A MOLECULAR INVESTIGATION OF POPULATION STRUCTURE AND PATERNITY IN THE COMMON GARTER SNAKE, *THAMNOPHIS SIRTALIS*

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We accept this thesis as conforming
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Abstract

The study of genetic variation within and among populations of a species is a cornerstone of evolutionary biology. Here, I use the wideranging species, *Thamnophis sirtalis*, to study genetic variation between two classes of study sites that are predicted to differ in genetic variation. I also test the role of multiple paternity in contributing to within population genetic variation in this species.

At salmon hatcheries on Vancouver Island, Common Garter Snakes often feed heavily on salmon smolts, which represent an abundant food source. The frequently high abundance of snakes at hatcheries thus may result either from snakes being attracted to them from surrounding areas or from explosion of a local population due to a rich source of food. If the former, genetic variation at hatcheries should be higher than at natural sites; if the latter, genetic variation should be similar to that of natural sites, or perhaps even lower. Microsatellite markers revealed little detectable genetic variation among sites, but genetic profiles from one locus suggest that hatcheries are not as stable as natural sites, providing slight support for the first hypothesis. Although genetic variation among populations was not closely related to geographic relationships, the data do suggest that snakes from southern Vancouver Island are distinct from more northern sites.

Based on single-locus microsatellite DNA analysis, there was clear evidence of multiple paternity in 37.5% of 16 litters born in the laboratory. However, the significance of this phenomenon is not clear. Snakes exhibiting multiple paternity were similar in body size and litter size to snakes showing only single paternity; they also did not differ in the size of their offspring or in the proportion of stillborn offspring they produced. Furthermore, there was no demonstrable difference in the frequency of multiple paternity between my sample and that from another study of eastern snakes, which produce much larger litters.

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INTRODUCTION

The study of genetic variation within and among populations plays a central role in evolutionary biology (Avise, 1994). Populations do differ in genetic structure, as do the individuals contained within a population (Mayr, 1963). This variation is directed by numerous processes, with natural selection as the major process that affects the phenotypic constitution of a population (Hedrick, 1983). Because phenotype is the result of the expression of genotypic traits (and, of course, environmental effects), the genetic variation of a population is also affected by selective processes.

Genetic variation within a population is dictated by the introduction of new alleles via migration, and introduction or loss of alleles through drift, mutation, selection and the mating system (Avise, 1994; Hedrick, 1983; Hartl, 1981). Mutation tends to increase variation but is often insignificant due to low rates (Avise, 1994), whereas drift tends to decrease variation (Hedrick, 1983). Mating system, selection and migration have varying effects depending on the situation (Hedrick, 1983). This study focuses on the effects of migration and mating system on genetic variation within populations of the Common Garter Snake, *Thamnophis sirtalis*. In particular, I am concerned with how many males may mate with a single female and how different foraging strategies may affect gene flow and population structure.

The Common Garter Snake, *Thamnophis sirtalis*, is the most widely distributed North American reptile, inhabits a wide array of environments and exhibits geographic variation in many life-history and other phenotypic characteristics (Gregory & Larsen, 1993; Fitch, 1980). Species that exhibit such broad distributions along with variations in habitat utilization and phenotype often prove to be diverse with respect to both ecology and genetic structure (Avise, 1994; Gregory & Larsen, 1993).

Multiple factors could influence the distribution of genetic variation within and among populations of Common Garter Snakes. Here, I focus on differences between two kinds of sites: "natural" sites and hatchery sites where snakes feed on artificially abundant salmon smolts (Gregory and Nelson, 1990). In particular, I ask whether hatcheries act as attractants, drawing snakes from surrounding areas or whether they simply allow small, local populations to expand. In the former case, genetic variation at hatcheries should be higher than that of natural sites, whereas in the latter case, it should be either similar or, possibly, lower than at natural sites.

Within populations, an important contributor to variation in genetic structure is the mating system (Hedrick, 1983). In *Thamnophis sirtalis*, males produce a copulatory plug shortly after mating, which in theory prevents multiple matings of a female (Halpert *et al.*, 1982; Ross and Crews, 1977; 1978; Devine, 1977). Whether this trait is a strategy of the female to reduce her attractiveness and allow her to move on to other critical activities or of males to prevent sperm competition is open to question, but the plug lasts only a few days (Devine, 1984). Because fertilization occurs several weeks after copulation, multiple insemination of an individual female is possible. In fact, multiple insemination is known to occur in Common Garter Snakes (Schwartz *et al.*, 1989), but its ecological significance and the factors that influence its occurrence are not well understood.

For example, multiple insemination might be especially likely at communal dens, where the extremely high density of males leads to intense competition for mates. The advantage to a male of mating with an already-mated female also might be especially high if that female is likely to produce many offspring. Litter size in *Thamnophis sirtalis* varies both with body size of females and geographically among populations (Gregory & Larsen, 1993). My aims in this study are: 1) to test whether multiple paternity occurs in *Thamnophis sirtalis* on Vancouver Island, British Columbia; and 2) if multiple paternity occurs, to test for the effect of potentially influential factors on its incidence.

For both parts of my study, I use single-locus polymerase chain reaction techniques to detect allelic variation at microsatellite DNA loci. These markers are well-known for their high degree of variability (Jones and Avise, 1997; Scribner *et al.*,1994), which makes them very amenable to studies of genetic variation both within families and between populations.

BACKGROUND

Population Structure and Gene Flow

Molecular Techniques and Snake Populations

Molecular investigations of genetic structure of populations and gene flow between populations originally focused on allozyme polymorphisms to quantify variability. Although many allozymes have multiple alleles, low levels of detectable polymorphism, along with the requirements of sample storage, make allozymes inappropriate nuclear markers in many cases (Scribner *et al.*, 1994). More recent techniques have measured polymorphism at the nucleic acid level (e.g., RFLPs, Karl and Avise, 1992, Karl *et al.*, 1992), and most recently, single-locus VNTR analyses have proven very effective at detecting structure and estimating gene flow (Goodman, 1997). Genetic differences between island and continental populations (Estoup *et al.*, 1996), deme size (Lehmann *et al.*, 1997), effective population size and gene flow (Tessier *et al.*, 1997) and estimates of the effective number of breeding adults (Scribner *et al.*, 1997) all have been revealed using single-locus microsatellite data.

Most of the research dealing with genetic variability of populations of squamate reptiles has been done using allozyme electrophoresis (e.g., Sasa, 1997; Sattler and Ries, 1995; Dessauer and Cole, 1991; Moritz *et al.*, 1990; Aguilars-S *et al.*, 1988; Sites *et al.*, 1988; Bock and McCracken, 1988; Murphy *et al.*, 1984). Although much research on trait variation in and between populations of snakes has been done at the phenotypic level (see Brodie and Garland, 1993, and references therein; King, 1988; Auffenberg, 1955), there is a paucity of molecular data. Those studies of snake populations that do include molecular data have focused on allozymes. Perhaps the most well-known study of population genetic structure in snakes is that of the Lake Erie water snake, *Nerodia sipedon insularum* (King and Lawson, 1997). Many of the early conclusions about gene flow between island and mainland populations were derived using colour polymorphisms (King, 1993a; 1993b; King, 1987), but the phenotypic evidence has been supported strongly by another study which sampled seven allozyme loci (King and Lawson, 1995). In a related study of Lake Erie garter snakes, gene flow was analyzed using twelve presumptive allozyme loci (Lawson and King, 1996).

Venom proteins of some species of venomous snakes have been shown to vary over geographic distances (Minton and Weinstein, 1986; Aragon and Gubensek, 1981). A recent study by Forstner *et al.* (1997) showed that venom profiles varied with geographically distinct populations, but such variation may not be representative of historical population divergences. Instead, geographic variation in venom composition may reflect diet specialization rather than phylogenetic relationships (Daltry *et al.*, 1996).

Population Ecology of Snakes

Long-term population studies of snakes are rare and dynamics of populations are poorly understood (Parker and Plummer, 1987). Parker and Plummer (1987) cite four reasons why this is so: snakes can be inconspicuous and nocturnal; individual snakes often have prolonged periods of inactivity which make them inaccessible to researchers; measurements of population densities suggest that many species exist at very low densities at most times; and snake movements can be extensive and irregular, which makes defining population boundaries problematic. Although some species do aggregate at certain times (e.g., communal egg laying in *Coluber rubriceps* and either mating or moisture conservation in Typhlops vermicularis, Amr *et al.*, 1997; garter snakes at communal hibernacula, Gregory, 1977a, b; yellow-bellied sea snakes at marine 'slicks', Kropach, 1971), for the most part, abundance estimates of less dense populations are usually associated with large standard errors, due to low recapture rates (Parker and Plummer, 1987). Furthermore, snakes often do not meet the assumption of equal catchability inherent in capture/mark/recapture models (Gregory *et al.*, 1987; Parker and Plummer, 1987). In a study of the feeding habits and habitat use of three species of garter snakes, Gregory (1984) noted that small snakes rarely were caught.

By contrast, snakes often concentrate in areas where prey availability is high or prey are abundant (e.g., Arnold and

Wassersug, 1978; Wharton, 1969). Some species move long distances to sufficiently prey-rich feeding locales. Temperate-zone snakes such as the Common Garter Snake undergo large-scale seasonal migrations to feeding areas from overwintering sites (Gregory *et al.*, 1987; Larsen, 1986).

The diet of a snake species often varies from site to site (Daltry *et al.*, 1996; Drummond and Macias Garcia, 1989; Gregory, 1984; Kephart, 1982), which is usually presumed to be a reflection of local specialization driven by availability of prey species (Gregory and Nelson, 1990; Kephart, 1982; White and Kolb, 1974). The Common Garter Snake reflects just such a variation. Over most of its wide range, earthworms and amphibians are predominant in stomach content analyses, but mammals, birds and fish also are consumed (Gregory, 1984; Drummond, 1983; Fitch, 1965). Fish are uncommon prey items in most populations studied (Gregory and Nelson, 1990), probably due to this species' poor aquatic foraging capability (Drummond and Burghardt, 1983; Kephart and Arnold, 1982). Salmonid fish are even rarer food items (Fitch, 1941), which may be due to the high levels of activity exhibited by salmonids and their fast swimming speed, which makes them difficult to capture (Gregory and Nelson, 1990).

Commercial fish hatcheries offer high densities of potential prey to a variety of fish predators (Pitt and Conover, 1996). Although the primary predators on fish at hatcheries are birds (Pitt and Conover, 1996), small mammals and snakes also forage for fish in rearing channels (Pitt and Conover, 1996;

Gregory and Nelson, 1990). *Nerodia rhombifer*, a natricine snake closely related to garter snakes also forages at hatcheries (Plummer and Goy, 1984). On Vancouver Island, Common Garter Snakes forage almost exclusively on salmon smolts at hatchery sites, whereas fish are rarely eaten at more natural sites (Gregory and Nelson, 1990; Gregory, 1984). This may be due to two different factors: food items common in garter snake diets, such as amphibians, are relatively rare at hatcheries (Gregory and Nelson, 1990; pers. obs.); or densities of fish in rearing channels are often extremely high (3-6 X 10^6 smolts in narrow rearing channels at Big Qualicum fish hatchery, Gregory and Nelson, 1990; pers. obs.). This artificial inflation of an uncommon food source, the rarity of more usual prey items, and the freedom from avian predators afforded snakes under predator nets that commonly cover rearing channels may direct garter snakes found near hatchery sites to become fish 'specialists'.

Because snakes congregate at food-rich areas, hatcheries may serve to attract common garter snakes from surrounding sites. In contrast, because most fish hatcheries are built on natural rivers that garter snakes often inhabit, the increased food availability associated with hatcheries may allow natural snake populations to exceed previous carrying capacities. In the first case (hatcheries attracting snakes), the genetic profiles of snakes captured at hatcheries should reflect an increased level of genetic diversity relative to natural populations. On the other hand, in the second case (hatcheries allow an already existing population to expand), then the genetic diversity of a hatchery population should be comparable to natural sites, or possibly show narrow genetic diversity. Furthermore, because geographic differences in food habits in species of garter snakes may have a genetic basis (Arnold, 1981a, 1981b), hatchery snakes may prove to be genetically distinct from nearby natural populations relative to the differences shown between natural sites, and snakes from different hatcheries may be genetically similar.

Paternity

Why Use Molecular Techniques?

Almost all studies of reproductive success in natural populations of vertebrates have restricted investigation to females (for examples, see studies of bird and mammal species in Clutton-Brock, 1988). Research on snakes also suffers from this bias. In their chapter on the reproductive ecology of snakes, Seigel and Ford (1987) do include information on the spermatogenic and hormonal cycles associated with male reproduction, but actual quantification of reproductive effort is restricted to measurements of litter or clutch size obtained from females. The convenience of sampling females over males is obvious; for males, matings need to be observed, but for females, only counts of eggs or young need be made. Exceptions to female-only assessments are usually studies of species in which paternal behaviour supports the hypothesis of monogamy (as once assumed in up to 90% of all bird species; see Lack, 1966).

The premise that paternal behaviour assures single paternity is no longer tenable, due primarily to investigations using molecular markers (e.g., Burley *et al.*, 1996; Schartl *et al.*, 1993; Harris *et al.*, 1991; Birkhead *et al.*, 1990). Molecular techniques have fostered a revolution in studies of reproductive success. Since the research of Jeffreys *et al.* (1985a, 1985b) first identified suitable hypervariable DNA markers useful for both identifying individual genotypes and quantifying relatedness among individuals, analyses of variable-number-of-tandem-repeat (VNTR) loci have been done in various taxa to determine patterns of reproductive success and to address numerous other, previously intractable, problems (Madsen *et al.*, 1996; Lubinski *et al.*, 1995; Galbraith *et al.*, 1993; Pfennig and Reeve, 1993; Marinelli *et al.*, 1992; Yamagishi *et al.*, 1992; Amos *et al.*, 1991).

Although components of reproductive cycles of male snakes, such as spermatogenesis (Saint Girons, 1982; Seigel and Ford, 1987), hormone production (Seigel and Ford, 1987; Crews *et al.*, 1984) and mating behaviour (Duvall *et al.*, 1993; Gillingham, 1987) have been studied in some species, actual quantification of the relative contribution of a single male to a given litter or clutch only recently has been examined in any detail (see Höggren and Tegelström, 1995). The difficulty with assessing annual reproductive success of a male snake in a natural population is due, in part, to the behaviour of snakes. Most snakes are secretive and cryptic and observations of snakes in a natural population are anything but comprehensive (Fitch, 1987). Conspecific snakes are also difficult to distinguish on the basis of external morphology, so that identification of individuals *in situ* in the wild is impossible without an obvious mark, such as clips of subcaudal scales (Gregory, 1977a), a paint mark (Madsen and Shine, 1993) or some naturally imposed, distinctive mark (e.g., "Short-tail" in Shine *et al.*, 1981). Therefore, reliable and repeated observations can be made only by disturbing snakes, which may bias further behaviours.

Observations of matings are an unreliable method of assessing fertilization for other reasons. For example, not all copulations result in fertilization and multiple matings can result in uneven fertilizations of a litter/clutch through sperm competition (Höggren, 1995). Furthermore, not all matings of a given female may be observed. Investigating paternity using molecular techniques avoids most of the difficulties associated with observations of matings. Methods comparing allelic distributions of allozymes, restriction fragment length polymorphisms (RFLPs) and multilocus DNA fingerprints of VNTRs all have been applied to questions of parentage (Avise, 1994). All of these techniques assume Mendelian transmission of genes and use some method of exclusion to estimate relatedness. Although Mendelian inheritance is a reasonable assumption in most cases, exclusion of individuals as parents based on genetic data requires that all potential parents be sampled. In a large population, this type of sampling is prohibitive. When simply determining numbers of fathers in a single litter or clutch, identifying the father becomes peripheral. Exclusion, therefore, is not necessary if the method applied can determine how many fathers contribute without necessarily genetically identifying who the fathers actually are.

Microsatellite DNA regions differ from other hypervariable regions on the basis of the size of the core repeat. Typically, microsatellites have a core repeat motif of one to six base pairs repeated up to one hundred times (Tautz, 1993). In-depth studies of the mutation rates of tandem repeat loci have found that rates can be as high as 10^{-4} (Henderson and Petes, 1992; Levinson and Gutman, 1987) or even 10^{-3} (Kelly *et al.*, 1991; Jeffreys *et al.*, 1988). The major mechanism responsible for these high rates is strand-slippage during DNA replication (Levinson and Gutman, 1987). Strand-slippage allows repeats to be added or subtracted to the replicating strand when either the replicating or template strand loops out somewhere in the repetitive region. Repeats are thought to be added or subtracted in a stepwise manner, so that only one or a few repeats are changed in a single mutational event (Schlötterer and Tautz, 1992).

Microsatellite loci, due to their hypervariable nature, often have many alleles present in a single population. In a recent study of the sleepy lizard, *Tiliqua rugosa*, two microsatellite loci (Tr5.20, Tr3.2) had 19 and 20 alleles, respectively, for 38 individuals sampled (Cooper *et al.*, 1997). In another study of Gulf pipefish, *Syngnathus scovelli*, each of the four loci examined had 19 or more alleles present (Jones and Avise, 1997). Most individuals in a given population can be heterozygous at a given locus if sufficient allelic diversity occurs at the locus. In the case of the two lizard loci cited above, observed levels of heterozygosity were 84% and 89%, respectively (Cooper *et al.*, 1997). Observed heterozygosities for the pipefish study ranged from 87.7% to 95.2% (Jones and Avise, 1997). If heterozygosity of most individuals in a population is assumed, distinguishing the number of males that contribute to a single litter does not require that parental genotypes be included in the analysis. Each parent that contributes to a litter also will contribute its two alleles, so the number of parents contributing will be equal to the number of alleles present in a litter divided by two. Of course, given that related individuals will exist in a single population and related individuals can carry the same alleles, measurement at a single locus will result in some underestimation of parental contributions. This shortcoming is overcome if sufficient loci are sampled. Under-estimation also can be minimized if the maternal genotype is known and included in the analysis.

Why Investigate the Common Garter Snake?

Several aspects of the mating behaviour of the Common Garter Snake, *Thamnophis sirtalis*, and other congeneric species suggest that multiple copulations and multiple paternity commonly occur in this species. As is the case with most temperate-zone ectothermic vertebrates, Common Garter Snakes hibernate for up to seven months (Gregory 1977a, b; Aleksiuk, 1976). Males emerge first from hibernacula (Gregory, 1977b) with abundant sperm in the vas deferens from the summer or fall of the previous year (Crews and Gartska, 1982). Females begin emerging a few days later and are actively courted by the available males (Gregory, 1977b). Because males emerge first, the operational sex ratio is heavily biased, and many males attempt intromission with a single female in what is called a mating ball (Seigel, 1996).

Although males stimulate females behaviourally during courtship (see Ford, 1996), females may control intromission through tail-lifting and/or cloaca-gaping (Ford, 1996), and there is evidence that females can hormonally direct the evacuation of stored sperm from previous matings (Halpert *et al.*, 1982). Sperm storage can occur, however, and although fall matings are infrequent, sperm from fall matings can be stored until the following spring (Ford, 1996). In a related species, *Thamnophis hammondii*, sperm have been stored up to four years (Stewart, 1972).

There are also aspects of garter snake mating behaviour that support the hypothesis of a single mating per female within a given season. Males insert a copulatory plug into mated females, usually within 20 minutes of intromission (Blanchard and Blanchard, 1942). This plug may last from four days to two weeks, and its persistence is temperature-dependent (Devine, 1984). The plug has been thought to act as a physical barrier to further suitors (Halpert *et al.*, 1982; Ross and Crews, 1977; 1978; Devine, 1977) and contains a pheromone that is unattractive to other males, further inhibiting multiple intromissions (Devine, 1984; Ross and Crews, 1977; 1978).

The male copulatory organ, the hemipenis, lacks rigid structure and cannot function in dislodging a copulatory plug (Devine, 1984). If a plug is in place and another male attempts intromission, it seems unlikely that sperm will be transferred to the female. Females also tend to leave the 'mating grounds' almost immediately after mating and aggregate in areas where males are in relatively low numbers or are absent (Schwartz *et al.*, 1989; Gregory, 1975; pers. obs.). This combination of copulatory plug and movement of females away from prospective mates after intromission has led many authors to assume that single paternity is the norm in the Common Garter Snake (Halliday and Arnold, 1987; Gartska and Crews, 1985; Gartska *et al.*, 1985; Whittier *et al.*, 1985; Halpert *et al.*, 1982; Gregory, 1974).

Research on other species of snakes clearly shows the occurrence of multiple paternity (e.g., Stille *et al.*, 1986; Schuett and Gillingham, 1986), but there is little published evidence that more than one male garter snake can contribute to a single litter of offspring. Gibson and Falls (1975) report proportions of melanistic offspring in litters of common garter snakes that deviate from expected Mendelian proportions. However, these morph frequencies, although suggestive of multiple paternity, are not solid evidence for multiple fertilizations, because colour patterns cannot be considered accurate indicators of parentage in this case. The authors assume that the genetic control of colour polymorphisms is simple Mendelian (Gibson and Falls, 1975) because of an earlier study by Blanchard and Blanchard (1941). Sample size in the latter study was inadequate, and frequencies of offspring morphs in wild-caught gravid females that depart from expected Mendelian ratios are considered to be indicative of multiple matings, rather than suggestive of an equally tenable alternative, a more complex genetic system involved in the inheritance of colour patterns (Blanchard and Blanchard, 1941). Ratios of offspring colour patterns that deviate from expected Mendelian ratios can be explained as something other than evidence for multiple inseminations. Melanism could be polygenic in nature, epistasis may play a role, or linkage could be a factor. Complex genetic systems controlling colour polymorphisms have been described in other species (e.g., mice and corn, as described in Tamarin, 1991). More recent work investigating the genetic mechanisms of melanism in this species suggest that the simple one locus, two allele system does not apply (Zweifel, 1998).

In a more recent investigation of paternity in *Thamnophis sirtalis*, Schwartz *et al.* (1989) used four polymorphic protein loci to measure the frequency of multiple paternity in wild-caught gravid females. The premise that multiple paternity occurs is well supported in this study, but the frequency estimates are likely to be underestimates of the actual frequencies. Allozymes are reasonable markers for population genetic analyses, but because complex gene products and not the genes themselves are being analyzed, there is high potential for loss of information. Utility of markers used for population genetic analysis depends on the extent of the polymorphism shown by the marker (Scribner *et al.*, 1994). In the case of proteins, mutations at the DNA level may occur that do not affect the activity or electrophoretic mobility of the protein encoded by the DNA (e.g., point mutations that occur at the third base position of a codon; see Stryer, 1988).

The different scale of variability inherent in proteins versus DNA is illustrated in a study of the population genetics of the Common Toad (Scribner *et al.*,1994). In that study intra- and interpopulation diversity was assessed using allozyme, single-locus microsatellite, single-locus minisatellite and multilocus minisatellite data (Scribner *et al.*, 1994). For all classes of markers, allozymes were found to have the lowest number of alleles per locus and, therefore, the lowest estimates of heterozygosity. This lack of information provided by allozymes led the authors to suggest that VNTR analyses were more appropriate for population studies that focus on population subdivision and breeding structure (Scribner *et al.*, 1994).

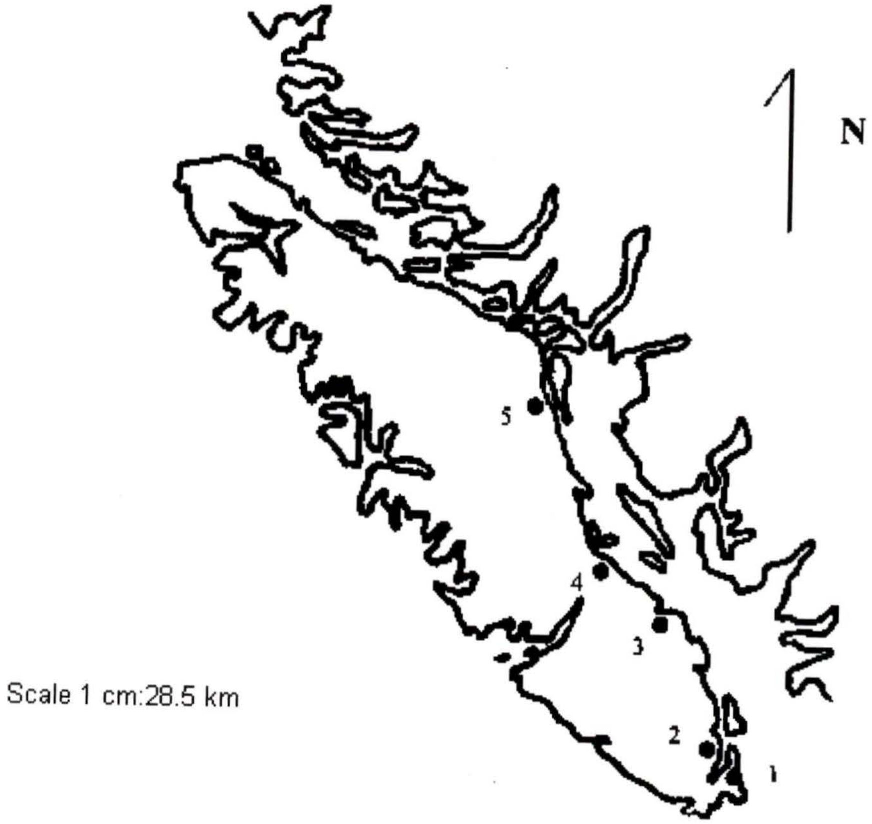
In this study, I use one VNTR analysis, single-locus microsatellite DNA polymorphisms, to tackle this problem.

Materials and Methods

Collection of Snakes

I captured snakes during the summers of 1996 and 1997 at five sites on Vancouver Island, British Columbia (Figure 1). One gravid female also was collected near Durrance Lake. I measured the snout-to-vent length (SVL) of each mother to the nearest 0.5 cm. Gravid females were held in the laboratory until they gave birth. Offspring were measured (SVL) and weighed immediately after birth and their sex was determined. The number of live versus dead offspring also was recorded. I sacrificed neonate snakes and several of the adult snakes for tissue samples, but the mothers used for the paternity sample were sampled non-destructively by clipping tail tips.

Figure 1. Locations of collection sites on Vancouver Island. 1=Elk Lake/Durrance Lake, 2=Malahat, 3=Island Highway, 4=Big Qualicum Hatchery, 5=Quinsam Hatchery.


N
5
4
3
2
1
Scale 1 cm:28.5 km

Genomic DNA Extractions

I used various snake tissues (liver, muscle, whole body sections of dead neonates and tail tips) for genomic DNA extractions. Genomic extractions were performed following Protocol I for isolating DNA from mammalian cells as outlined in Sambrook *et al.* (1989). A small (approx. 1 mg) amount of tissue was suspended in either 500 uL or 1 mL of cell extraction buffer (10 mM Tris/Cl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.5% SDS, 20-60 ug/mL RNAase), depending on the size of the tissue sample, and incubated for 1 hour at 37 degrees Celsius. Proteinase K then was added (100 mg/mL final concentration). Samples were extracted three times with equilibrated (pH 8.0) phenol, and once with chloroform/isoamyl alcohol (24:1). DNA was then precipitated using 1/10 volume 9 M ammonium acetate and one volume isopropanol and the resultant pellets were washed once with 70% ethanol. Pellets then were dried using a Speed Vac concen-trator and resuspended in either 20 or 30 uL of 1 M Tris/EDTA.

I determined concentration of DNA in solution using a spectrophotometer. If the ratio of A_{260} to A_{280} proved to be less than 1.45, the solution was increased in volume to 100 uL and was reextracted once more with equilibrated phenol. I stored DNA solutions at -20 degrees Celsius until used.

Construction and Screening of a Subgenomic Library

I used genomic DNA from one Vancouver Island Common Garter Snake for constructing a subgenomic library suitable for screening for microsatellite DNA inserts. The genomic DNA was prepared for ligation by overnight digestion at 37 degrees Celsius with a blunt-cutting restriction endonuclease [5 ug genomic DNA, 80 units Hinc II (New England Biolabs), 1X NEBuffer 3 (New England Biolabs) buffer, 1X Bovine Serum Albumin (New England Biolabs)]. After digestion, I reclaimed the 500-1,500 base-pair size range of the digest using agarose gel electrophoresis and activated DEAE-cellulose membrane (see Sambrook *et al.*, 1989 for procedure for activating membrane and reclaiming bound DNA from membrane).

I prepared the ligation vector (M13mp19 RF1, Pharmacia Biotech) for ligation as described above. Vector DNA was digested for only 1.5 hours at 37 degrees Celsius, extracted and precipitated as described above, and resuspended in 88 uL of double-distilled water. I then dephosphorylated the vector and precipitated it as described above.

I ligated insert DNA into vector DNA using T4 DNA ligase (New England Biolabs). Ligations were done at 14 degrees Celsius overnight. The most efficient ligation occurred using a 1:8 vector-to-insert molar ratio. I transformed ligations using electroporation, and library plates were screened using several simple sequence repeat oligonucleotides (GACA, CA, and GATA repeats) end-labelled with ^{32}P. Library screening followed the protocol outlined in the Clontech product protocol (#PR83281, 1994), with the following modifications: prehybridization was omitted (salmon sperm DNA blocking agent tends to bind small, repetitive oligonucleotides and prevent binding to positive clones); the initial posthybridization wash was omitted; and the final posthybridization wash was done as a series of 20-minute washes, testing between washes for high background using a Geiger counter. I exposed washed filters to autoradiograph film overnight at -70 degrees Celsius.

Development and Application of Polymerase Chain Reaction (PCR) Primers

I prepared and sequenced positive clones following the protocol for Dye Primer Cycle Sequencing from Perkin Elmer (1995). Sequences then were visually surveyed for useful repetitive regions. A total of 14 of sixty positive clones proved to have both repetitive regions and flanking regions for which primers could be constructed. I designed primers using OLIGO Primer Analysis Software, version 1 (National Biosciences, Inc, 1989). A total of 14 primer sets were synthesized and tested for PCR suitability. Of the 14 sets of primers, only two proved to generate consistent PCR products that appeared to be variable within populations and litters (see Table 1).

This primer set was augmented by a set of primers designed by Melanie Prosser (McMaster University) using *Nerodia sipedon* DNA and two primer pairs designed by Dr. Gary McCracken (University of Tennessee, Knoxville) using eastern North American *Thamnophis sirtalis* DNA. I used three sets of *Nerodia* primers for the population analysis, and only one of the eastern *Thamnophis* primer pairs for the paternity analysis. Both of the western *Thamnophis* primer pairs were used in both the population and paternity analyses.

I performed PCR of samples using the conditons specific for each primer set as found in Table 1. All PCR products were separated using 14.5 cm-by-17 cm non-denaturing 8% polyacrylamide (19:1 acrylamide:bis-acrylamide) gels. Gels were run using 2X TBE buffer and at 70V for 12-24 hours, depending on the size range of PCR products that a given primer set generated.

I loaded approximately 10 uL of PCR products for electrophoresis. I then stained gels with ethidium bromide for approximately 15 minutes and then digitally imaged the stained gels using the EagleEye system from Stratagene. Images were stored as TIF files and then analyzed as outlined below.

Table 1. Primer pairs, annealing temperature and number of alleles detected with each pair.

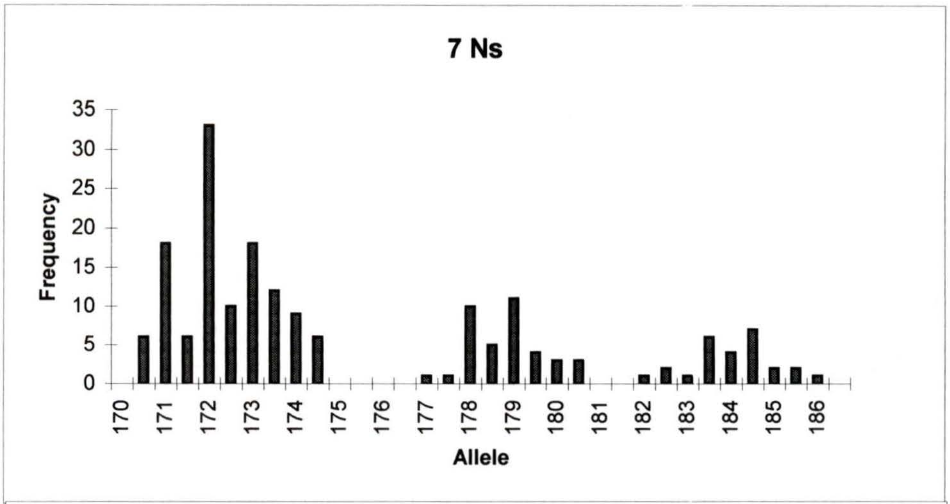
Primer	Sequence Forward/Reverse	Annealing Temp	#Alleles
2 Ts	5'TACACGTGCCGGAAATATGCTAG3' 5'TGAATAACACCTCTGGGTCAGTCTATC3'	54C	22
3 Ts	5'GGTCACTTAAATACAACGAAATTGGTTAGCT3' 5'CGGACAGCTCTGGCTCCCTTG3'	58C	12 +one null
7 Ns	Sequence not released	55C	3
8 Ns	Sequence not released	55C	2
9b Ns	Sequence not released	58C	2
5B Ts	Sequence unpublished	54C	13

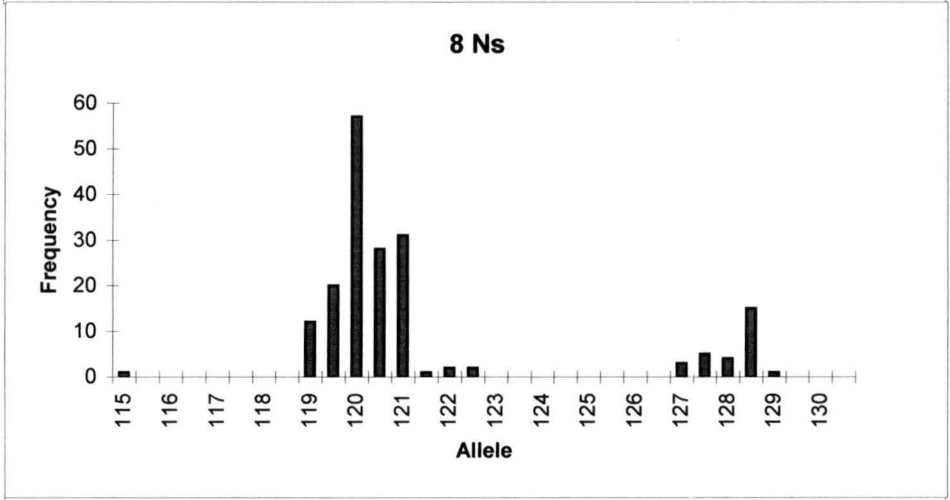
Data Analysis

I scored gel images using BioImage Whole Band software (Millipore Corp. Imaging Systems, Ann Arbor, Michigan). Allele sizes were estimated to the nearest base-pair using Whole Band analysis software and a molecular size grid created using either 50-bp or 20-bp size markers in at least three lanes of each gel image. I scored several of the litter gels by eye.

I identified alleles by binning scored alleles in an allele frequency histogram (Galbraith et al., 1991; Gill et al., 1990). Clustering of the scored alleles indicated bin limits, and the peak of each bin gave the most common allele size for each bin. For all of the *Nerodia* loci, clear bins were easily identified from the frequency histograms (Figure 2). For the two *Thamnophis* loci, some ambiguity existed as to where the bin limits occurred (Figure 3). I tested accuracy of binning for these two loci using the allele values generated for litter analysis. The number of parental alleles in each litter was established by generating allele frequency histograms for each litter scored with BioImage software (no strictly visually scored litter values were used for this analysis), and confirmation of the number of parental alleles was made by examining gel images visually. I then compared the scores for the alleles to the allele frequency histogram for all populations. Because repeated scores for single alleles were generated from single litters, the range of these repeat scores was used for comparison with the population frequency

Figure 2. Frequency histograms of raw allele scores for the three *Nerodia sipedon* loci.


7 Ns
Frequency
0
5
10
15
20
25
30
35
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
Allele


8 Ns
Frequency
0
10
20
30
40
50
60
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
Allele

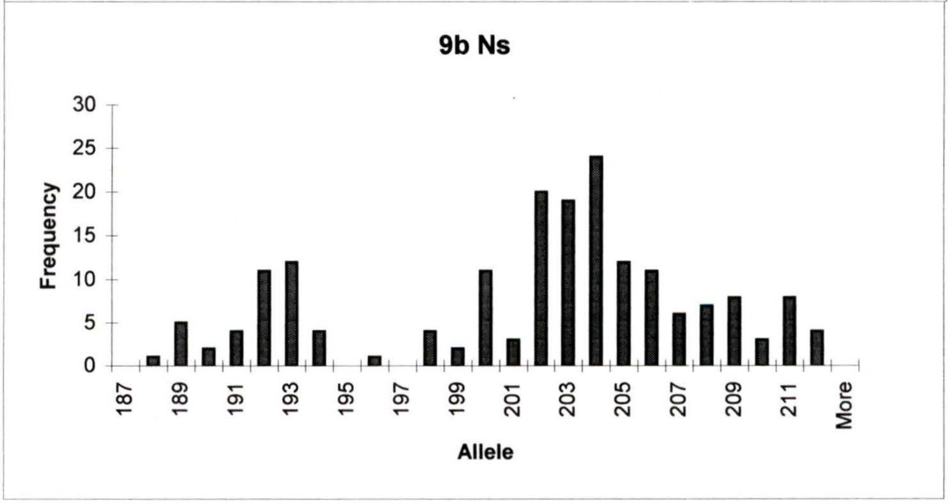
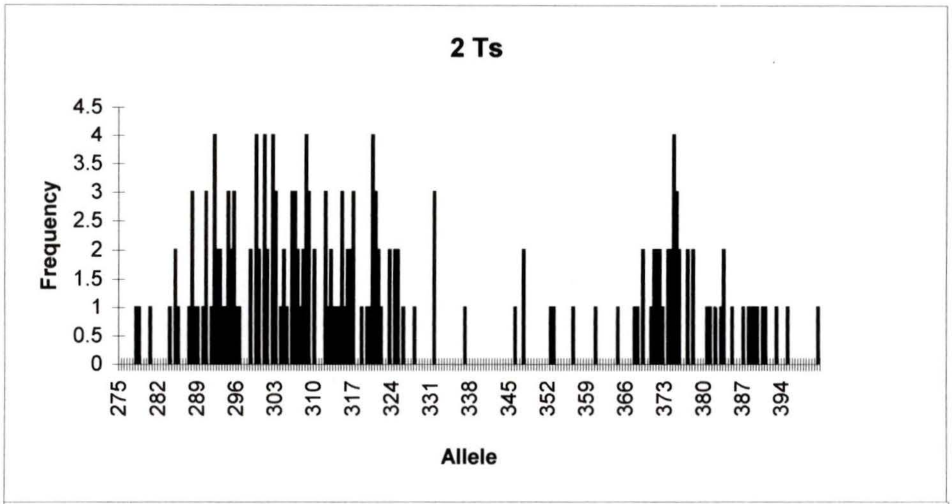
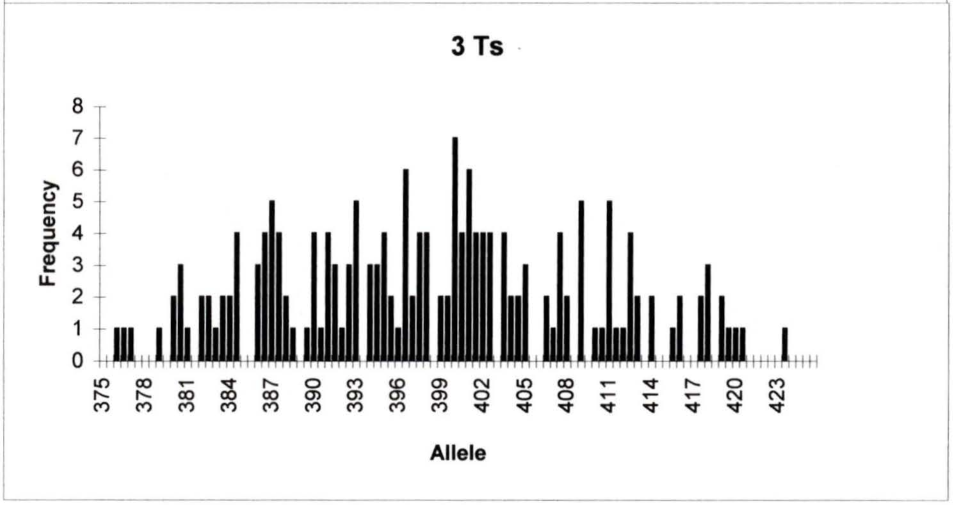

9b Ns
Frequency
0
5
10
15
20
25
30
187
189
191
193
195
197
199
201
203
205
207
209
211
More
Allele

Figure 3. Frequency histograms of raw allele scores for two *Thamnophis sirtalis* loci.


2 Ts
Frequency
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
275
282
289
296
303
310
317
324
331
338
345
352
359
366
373
380
387
394
Allele


3 Ts
Frequency
0
1
2
3
4
5
6
7
8
375
378
381
384
387
390
393
396
399
402
405
408
411
414
417
420
423
Allele

histogram to determine if each of these ranges fit an existing bin. For locus 2 Ts, nine scored litters containing 88 individuals were used in this comparison, with 97% of repeated alleles falling within a bin range. For locus 3 Ts, 11 litters containing 87 individuals were used, with 93% of repeated alleles falling within a bin range.

For the population study, allele frequencies and heterozygote vs. homozygote excess/deficiency were estimated for each population at each locus using GENEPOP version 1.2 (Raymond and Rousset, 1995b). Hardy-Weinberg equilibrium was tested at each locus in each population using a Markov chain method to calculate unbiased exact *P*-values (Guo and Thompson, 1992) when the number of alleles at a given locus exceeded four (2 Ts, 3 Ts). When alleles at a locus numbered four or fewer (7 Ns, 8 Ns, 9b Ns), I used complete enumeration. I calculated F-statistics and associated *P*-values according to Weir and Cockerham (1984), using both GENEPOP and FSTAT (Goudet, 1995). Populations were compared globally for differences in allele distributions using Fisher's exact test (Raymond and Rousset, 1995a) and also were tested for differences in genotype distributions using an F_{st}-based test (Raymond and Rousset, 1995b; Weir and Cockerham, 1984). Both tests use a Markov chain method to estimate *P*-values. In both tests, *P* was calculated for each locus across all populations, and tests were combined using Fisher's method (Raymond and Rousset, 1995a). For all data analyses, *P*-values were corrected for multiple tests (0.05/n, where n is the number of simultaneous tests, Lessios, 1992). Linkage was tested by pairwise comparisons of all loci in each population using a Markov chain method.

Relationships among populations were tested by generating a dendrogram using PHYLIP 3.5c (Felsenstein, 1993). I bootstrapped the allele frequency matrix 1000 times using SEQBOOT and genetic distances for each bootstrap were calculated using Cavalli-Sforza and Edward's (1967) chord distances. I used NEIGHBOR to generate unrooted neighbour-joining trees for each bootstrap, and CONSENSE was used to generate a single consensus tree. The consensus neighbor-joining tree was drawn using DRAWTREE. Pairwise comparisons of populations using F_{st} values was done using FSTAT (Goudet, 1995). F-statistics were estimated per allele, per locus, and over all loci. Estimated number of migrants between each pair of populations also was calculated.

I generated histograms and pie diagrams to compare allelic diversity of individual populations directly. Number of alleles in a given population, private alleles in each population, and the number of alleles shared between populations also were compared for locus 2 Ts and locus 3 Ts.

For paternity analysis, I determined allelic diversity for each of sixteen litters and mothers both visually and using the imaging procedure outlined above. Alleles present in littermates then were identified as either maternal or paternal, depending on litter allelic diversity and the maternal allele profile. In some cases, shared alleles between mothers and fathers were evident. For each locus used in this part of the study, litter alleles were counted and I estimated the number of parents per litter as the total number of observed alleles divided by two.

To test whether litters without observed multiple paternity met expected Mendelian genotype frequencies, genotype distributions of litters were tested at each locus using a Chi-square goodness-of-fit test (Zar, 1984). Expected frequencies were generated assuming one father. Only litters that contained at least 6 offspring were tested; I considered litters smaller than this to have too few offspring to use in this analysis. Litters in which multiple paternity was observed at at least one locus also were subjected to Chi-square goodness-of-fit tests, as a control. Tests were restricted to the loci at which multiple paternity was not detected.

Average relatedness within each litter was tested using Relatedness 4.2 (Queller & Goodnight, 1989). I separated litters with observed multiple paternity and those with no observed multiple paternity at any locus into two pools for this analysis. Average relatedness (R) was calculated at each locus over all litters, and for each litter over all loci. Jackknife values for each of the above also were generated. All R-values were generated by weighting all individuals equally.

I tested relationships between variables such as number of offspring, size of offspring, percentage of live offspring, maternal size (SVL) and observed paternity several ways. First, I compared mean SVL of mothers of singly sired litters to that of mothers of multiply sired litters using a two-sample *t* test (Zar, 1984). To examine the effects of paternity on number of offspring, I calculated residuals from regressions of number of offspring versus maternal SVL, in order to adjust for body size differences, and then compared average values of residuals between singly and multiply sired litters by using a two-sample *t* test (Zar, 1984). I tested for paternity effects on average mass of offspring in a litter and number of live offspring in the same way. All calculations were performed using SPSS Version 6.1.3 (SPSS Inc., 1994). I also did a power analysis if a test result was nonsignificant (Cohen, 1977). For all nonsignificant results, I also calculated the effect size (ES) and the minimal sample size (n_r) required for that effect to be found significant at a rejection level of 0.05 and a power (1-b) of 0.80.

Results

Population Structure

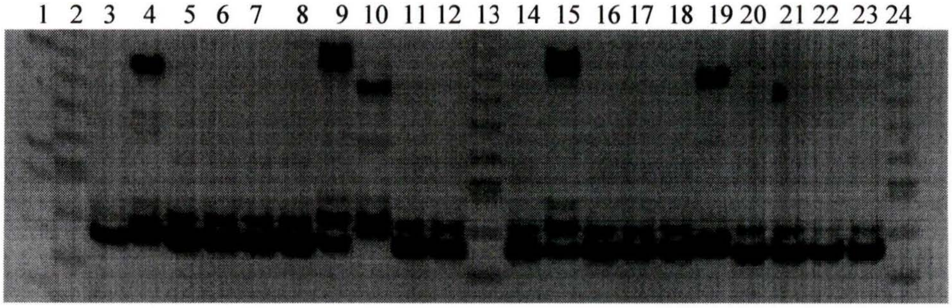
I sampled a total of 91 individuals from five populations for PCR analysis: 24 from the Island Highway site, 22 from the Elk Lake area, 10 from the Malahat region, 15 from Big Qualicum Hatchery and 20 from Quinsam Hatchery. I used three sets of PCR primers derived from *Nerodia sipedon* DNA and two sets derived from Vancouver Island garter snake DNA. Allelic diversity varied between the two sets of PCR primers (see Table 1). The *Nerodia* primers proved to be far less polymorphic than the *Thamnophis* primers (Figure 4).

Results of the Hardy-Weinberg exact tests by locus indicate that all loci over all populations met the requirements of Hardy-Weinberg equilibrium (corrected rejection level=P=0.05/25=0.002, Table 2). Results by population also met expectations of equilibrium. The combined probability (P=0.0097) across all populations and loci did not fall within the range of rejection.

Tests for homozygote and heterozygote excess/deficiency initially showed an excess of homozygotes in all populations for locus 3 Ts, most significantly in the Big Qualicum sample (Table 3). This excess of homozygotes suggested that a null allele was in operation at this locus (Summers & Amos, 1997). Null alleles are the result of a mutation in the DNA complementary to at least one of the primer binding sites.

Figure 4. PCR products of snakes captured at Quinsam Hatchery. a) Locus 7 Ns. Most lanes contain individuals homozygous for allele 1, but heterozygotes can be seen in lanes 4, 9, 10, 15, and 19. b) Locus 2 Ts. Note the greater level of polymorphism evident at this locus relative to locus 7 Ns.

a


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

b

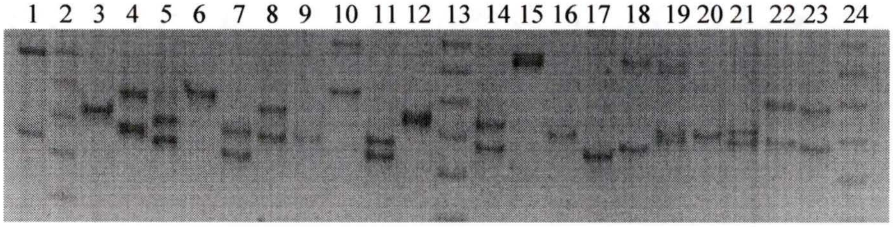

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Table 2. P-values and standard errors and combined probabilities (Fisher's method) of the probability test for Hardy-Weinberg equilibrium. P-values are for each locus in each population, and combined values are probabilities over each locus for all populations, and combined for all loci over all populations.

Locus	Population	P-value	SE	Combined P-value
7 Ns	BQ	0.067	/*	
	IH	0.398	/	
	EL	0.182	/	
	Ma	0.573	/	
	QH	0.354	/	
	combined			0.181
8 Ns	BQ	1.000	/	
	IH	1.000	/	
	EL	0.324	/	
	Ma	1.000	/	
	QH	no value[a]		
	combined			0.927
9b Ns	BQ	1.000	/	
	IH	0.004	/	
	EL	0.040	/	
	Ma	0.307	/	
	QH	0.129	/	
	combined			0.008
2 Ts	BQ	0.315	0.009	
	IH	0.167	0.007	
	EL	0.094	0.005	
	Ma	0.051	0.004	
	QH	0.517	0.008	
	combined			0.057
3 Ts	BQ	0.568	0.008	
	IH	0.328	0.007	
	EL	0.672	0.007	
	Ma	0.438	0.007	
	QH	0.874	0.003	
	combined			0.809
All	combined			0.010

* SE not computed if number of alleles at a locus was less than or equal to four, denoted as /.

[a] Locus 8 Ns is quasimonomorphic in QH population (only one copy of the second allele was detected in the population); therefore a *P*-value cannot be generated using GENEPOP.

Table 3. Observed and expected frequencies of homozygotes and heterozygotes at each locus in each population. Values in parentheses for locus 3Ts are original frequencies, without correction for a null allele.

Population	Locus	Heterozygosity obs/exp	Homozygosity obs/exp
BQ	7 Ns	5/8.03	10/6.97
	8 Ns	6/4.97	9/10.03
	9b Ns	5/5.55	10/9.45
	2 Ts	13/12.72	2/2.28
	3 Ts	(9/11.86)	(6/3.14)
		13/13.66	2/1.35
IH	7 Ns	6/6.36	18/17.64
	8 Ns	10/9.19	14/14.81
	9b Ns	3/8.66	21/15.34
	2 Ts	20/21.92	4/2.09
	3 Ts	(15/18.77)	(9/5.23)
		21/20.98	3/3.02
EL	7 Ns	13/14.44	9/7.56
	8 Ns	4/5.30	18/16.70
	9b Ns	4/7.91	18/14.09
	2 Ts	20/20.54	2/1.47
	3 Ts	(17/18.05)	(5/3.95)
		21/20.26	1/1.74
Ma	7 Ns	5/5.95	5/4.05
	8 Ns	3/2.68	7/7.32
	9b Ns	2/3.37	8/6.63
	2 Ts	7/9.37	3/0.63
	3 Ts	(6/8.16)	(4/1.84)
		9/9.16	1/0.84
QH	7 Ns	5/5.46	15/14.54
	8 Ns	1/1	19/19
	9b Ns	4/6.56	16/13.44
	2 Ts	16/18.10	4/1.90
	3 Ts	(9/15.15)	(11/4.85)
	3 Ts	18/17.41	2/2.59

This causes only one of the copies of the locus to amplify during PCR, and the resulting banding pattern appears to be homozygous for the amplifying allele. I used a likelihood approach proposed by Summers and Amos (1997) to estimate the number of null heterozygotes present at this locus for each population. For any allele, a, the true frequency of the allele can be described as follows:

$$n_a = Nf_a^2 + 2Nf_a(1 - f_a) \qquad (1)$$

where N is the total sample size and f_a is the frequency of allele a. The number of individuals that carry any of the amplifying alleles is known, so by solving the above quadratic equation, f_a can be calculated for each allele. The expected number of true aa homozygotes is given by Nf_a^2; by subtracting this from the observed number of aa homozygotes, an estimate of the number of ao heterozygotes (where o denotes a null allele) can be derived. This process was repeated for each allele of locus 3 Ts, and the total number of ao heterozygotes was estimated as the sum of each of the ao heterozygotes for each allele. No oo homozygotes were detected, as each individual amplified at least one band using primer set 3 Ts.

The frequency of the null allele also was calculated using the following relationship:

$$n_{xx} = N - n_{xo} \qquad (2)$$

where n_{xx} is the number of individuals that do not carry a null allele, and n_{xo} is the number of individuals that do carry a null, which, because there are no *oo* homozygotes in my data set, is the sum of *ao* heterozygotes determined above. The relationship between these two values and the frequency of the null allele has been derived by Summers and Amos (1997) as being:

$$f_o = n_{xo} / (2n_{xx} + n_{xo}) \qquad (3)$$

where f_o is the frequency of the null allele.

The total estimated number of *ao* heterozygotes was 26.5, with an f_o of 0.17. The individual allele values for each population were used to correct the original data set, so that a 13th (null) allele was included in the data set. After this correction, observed counts of homozygotes in each population for locus 3 Ts were equivalent to expected values (Table 3).

Only one pairwise comparison between loci suggested linkage (9b Ns and 2 Ts in the Big Qualicum population). Because only one of 50 tests resulted in a significant *P*-value (0.0274), all five loci are considered to be unlinked. Tests for allelic differences between populations were significant for three of the five loci (7 Ns, P=0.00, SE=0.00; 8 Ns, P=0.030, SE=0.00335; 2 Ts, P=0.00, SE=0.00), but, in the other two cases, it was impossible to reject the null hypothesis (9b Ns, P=0.996, SE=0.00055; 3 Ts, P=0.267, SE=0.0232). The combined test indicated that allelic diversity at all five loci was significantly different among populations (Chi-square= infinity, DF=10).

Results of tests for genotypic differentiation were similar to the results of the tests for allelic differentiation. Again, three loci were significantly different across populations (7 Ns, P=0.00, SE=0.00; 8 Ns, P=0.049, SE=0.00465; 2 Ts, P=0.001, SE=0.00134) and two did not show strong differences in population structure (9b Ns, P=0.998, SE=0.00038; 3 Ts, P=0.220, SE=0.02394). Combined test results matched those of the combined test for allelic differentiation (Chi-square= infinity, DF=10).

F-statistics suggested that, at locus 7 Ns, populations were structured significantly, whereas at loci 8 Ns, 2 Ts and 3 Ts, structural differences between populations were weaker, but still present (Table 4). The combined F_{st} test for all loci also suggested structural differences, albeit small ones, between populations (Table 4).

Cavalli-Sforza and Edward's (1967) genetic distances (Table 5) were relatively small, with Big Qualicum the most distant from all other populations. Genetic distances and geographical distance were not congruent. Population relationships inferred by the consensus neighbor-joining tree did not mimic geographical patterns, as the most northern population, Quinsam Hatchery, formed a node with the Island Highway sample, Elk Lake and Big Qualicum formed a second

Table 4. F-statistics estimates for each locus. Statistics generated using Wier and Cockerham's (1984) method.

Locus	F_{is}	F_{st}	F_{it}
7 Ns	+0.1578	+0.1770	+0.3069
8 Ns	-0.0382	+0.0370	+0.0002
9b Ns	+0.4455	-0.0407	+0.4229
2 Ts	+0.0826	+0.0244	+0.1050
3 Ts	-0.0070	+0.0038	-0.0031
All loci			+0.0395

Table 5. Genetic distance matrix. Distances calculated using Cavalli-Sforza and Edward's (1967) chord distances.

Population					
BQ	0.0000				
IH	0.0780	0.0000			
EL	0.0648	0.0425	0.0000		
Ma	0.0874	0.0661	0.0586	0.0000	
QH	0.0850	0.0352	0.0651	0.0586	0.0000

Figure 5. Consensus tree for the five populations. Values at each node are for 1000 bootstrap replicates of the original allele frequency matrix.

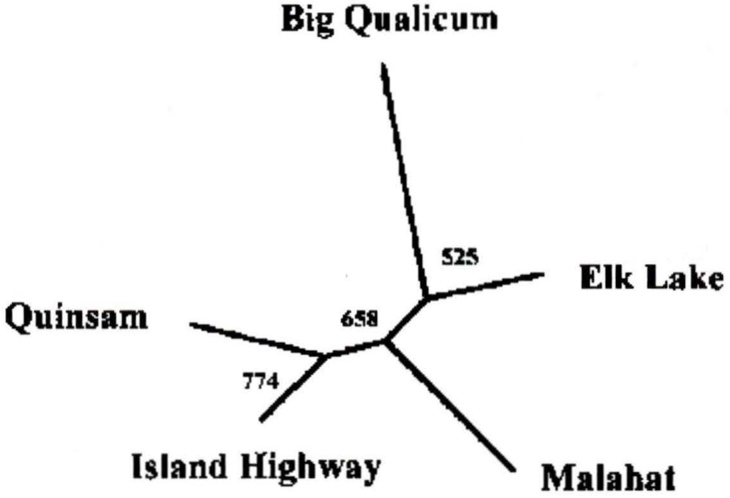

Big Qualicum
525
Elk Lake
Quinsam
658
774
Island Highway
Malahat

Table 6. Matrix of pairwise F_{st} comparisons.

Population	F_{st}				
BQ	0.0000				
IH	0.0337	0.0000			
EL	0.0274	0.0544	0.0000		
Ma	0.0495	0.0674	-0.0139	0.0000	
QH	0.0437	0.0081	0.0586	0.0695	0.0000

Table 7. Estimated number of migrants between each population.

Population	Comparison Population	Estimated # Migrants
BQ	IH	7.2
BQ	EL	8.9
BQ	Ma	4.8
BQ	QH	5.5
IH	EL	4.3
IH	Ma	3.5
IH	QH	30.7
EL	Ma	not calculated*
EL	QH	4.0
Ma	QH	3.3

* F_{st} value for the comparisons between these two populations was negative, making calculating an estimate of migrants impossible using FSTAT.

node, and the Malahat sample fell between these two nodes (Figure 5). The reliability of this relationship is questionable, due to low bootstrap values at two of the three nodes (Figure 5).

Results of the pairwise population F_{st} analysis showed only small amounts of structuring between populations, with the Malahat and Elk Lake populations virtually indistinguishable from each other (Table 6). Number of migrants between these two populations was not estimated, as the F_{st} value is negative (Table 7), but for almost all other comparisons, small structural differences are evident and supported by the low number of migrants expected between each population. The second smallest structural difference detected was between the Island Highway and Quinsam Hatchery populations, and the estimated number of migrants suggested substantial gene flow between these two populations (Tables 6, 7).

Direct counts of allelic diversity indicated that the Elk Lake and Island Highway samples were the most genetically diverse, whereas the Malahat and Quinsam Hatchery samples carried the fewest alleles combined between locus 2 Ts and 3 Ts (Table 8). Elk Lake and Island Highway also shared the largest number of alleles, whereas Quinsam Hatchery and Big Qualicum shared the fewest. Each population contained at least one private allele at locus 2 Ts, but Quinsam Hatchery was the only population that contained more than one private allele at that locus, and Big Qualicum was the only population that carried a private allele at locus 3 Ts.

Pie diagrams of allele frequencies illustrate that genetic differences at locus 9b Ns are almost absent, results supported by both the genic and genotypic differentiation tests (Figure 6). Diagrams generated using the 8 Ns locus data show that, again, differences in allelic frequencies are slight (Figure 7). Diagrams and histograms of locus 7 Ns showed that Big Qualicum, Quinsam Hatchery and Island Highway are heavily biased towards allele 1. In contrast, the Malahat and Elk Lake samples have much higher frequencies of allele 2 (Figures 8, 9, 10). Frequency histograms of allelic diversity showed little or no differences at loci 8 Ns, 9b Ns, or 3 Ts. Histograms of locus 2 Ts showed similar distributions of alleles between the Island Highway and Elk Lake populations, whereas the Malahat sample was strongly biased towards small molecular weight alleles (Figure 11). Quinsam Hatchery contained few large molecular weight alleles, and Big Qualicum contained several infrequent small molecular weight alleles. Allele 19 occurred 11 times out of a possible 30 in the Big Qualicum sample (Figure 12).

Table 8. Total number of alleles detected, number of private alleles and number of shared alleles between each population. Data presented for loci 2Ts/3Ts.

Pop'n	Total alleles detected	Private alleles	Comparison Population	Shared alleles
EL	14/12	#16/none	Ma	7/9
			IH	12/11
			BQ	7/10
			QH	8/10
Ma	11/9	#12/none	IH	7/9
			BQ	7/8
			QH	8/9
IH	14/11	#22/none	BQ	7/9
			QH	9/9
BQ	11/11	#14/#12	QH	8/6
QH	12/9	#15,#13/none		

Figure 6. Allele frequency pie diagrams for locus 9b Ns. a) Big Qualicum, b) Quinsam Hatchery, c) Island Highway, d) Elk Lake, e) Malahat.

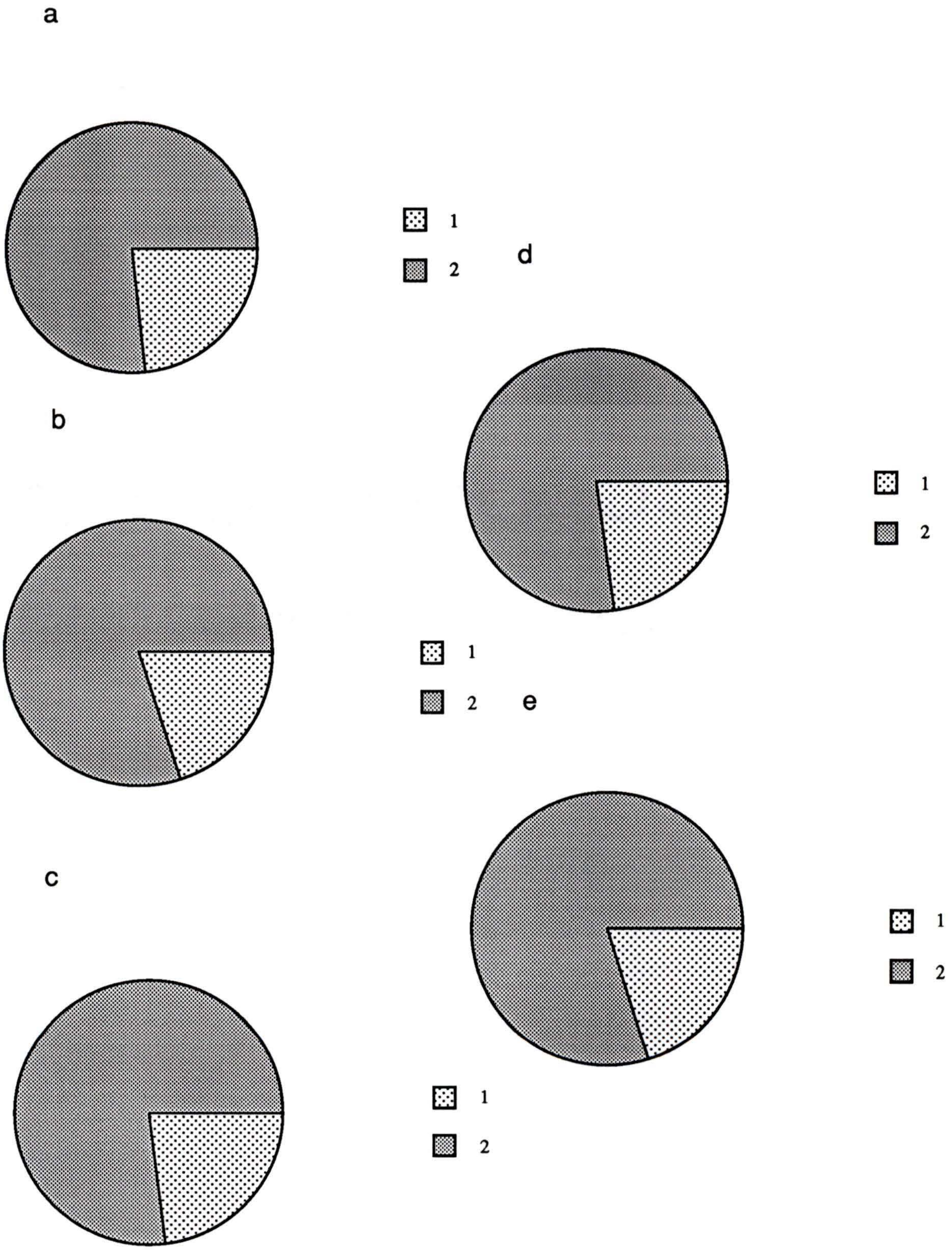
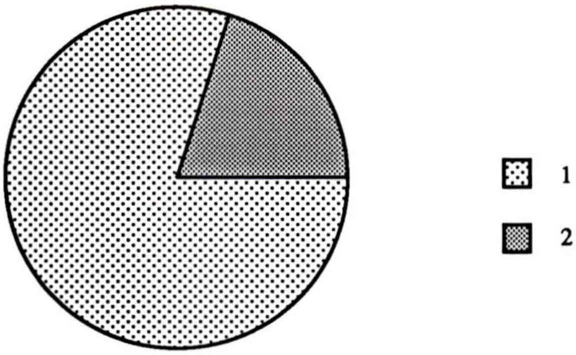
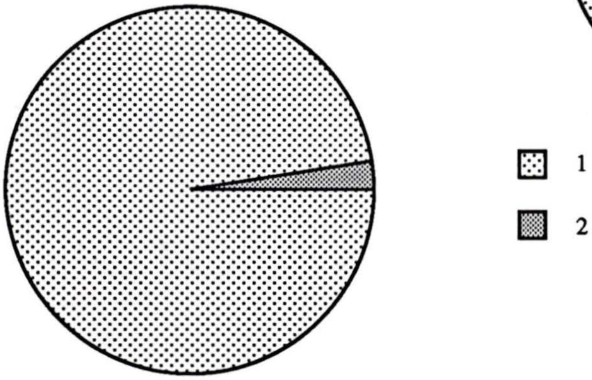

a
1
2
d
1
2
b
1
2
e
1
2
c
1
2

Figure 7. Allele frequency pie diagrams for locus 8 Ns. a) Big Qualicum, b) Quinsam Hatchery, c) Island Highway, d) Elk Lake, e) Malahat.

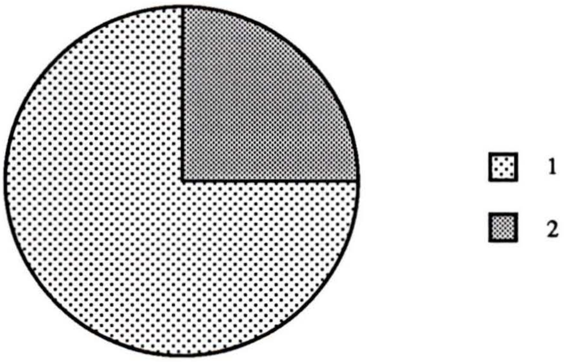
a



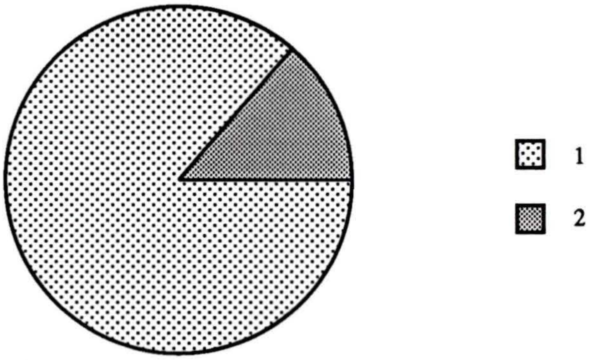
b



c



d



e

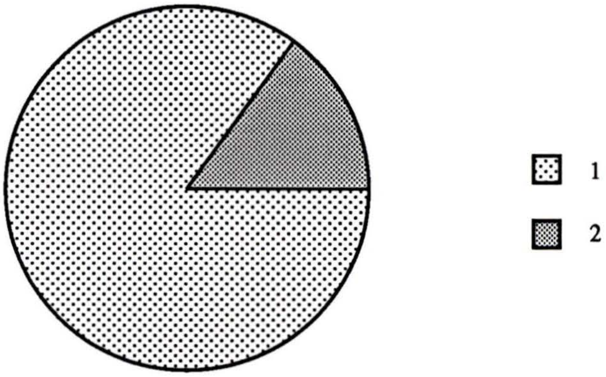
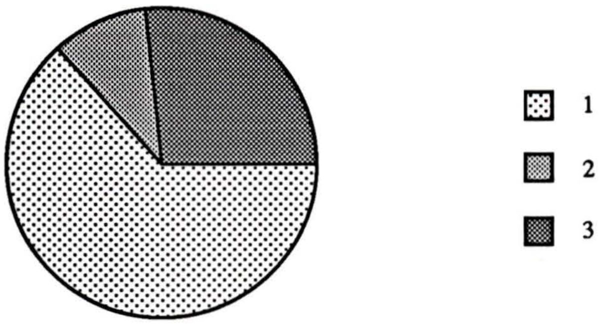
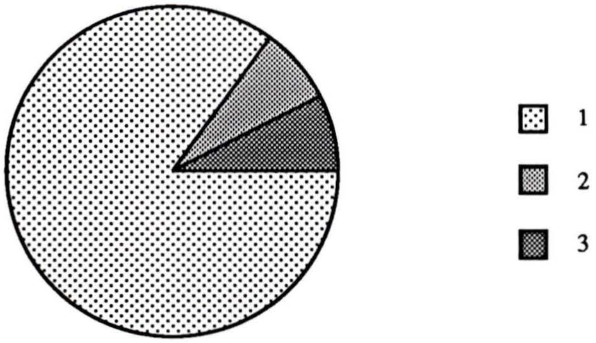


Figure 8. Allele frequency pie diagrams for locus 7 Ns. a) Big Qualicum, b) Quinsam Hatchery, c) Island Highway, d) Elk Lake, e) Malahat.

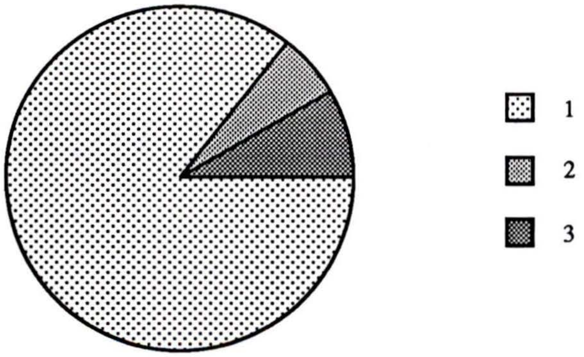
a



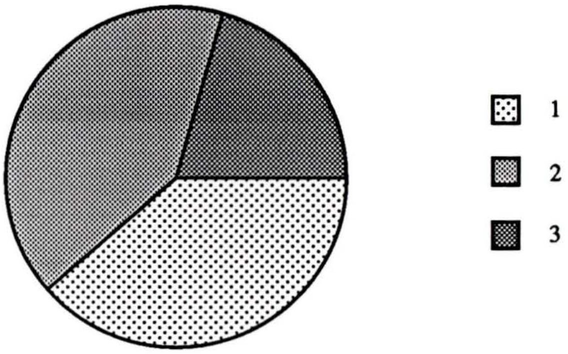
b



c



d



e

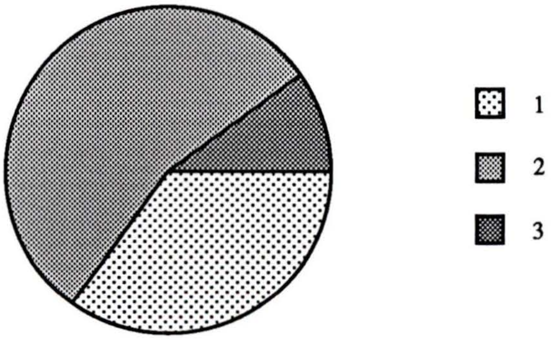
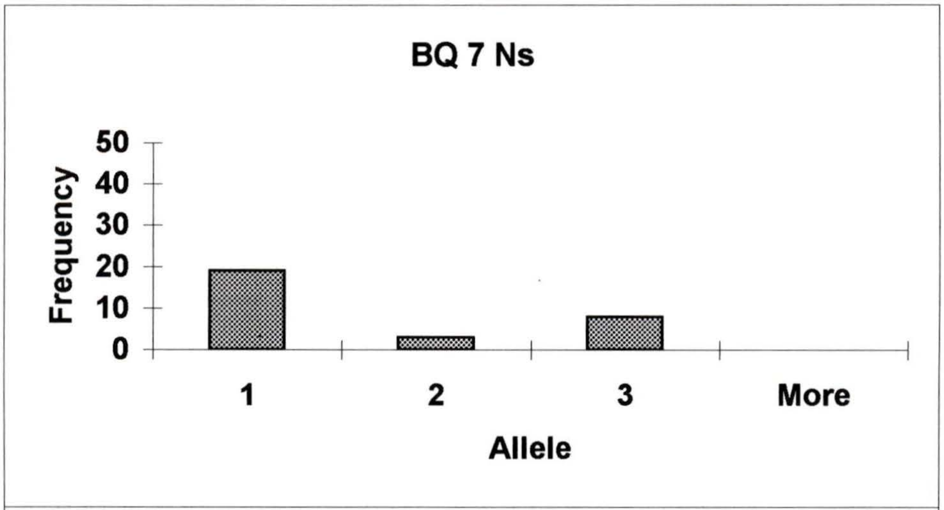
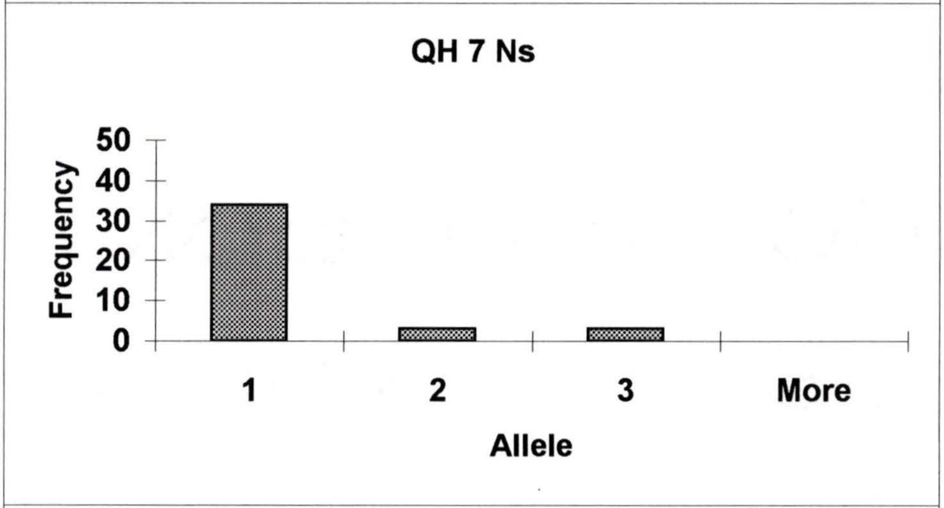


Figure 9. Allele frequency histograms for the two hatcheries and the Island Highway populations at locus 7 Ns. Note relative frequency of allele 1 in all three populations.


BQ 7 Ns
Frequency
50
40
30
20
10
0
1
2
3
More
Allele


QH 7 Ns
Frequency
50
40
30
20
10
0
1
2
3
More
Allele

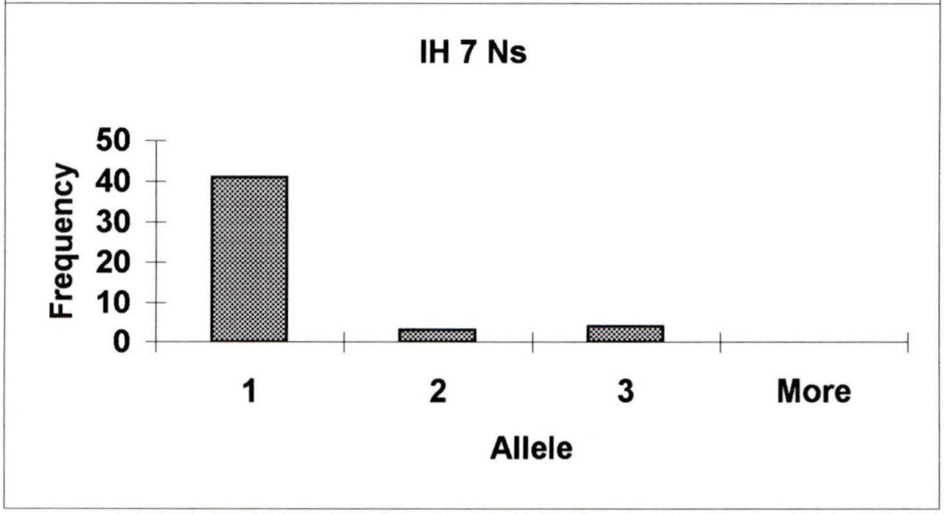
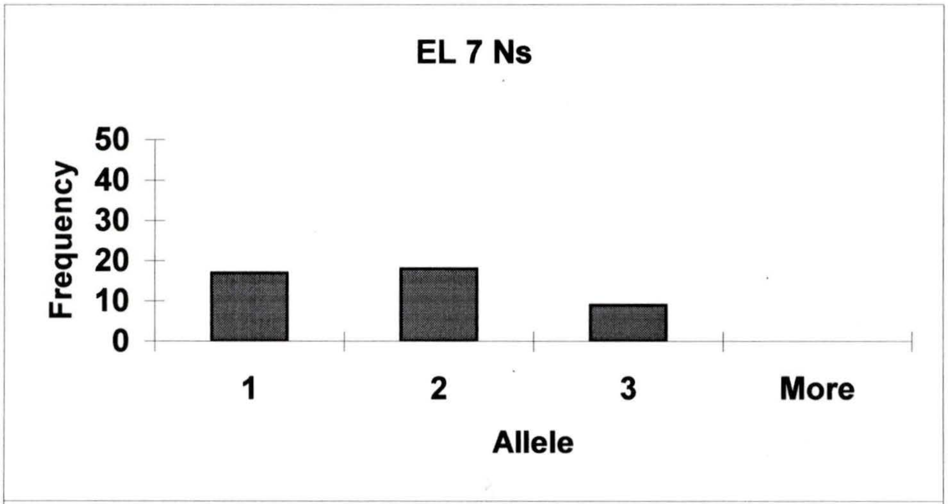
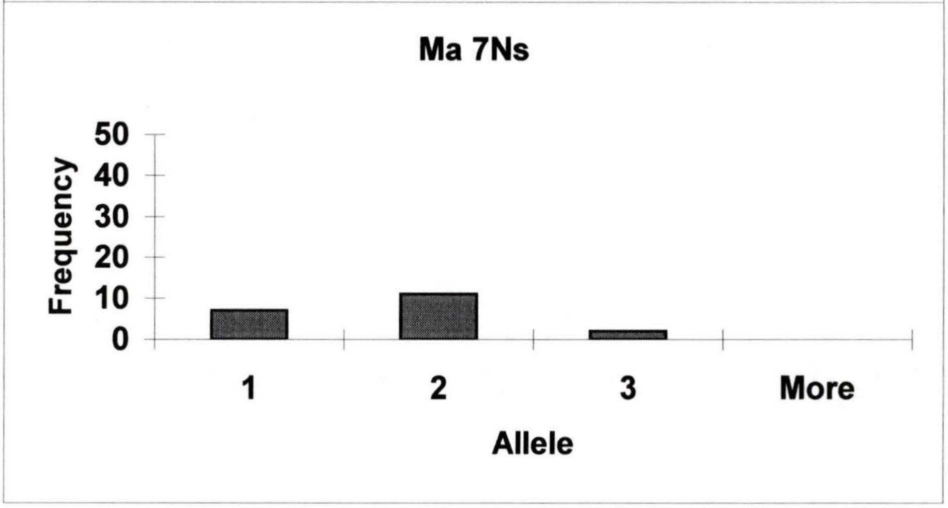

IH 7 Ns
Frequency
50
40
30
20
10
0
1
2
3
More
Allele

Figure 10. Allele frequency histograms for the Elk Lake and Malahat populations at locus 7 Ns.



EL 7 Ns

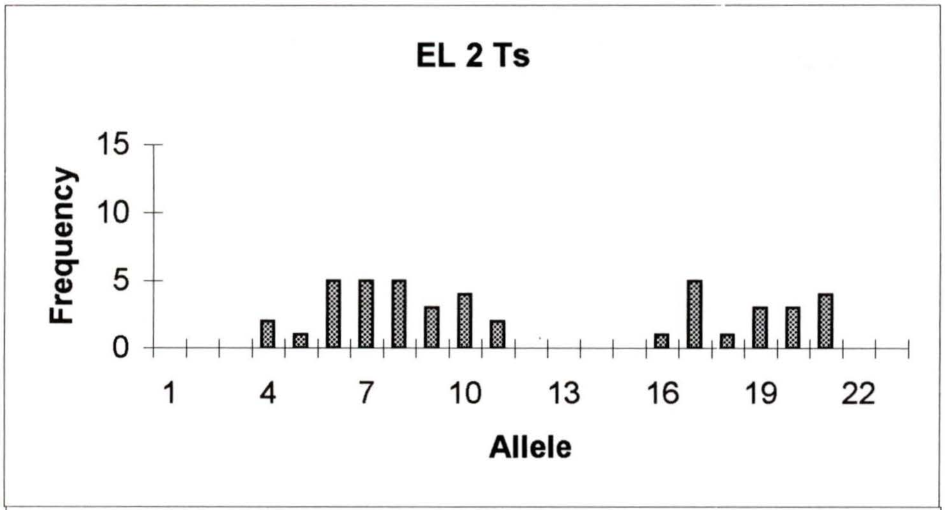
Frequency
50
40
30
20
10
0
1
2
3
More
Allele

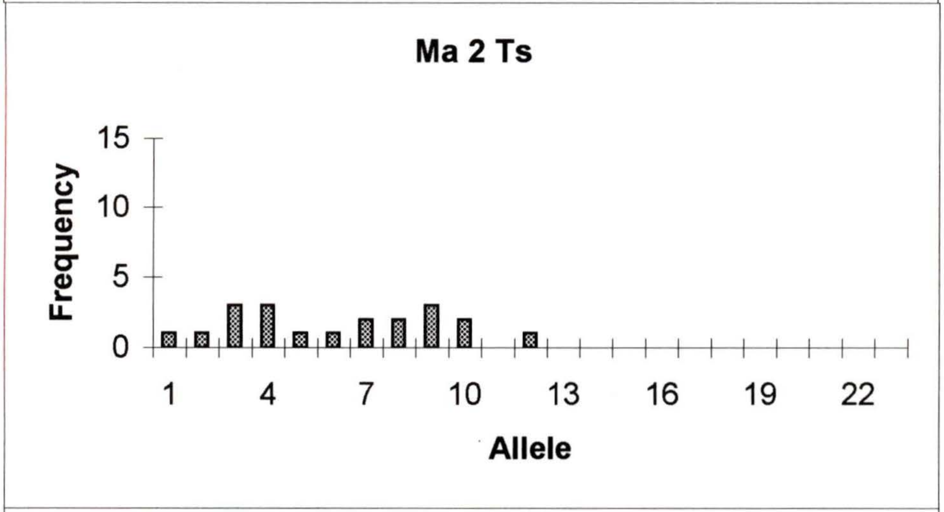


Ma 7Ns

Frequency
50
40
30
20
10
0
1
2
3
More
Allele

Figure 11. Allele frequency histograms for the three natural populations at locus 2 Ts.


EL 2 Ts


Ma 2 Ts

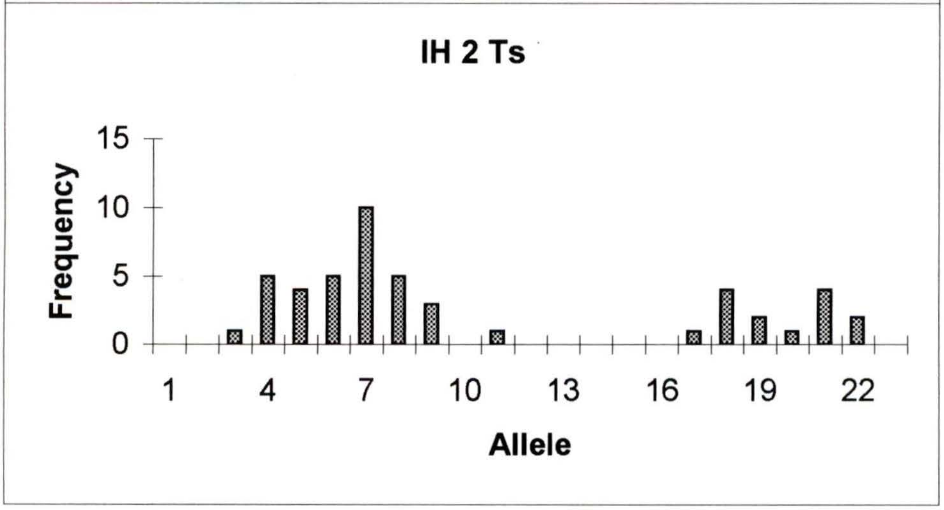
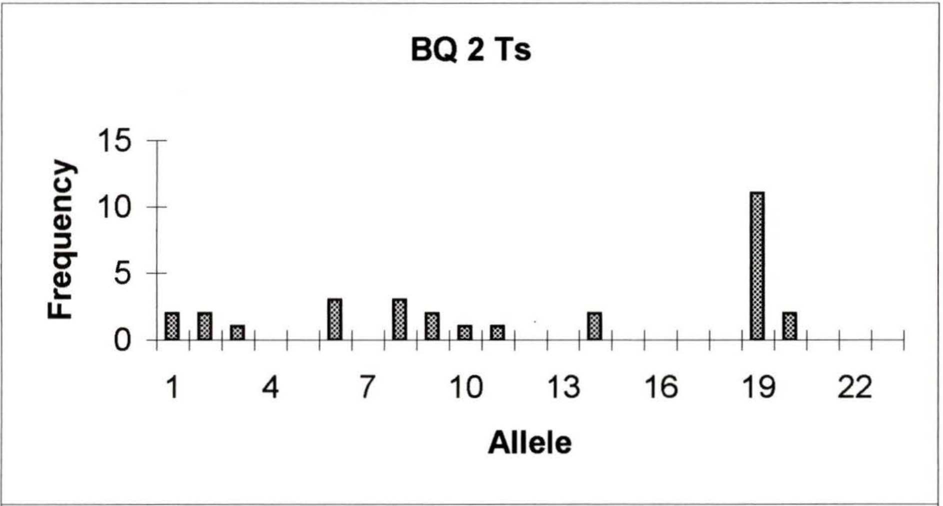
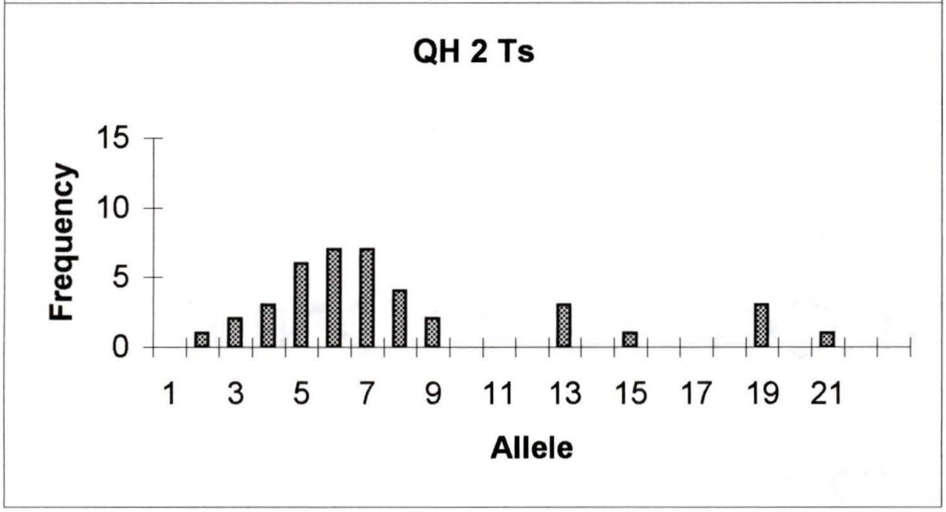

IH 2 Ts

Figure 12. Allele frequency histograms for the two hatchery populations at locus 2 Ts. Note the lack of structured distribution evident in the three natural populations in Figure 14.


BQ 2 Ts
Frequency
15
10
5
0
1
4
7
10
13
16
19
22
Allele


QH 2 Ts
Frequency
15
10
5
0
1
3
5
7
9
11
13
15
17
19
21
Allele

Paternity

A total of sixteen litters were sampled, one from Big Qualicum hatchery, one from Durrance Lake area, one from the Malahat region, three from the Island Highway site, two from the Elk Lake site, and eight from Quinsam Hatchery. Gravid snakes ranged in size from 480 to 730 mm. SVL. Litter sizes ranged from 3 to 19 (mean=8.5, *s*=5.073) and mean offspring size per litter varied from 1.60 to 2.81 grams. These counts include stillborn offspring, which ranged in number from 0 to 6; one litter of 3 was 100% stillborn. The maximum number of alleles detected in a single litter was 5 (e.g., BQ 1, locus 2 Ts, locus 3 Ts, Table 9), whereas the smallest was one (e.g., Q2, locus 3 Ts, Table 9). One individual from the litter of Quinsam 2 (Q2) which was mixed inadvertently in with the offspring of Quinsam 3 (Q3) during feeding was readily identified from the Q3 PCRs (Figure 13). Offspring 10 from the Q3 litter sample exhibited no maternal alleles for Q3 at locus 2 Ts, but carried a maternal allele from Q2 and a paternal allele scored from the Q2 sample. At both locus 3 Ts and 5 BTs, number 10 matched a Q2 genotype (Figure 14).

Six of the sixteen litters exhibited at least three paternal alleles at one of the loci scored, indicating that more than one male contributed to these litters (Table 9). Ambiguity at a locus occurred in several ways. First, offspring expressed the same genotype as the mother at a single locus with no homozygotes for either allele occurring within the litter.

Table 9. Alleles detected within each litter at each locus. Alleles in bold print also can be attributed directly to a paternal profile due to presence of an offspring homozygous for that allele.

Litter	Locus	Maternal alleles	Litter alleles	Multiple Paternity Detected
BQ 1	5B Ts	7,10	1,4,**7**,10	Yes
	2 Ts	7,20	3,**7**,16,20	Yes
	3 Ts	6,8	1,3,6,**8**,14	Yes
IH 4	5B Ts	3,8	3,7,**8**	No
	2 Ts	3,7	**3**,6,7,8	Yes
	3 Ts	3,6	**3**,6,10	No
IH 1	5B Ts	2,6	2,5,**6**	No
	2 Ts	6,19	3,6,16,19	No
	3 Ts	6,6	**6**,15	No
IH 3	5B Ts	2,6	1,2,3,6,7	Yes
	2 Ts	6,6	3,**6**,9	Yes
	3 Ts	6,10	**6,10**	No
EL 1	5B Ts	7,10	7,8	No
	2 Ts	6,8	6,8	No
	3 Ts	2,7	2,**7**	No
EL 2	5B Ts	5,8	**5**,6,8	No
	2 Ts	9,9	3,**9**,20	Yes
	3 Ts	4,7	4,**7**,9,11	Yes
Ma 1	5B Ts	1,6	1,2,6,9	No
	2 Ts	3,10	1,3,**10**	No
	3 Ts	6,11	**6,11**	No
Q2	5B Ts	4,6	1,2,4,6	No
	2 Ts	6,19	6,13,17,19	No
	3 Ts	8,8	**8**	No
Q8	5B Ts	5,5	5,6	No
	2 Ts	5,13	5,6,7,13	No
	3 Ts	6,10	**6,10**	No
Q6	5B Ts	1,5	1,**5**	No
	2 Ts	4,18	4,8,**18**	No
	3 Ts	7,10	**7,10**	No
Q4	5B Ts	2,5	2,5,6,8	No
	2 Ts	6,12	6,12	No
	3 Ts	6,6	**6**	No

Table 9. Con't.

Q9	5B Ts	2,6	1,2,4,6	No
	2 Ts	6,20	1,6,15	No
	3 Ts	4,9	4,6,**9**	No
Q5	5B Ts	2,3	2,3,6,7	No
	2 Ts	6,7	6,7,8,12	No
	3 Ts	6,10	**6,10**	No
Q1	5B Ts	1,7	1,3,4,6,**7**	Yes
	2 Ts	5,8	**5,8**	No
	3 Ts	3,6	**3,6**	No
Q3	5B Ts	4,6	2,3,4,6	No
	2 Ts	5,8	5,7,8,17,19	Yes
	3 Ts	3,6	3,**6**,8	No
DP 1	5B Ts	7,10	1,7,10	No
	2 Ts	10,10	6,10,16	No
	3 Ts	3,3	3,7	No

Figure 13. PCR products of litter Q3 generated using primers specific for locus 2 Ts. Lane 1: 1 kb DNA ladder; lane 2, 11, and 20: 20 bp DNA ladder; lane 3: maternal bands; lanes 4 to 8, 10, 12, 13, 15 to 19, 21 to 23: offspring bands; lane 9: empty lane; lane 14: offspring of Q2.

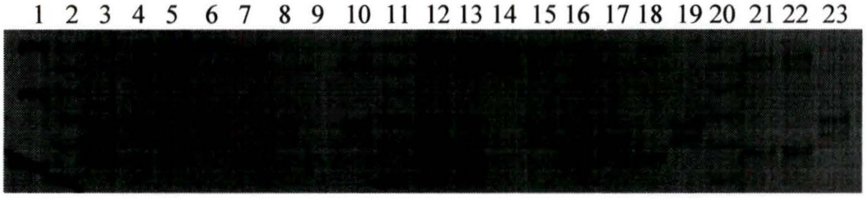
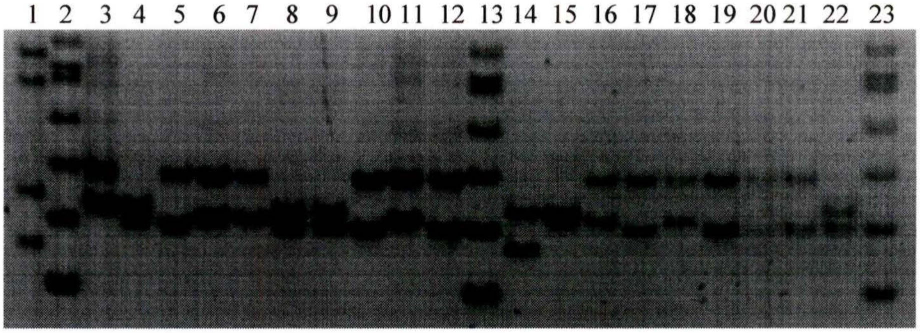

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Figure 14. PCR products of litter Q3 generated using primers specific for locus 5B Ts. Lane 1: 1 kb DNA ladder; lanes 2, 13, and 23: 20 bp DNA ladder; lane 3: maternal bands; lanes 4 to 12, 15 to 22: offspring bands; lane 14: offspring of Q2.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

This could indicate that both of the maternal alleles also were transmitted paternally, but also could indicate that only one of these alleles was paternally derived. This was the case for the Q3 litter at locus 2 Ts (Figure 13). In this case, however, three other paternal alleles were evident, so multiple paternity still is supported at this locus.

Second, offspring expressed the maternal genotype, but one of those alleles also occurred twice in a single offspring (homozygous for the allele). This eliminated ambiguity for the one allele expressed by the homozygote, but the second allele still could not be attributed directly to paternal contribution. This was the case at locus 3 Ts for Island Highway litter 4. In this case, locus 2 Ts supported multiple paternity.

The third way in which ambiguity affected scoring was limited to locus 3 Ts. This locus was determined to carry at least one null allele and litters exhibited allele distributions that suggested that a null may be operating. Quinsam 1 (Q1) littermates were all homozygous for one of two maternal alleles. Although this is possible with a father that carries one or both of the same alleles as the mother, the likelihood that none of the offspring would be heterozygous is small. A null allele is more likely, with a paternal contributor carrying either one maternal allele and a copy of the null, or being homozygous for the null allele. In this case, although more than one father is not directly supported at this locus, the prevalence of homozygotes in the litter and the strong possibility that a null allele is operating suggests something other than typical

Mendelian proportions. Because multiple paternity is supported at locus 5B Ts, the Q1 litter is considered to be multiply sired.

Only one of the litters in which multiple paternity was not observed failed to meet Hardy-Weinberg expectations of genotype frequencies at one locus (DP 1, Table 10). Tests of the multiply sired litters showed that two of the litters deviated from Hardy-Weinberg at a locus at which no multiple paternity could be detected (Q1, Q3; Table 10). In the other four litters, the loci that did not show direct evidence of multiple paternity all met expected genotype frequencies, but in two of those four litters, at least one locus only just missed falling within the range of rejection (BQ 1, locus 5B Ts; EL 2, locus 5B Ts; Table 10). This was the case in only one of the singly sired litters (SL 1, locus 5B Ts; Table 10).

Relatedness values for litters and loci differed between the two pools. Litters with observed multiple paternity averaged R=0.47506 and the three loci averaged R=0.480788 (Table 11). Litters in which single paternity could not be excluded directly averaged R=0.627422 and the loci averaged R=0.629756 (Table 11). Five of the single paternity litters did generate R-values closer to the expected range of the multiply sired litters (IH 1, SL 1, Q8, Q9, Q5, see Table 11), but each of these litters contained a maximum of only six offspring. Mean SVL of mothers of singly sired litters did not differ significantly from

Table 10. Results of tests for Mendelian proportions within litters. Chi-square and associated values are for each locus at which multiple paternity was not detected. Litters with fewer than six offspring are excluded.

Litter	Multiple/Single	Locus	Chi-square	DF	P
BQ1	Multiple	5B Ts	6.07	3	0.05<P<0.10
IH4	Multiple	3 Ts	2.00	3	0.50<P<0.75
		5B Ts	3.33	2	0.25<P<0.50
IH3	Multiple	3 Ts	3.86	2	0.10<P<0.25
EL2	Multiple	5B Ts	7.55	3	0.05<P<0.10
Q1	Multiple	2 Ts	0.14	2	0.90<P<0.95
		3 Ts	6.37	2	0.025<P<0.05
Q3	Multiple	3 Ts	19.94	3	P<0.001
		5B Ts	4.41	3	0.10<P<0.25
Q2	Single	2 Ts	3.11	3	0.25<P<0.50
		3 Ts	not calc*		
		5B Ts	4.37	3	0.10<P<0.25
SL1	Single	2 Ts	0.67	3	0.75<P<0.90
		3 Ts	0.17	2	0.75<P<0.90
		5B Ts	6.33	3	0.05<P<0.10
Q8	Single	2 Ts	0.17	3	0.75<P<0.90
		3 Ts	6.00	3	0.10<P<0.25
		5B Ts	0.00	1	0.99<P<0.995
Q6	Single	2 Ts	2.57	3	0.25<P<0.50
		3 Ts	1.79	2	0.25<P<0.50
		5B Ts	0.00	1	0.99<P<0.995
Q9	Single	2 Ts	6.00	3	0.10<P<0.25
		3 Ts	2.00	3	0.50<P<0.75
		5B Ts	0.67	3	0.75<P<0.90
DP1	Single	2 Ts	0.64	1	0.25<P<0.50
		3 Ts	0.00	1	0.99<P<0.995
		5B Ts	14.14	3	0.001<P<0.005

*all individuals homozygous for one maternal allele, sets degrees of freedom at 0, unable to calculate Chi-square.

Table 11. Relatedness values (R) and jackknife error estimates for each litter over all loci. Litters are separated into multiply and singly sired litters. R-values for each locus for both pools are reported at bottom.

Litter/Locus	R-value	Jackknife error estimate
Multiply sired pool:		
BQ1	0.487	0.147
IH4	0.387	0.149
IH3	0.543	0.082
EL2	0.637	0.068
Q1	0.368	0.089
Q3	0.429	0.087
Locus 2 Ts	0.527	0.097
Locus 3 Ts	0.404	0.130
Locus 5B Ts	0.512	0.090
Singly sired pool:		
IH1	0.557	0.141
EL1	0.879	0.115
SL1	0.451	0.083
Q2	0.724	0.294
Q8	0.513	0.167
Q6	0.677	0.066
Q4	0.789	0.261
Q9	0.476	0.122
Q5	0.352	0.054
DP1	0.856	0.076
Locus 2 Ts	0.547	0.083
Locus 3 Ts	0.747	0.171
Locus 5B Ts	0.579	0.130

Figure 15. Regression of number of offspring (litter size) on maternal SVL. Groups separated by paternity.

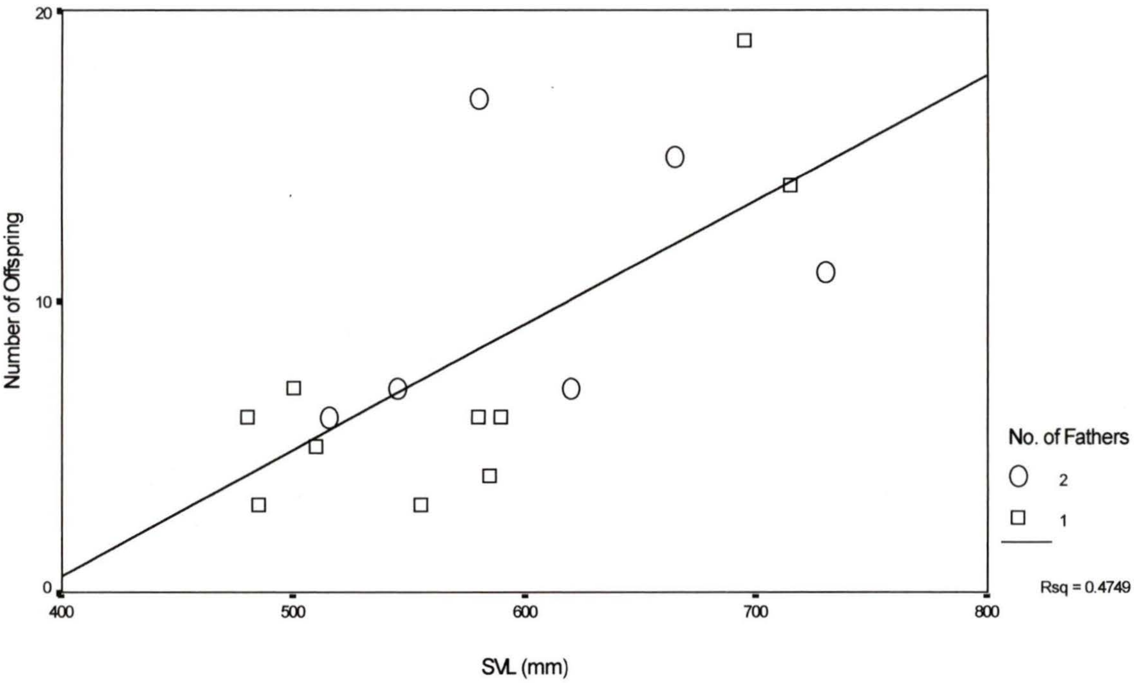
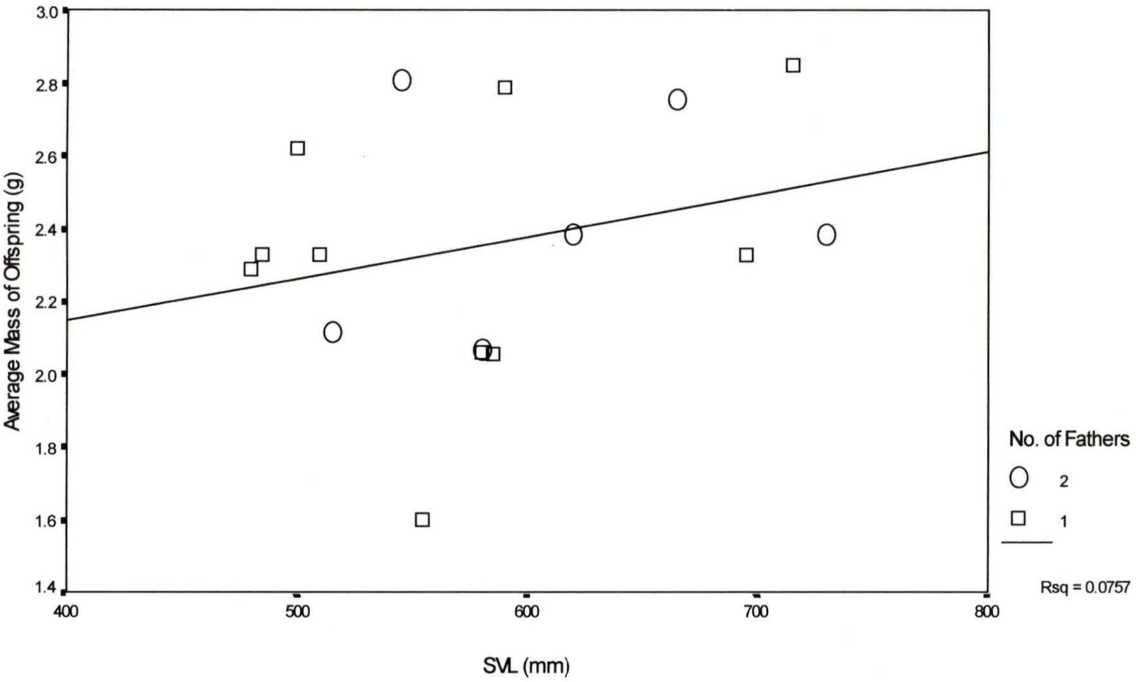
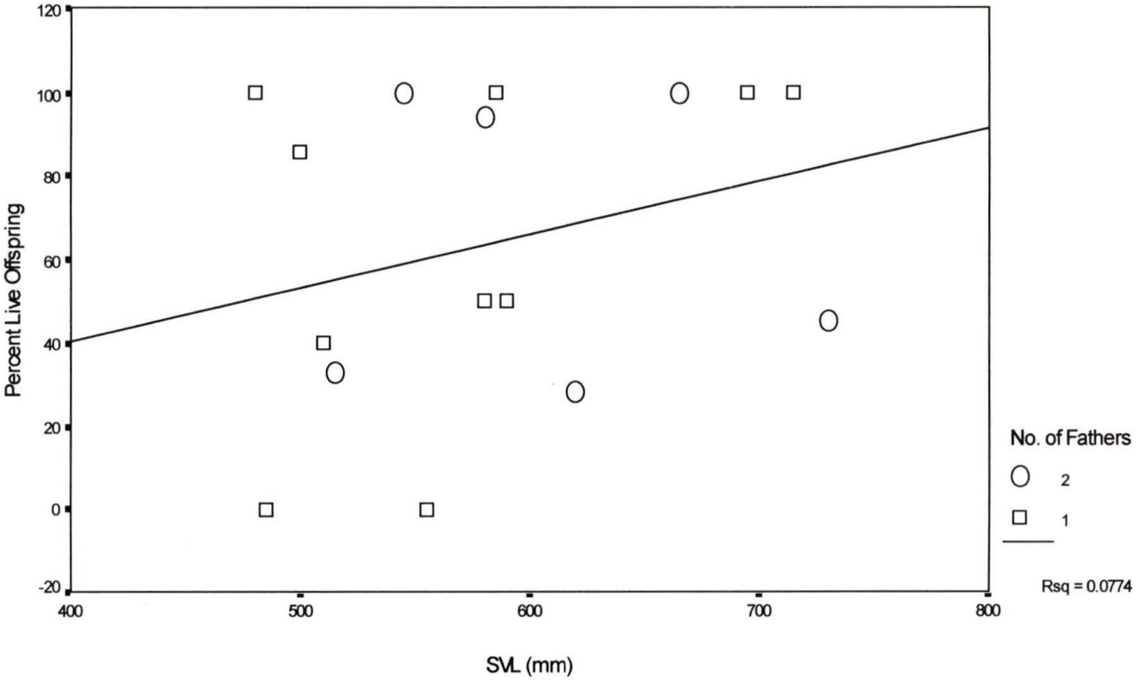

Number of Offspring
20
10
0
400
500
600
700
800
SVL (mm)
No. of Fathers
○ 2
□ 1
Rsq = 0.4749

Figure 16. Regression of average mass of offspring in a litter on maternal SVL. Groups separated by paternity.


Average Mass of Offspring (g)
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
400
500
600
700
800
SVL (mm)
No. of Fathers
○ 2
□ 1
Rsq = 0.0757

that of mothers of multiply sired litters [$P(t \geq 0.782) < 0.50$, $ES=0.48$, $1-\beta < 0.07$, $n_r > 1237$]. Overall, number of offspring was highly correlated with maternal SVL ($F_{(1,14)}=12.66$, Figure 15), but there was no significant difference between the mean residuals of litter sizes for singly or multiply sired litters [$P(t \geq 0.451) < 0.50$, $ES=0.41$, $1-\beta=0.11$, $n_r=78$]. Average offspring size was not correlated with maternal SVL ($F_{(1,14)}=1.15$, Figure 16) and there was no significant difference detected between residuals for singly and multiply sired litter[$P(t \geq 0.774) < 0.50$, $ES=0.16$, $1-\beta=0.06$, $n_r=770$]. Percentage of live offspring also was not correlated with maternal SVL ($F_{(1,14)}=1.18$, Figure 17) and residuals were not significantly different [$P(t \geq 0.970) < 0.50$, $ES=0.02$, $1-\beta < 0.05$, $n_r > 1237$].

Figure 17. Regression of percentage of live offspring on maternal SVL. Groups separated by paternity.


120
100
80
60
40
20
0
-20
Percent Live Offspring
400
500
600
700
800
SVL (mm)
No. of Fathers
2
1
Rsq = 0.0774

Discussion

Population Structure and Gene Flow

The small amount of genetic divergence detected among the five populations precludes any strong conclusions about population structure, but certain trends in the data still merit attention. Structuring does exist among populations, but the differences are small and subtle. Straight-forward biogeographical interpretations do not apply here, as the genetic relationships among the five populations suggested by both the consensus tree and the pairwise F_{st} comparisons do not map the geographic distribution of the five populations. Big Qualicum seems to be the most genetically distinct population, and this simply may be a function of its location relative to its nearest neighbour. However, certain results suggest that geographic proximity is not the best explanation.

First, the distribution of the 2 Ts locus alleles detected at Big Qualicum is almost the inverse of that at Quinsam Hatchery, its nearest neighbour to the north, whereas such drastic shifts in allele frequencies are not shown between other populations. Second, the number of shared alleles between the two hatchery sites is extremely low, much lower than that shared between the Island Highway and Elk Lake sites. The distinct differences in allelic distributions, the number of shared alleles and the fact that both of the hatchery sites have the greatest number of private alleles suggest that something novel affects genetic structure at hatchery sites.

The strongest effects are seen at the Big Qualicum site. Big Qualicum is the most genetically distant population relative to all other populations (Table 5) and has private alleles present at both the 2 Ts locus and the 3 Ts locus (Table 11). Furthermore, the distribution of alleles detected in this population consists of a scattering of smaller alleles and an overwhelming abundance of allele 19, an allele that is infrequent in all other populations (Figure 12).

The lack of polymorphism expressed at the two dimorphic *Nerodia* loci suggests that these two loci are inappropriate for detecting trends. Locus 3 Ts did not show significant differences in either allelic or genotypic distributions, which indicates that this locus may be inappropriate as well. This leaves only two loci apparently suitable for interpretation. Frequency histograms of locus 7 Ns are distinctly divided between populations that exhibit allele 2 frequently (Ma, EL) and those that do not (IH, QH, and BQ). Frequency histograms of locus 2 Ts are divided into populations that show either unimodal or bimodal allele distributions that could indicate stable structure (EL, Ma, IH) and those that show uneven distributions that indicate a less stable structure (BQ and QH). Interpreting these differences in light of the two proposed hypotheses (hatcheries attracting snakes versus hatcheries allowing local populations to expand) is more an exercise in supposition than conclusion. However, these differences perhaps could be used to generate hypotheses for future tests.

It is possible that the frequency distribution of locus 7 Ns indicates that the Malahat acts as some form of geographic barrier to gene flow. Elevation can affect the abundance and diversity of reptiles. Most of the literature dealing with elevational effects interpret differences in terms of climatic factors, such as precipitation and temperature (see Rogers, 1976; Scott, 1976). Furthermore, large mountains are effective barriers to gene dispersal (Avise, 1994).

This is an unlikely explanation for several reasons. The Common Garter Snake is abundant at lower elevations, but also has been found as high as 2,450 m. above sea level (Nussbaum *et al.*, 1983). The Malahat reaches a height of only 600 m and *T. sirtalis* are common at Spectacle Lake near the top of the Malahat (pers. obs.) Elevation, habitat and climate, all well-known restrictions to snake movement, do not seem to be limiting factors on the Malahat.

No differences were detectable between the Malahat sample and the Elk Lake sample (Table 6), so it is possible these sites are part of the same deme. If so, the Island Highway population can be identified tentatively as a separate population from the combined Elk Lake/Malahat population.

Although the allele distributions at locus 7 Ns supports genetic similarity between the two hatchery sites and the Island Highway sample, the data generated at locus 2 Ts indicate that the genetic structure of the two hatchery populations make them distinct from the three natural populations. The three natural populations are apparently genetically stable at the 2 Ts locus, whereas the unusual allele distributions of the two hatcheries could indicate that migrants are playing a role in the genetic diversity of the two hatcheries. The scattered distribution of infrequent alleles in both populations may be the result of the introduction of new alleles into each population via snakes migrating to the hatchery sites. This is speculative, and only further research can address this issue.

Determining if hatcheries are more genetically variable than natural sites requires a broader study than this one. It seems that the lack of genetic distinctiveness among southern Vancouver Island populations may preclude an answer to this question, but development of several new and highly polymorphic microsatellite markers may allow for enough information to be generated. Other suitable genetic markers, such as mitochondrial DNA genes, also could provide clues as to how hatcheries affect genetic structure of snake populations. Natural populations close to hatcheries should also be sampled, as they would be the most likely sites contributing to hatchery genetic profiles.

The inclusion of more natural sites should not be restricted to those near hatcheries. Distant sites, preferrably from more northern and eastern sites on the island, would allow for a more thorough treatment of the problem of population relationships. This would alleviate one problem I encountered with this study, that of analyzing natural populations independently of hatchery sites. I was unable to exclude hatcheries from any analysis that addressed relationships among populations (i.e., unrooted consensus tree) because this reduced the number of populations used for analysis to three. An unrooted tree of three populations provides no insight into relationships among populations. If biogeographical relationships are valid among natural sites, as is indicated by locus 7 Ns, more natural populations need to be included in an analysis that does not include hatchery sites.

Paternity

In this study, I obtained clear evidence of the occurrence of multiple paternity in *Thamnophis sirtalis* from Vancouver Island. At a minimum, multiple insemination was detected directly in 37.5% of the litters I examined. The Chi-square goodness-of-fit tests do not suggest that any of the purportedly singly sired litters were multiply sired. Although litter DP 1 did not meet expected genotype frequencies at one locus, the high R-value generated for this litter (0.856) supports the hypothesis that this litter contains full siblings. However, if the smaller-sized litters that had low R-values are included in the multiple paternity data set, my estimate of multiple paternity is raised to 68.75%. Thus, I have estimated the minimum frequency of multiple paternity in these snakes. Whatever the actual level of multiple paternity, we are left with the question of what factors influence the occurrence of multiple paternity. Is this just "noise" in the system and of no special significance or, as seems more likely, does it have behavioural and evolutionary importance?

First, we must distinguish clearly between multiple copulation and multiple fertilization. The latter obviously requires the former, but only the latter was tested in this study. It is in fact possible that all the females used in this study mated with two or more males but only one male was successful in fertilizing eggs in most cases. Madsen *et al.* (1992) point out that multiple mating may be significant even without, or independently of, multiple fertilization because it leads to sperm competition and presumably fertilization by the "best" male. I used molecular methods to detect multiple paternity in snakes because they are superior to simple observations of mating. However, if number of matings *per se* also is important, then both observational and molecular approaches are required to draw a complete picture.

The benefits of multiple paternity are obvious for males, which, in most species of animals, produce sufficient sperm to inseminate multiple females. Thus, it is better to have some offspring in a litter than to have none and males should not pass up opportunities to mate, even if the female has mated previously with another male. But what are the benefits to the female of being inseminated multiple times by different males? All else being equal, a female probably should not seek multiple mates but try to find the single "best" father for her offspring. However, the costs of avoiding multiple insemination may be too high to be worth paying, especially if "quality" of males is relatively invariant. Alternatively, multiple paternity could be interpreted as a "bet-hedging" strategy of females to maximize the probability that at least some of her offspring will survive to reproduce. In small populations, multiple mating by females also might be a way of reducing the risk of inbreeding (Stockley *et al.*, 1993); in a small population of the snake, *Vipera berus*, multiple mating (but not necessarily multiple fertilization) reduced the proportion of dead offspring in a litter, presumably due to sperm competition (Madsen *et al.*, 1992). In this study, I found no difference between singly and multiply sired litters in terms of proportion of live offspring per litter; furthermore, the small effect size and large minimum required sample size suggest that the lack of significance was not simply due to a lack of statistical power resulting from the small sample size.

Presumably, females that are attractive to males are more likely to have multiple mates. What features of females might make them more attractive to males? Because, all else being equal, longer females have bigger litters in most species, males might be more likely to mate with larger females. Again, however, I found no evidence of differences in body size between singly and multiply mated females nor any evidence that larger sample sizes would have changed this conclusion. However, body size is not just length, but mass as well. Thus, we should expect that females that are "fatter" at a given body length (or, in better "condition") preovulation should produce more offspring (Ford & Seigel, 1989) and therefore have a higher probability of being mated by multiple males. I did not measure body conditions of snakes, but comparison of sizes of singly versus multiply sired litters did not lend any support to this hypothesis, even with correction for body length of mothers. Once again, this lack of significance did not appear to be merely a function of small sample size. In any event, bearing in mind the distinction between multiple copulation and multiple fertilization, my data are not inconsistent with Madsen *et al.*'s (1992) observation that the number of copulations by individual female *Vipera berus* was not correlated with litter size.

Because *Thamnophis sirtalis* has been observed to mate in the autumn (Mendonça & Crews, 1989; Whittier & Crews, 1986; Aleksiuk & Gregory, 1974), multiple paternity could arise due to overlapping sperm from a fall and a spring mating, or even from matings in two consecutive spring seasons. In this case, the lack of efficacy of the copulatory plug is not in question. As argued by Schwartz *et al.* (1989), though, it is unlikely that multiple paternity is the result of a mixture of sperm from an autumn and a spring mating, or between two matings in different springs. Females are not found with viable sperm stores during mid-summer, probably due to complete use for fertilization (Gregory, 1977b; Fox, 1956; Blanchard & Blanchard, 1942). Sperm from fall matings, although effectively stored, are quickly evacuated from storage receptacles after a spring mating and degenerate within six hours (Halpert *et al.*, 1982). Gregory (1977b) has shown that ovulation and subsequent fertilization occurs six to eight weeks after spring mating, well after the period required for fall sperm to be evacuated and rendered useless.

Clearly, the mere occurrence of purported adaptations to prevent multiple paternity should not be taken as evidence that multiple paternity actually is prevented. Such characters also can be interpreted to mean that multiple insemination is in fact possible or even likely, all else being equal. When mating is under female control, as it is with *Thamnophis sirtalis*, it is reasonable to assume that male tactics to prevent multiple inseminations are not perfect.

The significance of multiple inseminations and sperm competition in snakes are poorly understood. Most research on mating systems in snakes is recent (e.g., Höggren & Tegelström, 1995; Duvall *et al.*, 1993; 1992) and only the very most recent studies actually assess male contributions at the molecular level (Höggren & Tegelström, 1995). Paternal care has not been shown in snakes (Shine, 1988), and physiological costs of reproduction for male snakes are limited to sperm production and mating behaviours (Höggren, 1995). Traditionally, the costs of sperm production in male vertebrates have been considered nominal in comparison to female ovum production and embryo maintenance (Charnov, 1982). This leaves searching for and inseminating mates as the main reproductive costs of males.

This may not be the case with snakes. Spermatogenesis in European adders, *Vipera berus*, is more costly than the mating behaviours themselves (Olsson *et al.*, 1997). Mathematical models of individual reproductive success show great sensitivity to the magnitude of reproductive costs, indicating that lower reproductive frequencies result when fixed costs are high (Olsson *et al.*, 1997). Because sperm production is a fixed cost, if males share reproductive success in single litters with other males, the benefits accrued by mating with many females can be outweighed by the costs associated with sperm competition.

The costs of sperm competition would be diminished if litter size is sufficiently large. If litter size is small, though, the potential for exclusion of the gametes of any one potential father increases. Akin *et al.* (1984) describe just such a situation when outlining sources of error associated with the estimation of multiple paternity. One source of error, called the progeny sampling error, is caused by the sample of offspring not expressing the entire range of alleles carried by the mother and all fathers. The progeny sampling problem is treated as a problem in subsampling extremely large litters or clutches (e.g., *Drosophila*), but it also can be considered a problem in sampling species with small total litter sizes. As litter size decreases, the likelihood that certain alleles present in the total sample of sperm will be excluded from the fertilization of ova increases. This bias is almost eliminated if only one male contributes sperm, but as the number of males that contribute sperm increases, so does the likelihood that all of the alleles carried by the sperm will not be found in the litter distribution; hence, the likelihood of detecting multiple paternity decreases.

Therefore, males that mate with previously mated females that carry only a few ova will be, in effect, wasting sperm. This premise is not supported directly from my research, because mean residuals of the number of offspring do not differ between multiply and singly sired litters, but evidence of progeny sampling error can be found in the allele distributions detected in some of the smaller, purportedly singly sired litters

(Table 9). For example, EL 1, at locus 5B Ts, carried two alleles within a litter of three offspring, but only one of those alleles could be attributed to maternal contributions. Not only does this show that progeny sampling error does have an effect, but it clearly illustrates that maternal alleles are affected as well.

Another scale on which to examine effects of body size or litter size is interpopulational or geographical. Although variation at such a scale is a different phenomenon from intrapopulation variation, its consequences for the likelihood of multiple paternity may be similar. In *Thamnophis sirtalis*, there is a fairly pronounced trend of increasing body size from eastern to western Canada (Gregory & Larsen, 1993). However, because snakes from western populations tend to produce larger offspring, they also have smaller litter sizes. Vancouver Island snakes are somewhat more similar to eastern populations than most of the other western populations studied by Gregory and Larsen (1993), but my data nonetheless offer an opportunity to test the hypothesis of litter-size mediated geographic variation in the occurrence of multiple paternity. Fortunately, Schwartz *et al.* (1989) collected similar data for *T. sirtalis* from Michigan and Wisconsin, within the range of Gregory and Larsen's "eastern phenotype". Certainly, the litter sizes they obtained (6-40) contrast strongly with mine (3-19). Schwartz *et al.* detected multiple paternity in 50% of the litters they tested, but felt that the true frequency might have been as high as 72%, using a correction factor from Akin *et al.* (1984). Thus, it is difficult to compare their figures with mine.

However, when I compared their minimum of 50% versus mine of 37.5% by Chi-square analysis, I found no significant difference (Chi-square=0.092, DF=1, $0.75<P<0.90$, ES=0.12, 1-ß=0.11, n_r=5499). Thus, there is no indication from a comparison of these two studies that eastern snakes with larger litters have a tendency towards more frequent multiple paternity than western snakes with smaller litters. Nonetheless, a broader array of samples of adequate size from more sites than examined here are needed to settle the issue.

However, given the finer scale of resolution afforded studies of genetic variability that use DNA markers rather than allozyme markers, the results of my study should show an even higher rate of multiple paternity relative to the allozyme study, assuming equal likelihood of multiple paternity in both cases. Polymorphism detected in the three loci used for this study also indicates that resolution is finer. The most polymorphic locus used in the Schwartz *et al.* (1989) study had only seven detectable alleles, and the other three expressed 5, 3, and 3, respectively. Thirteen alleles were detected at locus 5B Ts, the same at locus 3 Ts, and 22 were detected at locus 2 Ts in my population study (see Table 1). The lower frequency of occurrence of multiple paternity in my sample, coupled with the finer scale tested, suggests that there may be differences in frequency between the snakes tested here and those tested in the allozyme study. Sample sizes are small in both studies; therefore, further investigation of the effect of litter size on multiple inseminations and multiple fertilizations is warranted.

Frequency of multiple paternity also could be affected by population density. R. Shine (pers. comm.) has observed multiple copulatory plugs in the cloacas of garter snakes captured in the Interlake Region of Manitoba. The Interlake Region is well-known for its abundance of Common Garter Snakes: at a single hibernaculum in the Interlake region, Gregory (1977b) estimated population size to be as high as 5348 snakes during the spring of 1970, and even as high as 3407 during the fall of 1972. In such a densely populated competitive arena, multiple inseminations may be unavoidable. It is interesting to note that this region falls within the range of the eastern phenotype proposed by Gregory and Larsen (1993), so the effects of sperm competition may be depressed by large litter sizes. Multiple inseminations would be advantageous in this case, as many females are present in a small area and progeny sampling error is reduced.

Further research on multiple paternity in the Common Garter Snake should address the potential effects of population density and litter size on the frequency of realized multiple paternity. Although there are statistical questions as to whether eastern snakes show different levels of multiple paternity relative to western snakes, it seems a reasonable idea that should be investigated. If progeny sampling error is an issue, as seems likely, then the effect of sperm precedence on litter profiles needs to be studied. Progeny sampling error would be inflated if the first male enjoyed complete reproductive success over any subsequent male. If sperm precedence has no effect (sperm are randomly mixed), then progeny sampling error would strictly be a matter of random sampling. This is easily tested using controlled breeding experiments.

Density effects can be determined by combining controlled breeding experiments with field and molecular studies of populations with varying population densities. This could lead to further insight into the potential effects of progeny sampling error. In sufficiently dense populations, multiple paternity in small litters is more likely to be detected directly if density effects increase the likelihood of multiple inseminations. Of course, this should be undertaken only after potential differences in relevant life-history characteristics have been measured between study populations.

CONCLUSIONS

1. Genetic variation among five populations of the Common Garter Snake (*Thamnophis sirtalis*) was modest; however, based on one locus, there was some evidence that the two southernmost populations represent one deme.

2. Populations of snakes at salmon hatcheries had a less stable genetic structure at one locus than did populations at "natural" sites. This is consistent with the hypothesis that hatcheries, with their abundant supply of readily available food, attract snakes from surrounding areas. However, further research is needed to test this hypothesis properly.

3. Multiple paternity was observed in a minimum of 37.5% of litters examined. However, the ecological significance of multiple paternity is not clear. Its incidence was unrelated to length of mother, number of offspring in a litter, average mass of littermates, or percentage of live offspring. Furthermore, comparison with previous work yielded no evidence of geographic variation in the occurrence of multiple paternity. Although samples were small, power analyses suggested that these nonsignificant differences were real. Nonetheless, further study, using larger samples and accounting for other potentially influential factors, is warranted.

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Appendix I

Characterization of two microsatellite loci

Of the 14 positive clones that proved to have usable microsatellite sequences, only two sets of PCR primers designed from these clones generated PCR products that were variable both between populations and within litters. Primer set 2 Ts amplifies a complex microsatellite composed of a hexamer (CTCTAT), a tetramer (CTAT), another tetramer (CCAT), and the first tetramer again (CTAT). In the original sequence, 12 CTCTAT repeats, 14 CTAT repeats, 13 CCAT repeats, and another 9 CTAT repeats were found. Primer set 3 Ts amplifies a region composed primarily of a tetramer (TAGA), but is not a pure microsatellite. The repetitive region occasionally includes small regions where the repetitive sequence is not exact, but is composed of the three bases inherent in the repeat. For example, after the initial 19 TAGA repeats, the sequence reads as TAATAGATGATAGATAGATGA with similar variations for a total of 89 bases within the repetitive region. Another 17 TAGA repeats then occur, and then the repetitive sequence again deviates from simple repeats and the presence of cytosine bases becomes evident. Within the original clone, a total of 36 repeats were noted, with 89 bases of variant repeats included.

Size ranges for the PCR products generated with the two sets of primers are as follows; primer set 2 Ts amplified a total of

22 detectable alleles, ranging in size from 270-409 bases in size, while primer set 3 Ts amplified 12 alleles (one null detected, see Table 1 and methods section) ranging in size from 368-429 bases in size. Both sets of primers were used on template DNA derived from three congeneric species of garter snakes (*Thamnophis elegans*, *T. ordinoides* and *T. radix*) and one other colubrid (*Elaphe guttata*), but no amplification products were observed, indicating that these microsatellite loci are specific for *T. sirtalis*.

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