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Hybridization Between the Watersnakes *Nerodia sipedon* and *Nerodia fasciata,* in the Carolinas: A Morphological and Molecular Approach

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HYBRIDIZATION BETWEEN THE WATERSNAKES *NERODIA SIPEDON* AND *N. FASCIATA* IN THE CAROLINAS: A MORPHOLOGICAL AND MOLECULAR APPROACH

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ABSTRACT

HYBRIDIZATION BETWEEN THE WATERSMAKES *NERODIA SIPEDON* AND *N. FASCIATA* IN THE CAROLINAS: A MORPHOLOGICAL AND MOLECULAR APPROACH

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Old Dominion University, 2003
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A few traditionally applied diagnostic characters of color pattern were compared with an additional set of morphological and genetic characters to evaluate differences between *Nerodia sipedon* and *N. fasciata* and to study the dynamics across their hybrid zone in the Carolinas. Many of the morphological characters exhibited significant interspecific differences, although only the number of dorsally complete crossbands (CBa) was diagnostic by itself. A discriminant function analysis of morphological characters was successful in separating both taxa. Species-specific nuclear markers, identified by the AFLP (Amplified Fragment Length Polymorphism) technique were nearly fixed and served as diagnostic markers. They revealed extensive introgression between the two species not recognizable morphologically. Markers for *N. sipedon* and F_1 hybrids, backcrossed to a parental species exhibiting more than one introgressive marker, were less frequent than expected at Hardy-Weinberg equilibrium, imlpying selection against *N. sipedon* and hybrids with an increased introgression, respectively. This is consistent with a pronounced genetic and morphological dominance of *N. fasciata* in the hybrid zone. The genetic results show that the two taxa are independent entities and qualify for species status under both the Phylogenetic Species Concept and the Evolutionary Species Concept. *N. fasciata* and *N. sipedon* segregate ecologically along ecotones of salinity,

temperature, and water current. Variation in the width of these ecotones is reflected in the width of hybrid zones.

This thesis is dedicated to my late father, Dr. Werner Mebert, and my mother, Alice Mebert; without their generous and persistent support and commitment this project would have seen neither a beginning nor an end.

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TABLE OF CONTENTS

Page

Section

LIST OF TABLES

Table Page

LIST OF FIGURES

Figures Page

Figures Page

INTRODUCTION

The origin of new species can often be promoted by natural selection over several generations. Speciation involves the splitting of a population and the subsequent divergence into independent lineages by the accumulation of favorable mutations and recombinants (Mayr, 1970). Because these processes usually span more generations than an interested investigator can follow, scientists rely on studying the patterns of variation within and among contemporaneous populations or between different taxa in order to infer mechanisms of speciation (Coyne, 1992; Harrison, 1993). Hybridization and introgression are two such patterns of special interest.

Hybridization is the interbreeding between two taxa, usually closely related species. In the recent literature, the term hybridization has been applied in a broader context, referring to the offspring of two genetically distinct forms, and hence it could also include intergradation, which is the interbreeding of two subspecies (e.g., Hewitt, 1989). Harrison (1990) and Arnold (1997) modified Woodruff's (1973) definition of hybridization to "the interbreeding of individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters." Moreover, hybrids include interbreeding between F_1 hybrids, as well as the backcrosses between hybrids and their parental species or later-generation hybrids. Such individuals should be recognizable by being heterozygous for at least one of the diagnosable features

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of their parental species revealed by intermediacy in morphological characters or heterozygosity in molecular ones. The presence of different species-specific markers from genetic systems with dominant inheritance also can indicate hybridization. The dynamic aspect of hybridization events is termed introgression, which refers to the movement of genes mediated by backcrossing between two species or genetically well-distinguished populations (Avise, 1994). As a consequence of permanent introgression, hybrids may constitute a significant proportion of individuals in a contact area between two species, thereby developing a hybrid zone. The presence of hybrid zones conflicts with the traditional Biological Species Concept (see Mayr, 1963, 1970), under which two species should be reproductively isolated from each other. They represent an evolutionary window by revealing aspects of ecology, behavior, genetics and geography that contribute to speciation processes (Harrison, 1990).

How such hybrid zones originated and how long they persist are questions the evolutionary biologist addresses. Is a contact zone between two putative closely related taxa or morphs secondary, wherein allopatrically evolved species meet and hybridize, or is it primary, where populations diverge into separate lineages along an environmental gradient (a parapatric process) but remain reproductively compatible? Are hybrid zones spatially stable or do they shift with climatic change, favoring one of the involved species?

There are two principal models leading to the development of hybrid zones. First, the geographical-selection-gradient model assumes that two closely related species are adapted to a different environment in allopatry (rarely parapatry) and produce a secondary contact after expansion of their ranges. A hybrid zone is then formed through exogenous selection involving environmental parameters (Slatkin, 1973; May et al.,

1975; Endler, 1977; Moore and Price, 1993). Hybrids are often either sterile, less viable, or less fit in terms of offspring produced in the next generation as a consequence of their minor adaptation to the environment, or they show an increased fitness only in an intermediate or new environment uncharacteristic of either parental species (Moriya, 1954; Kawamura et al., 1981; Futuyma and Shapiro, 1995). The sex ratio of hybrid offspring may be heavily skewed (Sumida, 1996), which is one manifestation of the presence of a hybrid zone. Position and width of the hybrid zone may be variable and determined by structural habitat parameters. Predation is a special case of exogenous selection. For example, Müllerian mimicry among subspecies of *Heliconius* butterflies leads to narrow hybrid zones between adjacent races that are maintained by positive frequency-dependent selection through increased predation by birds on rare morphs in a population (Mallet, 1993).

In the second model a hybrid zone results solely from endogenous selection (internal genetic incompatibilities) and is not associated with an environmental gradient. In this case, the hybrid zone is called a tension zone because of the two opposing forces, dispersal of the parental species toward the hybrid zone and selection against hybrids (Key, 1968; Barton, 1979; Barton and Hewitt, 1985). The tension zone model predicts that a hybrid zone is relatively narrow, frequently correlated with a population density trough, and uniform in width (Moore and Price, 1993). In both models, integrity can be promoted by selection for individuals with characters enhancing prezygotic reproductive barriers, such as reduced mating between the two species due to differences in the anatomy of their copulatory organs, ecological differences (e.g., time and habitat selection for mating), behavioral differences in courtship, gametic mortality (Howard, 1993), or postzygotic barriers yielding hybrid inferiority. A third model combines the

characteristics from the first and second models, but endogenous and exogenous selections affect different hybrid genotypes (Arnold, 1997). In other examples, hybrids show increased developmental stability (Dosselman et al., 1998) or equal to better survivorship (Parris, 2000; Parris et al., 2001).

Hybrid zones often appear to be abrupt discontinuities recognizable as steep character clines between two differentiated populations that are themselves relatively homogenous over large areas (Harrison, 1993). Generally, narrow hybrid zones can be viewed as being produced by two species, whereas broad hybrid zones resemble the intergradation of two subspecies (Wiley, 1981; Sumida, 1996). However, the width of hybrid zones is related to the dispersal ability of the organisms and the strength of selection forces acting on the species involved. For example, the hybrid zone of a small animal, such as between two taxa of the Australian grasshopper *Caledia captiva* complex, can be over 250 km long but < 1 km wide (Shaw et al, 1993), whereas the hybrid zone between two fire-bellied toads (*Bombina* spp.) extends over several 1000s km and a width of 5-30 km (including low-level introgression) (Szymura, 1993). A mosaic hybrid zone may reflect patchy habitat (Harrison and Rand, 1989; MacCallum et al., 1998) or long-distance migrants that travel far beyond the initial lines of contact to establish new populations (Hewitt, 1989).

The study of hybrid zones has received increased attention over the last 25 years, particularly since the application of molecular techniques has allowed identification of introgressive genes (Rieseberg and Wendel, 1993). It is of interest for conservation purposes, when a vulnerable species is at risk by the hybridization with a closely related species (e.g., Evans et al., 1998; Arntzen and Thorpe, 1999). Interspecific and intergeneric hybridization are especially common in plants (Knobloch, 1972; Gill, 1989).

In vertebrates, hybridization is common in fishes (Schwartz, 1972, 1981), less frequent but still substantial in other groups such as amphibians, lizards, and birds (Mayr, 1963, 1970; Arnold, 1997; Highton, 1997, 1999), and rare in mammals and snakes, groups that will exhibit internal fertilization. Vertebrates are difficult subjects for evolutionary studies, due to their relatively long generation times and high laboratory maintenance costs. This contrasts with invertebrates, such as *Drosophila*, which can easily be maintained under laboratory conditions. However, in order to assure the influence of natural processes, one must rely on patterns observed under natural conditions to infer mechanisms of speciation. The occurrence of hybridization between two watersnake species, *Nerodia sipedon* and *N. fasciata*, provides an opportunity to study the process in a natural setting.

Background on Nerodia fasciata *and* N. sipedon.—The cosmopolitan colubrid subfamily Natricinae includes such conspicuous species as garter snakes, *Thamnophis* spp., and watersnakes, *Nerodia* spp., the latter containing 10 species ranging from Canada to Mexico. Interspecific relationships within *Nerodia* have been studied repeatedly (e.g., Blanchard, 1923; Taylor, 1929; Clay, 1938; Stejneger and Barbour, 1939; Cliburn, 1960; Malnate, 1960; Conant, 1963; Pearson, 1966; Mount and Schwaner, 1970; Mao and Dessauer, 1991; Rossman and Eberle, 1977; Scudder, 1977; Blaney and Blaney, 1979; Schwaner and Dessauer, 1982; Sanderson, 1983; Lawson, 1987; Morris, 1987; Rose and Selcer, 1989; Lawson et al., 1991; Densmore et al., 1992; Jansen, 2001). However, the relationship between the northern watersnake, *N. sipedon*, and the banded watersnake, *N. fasciata*, remains unclear. These two parapatric species meet along contact areas on the borders of the Mississippi River Valley, from Illinois to Louisiana

and Oklahoma, and from southern Mississippi farther east along the Lower Coastal Plain of the Gulf States to Georgia, and parallel to the Fall Zone (= Fall Line), north as far as northeastern North Carolina (Conant and Collins, 1991; Fig. 1). Both species are medium-sized to large watersnakes, with females the longer sex, reaching approximately 130 cm. Although color pattern is highly variable, most have a ground color from brown, gray, to almost black with dark dorsal crossbands or blotches that may become reddish on the side. Clay (1938) and Conant (1963) have summarized the distinguishing features of the two species in the eastern parts of their ranges, which include:

N. fasciata: dorsal pattern of complete crossbands throughout the body; ventral pattern of triangular to squarish blotches or wavy lines along the anterior edge of each ventral scale; postocular stripe.

N. sipedon: complete dorsal bands only anteriorly, changing to separate, alternating dorsal and lateral blotches posteriorly; half-moon blotches on the venter; no postocular stripe (see figures 32 and 33 in Appendix 1 for illustrations on color pattern differences between both species).

Many of the color pattern differences traditionally applied to distinguish *Nerodia sipedon* and *N. fasciata* (Conant and Collins, 1991) have led previous authors to distinguish small areas of sympatry from those where hybridization occurs (Fig. 1).

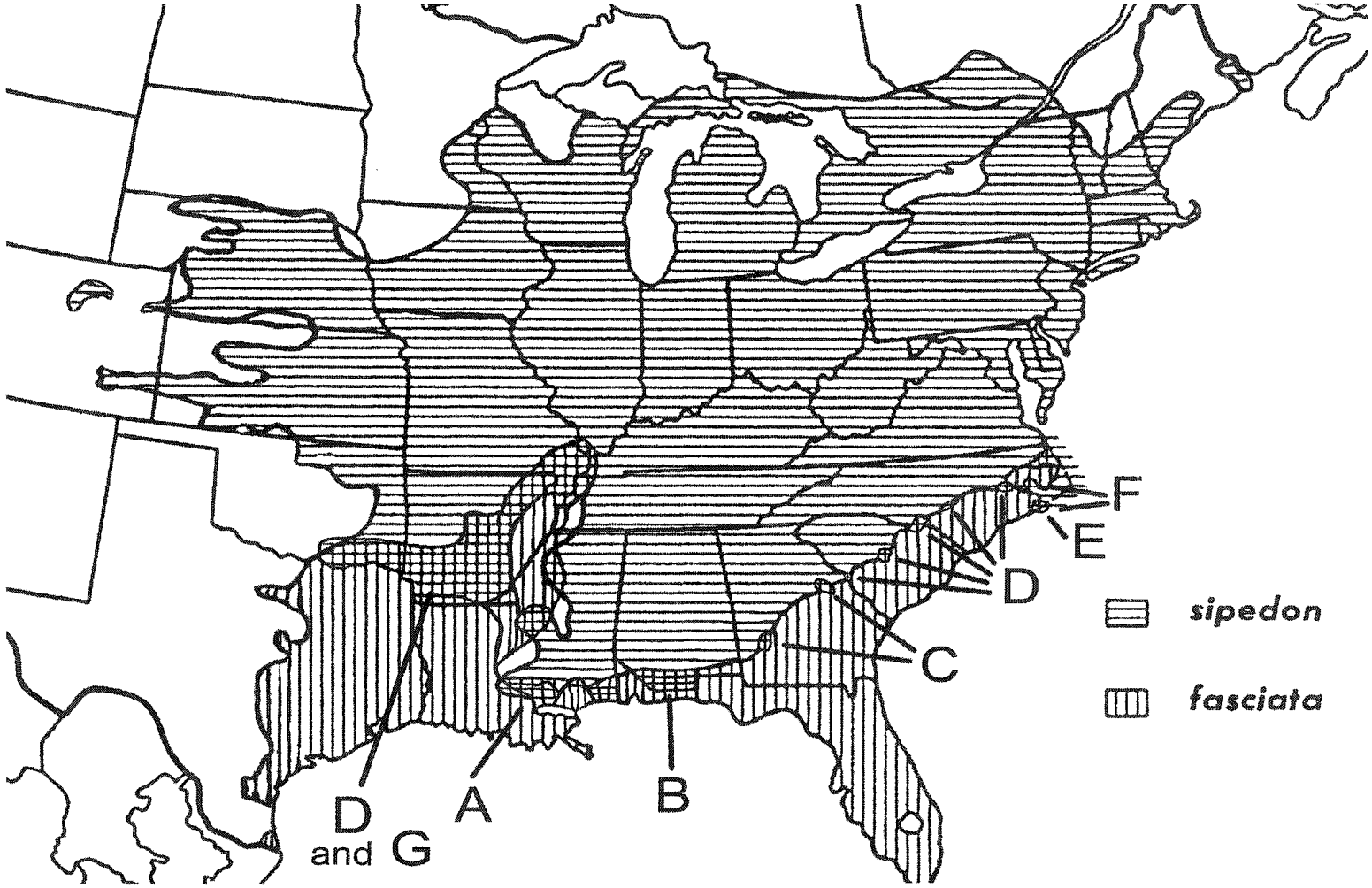


FIG. 1. Distribution of *Nerodia sipedon* and *N. fasciata*. Letters denote areas where hybrid zones or sympatry have been documented. **A**: Blaney and Blaney (1979), Schwaner et al. (1980); **B**: Schwaner and Mount (1976); **C**: Seyle (1980); **D**: Conant (1963); **E**: Conant and Lazell (1973); **F**: Gaul (1996); **G**: S. Trauth (unpubl. maps). Figure is modified from Conant (1958) with permission from Houghton Mifflin Company, New York.

Both species are viviparous. They readily enter the water to prey on fish and amphibians. All of the reported differences in their ecological requirements are anecdotal (e.g., Schwaner and Mount, 1976; Palmer and Braswell, 1995; Mebert, 1996a). *Nerodia fasciata* is considered to be a lowland species, inhabiting quiet waters such as ponds, lakes, sloughs, ditches, small streams and the environs of sluggish rivers, including bottomland swamps (Conant, 1963). North of its range, it is replaced in these habitats by *N. sipedon*, which in the southern states occupies more upland habitats, such as larger and faster-flowing rivers and streams (Schwaner and Mount, 1976; Blaney and Blaney, 1979; Seyle, 1980). However, the species differ markedly in their salinity tolerances along coastal areas of North Carolina, where *N. fasciata* is confined to freshwater and *N. sipedon* inhabits more saline environments (Conant and Lazell, 1973; Gaul, 1996).

Nerodia fasciata and *N. sipedon* occur sympatrically with other watersnake species within their ranges. For instance, in Louisiana, *N. fasciata* is found to be more opportunistic along the food, time, temperature and habitat-use axes than congeners (Mushinsky and Hebrard, 1977a, b; Hebrard and Mushinsky, 1978; Mushinsky et al. 1980). Similarly, *N. sipedon* can be regarded as a generalist due to the large variety of habitats it occupies (Conant and Collins, 1991). In the Midwest, *N. sipedon* appears to be more common in permanent and rapidly moving water bodies compared to the closely related *N. erythrogaster*, which inhabits more temporary ponds or slow-moving waters (Conant, 1934; Diener, 1957). Cagle (1942) reported that the *N. erythrogaster* abounds in sloughs and muddy ditches in southern Illinois, whereas *N. sipedon* inhabits clear rock-bottomed streams. However, in Indiana *N. sipedon* is abundant in quiet ponds adjacent to streams (Fraker, 1970). In Wisconsin, Tiebout and Cary (1987) termed *N. sipedon* an "edge species," as it was commonly found in open areas never farther than 6 m from the

lakeshore, which is consistent with observations made at other sites in the Midwest (Diener, 1957; King, 1986). Similar observations were made in the eastern Coastal Plain, where habitat differences between *N. erythrogaster* and either *N. sipedon* or *N. fasciata* appear to relate more to canopy closure than to water velocity (Holman and Hill, 1961; pers. obs.). *N. fasciata* and *N. sipedon* inhabit cypress swamps in northeastern North Carolina, but appear to be more common along the open edge of swamps and rivers, whereas *N. erythrogaster* inhabits the shaded areas within swamps or frequents small creeks with closed canopies (pers. obs.).

Although specimens that are morphologically intermediate between *Nerodia sipedon* and *N. fasciata* are known from sites along the contact zone, the two species generally maintain their identity in that area (Neill, 1946; Conant, 1963; Seyle, 1980; Mebert, 1996a). However, four studies have found morphological evidence of substantial interbreeding at several sites (Fig. 1): larger streams along the Alabama-Florida border (Schwaner and Mount, 1976); two sites along the Fall Zone in Georgia (Seyle, 1980), two sites along the Fall Zone in South Carolina (Conant, 1963); and a barrier island in North Carolina (Conant and Lazell, 1973). A few small areas of sympatry are also recognized in North Carolina (Conant, 1963). A zone of extensive mixing between *N. sipedon* and *N. fasciata* in Louisiana and Mississippi is interpreted as an intergradation between two subspecies (Blaney and Blaney, 1979), but was refuted after the discovery that each putative hybrid from that area possessed specific protein allelomorphs belonging to either one or the other species (Schwaner et al., 1980). Moreover, a clear phenetic allocation of most specimens to one or the other species could be achieved (Dundee and Rossmann, 1989). Wiley (1981) viewed this population as a case representing residual geographic variation (perhaps reinforced by local selection) of the common ancestor of the two

species. This raises the question of whether all currently recognized contact zones characterized by intermediate phenotypes actually are comprised of hybrids or whether many such intermediates belong genetically to one or the other species with the occasional aberrant specimen resembling superficially the sister species. If the latter is true, the cause of the morphological homoplasy of the two species within their contact zone is unclear.

Contact Zones between Nerodia fasciata *and* N. sipedon *in the Carolinas.*—Conant (1963) was the first to focus a study on the *Nerodia fasciata-sipedon* complex. He approached the problem by comparing scale and color patterns among large samples of watersnakes, mainly from the Carolinas. His investigation and field guide (Conant and Collins, 1991) show that most of the known distribution between the two species in the Carolinas is allopatric or parapatric (Fig. 2). *N. sipedon* generally occupies all kinds of freshwater habitats in the Piedmont and farther west, whereas *N. fasciata* inhabits equivalent environments on the Coastal Plain. Superposition of locality data of *N. fasciata* and *N. sipedon* from North Carolina (Palmer and Braswell, 1995) reveals a few areas of sympatry. For example, specimens from south-central North Carolina (Richmond and Moore counties; sites 1 and 2 in Fig. 2) indicate that the two species occur sympatrically or in close proximity without obvious introgression, whereas both species occur sympatrically along more than 20 km of the Tar River near Greenville, Pitt County (site 3 in Fig. 2).

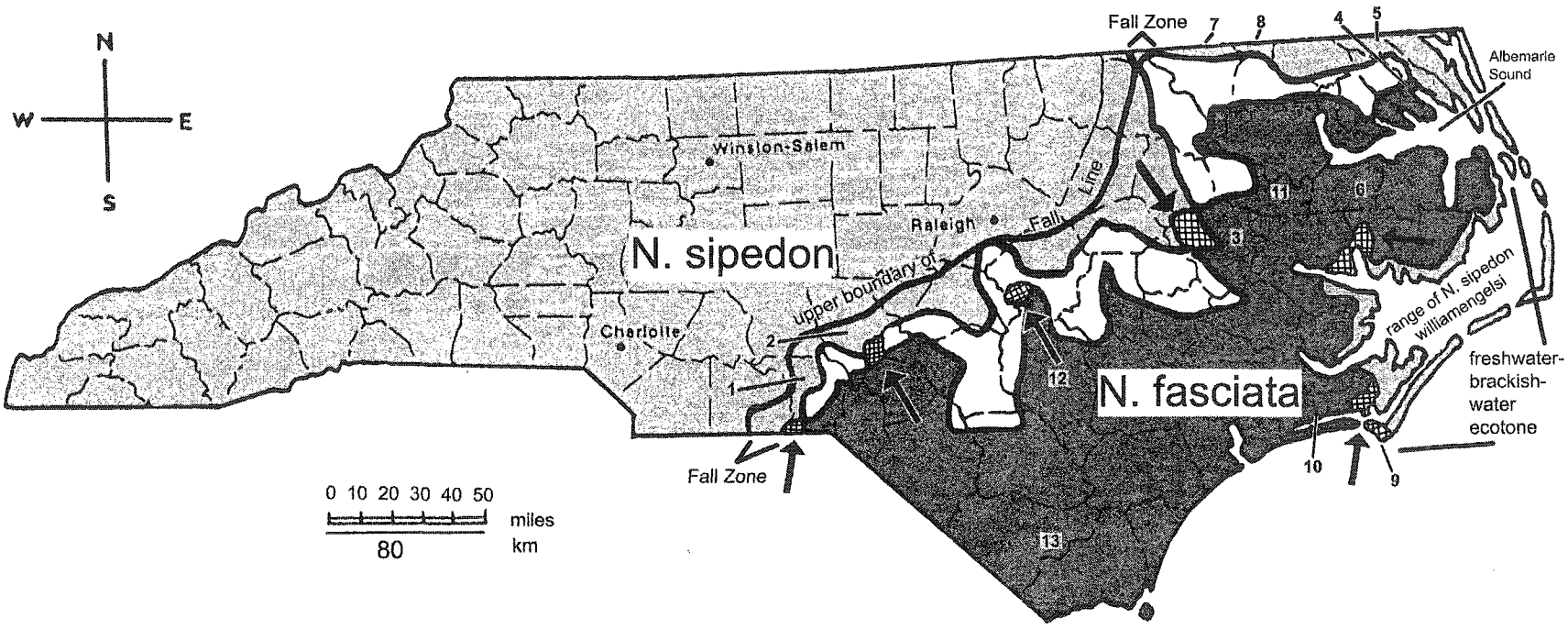


FIG. 2. Distribution of *Nerodia sipedon* and *N. fasciata* in North Carolina as known at the initiation of this project (1995). The white zone represents areas from which records o f either species are missing. Cross -hatching denotes areas where hybrid zones or sympatry have previously been documented (see text). In -text referred sites are (1) Richmond Co.; (2) Moore Co.; (3) Greenville, Pitt Co.; (4) Pasquotank Co.; (5) Camden Co.; (6) Phelps Lake, Washington Co.; (7) Northampton Co.; (8) Hertford Co.; (9) Shackleford Bank; (10) Carteret Co.; (11) Garner Creek,, Martin Co.; (12) Holts Lake, Johnston Co. (13) Lake Waccamaw, Columbus Co. Maps from 'Reptiles of North Carolina' (Palmer a nd Braswell, 1995) served to outline the initial range limits. The Fall Zone extends approximately 30 –50 km from the upper boundary toward the coast (adapted from Conant [1963]; Clay, Orr, and Stuart [1975]).

The current distribution of both species of watersnakes in North Carolina presumably reflects range expansion since the end of the Pleistocene. Several pairs of taxa of amphibians and reptiles show similar distributions along the Fall Zone, e.g., in snakes: *Regina septemvittata* and *R. rigida*, *Elaphe o. obsoleta* and *E. o. quadrivittata*; in salamanders: *Notophthalmus v. viridescens* and *N. v. dorsalis*, *Desmognathus fuscus* and *D. auriculatus*; and in anurans: *Bufo americanus* and *B. terrestris*, *Acris crepitans* and *A. gryllus*. Other species are restricted to the Coastal Plain, as is *Nerodia fasciata*; however, their northern boundaries vary, e.g., salamanders: *Siren intermedia*, *Amphiuma means*, *Eurycea quadridigitat*; anurans: *B. quercicus*, *Hyla squirella*, and *Pseudacris nigrita*; crocodilians: *Alligator mississippiensis*; turtles: *Deirochelys reticularia*, and *Pseudemys floridana*; lizards: *Ophisaurus ventralis*; and snakes: *Nerodia taxispilota*, *Seminatrix pygaea*, *Farancia abacura*, and *Agkistrodon piscivorus*, (Conant and Collins, 1991; Palmer and Braswell, 1995).

Such similar distributions and the location of the hybrid zone near the Fall Zone suggest that environmental discontinuities limit their ranges. However, recently published range maps for *N. fasciata* and *N. sipedon* from North Carolina and personal observations demonstrate that the northeastern contact area between the two species deviates substantially from the Fall Zone (Brothers, 1965; Palmer and Braswell, 1995; Mebert, 1996a; area between sites 3 and 4 in Fig. 2). The species meet north of the Albemarle Sound approximately along an east-west line stretching as far east as the area near Elizabeth City in Pasquotank and Camden counties (sites 4 and 5 in Fig. 2). This northern portion of the contact area does not correspond to any known gradient between habitats (Clay, Orr, and Stuart, 1975), suggesting a historical explanation for its location. For instance, the contact area may reflect a tension zone in which further dispersal of

each species into the other's range is precluded by reduced hybrid fitness, indicating substantial evolutionary divergence between the two taxa.

Another area of sympatry between the two species is based on one *Nerodia sipedon* from Lake Phelps (Conant, 1963; site 6 in Fig. 2) and a sighting of a typical *N. sipedon* from a freshwater canal south of Lake Mattamuskeet (R. Gaul, pers. comm.); both records are from the Peninsula between the Albemarle and Pamlico sounds, where freshwater habitats normally are inhabited by *N. fasciata*. However, these specimens may represent aberrant *N. fasciata* that possess dorsal patterns similar to that of *N. sipedon*. Further uncertainty results from the occurrence of *N. sipedon* in Northampton and Hertford counties, NC (sites 7 and 8 in Fig. 2), and from southeastern Virginia, as well as from several specimens near the contact zone with *N. fasciata* in southern Illinois, Indiana and Georgia, all of which show similarities to *N. fasciata* (Viosca, 1924; Neill, 1957; Smith, 1961; Conant, 1963; Conant et al., 1990). It may represent increased variability in both species where their ranges meet, e.g., as an adaptive response to a locally shared environment for the two closely related species. Such a response has been found in the gray treefrog species, *Hyla chrysoscelis* and *H. versicolor*, with the two sibling species showing convergence in various life-history traits in sympatry (Ptacek, 1996). Conversely, such convergence may also indicate an ongoing introgression of *N. fasciata* genes into the *N. sipedon* gene pool.

Preliminary collecting showed an increased frequency of intermediate color patterns in specimens from the contact area of *Nerodia sipedon* and *N. fasciata*, including areas from which no hybrids were known (Mebert, 1996a). For example, eight of 11 *N. fasciata* from Crystal Lake, Moore Co., NC, exhibited alternating dorsal and lateral blotches, a characteristic of *N. sipedon*. However, their ventral patterns consisted of

squarish blotches as in *N. fasciata*. This is an area for which Conant (1963) reported sympatry without hybridization. Two of six snakes from the Greenville area, Pitt Co., NC, also showed intermediate color patterns. Similarly, color pattern variation north and east of Elizabeth City, in Pasquotank and Camden counties, suggests introgression with various degrees of backcrossing.

A different situation is presented along coastal ecotones with their changing salinity gradients. The Outer Banks region of North Carolina is inhabited by the endemic salt-tolerant *Nerodia sipedon williamengelsi* and its intergrades with the nominate race (Conant and Lazell, 1973). Along the salt marshes of the nearby mainland, watersnakes from the *N. s. sipedon-williamengelsi* complex contact *N. fasciata*, which occupies inland freshwater habitats (Palmer and Braswell, 1995). This interspecific contact zone appears to be completely isolated from the contact between both species near the Fall Zone. Farther north (across the Albemarle Sound), road-killed specimens from Camden Point (a peninsula protruding into the Albemarle Sound) and its northern extension into the coastal (tidal) Great Swamp, Camden Co., resemble phenetic *N. sipedon* (pers. obs.). Therefore, the Albemarle Sound separates coastal contact zones to the north (*N. fasciata* with *N. s. sipedon*) and to the south (*N. fasciata* with *N. s. sipedon-williamengelsi* complex). Generally, *N. s. williamengelsi* from south of the Albemarle Sound seems to occupy brackish-to-saline environments, in contrast to the freshwater habitats occupied by *N. fasciata* (Conant, 1963; Conant and Lazell, 1973).

A few cases of hybridization between the *Nerodia s. sipedon-williamengelsi* complex and *N. fasciata* have been documented in the coastal area in North Carolina. For instance, a hybrid swarm was found inhabiting a freshwater pond on Shackleford Bank, an island approximately 3 km from the mainland (Conant and Lazell, 1973; site 9 in Fig.

2). A gravid *N. fasciata* from Ponzer, Hyde Co., gave birth to a litter of *N. fasciata* x *N. s. williamengelsi*, and a phenetically intermediate snake was found at Lennoxville Point, Carteret County (site 10 in Fig. 2), which is located on the mainland just across Shackleford Bank (Conant and Lazell, 1973). Conant (1963) suggested that Shackleford Bank originally was populated only by the salt-tolerant *N. s. williamengelsi*, as no freshwater ponds existed 10 years earlier when Engels (1952) studied that island. Recently, Gaul (1996), using morphological and genetic data, revealed further hybrids between *N. fasciata* and the *N. s. sipedon-williamengelsi* complex near Pamlico Sound, about 30 km farther north, and from other coastal localities in Carteret County.

Aside from the hybrid swarm on Shackleford Bank, *Nerodia fasciata* has been documented from only one other Outer Banks island, Bogue Banks, immediately west of Shackleford Bank (one voucher [Palmer and Braswell, 1995] and two sightings [R. Gaul, pers. comm.]). Farther south along coastal barrier islands *N. fasciata* has been reported only from Topsail Island and from barrier islands in Brunswick and New Hanover counties, NC (Palmer and Braswell, 1995). Although *N. fasciata* has been found in freshwater habitats on these islands, it is not known whether any form of *Nerodia* inhabits the salt marshes. Perhaps *N. fasciata* has ecologically replaced *N. s. williamengelsi* on more southern islands in North Carolina and South Carolina and has evolved into distinct salt-tolerant populations. That may be the case for salt-tolerant populations derived from *N. fasciata* along the coast of Florida and other Gulf states, which have been elevated to the species level as *N. clarkii* by Lawson et al. (1991), whereas Jansen (2001) denoted them subspecies status. The situations described above document continual confusion concerning the systematic arrangement of the *N. fasciata*-

sipedon complex to the present times and set the stage for a project that crosses the boundary between population genetics and species definitions.

Rationale for This Project.—The hybrid zone of *Nerodia sipedon* and *N. fasciata* in North Carolina constitutes a natural experiment for the study of evolutionary processes (Mebert, 1996a). It has several advantages for such a study, viz.:

1) Overall, the contact zone is narrow and over 3000 km long, supporting the hypothesis that the two are distinct species.
2) Unlike most snakes, *Nerodia* spp. are relatively easy to collect.
3) Previous studies of hybridization between the two species (Conant, 1963; Schwaner and Mount, 1976; Blaney and Blaney, 1979; Seyle, 1980) allow relevant comparisons.
4) Only a few interspecific hybrid zones in snakes have been documented: *Elaphe bairdi* and *E. obsoleta* in Texas (Lawson and Lieb, 1990); *Natrix n. natrix* and *Natrix n. helvetica* in Europe (deserving species status due to the narrowness of their hybrid zone; Thorpe, 1983); *Nerodia fasciata* and *N. sipedon* (Conant, 1963); *N. fasciata* with *N. clarkii* along the coast of the Gulf states in the southern U.S. (Lawson et al., 1991); several *Thamnophis* spp. (Rossman et al., 1996), and *Bothrops atrox* and *B. moojeni* (Wuester et al., 1996).
5) The situation in North Carolina is unique, where hybrid zones occur in at least two different ecological settings: (1) near the Fall Zone in the Carolinas and (2) the coastal brackish-freshwater ecotone; see references in Conant (1963) and

Figure 2. Thus, they offer an interesting opportunity to investigate various aspects of hybrid zones and speciation processes.

6) The suggestion that the position of the contact area near the Fall Zone relates to species differences in ecological preferences (Conant, 1963) could be re-evaluated in light of recent evidence that a considerable proportion of the hybrid zone in North Carolina lies outside a known environmental gradient in freshwater lowland swamps between the Fall Zone and the coast (Palmer and Braswell, 1995; Mebert, 1996a). However, methodological difficulties in measuring micro- and macrogeographic habitat attributes, and small sample sizes, have prevented a feasible on-site analysis.

Objectives.—Two objectives related to species concepts were established:

1) To determine whether the Phylogenetic Species Concept (Cracraft, 1983) is applicable to *Nerodia fasciata* and *N. sipedon*. This concept is a more practical approach to distinguish two species than the requirement of reproductive isolation, as in the Biological Species Concept (Mayr, 1963). The PSC emphasizes diagnosable characters such as fixed alleles or consistent morphological features, which imply a parental pattern of ancestry and descent. When hybridization occurs between two sister taxa, they can still be considered separate species if each taxon possesses diagnosable characters.

2) To evaluate whether the relationship between the species of two watersnakes within their contact zone in North Carolina conforms to the Evolutionary Species Concept (Simpson, 1961; Wiley, 1978; Frost and Hillis, 1990). Although this concept requires reproductive isolation between species, hybridization is permitted as long as it does not preclude the two taxa from maintaining their separate identities and evolutionary

fates (Grant, 1971; Wiley, 1981). Whereas Cracraft's phylogenetic species is based on the smallest detectable evolutionary entities, Wiley's evolutionary species consists of the largest evolving entities. As such, two allopatric lineages, whose members are diagnosably different but which share reproductive compatibility, would be considered distinct species (Frost and Hillis, 1990). For instance, hybrid inferiority and a narrow hybrid zone are indications that selection against hybrids occurs, hence the two species must represent distinct evolving entities and may differ in ecological requirements, behavior, or genetic compatibility (Dowling and Moore, 1985; Kocher and Sage, 1986).

I consider three derivative hypotheses concerning the maintenance of two distinct species due to morphological and genetic differences. First, I hypothesize that the two closely related taxa show substantial morphological and genetic divergence. Second, I hypothesize that the two taxa can be regarded as evolutionary species due to reduced gene flow via the weak nature of their hybridization (i.e., the hybrid zone is narrow and the proportion of hybrids in the contact zone is low). Emphasis is placed on determining species-specific genetic markers and the amount of hybridization. Decreased frequencies of backcrossing would be evidence of reduced hybrid fitness due to a genetic barrier, which prevents further introgression between the two species of watersnakes. Third, I hypothesize that the estimated time of divergence coincides with patterns of historical climate change and reveals the period when the two species were confined to separate refugia.

Specific Questions.—I address the following specific questions related to the foregoing hypothesis:

1) Are there any morphological characters that consistently distinguish *Nerodia sipedon* from *N. fasciata*? **H_0:** There are no significant quantitative and qualitative morphological differences between *N. sipedon* and *N. fasciata* that can be used as diagnostic characters. **H_1:** Such interspecific differences exist.

Several reports have previously attempted to address this question without finding characters that clearly separate between the two species (e.g., Clay, 1938; Cliburn, 1957, 1960; Conant, 1963; Schwaner and Mount, 1976; Blaney and Blaney, 1979; Seyle, 1980). Although no diagnostic features have been reported that apply in 100% of the cases, a few pattern characteristics frequently distinguish more than 90% of the specimens across their entire ranges. Those pattern characters that best distinguish the two taxa can be combined with scutellation characters studied in another natricine snake (Mebert, 1993; Gruschwitz et al., 1999). Furthermore, several measurements of head shape and pattern are evaluated with a modification of a subjective hybrid index developed by Anderson (1949). A similar index was used to characterize differences in head shape between *Nerodia sipedon* and *N. fasciata* (Schwaner and Mount, 1976; Seyle, 1980). Data are analyzed using statistical procedures such as ANOVA, ANCOVA and DFA (see Thorpe, 1976, 1979, 1983, for a comprehensive example applied to a natricine snake).

2) Are there fixed genetic differences between *Nerodia sipedon* and *N. fasciata* and can such markers be used to evaluate different hybrid categories (F_1 hybrids, F_1 hybrids backcrossed to a parental species, and later-generation hybrids)? **H_0:** There are no fixed

genetic differences between *N. sipedon* and *N. fasciata*; $\mathbf{H_1}$: There are fixed genetic differences between *N. sipedon* and *N. fasciata*.

Various molecular methods have been applied for the last 15 years to investigate the systematic relationships and population dynamics within the genus *Nerodia*. For example, allozymes and other protein classes were applied to study phylogenetic relationships among various *Nerodia* spp. (Lawson, 1987; Lawson et al. 1991; Rose and Selcer, 1989; Thompson and Crother, 1998) or gene flow among populations (King and Lawson, 2001). RFLP (Restriction Fragment Length Polymorphism) has been successfully applied on scnDNA to reveal highly variable regions with multiple alleles in birds (Quinn and White, 1987), turtles, and an oyster (Karl et al., 1992; Karl and Avise, 1993) and has been used to study hybridization (Keim et al., 1989; Hall, 1990). RFLPs were also used for restriction site mapping of mtDNA to study the relationships among a few *Nerodia* spp. (Densmore et al., 1992; Gaul, 1996). Simple Sequence Repeats (SSR or microsatellites), variable numbers of tandem repeats of nucleotides that generate a large genetic diversity due to high mutation rates, have been successfully applied to measure intraspecific population differences in *Nerodia* species. For example, 2 to19 alleles per locus detected with SSR produced sufficient genetic differentiation between local (< 2 km) populations of *N. sipedon* to infer limited dispersal abilities (Prosser et al., 1999). Similarly, SSR revealed high levels of variation among populations of the *N. clarkii*-complex from southern Florida with genetic sub-structuring among populations at a distance as small as 16 km (Jansen, 2001). SSR also have been used to detect hybridization in canids (Roy et al., 1994), but their highly polymorphic nature renders it difficult to determine the degree of hybridization or to study interspecific variation

(Russell et al., 1997). None of the studies above has addressed or resolved the issues concerning the close relationship and hybridization between *N. fasciata* and *N. sipedon.*

Another promising method is AFLP (Amplified Fragment Length Polymorphism), which combines the simplicity of RFLP and the resolving power of SSR. AFLP is a relatively novel method in the field of herpetology. Only two studies determining species' trees of fine taxonomic levels within the Asian pit viper *Trimeresurus albolabris* (Giannasi et al., 2001) and Caribbean *Anolis* lizards (Ogden and Thorpe, 2002) have been published. Both studies are associated with a herpetological laboratory at the University of Wales, Bangor, UK. AFLP can be used to generate fragments from genomic DNA to identify species-specific alleles. This technique involves cutting DNA with endonucleases, sequentially double-amplifying subsets of all available fragments, separating the resulting fragments according their molecular weights by electrophoresis, and visualizing the size-sorted fragments. At least five diagnostic loci for a codominant system and 10 for a dominant system are needed to keep the probability of misidentifications of true backcrosses below 5% (Lamb and Avise, 1986). Variation among individuals and taxa may result from base substitutions within cleavage sites or from additions or deletions of DNA. AFLP has a greater resolution than starch-gel electrophoresis of allozymes previously applied in a study of variation in *Nerodia* (Lawson, 1987) and is more sensitive in detecting hybrids than the analysis of conservative mtDNA.

3) Is there a small frequency of backcrossed hybrids? $\mathbf{H_0}$**:** Backcrossed hybrids, recognizable from fixed genetic differences between *Nerodia sipedon* and *N. fasciata*, are rare compared to specimens with the parental genome or early-generation hybrids; $\mathbf{H_1}$**:** backcrossed hybrids are abundant.

King (1986) followed cohorts of variably patterned *Nerodia sipedon* hatchlings to study their survival. Similarly, comparison of the survival of pure versus hybrid hatchlings could indicate the existence of a selective advantage for one or the other group. However, such a study is not feasible because (1) once hatchlings are released, it is unlikely that they could be resampled due to their secretive behavior and the inaccessibility of their habitat; and (2) the survivorship of neonates is low. However, an indirect alternative way to evaluate reduced fitness in hybrid snakes would be to count the frequency of backcrossed hybrids in a contact zone. If they are comparably rare, it indicates inferiority of hybrids (Lamb and Avise, 1986; Sharbel et al., 1995).

4) Are morphological and molecular polymorphisms correlated, and do hybrids show consistent intermediacy in such traits? $\mathbf{H_0}$: Morphological and genetic characters vary congruently; $\mathbf{H_1}$: Morphological and genetic variations do not exhibit congruent patterns. For instance, genetic variation has been found not to underlie morphological variation in several species of amphibians and reptiles (e.g., Highton et al., 1989; Larson, 1989; Rose and Selcer, 1989; Glenn and Straight, 1990; Knight et al., 1992). Furthermore, a female *Nerodia fasciata confluens* x *N. s. sipedon* hybrid (F_1) that was backcrossed to a pure *N. f. confluens* male yielded offspring that essentially lost all morphological traits of *N. sipedon* (Riches, 1976). These examples indicate that without molecular data, a substantial proportion of hybrids could potentially be misclassified as pure parental species, and the assignment of hybrids to the various genotype categories may not be reliably achieved (Lamb and Avise, 1987). Diverse morphological characters (scale counts, color pattern elements) could be tested for correlation with the genotypes in both species of *Nerodia*.

Other issues that are of interest in studies of hybrid zones concern the dynamic processes between the two species. For example, the mixing of diagnostic morphological and genetic markers increases toward the center of a hybrid zone. Such a geographic change from one to the other species (or character) can be expressed as a cline. A cline may be gradual, describing a smooth sigmoidal curve as expected for freely (neutral) diffusing alleles or for dispersal-independent models, in which hybrids are superior only in an ecotone and clines are maintained by selection alone (Slatkin, 1973; Endler, 1977; Barton and Hewitt, 1985; Hewitt, 1988, 1989). This contrasts a steep cline, in which selection is balanced against dispersal through abrupt environmental change or genetic barriers (Key, 1968). Such an example can be found in *Ensatina eschscholtzii platensis* and *E. e. xanthoptica* (Amphibia: Plethodontidae), where average gene frequencies of *platensis* alleles dropped from 0.88 to 0.2 over a distance of only 1400 m (Wake et al., 1989).

Barton and Gale (1993) summarized techniques to analyze spatial patterns of gene frequencies, linkage disequilibria, and quantitative characters to estimate selection and dispersal parameters in hybrid zones. For example, the strength of a barrier to gene flow can be estimated from the shape of the cline (Barton and Hewitt, 1989) or the magnitude of the cytonuclear disequilibria (Arnold, 1993). Alternatively, with the knowledge of the width of the hybrid zone and the dispersal rate of the organisms, one could estimate the number of genes under selection and the number of genes contributing to reduced hybrid fitness, after a model from Szymura and Barton (1991). Dispersal abilities of watersnakes may be deduced either from previous studies of movement involving *Nerodia sipedon* or *N. fasciata* (e.g., Stickel and Cope, 1947; Fitch, 1958; Fraker, 1970; Michot, 1981; Tiebout and Cary, 1987, Greshock, 2002) or indirectly from linkage

disequilibria between characters (molecular and morphological). The latter method supposedly is more accurate than direct measurements of dispersal, as it gives an estimate resulting from the period since the existence of the hybrid zone and therefore does not miss occasional long-distance migrants or sporadic movements of an entire population (Barton and Hewitt, 1989).

Another point of interest concerns the occurrence of "*Nerodia fasciata*-like" traits in *N. sipedon* from locations up to 100 km away from current contact areas (e.g., Smith, 1961; Conant, 1963; Conant et al., 1990). Differential selection on certain characters would lead to different dispersal abilities. For example, introgressive characters detectable outside the current zone of sympatry could be the result of such uncoupled characters (Arnold et al., 1987; Barton and Hewitt, 1989; Hewitt, 1989; Dowling and Hoeh, 1991; Dowling and DeMarais, 1993; Parsons et al., 1993). Alternatively, such characters could represent natural variation or the retention of a plesiomorphic character from an earlier ancestor (Wiley, 1981). However, if such a pattern occurs mainly near the hybrid zone, the pattern is most likely due to introgression (Harrison, 1990).

Finally, study of the hybrid zone should illuminate the evolutionary history of *Nerodia sipedon* and *N. fasciata* by including paleontological evidence, where available. Their current distribution reflects ecological and historical factors possibly associated with the southward progression of the Pleistocene glaciers that were responsible for the original separation of the common ancestor of *N. sipedon* and *N. fasciata* into isolated refugia. When the ice sheet retreated, range expansion northward may have brought the formerly isolated populations together, forming a zone of secondary contact that exhibits multiple concordant character clines. Although complete reproductive isolation did not evolve between *N. sipedon* and *N. fasciata*, the narrow hybrid zone indicates partial

incompatibility and implies a history of separation between the two species. The sequence divergence in mtDNA between *N. sipedon* and *N. fasciata* is at least 2.5% (Gaul, 1996) and coincides with a splitting event in the middle of the Pleistocene, which begun 2.48 million BP at the Gauss-Matuyama Chronozone. Several range contractions and expansions induced by glacial cycles would have accumulated multiple allelic differences in various character systems (Mayr, 1963; Thorpe, 1984, 1987; Hewitt, 1989).

Tectonic movements can have similar effects, such as the splitting of the range of the Japanese brown frog (*Rana japonica*) following the upheaval of a mountain barrier and the subsequent divergence and postglacial secondary contact resulting in the development of a broad hybrid zone due to negative heterosis (Sumida, 1996). Other patterns suggest secondary contact as the cause of a hybrid zone, including taxa that occupy a large variety of habitats (Patton, 1993) and concordance in the location of several hybrid zones (Thorpe, 1979; Bermingham and Avise, 1986; Moore and Price, 1993). I discuss the contribution of historical and ecological factors to the current distribution of *N. sipedon* and *N. fasciata* in the Carolinas by incorporating data from paleogeographic studies of the vegetation (Delcourt and Delcourt, 1981; Whitehead, 1981; Jackson et al., 1997), the herpetofauna (Fay, 1984; Holman, 1976, 1980, 1986), and Quaternary climates (Hibbard, 1960; Ruddiman and Wright, 1987; Pielou, 1992; Dawson, 1992).

SITES AND SAMPLES

Study Sites.—Collection of specimens was concentrated around known and anticipated sites of sympatry near the Fall Zone and the Atlantic Coast of the Carolinas as follows: (1) environs of Augusta in Richmond County, GA, and boundary between Aiken and Edgefield counties, SC; (2) Southern Pines, Moore County, NC; (3) Holts Lake, Johnston County, NC; (4) Greenville area, Pitt and Edgecombe counties, NC; (5) Fayetteville to Cambro Pond, Cumberland and Harnett counties; (6) Elizabeth City area, Camden and Pasquotank counties, NC; (7) lower Roanoke River near Oak City, Martin County, NC; and (8) a coastal contact area along highway 264 through Alligator River National Wildlife Refuge in Dare and Hyde counties, NC. The latter serves to investigate the influence of a salinity ecotone to separate *Nerodia sipedon* from *N. fasciata*. See the GEOGRAPHIC SECTION for maps showing these collection sites, except Holts Lake (3), which is displayed in Figure 2.

Due to time constraints, reevaluation of the published hybrid zone on Shackleford Bank and nearby mainland, Carteret County (Fig. 2), was not attempted. In an effort to reduce the distributional gaps between the ranges of both species, specimens were also collected from localities along potential contact zones between those mentioned above. However, unfavorable habitat and adverse weather conditions limited the collecting success. Putative pure specimens were collected from a distance of more than 25 km of any known hybrid zone or sympatry area (= distant sites). For example, multiple specimens of *Nerodia sipedon* were collected at (1) Pohick Recreational Area, Fairfax County, VA; (2) Mountain Lake Biological Station, Giles, and nearby localities in Craig,

Franklin, and Bland counties, western VA; (3) Tidewater area between the Great Dismal Swamp, Suffolk County, and the City of Virginia Beach, VA; and (4) Tar River Reservoir Lake and Falls Battle Park, Nash County, NC. Multiple *N. fasciata* were acquired from (1) Gardner Creek (lower Roanoke River) near Jamesville, Martin County, NC; (2) Lake Waccamaw, Columbus County, NC; (3) Jamestown, Berkeley County, SC; (4) Savannah River Site, Aiken and Barnwell counties, SC; and (5) localities in Sarasota and Manatee counties, FL. Distant *sipedon*-sites (3) and (4), as well as a distant *fasciata*-site (4) are displayed in the GEOGRAPHIC SECTION, whereas distant *fasciata*-sites (1) and (2) are shown in Figure 2. A list of all snakes with locality data is presented in Appendix 2.

Collecting and Handling of Snakes.—From 1995 to 1999, approximately 330 specimens were collected from selected sympatric and allopatric populations. Diurnal collecting of snakes began after their emergence from hibernation in March and April. By mid-May, collecting usually became more successful during their nocturnal foraging period, while habitats were still searched during daylight. When sighted at night, snakes were easily approached by foot or in a canoe and were captured by hands or tongs. Road cruising was the principal method of locating snakes in the Alligator River National Wildlife Refuge. Attempts to collect using minnow traps, as described by Gaul (1996) and Barbara Dietsch (pers. com.), were unsuccessful.

For each snake, time of capture, date, an identification number, and precise location were recorded. Ecological data were obtained for approximately 200 specimens before their transport to the laboratory. In the lab, a blood sample was taken from each specimen and numerous external morphological data were recorded. Most snakes were

photographed, scale-clipped with the individual identification number, and later returned to their sites of capture. Gravid snakes were maintained until parturition and then released at their capture sites with their neonates. Approximately 10% of snakes were preserved as voucher specimens and will be deposited in a recognized institutional collection.

MORPHOLOGICAL SECTION

MATERIALS AND METHODS

Forty morphological characters describing features of color pattern, body proportions, head shape, and scutellation were recorded from each specimen. Characters were selected that have been used as primary diagnostic features or were frequently found to distinguish *Nerodia fasciata* and *N. sipedon* (Clay, 1938; Conant, 1963; Schwaner and Mount, 1976; Behler and King, 1979; Blaney and Blaney, 1979; Seyle, 1980; Morris, 1987; Behler and King, 1979; Conant and Collins, 1991). Other characters were selected based upon a study by the author of another natricine snake (Mebert, 1993). For illustrations and definitions of characters, see list and figures 32-35 in Appendix 1.

Color Pattern.—Features of color pattern were evaluated, including: (1) prominence of postocular stripe; (2) prominence of shadow spots between the dorsal interspaces; (3) serration of dorsal bands and lateral blotches; (4) shape of ventral markings; (5) numbers of ventral scutes covered or touched by the extension of lateral bands/blotches; (6) number of complete dorsal bands; total number of (7) dorsal and (8) lateral blotches; (9) mean width of dorsolateral bands; (10) mean width of light interspaces across the first 10 crossbands or lateral blotches. Characters (9) and (10) were measured as the number of scales covered by the bands, or interspaces respectively, along the suture with the ventral scales. Characters 1–4 were evaluated with a modified hybrid index of Anderson (1949) using a scale from 1 (typical *Nerodia sipedon*) to 5 (typical *N. fasciata*).

A few diagnostic pattern characters for *Nerodia sipedon williamengelsi* (Conant and Lazell, 1973) were measured as well. However, low sample size (n = 13) and extensive

variation, especially among snakes collected from the subspecies' contact area with *N. fasciata,* rendered the measurements of minimal value in this analysis.

Scutellation.—Aspects of scutellation were examined, including: (11) number of postocular, (12) ventral, (13) preventral, and (14) subcaudal scales; (15) identity of supralabial scales contacting the eye; (16) number of dorsal scale rows at the 10th ventral scute; (17), (18), (19) reduction pattern of dorsal scale rows on trunk and (20), (21), (22) on tail, recorded as ventral or subcaudal level where reduction occurred. Many characters associated with the scale reduction pattern, such as scale row additions and identity of scale row being reduced, were recorded only for the preliminary sample (n = 115) due to time constraints.

Body Proportions.—Features of body proportions were measured, namely: (23) height and (24) width of rostral scale; (25) eye diameter; (26) maximum distance between the lateral surfaces of the eyes; (27) distance from eye to snout; (28) distance between eye and nostril; (29) height of canthus rostralis anterior to eyes; (30) intercanthal distance anterior to eyes; (31) height of canthus rostralis posterior to nostrils; (32) intercanthal distance posterior to nostrils; (33) distance between nostrils; (34) length and (35) width of lower jaws; (36) snout-vent length and (37) tail length.

Three characters of head shape -- (38) angle, (39) curvature, and (40) slope of the canthus rostralis -- were measured with a modified hybrid index adapted from Anderson (1949). The characters reflect the taller, broader head of *Nerodia sipedon.* However, these characters were recorded only for a limited sample size due to their imprecision and the lack of a clear definition in earlier studies (Conant, 1963; Schwaner and Mount, 1976; Seyle, 1980).

After a preliminary analysis of 115 specimens using one-way ANOVA, acquisition of subsequent data concentrated on 13 morphological characters that (1) revealed significant differences among the groups tested, (2) were accurate to measure, or (3) needed a larger sample size to analyze reliably. Morphological data were collected from 168 adult specimens. For the final analysis, the data were divided into meristic and metric sets to facilitate statistical handling and interpretation.

Initial assignment of captured snakes to a phenetic group was based mostly on a combination of perceived differences in color pattern and geographic location of capture (i.e., within or outside of a contact zone between the two species). Abbreviations used in the text for these phenetic groups are:

s (distant *sipedon*): *N. sipedon* from areas > 25 km away from the contact zone

cs (contact zone *sipedon*): phenetic *N. sipedon* from the contact zone

x (hybrids): phenetic intermediates (putative hybrids) from the contact zone

cf (contact zone *fasciata*): phenetic *N. fasciata* from the contact zone

f (distant *fasciata*): *Nerodia fasciata* from areas > 25 km away from the contact zone.

Statistical analyses of ANOVA, ANCOVA, Chi-square, and DFA were performed using SAS System for Windows (v. 8.02, SAS Institute, Inc., Cary, NC, 2002). Bilateral characters were averaged. Data were tested for normality and were transformed, if necessary, to achieve normality and homogenous variances (see RESULTS for transformations applied to specific characters). A few outliers were winsorized with their next closest values within their respective group to achieve normality. The df, mean squares and F-values were subsequently adjusted for winsorized data sets, but there was no observable difference in the interpretation of the original (unwinsorized) and winsorized results.

Most measurements of lengths were log-transformed (natural log) to render potential curvilinear relationships linear and to reduce heteroscedacticity of variances (Zar, 1999; King, 2000). For group comparisons, regression residuals were produced to reduce the effect of animals from different size classes within a group. For example, several head proportions were measured against the jaw length as a covariate, whereas the snout-vent length was used as a covariate for the tail length and the jaw length. In the latter measurement the jaw length was subtracted from the snout-vent length (covariate) before analysis.

After these adjustments, data sets for most phenetic groups separated by sex achieved normality. Only a few groups yielded some meristic characters with $P > 0.01$ and < 0.05, and thus the distribution of character values was sufficiently close to the 0.05 level of Type I error to reliably interpret the results with a robust ANOVA. Homogeneity of variances among groups (sex and phenetic groups) was tested with Bartlett's Test, if data were normally distributed, or with the Brown-Forsythe's Test, if data were slightly non-normal at the 0.05 level, i.e., $P > 0.03$ and < 0.05.

Sexual dimorphism was tested with ANCOVA for metric characters for each phenetic group (**s**, **cs**, **x**, **cf**, **f**), and with ANOVA for meristic characters, but only for **cf**, **f**, and **s**, because samples were too small ($n \leq 3$) for the males of **cs** and **x**. To increase sample size, data sets of both sexes were combined for characters that showed no significant sexual dimorphism within each phenetic group; otherwise, sexes were analyzed separately. Pooled samples were retested for normality.

For among-group testing, F-values of meristic data were calculated using ANOVA, whereas ANCOVA was applied to metric data. When there is a statistical linear relationship between two variables, the residual variances are smaller than the total

variances, making ANCOVA a more sensitive test to detect differences among groups than ANOVA (Jolicoeur, 1999). Welch's ANOVA was employed if variances were not homogenous, according the Brown-Forsythe calculation. In instances of severe deviation from the assumptions, the data were ranked and analyzed with a Kruskal-Wallis Test, which yields statistic H that approximates a chi-square$_{(a-1)}$ distribution (Sokal and Rohlf, 1995). Tukey's studentized range (HSD) tests, with adjustments for different among-group sample sizes, were applied to investigate mean differences among taxonomic groups.

A preliminary pairwise correlation analysis was conducted to investigate relationships among meristic characters and to identify potential redundancy. The large overlap of most character values between phenetic groups and sexes allowed the pooling of data for each character to improve normality requirements. Pearson correlation coefficients were calculated for all characters with normal distribution, but Spearman analysis was applied to the number of complete dorsal bands, which were not normally distributed.

Redundancy in the data set, relationships among groups of characters, and the value of characters to discern among phenetic groups were investigated with discriminant function analyses (DFA), a dimension-reduction technique (= canonical discriminant analysis, SAS System for Windows, v. 8.02, SAS Institute, Inc., Cary, NC, 2002). DFA with pooled within-group variances derives canonical variables, which are linear combinations of interval variables. The new variables of canonical components have mean scores of zero. The two most distinguishing groups yield scores on opposite sides of the mean. For instance, *Nerodia sipedon* would yield all positive scores of canonical variables and "pure" *N. fasciata* would reveal negative scores. Hybrids should produce scores intermediate (closer to zero) to those exhibited by their parental species.

The first canonical variable (= first component or axis) represents the linear combination of the original variables with the highest possible multiple correlation with the phenetic groups. The second canonical variable is obtained by finding a linear combination uncorrelated with the first canonical variable that produces the highest possible correlation with the groups. Canonical variables analyze between-group variation similar to total variation analyzed by a principal components analysis. Six different sets of canonical variables were generated, each containing a different sample size and combination of original and transformed variables. Canonical scores are calculated for each specimen to evaluate its position on a scale containing means and ranges of scores from all phenetic groups. Significance of differences of score means among phenetic groups and range of scores were analyzed with ANOVA.

MERISTIC RESULTS

Meristic characters were tested for their suitability to differentiate among *Nerodia sipedon* (**s**), *N. fasciata* (**f**), phenetic *sipedon* and *fasciata* from the contact zone (**cs**, **cf**) and putative hybrids (**x**). Historically, *N. sipedon* and *N. fasciata* have been most easily distinguished by the arrangement of posterior dorsal bands/blotches, which are complete crossbands in *N. fasciata* but consist of separated, alternating dorsal and lateral blotches in *N. sipedon* (Conant, 1963; Schwaner and Mount, 1976; Behler and King, 1979; Blaney and Blaney, 1979; Seyle, 1980; Tennant, 1997; Conant and Collins, 1991, see also figures 32-34 in Appendix 1). Other meristic characters were applied with little success by Conant (1963) and Seyle (1980) to distinguish *N. sipedon* and *N. fasciata*. In this study, the measurement of the reduction pattern of dorsal scale rows added to the differentiation

among phenetic groups. A character describing which supralabial scales contact the eye showed no among-group differences ((^{2} = 9.49, P = 0.6606), and was removed from further analysis.

Results of the correlation analyses are displayed in Table 1. Characters on caudal scale row reductions (e.g., Tred8, Tred6) were excluded from the correlation analysis due to their sex-dependent character expression. Normality tests revealed only the width of latero-ventral bands (VLBa) slightly non-normal (P = 0.048). LBa, the number of lateral bands/blotches from the neck to the vent, is omitted from the following section because it shows an overall high correlation (redundancy) with the number of dorsal bands/blotches (DBa) (r = 0.90, P < 0.0001, Table 1) and both characters constitute the same measurement in *Nerodia fasciata*. LBa-values were only slightly offset from DBa-values in *N. sipedon* due to the separation of the crossbands into dorsal and lateral blotches posteriorly. This feature added extra 2-3 lateral blotches in *N. sipedon*.

Several meristic characters showed significantly different distributions in the distant groups of *Nerodia sipedon* (**s**) and *N. fasciata* (**f**) and related groups from the contact zones (**cs**, **x**, **cf**). However, the number of complete crossbands posterior to the neck (CBa) remained the only external feature to easily distinguish distant *N. sipedon* from distant *N. fasciata* in the Carolinas (Fig. 3). Snakes superficially categorized as hybrids (**x**) constituted the only group to have values of this character overlapping with both

TABLE 1. Pairwise correlations among meristic characters (Pearson product-moment). Data for both sexes and phenetic groups pooled including *Nerodia sipedon* (**s**) and distant *N. fasciata* (**f**) from > 25 km distant from the contact zone, and phenetic *N. sipedon*, phenetic *N. fasciata*, and intermediately patterned snakes recognized as putative hybrids from the contact zone (**cs, cf, x**). Char. = Character

1. Char	2. Char	*N*	r	*P*
DBa	VPV	154	-0.3925	< 0.0001
IBa	VPV	122	0.4181	< 0.0001
IBa	DBa	123	-0.6726	< 0.0001
LBa	VPV	159	-0.4554	< 0.0001
LBa	DBa	158	0.9008	< 0.0001
LBa	IBa	126	-0.7197	< 0.0001
VLBa	VPV	158	-0.5413	< 0.0001
VLBa	DBa	158	0.3017	0.0001
VLBa	IBa	126	-0.6851	< 0.0001
VLBa	LBa	162	0.3722	< 0.0001
WBa	VPV	123	-0.4326	< 0.0001
WBa	DBa	124	0.1396	0.1221
WBa	IBa	126	-0.6533	< 0.0001
WBa	LBa	127	0.1935	0.0293
WBa	VLBa	127	0.8239	< 0.0001
Red19	VPV	158	-0.2681	0.0007
Red19	DBa	153	0.2542	0.0015
Red19	IBa	121	-0.0899	0.3269
Red19	LBa	158	0.2853	0.0003
Red19	VLBa	158	0.1327	0.0965
Red19	WBa	122	0.0903	0.3224
Red21	VPV	159	-0.2865	0.0003
Red21	DBa	154	0.2351	0.0033
Red21	IBa	122	-0.2053	0.0233
Red21	LBa	159	0.3351	< 0.0001
Red21	VLBa	159	0.2292	0.0037
Red21	WBa	123	0.1721	0.0570
Red21	Red19	161	0.7601	< 0.0001

parental species. The number of dorsal bands/blotches (DBa) showed a higher mean in **s** and **cs** than the other phenetic groups (Table 2). Although means of DBa in **s** and **cs** were inflated by the contribution of six *N. sipedon williamengelsi* with significantly higher numbers of DBa (two sample t-test for unequal variances: $t = -3.843$, $P = 0.0008$; mean: *N. s. sipedon* = 31.3, *N. s. williamengelsi* = 34.8), the small sample size from *N. sipedon williamengelsi* resulted in no relevant changes for the overall among-group comparison. DBa showed a negative correlation with the width of interspaces (IBa), indicating that the increase in the number of bands resulted in a decrease of the spaces between the bands (Table 1).

The width of dorsolateral bands (WBa; the number of dorsal scales covered by the band adjacent to the ventral scales) is positively correlated with the width of ventro-lateral bands (VLBa), which are the ventral extensions of the former character (Table 1). Both characters were significantly wider adjacent to the ventral scales in *sipedon*-like snakes (**cs**, **s**) than in the phenetic hybrids (**x**) or the distant *fasciata* (**f**), whereas only the distant *sipedon* (**s**) varied significantly from **cf** (Table 2 and Fig. 3). This pattern was approximatively reversed to the one found in interspaces IBa, as these two characters were mutually compensatory in closely related groups (wider bands with narrower interspaces, and vice versa). As in DBa, WBa and VLBa showed a strong negative correlation with IBa, i.e., they indicated that a higher number of bands resulted in narrower interspaces, corresponding with wider bands laterally and ventrally (Table 1).

Several characters of scutellation indicated that *Nerodia sipedon* has higher numbers of scales. For instance, its mean values were higher for the number of ventral scales (VPV), postoculars (PO) and dorsal scale rows at the 10th ventral scale (SR10) (Tables 2

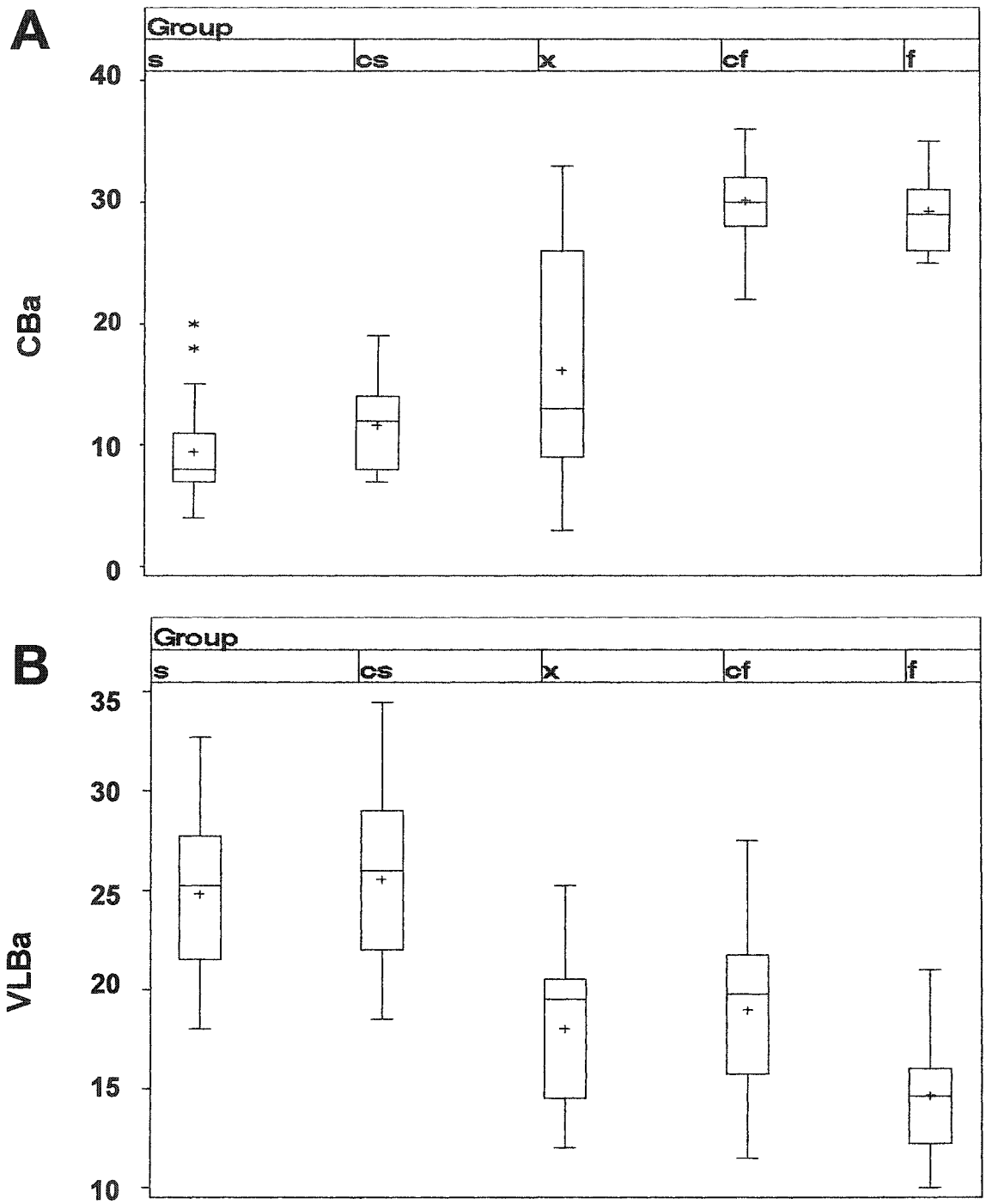


FIG. 3. Distribution of CBa (A) and VLBa (B) among phenetic groups. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**. Values exceeding a studentized residual of $\pm$ 3.64 at a significance level of 0.05 are considered outliers and marked as asterisks.

TABLE 2. Descriptive statistics and results of ANOVA of meristic characters for phenetic groups. Data for *Nerodia sipedon* (**s**); *N. fasciata* (**f**) from > 25 km distant from the contact zone; phenetic *N. sipedon* (**cs**), phenetic *N. fasciata* (**cf**); putative hybrids recognized as intermediately patterned snakes (**x**) from within the contact zone. Abbreviations for characters are defined in Appendix 1.

DBa							
	Original data				ANOVA / groups		
Phenetic group	Observed mean	SE	*N*	Range	Tukey HSD diff. from	F	*P*
s	32.00	0.64	32	22-38	x, cf, f	7.34	< 0.0001
cs	32.80	0.56	25	28-38	x, cf, f		
x	29.64	0.5	25	25-35	s, cs		
cf	30.14	0.36	57	22-36	s, cs		
f	29.20	0.69	20	25-35	s, cs		
CBa							
s	9.43	0.70	30	4-20	x, cf, f	Welch: 137.00	< 0.0001
cs	11.64	0.72	25	7-19	x, cf, f		
x	16.16	1.90	25	3-33	s, cs, x, cf, f		
cf	30.14	0.37	56	22-36	s, cs, x		
f	29.26	0.72	19	25-35	s, cs, x		
IBa							
s	23.95	1.18	25	14.00-37.00	x, cf, f	12.21	< 0.0001
cs	22.51	1.31	17	11.50-31.25	x, cf, f		
x	29.73	1.01	23	22.25-38.25	s, cs		
cf	28.70	0.74	40	18.00-38.50	s, cs		
f	31.65	1.06	21	23.75-40.75	s, cs		

TABLE 2. Continued.

Phenetic group	Original data: Observed mean	SE	*N*	Range	ANOVA / groups: Tukey HSD diff. from	F	*P*
WBa							
s	24.38	0.81	25	16.50-31.00	x, cf, f	12.39	< 0.0001
cs	23.41	1.00	17	18.75-30.25	x, f		
x	18.65	0.62	24	12.50-25.50	s, cs	Data transformation applied	
cf	20.54	0.57	40	12.00-28.50	s	log	
f	18.06	0.82	21	12.25-26.00	s, cs		
VLBa							
s	24.82	0.69	33	18.00-32.75	x, cf, f	37.30	< 0.0001
cs	25.56	0.81	26	18.50-34.50	x, cf, f		
x	18.00	0.78	25	12.00-25.25	s, cs, f		
cf	18.98	0.53	58	11.50-27.50	s, cs, f		
f	14.68	0.71	22	10.00-21.00	s, cs, x, cf, f		
V (incl. PV)							
s	134.88 (1.97)	0.44	32	133-139	x, cf, f	22.08	< 0.0001
cs	135.88 (1.88)	0.62	25	130-140	x, cf, f		
x	130.58 (1.76)	0.56	24	123-136	s, cs	Data transformation applied	
cf	132.88 (1.50)	0.42	59	127-143	s, cs, f	Reciprocal of sum (V+PV)	
f	129.08 (1.67)	0.65	24	122-136	s, cs, cf		

TABLE 2. Continued.

Phenetic group	Original data: Observed mean	SE	*N*	Range	ANOVA / groups: Tukey HSD diff. from	F	*P*	ANOVA / sex: F	*P*
Red21 females									
s	82.44	1.69	16	75-98	cf, f	4.67	0.0016	2.89	0.0994
cs	82.89	1.30	18	70-93	cf, f			3.19	0.0873
x	78.21	1.14	19	64-85	none			0.84	0.3691
cf	77.09	1.11	43	56-91	s, cs			7.01	0.0101
f	75.86	1.53	14	64-85	s, cs			6.31	0.0198
Red21 males									
s	79.31	1.23	16	70-89	cf, f	8.10	< 0.0001		
cs	78.86	1.32	7	72-83	cf, f				
x	75.67	3.47	6	66-86	none				
cf	71.75	0.79	14	68-77	s, cs				
f	70.00	1.74	10	63-82	s, cs				
Red19 females									
s	104.13	2.10	16	92-125	none	3.46	0.0106	6.55	0.0158
cs	111.39	2.00	18	93-126	x, cf, f			6.74	0.0162
x	102.39	1.91	18	85-121	cs			0.53	0.0738
cf	103.58	1.32	43	83-118	cs			14.22	0.0004
f	102.93	2.35	14	91-115	cs			15.12	0.0008
Red19 males									
s	98.81	1.60	16	86-111	none	2.89	0.0318		
cs	101.57	3.20	7	88-113	none				
x	98.83	4.04	6	86-109	none				
cf	93.29	2.56	14	71-113	none				
f	90.90	1.58	10	84-102	none				

TABLE 2. Continued.

Phenetic group	Observed mean	SE	*N*	Range	Tukey HSD diff. from	Chi-Square	*P*	F	*P*
	Original data				Kruskal-Wallis Test / groups			ANOVA / sex	
Tred8 females									
s	7.31	0.26	13	6-9	cs, x, cf	14.12	0.0069	45.45	< 0.0001
cs	8.93	0.46	15	6-12	s			.	.
x	9.25	0.41	8	4-5	s			.	.
cf	9.09	0.33	22	6-12	s			88.26	< 0.0001
f	8.33	0.40	12	6-10	none			79.71	< 0.0001
Tred8 males									
s	13.21	0.89	14	7-18	cf, f	11.22	0.0242		
cs	15.33	0.33	3	15-16	none				
x	14.00	.	1	6.00					
cf	17.25	0.73	8	14-20	s				
f	16.44	0.84	9	12-19	s			Kruskal-Wallis / sex	
Tred6 females									*P*
s	18.42	0.80	12	15-23	cs, cf, f	14.36	0.0062	11.35	0.0008
cs	24.00	1.59	15	16-39	s			.	.
x	24.00	1.10	8	18-29	none			.	.
cf	24.48	1.00	21	16-31	s			11.75	0.0006
f	23.50	1.25	12	15-30	s			10.77	0.0010
Tred6 males									
s	27.50	1.15	14	19-35	none	9.15	0.0575		
cs	28.00	1.00	3	27-30	none				
x	32.00	.	1	32.00					
cf	31.25	0.90	8	27-34	none				
f	31.56	1.38	9	25-39	none				

and 3). Ranking the PO data confirmed the previous results (Kruskal-Wallis: (^{2} = 19.85, P = 0.0005). *Sipedon*-like snakes received the highest ranks (mean: **cs** = 102.48, **s** = 93.53), *fasciata*-like snakes the lowest ranks (mean: **cf** = 71.20, **f** = 69.25), and the phenetic hybrids yielded intermediate values (mean for **x** = 80.31). SR10 ranged from 19 to 25 scale rows. Low values were expressed by one hybrid (19 rows) and a hybrid and a distant *fasciata* (20 rows), but high values were found in one **s** and **f** (25 rows), and one **cf** (24 rows). Table 3 shows that over 65% of **s**, **cs**, and **x** expressed at least 23 scale rows at SR10, but only half of **cf** and **f** yielded 23 rows.

Normally, snakes possessed an odd number of dorsal scale rows along the length of the trunk. However, short body segments between two ventral scale positions of scale row reductions yielded an even number of scale rows. For example, only one to two dorsal scales separated the even scale row segment between the scale row reductions from 22 to 21 rows. Mean scale distance between paired reductions Red20 and Red19 (mean ~ 1.5 scales, range = 0–28 scales) was twice that between the more anterior reductions Red22 and Red21 (mean ~ 3.0 scales, range = 0–12 scales). All scale row reductions were sexually dimorphic, with females reducing scale rows on the trunk closer to the vent and males reducing rows on the tail more posteriorly. P-values from an ANOVA comparing sex differences within phenetic groups ranged from 0.0101 (**cf**) to 0.0994 (**s**) in Red21, and from 0.0004 (**cf**) to 0.0738 (**x**) in Red19 (Table 2 and Fig. 4). In both sexes, **s** and **cs** had reduced dorsal scale rows more posteriorly than **f** or **cf**, albeit the situation was more pronounced in Red21 than in Red19.

TABLE 3. Proportions and results of chi-square analysis of postoculars (PO) and dorsal scale rows at the 10th ventral scale (SR10). Data for *Nerodia sipedon* (**s**); *N. fasciata* (**f**) from > 25 km distant from the contact zone; phenetic *N. sipedon* (**cs**), phenetic *N. fasciata* (**cf**); putative hybrids recognized as intermediately patterned snakes (**x**) from within the contact zone. Abbreviations for characters are defined in Appendix 1.

	PO					SR10				
Group	< 3	≥ 3	*N*	Chi-square	*P*	≤ 22	≥ 23	*N*	Chi-square	*P*
s	9.38	90.63	32	19.17	0.0007	28.12	71.88	57	21.31	< 0.0001
cs	0.00	100.00	25			29.17	70.83	24		
x	25.00	75.00	24			44.00	66.00	23		
cf	36.84	63.16	57			52.63	47.37	32		
f	37.50	62.50	24			47.82	52.18	25		

Compared to the F-tests, the weaker Tukey tests did not detect any group differences, although the power of the analysis (1-B) resulted in a 70% probability of detecting potential differences. To increase sensitivity of the analysis, two-sample t-tests were applied to test phenetic groups of males for differences. Only two pairings differed significantly: **cs-f** ($t = 3.278$, $P = 0.0051$), and **f-s** ($t = -3.192$, $P = 0.0041$). In conclusion, a further caudad position of scale row reductions in *sipedon*-like snakes resulted in a slightly higher dorsal scale counts in the posterior half of their trunk (Table 2 and Fig. 4). A strong relationship between the two positions for scale row reductions was demonstrated with a high correlation coefficient value (Table 1).

Sexual dimorphism in the position of caudal scale row reductions to 8 rows (Tred8) and 6 rows (Tred6) was tested only in phenetic groups **s**, **cf**, and **f**, which had sufficient sample sizes for statistical procedures. Males reduced scale rows significantly further posteriorly than females at both reduction positions with P-values < 0.0001 for Tred8 and P-values ranging from 0.0006 (**cf**) to 0.0010 (**f**) in Tred6. At both positions, **s** reduced caudal scale rows closer to the vent compared to specimens from the other groups, but differences among females were more pronounced than among males (Table 2 and Fig. 4).

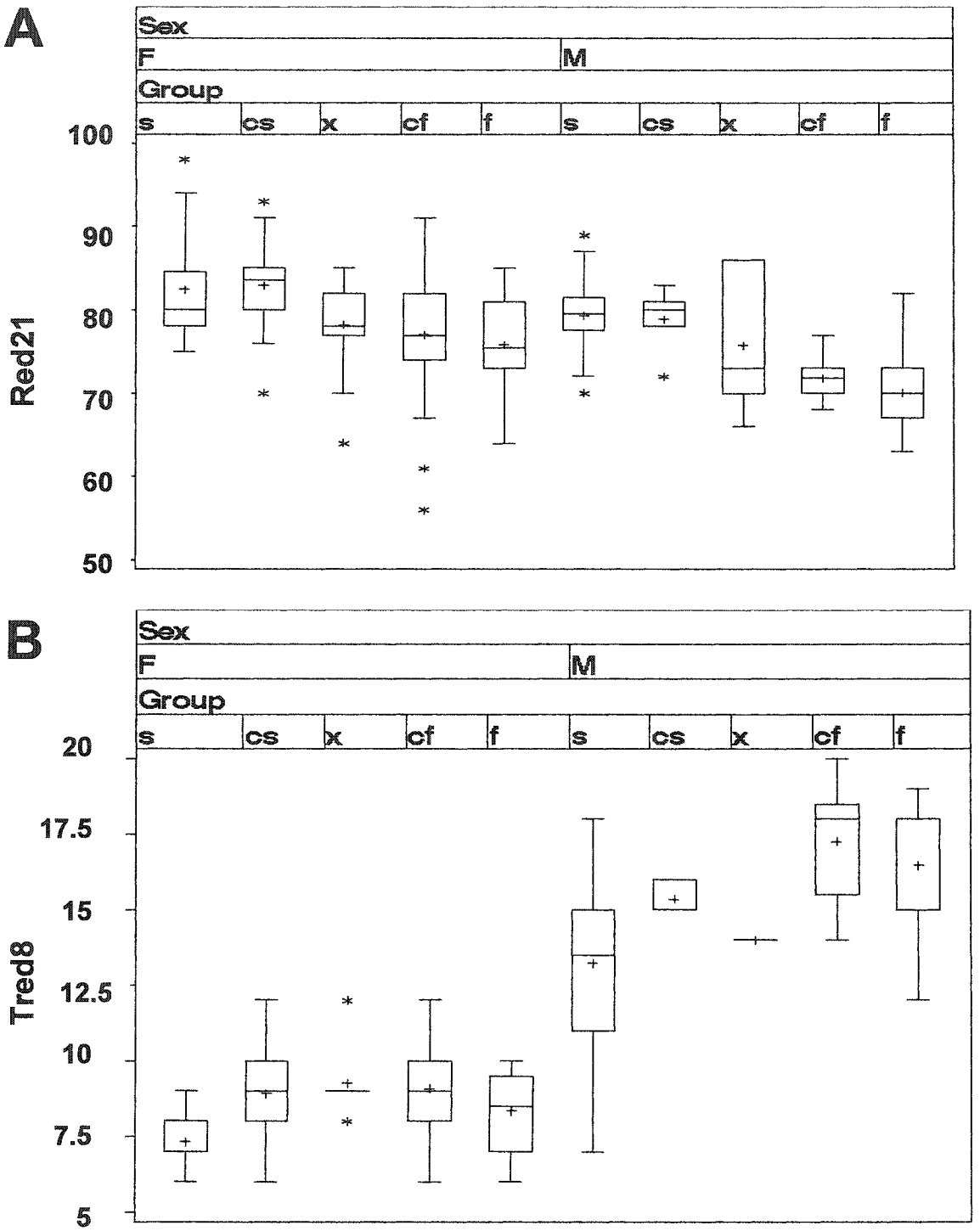


FIG. 4. Distribution of scale row reductions Red21 (**A**) and Tred8 (**B**) among phenetic groups and sexes. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**. Values exceeding a studentized residual of $\pm$ 3.64 at a significance level of 0.05 are considered outliers and marked as asterisks.

MORPHOMETRIC RESULTS

The analysis of cephalic measurements principally served to clarify the frequently observed differences in head shape and size between *Nerodia fasciata* and *N. sipedon*. The head of *N. sipedon* generally appears to be broader, with a higher and less sloping snout, smaller eyes, and a rounder canthus rostralis (Conant, 1963; Schwaner and Mount, 1976; Seyle, 1980). Blaney and Blaney (1979) and Morris (1987) reported larger eyes in *N. fasciata* compared to *N. sipedon*.

Pearson product-moment correlation analysis yielded relationships in a pairwise character analysis of cephalic proportions with high correlation coefficients (r-values) of ca. 70% or more. Such strong correlations can be expected among related length measurements, as the various head proportions likely grow in concert. However, variation may be introduced through allometric growth, as for the eye size, which is proportionately larger at birth.

Similar to the application of meristic data, the analysis of morphometric characters may identify potential differences between parental species and intermediate specimens, indicating potential hybridization. Such phenetic data can be compared with genetic data to reveal perceived versus true designation of differences between species and their intermediates. Results of descriptive statistics and ANCOVA are provided in Table 4.

Sexual Dimorphism.—Among three groups (**s**, **cf**, **f**) with sufficient sample sizes to test for sex differences in cephalic proportions, only snout length measurements (DiES and DiEN), jaw length (JawL), and the width of the snout (RoW) were sexually dimorphic in at least one group. For instance, *fasciata* males from the contact zone (**cf**) were the only

group to yield significantly longer distances between eyes and nostrils (slope: $F_{1,27}$ = 0.82, P = 0.3748; sex: $F_{1,27}$ = 13.97, P = 0.0009). However, such sexual dimorphism affected all three groups when the snout length was measured from the eyes to the snout tip (DiES) (**cf**-slope: $F_{1,27}$ = 0.01, P = 0.9332; sex: $F_{1,27}$ = 10.48, P = 0.0033; **f**-slope: $F_{1,15}$ = 0.57, P = 0.4646; sex: $F_{1,15}$ = 4.30, P = 0.0571; **s**-slope: $F_{1,26}$ = 0.21, P = 0.6495, sex: $F_{1,26}$ = 5.29, P = 0.0304). The males showed a wider rostral scale (RoW) in cf (slope: $F_{1,27}$ = 0.35, P = 0.5585; sex: $F_{1,27}$ = 5.65, P = 0.0249), but the differences were marginal in **s** (slope: $F_{1,15}$ = 2.10, P = 0.1648; sex: $F_{1,15}$ = 3.80, P = 0.0636). Females had longer jaws (JawL) in relation to the trunk length in **cf** (slope: $F_{1,29}$ = 0.07, P = 0.7879; sex: $F_{1,29}$ = 5.46, P = 0.0268), and **s** (slope: $F_{1,25}$ = 0.39, P = 0.5399; sex: $F_{1,25}$ = 7.79, P = 0.0101), whereas males produced relatively longer tails (**cf** slope: $F_{1,36}$ = 0.60, P = 0.4435; sex: $F_{1,36}$ = 68.49, P < 0.0001; **f**-slope: $F_{1,18}$ = 0.05, P = 0.8767; sex: $F_{1,18}$ = 54.33, P < 0.0001; **s**-slope: $F_{1,20}$ = 0.34, P = 0.5685; sex: $F_{1,20}$ = 39.42, P < 0.0001; Fig. 6).

Group Differences.—The results confirmed that *Nerodia sipedon* has proportionately larger cephalic measurements than *N. fasciata* in most of the selected characters (Table 4). Distant *N. sipedon* (**s**) showed significantly broader heads than distant *N. fasciata* (**f**) with regard to the posterior tips of the jaws (JawW; Fig. 5), the area enclosed by the canthus rostralis between the eyes and the nostrils (EyCW to SCW), and the snout tip (RoW; only in females). The anterior portion of the head was also significantly taller in *N. sipedon* than in *N. fasciata,* as shown by measurements taken at the snout tip (RoH; Fig. 6) and the nostrils (SCH). Conversely, analyses confirmed the larger eye

TABLE 4. Descriptive statistics and results of ANCOVA of morphometric characters for phenetic groups. Data for *Nerodia sipedon* (**s**); *N. fasciata* (**f**) from > 25 km distant from the contact zone; phenetic *N. sipedon* (**cs**), phenetic *N. fasciata* (**cf**); putative hybrids recognized as intermediately patterned snakes (**x**) from within the contact zone; character abbreviations in Appendix 1.

Phenetic group	Original data (mm) Observed mean	SE	*N*	Range	ANCOVA Tukey HSD diff. from	F_{slopes}	P_{slopes}	F_{group}	P_{group}	r^2	Transformations of orig. char. / covariate
JawW											
s	18.03	0.98	27	9.15-28.96	f	0.12	0.9736	4.94	0.0011	0.92	lnJawW / lnJawL
cs	20.24	0.76	19	12.30-25.22	f						
x	20.41	1.28	11	3.54-27.02	f						
cf	18.69	0.56	31	12.36-23.94	none						
f	19.50	1.04	17	12.40-28.80	s, cs, x						
DiET											
s	12.26	0.48	27	7.65-17.52	cf	0.38	0.8220	1.28	0.2855	0.88	lnDiET / lnJawL
cs	13.68	0.48	14	9.92-16.20	none	One outlier exceeding a studentized residual value of					
x	13.82	0.72	10	9.60-17.34	none	± 3.59 removed					
cf	13.43	0.40	25	9.06-16.36	s	0.55	0.6964	2.80	0.0309	0.92	
f	13.70	0.60	16	9.60-19.06	none						
EyCW											
s	7.86	0.33	27	4.2-11.3	f	0.46	0.7652	3.22	0.0155	0.83	EyCW / JawL
cs	8.72	0.30	19	5.6-11	none						
x	8.21	0.41	11	6.3-10.02	none						
cf	8.03	0.19	31	5.88-10.04	none						
f	8.38	0.39	17	5.08-11.24	s						
SCW											
s	4.73	0.23	27	2.50-7.30	x, cf, f	2.18	0.0775	11.85	< 0.0001	0.82	lnJawW / lnJawL
cs	4.93	0.19	15	3.44-6.18	x, f						
x	4.42	0.38	10	2.20-6.04	s, cs						
cf	4.66	0.14	28	3.16-5.90	s						
f	4.62	0.28	17	3.00-7.10	s, cs						

TABLE 4. Continued.

Phenetic group	Original data (mm) Observed mean	SE	*N*	Range	ANCOVA Tukey HSD diff. from	F_{slopes}	P_{slopes}	F_{group}	P_{group}	r^2	Transformations of orig. char. / covariate
					DiNo						
s	2.15	0.16	24	0.80-3.80	none	2.27	0.0678	1.77	0.1416	0.62	DiNO / lnJawL
cs	2.58	0.15	16	1.56-3.66	none						
x	2.52	0.20	10	1.52-3.30	none						
cf	2.23	0.09	29	1.10-2.94	none						
f	2.46	0.10	17	1.66-3.24	none						
					EyS						
s	3.98	0.14	27	2.28-5.70	cf, f	1.64	0.1710	5.60	0.0004	0.84	lnEyS / lnJawL
cs	4.63	0.17	19	3.24-5.99	none						
x	4.46	0.20	11	3.18-5.20	none						
cf	4.60	0.11	31	3.56-5.68	s						
f	4.70	0.17	17	3.70-6.38	s						
					EyCH						
s	7.32	0.35	27	3.88-11.19	none	0.62	0.6490	0.61	0.6554	0.93	lnEyCH / lnJawL
cs	8.30	0.29	19	5.50-10.59	none						
x	8.06	0.45	11	5.65-10.19	none						
cf	7.81	0.23	31	4.97-9.67	none						
f	8.37	0.40	17	5.47-11.26	none						
					SCH						
s	5.53	0.27	27	2.60-7.96	x, cf, f	0.97	0.4253	5.21	0.0008	0.86	lnSCH / lnJawL
cs	5.86	0.25	15	4.11-7.42	none						
x	5.60	0.46	10	3.57-8.28	s						
cf	5.50	0.17	28	3.63-6.89	s						
f	5.90	0.28	17	3.88-7.74	s						

TABLE 4. Continued.

Phenetic group	Original data (mm) Observed mean	SE	*N*	Range	ANCOVA Tukey HSD diff. from	F_{slopes}	P_{slopes}	F_{group}	P_{group}	r^2	Transformations of orig. char. / covariate
RoH											
s	3.51	0.17	26	1.60-5.38	x, f	0.75	0.5539	5.54	0.0005	0.77	lnRoH / lnJawL
cs	3.90	0.16	19	2.44-4.58	x, f						
x	3.34	0.31	11	1.74-5.00	s, cs						
cf	3.54	0.09	30	2.48-4.58	none						
f	3.58	0.17	17	2.26-4.76	s, cs						
RoW females											
s	6.46	0.33	13	5.00-9.14	none	0.76	0.5547	3.81	0.0077	0.77	lnRoW / lnJawL
cs	6.56	0.28	16	4.18-8.60	cf						
x	5.69	0.53	10	2.98-8.06	none						
cf	5.75	0.20	22	3.90-7.30	cs						
f	6.89	0.34	9	5.18-8.24	none						
RoW males											
s	4.86	0.29	13	2.85-7.00	none	0.62	0.6068	72.00	0.5837	0.83	lnRoW / lnJawL
cs	5.88	0.24	3	5.40-6.14	none						
x	5.72	0.00	1	5.72	none						
cf	5.49	0.27	8	4.30-6.90	none						
f	5.55	0.36	7	4.30-7.18	none						
DiES females											
s	9.09	0.39	13	7.16-12.05	none	0.13	0.9687	2.11	0.0901	0.94	lnDiES / lnJawL
cs	8.26	0.39	12	5.26--9.85	none						
x	8.21	0.60	9	5.27-10.01	none						
cf	8.40	0.27	22	5.48-9.98	none						
f	9.12	0.54	10	6.27-11.22	none						

TABLE 4. Continued.

Phenetic group	Original data (mm) Observed mean	SE	*N*	Range	ANCOVA Tukey HSD diff. from	F_{slopes}	P_{slopes}	F_{group}	P_{group}	r^2	Transformations of orig. char. / covariate
DiES males											
s	6.49	0.41	14	3.63-9.15	none	1.99	0.1439	0.79	0.5415	0.93	lnDiES / lnJawL
cs	7.73	0.86	3	6.65-9.44	none						
x	7.34	0.00	1	7.34	none						
cf	7.90	0.36	7	6.33-9.38	none						
f	8.07	0.51	7	6.15-9.66	none						
DiEN females											
s	5.73	0.25	13	4.46-7.48	none	0.54	0.7040	0.12	0.9756	0.86	lnDiEN / lnJawL
cs	5.35	0.20	12	3.59-6.06	none						
x	5.72	0.30	8	4.66-6.76	none						
cf	5.42	0.16	21	3.60-6.48	none						
f	5.54	0.42	12	2.45-7.91	none						
DiEN males											
s	4.10	0.23	14	2.43-5.72	cf	0.91	0.4527	2.84	0.0443	0.91	lnDiEN / lnJawL
cs	5.19	0.44	3	4.43-5.97	none						
x	4.96	0.00	1	4.96	none						
cf	5.20	0.30	7	4.16-6.72	s						
f	4.87	0.31	9	3.26-6.15	none						
JawL females											
s	37.98	1.48	13	30.59-48.79	none	0.88	0.4770	2.49	0.0524	0.81	lnJawL / lnSVLJ
cs	36.48	1.35	16	24.90-44.60	none						
x	36.18	2.19	10	23.02-43.05	none						lnSVLJ = ln (SVL - JawL)
cf	35.62	1.01	23	23.87-42.17	none						
f	39.74	2.28	10	29.06-50.29	none						

TABLE 4. Continued.

Phenetic group	Original data (mm, and cm for Tail and SVL) Observed mean	SE	N	Range	Tukey HSD diff. from	ANCOVA F_{slopes}	P_{slopes}	F_{group}	P_{group}	r^2	Transformations of orig. char. / covariate
					JawL males						
s	26.39	1.51	14	16.05-38.96	cf	0.09	0.9654	3.54	0.0197	0.92	lnJawL / lnSVLJ
cs	32.32	1.65	3	29.77-35.41	none						
x	31.61	0.00	1	31.61	none						lnSVLJ = ln (SVL - JawL)
cf	30.88	1.21	8	25.23-36.57	s						
f	32.92	2.12	7	24.63-38.96	none						
					Tail females (cm)						
s	18.95	1.03	11	14.0-24.8	x, cf	0.96	0.4328	5.70	0.0005	0.99	lnTail / lnSVL
cs	19.84	1.24	12	11.5-28.4	x						
x	18.15	1.45	13	10.2-28.1	s, cs						
cf	19.86	0.73	29	11.9-25.8	s						
f	19.20	1.98	11	8.3-27.4	none						
					Tail males (cm)						
s	16.06	1.10	11	8.1-19.7	none	1.15	0.3537	2.02	0.1153	0.99	lnTail / lnSVL
cs	18.87	1.62	3	17.0-22.1	none						
x	19.98	0.70	5	18.4-22.2	none						
cf	19.13	1.20	9	10.6-22.5	none						
f	18.86	1.39	9	12.3-25.3	none						
	SVL femles							SVL males			
s	71.16	3.17	16	47.0-100.7	see for	48.59	2.94	16	23.3-73.4		
cs	70.18	2.87	18	24.9-90.9	column	56.98	5.32	7	31.6-70.5		
x	63.48	3.74	19	33.0-86.0	labels	56.17	2.79	6	46.4-59.2		
cf	68.01	1.80	45	37.0-76.5	at SVL	53.38	2.93	12	26.5-68.3		
f	63.00	6.65	11	26.5-95.9	females	53.80	3.89	10	34.0-71.8		

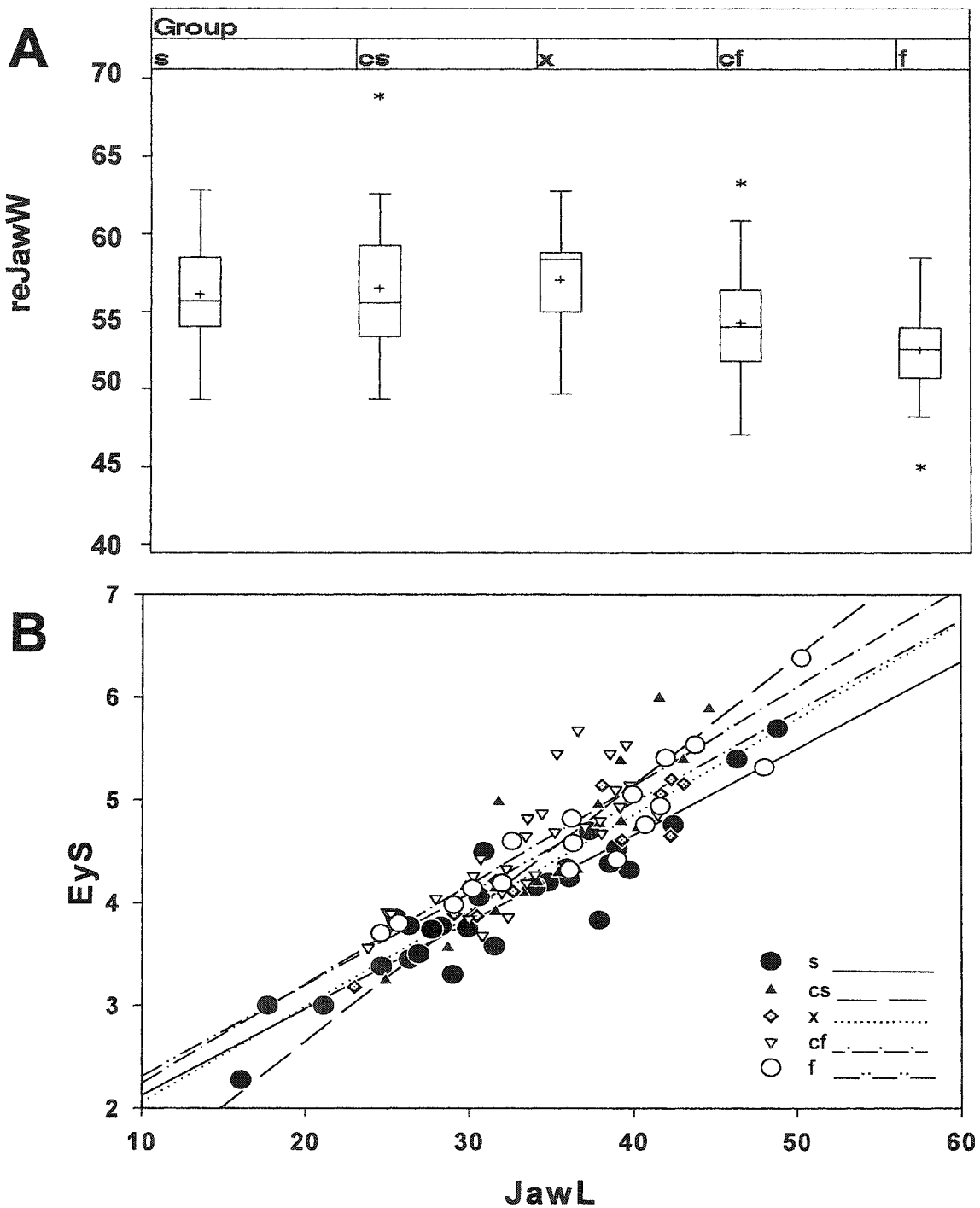


FIG. 5. Distribution of relative jaw width (reJawW) ([JawW/JawL] x 100) among phenetic groups (A) and relationship of eye size (EyS) on jaw length (JawL) in mm (B). Phenetic groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km away from the contact zone; and **x** for phenetic intermediates between **f** and **s**. Values exceeding a studentized residual of $\pm$ 3.64 at a significance level of 0.05 are considered outliers and marked as asterisks.

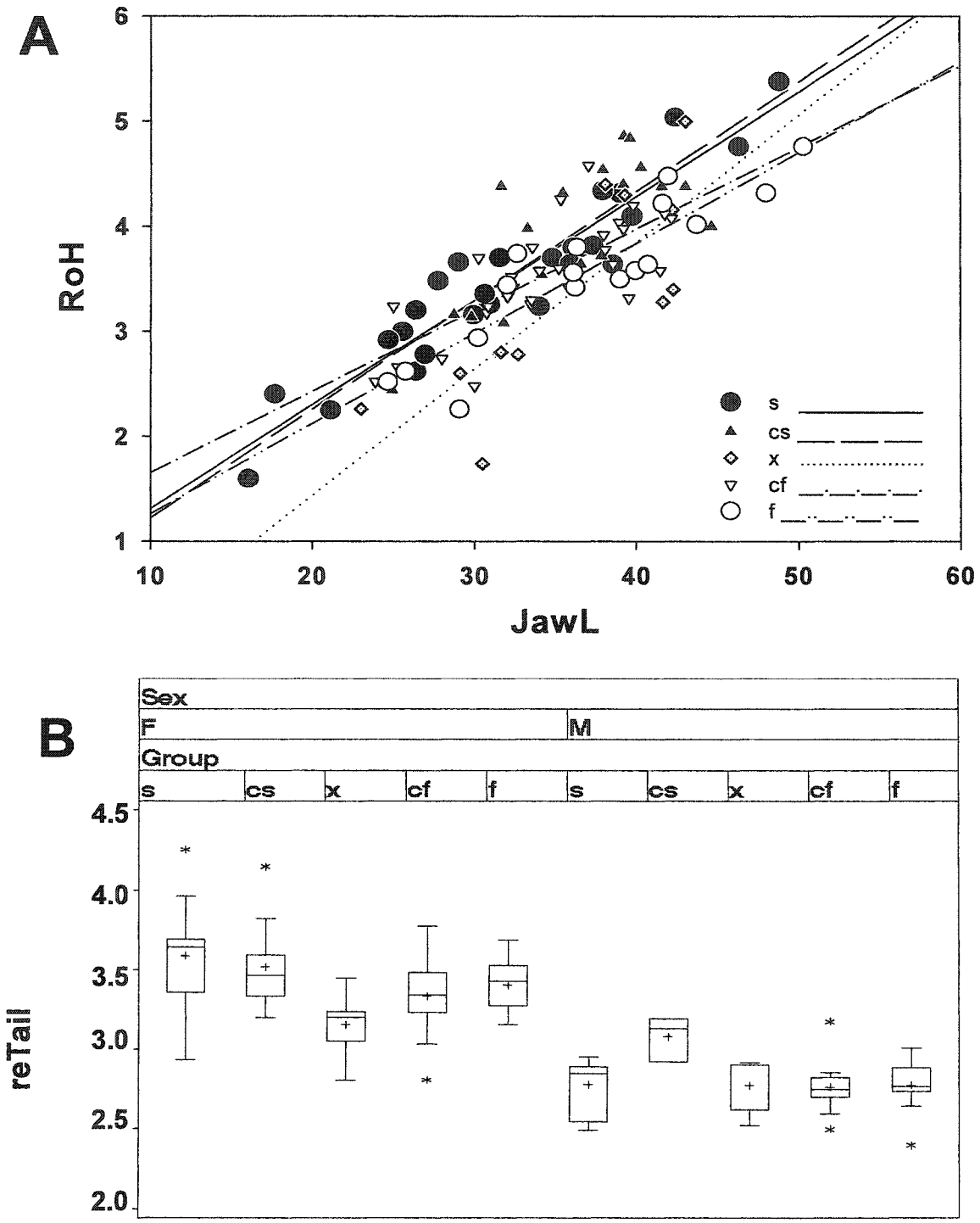


FIG. 6. Relationship of RoH on JawL in mm (A) and distribution of reTail (SVL/Tail) among phenetic groups and sexes. Phenetic groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km away from the contact zone; and **x** for phenetic intermediates between **f** and **s**. Values exceeding a studentized residual of ± 3.64 at a significance level of 0.05 are considered outliers and marked as asterisks.

diameter in (EyS; Fig. 5), distances between eye surfaces DiET (only females), jaw length (JawL; only in males), and relative tail length (Fig. 6) in *fasciata*-like snakes (**cf**, **f**).

In six of the seven distinctive cephalic proportions, the specimens from the distant categories showed the largest differences, whereas *fasciata*- and *sipedon*-like specimens from the contact areas (**cs**, **s**) had intermediate values usually positioned closer to the distant group with which they share a greater phenetic similarity (**f** or **s**). Although the overlap of morphological values between the phenetic groups was substantial, the differences in means and variances indicated that *Nerodia fasciata* and *N. sipedon* followed divergent trajectories of character expression. By contrast, the shift to a more intermediate position of character expression was small in snakes (from the contact zone; **cs** and **cf**) resembling their parental species. The phenetic hybrids (**x**) usually were grouped closer to *N. fasciata*, with only a few of their character means positioned approximately in the middle between both parental groups.

Hybrid Index Results

The presence of a dark postorbital stripe (PS), which is prominent and contrasting in *Nerodia fasciata* but light or absent in *N. sipedon*, and the shape of ventral blotches (SV), which are rectangular in *N. fasciata* but half-moon-shaped in *N. sipedon*, are characters that have been frequently applied to distinguish between the two species (Conant, 1963; Schwaner and Mount, 1976; Behler and King, 1979; Blaney and Blaney, 1979; Seyle, 1980; Tennant, 1997; Conant and Collins; 1991, see Appendix 1 for character descriptions). Differences in the shape of the canthus rostralis have also been

investigated for their usefulness to differentiate between *N. fasciata* and *N. sipedon* (e.g., Conant 1963; Morris, 1987). I evaluated data with a five-step hybrid index (**1** represents condition in a typical *N. sipedon*, **5** in a typical *N. fasciata*, with intermediate steps represented by conditions **2–4**; Anderson 1949). Results will be interpreted in order to allow comparisons with the work of authors mentioned above.

The results yielded significant differences, mostly between the distant *fasciata* (**f**) and *sipedon* (**s**). Phenetic *fasciata* and *sipedon* from the contact zone (**cs**, **cf**) usually revealed an extended range of values and means closer to the median value of the hybrid index (= 3), but the majority of such specimens still showed a stronger association to their presumed (phenetic) parental species (**s** or **f**). Although frequently possessing intermediate means, hybrids were too few (10-11 per analysis) to reveal a meaningful association with either *fasciata* or *sipedon* group.

Head Shape.—Head shape was assessed with three characters: AnCR, StCR, and SpCR (angle, curvature, and anterior height of canthus rostralis). A Kruskal-Wallis Test yielded significantly smaller AnCR (low index) values in both *sipedon* groups (**s**, **cs**) compared to either *fasciata* group (**f** or **cf**) or the hybrids (**x**) (chi-square = 30.86, $P < 0.0001$; see Fig. 7). The higher values in **x**, **f** and **cf** represented sharper angles in the canthus rostralis, rendering it an edgier appearance. The particularly large range in index values for **cf** snakes might indicate introgression from *Nerodia sipedon* into the *N. fasciata* gene pool.

The differences among phenetic groups were somewhat less pronounced for the canthus rostralis slope (SpCR) and curvature (StCR), but were still highly significant

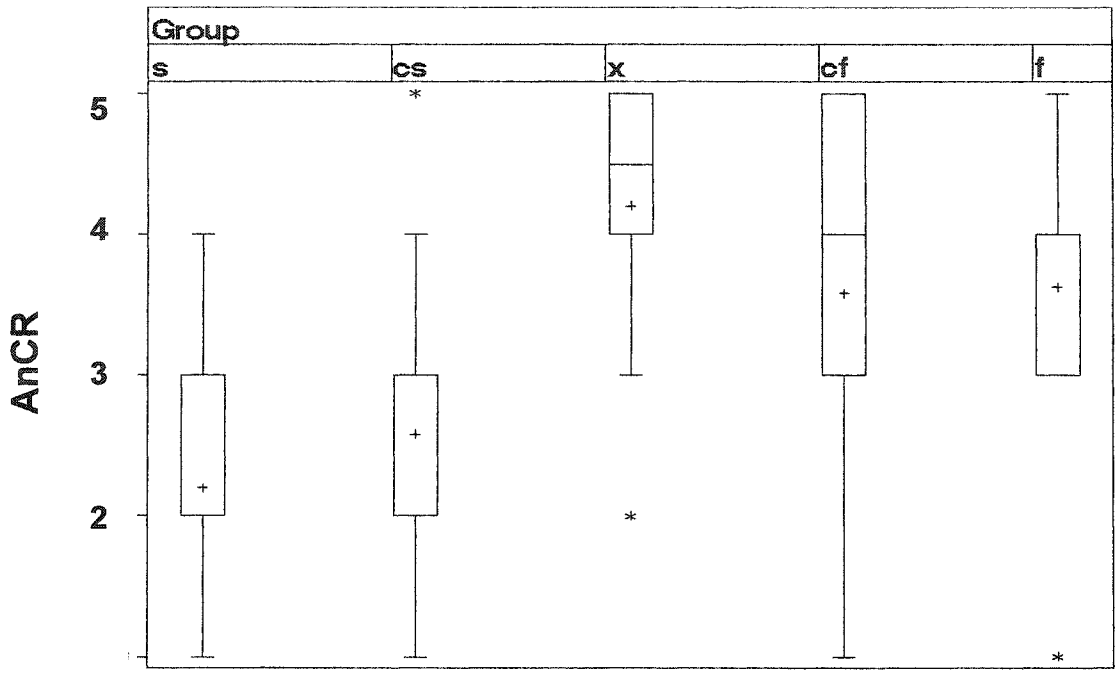


FIG. 7. Variation in the angle of the canthus rostralis AnCR estimated with a five-step hybrid index. Increments are: 1 = angle close to 45° as in a typical *N. sipedon*, 5 = angle close to 90° as in a typical *N. fasciata*, with 2 – 4 representing ascending intermediate stages. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively *N. sipedon* from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**. Values exceeding a studentized residual of $\pm$ 3.64 at a significance level of 0.05 are considered outliers and marked as asterisks.

(StCR: chi-square = 17.27, $P = 0.0017$; SpCR: chi-square = 16.99, $P = 0.0019$).

Fasciata-like snakes (**f**, **cf**) revealed a steeper (SpCR) and less curved (StCR) canthus rostralis than *sipedon*-like snakes (**s**, **cs**). Both characters had larger overlaps of indexed values, hence differentiated less well between both distant groups (**s**, **f**) than did AnCR. Both **cs** and **cf** yielded greatly enlarged ranges of values in StCR. The phenetic hybrids (**x**) revealed a canthus rostralis intermediate in shape to those expressed in **f** and **s**.

Color Pattern.—The prominence of the postocular stripe (PS) and the degree of serration along the lateral margin of bands/blotches (MB) yielded significant differences among groups, in particular between distant **f** and **s** (Kruskal-Wallis Test for PS: chi-square = 29.62, $P < 0.0001$; for MB: chi-square = 47.45, $P < 0.0001$). Both *fasciata* groups (**f** and **cf**) revealed a more prominent postocular stripe and a high degree of band serration, whereas **cf** displayed a large range of values. The *sipedon* groups (**s**, **cs**) had similar low values of **PS**, but approximately 20% in each group showed a prominent stripe (as in *Nerodia fasciata*). The distant *sipedon* (**s**) revealed the lowest degree of lateral band serration followed by those from **cs**. The phenetic hybrids (**x**) occupied an intermediate position in PS (Fig. 8) but had a tendency toward serration in lateral bands (MB), as in *N. fasciata*.

A general trend was found in the prominence of shadow spots between lateral bands/blotches **SS**, in which more prominent shadow spots occurred with a higher frequency in **f** and **cf**. However, very prominent shadow spots of an index value from 4 to 5 were infrequent. Only the difference between **f** and **s** was significant (Kruskal-Wallis: chi-square = 13.36, $P = 0.0096$). All groups had a considerable frequency of specimens without any or with only faint shadow spots (hybrid index values 1-2). The occurrence of shadow spots was too sporadic to render it as a useful tool to distinguish the various phenetic groups. A higher percentage of distant *fasciata* (**f:** 66%) lacked shadow spots than *sipedon* from the contact zone (**cs:** 40%).

The hybrid index revealed two distinctive sets in the shape of ventral spots **SV** (Kruskal-Wallis: chi-square = 59.22, $P < 0.0001$, Fig. 8). Half-moon-shaped spots were significantly more frequent (~ 68%) in *sipedon*-like snakes (**s**, **cs**), whereas rectangular

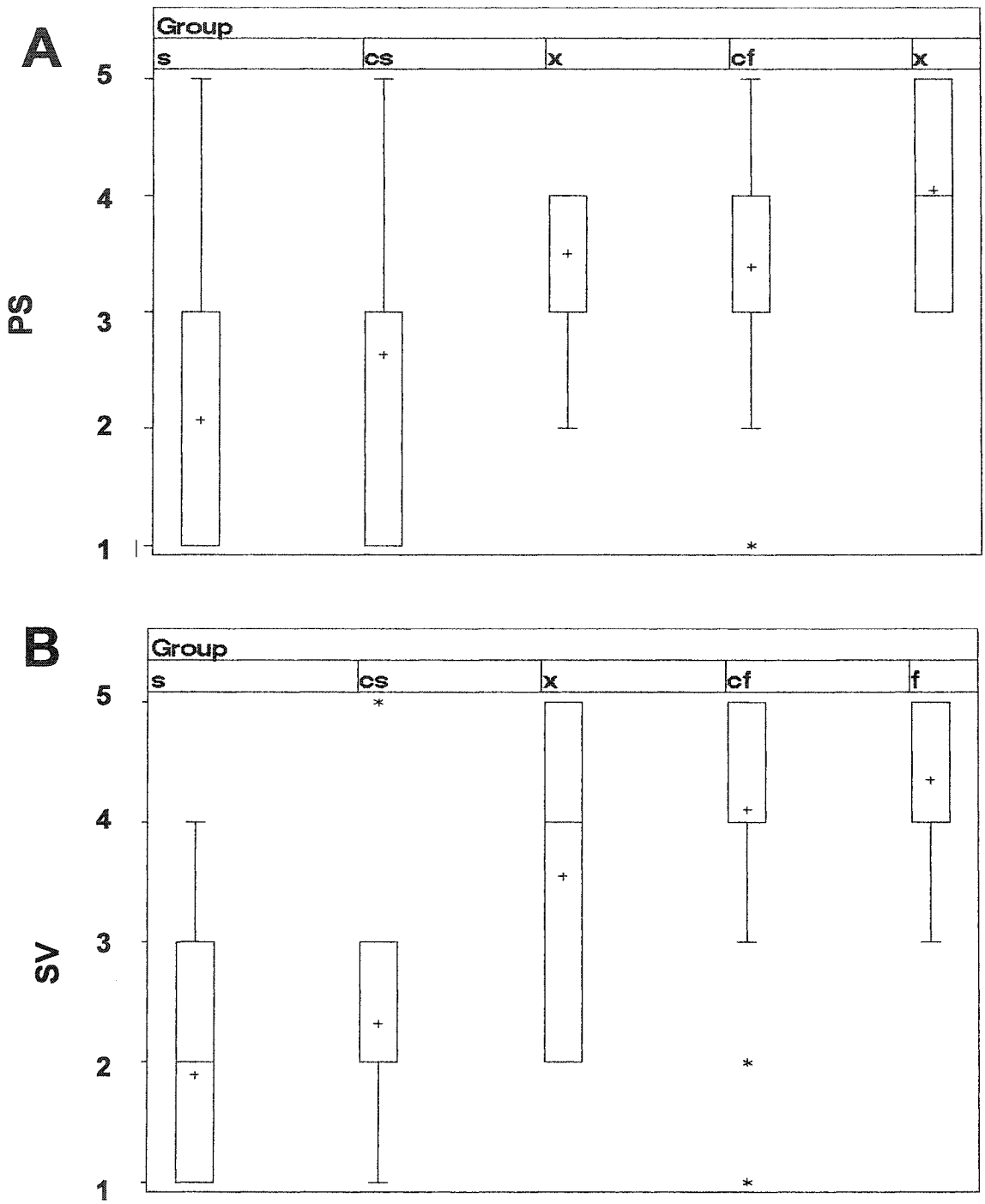


FIG. 8. Variation in the prominence of the postocular stripe (A) and shape of ventral spots (B) estimated with a five-step hybrid index. Increments are: 1 = half-moon shaped as in a typical *N. sipedon*, 5 = rectangular shaped as in a typical *N. fasciata*, with 2–4 representing ascending intermediate stages. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively *N. sipedon* from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**. Values exceeding a studentized residual of ± 3.64 at a significance level of 0.05 are considered outliers and marked as asterisks.

ones were common in **f** (80%), **cf** (77%), and **x** (54%). Snakes from groups **cf** and **x** yielded the largest variation in the shapes of ventral spots, which was consistent with their putative intermediate status.

Many distant *sipedon* (**s**) had spots of intermediate appearance or even rectangular shape. Aside from natural variation, such trends appeared to be enhanced by the influence of *Nerodia sipedon williamengelsi*, which revealed a significantly higher degree toward rectangular ventral spots than the nominate race (ANOVA of log-values $F_{43,2} = 4.0$, $P = 0.0277$). Although most *N. sipedon williamengelsi* in this analysis originated from the contact zone with *N. fasciata*, implying potential introgression, specimens from within the range of the presumed pure *N. sipedon williamengelsi* also revealed intermediate conditions or a trend toward rectangular ventral spots. For instance, three *N. sipedon williamengelsi* yielded index values of 3 or 4 (tendency toward rectangular ventral spots). One snake originated from Roanoke Island, a North Carolina barrier island, and two snakes were collected from farther northeast into the natural range of *N. sipedon* in Currituck County, NC, which is even farther away from the current range limits of *N. fasciata*.

Discriminant Function Analysis

Six discriminant function analyses (DFA = canonical discriminant analysis; SAS for Windows, v. 8.02) were applied to generate canonical variables (= component or axis) that enable the comparison of overall morphology among specimens and their allocations into categories (phenetic groups). DFA1 to DFA3 were formed from sexually non-dimorphic meristic and metric characters. Sexually nondimorphic cephalic characters

were also analyzed separately to find the most distinctive features of cephalic proportions between *Nerodia sipedon* and *N. fasciata* (DFA4). The effectiveness of sexually dimorphic characters to form species distinct canonical components was investigated with DFA5 and DFA6. These components were generated from a few cephalic measurements and caudal scale row reductions. The number of complete bands (CBa), which is the sole external diagnostic character differentiating between populations of *N. fasciata* and *N. sipedon* in the Carolinas and Virginia, was excluded due to its disproportionately large influence on score values. Also excluded from these DFAs were hybrid index, non-normal, and some redundant characters.

Although a DFA generates several components (canonical variables), only the first component of either DFA1 through DFA3 was used to compare phenotypes geographically and to evaluate similarities between phenotypic and genotypic expression (see GEOGRAPHIC SECTION). These first components (C1.1 through C3.1) were formed through distinct linear combinations of original character variables and represent the most influential variable in each of the three DFAs (see below).

C1.1 (DFA1): meristic characters only (VPV, LBa, VLBa), n = 159

C2.1 (DFA2): metric and meristic characters (VPV, LBa, IBa, VLBa, DiNo, DiET, JawW, SCW, EyCW, RoH, DiEN, SCH, EyCH, EyS; cephalic proportions were measured relative to the jaw length), n = 56

C3.1 (DFA3): metric and meristic characters as C2.1, but excluding IBa, n = 78; the purpose was to describe overall morphology of specimens missing IBa

C2.1 from the second DFA is the preferred component to compare phenotypes, as it includes the highest number of original variables. Thus, it represents the closest approach in describing the overall morphology of an individual snake. However, due to an incomplete data set consisting mostly of cephalic measurements (see MATERIAL AND METHODS), its sample size is small (n = 56) and could be only slightly increased by removing character IBa from a subsequent analysis (C3.1 from DFA3). The simultaneous application of meristic and metric characters in DFA2 and DFA3 reduces bias due to interdependency among characters. For instance, the same gene or gene complex may be responsible for the interspecific differences in cephalic proportions (see also correlation analysis). However, the DFAs involving meristic and metric data incorporated two independent character sets.

Although formed by three meristic characters only, the first component (C1.1) from the DFA1 will mostly be used to compare pheno- and genotypes locally, because twice as many snakes were scored with C1.1 as with C3.1 (see GEOGRAPHIC SECTION). The three original characters applied in DFA1 showed small correlation coefficients, revealing little association among each other (Table 1).

The two most distinct pairs of groups, the *fasciata*-like (**cf**, **f**) and *sipedon*-like (**cs**, **s**) groups, produced scores for canonical variables most divergent from the grand mean, which is zero. For example, **cs** and **s** mostly received positive scores between 1.0 and 3.0, whereas **f** and **cf** displayed a similar range of scores but with negative values (e.g., Fig. 9). The phenetic similarity of putative hybrids (**x**) to their parental species was revealed by the proximity of their canonical scores to those of their putative parental

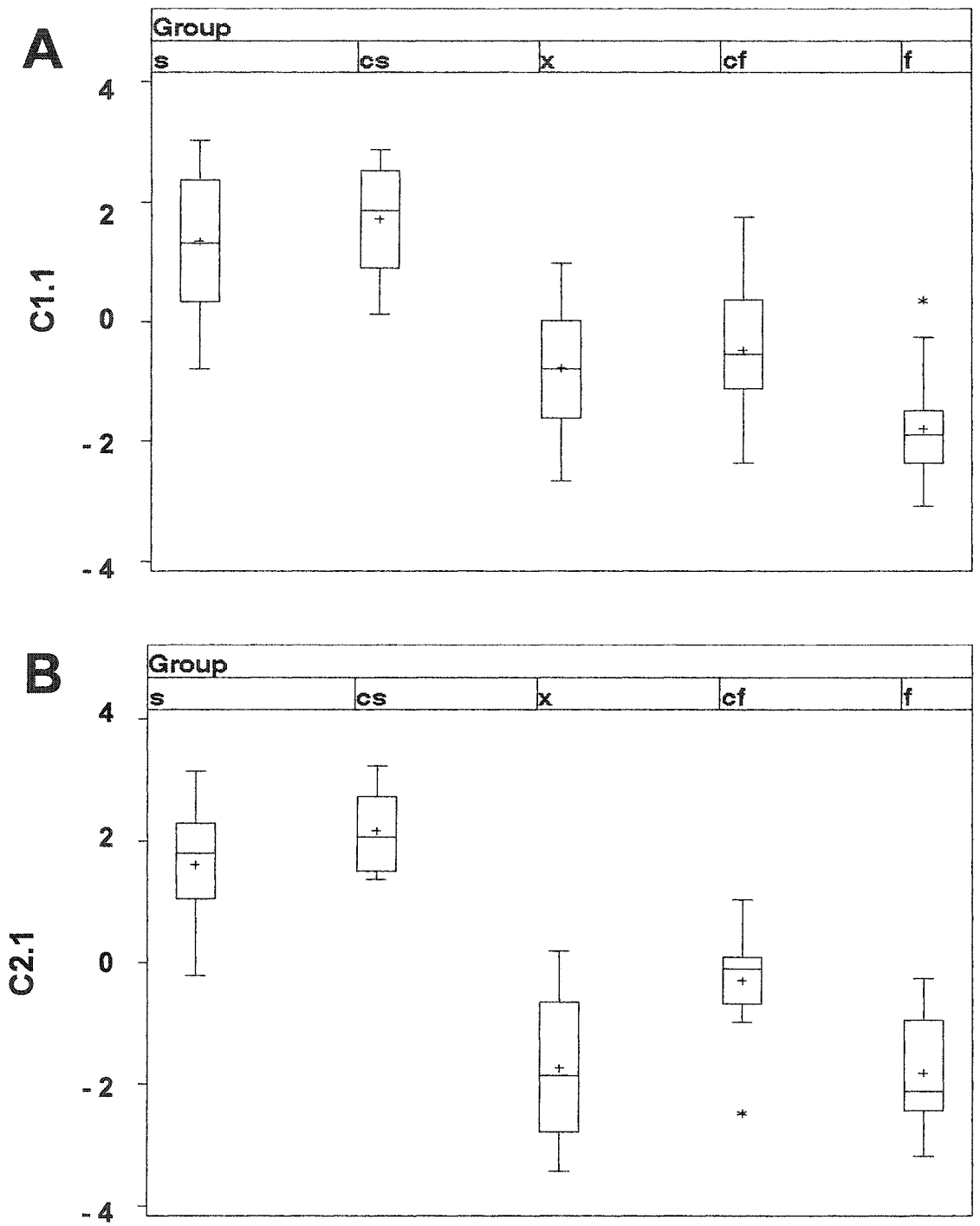


FIG. 9. Group range, quantiles, and means of canonical variable C1.1 derived from DFA1 on meristic variables (A) and C2.1 derived from DFA2 on meristic and metric variables combined (B). Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**. Values exceeding a studentized residual of $\pm$ 3.64 at a significance level of 0.05 are considered outliers and marked as asterisks.

species. Morphological intermediacy (score = 0) is indicated by the many individuals from the contact zone that received absolute scores below 1.0.

An ANOVA revealed significant differences among group means of canonical variable scores (Table 5). In all three variables, the two *sipedon* groups (**s**, **cs**) were significantly different from the two *fasciata* groups (**f**, **cf**) or the phenetic hybrids (**x**) (Fig. 9; variation in C3.1 closely resembled C2.1). In all three DFAs, meristic characters had the largest influence on forming the canonical variables. In DFA1, the width of the

TABLE 5. Descriptive statistics and results of ANOVA comparing scores of first components resulting from three discriminant function analyses on meristic and metric characters. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**.

C1 (first component of DFA1)							
	Descriptive statistics				Tukey HSD	ANOVA	
Group	mean	SE	*N*	Range	diff. from	F	*P*
s	1.35	0.19	31	-0.78-3.02	x, cf, f	56.75	< 0.0001
cs	1.71	0.18	25	0.13-2.87	x, cf, f		
x	-0.77	0.20	24	-2.66-0.99	s, cs, f		
cf	-0.47	0.14	9	-2.35-1.75	s, cs, f		
f	-1.79	0.18	22	-3.08-0.36	none		
C2 (first component of DFA2)							
s	1.61	0.19	31	-0.78-3.02	x, cf, f	38.85	< 0.0001
cs	2.17	0.26	9	1.37-3.23	x, cf, f		
x	-1.73	0.46	8	-3.42-0.75	s, cs, x, cf		
cf	-0.30	0.34	9	-2.47-1.03	none		
f	-1.82	0.25	15	-3.19-(-0.25)	s, cs, cf		
C3 (first component of DFA3)							
s	1.69	0.18	20	-0.13-2.88	x, cf, f	50.16	< 0.0001
cs	1.82	0.23	13	0.42-2.78	x, cf, f		
x	-1.94	0.50	9	-3.62-0.75	s, cs, cf		
cf	-0.45	0.18	21	-2.26-0.94	none		
f	-2.04	0.32	15	-4.00-0.09	s, cs, cf		

ventral extensions of lateral bands (VLBa) showed the strongest correlation (r = 0.91) with its first component (C1.1). The number of lateral bands (LBa) also yielded a high positive correlation (r = 0.69) with C1.1, whereas the number of ventral scales (VPV) resulted in a strong negative correlation (r = -0.75). Similar in DFA2, VLBa retained the strongest positive correlation (r = 0.88) with its first component (C2.2), followed by the anterior width of the canthus rostralis (SCW: r = 0.64). Two more characters had strong negative correlations, the number of ventral scales (VPV: r = -0.75) and the width of interspaces (IBA: r = -0.64).

The situation in DFA3 resembled that in DFA2, as it containd an analysis on the same characters except for IBa, which was excluded. Cephalic measurements generally contributed less than meristic characters to the formation of components. The width and height of the canthus rostralis caudad of the nostrils (SCW and SCH) and the height of the rostral scale (RoH) showed the largest influences on the first component, whereas jaw width (JawW) and lateral distance between eye surfaces (DiET) were negatively correlated with the second component.

In all three DFAs, the mean difference of first component scores was largest between the distant *fasciata* (**f**) and both *sipedon* groups (**cs** and **s**), whereas the phenetic *fasciata* (**cf**) and hybrids (**x**) from the contact zone produced more intermediate scores (Fig. 9; Table 5). Although the hybrids showed the largest range of C2.1 and C3.1 scores, the variation of C1.1 scores was equally great among **cf**. Contrary to expectations, the overlap of first component scores between the distant *sipedon* (**s**) and the *fasciata* groups (**f, cf**) was greater than between the *sipedon* from the contact zone (**cs**) and both *fasciata* groups. Two geographic factors are responsible for this. First, several distant *sipedon*

from western Virginia included in the analysis exhibited component scores close to zero. This is due to variation in VLBa, in which *sipedon* from localities closer to the mountains had exhibited values of VLBa approaching the low values in distant *fasciata* (**f**). Phenetic s*ipedon* from the contact zone (**cs**) generally produced higher VLBa values, despite some introgression from *Nerodia fasciata*. Second, many distant *sipedon* from the vicinity of Rocky Mount, Nash Co., NC, had low component scores due to low VLBa and LBa values, resembling those of in *N. fasciata*. Again, this may reflect geographic variation or it may result from introgression with *N. fasciata* (see DISCUSSION).

DFA1 based on three meristic characters produced a large range of overlapping scores (18.7%) between distant groups that included 7.7% of all **f**-values and 25.9% of all **s**-values with an average of 12.5% across both groups (Fig. 10; Table 5). Presumed pure snakes exhibiting overlapping scores were three distant *fasciata* from Gardner Creek along the lower Roanoke River, Martin Co., NC, and five distant *sipedon* from the vicinity of Rocky Mount, Nash Co., NC, as well as one distant *sipedon* from Bear Spring, Giles Co., VA, and Hampton City, VA. Natural variation likely is the cause for intermediate morphological expression at the latter two sites, which are far away from the nearest *fasciata* population (~ 300 km for Giles Co.; ~ 80 km for Hampton City). However, the overlapping morphology found at the other sites likely indicates that local introgression between *Nerodia fasciata* and *N. sipedon* reaches beyond the initially selected 25-km buffer zone to separate the hybrid zone from the parental species area. This original delineation of the hybrid zone was based mainly on the prime diagnostic feature, the occurrence of complete dorsal bands along the trunk in *N. fasciata* vs. alternating posterior dorsal/lateral blotches in *N. sipedon*. An extension of the

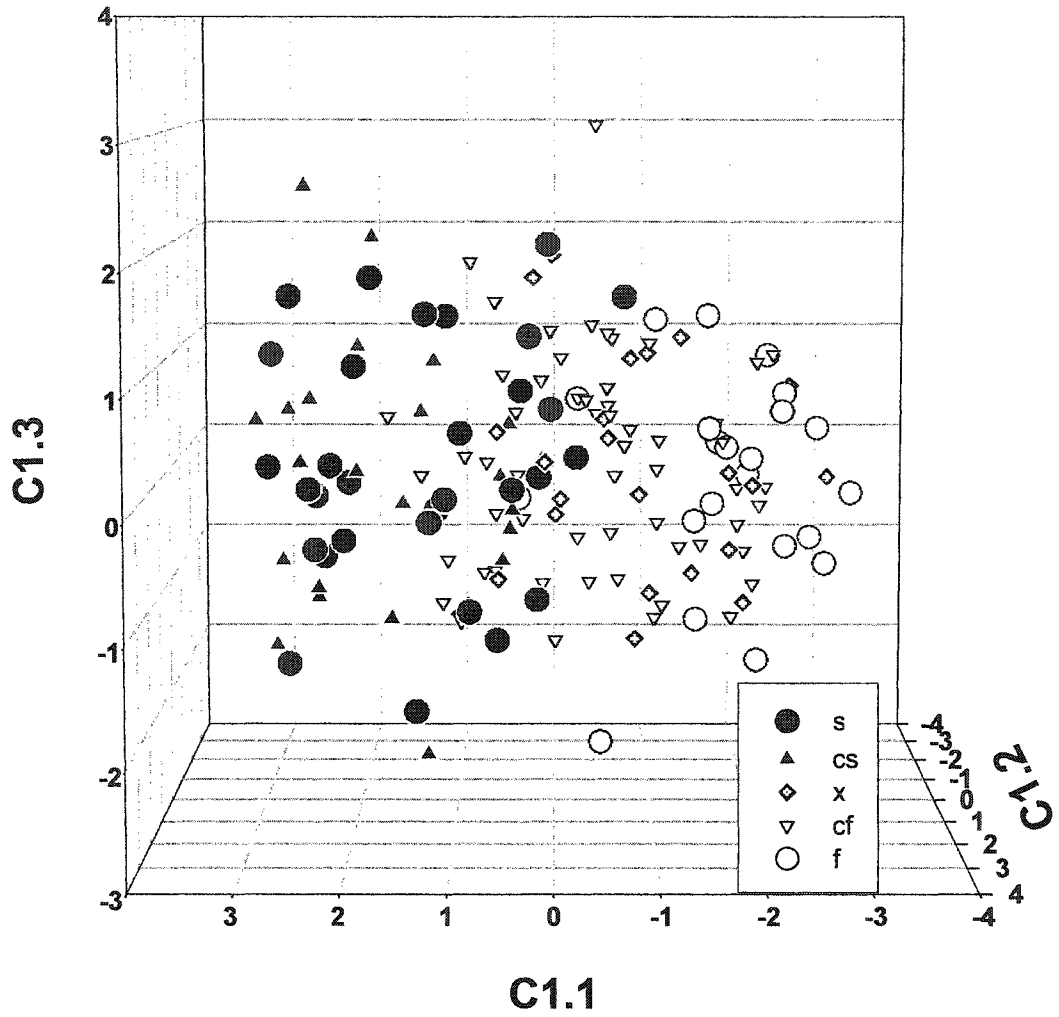


FIG 10. Distributions of canonical scores from DFA1 based on three components of meristic characters. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**.

delineation by an additional 25 km toward the previously presumed "pure" species' ranges would incorporate those populations into the contact zone boundaries that exhibit a morphological indication of introgression. Similar results from the genetic analysis corroborate these morphological findings (see GEOGRAPHIC SECTION).

Several metric characters were added to generate the second and third DFAs (DFA3 = DFA2 – IBa). DFA3 was most successful in separating the two distant groups (**s** and **f**), because overlapping scores between them constituted only 3.2% of the entire range of scores from all groups (Table. 5; Fig. 11). The overlapping range included 13.3% of all **f** values and 5.0% of all **s** values, which accounted for an average of 8.6% of specimens containing overlapping values across both groups. As in DFA1, character VLBa showed

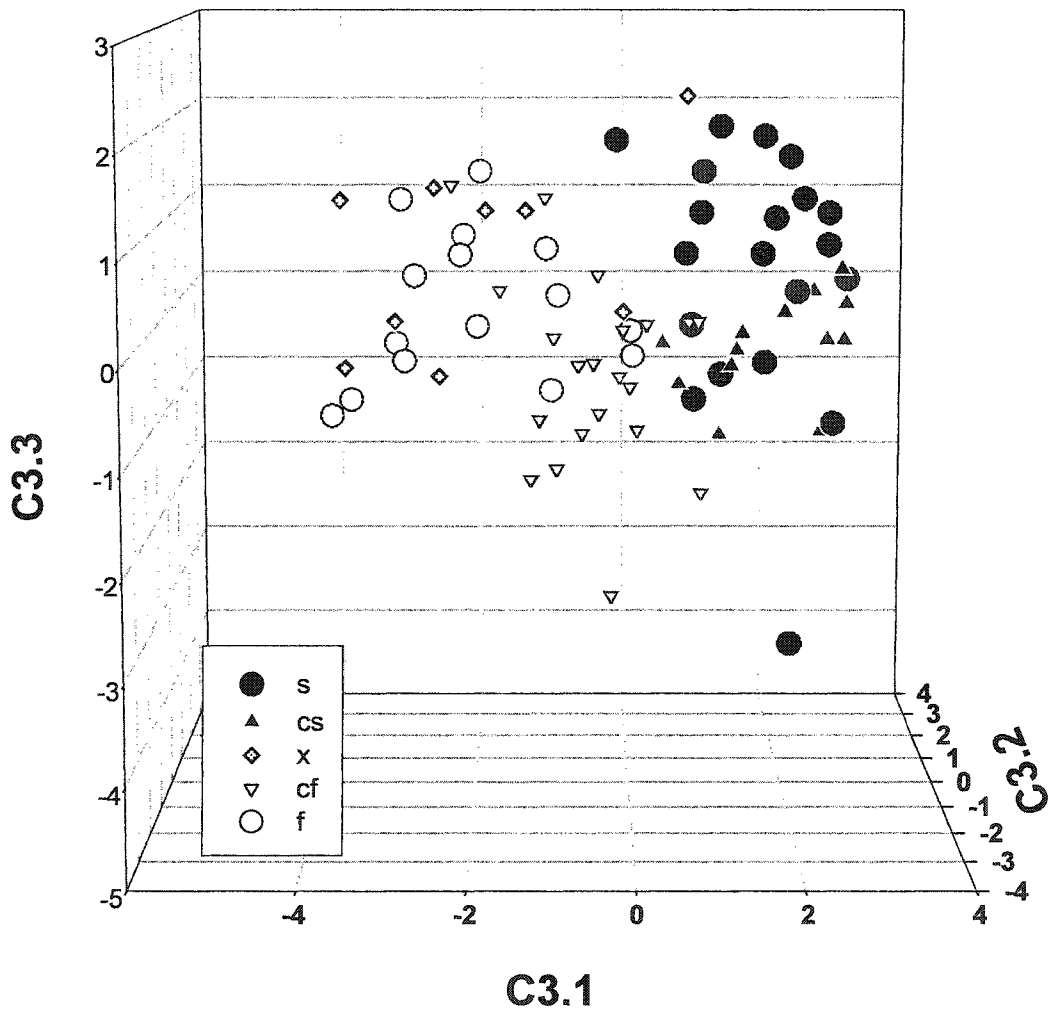


FIG 11. Distributions of canonical scores from DFA3 based on three components of metric and meristic characters. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**.

the strongest correlation for the first components of both DFAs (axes C2.1 and C3.1; r = 0.88 in both DFAs). The distance between the lateral surfaces of the eyes (DiET) was the only character that correlated strongly with the second component (discriminant axis C3.2; r = 0.62), whereas jaw width (JawW) had the largest influence on the third axis (r = - 0.52). Figure 11 also demonstrates the intermediate position of **cf** scores, and the *fasciata*-like distribution of **x** scores, whereas **cs** scores show less intermediacy. Elimination of the influential IBa character from the DFA3 resulted in a similar pattern. Which cephalic proportions best separated *Nerodia fasciata* from *N. sipedon* was investigated with DFA4. Figure 12 shows a good separation of both distant groups (**s** and **f**) with the first axis (C4.1), which is strongly correlated with the width of the canthus rostralis at the snout (SCW: r = 0.73) and the height of the rostral scale (RoH: r = 0.64), whereas C4.2 correlates most strongly with jaw width (JawW: r = -0.55).

DFA5 and DFA6 were performed to investigate which sexually dimorphic characters best distinguished between *Nerodia fasciata* and *N. sipedon*. For females, scale row reductions had the strongest influence in separating both species. The first component (axis C5.1) produced a strong correlation (r = 0.71) with the position of the dorsal scale row reduction to 21 rows (Red21), whereas the second component (axis C4.2) was mostly influenced (r = 0.71) by the caudal scale row reduction to eight rows (Tred8) (Fig 13). In males, both scale row reductions strongly correlated with the first component (Red21: r = 0.82; Tred8: r = -0.60), whereas the distance between the eyes and the snout tip (DiES) and the reduction to eight caudal scale rows mainly influenced the second component (DiES: r = 0.59; Tred8: r = 0.58; data not shown).

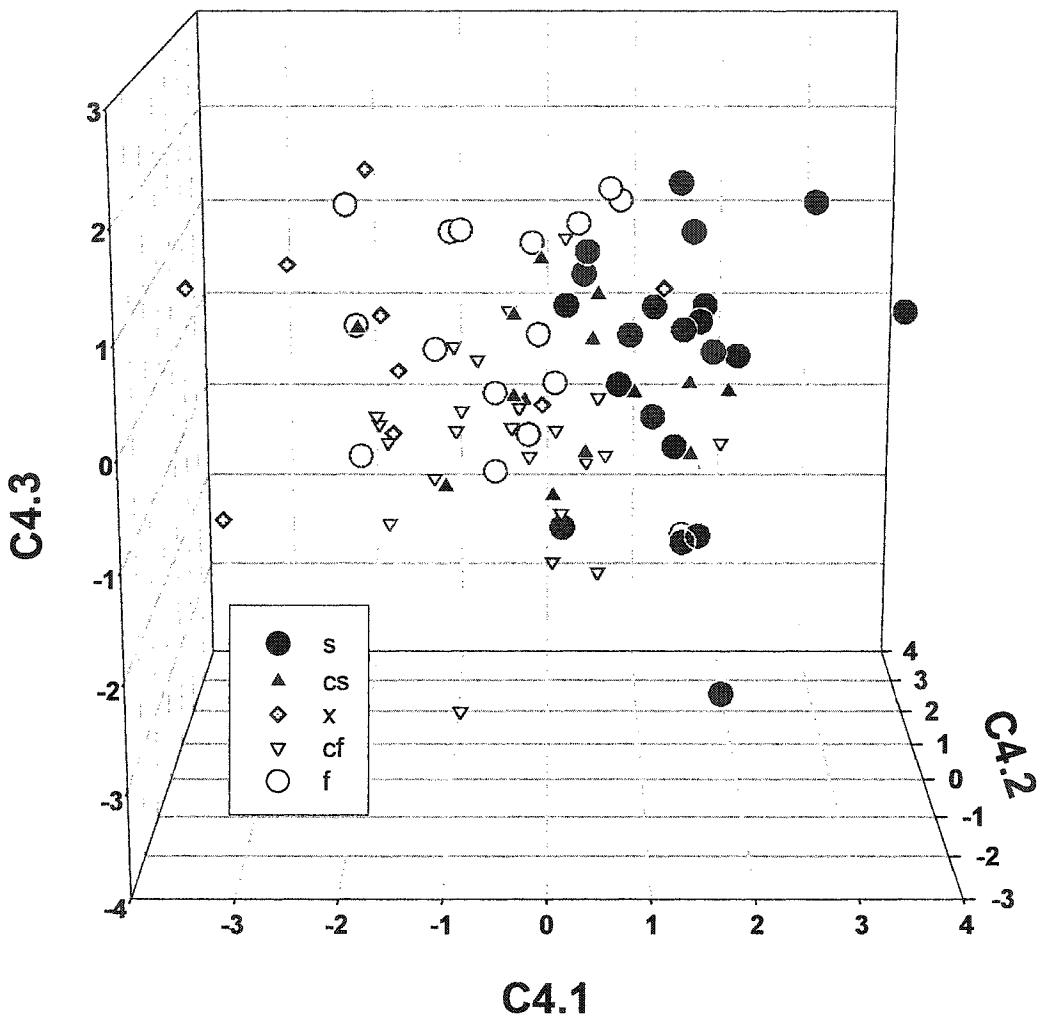


FIG 12. Distributions of canonical scores from DFA4 based on cephalic characters. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**.

In conclusion, all DFAs succeeded in separating most *Nerodia sipedon* from *N. fasciata*, with approximately 10% of snakes yielding scores overlapping between both distant groups. The width of the ventral extensions of lateral bands (VLBa) was the most influential character, but the number of lateral bands (LBa) and the number of ventral scales (VPV) also showed strong correlations to canonical variables. Width and height of the anterior canthus rostralis (SCW and SCH) and height of the rostral scale (RoH) were

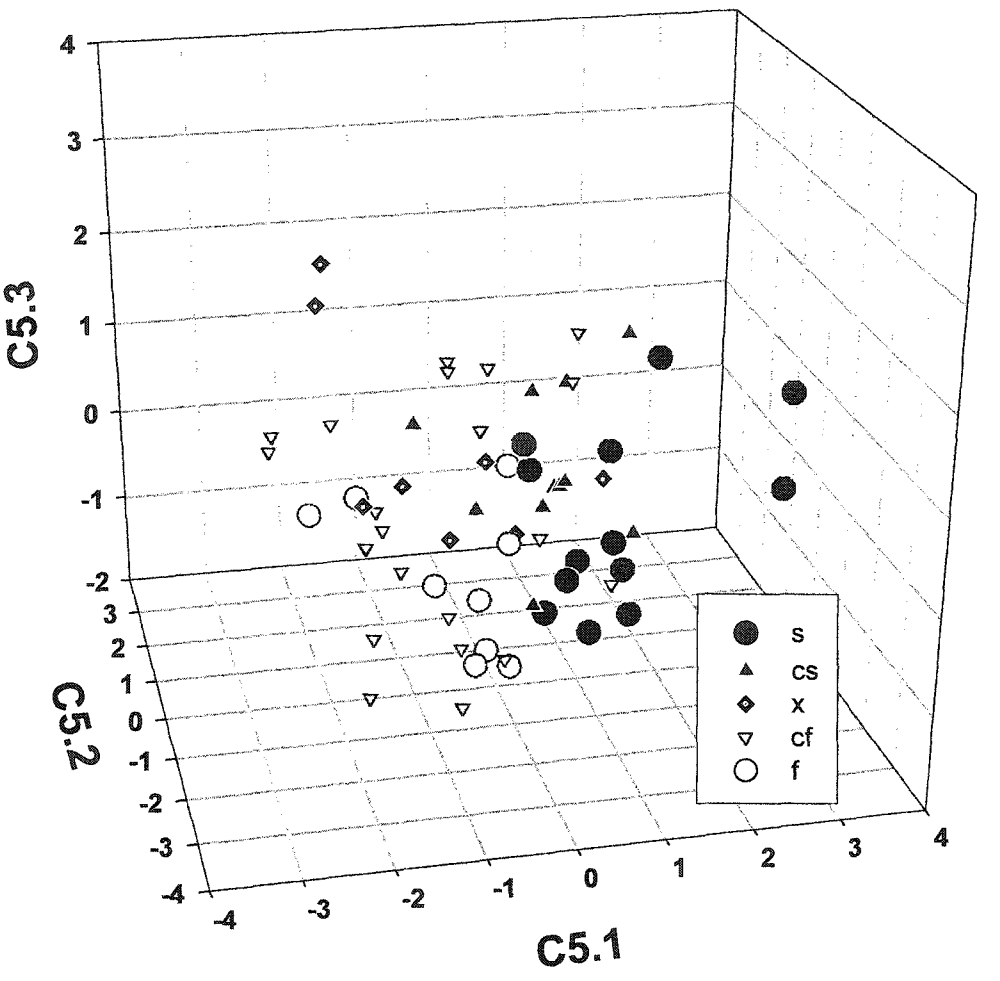


FIG 13. Distributions of canonical scores from DFA5 (females) based on sexually dimorphic characters. Groups are: **cf** and **cs** for phenetic *Nerodia fasciata* and *N. sipedon*, respectively from the contact zone; **f** and **s** for distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; and **x** for phenetic intermediates between **f** and **s**.

the most influential characters among the cephalic proportions in separating both species, whereas scale row reductions most clearly distinguished between both species among sexually dimorphic characters. Some distant *N. sipedon* yielded meristic canonical scores that approached values of *N. fasciata* due to geographic variation. By contrast, morphological intermediacy within 50 km of previously designated boundaries of the

contact zone indicated that introgression was farther-reaching than initially could be assessed with traditionally used diagnostic features only (e.g., the number of complete dorsal bands). The phenetic *sipedon* and *fasciata* from the contact zone were grouped with their respective parental species, whereas the phenetic hybrids were clustered with the *fasciata*-like snakes in all six DFAs, but exhibited an increased range of canonical variable scores.

SUMMARY

Most of the selected meristic characters revealed significantly different distributions between *Nerodia sipedon* and *N. fasciata* from the Carolinas, although only the number of dorsally complete crossbands (CBa) would qualify as a regional species-distinct diagnostic feature. *N. sipedon* tended to yield higher numbers of scales in several scutellation characters than *N. fasciata.* For instance, mean values were higher for the number of postocular scales, ventral scales, and the number of dorsal scale rows at the tenth ventral scale. Moreover, dorsal scale rows were reduced closer to the cloaca in *N. sipedon*-like snakes, rendering them higher scale counts in the posterior half of the trunk. Conversely, *N. fasciata* reduced their caudal scale rows significantly closer to the cloaca than *N. sipedon.* The number of dorsal and lateral band/blotches showed a higher mean in *N. sipedon*-like snakes, coinciding with a higher ventral scale count. The higher number of dorsal bands in *N. sipedon* correlated with narrower interspaces, which corresponded to wider bands laterally and ventrally. *N. sipedon* yielded larger cephalic proportions than *N. fasciata,* except for eye size. However, none of the measured metric characters was suitable as a diagnostic tool to distinguish between the two species.

Characters of color pattern and shape of the canthus rostralis yielded significant differences, mostly between the "pure" *N. fasciata* and *N. sipedon*. Phenetic intermediates (putative hybrids) resembled *N. fasciata* more closely in nearly all morphological characters. The discriminant function analyses demonstrated the partitioning of the two species into distinct phenetic groups even without the inclusion of the most distinctive character, the number of complete dorsal bands.

GENETIC SECTION

Materials and Methods

Blood was obtained from the caudal vein and processed following modifications of the methods of White and Densmore (1992), Jacobson (1993), and Brian Bowen (University of Florida, Gainesville, Florida, pers. comm. and unpubl.). Using 3- or 10-ml syringes, blood was extracted by inserting a 26-gauge (5/8) needle into the ventral midline between two subcaudal scales, approximately five scales posterior to the vent. After penetrating the subcaudal vein, not more than 1 ml of blood was extracted and stored in screw-cap tubes containing 9 ml lysis buffer (100mM Tris-HCL, pH 8; 100 mM EDTA, pH 8; 10 mM NaCl; and 1.0 % SDS weight:volume), which disrupts the blood cells, releasing the DNA.

The DNA of most samples was purified using the GFX^{TM} Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). A few samples were also purified with methods following slightly altered standard phenol/chloroform procedures in Sambrook et al. (1989) and Hillis et al. (1996), as modified by Gibbs et al. (1994). The DNA samples were then analyzed by the AFLP (amplified fragment length polymorphism) method (Zabeau and Vos, 1993; Vos et al., 1995).

Background of the AFLP Method.—The AFLP method selectively amplifies restriction fragments from a digest of total genomic DNA using the polymerase chain reaction, PCR (see reviews of AFLP in Vos and Kuiper, 1997; Blears et al., 1998; Mueller and Wolfenbarger, 1999; Savelkoul et al., 1999). AFLP was originally developed

by Keygene (Wageningen, The Netherlands) as a method for DNA fingerprinting that can be widely applied in a variety of fields, such as medical diagnostics, forensic analysis, plant and animal breeding, microbial typing, and population biology. The AFLP method is a high-resolution technique generating sufficient polymorphism that can be used to separate morphologically indistinguishable species (Anamthawat-Jonsson et al., 1999) and to differentiate among closely related bacterial strains (Rosendahl and Taylor, 1997). AFLP allows the study of paternity (Krauss, 1999; Questiau et al., 1999) and of clonal diversity in plants (Escaravage et al., 1998), but it is not ideally suited to phylogenetic studies on a level higher than species due to extensive homoplasy of fragment size (Janssen et al., 1997). However, O'Hanlon and Peakall (2000) found an average of only 2.5% size homoplasy for AFLP fragments among congeners.

The patterns of DNA fragments obtained by different individuals, populations, or species are polymorphic due to point mutations in the restriction sites or the adjacent target sequence (see below), and insertions and deletions within the amplified fragments. No knowledge of the target sequence is required because adapters (oligonucleotides) of known sequences are ligated to the restriction fragments. These adapter sequences serve as primer sites for follow-up PCRs.

The application of a limited set of universal primers (the same primers may be used for different animals and plants) and their annealing to adapters under stringent hybridization conditions render AFLP reproducible and reliable. The flexible technique can be modified to produce consistent banding patterns from DNA of any origin or complexity. Practically independent of template concentration, AFLP has been successfully used to amplify fragments ranging 0.05-0.5 μg (Blears et al., 1998) to as

little as 2.5 pg genomic DNA (Vos et al., 1995). Usually, 50-200 bands are generated, which then are visualized on an analytical polyacrylamide gel.

Normally, markers generated by AFLP are treated as dominant, since no information about their potential homo- or heterozygosity is available unless fragments were sequenced or their inheritance is revealed via breeding/pedigree analysis. Underlying assumptions are that there are no comigrating marker alleles from other loci creating size homoplasy and that each locus is a two-allele system, with the null allele failing to amplify. Methodological protocols and problems include: (1) only consistently bright, clearly distinguishable bands are scored, with poorly resolved bands being recorded as missing data (Yee et al., 1999); (2) lanes with visible obstacles due to contamination are disregarded; (3) contamination of the gel may occur due to unclean water and reagents, creating uneven staining and development of bands; (4) intensity of bands may be reduced due to thinning of gels as results of deformed glass plates; and (5) inconsistencies among amplified fragments may result from inefficient restriction-ligations or PCR procedures.

Although many AFLP loci are monomorphic (Russell et al., 1997), the large number of bands generated from the entire genome yields abundant genetic variation of mostly noncoding sequences. The AFLP technique is increasingly being applied to estimate genetic diversity in both cultivated and natural populations (e.g., Hill et al., 1996; Karp et al., 1996; Qamaruz-Zaman et al., 1997; Travis et al., 1996). AFLP was also successfully applied to demonstrate hybridization in plants (Beismann et al., 1997; O'Hanlon et al., 1999) and animals (Nijman et al., 1999).

Advantages of the AFLP technique are:

1. Polymorphism generated from the entire genome can be studied. Thus, fragments are more likely to be selectively neutral, reducing the bias of markers targeting specific coding sequences, where evolutionary rates can vary wildly under the influence of differing selection regimes (Avise, 1994; Murphy et al., 1996; Blears et al., 1998).
2. AFLP markers are inherited in a Mendelian pattern (Akerman et al., 1996).
3. One pair of primers readily yields up to 100 times more diagnostic markers than other fingerprinting techniques (e.g., isozymes, RFLPs, microsatellites; Sharma et al., 1996). AFLP is especially useful for studying the divergence of nuclear genomes in closely related species (Buntjer, 1999).
4. AFLP is not affected by potentially anomalous inheritance of the maternal lineage as in comparison of mitochondrial sequences (Savelkoul et al., 1999).
5. The technique is relatively cost-effective.
6. The technique is relatively quick. The process from DNA extraction to reading data from a gel can take 2-4 days.
7. Only small amounts of genomic DNA are required. Typically 0.05 - 0.5 μg of DNA, depending upon the size of the genome (Blears et al., 1998), is needed.
8. The technique is reproducible, allowing different researchers to work comparatively on the same taxa (Blears et al., 1998).
9. Unlike microsatellites, no extensive investigations on taxon-specific primer sets are required. Inexpensive universal primers are commercially available and are suitable for most organisms.

The modifications of the AFLP method applied in this study mostly followed a manual from an AFLP workshop co-sponsored by the ICBR and BEECS Genetic Analysis Laboratory, University of Florida, Gainesville (Brazeau et al., 2001). Sequences of primers and adapters are listed in Appendix 3. Most of the molecular work, including DNA purification, restriction-ligation, PCR, electrophoresis, fragment visualization, and image analysis, was conducted in the laboratory of Dr. Denise R. Cooper, Research Service, James A. Haley Veterans Administration Hospital, Tampa, which is affiliated with the Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, Tampa. The following is a brief description of the AFLP method as modified here.

Restriction.—The extracted DNA was cut into a large number of fragments with two restriction enzymes, MseI and EcoRI (Vos et al., 1995), which revealed sufficient polymorphism in preliminary trials. MseI is a 4-base pair (bp) cutter, recognizing the TTAA-sequence, whereas EcoRI is a 6-bp cutter (GTTAAC) and thus cuts genomic DNA less frequently. These two restriction enzymes generate many thousands of reproducible DNA fragments. In particular, the MseI generates small bands (100-1500 bp) that can be amplified efficiently and can be separated subsequently via electrophoresis. The two restriction enzymes produce three types of fragments in reference to their cutting sites on each end: MseI-MseI, EcoRI-EcoRI, and MseI-EcoRI fragments. More than 90% of the fragments have an MseI site at both ends, whereas most of the remaining fragments have an MseI and an EcoRI site on either end. Only the latter classes of fragments were targeted for subsequent amplifications (see below).

Ligation.—Simultaneous with the digestion of genomic DNA, the freshly cut fragments were ligated to small double-stranded adapters (oligonucleotides < 20 bp, see Fig. 14), which have sequences homologous to the 5'-end of the restricted fragments. That is, one strand per adapter partly shares a sequence identical to the one recognized by the restriction enzymes. The restriction sites are not restored after ligation, because the adapters contain one base within the recognition sequence that is different from the original restriction site (Fig. 14). This prevents further digestion.

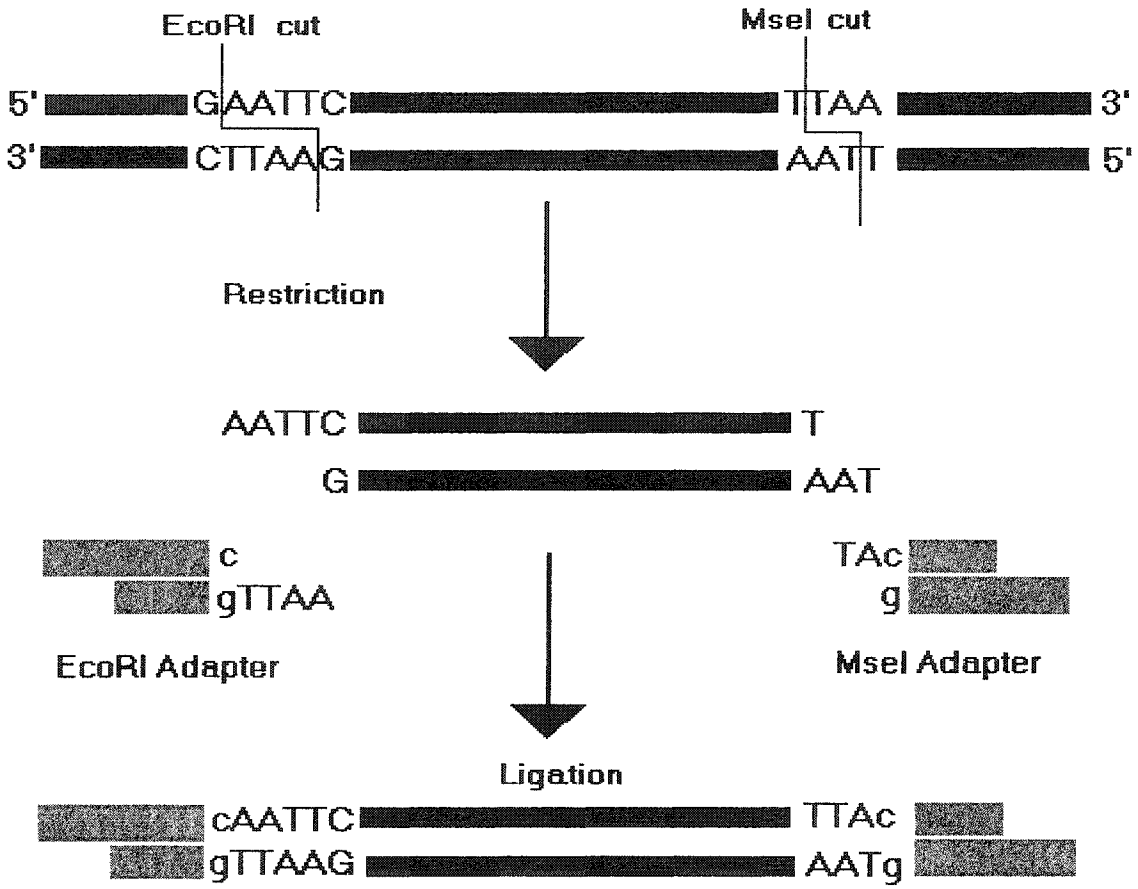


FIG. 14. Restriction of genomic DNA with EcoRI and MseI and subsequent ligation of double-stranded adapters to the ends of restricted fragments. One strand of each adapter (gray) is complementary to a short single-strand extension on the restriction fragment. Both restriction sites are masked by a base change (shown in lower case) to prevent further digestion.

Preselective Amplification (PCR): Two consecutive amplifications (PCRs) are applied to the mixture of fragments obtained from the restriction-ligation step. The two primers for the first amplification, termed preselective amplification, consist of the complementary sequence of the adapter strand containing restriction recognition sites for either MseI or EcoRI plus one additional selective base (nucleotide) at the 3'-end of the primers, which extends toward the center of a fragment (Fig. 15). The high specificity of Taq DNA polymerase amplifies only the subset of available fragments that contain the selective bases, unlike some other DNA polymerases that tolerate some mismatches occurring at the 3'-end of the synthesizing strand (Vos et al., 1995). An adenine was selected for the EcoRI (preselective) primer and a cytosine for the MseI (preselective) primer. The extra base extension in the preselective primers reduces the number of available fragments for amplification by approximately 75%, because the application of one of four possible selective nucleotides (dA, dT, dC, dG) reduces amplification to only those fragments that match the extra base next to their primer sequence.

Although MseI-MseI fragments account for over 90% of all fragments after digestion, the fragments cut by both enzymes (MseI-EcoRI) are preferentially amplified (Fig. 15) because of (1) a higher annealing temperature is required for the EcoRI primers compared to shorter MseI primers, which remain mostly resolved from the adapters at that temperature, and (2) fragments containing modified MseI and EcoRI sequences on opposite ends are prevented from forming inverted repeats via stem loop structures, which compete with primer annealing (Vos et al., 1995). Thus, stringent PCR conditions reduce the number of fragments initially available for amplification to less than 10%, of which an additional reduction of 75% is generated by the preselective base extension.

Therefore, only 1-2% of the initial variation of fragments is amplified in the preselective PCR, still providing thousands of fragments.

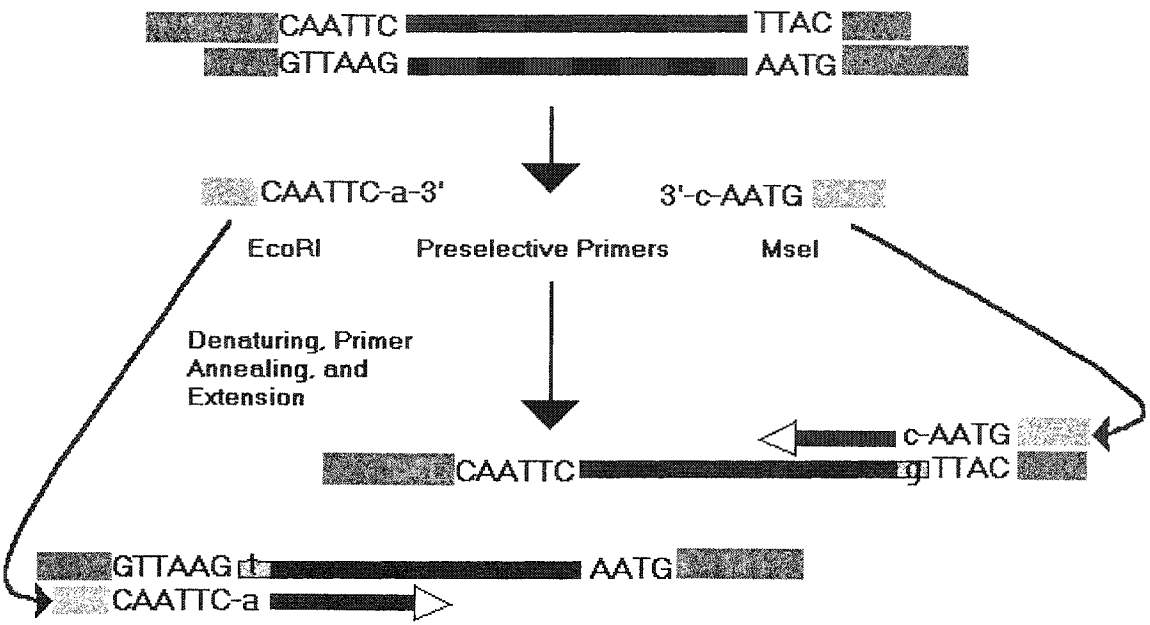


FIG. 15. Preselective amplification of fragments with two primers, one annealing to the EcoRI adapter and one to the MseI adapter. Primers are shown in light gray; adapters in dark gray. Both primers have one additional, preselective base (show in lower case) at the 3'-end directed toward the center of the fragment (direction of extension). Only fragments that contain a base complementary to the preselective base (shown as a base in a light gray shaded box) are amplified.

Selective Amplification (PCR).—A second selective amplification follows, which applies primers with the same sequences as the preselective primers but adds two selective bases to each of the new primers (Fig. 16). Two cytosine bases were selected to extend the new EcoRI primer, which then were paired with either of two new MseI primers, one extended with two thymines and a second with a thymine and an adenine. This decreased the number of amplified fragments for each of the two selective amplifications to a manageable 50-100 fragments.

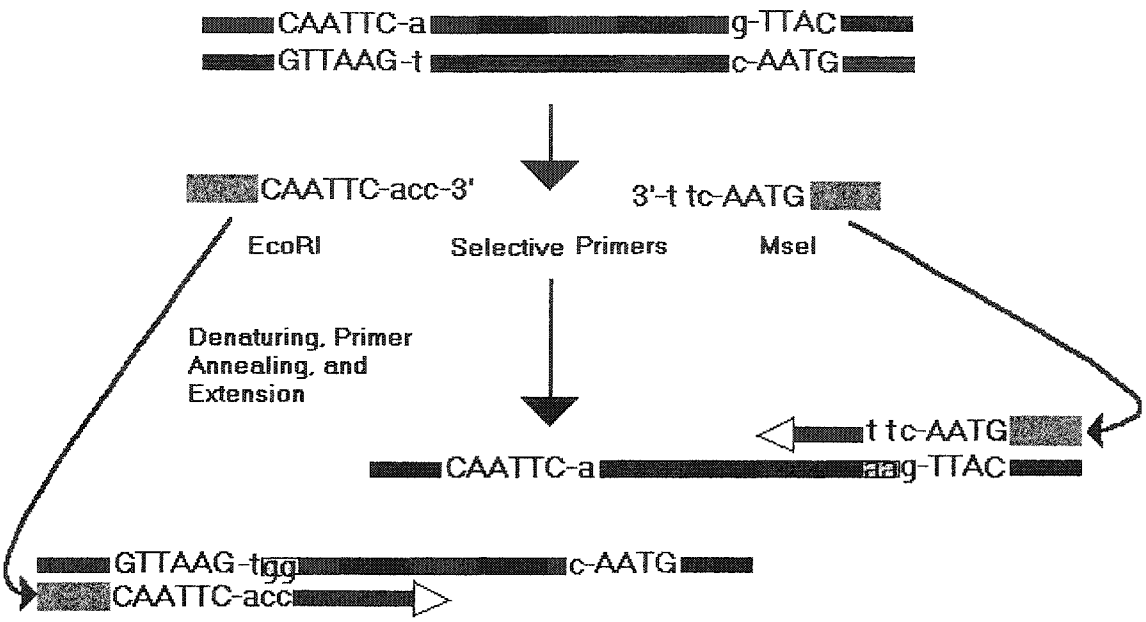


FIG. 16. Selective amplification of fragments with two primers. Both primers (dark gray) have two additional, selective bases (show in lower case next to the preselective base, also in lower case) at the 3'-end directed toward the center of the fragment (direction of extension). Only fragments that contain bases complementary to both selective bases (shown in a light gray shaded box) are amplified. A second selective MseI primer containing a –TA extension was also used in this research (not shown).

Analysis of Gels.—The doubly amplified fragments were separated with polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining. Samples were not heated prior to loading the gel. Instead, urea was added to the gel solution and formamide was added to the loading buffer to separate the double strands. No doublets were observed. The 6% acrylamide solution appeared optimal for the resolution of bands in the range of 100-1000 bp. A current of 60 watts was applied to the gel for 2-3 hours. To visualize the bands after electrophoresis, gels were fixed, stained with silver nitrate, and developed with sodium carbonate, according a technique modified from Promega (Technical Manual: Silver Sequence™ DNA Sequencing System, Promega Corporation, Madison, Wisconsin). For permanent preservation, many gels were scanned at 2000 dpi

and photographed with 100 or 200 ISO film. Initially, more than 50 potential species-specific markers were identified with two different banding patterns generated by two selective primer pairings; EcoRI primer with -ACC (bp) extension paired with either the MseI primer with CTA-extension (Pattern 1), or MseI primer with CTT-extension (Pattern 2). The AFLP method mainly produces bands that exhibit dominant inheritance, and the bands are scored as absent or present. However, some pairs of markers were suspected of being codominant, as for example when both species yielded a band of nearly the same size and apparently differing by only a few base pairs. However, since the sequences remained unknown, such potentially codominant marker pairs were scored independently. Mueller and Wolfenbarger (1999) report that codominant markers can occur in a frequency of 4-15%. A Sigma PCR Low Ladder Set containing fragments with 100 bp and 20 bp increments was used as a size reference for species markers (SIGMA, St. Louis, Missouri).

Primary Markers:— In all, 286 specimens from all five phenetic groups (**s** = distant *sipedon*, n = 83; **cs** = contact zone *sipedon*, n = 37; **x** = hybrids, n = 36; **f** = distant *fasciata*, n = 50; and **cf** = contact zone *fasciata*, n = 80) were initially scored for the presence or absence of over 50 species markers. Of those, the 10 most useful markers (five per species) were selected for the main analysis. Hence, each specimen was scored for 10 loci composed of five positive states (presence of markers of one species) and five negative states (absence of markers of the other species). For example, a *sipedon*-like snake (**cs** or **s**) was screened for the presence of five *N. sipedon* markers and the lack of five *N. fasciata* markers. Criteria for the choice of primary markers were:

1. Visual identification: Darker and sharper bands were more suitable for scoring.
2. Fixation rate: Markers were preferred, if they were fixed or nearly fixed in a species, and occurred at < 5% in the other species. Only animals from localities > 25 km from the center of a putative contact zone were used to test the fixation rate.
3. Reproducibility: Reproduction of bands was achieved with a second AFLP trial. Approximately 40 specimens were reprocessed with new reagents to compare the repeatability of the banding pattern.
4. Sample size: Markers for which a large number of snakes could be scored were preferred.

These preferred markers were labeled with a letter and number (S = marker expressed by *Nerodia sipedon*, F = marker expressed by *N. fasciata*). Following is a list of these 10 primary markers and their size.

Pattern 1: selective primer pairing with -ACC and –CTA base extensions (Fig. 17).

F3: band at ~ 3000 bp

S4: band just below F3. Together F3 and S4 comprise a pair of contrasting bands, frequently accompanied by fainter bands

F4: band at ~ 1100 bp; in some specimens on top of a contrasting band shared by both species

S5: band at ~ 830 bp; occasionally a slight size shift was observed

F5: band at ~ 780 bp, the middle of a triplet of bands

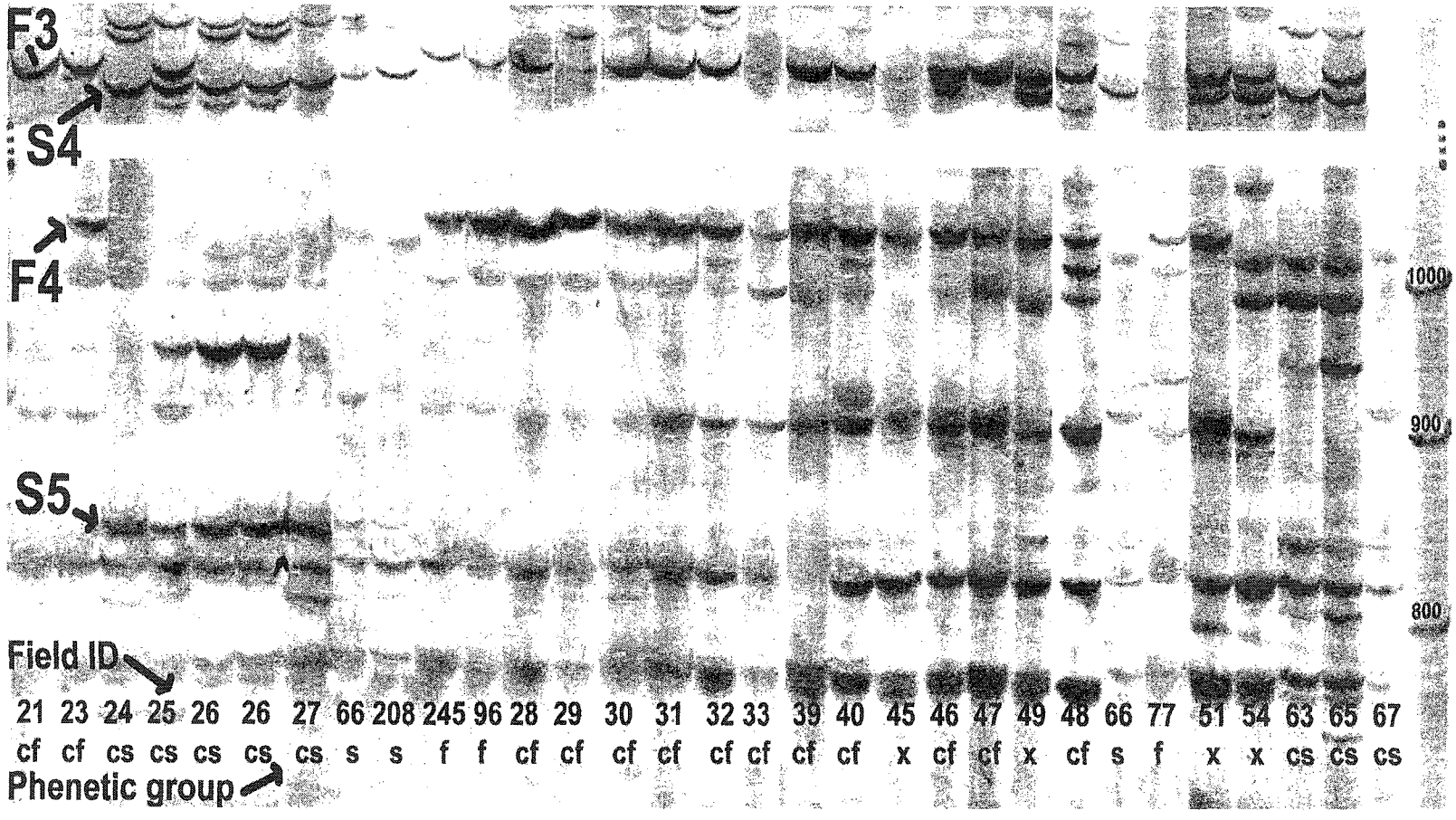


FIG. 17. Gel (PAGE) Pattern 1: Selective primer pairing with -ACC and –CTA base extensions. S_x = marker expressed by *Nerodia sipedon*, F_x = marker expressed by *N. fasciata*. Each lane shows the individual field ID -number assigned to that particular snake (Appendix 2) and the associated phenetic group. Phenetic groups: **cf** and **cs**, phenetic *Nerodia fasciata* and *N. sipedon*, respectively, from the contact zone; **f** and **s**, distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; **x**, phenetic intermediates between **f** and **s**. Ladder indicating size range (bp) is in lane far left. Markers F3 and S4 are a ppended from size range ~ 3000 bp.

Pattern 2: selective primer pairing with -ACC and –CTT base extensions (Fig. 18).

S1: band at ~ 730 bp

S2: band at ~ 880 bp

S3: band at ~ 790 bp

F1: band at ~ 780 bp, just below S3

F2: band at ~ 3000 bp

To examine the extent of hybridization and introgression, every individual was assigned to one of six categories depending on its combination of species-specific primary markers (Lamb and Avise, 1986):

1. Genotypic *Nerodia fasciata*, having all of the *N. fasciata* markers but none of *N. sipedon*
2. Genotypic *N. sipedon*, having all of the *N. sipedon* markers but none of *N. fasciata*
3. F_1 hybrid, having all *N. sipedon* and *N. fasciata* markers
4. *N. fasciata* backcross, having all five *N. fasciata* markers plus one to four *N. sipedon* marker(s)
5. *N. sipedon* backcross, having all five *N. sipedon* markers plus one to four *N. fasciata* marker(s)
6. Later-generation hybrid, having fewer than five markers of either species, but otherwise a varying combination of *N. fasciata* and *N. sipedon* markers not described above (e.g., two *N. fasciata* with four *N. sipedon* markers). These specimens may represent F_2 from F_1 hybrid matings or any other later-generation hybridization that involves a backcross.

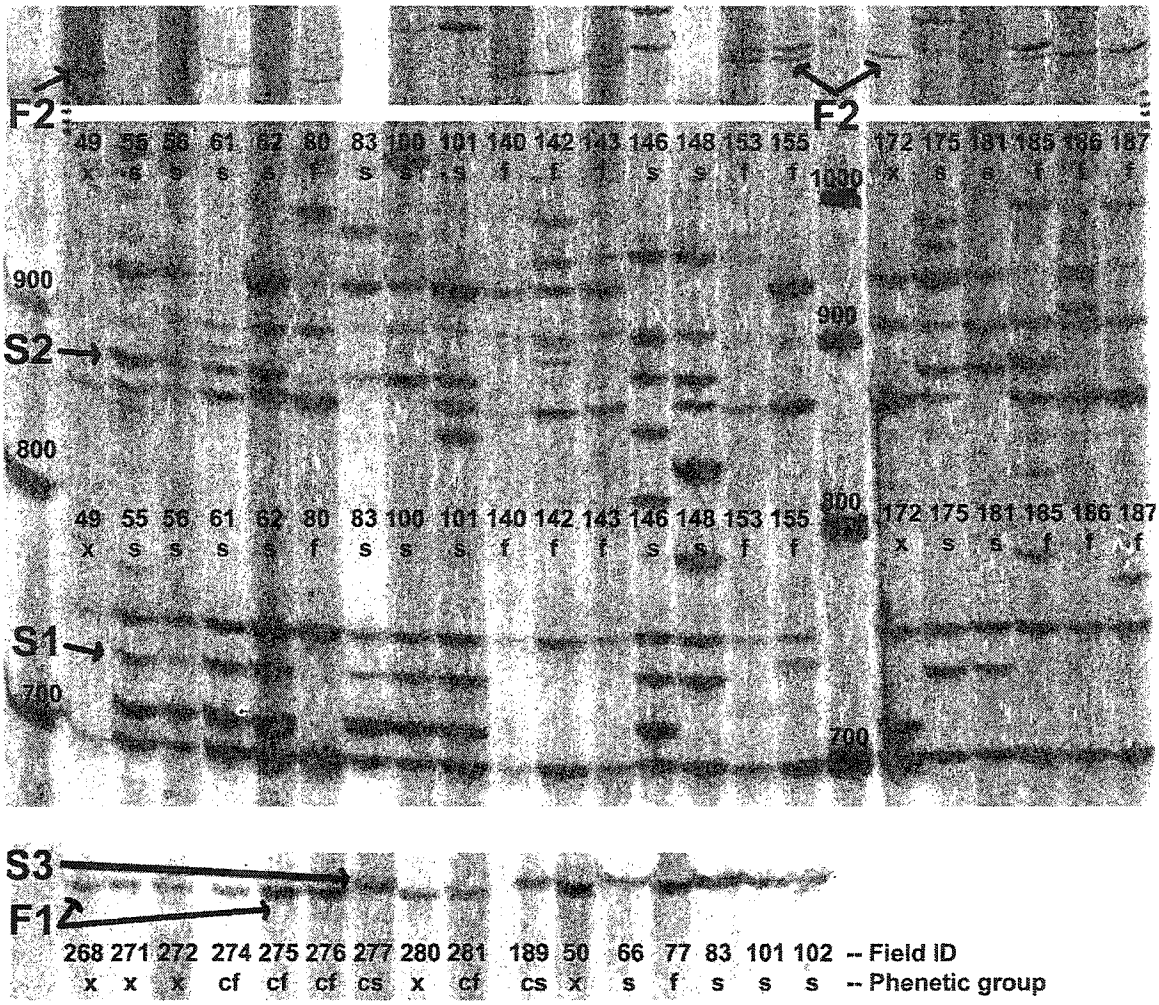


FIG. 18. Gel (PAGE) Pattern 2: Selective primer pairing with -ACC and –CTT base extensions. S_x = marker expressed by *Nerodia sipedon*, F_x = marker expressed by *N. fasciata*. Each lane shows the individual field ID-number assigned to that particular snake (see also Appendix 2) and the associated phenetic group. Phenetic groups: **cf** and **cs**, phenetic *N. fasciata*, and *N. sipedon*, respectively, from the contact zone; **f** and **s**, distant *N. fasciata*, and *N. sipedon*, respectively, from an area > 25 km from the contact zone; **x**, phenetic intermediates between f and s. Ladder indicating size range (bp) is in lane far right and seventh from the left. Marker F2 is appended from size range ~ 3000 bp. Markers S3 and F1 are appended from a different gel.

Mendelian considerations allow the calculations of the probabilities of misclassifying a specimen into the wrong category (Avise, 1994). For example, the probability of mistaking a true F_1 backcross to *Nerodia fasciata* for a pure *N. fasciata* is 0.031 (one out

of 32 possible genotypic combinations of backcrossing with a pure *N. fasciata*) or k = $(0.5)^n$, where n represents the number of fixed markers per species. Similarly, the probability that a first generation backcross shows the same genotype as an F_1 is 0.031.

Frequencies of markers (including null markers = absent bands) and heterozygosity levels among putative backcrosses of F_1 hybrids to pure species were compared to the Hardy-Weinberg equilibrium (Nei, 1987). The calculations of gene frequencies were adjusted following the procedure of Lynch and Milligan (1994) for dominant markers, but yielded no deviations from Hardy-Weinberg expectation. Hence, the frequency of the null allele of a particular marker (bi-allelic) can be derived simply from the square root of the observed number of specimens missing that species marker. An individual's level of heterozygosity is determined by the number of markers for its closely related species that are present in an individual that also exhibits all five markers from the one species with which it backcrossed. This corresponds to calculations of heterozygosity in a codominant system with five diagnostic nuclear markers. Corrections were made for the absence of specimens containing all or no sister species' markers, which would have been scored as F_1 hybrids or "pure" genotypes.

Secondary Markers.—In some specimens, all 10 primary markers could not be properly scored due to procedural artifacts. An assumption is made that the likelihood of inheriting any of the loci is equal for hybridizing animals. Consequently, the probability of correctly classifying a presumptive "pure" individual based on nine loci, with data for one locus missing, is reduced by 50%. In other words, if a snake appears to be a "pure" species based on those nine loci, the unknown expression at the 10^{th} locus would

determine whether that snake would be classified either as a "pure" or as one of the hybrid categories. Missing information on five markers reduces that probability to 3.1%. Therefore, a secondary set of nine markers was applied to specimens that did not yield the full set of 10 primary markers. Most of these secondary markers were drawn from a smaller sample of individuals and labeled as (sample size in parentheses) F50 (208), F67 (113), F69 (120), and F71 (116) for markers predominantly expressed by *Nerodia fasciata* and S49 (253), S53 (263), S60 (200), S65 (141), and S68 (119) for markers predominantly expressed by *N. sipedon.*

F67 presented the best resolution, as it was nearly fixed in *N. fasciata* (> 95%) but was rare in *N. sipedon* (< 5%). The other markers showed either a reduced fixation rate (as low as 91%) and/or an increased frequency in the atypical species (as high as 9%). However, the simultaneous application of at least two such markers to replace one missing primary marker assisted in a fairly accurate classification of species genotypes (> 84% probability of correct classification). More importantly, it reduced the Type I error (the probability of misclassifying it as a particular species) to < 1%. For example, S60 and F71 were the two secondary markers with the lowest fixation rates and highest frequencies in the atypical species. Marker S60 was fixed at 100% in *N. sipedon* but also occurred at a frequency of 9% in *N. fasciata*, whereas F71 was fixed at 92% in *N. fasciata* and occurred at a frequency of 6% in *N. sipedon.*

Binomial calculations allow the determination of probabilities of correctly classifying the paired expression of two markers. Thus, a band at S60 and the absence of a band at F71 would be correctly interpreted as an expression typical for *N. sipedon* with 84% probability, whereas the absence of a band at S60 and the presence of a band at F71

would be correctly interpreted as an expression typical for *N. fasciata* with 94% probability. The hybrid condition would be interpreted as either the presence of bands at both loci (correct at > 92% probability), or as the absence of both bands (correct at > 86% probability).

Most of the misclassifications do not involve individuals representing "pure" species, but rather are later-generation hybrids that would be misclassified as "pure" species. For example, the presence of bands at two secondary markers for *Nerodia sipedon* would be classified as an expression typical for *N. sipedon* expression, although there is a low probability that one of the presumptive *N. sipedon* markers actually represents a rare *N. fasciata* marker of similar size (size homoplasy of different or same locus). The probability of misclassifying such later-generation hybrids is 5-8% per locus or 10-16% for two loci. Therefore, more of the later-generation hybrids may exist than can be detected with the current classification system.

Despite some shortcomings, these secondary markers were useful for classifying animals missing data in some of the 10 primary markers or for investigating the status of supposedly "pure" specimens from within and near the contact zone. The detection of rare mutations and the low number of potentially relictual markers from a sister species are positively correlated with the number of loci screened. In summary, with the above binomial considerations in mind, the application of at least two secondary markers as a replacement for a missing primary marker resulted in the correct classification of approximately 85% of corresponding individuals, whereas approximately 10-15% may represent misclassifications of later-generation hybrids as parental species genotype or vice versa, and < 1% constitute misclassified "pure" individuals.

RESULTS

Frequency of Species Markers.—Most primary markers were fixed or nearly fixed (98–100%) for their respective species (e.g., Fig. 19-20 and Table 6). In contrast, the average frequency of a *sipedon* marker in distant *N. fasciata* (**f**) was 1.6% and the average frequency of a *fasciata* marker in distant *N. sipedon* (**s**) was only slightly higher (2.2%). The maximum frequency of finding a particular species' marker in the other (wrong) species group never exceeded 4% (2–3 specimens per group) among the 10 primary loci. Only three contact zone *sipedon* (**cs**) were misclassified, as they contained a majority of *fasciata* genetic states, whereas four other **cs** possessed five genetic states from each species. This demonstrates the accuracy of the initial phenetic classification, in that specimens were correctly associated with the species with which they also shared greater genetic similarity.

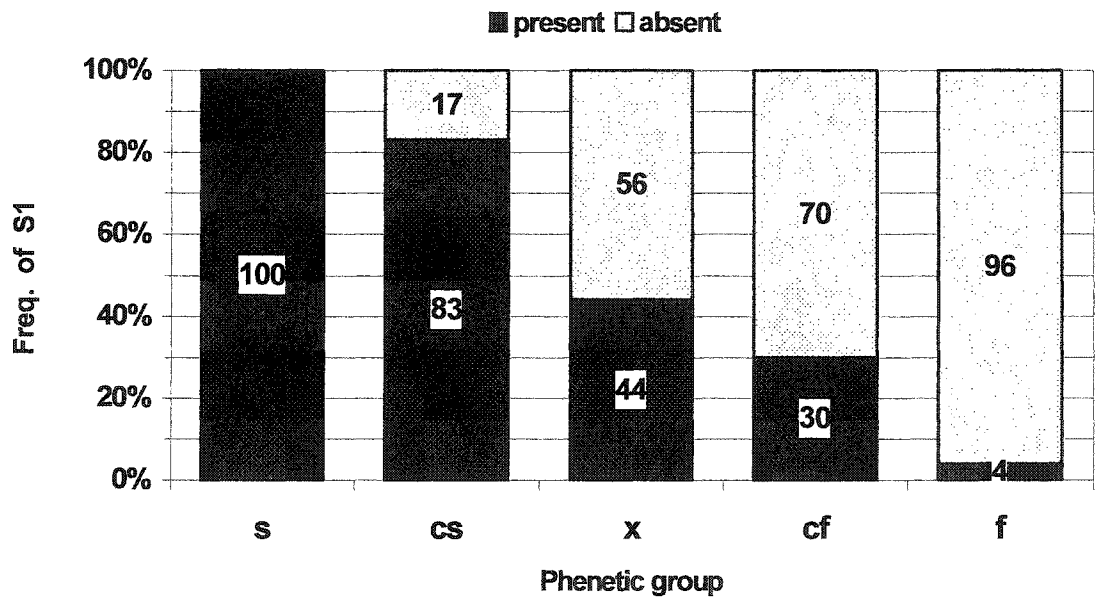


FIG. 19. Proportions of *sipedon* marker S1 among phenetic groups. Groups: **cf** and **cs**, phenetic *Nerodia fasciata* and *N. sipedon*, respectively, from the contact zone; **f** and **s**, distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; **x**, phenetic intermediates between **f** and **s**.

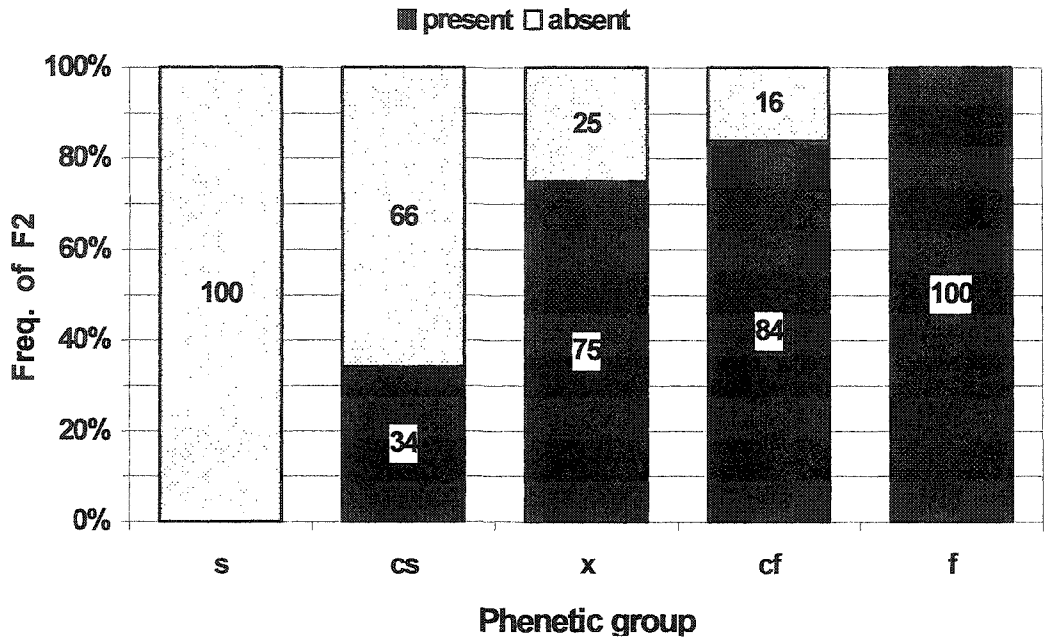


FIG. 20. Proportions of *fasciata* marker F2 among phenetic groups. Groups: **cf** and **cs**, phenetic *Nerodia fasciata*, and *N. sipedon*, respectively, from the contact zone; **f** and **s**, distant *N. fasciata*, and *N. sipedon*, respectively, from an area > 25 km from the contact zone; **x**, phenetic intermediates between **f** and **s**.

TABLE 6. Absolute number and frequency (in parentheses) of AFLP genetic markers among phenetic groups; S_x = marker specific number for *Nerodia sipedon*; F_x = marker specific number for *N. fasciata*. Groups: **cf** and **cs**, phenetic *Nerodia fasciata* and *N. sipedon*, respectively, from the contact zone; **f** and **s**, distant *N. fasciata* and *N. sipedon*, respectively, from an area > 25 km from the contact zone; **x**, phenetic intermediates between **f** and **s**.

sipedon markers										
	S1		S2		S3		S4		S5	
Group	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent
s	83(100)	0	83(100)	0	82(99)	1(1)	82(99)	1(1)	82(99)	1(1)
cs	29(83)	6(17)	31(86)	5(14)	26(74)	74(26)	29(81)	6(19)	29(81)	6(19)
x	16(44)	20(56)	18(50)	18(50)	12(35)	22(65)	13(38)	21(62)	18(50)	18(50)
cf	29(30)	54(70)	9(12)	68(88)	10(13)	66(87)	4(5)	73(95)	12(18)	56(82)
f	2(4)	48(96)	0	50(100)	0	50(100)	1(2)	49(98)	1(2)	49(98)
fasciata markers										
	F1		F2		F3		F4		F5	
s	3(4)	80(96)	0	83(100)	2(2)	81(98)	1(1)	82(99)	3(4)	80(96)
cs	9(26)	26(74)	12(34)	23(66)	20(56)	16(44)	9(29)	22(71)	9(29)	22(71)
x	29(83)	6(17)	27(75)	9(25)	28(82)	6(18)	27(79)	7(21)	17(68)	8(32)
cf	72(95)	4(5)	64(84)	12(16)	75(97)	2(3)	72(95)	4(5)	57(92)	5(8)
f	50(100)	0	50(100)	0	50(100)	0	49(98)	1(2)	50(100)	0

Genetic Analysis of the Contact Zone.—Within the contact zone, frequencies for *fasciata* markers ranged from 70.6-86.2% and were in Hardy-Weinberg equilibrium (75.0%), as expected for a dominant system in a freely interbreeding population (χ^2 = 0.062 [F1] – 1.835 [F3], all with $P < 0.0001$). Null alleles of the *fasciata* markers (snakes missing the markers, and thus homozygous at those loci) also met Hardy-Weinberg expectations (χ^2 = 0.83 [null-F2] – 5.51 [null-F3], $P < 0.0001$, except marker F3 with $P < 0.025$). In contrast, all *sipedon* markers revealed lower than expected frequencies, 29.4 to 50.0%, while their corresponding null alleles had unexpectedly high frequencies of 56.1 to 70.6% (χ^2 = 12.33 [S1]-30.26 [S4] for markers, and 36.99 [null-S1] – 90.82 [null-S4] for associated null alleles; all $P < 0.0001$).

Generally, phenetic *sipedon* (**cs**) and *fasciata* (**cf**) from the contact zones yielded lower frequencies of *sipedon* markers or *fasciata* markers, respectively, than phenetic *sipedon* and *fasciata* from the distant groups (**s** and **f**; Table 6). Markers for *fasciata* yielded higher frequencies than those for *sipedon* in equivalent cross-comparisons among phenetic groups. For example, *fasciata* markers in **cf** had a higher relative proportion than *sipedon* markers in **cs** (Table 6). Furthermore, the frequencies of *fasciata* markers in **cs** were higher than the comparable frequencies of *sipedon* markers in **cf**. A detailed description of the genetic composition of each phenetic group in the contact zone follows.

Contact Zone fasciata (**cf**).—Phenetic *fasciata* from the contact zone not only resemble *Nerodia fasciata* externally, but also exhibit a substantially higher proportion of genetic *fasciata* markers than *sipedon* markers (Table 6). The frequency of *fasciata* markers was only slightly reduced in **cf** compared to distant *fasciata* (**f**), which

presumably represents "pure" *N. fasciata*. Introgression by *N. sipedon* into the **cf** genome is slight but evident, with a maximum of 30% of phenetic *fasciata* possessing a specific *sipedon* marker (see S1: Table 6). Figure 21 shows the preponderance of genetic contribution of *N. fasciata* to **cf**, which possessed 7.7 times higher frequency of genetic *fasciata* states than *sipedon* states.

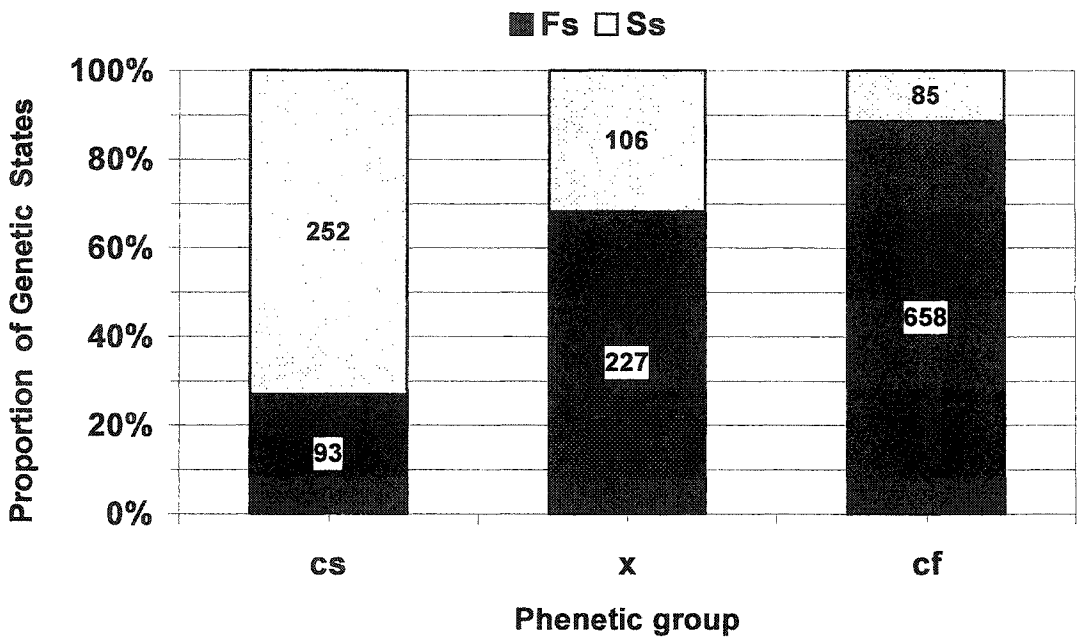


FIG. 21. Proportions and absolute counts (inside bars) of genetic *fasciata* states (Fs) and *sipedon* states (Ss) among groups from the contact zone. Groups: **cf** and **cs**, phenetic *Nerodia fasciata* and *N. sipedon*, respectively, from the contact zone; **x**, phenetic intermediately patterned snakes.

A total of 33 phenetic *fasciata* (**cf**) possessed only *fasciata* markers, as expected for "pure" *Nerodia fasciata* (Fig. 22). However, nine of these animals had missing data for up to five of 10 primary species markers. To verify the absence of hybridization for all 33 specimens, secondary markers were evaluated. A snake initially classified as *N. fasciata* was switched to a category of later-generation hybrid when it expressed at least

one genetic *sipedon* state at either of two secondary genetic markers. As a result, only nine of 24 *N. fasciata* with the full set of primary markers and four of nine with missing primary data remained in the category of "pure" *N. fasciata* after evaluating the secondary markers. This indicates that only approximately 16% of all *fasciata*-like snakes in the contact zone are "pure" *N. fasciata*.

Thirty-six snakes from the contact zone appeared phenotypically to be "pure" *Nerodia fasciata*, but revealed a genotype of mixed ancestry after scoring all 10 primary species markers. They contribute to a group of cryptic hybrids, as their hybridization status was not recognizable using traditional color pattern characteristics. Half of those cryptic hybrids that possessed a full set of markers exhibited one genetic *sipedon* state (presence of a *sipedon* marker or absence of a *fasciata* marker; Fig. 22). Most other cryptic hybrids (44%) yielded two or three *sipedon* states, whereas only two **cf** had four *sipedon* states. An additional 11 **cf** did not yield the full set of primary markers, but clearly showed introgression based on at least one *sipedon* state. Overall, the frequencies of incorporating *sipedon* markers for **cf** were substantially greater than losing one *fasciata* marker (40:22; ratio ~ 2:1).

Contact Zone sipedon (**cs**).—A total of 345 genetic positions across the 10 primary loci was scored in phenetic *Nerodia sipedon* from the contact zone (**cs**). The introgression of *N. fasciata* genes into **cs** appears to be larger than introgression in the opposite direction (from *N. sipedon* into *N. fasciata*), because **cs** had decreased frequencies of *sipedon* markers and increased frequency of *fasciata* markers compared to marker frequencies in **cf** (Table 6). Similarly, the proportion of genetic *sipedon* states in **cs**,

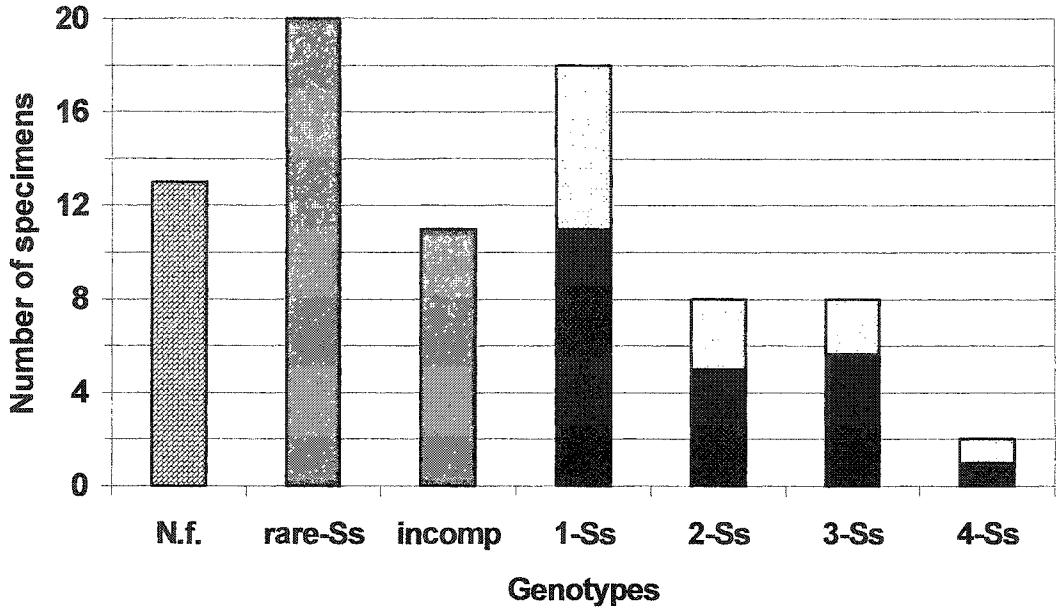


Fig. 22: Distribution of genotypes for phenetic *fasciata* (**cf**) from the contact zone. Genotypes are shown as frequencies of *sipedon* states (Ss), which are defined by the presence of *sipedon* markers and the absence of *fasciata* markers. The degree of hybridization increases from left to right. Genotypic categories: N.f. ("pure" *Nerodia fasciata* genotype, **cf** lacking any genetic *sipedon* states); rare-Ss (rarely a *sipedon* state; specimens with a *N. fasciata* genotype based on the 10 primary markers, but exhibiting low degree of introgression by *N. sipedon* based on secondary markers); incomp (**cf** with an incomplete set of primary markers, but yielding at least one *sipedon* state); 1- to 4-Ss (specimens with 1- to 4 *sipedon* state[s] based on a full set of 10 primary markers). The shading in the 1-Ss to 4-Ss categories represent the proportions of the presence of *sipedon* markers (dark) vs. the absence of *fasciata* markers (light).

including the summation of null alleles (absence of markers), was markedly lower compared to genetic *fasciata* states in **cf** (Fig. 21). Nonetheless, genetic *sipedon* states in **cs** were 2.7 times higher than *fasciata* states, confirming their proper classification as **cs**.

Only five **cs** appeared to be "pure" *Nerodia sipedon* based on the primary markers (one specimen had missing data for three loci). Furthermore, all appeared to represent later-generation hybrids with at least one genetic expression typical for *N. fasciata* among

the secondary markers evaluated that yielded fixation rates ranging between 92–100% (Fig. 23).

Twenty-two **cs** revealed a mixed genotype of primary markers. Forty-five percent of those cryptic hybrids expressed one *fasciata* state, whereas other proportions of *fasciata* states were far less common (Fig. 23). One phenetic *sipedon* possessed six genetic *fasciata* states and thus closely resembled *Nerodia fasciata* genetically. As in **cf**, **cs** was more likely to gain a *fasciata* marker than to lose a *sipedon* marker (39:25; ratio ~ 1.6:1).

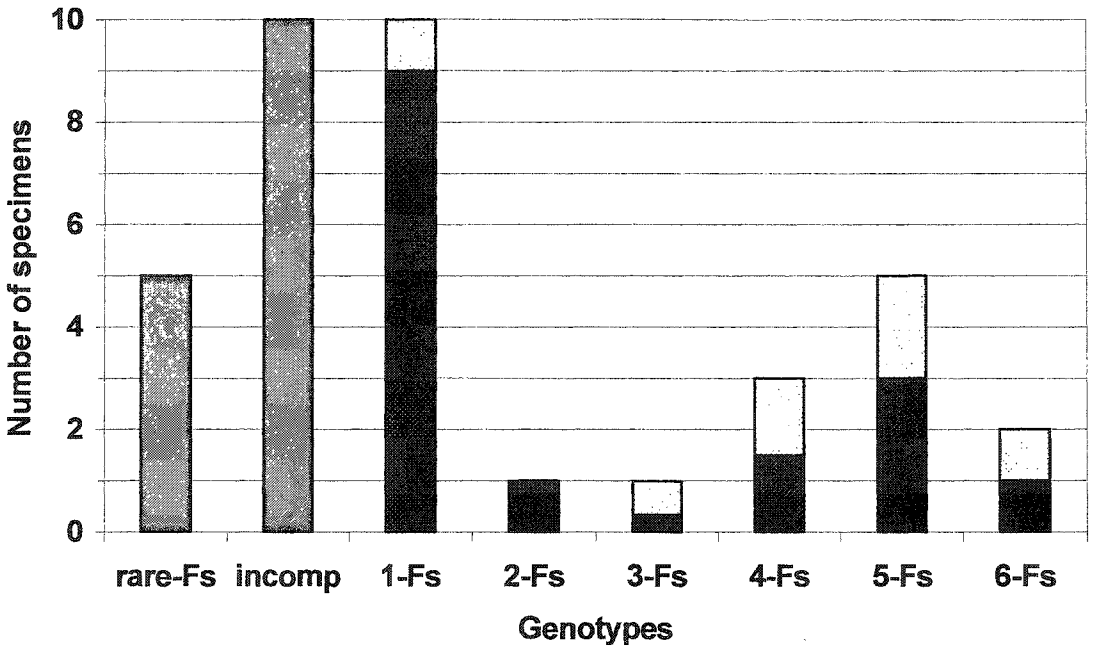


Fig. 23: Distribution of genotypes for phenetic *sipedon* (**cs**) from the contact zone. Genotypes are shown as frequencies of *fasciata* states (Fs), which are defined by the presence of *fasciata* markers, and the absence of *sipedon* markers. The degree of hybridization increases from left to right. Genotypic categories: rare-Fs (rarely a *fasciata* state; specimens with a *Nerodia sipedon* genotype based on the 10 primary markers, but exhibiting low degree of introgression from *N. fasciata* based on secondary markers); incomp (**cs** with an incomplete set of primary markers, but yielding at least one *fasciata* state); 1- to 6-Fs (specimens with 1-6 *fasciata* state(s) based on a full set of 10 primary markers). The shaded areas in the 1-Fs to 6-Fs categories represent the proportions of the presence of *fasciata* markers (dark) and the absence of *sipedon* markers (light).

Phenetic Hybrids from the Contact Zone (**x**).—Frequencies of the primary markers in **x** had intermediate ranges compared to those in **cf** and **cs** (Figs. 19-20). However, the general tendency toward increased expression of *fasciata* markers in snakes from the contact zone is repeated for **x**, which expressed close to twice as many *fasciata* markers than *sipedon* markers (Table 6). Genetic *fasciata* states were 2.1 times more frequent than *sipedon* states in **x** (156:74). Four snakes that appeared to be *Nerodia fasciata* based on the primary markers proved to be backcrosses after examining several secondary markers.

The phenetic hybrids (**x**) yielded the widest range of genetic variation, from snakes with one *sipedon* state and nine *fasciata* states (category 9-Fs in Fig. 24) to those with seven *sipedon* states and three *fasciata* states (category 3-Fs in Fig. 24). However, Figure 24 also illustrates that **x** with a majority of *fasciata* states (> 5-Fs) predominated among the phenetic hybrids. In most categories, the majority of *fasciata* or *sipedon* states involved the presence of *fasciata* or *sipedon* markers, respectively, rather than the absence of the other species' marker (Fig. 24). No true F_1 hybrids, expressing all 10 primary species markers, were identified, although three snakes (two **x** and one **cs**) exhibited nine markers. One phenetic hybrid exhibited a "pure" *fasciata* genotype, including secondary markers.

Frequencies of Genotypes among Phenetic Groups from the Contact Zone (**cf**, **cs**, **x**).—The contact zone primarily contained individuals of hybrid ancestry. Only *Nerodia fasciata* was represented by a few (< 10%) "pure" genotypes (Table 7). The majority of

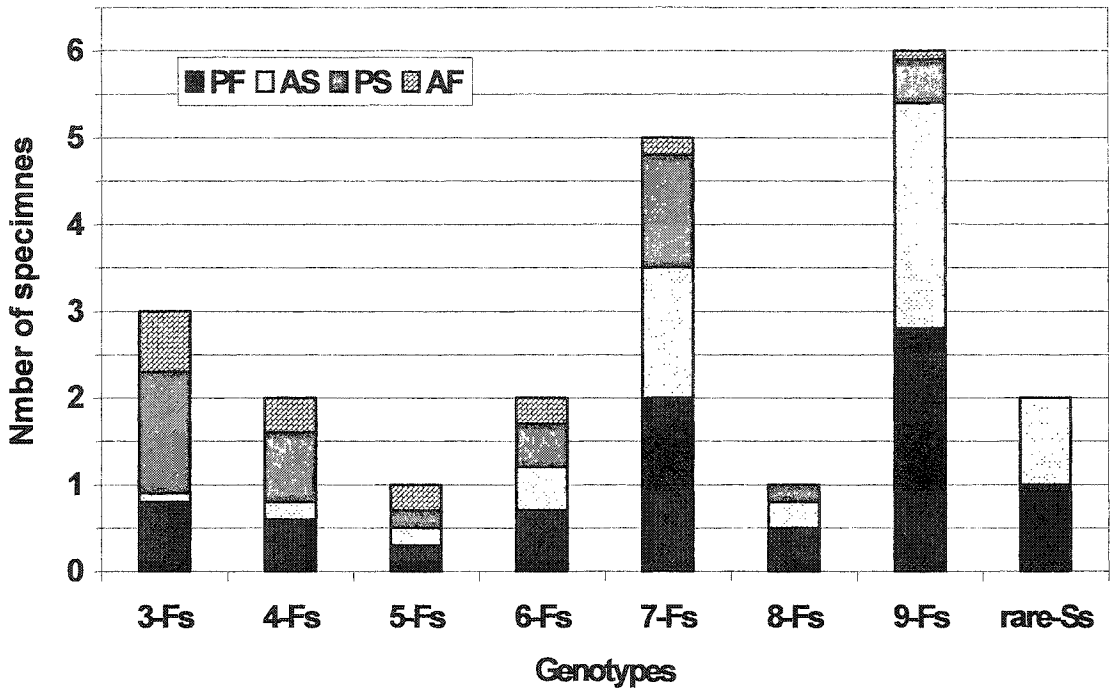


Fig. 24: Numbers of specimens by genotype among phenetic hybrids (**x**). Categories represent the number of genetic *fasciata* states (Fs). Ten Fs would represent a "pure" *Nerodia fasciata* (not shown). The *fasciata* states are defined by the presence of *fasciata* markers and/or the absence of *sipedon* markers. Genotypic categories: 3- to 9-Fs (specimens with 3-9 *fasciata* states based on a full set of 10 primary markers); rare-Ss (rarely a *sipedon* state; specimens with a "pure" *N. fasciata* genotype based on the 10 primary markers, but revealing level of low introgression from *N. sipedon* based on secondary markers). Shading represents the proportions of *fasciata* states (PF and AS) and *sipedon* states (PS and AF) in each category (PF, presence of *fasciata* markers; AS, absence of *sipedon* markers; PS, presence of *sipedon* markers; AF, absence of *fasciata* markers).

snakes (55%) were late-generation hybrids, which included crosses between F_1 hybrids and either parental or hybrid genotypes. No F_1 hybrids were found in the contact zone. Backcrosses of F_1 hybrids to *N. fasciata* were twice as frequent as backcrosses to *N. sipedon*. Although the initial phenetic classification of individuals was fairly accurate (*fasciata* phenotypes generally exhibited more *fasciata*-like genotypes; *sipedon* phenotypes showed a similar but less pronounced pattern), the traditional set of

diagnostic morphological characters did not detect the vast majority of hybrids (~ 90%) found in the contact zone.

TABLE 7. Comparisons of phenetic groups of snakes from the contact zone with categories of genotypes. Phenetic groups: **cf** and **cs**, phenetic *Nerodia fasciata*, and *N. sipedon*, respectively, from the contact zone; **x**, phenetic intermediates between *N. fasciata* and *N. sipedon*. Genotype categories: **Nf**, "pure" genotypes of *N. fasciata* (five *fasciata* markers, no *sipedon* markers); **Ns**, "pure" genotypes of *N. sipedon* (five *sipedon* markers, no *fasciata* markers); F_1 hybrid between **Nf** and **Ns** (five *sipedon* and five *fasciata* markers); F_1 hybrid backcross to **Nf** (five *fasciata* markers, 1-4 sipedon markers); F_1 hybrid backcross to **Ns** (five *sipedon* markers, 1-4 markers *fasciata*); later-generation hybrid (1-4 *fasciata* markers, 1-4 *sipedon* markers). The proportional contribution of each subgroup within the contact zone is indicated in parenthesis.

Genotype category	**cs**	**x**	**cf**	Total
Nerodia fasciata (Nf)	0	1(0.9)	9(8.3)	10(9.1)
Nerodia sipedon (Ns)	0	0	0	0
F_1 hybrid between Nf and Ns	0	0	0	0
F_1 hybrid backcross to Nf	1(0.9)	9(8.3)	16(14.7)	26(23.9)
F_1 hybrid backcross to Ns	11(10.1)	2(1.8)	0.00	13(11.9)
Later generation hybrid	14(13.8)	11(10.1)	35(32.1)	60(55.0)

Distribution of Heterozygotes in Backcrosses of F_1 Hybrids to Parental Species.—The distribution of species markers within both genotypic categories of presumptive backcrossed F_1 hybrids to one of their parental species (see Table 7) deviated significantly from Hardy-Weinberg equilibrium (backcross to *Nerodia fasciata*: (χ^2 = 45.2, $P < 0.0001$; backcross to *N. sipedon*: $\chi^2 = 26.4$, $P < 0.0001$). For example, there was a preponderance of single-locus heterozygotes, such as F_1 hybrids backcrossed to *N. fasciata* that revealed only one *sipedon* marker (Table 8). The situation was similar with F_1 hybrids backcrossed to *N. sipedon*. Perfect Hardy-Weinberg expectations would predict a proportion of 16.7% for a subpopulation exhibiting only one introgressive

genetic marker (single-locus heterozygotes). However, the proportions were 65.4% in presumptive backcrosses to *N. fasciata* and 69.2% in presumptive backcrosses to *N. sipedon*. There was a simultaneous deficit of two- to four-locus heterozygotes (Table 8). Therefore, specimens that genetically most closely resembled the "pure" genotype of either parental species were abundant, whereas others were increasingly uncommon the higher the number of heterozygous loci they contained (= increased heterozygosity). This suggests that some type of negative heterosis (reduction in hybrid fitness) affects offspring with a higher degree of heterozygosity.

TABLE 8. Proportions (%) of introgressive species markers (heterozygotes) in individuals resulting from backcrosses between F_1 hybrids to one parental species. Corrections are made for the absence of five- and zero-locus heterozygotes, which would have been scored as F_1 hybrids or "pure" parental genotype, respectively. Thus, the proportion of heterozygotes was calculated for 30 different genotypic combinations rather than for 32, as predicted by Mendelian considerations, because ~ 3% (1/32) of such backcrosses would be indistinct from a "pure" genotype, and another ~ 3% would be indistinct from an F_1 hybrid.

	Number of marker loci heterozygous			
	1	2	3	4
	Nerodia fasciata backcross			
Observed	65	15	12	8
Expected	17	33	33	17
	Nerodia sipedon backcross			
Observed	69	8	15	8
Expected	17	33	33	17

No linkage between markers was found after screening frequencies for all possible pairwise combinations within a species in the presumptive first-generation backcross to either species. Recombinant genotypes were observed with the relatively high

frequencies of 31% (between markers S2 and S3) to 69% (between markers S2 and S5, as well as between F3 and F5). Only the pairing of S3 and S4 yielded a low recombination frequency of 15%, possibly indicating a linkage disequilibrium.

SUMMARY

Two different primer pair combinations were used with the AFLP to produce five primary and dominant species markers of high scoring quality for each species. Secondary markers were amplified to assist in characterizing genotypes. Species-specific fixation rates for the primary markers in snakes from localities at least 25 km from the boundary of a contact zone were close to 100%, and the markers did not exceed frequencies of 4% in the second species.

Fixation rates of *fasciata* markers in phenetically *fasciata*-like snakes from the contact zone (**cf**) were only slightly reduced compared to "pure" *Nerodia fasciata*, whereas corresponding rates for *sipedon* markers in *sipedon*-like snakes (**cs**) were as low as 74%. Conversely, **cs** showed a higher rate of incorporating *fasciata* markers (as high as 56%) than **cf** showed of incorporating *sipedon* markers. Phenetic hybrids (**x**) more closely resembled *N. fasciata* genetically. Phenetic *fasciata* (**cf**) showed a higher frequency of incorporating a *sipedon* marker versus losing a *fasciata* marker than did **cs** of incorporating a *fasciata* marker versus losing a *sipedon* marker.

The pronounced influence of *Nerodia fasciata* in the contact zone was also manifested by higher frequencies of genetic *fasciata* states than *sipedon* states. Furthermore, the only snakes found in the contact zone that exhibited all 10 loci of one parental species were *N. fasciata*, constituting about 9% of all snakes in the contact zone.

Another third consisted of first-generation backcrosses to a parental species, whereas over 50% are later-generation hybrids. Backcrosses of F_1 hybrids to a parental species exhibited predominantly only one of four possible heterozygous loci, suggesting reduced fitnesses of backcrosses with a greater degree of hybridization (two- to four-locus heterozygotes). Such individuals would show the greatest degree of disruption for species-specific coadadpted gene complexes.

GEOGRAPHIC SECTION

This section describes the phenotypes and genotypes of *Nerodia sipedon* and *N. fasciata* in and near their contact zone in the Carolinas. Epithets (*sipedon* or *fasciata*) are used to refer to species-typical attributes of genetic or morphological characters. For this discussion, snakes were categorized in several ways. First, phenotypes of individuals were compared using traditional diagnostic features of color pattern (dorsal banding pattern, postocular stripe, and ventral pattern: Blanchard, 1925; Clay, 1938; Conant, 1963; Conant and Collins, 1991). The expressions "phenetic *fasciata*" and "phenetic *sipedon*" (*fasciata*-like or *sipedon*-like, respectively) refer to this traditional classification system.

Second, phenotypes were compared with canonical variable scores resulting from three discriminant function analyses (DFA1-DFA3). The first components (canonical variables) of the three DFAs describe overall morphological expression and allow allocation of individuals to the nominal species with which they share the greatest morphological similarity (Fig. 9; Table 5). Redundant, hybrid-index, and sexually dimorphic characters were excluded from the DFAs because of low sample sizes and non-normal distribution of values. In addition, the only fully diagnostic morphological character for the Carolinas, the number of complete dorsal crossbands (CBa), was removed from the DFA because it exerted a disproportionately large influence in determining group allocation and thus masked the influence of other characters. For example, an individual with a *sipedon*-like dorsal banding pattern (CBa) may have been classified as *N. sipedon*, even though most other characters placed it closer to *N. fasciata*.

Presumably phenetic classification based on many characters is more reliable than classification based on a single character. The scores of DFA2 (C2.1) and DFA3 (C3.1) therefore are preferred because they combine the largest number of meristic and morphometric characters. Missing C2.1 or C3.1 scores were complemented with meristic scores of DFA1 (C1.1). All three DFAs are comparable, as the ventral extension of lateral bands (VLBa) and the number of ventral scales (VPV) showed the strongest correlations with the first component of each DFA. DFA scores and a list of specimens, localities and genotypes are listed in Appendix 2.

Third, genotypes were compared with presence/absence data from 10 primary diagnostic species markers (five markers for each species). The two genetic markers with the highest number of scored snakes (S49: n = 253; S53: n = 263) were selected from the set of secondary markers to screen for potential heterozygous (hybrid) conditions in loci of snakes that otherwise appeared to represent a "pure" species based on the 10 primary markers only (see GENETIC SECTION). Such snakes would be classified as later-generation hybrids and exhibit low levels of introgression.

Figure 25 displays the distribution of *Nerodia sipedon* and *N. fasciata* and hybrid zones between them in central and eastern North Carolina based on this and previous studies. More detailed maps display the genetic contribution of both species to single specimens (represented as a percentage) based on the primary and selected secondary markers within six areas in the Carolinas (Figs. 26-31). Additional snakes classified only on the basis of color pattern characters include live specimens, road kills (DORs), museum specimens, literature records, and photographic records. The initial criterion to

delineate the periphery of an active hybrid zone was to include sites with a frequency ≥ 5% of introgressive genetic markers in multiple specimens.

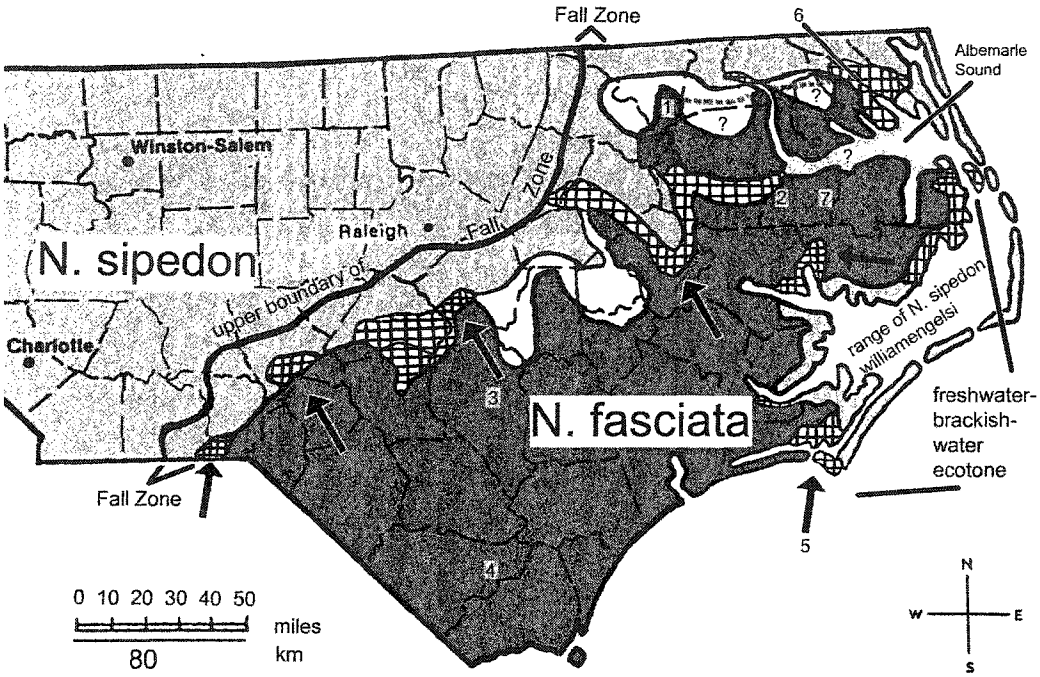


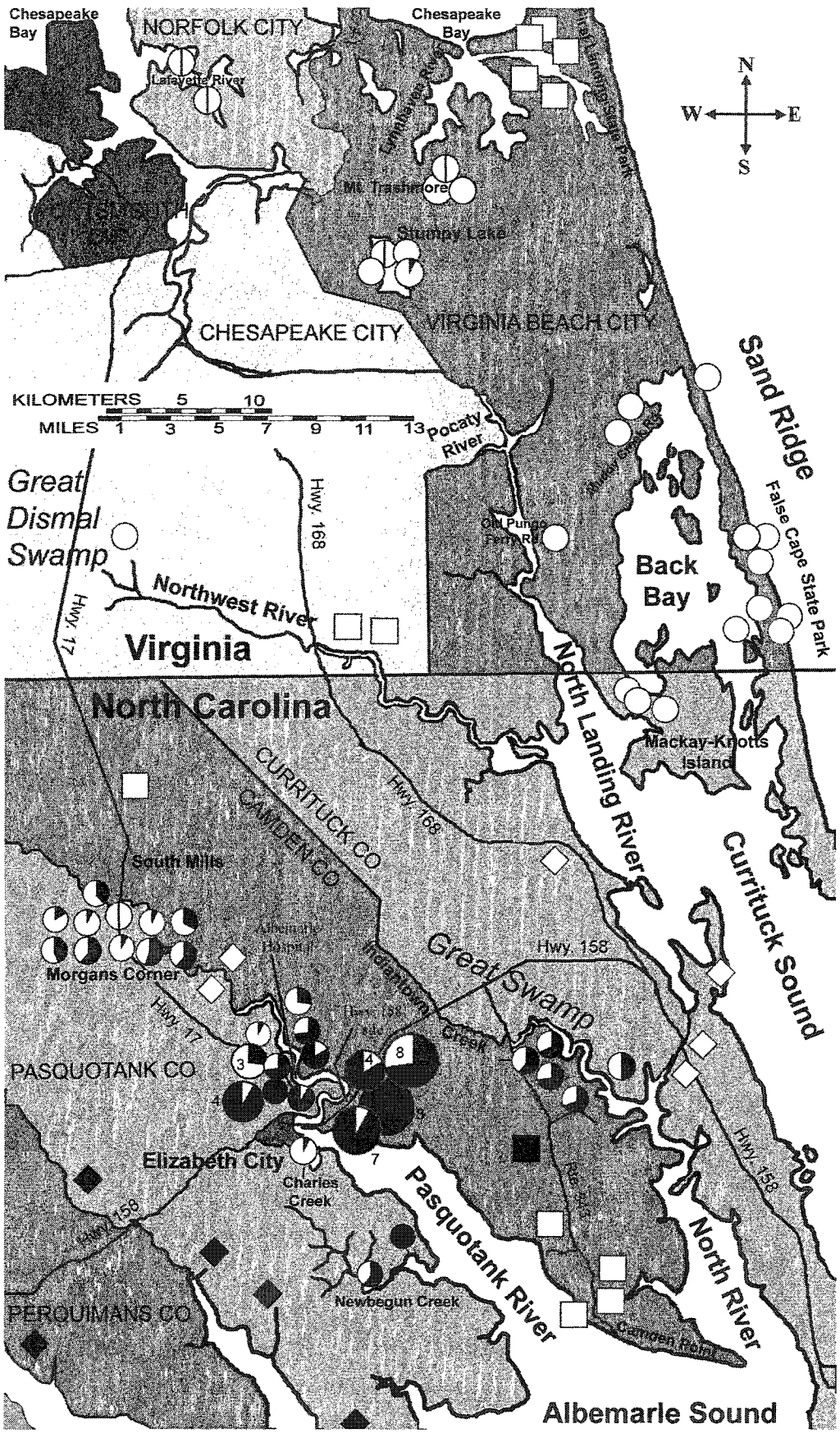
FIG. 25. Distribution of *Nerodia sipedon* and *N. fasciata* and hybrid zones in North Carolina. Range limits are drawn from personal observation and from data from Palmer and Braswell (1995). Cross-hatching shows the extent of hybrid zones and sympatry currently documented with arrows pointing to sites of previous studies (see text). The white areas represent regions from which records of either species are missing. Numbers indicate: (1) Urahaw Creek, Northampton Co. (2) Plymouth, Washington Co., (3) Holts Lake, Johnston Co. (4) Lake Waccamaw, Columbus Co., (5) Shackleford Bank, Carteret Co., (6) Elizabeth City, Pasquotank Co., (7) Phelps Lake, Washington County. The Fall Zone extends approximately 30–50 km from the upper boundary toward the coast (adapted from Conant [1963]; Clay, Orr, and Stuart [1975]).

PRIMARY STUDY AREAS

Area 1 – Coastal Lowland Between the Chesapeake Bay, Virginia, and the Albemarle Sound, North Carolina.—This region (Fig. 26) encompasses an area of 88 x 53 km from the mouth of Chesapeake Bay, Virginia, south to the Albemarle Sound, North Carolina, and from the Dismal Swamp east to the Atlantic Coast. Most specimens collected on the Virginia Coastal Plain and near the Currituck Sound, North Carolina, were predominantly phenetic *Nerodia sipedon* based on overall morphology (canonical scores C1.1 and C2.1) and most color pattern characters. However, individuals with complete dorsal crossbands, a trait typical for *N. fasciata* in the Carolinas, were common in First Landing State Park (FLSP), near the northeastern corner of Area 1. Furthermore, individuals from near the coast of southeastern Virginia more frequently exhibited indications of a *fasciata*-like dark brown postorbital stripe and a few squarish ventral markings. The expression of *fasciata*-like cephalic and ventral patterns also typifies populations of *N. sipedon williamengelsi*, a coastal endemic from North Carolina (Conant and Lazell, 1973; Gaul, 1996), farther south within Area 1. Coastal populations in both Virginia and North Carolina showed also a tendency to develop all-black posterior ventral markings, a diagnostic feature of *N. s. williamengelsi* (Conant and Lazell, 1973). One individual each, from False Cape State Park, Virginia Beach, from Norfolk, VA, and from Northwest River Park, Chesapeake, VA, exhibited a strong tendency toward a *fasciata*-like dorsal banding pattern with 70-80% complete bands.

Genotypes from southeastern Virginia revealed 100% *Nerodia sipedon* expression of the 10 primary species markers except for the occurrence of one *fasciata* marker in an individual from Stumpy Lake, Virginia Beach. Two individuals each from Norfolk and

FIG. 26. Distribution of genotypes in southeastern Virginia and northeastern North Carolina. Individual genotypes are based mainly on 10 primary species markers and illustrated with circles, indicating the proportional contribution of each species; genetic states (presence/absence of species markers) from *Nerodia fasciata* are shown in black and from *N. sipedon* in white: ○ pure *N. sipedon*, ● pure *N. fasciata*, ◐ *N. fasciata* with one of the secondary *sipedon* marker, ⓘ *N. sipedon* missing one of the secondary *sipedon* markers. Numbers in or next to larger circles near Elizabeth City represent the sample size exhibiting that genotype. Those circles are enlarged to emphasize their increased contribution, but are not in proportion to the sample size. Many genotypes from sites along the Pasquotank River are shown slightly displaced for clarity. Squares represent snakes evaluated with phenotypic features of color pattern, such as dorsal banding pattern, postocular stripe, and ventral marking: ■ phenetic *fasciata* and □ phenetic *sipedon* (this study), ◆ phenetic *fasciata* and ◇ phenetic *sipedon* (Brothers, 1965; Palmer and Braswell, 1995).



two from the area between Mt. Trashmore and Stumpy Lake were lacking one of the two secondary markers for *N. sipedon.*

Most snakes collected from borrow pits adjacent to lowland swamps bordering the narrow (10-20 m) Pasquotank River north of Morgans Corner, Pasquotank Co., NC, exhibited a dorsal pattern and canonical scores of overall morphology typical of *Nerodia sipedon* (n = 12). However, Morgans Corner also constitutes the northernmost locality with substantial influence of *N. fasciata*; most specimens exhibited an intermediate ventral pattern and one individual possessed complete dorsal crossbands. Furthermore, the preponderance of *sipedon* phenotypes contrasted with the strong genetic influence from *N. fasciata.* Only one individual revealed a "pure" *sipedon* genotype across all 10 primary markers, although it was missing one of the secondary *sipedon* markers. Three individuals yielded 10% and one each exhibited 20%, 33%, 40%, 43%, 50%, 55%, and 60% (mean = 31%) genetic contribution from *N. fasciata.* There was no relationship between the expression of *fasciata* genes and the presence of complete dorsal bands, as the snake with complete bands yielded only one *fasciata* marker.

The average genetic contribution from *Nerodia fasciata* increases to 63.3% (n = 14) approximately 15 km farther south, near the Albemarle Hospital in Elizabeth City, Pasquotank Co., where the Pasquotank River expands to a width of 200–300 m. One individual revealed 10% genetic influence of *N. fasciata*, four 30%, and nine between 70-100%. *N. fasciata* traits also dominated the color pattern and head shape. Only one specimen near the hospital and three from a stretch 3 km upstream exhibited *sipedon*-like partially broken dorsal bands, a large head, and lack of a postocular stripe. However, the ventral patterns of many specimens received moderate hybrid index scores, and overall

morphology (C3.1 and C1.1) produced values close to zero for eight snakes, indicating intermediacy (hybridization); only two individuals received typical *sipedon* scores. Two phenetic *sipedon* reported from several km upstream from the Albemarle Hospital confirmed the increasing *sipedon* influence to the north (Palmer and Braswell, 1995).

The average genetic influence from *Nerodia fasciata* peaked at 85.5% (n = 27) 3 km downstream from the Albemarle Hospital and 3.8 km east of Elizabeth City, Camden Co., at the edge of a cypress swamp next to Hwy. 158. That genetic expression coincided with predominantly *fasciata*-like dorsal color patterns, but two individuals exhibited broken dorsal bands as in *N. sipedon*. The ventral patterns and overall morphological phenotypes (C3.1) were intermediate, with 15 individuals classified closer to *N. fasciata* and 10 closer to *N. sipedon*. The natural habitat between Morgans Corner, a predominantly *sipedon* site, and Hwy. 158 mainly consists of cypress swamps and shows no vegetational or topographic change except widening of the Pasquotank River.

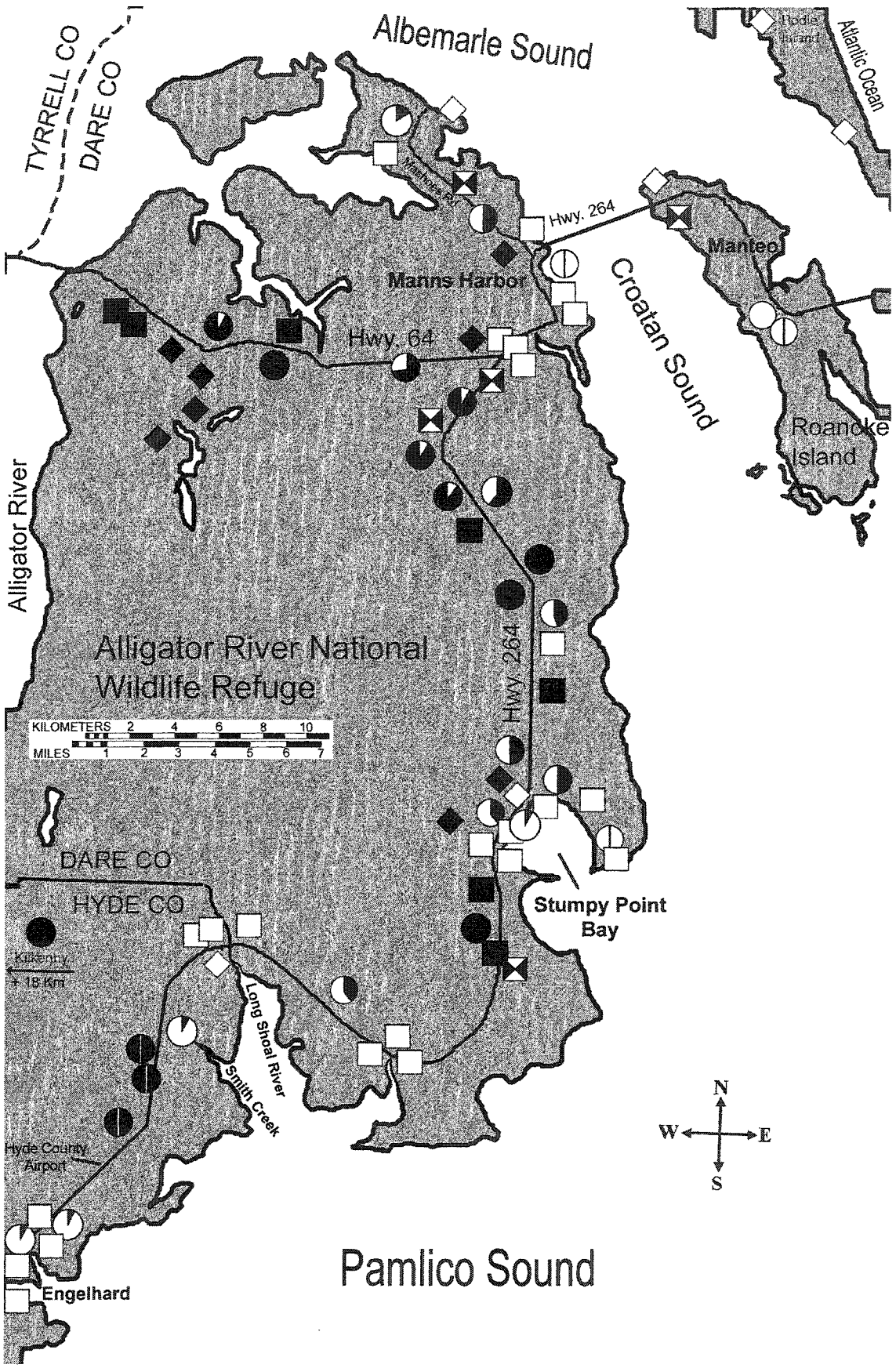
A snake with a dorsal color pattern and meristic canonical scores (C1.1) typical of *Nerodia sipedon* was collected from Charles Creek in southern Elizabeth City, 3.5 km south of the Albemarle Hospital. Its ventral pattern exhibited the intermediate condition commonly found in coastal *N. sipedon* of VA and NC (see above), whereas its genotype revealed 10% *fasciata* influence. Farther south, influence of *N. sipedon* decreased again, as two snakes collected from Newbegun Creek, approximately 10 km south of Elizabeth City, exhibited intermediate and *fasciata*-like meristic scores (C1.1). One of the individuals revealed a "pure" *fasciata* genotype, whereas the other's genotype exhibited 40% *sipedon* influence. Other phenetic *fasciata* were reported from several locations farther southwest of Elizabeth City (Brothers, 1965; Palmer and Braswell, 1995).

Conversely, to the east the genetic influence of *Nerodia fasciata* decreased to ~ 65%, as revealed by four snakes collected from borrow pits southeast of a site where NC Rte. 343 crosses Indiantown Creek (= Currituck-Camden county line). All individuals possessed complete dorsal crossbands but they exhibited intermediate values in ventral pattern, postocular stripe, cephalic shape and meristic characters. About 4.8 km farther east at the North River, a snake with color pattern typical of *N. sipedon* still yielded 50% genetic *fasciata* influence. This river is the southward extension of Indiantown Creek and meanders through the Great Swamp and empties into Albemarle Sound. Individuals from the area farther northeast appeared to be typical phenetic *sipedon* (Palmer and Braswell, 1995), although their genotypes are not known. On Camden Point approximately 10-15 km south of the borrow pits, several specimens exhibited a typical *sipedon* pattern.

Thus, morphological and genetic data show that the Virginia section of Area 1 is occupied by *Nerodia sipedon,* with a transition to *N. fasciata* beginning < 15 km south of the state line with North Carolina. Traits typical of *N. fasciata* become dominant another 15 km farther south near Elizabeth City, but *sipedon* influence remains measurable for at least another 10 km farther south. *N. fasciata* is known from sites west of Elizabeth City, but its influence decreases 20 km to the east and southeast of the city, where *N. sipedon* influence becomes dominant along the North River.

Area 2 – Coastal Zone of the Peninsula Between the Albermarle and Pamlico Sounds; Dare and Hyde Counties, North Carolina.—The eastern region of the peninsula(Fig. 27) bordered by the Albemarle Sound to the north and the Pamlico Sound

FIG. 27. Distribution of genotypes in the coastal zone of the peninsula between the Albemarle and Pamlico sounds in Dare and Hyde counties, North Carolina. Individual genotypes are based mainly on 10 primary species markers and illustrated with circles, indicating the proportional contributions of each species; genetic states (presence/absence of species markers) from *Nerodia fasciata* are shown in black and from *N. sipedon* in white: ○ pure *N. sipedon*, ● pure *N. fasciata*, ◐ *N. fasciata* with one of the secondary *sipedon* markers, ◑ *N. sipedon* missing one of the secondary *sipedon* markers. Squares represent snakes evaluated with phenotypic features of color pattern, such as dorsal banding pattern, postocular stripe, and ventral marking: ■ phenetic *fasciata*, □ phenetic *sipedon*, and ⊠ phenetic intermediate (this study), ◆ phenetic *fasciata* and ◇ phenetic *sipedon* (Palmer and Braswell, 1995).



to the south, herein referred as the Albemarle Peninsula, encompasses an area of 37 x 60 km. Sampling was conducted mainly along Hwy. 264 and Hwy. 64 within the Alligator River National Wildlife Refuge (ARNWR). Extensive freshwater swamps flank the roads but are replaced by salt marshes where bays and creeks carrying saline water extend into the peninsula. Salinity is high near its eastern shores, but decreases to an annual average of 2 ppt toward the western (inner) ends of the sounds.

Two phenetic *sipedon* were collected on Roanoke Island, Dare Co., NC. Each exhibited a postocular stripe and a ventral pattern with a mixture of half-moon-shaped, triangular, and rectangular markings typical of coastal *Nerodia sipedon* (see Area 1). Overall morphology (C2.1) and genotype were also typical for *N. sipedon*, although one individual missed one of the secondary *sipedon* markers. A hatchling recently collected from the island (A. Braswell, courtesy N.C. State Museum of Natural Sciences collection) indicates that *N. fasciata* occasionally reaches the island despite the saline barrier posed by the Croatan Sound separating it from the mainland.

On the mainland only *Nerodia fasciata* was collected as a "pure" genotype along Hwys. 64 and 264 crossing through stretches of lowland freshwater swamps. A transition to *N. sipedon* becomes significant where the highways approach or cross saline environments, especially near Engelhard, Long Shoal River, and Smith Creek in Hyde Co., as well as the area near Stumpy Point Bay, Manns Harbor, and Mashoes, Dare County. Dominance of *sipedon* genetic expression could not be found > 3 km from major saline habitats (bays, lakes, large creeks). However, minor genetic *sipedon* influence reached as far as 6 km inland, possibly facilitated by the inland migration of hybrids along the numerous man-made channels and roadside ditches.

Area 3 - Middle/Lower Tar and Roanoke Rivers; Nash, Edgecombe, Wilson, Pitt, Greene, Beaufort, Martin, Bertie, and Halifax Counties, North Carolina.—This region encompasses an area of 61 x 60 km (Fig. 28). The major feature is the 50-m wide Tar River, which enters the northwest of Area 3 shortly after leaving the Piedmont. The strong flow of the Tar River precludes the development of impoundments, but many small depressions along its tributaries support small cypress stands. Conversely, the 100-m wide Roanoke River in the northeast slowly meanders through the Coastal Plain, supporting substantial lowland swamps.

Most snakes from the northwestern section of Area 3 exhibited color patterns characteristic of *Nerodia sipedon*. Four phenetic *sipedon* were collected from Falls Battle Park, Rocky Mount, Nash Co., and an additional 35 phenetic *sipedon* were sampled at Tar River Reservoir Lake (TRRL), 10.6 km farther southwest. Influence from *N. fasciata* was visible in two individuals from Falls Battle Park that yielded a pronounced postocular stripe and an intermediate ventral pattern, whereas two of six snakes from TRRL revealed an intermediate overall morphology (C2.2). Genotypic expression corroborates the small influence of *N. fasciata*, as four individuals possessed one *fasciata* marker and another snake lacked a secondary *sipedon* marker (overall genetic *fasciata* influence = 1.2%, n = 31).

The influence of *Nerodia fasciata* increased to 11% at Nobles Millpond, Edgecombe Co., approximately 21 km east of TRRL. Two snakes collected at that manmade pond revealed one *fasciata* marker. Introgression was also visible in their color pattern, as they revealed a high degree of complete dorsal crossbands, a postocular stripe and an intermediate ventral pattern. However, overall morphology based on meristic and metric

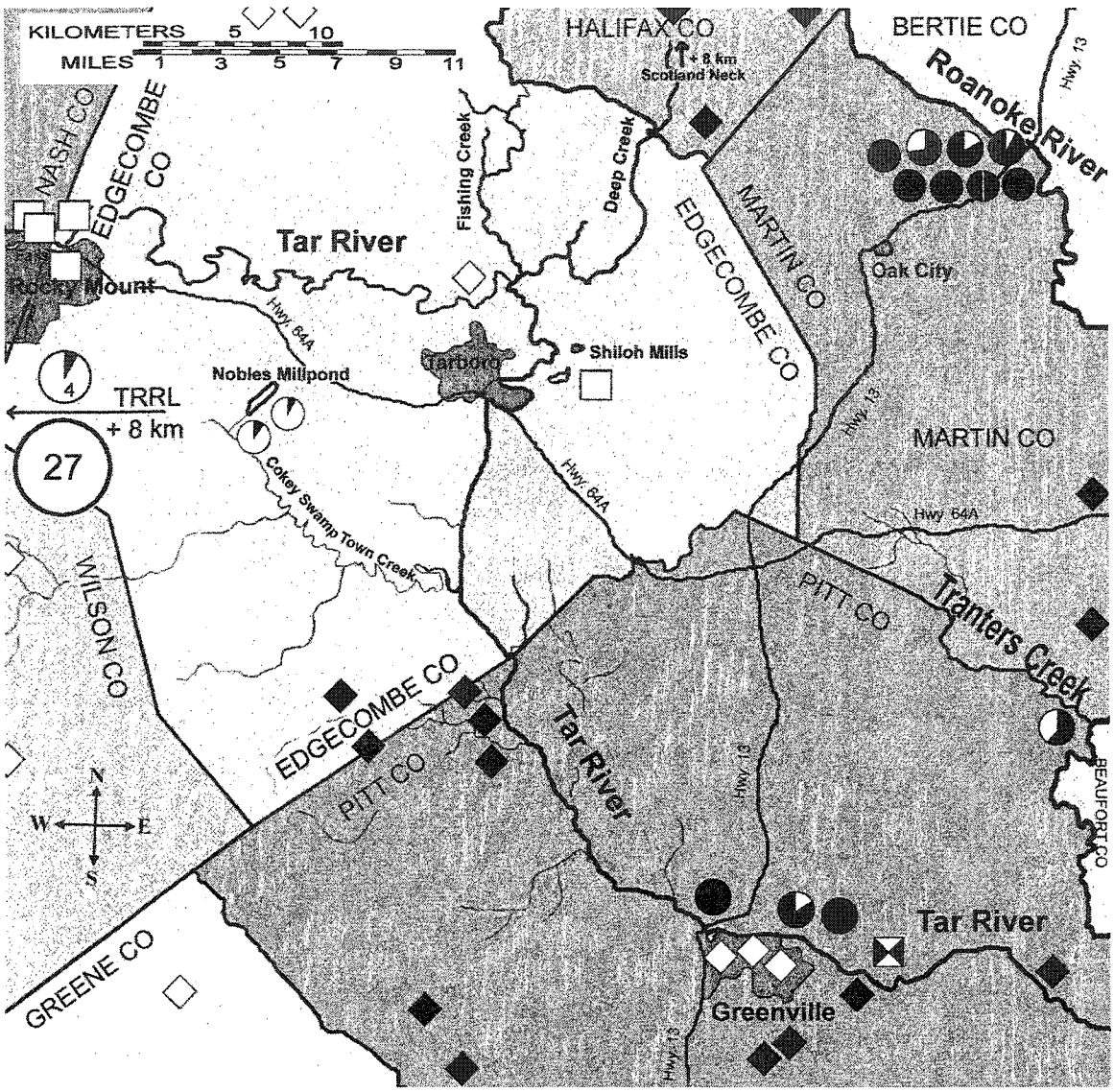


FIG. 28. Distribution of genotypes in the middle/lower sections of the Tar and Roanoke rivers in Nash, Edgecombe, Wilson, Pitt, Greene, Beaufort, Martin, Bertie, and Halifax counties, North Carolina. Individual genotypes are based mainly on 10 primary species markers and illustrated with circles, indicating the proportional contribution of each species; genetic states (presence/absence of species markers) from *Nerodia fasciata* are shown in black and from *N. sipedon* in white; ○ "pure" *N. sipedon*, ● "pure" *N. fasciata*, ◐ *N. fasciata* with one of the secondary *sipedon* markers. Numbers in or next to circles near Rocky Mount and pointing to TRRL (Tar River Reservoir Lake) represent the sample size exhibiting that genotype. Those circles are enlarged to emphasize their increased contribution, but are not in proportion to the sample size. Squares represent snakes evaluated with traditional diagnostic features of color pattern, such as dorsal banding pattern, postocular stripe, and ventral marking; ■ phenetic *fasciata*, □ phenetic *sipedon*, and ⧓ phenetic intermediate (this study), ◆ phenetic *fasciata* and ◇ phenetic *sipedon* (Conant, 1963; Palmer and Braswell, 1995).

characters (C2.2) was typical of *N. sipedon*. Phenetic *sipedon* have been found 18 km farther east from Nobles Millpond near Tarboro, but no genetic data are available (Palmer and Braswell, 1995; this study). Phenetic *fasciata* were reported from small creeks and swamps between Nobles Millpond and their influxes into the Tar River, approximately 10 to 20 km south of Tarboro (Palmer and Braswell, 1995; R. Gaul, pers. comm.).

Snakes collected near Greenville yielded a color pattern and overall morphology (C3.3) predominantly of *Nerodia fasciata* (n = 5). However, one individual exhibited *sipedon* influence with an intermediate ventral pattern, and two other individuals had a few interrupted dorsal blotches near the vent. Genotypes of two snakes were 100% *N. fasciata*, whereas a third individual exhibited 20% genetic influence of *N. sipedon*. Introgression has also been detected along Tranters Creek 21 km farther northeast. Several *fasciata*-like snakes were sighted, but only one could be collected; it exhibited intermediate color pattern and a genotype of 60% *fasciata* influence. These accounts from the Tar River drainage indicate a large hybrid zone, extending from Tranters Creek over 70 km (straight line) to Tar River Reservoir Lake east of Rocky Mount, and possibly beyond that, because "pure" *fasciata* and *sipedon* populations have not been detected along the Tar River drainage within the limits of Area 3.

Farther north, specimens collected along the Roanoke River indicate that *Nerodia fasciata* occupies the extensive lentic habitat as far inland as Scotland Neck, Halifax Co., and Urahaw Creek in southern Northampton County (Palmer and Braswell, 1995; fig. 25). One of 13 phenetic *fasciata* collected in a swamp clearing near the river, 8.8 km northeast of Oak City in Martin Co., exhibited a few *sipedon*-like alternations of the dorsal and lateral blotches. Overall meristic scores (C1.1) generally were close to zero,

indicating intermediate morphology. Five individuals yielded a 100% *fasciata* genotype across the 10 primary markers, whereas one individual revealed also a secondary *sipedon* marker. One snake each had 10, 20, and 30% genetic influence from *N. sipedon*. This *sipedon* influence extends 34 km south of the phenetic *fasciata* record from Urahaw Creek. However, low levels of introgression from *N. sipedon* were still measurable in predominantly phenetic and genetic *N. fasciata* at sites 35 km (Devil's Gut Creek, Martin County) and 50 km (Plymouth, Washington Co.) southeast of the hybrid site near Oak City (Fig. 25). Plymouth lies 8 km west of the confluence of the Roanoke River with the Albemarle Sound, a potential site for *N. sipedon* due to seasonally high salinity (see DISCUSSION). Two of eight individuals from Devil's Gut Creek produced one primary *sipedon* marker and showed influence from *N. sipedon* either in ventral markings or width of interspaces (dorsal bands). The specimen from Plymouth revealed intermediacy in all color pattern characters but no genetic data could be collected. Therefore, the extensive lowland swamps of the Roanoke River drainage between Scotland Neck and the river mouth 70 km downstream are inhabited by *N. fasciata*, which is marginally introgressed by *N. sipedon* from two directions, the Piedmont upstream and the Albemarle Sound downstream.

Area 4 - Fayetteville and Fort Bragg Area; Cumberland and Harnett Counties, North Carolina.—This area north of Fort Bragg (military reservation and city) and Fayetteville encompasses an area of 22.5 x 22.5 km (Fig. 29). The northwest of Area 4 contains many clear ponds situated among sandy hills covered by coniferous forest, such as the Buffalo Lakes, Harnett County. A single individual was collected at that lake. It

possessed a *sipedon*-like dorsal pattern but showed influence from *Nerodia fasciata* in its cephalic and ventral patterns. Overall meristic characters (C1.1) and genotype were intermediate (62.5% contribution from *N. fasciata*). A phenetic *sipedon* voucher specimen was collected approximately 3 km north of Buffalo Lakes (Palmer and Braswell, 1995). Cypress stands become more frequent at ponds toward the east and south, such as the Cambro Pond, 12.8 km east of Buffalo Lakes, where the influence of *N. fasciata* increased to > 90%. The preponderance of *fasciata* traits was also evident in overall morphology of meristic characters (C1.1) and color pattern, as only four of 10 individuals exhibited some influence of *N. sipedon*.

To the south, two snakes from Carvers Creek, a tributary of the 150-m wide Cape Fear River east of Fayetteville, exhibited typical *sipedon* color pattern without externally visible *fasciata* influence (photographs by Bob Flook, North Carolina Herpetological Society). They represent the farthest downstream occurrence of *Nerodia sipedon* along the Cape Fear River, which continues to flow southward through the Coastal Plain. Carvers Creek and other nearby creeks are lotic systems typical of *N. sipedon* with strong currents, cutting their way through clay, creating steep banks interspersed with riffles, cascades and rocky shores for a distance of 1-2 km before joining the Cape Fear River.

Farther west, three snakes from the area of Ft. Bragg exhibited typical *fasciata* color pattern and meristic expression (C1.1). Genotypes were "pure" *fasciata* for two snakes, whereas the third possessed one *sipedon* marker. Therefore, the hybrid zone west of Fayetteville extends at least 16 km from Ft. Bragg north to the Buffalo Lakes. However, introgression of *N. fasciata* into the range of *N. sipedon* probably extends beyond the

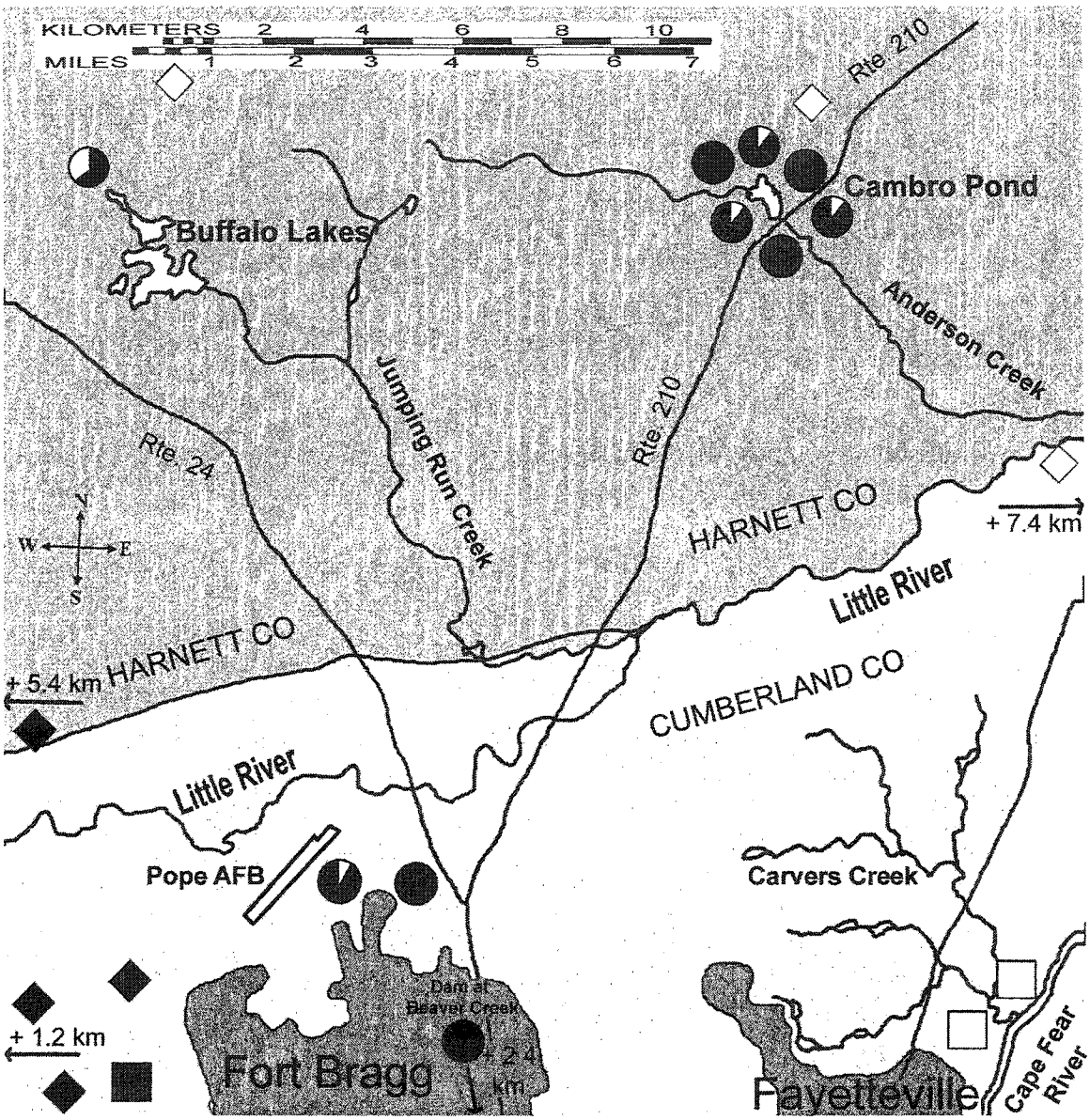


FIG. 29. Distribution of genotypes in the Sandhill area north of Fayetteville and Ft. Bragg in Cumberland and Harnett counties, North Carolina. Individual genotypes are based mainly on 10 primary species markers and illustrated with circles, indicating the proportional contribution of each species; genetic states (presence/absence of species markers) from *Nerodia fasciata* are shown in black and from *N. sipedon* in white; ● "pure" *N. fasciata*. Squares represent snakes evaluated with traditional diagnostic features of color pattern, such as dorsal banding pattern, postocular stripe, and ventral marking; ■ phenetic *fasciata* and □ phenetic *sipedon* (this study), ◆ phenetic *fasciata* and ◇ phenetic *sipedon* (Palmer and Braswell, 1995).

Buffalo Lakes, Harnett Co., where the genetic contribution of *N. fasciata* was still dominant.

Area 5 - Southern Pines; Moore Co., North Carolina.—Area 5 covers approximately 13 x 17 km (Fig. 30) and begins 30 km west of Fayetteville (Area 4). It includes a section of the Little River, which connects both areas. Between those regions several phenetic *fasciata* have been documented from the vicinity of the Little River (Palmer and Braswell, 1995), which, together with the James Creek, flows in an easterly direction through the Sandhills.

Seven predominantly phenetic *Nerodia sipedon* were collected at Thagards Lake and Heritage Camping site near Whispering Pines in the northwest of Area 5, where sandhills, coniferous forests and clear sandbottom ponds shape the landscape. One snake yielded a complete dorsal banding pattern, as in *N. fasciata*, but ventral pattern and meristic characters (C1.1) of all specimens were either intermediate or typical of *N. sipedon*. Genotypic contribution was dominated by *N. sipedon*, but influence from *N. fasciata* was substantial and ranged from 10-60% (mean = 40%). Another snake from a pond 2 km south of Whispering Pines combined a *sipedon* phenotype with a 90% *fasciata* genotype. The pond is approximately 1.5 km north of a reported site of *N. fasciata* (Palmer and Braswell, 1995) and 6 km north of Southern Pines, where a typical *N. fasciata* was collected (phenotype and genotype).

Approximately 6 km southeast of Whispering Pines, the genetic influence of *Nerodia fasciata* doubled to 80% at Crystal Lake. Small creeks 3-5 m in width connect the two sites. However, Crystal Lake represents a typical Coastal Plain habitat, with dense

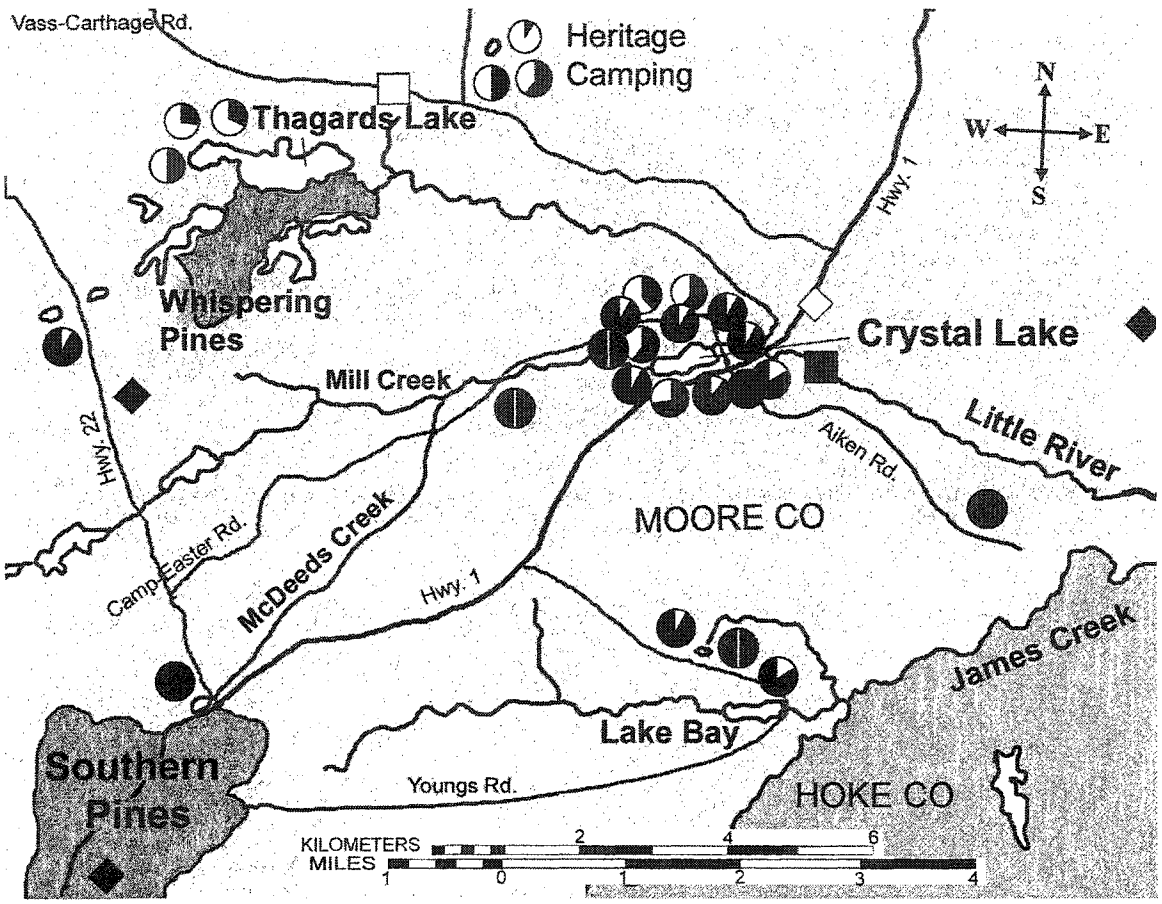


FIG. 30. Distribution of genotypes in the Sandhills northeast of Southern Pines, Moore County, North Carolina. Individual genotypes are based mainly on 10 primary species markers and illustrated with circles, indicating the proportional contribution of each species; genetic states (presence/absence of species markers) from *Nerodia fasciata* are shown in black and from *N. sipedon* in white; ● "pure" *N. fasciata*, ◐ *N. fasciata* with one of the secondary sipedon markers. Squares represent snakes evaluated with traditional diagnostic features of color pattern such as dorsal banding pattern, postocular stripe, and ventral marking; ■ phenetic *fasciata* and □ phenetic *sipedon* (this study), ◆ phenetic *fasciata* and ◇ phenetic *sipedon* (Palmer and Braswell, 1995).

cypress stands. Nine of 14 specimens collected from Crystal Lake and a swampy depression 3 km upstream (Mill Creek) revealed mixed phenetic expression, whereas five individuals were typical of *N. fasciata*. Expression of color pattern characters and genotypes were not concordant. For example, one snake appeared to be a hybrid, with a *sipedon*-like dorsal pattern and a *fasciata*-like ventral pattern, but exhibiting a 100%

fasciata genotype. Its overall morphology (C2.1) was clearly associated with *N. fasciata*. Broods from four gravid individuals from Crystal Lake exhibited character variation as great as in their dams.

Influence of *Nerodia sipedon* is still present near Lake Bay at Hog Island (a residential community), 5 km south of Crystal Lake. Two phenetic *fasciata* from a small pond surrounded by pine forest and one individual from a nearby creek exhibited 80, 90, and 100% *fasciata* genotypes, respectively, although one specimen possessed also one secondary *sipedon* marker. An individual from the creek exhibited alternating dorsal and lateral blotches as in *N. sipedon*, but it displayed a *fasciata*-like ventral pattern. Another specimen collected near Little River, 3.7 km to the northeast, possessed typical *N. fasciata* genotype and phenotype. Therefore, the transition from predominantly *N. sipedon* in the northwest to predominantly *N. fasciata* in the southeast stretches at least 13 km along the Little River and its tributaries and resembles the shape of a stepped cline.

Area 6 – Augusta, Aiken, and the Savannah River Site; Georgia and South Carolina.—This region encompasses an area of 80 x 80 km from the Sumter National Forest southeast to the SRS (Savannah River Site), a federal property that includes a laboratory for field research (Fig. 31). The composition of habitats and topography is somewhat similar to that of the Sandhills in North Carolina (Areas 4 and 5), but with a greater relief in the northern section, resulting in deeper valleys and steeper slopes up to the Aiken-Edgefield county line, from where a flat plateau continues northwestward.

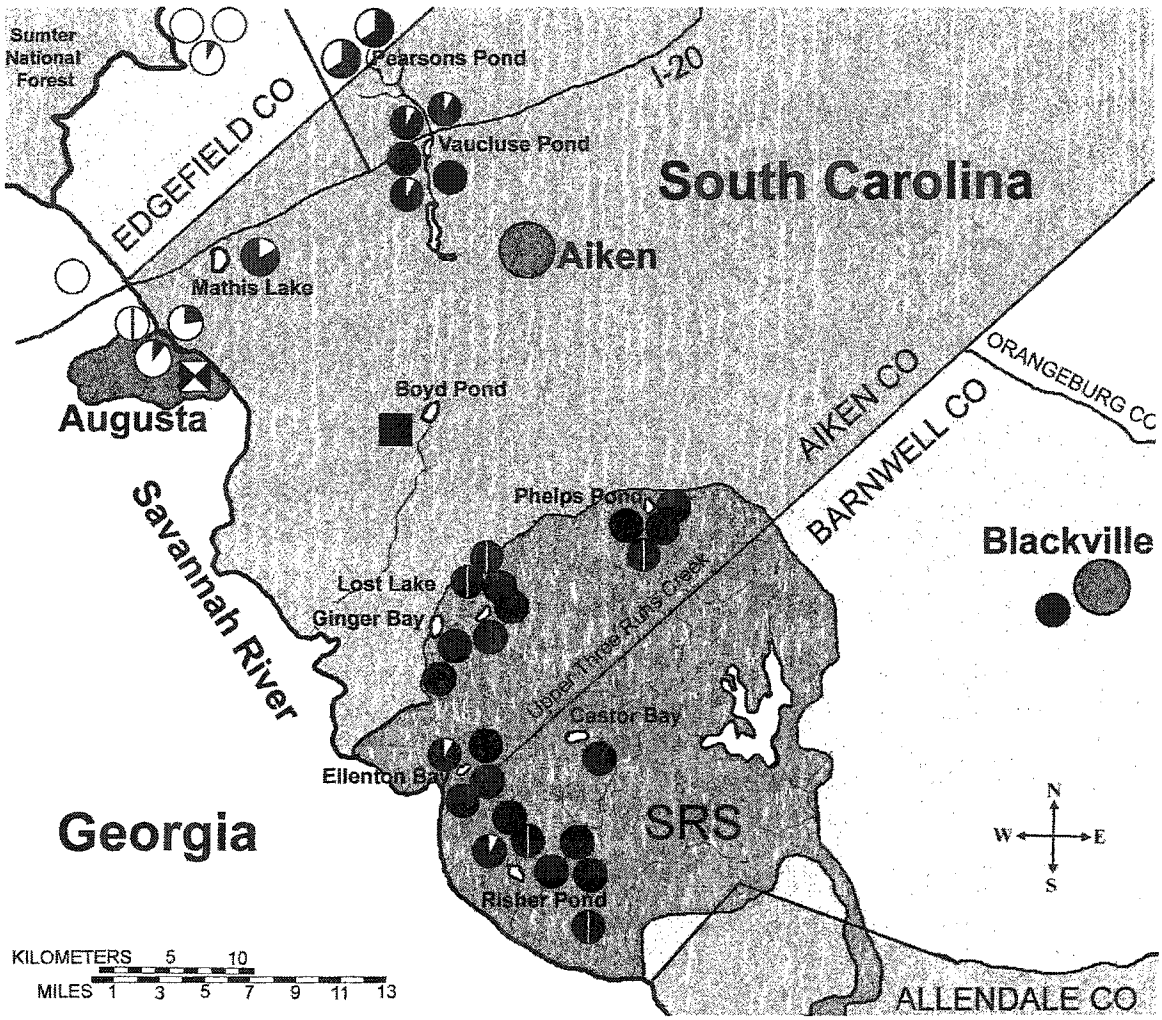


FIG. 31. Distribution of genotypes between the Savannah River Site (SRS) and the Sumter National Forest, South Carolina. Individual genotypes are based mainly on 10 primary species markers and illustrated with circles, indicating the proportional contribution of each species; genetic states (presence/absence of species markers) from *Nerodia fasciata* are shown in black and from *N. sipedon* in white; ○ "pure" *N. sipedon*, ● "pure" *N. fasciata*, ◐ *N. fasciata* with one of the secondary *sipedon* markers, ⓘ *N. sipedon* missing one of the secondary *sipedon* markers. Squares represent snakes evaluated with traditional diagnostic features of color pattern such as dorsal banding pattern, postocular stripe, and ventral marking; ■ phenetic *fasciata* and ⧓ phenetic intermediate (Conant, 1963; this study).

Genetic analysis of 23 specimens from the SRS showed that all but two represented typical (100%) *Nerodia fasciata,* in concordance with their overall morphology (C2.1). The two exceptions each revealed one genetic *sipedon* state. Both were collected near

the Savannah River (~ 4-5 km), a potential source for *N. sipedon*, a snake known to expand its range downstream along large rivers (e.g., Areas 3 and 4). Another five snakes from the SRS revealed one secondary *sipedon* marker, indicating the existence of low levels of introgression farther away from the Savannah River. Typical phenetic *fasciata* traits were found in specimens 11 km farther north at Boyd Pond, Aiken Co., and 16 km east at Blackville, Barnwell County. A specimen from Blackville, the only individual genotype analyzed in this group, exhibited a 100% *fasciata* expression.

Four snakes with predominantly *sipedon* pattern elements were sampled from northern Augusta, GA, 29 km northwest of the SRS. Two individuals were collected within a 30-m stretch of lotic habitat, with rapidly flowing water and a rocky bed, that connects the Savannah River with the parallel running, but 10-m higher (elevated) Augusta Canal. Both snakes revealed some genetic *fasciata* influence, one 12.5% and the other 25%, but only one individual showed intermediacy in dorsal pattern and meristic score (C1.1). Another phenetic *sipedon* collected from the Augusta Canal yielded dorsal and ventral patterns suggestive of introgression by *N. fasciata*. Its genotype was 100% *N. sipedon* across the 10 primary markers, but it lacked one of the secondary *sipedon* markers. A fourth individual from 6 km farther north was a typical *N. sipedon* in pattern characters and genotype. Other putative hybrids have been reported from Augusta, but more precise locality designations were not provided (Neil, 1946; Conant, 1963). The influence of *N. fasciata* increases in a northeasterly direction. A phenetic *fasciata* that exhibited 20% genetic influence of *N. sipedon* was collected at Mathis Lake, 8 km northeast of Augusta, just south of Interstate I-20 in Aiken Co., SC. The lake resembles a marsh, with much emergent vegetation interspersed among smaller pools.

The increasing influence of *Nerodia fasciata* continues beyond the Savannah River. At Vaucluse Pond in Aiken Co., a reservoir located 22 km northeast of the river, five phenetic *fasciata* were collected with *fasciata*-like meristic scores (C1.1) and genotypes with 90-100% *fasciata* contribution. North of Vaucluse Pond the slope of the terrain increases over the next 8 km into Edgefield Co., where two individuals were collected at Pearsons Pond. One resembled a typical *N. fasciata* in color pattern, whereas the other one exhibited intermediate characteristics. Genotypes revealed an increased *sipedon* influence of 37.5% in both individuals. Another 12 km to the west, the genetic influence from *N. sipedon* increased to 97% in three phenetic *sipedon* from a residential pond close to the Sumter National Forest. Two of those individuals exhibited intermediate ventral patterns.

Aside from indications of an expansion of *Nerodia sipedon* from Augusta over 32 km downstream along the Savannah River as far as the SRS, the above localities with moderate *sipedon* influence show that the hybrid zone between *N. fasciata* and *N. sipedon* runs along the Fall Zone, which is paralleled by Interstate I-20. The width of the hybrid zone, estimated by the low *sipedon* influence (< 10%) at Vaucluse Pond and the low *fasciata* influence 18 km (straight distance) farther northwest in Edgefield Co., coincides with a change of landscape associated with the Fall Zone. In this hybrid zone, the transition from a relatively flat area to a more heterogeneous topography and an increased terrain slope is more drastic than across topography-related hybrid zones in North Carolina. For example, the genetic *fasciata* contribution decreased from 94% to 3% across 18 km in Area 6 (South Carolina), whereas it decreased from 90% to 40% over a distance of 13 km in the North Carolina Sandhills (Area 5), and from 85% to 11% over

a distance of 50 km along the Tar River (Area 3). Standardized to a distance of 10 km, the *fasciata* contribution in the hybrid zone in South Carolina decreased approximately by 50%, the one in the North Carolina Sandhills by 43%, and the one within the Tar River drainage by 10%.

Additional Study Areas

Area 7 – Holts Lake, Johnston Co., North Carolina.— A drastic topographic change can be found at Holts Lake, near Smithfield, Johnston Co., NC (Fig. 25), 50 km northeast of Fayetteville. The lake has a sloped bank near its outflow, while dense cypress stands grow along the flat shores at the upper section. Three predominantly phenetic *sipedon* were collected within a section 80 m downstream of the spillway, a segment with many boulders and gravel-bordered pools. Introgression from *Nerodia fasciata* was detected in two individuals which exhibited mostly complete dorsal crossbands and intermediate meristic scores (C1.1). Genotypes revealed *fasciata* contribution of 25% in two individuals and 40% in one. A typical phenetic *fasciata* collected among cypress trees at the upper end of Holts Lake revealed 50% genetic influence of *N. sipedon*.

Other Sites Near the Contact Zone.—A single *Nerodia fasciata* was collected at Lake McKinney, Richmond Co., NC., approximately 30 km southwest of Southern Pines. It revealed a "pure" *fasciata* genotype. The closest phenetic *sipedon* was documented from Millstone Lake 7 km to the northwest (Palmer and Braswell, 1995). Farther south, three *fasciata*-like snakes from the Cheraw Fish Hatchery, Chesterfield Co., SC, revealed

"pure" *fasciata* genotypes. The hatchery lies only 10.6 km southwest of a reported hybrid site north of Cheraw (Conant, 1963).

Other Sites Distant from the Contact Zone.—Snakes from distant areas were used to calculate the fixation rates of genetic species markers. *Nerodia sipedon* from western Virginia and adjacent North Carolina served as a reference for "pure" *sipedon* genotypes. Although they exhibited a few differences in color pattern compared to *N. sipedon* from the Coastal Plain only; similar canonical scores of morphology (C1.1 and C3.1) indicated their close relationship. One *N. sipedon* from Hanging Rock State Park, Stokes Co., NC, and two from Giles Co., VA, lacked one of the secondary *sipedon* markers, and one of them also expressed a *fasciata* marker (total n =7). The regional average of genetic *fasciata* states was < 5% and may be attributed to homoplasy or methodological artifacts (see GENETIC SECTION-MATERIAL AND METHODS). One *N. sipedon* from Bear Creek Lake, Cumberland Co. in central Virginia, revealed a "pure" *sipedon* genotype.

In northeastern Virginia, 12 *Nerodia sipedon* were collected from Pohick Bay Recreation Park, Fairfax County. Genotypes of nine specimens revealed "pure" *sipedon* expressions across all primary markers, and one snake produced a *fasciata* marker. Farther south, four typical *N. sipedon* from the vicinity of the James River in Virginia yielded "pure" *sipedon* genotypes, although two individuals (one from New Kent Co. and one from Prince George Co.) were lacking a secondary *sipedon* marker. Generally, genetic *fasciata* states in those distant *sipedon* from NC to VA occurred in a frequency < 3%.

Distant *Nerodia fasciata* were collected from Lake Waccamaw in Columbus Co. (Fig. 25), NC, approximately 95 km southeast of the nearest site for *N. sipedon* in Fayetteville,

Cumberland County. Four snakes from the lake's environs exhibited typical *fasciata* color pattern and overall morphology (C1.1 and C3.1). All exhibited a 100% *fasciata* genotype across the 10 primary markers, although one snake revealed a secondary *sipedon* marker. In North Myrtle Beach, near the Atlantic coast of SC, two snakes revealed a "pure" *fasciata* genotype across the primary markers, but one individual possessed also a secondary *sipedon* marker. Farther south, seven *N. fasciata* from Cypress Garden near Jamestown, Berkeley Co., SC, and from Sarasota and Manatee counties, FL, revealed "pure" *fasciata* genotypes.

DISCUSSION

MORPHOLOGICAL SECTION

Although many of the morphological characters showed significantly different distributions between *Nerodia sipedon* and *N. fasciata* from the Carolinas, they do not qualify as diagnostic features (with the exception of Cba, see below). In the following discussion the interspecific and intraspecific variation of morphological characters is compared to that found in other studies and is interpreted in regard to their possible underlying biological bases. These interspecific differences are discussed in an ecological context, which is related to the geographic location of the hybrid zone.

Meristic Data

Number of Ventral Scales (VEN), Postocular Scales (PO), and Reductions of Dorsal Scale Rows (SR10, Red21/19, Tred10/8/6).— Generally, females exhibited greater mean VEN than males in both species, and *Nerodia sipedon* had a higher VEN compared to *N. fasciata*. Both differences are accompanied by a similar pattern in the number of scale rows at positions behind the neck (SR10), posterior the midbody (Red21), and anterior the cloaca (Red19). Similar trends for some of those characters have been found by Conant (1963), Schwaner and Mount (1976), Seyle (1980), Mitchell (1994), and Palmer and Braswell (1995).

Sexual dimorphism in VEN has been related primarily to fecundity selection, which favors an increase in maternal abdominal volume, reflected by an increase in VEN (Pope, 1935) and correlated positively with the number of vertebrae (Ruthven and Thompson,

1913; Kramer, 1961). This is a common trait in natricines (Fitch, 1981; Semlitsch and Gibbons, 1982; Mebert, 1993, 1996b). Similarly, relative positions of scale row reductions may relate to sexually dimorphic body shape (Shine, 1993), resulting from adaptations to different reproductive strategies and diets between the sexes. Although Mushinsky et al. (1982) found that females in closely related species of *Nerodia* consume larger prey than males and King (1986) documented the increased fitness of larger compared to smaller female *N. sipedon*, it is not clear how much the increased abdominal volume of females contributes to fitness. A greater food intake allows more energy to be allocated to developing embryos and relates to the increased foraging activity of female *N. sipedon* and *N. fasciata* during and after the mating season (King, 1986; Greshock, 1998; pers. obs.).

Differences associated with climate may intertwine with the selective pressures leading to sexual dimorphism, producing additional interspecific differences in VEN and scale row reductions between *Nerodia sipedon* and *N. fasciata*. Fitch (1981) demonstrated that squamates from the temperate zone exhibit relatively larger females than squamates living in a tropical climate, possibly resulting in higher fitness. He associated this with the reduced time available to complete a reproductive cycle in cooler temperate regions (see also Peterson et al., 1993). Activity season differs between the more northern *N. sipedon* and the southern *N. fasciata*. For example, the activity season of *N. sipedon* in northern Ohio is 3-5 months shorter than that of *N. fasciata* in southern Florida (King, 1986; Dalrymple et al., 1991; pers. obs.). A direct effect of gestation temperature on counts of ventral scales in *N. fasciata* has been observed by Osgood (1978), who found that gestation at 26 °C yielded the lowest number of ventral scales,

whereas either higher or lower temperatures produced higher counts. A cool climate may also promote larger body volume in *N. sipedon* to decrease heat loss due to reduced surface/volume ratio and to accumulate more body fat for increased survival at low hibernation temperatures (Blem and Blem, 1995).

A greater number of scales in *Nerodia sipedon* also leads to more interscutellar skin, resulting in an increased stretching capacity. Mell (1929) noted that snake species that eat large prey (e.g., mammals vs. reptiles) possess higher numbers of midbody scale rows and supralabial scales, and associated with this is the ability to stretch the skin over larger prey, an idea repeated by several authors (Gans, 1974; Pough and Groves, 1983). However, others pointed out that Mell's hypothesis does not account for different aspects of prey size (Greene, 1983). For example, ophiophagous and eel-eating snakes can ingest large, elongated prey without significantly compromising their stretching capacity. Shine (2002) recognized Mell's idea of a general association between scale counts and diets, especially the idea that larger snakes have more scales and eat larger prey. However, Shine did not demonstrate a causal effect due to size of the prey, perhaps because prey categories (birds, mammals, reptiles, others) were presented rather than actual diameter of prey. Although *N. sipedon* and *N. fasciata* are similarly indiscriminate in consuming aquatic prey (Clark, 1949; Diener, 1957; Huheey and Stupka, 1967; Mushinsky and Hebrard, 1977a; Brown, 1979, 1992; Collins, 1980; King, 1993c; Mitchell, 1994; Palmer and Braswell, 1995), the relatively larger head in *N. sipedon* indicates the potential that the species may consume larger prey (see below). Similar geographic and sexual variations in scale numbers have been observed in other groups of snakes (e.g., Crotalinae: Klauber, 1941; Colubrinae: Williams, 1978; Dixon, 1983; Savage and

Lahanas, 1991; and Natricinae: Mebert, 1993) and indicate an association with reproduction and dietary shifts. Moreover, a comparable geographic variation for VEN exists intraspecifically in both species, with greater numbers of ventral scales in populations from cooler, more northerly or mountainous (only *N. sipedon*) regions (Boyles, 1952; Cliburn, 1960; Conant, 1963; Schwaner and Mount, 1976; Seyle, 1980; Morris, 1987; Mitchell, 1994; Palmer and Braswell, 1995; this study). Only *N. s. williamengelsi* from coastal North Carolina shows a clinal decrease northward in VEN (Gaul, 1996). In summary, a higher VEN and position of scale row reductions appear to correlate with populations and species from cooler regions, suggesting the evolution of greater body volume to increase reproduction and food intake to compensate for a shorter activity season.

The interspecific difference in caudal reduction of scale rows is reversed from that on the trunk, as *Nerodia sipedon* reduces scale rows more anteriorly than does *N. fasciata*. In addition, the males of both species reduce caudal scale rows significantly more posteriorly in order to accommodate the hemipenis (Semlitsch and Gibbons, 1982; Mebert, 1993). Whether larger volume of the tail base of *N. fasciata* reflects with a relatively larger hemipenis is not known.

Number of Dorsal and Lateral Bands (DBa, LBa).—Similar to VEN, the mean DBa and related LBa (King, 1993a) increased clinally northward and toward the mountains, corroborating similar findings in other studies (Conant, 1963; Seyle, 1980; Palmer and Braswell, 1995). *Nerodia sipedon williamengelsi* again shows a reversed trend (Gaul, 1996). Clinal decrease in DBa and LBa continues in a westerly direction, where the

ranges of both species include or approach those of the subspecies *N. fasciata confluens* and *N. s. pleuralis*, which have lower band numbers (Clay, 1938; Cliburn, 1957; Schwaner and Mount, 1976).

The biological significance underlying variation in the number of dorsal and lateral bands/blotches, if any, is unknown, but cryptic and distractive coloration are means by which snakes reduce exposure to predation (Lillywhite and Henderson, 1993; Mushinsky and Miller, 1993). For example, cryptic coloration as a protection against avian predation has been inferred from the increased survival frequency of unpatterned young Lake Erie watersnakes, *Nerodia sipedon insularum*, in their rocky shore habitat (King, 1993b). Conversely, the bright contrast in banded specimens may reduce predation by inducing "flicker fusion" in the visual systems of diurnal vertebrate predators through the rapid movement of alternating dark and light bands in a moving snake (Shine, 1980a, 1993), or it may disrupt the snake's outline against a background of mixed colors and patterns (Pough et al., 1998; Jansen, 2001; Zug et al., 2001). Beatson (1976) demonstrated that directional selection in *N. sipedon* increases DBa and LBa by 0.09 and 0.12 per generation, respectively; i.e., hatchlings with higher DBa and LBa had a better chance of survival. Beatson suggests that the stronger selection pressure on LBa in the youngest class of snakes and their disruptive ventral coloration may relate to their frequent exposure to small aquatic predators attacking from the side or below. Conversely, *N. fasciata* retained the ancestral condition of lower DBa and complete dorsal bands (see comments on phylogeny in SYSTEMATIC ASPECTS OF *NERODIA SIPEDON* AND *N. FASCIATA*), perhaps because it experienced a reduced selection pressure from visually oriented diurnal predators due to increased nocturnal activity in its warmer range, which is

consistent with increased frequency of melanism as a cryptic protection during night hours (see below).

The trend to a higher DBa (and melanism) in coastal *Nerodia sipedon williamengelsi* may be a selective response to blend with its preferred habitat of black needlebush, *Juncus romerianus*, and grasses of the genus *Spartina* growing on relatively dark, muddy soil, compared to the patchily colored background of the swamps and rocky shores frequented by *N. s. sipedon* farther inland. Such visual protection has been suggested for the striped (longitudinal) color pattern of the closely related salt marsh snake *N. clarkii compressicauda* (Myer, 1988). Meyer suggests that intense predation pressure from wading birds prevents snakes with the conspicuous color pattern of *N. clarkii* from invading the freshwater habitat occupied by *N. fasciata*. The position of the narrow hybrid zone lies where either man-made or climatic disturbances have created a mosaic habitat (e.g., small thickets of *Spartina* within a freshwater marsh). Comparable dorsal patterns (high DBa numbers, stripes, melanism) have been found in salt marsh populations of *N. sipedon* from Virginia to Long Island, NY (Morris, 1987; Conant et al., 1990; Martin, 1998), indicating reduced gene flow from adjacent conspecific freshwater populations, thus preserving locally adapted color patterns along the coast.

Number of Complete Crossbands Dorsally (CBa).—CBa was the only externally visible diagnostic feature to consistently distinguish *Nerodia sipedon* and *N. fasciata* from the Carolinas (Figs. 3 and 33). Phenotypically intermediate snakes from the contact zone (presumptive hybrids) constituted the only snake group to produce CBa values overlapping those of both parental species. The unusual occurrence of *fasciata*-like

completely banded *N. sipedon* has been reported by Werler and McCallion (1951) and Conant (1963) from First Landing State Park (FLSP, formerly Seashore State Park) in extreme southeastern Virginia. Recent photographs of five specimens demonstrate the persistence of such snakes at FLSP (Erik Molleen, FLSP, pers. comm.). Two of those individuals revealed complete dorsal bands throughout, and one showed an unusually high CBa (> 20). The presence of such specimens probably has led to suggestions of *N. fasciata* influence into southeastern Virginia (Conant, 1963; Mitchell, 1994).

However, other findings do not support the hypothesis of current introgression. First, the shape of the posterior complete bands in specimens from FLSP suggests a relationship with *N. sipedon*, because the lateral sections of bands are as wide (along the body axis) as the separate lateral blotches in normally patterned *N. sipedon* and are unlike the narrower lateral markings usually found in *N. fasciata*. Conversely, a few museum specimens (NC State Museum of Natural Sciences) demonstrate that *N. fasciata* with substantial sections of alternating dorsal and lateral blotches occurred at sites distant from *N. sipedon*, such as Lake Waccamaw, Columbus Co., NC, indicating that this trait is not stable either. Second, none of 25 *N. sipedon* sampled between FLSP and the nearest population with substantial influence of *N. fasciata*, 65 km away, exhibited complete crossbanding, or any of the genetic primary markers for *N. fasciata* (Fig. 26). Third, completely banded *N. sipedon* occur naturally along coastal Virginia. One was reported from Smith Island, VA, farther north (Conant et al., 1990), although they are rare elsewhere in Virginia and North Carolina (Morris, 1987; Mitchell, 1994; Palmer and Braswell, 1995; this study). Higher frequencies (23-48%) of completely banded *N. s. sipedon* are also reported from other coastal populations between Maine and New York

(Brown, 1940; Morris, 1987). Completely banded specimens are also regularly found throughout the more southern range of *N. s. pleuralis* (Clay, 1938; Boyles, 1952; Cliburn, 1957; Conant, 1963; Schwaner and Mount, 1976; Seyle, 1980), although some specimens collected near the range of *N. fasciata* probably show complete bands due to introgressive hybridization with *N. fasciata*, e.g., in central South Carolina (Morris, 1987) and in Louisiana and Mississippi (Woodman, 1959; Blaney and Blaney, 1979). Alternatively, complete crossbands at FLSP may be retained from an earlier (hundreds to thousands ago) range shift of *N. fasciata* northward to FLSP. However, such a trait would be unlikely to persist to the present time, as it would be swamped by gene flow of *N. sipedon* from adjacent populations (e.g., Lynnhaven Bay), unless complete bands are locally advantageous or genetically linked to a *sipedon* character that is under positive selection. The hypothesis of ancient introgression receives support from a similar case in southern Illinois (see ECOLOGICAL SECTION, below).

MORPHOMETRIC DATA

Cephalic Proportions.— In reptiles, cephalic dimensions can be an indicator of maximal food size for a species (Schoener, 1977; Vitt, 1983; Voris and Voris, 1983), can correlate with geographic variation of available prey size (Barnett and Schwaner, 1985; Madsen and Shine, 1993), and can correspond to sexually dimorphic dietary habits (Shine, 1993). King (1989) found that some populations of *Nerodia sipedon* and the gartersnake *Thamnophis sirtalis* exhibit a relatively larger body and head due to differences in individual growth rate and demographic composition. Furthermore, Queral-Regil and King (1998) experimentally confirmed a phenotypically plastic

response to feeding experience in neonatal *N. sipedon*. Snakes raised on a diet of fishes of larger diameter and mass developed relatively larger heads (jaw length) than snakes fed the same mass of smaller but more numerous fishes. Although there were significant effects among litter and sex, there was also a clear effect of prey size on jaw length (with body length held constant). Increased mechanical stress on the mandibles during ingestion may cause jaw length to growth more when fed larger fish (Emerson and Bramble, 1993). Consequently, the larger head, and in particular the wider jaw, in *N. sipedon* increases gape size, which could facilitate the ingestion of larger prey when compared to *N. fasciata*. As mentioned above, selective pressures may have existed for *N. sipedon* to ingest larger prey to compensate for a shorter activity season in northern and montane populations.

Eye Size (EyS).—Snakes are known for their keen sense of vomerolfaction, which they use to detect potential prey, but the extensive use of vision is also apparent from the number of diurnal species with large eyes that visually target and pursue potential prey (Shine, 1980b; Drummond, 1985; Theater, 1991; Mullin and Mushinsky, 1997; Zug et al., 2001). Field and laboratory observations by Brown (1940) and in this study confirm the importance of sight for *Nerodia sipedon* and *N. fasciata*, both of which forage terrestrially or above the water surface. Snakes frequently caught leaping frogs in midair, whereas frogs remaining motionless were ignored.

Many nocturnal snakes (e.g., *Imantodes* and *Dipsas*) presumably evolved large eyes to maximize light entry for locating prey above ground under low-light conditions. The large eyes of adult *Nerodia fasciata* (SVL > 30 cm) (Blaney and Blaney, 1979; Morris,

1987; this study) suggest that similar selective forces act to enhance visual foraging at night. Although there are no data on interspecific differences in the visual systems and their relation to success rates in nocturnal foraging, habitat and climatic characteristics probably promote the frequently observed nocturnal activity in *N. fasciata* (Cliburn; 1960; Mushinsky et al., 1980; Dalrymple et al., 1991; this study). Personal observations confirm the visually triggered predatory strike in nocturnally foraging *N. fasciata*, which often slowly approached a floating frog within a few centimeters, not attempting an attack until the frog suddenly moved.

Field observations of both species indicate a decrease in the frequency of nocturnal activity with increasing latitude. For example, *Nerodia fasciata* in Louisiana is predominantly nocturnal from April through October (Mushinsky et al., 1980). In contrast, *N. sipedon* in Michigan, New York, Wisconsin, and Virginia increases diurnal foraging during the cooler spring and fall (Brown, 1940; Cary and Tiebout, 1987; Mitchell, 1994), whereas populations from higher elevations of the Allegheny and Appalachian Mountains remain diurnal even in the summer (pers. obs.). Snakes in northern regions and at higher elevations require solar radiation to compensate for the generally cooler temperatures, especially when foraging in cold water (see ECOLOGICAL SECTION). The snakes likely require elevated temperatures (direct or indirect solar radiation) after exiting the water to thermoregulate at their preferred temperatures (Nelson and Gregory, 2000). Although swimming speed is relatively unaffected by low temperatures, crawling on a solid surface is, leaving cool snakes more vulnerable to predators and decreasing digestive efficiency (Stevenson et al., 1985). Finally, the daylight period increases toward the north during the peak of the activity

season between May and August, which further abbreviates the time available for nocturnal foraging in many populations of *N. sipedon.*

Thus, habitat, climate, and seasonal photoperiod in southern regions are more favorable for nocturnal foraging and may have promoted the evolution of large eyes and melanism in *Nerodia fasciata.* The benefits of nocturnal foraging are related to predator avoidance and prey availability (Gibbons and Semlitsch, 1987; Grothe, 1992), as well as to a decreased risk of dehydration and overheating. Conversely, *N. sipedon* in northern regions may be diurnal and possess smaller eyes, as benefits from visual foraging are reduced in its lotic habitat and smaller eyes are less vulnerable to physical damage while searching for prey in rocky crevices. This likely accounts also for small eyes in the increased nocturnality of *N. sipedon pleuralis* from the Gulf Coastal Plain.

Hybrid Index Data

Shape of Ventral Spots and Prominence of Postocular Stripe (SV, PS).— Despite the extensive variation in ventral pattern (SV), the majority of *Nerodia sipedon* exhibited semilunar markings, whereas rectangular markings occurred commonly in *N. fasciata.* However, many specimens near the contact zone showed intermediate ventral markings, e.g., *N. fasciata* from 15 km west of Greenville, Pitt Co., NC (Robertson and Tyson, 1950; this study) and *N. sipedon* from southeastern Virginia and adjacent North Carolina, which frequently exhibited two rows of *fasciata*-like, laterally compressed rectangular markings in the midventer section. Many of the latter specimens also possessed a postocular stripe (PS) as pronounced as that of *N. fasciata*, confirming previous observations of intermediacy in that area (e.g., Conant, 1963). The simultaneous

appearance of two *fasciata* traits in those *N. sipedon* suggests current introgression from nearby populations of *N. fasciata* (Fig. 26). Recent discovery of a hatchling with intermediate color pattern on Roanoke Island, a barrier island in North Carolina inhabited by *N. s. williamengelsi* (A. Braswell, pers. comm.; Palmer and Braswell, 1995) provides additional evidence of introgression from *N. fasciata* and shows that *N. fasciata* occasionally crosses the 5-6 km distance across the sound from the nearby mainland. Similar overwater dispersal was proposed for the occurrence of a hybrid swarm farther south on Shackleford Bank, another island normally inhabited by *N. s. williamengelsi*, located 2-3 km distant from the mainland (Conant, 1963; Conant and Lazell, 1973). However, the molecular data from southeastern Virginia do not support introgression of *N. fasciata* into the range of *N. sipedon*, as presumed with the common occurrence of *fasciata*-like color pattern in that area; only a single primary *fasciata* marker was found among *N. sipedon* in southeastern Virginia and one secondary *sipedon* marker was absent. As with the occasional occurrence of complete dorsal bands in *N. sipedon*, *fasciata*-like traits may have evolved as regional natural variation or may represent relicts from earlier introgression.

Alternatively, *fasciata* introgression of color pattern characters may be decoupled from the neutral genetic markers. Directional selection of *fasciata* color pattern could promote the introgression of *fasciata*-like color pattern into *N. sipedon* from southeastern Virginia and coastal North Carolina. A combination of these processes may also be responsible for the complex color pattern phenotypes among coastal *N. sipedon*. Interestingly, a similar situation may exist among the snakes of the *Lampropeltis triangulum* complex. The coastal taxon *L. t. elapsoides* from southeastern Virginia and

northeastern North Carolina exhibits a trend toward intergradation in pattern characters with the very different *L. t. triangulum* from the Piedmont and Appalachians (Williams, 1978). Armstrong et al. (2001) suggest that the situation between the two subspecies of *Lampropeltis triangulum* in Virginia may be similar to the one they found in western Kentucky, where two subspecies of *Lampropeltis triangulum* are sympatric but show little morphological evidence of genetic exchange. Regardless of phylogenetic studies in the future that may split the *Lampropeltis triangulum* complex into several species, it remains currently impossible to differentiate between natural variation and current, relictual, or decoupled introgression in the above examples.

Head Shape (AnCR, StCR, SpCR).—Nerodia fasciata has a sharper, steeper, and less curved canthus rostralis than *N. sipedon* (Schwaner and Mount, 1976; Seyle, 1980; Conant, 1963; Morris, 1987; this study). Those features are also evident from skulls of the two species, in which the prefrontal bone in *N. fasciata* shows a steeper slope than in *N. sipedon* (Cope, 1900; Conant, 1963). The sharper canthus and more sloping snout of *N. fasciata* may reduce resistance and facilitate movement through its lentic habitat, which frequently contains dense mats of emergent, floating, and submergent vegetation (pers. obs.).

Distribution of Phenotypes

The initial use of traditional diagnostic characters of color pattern (Conant and Collins, 1991) to classify snakes from the contact zone yielded three phenetic groups; *Nerodia sipedon*, *N. fasciata*, and phenetic intermediates (putative hybrids). Contrary to

Conant (1963), phenetic hybrids could be found where the ranges of the two species came into contact. The higher frequency of morphological hybrids in this study may be the result of a greater effort to sample within the contact zone, thereby uncovering a larger number of hybrids. Despite the ubiquity of phenetic hybrids within the Carolina contact zone, their proportion remained below 24%, reflecting on the numbers of intermediately patterned snakes in previously studied hybrid zones from North Carolina to Louisiana (Conant and Lazell, 1973; Schwaner and Mount, 1976; Blaney and Blaney, 1979; Schwaner et al., 1980; Seyle, 1980; Gaul, 1996). The relatively low proportion of phenetic hybrids and lack of any recognizable physiographic transition paralleling the hybrid zone north of Albemarle Sound initially suggested that the hybrid zone is a tension zone, the position of which is determined solely by a balance of selection against hybrids and influx of parental genes (e.g., Key, 1968; Barton, 1979; Lamb and Avise, 1986). However, environmental factors for the occurrence of hybrids between *N. sipedon* and *N. fasciata* also have been suggested in explanation (e.g., Conant, 1963; Blaney and Blaney, 1979).

The application of traditional diagnostic features of color pattern revealed that individuals phenotypically resembling *Nerodia fasciata* were twice as common as phenetic *N. sipedon* in the contact zone. However, neither phenetic group was completely free from introgression, as indicated by the extended range of values of nondiagnostic morphological characters, which were also biased toward *fasciata*-like expression in the phenetic hybrids.

GENETIC SECTION

Efficacy of Genetic Markers

The molecular methods attempted early in this project included RFLP (Restriction Fragment Length Polymorphism) and sequence analysis of a few conservative genes (ZFY, ZNF6 gene families: Johnston et al., 1998; REP-gene: Kordis and Gubensek, 1998). Both revealed little variation between *Nerodia sipedon* and *N. fasciata.* Only the AFLP method generated a sufficiently resolved genetic pattern of over 10 reliable and selectively neutral (noncoding sequences of the genome) species' markers.

No linkage between markers was found in presumptive first-generation backcrosses to either species, confirming the independence among genetic markers (Mendelian inheritance), except for the *sipedon* markers S3 and S4. These two markers are amplified by different primer pair combinations, sharing the EcoRI primer but differing in their MseI primer by one base pair. These markers are vastly different in size, with S3 ~ 790 bp and S4 ~ 3000 bp long. Hence, they are unlikely to be locus-specific mutations or alleles. Rather, the two markers are in close proximity to each other, increasing the probability of linked crossovers during recombination. In spite of this potential linkage between these two *sipedon* markers, their parallel introgression into phenetic *N. fasciata* was low. Overall, AFLP emerged as a highly efficient method for distinguishing the two closely related species.

Distribution of Genetic Markers

Each AFLP species marker was nearly fixed in its corresponding species (based on comparisons with specimens from outside the contact zone), but introgression resulted in

a slightly decreased frequency of genetic markers in the two parental groups from the contact zone and a greater mixing of markers in the phenetic hybrids. Dominance of *fasciata* markers paralleled the situation for morphological characters. The combination of 10 genetic species markers revealed that more than 90% of the specimens from the contact zone represented some type of hybrid.

The abundance of hybrids in the contact zone initially suggested that the two species created a hybrid swarm with freely interbreeding members similar to a panmictic population. Although the genetic influx from peripheral parental populations appears to have been small, continued introgression could result in a complete convergence of their genomes. Moreover, bidirectional mating between both species has been confirmed with mitochondrial data (Gaul, 1996) and under controlled laboratory conditions (N. Ford, pers. comm.). Survival and fertility of second-generation hybrids have been demonstrated by Riches (1976), corroborating the high fertility of wild-caught hybrids collected in this study (Mebert, unpubl. data).

Nontheless, the distribution of genetic markers within the contact zone is asymmetrical, rejecting the hypothesis of a panmictic population. First, all genetic markers for *Nerodia sipedon* showed frequencies lower (< 46%) than expected at Hardy-Weinberg equilibrium (~ 75%), whereas frequencies of markers for *N. fasciata* were near equilibrium or higher, indicating selection against *N. sipedon*. Genetic drift, assortative mating, migration, and mutation pressure are unlikely mechanisms to select against *N. sipedon* in the contact zone, in part because extensive habitat is available, mating is bidirectional, and hybrids are abundant. Second, the frequency of genetic *fasciata* states (presence of *fasciata* markers and absence of *sipedon* markers) in phenetic *sipedon* is

higher than the reverse, genetic *sipedon* states in phenetic *fasciata*. In both phenetic groups this genetic exchange is achieved by incorporating introgressive markers rather than by releasing their own markers, but the difference was slightly more pronounced in phenetic *fasciata*. This suggests that genes of *N. sipedon* disrupt the phenotype of *N. fasciata* less than the phenotype of *N. sipedon* is affected by the introgression of *N. fasciata*. Third, phenetic hybrids show dominance of *fasciata* genetic traits. Finally, *N. fasciata* was the only species contributing "pure" genotypes into the contact zone from adjacent parental populations.

The preponderance of morphological and genetic *fasciata* traits within the contact zone implies two possible scenarios. First, the contact zone may represent an area of introgression of *fasciata* genes into the former range of *Nerodia sipedon* -- i.e., *N. fasciata* is in the process of displacing *N. sipedon*. Already Seyle (1980) suggested that the occurrence of a few *fasciata*-like traits of color pattern in *N. sipedon* from near the contact zone in Georgia and the lack of the reverse situation indicate introgression from *N. fasciata*. This process was termed allopatric introgression by Arnold and Bennett (1993), whereby directional selection results in the local predominance of one of the taxa. Second, the area may represent a stable contact zone with a heterogeneous habitat, thereby allowing both species and their hybrids to coexist. A local selective advantage accorded to *N. fasciata*, together with limited intrusion of *N. sipedon*, could explain the observed pattern under either scenario.

Distribution of Hybrid Genotypes

Specimens from the contact zone showed an unequal distribution when divided into categories containing different genotypes, including parental species and hybrid genotypes. The category containing later-generation hybrids was dominant in the contact zones (55%). No true F_1 hybrid could be detected, but backcrosses to a parental species included about 35% of all specimens within the contact zone, whereas "pure" parental genotypes were uncommon (< 10%). The lack of F_1 hybrids likely is the result of the wide hybrid zone (> 20 km, except for the coastal zone in Area 2), which reduces the probability of reproduction between *Nerodia sipedon* and *N. fasciata* from populations adjacent to the contact zone (Szymura, 1993). A low dispersal rate (e.g., Fraker, 1970; Fitch and Shirer, 1971; Michot, 1980; Cary and Tiebout, 1987; Prosser et al., 1999) supports this interpretation. A similar pattern of hybridization was documented in two hybridizing species of treefrogs. At one pond close to 40% of the population constituted first generation backcrosses and later-generation hybrids were rare (3.6%), indicating either selective disadvantage against that group (Lamb and Avise, 1986) or genetic swamping by parental genotypes.

Possible selection against increased hybridization has been detected in this study among putative offspring from backcrosses involving F_1 hybrids and parental species. The frequency of snakes containing only one introgressive marker is about twice that of all other categories combined (containing two, three, or four introgressive markers) and deviates significantly from Hardy-Weinberg expectations. Similarly, Lamb and Avise (1986) found significant deviations among heterozygotes of presumptive backcross progeny in treefrog hybrids, albeit not as strong as among *Nerodia* backcrosses. The

selective disadvantage of specimens with a greater degree of hybridization (increased heterozygosity) may be mediated by epistasis between loci due to the disruption of coadapted gene complexes within each species (Hewitt, 1993; Arnold and Hodges, 1995). The apparent reduction in fitness of some *Nerodia* hybrids may reflect the distinctive adaptations of two species, similar to the habitat-related reduced fitness of hybrids between the fire-bellied toads *Bombina variegata* and *B. bombina* in Kostanjnica, Croatia (Szymura, 1993).

In summary, my study revealed extensive introgression between *Nerodia sipedon* and *N. fasciata*, with a dominance of *fasciata* traits and certain hybrid types as well as a possible selective disadvantage for specimens with an increased hybridization. This pattern of a mixed selection for and against certain genotypes in the hybrid zone resembles a structure proposed with the Evolutionary Novelty Model by Arnold (1997). Although new hybrid types would be formed every new season, the presumed persistence of the hybrid zone over four million years (see *Phylogeny* below) suggests that the term stability is more appropriate than novelty in this case.

Cryptic Introgression

The traditional set of color pattern characters usually sufficed to differentiate between *Nerodia sipedon* and *N. fasciata* from outside (> 15 km) the contact zone in the Carolinas. Within the contact zone those characters conformed well to the majority of genetic markers expressed by an individual. For example, a snake classified as phenetic *N. sipedon* revealed also a majority of *sipedon* genes. These traditional color pattern characters likely are based on only a few genes and do not adequately represent the

overall genetic composition of an individual but are heavily weighted in herpetological taxonomy (Rose and Selcer, 1989). Conversely, the genetic markers selected presumably are not involved in coding for color pattern, as indicated by three phenetic *sipedon* that contained genotypes with 60-90% *fasciata* states, and two snakes with introgressive color pattern that revealed "pure" *fasciata* genotypes. Due to the inconsistencies between color pattern characters and genetic data, 67.5% of the hybrids were not detected when using the traditional pattern characters. Such cryptic introgression can include specimens from all categories of presumptive backcrosses, including F_1 hybrids to a parental species, later-generation hybrids, and specimens exhibiting all 10 primary loci of one species but having an introgressive expression at one of the secondary markers (11^{th} or 12^{th} locus). Such cryptic introgression demonstrates that the superficial perception of an individual's taxonomic allocation can be deceived by the choice of a few color pattern characters, at least for snakes from the contact zone (Rose and Selcer, 1989).

In contrast, genotyping by AFLP method or use of canonical scores gathered over many morphological characters reflects an average genetic expression across a wider sample of the genome, and thus better reflects individual similarity to either species than the few traditional characters of color pattern. Thus, in previous morphological studies many hybrids may have gone undetected in the absence of molecular data (Conant, 1963; Schwaner and Mount, 1976; Blaney and Blaney, 1979; Seyle, 1980). A few other amphibian and reptilian studies describe the inconsistency between morphological and genetic variation. Examples include the subspecies of the copperhead, *Agkistrodon contortrix* (Gloyd and Conant, 1990; Knight et al., 1992), and the frog *Rana japonica* (Sumida, 1996), and cases of cryptic introgression between *N. fasciata* and *N. clarkii*

(Jansen, 2001), the toads *Bufo americanus* and *B. hemiophrys* (Green and Pustowka, 1997), and the salamanders *Eurycea longicauda* and *E. guttolineata* (Carlin, 1997) and *Triturus carnifex* and *T. cristatus* (Arntzen and Thorpe, 1999).

Cryptic introgression probably also is involved in the replacement of *Nerodia sipedon* by *N. fasciata* along the Bogue Chitto River in southeastern Louisiana. Blaney and Blaney (1979) suggest that the extensive variation and frequent intermediate expression of color morphs between the species reflect abundant gene flow between two subspecies rather than restricted hybridization between species. Schwaner et al. (1980) also found a few specimens with introgressive color pattern in the Bogue Chitto and Tchefuncte rivers, but argued for species status for snakes from that area based on clear genetic differences, including (1) species-specific alleles in two allozymes, (2) the occurrence of four unique alleles at frequencies > 0.05, and (3) marked frequency differences of common alleles at two additional loci.

However, the investigation of species-specific alleles of two codominant allozyme markers is insufficient to detect many backcrossed and later-generation hybrids, as Mendelian criteria require five codominant species markers to keep the probability of misclassification < 5% (Avise, 1994). With only two fixed markers, the probability of such misclassifications rises to 25% for F_1 hybrids and higher for later-generation hybrids. A comparable calculation generated with AFLP markers showed that 44-62% of hybrids in the Carolina contact zones would have been classified as "pure" parental species, if based upon only two codominant markers rather than five. Furthermore, unique alleles would not detect many of the later-generation hybrids, and differences in the frequencies of common alleles actually could be interpreted as evidence of low levels

of introgression. For example, the co-occurrence of the same transferrin alleles in *N. sipedon* and *N. fasciata* from those rivers (Schwaner et al., 1980) indicates introgression; Lawson (1987) found that none of those transferrin alleles were shared between "pure" specimens of both species. The controversy between Blaney and Blaney (1979) and Schwaner et al. (1980) appears to be one of arguing for substantial or little introgression, respectively.

Ecological Section

A parapatric distribution of two closely related species may correlate with environmental parameters, indicating distinctive ecological requirements for each. Disturbances near their contact zone could lead to a breakdown of ecological barriers and promote hybridization (Mayr, 1963, 1970). Examples include hybrids between the toads *Bufo americanus* and *B. fowleri* (Blair, 1941) and coyotes (*Canis latrans*) and gray wolves (*C. lupus*) (Roy et al., 1994). Hybrid zones may shift in response to fluctuating population sizes, as in toads *B. americanus* and *B. hemiophrys* (Green and Pustowka, 1997). This was also the original interpretation for the occurrence of hybrids between *Nerodia fasciata* and *N. sipedon* in disturbed and anthropogenic habitats in the Carolinas (Conant, 1963; Conant and Lazell, 1973). However, habitat disturbance is not required, because *Nerodia* and *Bufo* hybrids have been documented from relatively undisturbed habitats (Schwaner and Mount, 1976; Blaney and Blaney, 1979; Seyle, 1980; Green, 1984; Gaul, 1996).

Distinctive ecological requirements can be difficult to demonstrate and may require experimental studies such as reciprocal transplantation (Futuyma and Shapiro, 1995),

although they can be inferred from the concordance between range limits or hybrid zones and environmental transitions such as climatic gradients or vegetational ecotones (e.g., Moore and Price, 1993; Patton, 1993; Goyenechea et al., 1996). Resource partitioning in sympatric snake species is most commonly attributed to food (Carpenter, 1952; Mushinsky and Hebrard, 1977a; Shine, 1977; Brown and Parker, 1982; Reinert, 1993), but literature records indicate no distinction in prey selection between *Nerodia sipedon* and *N. fasciata* (Clark, 1949; Neill, 1951; Diener, 1957; Huheey and Stupka, 1967; Mushinsky and Hebrard, 1977a; Brown, 1979; 1992; Collins, 1980; King, 1993c; Mitchell, 1994; Palmer and Braswell, 1995). However, the two species do exhibit distinctive habitat preferences (Parker, 1948; Gordon, 1952; Cliburn, 1957; Anderson, 1965; Smith, 1961; Conant, 1963; Barbour, 1971; Schwaner and Mount, 1976; Hebrard and Mushinsky, 1978; Blaney and Blaney, 1979; Seyle, 1980). Generally, *N. sipedon* occupies temperate lentic and lotic water systems with swiftly flowing, shallow streams, inorganic bottom (e.g., sand, bedrock), steep banks, frequent riffles, shoals in hilly and mountainous areas. In contrast, *N. fasciata* is restricted to subtropical lentic systems along coastal and alluvial plains where streams widen, deepen, and become sluggish, flowing over softer sediments frequently accompanied by swampy floodplains and cypress stands. The two species may form a hybrid zone where the transition between those habitats is not sharp.

I suggest that three environmental factors may limit *Nerodia fasciata*'s expansion into the range of *N. sipedon*: (1) salinity along the coast, (2) temperature along the northernmost range limit, and (3) water current related to topography in areas near the Fall Zone.

Salinity.—Intolerance to saline water appears to impose a barrier for *Nerodia fasciata* to advance substantially into coastal salt marshes occupied by *N. sipedon.* Adaptations of *N. sipedon* against the risk of dehydration in a highly saline environment may be similar to those evolved by the closely related *N. clarkii* (*N. fasciata clarkii* complex; Jansen, 2001), which inhabits salt marshes and mangroves along the Florida and Gulf coasts. *N. clarkii* has a lower skin permeability, resulting in lower body water efflux and lower body sodium influx than *N. fasciata* and freshwater *N. sipedon* (Krakauer, 1970; Kochman, 1977; Dunson, 1980). *N. clarkii* suffers little from dehydration in saline conditions and avoids drinking saltwater, whereas *N. fasciata* drinks saltwater to compensate for the water loss, leading to a deadly cycle of increasing body sodium influx and drinking more saltwater (Pettus, 1958, 1963). Zug and Dunson (1979) also found that *N. clarkii* consistently prefers freshwater over saltwater ranging between 3.5 ppt to 30 ppt (= sea water), whereas *N. fasciata* showed a random distribution across all salinity levels. This suggests that *N. clarkii* exhibits an innate opportunistic behavior to seek freshwater. The experimentally evaluated differences reflect habitat preferences of *N. fasciata* and *N. clarkii.* Pettus (1958, 1963) found that although morphological traits of *N. fasciata* have been detected in snakes living at sites with salinities of up to 3.0 ppt, *N. fasciata* generally inhabited waters with an average of 0.8 ppt. Conversely, traits of *N. clarkii* dominated at salinity levels > 5.0 ppt.

Recently, Martin (1998) found similar physiological differences between populations of *Nerodia sipedon* from saltwater and freshwater habitats in Maryland and Ohio. Offspring of females from saltwater sites showed oxygen consumption up to 35% higher than freshwater offspring, suggesting increased metabolic functions such as

osmoregulation to maintain the ionic pumps for salt efflux or the production of metabolic water. Freshwater offspring became stressed in saline water and died in 4–8 weeks. Their bodies swelled from drinking saltwater, whereas no negative effects were observed in the saltwater offspring raised under equivalent conditions. Perhaps similar differences characterize *N. s. williamengelsi* from the salt marshes of North Carolina and *N. fasciata* from nearby freshwater habitats. *N. s. williamengelsi* also avoids drinking saltwater (Conant and Lazell, 1973).

The interspecific differences described above can be applied to identify the location of the salinity-related hybrid zone between *Nerodia sipedon* and *N. fasciata*. Hybrids between the two species in coastal North Carolina are best known from ecotones between salt marsh and freshwater habitats in the Croatan Sound, the Pamlico Sound, Shackleford Slue, and the wide, saline lower stretch of the Neuse River (Conant, 1963; Conant and Lazell, 1973; Palmer and Braswell, 1995; Gaul, 1996; this study). Lack of sampling success renders the situation less clear in the Albemarle Sound of extreme northeastern North Carolina. Due to large freshwater influx from five rivers, salinity varies from 0.5-2.0 ppt between April and August (Geise et al., 1979; Environmental Sciences Branch, North Carolina Department of Environment and Natural Resources, unpubl. data, 2002). Extensive salt marshes preferred by *N. sipedon* occur only along the outer (eastern) shores.

Although *N. fasciata* could survive along the inner shores of the Albemarle Sound throughout most of an average year (salinity < 1.0 ppt), a salinity increase to 2.0-4.0 ppt during the period of parturition poses potentially hazardous condition for neonates. Moreover, the diluting effect from freshwater rivers is substantially reduced in dry years

such as 1999, when salinity increased to 3.5 ppt between April and August at the inner end of the sound and to 8.5 ppt between March and August at the outer third of the sound (Environmental Sciences Branch, North Carolina Department of Environment and Natural Resources, unpubl. data, 2002). Occasional dry years with intolerable high salinity levels may be sufficient to prevent *N. fasciata* from permanently colonizing even the inner shores of the sound, which then could be occupied by *N. sipedon* (Fig. 25). Furthermore, the wind and tidal dynamics that are characteristic of northeastern North Carolina promote the transport of saline waters far inland along the wide branches and tributaries of the Albemarle Sound.

A few specimens with typical *sipedon* color patterns from Camden Point, a peninsula protruding into the eastern, more saline area of the Albemarle Sound, and the occurrence of pure *Nerodia fasciata* genotypes only at sites with salinities < 1.15 ppt, support a shoreline distribution of *N. sipedon* along the eastern section of the sound. The closest evidence of *N. sipedon* from farther west in Albemarle Sound is an intermediately patterned snake collected at Plymouth, Washington Co., 8 km upstream from where the Roanoke River flows into the sound (Fig. 25). The extensive freshwater swamps near Plymouth are usually inhabited by *N. fasciata*, but the shoreline of the nearby sound may be inhabited by *N. sipedon* due to the slightly elevated salinity levels, promoting occasional introgression.

Similar inland migration of *Nerodia sipedon* upstream along the Scuppernong River from the Albemarle Sound at Columbia could explain the putative sympatry at Lake Phelps, Washington Co. (Conant, 1963). Although potential dispersal is regarded as low for both species (Michot, 1980; Cary and Tiebout, 1987; Prosser et al., 1999), occasional

long-distance movements may occur along the coast, associated with daily tidal movements or seasonal flooding. Hybridization with occasional migrants would have little effect on the local *N. fasciata* population due to the swamping effect of gene flow from surrounding conspecifics.

A situation similar to that in Albemarle Sound occurs farther south in Pamlico Sound, where surface salinity levels are twice as high (1.0-4.0 ppt; data Environmental Sciences Branch, North Carolina Department of Environment and Natural Resources, 2002). Salinity at the inner stretches, up to 20 km east of Washington, Beaufort Co., rise even more during spring and summer and peak at higher values (16.0-20.0 ppt, August 2002) than at a comparable longitude in Albemarle Sound. The higher salinity levels in Pamlico Sound probably result from decreased freshwater influx from its only tributary, the Tar River, compared to five larger tributaries of Albemarle Sound. This promotes the distribution of salt marshes farther inland and allows *N. sipedon* to penetrate deep into the fingers of Pamlico Sound (Gaul, 1996), although *N. fasciata* inhabits the nearby freshwater creeks (Palmer and Braswell, 1995).

South of Pamlico Sound a hybrid swarm has been reported from Shackleford Bank, and hybrids have been collected from shoreline localities on the nearby mainland (Gaul, 1996). Conant (1963) and Conant and Lazell (1973) suggested that hybridization on Shackleford Bank may have been promoted by immigration of *Nerodia fasciata* floating on debris from the mainland via freshwater currents resulting from occasional torrential rains. *N. fasciata* might survive in the newly created freshwater ponds, subsequently hybridizing with local *N. sipedon*. Alternatively, introgression along the shores of Pamlico Sound and the Pungo River may be facilitated by frequent cyclonic storms that

sweep the coast of North Carolina, mixing fresh and saltwaters. However, the occurrence of hybrids along the mainland ecotones between salt- and freshwater probably represents a stable hybrid zone and is not based on erratic weather events, although such events may increase contact between the species. In undisturbed coastal areas on the Alligator River National Wildlife Refuge (ARNWR) of the Albemarle Peninsula, the ecotone from freshwater to salt marsh habitats often is a short (< 2 km: pers. obs.) zone populated by communities tolerating exposure to constant low salinity or occasional high salinity. *N. fasciata* (or *fasciata* genes) advancing from freshwater would lose their presumed advantage farther into that ecotone, i.e., closer to brackish water. Thus, *N. sipedon* becomes dominant in the more saline habitats. The ecotone may allow only restricted contact between pure genotypes of both species and establishes a natural barrier that is occupied primarily by hybrids. Salinity data recorded while sampling snakes at ARNWR generally corroborates this scenario. Pure *N. fasciata* genotypes could not be found at salinities greater than 0.5 ppt. Conversely, *N. sipedon* with little *fasciata* influence (10%; "pure" *N. sipedon* were not found on the mainland of the ARNWR) were usually collected at salinities > 1.0. *N. sipedon* individuals with less *fasciata* influence were collected at salinities > 4.0 ppt.

The present distribution of ecotones probably is different than in the past, as daily tidal movements transport saline water along many man-made channels deep into historically freshwater habitats. These channels may generate enough mixing to allow *sipedon* influence to reach farther inland along Hwy. 264 in ARNWR than under natural conditions. For example, salinity measurements in channels and ditches along Hwy. 264 revealed freshwater (0.0-0.4 ppt) on one side of the road and brackish water (0.5-10.6

ppt) on the other side. Six hours later, such salinity values change again, due to tidal movement. Nonetheless, the barrier presented by salinity gradients appears to be particularly effective, creating the narrowest hybrid zone in this study (< 3 km).

Temperature.—Interspecific differences in temperature tolerance are related to geographic range and features of the habitat, suggesting an adaptive basis of thermal tolerances (Lillywhite, 1987). *Nerodia sipedon* occupies a colder climate in northern or mountainous regions. Physiological adaptation to cool temperature in *N. sipedon* has been observed by Blem and Blem (1990), as it acclimates more rapidly to varying temperatures than its congener *N. taxispilota*. No comparable data are available for *N. fasciata*.

Some experimental and observational data indicate that *Nerodia sipedon* prefers a lower mean temperature (25°C) than *N. fasciata* (27-28°C) (Mushinsky et al., 1980; Michot, 1981; Blem and Blem, 1990). However, other studies reported a preferred temperature for *N. sipedon* of 28 °C, similar to that of *N. fasciata* (Kitchell, 1969; Lutterschmidt and Reinert, 1990). Furthermore, Mushinsky et al. (1980) found that monthly profiles of body temperatures in *N. fasciata* from Louisiana closely reflect the changes of temperatures of water in which the majority of individuals were captured. In both species, individuals shuttle between air and water, selecting the warmer medium (Brown, 1940; Osgood, 1970), thus influencing diurnal and nocturnal activity (Swanson, 1952; pers. obs.).

In temperate regions, *Nerodia sipedon* may thermoregulate and forage under suboptimal thermal conditions due to prey availability, predator avoidance, feeding

status, dehydration risk, mating season and location (e.g., Huey et al., 1989; Knowles and Weigl, 1990; Dalrymple et al., 1991). For example, *N. sipedon* emerges within days of the snowmelt in Canada (Scribner and Weatherhead, 1994) and has been observed to be active at temperatures as low as 13.0°C (Mitchell, 1994), 11.0°C (this study), 10.0°C (Brown, 1940), and entering water of 5.0°C (King, 1986). Active *N. fasciata* have not been recorded at such low temperatures (Mushinsky et al., 1980; Michot, 1981; this study). Hence, *N. fasciata* may be intolerant of cooler temperatures during the active season, thus limiting its expansion into the range of *N. sipedon.*

Although mean annual air temperature is only one measure of the temperatures experienced by snakes in a given area, it correlates positively with body temperature in snakes (Gregory, 1984; Mushinsky et al., 1980). Hence, annual average air temperature is used as a simple index of temperature regime in an area. The northern limit of the range of *Nerodia fasciata* in North Carolina (Fig. 25) coincides with a mean annual temperature of 15.0-15.5°C (1961-1991: National Climate Data Center, 2002) and the isopleth of 230 days activity season (Cliburn, 1960). The isotherm extend from Currituck Co., east of Elizabeth City, in a westerly direction over 100 km to southern Northampton Co., where Urahaw Creek near Rich Square marks the northwesternmost *N. fasciata* voucher specimen (Palmer and Braswell, 1995; Fig. 25). *N. sipedon* occupies the area north of these isolines. A more detailed map produced by the Southeast Regional Climate Center (2002) with data from 1971–2001 and simultaneous city weather data from Norfolk (VA) and Cape Hatteras (NC) shows that the Tidewater area in southeastern Virginia and northeastern North Carolina shares the same range of annual mean temperatures (15.5-17°C), due to a slight temperature increase in recent years.

Higher temperatures in the current contact zone could reduce the putative advantage of *N. sipedon* in a cooler climate and explain the observed dominance of *N. fasciata* traits in that region.

A similar situation exists at the northern limit of the range of *Nerodia fasciata* in southern Illinois, where the mean annual temperature was measured at 14.7°C (Smith, 1961). Although mean temperatures during 1971-2000 were lower than in northeastern North Carolina, the annual temperatures averaged over the last 70 years (1931-2002) indicate a warm pocket reaching into southern Illinois that was equivalent to the temperatures in northeastern North Carolina (National Climate Data Center, 2002). In a comparable case, the northeastern range limit of the semi-aquatic pitviper *Agkistrodon piscivorus* near Richmond, VA, closely coincides with another isotherm (Blem and Blem, 1995).

The isolated occurrence of completely banded *Nerodia sipedon* at distances of 65-80 km from the closest populations of *N. fasciata* near both the northeastern and northwestern limits of the range of *N. fasciata* may represent a historical, temperature-related range shift. *N. sipedon* with complete crossbands and some other color pattern characteristics typical of *N. fasciata* occur with a frequency of 20-40% at FLSP, Virginia Beach, VA, and near Energy, Williamson Co., and Jonesboro, Union Co., IL, at distances of 65-80 km from the closest populations of *N. fasciata* away (Viosca, 1924; Cagle, 1942; Smith, 1961; Werler and McCallion, 1951; Conant, 1963; this study). Morris (1987) suggested that the *fasciata*-like *N. sipedon* from southern Illinois exhibit extremes of a regional variation, inasmuch as completely banded *N. sipedon* are not uncommon in southern and southwestern Illinois (frequency of approximately 10-25%). However, the

fasciata-like character expression in these *N. sipedon* may constitute a relictual trait associated with an earlier climatic optimum during the Holocene, when the interspecific contact zone shifted northward. The mean temperatures of 5000–8000 years BP were 1–2°C warmer than today (Pielou, 1992; Dawson, 1992). Many currently southern taxa were more widely distributed at northern latitudes during this period, but became locally extinct later (Smith, 1961; Szyndlar and Böhme, 1993). Movement of up to 450 km over at least the past 20,000 years has been suggested for a hybrid zone between two taxa of the Australian grasshopper *Caledia captiva* complex, based on climatic reconstructions and the asymmetric distribution of genetic markers (Shaw et al., 1993). On a smaller scale, the center of a hybrid zone between the toads *Bufo americanus* and *B. hemiophrys* shifted 9.6 km westward over at least 11 years, indicating that measurable changes in distance can be achieved relatively quickly (Green and Pustowka, 1997). Similarly, *N. fasciata* may have expanded northward into lowland swamps of southern Virginia and Illinois during warmer periods in the past, due to a thermal advantage over *N. sipedon*. With the onset of cooler temperatures, *N. fasciata* may have regressed southward, but previous hybridization would have incorporated the *fasciata*-like dorsal pattern into *N. sipedon* populations.

In summary, *Nerodia sipedon* apparently evolved to occupy a cooler climate than *N. fasciata*. The different climatic regimes of the two species influence their current distributions along their northernmost contact zone. Frequent climatic fluctuations may contribute to a relatively broad (> 30 km) hybrid zone in northeastern North Carolina, where other sharp environmental changes (climatic, physiographic, or vegetational) are absent (Clay et al., 1975; Thompson et al., 1999).

Topography and Water Current.—Most of the locations of the contact zone between the two species seemingly result from different preferences for lotic (*Nerodia sipedon*) and lentic (*N. fasciata*) water systems that parallel the changes of landscape relief (see above) associated with the Fall Zone in the eastern contact zone and similar topographic changes in the southern and western contact zones (e.g., Conant, 1963; Schwaner and Mount, 1976; Blaney and Blaney, 1979; Seyle, 1980; S. Trauth, unpubl. maps; this study). Diener (1957) found similarly distinctive preferences for water currents between *N. sipedon* and *N. erythrogaster*. The latter species is ecologically comparable to *N. fasciata*. Although *N. erythrogaster* and *N. fasciata* do not hybridize, several traits, including general range, habitat selection, fossil, morphological, and genetic data, demonstrate that the relationship between them is even closer than between *N. sipedon* and *N. fasciata* (see below). *N. erythrogaster* prefers water currents below an average of 4 m x min^{-1}, and rarely is found above a current of 8 m x min^{-1}. Conversely, *N. sipedon* prefers water currents with an average of 11 m x min^{-1}. In the Coastal Plain of North Carolina, the greater genetic contribution of *N. fasciata* into the contact zone near the Fall Zone appears to relate to an abundance of lentic aquatic systems, typical of the Atlantic Coastal Plain. The following section discusses the regional position of the eastern, southern, and western contact zones in relation to topography and water gradient.

Eastern Contact Zone (Atlantic Coastal Plain).— Topographic gradients increasingly impose an influence on the position of the contact zone between *Nerodia fasciata* and *N. sipedon* northwest of the Chowan River, NC, where stronger currents and increased linearity of creeks and rivers coincide with the reduction of adjoining swampland, perhaps reducing the ability of *N. fasciata* to compete against *N. sipedon*. This transition

is concordant with the limits of introgression of morphological *fasciata* traits upstream along the Chowan River to Winton, 20 km west from the nearest voucher specimen of a *N. fasciata* phenotype, near Harrellsville, Hertford Co. (Conant, 1963; Conant et al., 1990).

Approximately 20 km farther west, the contact zone turns southwest from Urahaw Creek in southern Northampton Co., NC, and continues along the lower end of the Fall Zone, which represents the transition between the Piedmont and the Coastal Plain physiographic regions (Conant, 1963; Seyle, 1980; Palmer and Braswell, 1995). This topographic change is relatively gradual in northern North Carolina, corresponding to the wide hybrid zone between *N. fasciata* and *N. sipedon.*

Nerodia sipedon usually inhabits the upstream section within a drainage system, but it has colonized a few lotic systems many kilometers downstream of the lentic systems occupied by *N. fasciata*, emphasizing the strict relationship of each species with water current. Such an unusual, reversed situation was observed south of Scotland Neck, Halifax Co., approximately 20 km south of Urahaw Creek, where several typical *N. fasciata* have been recorded from Deep Creek, which slowly meanders southward through dense cypress stands (Palmer and Braswell, 1995; R. Gaul, pers. comm.). As the creek enters Edgecombe Co., its velocity increases drastically while running approximately11 km through deciduous forest, creating abrupt upland habitat alongside its banks. Although no specimens could be collected in that section, the habitat is atypical for *N. fasciata* and may constitute a natural barrier for its downstream expansion. The exclusive occurrence of *N. sipedon* farther downstream near Tarboro and 7-11 km farther west in Fishing Creek correlates with the lack of extensive lowland swamps,

consisitent with the segregation of the two species by habitats with different water gradients (Palmer and Braswell, 1995; this study).

Similar situations occur at Holts Lake, near Smithfield, Johnston Co., NC, and at Carvers Creek, a tributary to the Cape Fear River near Fayetteville, Cumberland Co. Streams near Fayetteville normally exhibit a lentic character typical of the Coastal Plain, including the blackwater ponds and cypress stands preferred by *Nerodia fasciata*. However, a few creeks are lotic. For example, Carvers Creek runs 4-6 km across a flat landscape before it drops 30 m within 2 km and joins with the Cape Fear River. The abrupt slope produces strong water currents and the heterogeneous topography typically preferred by *N. sipedon*. Although *N. fasciata* has been confirmed 7 km upstream within from Carvers Creek, only *N. sipedon* has been reported from the creek itself. *N. sipedon* may once have expanded historically into the Coastal Plain along the Cape Fear River and migrated into the smaller tributaries, based on a voucher specimen from south of Linden, Cumberland Co. (Palmer and Braswell, 1995). A few kilometers upstream they came in contact with *N. fasciata* to produce a hybrid zone, or else the drastic topographic changes may have restricted or prevented contact and subsequent hybridization.

Alternatively, *Nerodia fasciata* did not use the Cape Fear River to expand northward and enter the Little River drainage north of Fayetteville and Ft. Bragg. Rather, the species traveled upstream along the numerous streams flanked by extensive lowland swamps farther southwest to reach the Sandhills and Little River, which could be colonized by a short overland dispersal of 1-2 km, a behavioral trait associated with *N. fasciata* (Holman and Hill, 1961; Schwaner and Mount, 1976; Seyle, 1980; Seigel et al., 1995). *N. fasciata* was collected from an isolated upland pond near Lake Bay, Moore

Co., NC, that could have been accessed by migrating 2 km overland from the nearest lowland swamp.

Introgression between *Nerodia sipedon* and *N. fasciata* in the Sandhills west of Fayetteville is promoted by the variable topography, with habitats suitable for either species. The area consists mainly of rolling hills with numerous high gradient streams that dissect the landscape. For example, the mineralized soil supports many permanent ponds surrounded by coniferous forest near Whispering Pines. *Nerodia sipedon* exerts the major influence in these ponds and streams. But the 40% genetic contribution of *N. fasciata* at that location is relatively high and indicates that introgression likely reaches a few more km beyond Whispering Pines. As the streams slow about 6 km to the east, they become interspersed with natural depressions, anthropogenic ponds and reservoirs. The lentic systems favor development of lowland swamps with cypress stands, which are preferred by *N. fasciata*. Introgression from *N. sipedon* probably does not reach far across the Little River into Hoke Co., as its influence is already weak at Lake Bay near the county boundary.

The situation in South Carolina and Georgia is similar to that in North Carolina. The distribution of *Nerodia sipedon* is concordant with the Piedmont, whereas that of *N. fasciata* overlaps with the Coastal Plain or the Dougherty Plain in southwestern Georgia (Conant, 1963; Seyle, 1980). Several of Conant's hybrid sites in South Carolina were revisited, but they produced little sampling success (e.g., two *N. fasciata* from Cheraw, Chesterfield Co.; none from Fairwold Pond, Columbia, Richland Co.). However, I suggest that specimens from two sites reported by Conant (1963) as areas of sympatry along the Fall Zone at Leesville and Gilbert, Lexington Co., may represent members of a

hybrid population not recognized due to cryptic introgression. The hybrid zone may also include sites of sympatry and parapatry near Columbia (Conant, 1963). That the hybrid zone may continue along the Fall Zone in a southwesterly direction into Georgia, where the occurrence of previously reported hybrids in Augusta, Richmond Co. (Neil, 1946; Conant, 1963), could be confirmed with genetic data.

Farther south in Georgia, Seyle (1980) found *N. sipedon* entering the Upper Coastal and Dougherty Plains along larger creeks and rivers, similar to the situation in the Carolinas. Seyle also reported hybrids from the Oggeechee River and Kinchafoonee Creek, several kilometers below the Piedmont in central and southwestern Georgia. *N. fasciata* was common in nearby limestone sink ponds. The two hybrid sites are topographically similar to the Fall Zone in the Carolinas. An isolated *N. fasciata* from Dry Fork Creek, Oglethorpe Co., GA, approximately 75 km from its supposed range limit near the Fall Line (Neil, 1957), may represent a misclassified *N. sipedon* with complete dorsal bands. This locality lies within the Piedmont but exhibits a habitat and several relict species similar to those of the Coastal Plain. Seyle (1980) found mentioned that the status of completely banded *N. sipedon* in Georgia is unclear, whereas Morris (1987) has not found such specimens from that state.

Southern Contact Zone (Lower Gulf Coastal Plain).— An ecological segregation between *Nerodia sipedon* and *N. fasciata* similar to that along the Atlantic Fall Zone has been found in the Gulf Coast states. However, the contact zone between the two species follows the transition between the Red Hills and the Lower Gulf Coastal Plain east of the Mississippi River, which constitutes the topographically equivalent of the Fall Zone of

the Atlantic Coastal Plain. The Upper Gulf Coastal Plain generally is more strongly dissected and resembles the Piedmont in this gradient (Fenneman, 1938).

Replacement of *Nerodia sipedon* by *N. fasciata* includes intermediary phenotypes and has been observed across southern Alabama and along the drainages of the Escambia-Conecuh and Yellow rivers near the border with Florida (Boyles, 1952; Schwaner and Mount, 1976). Similar to the eastern contact zone, *N. sipedon* extends south into the Lower Gulf Coastal Plain along the larger rivers, whereas *N. fasciata* occupies aquatic systems in the intervening areas (Neill, 1954; Schwaner and Mount, 1976). Interbreeding occurs near the large rivers and the apparent scarcity of typical *N. fasciata* in larger streams is due to genetic swamping by *N. sipedon*.

Blaney and Blaney (1979) found a similar situation along the Pascagoula River and its tributaries in southeastern Mississippi and the Bogue Chitto River in southeastern Louisiana, where the gradual change of topography and associated stream current correlate with the replacement of *Nerodia sipedon* by *N. fasciata*. In addition, sympatry and hybrids were reported from Stone and Forrest counties to the west (Cliburn, 1957). Habitat associations of *N. fasciata* and *N. sipedon* become more distinct farther west, as the topography changes from hilly southwestern Mississippi to the flat alluvial floodplain in adjacent Louisiana is abrupt (JHUAPL and Ray Sterner, Color Landforms Atlas of the United States, 2002). There, *N. sipedon* penetrates the range of *N. fasciata* along north-south streams that cut through sand and clay hills across southwestern Mississippi and southeastern Louisiana, with no morphological indication of introgression (Gordon, 1952; Conant, 1963; Blaney and Blaney, 1979; Schwaner et al., 1980; Rossman, per. comm.). For example, *N. sipedon* was collected within the Amite River drainage,

whereas *N. fasciata* was observed in adjacent ponds (Blaney and Blaney, 1979) and in a backwater swamp only 50 m on the other side of a sandbar (D. A. Rossman, per. comm.). However, hybrids may occur in Livingston Parish, as indicated by a specimen illustrated by Blaney and Blaney (1979: Fig. 4b) and in the vicinity of Clinton and Wakefield, East Feliciana Parish (Cliburn, 1957; Conant, 1963).

Western Contact Zone (Mississippi River Valley).— Conant (1963) originally assumed that *Nerodia sipedon* and *N. fasciata* overlap without hybridization over a large area from southern Illinois and southeastern Oklahoma to southern Alabama and the Florida Panhandle. Later, he substantially reduced this area of sympatry, with a relatively large range overlap remaining only in Arkansas (Conant and Collins, 1991). Maps prepared for an upcoming field guide (S. Trauth, pers. comm., Arkansas State University) confirm the unusually extensive overlap between *N. sipedon pleuralis* and *N. fasciata confluens* in Arkansas.

As in Louisiana, increased topographic heterogeneity appears to separate the two species farther north along the eastern alluvial plain of the Mississippi River Valley. For example, the bluffs just east of the Mississippi River near Vicksburg, Warren Co., MS, provide a drastic topographic change and form a barrier between both species that continues northward through western Mississippi (Blaney and Blaney, 1979; Conant and Collins, 1991). In adjacent Tennessee, *Nerodia sipedon* occupies the gravel-bottomed streams but is replaced by *N. fasciata* in the sand-bottomed streams, sloughs and ponds adjoining the Mississippi River near Memphis, Shelby Co., and Reelfoot Lake, Lake and Obion counties (Parker, 1939, 1948; Conant, 1963). Barbour (1971) noted similar ecological differences between *N. fasciata* and *N. sipedon* in southwestern Kentucky. In

southern Illinois, Conant (1963) and Smith (1961) reported sympatry restricted to Horseshoe Lake, Alexander Co., at the northern range limit of *N. fasciata*, associated with the occurrence of rocky outcrops of the Shawnee Hills region.

A large area of sympatry occurs along the western alluvial plain of the Mississippi River (Cliburn, 1960) and will be discussed further below. Generally, *Nerodia fasciata* occupies the lentic systems of the flat Mississippi River Alluvial Plain, which expands in a southwesterly direction from southern Illinois into southeastern Missouri and covers most of eastern Arkansas. *N. fasciata* also inhabits the scattered lentic systems between the rolling hills in the Upper West Gulf Coastal Plain in southern Arkansas and small areas of adjacent southeastern Oklahoma and invades the mountain ranges along low elevated valleys (< 200 m elevation), such as the Arkansas River Valley between the Boston and Ouachita Mountains and valleys along the Saline, Ouachita, and Red rivers at the southern fringe of the Ouachita Mountains (Anderson, 1965; Webb, 1971; S. Trauth, pers. comm. and unpubl. maps). Conversely, *N. sipedon* inhabits all the mountainous regions to the west in both states, but expands deep into the Gulf Coastal Plain along larger rivers. For example, it extends 48 km (White River), 130 km (Arkansas River), 95 km (Ouachita River), and 145 km (Saline River and Bayou Bartholomew) straight distance from the points where the rivers exit the mountains. The ability of *N. sipedon* to occupy aquatic systems with substantial currents probably has favored expansion along those rivers, as it seemingly has in the eastern and southern contact zones (Conant, 1963; Schwaner and Mount, 1976; Blaney and Blaney, 1979; this study). However, the comparably much larger expansions along rivers in the Mississippi River Alluvial Plain of Arkansas suggest that *N. sipedon* may have dispersed from mountains and hills

adjoining the White and Arkansas rivers, distances of 15-40 km. Other reported localities for *N. sipedon* in syntopy with *N. fasciata* on the alluvial plain include the Black River in Randolph Co., AK (Woodman, 1959), the Bald Knob NWR, White Co., and Overflow NWR, Ashley Co. (L. Irwin, pers. comm.), all of which are close to upland habitat more typical of *N. sipedon.*

A possibly isolated population of *Nerodia sipedon* occurs along the Crowley's Ridge in northern Arkansas, a small upland region that is completely surrounded by flat bottomlands inhabited by *N. fasciata* (unpubl. maps, S. Trauth; JHUAPL and Ray Sterner, Color Landforms Atlas of the United States, 2002). Sympatry in the bottomlands of the Mississippi River is indicated by single records of *N. sipedon* from the Wapanocca NWR, Crittenden Co., AK, two records from Dunklin Co., MI, and one from Mississippi Co., MI (Johnson, 2000). These records are < 20 km from upland habitats on Crowley's Ridge and in Kentucky and Tennessee.

Although the zone of sympatry between *Nerodia sipedon* and *N. fasciata* in Arkansas is extensive, reaching a maximum width of 190 km, and despite the hybridization in captivity of *N. f. confluens* and the eastern *N. s. sipedon* (Riches, 1976; N. Ford, pers. comm.), no natural hybrids have been verified from Arkansas, Oklahoma, or Missouri except one, a potential hybrid from the Black River drainage along the Ozark Escarpment in northern Arkansas (Terry Schwaner, pers. comm. in Seyle, 1980). Color pattern characters (Cliburn, 1960) and allozyme data (Lawson et al., 1991) indicate that *N. f. confluens* has diverged substantially from the eastern *N. f. fasciata* and and southern *N. f. pictiventris* (eastern and Florida ssp.), which among themselves exhibit little pattern and no genetic differences. That divergence of *N. f. confluens* may be reflected in reduced

gene flow between *N. sipedon* and *N. fasciata* in the central Mississippi Valley, although further genetic studies of snakes from that region are needed.

In summary, mine and other studies consistently describe ecological segregation of *Nerodia fasciata* and *N. sipedon* based on several environmental factors, including salinity, temperature, and topography and water current. *N. fasciata* avoids water with salinity > 1.0 ppt, regions with a mean annual temperature below 14.7-15.5°C, and lotic systems, but dominates in the lentic system of the southern coastal and alluvial plains. Interspecific differences in ecological preferences and tolerances may impose limitations on genetic exchange due to hybrid inferiority outside the core of the hybrid zone. Selection against certain hybrids probably involves the disruption of coadapted gene complexes, negatively affecting the ecological adaptations of the parental species. However, the extensive introgression uncovered in this study suggests that the two species form hybrid swarms that successfully occupy habitats intermediate between those of the parental species. The position of hybrid zones reflects habitat alterations and temporal fluctuations over shorter (salinity) or longer (temperature) intervals. The current position of a hybrid zone is likely to be the result of several environmental factors, affecting the fitnesses of hybrids over many generations.

A mosaic of lotic and lentic systems occurs along the Fall Zone. There the hybrid zone probably occurs where isolated pockets of lowland swamps become too few and the distances between them too great to be colonized by *Nerodia fasciata*, adapted to those lentic systems. Increasing lotic systems upstream favor *N. sipedon*. Although the underlying topographic features generally are more stable than climatic variables,

anthropogenic influences may play a major role in the future position (and movements) of the hybrid zone. Such influences may include deforestation and agriculture (which affect hydrology and shoreline alterations) or increasing global temperatures. Hybrid zones can also move with changes in environmental factors. Dynamic shifts of the hybrid zone associated with past fluctuations in environmental parameters may be responsible for apparent introgression by *N. fasciata* 40-80 km from the current hybrid zone.

The width of hybrid zones reflects the width of the topographic transition, a pattern that corresponds to the geographical-selection-gradient model (Slatkin, 1973; May et al., 1975; Endler, 1977; Moore and Price, 1993). For example, the frequency of genetic markers between the species exhibits a steep cline in the hybrid zones of South Carolina (Area 6) and the Sandhills of North Carolina (Area 5), correlated with a heterogeneous topography, whereas the smooth cline seen along the Tar River drainage in northern North Carolina (Area 3) is consistent with a gradual transition of topography.

Although hypotheses about the role of ecological variables and their morphological correlates in determining the position of the hybrid zones require experimental testing (Reinert, 1993), the concordance between transitions of environmental features and genetic species markers strongly suggest a causal relationship. This is unlike the tension zone or dynamic equilibrium model, in which hybrids are less fit and selection is balanced against dispersal, producing a hybrid zone of constant width, located in regions of low population densities (Barton, 1979; Harrison, 1993). However, the possible selection against increased hybridization in certain backcrosses may fit the tension zone model. The environmental data underlying the contact zones, and the selection for and

against different hybrid genotypes correspond to the Evolutionary Novelty Model proposed by Arnold (1997).

SYSTEMATIC IMPLICATIONS FOR *NERODIA SIPEDON* AND *N. FASCIATA*

Species Concepts

One of the objectives in this study was to determine which species concept best describes the relationship between *Nerodia fasciata* and *N. sipedon*. First, the primary genetic species markers with nearly 100% fixation rates produced with the AFLP method fulfill the requirement of the Phylogenetic Species Concept (Cracraft, 1983) by demonstrating diagnosable characters clearly distinguishing two species. All *N. sipedon* from localities outside the contact zone were unified by at least five diagnostic species markers, including individuals from localities as distant as Pohick Bay Regional Park, Fairfax Co., VA; Augusta, GA; Manteo, Roanoke Island, Dare Co., NC; and Grapefield, Bland Co., VA. *N. fasciata* was also identified by at least five diagnosable genetic markers that unify individuals from north of the Albemarle Sound, NC, to those from Sarasota Co., FL. There was a transition between these species markers across a relatively narrow hybrid zone that varied between 5-70 km in width. The reliability of these species markers was not tested on specimens outside the study area (the southeastern US), but the high frequency of secondary species markers corroborates the validity of the primary genetic species markers and is consistent with substantial divergence between *N. fasciata* and *N. sipedon*

Second, despite the new genetic evidence supporting the specific status of *Nerodia fasciata* and *N. sipedon*, substantial gene flow between those species in the Carolinas

confirms previously suspected introgression based on morphological intermediacy (e.g., Conant, 1963; Schwaner et al., 1976; Blaney and Blaney, 1979). However, unbalanced genotype frequencies (deviation from Hardy Weinberg equilibrium for heterozygous conditions of AFLP markers) and relatively narrow hybrid zones indicate that mutual gene flow is reduced. Gene flow can be reduced by factors such as geographic distance (Wright, 1943, 1969), dispersal capability (Palumbi, 1992), ecological specialization (Futuyama and Moreno, 1988), and habitat patchiness (Rodderick, 1996). Distinct habitats and small dispersal rates are likely factors delimiting the ranges of *N. sipedon* and *N. fasciata* and preventing their complete introgression. Limited gene flow could promote further divergence and accelerate the process of speciation (Mayr, 1963; Bush, 1975; Rice and Hostert, 1993) or at least maintain the species' integrity over most of their ranges. In summary, the largely parapatric distribution, presence of diagnosable genetic characters (allozymes: Lawson, 1987; AFLP markers: this study), significant morphological differences, distinctive habitat preferences, and indirect indication of hybrid inferiority suggest that *N. sipedon* and *N. fasciata* are on independent evolutionary trajectories. As such, the two lineages conform to the Evolutionary Species Concept (Simpson, 1961; Wiley, 1978; Frost and Hillis, 1990). Reproductive compatibility is regarded as a symplesiomorphic trait, which has been maintained for over 4 million years (see below). Gergus et al. (1999) provide a comparable example among bufonids.

Phylogeny

The relationship between *Nerodia fasciata* and *N. sipedon* has had a long scientific history. Boulenger (1893) lumped both taxa into one species that also included *N.*

clarkii, *N. erythrogaster* and *N. rhombifer*. Conant (1963) elevated *N. fasciata* and *N. sipedon* to species based on rarity of hybrids and the apparent lack of a natural transition zone. However, the discovery of intermediate phenotypes along several contact zones has continued to generate uncertainty concerning the status of *N. fasciata* and *N. sipedon* (Schwaner and Mount, 1976; Blaney and Blaney, 1979; Schwaner et al., 1980; Seyle, 1980; Morris, 1987). Lawson (1987) employed allozyme data to resolve the phylogenetic relationships within the genus *Nerodia*. He found a close relationship between *N. fasciata*, *N. sipedon*, *N. clarkii*, *N. erythrogaster*, and *N. harteri* (including *N. paucimaculata*), which he termed the *sipedon* group. However, the relationship between *N. sipedon* and *N. fasciata* has remained unresolved.

Frequencies of 10 informative allozymes (Lawson, 1987) were reanalyzed for this research to evaluate the relationship within his *sipedon* group. Allozymes were selected that yield a substantial variation within the *sipedon* group, with allele frequencies > 5%, which reduces the influence of local mutations. The most parsimonious arrangement of synapomorphic alleles indicated that *N. erythrogaster* is basal within the *sipedon* group, a conclusion also supported by mtDNA sequence data (Densmore et al., 1992) and by the oldest fossils within the *sipedon* group (Holman, 2000). The sharp canthus rostralis, large eyes (Morris, 1987) and vertebral shape (Holman, 2000) of *N. fasciata* are symplesiomorphies shared with *N. erythrogaster*, but *N. fasciata* shares one synapomorphic allele with the remaining species in the *sipedon* group (Lawson, 1987). *N. fasciata* has also higher interdemic genetic differentiation than *N. sipedon*, possibly reflecting a longer period of isolation (Lawson et al., 1991; King and Lawson, 2001).

The two youngest lineages, *N. sipedon* and *N. harteri* (including *N. paucimaculata*), are defined by two synapomorphic alleles.

The largest discrepancy in this phylogenetic synopsis involves the presence of an allele (Fum b; Lawson, 1987) in a substantial proportion of *Nerodia sipedon* (29%) that also occurs in 85% of *N. clarkii*. However, that allele is absent in *N. fasciata confluens* or occurs in < 4% in *N. f. fasciata* and *N. f. pictiventris* (Lawson et al., 1991). The co-occurrence of allele b in *N. sipedon* and *N. clarkii* may represent homoplasious evolution of co-migrating Fum alleles.

Several rare alleles shared between *Nerodia fasciata* and *N. sipedon* may indicate the unintentional inclusion of backcrossed hybrids phenotypically indistinguishable from the parental species by Lawson (1987). Many of his *N. fasciata* originate from localities within 20-50 km of potential areas of introgression by *N. sipedon*. Such alleles usually occur at frequencies of < 5% in one or both species. Rare alleles often are more frequent in hybrid zones than in parental populations (e.g., Barton et al., 1983; Case and Williams, 1984).

A sequence divergence of ~ 2.5% in mtDNA between *Nerodia sipedon* and *N. fasciata* dates their split from a common ancestor 1-2 million years ago (Gaul, 1996), applying the generalized mtDNA molecular clock of 2% sequence divergence per million years (Brown et al., 1979; Wilson et al., 1985). However, Gaul's (1996) calculated divergence may be an underestimation resulting from a small sample size (n = 4, including two hybrids), missing RFLP fragments (Appendix in Gaul, 1996), and the large sequence divergence of 7.7% between *N. fasciata* and *N. harteri* (including *N. paucimaculata*), which is closely related to *N. sipedon* (Lawson, 1987; Densmore et al.,

1992). Moreover, Gaul (1996) applied a smaller and different combination of restriction enzymes than Densmore et al. (1992) did, which may reduce the probability of finding divergent mtDNA sequences. The application of different sets of restriction enzymes among those authors complicates comparisons. Different nucleotide positions within mtDNA evolve at varying rates even within a lineage (Avise, 1994), and a relatively slow divergence in mtDNA has been detected in several vertebrate groups (Avise, 1989; Tan and Wake, 1995; Fleischer and McIntosh, 2001, Grant and Grant, 2002). Therefore, the results of Gaul (1996) and Densmore et al. (1992) should be interpreted qualitatively (in terms of cladistic relationships) rather than quantitatively (divergence times between lineages).

Paleoherpetological Aspects

The glacial periods of the Pleistocene (ca. 2.5 million BP) are considered to have been driving forces shaping the current distributions of some vertebrates (e.g., Bermingham and Avise, 1986; Riddle et al., 1993; Joseph et al., 1995; Avise and Walker, 1998; Arbogast, 1999). Climatic fluctuations and changing coastlines due to changes in sea level had profound effects on the biota. Vicariant events were responsible for splitting populations, which either recombined during interglacial periods or differentiated sufficiently to be considered separate species.

Natricine snakes are known from the Early Miocene (~ 24-20 million BP) of Wyoming and the earliest *Nerodia* date from the Middle and Late Barstovian of Nebraska, ~ 14.5-11.5 million BP (Holman, 2000). Fossils and current distribution suggest that the ancestor of the *sipedon* group probably resembled *N. erythrogaster*,

which inhabited aquatic systems in wooded and semi-arid areas of central and eastern North America and Mexico during the Late Miocene; ~ 10-5 million BP (Preston, 1970; Conant and Collins, 1991; Holman, 2000; pers. obs.). Toward the end of the Miocene, some of the ancestral populations may have diverged into early *N. fasciata*, occupying open areas in lowland swamps and marshlands. The eastern population of early *N. fasciata* may have diverged into the *N. f. fasciata-f. pictiventris* clade, with the oldest fossils from Texas dating back between 0.9-0.4 million BP $\pm$ 25k (Holman, 2000).

In the Pliocene, ~ 5-2 million BP, temporarily isolated populations of early *Nerodia fasciata* along its northern range limit may have become adapted to a cooler, drier environment with lotic systems, giving rise to *N. sipedon*. The earliest fossils of *N. sipedon* are known from Nebraska and perhaps Kansas between 4.25-3.7 million BP (Holman, 2000). At the end of the Pliocene, the climate began to cool and communities became more mixed in the mid-latitudes (Holman, 2000). The common ancestor of *N. harteri* and *N. paucimaculata* may have diverged at that time from early *N. sipedon*.

The Pleistocene produced a series of up to 44 glacial cycles beginning at Gauss-Matuyama magnetic reversal the 2.48 million BP (Morrison, 1991). Many warm periods (interglacials) exhibited average temperatures similar to or higher than those of the Holocene, promoting range expansions of some taxa. For example, *Nerodia sipedon* is recorded from Walworth Co., South Dakota, a location 300 km north of its present distribution, 1.9-0.9 million BP (Holman, 2000). Glacial periods were characterized by a drier climate and ice sheets up to 3 km thick that advanced as far as southern Illinois, Indiana, and Ohio during the Illinoian glacial and nearly as far during the Late Wisconsinan glacial (Dawson, 1992; Holman, 2000). The ice sheet and periglacial flora

(a tundralike ecosystem promoted by permafrost) reached 80-200 km south of the ice front into Kentucky and Missouri (Dawson, 1992), bordered by spruce forest (Delcourt and Delcourt, 1981). That cold climate presumably caused the extinction of temperate snake species in northern regions. However, communities did not simply shift southward as intact ecosystems. Whitehead (1981) and Blondel and Vigne (1993) suggest that changes in vegetation structure and composition allowed boreal and temperate species to co-occur. Increased climatic equability has been postulated based on the co-occurrence of cold-adapted Pleistocene mammals and giant tortoises on the central and southern Great Plains (Hibbard, 1960; Holman, 1976, 1980). Mild winters were hypothesized to have enabled the survival of extralimital southern species and cooler summers accounted for the occurrence of extralimital northern species. A comparable herpetofaunal mixture of northern and southern species has been found from the latitudes of Nebraska and western Virginia south to Texas and Florida (Wilson, 1975; Eshelman and Grady, 1986; Holman, 1976, 1980, 2000; Knight, pers. comm.).

As the ranges of temperate species shifted southward, they may have fragmented into several refugia and may have become mixed with southern species. In contrast, the range reductions of subtropical species were less pronounced, and most may have occupied similar ranges during the Pleistocene as today (Holman, 2000). Climatic extremes of the glacial periods (aridity, cold temperatures) probably intensified selection pressures on northern and higher elevation populations, promoting their divergence and differentiation. Morris (1987) inferred from the ranges of current subspecies that *N. s. sipedon* differentiated in the Appalachian Mountains and areas re-occupied by the species following the Wisconsinan glaciation, whereas *N. s. pleuralis* differentiated in areas south

of the Wisconsinan glacial maximum. This would explain the primary intergradation zone evident today, which occurs roughly along the Wisconsinan glacial maximum and just west of the Appalachians. However, the harsh climatic conditions during the glacial maxima probably split the range of *N. s. sipedon* into eastern and western refugia. Biotic and abiotic elements in the proposed Appalachian refugia indicate a periglacial climate typical of tundra and boreal zones during the Wisconsinan glacial (Delcout and Delcourt, 1981; Whitehead, 1981; Eshelman and Grady, 1986; Wright, 1987; Clark and Ciolkosz, 1988; Braun, 1989; Mills and Delcourt, 1991), rendering the mountains inhospitable for watersnakes. Therefore, the Atlantic Coastal Plain in Virginia and North Carolina, greatly enlarged due to lowering sea level (e.g., Blanchon and Shaw, 1995) could have served as the eastern center of divergence for *N. s. sipedon* during the glacials.

With the arrival of each interglacial period, thermophilic reptiles expanded northward to colonize land exposed by the retreating ice sheet. Previously separated ranges of conspecific populations may have been reconnected if divergence during the glacial period did not result in the evolution of reproductive barriers. For example, allopatric western populations of *Nerodia s. sipedon* may have been located in Kansas and Missouri, along the northwestern fringe of the Ozark Mountains. Gene flow between the eastern and western populations probably was maintained along the Peninsula Prairie Corridor south of the Great Lakes during moderate glacial periods (e.g., the Eowisconsin and Early Wisconsinan glacials, ~ 122,000-79,000 BP; Roberts, 1989; Dawson, 1992). The postulated corridor, between coniferous forest to the north and deciduous forest to the south, and has been suggested to have supported the expansion of snake species such as *Elaphe vulpina* and *Sistrurus catenatus* as far east as Maryland between 900,000-

400,000 BP (Holman, 2000). Similarly, prairie corridors south of the Great Lakes allowed the relatively rapid (< 5,000 years) reconnection of the eastern and western ranges of *Nerodia s. sipedon*, because the increased availability of solar radiation in the open areas favored the survival of advancing populations during cool periods. *N. sipedon*, and to a lesser degree *N. fasciata*, may have evolved in relatively open marsh and prairie-like areas compared to other congeners (Conant, 1934; Carr 1940; Diener, 1957; Holman and Hill, 1961; Hebrard and Mushinsky, 1978; Mushinsky et al., 1980; King, 1986; Tiebout and Cary, 1987; Greshock, 1998). *N. s. sipedon* may have been better adapted to the arid and cold but sunny climate of the prairie corridor during the glacial maxima (Dawson, 1992) than *N. s. pleuralis*, which was restricted to wooded areas farther south. The glacial refugium of *N. s. pleuralis* may have been located in the geographic center of its current range, in Tennessee, Mississippi, Alabama, and Georgia. That refugium would have been isolated from populations of *N. s. sipedon* by the Appalachians in the east, the prairie corridor and tundra belt in the north, and the Mississippi River Valley in the west. Other subspecies of *N. sipedon* have a younger history. For example, *N. s. insularum* diverged within the last 3,000-4,000 years (Morris, 1987; King and Lawson, 2001), whereas *N. s. williamengelsi* diverged from the nominate race along the Atlantic coast in the last 10,000 years or during an earlier interglacial, when the Outer Banks became isolated (Hocutt et al., 1986).

In the south, divergence of *Nerodia fasciata confluens* from its nominate race occurred along the Mississippi River, whereas *N. f. fasciata* and *N. f. pictiventris* in the east diverged little (morphological and genetic data do not coincide with the current subspecies ranges; Lawson et al., 1991; this study). *N. fasciata* apparently is less well

adapted to arid areas than *N. sipedon*, as its eastern range limit follows the 76.2 cm precipitation isopleth (Cliburn, 1960). Frequent eustatic changes in sea level, including a maximum lowering of 120 m during the Pleistocene (Dawson, 1992), fragmented peripheral populations of *N. fasciata* along the coast of the Gulf of Mexico. Some populations became sufficiently isolated from those of the mainland to differentiate into *N. clarkii*, which adapted to more saline conditions.

Morris (1987) suggested that the lack of hybridization between *Nerodia fasciata confluens* and *N. sipedon pleuralis* implies that their sympatry along the Mississippi River Valley lasted long enough for them to have evolved reproductive isolation in the sense of speciation by reinforcement in sympatry (Dobzhansky, 1940), whereas the contact zones in the south and west are more recent. My explanation is that the substantial sympatry of *N. fasciata* and *N. sipedon* in the Mississippi River Valley indicates secondary contact after the divergence in allopatry existed for longer periods than between the two species in the east, where introgression persisted into the present period. Repeated range expansion and contraction during the Pleistocene may have promoted further genetic differences, producing increasingly distinct hybrid zones (Hewitt, 1989).

CONCLUSIONS

The study of morphological characters and genetic markers has contributed valuable information regarding the relationship between *Nerodia fasciata* and *N. sipedon.* Traditional diagnostic features of color pattern serve well to distinguish specimens of both taxa from allopatric populations, but they are inadequate to detect the extent of introgression in the contact zone. An enlarged set of morphological characters emphasized the distinction between the two species, albeit without great diagnostic efficacy. However, discriminant function analyses of morphological characters successfully separated the two taxa.

The identification of ten nuclear markers using the AFLP technique yielded diagnostic characters that were nearly fixed in one species and nearly absent in the other. Those markers revealed a previously unrecognized degree of introgression between *Nerodia sipedon* and *N. fasciata* in their easternmost contact zone. Many snakes that externally resemble one of the parental species exhibited a genotype typical of backcrossed hybrids. This suggests that a similar degree of introgression might be uncovered in other contact zones where few or no hybrids have been reported (Conant, 1963; Schwaner and Mount, 1976; Schwaner et al., 1980; Seyle, 1980). Although both taxa appear to interbreed extensively in the easternmost contact zone, markers for *N. sipedon* and F_1 hybrids, backcrossed to a parental species exhibiting more than one introgressive marker, were less frequent than expected at Hardy-Weinberg equilibrium, imlpying selection against *N. sipedon* and specimens with increased introgression, respectively. This is consistent with a pronounced genetic and morphological

dominance of *N. fasciata* in the hybrid zone and likely reflects the competitive superiority of its morphotype in the Coastal Plain of the south.

Interspecific differences in morphology and distribution apparently reflect distinct habitat preferences and physiological tolerances in *Nerodia sipedon* and *N. fasciata* that correlate with water current, salinity, and temperature. Transitions between relevant environmental parameters (ecotones) determine the position of the hybrid zone. Variation in the width of those ecotones relates to the variable width of hybrid zones, a pattern that corresponds to the geographical-selection-gradient model (Slatkin, 1973; Endler, 1977). The rarity of pure parental genotypes in those ecotones suggests that many hybrids perform better than the parental species. This hybrid superiority, the environmental data underlying the contact zones, the lack of F_1 hybrids, and the selection against hybrids with more than one introgressive marker correspond to the Evolutionary Novelty Model by Arnold (1997).

Reproductive isolation is a precondition for two taxa to reproduce successfully as separate entities and is the pillar of the Biological Species Concept (Mayr, 1963). The ease with which *Nerodia fasciata* and *N. sipedon* establish hybrid zones has left some authors uncertain about their taxonomic status (e.g., Schwaner and Mount, 1976; Blaney and Blaney, 1979; Lawson et al., 1987). Reports of bidirectional mating (Riches, 1976; Gaul, 1996) have further eroded support for their specific status. However, the genetic results presented here indicate that the two taxa are independent entities and qualify as species under both the Phylogenetic Species Concept (Cracraft, 1983) and the Evolutionary Species Concept (Simpson, 1961; Frost and Hillis, 1990). Reproductive

compatibility between *N. fasciata* and *N. sipedon* is a symplesiomorphic character, retained from their common ancestor (Wiley, 1981).

This study has extended our knowledge of natural hybridization in snakes, a group with only a few known hybrid zones (Thorpe, 1983; Lawson and Lieb, 1990; Lawson et al., 1991; Wuester et al., 1996). I have demonstrated the utility of AFLP, a method still largely neglected in zoological studies, for the detection of diagnostic markers of two closely related but morphologically similar species, and for investigating the dynamics of genetic exchange between them. This study, encompassing genetic, morphological, and ecological data, represents an integrated approach to understanding the pattern of selection in hybrid zones (Arnold and Bennett, 1993; Arnold and Hodges, 1995). As Futuyma and Shapiro (1995) wrote, "as part of such a program, the importance of studying multiple contact zones cannot be overemphasized." The study of this species pair has provided an excellent opportunity to investigate the consequences of incomplete speciation, to examine the applicability of various species concepts under natural conditions, and to test for congruence between genotypic and phenotypic variation. I believe this study has contributed empirical data that address some of the persistent problems of species concepts in general and elucidate the relationship between *Nerodia fasciata* and *N. sipedon* in particular.

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APPENDIX 1

DEFINITION OF MORPHOLOGICAL CHARACTERS

Color Pattern

The first five characters in the list were visually estimated with an interval index. The value 1 represents the typical state found in *Nerodia sipedon* and 5 the one found in a typical *N. fasciata*, with a gradadation of states for the intermediary values. Figures illustrating the characters follow the different sections of the text below.

1) **PS:** Prominence of postocular stripe that extends from the eye posterior toward the last supralabial scale; (1 = none, 5 = saturated black).
2) **SS:** Prominence of dark shadow spots located between the lateral bands; (1 = none, 5 = saturated black).
3) **MB:** Degree of serration along the margins of lateral bands or blotches; (1 = smoothly, 5 = strongly serrated).
4) **SV:** Shape of ventral spots; (1 = half-moon shaped, 5 = rectangle shaped).
5) **VLBa:** Mean number of ventral scales that are covered by a lateral band, measured along the contact with the first dorsal scale row, but averaged across both sides and over the first 10 bands posterior the neck; if a lateral band covers < 50% of ventral scale, it contributed half a value, else it contributed one value.
6) **CBa:** Number of complete bands posterior the neck; both species have complete bands beginning posterior the neck, but in *N. sipedon* the lateral portions of crossbands shift gradually toward the tail in more posterior bands until they become separated from the dorsal portion; a band is considered to be complete (a crossband) as long as at least half of the upper edge of a lateral portion is in contact with a dorsal portion; occasional complete bands in the section of alternating dorsal and lateral bands in a *N. sipedon*, and vice versa, occasional interrupted bands in the section of complete bands in both species were not counted, if those irregularities didn't span across more than 3 bands/blotches.
7) **DBa:** Number of dorsal bands and blotches from the neck to the cloaca.
8) **LBa:** Number of lateral bands from the neck to the cloaca; values of both sides averaged.
9) **WBa:** Mean length (scale counts along the body axis) of the first 10 lateral bands posterior the neck measured along the first dosal scale row adjacent to the ventral scales, averaged between left and right; scales with a coverage of < 50% count as half values, else it contributed one value.
10) **IBa:** Mean length of the first 10 interspaces; methods of counting as for WBa, but for light scales in between lateral bands; in some *N. fasciata* the interspaces can be darker (blackish) than the actual lateral bands, which may are yellowish to reddish.

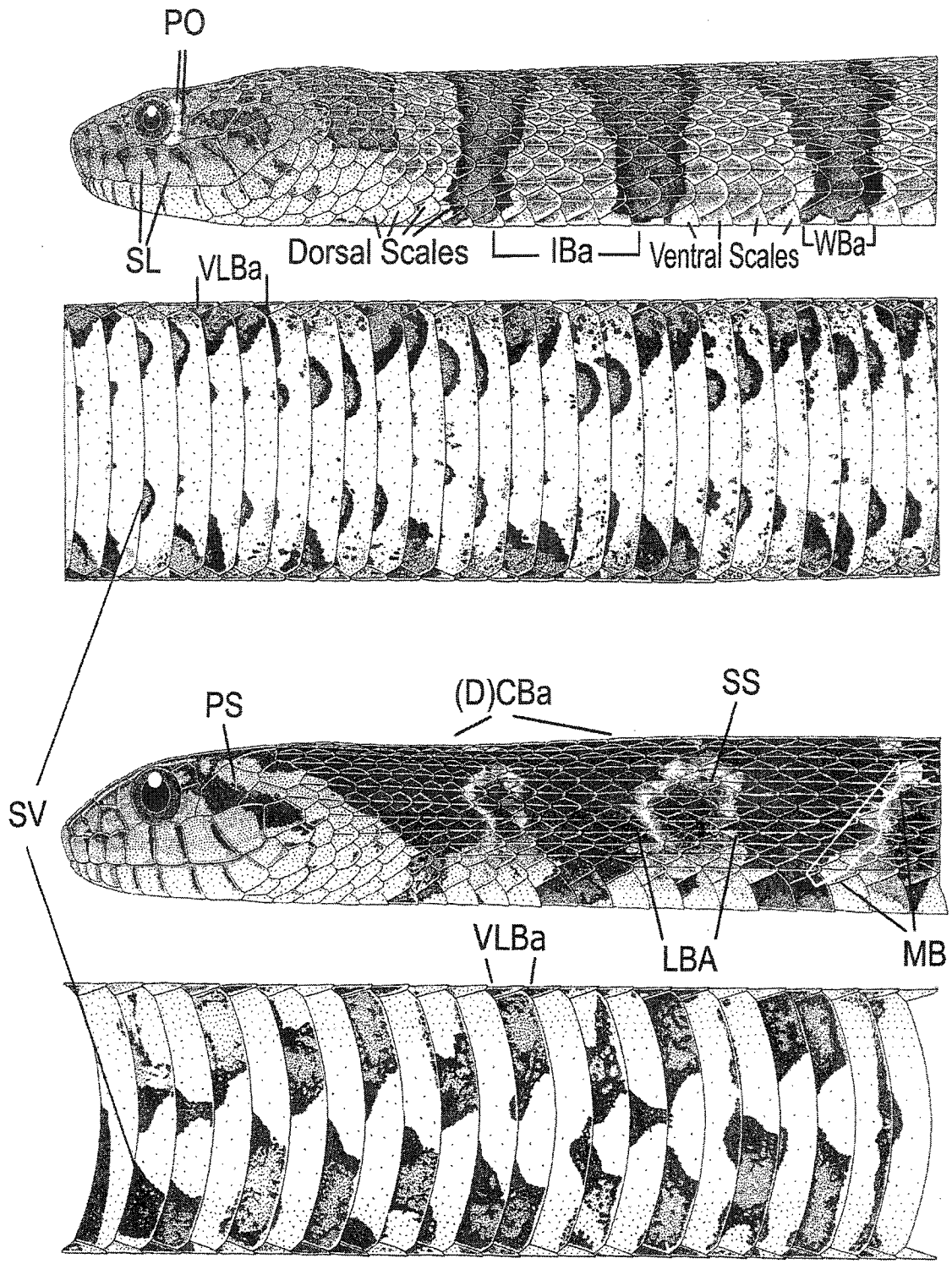

FIG. 32. *Color Pattern.* Lateral view of anterior body and ventral view of midbody of *Nerodia sipedon* (upper half) and *N. fasciata* (lower half). Abbreviations and descriptions of characters are explained in text above. Both species represent specimens from North Carolina (Palmer and Braswell, 1995). Modified from Ronaldo G. Kuhler after NCSM 9542/1436; courtesy N.C. State Museum of Natural Sciences.

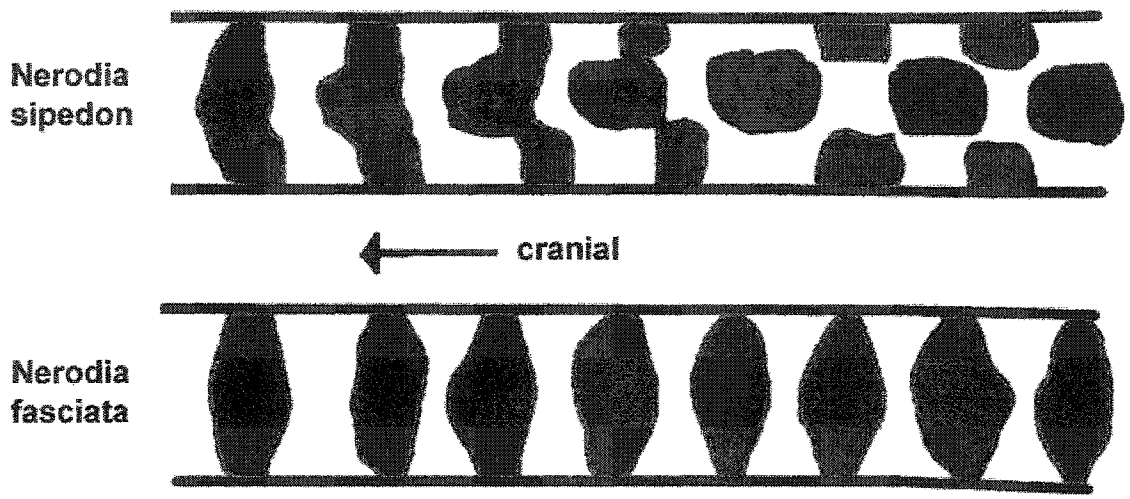

FIG. 33. *Dorsal Band Pattern.* Schematic display: dorsal view of anterior and mid body sections of *Nerodia sipedon* (upper half) and *N. fasciata* (lower half).

Scutellation

11) **PO:** Number of postocular scales, averaged between left and right side (Fig. 32).
12) **V:** Number of ventral scales, beginning with the first ventral scale that is in contact with the lowest scale row on both sides (Dowling, 1951).
13) **PV:** Number of preventral scales; any scale anterior the ventral scales that is wider than long (parallel the body axis).
14) **SC:** Number of subcaudal scales for tails with tailtip present
15) **SL:** i^{th}-number of supralabial scales contacting the left and right eye.
16) **SR10:** Number of scale rows at the 10^{th} ventral scale.
17) **Red21:** Ventral scale position of the reduction to 21 scale rows.
18) **Red19:** Ventral scale position of the reduction to 19 scale rows.
19) **Red17:** Ventral scale position of the reduction to 17 scale rows; if a reduction to 17 (or 18) scale rows has not occurred before the cloaca, the total ventral scale count plus one was used as a ventral position value to indicate the retention of a higher scale row number.
20) **Tred10:** Subcaudal scale position of the scale row reduction to 10 caudal rows.
21) **Tred8:** Subcaudal position of the scale row reduction to 8 caudal rows.
22) **Tred6:** Subcaudal position of the scale row reduction to 6 caudal rows.

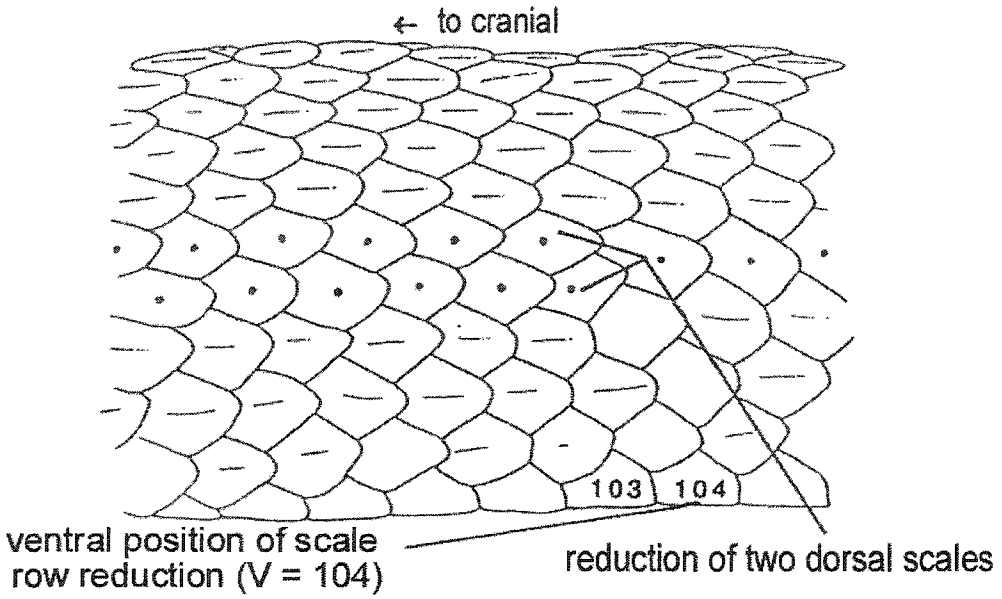

FIG. 34. *Scale Row Reduction Pattern.* Displayed is the positon where two dorsal scale rows are continued by only one scale row caudad. This method has been applied for all characters involving scale row reductions on the trunk and the tail.

Body Proportions

Cephalic measurements were made with a caliper. Head proportions were measured to 0.01 mm accuracy, SVL and TL to 0.1 cm. The snake's head was hold loosely behind the neck and the caliper positioned over the endpoints of the length to be measured, barely touching the head. This technique prevents compressing the soft tissue and the flexible structure of the head, and hence, reduces the incorporation of distorted values.

23) **RoH:** Height of rostral scale.
24) **RoW:** Width of rostral scale.
25) **Eys:** Diameter of eyes, averaged between left and right.
26) **DiET:** Distance between lateral surfaces of eyes.
27) **DiES:** Distance between eye and snout-tip; average between left and right.
28) **DEN:** Distance between eyes and nostrils; average between left and right.
29) **EyCH:** Height of canthus rostralis anterior the eyes; average between left and right.
30) **EyCW:** Width between both canthus rostralis anterior the eyes.
31) **SCH:** Height of snout measured as height of canthus rostralis posterior the nostrils; average between left and right.
32) **SCW:** Width of snout measured as distance between both canthus rostralis posterior the nostrils.
33) **DiNo:** Distance between medial edge of nostrils.
34) **JawL:** Length of lower jaws; averaged between left and right.
35) **JawW:** Width of lower jaw measured between the posterior ends.

36) **SVL:** Snout-vent length
37) **TL:** Tail length
38) **AnCR:** Angle of canthus rostralis, crossection frontally viewed (1 = 45 °, 5 = 90 °).
39) **StCR:** Curavture of the canthus rostralis; laterally viewed (1 = heavily curved, 5 = straight line.
40) **SpCR:** Depth of slope (= vertical distance laterally viewed) between endpoints of the canthus rostralis (anterior eyes to posterior nostrils); 1 = endpoints on same level, 5 = increased vertical distance between endpoints).

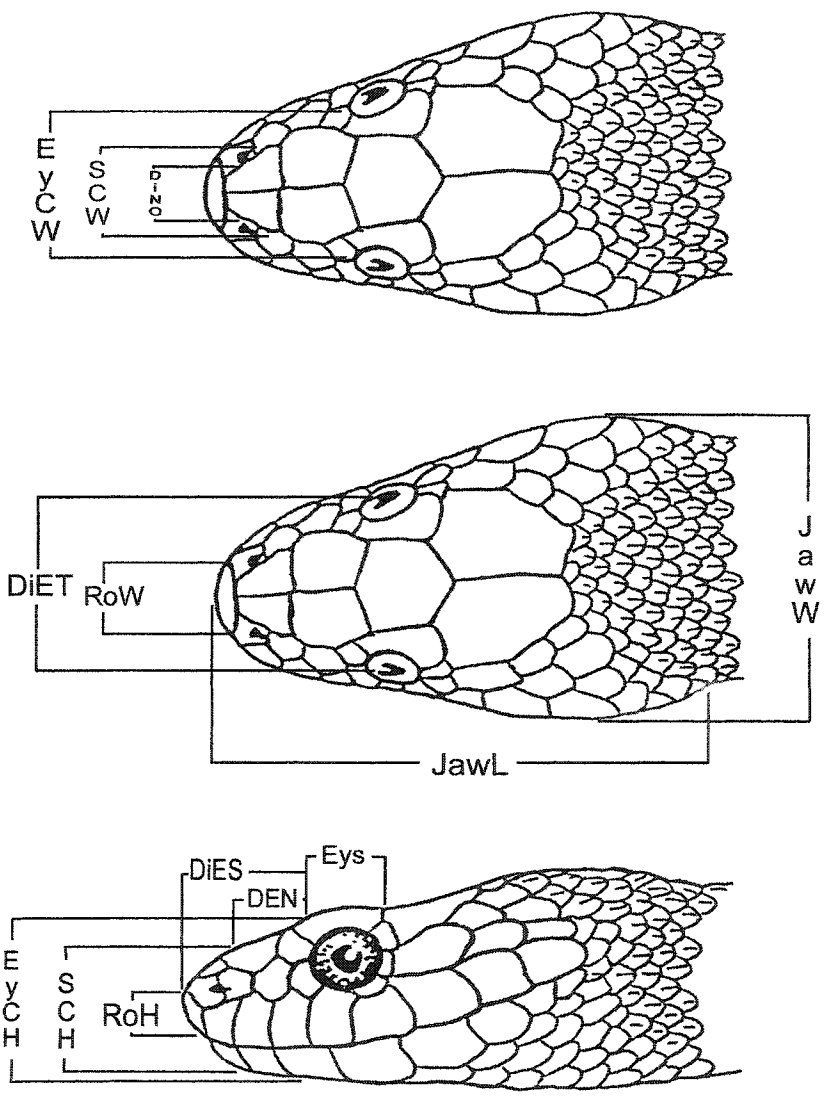

FIG. 35. *Head Proportions.* A schematic head of a natricine snake showing various cephalic lengths.

APPENDIX 2

SPECIMENS EXAMINED

List of specimens grouped by geographic area (see GEOGRAPHIC SECTION): ID (individual field number), Group (phenetic group; see MORPHOLOGICAL SECTION), Sex (sex), Date (collection date), Location (collection site), % N. f. (proportion of genetic contribution of *Nerodia fasciata*), C1.1 (canonical score of first component of DFA1), and C3.1 (canonical score of first component of DFA3).

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
1	1	x	F	10/15/95	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	90	0.027	-
1	2	cf	M	10/15/95	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	-	-	-
1	3	cs	F	10/24/95	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	90	0.564	-
1	4	cf	M	4/25/96	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	-	-	-
1	5	cs	-	4/24/96	Culpepper Rd. 110 North, South Mills, Camden Co., NC	-	-	-
1	6	cf	F	5/1/96	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	70	0.599	-
1	7	cs	F	5/1/96	Charles Creek, Elizabeth City, Pasquotank Co., NC	10	2.513	-
1	8	cf	M	5/2/96	Newbegun Creek, 5 km South of Elizabeth City Airport, Pasquotank Co., NC	100	-0.4	-
1	13	cs	M	5/14/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	40	1.4	-
1	14	cf	M	5/14/96	Salem Church Rd. x Symonds Creek Rd., Weeksville, Pasquotank Co, NC	60	-1.53	-
1	18	cf	M	5/18/96	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	100	-0.46	-
1	19	cs	F	5/18/96	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	30	2.051	2.78
1	20	x	F	5/18/96	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	30	0.987	-0.11
1	21	cf	M	5/21/96	200 m se of Albemarle Hospital, Elizabeth City, Camden Co., NC	90	1.118	-
1	24	cs	F	6/6/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	10	1.335	1.31
1	25	cs	F	6/7/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	50	2.01	2.35
1	26	cs	F	6/9/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	33	2.804	2.6
1	27	cs	F	6/9/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	10	1.499	-
1	28	cf	F	6/10/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	0.414	-0.12
1	29	cf	F	6/10/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	70	1.136	0.1
1	30	cf	F	6/10/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	90	0.598	-0.01
1	31	cf	F	6/10/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	-0.74	-1.09
1	32	cf	F	6/12/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	90	1.752	0.94
1	33	cf	F	6/12/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	80	-0.25	-1.26
1	34	cf	F	6/12/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	-1.02	-0.46
1	39	cf	F	6/19/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	70	-0.33	-0.26

APPENDIX 2. Continued.

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
1	40	cf	F	6/19/96	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	80	-1.11	-0.39
1	41	s	F	6/3/96	Env. Edu. Ctr., Wash Woods Rd., False Cape St. Pk., Virginia Beach City, VA	-	1.317	1.68
1	42	s	M	5/24/96	Barbour Hill impoundment, False Cape State Park, Virginia Beach City, VA	0	-	-
1	43	s	M	6/7/96	Env. Edu. Ctr., Wash Woods Rd., False Cape St. Pk., Virginia Beach City, VA	0	-	-
1	44	s	M	5/29/96	Barbour Hill Nature Trail, False Cape State Park, Virginia Beach City, VA	0	2.139	-
1	63	cs	F	7/17/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	0	2.294	2.5
1	64	cs	F	7/21/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	43	1.848	2.08
1	65	cs	F	7/21/96	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	10	2.492	1.47
1	83	s	F	2/24/97	304 Ruddy Crsc., Sandbridge, Virginia Beach City, VA	0	-	-
1	84	s	F	2/27/97	3 km east Douglas Rd. x Hwy. 17, Chesapeake City, VA	0	-	-
1	85	s	F	3/27/97	Stumpy Lake, Virginia Beach City, VA	0	2.715	2.6
1	86	x	F	3/29/97	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	30	0.019	0.75
1	87	cf	F	3/29/97	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	80	-0.02	0.82
1	88	s	M	3/30/97	Wash Woods Rd. near Back Bay, False Cape State Park, Virginia Beach City, VA	0	-	-
1	89	s	F	4/13/97	Stumpy Lake, Virginia Beach City, VA	10	-	-
1	98	s	M	5/16/97	northeast Rd. 1722 x of Rd. 1724, Suffolk Co., VA	-	-	-
1	106	s	M	5/30/97	Wash Woods Landing, False Cape State Park, Virginia Beach City, VA	0	2.966	1.8
1	107	s	F	6/1/97	400m southeast of Back Bay outlet, False Cape State Park, Virginia Beach City, V	0	2.368	1.79
1	138	x	F	6/12/97	800 m north Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	70	-	-
1	139	cs	M	6/12/97	2.5 km north Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	30	-	-
1	158	s	F	6/29/97	near Rte. 4, western shore of Back Bay, Mackay Island, Currituck Co., NC	0	2.528	2.07
1	159	s	M	7/2/97	Muddy Creek Rd. at Back Bay, Virginia Beach City, VA	0	-	-
1	161	cs	F	7/19/97	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	20	1.326	1.33
1	162	cs	F	7/19/97	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	60	2.673	2.76
1	163	x	F	7/26/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	70	-	-
1	164	cf	F	7/26/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	90	0.331	-
1	165	cf	F	7/26/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	70	-0.26	-
1	166	cf	F	7/27/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	70	0.703	0.83
1	167	cf	M	7/27/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	90	0.522	0.27
1	168	cf	F	8/1/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	0.114	-0.61
1	169	cf	F	8/1/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	-0.54	-0.39
1	170	cf	F	8/2/97	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	0.376	-0.13
1	175	s	F	11/22/97	dam, Mackay Island, Currituck Co., NC	0	3.02	-

APPENDIX 2. Continued.

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
1	176	s	F	11/26/97	dam, Mackay Island, Currituck Co., NC	0	2.444	2.64
1	177	s	F	3/6/98	Env. Edu. Ctr., Wash Woods Rd., False Cape St. Pk., Virginia Beach City, VA	0	2.27	-
1	178	s	M	3/14/98	dam, Mackay Island, Currituck Co., NC	-	-	-
1	179	x	F	3/26/98	Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	90	0.565	-
1	180	s	F	8/1/98	Muddy Creek Rd., Virginia Beach City, VA	0	-	-
1	197	cf	F	5/5/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	80	-0.59	-
1	198	cf	F	5/6/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	0.139	-
1	199	s	M	5/5/98	residential pond at Shoreline Circle, Virginia Beach City, VA	0	2.017	-
1	200	s	M	5/7/98	residential pond at Shoreline Circle, Virginia Beach City, VA	0	1.879	-
1	201	cf	F	5/7/98	500 m west of Albemarle Hospital, Elizabeth City, Camden Co., NC	90	-	-
1	202	cs	M	5/7/98	1.5 km north of Albemarle Hospital, Elizabeth City, Pasquotank Co., NC	10	-	-
1	220	s	F	5/30/98	Stumpy Lake, Virginia Beach City, VA	0	0.856	-
1	221	cf	F	6/4/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	75	0.75	-
1	222	cf	F	6/4/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	100	0.95	-
1	223	cs	F	6/4/98	adjacent to Hwy. 17, 300m north of Morgans Corner, Pasquotank Co., NC	55	2.864	-
1	248	s	F	6/22/98	Old Pungo Ferry Rd., North Landing River, Virginia Beach City, VA	0	-	-
1	249	x	F	6/24/98	near Indiantown Creek, 800 m north Garlingtons Island Rd., Camden Co., NC	67	-	-
1	250	cs	F	6/24/98	North River, 4.8 km southwest Coinjock, Currituck Co., NC	50	1.616	-
1	252	cf	M	6/30/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	89	-0.44	-
1	253	cf	F	6/30/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	89	-	-
1	254	cf	F	6/30/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	78	-	-
1	255	cf	F	6/30/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	70	-0.57	-
1	256	cf	F	6/30/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	90	0.953	-
1	257	x	F	6/30/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	70	-0.02	-
1	258	cf	F	6/30/98	adjacent to Hwy. 158, 2 km east of Elizabeth City, Camden Co., NC	80	1.37	-
1	259	x	F	6/30/98	640 m southeast of Indiantown Creek x Sandy Hook Rd., Camden Co., NC	67	0.09	-
1	264	x	F	7/14/98	southeast of Sandy Hook Rd. x Indiantown Creek, Camden Co., NC	60	-	-
1	265	x	M	7/14/98	southeast of Sandy Hook Rd. x Indiantown Creek, Camden Co., NC	70	-	-
1	287	s	F	4/16/99	Stumpy Lake, Virginia Beach City, VA	0	-	-
1	288	s	F	4/16/99	Stumpy Lake, Virginia Beach City, VA	-	-	-
1	289	s	F	5/4/99	bridge (Colley Ave.) across Lafayette River, Norfolk City, VA	0	-	-
1	290	s	F	5/4/99	bridge (26th str.) across Lafayette River, Norfolk City, VA	0	-	-
1	291	s	M	5/4/99	residential pond at Shoreline Circle, Virginia Beach City, VA	0	-	-

APPENDIX 2. Continued.

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
1	292	s	F	5/4/99	residential pond at Shoreline Circle, Virginia Beach City, VA	-	-	-
2	22	cf	M	5/20/96	Hwy. 264, 6.4 km south Stumpy Point, Dare Co., NC	-	-	-
2	23	cf	M	5/20/96	Hwy. 264, 6.4 km south Stumpy Point, Dare Co., NC	100	-0.61	-
2	67	cs	M	8/24/96	Pier of crab port at Manns Harbor, Dare Co., NC	0	-	-
2	68	cs	F	8/25/96	harbor at Stumpy Point, Dare Co., NC	0	2.858	2.39
2	69	s	F	8/25/96	Cypress Cove Camping, Manteo, Roanoke Island, Dare Co., NC	0	2.445	2.06
2	74	cs	F	9/16/96	Montgomery Rd. near intersection with Smith Creek, Hyde Co., NC	10	2.322	1.09
2	160	s	F	7/12/97	Cypress Cove Campground, Roanoke Island, Manteo, Dare Co., NC	0	2.689	2.89
2	218	x	F	5/29/98	Hwy. 264, 5.8 km south Lake Worth (Stumpy Point), ARNWR, Dare Co., NC	-	-	-
2	260	cf	F	6/30/98	Hwy. 264, 8 km north of Lake Worth (Stumpy Point), ARNWR, Dare Co., NC	100	-2.15	-
2	261	cs	M	7/1/98	Stumpy Point Rd., 1.1 km west of Hwy. 264, Stumpy Point, Dare Co., NC	50	0.895	-
2	262	cf	M	7/1/98	Hwy. 264, 1.3 km north of intersection with Airport Rd., ARNWR, Hyde Co., NC	100	-0.99	-
2	263	cs	-	7/1/98	Hwy. 264, 1.8 km north of intersection with Swamp Rd., Hyde Co., NC	-	-	-
2	266	x	F	7/16/98	Hwy. 264, 5.6 km north of Stomper Rd. at Air Force Bombing Range, Dare Co., N	44	0.582	-
2	267	cf	M	7/25/98	Hwy. 264, 1.6 km north of Lake Worth (Stumpy Point), ARNWR, Dare Co., NC	50	-	-
2	276	cf	F	8/8/98	Hwy. 264, 4.8 km south from intersection with Hwy. 64, ARNWR, Dare Co., NC	90	-	-
2	277	cs	M	8/11/98	Hwy. 264, intersection Crab Bridge Rd. x Log Storage Rd., ARNWR, Dare Co., N	10	2.868	-
2	278	f	F	8/8/98	Hwy. 94, Millkenny, Tyrell Co., NC	100	-	-
2	279	cf	M	9/4/98	Hwy. 264, 800 m west of Engelhard, Hyde Co., NC	-	-	-
2	280	x	F	9/4/98	Hwy. 64, 10.6 km east of bridge over Alligator River, Dare Co., NC	100	-	-
2	281	cf	F	9/4/98	Hwy. 64, 1 km east of Milltail Rd., ARNWR, Dare Co., NC	90	-	-
2	283	cf	F	10/6/98	Hwy. 264, 4.5 km north of Hyde County Airport, Hyde Co., NC	100	-	-
2	284	cs	-	10/6/98	Hwy. 264, 1.6 km north of Swamp Rd., Hyde Co., NC	10	-	-
2	285	cs	F	10/9/98	end of Mashoes Rd., ARNWR, Dare Co., NC	20	-	-
2	307	cs	M	6/9/99	Hwy. 264, Lake Worth (Stumpy Point), Dare Co., NC	40	-	-
2	308	cs	-	6/10/99	Hwy. 264 x 64, ARNWR, Dare Co., NC	-	-	-
2	309	cs	M	6/10/99	residential road, Manns Harbor, Dare Co., NC	-	-	-
2	310	x	F	6/9/99	Hwy. 264, 4.5 km north of Hyde County Airport, Hyde Co., NC	100	-0.55	-
2	312	x	-	6/15/99	2.4 km south of Hwy. 264 x Hwy. 64, ARNWR, Dare Co., NC	90	-	-
2	313	cf	-	6/15/99	6.4 km south of Hwy. 264 x Hwy. 64, ARNWR, Dare Co., NC	88	-	-
2	314	x	-	6/15/99	10.3 km south of Hwy. 264 x Hwy. 64, ARNWR, Dare Co., NC	40	-	-
2	315	cf	-	6/15/99	8.8 km south of Hwy. 264 x Hwy. 64, ARNWR, Dare Co., NC	100	-	-
2	320	cs	-	7/10/99	Mashoes Rd., 3.2 km south of Mashoes, ARNWR, Dare Co., NC	50	-	-

APPENDIX 2. Continued.

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
2	321	cf	-	7/10/99	6.4 km south of Hwy. 264 x Hwy. 64, ARNWR, Dare Co., NC	60	-	-
2	322	cs	-	7/10/99	800 m north of Hwy. 264 x Deep Creek, ARNWR, Dare Co., NC	-	-	-
2	323	cs	F	7/10/99	Hwy. 264, 2.6 km north of Engelhard, Hyde Co., NC	10	-	-
2	324	cs	-	7/10/99	1.9 km west of Hwy. 264 x Stumpy Point Rd., Stumpy Point, Dare Co., NC	-	-	-
2	325	cf	-	7/10/99	4.1 km west of Hwy. 64 x Hwy. 264, ARNWR, Dare Co., NC	70	-	-
3	9	s	F	5/9/96	Battle Park, Tar River, Rocky Mount, Nash Co, NC	-	-	-
3	10	s	F	5/4/96	Battle Park, Tar River, Rocky Mount, Nash Co, NC	-	-	-
3	11	s	F	5/4/96	Battle Park, Tar River, Rocky Mount, Nash Co, NC	-	-	-
3	12	s	F	5/10/96	Battle Park, Tar River, Rocky Mount, Nash Co, NC	-	-	-
3	35	cf	M	6/13/96	pond between Tar River and Hwy. 264A, northeast of Greenville, Pitt Co., NC	80	-0.07	-0.94
3	36	cf	F	6/13/96	pond between Tar River and Hwy. 264A, northeast of Greenville, Pitt Co., NC	100	-1.94	-1.64
3	37	x	F	6/14/96	southwest of Bactolus Rd. and Possum Town Rd., ne. of Greeneville, Pitt Co., NC	-	-0.81	-
3	38	cf	F	6/14/96	south of Greenville Airport, adjacent Hwy. 11, Greenville, Pitt Co., NC	100	-0.65	-0.98
3	70	cs	M	9/13/96	Nobles Millpond, Edgecombe Co., NC	11	0.534	0.61
3	71	cs	M	8/13/96	Nobles Millpond, Edgecombe Co., NC	10	0.462	0.42
3	75	s	M	10/11/96	Tar River Reservoir Lake, Nash Co., NC	10	0.067	0.9
3	99	cs	F	5/23/97	1 km south of Shiloh Mills, east of Princeville, Edgecombe Co, NC	-	-	-
3	100	s	M	5/23/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	101	s	M	5/23/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	102	s	M	5/23/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	103	s	F	5/23/97	Tar River Reservoir Lake, Nash Co., NC	0	0.031	0.95
3	104	s	F	5/23/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	108	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	10	-	-
3	109	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	110	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	10	0.577	0.89
3	111	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	112	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	1.001	2.52
3	113	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	114	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	115	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	116	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	117	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	119	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-

APPENDIX 2. Continued,

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
3	120	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	-	-	-
3	121	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	10	-	-
3	122	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	-	-	-
3	123	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	124	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	125	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	126	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	127	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	-	-	-
3	128	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	-	0.246	1.18
3	129	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	1.104	-
3	130	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-0.24	0.84
3	131	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	132	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	133	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-0.78	-0.13
3	134	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	0.156	1.04
3	135	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	136	s	M	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	137	s	F	6/8/97	Tar River Reservoir Lake, Nash Co., NC	0	-	-
3	215	x	F	5/18/98	near Tranters Creek x Beargrass Rd., Pitt Co., NC	60	-	-
3	216	cf	F	5/18/98	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	80	-	-
3	224	cf	F	6/10/98	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	100	0.66	-
3	225	cf	F	6/10/98	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	100	-1.12	-
3	226	cf	F	6/10/98	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	90	0.036	-
3	227	cf	F	6/10/98	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	100	-0.39	-
3	228	cf	F	6/10/98	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	70	-2.3	-
3	326	cf	M	7/16/99	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	-	-	-
3	327	cf	F	7/16/99	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	-	-	-
3	328	cf	F	7/16/99	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	-	-	-
3	329	cf	F	7/16/99	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	-	-	-
3	330	cf	F	7/16/99	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	100	-	-
3	331	cf	F	7/16/99	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	100	-	-
3	332	x	F	7/16/99	near Roanoke River x Rd. 18.5 km northeast of Oak City, Martin Co., NC	-	-	-
4	17	f	M	4/10/96	dam of Beaver Creek, Fayetteville, Cumberland Co., NC	100	-2.27	-

APPENDIX 2. Continued.

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
4	149	f	M	6/8/97	Pope Air Force Base, north of golf course, Fayetteville, Cumberland Co., NC	90	-	-
4	172	cf	F	6/29/97	Pope Air Force Base, Fayetteville, Cumberland Co., NC	100	-0.5	-1.98
4	296	x	F	5/22/99	Buffalo Lakes, Harnett Co., NC	62	-0.07	-
4	298	x	F	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	-	-	-
4	299	x	F	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	100	-	-
4	300	cf	F	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	100	-0.64	-
4	301	cf	M	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	87	-0.56	-
4	302	x	M	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	87	-1.32	-
4	303	cf	F	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	87	-2.35	-
4	304	cf	F	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	100	-1.85	-
4	305	cf	F	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	-	0.878	-
4	306	x	M	5/23/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	-	-1	-
4	311	cf	F	5/27/99	Cambro Pond, 13.7 km southwest of Lillington, Harnett Co., NC	-	-0.8	-
5	15	cs	F	5/17/96	Heritage Camping, north of Wispering Pines, Moore Co., NC	60	2.549	-
5	16	cs	F	5/18/96	Heritage Farm Rd., north of Heritage Camping, Moore Co., NC	50	-	-
5	45	x	M	7/2/96	Crystal Lake, Lakeview, Moore Co., NC	90	-2.02	-2.43
5	46	cf	F	7/2/96	Crystal Lake, Lakeview, Moore Co., NC	90	-	-
5	47	cf	F	7/2/96	Crystal Lake, Lakeview, Moore Co., NC	90	-0.56	-1.16
5	48	cf	M	7/3/96	Crystal Lake, Lakeview, Moore Co., NC	80	-1.04	-0.65
5	49	x	F	7/3/96	Crystal Lake, Lakeview, Moore Co., NC	90	-1.91	-2.97
5	50	x	F	7/3/96	Crystal Lake, Lakeview, Moore Co., NC	100	-1.8	-2.49
5	51	x	F	7/3/96	Crystal Lake, Lakeview, Moore Co., NC	60	-2.44	-3.49
5	52	x	F	7/3/96	Crystal Lake, Lakeview, Moore Co., NC	40	-0.86	-1.32
5	53	x	F	7/3/96	Crystal Lake, Lakeview, Moore Co., NC	60	-1	-1.76
5	54	x	F	7/3/96	Crystal Lake, Lakeview, Moore Co., NC	70	-2.66	-3.62
5	73	cf	F	9/21/96	Crystal Lake, Lakeview, Moore Co., NC	100	-1.96	-2.26
5	157	cf	F	6/23/97	Rte. 2 x Rte. 1, north of Southern Pines, Moore Co., NC	100	-2.38	-2.2
5	251	cs	M	6/28/98	Heritage Camping, Moore Co., NC	11	0.427	-
5	268	x	F	7/28/98	Crystal Lake, Lakeview, Moore Co., NC	90	-	-
5	269	x	F	7/29/98	Crystal Lake, Lakeview, Moore Co., NC	87	-1.83	-
5	270	cs	F	7/29/98	Thagards Lake, Whispering Pines, Moore Co., NC	-	0.431	-
5	271	x	F	7/29/98	Thagards Lake, Whispering Pines, Moore Co., NC	50	-0.52	-
5	272	x	F	7/30/98	Youngs Rd., 1.6 km north of Lake Bay, Moore Co	80	-1.41	-

APPENDIX 2. Continued.

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
5	273	cf	F	7/30/98	Youngs Rd., 1.6 km north of Lake Bay, Moore Co	100	-1.1	-
5	274	cf	M	7/30/98	Youngs Rd., 1.6 km north of Lake Bay, Moore Co	90	-2.16	-
5	275	cf	F	7/30/98	0.5 km north Aiken Rd. x Lake Bay Rd., Moore Co., NC	100	-1.97	-
5	297	cf	F	5/22/99	McDeeds Creek x Camp Easter Rd., Moore Co., NC	100	-1.37	-
5	333	cf	F	7/17/99	3.7 km west Manchester Rd. x Connecticut Ave., Hoke Co., NC	-	-	-
5	334	cs	F	7/17/99	0.3 km south of Rays Bridge Rd. x Rte. 22, Moore Co., NC	90	-	-
5	335	x	F	7/18/99	Thagards Lake, Whispering Pines, Moore Co., NC	33	-	-
5	336	x	F	7/18/99	Thagards Lake, Whispering Pines, Moore Co., NC	30	-	-
6	77	f	F	8/26/96	Lost Lake, Savannah River Site, Aiken Co., SC	100	-1.49	-2.86
6	78	f	F	8/26/96	Lost Lake, Savannah River Site, Aiken Co., SC	100	-	-
6	79	f	M	8/26/96	Lost Lake, Savannah River Site, Aiken Co., SC	100	-2.8	-3.61
6	80	f	M	8/6/96	Castor Bay (Wodd Duck Bay), Savannah River Site, Aiken Co., SC	100	-2.68	-4
6	81	f	F	8/17/96	Lost Lake, Savannah River Site, Aiken Co., SC	100	-1.98	-2.96
6	82	f	F	8/17/96	Lost Lake, Savannah River Site, Aiken Co., SC	100	-1.72	-0.91
6	143	f	F	6/18/97	Phelps Pond, Savannah River Site, Aiken Co., SC	100	-	-
6	144	f	M	6/18/97	Phelps Pond, Savannah River Site, Aiken Co., SC	100	-	-
6	145	cs	F	6/10/97	200 m se of Riverwatch Pkwy. x Augusta Canal, Augusta, Richmond Co., GA	0	-	-
6	146	s	M	6/2/97	Sweetwater Rd. x Burkhalter Branch, Edgefield Co., SC	10	-	-
6	147	s	M	6/2/97	Sweetwater Rd. x Burkhalter Branch, Edgefield Co., SC	0	-	-
6	148	s	M	6/2/97	Sweetwater Rd. x Burkhalter Branch, Edgefield Co., SC	0	-	-
6	151	f	M	6/16/97	near Rd.-C, Upper Three Runs Creek, Savannah River Site, Aiken Co., SC	100	-	-
6	152	f	F	6/22/97	Boyd Pond, south of Aiken, Aiken Co., SC	-	-	-
6	153	f	M	6/18/97	Macedonia Elementary School, Blackville, Barnwell Co., SC	100	-	-
6	154	f	F	6/18/97	Macedonia Elementary School, Blackville, Barnwell Co., SC	-	-	-
6	182	f	F	4/1/98	Risher Pond Rd., Savannah River Site, Barnwell Co., SC	100	-1.49	-
6	183	f	F	4/1/98	Risher Pond Rd., Savannah River Site, Barnwell Co., SC	100	-2.35	-
6	184	f	M	4/1/98	Risher Pond Rd., Savannah River Site, Barnwell Co., SC	100	-	-
6	185	f	F	4/1/98	Risher Pond Rd., Savannah River Site, Barnwell Co., SC	90	-	-
6	186	f	F	4/1/98	Risher Pond Rd., Savannah River Site, Barnwell Co., SC	100	-	-
6	187	f	M	4/1/98	Risher Pond Rd., Savannah River Site, Barnwell Co., SC	100	-	-
6	188	f	M	4/1/98	Risher Pond Rd., Savannah River Site, Barnwell Co., SC	100	-2.76	-
6	189	cs	M	4/17/98	Evans, residental area north Augusta, Columbia Co., GA	0	-	-
6	190	cf	F	4/24/98	upper Vaucluse Pond, Aiken Co., SC	100	-	-

APPENDIX 2. Continued.

Area	ID	Group	Sex	Date	Location	% *N. f.*	C1.1	C3.1
6	191	cf	F	4/24/98	upper Vaucluse Pond, Aiken Co., SC	100	-	-
6	192	cf	M	4/24/98	upper Vaucluse Pond, Aiken Co., SC	90	-1.72	-
6	193	cf	F	4/25/98	upper Vaucluse Pond, Aiken Co., SC	90	-	-
6	194	cf	F	4/25/98	upper Vaucluse Pond, Aiken Co., SC	90	-	-
6	195	cf	F	4/26/98	Mathis Lake, Interstate 20 x Hwy. 25, Aiken Co., SC	80	-	-
6	232	f	M	6/1/98	Ginger Bay, Savannah River Site, Aiken Co., SC	100	-	-
6	233	f	F	6/1/98	Ginger Bay, Savannah River Site, Aiken Co., SC	100	-	-
6	235	f	F	6/12/98	Upper Three Runs Creek, approx. 500 m upstream of Hwy. 278	100	-1.8	-
6	236	x	F	6/13/98	Pearsons Pond, Edgefield Co., SC	62	-	-
6	237	cf	F	6/13/98	Pearsons Pond, Edgefield Co., SC	62	-	-
6	238	cs	F	6/14/98	Augusta Canal, Augusta, Richmond Co., GA	12	0.129	-
6	239	cs	M	6/14/98	Augusta Canal, Augusta, Richmond Co., GA	25	-	-
6	240	f	M	6/15/98	Ellenton Bay, Savannah River Site, Aiken Co., SC	100	-	-
6	241	f	M	6/15/98	Ellenton Bay, Savannah River Site, Aiken Co., SC	90	-	-
6	242	f	F	6/15/98	Ellenton Bay, Savannah River Site, Aiken Co., SC	100	-	-
w VA	55	s	-	7/21/96	Mountain Lake Biological Station, Giles Co., VA	0	-	-
w VA	56	s	F	7/20/96	Walker Creek, 1.6 km north of Staffordsville, Giles Co., VA	0	1.305	-
w VA	57	s	M	7/23/96	bridge over Wolf Creek, Grapefield, Bland Co., VA	-	1.231	2.59
w VA	58	s	M	7/25/96	Bear Spring Branch 8 km west New River, Giles Co., VA	20	0.166	2.03
w VA	59	s	M	8/3/96	Craig Creek, Rte. 311 x Rte. 691, 4.8 km south of New Castle, Craig Co., VA	0	2.104	2.25
	60	s	M	8/4/96	spillway of lake in Hanging Rock State Park, Stolkes Co., NC	0	0.428	1.19
s VA	61	s	F	8/5/96	Mountain Lake Biological Station, Giles Co., VA	10	-	-
s VA	62	s	M	8/13/96	Madcap Creek x Blackwater River, 6.4 km west Rocky Mount, Franklin Co.,VA	0	1.138	-
s VA	66	s	F	8/21/96	Yacht Harbor of Hopewell, Prince George Co., VA	0	1.428	-
s VA	181	s	M	4/16/98	Diascund Creek Reservoir, New Kent Co., VA	0	-	-
s VA	217	s	F	5/20/98	James River near Benjamin Harrison Memorial Bridge, Prince George Co., VA	0	-	-
s VA	219	s	F	5/27/98	creek at Cooper Automotive, Hampton Co., VA	0	0.328	-
c VA	282	s	F	6/23/98	spillway of Bear Creek Lake, Cumberland State Forest, Cumberland Co., VA	0	-	-
n VA	203	s	M	5/17/98	Pohick Bay Regional Park, Fairfax Co., VA	0	-	-
n VA	204	s	F	5/17/98	Pohick Bay Regional Park, Fairfax Co., VA	0	-	-
n VA	205	s	M	5/17/98	Pohick Bay Regional Park, Fairfax Co., VA	0	-	-
n VA	206	s	F	5/17/98	Pohick Bay Regional Park, Fairfax Co., VA	10	-	-
n VA	207	s	F	5/17/98	Pohick Bay Regional Park, Fairfax Co., VA	0	-	-

APPENDIX 3

AFLP PROTOCOL

This AFLP protocol is slightly modified after the method applied during the AFLP workshop cosponsored by the ICBR and BEECS Genetic Analysis Laboratory, both at the University of Florida, Gainesville (Brazeau et al., 2001).

Restriction Ligation

1. Adapters were preheated to 95°C for 5 minutes, spun and cooled to room temperature for at least 10 minutes.
2. Set up of a restriction ligation mixture (Table 9)

TABLE 9. Recipe for the restriction ligation.

Reagents	For 1 reaction of 11 µl vol.	Final concentration
10X T_4 Ligase buffer (incl. ATP)	1.11 µl	1X
0.5 M NaCl	0.63 µl	0.05 M
BSA (1 mg/ml)	0.5 µl	.045 M
MseI adapters (50 µM)	2.0 µl	10 µM
EcoRI adapters (20 µM)	0.5 µl	1.0 µM
*Eco*RI (12 U/µl)	0.42 µl	5 U
*Mse*I (10 U/µl)	0.1 µl	1 U
T_4 DNA Ligase (2.75 U/µl)	0.35 µl	1 U
Total	5.61 µl	

3. 5.5 µl of template (genomic DNA) were combined with 5.61 µl of restriction ligation mixture.
4. Samples were spun and incubated for 4 hours at 37 °C or overnight at room temperature.
5. 189 µl of double destilled water added and stored at –20 °C if not immediately processed for amplicfication.

Preselective Amplification

1. Preparation of a preselective PCR master mix (Table 10):

TABLE 10. Recipe for the preselective PCR.

Reagents	For 1 rxn of 20 µl vol.	Final concentration
double destilled Water	6.1 µl	-
10X PCR buffer	2.0 µl	1X
$MgCl_2$ (25 mM)	1.2 µl	1.5 mM
2.5 mM dNTP's each	1.6 µl	200 µM each
EcoRI-A PS primer (2.75 µM)	2.0 µl	0.275 µM
MseI-C PS primer (2.75 µM)	2.0 µl	0.275 µM
Thermostable DNA polymerase (5 U/µl)	0.1 µl	0.5 U/rxn
Total	15.0 µl	

2. 5 µl of restriction ligation product (new template) were combined with 15 µl of PCR master mix, mixed and briefly spun.
3. Amplification was performed with following cycle profile (Table 11):

TABLE 11. Cycling program for the preselective PCR.

72°C	2 min		initial incubation
94°C	20 sec	denaturation	20 cycles
56°C	30 sec	annealing	
72°C	2 min	extension	
72°C	2 min		final extension
60°C	30 min		final incubation

4. After PCR completion, 90 µl ddi water was added to each sample, which will constitute the new template for the selective amplification.

Selective Amplification

1. Preparation of a selective PCR master mix (Table 12):

TABLE 12. Recipe for the selective PCR.

Reagents	For 1 rxn of 20 µl vol.	Final concentration
double destilled Water	6.1 µl	-
10X PCR buffer (w/ 15 mM $MgCl_2$)	2.0 µl	1X
$MgCl_2$ (25 mM)	1.2 µl	1.5 mM
5 mM dNTP's each	1.6 µl	200 µM each
EcoRI-A## primer (0.92 µM)	2.0 µl	0.092 µM
MseI-C## primer (5.5 µM)	2.0 µl	0.550 µM
Thermostable DNA polymerase (5 U/µl)	0.1 µl	0.5 U/rxn
Total	15.0 µl	

2. 5 µl of new template from preselective amplification were combined with 15 µl of selective PCR master mix, briefly mixed and spun.
3. Selective amplification was performed with following cycle profile (Table 13):

TABLE 13. Cycling program for the selective PCR.

94°C	2 min.	initial denaturation		
94°C	20 sec.	denaturation		
66°C	30 sec.	annealing		
72°C	2 min.	extension		
94°C		20 sec.	denaturation	
Decrease 1°C/cycle		30 sec.	annealing	9 cycles
72°C		2 min.	extension	
94°C	20 sec.	denaturation		
56°C	30 sec.	annealing		20 cycles
72°C	2 min.	extension		
60°C	30 min.	final incubation		

Applied AFLP Oligonucleotides

Adapters:

EcoRI-adapter	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
MseI-adapter	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'

Preselective Primers (preselective nucleotide extension in **bold**):

EcoRI-**A**	5'-GACTGCGTACCAATTC-**A**-3'
MseI-**C**	5'-GATGAGTCCTGAGTAA-**C**-3'

Selective Primers (selective nucleotide extensions in **bold**). Two different selective MseI-primers were used, one with a T- and one with an A-nucleotide at the third extension site:

EcoRI-**ACC**	5'-GACTGCGTACCAATTC-**ACC**-3'
MseI-**CTT(A)**	5'-GATGAGTCCTGAGTAA-**CTT(A)**-3'

APPENDIX 4

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11 March 2002

Konrad Mebert
c/o Dr. Denise R. Cooper
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August 7, 2002

Konrad Mebert
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EDUCATION

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EXTRA CURRICULAR TRAINING

- GCG/Seqlab, University of Florida, ICBR (Interdisciplinary Center for Biotechnology Research), 1999
- PCR Primer design, University of Florida, ICBR, 1999
- Marine Populations Genetics, Virginia Institute of Marine Science (Coll. of William and Mary), Spring 1999
- Molecular Biology, University of Virginia, Mountain Lake Biological Station, Summer 1996

PROFESSIONAL SERVICES

- 1991-1994; state employed field biologist, Switzerland
- 1995-1999; research assistant, ODU Research Foundation, Chesapeake Bay Monitoring Program
- 2000; lab manager, Dept. of Biochemistry and Molecular Biology, University of Florida, Gainesville. PI: Dr. Tom O'Brien
- 2001-2002; research assistant, Dept. of Biochemistry and Molecular Biology, Univ. of South Florida, at JAHVA Medical Center, Tampa, FL; PI: Dr. Denise R. Cooper.

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