Specific and subspecific differentiation may be facilitated by changes in C-values (the amount of DNA per haploid genome). Olmo (1973, 1974) and Olmo and Morescalchi (1975) found differences in nuclear DNA values between different Triturus species and subspecies. Structural rearrangements may also play some role in the differentiation of Triturus groups. Although widespread translocational differences now seem unlikely, there is certainly good evidence for the occurrence of inversions in newts. In lampbrush chromosomes, inversion heterozygosities have been seen in Notophthalmus viridescens and T. vulgaris meridionalis (Hartley and Callan, 1977; Mancino et al., 1970). Inversion heterozygosities have also been seen in mitotic chromosomes of T. c. cristatus (present study, section 2.3.10), and in T. italicus which may be polymorphic for three forms of
chromosome 12 (Bucci-Innocenti et al., 1983a; Ragghianti et al., 1980). Inversions may serve to redistribute certain cytological landmarks, although their speciation value is dependent on their effects on meiotic pairing and their frequency in a breeding population.

The aim of the present work was to examine the extent of homologies/differences between different *Triturus* species in terms of amount and distribution of C-heterochromatin and overall chromosome morphology. The extent of differences largely depends on the way in which one looks at a karyotype.
TRITURUS INTERSPECIFIC CYTOLOGY.

4.2 RESULTS

Species examined in addition to the cristatus group:—

**T. alpestris apuanus**

**T. boscai**

**T. helveticus helveticus**

**T. vulgaris vulgaris**

**T. vittatus ophryticus**

Note: **T. italicus** and **T. montandoni** were not available for this study.

4.2.1 General Karyotypic Features

The 24 diploid chromosomes are arranged into three groups on the basis of size.

(i) Large group (chromosomes 1-4). All are metacentric elements.

(ii) Medium-sized group (chromosomes 5-8). Two chromosomes are metacentric and two are submetacentric. In **T. vittatus**, the submetacentric elements are chromosomes 5 and 6. In all other species, chromosomes 6 and 7 are submetacentric.

(iii) Small group (chromosomes 9-12). The small chromosomes show the greatest variation between species and may be metacentric or submetacentric. In **T. vittatus**, chromosome 11 is nearly acrocentric and has the smallest centromere index of all **Triturus** chromosomes (C.I. = 0.149).
4.2.2 Distribution of C-Heterochromatin

C-staining constitutive heterochromatin appears in different forms, all staining more strongly than euchromatin with Giemsa. (i) Intensely staining, discrete blocks of heterochromatin. (ii) Grey heterochromatin which may be present as a discrete block or appear 'diffuse', merging-in with adjacent chromatin. The grey heterochromatin may vary slightly in staining intensity.

Most of the major sites of heterochromatin are in centric, pericentric, terminal and subterminal positions. Most interstitial C-bands are small and 'grey'.

4.2.2.1 Centromeric and Pericentric Heterochromatin

In no species does the size of the centromeric heterochromatin approach the size of the large blocks of centric material seen in T. c. karelinii.

T. alpestris apuanus. Centric C-bands vary considerably in size within the karyotype (Fig. 59). Some centric heterochromatin is barely visible. Pericentric heterochromatin is present on both sides of the centromere in all chromosomes. The staining intensity of the pericentric bands may vary between the arms of a chromosome. Most chromosomes possess 'double' or 'multiple' pericentric bands in close apposition.
**T. boscai.** Centric heterochromatin is evident on all chromosomes, although in relatively small amounts on many (Fig. 60). Pericentric heterochromatin, on the other hand, is particularly striking. *T. boscai* has more pericentric heterochromatin on its chromosomes than any other *Triturus* species. Pericentric C-bands are present on both arms of all chromosomes as double or multiple blocks of heterochromatin. It is the size and multiple nature of these bands that makes them so distinctive.

**T. helveticus helveticus.** Centric heterochromatin is prominent on all but two chromosomes (Fig. 61). Chromosome 8 has very small centric bands which are often visible as two small dots, one on each chromatid. Chromosome 3 does not appear to have C-staining centromeres. Pericentric heterochromatin is present on all chromosomes. Chromosome 12 has only a very small grey pericentric band in the long arm, which may be absent leaving the centromere as the site of heterochromatin. Chromosome 6 may have pericentric C-bands in the short arm only or, in some individuals, an additional small grey pericentric band in the long arm. All other chromosomes have pericentric bands in both arms. Most pericentric bands are prominent single heterochromatic blocks, although some chromosomes have double C-bands.

**T. vulgaris vulgaris.** Centromeric heterochromatin is present on all chromosomes (Fig. 62). Only chromosome 9 has a small centric C-band. Pericentric heterochromatin is prominent and most often present as double bands. The amount of
pericentric C-heterochromatin is not quite as much as in *T. boscai*.

*T. vittatus ophryticus*. Centromeric C-bands are relatively large on all chromosomes (Fig. 63). Pericentric heterochromatin is present on all chromosomes, most often as a distinctive, strongly staining single band. Few are double bands. The small group chromosomes tend to have small pericentric bands.

**4.2.2.2 Terminal, Subterminal and Interstitial Heterochromatin.**

This section will take the form of an interspecific comparison of homologous positions of C-band heterochromatin. The karyotypes have been arranged with long arms facing downwards. Metacentric chromosomes rarely have a centromere index of exactly 0.5, thus the longer of the two arms is orientated down in the karyotypes. Speciation has probably involved slight growth or reduction of some chromosome arms. Therefore, a metacentric chromosome of one species may be equivalent to one in another species, but orientated upside-down between the different karyotypes. Without detailing the position of every C-band in a chromosome, the following considers certain chromosomes together, between species, based on obvious similarities in the distribution of heterochromatin. The size of a particular band may vary between species, but the comparison is made largely on the positions of terminal, subterminal and interstitial C-bands.
of a chromosome.

(i) The chromosomes 1 of helveticus, vulgaris and vittatus appear similar. Also, the chromosome 1 of alpestris resembles chromosome 2 of the cristatus species group, especially that of T. c. cristatus and T. c. carnifex.

(ii) The chromosome 2 of helveticus and vulgaris and possibly boscai are very similar. T. boscai possess no terminal heterochromatin in the shorter arm, unlike the other two species, but has homologies in the longer arm.

(iii) Chromosomes 3 of helveticus, vulgaris and boscai, and chromosome 4 of vittatus are similar. The chromosome 3 of alpestris could, tentatively, be grouped with the above species and/or with the chromosome 3 of the cristatus species group.

(iv) Chromosomes 4 of helveticus and vulgaris are similar, but do not resemble the similar sex chromosomes (4) of alpestris, cristatus, marmoratus and possibly boscai. Also, the similar sex chromosomes (5) of helveticus and vulgaris (ignoring the Y-heterochromatin in vulgaris) do not share homologies with the sex chromosomes of the above species.

(v) Perhaps the strongest homologies are seen between chromosomes 6, 7 and 8. Chromosome 6 in all Triturus species is a submetacentric element, showing a similar distribution of interstitial C-bands in the long arm. The amount of terminal, centric and pericentric heterochromatin varies between the species. Chromosome 7 is more submetacentric than chromosome 6 in all but cristatus, marmoratus and vittatus. Chromosome 8 is remarkably conserved in all
Triturus species, in terms of C.I. and distribution of C-bands.

(vi) The small group chromosomes show the greatest variations between species, such that an interspecific grouping may be of little value. Nevertheless, similarities are apparent between chromosomes 11 of *alpestris* and *vulgaris*; chromosome 10 of *alpestris* and chromosome 11 of *boscai*; and chromosome 9 of *vulgaris* and 10 of *boscai*. With the exception of the distinctive chromosome 12 of *helveticus*, all other species have a very similar smallest chromosome.

The positions of NORs have not been confirmed in *T. boscai* or *T. vittatus*. The large subterminal band in the long arm of chromosome 9, in *T. vittatus*, is GC-rich (Fig. 64) and shows heavy labelling with in situ hybridisation of a tritiated *Xenopus laevis* ribosomal gene probe, pXlr101 (personal observation). So, the C-band at this site can be considered as the NOR. The band often shows a close apposition between the sister chromatids, as is often the case with nucleoli in interphase nuclei. In *T. boscai*, a small group chromosome (10) has a GC-rich band adjacent to the pericentric region in the long arm (Fig. 65). This band corresponds to a 'silver-positive' band on chromosome 10 in *boscai* chromosomes subjected to the AS-SAT technique (Bucci-Innocenti et al., 1983b). The AS-SAT method can be used to stain NORs, but often stains other regions in a variable way. The silver-band on chromosome 10 was a consistent feature, although other sites in the *boscai* karyotype also
stained, the positions of which varied between individuals. The GC-rich, silver-positive band on chromosome 10 of *T. boscai* is most probably the site of the NOR.

4.2.3 Cytology of an F₁ Hybrid between *T. h. helveticus* and *T. v. vulgaris*.

No natural hybrids had ever been recorded between sympatric populations of *T. h. helveticus* and *T. v. vulgaris*. However, Mr. R. Griffiths of the Llandrindod Field Centre, U.W.I.S.T., Powis, Wales found a potential F₁ hybrid at a locality in S. Wales. Ms. J. Roberts of the Department of Biology, Open University, Milton Keynes confirmed that the newt was a natural hybrid, on the basis of morphological criteria. The newt was given to me to examine its chromosome constitution.

The newt was a sexually mature male, aged between 2 and 3 years, showing secondary sexual characteristics of both *T. helveticus* and *T. vulgaris*. Spermatogonial mitoses were obtained from the testes. However, no meiotic divisions were present in either testis. So the extent of pairing and chiasma formation could not be analysed.

The newt was clearly an F₁ hybrid, possessing a haploid chromosome set from *T. h. helveticus* and a haploid set from *T. v. vulgaris*. Figure 66 shows a diploid karyotype from the hybrid. Figure 68 shows four examples of each chromosome from spermatogonial metaphases.
Despite the fact that meiotic divisions were not found, it was clear that meiotic anomalies were not so great as to upset normal sperm formation. Numerous mature sperm bundles were present in both testes (Fig. 67). The sperm bundles appeared quite normal with no evidence of extensive spermatid degeneration or anomalous sperm, as is often the case with experimentally produced hybrids (Spurway and Callan, 1960).

This is the first clear demonstration of a natural $F_1$ hybrid between $T._{helveticus}$ and $T._{vulgaris}$. Interestingly, one can deduce the sex of the parents by examining the sex pair, chromosome 5. The hybrid possesses an X-chromosome from vulgaris (no telomeric C-band in the long arm). Therefore, the Y chromosome must have come from the helveticus father. The situation is unusual in that vulgaris females are normally more discriminating in choosing conspecific rather than heterospecific male partners, than helveticus females are (Roberts, unpublished observations).

4.2.4 Chromosome Polymorphisms.

Some chromosomes may show differences between homologues in the size or even absence of certain C-bands in some individuals:

In one individual of $T._{h.}$ helveticus, a prominent C-band was present at the telomere of the short arm of one homologue of chromosome 7 (Fig. 69). The sex chromosomes (5) may
have either no (or at least a small amount) of telomeric heterochromatin in the long arm. One individual was found with quite distinct telomeric heterochromatin in both homologues. Chromosome 8 may possess an additional C-band in a distal position along the long arm.

In *T. v. vulgaris*, chromosome 8 may or may not possess a distinct intercalary band in a median position in the long arm. The previous section describes an F hybrid between *vulgaris* and *helveticus*. The *vulgaris* chromosome 6 from the hybrid showed a difference in that the pericentric heterochromatin of the long arm consists of two strongly staining blocks, the short arm having only one small pericentric band. The reverse is true for all *vulgaris* individuals examined. This probably signifies that a small pericentric inversion has taken place, possibly involving a chromosome segment within the limits of the pericentric heterochromatin.
TRITURUS INTERSPECIFIC CYTOLOGY

4.3 DISCUSSION

The chromosome banding patterns of most of the Triturus species has been examined in this study. Can the banding patterns help in assessing evolution, speciation and relationships within Triturus?

The current understanding of species relationships and genetic distances within Triturus is based on morphology, osteology, starch gel electrophoresis and behavioural data. Fragmentary fossil records help to assign an age to some of the lineages. All data generally agree, with slight disparities.

Osteological studies have shown that the genus can be divided into three species groups: 'Paleotriton' comprising boscai, helveticus and vulgaris; 'Mesotriton' which has just one species, alpestris; and 'Neotriton' which contains cristatus, marmoratus and vittatus (Bolkay, 1928).

The grouping of Bolkay (1928) generally agrees with the 'vulgaris', 'alpestris' and 'cristatus' groups of Lantz (1947). Lantz based his grouping on morphology and hybrid experiments and it differs from the osteological scheme only in the allocation of vittatus to the vulgaris species group, rather than to the cristatus group. T. boscai was recognised as having some affinity to the alpestris group as the two
species freely court in captivity. However, *boscai* shows a greater morphological resemblance to *vulgaris*. Later studies show that although *boscai* and *alpestris* court in captivity, the hybrid zygotes do not cleave (Bucci-Innocenti et al., 1983b), suggesting post-zygotic reproductive isolation.

Bucci-Innocenti et al. (1983b) show that in captivity *alpestris* can successfully court with *vittatus* and produce viable hybrid offspring. The natural ranges of these two species do not overlap, thus the species would not normally have the potential to hybridise. Bucci-Innocenti et al. (1983b) suggest that *Triturus* should be divided into two species groups: the *cristatus* and *vulgaris* groups (after Lantz, 1947), but the latter including *alpestris*.

From details of the elaborate courtship displays, Halliday (1977) was able to distinguish three species groups like those of Lantz. *T. helveticus* and *vulgaris* have similar courtship displays, as do *cristatus* and *marmoratus*. *T. alpestris* was considered as separate from the other two groups. Behavioural details from the remaining *Triturus* species have not been fully characterised, but show general similarities with the *vulgaris* group.

Kalezic and Hedgecock (1980) used starch gel electrophoresis to determine genetic variation and distance between eastern populations of *alpestris*, *vulgaris* and *cristatus* (subspecies *karelinii* and *dobrogicus*). The overall conclusions were that *alpestris* is more variable than *vulgaris*, which in turn is
more variable than *cristatus*. Also, *alpestris* and *vulgaris* are more closely related than either is to *cristatus*. These findings do not entirely correspond with genetic distance data determined by Frelow, Macgregor and Wake (unpublished observations). This work involved slightly different methods and different populations to that of Kalezic and Hedgecock (1980). The overall findings were that the *cristatus* group (*marmoratus* and the *cristatus* subspecies) is well differentiated from the *vulgaris* group (*boscai*, *helveticus* and *vulgaris*). *T. alpestris* is considered to be somewhat closer to the *cristatus* group than the *vulgaris* group. However, this affinity is not so close as to prevent *alpestris* from being placed in a group on its own (the formerly recognised 'mesotriton' or 'alpestris' group). In addition, the results show that previous tenuous links of *boscai* with *alpestris* were not apparent and *boscai* shows a closer affinity to *helveticus*, in the *vulgaris* group.

Frelow, Macgregor and Wake, Kalezic and Hedgecock (1980), and Bucci-Innocenti et al. (1983b) all consider that the subspecies of *cristatus* should be regarded as full species.

Fossils recognised as *Triturus* date back to the Belgian middle Oligocene, about 30 million years ago (Hecht and Hoffstetter, 1962). Representatives from the *vulgaris* and *cristatus* groups have been found in the European middle Miocene, about 18 million years ago (Estes, 1981; Estes and Hoffstetter, 1976), by which time most of the present day species were probably distinct.
Can any useful information be gleaned from chromosome studies to add to the above picture of relationships?

From the outset, the *cristatus* and *marmoratus* can be considered as distinct from all other *Triturus* species, primarily because of the chromosome 1 situation. There are other karyological details that further support the separation of the *cristatus* group, which will be discussed later.

Taking the karyotype as a whole, it can be seen that certain features have been conserved. All species possess 24 (2n) biarmed chromosomes. An NOR is present on a small group chromosome in all species (except *helveticus*, where the NOR has not yet been located). Other chromosomal NOR sites may be present in the karyotype, the positions of which may vary between species (Mancino et al., 1977) and between individuals (Batistoni et al., 1978; Nardi et al., 1977). The centromere position and distribution of C-bands are remarkably similar in both chromosomes 6 and 8 of all *Triturus* species examined, although the amount of C-heterochromatin at any one site may vary between species. This conservation at a gross level is not maintained when one looks at the karyotypes in detail, employing chromosome banding techniques.

Karyological changes that have clearly happened include: (i) structural rearrangements; (ii) duplication of C-bands;
(iii) quantitative changes in C-heterochromatin; and (iv) de novo accumulation of heterochromatin.

Consider the cristatus group. All members have essentially the same centromere positions and relative lengths on corresponding chromosomes, discounting chromosome 1. Also, all members share most of the major sites of C-heterochromatin. The only appreciable differences between members of the cristatus group are with respect to amounts of C-heterochromatin at specific sites.

As for the remaining Triturus species, helveticus and vulgaris show the greatest number of homologies in terms of centromere positions and distribution of C-bands. Although, vulgaris shows more evidence of duplication of pericentric heterochromatin than helveticus. T. alpestris, boscai and vittatus are all dissimilar from each other and from helveticus and vulgaris. The differences take the form of structural rearrangements and heterochromatic changes, resulting in very few whole chromosome homologies. T. alpestris is characterised by multiplication of pericentric C-bands and apparent accumulation of diffuse heterochromatin at the telomeres of all chromosomes. T. boscai has large, multiple blocks of pericentric heterochromatin. T. vittatus has prominent centric and pericentric heterochromatin, and has the greatest number of centromeric positional differences compared to the other species.

Are there any trends in chromosomal changes within Triturus?
Different factors appear to be operating within the *cristatus* group compared to the other species. Among the members of the *cristatus* group, structural rearrangements appear to have played little part in chromosome change. Centromere indices and the main distribution of C-bands have not changed significantly through specific/subspecific differentiation. Quantitative change in the amount of heterochromatin has shown two different trends within the *cristatus* subspecies. The amounts of heterochromatin has remained approximately the same at most sites in *cristatus* and *carnifex*. However, in *karelinii* all centromeres have substantially increased their amounts of centric heterochromatin. *T. marmoratus* appears intermediate in that only some centromeric sites have accumulated heterochromatin. This, of course, assumes that the ancestral condition was close to that of *cristatus* and *carnifex* with relatively small centric C-bands.

So, there may be factors preventing any quantitative change in C-band heterochromatin in *cristatus* and *carnifex*, and conversely, we may suppose that there has been a tendency for all chromosomes to accumulate centric heterochromatin in *karelinii*.

In the remaining species, there is no clear evidence for any particular common trend in chromosomal change. There is evidence for structural rearrangement differences, the rate of which may vary between species, perhaps being most evident in *vittatus*. The rate of structural change is in no
way related to the rate of quantitative change in C-heterochromatin. Heterochromatic change may vary within and between species, apparently being either random or non-random. For example, *boscai* shows duplication and growth of nearly all sites of pericentric heterochromatin. *T. alpestris*, on the other hand, shows duplication of pericentric C-bands, but apparently random growth/depletion of these bands both within and between chromosomes. *T. alpestris* may be accumulating heterochromatin to eventually resemble *boscai*, and may therefore represent an intermediate evolutionary stage. Conversely, it may be argued that *boscai* may lose pericentric heterochromatin ultimately to resemble *alpestris*.

Whatever the case, it may be concluded that several different factors of karyological change have operated independently and not consistently throughout the genus. The gross observation of karyotypic conservation is certainly not applicable to lower levels of chromosome organisation and other cytological features. Chromosome studies on *Triturus* cannot usefully contribute to what is already known about the relationships within the genus. The studies do confirm the cristatus group distinction, but cannot help in deducing affinities between the remaining species. Although, the allocation of *vittatus* to the cristatus group on osteological grounds (Bolkay, 1928) can be firmly ruled out from the chromosome study.

All species have distinct C-banding patterns in terms of
size and distribution of heterochromatin. For example, one can easily distinguish between vulgaris and helveticus chromosomes in an F hybrid (section 4.2.3), despite many homologies between the karyotypes. It is likely that most quantitative change in C-bands has followed speciation or subspecific differentiation. Some changes may be trivial in the sense that populational polymorphisms may be observed, probably without conferring any speciation value. Also, chromosome 8 shows considerable C-band size differences between species, but the actual sites of heterochromatin have been highly conserved. It is difficult to assess whether changes in C-band size contribute to speciation. Translocations are potential speciating agents in particular colonising individuals, as discussed in section 2.4.2. Small inversions probably have little speciation value as they often do not greatly disturb meiotic pairing and several examples have already been mentioned, giving rise to populations polymorphic for a chromosome with different centromere indices. Larger inversions may have more of an effect on disturbing normal meiotic pairing, and have been known to redistribute chiasma positions in a bivalent heterozygous for the inversion, in Notophthalmus viridescens (Hartley and Callan, 1977). This could potentially give the colonising newts different and possibly adaptively favourable gene combinations in their gametes. However, it is more difficult to comment on heterochromatic change conferring speciation value. In Triturus, it would seem unlikely that any heterochromatic change could have significant speciation/adaptive value. It is more
likely that heterochromatic changes follow the isolation of a breeding group that ultimately differentiates from the main stock by adaptation to its new environment. Heterochromatic change in the new lineage may or may not be caused by, or confer adaptive advantage in the new environment.

For the purpose of convenient interspecific evaluation of heterochromatic change, subsequent discussion will focus on centromeric heterochromatin which is common to all species and can show a high level of quantitative variation.

Centromeric heterochromatin may be considered as distinct from, and to have evolved independently from pericentric heterochromatin. This distinction comes largely from the fluorochrome studies which show centric and pericentric heterochromatin to differ widely in base-pair content.

Leaving aside the cristatus group, for the moment, alpestris shows the greatest variation in size of centromeric heterochromatin. Within each of the long, medium and small chromosome groups, variation includes chromosomes with virtually no centromeric heterochromatin to those with substantial blocks. T. helveticus similarly shows variation from virtually no heterochromatin (e.g. on chromosome 3) to relatively large centromeric bands. The largest helveticus band is not as big as the larger alpestris C-bands. T. vulgaris shows a similar range of size variation as helveticus. T. boscai shows generally small centromeric C-bands. In vittatus, the centromeric heterochromatin is
prominent on all chromosomes, with some size variation. Most centromeric bands in *vittatus* are as large as or larger than the largest *alpestris* C-bands.

In none of the above cases can the size of the centromeric bands be correlated with the relative length or any other feature of the chromosome. Likewise, there is no evidence that any one chromosome has maintained a large or a small centromeric C-band throughout speciation. Centromeric changes appear random.

Within the *cristatus* group, a similar range of C-band size variation is seen in *cristatus* and *carnifex*. The chromosome with the largest amount of centric heterochromatin is a small group chromosome (10). Some chromosomes may have very small centric bands, often visible as discrete dots over each chromatid. However, despite a somewhat random range in size throughout the set, it is interesting to note that both subspecies do not show significant size differences between centromeres of corresponding chromosomes. *T. marmoratus* shows a much wider range of centric C-band sizes, with a tendency for those on about half of the chromosomes to have grown disproportionately. Of the remaining chromosomes with smaller amounts of C-heterochromatin, the sizes of the centromeres are roughly similar to the corresponding chromosomes of *cristatus* and *carnifex*.

Nearly all of the centromeres of *karelinii* chromosomes have grown to massive proportions. Even the C-band of chromosome
6 (actual chromosome 7 in relative length), which is the smallest in the set, is larger than the C-bands on chromosomes 6 of the other group members. Most of the C-bands are of equivalent massive size.

Assuming cristatus and carnifex to represent the near ancestral condition, then one can see a degree of size variation in C-bands between different chromosomes. However, constancy of the relative sizes of centric C-bands within chromosome sets signifies conservation between subspecies and species. Superimposed on the basic pattern of size variation is the tendency for growth of centromeric heterochromatin, which has reached a present-day maximum in karelinii. There is probably a limit to C-heterochromatin growth as may be tentatively inferred from the similar size of the largest centric bands on large and small chromosomes. T. marmoratus represents an transitional stage between the minimal change of cristatus and carnifex, and the maximal change of karelinii. The general growth of centric C-bands on all karelinii chromosomes would seem to be independent of changes in heterochromatin at any other site in the chromosome set.

In summary, it is clear that the size of centromeric heterochromatin is independent of chromosome size and tends towards growth, the extent of which is again independent of chromosome size.

The situation can be contrasted with that in the north
American plethodontid salamanders of the genus *Plethodon*. The karyotypes within *Plethodon* are extremely conserved in terms of number (2n=28), relative lengths and centromere positions (Mizuno and Macgregor, 1974). In *Plethodon* there is a clear correlation between the size of a chromosome and the amount of centric heterochromatin that it possesses (Macgregor et al., 1973). *Plethodon* species can be divided into several groups on the basis of morphology and geographical distribution (Highton, 1962). In the eastern small group, only one subspecies, *P. cinereus cinereus*, out of at least eight closely related members has large amounts of centric heterochromatin that form large chromocentres in interphase nuclei (Kezer and Macgregor, 1971).

What is known about the composition of the centromeres in *T. c. karelinii* and *P. c. cinereus*? Baldwin and Macgregor (unpublished observations) have made some investigations into the DNA composition of *karelinii* centromeres. Digestion of *karelinii* genomic DNA with Hae III revealed the presence of two highly repeated DNA fragments of about 70 and 90 b.p.. In situ hybridisation to mitotic and lampbrush chromosomes showed both fragments to be localised exclusively at the region of the centromere on all chromosomes. The 70 and 90 b.p. fragments comprise between 5 and 10%, and 0.1 and 1% of the genome, respectively.

Interestingly, the total amount of centromeric heterochromatin makes up about 18% of the entire length of the chromosome set (calculated from chromosome measurements). Southern blotting and dot hybridisation show that
sequences homologous to both fragments are also present in cristatus, carnifex and marmoratus, but not in alpestris, vittatus or helveticus genomic DNA. It would appear that these centromere-specific sequences are confined to the cristatus group. Although hybridisation of the fragments was confirmed in other members of the cristatus group, the relative amount of hybridisation was much lower than in karelinii. Of the three other members, cristatus has more sequences homologous to both fragments than carnifex, and carnifex more than marmoratus. It is likely that the differences in levels of hybridisation represent differences in copy number rather than sequence divergence.

In P. c. cinereus, Macgregor et al. (1973) found a heavy satellite by neutral caesium chloride centrifugation of genomic DNA. The satellite is localised near the centromere on all chromosomes. It represents about 2% of the genome and about 25% of the DNA of the centromeric heterochromatin. Satellite sequences from P. c. cinereus are present in smaller amounts in closely related species that do not form large interphase chromocentres of centric heterochromatin. In situ hybridisation to mitotic and meiotic chromosomes shows that the amount of hybridisation depends on the size of the chromosome, larger chromosomes have more centric heterochromatin.

Centromeric heterochromatin of this type is distinctive in that it stains strongly with Giemsa, has a largely homogeneous sequence composition, and has adhesive
properties, fusing with heterochromatin of similar composition.

The situation of large amounts of centromeric heterochromatin is probably derived from an ancestral condition of smaller amounts of centric material. This point will be discussed later. Could the expansion of centromeric heterochromatin be an example of "selfish" DNA where sequences have an intrinsic property of self propagation (Doolittle and Sapienza, 1980; Orgel and Crick, 1980)? This would seem unlikely as sequences homologous to the karelinii centromeric sequences are also present at the centromeres of cristatus and carnifex in much smaller amounts, but extensive amplification has only occurred in karelinii.

How can growth of centric heterochromatin have been achieved in T. c. karelinii and P. c. cinereus? It is probable that different mechanisms of heterochromatic expansion have operated separately in both species. In P. c. cinereus, the amount of centromeric heterochromatin is proportional to chromosome size. P. c. cinereus has more centric material than any other closely related species, but a comparable C-value. Thus, all centromeres appear to have grown in step, but at a faster rate than in other species. Other Plethodon species may have over twice the C-value of P. c. cinereus, but have retained the karyotypic features of 28 diploid chromosomes, with similar relative lengths and centromere indices as P. c. cinereus (Mizuno and Macgregor, 1974). Genome growth in Plethodon has been achieved by a general
and uniform expansion of moderately and highly repeated sequences. In *P. c. cinereus*, a uniform expansion of all centromeres has occurred, but at a faster rate than the rest of the genome (euchromatin). Growth of the genome as a whole can be accounted for by random unequal cross-over events. However, in *P. c. cinereus*, there may be some factor which has caused random exchange events to occur preferentially in or adjacent to heterochromatin rather than in euchromatin (N.B. the only major sites of heterochromatin in *Plethodon*, are at the centromeres). The net result is that families of sequences have been homogenised within the centric heterochromatin giving the property of fusion of similar heterochromatic blocks.

The situation is somewhat different in *karelinii* where the size of centromeric heterochromatin is not proportional to chromosome size. Growth in all centromeres could easily be explained by unequal cross-over events, which may be preferentially located in or near centric heterochromatin, but probably not uniformly throughout the karyotype.

Many of the species of *Triturus* show a range of different sized heterochromatic blocks in interphase nuclei. The sizes of some blocks suggest that they represent fusions of two or more smaller blocks. With the capacity for fusion of blocks of similar composition, there is a greater opportunity for exchanges between similar heterochromatic sequences from different chromosomes, which may aid growth or depletion of a block. The ancestral condition may have been one of
similar sequences in the heterochromatin of all centromeres of the karyotype. The presence of fused centromeric heterochromatin in premeiotic nuclei in \textit{P. c. cinereus} has been suggested to play some role in chromosome recognition and pairing in the meiotic process (Kezer and Macgregor, 1971). The actual value of centromeric pairing before zygotene is questionable. The initiation of chromosome pairing begins at chromosome ends and progresses towards the centromeres. Premeiotic centromeric pairing may serve to disrupt pairing, if nucleotide sequence is important in pairing recognition. The homogeneous composition of the large blocks of centromeric heterochromatin may promote mismatching of homologues around the region of the centromeres. The very nature of a nuclear division always brings centromeres into close apposition during ana phase when chromosomes are drawn to opposite poles of the cell. This would promote the likelihood of centromeric fusion. Heterochromatic regions generally replicate late in S-phase of the cell cycle. If \textit{karelinii} centromeres replicate late in the premeiotic S-phase, then replication may not be complete when breakage/repair mechanisms are coming into play for the zygotene stage. This may promote the chances of replication of mismatched sequences in centromeric heterochromatin both within and between chromosomes. The process could result in rapid expansion and contraction of blocks of centric heterochromatin. Heterochromatin in the region of the centromere would primarily be affected as these regions are always brought into close proximity at the end of every division, promoting the chances of heterochromatic fusion.
The associations are more likely to facilitate net growth rather than depletion, because fusions are encouraged to occur with great frequency. If a block becomes reduced by a major unequal exchange event, then it will still have opportunities to re-expand since it will always be brought into close apposition with other blocks at the end of every division. The first meiotic division rather than mitotic divisions may cause greater exchanges and/or greater opportunities for exchange. The chances of centric exchanges within chromocentric fusions should cause similar net increases in the size of all centromeres involved in the fusion over the course of time, such that all centromeres will attain a comparable size. Accordingly, it is to be expected that the centric C-bands of species with large amounts of centric heterochromatin will be more uniform in size than those in species with little centric heterochromatin; which is exactly what we find when comparing karelinii with cristatus, carnifex or marmoratus (Fig. 53).

But why have cristatus and carnifex not followed the same expansion of centric material, and why has marmoratus partly done so? Also, why have Plethodon centromeres shown a more regular pattern of growth that is in some way related to chromosome size? Other factors are probably important, such as homogeneity of sequence composition and organisation of sequence repeats. Heterochromatic blocks may fuse with only a general sequence homology between blocks, a property which may not strictly be the result of sequence similarity but, rather a function of certain proteins associated with
certain repeated sequences.

About one quarter of the DNA of centric heterochromatin in *P. c. cinereus* is composed of a heavy satellite. The satellite is likely to be composed of range of related sequences. These sequences may be evenly spread throughout the centromeres, sufficient to promote general fusion, but not homologous enough (or sufficiently clustered) to encourage inter- and intrachromosomal exchanges within the chromocentre. Thus, each chromosome will maintain independent centric expansion which is relative to the chromosome size.

One of the *karelinii* repeated fragments comprises 5-10% of the genome and possibly up to half of the centric material (about 18% of the entire karyotype length). Thus, much more of the centromeres are known to be homogeneous than in *Plethodon*. These sequences are likely to be organised in tandem arrays and dispersed throughout the centric heterochromatin, encouraging sequence pairing and unequal exchange in chromocentres.

But why have *cristatus* and *carnifex* not shown similar expansion of their sequences known to be homologous to *karelinii* sequences? *T. c. karelinii* and possibly *marmoratus* may have independently amplified certain centric sequences to a point where heterochromatic fusion was encouraged. From this point on, expansion may be rapid. So, sequence organisation, repetition and homogeneity may be important in
growth of centromeric heterochromatin.

Thus, it can be envisaged that centric and/or pericentric heterochromatin may show rapid changes, a situation which is enhanced by the drawing together of centric regions at the poles of the mitotic or meiotic spindle. Similar uniform growth and multiplication of pericentric heterochromatin is seen in boscai. T. alpestris also shows multiplication of pericentric bands, but random growth of any particular one: i.e. all bands have not grown in unison. Similar changes are not seen at chromosome termini or interstitial regions. Interstitial heterochromatin shows a much narrower range in size and changes here could be accounted for by unequal SCEs, but not approaching the frequency or magnitude of those in centric regions. Telomeric heterochromatin is most often 'diffuse', suggesting a dispersed accumulation of repeated sequences, separated by short stretches of euchromatin. Growth of heterochromatin at the telomeres is not under the same conditions as those causing centric growth.

Heterochromatic changes are influenced by several factors, such as composition, position and ability to fuse. Do changes occur, and are they allowed to occur, because of some general property of the material to show quantitative change? Are changes allowed because they confer no particular advantage or disadvantage on the organism? The lack of evidence for change in cristatus and carnifex could suggest that any heterochromatic change in these subspecies
is selectively disadvantageous. Conversely, the nature of their heterochromatin may be such that changes are not encouraged. The frequent occurrence of change elsewhere in *Triturus* may suggest something more than just indiscriminate accumulation of heterochromatin. Perhaps one of the most important properties held by heterochromatin is its affect on chiasmata. The only correlation that can be made with the massive centromeric heterochromatin in *karelinii* is that this subspecies has a higher chiasma frequency than other *cristatus* subspecies. The significantly higher chiasma frequency may not be related to the amount of centric heterochromatin, but it would be interesting to compare chiasma frequencies and distributions with amounts of heterochromatin in other subspecies of *Triturus*. Likewise, the sequence composition and organisation of centric heterochromatin needs further study, along with timing of replication in relation to other events in the cell cycle and sequence-specific protein associations.
SUMMARY

Of the 9 species of European newts, 7 are examined in this study. All have 24 (2n) bi-armed chromosomes. Giemsa C-banding and base-pair specific fluorochrome banding are employed to characterise the karyotypes.

*Triturus cristatus* and *T. marmoratus* have an unusual chromosomal situation. All individuals are heteromorphic for the largest chromosome (chromosome 1). Chromosomes 1 are heteromorphic for a region comprising most of the long arm which shows different banding patterns between the two homologues (chromosomes 1A and 1B). The heteromorphic regions are largely heterochromatic and are asynaptic and achiasmate at meiosis in both sexes. Banding patterns of the heteromorphic regions varies between the subspecies of *T. cristatus* and *T. marmoratus*. Individuals which are homomorphic for either chromosomes 1A or 1B show arrested development and eventual death at tail bud stage of embryogenesis, resulting in a 50% mortality of all offspring. A model for the evolution of the heteromorphism is suggested.

The sex chromosomes of *Triturus* are in an early stage of differentiation. The male is the heterogametic sex. Most species show a C-band heteromorphism between the tips of the long arms of the X and Y chromosomes. Evidence is presented which suggests that the Y-sex factors are contained within the small, often heterochromatic, region at the tip of the
long arm of the Y chromosome.

Subspecies of *T. cristatus* and *T. marmoratus* are considered as a separate species group from the other *Triturus* species. The *T. cristatus* subspecies and *T. marmoratus* have karyotypes which share many homologies in the distribution of C-bands. There is little evidence for much structural rearrangements differentiating the species/subspecies, but quantitative C-heterochromatic change can be extensive. The remaining *Triturus* species show evidence for both structural and heterochromatic change. Chromosome studies can not usefully assess taxonomic affinities within *Triturus*, but have shown some interesting lines of karyotypic evolution.
REFERENCES


Batistoni, R.; Andronico, F.; Nardi, I.; Barsacchi-Pilone, G.: Chromosomal location of the ribosomal genes in *Triturus vulgaris meridionalis* (Amphibia Urodela). III. Inheritance of the chromosomal sites for 18S + 28S
rRNA. Chromosoma (Berl.) 65, 231-240 (1978).


Bradshaw, W.N.; Hsu, T.C.: Chromosomes of Peromyscus (Rodentia, Cricetidae) III. Polymorphism in Peromyscus


Burkholder, G.D.; Duczek, L.L.: The effect of chromosome


Callan, H.G.; Taylor, J.H.: A radioautographic study of the time course of male meiosis in the newt *Triturus*


Drets, M.E.; Folle, G.A.; Comings, D.E.: Mechanisms of chromosome banding. X. Chromosome and nuclear changes induced by photo-oxidation and their relation to R-banding with anti-C bodies. Chromosoma (Berl.) 69, 101-


Lacroix, J.C.: Variations expérimentales ou spontanées de la morphologie et de l'organisation des chromosomes en


Mancino, G.; Ragghianti, M.; Innocenti, S.B.: I cariotipi di Triturus marmoratus e T. cristatus studiati con il "C-


Mengden, G.A.: Linear differentiation of the C-band pattern of the W chromosome in snakes and birds. Chromosoma


Moritz, C.: The evolution of a highly variable sex chromosome in Gehyra purpurascens (Gekkonidae).


Musich, P.R.; Brown, F.L.; Maio, J.J.: Subunit structure of


Natarajan, A.T.; Klasterska, I.: Heterochromatin and sister chromatid exchanges in the chromosomes of *Microtus*


Ragghianti, M.; Bucci-Innocenti, S.; Mancino, G.: An


Sahar, E.; Latt, S.A.: Enhancement of banding patterns in


Schweizer, D.: Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma (Berl.) 56, 307-324


Sumner, A.T.; Evans, H.J.; Buckland, R.A.: Mechanisms


Vistorin, G.; Gamperl, R.; Rosenkranz, W.: Studies on sex


Some Aspects of the Cytology of European Newts (Genus Triturus).

Simon H. Sims.

Thesis for the degree of Doctor of Philosophy.

ABSTRACT

Of the 9 species of European newts, 7 are examined in this study. All have 24 (2n) bi-armed chromosomes. Giemsa C-banding and base-pair specific fluorochrome banding are employed to characterise the karyotypes.

Triturus cristatus and T. marmoratus have an unusual chromosomal situation. All individuals are heteromorphic for the largest chromosome (chromosome 1). Chromosomes 1 are heteromorphic for a region comprising most of the long arm which shows different banding patterns between the two homologues (chromosomes 1A and 1B). The heteromorphic regions are largely heterochromatic and are asynaptic and achiasmate at meiosis in both sexes. Banding patterns of the heteromorphic regions varies between the subspecies of T. cristatus and T. marmoratus. Individuals which are homomorphic for either chromosomes 1A or 1B show arrested development and eventual death at tail bud stage of embryogenesis, resulting in a 50% mortality of all offspring. A model for the evolution of the heteromorphism is suggested.

The sex chromosomes of Triturus are in an early stage of differentiation. The male is the heterogametic sex. Most species show a C-band heteromorphism between the tips of the long arms of the X and Y chromosomes. Evidence is presented which suggests that the Y-sex factors are contained within the small, often heterochromatic, region at the tip of the long arm of the Y chromosome.

Subspecies of T. cristatus and T. marmoratus are considered as a separate species group from the other Triturus species. The T. cristatus subspecies and T. marmoratus have karyotypes which share many homologies in the distribution of C-bands. There is little evidence for much structural rearrangements differentiating the species/subspecies, but quantitative C-heterochromatic change can be extensive. The remaining Triturus species show evidence for both structural and heterochromatic change. Chromosome studies can not usefully assess taxonomic affinities within Triturus, but have shown some interesting lines of karyotypic evolution.
SOME ASPECTS OF THE CYTOLOGY OF EUROPEAN NEWTS
GENUS (TRITURUS)

by

SIMON H. SIMS

Volume 2

TABLES AND FIGURES

A thesis submitted for the degree of
Doctor of Philosophy of the
University of Leicester

August, 1984
TABLE 1. The three Triturus species groups (after Lantz, 1947).

**THE ALPESTRIS GROUP.**

<table>
<thead>
<tr>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. alpestris alpestris</em></td>
</tr>
<tr>
<td><em>T. a. apuanus</em></td>
</tr>
<tr>
<td><em>T. a. cyreni</em></td>
</tr>
<tr>
<td><em>T. a. lacus-nigri</em></td>
</tr>
<tr>
<td><em>T. a. montenegrinus</em></td>
</tr>
<tr>
<td><em>T. a. piperianus</em></td>
</tr>
<tr>
<td><em>T. a. reiseri</em></td>
</tr>
<tr>
<td><em>T. a. serdarius</em></td>
</tr>
<tr>
<td><em>T. a. veluchiensis</em></td>
</tr>
</tbody>
</table>

**THE CRISTATUS GROUP.**

<table>
<thead>
<tr>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. cristatus cristatus</em></td>
</tr>
<tr>
<td><em>T. c. carnifex</em></td>
</tr>
<tr>
<td><em>T. c. danubialis (=dobrogicus)</em></td>
</tr>
<tr>
<td><em>T. c. karelinii</em></td>
</tr>
<tr>
<td><em>T. marmoratus marmoratus</em></td>
</tr>
<tr>
<td><em>T. m. pygmaeus</em></td>
</tr>
</tbody>
</table>

**THE VULGARIS GROUP.**

<table>
<thead>
<tr>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. boscai</em></td>
</tr>
<tr>
<td><em>T. helveticus helveticus</em></td>
</tr>
<tr>
<td><em>T. h. punctillatus</em></td>
</tr>
<tr>
<td><em>T. h. sequeirai</em></td>
</tr>
<tr>
<td><em>T. italicus</em></td>
</tr>
<tr>
<td><em>T. montandoni</em></td>
</tr>
<tr>
<td><em>T. vittatus vittatus</em></td>
</tr>
<tr>
<td><em>T. v. ophryticus</em></td>
</tr>
<tr>
<td><em>T. vulgaris vulgaris</em></td>
</tr>
<tr>
<td><em>T. v. ampelensis</em></td>
</tr>
<tr>
<td><em>T. v. borealis</em></td>
</tr>
<tr>
<td><em>T. v. graecus</em></td>
</tr>
<tr>
<td><em>T. v. meridionalis</em></td>
</tr>
</tbody>
</table>

TABLE 2. The relative lengths of the haploid karyotypes of T. cristatus cristatus, T. c. carnifex, T. c. karelinii and T. marmoratus.

Relative length = (length of chromosome / entire length of chromosome set) x 100

<table>
<thead>
<tr>
<th></th>
<th>marm</th>
<th>crist</th>
<th>carn</th>
<th>karel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.95(A)</td>
<td>11.89</td>
<td>11.02</td>
<td>13.11</td>
</tr>
<tr>
<td></td>
<td>11.64(B)</td>
<td>(0.13)</td>
<td>(0.03)</td>
<td>(0.15)</td>
</tr>
<tr>
<td>2</td>
<td>11.05</td>
<td>11.33</td>
<td>10.81</td>
<td>11.21</td>
</tr>
<tr>
<td></td>
<td>(0.12)</td>
<td>(0.10)</td>
<td>(0.12)</td>
<td>(0.08)</td>
</tr>
<tr>
<td>3</td>
<td>10.42</td>
<td>10.34</td>
<td>10.69</td>
<td>10.64</td>
</tr>
<tr>
<td></td>
<td>(0.11)</td>
<td>(0.09)</td>
<td>(0.11)</td>
<td>(0.13)</td>
</tr>
<tr>
<td>4</td>
<td>10.20</td>
<td>10.25</td>
<td>10.33</td>
<td>9.72</td>
</tr>
<tr>
<td></td>
<td>(0.13)</td>
<td>(0.12)</td>
<td>(0.10)</td>
<td>(0.13)</td>
</tr>
<tr>
<td>5</td>
<td>10.19</td>
<td>10.00</td>
<td>9.54</td>
<td>9.89</td>
</tr>
<tr>
<td></td>
<td>(0.09)</td>
<td>(0.11)</td>
<td>(0.06)</td>
<td>(0.12)</td>
</tr>
<tr>
<td>6</td>
<td>9.32</td>
<td>8.79</td>
<td>8.78</td>
<td>8.95</td>
</tr>
<tr>
<td></td>
<td>(0.08)</td>
<td>(0.08)</td>
<td>(0.09)</td>
<td>(0.07)</td>
</tr>
<tr>
<td>7</td>
<td>8.08</td>
<td>8.57</td>
<td>8.33</td>
<td>8.78</td>
</tr>
<tr>
<td></td>
<td>(0.06)</td>
<td>(0.08)</td>
<td>(0.07)</td>
<td>(0.08)</td>
</tr>
<tr>
<td>8</td>
<td>7.20</td>
<td>7.42</td>
<td>7.52</td>
<td>7.33</td>
</tr>
<tr>
<td></td>
<td>(0.08)</td>
<td>(0.11)</td>
<td>(0.06)</td>
<td>(0.08)</td>
</tr>
<tr>
<td>9</td>
<td>5.82</td>
<td>5.73</td>
<td>6.56</td>
<td>5.81</td>
</tr>
<tr>
<td></td>
<td>(0.06)</td>
<td>(0.04)</td>
<td>(0.07)</td>
<td>(0.07)</td>
</tr>
<tr>
<td>10</td>
<td>5.58</td>
<td>5.53</td>
<td>6.35</td>
<td>5.76</td>
</tr>
<tr>
<td></td>
<td>(0.09)</td>
<td>(0.04)</td>
<td>(0.03)</td>
<td>(0.05)</td>
</tr>
<tr>
<td>11</td>
<td>5.48</td>
<td>5.73</td>
<td>5.78</td>
<td>5.30</td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
<td>(0.08)</td>
<td>(0.03)</td>
<td>(0.04)</td>
</tr>
<tr>
<td>12</td>
<td>4.37</td>
<td>4.42</td>
<td>4.72</td>
<td>4.17</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.09)</td>
<td>(0.04)</td>
<td>(0.06)</td>
</tr>
</tbody>
</table>

Figures in brackets indicate the standard error. The standard error for marmoratus chromosome 1A = 0.12 and for chromosome 1B = 0.13.

marm = T. marmoratus, n = 10
crist = T. c. cristatus, n = 8
carn = T. c. carnifex, n = 10
karel = T. c. karelinii, n = 10
TABLE 3. The centromere indices of the haploid karyotypes of *T. cristatus cristatus*, *T. c. carnifex*, *T. c. karelinii* and *T. marmoratus*.

<table>
<thead>
<tr>
<th>Centromere Index = length of short arm/entire length of chromosome</th>
<th>marm</th>
<th>crist</th>
<th>carn</th>
<th>karelini</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 0.23(A) 0.26(B)</td>
<td>0.36</td>
<td>0.38</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>2 0.46 0.47</td>
<td>0.46</td>
<td>0.46</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>3 0.43 0.48</td>
<td>0.45</td>
<td>0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 0.48 0.48</td>
<td>0.47</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 0.42 0.44</td>
<td>0.45</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 0.46 0.34</td>
<td>0.36</td>
<td>0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 0.32 0.45</td>
<td>0.43</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 0.43 0.48</td>
<td>0.47</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 0.44 0.40</td>
<td>0.47</td>
<td>0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 0.40 0.42</td>
<td>0.44</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 0.43 0.41</td>
<td>0.43</td>
<td>0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 0.35 0.37</td>
<td>0.37</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The standard error for all values is less than or equal to 0.01.

marm = *T. marmoratus*, n = 10  
crist = *T. c. cristatus*, n = 8  
carn = *T. c. carnifex*, n = 10  
karelini = *T. c. karelinii*, n = 10
TABLE 4. The length of the heteromorphic region as a percentage of the length of chromosome 1.

<table>
<thead>
<tr>
<th></th>
<th>marm</th>
<th>crist</th>
<th>carn</th>
<th>karel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>54.17% (0.54)</td>
<td>51.69% (0.65)</td>
<td>46.74% (0.61)</td>
<td>51.26% (0.48)</td>
</tr>
<tr>
<td>1B</td>
<td>44.31% (0.67)</td>
<td>47.24% (0.63)</td>
<td>46.28% (0.63)</td>
<td>46.31% (0.35)</td>
</tr>
<tr>
<td>Average</td>
<td>49.23%</td>
<td>49.47%</td>
<td>46.51%</td>
<td>48.79%</td>
</tr>
</tbody>
</table>

Figures in brackets indicate the standard error.

marm = *T. marmoratus*, n = 10  
crist = *T. c. cristatus*, n = 8  
carn = *T. c. carnifex*, n = 10  
karel = *T. c. karelinii*, n = 10

Note: Measurements for tables 2-4 were taken from between 2 and 5 individuals, depending on species/subspecies. Chromosome spreads from 5 individuals were used for *marmoratus* and *carnifex*, 3 for *cristatus* and 2 for *karelinii*. For each chromosome, both chromatids were measured and averaged to give a value for that chromosome.
**TABLE 5. Distribution of chiasmata in both sexes of *Triturus* species.**

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T. alpestris</strong> apuanus</td>
<td>No localisation (P.S.)</td>
<td>Frequently procentric (4)</td>
</tr>
<tr>
<td><strong>T. boscai</strong></td>
<td>Mainly terminal, some subterminal or intercalary (1)</td>
<td>Preferentially procentric (1)</td>
</tr>
<tr>
<td><strong>T. cristatus</strong> subspecies</td>
<td>No localisation (5 &amp; P.S.)</td>
<td>Frequently procentric (2 &amp; 5)</td>
</tr>
<tr>
<td><strong>T. helveticus</strong></td>
<td>Terminal localisation (5 &amp; 9)</td>
<td>No localisation (5 &amp; 9)</td>
</tr>
<tr>
<td><strong>T. italicus</strong></td>
<td>No localisation (3)</td>
<td>Frequently procentric (6)</td>
</tr>
<tr>
<td><strong>T. marmoratus</strong></td>
<td>No localisation, but often distal (7 &amp; P.S.)</td>
<td>Frequently procentric (8)</td>
</tr>
<tr>
<td><strong>T. montandoni</strong></td>
<td>Not recorded</td>
<td>Not recorded</td>
</tr>
<tr>
<td><strong>T. vittatus</strong></td>
<td>Not recorded</td>
<td>Procentric localisation (1)</td>
</tr>
<tr>
<td><strong>T. vulgaris</strong></td>
<td>Terminal localisation (5, 9 &amp; P.S.)</td>
<td>No localisation (5 &amp; 9)</td>
</tr>
</tbody>
</table>

P.S. - present study

(1) Bucci-Innocenti et al., 1983b
(2) Callan and Lloyd, 1960
(3) Mancino et al., 1977
(4) Mancino and Barsacchi, 1965
(5) Watson and Callan, 1963
(6) Mancino and Barsacchi, 1969
(7) Lantz and Callan, 1954
(8) Nardi et al., 1972a
(9) Spurway and Callan, 1960
Spermatogonial mitoses from *T. cristatus cristatus* stained with orcein. Secondary constrictions are normally seen at the site of the NOR, but cannot be resolved in these newt chromosomes. The site of the major NOR is in a subterminal position of the long arm of chromosome 9, a 'small group' chromosome.

Scale bar = 10 um
Fig. 2  
T. marmoratus: Mitotic chromosomes from a larva that had been kept at 0-4 degrees C for several days.

a) Phase-contrast micrograph of fixed chromosomes squashed in 45% acetic acid. Cold-induced constrictions are present at many chromosomal sites.

b) The same mitotic chromosomes after C-banding. All cold-induced constrictions occur at sites of C-positive constitutive heterochromatin.

Scale bar = 10 um.
Fig. 3  C-banded karyotype of *T. cristatus cristatus* from a spermatogonial mitotic metaphase.

Fig. 4  C-banded karyotype of *T. cristatus carnifex* from a spermatogonial mitotic metaphase.

Scale bar = 10 um.
Fig. 5  C-banded karyotype of *T. cristatus karelinii* from an intestinal epithelial metaphase.

Fig. 6  C-banded karyotype of *T. marmoratus* from an intestinal epithelial metaphase.

Scale bar = 10 um.
Figs. 7 & 8  C-banded mitotic karyotypes of *T. cristatus carnifex* from spermatogonial metaphases. The karyotypes are taken from different individuals of the same population. The chromosomes show the types of C-band differences between individuals. Some chromosomes may show diminished, enlarged, duplicated or even absence of C-bands at any one site.

Scale bar = 10 um.
Figs. 9 & 10  C-banded mitotic karyotypes of *T. cristatus carnifex* from spermatogonial metaphases. The karyotypes show further C-band polymorphisms within a population.

Scale bar = 10 um.
Fig. 11  C-banding patterns of chromosomes 1A (upper row) and the corresponding chromosomes 1B (lower row) of *T. marmoratus*.

Fig. 12  C-banding patterns of chromosomes 1A (upper row) and the corresponding chromosomes 1B (lower row) of *T. c. carnifex*. 
Fig. 13  C-banding patterns of chromosomes 1 from T. c. karelinii. Corresponding homologues are arranged in vertical pairs.

Fig. 14  C-banding patterns of chromosomes 1 from T. c. cristatus. Corresponding homologues are arranged in vertical pairs.
Fig. 15  Three pairs of chromosomes from *T. c. carnifex*. The chromosome 1A in these pairs (left) differs from the typical form in that the heterochromatic, heteromorphic region extends to the tip of the long arm and the distal segment is heavily stained. This variant form of chromosome 1A was found in only two individuals of the population, with the typical form of chromosome 1B (right).

Fig. 16  a–d  C-banded interphase nuclei showing the sizes of heterochromatic blocks in: a) *T. c. cristatus*, b) *T. c. carnifex*, c) *T. c. karelinii* and d) *T. marmoratus*. The C-positive heteromorphic regions of chromosomes 1 can not be identified in interphase nuclei. Note the large chromocentre of fused centromeric heterochromatin in *T. c. karelinii*.

Scale bar = 10 um.
Fig. 17  C-banded spermatogonial nuclei entering mitotic prophase from *T. c. cristatus*. a)-f) show a progression in chromatin condensation. As the chromatin condenses, the heterochromatic blocks of the heteromorphic region come together, making it clearly distinguishable.

Scale bar = 10 um.
Fig. 18 Orcein staining of male meiotic prophase in *T. c. cristatus*. a) and b) show leptotene nuclei, where chromatin is beginning to condense. c), d) and e) show zygotene nuclei with different degrees of homologous pairing. The chromosome tips pair first, at one pole of the nucleus. Pairing proceeds down the chromosome arms towards the centromeres at the opposite pole. The arrow in e) indicates a small region of a chromosome pair which has not yet completed synapsis. f)-h) show pachytene nuclei. The paired nature of homologous chromomeres is evident in f). Arrows in g) and h) show a chromosomal region which has remained asynaptic. This region is likely to be the heteromorphic region of chromosome 1.

Scale bar = 10 um.
Fig. 19  Orcein staining of male meiosis in *T. c. cristatus*. a) and b) show diplotene/diakinesis stages. Crossing-over between chromatids is clearly visible. c) shows a metaphase II spread, before the final meiotic division. The metaphase II chromosomes are highly condensed and adopt an 'X' conformation.

Scale bars = 10 um.
Fig. 20 Longitudinal section of an immature testis from Trichuris c. carnifex, stained with crystal violet.

a) Low power montage of the entire section. The section comprises one lobe of the testis. The lobe is subdivided into lobules. Within each lobule are a number of follicles which comprise many germ cells. The cells within a follicle are all synchronised at the same stage i.e. mitotic, meiotic, maturing spermatids, etc.. The spermatogenic wave is passing from the bottom right to the top left of the picture. Spermatogonial mitoses are found in the bottom left of the section (b). Meiotic stages are found in the middle region of the testis (c and d). c) shows a pachytene nucleus and d) shows a first meiotic metaphase. Towards the top left of the section, follicles are found with differing stages of sperm maturation. No completely mature sperm bundles were seen in this testis. e) shows some elongating spermatids.

Scale bar for 'a' = 1 mm.
Scale bar for 'b-e' = 10 um.
Longitudinal section through a mature testis from *T. c. carnifex*, stained with Mayer's haematoxylin and eosin.

a) Low power montage of the testis section. The testis is composed of two lobes. The upper lobe contains essentially spermatogonia undergoing mitotic divisions. The lower lobe is composed of numerous bundles of completely mature sperm. No meiotic stages were present in the testis. The animal was in good breeding condition with a high dorsal crest. The breeding condition of the animal is evident from the presence of sperm in the vas deferens (arrow). Prior to excising the testes, the animal was treated with colchicine to arrest chromosomes at metaphase. Colchicine depolymerises the microtubules of the mitotic spindle. Hence, mitotic chromosomes adopt a random orientation in the nucleus (b). All sperm heads align themselves within a follicle. The elongate sperm heads are distinguished from the tails because of their intense staining with Mayer's haematoxylin (c).

Scale bar for 'a' = 1 mm.
Scale bars for 'b & c' = 10 um.
Fig. 22  C-banded male meiotic stages in *T. c. cristatus*.
a) Two spermatocyte nuclei after a mitotic division. The heterochromatic blocks of the centric and pericentric regions are concentrated at one pole of the nuclei.
b) and c) Leptotene nuclei.
d) A late zygotene to early pachytene nucleus. Paired chromosomes have adopted a bouquet arrangement.
e) and f) Zygotene/pachytene nuclei showing that the deeply staining heteromorphic region of chromosome 1 have not synapsed (arrows).

Scale bar = 10 um.
Fig. 23  C-banded male meiotic stages in *T. c. cristatus*.

a) A late zygotene to early pachytene nucleus, where the euchromatic tips of the long arms of chromosomes 1 have paired (arrows), but the heteromorphic region has remained asynaptic.

b) and c) Metaphase I chromosomes. Bivalents have condensed considerably and chiasma positions are easily distinguished. In 'b', many bivalents are ring-shaped because of terminally located chiasmata. In instances like this one, the ring-shaped bivalents tend to interlock, resulting in chain conformations.

d) Anaphase I. Half-bivalents have just separated from the metaphase 1 plate.

Scale bar = 10 um.
Fig. 24 Chromosomes 1 in male meiosis. Chiasmata occur in different positions of the euchromatic regions, but never within the heteromorphic region.

Column a) *T. c. cristatus*. The arrow indicates where a rod shaped bivalent 1 is threaded through a smaller ring-shaped bivalent.

Column b) *T. c. carnifex*.

Column c) *T. marmoratus*. Chiasmata are also absent from the euchromatic region proximal to the centromere in the long arm.
Fig. 25 A first meiotic metaphase from T. marmoratus. On the rare occasion, chromosomes 1 appear as univalents.

Fig. 26 A first meiotic metaphase from T. c. cristatus.

Fig. 27 A first meiotic metaphase from T. c. carnifex.

Scale bars = 10 um.
Figs. 28 & 29  Chromosomes from *T. c. carnifex* embryos showing arrested development before hatching. In both cases, two chromosomes 1A were found together in all metaphases.

Fig. 30  Chromosomes from a *T. marmoratus* embryo that showed arresting development. In this case, the embryo was homomorphic for chromosome 1B.

Scale bar = 10 um.
Fig. 31 Chromosomes 1 of an arresting embryo from a hybrid mating between *T. c. carnifex* and *T. marmoratus*. The embryo has a chromosome 1A from both parental species.

Fig. 32 Chromosomes from another 'hybrid' arresting embryo. Chromosomes 1A from both parental species are evident. It is interesting to note that the chromosome 1A from *T. c. carnifex* is a variant form, as shown in Fig. 15.

Scale bar = 10 um.
Fig. 33 Fluorochrome banding of chromosomes 1 with actinomycin D/DAPI. AT-rich regions fluoresce brighter than euchromatin. Corresponding homologues are arranged in vertical pairs.

a) *T. c. cristatus*,
b) *T. c. carnifex*,
c) *T. c. karelinii*,
d) *T. marmoratus*. 
Fluorochrome banding of chromosomes 1 using mithramycin counterstained with either distamycin-A or methyl green. The heteromorphic region fluoresces slightly brighter than euchromatin, indicating a bias towards GC-richness. Two chromosome pairs are shown for:

a) *T. c. cristatus*,
b) *T. c. carnifex*,
c) *T. c. karelinii* and
d) *T. marmoratus*.

Some individuals from *T. c. carnifex* show a GC-rich, brightly fluorescing band in the heteromorphic region of one homologue.
Fig. 35  
A mitotic chromosome set from *T. c. cristatus* stained to show GC-rich regions with distamycin-A/mithramycin (a). In (b), the same preparation has been destained and restained to show AT-rich regions with actinomycin D/DAPI. Mithramycin bright bands appear as DAPI dull bands and vice-versa.

Scale bar = 10 um.
**Fig. 36**  
*T. c. karelinii* chromosomes at different stages throughout the C-banding procedure.

a) Fixed, squashed chromosomes (phase-contrast).

b) The same chromosomes after barium hydroxide and hot 2xSSC treatments. The chromosomes appear swollen under phase-contrast optics.

c) Phase-contrast micrograph after Giemsa staining. 'Bulky' regions appear refractile and correspond to Giemsa C-positive regions (d).

Scale bar = 10 um.
Fig. 37  
(a) Phase-contrast micrograph of fixed, squashed chromosomes from *T. c. karelinii*, showing distinct centromeric constrictions.  
(b) The same chromosomes after C-banding. Centromeric constrictions are obscured by the large centromeric C-bands.

Fig. 38  
Spermatogonial mitotic chromosomes from *T. c. carnifex* after C-banding using sodium hydroxide rather than barium hydroxide in the alkali denaturation step. The chromosomes are weakly stained, except for a 'dot' over each chromatid at the site of the centromere. Some chromosomes show dots in intercalary regions. Nucleoli in interphase nuclei (arrows) are also stained. Nucleoli are not normally visible after C-banding.

Scale bars = 10 µm.
Fig. 39  Mitotic karyotype from *T. c. carnifex*. The chromosomes were taken through the C-banding procedure, but stained with quinacrine rather than Giemsa. Most C-band positive regions fluoresce brightly, suggesting AT-richness, except the NORs on chromosome 9, which quench the fluorescence (arrows). All centromeric heterochromatin fluoresces brightly.

Fig. 40  Mitotic chromosomes from *T. c. carnifex* stained with actinomycin D/quinacrine. The centric heterochromatin of some chromosomes stains brighter than euchromatin.

Scale bars = 10 um.
Fig. 41 Mitotic karyotype from *T. c. cristatus* stained with distamycin A/mithramycin. GC-rich regions fluoresce brightly. Arrow marks the NOR.

Fig. 42 Mitotic karyotype from *T. c. cristatus* stained with actinomycin D/DAPI. AT-rich regions fluoresce brightly.

Scale bars = 10 um.
Fig. 43  
Mitotic chromosomes from *T. c. carnifex* stained with actinomycin D/DAPI. AT-rich regions fluoresce brightly.

Fig. 44  
Mitotic chromosomes from *T. c. carnifex* stained with distamycin A/mithramycin. GC-rich regions fluoresce brightly. Arrows show the NOR.

Scale bars = 10 um.
Fig. 45  
a & b  
Mitotic chromosomes from T. c. karelinii stained with distamycin A/mithramycin. GC-rich regions fluoresce brightly. NORs fluoresce brightly (arrows).

Scale bars = 10 um.
Fig. 46  Mitotic chromosomes from *T. c. karelinii* stained with actinomycin D/DAPI. AT-rich regions fluoresce brightly.

Scale bars = 10 um.
Fig. 47 Mitotic chromosomes from *T. marmoratus* stained with methyl green/mithramycin. The GC-rich NOR fluoresces brightly (arrows).

Scale bars = 10 um.
Fig. 48 a & b Mitotic chromosomes from *T. marmoratus* stained with actinomycin D/DAPI. AT-rich regions fluoresce brightly.

Scale bars = 10 um.
Fig. 49 Mitotic chromosomes of *T. marmoratus* from the intestinal epithelium of a male newt. Many chromosome spreads from the intestine showed structural rearrangements. Most of the translocational rearrangements involved a chromosome 1B (a-g). However, one rearrangement was found to involve a chromosome 1A (h). Some dicentric chromosomes were found (g and h).
Fig. 50 Mitotic chromosomes 6 from an individual of *T. c. cristatus*. Chromosome 6 is typically a submetacentric element (upper row). However, a pericentric inversion has caused the homologue to become more metacentric (lower row). The chromosomes are C-banded and stained with actinomycin D/DAPI to highlight the centric and pericentric heterochromatin. The inversion has involved a small chromosomal region extending from a position below the pericentric heterochromatin of the short arm, down into the long arm, including the pericentric heterochromatin of the long arm.

Figs. 51 and 52 Metaphase I spreads from the same individual of *T. c. cristatus*. The inversion heterozygosity of chromosome 6 does not greatly affect the formation of chiasmata in the meiotic bivalent (arrows). However, chiasmata are not seen within the region of the pericentric inversion.

Scale bars = 10 um.
Fig. 53  Haploid mitotic karyotypes from members of the *cristatus* species group. From left to right, the corresponding chromosomes are of *T. c. cristatus*, *T. c. carnifex*, *T. c. karelinii* and *T. marmoratus*.

The corresponding Y chromosomes (chromosome 4) from the above karyotypes:
Fig. 54 Scheme for the evolution of marmoratus-type chromosomes 1A and 1B from a more metacentric cristatus-type ancestral heteromorphic pair, Aa and Ba (i). A major pericentric inversion (INV1)(ii) produced a more submetacentric, marmoratus-type chromosome 1B (Bm(a)); crossing-over in the remaining interstitial euchromatin (iii-v) generated ancestral marmoratus-type chromosomes 1A (Am(a)) and 1B (Bm(a)); and a further small paracentric inversion in the ancestral marmoratus chromosome 1B (INV2) moved the pericentric heterochromatin (pc) of this chromosome down towards the start of the heteromorphic region of the long arm and generated the present day situation (Am and Bm, vi). This diagram does not take into account changes in length of either the heteromorphic regions or the chromosomes themselves.
Fig. 55 Two pairs of C-banded mitotic sex chromosomes from:

a) *T. helveticus helveticus*,
b) *T. vulgaris vulgaris*,
c) *T. boscai*,
d) *T. alpestris apuanus*,
e) *T. cristatus cristatus*,
f) *T. c. carnifex*,
g) *T. c. karelinii*,
h) *T. marmoratus* and
i) *T. vittatus ophryticus*.

Meiotic stages were found in some species. In these cases, sex-bivalents are shown below the mitotic chromosomes.
Fig. 56  C-banded first meiotic metaphase from *T. alpestris apuanus*. Chiasmata show no localisation in distribution.

Fig. 57  C- banded first meiotic metaphase from *T. vulgaris vulgaris*. Chiasmata show terminal localisation.

Scale bars = 10 um.
Fig. 58  C-banding of first meiotic anaphase of male meiosis in *T. c. cristatus*. A chiasma has taken place in the long arm of the sex bivalent, exchanging the terminal heterochromatin between chromatids of each half-bivalent (arrows).

Scale bar = 10 um.
Fig. 59  C-banded mitotic karyotype from a spermatogonial metaphase of *T. alpestris apuanus*.

Fig. 60  C-banded mitotic karyotype from a spermatogonial metaphase of *T. boscai*.

Scale bars = 10 um.
Fig. 61  C-banded mitotic karyotype from a spermatogonial metaphase of *T. helveticus helveticus*.

Fig. 62a  C-banded mitotic karyotype from a spermatogonial metaphase of *T. vulgaris vulgaris*. An additional *vulgaris* karyotype is shown in Fig. 62b (next page).

Scale bars = 10 um.
Fig. 62b  C-banded mitotic karyotype from a spermatogonial metaphase of *T. vulgaris vulgaris*.

Fig. 63  C-banded mitotic karyotype from an intestinal epithelial metaphase of *T. vittatus ophryticus*.

Scale bars = 10 μm.
Fig. 64 Mitotic chromosomes from the intestinal epithelium of *T. vittatus*, stained with methyl green/mithramycin. The NOR appears as a GC-rich, bright band on chromosome 9 (arrow).

Fig. 65 Mitotic chromosomes from spermatogonial mitoses of *T. boscai*, stained with methyl green/mithramycin. The NOR appears as a bright band in a pericentric region on the long arm of chromosome 10 (arrows).

Scale bars = 10 μm.
Fig. 66  C-banded mitotic karyotype from a spermatogonial metaphase of a male F1 hybrid between *T. h. helveticus* and *T. v. vulgaris*. The *helveticus* chromosomes are on the left in each pair, *vulgaris* are on the right.

Fig. 67  Sperm bundle from the hybrid, indicating that the animal was fertile.

Scale bars = 10 um.
Fig. 68 Examples of each chromosome in the C-banded mitotic karyotype of the hybrid between *helveticus* and *vulgaris*. The chromosomes were taken from spermatogonial metaphases. *T. helveticus* chromosomes are in the upper row and *T. vulgaris* in the lower row for each chromosome.
Fig. 69 Partial C-banded karyotype taken from an intestinal mitosis of *T. h. helveticus*. Chromosome 7 may show a distinct C-band in the terminal region of the short arm (arrow).

Scale bar = 10 um.