ABSTRACT

Rufus Wharton Gaul, Jr. AN INVESTIGATION OF THE GENETIC AND ECOLOGICAL STATUS OF THE CAROLINA SALT MARSH SNAKE, *NERODIA SIPEDON WILLIAMENGELSI*. (Under the direction of Dr. Trip Lamb) Department of Biology, April, 1996.

The Carolina salt marsh snake (*Nerodia sipedon williamengelsi*) a melanistic water snake endemic to estuarine habitats in coastal North Carolina, is currently listed by the state as a taxon of special concern. A combination of molecular and morphological techniques was employed to examine genetic relationships between the salt marsh snake and the nominate subspecies *Nerodia sipedon sipedon*, as well as the dynamics of hybridization between the salt marsh snake and a closely related species, the banded water snake, *Nerodia fasciata*. Salt marsh snakes were trapped in large numbers in *Juncus* marsh, but only infrequently in other habitats. Restriction endonuclease digests of mitochondrial DNA produced six unique haplotypes in coastal *N. sipedon*, but no clear distinction was detected between *N. s. williamengelsi* and *N. s. sipedon*. Analysis of morphological characters revealed statistically significant differences between the two subspecies in numbers of ventral scales, subcaudal scales, and lateral bars. Two morphological characters, ventral scales and lateral bars, showed evidence of clinal variation and appear to correspond closely to estuarine salinity gradients. Evidence for hybridization between *N. s. williamengelsi* and *N. fasciata* was observed in five specimens; mtDNA variation observed in these hybrids suggests that hybridization events between these two species are bi-directional.

AN INVESTIGATION OF THE GENETIC AND ECOLOGICAL STATUS OF THE CAROLINA SALT MARSH SNAKE, *NERODIA SIPEDON WILLIAMENGELSI*

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Rufus Wharton Gaul, Jr.

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STATUS OF THE CAROLINA SALT MARSH SNAKE,

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DEDICATION

This thesis is dedicated to my wife, Lisa, without whose love, patience and support it never could have been attempted; to my parents, Dr. and Mrs. R. Wharton Gaul, Sr., for encouraging my interest in biology; and to the memory of Detlev M. Bunger, friend and colleague.

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INTRODUCTION

The Subspecies Concept and Its Role in Taxonomy

During the early 1900's a great amount of research effort was directed towards the identification and formal taxonomic description of intraspecific variation. Often, such investigations resulted in the naming of one or more geographic "races" or "subspecies." Subspecies were perceived as being genetically distinct, geographically separate populations belonging to the same species and therefore interbreeding freely in zones of contact (Wilson and Brown, 1953). Mayr (1942) gave new impetus to the geographic race as a valid taxonomic category, emphasizing the evolutionary significance of subspecies when correlated with geography. Taxonomists readily exploited the opportunities opened by acceptance of the subspecies concept, concentrating especially on groups of organisms that were already well-known at the species level, and often combining many populations formerly known as full species into subspecies. Wilson and Brown (1953) criticized the subspecies concept as "illusory and superfluous," citing the difficulty of establishing viable lower limits for the recognition of a race. In addition, they noted other difficulties with subspecies, including "polytopic races," in which a single diagnostic character arises in more than one population and cannot be geographically coordinated, and "microgeographic races," in which one or a number of characters vary so extensively that nearly every local population is distinguishable from all others. These criticisms aside, Wilson and Brown (1953) did provide some general criteria bearing on the taxonomic application of the subspecies concept:

1. Where one character varies geographically, other genetically variable characters can be found to vary also.

2. The geographical variation of independent characters tends to be discordant to some degree. The degree of concordance increases with the degree of isolation of populations.

3. The greater the number of characters, the greater will be the total discordance.

4. The greater the geographical area encompassed, the less homogeneous will be the population.

Henning (1966), while acknowledging a role for subspecies in the realm of taxonomy, cautioned that there are practically no set rules for differentiating subspecies. Moreover, he stressed that variation in the extent of differences between subspecies often compromises efforts to define their true relationships.

Biologists today must contend with the nomenclatorial legacies left by the zealous taxonomic activities brought on by acceptance of the subspecies concept. For certain species, long-standing trinomial taxa, often based on subtle morphological distinctions, can be seriously questioned as valid evolutionary units. In others, original subspecific designations have assumed greater legitimacy, by virtue of the subsequent discovery of distinctive character complexes (genetic and morphological) that have long-term adaptive or historical bases. Increasingly, studies of intraspecific variation are being brought to bear upon species faces with extinction or habitat loss (e.g.: Osentoski and Lamb, 1995; Reichling, 1995).

The advent of sophisticated molecular techniques has resulted in a greater understanding of the geographic structure and variation within species. Studies of allozymic and mitochondrial DNA variation have often helped to answer questions both about the status of subspecific taxa and the geological history that may have shaped them.

Mitochondrial DNA and Evolutionary Genetics

The value of animal mitochondrial DNA (mtDNA) as a tool for studying evolutionary biology has been well established. In particular mtDNA has become a powerful tool for the study of intraspecific phylogeny (Avise et al., 1987). Increasingly, studies involving mtDNA have helped bridge the gap between microevolutionary processes and macroevolutionary ones.

The vertebrate mitochondrial genome is a maternally inherited, covalently closed circular molecule. Genome size averages 16,000 to 22,000 base pairs (bp) although Densmore et al. (1992) documented mtDNA molecules ranging from 22,000 to 26,000 bp in several species of water snakes (*Nerodia)*, among the largest reported in any vertebrate. Within vertebrates the gene content and gene arrangement of the molecule are highly conserved, encoding 2 ribosomal RNA genes, 22 transfer RNA genes, and 13 protein genes coding for subunits of enzymes associated with the electron transport chain. Vertebrate mtDNA lacks the introns, transposable elements, and pseudogenes commonly found in nuclear DNA.

Animal mtDNA evolves 1-10 times more rapidly at the nucleotide level than single copy nuclear DNA (Avise et al., 1979), averaging an estimated 2% sequence

divergence per million years (Brown et al., 1979). Two possible reasons for this rapid rate of evolution are 1) an enhanced mutation rate; and 2) relaxed selective pressure upon the molecule's functional products (Avise, 1986). Although neither hypothesis has been strongly supported, there is some evidence that mtDNA has a less efficient repair mechanism than nuclear DNA, exhibiting a large number of simple base substitutions (Moritz et al., 1987).

An important characteristic that facilitates the use of mtDNA in phylogenetic studies is its maternally mediated mode of inheritance. Cytoplasmic organelles are contributed to offspring via the egg (Wilson et al., 1985). Consequently, non-deleterious mutations tend to be maintained within this maternal lineage. A maternal lineage thus contains a record of the mutational history of mtDNA that can often be traced back many generations. In addition, the maternal inheritance of mtDNA makes it more sensitive to factors affecting population structure, such as bottlenecks and female survivorship (Wilson et al., 1985). Thus, populations tend to be characterized by a small number of mtDNA genotypes, due to the stochastic loss of those that are rare. Neigel and Avise (1986) concluded that for stable populations with initial sizes of N females, there was a high probability that within 4N generations all descendents could be traced to a single female. The same principle applies to geographic isolation, where the number of generations required for the isolated populations to become genetically distinct is 2-4 times their effective population size (Neigel and Avise, 1986).

Often, genetically distinct populations display a distinct geographic arrangement,

reflecting the effects of significant isolation events. This phenomenon has been termed *intraspecific phylogeography* (Avise et al., 1987). Five distinct phylogeographic patterns were identified by Avise et al. (1987). They are: 1) phylogenetic discontinuities with spatial separation; 2) phylogenetic discontinuities without spatial separation; 3) phylogenetic continuity with spatial separation; 4) phylogenetic continuity without spatial separation; and 5) phylogenetic continuity with partial spatial separation. The first pattern, phylogenetic discontinuities with spatial separation, is the one most commonly observed. It is characterized by groups of related but distinct mtDNA genotypes separated from each other by long-term zoogeographic barriers. Such barriers create periods of reduced gene flow of sufficient time to allow two isolated populations to attain the loss of shared ancestral genotypes, or *reciprocal monophyly.* This pattern has been identified in a number of vertebrate species, including three species of sunfishes, *Lepomis,* (Bermingham and Avise, 1986), the desert tortoise, *Gopherus agassizii,* (Lamb et al., 1989), and the gopher tortoise, *Gopherus polyphemus,* (Osentoski and Lamb, 1995).

The second category, phylogenetic discontinuities without spatial separation, has not been documented to date. Such a pattern would require the evolution and maintenance of two or more distinct, sympatric groups of genotypes. In theory, this pattern could arise through the spontaneous occurrence of intrinsic reproductive barriers within a species.

Phylogenetic continuity with spatial separation is the third category. This pattern is characterized by little to no genotypic variation among geographically disjunct

populations. Lamb and Avise (1992) documented this pattern in the diamondback terrapin, *Malaclemys terrapin,* and Avise et al. (1983) described it in the old field mouse, *Peromyscus polionotus.* Such a pattern could be maintained by frequent disruption and reestablishment of gene flow among populations.

The fourth category, phylogenetic continuity with no spatial separation, is seen in species exhibiting high levels of gene flow. It has been documented in American eels (Avise et al., 1986), sea urchins (Palumbri and Wilson, 1990), and red-winged blackbirds (Ball et al., 1988). Species fitting this category are often far-ranging, permitting a high level of mixing throughout their ranges; or they may have undergone rapid population or range expansion.

Phylogenetic continuity with partial spatial separation is the final category. This pattern is characterized by the co-occurrence of both localized and widespread genotypes. Berminghan and Avise (1986) documented this pattern in the bowfin, *Amia calva,* in which a set of localized genotypes is found within the range of a larger, more widespread one.

Methods of mtDNA analysis ---Surveys of mtDNA variation routinely involve restriction endonuclease assay or DNA sequencing. Restriction endonucleases are bacterial enzymes that recognize a specific sequence of bases (usually four or six bases in length) along a strand of DNA and cut the molecule wherever that sequence occurs, resulting in a collection of fragments of varying size. The resulting fragments can be detected by attaching radioactive phosphate to their ends, by Southern blotting, or by

ethidium staining. Restriction endonucleases generate restriction fragment length polymorphisms (RFLPs) or restriction site data, which is compared among closely related mtDNAs. Restriction site data is typically analyzed by one of two approaches, one quantitative, the other qualitative.

In the quantitative approach, restriction sites are converted to an estimate of nucleotide sequence divergence, e.g., a *p*-value (Nei and Miller, 1990), representing a pair-wise genetic distance between two distinct mtDNA genotypes. The resultant *p*-values from a collection of different mtDNA samples can then be subjected to UPGMA (unweighted pair-group method using arithmetic averages) analysis. UPGMA analysis generates a tree or "phenogram," indicating phenetic relationships.

In the qualitative analysis, restriction sites are scored as either present (1) or absent (0) for each genotype. By comparing these data using the method of maximum parsimony, a tree can be constructed allowing one to infer relationships among the haplotypes examined. An "outgroup" is usually used to polarize restriction sites as either ancestral or derived. In the absence of an outgroup, the tree remains unpolarized and is termed simply as a "network." A network, while lacking the resolving power of a "rooted" parsimonious tree, can nevertheless provide information regarding the relative relationships among different haplotypes.

The Carolina salt marsh snake, *Nerodia sipedon williamengelsi*

My study focused on the Carolina salt marsh snake, *Nerodia sipedon williamengelsi,* a form occurring in estuarine habitats in coastal North Carolina.

Natricine water snakes of the *Nerodia sipedon-fasciata* complex occur in a wide variety of aquatic and semi-aquatic habitats throughout eastern North America. The complex comprises two polytypic species, *N. sipedon* with four subspecies (*sipedon, insularum, pleuralis,* and *williamengelsi),* occurring in the northern part of the range of the complex; and *N. fasciata* with three subspecies *(fasciata, confluens,* and *pictiventris),* occurring primarily on the Atlantic and Gulf coastal plains.

Nerodia sipedon is a large, stout-bodied water snake with crossbands on the neck and anterior body breaking up posteriorly into dorsal blotches and alternating lateral bars. Dorsal coloration is extremely variable. Ground color ranges from gray to dark brown, with the dorsal markings ranging from red to black. Ventral coloration typically ranges from cream to reddish. Ventral markings are normally brown, black, or reddish semi-circular spots. Most populations of *N. sipedon* lack a prominent post-orbital dark bar running from the eye to the angle of the jaw. Specimens are occasionally virtually patternless, ranging in color from dark brown to black.

Nerodia fasciata, like *N. sipedon,* is a large, heavy bodied water snake with a highly variable coloration. In contrast to *N. sipedon,* however, *N. fasciata* normally exhibits a dorsal pattern consisting entirely of complete crossbands throughout the length of the body, possesses ventral markings that are usually squarish or triangular-shaped, and has a prominant postorbital dark bar.

Systematic relationships within the complex are poorly understood, with different taxonomic studies yielding conflicting interpretations. Formerly, all

subspecies of *N. fasciata* were considered subspecies of *N. sipedon.* Conant (1963) divided the complex into two polytypic species. Schwaner and Mount (1976) reached the same conclusion but noted extensive interbreeding between the two species. In a study of the complex in Louisiana and Mississippi, Blaney and Blaney (1979) concluded that *N. sipedon* and *N. fasciata* were conspecific.

In North Carolina, *N. fasciata* occurs in freshwater habitats on the Coastal Plain and has only recently been reported from north of the Albemarle Sound (Brothers, 1992). *Nerodia sipedon* is common throughout the mountains and Piedmont but also occurs in the Coastal Plain north of the Albemarle Sound, inhabits the peninsula between Albemarle and Pamlico Sounds, and occurs on the islands of the Outer Banks (Fig. 3). Populations of *N. sipedon* occupying brackish-water localities represent a distinct color morph, a characteristic that led to the description of the Carolina salt marsh snake as a third form of the *Nerodia sipedon-fasciata* complex in the state.

Water snakes have been known from brackish habitats in coastal North Carolina since at least 1905, when a specimen was collected at Cape Hatteras (Conant, 1963). Formal recognition of the coastal population as a distinct taxon began with the work of Barbour (1943), who described *Natrix sipedon engelsi* on the basis of a single specimen from Mullet Pond on the Shackleford Banks. Robertson and Tyson (1950) examined a small series of specimens from Mullet Pond, Core Banks, and Ocracoke Island and concluded that while the Shackleford Banks population exhibited characteristics intermediate between *sipedon* and *fasciata,* other populations on the

offshore banks to the north merited recognition as a distinct, dark subspecies, thus supporting Barbour's original description. Conant (1961) examined the *engelsi* specimen and concluded that it was identifiable as *N. fasciata* and that all brackish-water populations to the north were *N. sipedon.* Upon examination of some 50 specimens of *N. sipedon* from the Outer Banks and adjacent mainland, Conant and Lazell (1973) formally described *N. sipedon williamengelsi.*

Conant and Lazell diagnosed *N. s. williamengelsi* as a strongly melanistic race in which: 1) the dorsum in adults is essentially black with interspaces between the crossbands and blotches so dark as to obscure pattern details; 2) the venter posterior to midbody is predominantly black; 3) there are no reddish- or brown-centered ventral markings posterior to the 50th ventral; and 4) the light scales between the dark cross-bands average 1.5 on the neck at the level of the second dorsal scale row. In general the subspecies exhibits much less variation than the nominate race. Although melanistic individuals occur throughout the range of *N. sipedon,* no other population has been documented to exhibit all four diagnostic characteristics of *N. s. williamengelsi*

Intergradation between *N. s. sipedon* (Fig. 1) and *N. s. williamengelsi* (Fig. 2) is extensive, with specimens showing intergradient characteristics occurring over much of the Outer Banks north of Cape Hatteras and at many localities along the mainland shore of Pamlico Sound (Conant and Lazell, 1973). Intergradation has also been noted by other researchers, e. g., Robertson and Tyson (1950) and Palmer and Braswell (1995).

Hybridization between *N. sipedon* and *N. fasciata* has long been recognized as an

important factor in the biology of the two species. Cliburn (1957) noted evidence of extensive interbreeding between the two forms on the Gulf Coastal Plain but considered it to represent secondary intergradation between subspecies, a view shared by Blaney and Blaney (1979). In his taxonomic review of the complex, Conant (1963) examined several specimens that were intermediate between *sipedon* and *fasciata*. He concluded that interbreeding between the two forms represented introgressive hybridization, resulting from habitat alteration by man rather than evidence of conspecificity. Schwaner and Mount (1976) surveyed the complex in Alabama and northern Florida and suggested that *sipedon* and *fasciata* were conspecific but conceded that data were insufficient to resurrect *fasciata* as a subspecies of *N. sipedon.*

Conant and Lazell (1973) documented three localities from which hybrid *N. s. williamengelsi* x *fasciata* were collected. Two of these, Mullet Pond and Lennoxville Point in Carteret County, lie in an area where severe storms may play a role in bringing individuals of the two species into contact with one another (Engels, 1952). Several other localities have produced hybrids between *N. s. williamengelsi* and *N. fasciata* (Palmer and Braswell, 1995).

Nerodia s. williamengelsi is closely associated with salt marshes dominated by black needlerush, *Juncus romerianus*, and marsh grasses of the genus *Spartina.* It is also found in tidal creeks, various man-made freshwater impoundments along the Outer Banks, and along the mainland shore of Pamlico and Core Sounds. Conant and Lazell (1973) determined that *williamengelsi* would not drink salt water, a finding similar to that noted

by Pettus (1963) in a study of the Gulf salt marsh snake, *Nerodia fasciata clarki*. Dunson (1980) studied *N. f. clarki* and several freshwater *Nerodia* in Florida and found important physiological differences between the brackish and freshwater taxa. Freshwater *Nerodia* were found to have skins that were more permeable to water and salt than *N. f. clarki*.

Aside from Conant and Lazell's (1973) description of the subspecies and a few brief notes on local abundance and natural history (Willson, 1992; Palmer and Braswell, 1995), no formal study of the Carolina salt marsh snake has been attempted. *Nerodia s. williamengelsi* is listed by the state of North Carolina as a taxon of special concern due to its endemism and the lack of published data on its ecology and systematic relationship with the nominate subspecies.

RESEARCH OBJECTIVES

In this study I used both genetic and morphological techniques to examine variation in *Nerodia sipedon* in coastal North Carolina. Restriction analysis of mtDNA and statistical analysis of morphological and morphometric traits were performed on snakes collected throughout the putative range of *N. s. williamengelsi*. Specifically, my objectives were 1) to determine the current distribution of populations of snakes assignable to *williamengelsi*; 2) determine whether *williamengelsi* can be distinguished genetically from *N. s. sipedon*; 3) assess the extent and direction of hybridization between *williamengelsi* and *N. fasciata*; and 4) collect further information on the ecology and natural history of *williamengelsi*.

MATERIALS AND METHODS

Snake Collection

A total of 176 specimens of *N. s. williamengelsi, N. s. sipedon,* and *N. fasciata* was examined during the project (Appendix A). Snakes were collected by trapping or by hand capture. "Gee" brand minnow traps and eel pots were placed within suitable habitat. In *Juncus* marsh, traps were set in a 1 x 1 meter pit or other area containing standing water. In canals and creeks, traps were set along banks in areas of heavy vegetation. All traps were staked and equipped with floatation devices to prevent snakes from drowning during periods of high water. The number of traps employed during any one trapping effort ranged from five to thirty-five, and the duration of individual trapping attempts ranged from 24 hours to 89 days. A total of 19 sites was sampled by trapping. Snakes were also hand-captured by "road riding" through suitable habitat and by boating in suitable habitat. Road-kill snakes were salvaged when possible and preserved for morphological analysis. In addition to snakes collected during the study, a number of specimens were provided by other individuals. Preserved specimens from the collection of the N. C. State Museum of Natural Sciences (NCSM) and my private collection were also examined (Fig. 4). All specimens from which mtDNA was isolated were preserved as vouchers and subsequently used in morphological analysis. In order to minimize the effects of collecting on wild populations, litters totaling 71 neonates born to three females in captivity were released at their original sites of capture, and several other gravid females were released immediately upon capture.

Mitochondrial DNA Analysis

Mitochondrial DNA was isolated from whole liver tissue, which was homogenized and centrifuged in 1 X MSB buffer (Lansman et al., 1981, Appendix B). Mitochondria were lysed with SDS and centrifuged at 40,000 rpm in a cesium chloride gradient. The resultant mtDNA band was dripped under UV light.

Samples were mixed with 100% isopropyl alcohol and shaken vigorously to remove excess cesium chloride, ethidium bromide, and EDTA. Samples were then dialysed against 1.4 L sodium acetate, 1M Tris-HCL, and 0.2M EDTA for 48 hours, with one change of buffer after the first 24 hours. Samples were stored at 4^{O} C until use. Restriction digests of mtDNA were generated with the following fourteen restriction endonucleases: *Acc*I, *Ava*I, *Ava*II, *Bgl*II, *Bcl*I, *Bst*eI, *EcoR*I, *Eco*O109, *Eco*R5, *Hha*I, *Hin*cII, *Hin*dIII, *Sac*I, and *Spe*I. Restriction digests were conducted according to conditions suggested by the supplier (New England Biolabs, Beverly, MA). In general the reaction volume of the digests totaled 20 ul (10ul DNA, 2 ul 10X buffer, 8 ul distilled water). Digestion fragments were separated according to molecular weight by electrophoresis thorough 1.2% agarose (4.2g agarose, 35 ml 10X TBE buffer, 315 ml distilled water) gels. Gels were stained in 1 ug/ml ethidium bromide for 10-20 min. to allow fragments to be visualized and photographed under UV light. Photographs were made using Polaroid type 55 pos./neg. ASA 50 film. Fragment lengths were determined by comparison to a 1-kb molecular weight standard (Bethesda Research Labs). Each restriction profile produced by a given enzyme was assigned an uppercase letter code.

Following convention, the most common profile was designated "C"; profiles that differed by a single restriction site loss or gain were designated "B" or "D" respectively. Non-adjacent letter designations represent more than one site gain/loss relative to the "C" profile. Letter codes were compiled for all enzymes to compile a composite mtDNA genotype or "haplotype" for each specimen. Each haplotype was assigned a numerical designation (e.g., haplotype "1", etc.). Haplotypes combined into a composite data input file and enzyme profiles were compiled. The two data sets thus created were treated using the Restriction Enzyme Analysis Package (REAP) version 4.0 (McElroy et al., 1990) resulting in a binary character and genetic distance matrix (Table 3). UPGMA analysis of the genetic distance data was conducted using the NTSYS software package.

Morphological Analysis

All specimens were examined and scored on an initial classification scale ranging from pure *N. s. sipedon* (1) to pure *N. s. williamengelsi* (5). Following this cursory examination, specimens were separated into *N. s. sipedon* or *N. s. williamengelsi* populations and examined for morphological characters using the methods detailed below.

Ventral scales were counted in the manner proposed by Dowling (1951). Subcaudal scales were counted in pairs beginning at the anal plate and continuing to but not including the terminal spine. Subcaudals were counted only in specimens with complete tails, as evidenced by intact terminal spines. Subcaudal counts for males and females were averaged separately. Dorsal scale rows were counted diagonally at three points along the body: anterior, mid-body, and posterior. The number of lateral bars

coming into contact with ventral scales was noted for the right and left side of each specimen and averaged. The presence of red- or brown-centered ventral markings posterior to the 50th ventral scale was noted for each specimen.

Mean values of morphological characteristics for *N. s. sipedon* and *N. s. williamengelsi* were treated statistically using a Students T test, F test, and One-way Analysis-of-Variance (ANOVA) to assess possible differences between the two subspecies. In addition, populations of *N. sipedon* were grouped together into six geographic subunits (Northeastern: Currituck Co. and SE Virginia; Eastern: Dare and E Hyde Cos.; Central: Beaufort, Pamlico, and W Hyde Cos.; Southern: Carteret Co. and Core Banks; Northwestern: Nash, Wilson, and Pitt Cos.; and Piedmont: localities west of the Coastal Plain) and subjected to comparison of means in order to determine the presence or absence of gradients in morphological characters.

RESULTS

Collecting Results

During the course of the study, 56 *N. s. williamengelsi* were trapped. Trapping was carried out from 15 May until 8 Dec. 1992 and from 10 Apr. until 4 Nov. 1993. Trapping was most successful in *Juncus* marsh, where 82.1% of all *williamengelsi* trapped were caught (Table 1). Twenty-four *N. s. sipedon* were collected by hand and 14 *N. fasciata* were also collected during 1992-1994 (13 by hand-capture, 1 by trapping).

Mitochondrial DNA Analysis

Of the 14 restriction endonucleases used, nine produced variation in *Nerodia sipedon* and *N. fasciata* (Table 2). Comparison of mtDNA restriction patterns from 14 individuals of *Nerodia* (seven *N. s. williamengelsi*, two *N. s. sipedon*, two *N. fasciata*, and three putative *N. s. williamengelsi* x *fasciata* hybrids) resulted in the identification of ten unique mtDNA haplotypes (Table 2). Four snakes (two *N. s. williamengelsi,* one *N. s. sipedon,* and one putative *N. s. williamengelsi* x *fasciata* hybrid) all exhibited the most common haplotype (*a*). Haplotype (*e*) was found to occur in two specimens, a *N. s. williamengelsi* from Wyesocking Bay, Hyde County, and a *N. s. sipedon* from Rutherford County in the North Carolina foothills. Four other *williamengelsi* each possessed their own unique haplotype. Two *N. fasciata* and two putative *N. s. williamengelsi* x *fasciata* hybrids each exhibited a unique haplotype (*g, h, i,* and *j*). Overall, restriction fragment polymorphism was extensive in *N. s. williamengelsi.*

The UPGMA analysis of *p*-values separated the ten unique haplotypes into three

lineages (Fig. 5). Four haplotypes, two from putative *N. s. williamengelsi* x *fasciata* hybrids and two from *N. fasciata,* were separated from all *N. sipedon* haplotypes by a sequence divergence of 2.0%. In addition, one of the hybrid haplotypes (*h*) separated out from the other hybrid and the two *N. fasciata* haplotypes at an overall sequence divergence of 2.5%. All *N. sipedon* haplotypes (*a-f*) clustered together; there was no clear distinction between *N. s. sipedon* and *N. s. williamengelsi.* The third putative hybrid collected fell within this range as well, exhibiting haplotype *a.*

Morphological Analysis

A number morphological characters exhibited variation (Table 4). *Nerodia s. williamengelsi* exhibited a significantly higher number of ventral scales (with males averaging 136.6; females 136.9) than *N. s. sipedon* (males: 134.2; females 133.6, Table 5). Differences were also noted in counts of scale rows: male *N. s. sipedon* averaged 21.1 scale rows whereas male *N. s. williamengelsi* averaged 20.7 scale rows. For females, the number of scale rows averaged 20 and 21.0, respectively. However, the only statistically significant difference found in scale row counts was between male and female *N. s. sipedon* (Table 6).

Nerodia s. sipedon exhibited significantly higher subcaudal scale counts for both sexes (Table 7). In male *N. s. sipedon* subcaudals averaged 73.7 and females averaged 64.3; whereas *N. s. williamengelsi* had subcaudal counts of 72.5 (males) and 63.0 (females). The third and final significant difference between subspecies involved lateral bar counts. Both male and female *N. s. williamengelsi* had higher numbers of lateral bars

(37.0 and 36.9, respectively) than did male and female *N. s. sipedon* (32.1 and 28.6, see Table 8).

The presence of red- or brown-centered ventral markings posterior to the 50th ventral scale could not be accurately determined for the majority of specimens examined, due to fading of pigments in preserved material. Consequently, statistical treatment of this morphological character was not attempted. However, based on a small number of live specimens examined during the study, it was possible to note that red-centered ventral markings posterior to the 50th ventral occurred in some specimens throughout the range of *williamengelsi*.

Clinal variation was evident for two of the morphological characters surveyed. Populations of *N. sipedon* exhibited clinal gradients in ventral scale counts (Fig. 6) and lateral bar counts (Fig. 7). Mean ventral scale counts ranged from 133.0 in populations from the Piedmont to 138.6 in the population occurring in eastern Carteret County. Intermediate populations had ventral counts ranging from 134.9 to 136.6 (Fig. 6). Lateral bar counts showed a similar trend, with Piedmont and inner Coastal Plain populations exhibiting lower counts ($x = 30.4$) and populations in eastern Carteret County showing the highest counts ($x = 38.1$, Fig. 7). For coastal populations of *N. sipedon*, these gradients appear to correspond to salinity gradients that have been documented in the sounds of North Carolina (Fig. 8).

Assessment of Hybridization

Three snakes morphologically resembling hybrids between *N. s. williamengelsi* and

N. fasciata were collected. Two of these (92-1237, 92-1238) were collected in a ditch approximately 9.3 km NW of Otway, Carteret County, in an area where other snakes believed to be hybrids have been found (L. R. Settle, pers. comm.). Mitochondrial DNA restriction patterns for these two snakes are similar to *N. fasciata* for several enzymes (Fig. 9). The third hybrid specimen, 91-2044, was collected in Saint Clair Creek, Beaufort County. Mitochondrial DNA restriction patterns for this specimen are identical to those of *N. s. williamengelsi* as generated by all restriction enzymes used (see Fig. 10).

Figure 1. *Nerodia sipedon williamengelsi*, adult female from Cedar Island, Carteret County, NC.

Figure 2. *Nerodia sipedon sipedon*, adult male from the Tar river, Nash County, NC.

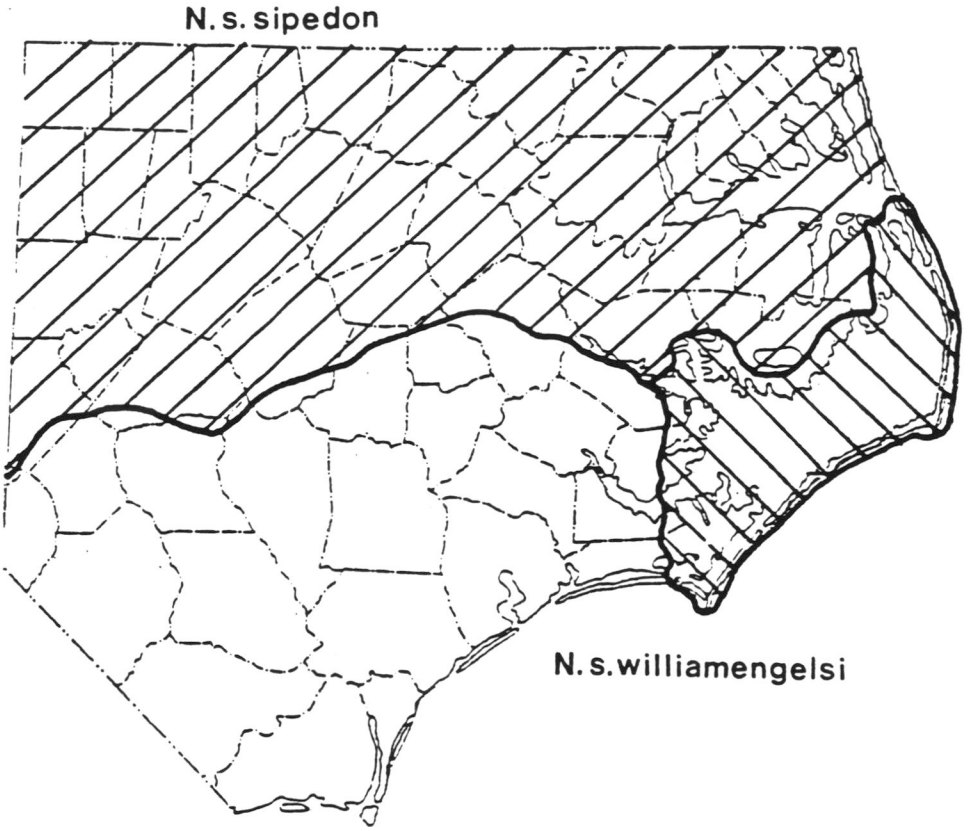

Figure 3. Ranges of *Nerodia sipedon sipedon* and *Nerodia sipedon williamengelsi* in eastern North Carolina.

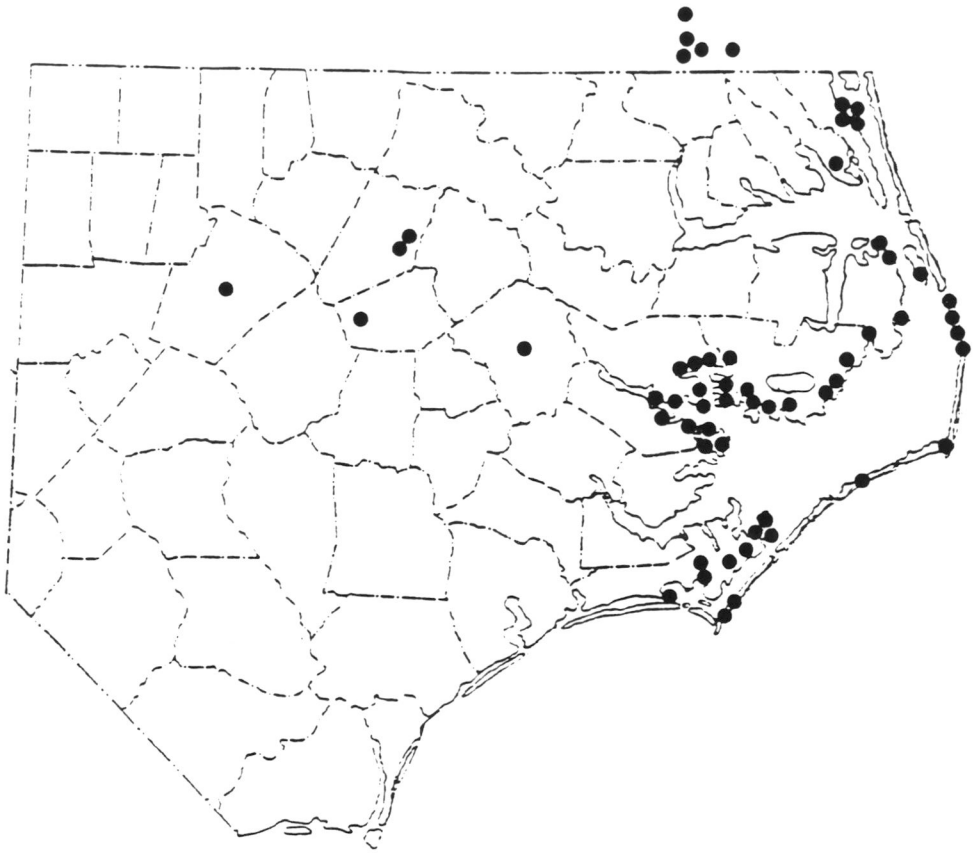

Figure 4. Map of collecting localities for *Nerodia sipedon sipedon* and *Nerodia sipedon williamengelsi* in eastern North Carolina and southeastern Virginia.

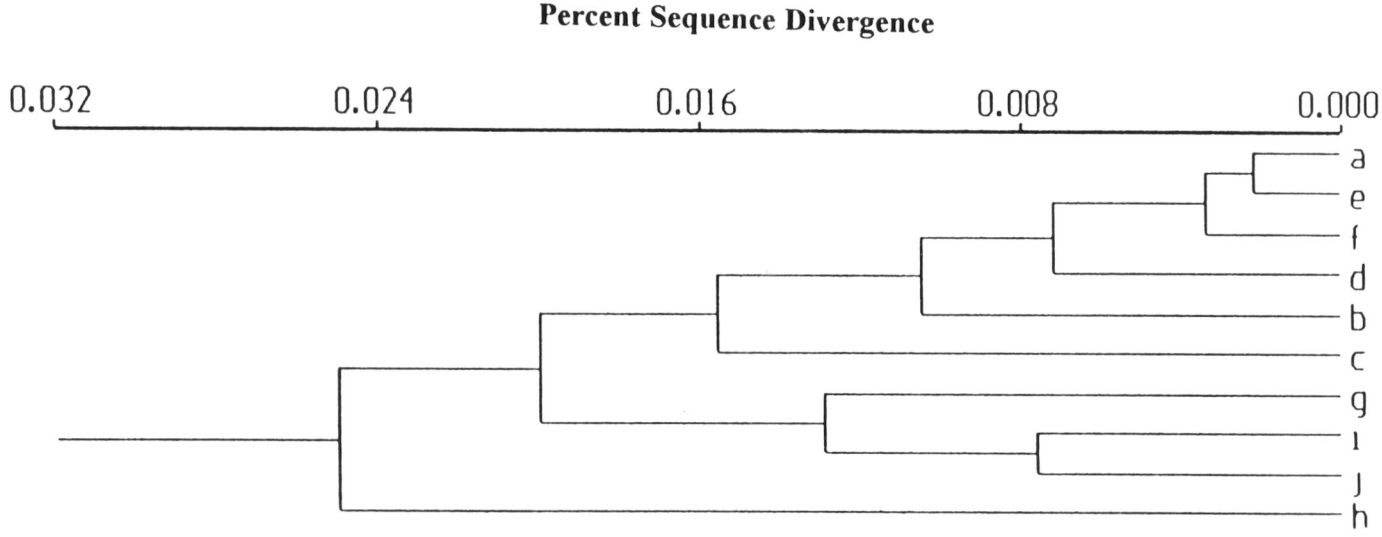

Figure 5. A UPGMA phenogram depicting estimates of nucleotide sequence divergence (*p*) among ten mtDNA haplotypes of *Nerodia sipedon sipedon*, *Nerodia sipedon williamengelsi*, *Nerodia fasciata*, and *N. s. williamengelsi* x *fasciata* hybrids. Haplotypes correspond to those in Table 2.

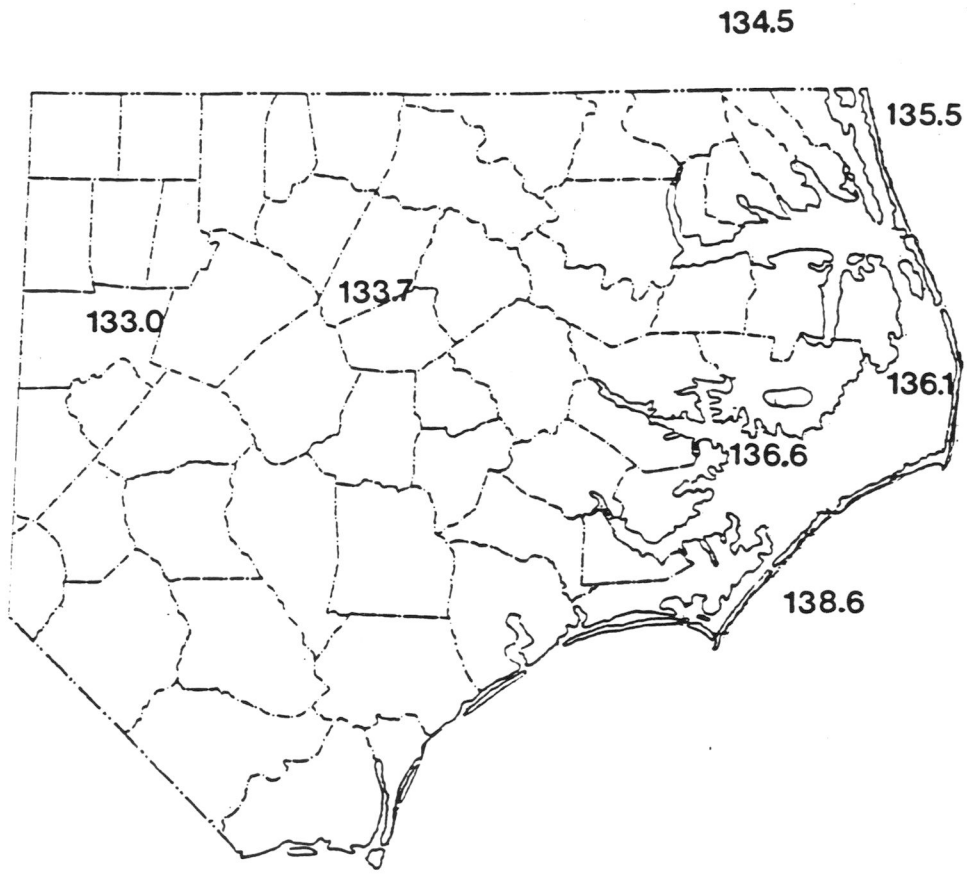

Figure 6. Geographic distribution of mean ventral scale counts in populations of *Nerodia sipedon* in eastern and coastal North Carolina.

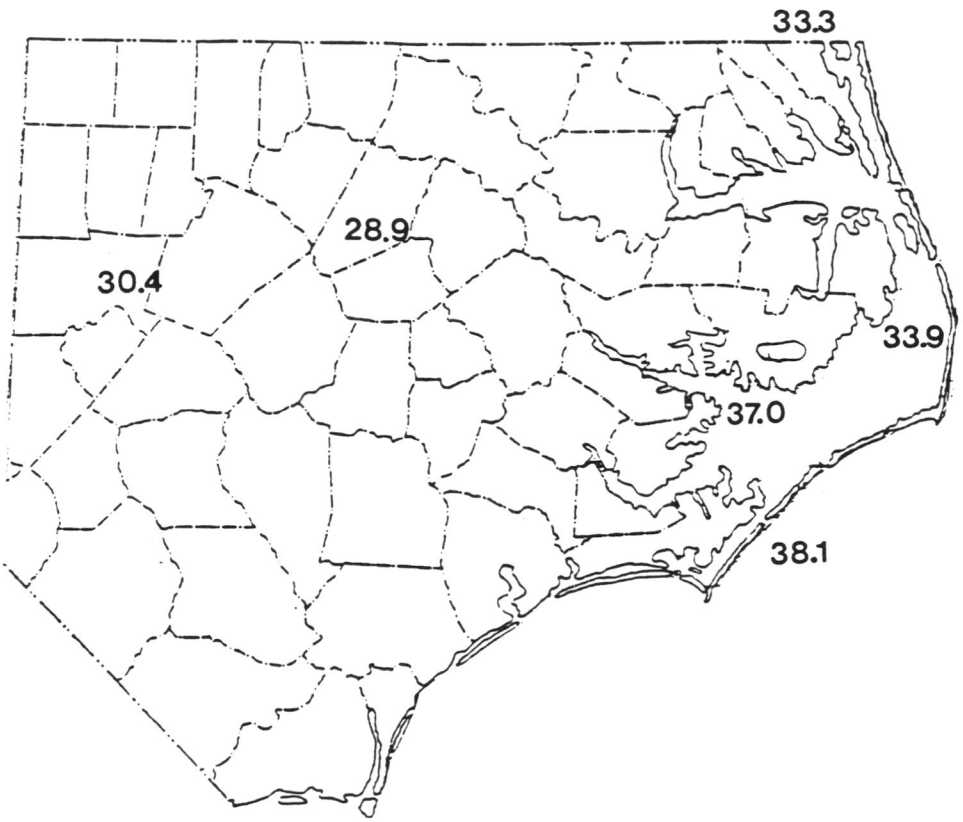

Figure 7. Geographic distribution of mean lateral bar counts in populations of *Nerodia sipedon* in eastern and coastal North Carolina.

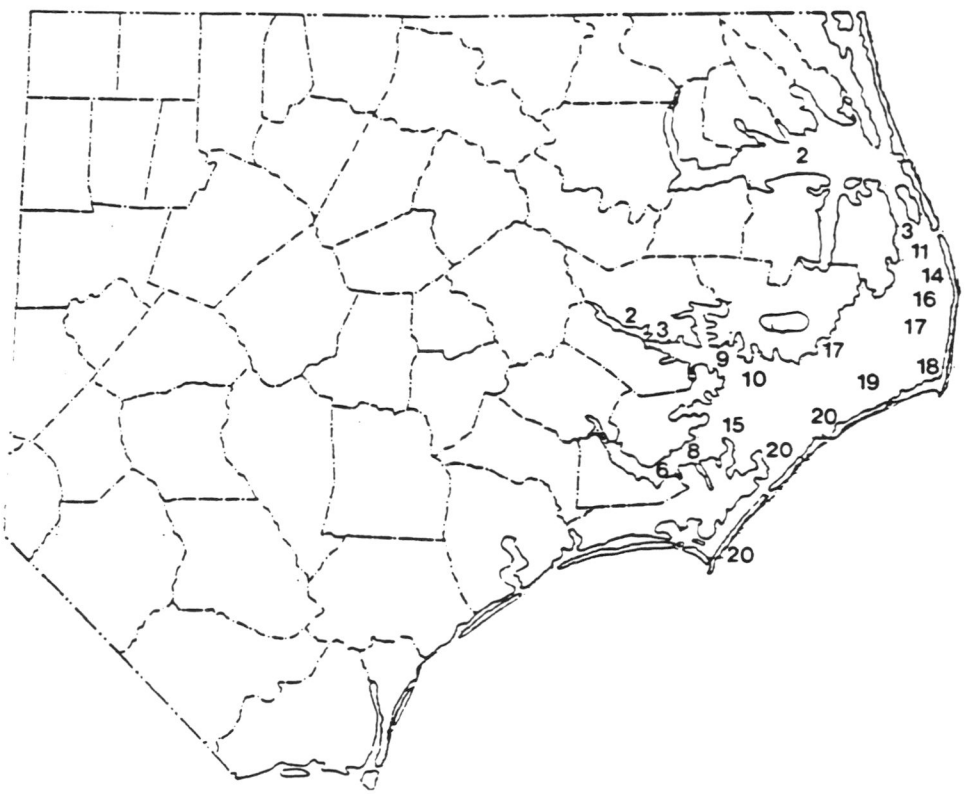

Figure 8. Average salinity of the coastal Sounds of North Carolina. Units are grams per kilogram. (Adapted from Giese et al., 1979).

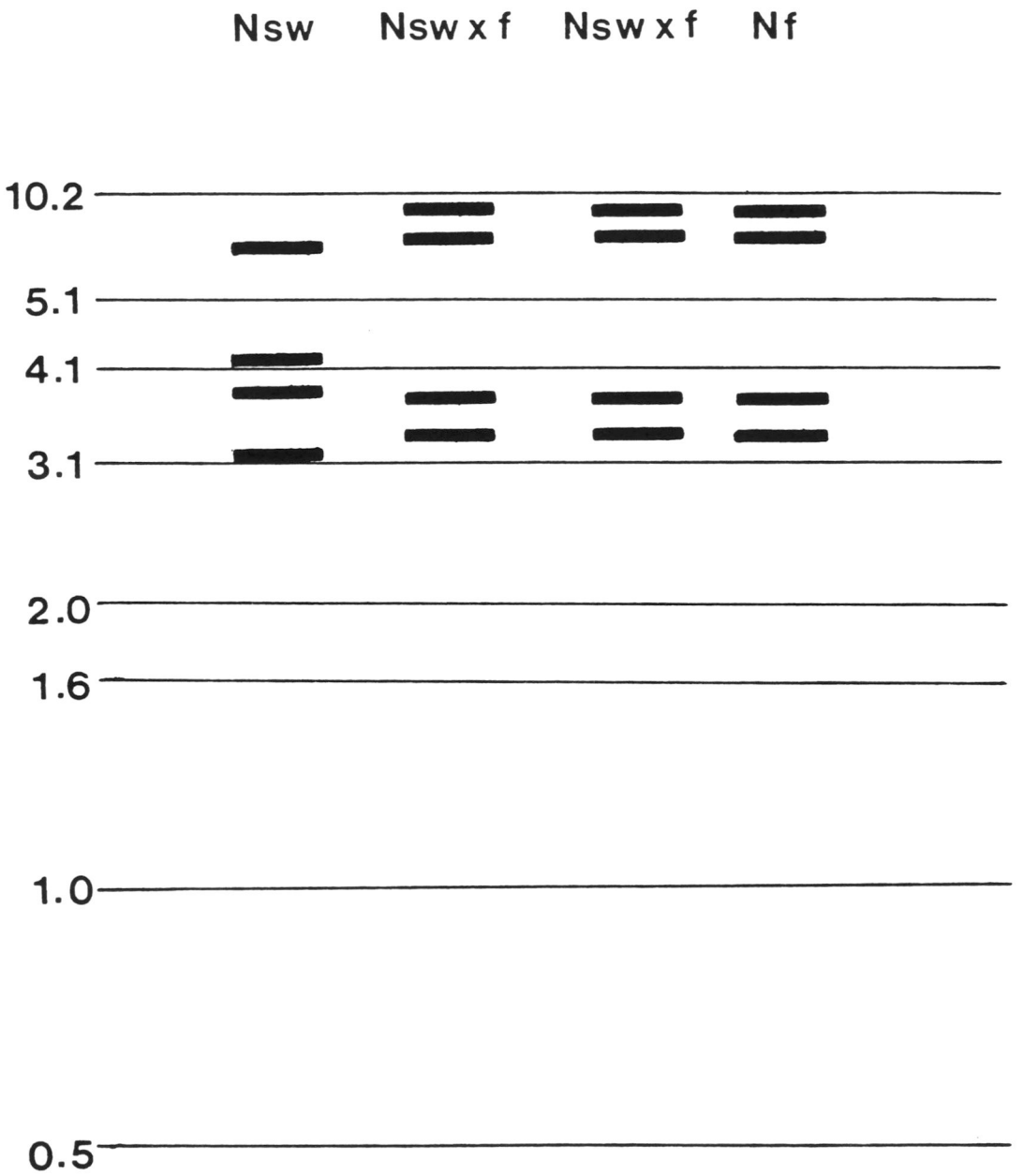

Figure 9. Representative mtDNA digestion profiles for *N. s. williamengelsi*, *N. fasciata*, and two *N. s. williamengelsi* x *fasciata* hybrids using the restriction enzyme *Bcl*I.

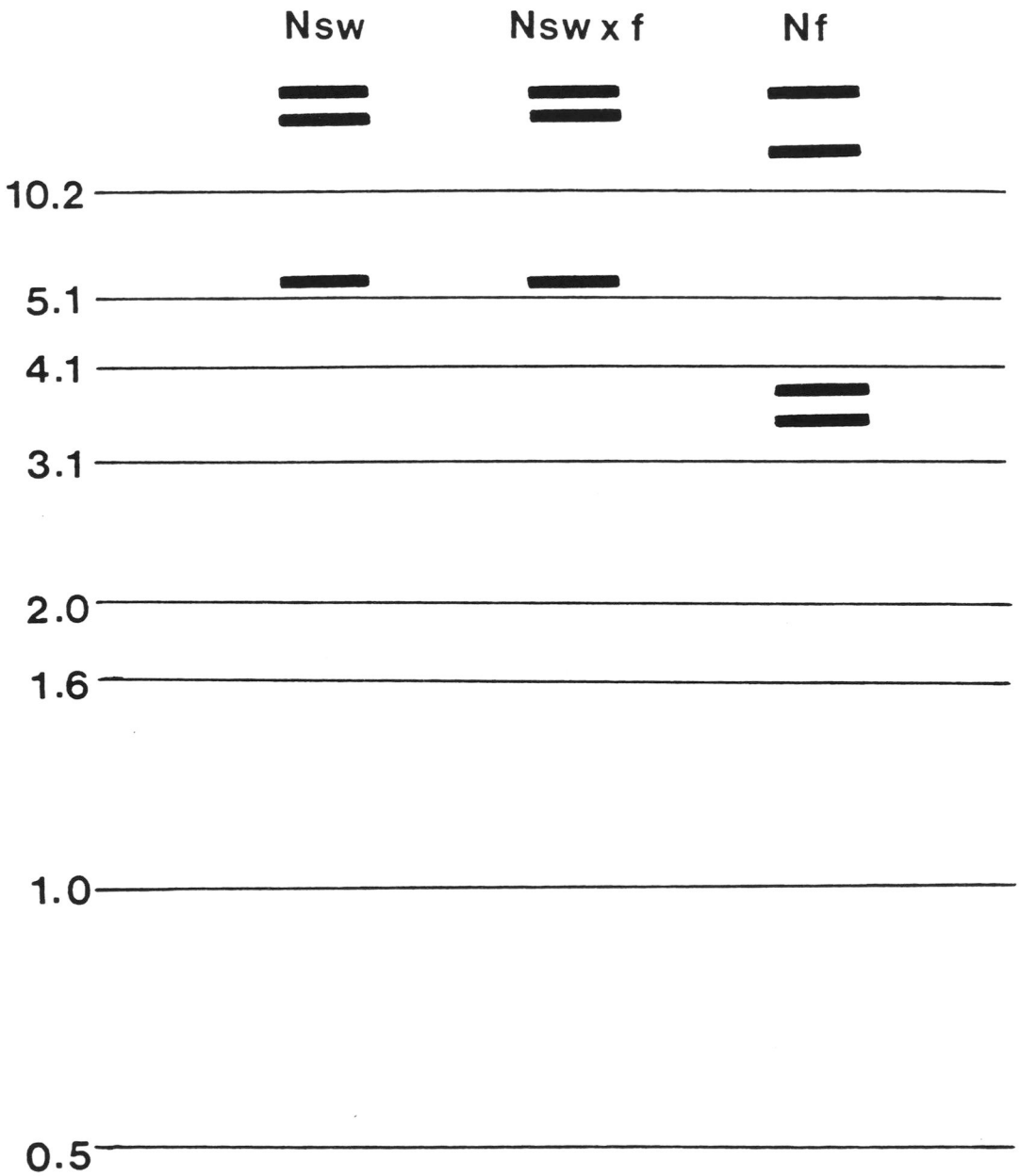

Figure 10. Representative mtDNA digestion profiles for *N. s. williamengelsi*, *N. fasciata*, and a *N. s williamengelsi* x *fasciata* hybrid using the restriction enzyme *Ava*I.

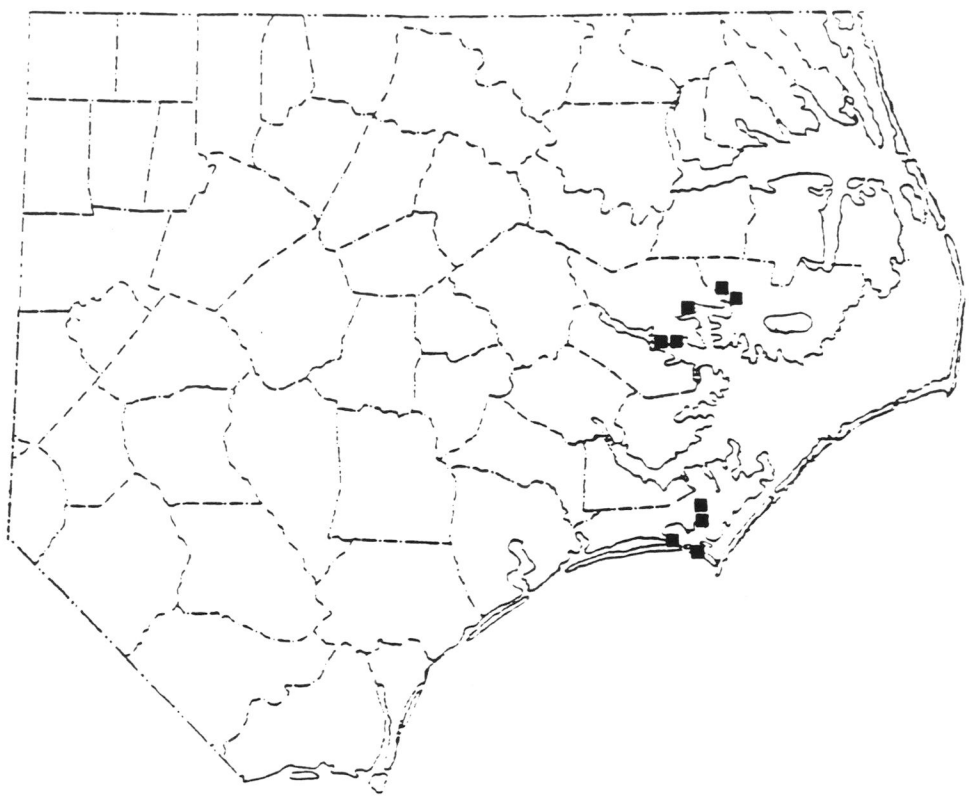

Figure 11. Map showing localities for *Nerodia sipedon williamengelsi* x *fasciata* hybrids in coastal North Carolina.

Table 1. Trapping results for *Nerodia sipedon williamengelsi* in different habitats.

Habitat	No. efforts	No. traps mean (range)	No. snakes	%
Canals	14	16.2 (7-30)	3	5.4
Creeks	9	11.2 (5-28)	4	7.1
Ditches	10	9.6 (5-16)	3	5.4
Juncus	18	9.9 (5-20)	46	82.1
Totals	51		56	100

Table 2. MtDNA haplotypes observed in *Nerodia sipedon*, *Nerodia fasciata*, and *Nerodia sipedon* x *fasciata* hybrids. Letters refer to digestion profiles produced by restriction endonucleases.

Haplotype	Code
a	CCCCCCCCCCCCC
b	CCCCCCCCCCBCCC
c	CCCCCCCCCBCCCC
d	CCCCCCCCCCCCCD
e	CCCCCCCCCCCBCC
f	CCCCCCCCCDCCCC
g	CDCBCCCCCCCCCC
h	CBCBCCCCCCBCCC
i	CDCBBBCCCDCCCC
j	CDCBBBCCCDCCCC

Letter codes depict profiles produced by the following fourteen restriction enzymes (in order from left to right) : *Acc*I, *Ava*I, *Ava*II, *Bgl*II, *Bcl*I, *Bst*eI, *Eco*RI, *Eco*R5, *Eco*O109, *Hha*I, *Hin*cII, *Hin*dIII, *Sac*I, *Spe*I.

Table 3. Estimates of base substitutions per nucleotide (*p*) among mtDNA haplotypes of *Nerodia sipedon sipedon*, *N. s. williamengelsi*, *N. fasciata*, and *N. s. williamengelsi* x *fasciata* hybrids.

	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*
a	0.0000									
b	0.0075	0.0000								
c	0.0016	0.0193	0.0000							
d	0.0073	0.0150	0.0191	0.0000						
e	0.0021	0.0095	0.0138	0.0094	0.0000					
f	0.0023	0.0098	0.0139	0.0047	0.0044	0.0000				
g	0.0096	0.0172	0.0219	0.0171	0.0118	0.0120	0.0000			
h	0.0222	0.0148	0.0349	0.0299	0.0242	0.0247	0.0177	0.0000		
i	0.0191	0.0267	0.0327	0.0266	0.0213	0.0215	0.0090	0.0276	0.0000	
j	0.0162	0.0239	0.0298	0.0188	0.0184	0.0140	0.0166	0.0294	0.0075	0.0000

Table 4. Variation in morphological characters in *Nerodia sipedon sipedon* and *Nerodia sipedon williamengelsi*. (N = number of specimens).

Character	*N. s. sipedon*			*N. s. williamengelsi*		
	N	Range	Mean	N	Range	Mean
Ventrals						
males	20	129-140	134.2	40	129-147	136.6
females	15	129-138	133.6	44	131-144	136.9
Scale rows						
males	20	20.3-24.3	21.1	40	19.6-21.6	20.7
females	14	21.0-22.3	21.3	43	19.0-23.0	21.0
Subcaudals						
males	18	61-80	73.7	34	58-77	72.5
females	13	50-71	64.3	36	52-70	63.0
Lateral bars						
males	19	25.0-39.0	32.1	39	29.5-45.5	37.0
females	14	25.5-36.5	28.6	43	32.5-45.0	36.9

Table 5. Pairwise mean differences in ventral scale counts in *Nerodia s. sipedon* (Nss) and *Nerodia s. williamengelsi* (Nsw). Significant differences are marked with an asterisk (*). M = male, F = female. Differences are given as absolute values.

	Nsw (M)	Nsw (F)
Nss (M)	2.425*	2.693*
Nss (F)	3.008*	3.277*

Table 6. Pairwise mean differences in counts of scale rows in *Nerodia s. sipedon* (Nss) and *Nerodia s. williamengelsi* (Nsw). Significant differences are marked with an asterisk (*). M = male, F = female. Differences are given as absolute values.

	Nsw (M)	Nsw (F)
Nss (M)	0.455	0.136
Nss (F)	0.657	0.339

Table 7. Pairwise mean differences in counts of subcaudal scales in *Nerodia s. sipedon* (Nss) and *Nerodia s. williamengelsi* (Nsw). Significant differences are marked with an asterisk (*). M = male, F = female. Differences are given as absolute values.

	Nsw (M)	Nsw (F)
Nss (M)	9.559*	10.722*
Nss (F)	8.251*	9.415*

Table 8. Pairwise mean differences in lateral bar counts in *Nerodia s. sipedon* (Nss) and *Nerodia s. williamengelsi* (Nsw). Significant differences are marked with an asterisk (*). M = male, F = female. Differences are given as absolute values.

	Nsw (M)	Nsw (F)
Nss (M)	4.945*	4.775*
Nss (F)	8.434*	8.264*

DISCUSSION

Mitochondrial DNA Variation

Traditionally, studies of variation among natricine snakes have involved comparison of morphological characters or, at the molecular level, analysis of allozyme frequency data (e.g., Lawson, 1987). My study attempted to combine a morphological approach with a molecular one, in an effort to gain a better understanding of the status of the Carolina salt marsh snake as it relates to the subspecies concept, as well as to gain some knowledge of the dynamics of its relationship to a closely related but genetically distinct species, the banded water snake.

The mitochondrial genome of *Nerodia sipedon* and *N. fasciata* was large, approaching 20+ kb in length (see Appendix C). This compares closely to the large genomes found by Densmore et al. (1992) in a study of six taxa of *Nerodia* in Texas.

My study failed to detect a distinction between *N. s. sipedon* and *N. s. williamengelsi* on the basis of restriction fragment variation of mtDNA. Two of the six haplotypes identified in *Nerodia sipedon* (*a* and *e*) were shared by both subspecies. Haplotype *a*, the most common haplotype, was observed in a specimen of *N. s. sipedon* from Nash County, a specimen of *N. s. williamengelsi* from Beaufort County, and in another *williamengelsi* from Hyde County. In addition, haplotype *a* was identified in one of the three *N. s. williamengelsi* x *fasciata* hybrids. Another haplotype (*e*) occurred in two widespread localities, eastern Hyde County and in a specimen from the NC foothills. The locality in eastern Hyde County, at Wyesocking Bay adjacent to Pamlico Sound

supported two different Haplotypes (*e* and *f*). Sequence divergence within *N. sipedon* from this survey ranged from 0.2% to 1.58% (Fig. 5).

The few studies of mtDNA variation in snakes have concentrated on taxa at the species level (e.g.: Knight et al., 1992) or on subspecies that occur allopatrically (e.g.: Densmore et al., 1992). Knight et al. (1992) compared mtDNA variation in New World pitvipers of the *Agkistrodon* complex, including all currently recognized subspecies of the copperhead (*Agkistrodon contortrix*) and the cottonmouth (*Agkistrodon piscivorus*). Knight et al. (1992) observed little mtDNA variation among the five subspecies of copperheads which exhibited an overall sequence divergence of 1.26%. However Knight et al. (1992) detected a much higher sequence divergence (3.70%) between the cottonmouth (*A. p. piscivorus* and *A. p. leucostoma*). The high level of divergence between *piscivorus* and *leucostoma*, which may reflect a prior isolation event, exceeds that observed between some taxa recognized as full species (Knight et al., 1992). However, Knight et al. (1992) cautioned against premature recognition of *leucostoma* as a full species pending further genetic studies of specimens from areas of intergradation between *piscivorus* and *leucostoma*. Indeed, the zone of intergradation between these two subspecies may be much wider than has been previously recognized (Gloyd and Conant, 1990).

Densmore et al. (1992) studied the genetic relationship between two allopatric subspecies of *Nerodia harteri* in Texas. Both subspecies, *N. harteri harteri* and *N. harteri paucimaculata*, occur in similar habitats and are separated by a short distance of

approximately 90 km. Densmore et al. (1992) observed an mtDNA sequence divergence of approximately 2.5% between the two subspecies and, on the basis of meristic as well as genetic data, concluded that no genetic exchange had occurred between them for some time. Thus, they concluded that *N. harteri paucimaculata* should be elevated to species status.

My mtDNA data showed high levels of restriction pattern polymorphism in populations of *N. sipedon* in coastal North Carolina. Within the relatively small geographic area defining the range of *williamengelsi*, six haplotypes (two shared with *N. s. sipedon* and four seen only in *williamengelsi*) were identified. Whether *N. s. williamengelsi* exhibits higher levels of polymorphism than *N. s. sipedon* cannot be determined from my data due to the small sample size of *N. s. sipedon*. *Nerodia s. williamengelsi* exhibited within-population variation, unlike the copperhead which in Knight et al's. (1992) study showed no detectable variation within populations. The high levels of polymorphism in coastal *N. sipedon* may reflect the stochastic sorting of mtDNA lineages. Such sorting has been seen in computer simulations (Neigel and Avise, 1985). Factors that may contribute to the sorting of mtDNA lineages include population size and differential survivorship (Hartl, 1980), vagility and dispersal of females (Moritz et al., 1987), and changes in habitats (Hedrick et al., 1976).

Morphological Variation

Previous studies of *N. s. williamengelsi* have concluded that little to no variation in scalation exists between *williamengelsi* and *N. s. sipedon* (Conant and Lazell, 1973;

Palmer and Braswell, 1995). In contrast to these earlier studies, my data revealed significant statistical differences in two scale counts: ventrals and subcaudals. In addition, I found statistically significant differences in lateral bar counts. Palmer and Braswell (1995) documented similar trends in ventral scale counts, subcaudal scale counts, and lateral bar counts but did not examine these characters statistically. The low number of characters exhibiting statistically significant variation is not unusual for the *Nerodia sipedon-fasciata* complex. In a study of the complex in Georgia, Seyle (1980) found that only three of nine scale counts displayed significant variation. The degree of intraspecific morphological variation may be greater for other species. Reichling (1995) examined 14 morphological characters in a study of the Louisiana pine snake, *Pituophis melanoleucus ruthveni*, and recommended that it be elevated to species status on the basis of morphological variation. Brown and Ernst (1986) surveyed nineteen morphological characters in the timber rattlesnake, *Crotalus horridus*, of which six showed evidence of statistically significant variation. Corn and Bury (1986) studied morphological variation in racers, *Coluber constrictor*, in the central Rocky Mountains and found statistically significant differences in several characters among two subspecies but also noted that the variation was clinal.

Coastal populations of *Nerodia sipedon* exhibit clinal variation in ventral scale counts and lateral bar counts. This gradation corresponds closely to salinity gradients in the sounds of North Carolina. The taxonomic significance of clines has been a subject of much debate among biologists. In some instances, researchers have called for taxonomic

recognition of clines in lieu of formal recognition of subspecies (Simpson, 1961). Others have called for recognition of clines only if they are correlated with species formation (Mayr, 1982). In coastal North Carolina clines have been identified in pigmy rattlesnakes (Palmer, 1971), which display a marked trend towards red coloration at the northeastern part of their range along Pamlico Sound and a gradual trend toward a grayish coloration in populations in the southeastern corner of the state. The red morph of the pigmy rattlesnake was not afforded taxonomic recognition due to its limited range and a wide zone of intergradation (Palmer, 1971). *Nerodia s. williamengelsi* exhibits similar clines and warrants further investigation with regard to its ecology and physiology in order to better gauge its true subspecific status.

Hybridization

Hybridization between *N. sipedon* and *N. fasciata* has been recorded from throughout the zone of contact between the two species. Hybridization on the Gulf Coastal Plain was noted by Cliburn (1957), Schwaner and Mount (1976), and Blaney and Blaney (1979). Neill (1946) and Seyle (1980) reported on the occurrence of intermediates in Georgia. In North and South Carolina specimens showing intermediate characteristics were noted by Viosca (1924) and Conant (1963).

Conant (1963) found hybridization occurring only where habitats had been altered by man, allowing the two species to come into contact with each other, or in areas where sudden natural phenomena (e. g., severe storms) transported individuals of one species into habitats occupied by the other. In contrast, other studies (e. g., Cliburn, 1957;

Schwaner and Mount, 1976; Blaney and Blaney, 1979) have reported hybrid specimens from unaltered habitats.

One of the goals of this study was to document any incident of hybridization and, based on genetic and morphological data, gain an understanding of the dynamics of hybridization events between the two species.

During the course of this study three live hybrid snakes were collected. Two other specimens showing evidence of hybridization were collected dead on roads. These data, along with the localities provided by Conant and Lazell (1973) and Palmer and Braswell (1995), indicate that hybridization between *N. s. williamengelsi* and *N. fasciata* has occurred in at least nine localities in three counties (Fig. 11).

Of the five localities from which hybrid snakes were collected during this study, only two can be considered unaltered habitats. One hybrid was trapped in Saint Clair Creek, Beaufort County. Both *N. s. williamengelsi* and *N. fasciata* were collected at this site as well. Saint Clair Creek lies in close proximity to a hybrid locality noted by Palmer and Braswell (1995) along the shore of Pamlico River. The second hybrid specimen from an unaltered locality was collected dead on U. S. highway 264 in Hyde County at the Beaufort County line. The habitat at this locality is *Juncus* marsh along Pungo River. Conant and Lazell (1973) noted a hybrid from near the town of Ponzer, approximately 3 km to the north. However, they did not note the condition of the habitat from which the specimen was collected.

Two specimens trapped in central Carteret County and a third collected dead on a

road in Beaufort County represent hybrids from altered habitats. The Carteret County specimens were trapped in a man-made ditch along state road 1300 approximately 9.3 km northwest of the town of Otway. The ditch drains a large area of freshwater south into an area of *Juncus* marsh adjacent to North River. *Nerodia fasciata* is common in freshwater habitats immediately to the north (Palmer and Braswell, 1995) and *N. s. williamengelsi* was collected in *Juncus* habitat 0.3 km to the southeast during the course of this study.

The third hybrid specimen collected from an altered habitat was collected dead on NC highway 99 southwest of Belhaven, Beaufort County. The habitat along the road at this locality is a large man-made canal which, like the Carteret County locality, drains an area of freshwater into brackish marsh.

Hybridization between *N. s. williamengelsi* and *N. fasciata* may be bi-directional, with males and females of each species being involved. Each of the two hybrids collected in Carteret County exhibited mtDNA restriction patterns identical to *N. fasciata* for a number of enzymes (Fig. 9). Restriction patterns for the third specimen are identical to *N. sipedon.* These data, coupled with the maternal inheritance of mtDNA, suggest the strong possibility of bi-directionality for hybridization events between *N. s. williamengelsi* and *N. fasciata*. However, since my sample size for hybrid snakes is small and it is not known if the specimens examined represent F_1 or later generations hybrids such a conclusion must considered tentative. Conant and Lazell (1973) concluded that the *Nerodia* population at Mullet Pond on the Shackleford Banks constituted a "hybrid swarm," resulting from directional introgression of *N. fasciata* genes into *N. s. williamengelsi.*

Fifteen of 22 specimens from Mullet Pond examined by Conant and Lazell showed intermediate characteristics between the two species.

Summary: *Nerodia sipedon williamengelsi* and the Subspecies Concept

The subspecies concept, despite questions regarding its validity, has had profound effects in conservation biology, and numerous examples can be cited of state and federal programs or legislation geared towards subspecies of otherwise secure species (Reichling, 1995). The Carolina salt marsh snake is such an example, having been listed by the State of North Carolina as a taxon of "Special Concern" in 1987 (Palmer and Braswell, 1995).

The acceptance of newer, evolution-based species concepts has changed the manner in which the subspecies concept is applied. In particular, the Evolutionary Species Concept (ESC) of Wiley (1978) and, to a lesser extent, the Phylogenetic Species Concept (PSC) discussed by Rosen (1978), have led to a decrease in the formal naming of subspecies. Increasingly, the trend has been to recognize diagnosable, allopatric subspecies as full species. Following this pattern, Collins (1991) proposed full species status for 55 allopatric subspecies of amphibians and reptiles. Collins' proposals did not constitute an abandonment of the subspecies concept, despite opinions to the contrary (e. g., Van Devender et al., 1992; Montanucci, 1992). Rather, the proposals limited subspecies to populations that 1) are distinguishable from all other populations of the same species, and 2) maintain a zone of contact, hence genetic exchange, with other populations.

In summation no distinct genetic differences exist between *N. s. williamengelsi*

and *N. s. sipedon.* However *williamengelsi* does possess several clearly defined morphological features that distinguish it from the nominate subspecies. Further investigation, particularly of the role that salinity plays in the ecology and physiology of *williamengelsi*, may clarify its relationship with *N. s. sipedon.* Pending further study, it is recommended that the subspecific status of *N. s. williamengelsi* remain the same.

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APPENDIX A. List of specific collection localities for *Nerodia sipedon williamengelsi, N. s. sipedon,* and *N. fasciata.* Specimens borrowed from the North Carolina State Museum of Natural Sciences (NCSM) are denoted by museum collection numbers.

Nerodia sipedon williamengelsi (All from North Carolina)

County	N	NCSM #	Locality
Beaufort	1		1.6 km SW Belhaven
	1		3.7 km WSW Belhaven
	1		5.1 km NE Belhaven
	1		3.8 km NE Belhaven
	1		2.5 km NE Belhaven
	1		8.0 km SE Bath
	4	12410, 12841, 13860, 12412	3.2 km E Bayview
	1		1.44 km WNW Pamlico Beach
	3		0.4 km SE Pamlico Beach
	1		3.7 km S Ransomville
	2		3.2 km SW Ransomville
	2		5.7 km NNE Ransomville
	1		3.4 km S Ransomville
	2	12020, 12413	4.0 km NW South Creek
	1	12411	6.4 km NW South Creek
	1	13545	3.2 km NW South Creek
	1	15466	5.2 km NW South Creek
	1	17964	3.2 km ENE South Creek
	1		1.6 km ESE Winsteadville
	1		7.2 km SSE Winsteadville
	1		2.4 km SSE Winsteadville
Carteret	13	12382	Cedar Island NWR
	1		7.0 km W Atlantic
	1		8.6 km NW Otway
	1	8003	17.6 km S Merrimon
	3	11796, 12406, 12405	Core Banks
Currituck	1		4.3 km NE Coinjock
Dare	1	17926	Roanoke Island
	1		3.5 km WSW Stumpy Point
	1		3.2 km WSW Stumpy Point
	1		1.3 km SE Stumpy Point

APPENDIX A (Con'd.). List of specific collection localities for *Nerodia sipedon williamengelsi, N. s. sipedon,* and *Nerodia fasciata.*

County	N	NCSM #	Locality
Dare	1	2199	Near Hyde Co. line, US 264
	1		8.0 km SW Stumpy Point
	1		7.2 km NNW Rodanthe
	1	20814	16.8 km NNW Rodanthe
	1	13957	Pea Island NWR
	3	23040, 23007, 23041	1.2 km E Buxton
	1	3792	Hatteras Island (Waves)
	1	11800	Cape Hatteras
Hyde	1	11797	Ocracoke Island
	1		2.7 km NE Engelhard
	1		2.4 km NE Engelhard
	1		0.96 km NE Engelhard
	5		3.1 km NE Gull Rock
	1		3.6 km NE Gull Rock
	1		Near Beaufort Co. line, US 264
	1		1.1 km W Rose Bay
	1		0.4 km SE Rose Bay
	9		2.4 km W Rose Bay
	1		3.8 km SSE Scranton
	1		4.4 km E Sladesville
	1		1.1 km SSW Sladesville
	1	11475	Near Sladesville
	1		1.8 km SSE Sladesville
	1		2.4 km ESE Sladesville
	1		0.32 km SSE Swanquarter
	1		7.0 km E Swanquarter
	1		7.5 km E Swanquarter
	1		2.4 km ESE Swanquarter
Pamlico	7		1.6 km E Hobucken
	2	12793, 12794	6.0 km E Lowland
	1	23590	4.8 km NNW Hobucken

APPENDIX A (con'd.). List of specific collection localities for *Nerodia sipedon williamengelsi, N. s. sipedon,* and *N. fasciata.*

Nerodia s. sipedon (North Carolina, Virginia)

County	N	NCSM #	Locality
Currituck	1	19400	Coinjock
	1	7262	Church Island
	1	12210	1.6 km S Coinjock
Guilford	1		Lake Higgins
Lincoln	1		1.6 km W Lowesville
Mecklenburg	1		6.4 km ESE Pineville
	3		Hickory Grove
	1		1.6 km N Weddington
	1		Mint Hill
Nash	6		9.6 km SW Rocky Mount
	2		Rocky Mount (Battle Park)
	1		1.9 km NNW Stanhope
Randolph	2		Ramseur
	1		12.4 km SSE Farmer
Rutherford	1		Chimney Rock
Stanly	1		8.0 km SSW Stanfield
Wake	1		Raleigh
Wilson	1		Rock Ridge
Hanover Co., VA	9		Mechanicsville

APPENDIX A (cont'd.). List of specific collection localities for *Nerodia sipedon williamengelsi, N. s. sipedon,* and *N. fasciata.*

Nerodia fasciata (All from North Carolina)

County	N	Locality
Beaufort	1	6.4 km SW Belhaven
	1	3.2 km WSW Belhaven
	1	5.6 km SW Belhaven
	3	3.2 km SW Ransomville
	1	Ransomville
	1	3.4 km WSW Ransomville
	1	Goose Creek State Park
	1	Leechville
Craven	1	6.7 km SSW Ernul
	1	3.7 km WSW Croatan
Dare	1	Manns Harbor
	2	8.0 km SSW Manns Harbor
	1	4.0 km WSW Stumpy Point
Hyde	2	12.6 km NNW Engelhard
	1	5.4 km SSE Fairfield
	1	3.5 km WNW Lake Comfort
	2	4.8 km ESE Ponzer
	1	1.3 km SSE Ponzer
	1	9.6 km NE Ponzer
	1	0.64 km S Sladesville
	1	6.2 km NE Swanquarter
Pitt	1	5.6 km E Pactolus
	1	Clarks Neck
Tyrrell	2	12.8 km NE Kilkenny
	1	9.6 km NNE Kilkenny
	3	8.0 km WNW Gum Neck
	1	Near Hyde Co. line, NC 94

Appendix B

MtDNA Extraction (CsCl-EtBr Gradient)

Reagents
1x MSB buffer
3mM CaCl
EDTA
STE (100mM NaCl, 50ml Tris pH 8.0, 10 mM EDTA pH 8.0)
25% SDS
Ethidium Bromide (EtBr)

For each sample:
1. Rinse tissue with 1x MSB with 3 mM CaCl and EDTA. Then mince tissue; homogenize tissue in 1x MSB with Ca++ and EDTA. Volume depends on size of sample, usually ~20-25 ml. Keep sample and buffer on ice (4 deg. C).

2. Pour homogenate into 50 ml nalgene conical tubes and centrifuge in Sorval GLC-2 at setting 6.5 (1,700 rpm) for 5 min., 4 deg. C.

3. Pour supernatant (save), avoiding pellet, into second set of conical tubes and recentrifuge in GLC at setting 7.0 (2,000 rpm) for 5 min.

4. Pour supernatant into 50 ml round-bottom polycarbonate tubes and spin at 13,000 rpm in Sorvall (SS34 rotor) for 20 min., 4 deg. C.

5. Discard supernatant; mitochondria are pelleted. Wipe out fat on sides of tube. Re-suspend pellet in 15 ml 1x MSB with EDTA and recentrifuge as above for 20 min.

6. Pour off supernatant (wipe out tube) and add 3ml of STE. Resuspend gently with pasteur pipette.

7. Add 2-4 drops of 25% SDS to lyse the mitochondria and mix by gently swirling (the suspension should clarify in seconds).

8. Add CsCl (solid-1.1g/ml of STE). Dissolve completely with a pipette.

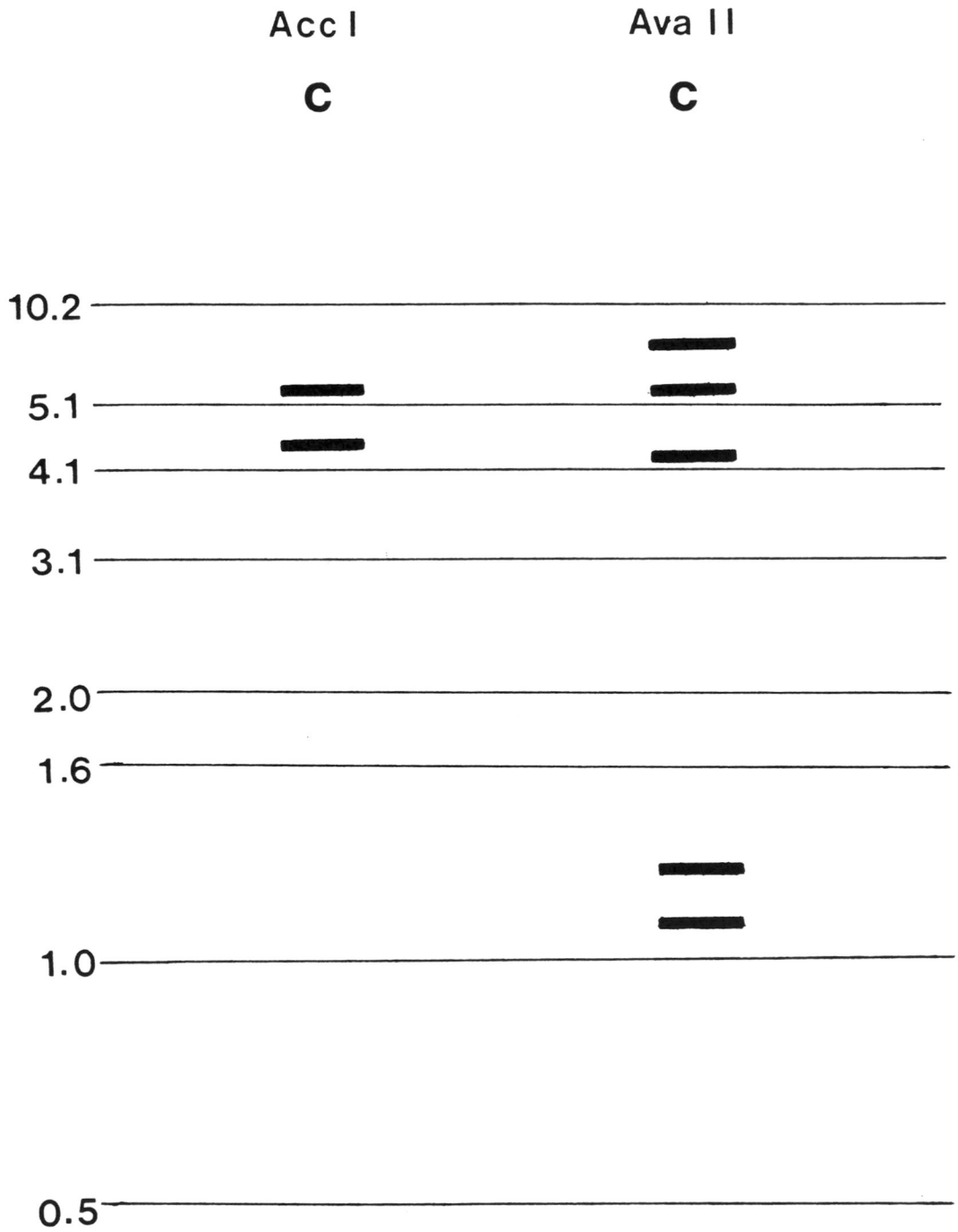
9. Cover and refrigerate samples ~6 hr. or even overnight. Centrifuge in Sorvall for 10 min. at 12,000 rpm. at 4 deg. C.

10. Carefully pipette supernatant from SDS-protein-nuclear DNA film. Transfer clear to amber supernatant to test tubes or storage tubes. MtDNA is stable in CsCl and can be refrigerated for months.

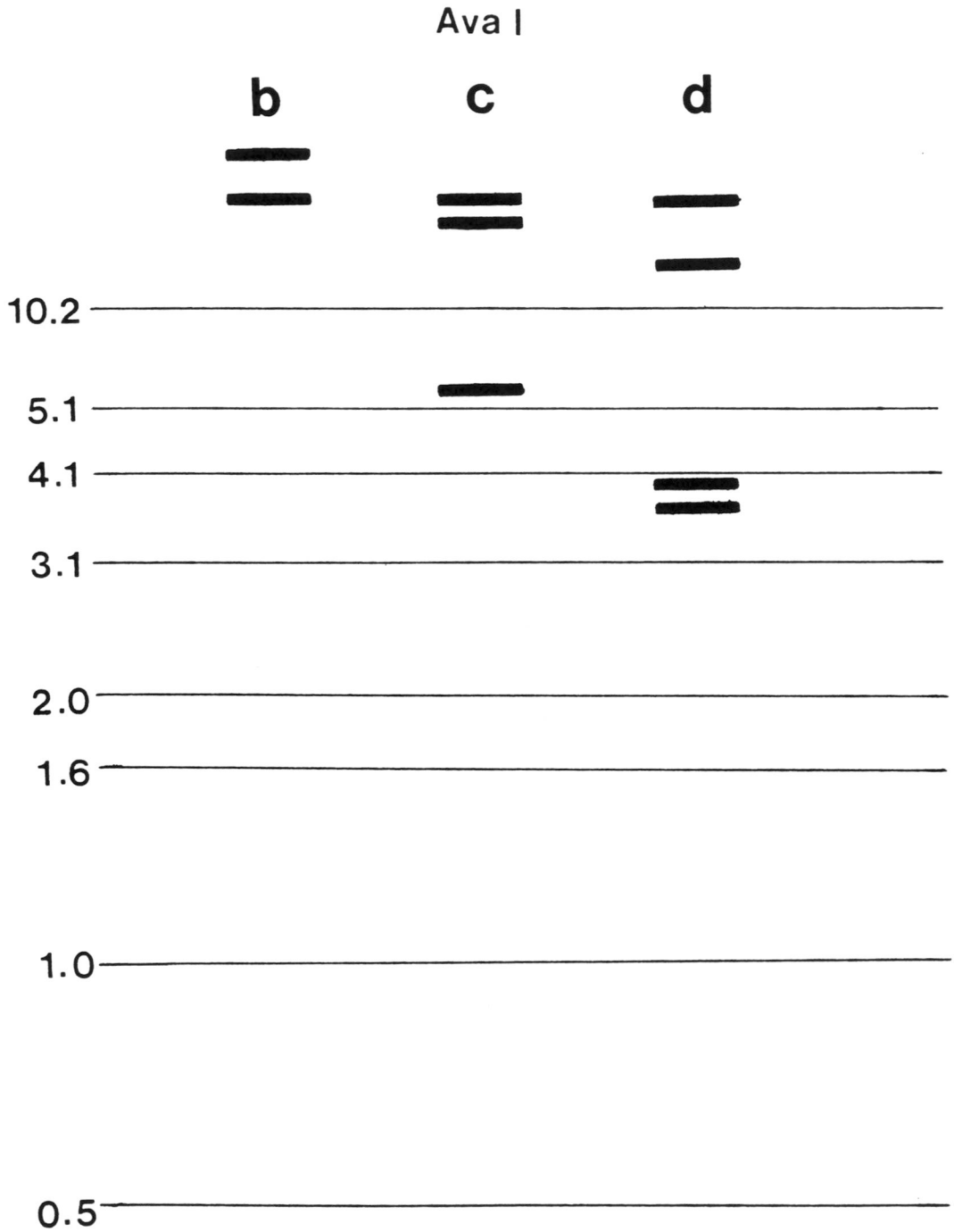
11. Check refractive index; should be between 1.392-1.395. Adjust as necessary with more CsCl to increase or STE to decrease.

12. Add 20 microliters Ethidium Bromide, mix with pipette.

13. Transfer to Sarstedt cellulose ultra-centrifuge tubes. Spin in Beckman SW 501 swinging bucket rotor for 36-40- hrs. at 40,000 rpm. Cut back to 36,000 rpm three hrs. prior to termination of run. Drip samples under UV light source.



APPENDIX C: Mitochondrial DNA restriction profiles from *Nerodia sipedon sipedon, Nerodia sipedon williamengelsi, Nerodia fasciata,* and *N. s. williamengelsi* x *fasciata* hybrids.



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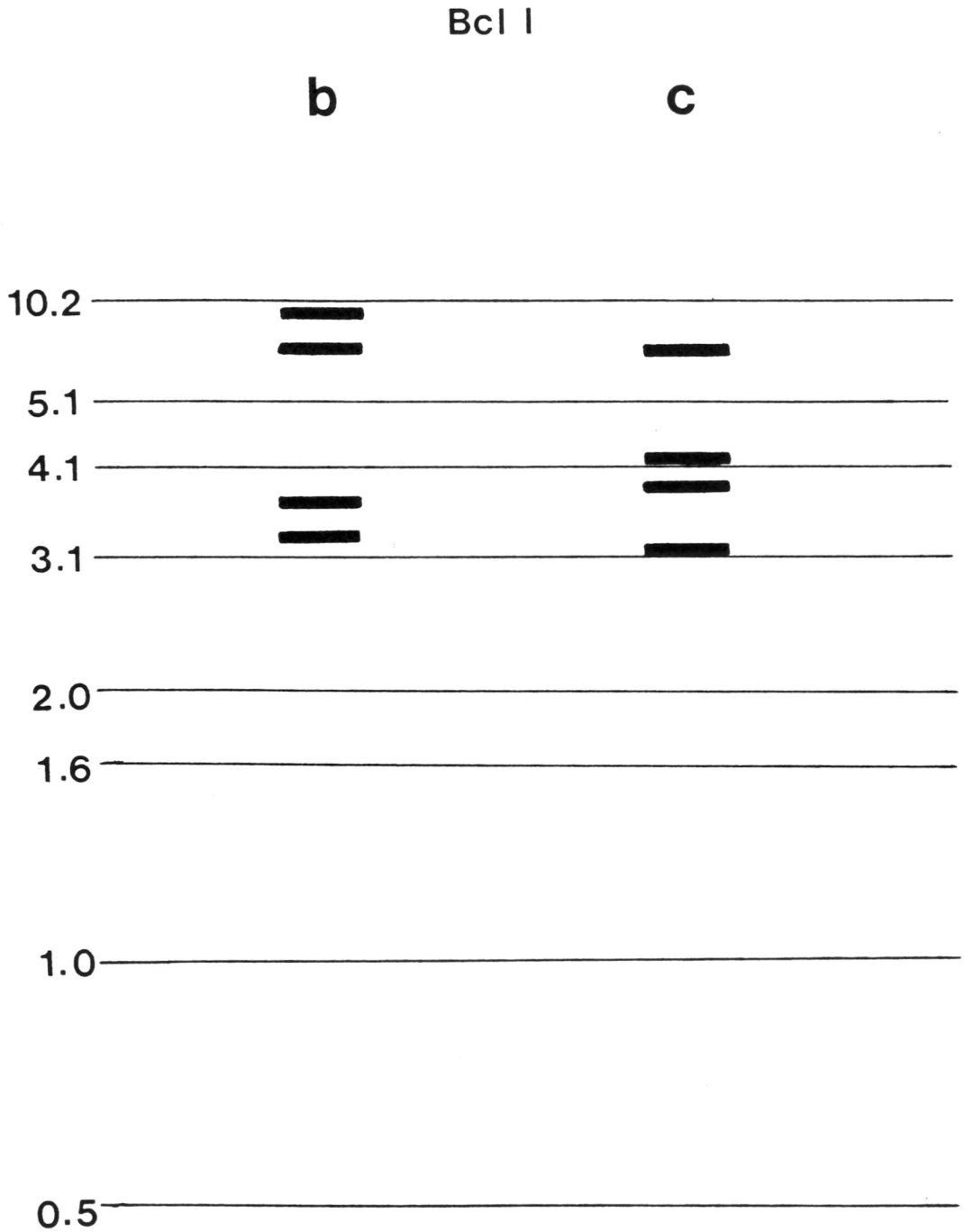

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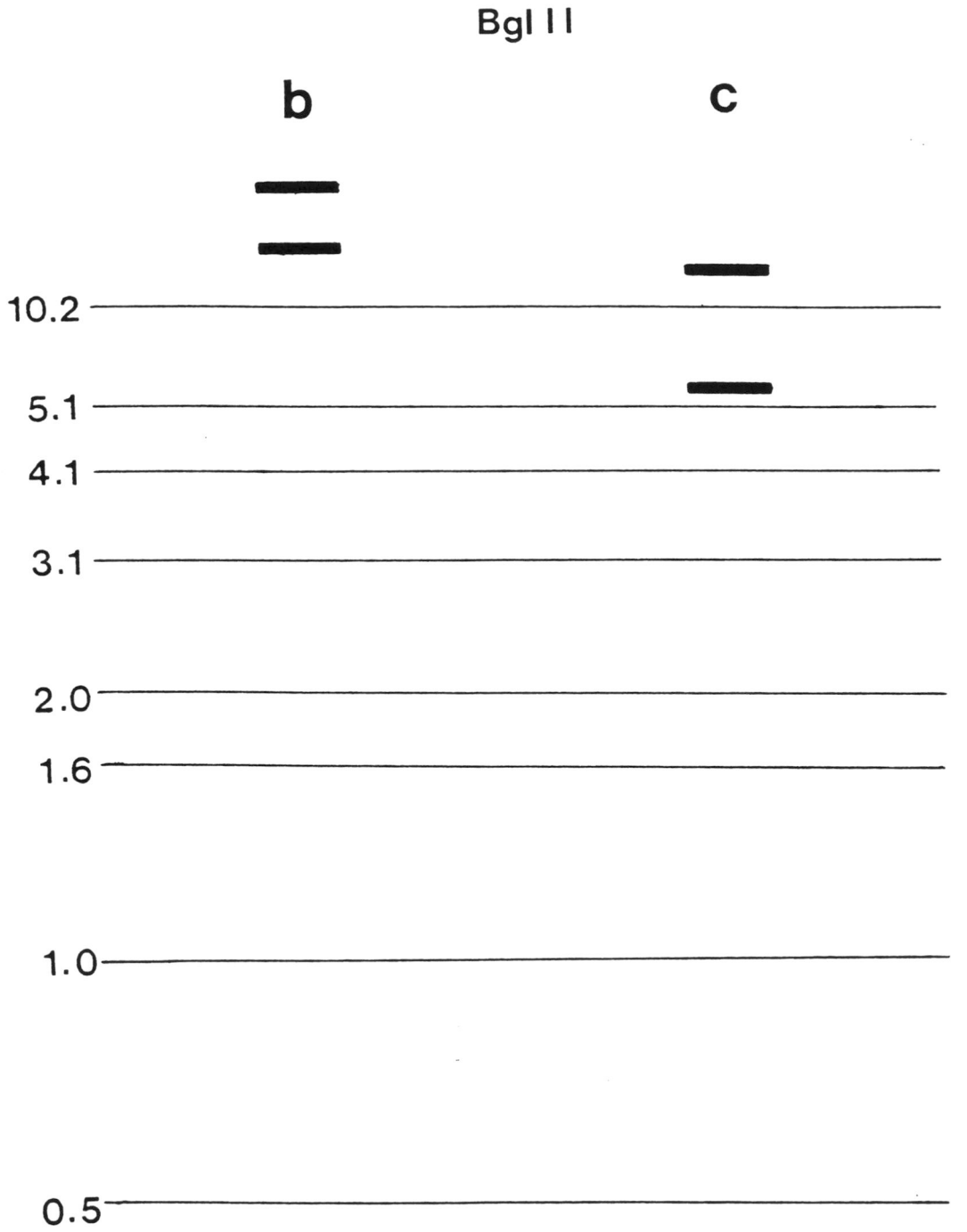

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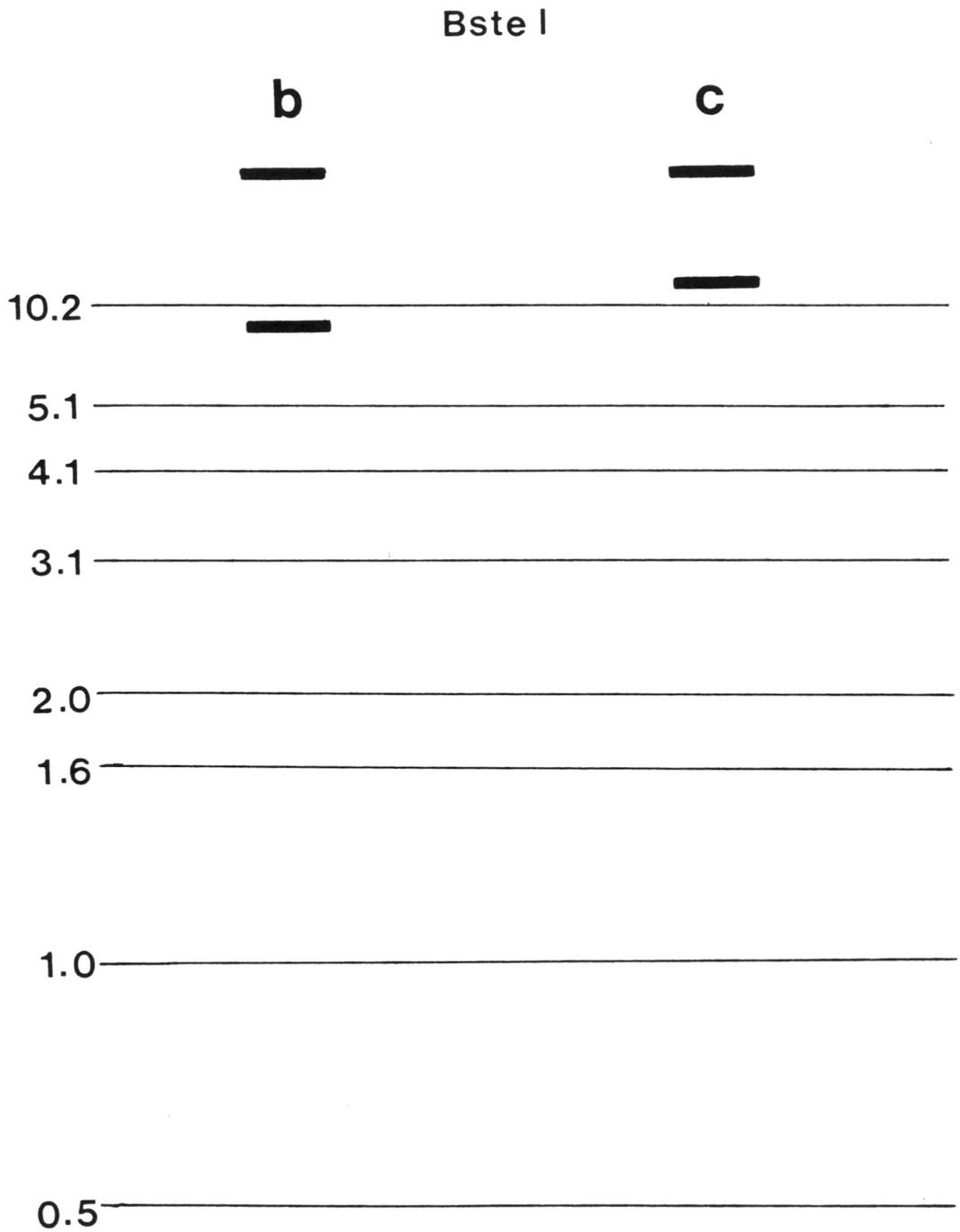

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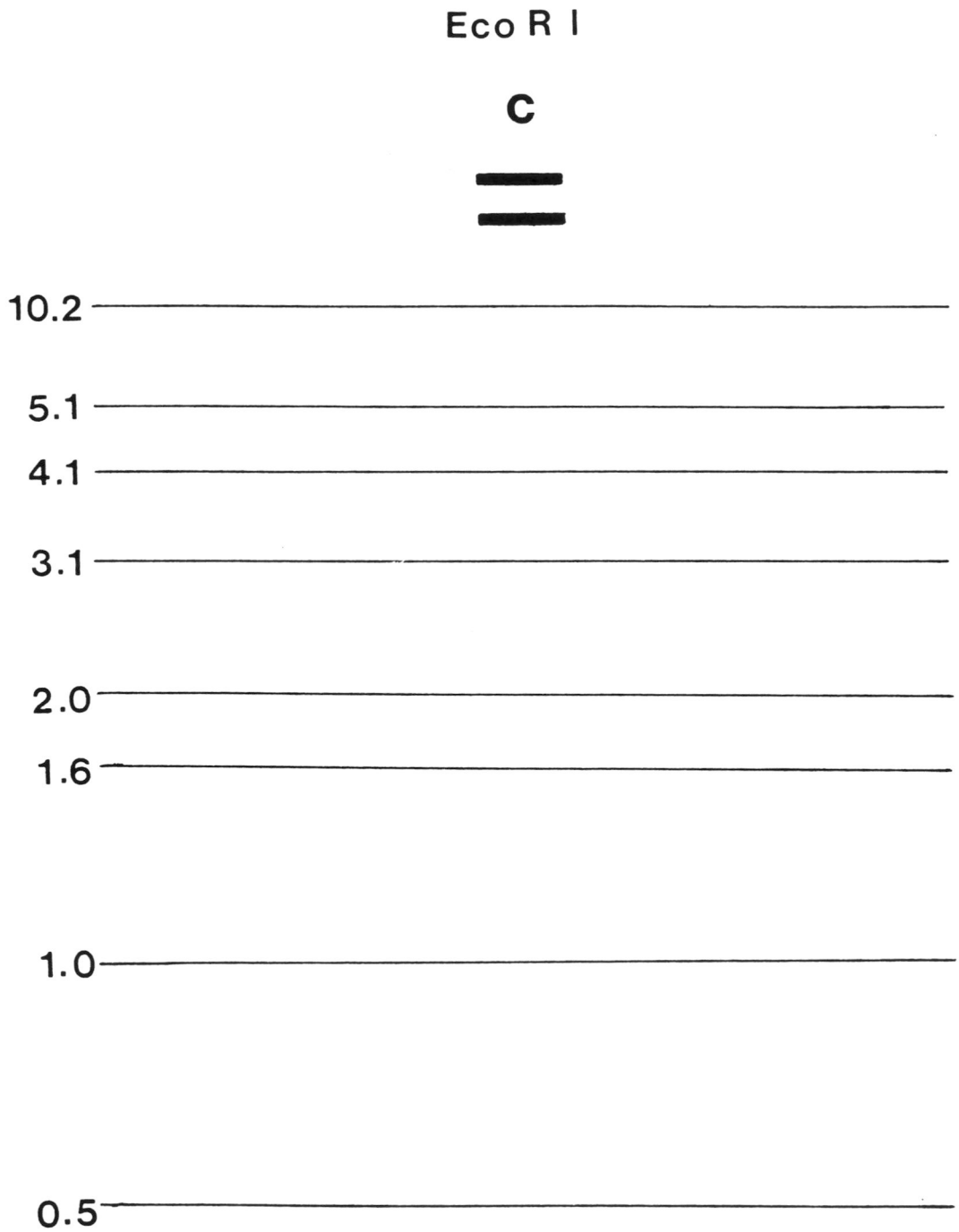

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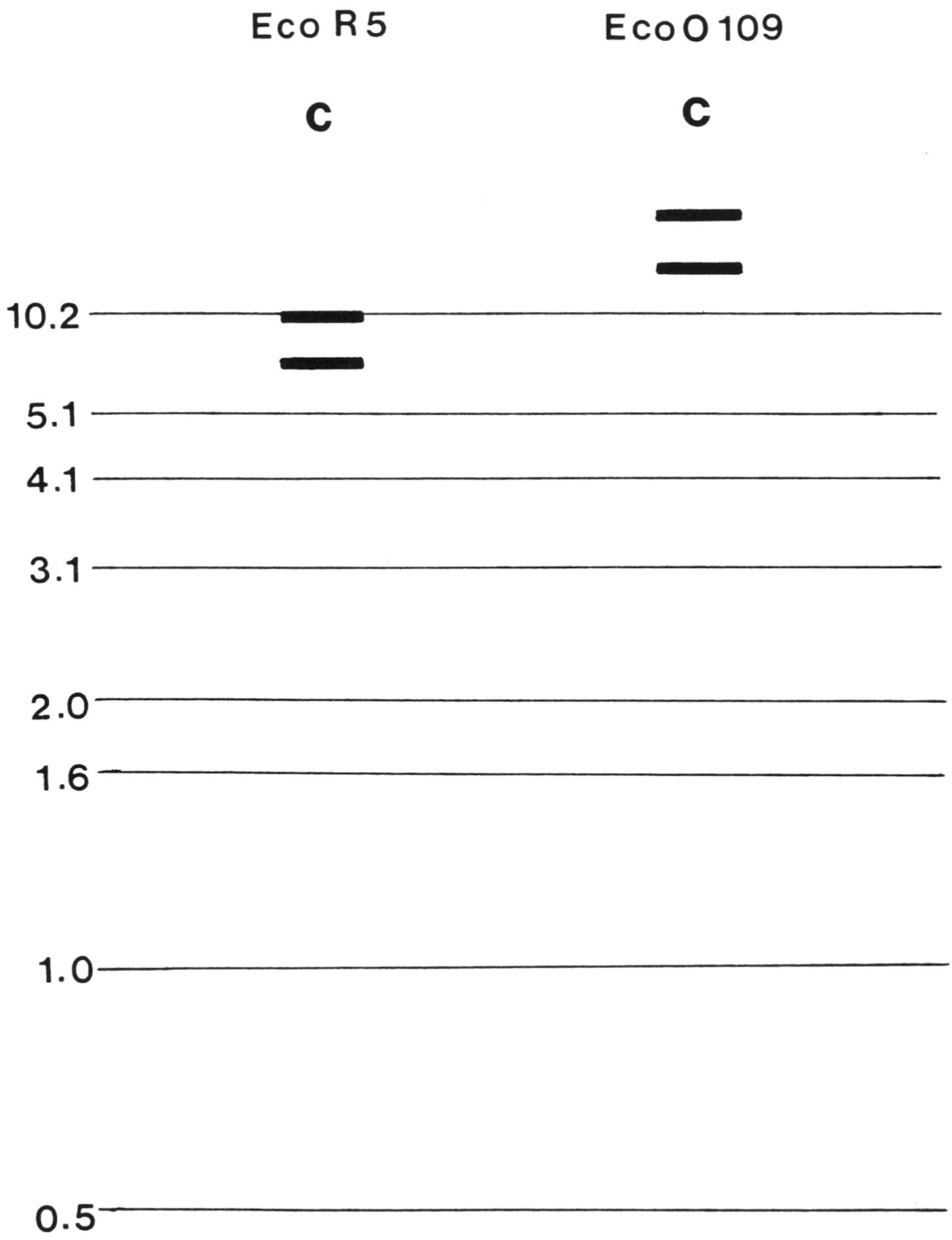

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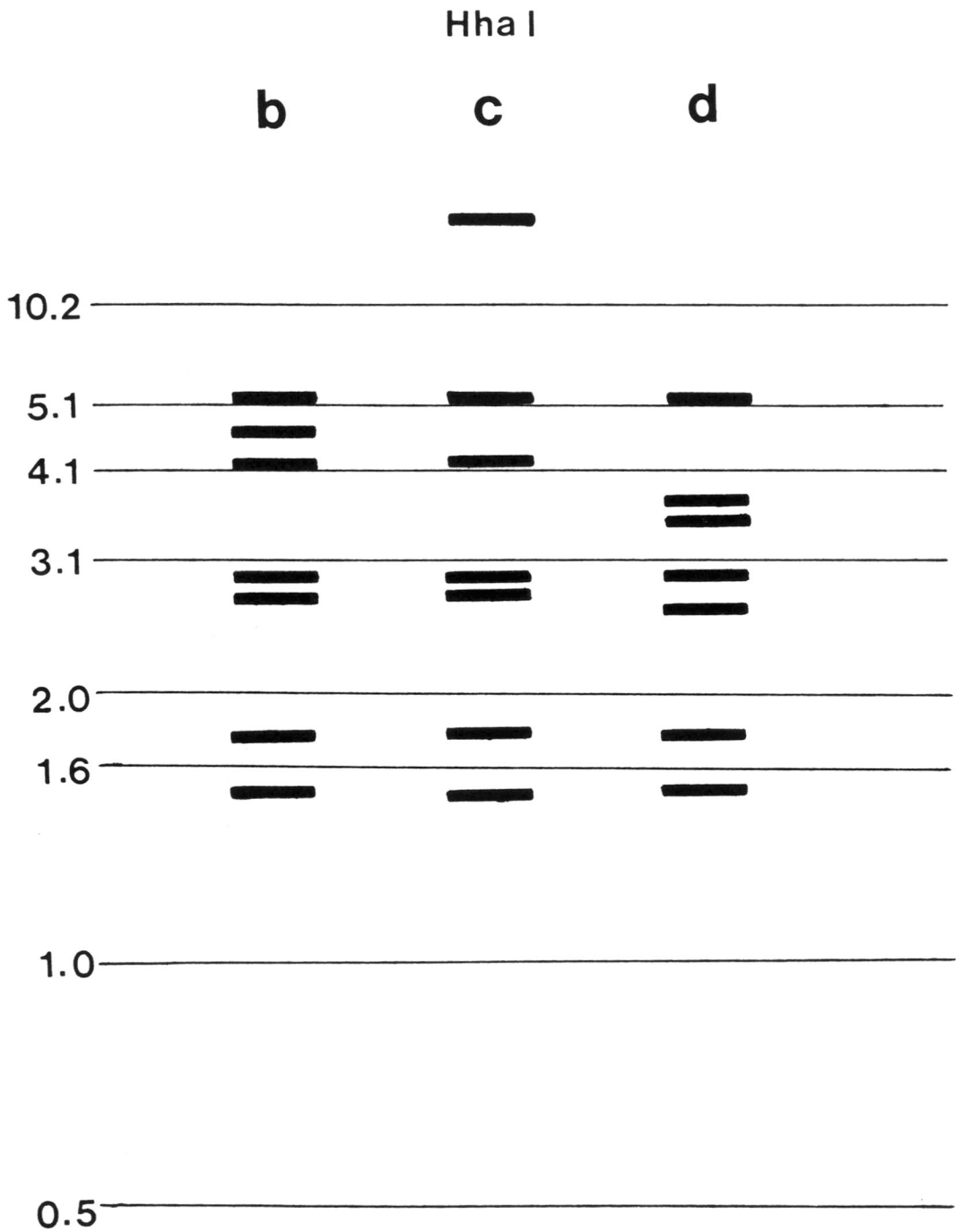

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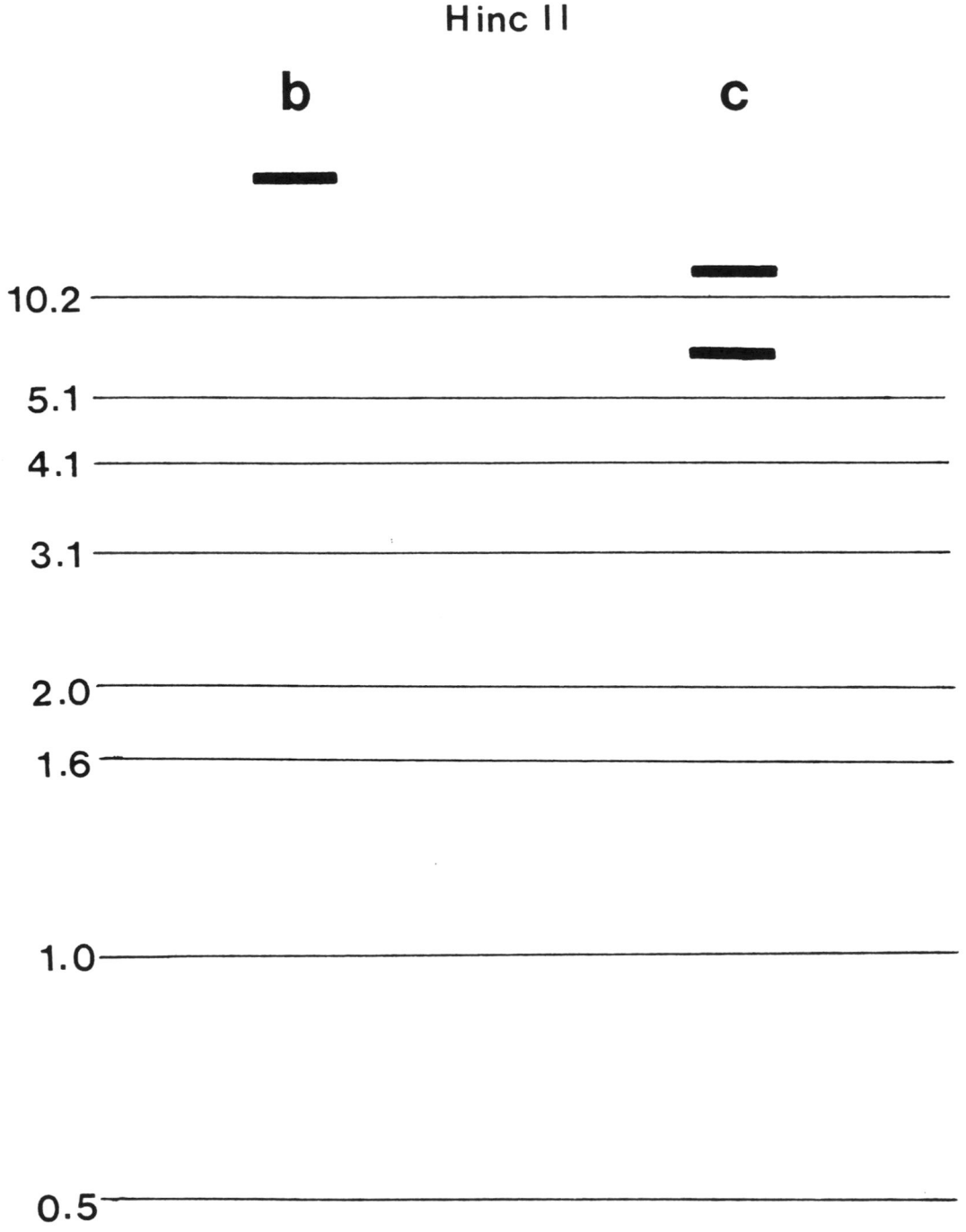

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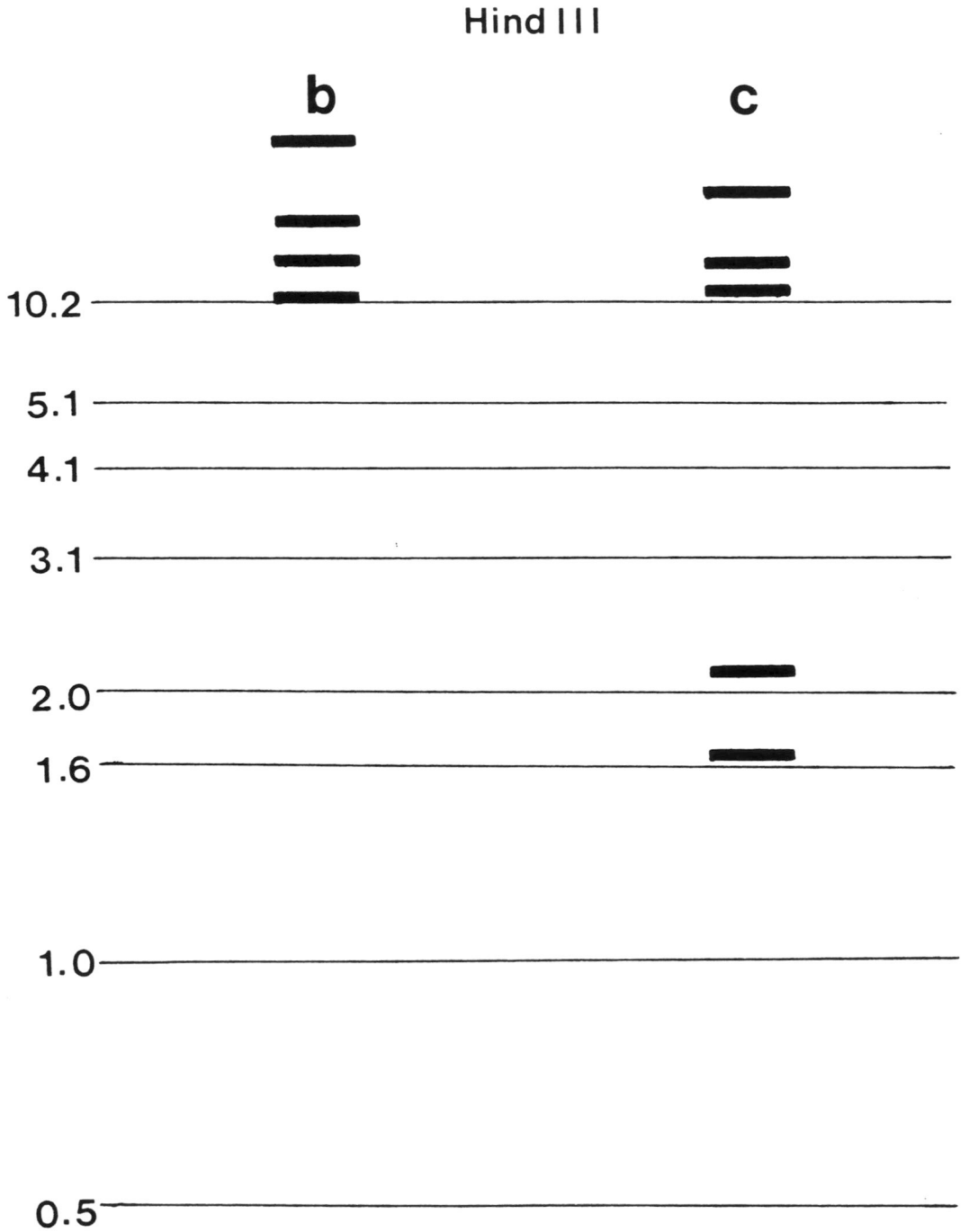

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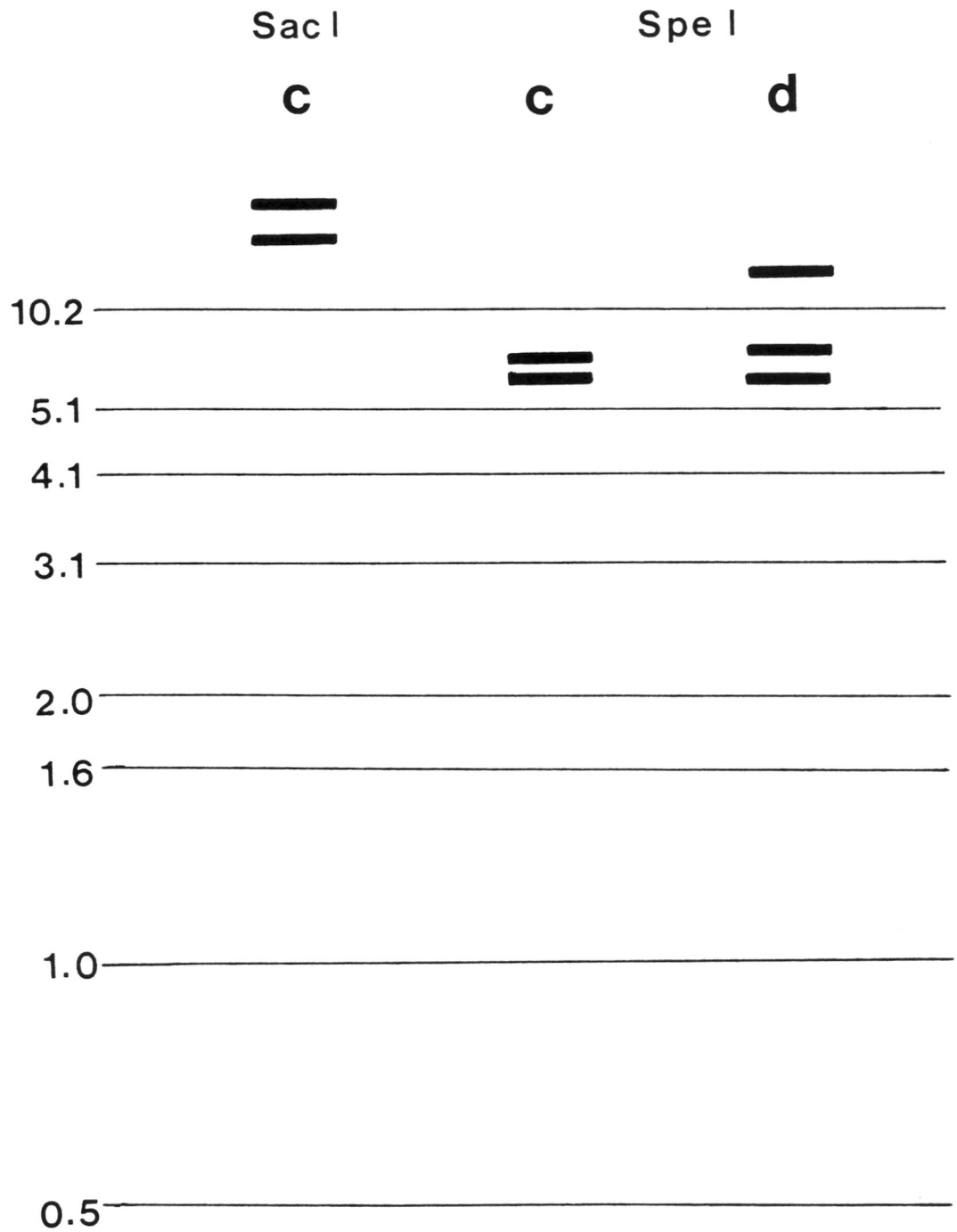

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