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Structure and Evolution of Supernumerary Chromosomes in the Pacific Giant Salamander *Dicamptodon tenebrosus*.

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Abstract

The Pacific Giant salamander, *Dicamptodon tenebrosus*, from the Pacific Northwest of North America has variable numbers of supernumerary chromosomes, from 0 to 10, per individual. B chromosome frequency among populations varies geographically such that salamanders from the most southern and northern regions have lower average numbers of B chromosomes than salamanders in the middle of the range. This variation in B frequency may be correlated with both historical and climatic factors. To assess how the supernumerary chromosomes originated in *D. tenebrosus*, B chromosome DNA was isolated by microdissection and amplified by degenerate oligonucleotide-primed PCR. The B DNA hybridized similarly to genomic DNA from individuals of *D. tenebrosus* and the related species *D. copei* and *D. ensatus* demonstrating that the supernumerary chromosomes were derived from the normal chromosome complement. Unique hybridization bands in both *D. copei* and *D. tenebrosus* suggest that the shared sequences have evolved independently.

Résumé

La salamandre géante du Pacifique, *Dicamptodon tenebrosus* qui habite le Nord-Ouest Pacifique de l'Amérique du Nord possède un nombre de chromosomes surnuméraires variant de 0 à 10 par individu. La fréquence de ces chromosomes B varie selon la distribution géographique, de sorte que les salamandres se trouvant dans les régions plus au nord et plus au sud possèdent en moyenne moins de chromosomes B que les salamandres provenant de la région intermédiaire. Cette variation dans le nombre de chromosomes B peut être corrélée à des facteurs historiques ainsi que climatiques. Afin de déterminer l'origine des chromosomes surnuméraires chez *D. tenebrosus*, nous avons isolé puis amplifié l'ADN des chromosomes B par microdissection et par PCR utilisant des oligonucléotides dégénérés. L'ADN B s'hybride de façon similaire à l'ADN provenant de *D. tenebrosus* et aux espèces apparentées *D. copei* et *D. ensatus*, ce qui démontre que les chromosomes surnuméraires sont dérivés du complément chromosomique normal. La présence de bandes d'hybridation uniques observée chez *D. copei* et *D. tenebrosus* suggère que les séquences qui sont partagées par les deux espèces ont eu une évolution indépendante.

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1. Introduction and Literature Review

This thesis is an exploration of the structure, abundance and evolution of supernumerary, or B, chromosomes in the Pacific Giant salamander, *Dicamptodon tenebrosus* (Caudata: Dicamptodontidae). Beyond the karyology, little is known about the B chromosomes in this species. However, recent technological advances have allowed us to broaden our examination of the B chromosomes in *D. tenebrosus*. I present here a detailed evaluation of the molecular structure of the supernumerary chromosomes which is used to determine the possible modes of origin and evolution of the B chromosomes in *D. tenebrosus*. As well, I review the geographic distribution of supernumerary chromosomes in populations of this salamander in relation to both historical and climatic factors.

Supernumerary, or B, chromosomes have been found in 10-15% of karyotyped living species (Beukeboom, 1994; Jones, 1995). Some species are more thoroughly studied than others since some are much more difficult to analyze. In amphibians, B chromosomes occur in approximately 2% of species thus far examined: 11% of 156 karyotyped salamander species, 1% of 1000 anuran species, and no caecilians in 21 species (Kuramoto, 1990; King, 1990; Green, 1991). Species with B chromosomes are continuously being discovered. The advent of new cytological techniques (Beukeboom, 1994; Jones, 1995), for example pulsed-field gel electrophoresis (PFGE), has recently allowed researchers to identify B chromosomes even in fungi (Covert, 1998).

Supernumerary chromosomes were defined by Jones and Rees (1982) as dispensable, extra chromosomal elements which appear in addition to the normal chromosome complement, or A chromosomes. B chromosomes are often morphologically unlike the A chromosomes and are unable to pair with them at meiosis. B chromosomes exhibit non-Mendelian inheritance, vary in number among and within individuals and, in many cases, lack major gene loci. They usually appear to have no qualitative effect upon the host's phenotype yet may depress fertility and growth when present in high numbers (Jones and Rees, 1982; Jones, 1995). When B chromosomes are present in odd, rather than in even numbers their effects are often more pronounced (Beukeboom, 1994; Jones, 1995). B chromosomes, in some instances, have been found to affect the frequency of cross-over events and alter their location (Beukeboom, 1994). But extreme variation in the characteristics of these chromosomes exists. Due to their variability, J. P. M. Camacho and J. S. Parker (quoted in Beukeboom, 1994) updated the definition of a B chromosome to "a dispensable supernumerary chromosome that does not recombine with the A chromosomes and follows its own evolutionary pathway". Similarly, Jones (1995) defined B chromosomes as "dispensable supernumeraries which do not recombine with any members of the basic A chromosome set and which have irregular and non-Mendelian modes of inheritance".

1.1 Variation in B Chromosomes

In natural populations, environmental variables such as altitude, latitude, rainfall and temperature have been correlated with B chromosome distribution (Jones and Rees, 1982; Beukeboom, 1994). The effects of B chromosomes appear to be tolerable only under environmental conditions favorable for the host species' growth where selection pressure is low (Jones and Rees, 1982). Hewitt and Brown (1970) observed a cline in B frequency correlated with environmental conditions in the grasshopper *Myrmeleotettix maculatus* (Orthoptera: Acrididae) in Britain. Several climatic factors such as cloud, rainfall, onshore winds and/or summer temperature were the predominant factors correlated with B chromosome distribution. In populations of maize (*Zea mays mays*; Poaceae) from Northern Argentina, the mean number of Bs was found to correlate positively with the altitude of cultivation and negatively with the A-DNA content (Rosato et al., 1998; Poggio et al., 1998). However, the A-DNA content is negatively correlated with altitude. Thus, although the B frequency was positively correlated with altitude, there is a maximum limit to nuclear DNA mass such that Bs at high frequencies are better tolerated in populations with low A-DNA content (Rosato et al., 1998; Poggio et al., 1998).

Extensive geographic supernumerary chromosome variation also appears in the New Zealand frog *Leiopelma hochstetteri* (Anura: Leiopelmatidae; Green, 1988). This species' range consists of several geographically and genetically isolated populations which exhibit variation in

the number and form of supernumerary chromosomes present. There are small supernumerary chromosomes that vary in number, from 0 to 15, among some populations (Green, 1988). One large, frequently distinctive supernumerary chromosome (W) is present in females yet absent from males in North Island populations. The W chromosome is not found in Great Barrier Island populations (Green, 1988). This chromosome variation is so drastic it has been used to identify important subdivisions of the species which merit individual consideration in conservation practices (Green, 1994).

B chromosome presence in the grasshopper *Eyprepocnemis plorans* (Orthoptera: Acrididae) ends abruptly at the same location along four different rivers in Spain (Cabrero et al., 1997). At this location a narrow pass that is surrounded by dry areas exists. This region is inhospitable for this grasshopper (Cabrero et al., 1997). Previous studies have shown that supernumerary chromosomes in this species are lowest in frequency in dry areas where populations are scarce and small (Henriques-Gil et al., 1984). From this example, it appears that B chromosome distribution and frequency is dependent upon environmental selection pressures. However, rather than the environment regulating the spread of B chromosomes, Bs could regulate their own spread to new niches by inducing genetic variability in their carriers such that populations with different ecological preferences could arise (Sapre and Deshpande, 1987). The absence of B chromosomes in some populations may also be determined historically, with B chromosomes not having reached those populations from their centre of origin (Beukeboom, 1994). In the case of *E. plorans*, Cabrero et

al. (1997) explain the absence of B chromosomes in six of the locations sampled by the fact that, in its advance from the coast, the B chromosome has not yet reached these locations.

1.2 Chromosome Structure

Tandemly repeated DNA sequences, or 'satellite DNA', are very common within both coding and noncoding regions of eukaryotic genomes (Singer and Berg, 1991; Stephan and Cho, 1994). These sequences are generally found in high copy number in the centromeres and telomeres of animal and plant chromosomes and usually constitute much of the total genomic DNA (Singer and Berg, 1991; Stephan and Cho, 1994).

Tandem repeats, often associated with heterochromatin and genetically silent, may accumulate or disappear without disturbing the function of important coding or regulatory segments (Singer and Berg, 1991). These sequences may be present in homologous chromosome regions in closely related species yet may vary in copy number between species (Stephan and Cho, 1994; Vershinin et al., 1996). The evolution of these satellite sequences may occur through unequal recombination which, by introducing similar or identical sequences, can cause rapid changes in the number of repeat units in a chromosome region (Brutlag, 1980). Tandem repeats may diverge independently of each other and accumulate changes at different rates when compared to gene rich regions of the genome through single base changes, long insertions or deletions, homogenization between copies, or mutation of the

primary sequence (Brutlag, 1980; Vershinin et al., 1996). Mobile genetic elements found adjacent to, or within, highly repeated satellite DNA arrays may provide a mechanism whereby these sequences may move from chromosome to chromosome and increase genetic variation among closely related species (Brutlag, 1980; Vershinin et al., 1996).

In the wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae), palindromic regions associated with paternal sex ratio (PSR)-specific repeat DNA may play a role in the amplification of the repeat DNA (Reed et al., 1994). The amplification of repeats on supernumerary chromosomes such as PSR could be a major contributor to the evolution of B chromosomes in several species. This amplification could increase the amount of centromeric heterochromatin thus conveying a selective advantage to the chromosome by increasing its stability and likelihood of transmission (Reed et al., 1994).

Many families of dispersed repetitive DNA, including transposable elements, retrotransposons, and retrogenes, are mobile within the genome. Short (SINES) or long interspersed repetitive sequences (LINES) are other types of repetitive units that constitute a large part of many mammalian genomes (Singer and Berg, 1991). LINES are often capable of moving, via amplification and transposition, to new genomic sites. Their effects are unclear although these repetitive units may be considered selfish because of their ability to multiply themselves and disperse. SINE- and LINE-like sequences are also found in other organisms including amphibians (Singer and Berg, 1991).

The European plethodontid salamander, *Hydromantes* (Caudata: Plethodontidae), has a 513-base pair (bp) highly repeated centromeric satellite DNA (Hy500) that represents about 1% of its genome (Batistoni et al., 1991). This sequence is highly conserved between species suggesting that it has not changed considerably for approximately 20 million years (Batistoni et al., 1991). Another highly repetitive family (Hy/Pol III) is present in about 6% of the genome of this genus. The repeat units of the Hy/Pol III family consist of short (about 200-bp) tandemly repeated sequences that are flanked by two short direct repeats and are dispersed in clusters throughout the genome (Batistoni et al., 1995). The structure of this repeat unit likely arose through the integration of two similar retroposons into a transposition 'hot spot', potentially the RNA polymerase III promoter motif, on the sequence. This mechanism may, without adversely affecting the host's genetic make-up, allow the integration of transposable elements in genomic regions that are genetically silent. Amphibian genomes do have a high tendency for tandem duplication of short retroposons (Batistoni et al., 1995).

A family of long repetitive DNA sequences (Hsr 1), some having characteristics of retrotransposons, have also been found in approximately 10^6 copies interspersed throughout the genome of *Hydromantes* (Marracci et al., 1996). These sequences are highly conserved in closely related species and also appear in distantly related species. This suggests that Hsr 1 elements arose, before speciation occurred, from a common ancestor that carried its progenitor sequence. These retrotransposon sequences, likely transcriptionally silent since

most are methylated in the genome, are efficient at amplification and thus may be able influence the host genome's evolution by increasing the total mass of DNA or generating allelic variants (Marracci et al., 1996).

Hydromantes is one example of an amphibian genus that has several different repetitive sequences which make up much of its very large genome, one of the largest among vertebrates (Batistoni et al., 1991; Batistoni et al., 1995; Marracci et al., 1996). Some of these are transposon-like sequences and may, therefore, be candidates for sequences which could move and amplify in the specific regions of the genome.

Repetitive sequences have also been found in abundance in other amphibians such as the red spotted newt, *Notophthalmus viridescens* (Caudata: Salamandridae; Diaz et al., 1981; Epstein et al., 1986). Two different tandemly repeated sequences have been characterized in this species; one is found in both the pericentric heterochromatin and the spacer regions between histone gene clusters while the other occurs in clusters throughout the genome (Diaz et al., 1981; Epstein et al., 1986).

1.3 Function of B Chromosomes

It has been thought that supernumerary chromosomes generally lack genes or gene activity (Jones and Rees, 1982) although this is now known not always to be true (Green, 1990; Jones, 1995; Covert, 1998). For example, supernumerary chromosomes in both of the fungi ***Cochliobolus carbonum*** (Ascomycota: Pleiosporaceae) and *Nectria haematococca* (Ascomycota:

Hypocreaceae) carry genes that contribute to the disease-causing capacity of these pathogenic fungi on specific host plants and appear to be functionally analogous to bacterial plasmids (Covert, 1998). B chromosomes in rye and maize, specifically, may contain genes which enable them to control their own transmission (Jones, 1995; Puertas et al., 1998).

In many species including *Dicamptodon tenebrosus*, B chromosomes carrying ribosomal DNA genes have been detected through the ability of the B chromosomes to organize extra nucleoli (Sessions, 1984; Green, 1990). The presence of these nucleolar organizer regions (NORs) does not appear to affect the phenotype but may alter autosomal NOR expression (Green, 1990; Jones, 1995). An active NOR present on a supernumerary segment in the grasshopper *Oedipoda fuscocincta* (Orthoptera: Acrididae) has been shown to potentially increase the carrier's capacity for protein synthesis and could, therefore, be adaptively significant (Camacho et al., 1986).

Since many B chromosomes do lack major gene activity, any genes on these B chromosomes must be inactivated through such modifications as deletions, mutations, 'switching off', or heterochromatinization (Jones and Rees, 1982). One B chromosome of the grasshopper *Eyprepocnemis plorans* has an NOR which is normally inactive yet, when fused to the longest autosome, is activated in many cells (Cabrero et al., 1987). Determination of DNA methylation levels between these two situations reveals that the NOR is methylated in the standard B and is not methylated in the fused B indicating that

methylation likely results in inactivity of the NOR. This gene inactivation mechanism is not utilized by the standard chromosomes of this species (López-León et al., 1991). Tandemly repeated sequences found on the B chromosomes of the Australian daisy ***Brachycome dichromosomatica*** (Asteraceae) are also highly methylated and have no evident transcriptional activity providing further evidence that methylation is a mechanism which could effectively inactivate genes located on supernumerary chromosomes (Leach et al., 1995).

Transcriptional activity is often associated with loosened chromatin (Darnell et al., 1990). In higher order chromatin formation the acetylation of histone proteins likely prevents their tight packing. Thus, transcriptional activity could be aided by acetylation or some other modification of histones (Darnell et al., 1990). Houben et al. (1997a) suggest that gene inactivation of the supernumerary chromosomes in *B. dichromosomatica* may be controlled by both underacetylation of the histone protein H4 and late replication of DNA on the B chromosome. This underacetylation and late replication probably affects B chromosome chromatin formation and resultant transcriptional activity.

1.4 Maintenance of B Chromosomes

There are two main schools of thought on how B chromosomes are maintained in populations: the heterotic model and the parasitic or selfish model (Beukeboom, 1994). Both of these models discuss the advantages or disadvantages of having B chromosomes in relation to the absence or presence of accumulation or drive mechanisms (Beukeboom, 1994). Drive mechanisms

are processes that alter the transmission of B chromosomes such that increases in their numbers in certain tissues or in the germ line occurs (Jones and Rees, 1982). Germ line alterations will affect the transmission of B chromosomes in heredity.

The most common drive mechanism is nondisjunction at mitosis or meiosis followed by preferential segregation of the B chromosomes to the daughter cells destined to give rise to the germ line (Jones and Rees, 1982; Beukeboom, 1994). For example, in maize, nondisjunction in pollen grain mitosis results in two gametes, one with two B chromosomes and the other with none (Roman, 1948a,b). Roman (1948a,b) demonstrated that the two B chromosome gamete fertilized the egg much more frequently than the gamete lacking a B chromosome via selective or directed fertilization. Through this mechanism, all B chromosomes present are heritable thus counteracting the fact that this B chromosome is normally subject to fragmentation and loss in meiosis (Roman, 1948a,b). A supernumerary chromosome segment (SCS) in the lily *Muscari comosum* (Hyancinthaceae) preferentially segregates to the egg cell and shows substantial drive (Garrido-Ramos et al., 1998).

Other drive mechanisms include paternal genome elimination (*Nasonia vitripennis*; Werren, 1991) and paternal inheritance (*Polycelis nigra*; Turbellaria: Planariidae; Beukeboom et al. 1996). In both of these situations females that do not carry B chromosomes can produce progeny with B chromosomes. In the parasitic wasp *N. vitripennis*, the supernumerary

chromosome, PSR, has a transmission advantage in that it is inherited from the male and is maintained although the remaining male genetic material is eliminated (Werren, 1991). Similarly, in *P. nigra*, a pseudogamous parthenogenetic freshwater flatworm where females require sperm to initiate egg development but then eliminate the sperm, the B chromosome is inherited from the male but it is not destroyed with all other paternal chromosomes (Beukeboom et al., 1996).

A drive mechanism where B chromosome frequency does not increase but is maintained because it is stabilized occurs in *Eyprepocnemis plorans* and *Chorthippus jacobsi* (Orthoptera: Acrididae; López-León et al., 1996). These grasshoppers have nonrandom, negatively assortative gamete fertilization where ova and sperm preferentially fuse with gametes carrying alleles other than their own such that a mating between a male and female both carrying one B chromosome or SCS results in offspring with one B chromosome or SCS (López-León et al., 1996).

One theory of how supernumerary chromosomes are maintained, the heterotic model (White, 1973), proposes that in the absence of accumulation mechanisms B chromosomes at low frequencies confer an adaptive advantage to an organism yet, when present in high numbers, cause a decrease in host fitness. A clear example of this is in *Allium schoenoprasum* (Alliaceae) that, on the banks of the river Wye, UK, has abundant B chromosomes. Germination experiments (Plowman and Bougourd, 1994) demonstrated that seedlings

carrying Bs are able to germinate more rapidly and better under drought conditions than individuals lacking B chromosomes. These traits are beneficial since this plant grows in rocky crevices above the summer river level where near-drought conditions occur. Seeds unable to germinate promptly and anchor themselves in these conditions could be washed away since the river Wye can rise suddenly, covering the crevices. The B chromosomes did not have demonstrable accumulation mechanisms since they did not significantly increase in number within individual seedlings (Plowman and Bougourd, 1994).

The more widely accepted view is the parasitic model which proposes that Bs are selfish and are maintained as genomic parasites by way of an accumulation or drive mechanism that is counter balanced by their deleterious effects on host fitness (Östergren, 1945; Nur, 1977; Jones, 1985; Shaw and Hewitt, 1990). In *P. nigra*, accumulation of the B chromosome via paternal inheritance (Beukeboom et al., 1996) balances its negative effects on both cocoon production and juvenile growth (Beukeboom et al., 1998). The most selfish genetic element thus far examined is the paternal sex ratio (PSR) chromosome of *N. vitripennis* (Werren, 1991). This parasitic supernumerary chromosome is transmitted via sperm 94 to 100% of the time and is expressed whenever it is transmitted (Beukeboom and Werren, 1993). PSR supercondenses and destroys all other paternal chromosomes just after fertilization resulting in, due to a haplodiploid sex determining system in this species, all haploid male PSR-bearing offspring and no diploid females (Werren, 1991).

Both of the above models are now considered valid although most research supports the latter (Beukeboom, 1994). A slight variation to this parasitic or selfish model suggests that selfishness, the presence of B-drive, and parasitism, the harmful effects of B chromosomes on carrier fitness, be analyzed separately since the selfish B chromosome of *Locusta migratoria* (Orthoptera: Acrididae) has no significant harmful effects (Castro et al., 1998). The accumulation and elimination mechanisms which keep Bs in a state of equilibrium according to the selfish or parasitic model of maintenance are based on normal biological processes and are thus modulated by environmental factors (Jones, 1985).

A third model, the near-neutral model of supernumerary chromosome maintenance and evolution, has recently been proposed (Camacho et al., 1997a,b). While studying the grasshopper *Eyprepocnemis plorans* researchers found that B chromosomes initially have substantial drive but are then forced to near-neutrality by drive suppressor genes evolved on the A chromosomes. These near-neutral B chromosomes should disappear from populations because, without drive, the negative effects of these B chromosomes coupled with lack of accumulation would force them from populations. However, the Bs maintain themselves by the establishment of new B chromosome variants which have greater drive and are able to restart a cycle of drive, suppression and drift to extinction (Camacho et al., 1997a,b). In support of this model, *E. plorans* has B chromosomes that have no tendency toward accumulation or loss from either sex and, therefore, appear neutral and lacking meiotic drive (López-León et al.,

1992). Also, B-chromosome containing individuals contain possible drive suppressor genes (Herrera et al., 1996) and new B chromosome variants are frequently generated in populations of this species (López-León et al., 1993; Zurita et al., 1998). Zurita et al. (1998) found a new B chromosome variant that shows drive, replacing a B chromosome without drive, and is more parasitic than its ancestor since it is harmful to female fertility where the preexisting B chromosome was not. This theory could represent the evolutionary pathway of selfish B chromosomes in many species (Camacho et al., 1997a,b).

Contrary to this near-neutral model, Puertas et al. (1998) report the finding of B transmission control genes on the B chromosomes in rye (*Secale cereale*; Poaceae) rather than associated with the A chromosomes. Thus, instead of suppressor genes evolved on the A chromosomes counteracting B chromosome accumulation, the B chromosome would moderate itself.

1.5 Origin of B Chromosomes

Since B chromosomes are present in only 10-15% of plants and animals (Beukeboom, 1994; Jones, 1995), the origination of supernumerary chromosomes in populations must be rare occurrences in history. But, how does this happen? What mechanisms are involved in the origination of B chromosomes? Where do they come from and how do they establish themselves as supernumerary chromosomes?

Supernumerary chromosomes may originate through interspecific hybridization (Sapre and Deshpande, 1987). Chromatin fracture in the species

donating the chromosome fragment is necessary for interspecific hybridization to generate supernumerary chromosomes (McVean, 1995). A cause of chromosomal fragmentation may be exposure to foreign cytoplasm. There could be intrinsic cytoplasmic components that are able to fracture chromosomes in certain instances. In hybrid situations, donor chromatin fracture consistently occurs in one species and not the other suggesting it is not merely a random coincidence (Ringertz and Savage, 1976; McVean, 1995). Sapre and Deshpande (1987) cytologically screened individuals of an oriental wild relative of cultivated maize, *Coix* L. (Poaceae) from populations containing hybrids and demonstrated that chromosomes could arise and establish themselves as supernumerary chromosomes through spontaneous interspecific hybridization.

Molecular evidence indicates that the large B chromosome in the daisy *Brachycome dichromosomatica* contains numerous copies of a B-specific tandem repeat sequence (Bd49) which is also found to exist in high copy number in the genomes of closely related species, none of which have B chromosomes (John et al., 1991; Leach et al., 1995). In the autosomal complement of *B. dichromosomatica* as well as in other related species, the sequence appears in few or no copies. This suggests interspecific hybridization is a possible source of origin of the B chromosome (John et al., 1991; Leach et al., 1995).

The paternal sex ratio (PSR) chromosome of *Nasonia vitripennis* is thought to have arisen from interspecific hybridization between *N. vitripennis* and one of its sibling species or from a region that was conserved in all three sibling species examined (Eickbush et al., 1992). Other evidence suggests that, although PSR shares homologous sequences with the sibling species, it likely arose through hybridization between *N. vitripennis* and a wasp from the genus *Trichomalopsis* (Hymenoptera: Pteromalidae; McAllister and Werren, 1997). Regardless of its exact origin, it has been shown that PSR is able to survive in sibling species after interspecific crosses and maintain its ability to induce paternal autosome loss (Dobson and Tanouye, 1998). This is consistent with interspecific hybridization as PSR's mode of origination in *N. vitripennis*.

When sequences are found on the B chromosomes of one species and on the normal chromosome complement of a closely related species it is also possible that the B chromosome arose from a region that was present in an ancestral species and has been conserved in both species. These regions could be remnants of an ancestral sequence that was present on the A chromosomes but has diverged on both the As and Bs since the origin of the B chromosome (Eickbush et al., 1992; McVean, 1995; Houben et al., 1997b). For instance, dot-like micro B chromosomes of *B. dichromosomatica* contain a B-specific, highly methylated, tandem repeat (Bm29) which, along with a ribosomal DNA sequence, is also present on the larger B chromosome and in the host genome suggesting the possibility that both Bs are derived from a common ancestral B chromosome (Houben et al., 1997b).

Most evidence, however, supports a theory that B chromosomes originate intraspecifically from the normal chromosome complement (Amos and Dover, 1981; Jones and Rees, 1982). A likely source of a B chromosome is the sex chromosomes. In the fly, *Glossina* (Diptera: Glossinidae), distribution patterns of satellite DNA on both A and B chromosomes reveals that the B chromosome likely originated by duplication of the Y sex chromosome followed by both repetitive DNA amplification at the B chromosome telomeres and heterochromatinization (Amos and Dover, 1981).

Both morphological and molecular evidence reveals that supernumerary chromosomes in the frog *Leiopelma hochstetteri* are derived from a univalent W sex chromosome. The B chromosomes themselves show high levels of variability in structure, morphology and sequence diversity suggesting possible multiple derivation events from the W chromosome although homologous B chromosome-specific sequences found on the Bs of individuals in geographically isolated populations indicate a single origin (Green et al., 1993; Sharbel et al., 1998).

The most striking example of a sex chromosome B origin is in the grasshopper *Eyprepocnemis plorans*. Direct chromosomal hybridization with two sequences labeled with different colours (fluorescence *in situ* hybridization; FISH; Trask, 1991) revealed that, although these two DNA sequences are present throughout the standard chromosomes, they are the main constituents of both the B and X chromosomes (López-León et al., 1994). The location of

these DNA sequences on one B chromosome, in relation to the centromere, was the same as that in the X chromosome, only confirming the X as this B chromosome's progenitor (López-León et al., 1994).

For species where the sequences on the B chromosome do not resemble any one chromosome, yet still share repetitive DNA with the A chromosomes, it is possible that the B chromosome could have accumulated DNA from various sources and as such exist as an assortment of transposable DNA (Beukeboom, 1994; Franks et al., 1996). A B-specific repeat sequence of *Brachycome dichromosomatica* (Bd49) is flanked by a retrotransposon-like sequence suggesting that, over time, mobile elements were transposed into and disrupted this Bd49 tandem array. Since Bs are often genetically silent, repetitive DNA sequences on the B chromosomes are expected to be highly tolerant of transposition and thus, via such mechanisms, may show a high rate of sequence change due to the accumulation or deletion of transposed repetitive sequences (Franks et al., 1996).

1.6 Comparison of A and B Chromosome Structure

To determine a precise origin of supernumerary chromosomes in both species and populations it is valuable to assess the presence or absence of sequences shared between the A and B chromosomes (Beukeboom, 1994). Repetitive sequences that are shared between the A and B chromosomes or are B-specific have been found on many occasions (Table 1; Beukeboom, 1994). Utilization of molecular biology tools is very effective way to analyze

chromosome structure. Degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR; Telenius et al., 1992) in combination with microdissection (Schondelmaier et al., 1993; Pich et al., 1994), a process whereby specific chromosomes or chromosome parts are excised from a slide, is an efficient method of amplifying chromosome specific DNA (Jamilena et al., 1995; Houben et al., 1996; Potz et al., 1996; Sharbel et al., 1998). This chromosome-specific DNA may be cloned such that small fragments of the DNA may be amplified independently and used as 'probe' DNA. Once probes are available, Southern hybridization (Jamilena et al., 1995; Houben et al., 1996; Sharbel et al., 1998) and FISH (Cuadrado and Jouve, 1994; López-León et al., 1994; McQuade et al., 1994; Morais-Cecílio et al., 1997; Peppers et al., 1997) are suitable techniques for the analysis of B chromosome DNA composition and structure. Both of these techniques involve the hybridization of the chromosome-specific probe DNA to genomic DNA of the species from which the sequences were obtained or closely related species for comparison. The probe DNA will align to regions of the genomic DNA with which it is homologous thus allowing comparison of sequence structure between them (Singer and Berg, 1991; Trask, 1991).

Jamilena et al. (1995) used these techniques to determine that the B chromosomes of *Crepis capillaris* (Asteraceae) share dispersed repeated sequences with the A chromosomes. They have one sequence family in particular (B134) that is 20,000 copies per micron of chromosome more concentrated on the B chromosomes suggesting it has been amplified on the B

chromosomes after its origin. McQuade et al. (1994) used a combination of microdissection, DOP-PCR and FISH to determine that the supernumerary chromosomes in the gliding marsupial possum *Petauroides volans* (Metatheria: Pseudocheiridae) are composed of a heterogeneous mixture of repetitive DNA which is also present in the autosomal centromeric regions. FISH enables researchers to visualize the precise chromosomal location of the sequence of interest (Trask, 1991).

1.7 Evolution of B Chromosomes

As evident by the inability of supernumerary chromosomes to pair with A chromosomes at meiosis, even assuming they were derived from the normal chromosome complement, any initial homology between the A and B chromosomes must be eventually lost in the genesis of a supernumerary chromosome (Jones and Rees, 1982; Green, 1990). Initially, they must carry genes from their progenitor chromosomes resulting in a trisomic state for those genes (Jones and Rees, 1982). This genetic imbalance could result in deleterious effects such as abnormal development and so gene alteration or inactivation through B chromosome sequence differentiation (Green, 1990) or modifications such as methylation (Cabrero et al., 1987; López-León et al., 1991; Leach et al. 1995) or underacetylation (Houben et al., 1997a) often occurs.

Differentiation of supernumerary chromosomes, via acquisition of heterochromatin or loss of sequence homology with the A chromosomes, may

be due to Muller's Ratchet (Green, 1990). The evolution of sex chromosome dimorphism has been accounted for by this process (Charlesworth, 1978). The X chromosome, constantly present due to the evolutionary constraints of homozygosity in one sex, contains genes that will function for the Y chromosome thereby permitting, in the absence of recombination, the accumulation of recessive mutations, deletions or heterochromatin on the Y chromosome. The ratchet has turned one click when a class of Y chromosomes with the least number of mutations is lost from a population through random sampling or relaxed selection on Y-linked sequences, so that it cannot be passed to future generations. This process will continue and, in time, result in a gradual increase in the number of mutations on the populations of Y chromosomes (Charlesworth, 1978). Rice (1994) demonstrates that the Y chromosome of *Drosophila melanogaster* (Diptera: Drosophilidae) populations decays, in the absence of recombination, via mutation accumulation and that one possible mechanism for this is Muller's Ratchet.

If supernumerary chromosomes are not required to function due to genetic activity of the A chromosomes from which they were derived they, like Y chromosomes, are free to accumulate deletions and heterochromatin through Muller's Ratchet (Green, 1990). Lack of recombination between the A and B chromosomes is essential for the ratchet to proceed since this prevents any possible repair of accumulated mutations through recombination so that they may be maintained (Green, 1990). The action of Muller's Ratchet has generally been accepted as a way in which B chromosomes may accumulate mutations

(Beukeboom, 1994). However, Jones (1995) argued against this theory of B chromosomes evolution, stating that young B chromosomes in different stages of degeneracy and genetic silencing should be present if Muller's Ratchet was operating and that this is not evident. Eventually, regardless of the mechanism, B chromosomes must be modified such that they differ greatly in both molecular composition and organization from their progenitor sequence (Jones and Rees, 1982).

1.8 B Chromosomes in *Dicamptodon*

Among salamanders of the genus *Dicamptodon* (Caudata: Dicamptodontidae), one species has supernumerary chromosomes (Sessions, 1984). *Dicamptodon* is distributed in the Pacific Northwest from southern British Columbia to Santa Cruz County, California as well as in an isolated region of Idaho (Figure 1). This genus is not closely related to other salamander groups (Good, 1989) and was for a time thought to contain but one species, *Dicamptodon ensatus*. Using morphological evidence, Nussbaum (1970), recognized *D. copei* as a distinct species restricted to western Washington and northwestern Oregon. Nussbaum (1976) later characterized *Dicamptodon* into four groups including *D. copei*, in western Washington and northwestern Oregon, and three groups of *D. ensatus* which he declined to name; a California group, a Cascade and Oregon Coast range group, and a group from the Rocky mountains of Idaho. Allozyme data identified the Idaho populations as a genetically homogeneous cluster greatly divergent from the Coastal-Cascade *D.*

ensatus thereby recognizing the Idaho populations as an independent evolutionary lineage which Daugherty et al. (1983) classified as *D. aterrimus*. Good (1989) further characterized these groups into four distinct species; *D.copei*, *D. aterrimus*, *D. tenebrosus*, and *D. ensatus*. *D. ensatus* is restricted to populations south of Medicino county in California while all coastal populations to the north are *D. tenebrosus*.

Within the four species of *Dicamptodon* there are two geographic regions where interspecies contact occurs (Good, 1989). *D. copei* and *D. tenebrosus* are sympatric in northwestern Oregon and southwestern Washington. *D. tenebrosus* and *D. ensatus* contact each other in central California where a small hybrid zone is located. Also, within *D. tenebrosus*, populations are divergent and cluster into three distinct geographic groups (Good, 1989).

Dicamptodon tenebrosus is the one species that has supernumerary chromosomes (Sessions, 1984). This species has 14 pairs of A chromosomes and small, telocentric supernumeraries which vary in number, from 0 to 10, between individuals and constitute up to 1% of the genome. As characterized by Sessions (1984), the B chromosomes of *D. tenebrosus* are generally mitotically stable yet meiotically unstable and, therefore, susceptible to nondisjunction. The B chromosomes are non-heterochromatic, even at the centromeres. Active nucleolar organizer regions (NORs), detected by silver staining and examination of meiotic prophase preparations, are present and may

be functionally significant due to the increased potential for protein synthesis (Sessions, 1984). This synthesis could possible enhance some stage of development thereby increasing the salamanders' ability to grow well in colder environments. Sessions (1984) notes that an increase in the frequency of B chromosomes in populations from south to north is discernible.

Supernumerary chromosomes appear to be a derived feature of *D. tenebrosus* since they are absent from other species of *Dicamptodon.* A possible origin of these chromosomes is from one of the A chromosomes through a chromatin break distal to an NOR secondary constriction (Sessions, 1984). According to Sessions (1984), this chromatin fragment, comprised of an NOR as well as satellite DNA, would have differentiated from its progenitor chromosome to establish itself as a supernumerary chromosome. Another possible origin is from a B-like ancestor which itself was derived from the microchromosomes of hynobiid species. *Dicamptodon*s also have a pair of telocentrics which are a hynobiid-like feature (Sessions, 1984).

Since much of the cytogenetic work on the supernumerary chromosomes in *D. tenebrosus* has been completed (Sessions, 1984), I have concentrated on the molecular characterization of the structure of the B chromosomes. To determine the possible mechanisms of how these chromosomes originated in *D. tenebrosus*, I have compiled information on the similarity or difference in sequence composition between the A and B chromosomes in *D. tenebrosus.* By concluding whether or not the B chromosomes share DNA sequences with the

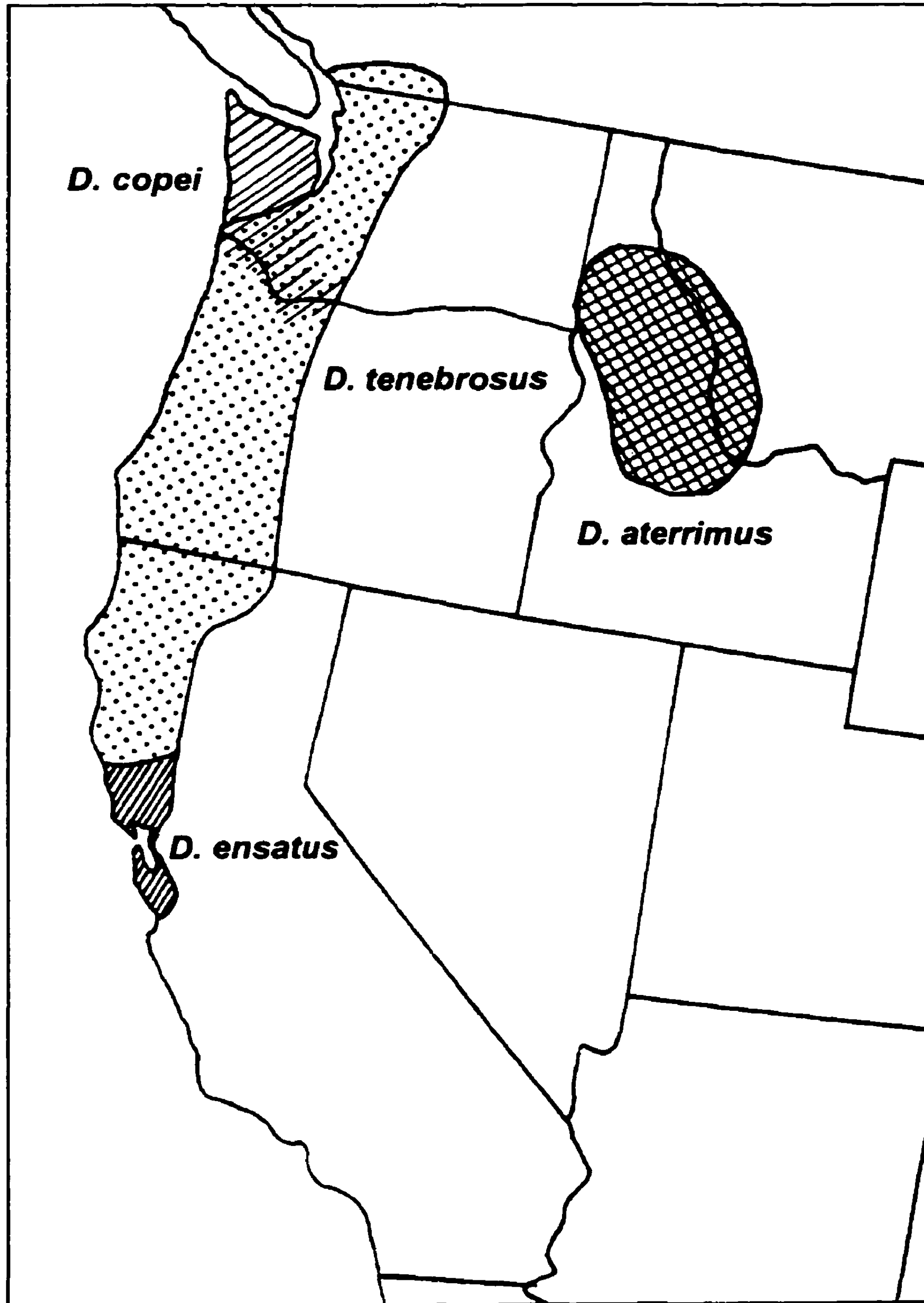
autosomal complement, I can propose whether the B chromosomes in *D. tenebrosus* likely originated from a member of the normal chromosome complement or if they were derived through interspecific hybridization with another *Dicamptodon* species. If the B chromosomes have originated from the normal chromosome complement, have they diverged from their progenitor sequence and by what mechanisms did this evolution occur? Thus, are the DNA sequences on the Bs different from the sequences on the autosomal complement. In other words, is Muller's Ratchet applicable to the supernumerary chromosomes in *D. tenebrosus*?

Evidence of a geographic pattern in supernumerary chromosome number in populations of *D. tenebrosus* exists (Sessions, 1984). By analyzing seven more populations from northern regions, I have assessed the level of diversity in supernumerary chromosome number geographically. Variation in B chromosome frequency between *D. tenebrosus* populations could help explain how the B chromosomes originated and evolved in *D. tenebrosus*.

Table 1. DNA homologies between A and B chromosomes. * = B-specific sequences not present on the A chromosomes.

SPECIES	B CHROMOSOME SEQUENCES	REFERENCE
Brachycome dichromosomatica	-176 bp tandem repeat DNA	John et al. (1991); Leach et al. (1995)
(daisy; Asteraceae)	-Ribosomal RNA gene cluster	Donald et al. (1995)
	-sequences linked to repeats	Franks et al. (1996)
	-B-specific tandem repeat	Houben et al. (1997b)
Crepis capillaris (plant; Asteraceae)	-repeat DNA family (B134)	Jamilena et al. (1995)
Eyprepocnemis plorans (grasshopper; Orthoptera: Acrididae)	-180 bp tandem repeat	López-León et al. (1994)
Glossina spp. (tsetse fly; Diptera: Glossinidae)	-telomeric and centromeric satellite DNA	Amos and Dover (1981)
Leiopelma hochstetteri (frog; Anura: Leiopelmatidae)	-repeat DNA sequences	Zeyl and Green (1992); Sharbel (1996); Sharbel et al. (1998)
Nasonia vitripennis (parasitic wasp;	-three families of tandem repeats*, 94 bp tandem repeat	Eickbush et al. (1992)
Hymenoptera: Pteromalidae)	-retrotransposable element (NATE)	McAllister and Werren (1997)
Petauroides volans (marsupial; Metatheria; Pseudocheiridae)	- repetitive DNA homologous to centromeric regions of A -B-specific DNA sequences*	McQuade et al. (1994)
Reithrodontomys megalotis (mouse; Rodentia: Muridae)	-LINE elements, telomeric repeat sequences and centromeric heterochromatin	Peppers et al. (1997)
Secale cereale (rye; Poaceae)	-two terminal heterochromatic repetitive DNA families (some B-specific sequences*)	Sandery et al. (1990); Blunden et al. (1993); Houben et al. (1996)
	-euchromatin sequences -telomeric heterochromatin*	Tsujimoto and Niwa (1992)
	-two highly repetitive telomeric DNA sequences	Cuadrado and Jouve (1994)

Figure 1. The geographic location of *Dicamptodon* species in the Pacific Northwest of North America.


D. copei
D. tenebrosus
D. aterrimus
D. ensatus

2. Methods

2.1 Chromosome Squashes

Dicamptodon tenebrosus were collected by Jacqui Brinkman, Trenton Garner and Jason Irwin in July of 1997 from 6 localities in Washington state, USA and by Dr. David Green in August of 1997 from one locality in Oregon state, USA. Populations were sampled at the Washington collection sites of Cold Creek, Kittitas County (Co.), Prairie Creek, Pierce Co., Miller River, King Co., Mallardy Creek, Snohomish Co., and both Cumberland Creek and Miller Creek in Skagit Co. The Oregon site was Oneonta Gorge, Multnomah Co. Salamanders were transported to the Redpath Museum at McGill University where they were kept in individual plastic containers at 4°C in the dark. The salamanders were fed worms and given fresh, dechlorinated water once weekly.

Two days prior to use, animals were moved to an environmentally-controlled chamber maintained at treatment temperature and a 12 hour light/ 12 hour dark cycle. Small salamanders were immersed in 0.2% colchicine solution and left at 4°C; mid-size salamanders were injected with, and immersed in, 0.2% colchicine solution and incubated at 10 and 15°C; large salamanders were injected with 0.4% colchicine solution and left in water at 15°C. All salamanders were treated for 14-21 days.

Individuals were sacrificed in 1% MS-222 (3-aminobenzoic acid ethyl ester). Tissues (heart, liver, spleen and body wall muscle) were collected and

stored at -80°C for future DNA work. Gut (stomach and intestine) and gill tissue was removed and prepared for chromosome work with a 30-minute hypotonic treatment followed by submersion in ice-cold 3:1 fixative (3 parts ethanol, 1 part glacial acetic acid). These tissues are stored indefinitely in 3:1 fixative at -20°C.

Squashes were prepared as in Sessions (1996). A small piece of tissue was removed from the 3:1 fixative, placed in 40% acetic acid briefly, removed to a clean glass slide and scraped to remove cells. A drop of fresh 40% acetic acid was placed over the cells and an even suspension of cells was attempted by removing any tissue clumps or debris. A freshly siliconized coverslip was placed on top and the cells were squashed by placing the slide between two pieces of blotting paper and pressing down firmly with thumb pressure. Slides were examined using an Olympus BX50 microscope equipped with a camera (Cansen group, Inc.) and pictures were taken using black and white slide film. Each microscope slide was made permanent by immersing it in liquid nitrogen until frozen, removing it and popping off the coverslip with a razor. The slide was immersed in 95% ethanol for 5 minutes, removed to fresh 95% ethanol for another 5 minutes, removed to air dry and stored in a sealed slide box at room temperature.

Slides of chromosome squashes prepared from the gut epithelium of one salamander, Redpath Museum (RM) number 5465, from Prairie Creek, Washington, were made permanent immediately after exposure to 40% acetic

acid. This was to prevent damage to the chromosome DNA by acetic acid while examining the chromosomes under the microscope. The slides were then observed and nuclei containing isolated supernumerary chromosomes were marked by circling their location on the underside of the slide with a waterproof marker. Several of the slides prepared for this salamander were shipped, dry to Dr. Andreas Houben (Department of Genetics, University of Adelaide, Australia) for further processing.

2.2 Microdissection and DOP-PCR

Supernumerary chromosomes from metaphase chromosome spreads of one salamander (RM 5465; Prairie Creek) were microdissected by Dr. Andreas Houben using a Zeiss Axiovert 35 inverted microscope equipped with a micromanipulator (model No. 5170, Eppendorf). Approximately 20 metaphase B chromosomes were isolated and collected in a 1 μL droplet containing proteinase K (Boehringer; 0.5 mg/ml) in 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 0.1% (w/v) SDS and overlaid with water-saturated paraffin oil (Schondelmaier et al., 1993; Sharbel et al., 1998). Nonspecific DNA from the isolated chromosomes was then amplified using the degenerate 22 nucleotide primer 6-MW (5' CCGACTCGAGNNNNNNATGTGG 3', Telenius et al., 1992; Pich et al., 1994). DOP-PCR (degenerate oligonucleotide primed-polymerase chain reaction) reactions containing B-DNA, 2 mM primer, 0.8. mM dNTPs, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.25 U Taq polymerase (Pharmacia) in a final volume of 50 μL were overlaid with

mineral oil and amplified as follows: 1 cycle at 94°C for 5 min; 5 cycles at 94°C for 1 min, 30°C for 1.5 min, and 72°C for 3 min, with a 3 min transition time to 72°C; 35 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 3 min; and one extension cycle at 72°C for 10 min. Negative control reactions without template DNA were amplified using precisely the same protocol (Sharbel et al., 1998). DOP-PCR is a protocol which non-specifically amplifies any DNA present in a sample (Telenius et al., 1992; Schondelmaier et al., 1993; Pich et al., 1994). At this point, the DOP-PCR products were returned to McGill University for further DNA amplification and cloning by Jacqui Brinkman.

2.3 Cloning

DOP-PCR products were further amplified using the 6-MW primer as follows: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 3 min; and one extension cycle at 72°C for 10 min. PCR on DNA-containing samples yielded a DNA smear ranging from 200 to 700 base pairs (bp) whereas control samples without DNA resulted in no smear. These PCR products were run on a 1.5% agarose gel and the region of the smear ranging from 250-600 bp was excised and purified using the QiaEx II Gel Extraction Kit (Qiagen). Purified DOP-PCR products were cloned into the PCR-Script Amp SK(+) vector (Stratagene). Thirty-one positive colonies were obtained by transformation. These colonies were restreaked to ensure isolation of pure clones. Plasmids contained in each colony were purified using the QIAprep miniprep kit (Qiagen). The recombinant clones were screened for inserts by

digestion with *Pvu*II. Inserts ranged in size from 250-500 bp with an average insert size of 350 bp. Cloned DNA was sequenced manually using the SequiTherm EXCEL™ II DNA Sequencing kit (Epicentre Technologies) or automatically on an ALFexpress automated sequencer (Sheldon Biotechnology; Appendix 1). Sequences were analyzed at the nucleotide level (BLASTN 2.0.8) and, if potential coding regions were identified, these were analyzed at the amino acid level (TBLASTN 2.0.7). Sequences were aligned against themselves and all other sequences using the Blast 2 sequences version BlastN 2.0.8 program (Altschul et al. 1997).

2.4 Genomic DNA Isolation

For each sample, 25 mg of organ (liver and heart) tissue was ground in liquid nitrogen and genomic DNA, for use in dot blot and Southern hybridizations, was isolated using the QIAamp tissue kit (Qiagen). Samples were lysed for 1 hour and treated with RNase A (Pharmacia) as stated in the protocol. All other steps of the QIAamp procedure were precisely followed.

2.5 Dot Blot Hybridization

Two μg of each undigested recombinant plasmid was dotted onto a BIOTRANS™ nylon membrane (ICN). Controls including two μg of both gDNA and B chromosome DNA as well as two μg of plasmid DNA without insert were also dotted on the membrane. The membrane was then placed on filter paper saturated with high salt denaturing solution (0.5M NaOH/1.5M

NaCl) for 5 min, neutralizing solution (0.5M Tris/HCL (pH 7.5)/1.5M NaCl) for 2 min, and 2X SSC for 2 min. The membrane was UV irradiated and rinsed with 2X SSC before proceeding to prehybridization and hybridization in 5X Denhardt's solution, 5X SSC, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. The blots were hybridized with labeled *D. tenebrosus* genomic DNA (gDNA) isolated from a 2 B chromosome-containing salamander from Prairie Creek, Washington (Redpath Museum specimen 5427). The gDNA was radiolabeled by random priming in the presence of ^{32}P-dCTP using the T7 QuickPrime™ kit (Pharmacia). This membrane was stripped using boiling 0.1% SDS and reprobed with 25 ηg fully labeled plasmid DNA without insert (labeled as above) to ensure that each plasmid 'dot' contained equal amounts of DNA.

2.6 Southern Hybridization Probes

Recombinant plasmid DNA was digested with *Pvu*II and run on a 0.7% agarose gel. The band containing the insert DNA plus 450 bp of plasmid was excised and purified using the QiaEx II Gel Extraction Kit (Qiagen). 25-50 ηg of this linearized DNA was denatured and labeled in the presence of ^{32}P-dCTP using the T7 QuickPrime™ kit (Pharmacia). The probe was again denatured prior to hybridization.

2.7 Southern Blot Hybridization

Genomic DNA for Southern hybridizations was isolated from tissue of Museum of Vertebrate Zoology (MVZ) specimens of *D. copei* (MVZ PC13800) and *D. ensatus* (MVZ R13870) as well as Redpath Museum (RM) specimens of *D. tenebrosus* with 0 Bs (RM 5479), 1 B (RM 5475), 3 Bs (RM 5489), 5 Bs (RM 5499) and 8 Bs (RM 5501). *D. tenebrosus* specimens with 0, 3 and 8 B chromosomes were from Prairie Creek, Washington while the 1 B salamander was from Cold Creek, Washington and the 5 B individual was from Mallardy Creek, Washington.

Genomic DNA was double digested with *Eco*R1 and *Hind*III overnight at 37°C. These samples were then run on a 0.7% agarose gel at 20V for 17 hours. The gel was photographed, rinsed in 0.25M NaOH and alkaline transferred onto an uncharged BIOTRANS™ nylon membrane (ICN) using 0.25M NaOH/ 1.5M NaCl for 6 hours to overnight (Ausubel et al., 1995). The membrane was removed from the transfer set-up and placed on filter paper wetted with 2X SSC for 5 min. The membrane was UV irradiated and put directly in prehybridization buffer or wrapped in saran wrap. At the same time, a small square of membrane was dotted with 1 µL of a 10^1 dilution of the insert DNA probe as a positive control. This membrane piece was prepared in the same manner as the dot blot hybridization membrane. The membranes were prehybridized at 65°C for 1 hour before addition of the labeled probe DNA (T7 QuickPrime™ kit; Pharmacia). Hybridization was at 65°C overnight.

Membranes were washed twice with 2X SSC/ 0.1% SDS and once with 1X SSC/ 1% SDS prior to autoradiography. These stringent washes were performed to ensure that when the probe hybridization was strong, the probe could not be removed and was thus tightly bound to the gDNA.

3. Results

3.1 Chromosome Squashes

Chromosome squashes using stomach and intestinal epithelium were performed for 44 individuals collected in 7 locations. For each individual, 10 to 20 isolated nuclei were examined for the presence of B chromosomes as well as the complete complement of 28 chromosomes. The number of B chromosomes per individual ranged from 0 to 8 such that the average number of B chromosomes per individual was 2.18 (Figure 2). The population means ranged from 0.5 to 2.67 B chromosomes per individual (Table 2). The data I have compiled (Table 2) was combined with data previously obtained for this species (Sessions, 1984). The cumulative data to date suggests that these salamanders have 0 to 10 B chromosomes per individual with population means ranging from 0 to 3.4 B chromosomes per individual (Table 3; Figure 3). The combined data set includes 149 individuals from 17 different geographical locations.

3.2 Geographic Variation

Examination of the number of B chromosomes in populations from different geographical regions (Table 3; Figure 3) reveals a trend where south of, and including, Corvallis the populations had a lower average number of B chromosomes (means < 1.5) than the populations north of, and including, Oak Springs, Oregon (means > 1.7). The exceptions to this trend are the populations

from the three most northern regions, beginning with Cumberland Creek, which had means of 0.5 to 1.8 B chromosomes per individual (Table 3; Figure 3). These variations are noticeable when comparing the population means yet are not statistically significant. In several of the populations few individuals were analyzed. However, populations in which many individuals were examined show that the geographic distribution pattern is still evident. For example, 22 individuals from Corvallis, Oregon were examined. The number of B chromosomes ranged from 0-2 per individual with a mean of 0.6 B chromosomes per individual. Just north of this region in the Columbia River Gorge, 20 individuals were examined. The number of supernumerary chromosomes ranged from 0-10 with a mean of 2.2 B chromosomes per individual (Table 3). The range of B chromosome number in populations from the most northern and southern locations was narrower and individuals had fewer B chromosomes than populations between, and including Oak Springs, Oregon and Mt. Pilchuck, Washington (Table 3).

3.3 Microdissection and DOP-PCR

It is evident that recovery of DNA from supernumerary chromosomes obtained through microdissection is possible since the amplification of the isolated chromosomes with degenerate primers (DOP-PCR) resulted in a smear of DNA from 200-700 bp. Chromosome squash preparations during which minimal chromosome exposure to acetic acid occurred and the resultant slides are stored dry, followed by the removal of isolated chromosomes from the slide

by microdissection resulted in DNA that was suitable for further amplification, cloning, sequencing and Southern blot experiments.

3.4 Dot Blot Hybridization and Sequencing

To determine whether the sequences obtained from the supernumerary chromosomes were represented in genomic DNA of *D. tenebrosus*, a dot blot hybridization of fully labeled genomic DNA (gDNA) from a 2 B chromosome-containing individual to the recombinant plasmids was performed. The results indicated that the recombinant plasmids had varied signals which reflected their representation within the salamander's genome. The recombinant plasmids contain DNA amplified directly from isolated supernumerary chromosomes using degenerate primers (DOP-PCR; Telenius et al., 1992; Pich et al., 1994). Three of the plasmids (E1, F17 and F20) hybridized very strongly, 9 (E8, F1, F2, F9, F11, F18, F21, F28 and F29) hybridized strongly and the remaining 19 recombinants hybridized very weakly, if at all, to the gDNA probe (Figure 4). Thus 9.7% of the clones hybridized very strongly, 29% strongly and 61.3% weakly if at all (Figure 5). DOP-PCR products and gDNA hybridized strongly to the probe while vector DNA did not hybridize to the probe at all (Figure 4).

Fourteen of the recombinant plasmids were randomly chosen for sequencing (Appendix 1). The sequence analysis showed that two clones had repeated sequences (Figure 6). Plasmid E3, which hybridized weakly with gDNA, encoded two repeats of 118 bp (96% identical sequence). F20, which hybridized strongly in the dot blot, encoded two tandem repeats of a nearly

identical 37 bp sequence (Figure 6). This sequence was purine rich, with G or A in 211 bp of a 274 bp region (77%).

Seven of the clones had sequences in common (Appendix 2). Clones E5 and E7, 436 bp and 433 bp inserts respectively, were identical over 433 bp (Appendix 2). Clone F6, a 436 bp insert, encoded a portion of this insert (418 bp) with two nucleotide differences (99% identical). Clones E6 and F7, 312 bp and 372 bp inserts, share 294 bp of a 296 bp region (99% identical) while clones E8 and F9, 487 bp and 336 bp inserts, have 314 bp of a 319 bp region in common (98% similar; Appendix 2).

GenBank searches using the fourteen sequences as a query at both the nucleotide and amino acid level showed no significant matches (Altschul et al. 1997).

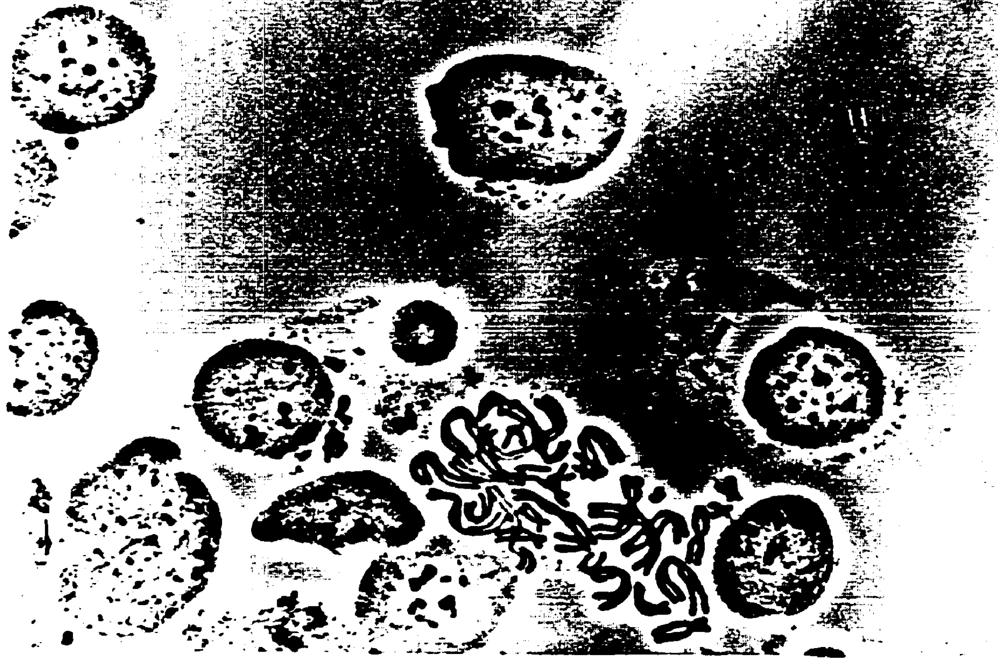
3.5 Southern Hybridization

Genomic Southern blots of *Dicamptodon copei*, *D. ensatus* and *D. tenebrosus* with 0, 1, 3, 5 or 8 B chromosomes hybridized to probes E1 and F17 are shown in Figure 7. Clone E1 hybridized to 2.2 kb and 1.6 kb fragments of gDNA of all salamanders. A unique 2 kb fragment hybridized in *D. copei* (with no B chromosomes) and a 1.4 kb band hybridized in individuals of *D. tenebrosus* (with 5 or 8 B chromosomes). Clone F17 hybridized to 4, 2.7, 2.3, 1.8, and 1.4 kb bands in gDNA of all salamanders. The 2.7 and 2.3 kb bands were more intense than the other fragments. The 4.0 kb band is most intense in

gDNA of *D. tenebrosus* with 5 B chromosomes (lane 7) although it is also intense in *D. ensatus* which does not have B chromosomes (lane 2; Figure 7).

Probes E8 and F20 were also used for Southern hybridization yet neither probe resulted in any specific binding (data not shown).

Figure 2. Nuclei of two *Dicamptodon tenebrosus* individuals with: A) 1 supernumerary chromosome and B) 8 supernumerary chromosomes. Bar represents 50 µm.


A

B

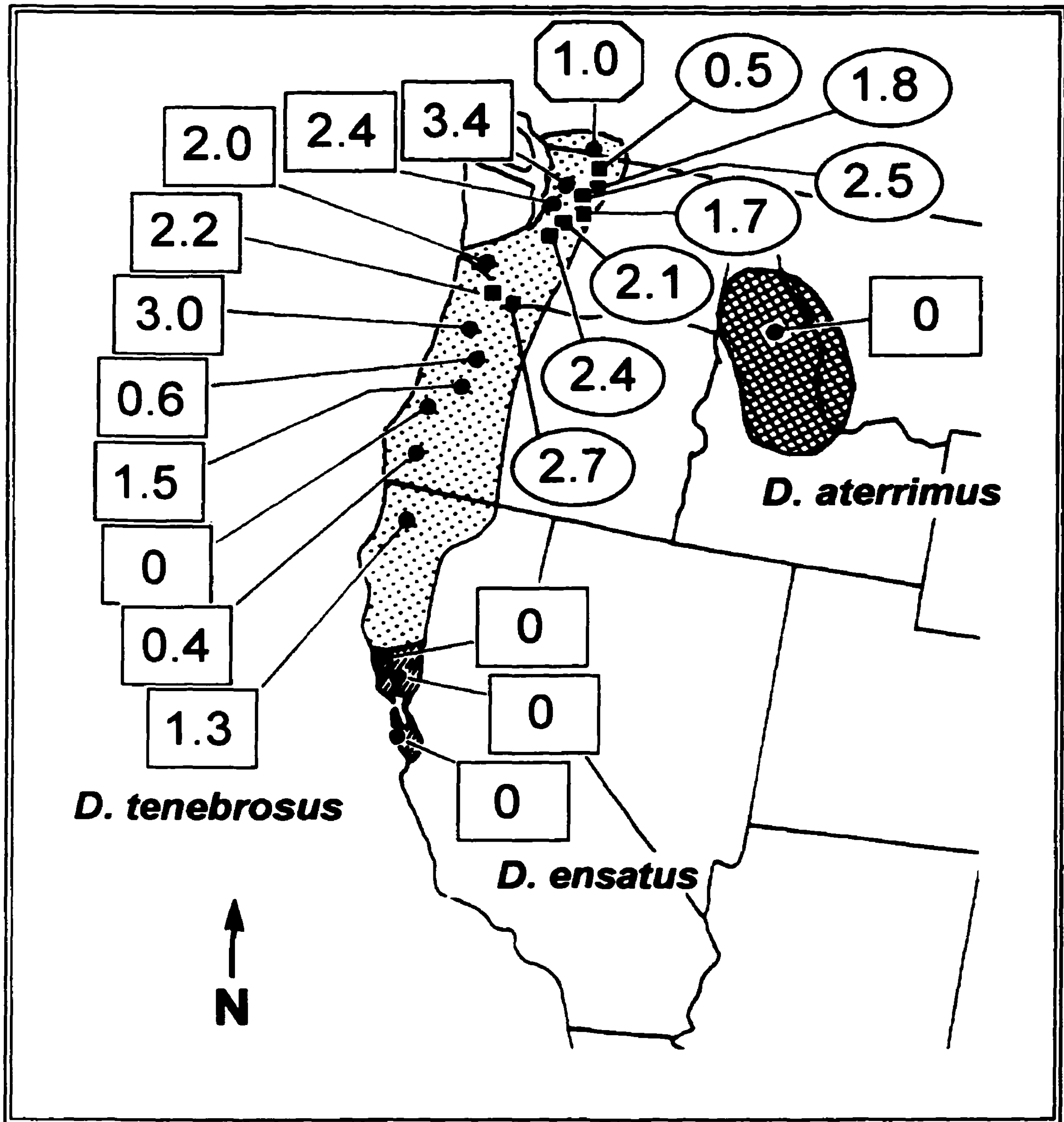
Table 2. Frequency of supernumerary (B) chromosomes in *Dicamptodon tenebrosus* populations (1997 collection).

COLLECTION SITE	N	NUMBER OF Bs	
		RANGE	MEAN
CUMBERLAND CREEK	2	0 - 1	0.5
MILLER CREEK	6	1 - 3	1.8
MALLARDY CREEK	8	1 - 5	2.5
MILLER RIVER	3	1 - 3	1.7
COLD CREEK	4	1 - 6	2.1
PRAIRIE CREEK	18	0 - 8	2.4
ONEONTA GORGE	3	2 - 3	2.7

Table 3. Frequency of supernumerary (B) chromosomes in *Dicamptodon tenebrosus* populations listed from north to south. †=data from Sessions (1984), *=data from Green (1991), and ‡=data from Brinkman (1999).

COLLECTION SITE	N	NUMBER OF Bs	
		RANGE	MEAN
CHILLIWACK*	1	---	1.0
CUMBERLAND CREEK‡	2	0 - 1	0.5
MILLER CREEK‡	6	1 - 3	1.8
MALLARDY CREEK‡	8	1 - 5	2.5
MT. PILCHUCK†	4	2 - 4	3.4
MILLER RIVER‡	3	1 - 3	1.7
SEATTLE†	1	1 - 5	2.4
COLD CREEK‡	4	1 - 6	2.1
PRAIRIE CREEK‡	18	0 - 8	2.4
MARATTA CREEK†	1	---	2.0
ONEONTA GORGE‡	3	2 - 3	2.7
COLUMBIA RIVER GORGE†	20	0 - 10	2.2
OAK SPRINGS†	1	---	3.0
CORVALLIS†	22	0 - 2	0.6
WILLAMETTE RIVER†	17	0 - 6	1.5
LOON LAKE†	19	---	0
HUMBUG MT.†	13	0 - 2	0.4
SISKIYOU-TRINITY†	7	0 - 4	1.3

Figure 3. Mean number of supernumerary chromosomes in *Dicamptodon* populations. Squares = data from Sessions (1984), ovals = data from Brinkman (1999), and the octagon = data from Green (1991).



1.0
0.5
1.8
2.4
3.4
2.0
2.5
1.7
2.2
2.1
0
3.0
2.4
0.6
2.7
1.5
D. aterrimus
0
0
0.4
0
1.3
0
D. tenebrosus
D. ensatus
N

Figure 4. Dot blot hybridization showing recombinant plasmid DNA hybridized with fully labeled genomic DNA. A= 29 hour exposure and B= 3 day exposure. Dots represent clones as follows, from top left hand corner:

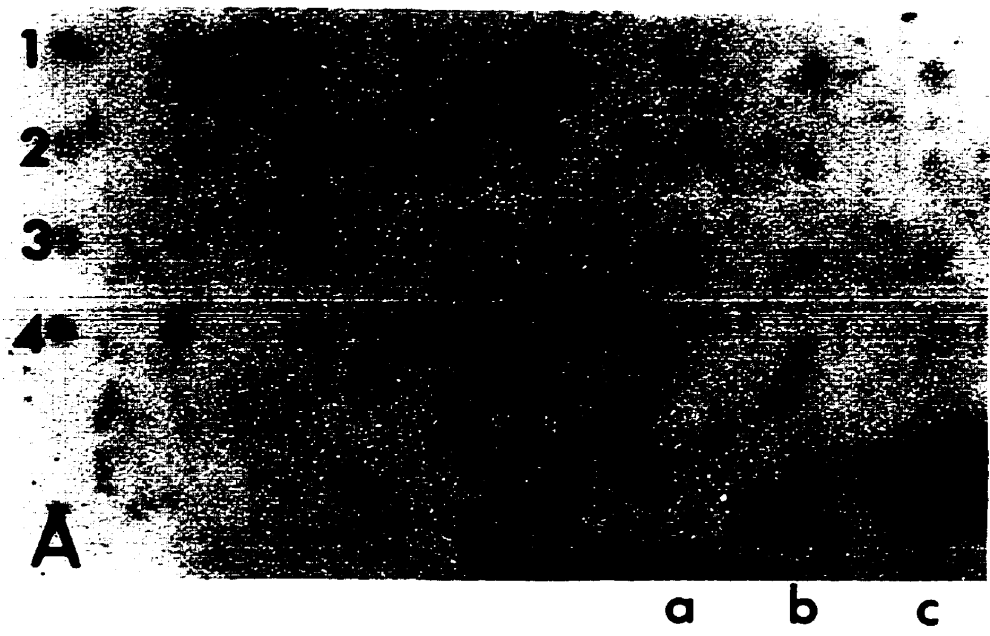
Row 1: E1 E3 E4 E5 E6 E7 E8 F1

Row 2: F2 F3 F4 F6 F7 F8 F9 F10

Row 3: F11 F12 F13 F15 F16 F17 F18 F19

Row 4: F20 F21 F22 F23 F27 F28 F29

Controls in the bottom right of each picture are: a. PCR-script (vector), b. DOP-PCR products, c. genomic DNA.


1
2
3
4
A
a
b
c

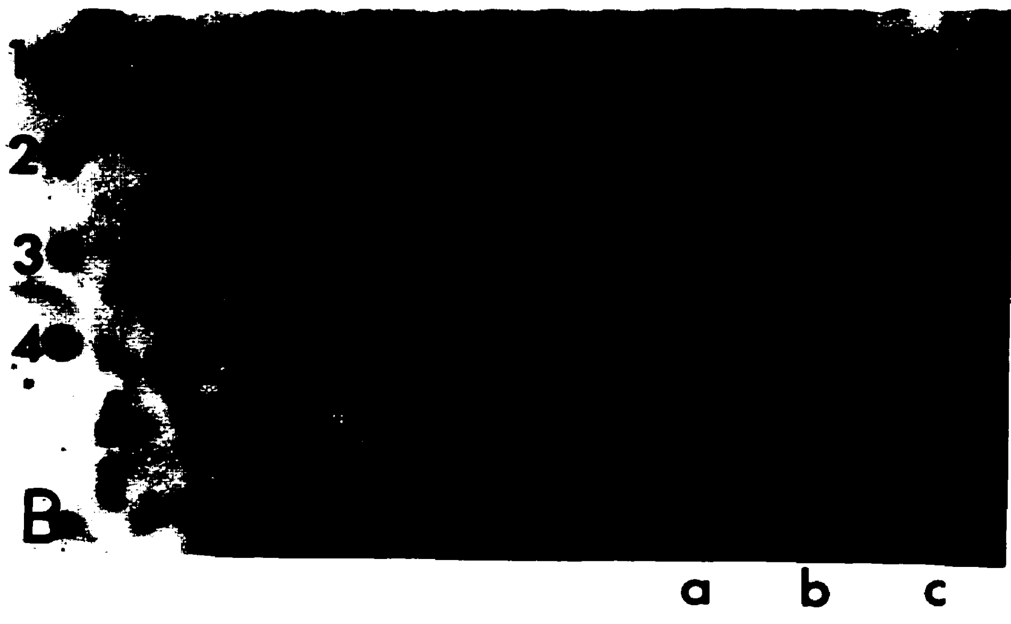

2
3
4
B
a
b
c

Figure 5. Distribution of insert classes for 31 inserts categorized by dot blot hybridization.

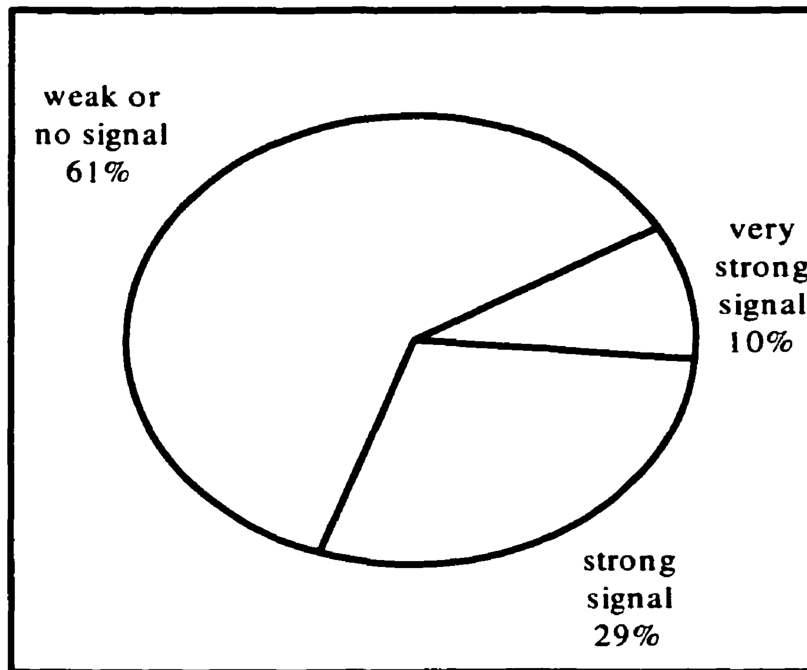


Figure 6. Supernumerary chromosome DNA with repeat sequences. The repeat sequences are in bold lettering with underlining to indicate nucleotide changes between the repeats. E3 and F20 = clone number. Sequences are continuous from 3' to 5'.

>E3

3'-gtgggga

atgtggtacggtaaaaattcgggggcttaataggtgcatgaataagacgctttgtgttagggtggtatatca
tacaggacacttgaaaattggggtttattaagtggttttgtaggcc

gcatgatatcggggtcatattac

atgtggtacggtaaaaattaggggcttaataggtgcatgaataagacgctttgtgttagggtggtaatatca
tacaggacacttgaaaattggggtttattaagtggttttataggcc

acattcgcccctcgagt-5'

>F20

3'-ccgactcgagggggatgtgga

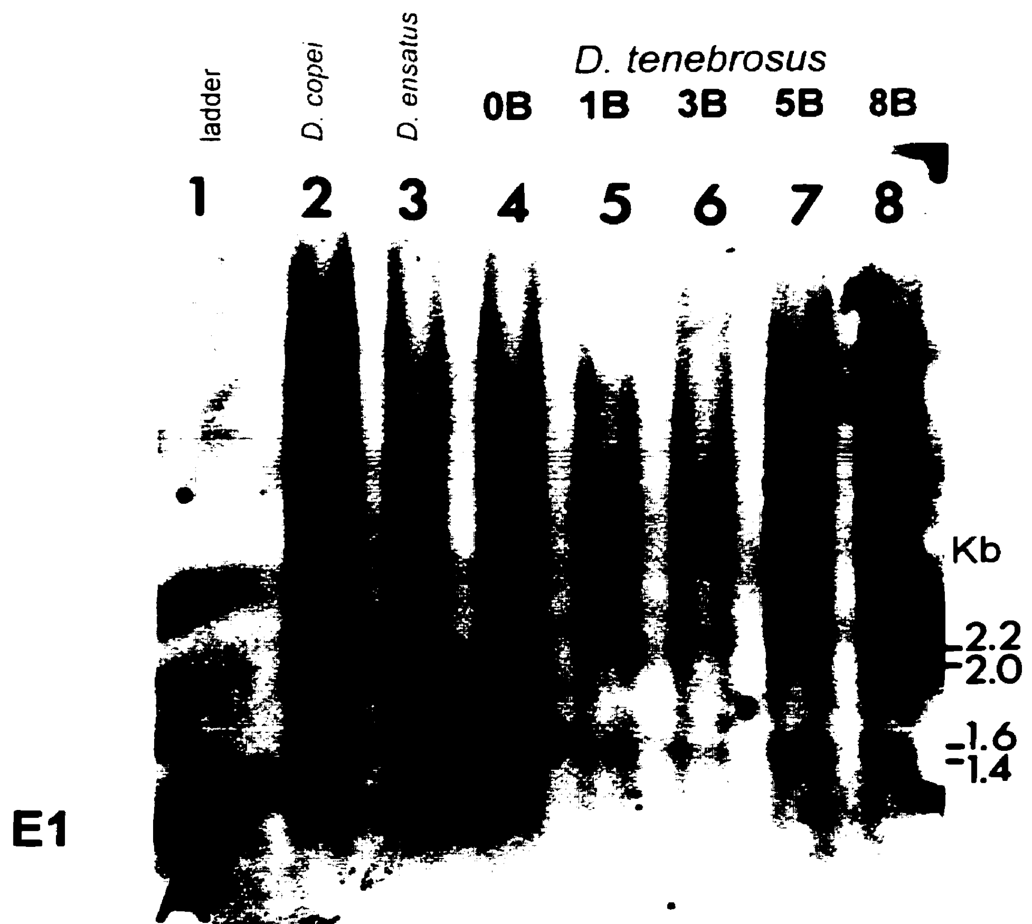
acagagaggaggaggagaggagagtgagataccatca

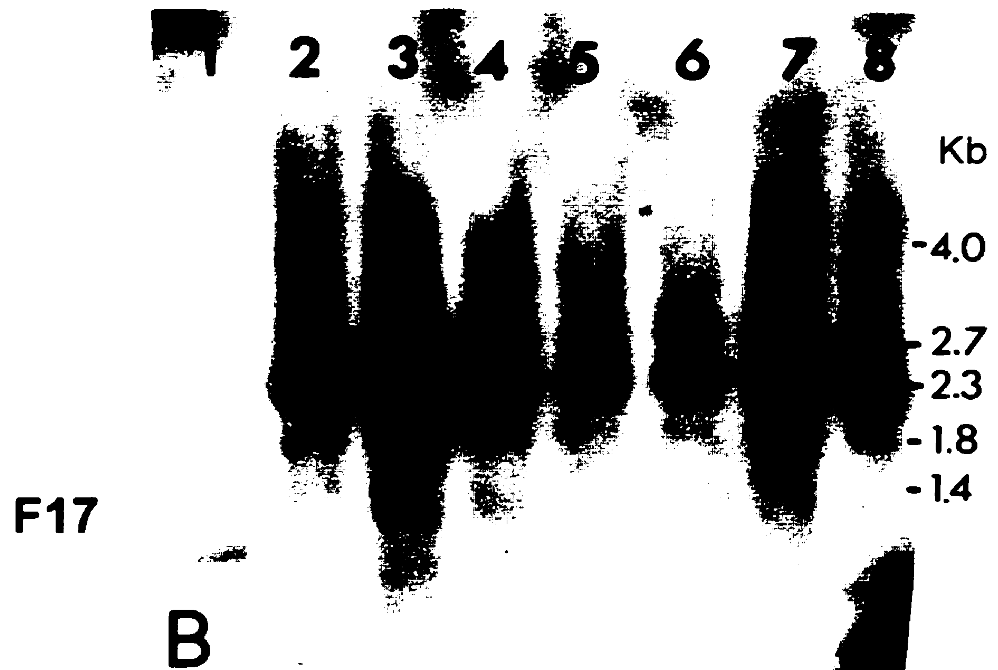
acgggaggggag

acagagcggaggacaaggggagagtgagataccatca

gaggaagagagacagagagcagaagaggagaggaaggagagctaccatcagagagagagagcagaggagg
agaagagagcgaggtaccattggagcaaaatacagagatcagaggaggaggatagagagagacatattcagag
gagagatgtaccaccacatcccgct-5'

Figure 7. Southern hybridizations probed with clones E1 (A) and F17 (B). Lanes represent kb ladder (1) and genomic DNA from *Dicamptodon copei* (2), *D. ensatus* (3) and *D. tenebrosus* with 0 Bs (4), 1 B (5), 3 Bs (6), 5 Bs (7) and 8 Bs (8).


ladder
D. copei
D. ensatus
D. tenebrosus
0B
1B
3B
5B
8B
1
2
3
4
5
6
7
8
Kb
2.2
2.0
1.6
1.4
E1


2
3
4
5
6
7
8
Kb
4.0
2.7
2.3
1.8
1.4
F17
B

4. Discussion

4.1 B Chromosome Structure

My Southern hybridization experiments with two B chromosome-specific clones from *Dicamptodon tenebrosus* demonstrate that these sequences are represented in the genomes of *D. tenebrosus* individuals regardless of the presence of B chromosomes. These sequences are also found in the sibling species *D. copei* and *D. ensatus* (Figure 7) suggesting that the supernumerary chromosomes in *D. tenebrosus* are composed of DNA sequences that are present in high copy number in the normal chromosome complement of *D. tenebrosus*, *D. ensatus* and *D. copei*. Variation in the number of genomic DNA (gDNA) bands that hybridized with clone E1 (Figure 7) suggests that this sequence may be present in different copy numbers in related individuals and thus has evolved independently. As I found, tandem repeat DNA shows varying intensities of signal between related species in hybridization experiments which is indicative of copy number fluctuation within a sequence family in the different genomes (Vershinin et al., 1996).

E1 DNA hybridized to a unique 2 kb band in *D. copei* and a unique 1.4 kb band in both 5B-containing and 8B-containing *D. tenebrosus* individuals (Figure 7). To account for these observations, one could hypothesize that after the isolation of these two species, this DNA sequence was presumably amplified in different chromosome regions in these individuals. The presence

of an extra band in only the 5B and 8B *D. tenebrosus* individuals, and not *D. tenebrosus* individuals with fewer B chromosomes or *D. copei* or *D. ensatus*, strongly suggests that amplification of this sequence occurred on the supernumerary chromosomes. It was difficult to determine whether an actual band exists or if the increase in signal was simply due to a more prominent 1.6 kb band. The increase in signal was more pronounced in the 8B rather than the 5B individual and was absent from all other *D. tenebrosus* individuals examined (Figure 7). This is to be expected as the presence of more B chromosomes in these individuals would result in higher copies of the sequence and, presumably, a stronger signal. This also suggests that all of the B chromosomes are alike in that they are comprised of the same sequences. The band sizes on Southern blots indicate regions of endonuclease restricted gDNA and are thus representative of the size of gDNA fragments and not precise chromosome regions. Chromosomal *in situ* hybridization of this sequence to the three individuals with unique bands on Southern blots as well as to an individual without the unique bands would reveal the precise location of the sequence amplification.

To address the question of how repetitive the B chromosome DNA fragments were, a dot blot experiment was performed. The results of a dot blot hybridization allow one to deduce how well represented a sequence is in the genome; clones will show signal only if they are at least moderately repeated (Schondelmaier et al., 1993; Avise, 1994; Potz et al., 1996). Dot blot hybridization of genomic DNA from *D. tenebrosus* to clones containing B

chromosome-derived DNA found that 39% of the clones hybridized strongly indicating they were represented in high copy number within the genome of *D. tenebrosus*. The other 61% of the clones hybridized with gDNA weakly or not at all and, thus, contained low or single copy sequences (Figure 4; Figure 5). These results suggest that the B chromosomes of *D. tenebrosus* are composed of both low and high abundance sequences.

In comparison, Schondelmaier et al. (1993) microdissected and cloned DNA from a barley (*Hordeum vulgare* L.; Poaceae) chromosome and found that 60% of the clones were repetitive. Similarly, Potz et al. (1996) found that DNA from a chromosome of the wheat *Aegilops markgrafii* (Poaceae) produced clones with 62% repetitive sequences. B chromosomes are also comprised of mainly repetitive DNA in many species. In the grasshopper *Eyprepocnemis plorans*, two thirds of the B chromosome is primarily composed of a 180 bp tandem repeat (López-León et al., 1994). The terminal, heterochromatic segment of the B chromosome of rye, *Secale cereale* is mainly made up of several high copy tandem repeat families (Houben et al., 1996).

Since B chromosomes generally have such high amounts of repetitive DNA then why are the supernumerary chromosomes of *D. tenebrosus* comprised of only 39% repetitive DNA? The most likely explanation is that B chromosomes in *D. tenebrosus* are non-heterochromatic throughout and would thus not be expected to have tandem repeats in high concentration. If the B chromosomes originated from an autosomal chromosome region that contained

little heterochromatin, perhaps they have not yet evolved high copy numbers of repetitive DNA. Heterochromatin is a chromosomal area with high numbers of tandem repeats (Vershinin et al., 1996).

Conversely, the estimate of 39% highly repetitive DNA may be conservative. This number is representative of the copy number of different repeat DNA sequences and not the overall repetitiveness. Thus, only a few repeat families may be present in *D. tenebrosus* but these may be very abundant throughout the genome. Also, the 31 B chromosome clones examined in the present analysis may not be representative of the entire chromosome. Inclusion of more clones in a dot blot would help to confirm my estimate of 39% highly repetitive DNA in the supernumerary chromosomes of *D. tenebrosus*. Chromosomal *in situ* hybridization with the repetitive clones would clarify how much of the total chromosome each sequence represents.

Sequence analysis supported the hypothesis that repetitive DNA is present on the B chromosomes in *D. tenebrosus*. Two of the sequences had repeats internal to the cloned fragment (Figure 6). One of these sequences, clone F20, hybridized very strongly to gDNA in the dot blot suggesting the repeat is present in the A chromosomes as well (Figure 4). However, when this sequence was used as a probe for Southern hybridization it did not bind specifically to any region of gDNA (data not shown). The genomic Southern may simply not have worked although strong hybridization of probe DNA to itself indicated hybridization did occur. A more probable explanation for these

observations is that the sequence is so interspersed throughout the genome that it does not appear in the Southern hybridization. As well, the gDNA may have been digested such that fragments to which this probe could have hybridized were either too small or not clearly isolated on the gel. Digestion of the gDNA with different restriction endonucleases in other Southern hybridizations with this same probe could potentially result in some hybridization. The same reasoning applies to probe E8 which hybridized strongly in the dot blot (Figure 4) but did not bind specifically in a Southern blot hybridization (data not shown).

Several of the clones were 98 to 100% identical in sequence for much of their length (Appendix 2). Clones E5 and E7 share 433 bp and match a 418 bp region of clone F6 for all but two nucleotides. Clones E6 and F7 are 99% identical over 296 bp while clones E8 and F9 are 98% similar over 319 bp (Appendix 2). These clones could be representative of duplicates generated by the DOP-PCR reaction that preferentially ligated during PCR product cloning or they could denote repetitive DNA sequence families on the supernumerary chromosomes of *D. tenebrosus*. The latter hypothesis is feasible since the small nucleotide differences between repeats could represent point mutations in the repeats. Single base changes are a common way through which tandem repeat sequences are able to diverge (Brutlag, 1980; Vershinin et al., 1996). However, the nucleotide differences could also be PCR incorporated errors. PCR nucleotide substitution errors are known to accumulate at a rate of 0.22% over

450 bp of clone sequence in the fly *Drosophila melanogaster* (Eickbush and Eickbush, 1995).

Clones E5, E7 and F6 as well as E6 and F7 show weak signal when hybridized with gDNA (Figure 4) and could thus represent two different low copy number DNA sequences while clones E8 and F9 show strong signal when hybridized with gDNA (Figure 4) and could represent a high copy number repeat. Sequencing of more clones could potentially uncover more repeat sequences and help to clarify if the nearly identical clones are in fact artifacts of PCR or if they represent repeat families.

4.2 A History of *Dicamptodon*

Before tackling the question of how B chromosomes have originated and evolved in *Dicamptodon tenebrosus*, it is useful to review current ideas concerning the evolution of the genus *Dicamptodon* in the North American Pacific Northwest. Nussbaum (1976) examined morphological characters and proposed that modern *Dicamptodon* are descendants of a salamander which originated from a hynobiid-like stock found in the North American arctic during the Cretaceous period. During this time, dicamptodontines would have ranged over what is now western Canada and Alaska and then spread down into the Pacific Northwest. By the Pleistocene period, the ancestral range would have been reduced to a small area in northern coastal California and southwestern Oregon (Nussbaum, 1976).

The Pacific Northwest has undergone five glacial advances over the past 100,000 years, with the most recent glacial advance occurring 25,000 to 10,000 years before present (ybp; Richmond, 1965). Nussbaum (1976) hypothesized that, during the last glacial maximum, *D. copei* was isolated in a Washington refugium. Other *Dicamptodon* were probably restricted to southern latitudes and low elevations since alpine glaciers covered much of the Rocky Mountains of Idaho (Richmond et al., 1965), the Cascade Range of Washington and Oregon, the Olympic Range of Washington and portions of the Coast Range and Klamath Mountains of Oregon (Crandell, 1965).

Until approximately 12,000 years ago, the populations likely ranged from the lower eastern slopes of the Cascades, across central Washington, and to the Rockies with populations on the western side of the Cascades in Oregon linked to those on the east side by the Columbia River Gorge in northern Oregon (Nussbaum, 1976). This continuous range would have allowed for genetic exchange between these populations. Populations from Oregon to northern California were probably genetically isolated by mountain glaciers in the Klamath-Siskiyou region of southern Oregon (Nussbaum, 1976). The more southern coastal Californian populations were not likely affected by this glaciation resulting in their being more similar to ancestral *Dicamptodon* than individuals from populations more north based on morphology, evidence of vocalization, and lower levels of neoteny (Nussbaum, 1976).

Neoteny is the ability of an animal to reproduce in the larval form. The fixation of paedogenesis in *D. copei* probably occurred in the Washington refugium where there was abundant moisture and reliable waterways and a relatively small gene pool. This trait was also common in other *Dicamptodon* species near the glacial borders where the terrestrial environment was dry and unsuitable forcing salamanders to remain in their aquatic environment.

Daugherty et al. (1983) argued that the morphological evidence described by Nussbaum (1976) was insufficient to date these phylogenetic events. They assessed allozyme characters and proposed that *Dicamptodon* populations from coastal and inland regions were isolated long before the most recent Pleistocene glacial advance (Daugherty et al., 1983). The presence of fossil remains of *Dicamptodon* from as early as the lower Pliocene lends support to this hypothesis (Peabody, 1954). Daugherty et al. (1983) also propose that the Coastal-Cascade and Rocky mountain populations diverged from one another 7.5 million ybp and that *D. aterrimus* has been a genetically isolated species since this time. Good et al. (1989) used allozyme variation to estimate that *D. tenebrosus* and *D. ensatus* were isolated from each other 7 million ybp although they did not devise a hypothetical scenario for this separation and if, or when, secondary contact between these species occurred.

The best evidence from these three reports suggests a history in which *D. tenebrosus* has been genetically isolated from *D. aterrimus* for 7.5 million years (Daugherty et al., 1983), from *D. ensatus* for 7 million years with

subsequent secondary contact (Good et al., 1989), and from *D. copei* for 25,000 years (Nussbaum, 1976).

4.3 B Chromosome Origin and Evolution in *Dicamptodon*

Among *Dicamptodon*, supernumerary chromosomes have been found only in *D. tenebrosus* (Sessions, 1984). Based on the hypothetical history of *Dicamptodon* proposed by Nussbaum (1976), it is possible that supernumerary chromosomes originated in *D. tenebrosus* during the last Pleistocene glacial advance. There are two main ways this may have occurred. Supernumerary chromosomes may have arisen in an ancestral genome and were subsequently lost from *D. copei*, *D. ensatus* and *D. aterrimus* or supernumerary chromosomes may have arisen in *D. tenebrosus* just prior to or during the last glaciation followed by isolation of this species from *D. copei* to the west and *D. ensatus* in the south.

Green (1991) disputed the idea of amphibian supernumerary chromosomes being evolutionary vestiges from ancestral karyotypes since they occur at low frequencies across species. He suggested that they are products of normal chromosomal aberrations that have persisted in populations and that the capturing of B chromosomes from chromosomal anomalies is a rare, random event. However, the persistence of B chromosomes as remnants of an ancestral sequence is supported in many species where the B chromosomes closely resemble DNA in the genome of sibling species that do not have B chromosomes (Eickbush et al., 1992; McVean, 1995; Houben et al., 1997b).

A slightly altered scenario is possible when considering the hypotheses concerning *Dicamptodon* evolution from both Daugherty et al. (1983) and Good (1989). If Rocky Mountain and Coastal-Cascade populations have been separated for 7.5 million years (Daugherty et al., 1983), then *D. aterrimus* presumably diverged from other *Dicamptodon* species before supernumerary chromosomes originated. Similarly, if *D. ensatus* and *D. tenebrosus* diverged from each other 7 million years ago (Good, 1989) then supernumerary chromosomes could have originated independently in *D. tenebrosus* after this time, with secondary contact having no effect. Conversely, the supernumerary chromosomes in *D. tenebrosus* could be products of interspecific hybridization with *D. ensatus* resulting from secondary contact. Interspecific hybridization is proposed as the mechanism of B chromosome origin in cultivated maize *Coix* L. (Sapre and Deshpande, 1987), the daisy *Brachycome dichromosomatica* (John et al., 1991; Leach et al., 1995) and the wasp *Nasonia vitripennis* (Eickbush et al., 1992; McAllister and Werren, 1997).

During the last glacial maximum, the restriction of *D. copei* to western Washington would have resulted in the genetic isolation of this species from *D. tenebrosus*. Either supernumerary chromosomes were never present in *D. copei* or they were lost during *D. copei*'s isolation in Washington. Since B chromosomes in *D. tenebrosus* are meiotically unstable, their accumulation or loss during meiosis is possible (Sessions, 1984).

The supernumerary chromosomes in *D. tenebrosus* most likely arose from an autosomal chromosome region where a nucleolar organizer region (NOR) was present (Sessions, 1984). The ancestral B chromosome in this species would thus represent a fragment of chromatin that not only included an NOR but was non-heterochromatic because the supernumerary chromosomes in *D. tenebrosus* are non-heterochromatic throughout (Sessions, 1984).

My results from Southern hybridizations indicate that there is similarity between B chromosome DNA of *D. tenebrosus* and genomic DNA (gDNA) from *D. copei*, *D. ensatus*, and *D. tenebrosus* (Figure 7). This similarity, and their lack of heterochromatin, argues for a relatively recent intraspecific origin of supernumerary chromosomes in *D. tenebrosus*. This would be expected, especially of young B chromosomes, because long-established supernumerary chromosomes should have lost sequence homology with the autosomes (Green, 1990). Any initial homology between the B chromosome and its progenitor sequence must be lost in the evolution of a supernumerary chromosome since they are no longer able to pair with the autosomes at meiosis (Jones and Rees, 1982; Green, 1990). This evolution may occur by Muller's Ratchet whereby B chromosomes that are not required for normal organismal function will, if they do not recombine, accumulate mutations and gain heterochromatin (Green, 1990). Thus, the B chromosomes in *D. tenebrosus* may be in the initial stages of diverging from their progenitor chromosome by accumulating repeat DNA.

High sequence similarity between the B chromosomes and the autosomes in species such as the grasshopper *Eyprepocnemis plorans* (López-León et al., 1994) and the plant *Crepis capillaris* (Jamilena et al., 1995) also support a hypothesis of recent intraspecific B chromosome origin since the B chromosomes still share a high degree of homology with their presumed progenitor sequence.

In *E. plorans*, the B chromosome shares a high degree of sequence similarity with the A chromosomes for both ribosomal DNA (rDNA) and a tandem repeat sequence (López-León et al., 1994). The rRNA genes on the B chromosome retain their expression competence even though they are typically transcriptionally inactive, suggesting they have not changed from the autosome rDNA.

In *C. capillaris*, the B chromosome is mainly composed of sequences shared with the A chromosomes (Jamilena et al., 1995). One shared sequence is present on the B chromosome in 20 000 more copies per micron of chromosome than on the A chromosomes, clearly indicating that B chromosome evolution is independent of A chromosome evolution (Jamilena et al., 1995).

Alternatively, the similarity between the B chromosome DNA of *D. tenebrosus* and genomic DNA (gDNA) from *D. copei*, *D. ensatus*, and *D. tenebrosus* (Figure 7) could be a result of the homogenization of the chromosomes. Batistoni et al. (1995) report that amphibian genomes are permissive and appear to have a strong tendency for both the tandem

duplication of short retroposons and the accumulation of noncoding DNA sequences. Furthermore, homogenization between copies of repeat DNA sequences on different chromosomes is reported by Vershinin et al. (1996) as a mechanism of repeat sequence evolution. Thus, the genome of *D. tenebrosus*, including the B chromosomes, may be composed of a large number of genetically dispensable DNA components that are capable of amplification and transposition to different chromosomes resulting in the high sequence similarity seen between the A and B chromosomes. The non-heterochromatic nature of the B chromosomes could be explained in that the accumulated repeat sequences are dispersed, rather than tandemly distributed, throughout the chromosome. The detection of transposon-like sequences on the B chromosomes of *D. tenebrosus* would further support this hypothesis.

4.4 Geographic Variation in *Dicamptodon*

Using allozyme variation, Good (1989) divided *Dicamptodon* into 4 distinct species. *Dicamptodon tenebrosus* was most closely related to *D. copei* while *D. aterrimus* was the most divergent species. This relationship concurs with the history of *Dicamptodon* where *D. aterrimus* has long been genetically isolated from the remainder of the populations while the isolation of *D. ensatus*, *D. copei* and *D. tenebrosus* was more recent (Nussbaum, 1976; Daugherty et al., 1983; Good, 1989). Geographic variation within *D. tenebrosus* populations is evident such that two large distinct assemblages form a definite coastal versus inland demarcation (Good, 1989). The coastal populations range from mid-

California to northwestern Oregon while the remaining populations range from inland mid-Oregon to northern Washington on the west slope of the Cascade Range. One population from the east coast of the Cascade range forms a third distinct group that is more closely related to the coastal, than inland, populations (Good, 1989).

Could the analysis of B chromosome distribution shed any light onto current ideas concerning speciation in *Dicamptodon*? Geographic clustering of *D. tenebrosus* populations based on their mean number of B chromosomes (Table 3; Figure 3) follows a similar trend as the clustering based on allozyme variation (Good et al., 1989) although the pattern is more south/north than west/east. Initial examination of supernumerary chromosome abundance in *D. tenebrosus* populations showed a gradual south to north cline of increasing B chromosome number (Sessions, 1984). This study was completed prior to Good's (1989) separation of *Dicamptodon* into four species and so was based on the conviction that only two species of *Dicamptodon* were present: *D. copei* and *D. ensatus* (Nussbaum, 1970, 1976). Thus, several of the most southern populations containing no B chromosomes were *D. ensatus* rather than *D. tenebrosus* (Green, 1991). With these populations removed from the data set, a different trend is apparent where populations of *D. tenebrosus* south of Corvallis, Oregon and north of Cumberland Creek, Washington have lower average numbers of B chromosomes than do populations in between (Green, 1991; Table 3; Figure 3). In the present work, the addition of B chromosome

numbers for several populations from northern areas (Table 2) further clarifies this geographic pattern, which closely resembles that proposed by Good (1989).

The region separating Corvallis from the Columbia River Gorge lies very close to the line demarcating the populations examined by Good (1989). From northwest to mid-Oregon at about the same latitude there is an area where both variation in allozymes and B chromosome abundance occurs. This region is close to where *D. tenebrosus* is sympatric with *D. copei* (Good, 1989). Oak Springs is also located in this zone and is where Daugherty et al. (1983) proposed neoteny originated. This event may have been causally related to the reproductive isolation of *D. copei* and *D. tenebrosus* (Daugherty et al., 1983) as well as to the preservation of B chromosomes in *D. tenebrosus*.

Historically, a warm, dry period followed the last glacial maximum during which lifezones were more northern and at higher elevations than today (Richmond, 1965). The coastal and inland populations were probably separated and the east side of the Cascade mountains was abandoned. Thus, the independent evolution of B chromosomes in coastal versus inland populations of *D. tenebrosus* during this period could have resulted in higher frequencies of Bs in inland populations (Figure 3, Table 3). It is possible that the B chromosomes in coastal populations are much more detrimental to their host than those in inland populations. B chromosome effects on host fitness in many species are unfavorable at higher B frequencies and put an upper limit to the number of B chromosomes an individual can have (Jones and Rees, 1982;

Jones, 1995). For *Dicamptodon*, more harmful effects of B chromosomes in coastal populations would limit the number of B chromosomes they could tolerate.

The present day geographic variation could also be, in part, correlated with climate. Clines correlated with climatic factors have been reported in the grasshoppers *Myrmeleotettix maculatus* and *Eyprepocnemis plorans* (Hewitt and Brown, 1970; Henriques-Gil et al., 1984; Cabrero et al., 1997) and in maize (*Zea mays mays*; Rosato et al., 1998; Poggio et al., 1998). Among the *Dicamptodon* populations sampled, Corvallis and more southern populations are located west of the Cascades, Oak Springs is east of the Cascades and most of the northern populations are situated on the west slope of the Cascades or within low-lying areas. In Oregon, the east slope of the Cascades receives low levels of precipitation and has high summer and low winter temperatures while the west slopes have much higher precipitation levels and less drastic seasonal temperature fluctuations (Nussbaum, 1976). This differentiation in climate is less defined in northern Washington than it is in Oregon (Nussbaum, 1976) which could be correlated with the change in B chromosome numbers in populations found in the extreme north of Washington and southern British Columbia. Also, many of the west/south low B frequency populations are closer to the coast and so could experience slightly more rainfall than the Cascades' west slope. Milder average temperatures would also be expected in more southern locations. Exactly how these climate differences would effect B chromosome frequency in *D. tenebrosus* has yet to be determined.

Examination of more populations near the south/north transition zone, on the Cascades' east slope in Oregon, and in northern Washington and British Columbia would help to localize the regions where changes in supernumerary chromosome number in *D. tenebrosus* occur. Assessment of morphological or genetic variation in other amphibian, reptile, or mammalian species near these areas would be useful in determining if this pattern holds in other species. Furthermore, although extremely difficult if not impossible, breeding experiments to determine the frequency of, and the mechanism involved in, B chromosome transmission in *D. tenebrosus* populations would help to explain how this chromosome polymorphism is maintained and spread in this species.

4.5 Conclusions

The most parsimonious explanation of supernumerary chromosome origin in *D. tenebrosus* is that the B chromosome arose from the normal chromosome complement before the last glacial advance of the Pleistocene. This would have occurred after *D. tenebrosus* was separated from *D. ensatus* and *D. aterrimus* and just prior to isolation from *D. copei*. The progenitor chromosome would have been non-heterochromatic and have had an NOR.

The B chromosomes in *D. tenebrosus* contain repetitive DNA. Amplification of sequence copy number is evident in the genome of *D. copei* and on the supernumerary chromosomes in *D. tenebrosus*.

The mean number of supernumerary chromosomes per population varies geographically such that the most southern and northern populations have lower

average numbers of B chromosomes than populations in the middle of the range. Both historical and climatic factors could be correlated with this pattern.

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6. Appendices

6.1 Appendix 1: Sequence Data

>E1

3'-ggaggggatgtggtatgttttggactctgtcttctttgttctttgttgcagtggttgagcatttttctcacccactggg

tttttctcagcactttttgcacagtggggcaccgggtagtccttatggctcccagtgctgtgtttttcccatagccttcctt
tttgtatcctgacataacccatgttaagtggagtgccaggggggacatatgcttgtgtccccataactttttaaagagcct
aacaactcttaccctttttggttgcccacatcgacctc-5'

>E3

3'-gtggggaatgtggtacggtaaaaattcgggggcttaataggtgcatgaataagacgctttgtgttagggtggta

tatcatacaggacacttgaaaattggggtttattaagtggttttgtaggccgcatgatatcggggtcatattacatgtgg
tacggtaaaaattaggggcttaataggtgcatgaataagacgctttgtgttagggtggtaatatcatacaggacactt
gaaaattggggtttattaagtggttttataggccacattcgcccctcgagt-5'

>E4

3'-ggaggggatgtggctatttgaagttgcctaagttgttttatgcaaaggcattgcttcatactcagaatgttaatca

cattgctttaattaagatggtgcaagatgcactctctcttcgataccgttgacaccatacccaagtcctaaaatactgg
gggactggttggtggaagacagtttgctgaagcctcccagatatggtctgctaaaagtgttcttgttgtattcctttgtt
atagctcccgcactcccacttgttgcctgcccctactacctgtcacactgaccactaccgattacaaacagatagcaa
gaccatttggactattccacttttcaaccctcgaggaccacatccctccc-5'

>E5

3'-gaagggaatgtggcctaaactctggtaacaatggttcacaaaatgctggacctttaactgattcgtcggaaccc

aatcaccaacaaacctcagcttatcattaaactgcccgatcaaatcatcaatgtcagtattttcctccatcttgatggct
aaaaactgtctcctcatttcctcaatctccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagt
cactgagtccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgactcttcaacaactg

agtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaaagctctccatttccctcagccaatccaaa
gaaacagacggatcattactctcataatactccacattcctccc-5'

>E6

3'-ggggaggatgtgggtagcagaatagagatacattgagcttgttgagtgttggttttacgccgatagattcccaa

aactgtgtttgcatcggaatcgactgctgaaataaatctcgctcgatagtgagagactgttcacgaattaagccgcct
gtagctccattaaaacccggaaagaagaagtgtttgattaagccattacgtggatgaggcgagcttttgcgtgaaatt
cattgacccaggcttcggcagataaatattccaagtttagccaaatgggtttttgctaatcatggcagccacattccc
ccc-5'

>E7

3'-gaagggaatgtggcctaaactctggtaacaatggttcacaaaatgctggacctttaactgattcgtcggaaccc

aatcaccaacaaacctcagcttatcattaaactgcccgatcaaatcatcaatgtcagtattttcctccatcttgatggct
aaaaactgtctcctcatttcctcaatctccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagt
cactgagtccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgactcttcaacaactg
agtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaaagctctccatttccctcagccaatccaaa
gaaacagacggatcattactctcataatactccacattcctccc-5'

>E8

3'-gaaggggatgtgggtaaggcaacccttaaatcctacagctcgctgttaaaacaaatctcctatgtgggcgaga

cgatggtcgaacaatgtcacctccaacccaacatcccaatcttgggaatcttaccacctgtacacagcccaaacctg
taacccattgtgactccgctcctaggcgtaaccctggcccaacgtaactccactctcacctggcctacctactaatcc
accctcgcaatctcagctctcctccctcgcaccatgtacaacttcacagcactttacataacactgaatatcatacgg
aaggcatctaccttatgccccgttttacctacttatgctcgggactccccatgaataacagcatccaccccactcacat
ctcccacagcaaccgctttgcagagcagtaacgcacaccttttaactcttgcctagcctgatttccctctaatgaccat
taccctgcactacagaggaccacattcccccc-5'

>F1

3'-ggaggagtgtgggactaccactacttcaactactcacctcatcaatcaaaacccaaattataaccgtacatatc

aactatcatattcatcttgcattatcactaaccaactacccgatcttcttataacgaataaaattttccactctacgaataa
acatcacaaataatcacataggtcgaccgaactttaagtcaaaccaacccggaacttactattacaatcccattcaatt

ccggaaaaatcaacccgcggctcaaatccccaaactcactgcctttacaaaatcataactgaatcaacaatacaata
gtctctacacgaaacttcactgccacatccctgcc-5'

>F2

3'-ctgntcgagagcgggatgtggcatgagaaagggggatgctacncatacatgggcagagtcagcaggattac

aagggcaacactttacattaaggagcagagtcatacgggggaaattgcatccgggagcagagattgcaaggggа
acactacatcaggggaaattcagcggtaggatcacatggggagcagaaggcattcagcaaaatctcataagtctgt
gagcagagacattcttggcacagggtaacactttaaagtctaggagcagtgtcagctacaaggttgcatggcatgg
caaacaagtttggtgcagagtcagcatgatgtatcagcaaggcggccgctagtcagtgaccaaaggtgtagcgtct
aggcccacatcccgccctcgagt-5'

>F6

3'-ggaggggatgtggaagtattatgagagtaatgatccgtctgtttctttggattggctgagggaaatggagagctt

ttgatgttagtcactgtgagcctgagctcaaggttttgtatgctactcagttgttgaagagtcatgcttacagatggtgg
gattctcttaccggtcattttactcaggagcagaaggactcagtgacttgggaaatgttcaaggagcagtttatggcc
aagtattgttcattggtggagattgaggaaatgaggagacagtttttagccatcaagatggaggaaaatactgacatt
gatgatttgatcgggcagtttaatgataagctgaggtttgttggtgattgggttccgacgaatcagttaaaggtccagc
attttgtgaaccatcgttaccagagtttaggccacatcccctcc-5'

>F7

3'-ccgactcgagggaggaatgtgggtagcagaatagagatacattgagcttgttgagtgttggttttacgccgata

gattcccaaaactgtgtttgcatcggaatcgactgctgaaataaatctcgctcgatagtgagagactgttcacgaatta
agccgcctgtagctccattaaaacccggaaagaagaagtgtttgattaagccattacgttgatgaggcgagcttttg
cgtgaaattcattgacccaggcttcggcagataaatattccaagtttaggcaaatgggtttttgctaatcatggcagc
cacgtaactgctcggtagttcgcatgcgaacgcttcaattaccacatcccttcctcgagt-5'

>F9

3'-gcggagaatgtgggtaaggcaacccttaaatcctacagctcgctgttaaaacaaatctcctatgtgggcgaga

cgttggtcgaacaatgtcacctccaacccaacatcccaatcttgggaatcttaccacctgtacacagcccaaacctg
taacccattgtgactccgctcctaggcgtaaccctggcccaatttaactccactcacacctggcctacctactaatcc

accctcgcaatctcagctctcctccctcgcaccattgtacaacttcacagcactttacataacactgaatatcatacgg
aaggcatctaccttatgccacatccccacc-5'

>F17

3'-cccgactcgagcggggratgtggaaactatttacaacaacctgctgcaccttgctactctcaacttccgctctg

cctctaagcactcctttgatatctgcctcatccttgaagactttgacctcgatgtcctcgatcttaccgagacatggttc
aatgccagctctgtcactgctttcggcactatcctccctgacggctacaagatcctctccaaacccagacctaaaca
gtcttggggcggagtggccctgattttccctgaatgggttactgcctctaccatatccactcagtcctactcctatttcg
agtcccttatctgcagactcaatctctcccctaactcctctatcactctcgctaccatctactgaccttcctgaccccac
atccttg-5'

>F20

3'-ccgactcgagggggggatgtggaacagagaggaggaggagaggagagtgagataccatcaacgggaggg

agacagagcggaggacaaggggagagtgagataccatcagaggaagagagacagagagcagaagaggaga
ggaaggagagctaccatcagagagagagagcagaggaggagaagagagcgaggtaccattggagcaaaatac
agagatcagaggaggaggatagagagagacatattcagaggagagatgtaccaccacatcccgct-5'

6.2 Appendix 2: Sequence Alignments

The following data are BLAST 2 sequences results from BLASTN 2.0.8. Dashes (-) represent missed nucleotides in one sequence and bold, underlined nucleotides (**n**) represent base differences between sequences. Numbers at either end of each row of sequence represents the nucleotide number within each insert in the 3' to 5' direction.

1. Sequence 1: E5 (433bp) vs. Sequence 2: F6 (434bp)

Identities = 416/418 (99%), Positives = 416/418 (99%)

Seq. 1: 3 aggg**a**atgtggcctaaactctggtaac**a**atggttcacaaaatgctggacctttaactgat 62

Seq. 2: 432 aggg**g**atgtggcctaaactctggtaac**g**atggttcacaaaatgctggacctttaactgat 373

Seq. 1: 63 tcgtcggaacccaatcaccaacaaacctcagcttatcattaaactgcccgatcaaatcat 122

Seq. 2: 372 tcgtcggaacccaatcaccaacaaacctcagcttatcattaaactgcccgatcaaatcat 313

Seq. 1: 123 caatgtcagtattttcctccatcttgatggctaaaaactgtctcctcatttcctcaatct 182

Seq. 2: 312 caatgtcagtattttcctccatcttgatggctaaaaactgtctcctcatttcctcaatct 253

Seq. 1: 183 ccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagtcactgagt 242

Seq. 2: 252 ccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagtcactgagt 193

Seq. 1: 243 ccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgactct 302

Seq. 2: 192 ccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgactct 133

Seq. 1: 303 tcaacaactgagtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaaag 362

Seq. 2: 132 tcaacaactgagtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaaag 73

Seq. 1: 363 ctctccatttccctcagccaatccaaagaaacagacggatcattactctcataatact 420

Seq. 2: 72 ctctccatttccctcagccaatccaaagaaacagacggatcattactctcataatact 15

2. Sequence 1: E5 (433bp) vs. Sequence 2: E7 (433bp)

Identities = 433/433 (100%), Positives = 433/433 (100%)

Seq. 1: 1 gaagggaatgtggcctaaactctggtaacaatggttcacaaaatgctggacctttaactg 60

Seq. 2: 1 gaagggaatgtggcctaaactctggtaacaatggttcacaaaatgctggacctttaactg 60

Seq. 1: 61 attcgtcggaacccaatcaccaacaaacctcagcttatcattaaactgcccgatcaaatc 120

Seq. 2: 61 attcgtcggaacccaatcaccaacaaacctcagcttatcattaaactgcccgatcaaatc 120

Seq. 1: 121 atcaatgtcagtattttcctccatcttgatggctaaaaactgtctcctcatttcctcaat 180

Seq. 2: 121 atcaatgtcagtattttcctccatcttgatggctaaaaactgtctcctcatttcctcaat 180

Seq. 1: 181 ctccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagtcactga 240

Seq. 2: 181 ctccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagtcactga 240

Seq. 1: 241 gtccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgact 300

Seq. 2: 241 gtccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgact 300

Seq. 1: 301 cttcaacaactgagtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaa 360

Seq. 2: 301 cttcaacaactgagtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaa 360

Seq. 1: 361 agctctccatttccctcagccaatccaaagaaacagacggatcattactctcataatact 420

Seq. 2: 361 agctctccatttccctcagccaatccaaagaaacagacggatcattactctcataatact 420

Seq. 1: 421 ccacattcctccc 433

Seq. 2: 421 ccacattcctccc 433

3. Sequence 1: E7 (433bp) vs. Sequence 2: F6 (434bp)

Identities = 416/418 (99%), Positives = 416/418 (99%)

Seq. 1: 3 aggg**a**atgtggcctaaactctggtaac**a**atggttcacaaaatgctggacctttaactgat 62

Seq. 2: 432 aggg**g**atgtggcctaaactctggtaac**g**atggttcacaaaatgctggacctttaactgat 373

Seq. 1: 63 tcgtcggaacccaatcaccaacaaacctcagcttatcattaaactgcccgatcaaatcat 122

Seq. 2: 372 tcgtcggaacccaatcaccaacaaacctcagcttatcattaaactgcccgatcaaatcat 313

Seq. 1: 123 caatgtcagtattttcctccatcttgatggctaaaaactgtctcctcatttcctcaatct 182

Seq. 2: 312 caatgtcagtattttcctccatcttgatggctaaaaactgtctcctcatttcctcaatct 253

Seq. 1: 183 ccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagtcactgagt 242

Seq. 2: 252 ccaccaatgaacaatacttggccataaactgctccttgaacatttcccaagtcactgagt 193

Seq. 1: 243 ccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgactct 302

Seq. 2: 192 ccttctgctcctgagtaaaatgaccggtaagagaatcccaccatctgtaagcatgactct 133

Seq. 1: 303 tcaacaactgagtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaaag 362

Seq. 2: 132 tcaacaactgagtagcatacaaaaccttgagctcaggctcacagtgactaacatcaaaag 73

Seq. 1: 363 ctctccatttccctcagccaatccaaagaaacagacggatcattactctcataatact 420

Seq. 2: 72 ctctccatttccctcagccaatccaaagaaacagacggatcattactctcataatact 15

4. Sequence 1: E6 (312bp) vs. Sequence 2: F7 (369bp)

Identities = 294/296 (99%), Positives = 294/296 (99%)

Seq. 1: 8 atgtgggtagcagaatagagatacattgagcttgttgagtgttggttttacgccgataga 67

Seq. 2: 17 atgtgggtagcagaatagagatacattgagcttgttgagtgttggttttacgccgataga 76

Seq. 1: 68 ttcccaaaactgtgtttgcatcggaatcgactgctgaaataaatctcgctcgatagtgag 127

Seq. 2: 77 ttcccaaaactgtgtttgcatcggaatcgactgctgaaataaatctcgctcgatagtgag 136

Seq. 1: 128 agactgttcacgaattaagccgcctgtagctccattaaaacccggaaagaagaagtgttt 187

Seq. 2: 137 agactgttcacgaattaagccgcctgtagctccattaaaacccggaaagaagaagtgttt 196

Seq. 1: 188 gattaagccattacgt**g**gatgaggcgagcttttgcgtgaaattcattgacccaggcttcg 247

Seq. 2: 197 gattaagccattacgt**t**gatgaggcgagcttttgcgtgaaattcattgacccaggcttcg 256

Seq. 1: 248 gcagataaatattccaagtttag**c**caaatgggttttttgctaatcatggcagccac 303

Seq. 2: 257 gcagataaatattccaagtttag**g**caaatgggttttttgctaatcatggcagccac 312

5. Sequence 1: E8 (496bp) vs. Sequence 2: F9 (337bp)

Identities = 314/319 (98%), Positives = 314/319 (98%), Gaps = 1/319 (0%)

Seq. 1: 8 atgtgggtaaggcaacccttaaatcctacagctcgctgttaaaacaaatctcctatgtgg 67

Seq. 2: 8 atgtgggtaaggcaacccttaaatcctacagctcgctgttaaaacaaatctcctatgtgg 67

Seq. 1: 68 gcgagacg**a**tggtcgaacaatgtcacctccaacccaacatcccaatcttgggaatcttac 127

Seq. 2: 68 gcgagacg**t**tggtcgaacaatgtcacctccaacccaacatcccaatcttgggaatcttac 127

Seq. 1: 128 cacctgtacacagcccaaacctgtaacccattgtgactccgctcctaggcgtaaccctgg 187

Seq. 2: 128 cacctgtacacagcccaaacctgtaacccattgtgactccgctcctaggcgtaaccctgg 187

Seq. 1: 188 cccaa**cg**taactccactc**t**cacctggcctacctactaatccaccctcgcaatctcagctc 247

Seq. 2: 188 cccaa**tt**taactccactc**a**cacctggcctacctactaatccaccctcgcaatctcagctc 247

Seq. 1: 248 tcctccctcgcacca-tgtacaacttcacagcactttacataacactgaatatcatacgg 306

Seq. 2: 248 tcctccctcgcaccattgtacaacttcacagcactttacataacactgaatatcatacgg 307

Seq. 1: 307 aaggcatctaccttatgcc 325

Seq. 2: 308 aaggcatctaccttatgcc 326