THE DYNAMICS OF VOCAL, MORPHOLOGICAL, AND MOLECULAR INTERACTION BETWEEN HYBRIDIZING BLACK-CAPPED AND CAROLINA CHICKADEES

by
EUGENE DONALD SATTLER

A dissertation submitted to the Faculty of the Graduate School
of the University of Maryland in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
1996

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ABSTRACT

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Eugene Donald Sattler, Doctor of Philosophy, 1996

Dissertation directed by: Michael J. Braun, Adjunct Assistant Professor, Department of Zoology, and Lin Chao, Associate Professor, Department of Zoology

Previous investigation of genetic interactions between black-capped and Carolina chickadees (Parus atricapillus and P. carolinensis) has been hindered by their morphological similarity, and by a paucity of differentiated genetic markers distinguishing them. Nine fixed or strongly differentiated restriction fragment length polymorphism (RFLP) markers were developed, and one strongly differentiated allozyme locus was detected. These markers were used in conjunction with one fixed allozyme marker and three fixed RFLP markers previously available for these birds to examine interactions along their contact zone at three locations. A principal component analysis of mass, wing length and tail length revealed minimal morphological intermediacy at the contact zone in Virginia, in contrast with more extensive

intermediacy at the contact zone in West Virginia, despite high levels of hybridization at both locations. This reflects the unreliable nature of these morphometric characters in reflecting genetic interactions occurring along this hybrid zone, due to the poor morphometric resolution of P. atricapillus and P. carolinensis. Principal component and discriminant analysis of eight frequency and note duration variables showed songs of intermediate nature to be present only at the contact zone in Missouri, while bilingual singing was widespread both in Missouri and West Virginia, but limited in Virginia. The proportion of hybrids detected by the diagnostic genetic markers was high at all three of these regions, demonstrating that like morphology, use of song is unreliable in assessing genetic interactions between P. atricapillus and P.carolinensis. Heterospecific song learning between these chickadees is a potential explanation for this result. Introgression of mitochondrial DNA across the hybrid zone was limited relative to autosomal introgression at all three locations. This observation is consistent with the potential operation of Haldane's rule in F_1 hybrids. Introgression of sex-linked markers was likewise limited, suggesting that epistatic interactions involving sex-linked genes contribute to reproductive isolation between these chickadees. In contrast, introgression at

autosomal loci appears to be more substantial overall, reflecting the semipermeable nature of this hybrid zone. A correlation between allele frequency and elevation suggests that ecological factors are also important to this hybrid zone's dynamics.

ACKNOWLEDGEMENTS

I thank T. Parsons, C. Sattler, P. Sattler, and M. Hayslett for field assistance, and am grateful to the following for granting permission or permits for collection of specimens: R. Glasgow, George Washington National Forest; S. Griep, Jefferson National Forest; J. Page, Monongahela National Forest; D. Schiller, Susquehannock State Forest; J. Bearer and J. Nelson, Bureau of Forestry, Pennsylvania Department of Environmental Resources; M. J. Scanlan, Wildlife Division, Maryland Department of Natural Resources; R. Wajda and D. Bradshaw, Virginia Department of Inland Game and Fisheries; R. Ross, Wildlife Resources Section, West Virginia Department of Natural Resources; E. Camp, Pennsylvania Game Commission; R. Pierce, Division of Wildlife, Ohio Department of Natural Resources; the U. S. Fish and Wildlife Service; N. Krueger, Wayne National Forest, K. Sexton, Gathright Wildlife Management Area; and S. Brock, Chickahominy Wildlife Management Area. J. P. Angle, C. Angle, J. Dean, R. Clapp, and the Division of Birds, U. S. Museum of Natural History provided logistic support for field work, and assisted in the preparation and cataloging of specimens, while T. Kain, Y. Larner, C. Wilds, C. Cabe, D. Johnston, R. Blum, D. Browning and G. Hall

supplied helpful information on chickadee distribution. P. Sawaya, K. Zeller, T. Parsons, D. Albright, J. Blake, T. Glenn, C. Huddleston, R. Brumfeld, D. Emery, L. Lopez, A. Fulwood and others associated with the Smithsonian Institution's Laboratory of Molecular Systematics provided invaluable technical assistance, and R. Jernigan, D. Dance, D. Styer, G. Graves, K. Winker, E. Morton, M. Isler, and P. Isler gave valuable assistance in data analysis. M. Steffan cloned DPAC121. This dissertation was submitted in partial fulfillment of the requirements for the Ph.D. in the Department of Zoology, University of Maryland, College Park. The guidance of my supervising coadvisor, Michael J. Braun, was invaluable to the completion of this work. The support of my departmental coadvisor, Lin Chao, as well as that of the other committee members, Richard Highton, Douglas E. Gill, Gerald S. Wilkinson, and Charles B. Fenster, is also gratefully acknowledged. This research was supported by a Smithsonian Institution Predoctoral Fellowship, the Alexander Wetmore Memorial Fund of the American Ornithologists' Union, and the J. J. Murray Award of the Virginia Society of Ornithology.

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1. INTRODUCTION

Hybridization in a very broad sense is simply, "the interbreeding of individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters" (Woodruff 1973, Harrison 1990). While often thought of as a rare phenomenon, hybridization between two taxa is sometimes prevalent where they come into contact, and can result in a zone of hybridization where the ranges of the two taxa meet and hybridization occurs. The term "hybrid zone" is typically applied to such a situation, and has sometimes been used to specify a particular historical context such as secondary contact (e.g. Moore 1977), or a narrowly defined genotypic composition, such as only hybrids and no parental forms (i.e. a hybrid swarm; Short 1969). However, I will be using a broad definition of hybrid zones as proposed by Harrison (1990), which defines them as, "interactions between genetically distinct groups of individuals resulting in at least some offspring of mixed ancestry. Pure populations of the two genetically distinct groups are found outside of the zone of interaction." Such hybrid zones often exhibit a clinal structure for characters differentiating the hybridizing taxa. A cline, as dealt with theoretically by Haldane (1948), is simply a continuous gradient (such as in

allele frequency or a morphological polymorphism) along a geographical line or transect. Genetic interactions between two taxa can also result in the phenomenon of "introgressive hybridization", or simply "introgression", as detailed by Anderson (1949), which entails the gradual infiltration of the genome of one species into that of another through the process of successive backcrossing of hybrids to one or both of the parental species.

Hybrid zone analysis and theory is currently in a state of rapid growth, and the development of hybrid zone theory has revolved around three fundamental issues (Arnold 1992). These are 1) the relationship of hybrid zones to reproductive isolation and incipient speciation, 2) the evolutionary significance attributable to such zones, and 3) their taxonomic or systematic significance. The first of these issues takes its perspective from the biological species concept, which emphasizes the importance of gene flow in unifying a species. Hybrid zones are thus seen as "natural laboratories for evolutionary studies", allowing investigation into how geographic variation within species might be translated into new species, and what the nature of these species boundaries are (Barton and Hewitt 1981, 1989, Hewitt 1988, Harrison 1990). The study of hybrid zone structure and the outcome of hybrid interactions between taxa has the potential to reveal much about the "genetic

architecture" of species differences, such as the number of genes involved, their nature, location, and relationship to one another (Barton and Hewitt 1981, 1985, Harrison 1990). A primary focus of this study will be to address some issues related to potential differences in relative levels of introgression among different molecular markers, and what these differences might suggest regarding reproductive isolation and speciation in these chickadees.

Evolutionary significance of hybrid zones.-A second fundamental issue in hybrid zone research seeks to resolve the evolutionary significance they might have in their own right. The origin of hybrid zones has been an important question; whether they typically reflect secondary contact of two populations that diverged in allopatry, or formed in many cases via primary differentiation along a selective gradient, as might presage parapatric speciation. Quantitative models of clines show that both types of origins will result in similar clinal structures (Clarke 1966, Slatkin 1973, Endler 1973, 1977), so this issue has remained contentious. It has been argued that multiple concordant clines favor a secondary origin, because clines for different kinds of characters are not expected to all have selective null points that are coincident in position (Hewitt 1988, 1989). But cline theory also predicts that linkage disequilibrium can cause clines to coalesce (Slatkin

1975, Barton 1983). In addition, a secondary contact origin for a current hybrid zone does not exclude the possibility that the original differentiation might have developed sympatrically, with a subsequent range disjunction, followed by later reunion (Barton and Hewitt 1985).

With the question of hybrid zone origins often ambiguous, attention has focused on their fate. According to the classic allopatric scenario, hybrid zones are ephemeral phenomena. Following allopatric divergence and secondary contact, the two forms will either merge if a sufficient reproductive barrier has not formed, or reproductive isolation will be perfected through the process of reinforcement and the elimination of individuals prone to hybridize (Moore 1977). Reinforcement has received limited empirical support (Butlin 1987, 1989, Rice and Hostert 1993, but see Coyne and Orr 1989), and fusion of two taxa following secondary contact might often proceed quickly and so be difficult to detect. Though hard to verify, the majority of hybrid zones that currently exist and that have been investigated appear to be relatively stable phenomena, possibly thousands of years old (Barton and Hewitt 1985, Hewitt 1989, Harrison 1990). Attention has therefore shifted to the factors that might maintain stable hybrid zones.

Models to explain the maintenance of stable hybrid zones fall into two general classes (Moore and Price 1993). Endogenous selection models encompass those in which a hybrid zone is maintained by a balance between selection against hybrids and dispersal of parental genotypes into the hybrid zone (Bazykin 1969, Barton 1979a, b, 1983; Barton and Hewitt 1981, 1985, 1989). Selection in these models is dependent only on genetic interactions between disharmonious combinations of the two taxa's alleles in hybrids. Cline theory predicts that such a hybrid zone will tend to straighten because of dispersal pressure on either side of it; the term "tension zone" has thus been applied to them. Such hybrid zones can occur anywhere irrespective of ecological factors, but will tend to move down population density gradients until they are trapped in a density trough, often at an ecotone. Their width will vary with dispersal rate, but will typically be narrow with respect to the overall range and dispersal ability of the taxa. The majority of hybrid zones appear to be consistent with this class of model (Barton and Hewitt 1985).

Exogenous selection or geographic selection gradient models make up the second general class of hybrid zone model (Slatkin 1973, 1975, May et al. 1975, Endler 1977, Moore 1977). Here, the strength of selection is dependent on a selection gradient, with the two parental forms occupying

opposite ends of the gradient, and having highest fitness here. Hybrids might be equally fit as both parentals in the center of the hybrid zone, or could even have higher fitness than either one in the hybrid zone (Moore 1977). However, hybrids are not expected to be less fit than both parental forms unless endogenous selection is operating. A key feature of exogenous selection models is that the hybrid zone will be positioned by the environment selection gradient, often at an ecotone.

One of the most direct ways to test these two models is to measure hybrid fitness directly. In many cases the fitness of hybrids relative to parental forms is lower, thus supporting endogenous selection models (Barton and Hewitt 1985, but see Arnold and Hedges 1995). The two models are not mutually exclusive, however; ecological selection might lead to coadaptation of gene complexes with each taxon, the disruption of which could result in endogenous selection against hybrids (Hewitt 1988).

It is not always convenient to measure the fitness of hybrids directly, in which case inferences concerning the selective forces maintaining a hybrid zone might be made from its structure. The overall width of a hybrid zone under the two models is essentially the same, and does not allow a means of discriminating between the two (Barton and Gale 1993, Moore and Price 1993). Under the endogenous

selection model, however, the width of a particular character cline should not vary significantly along the length of the hybrid zone unless dispersal rate does, or unless the epistatic interactions involved vary geographically. In contrast, the width of a hybrid zone maintained by exogenous selection would be expected to vary in concert with variation in the width of the ecological selection gradient maintaining it. Such variation has been seen in a number of hybrid zones (e.g. Hunt and Selander 1973, Cook 1975, Yang and Selander 1968, Moore and Price 1993). Another clue to the operation of exogenous selection would be a fine-grained mosaic structure to the hybrid zone paralleling an environmental mosaicism (e.g. Harrison 1986, Howard and Waring 1991). In examining cline structure at multiple locations differing in ecological variables, I will have an opportunity to look for environmental influences on the hybrid zone's structure. Inferences from such correlations are necessarily weaker than more direct tests addressing the selection pressures operating, but in combination with other evidence can provide useful insights into hybrid zone dynamics.

Teasing apart the many factors operating on a hybrid zone is a monumental task that has not been accomplished for any one hybrid zone. With respect to this hybrid zone, the greatest need is for a more comprehensive set of molecular

markers differentiating P. atricapillus and P. carolinensis. With the acquisition of such markers, I believe the best strategy is then to examine cline structure in detail. This will be done comparatively at three levels: 1) comparisons will be made among different classes of marker, both non-molecular and molecular, 2) comparisons will be made among different geographic locations differing in ecology, and 3) comparisons will be made between this hybrid zone and non-avian hybrid zones, which might differ in structure as a result of differences between avian and non-avian taxa in important characteristics such as dispersal rate.

Taxonomic significance of hybrid zones.-The final fundamental issue addressed by the study of hybrid zones is their taxonomic significance. Much of the early incentive to study hybrid zones was as a means of resolving the taxonomic ambiguity of the taxa involved. At an evolutionary level, this issue revolves around the concept of what a species is.

Species concepts.-Hybrid zones pose a problem for the biological species concept (Mayr 1963), based as it is on reproductive isolation. Mayr (1951) acknowledged the relative nature of reproductive isolation, and did not believe that isolation had to be absolute. However, the differential permeability of hybrid zones to various characters and loci could justify the view that species need

to be defined on a gene by gene basis (Barton and Hewitt 1981). The recognition species concept (Paterson 1985) is similarly based on reproductive criteria, although its implications for hybrid zone theory can differ from that of the biological species concept. The evolutionary species concept (Wiley 1981) and the phylogenetic species concept (Cracraft 1983) both dismiss reproductive isolation as a pleisiomorphic trait of little relevance to the definition of species, and instead view species within a cladistic framework emphasizing shared derived characters uniting monophyletic clades. Hybridization will obviously impact the composition of species defined by these criteria, but the analysis of hybrid zones is only peripheral to the demarcation or recognition of species under either of these concepts. Finally, the cohesion species concept, which defines species on the basis of potential phenotypic cohesion through intrinsic cohesion mechanisms (Templeton 1989), and the genealogical concordance species concept, which recognizes species on the basis of intrinsic reproductive barriers that are detected on the grounds of concordant genetic differences (Avise and Ball 1990), attempt to blend certain elements of these other species concepts and arrive at a satisfactory synthesis. P. atricapillus and P. carolinensis have traditionally been considered good biological species, which in spite of the

apparently frequent occurrence of hybridization at their range interface, maintain distinct genetic identities throughout the bulk of their ranges. These conclusions are based, however, on an evaluation of introgression that may be inaccurate, based as it is on song, which is culturally transmitted in large part (Kroodsma et al. 1995), and morphology, which lacks resolution (Rising 1968, Robbins et al. 1986). This molecular analysis of their genetic interactions will attempt to place its results in the context of differing species concepts.

Prevalence of hybridization.-The phenomenon of hybridization has come to be recognized as a common event in nature, more frequent in some taxa than others. It has been estimated that 40-50% of all vascular plants arose as a consequence of hybridization followed by polyploidy (Ehrlich and Wilson 1991, Stace 1993). Hybridization is much less frequent among animals, but Hewitt (1989) cites 170 hybrid zones, the majority of them among animals, that have been more thoroughly studied. Approximately 10% of the world's bird species have been documented as hybridizing (Grant and Grant 1992), and Ford (1987) reviewed about 80 hybrid zones known among bird species in Australia alone.

Avian hybridization and hybrid zones.-Bird species appear to have lost the ability to hybridize relatively slowly, as over 40% of such events occur intergenerically (Prager and

Wilson 1975). It has also been noted that birds share smaller protein and mtDNA distances than other vertebrates of comparable taxonomic rank (Avise et al. 1980a, b, c, Kessler and Avise 1985). One explanation for these observations is that birds are oversplit taxonomically, and share common ancestors more recently than other taxa. If so, hybridization might be expected to be more frequent among non-sister bird taxa.

Some of the earliest work done on animal hybrid zones was with birds, by virtue of their conspicuousness, frequently obvious plumage differences, aesthetic appeal, and this propensity to hybridize (e.g. Meise 1928a, Sutton 1938, Cockrum 1952, Mayr and Gilliard 1952, references in Mayr 1963). Early studies of avian hybridization have also elucidated some fundamental concepts relating to hybrid zones. The location of hybrid zones where taxa met following expansion from refuges caused by glacial or drought periods, and the concentration of multiple hybrid zones at "suture zones" because of such historical factors, were recognized in large part due to early ornithological study (Gentilli 1949, Sibley 1959, Remington 1969). Bird hybrid zones also revealed width variation associated with ecological factors (Huntington 1952, Meise 1928b) and the role of man-induced habitat disturbance in promoting hybridization (Chapin 1948, Sibley 1954).

Molecular markers and cline models in hybrid zone research.-Major advances in understanding hybrid zones have come in recent decades as a result of two factors: the availability of molecular characters as markers in hybrid zone analysis, and the development of a theoretical basis underlying hybrid zones. One advantage of molecular markers such as allozymes and restriction fragment length polymorphisms (RFLP's) of DNA is that they are a potentially rich source of diagnostic traits that are necessary for the identification of hybrids and that allow levels of hybridization and introgression to be evaluated. Even when substantial morphological differentiation exists between taxa, it is difficult to determine the extent of genetic introgression underlying these traits (e.g. Hubbard 1969, Rohwer 1972), and the difficulty is exacerbated for instances such as these chickadees in which morphological differentiation is poorly developed (Rising 1968, Robbins et al. 1986). Molecular markers are thus especially helpful in cases where sibling species or morphologically similar subspecies are present.

Sibling species and the subspecies concept.-Morphologically similar species are often referred to as sibling species or cryptic species. Sibling species are sometimes recognizable on other grounds such as vocalizations or behavior (e.g. Barber 1951, Rising and

Schueler 1980, Capparella and Lanyon 1985), and upon closer analysis are usually recognizable on morphological grounds, as for instance in the case of Drosophila pseudoobscura and D. persimilis (references in Mayr 1963). Detection sometimes occurs as a result of biochemical surveys, however. Analysis of allozyme variation in the slimy salamander (Plethodon glutinosus) led Highton (1989) to recognize 16 distinct species or semispecies. Some of these forms showed no evidence of current gene flow between them, while hybrid zones were evident between others. Depending on the degree of genetic exchange occurring, some might therefore prefer to use the subspecies designation in some of these cases. While the subspecies concept makes a useful distinction between cases in which reproductive isolation is essentially complete (sibling species) and those in which it is not, it is sometimes incorrectly applied. For instance, upwards of 20,000 avian subspecies, many recognized on poor criteria, were accorded full species status for a time in the 19th century (Fjeldså 1985). Less than half of these are now considered full species. A primary weakness in the use of the subspecies unit has been the blurring of a distinction between entities showing well-defined reductions in gene flow and so representing incipient speciation events, and merely clinal variation (Fjeldså 1985). This ambiguity has led many to consider abandoning the concept,

but what appears to be necessary is more rigid criteria for recognizing subspecies, such as concordance among multiple independent characters in reflecting a valid distinction (Gill 1982, Barrowclough 1982, Avise and Ball 1990). Molecular markers, by providing many such independent characters, are a promising tool for establishing more objectively-defined subspecies (e.g. Ball and Avise 1992, Zink and Dittmann 1993, Zink 1994).

Value of molecular markers in assessing gene flow.-The ability of molecular markers to unambiguously identify hybrids, even in cases where sibling species or morphologically similar subspecies are involved, provides a means of assessing the significance of variation in characters whose genetic basis may be open to question. In the case of P. atricapillus and P. carolinensis, both morphology and song have been used to diagnose species identity, and to evaluate the extent of hybridization at their contact zone. However, morphological characters can have limited resolution for diagnosis when the taxa are as similar morphologically as are P. atricapillus and P. carolinensis (Rising 1968, Robbins et al. 1986). In addition, both morphological and vocal characters in birds can have a significant nongenetic component to their expression (Berven et al. 1979, James 1983, Kroodsma et al. 1995). The availability of diagnostic molecular markers

will provide in this study the means of assessing the significance of both morphological and song variation in these chickadees.

Molecular markers not only provide markers for hybrid zone analysis that aid in the identification of hybrids, but their use can yield many insights when used in a comparative way to analyze a hybrid zone. Because these markers vary in their transmission genetics, in selection levels they are exposed to, in their linkage relationships, and in other important ways, they can provide insights into issues such as which genes might be important in establishing reproductive isolation, the operation of selection, and the importance of other microevolutionary processes such as drift, gene flow, and mating patterns.

As a brief background on P. atricapillus and P. carolinensis, they are small songbirds parapatrically distributed across eastern North America. Their contact zone stretches from New Jersey to Kansas, with the range of the northerly distributed P. atricapillus dipping south in a peninsular fashion through the Appalachian Mountains as far as Tennessee and North Carolina. The two are morphologically similar in many respects. Both having grey backs, pale underparts, a black cap and bib, and white cheeks. However, P. atricapillus averages larger both in overall size and in the ratio of tail length to wing length,

has a greater degree of white edging to the greater wing coverts and rectrices, and has a more ragged border to the edge of its black bib (Tanner 1952, Brewer 1963, Rising 1968, Robbins et al. 1986). Morphological intermediacy is extensive at the P. atricapillus/P. carolinensis contact zone (Rising 1968, Johnston 1971, Robbins et al. 1986), but many of these characters converge in both towards their range interface (Duval 1945, Lunk 1952, James 1970).

The song of the two differs. P. atricapillus has a uniform two-noted song across its range. P. carolinensis typically sings a four-noted song, but greater song variation exists across its range (Kroodsma et al. 1995). Bilingual birds and intermediate songs are commonly heard at their range interface (Brewer 1963, Johnston 1971, Ward and Ward 1974, Robbins et al. 1986), suggesting the occurrence of hybridization. Learning plays a strong role in their song ontogeny, however, so song may not be a reliable marker of genetic interactions between them (Kroodsma et al. 1995).

The two are virtually identical in all ecological traits that have been examined (Brewer 1961, 1963); each is a hole-nesting woodland inhabitant, preferring openings and edges, but is also comfortable in fairly urban settings where sufficient trees are present. P. atricapillus has somewhat smaller clutches and population density, but these characters show clinal variation (Brewer 1963). Both are

non-migratory, though P. atricapillus engages in periodic irruptions southward (Bagg 1969).

The two were considered sister taxa until recently, when allozyme and mtDNA phylogenies both suggested that each was not the other's closest relative (Gill et al. 1989, 1993). Morphological and vocal analyses have not been able to resolve the question of how much hybridization and introgression occurs between them because of their morphological similarity and the potential nongenetic component to both morphology and song (Rising 1968, James 1983, Robbins et al. 1986, Kroodsma et al. 1995). An initial search for allozyme markers was unsuccessful because of their extensive protein similarity (Braun and Robbins 1986). One diagnostic allozyme difference was subsequently discovered (Gill et al. 1989). Efforts to develop DNA-based nuclear markers were more successful, and resulted in the discovery of two diagnostic single copy nuclear RFLP's. Diagnostic restriction fragment differences in their mitochondrial genome (Mack et al. 1986, Sawaya 1990, Sawaya and Braun in prep) have also been found. Use of these molecular markers to analyze hybrid zone interactions in Missouri revealed a significant level of hybridization, but a limited level of introgression (Sawaya 1990, Sawaya and Braun in prep). This analysis suggested that the level of mtDNA and sex-linked introgression was more limited than

that of autosomal introgression, and that females were under-represented among F_1's, in conformance to Haldane's rule (Haldane 1922).

The present study builds upon these earlier efforts with a search for additional molecular markers and an analysis of two additional transects across the hybrid zone through the Appalachian Mountains in West Virginia and Virginia. Results are reported here as three primary chapters. The first assesses morphological variation across the hybrid zone in conjunction with information on levels of hybridization and introgression, to evaluate the reliability of morphological variation in reflecting the genetic interactions taking place. The second examines song variation across the hybrid zone in conjunction with information on hybridization and introgression to assess the reliability of song as a marker of genetic interactions. The final paper uses more complete information obtained from newly developed genetic markers to examine patterns of introgression across the hybrid zone. Inferences are drawn regarding the importance of different modes of selection in contributing to reproductive isolation between these chickadees and in maintaining their hybrid zone.

Five questions regarding interactions between P. atricapillus and P. carolinensis are addressed here. First, how reliably does song reflect genetic interactions?

Second, how reliably does morphology reflect genetic interactions? Third, how uniform is the level of hybridization along this contact zone? Fourth, is the extent of introgression among different genetic markers comparable, or are there differences reflective of the hybrid zone's dynamics? And finally, is there any evidence that ecological factors play a role in the hybrid zone's dynamics?

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2. MORPHOMETRIC VARIATION IN BLACK-CAPPED AND CAROLINA CHICKADEES ACROSS THEIR APPALACHIAN CONTACT ZONE

Introduction

Black-capped and Carolina chickadees (Parus atricapillus and P. carolinensis) meet along an extensive contact zone where a narrow band of hybridization occurs (Brewer 1963, Rising 1968, Johnston 1971, Robbins et al. 1986). Such hybrid zones are useful for investigating the development of reproductive isolation, and for resolving the taxonomic status of the hybridizing taxa. Hybrid zones are also viewed as natural laboratories where the interaction of populations possessing differentiated genetic markers can be used to study population genetic processes, where insights can be provided into the process of speciation, and where evolutionary events of significance in their own right can occur (Hewitt 1988, Harrison 1990, 1993, Arnold 1992, Barton and Gale 1993).

Plumage differences between two taxa often aid in the recognition and study of avian hybrid zones. Phenotypic intermediacy in parental traits and increased variability typically mark the occurrence of hybridization (Anderson 1949, Schueler and Rising 1975). Other phenomena can display these characteristics, however, including character convergence and clinal variation. Thus it is important to

use multiple independent characters in assessing evidence for hybridization. The presence of hybrids can also be masked when such phenotypic evidence is rare or lacking. Therefore, the lack of morphological intermediacy and variability cannot be taken as conclusive evidence that hybridization is absent.

In a number of cases, plumage differences have enabled the detection of assortative mating or other evidence of limited hybridization or introgression that has resulted in hybridizing avian taxa being considered distinct biological species (Short 1963, Anderson and Daugherty 1974, Emlen et al. 1975, Johnson and Johnson 1985). In other cases, essentially random mating and high levels of hybridization have been detected, and the hybridizing pairs have been classified as a single biological species (Huntington 1952, Dixon 1955, Sibley and Short 1964, Short 1965, Barrowclough 1980). In some of the latter instances, subsequent evidence of limited introgression, significant genetic differentiation, or changes in taxonomic philosophy, have led to a reconsideration of the grounds for lumping the two forms. When two avian taxa meeting along a common zone are morphologically similar, determination of genetic interaction is more difficult. A case in point are the Eastern and Western meadowlarks (Sturnella magna and S. neglecta), for which careful documentation of vocalizations

and univariate and multivariate analyses of morphology were necessary to resolve that hybridization between them is relatively infrequent (Lanyon 1957, 1966, Szijj 1966, Rohwer 1972).

Determining how much genetic admixture occurs between Parus atricapillus and P. carolinensis has been even more difficult. Although the two chickadees differ in several mensural and plumage traits at the extremes of their ranges (Simon 1956, James and Rising 1985, Kaufmann 1990), clinal variation and subspecies differences in both minimize these phenotypic differences where the two meet (Duval 1945, Lunk 1952, James 1970). Consequently, although several studies of morphology have suggested that a significant number of hybrids are present at the contact zone, it has not been possible to establish with certainty the level of hybridization or introgression that might be present (Brewer 1963, Rising 1968, Johnston 1971, Robbins et al. 1986, Ballard 1988).

Direct molecular assays of genetic differences among taxa constitute a new method for studying hybrid zones that can provide discrete genetic markers allowing the identification of hybrids and revealing the structure of a hybrid zone in great detail; these methods are especially useful where morphological differentiation is weak. Four diagnostic molecular markers have recently been developed for these two

chickadees (Mack et al. 1986, Gill et al. 1989, Sawaya 1990, Gill et al. 1993, Sawaya and Braun in prep). Here we use these four genetic markers to assess levels of hybridization along two transects crossing the atricapillus/carolinensis contact zone in the Appalachian Mountains. Patterns of mensural variation are also quantified in these populations. Comparison of these two data sets allows us to evaluate the correlation of morphometric and genetic variation in these chickadees, and to assess the reliability of these morphometric variables in reflecting genetic interactions taking place in this hybrid zone.

Materials and Methods

Study sites and population samples.

Seventy-five birds collected at 5 sites comprised the Virginia transect (VA1-VA5) and 69 birds collected at 5 sites comprised the West Virginia transect (WV1-WV5) (Fig. 1, Table 1). The two transects share one of these populations in common (VA1/WV1). Parental populations of atricapillus (PA) and carolinensis (OH) were also collected, and represent the terminal populations of the West Virginia transect, while PA and a second carolinensis parental population (VA) constitute the terminal populations for the Virginia transect.

These species are sexually dimorphic in size, so only

males were included in morphological analyses. To allow comparison of morphometric and genetic variation, females were excluded from genetic analyses as well. All birds were collected with shotguns, frozen within a few hours on either dry ice or in liquid nitrogen, and later transferred to a -80°C freezer. Collecting was done during the breeding season between 1989 and 1992 (Table 1), and both study skins and tissue specimens were deposited at the U. S. National Museum of Natural History.

Morphometric analysis.

Specimens were thawed at the laboratory and weighed to the nearest 0.1 g. Wing chord and tail length were measured to the nearest 0.5 mm by ruler, and specimens showing excessive wear or damage were eliminated from morphological, but not genetic, analysis. Each bird was sexed by examination of gonads and aged by examining skull pneumatization. Populations VA2, VA3 and VA4 each contained four to eight immatures. No significant differences were found between adults and immatures for the three morphometric variables (Mann-Whitney U-tests, all $P > 0.10$), so the two age classes were combined in each population.

A principal component analysis (PCA) was performed on the untransformed data using the correlation matrix, thus weighting all variables equally (PROC PRINCOMP; SAS 1987). All 12 populations of the Appalachian transects including

terminal parental populations were included in the analysis. The three morphometric variables were distributed normally in each population with the following exceptions: mass, wing length and tail length were non-normally distributed in VA4 due to the presence of a single individual characterized by our genetic markers as a pure atricapillus in this predominantly carolinensis population. PCA was performed both with and without this individual. Mass was also non-normally distributed in VA, VA5 and WV4, as was wing length in VA3. Transformations failed to normalize the variables in these populations, so untransformed values were retained in the PC analyses. Extracted components were distributed normally in each population; one-way analyses of variance (ANOVA) tests of the components were done for the West Virginia and Virginia transects separately (PROC GLM; SAS 1987), followed by a posteriori Tukey tests (SAS 1987).

Genetic analysis.

Isozyme analysis.-Liver tissue was thawed and 0.05-0.2 g homogenized in 150 µl water with a pestle. Samples were centrifuged for two min and supernatant aliquoted and stored at -80°C until use. Isozymes were separated on Titan III thin layer cellulose acetate plates using Zip Zone electrophoresis chambers (Helena Laboratories, Beaumont, Texas). Gels were run at 200 V for 50-120 min using a 50mM Tris/20mM Maleate buffer (pH 7.8), and stained by agar

overlay using the guanine deaminase (GDA) staining recipe of Richardson et al. (1986).

DNA extraction and restriction analysis. - Pectoral muscle was thawed and 0.7 g from each bird mechanically homogenized in 7.0 ml of extraction buffer (0.1 M NaCl, 0.1 M EDTA, 0.01 M Tris, pH=8.0). The homogenate was digested overnight at 55°C with proteinase K (200 µg/ml) in the presence of 0.5% SDS, then digested with RNAse (100 µg/ml) for 1 h at room temperature. NaCl was added to a 0.2 M concentration, and samples extracted once in an equal volume of a phenol-chloroform-isoamyl alcohol solution (PCI; 25:24:1), and twice in an equal volume of a chloroform-isoamyl alcohol solution (CI; 24:1), incubating each extraction at 55°C for 20 min. Total DNA was recovered by overlaying the aqueous solution with 2 volumes of cold 95% ethanol and spooling the high molecular weight DNA onto a pasteur pipette, rinsing in 70% ethanol, and resuspending in 800 µl of 1X TE (10 mM Tris, 1 mM EDTA). Mitochondrial DNA (mtDNA) from carolinensis was also purified for use as a probe of Southern blots, following the subcellular fractionation and CsCl equilibrium gradient centrifugation protocol of Dowling et al. (1990).

Restriction analysis.- Restriction enzyme digestions were carried out overnight according to manufacturer's recommendations. Four micrograms of total genomic DNA were

digested with 20 units of restriction enzyme, and electrophoresed in 0.6% agarose gels overnight at 40-50 V. Gels were soaked for 30-45 min in 1 liter of 0.4 M NaOH, 0.8 M NaCl under gentle agitation to denature the DNA, then soaked in 1 liter of 1.5 M NaCl, 0.5 M Tris HCl for 30-60 min prior to blotting onto MSI Magnagraph nylon membrane (Southern 1975). Transfer was accomplished over 6-20 h using 10X SSC (1.5 M NaCl, 0.15 M sodium citrate). DNA was crosslinked to membranes using a Strata-gene UV Stratalinker 1800; membranes were rinsed in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate), then air dried and stored at -20°C. Probes were labelled to high specific activity (10^8-10^9 dpm/μg) with alpha ^{32}P dATP using a random priming reaction (Feinberg and Vogelstein 1983). Transfer membranes were prehybridized in glass hybridization tubes (1-5 membranes/tube) with a solution of 1 M NaCl, 1.0% SDS, 10.0% dextran sulfate for 1-3 h at 65°C, using a Robbins Scientific Hybridization Incubator (Model 310). Labelled probe was then added to a concentration of $2x10^6$-$2x10^7$ dpm/ml ($1-2x10^5$ dpm/ml for mitochondrial probe), and hybridization carried out for 18-24 h. One low stringency wash (1.0X SSC, 0.5% SDS, 1 mM EDTA) and two high stringency washes (0.2X SSC, 0.1% SDS, 1 mM EDTA) were done at 48°C. Membranes were then wrapped in cellophane without drying and exposed to Kodak XRP film for 20-200 h using two Dupont

Cronex intensifying screens. After autoradiography, some membranes were stripped of radioactivity in two changes of boiling 0.1% SDS (1000 ml each) and reprobed with mtDNA.

Three probes were used to detect restriction fragment length variants diagnostic for atricapillus and carolinensis (Sawaya 1990). A 1.2 kb fragment of the chicken oncogene ski (Li et al. 1986) was used to probe Eco RI digests, while a randomly cloned 4.0 kb fragment of Tufted Titmouse (Parus bicolor) DNA designated DPAC121 was used to probe Pst I digests. Carolina Chickadee mtDNA served as the third probe, using three separate restriction enzymes (Pst I, Pvu II, and Ava II) for haplotype determination. Fragment lengths were estimated by comparison with a size marker consisting of Hind III digested bacteriophage lambda DNA and Hae III digested bacteriophage ϕX174 DNA. We did not attempt to score fragments smaller than 400 bp.

These four diagnostic markers allowed us to make estimates of the frequency of hybrids and the relative contribution of the two forms to each population (Table 2). A hybrid was defined as any individual possessing a mixture of atricapillus and carolinensis alleles among these four loci. Individuals heterozygous at each of the three diploid loci were identified as potential F_1's, while those individuals characterized by some other mixture of the two parental alleles at the four diagnostic loci must be

backcross or later-generation hybrids. Estimates of hybrid frequency are conservative, as backcross or later-generation hybrid matings can produce some triple homozygotes as well. Ski is autosomal in these chickadees, while GDA has been observed to exhibit variation that is consistent with sex-linkage; only males display a heterozygous pattern (Sawaya 1990, Sawaya and Braun in prep). Our results support this inference, with 14 male and no female heterozygotes being detected among 61 and 28 hybrids respectively. The possibility of physical linkage of GDA and DPAC121 on the Z chromosome could result in non-independence of these markers, further increasing the chances of misclassifying later-generation hybrids as parentals.

Results

Genetic analysis.

Restriction fragment sizes for ski and DPAC121 and allelic mobilities for GDA agreed with those reported earlier (Gill et al. 1989, Sawaya 1990, Sawaya and Braun in prep.). Screening of mtDNA haplotypes with Pst I, Pvu II, and Ava II produced a size estimate for the mtDNA genome of these chickadees of 16.2 kb. DPAC121 and MtDNA revealed intraspecific polymorphisms that could be unambiguously assigned to one or the other species based on their distribution in parental populations and/or their

relationship to parental haplotypes. Intraspecific variation will be analyzed in detail elsewhere. Ski did not reveal any such variation, nor did GDA. MtDNA fragment profiles produced by each enzyme were concordant in all individuals.

Based on the four diagnostic markers, parental population PA contained only individuals classified as pure atricapillus, and parental population VA consisted only of individuals classified as pure carolinensis (Table 2). However, more than 40% of population OH individuals were classified as hybrids, although OH was collected to represent parental carolinensis in southern Ohio, 170 km from the contact zone. All hybrids in OH were so classified on the basis of single atricapillus alleles for the marker ski (see Discussion). The remaining populations of both the Virginia and West Virginia transects all contained some hybrids. WV3 and WV4 straddle the center of the hybrid zone along the West Virginia transect, while in Virginia the center of the hybrid zone lies between VA2 and VA3 (Table 2). In these four populations, a minimum of 35% to nearly 70% of the birds sampled were of hybrid ancestry. With the exception of WV3, one species' alleles strongly predominated in any population (Table 2), and these skewed frequencies also predominated within individuals. In all populations, backcross or later-generation hybrids predominated among

hybrids (Table 2). In the West Virginia transect, potential F_1's comprised less than 20% of any population. In the Virginia transect, no potential F_1's were detected (but see Discussion), and all but four of the hybrids were identified as such on the basis of a single foreign allele at the loci surveyed. The frequency of hybrids declined rapidly away from the range interface, except in populations on the carolinensis side of the West Virginia transect. Non-F_1 hybrids remained at frequencies near 50% through WV4 and WV5 to OH. However, all hybrids found greater than 20 km from the contact zone in either transect were classified as such on the basis of a single foreign allele at the marker ski, with the exception of a single bird in WV5, 40 km from the range interface. Genetic data on hybridization and introgression will be treated in greater detail elsewhere (Sattler and Braun in prep).

Morphometric analysis.

Both parental populations of carolinensis (OH, VA) averaged smaller than the parental sample of atricapillus (PA) in all univariate measurements, and in the ratio of tail length to wing length (Table 3), one of the most reliable features distinguishing these species (Tanner 1952, Simon 1959, Johnston 1971, Merritt 1978, 1981). Other populations from the two transects were intermediate between the appropriate parental populations in these measures, with

the exception of genetically atricapillus-like populations WV1/VA1 and WV2, which were larger than parental atricapillus (PA) in most of these variables. WV1/VA1 and WV2 were each collected at higher elevations (564 ± 35 m and 708 ± 22 m respectively) than was PA (465 ± 14 m). Thus, their larger size relative to PA is compatible with a proposed modification of Bergmann's rule, which takes into account the effect of elevation as well as temperature on clinal size variation in homeotherms (Snow 1954, Moreau 1957, James 1970). Populations overlapped extensively in each of these measurements, so principal component analyses (PCA) were performed on mass, wing length and tail length to improve resolution.

The first principal component (PC 1) accounted for 80.5% of the total variance, while the second and third components (PC 2 and PC 3) explained 13.5% and 6.0%, respectively (Table 4). PC 1 had positive factor loadings for all three variables and thus is closely related to overall body size.

Along the Virginia transect, PC 1 varied significantly among populations (ANOVA $F=47.9$, $df=6$ and 89, two-tailed $P<0.0001$), with an abrupt transition occurring between VA2 and VA3 (Fig. 2). There were no significant differences in PC 1 either among populations in which atricapillus alleles predominated (PA, VA1, VA2) or among populations in which carolinensis alleles predominated (VA, VA3-VA5). However,

all genetically atricapillus-like populations were significantly larger (higher PC 1) than all genetically carolinensis-like populations ($P<0.05$, Tukey tests).

Size, as gauged by PC 1, also varied significantly along the West Virginia transect (ANOVA $F=16.6$, $df=6$ and 91, two-tailed $P<0.0001$). Genetically atricapillus-like populations (PA, WV1-WV3) again averaged larger than genetically carolinensis-like populations (OH, WV4, WV5), although the difference was not as great or the transition as pronounced as along the Virginia transect (Fig. 2). The atricapillus-like populations were all significantly larger than the carolinensis-like populations ($P<0.05$, Tukey tests), with the exception that WV3 and WV5 did not differ significantly. PC 1 did not vary significantly among the genetically atricapillus-like populations of this transect, and among the genetically carolinensis-like populations, only OH and WV5 differed significantly ($P<0.05$, Tukey test).

Considering the two transects as a whole, there was no size variation (PC 1) among genetically atricapillus-like populations (PA, WV1/VA1, WV2, WV3, VA2; $F=2.40$, $df=4$ and 70, two-tailed $P>0.05$), while such size variation did exist among genetically carolinensis-like populations (OH, WV4, WV5, VA3-VA5, VA; ANOVA $F=10.99$, $df=6$ and 86, two-tailed $P<0.0001$). Most of this variation in size occurred between populations on opposite sides of the Appalachian Mountains

(Table 3, Fig. 2), with carolinensis-like populations of the West Virginia transect being significantly larger (ie more atricapillus-like) than those in Virginia in most pairwise comparisons. OH, the smallest-bodied population west of the Appalachian Mountains, however, differed significantly only from VA among genetically carolinensis-like populations in Virginia ($P<0.05$, Tukey tests).

The PC 2 axis contrasts mass with measures of wing and tail length; thus, birds with large PC 2 scores had a higher mass relative to wing and tail length (Table 4). This component showed no consistent differences between the two species (Fig. 2), and the only significant differences among populations occurred between WV1/VA1 and both PA and WV5 ($P<0.05$, Tukey tests).

PC 3 primarily contrasts wing and tail length (Table 4), and did show a consistent trend between species (Fig. 2). Birds of predominantly carolinensis ancestry had shorter tails relative to wing lengths, which was reflected in larger PC 3 scores. As with PC 1, there was a transition in PC 3 scores at the range interface along both transects. Differences among populations of the Virginia transect were not significant, however (ANOVA $F=0.79$, $df=6$ and 89, two-tailed $P>0.50$), and within the West Virginia transect the only significant pairwise differences in PC 3 was between OH and all populations of predominantly atricapillus genetics

(WV1-WV3; P<0.05, Tukey tests). Across the two transects as a whole, genetically atricapillus-like populations did not differ significantly in PC 3. Among genetically carolinensis-like populations, those east of the Appalachian Mountains had smaller average PC 3 scores (ie more atricapillus-like) than those west of the Appalachians, although only OH and VA differed significantly (P<0.05, Tukey tests).

The best separation of parental populations was achieved with a scatterplot of PC 1 and PC 3. In the Virginia transect, the parental populations of atricapillus (PA) and carolinensis (VA) were well resolved morphometrically from one another (Fig. 3). Among the non-parental populations of this transect, genetically atricapillus-like populations (VA1-VA2) also separated morphometrically from genetically carolinensis-like populations (VA3-VA5) with the exception of one individual in VA4 that possessed only atricapillus alleles at the marker loci, and fell among the atricapillus-like populations (Fig. 4). Defining morphological intermediacy on the basis of an intermediate position between parental polygons in the scatterplot, about 22 individuals in the Virginia transect were intermediate. These birds represented 32.4% of the individuals in VA1-VA5, a proportion similar to the proportion of hybrids shown genetically to be present in these populations (21 of 68

individuals, or 30.9%). However, over half of these morphologically intermediate individuals were classified genetically as pure atricapillus or carolinensis, and birds of hybrid ancestry in the Virginia transect exhibited a bimodal distribution in PC 1 scores similar to that of the pure atricapillus and carolinensis individuals (Fig. 4). The majority of these hybrids are later-generation backcross progeny (see Genetic Analysis above), and each falls morphologically among the appropriate parental species based on the alleles dominating its genome.

The bimodality of PC 1 scores in the Virginia transect falls between VA2 and VA3. The centroids of these two populations are about eleven kilometers apart, but their borders abut one another (Fig. 10). VA3 spans the width of the Shenandoah Valley because of the dispersed nature of woodlot habitat suitable for chickadees there, while VA2 was collected on the first few ridges of Little North and Great North Mountains, immediately west of the Shenandoah Valley. The transition in PC 1 (and PC 3) score along the Virginia transect therefore occurs over a short distance.

For the West Virginia transect, parental populations of atricapillus (PA) and carolinensis (OH) were separated on the scatterplot of PC 1 and PC 3 (Fig. 3). However, the degree of separation was less than that of parental populations of the Virginia transect, due to more

atricapillus-like PC 1 scores in OH. Because of this greater morphometric similarity between PA and OH, the region between them that defines morphometric "intermediacy" is narrow. Only six of 69 birds (8.7%) in WV1-WV5 fall within this morphometric space, compared with 28 birds (40.6%) genetically defined as hybrids among WV1-WV5 (Fig. 5). As in Virginia, however, allele frequencies in four of these populations are strongly skewed towards either atricapillus (WV1, WV2) or carolinensis (WV4, WV5) types. Hybrids in these four populations are predominantly backcrosses or later-generation hybrid progeny (one potential F_1 in WV4 and three individuals overall with more than a single foreign allele). As in the Virginia transect, they showed a strong tendency to fall morphometrically among the appropriate parental species as expected on the basis of their genetic makeup, although there was some overlap between WV1-WV2 and WV4-WV5 because of the atricapillus-like PC 1 scores of WV4 and WV5.

In WV3, where the representation of atricapillus and carolinensis alleles was more evenly balanced (68.8% and 31.2% respectively), hybrids were more genetically intermediate. Four potential F_1's were found in WV3, as well as five additional individuals with two or more foreign alleles classifying them as hybrids. WV3 hybrids showed the broadest range of morphological overlap with the other

populations of the West Virginia transect. (Fig. 5). As a result, the distribution of PC 1 scores for hybrids in WV1-WV5 was unimodal (Fig. 5). The transition in PC 1 score between WV3 and either adjacent population was less than that occurring between VA2 and VA3, and occurred over a greater distance.

Because of the more balanced representation of species' alleles in WV3, we were able to directly assess the relationship between morphometric and genetic variation. We examined the correlation among PC scores and the number of atricapillus alleles possessed by individuals of WV3 using a Spearman's rank test (Fig. 6). For PC 1 and PC 3 this correlation was significant (r_s=0.62, one-tailed P<0.005 and r_s=-0.50, one tailed P=0.01, respectively; n=21), while for PC 2 the correlation was not significant.

Discussion

Levels of hybridization and introgression.

The morphological similarity of atricapillus and carolinensis has prevented assessment of the degree of hybridization and introgression occurring between them. While morphological studies conducted at some locations found little evidence of genetic mixing (Tanner 1952, Merritt 1978, 1981), others indicated that a more substantial number of hybrids including backcrosses were

present (Rising 1968, Johnston 1971, Robbins et al. 1986, Ballard 1988).

With the use of four diagnostic molecular markers, we have confirmed the presence of a high proportion of hybrids at two transects of the atricapillus/carolinensis contact zone in Virginia and West Virginia. The estimated proportion of hybrids in the zone's center at these two locations are comparable to that of an earlier study of the hybrid zone in Missouri using these same markers (Sawaya 1990, Sawaya and Braun in prep), in which 56.0% of 25 males sampled in the hybrid zone's center were found to be of mixed ancestry. The proportion of F_1's in central populations of the West Virginia and Virginia transect ranged from 0 to 19% (Table 2), but these differences were not significant ($P > 0.10$, Fisher's two-tailed exact tests). In the central hybrid population in Missouri, 24.0% of 25 males were F_1's, which differed only from VA3 among central Appalachian hybrid populations ($P < 0.02$, Fisher's two-tailed exact test).

The presence of a majority of non-F_1 hybrids among progeny of mixed ancestry at each of the three locations substantiates most previous morphological analyses of this hybrid zone, which found a continuum in the range of morphological variation seen, suggesting that substantial successful backcrossing was taking place (Rising 1968,

Johnston 1971, Robbins et al. 1986, Ballard 1988). The consequences of this extensive backcrossing has not been uniform with respect to loci, as seen in the more extensive introgression of ski alleles. The concept of the semipermeability of hybrid zones to the movement of different markers and characters was an early feature of hybrid zone theory (Key 1968) that has been borne out in many cases (Harrison 1990 and references therein). Levels of selection that vary among loci, combined with differing degrees of physical linkage between loci, can potentially explain such semipermeability.

Correlation of morphometric variation with genetic ancestry.

Many morphological traits in birds are under polygenic control (Buckley 1987), making them potentially useful for assessing genetic interactions within a hybrid zone. Atricapillus averages larger in overall size (PC 1) than carolinensis (Duvall 1945, Lunk 1952, Simon 1959, Hubbard 1970, James 1970). Likewise, the ratio of tail length to wing length (closely related to PC 3 in our PCA) has traditionally been used as a reliable feature distinguishing atricapillus and carolinensis (Tanner 1952, Simon 1959, Johnston 1971, Merritt 1978, 1981). Both PC 1 and PC 3 exhibited an abrupt transition across the contact zone that was concordant in position with change in allele frequency at the four marker loci. Such concordance is not

necessarily strong evidence of a direct relationship between phenotype and genotype, however. A similar transition is also expected for a culturally transmitted trait such as song (see below). Significantly, however, the rank correlation of morphological PC score with number of atricapillus alleles for individuals in WV3 revealed highly significant relationships of both PC 1 and PC 3 with an individual's genetic composition (Fig. 6). In addition, allele frequencies in WV3 were skewed towards representation of atricapillus alleles, and both PC 1 and PC 3 displayed a similar skewing of scores in WV3 towards atricapillus-like values (Fig. 5). While there was a significant correlation between individuals' PC scores for song and number of atricapillus alleles in WV3, the relationship was much weaker than between morphological PC score and genetics (r_s=-0.36, one-tailed P=0.025, n=30; Fig. 13).

Song is a primary diagnostic feature of atricapillus and carolinensis in allopatry, differing to a greater extent than morphology. However, song has not been found to be a reliable genetic marker in this or other songbird hybrid zones (Ficken and Ficken 1967, Gill and Murray 1972, Emlen el al. 1975, Morrison and Hardy 1983, Sorjonen 1986, Gelter 1987, Lein and Corbin 1990, Chapter 3). This failure has been attributed to the importance of learning in the development of song in oscine songbirds. In allopatry,

cultural transmission of a species diagnostic trait will be as faithful as genetic transmission, because there is no opportunity for interspecific transmission of the trait via learning. But in a sympatric context, the reliability of a culturally transmitted trait as a species' marker can break down because of the possibility for interspecific learning in the absence of genetic exchange.

Assessment of genetic interactions from morphometric variation.

Given a strong correlation between morphometric and genetic variation in these chickadees, can morphological analyses provide reliable information on the genetic interactions taking place in instances where genetic data is lacking? Character intermediacy and increased character variability in a population can be a reliable means of phenetically identifying the occurrence of hybridization (Schueler and Rising 1975). Extensive and continuous morphometric intermediacy was seen in our West Virginia transect, suggesting the presence of a considerable number of hybrids, including non-F_1 progeny. Genetic analysis of these birds confirmed this hypothesis. Likewise, the prediction by Robbins et al. (1986) that extensive hybridization, including the production of advanced generation hybrids, was taking place at the atricapillus/ carolinensis contact zone in Missouri on the basis of the

continuum of morphometric variation, was borne out by genetic data (Sawaya 1990, Sawaya and Braun in prep). Other morphological analyses of this hybrid zone have similarly suggested extensive genetic interactions (Rising 1968, Johnston 1971, Ballard 1988). These conclusions seem justifiable.

On the other hand, some morphological investigations of this hybrid zone have found little or no evidence for the presence of hybrids at certain portions of the atricapillus/ carolinensis contact zone (Tanner 1952, Merritt 1978, 1981). The pronounced bimodal distribution of PC 1 scores in the Virginia transect, in contrast to the unimodal distribution of PC 1 scores in the West Virginia transect, lends itself to the conclusion that hybridization is significantly reduced in the Virginia transect. Such is not the case. The bimodal PC 1 distribution in the Virginia transect is due in part to greater differentiation between the parental forms here. While the genetic interface between atricapillus and carolinensis along the Virginia transect also shows evidence of being sharper than along the West Virginia transect, this might be the result of lower production of F_1 progeny here. Reduced levels of F_1 production, but free production of advanced generation hybrids, has been found to phenotypically mask extensive levels of hybridization and introgression in an iris hybrid

zone (Arnold 1993, Arnold et al. 1993). Whether due to a real biological phenomenon or a result of sampling error, under-representation of F_1 progeny in the Virginia transect has likely reduced the extent of morphometric intermediacy seen here relative to the West Virginia transect.

Finally, selection against some recombinant genotypes in a hybrid zone and the evolution of genes modifying the phenotype of hybrids can minimize morphological evidence of hybridization (Schueler and Rising 1975). The paucity of morphological intermediacy between atricapillus and carolinensis found by Tanner (1952), and Merritt (1978, 1981) may reflect a low level of hybridization at these locations, but we have shown that morphometric characters in these birds can give a misleading picture of their genetic interactions in some circumstances. It may well be that hybridization is fairly common at localities studied by Merritt and Tanner. Morphological evidence against substantial hybridization and introgression between atricapillus and carolinensis should be treated cautiously.

Significance of long-distance introgression to morphometric variation.

The correlation between morphometric and genetic variation in atricapillus and carolinensis raises another question. Is the larger, more atricapillus-like morphology of genetically carolinensis-like chickadees in the West

Virginia transect a result of the greater genetic introgression they have experienced from atricapillus? Or is this morphometric variation the expression of geographic variation in carolinensis unrelated to the contribution of genes from atricapillus? While OH was collected as a presumed parental population of carolinensis, we found a high proportion of alleles there characterized as atricapillus for the autosomal marker ski. Populations WV4 and WV5 of the West Virginia transect also contained a high proportion of atricapillus ski alleles. This pattern contrasts with that seen in the Virginia transect (present study), and in the Missouri transect analyzed by Sawaya and Braun (in prep), where distant allopatric populations of carolinensis were fixed for an alternate allele. In both of these latter cases, the frequency of the atricapillus ski allele quickly declined into the range of carolinensis, and was not detected in parental carolinensis populations of these transects. Thus, the high frequency of C alleles in OH, WV5 and WV4 are presumed present as a result of introgression. Other reasons for suspecting gene flow to be the source of these alleles include a more southerly position of the range interface in Ohio during historic times (Wheaton 1882), which would have put atricapillus closer to carolinensis populations in southern Ohio and West Virginia. Also, occasional incursions of atricapillus in

winter as far as southern Ohio are known (Peterjohn 1989). If some individuals undertaking these winter movements remain and breed successfully, they would provide another source for the alleles found within the range of carolinensis. Such winter occurrences of atricapillus are virtually unknown along the coastal plain of Virginia (VSO 1987), and have not been recorded as far south as Missouri or Louisiana (AOU 1983).

Introgression of atricapillus alleles therefore appears correlated with, and could be the cause of, the more atricapillus-like PC 1 scores found in WV4, WV5 and OH. However, geographic variation in carolinensis independent of genetic influence from atricapillus must also be considered as a possible cause of this trend. Both atricapillus and carolinensis increase in size from south to north across their ranges, in accordance with Bergmann's rule (Duvall 1945, Lunk 1952, James 1970). Such clinal variation is typically interpreted as an adaptive response to ecological variables such as temperature and humidity, and so is unlikely in atricapillus and carolinensis to result from introgression between them. The data of Lunk (1952) also indicates some increase in size of carolinensis from east to west across the southern portion of its range. This tendency for size in carolinensis to increase from east to west in the south, some distance from the probable genetic

influence of atricapillus, likewise suggests that similar size variation in the north could be unrelated to introgression from atricapillus.

Lunk (1952) noted similar geographic variation in the ratio of tail length to wing length in carolinensis. This ratio is typically used to discriminate atricapillus and carolinensis, and is related to PC 3 of our multivariate analysis. In Lunk's study tail length increased proportionally from east to west in carolinensis, indicating that variation in this ratio varies intraspecifically, as does overall size. Considering variation in PC 3 within our populations, if introgression of atricapillus alleles in West Virginia and southern Ohio is responsible for a more atricapillus-like morphology of carolinensis populations there, as reflected in PC 1 scores, one might expect a similar trend to be seen in PC 3 scores. However, the opposite is true. Genetically carolinensis-like populations of the West Virginia transect were more distinct in PC 3 from atricapillus than were carolinensis-like populations of the Virginia transect.

The correlation of atricapillus ski introgression in West Virginia with atricapillus-like PC 1 scores there may reflect a cause-and-effect relationship. However, other evidence suggests that the apparent introgression at the ski locus does not represent genetic material governing

morphological development in these chickadees. Does then the limited genetic introgression exhibited by DPAC121 (which is sex-linked), Gda (which is also probably sex-linked), and the mitochondrial haplotype of these chickadees (which is maternally transmitted), reflect the overall picture of genetic interaction between atricapillus and carolinensis? Or does the extensive autosomal introgression seen at the ski locus more accurately portray the level of genetic exchange between these two taxa? With a more complete survey of the genome of atricapillus and carolinensis for differentiated molecular markers, we hope to gain a broader picture of the genetic exchange occurring between them, and to establish whether the substantial introgression observed at the autosomal marker ski is the exception or the rule in these chickadees.

Table 1. Sample size, collecting locality, distance along transect, U. S. National Museum catalog numbers, and year(s) collected for populations comprising the West Virginia and Virginia transects.

Population	No. birds[a]	Location[b]	Distance (km)	USNM No.[c]	Year(s) Collected
West Virginia transect					
PA[d]	14(13)	PA: Potter Co., 2.5 km S and 4.5 km E of Ole Bull State Park, PA, 41° 31'N, 77° 39'W.	0	600060-600077	1991
WV1[e]	13	WV: Pendleton and Tucker Co., 9 km S and 11 km W of Petersburg, WV, Monongahela N. F., 38° 54'N, 79° 15'W.	172.3[f]	600078-600094	1990
WV2	13	WV: Randolph Co., 2 km S and 3.5 km E of Belington, WV, Laurel Mtn., 38° 59'N, 79° 54'W.	227.7	600114-600131	1990
WV3	21	WV: Upshur Co., 3 km S and 9 km E of Buckhannon, WV, 38° 57'N, 80° 8'W.	245.0	600132-600162	1990 1992
WV4	11	WV: Upshur Co., 3 km S and 7.5 km W of Buckhannon, WV, Stonecoal Reservoir, 38° 57'N, 80° 20'W.	261.0	600212-600229	1990

Table 1 continued

WV5	11	WV: Lewis Co., 10 km S and 13 km W of Weston, WV, Butchers Fork R., 38° 56'N, 80° 37'W.	284.8	600230-600247	1990
OH	16	OH: Lawrence Co., 9 km S and 5 km E of Lawrence, OH, Wayne N. F., 38° 43'N, 82° 34'W.	417.2	597882-597900	1991
Virginia transect					
PA[d]	14	PA: Potter Co., 2.5 km S and 4.5 km E of Ole Bull State Park, PA, 41° 31'N, 77° 39'W.	0	600060-600077	1991
VA1[e]	13	WV: Pendleton and Tucker Co., 9 km S and 11 km W of Petersburg, WV, Monongahela N. F., 38° 54'N, 79° 15'W.	100.0[f]	600078-600094	1990
VA2	17(15)	VA: Shenandoah Co., 2.5 km N and 2 km E of Liberty Furnace, VA, Geo. Washington N. F., 38° 54'N, 78° 41'W.	153.6	600288-600319	1989 1991
VA3	17(12)	VA: Shenandoah Co., 1 km S and 3 km W of Woodstock, VA, 38° 52'N, 78° 33'W.	164.5	600320-600342	1989

Table 1 continued

VA4	14	VA: Shenandoah Co., 6 km E of Edinburg, VA, Geo. Washington N. F., 38° 50'W, 78° 30'W.	171.4	600267-600287	1989
VA5	14	VA: Rappahannock Co., 2 km S and 3.5 km E of Flint Hill, VA, 38° 45'N, 78° 3'W.	205.5	600095-600113	1990
VA	16(15)	VA: Charles City Co., 5.5 km N and 17.5 km W of Williamsburg, VA, Chickahominy WMA, 37° 20'N, 77° 51'W.	399.5	600039-600059	1991

Table 1 Continued

[a] Males only, with sample size for morphometric analyses in parentheses if different from genetic analyses because of incomplete morphometric data.

[b] Location is approximate center of area in which collection was made. Population diameters spanned from a few kilometers to a few tens of kilometers. Some distances along transects are adjusted because transects were not perpendicular to the hybrid zone interface, and reflect straight line distances to the nearest portion of the range interface as determined from Breeding Bird Atlas data.

[c] Catalog numbers for all individuals deposited at the U. S. National Museum of Natural History, including females and unsexed individuals.

[d] Serves as the atricapillus parental population for both transects.

Table 1 continued

[e] WV1 and VA1 represent the same population, serving as the second population at the atricapillus end of both transects.

f Linear distance between PA and WV1 corrected for the fact that PA is displaced from the east/west oriented West Virginia transect. It was estimated by measuring the distance from PA to the closest point of the range interface in southwestern Pennsylvania, and subtracting the distance between WV1 and the range interface in West Virginia. Same procedure used to correct the linear distance between PA and VA1, except that the closest point to the range interface in southeastern Pennsylvania was used.

TABLE 2. Sample size, and percentage of hybrids, F_1's and *atricapillus* alleles, based on five diagnostic genetic markers.

Population	No. birds	Percent hybrids	Percent potential F_1's[a]	Percent *atricapillus* alleles[b]
West Virginia transect				
PA	14	0	0	100.0
WV1	13	15.4	0	98.3
WV2	13	15.4	0	98.3
WV3	21	66.7	19.0	68.9
WV4	11	54.5	9.1	13.1
WV5	11	54.5	0	7.1
OH	17	41.2	0	4.6
Virginia transect				
PA	14	0	0	100.0
VA1	13	15.4	0	98.3
VA2	17	35.3	0	92.8
VA3	17	64.7	0	12.4
VA4	14	28.6	0	11.1
VA5	14	14.3	0	1.6
VA	16	0	0	0

TABLE 2 Continued

[a] Individuals heterozygous at all four diploid loci.

[b] Percentages of A alleles pooling across the five diagnostic markers for all males.

Table 3. Sample size, morphological measures, and the three principal components of populations comprising the West Virginia and Virginia transects. Values are x ± 1 SE.

Population	No. birds[a]	Mass (g)	Wing length (mm)	Tail length (mm)	Tail/Wing Ratio	PC1	PC2	PC3
West Virginia transect								
PA[b]	13	11.3 ± 0.2	66.0 ± 0.6	61.1 ± 0.6	0.927 ± 0.008	1.39 ± 0.31	0.30 ± 0.18	-0.20 ± 0.16
WV1[c]	13	11.2 ± 0.1	66.9 ± 0.4	62.7 ± 0.6	0.936 ± 0.007	1.62 ± 0.20	-0.44 ± 0.14	-0.18 ± 0.11
WV2	13	11.7 ± 0.2	66.6 ± 0.6	62.1 ± 0.7	0.933 ± 0.006	1.83 ± 0.31	0.27 ± 0.19	-0.18 ± 0.12
WV3	21	11.1 ± 0.1	65.8 ± 0.4	60.1 ± 0.7	0.913 ± 0.007	0.91 ± 0.22	-0.01 ± 0.13	-0.08 ± 0.09
WV4	11	10.6 ± 0.2	64.5 ± 0.6	56.9 ± 0.5	0.881 ± 0.008	-0.25 ± 0.28	-0.02 ± 0.27	0.09 ± 0.16
WV5	11	11.1 ± 0.1	65.0 ± 0.4	56.9 ± 0.5	0.875 ± 0.005	0.17 ± 0.21	0.37 ± 0.15	0.25 ± 0.07
OH	16	10.2 ± 0.1	64.4 ± 0.4	54.8 ± 0.4	0.852 ± 0.004	-0.90 ± 0.21	-0.27 ± 0.08	0.39 ± 0.09

Table 3 continued

Virginia transect								
PA[b]	13	11.3 ± 0.2	66.0 ± 0.6	61.1 ± 0.6	0.927 ± 0.008	1.39 ± 0.31	0.30 ± 0.18	-0.20 ± 0.16
VA1[c]	13	11.2 ± 0.1	66.9 ± 0.4	62.7 ± 0.6	0.936 ± 0.007	1.62 ± 0.20	-0.44 ± 0.14	-0.18 ± 0.11
VA2	15	11.1 ± 0.2	66.1 ± 0.4	60.7 ± 0.7	0.919 ± 0.009	1.06 ± 0.22	-0.18 ± 0.22	-0.10 ± 0.13
VA3	12	10.3 ± 0.1	63.0 ± 0.2	54.6 ± 0.5	0.866 ± 0.007	-1.25 ± 0.11	0.08 ± 0.17	0.00 ± 0.07
VA4	14	10.2 ± 0.2	63.1 ± 0.4	54.6 ± 0.7	0.865 ± 0.009	-1.30 ± 0.31	-0.04 ± 0.12	0.02 ± 0.11
VA4[d]	13	10.0 ± 0.1	62.8 ± 0.3	54.0 ± 0.5	0.861 ± 0.008	-1.58 ± 0.17	-0.10 ± 0.11	0.02 ± 0.12
VA5	14	10.4 ± 0.1	62.9 ± 0.3	54.1 ± 0.4	0.860 ± 0.005	-1.31 ± 0.17	0.26 ± 0.11	0.05 ± 0.09
VA	15	9.7 ± 0.2	62.1 ± 0.3	53.1 ± 0.3	0.855 ± 0.003	-2.08 ± 0.16	-0.17 ± 0.18	-0.04 ± 0.06

[a] Males only.

[b] Serves as the atricapillus parental population for both transects.

[c] WV1 and VA1 represent the same population.

[d] Omits one atricapillus individual from population.

TABLE 4. Eigenvectors generated by a principal component analysis of three morphometric variables for all individuals comprising the West Virginia and Virginia transects.

Character	PC 1	PC 2	PC 3
Mass	0.54	0.84	0.02
Wing Chord	0.59	-0.40	0.70
Tail Length	0.59	-0.37	-0.72
Eigenvalue	2.41	0.41	0.18
Variation Explained	.805	.135	.060

FIGURE LEGENDS

Fig. 1 Distribution of P. atricapillus and P. carolinensis in the Appalachian region, with locations of populations comprising the West Virginia and Virginia transects, including parental populations. Exact localities are given in Table 1.

Fig. 2. Population averages (± 1 SE) of principal component scores and number of atricapillus alleles for individuals in each population along linear series forming the West Virginia and Virginia transects. PA and WV1/VA1 each constitute part of both transects. PA appears once as the central atricapillus origin of each transect, while WV1/VA1 appears twice as the second population of both transects. Distance of PA to WV1/VA1 calculated separately for each transect as described in Table 1.

Fig. 3. Scatterplots of individual PC 1 and PC 3 scores for parental populations PA, VA and OH from a PCA of three morphometric variables (mass, wing length and tail length) using all males from the Virginia and West Virginia transects. In the left figure symbols indicate local populations; in the right, symbols denote genetic classification.

Fig. 4. Scatterplots of individual PC 1 and PC 3 scores for populations of the Virginia transect from a PCA of three morphometric variables (mass, wing length and tail length) using all males from the Virginia and West Virginia transects. In the left figure symbols indicate local populations; in the right, symbols denote genetic classification.

Fig. 5. Scatterplots of individual PC 1 and PC 3 scores for populations of the West Virginia transect from a PCA of three morphometric variables (mass, wing length and tail length) using all males from the Virginia and West Virginia transects. In the left figure symbols indicate local populations; in the right; symbols denote genetic classification.

Fig. 6. Plots of principal component scores and number of atricapillus alleles for individuals in WV3. Spearman rank correlation: PC 1, $r_s = 0.62$, $P < 0.005$, $n = 21$; PC 2, $r_s = -0.03$, $P > 0.25$, $n = 21$; PC 3, $r_s = -0.50$, $P = 0.01$, $n = 21$.

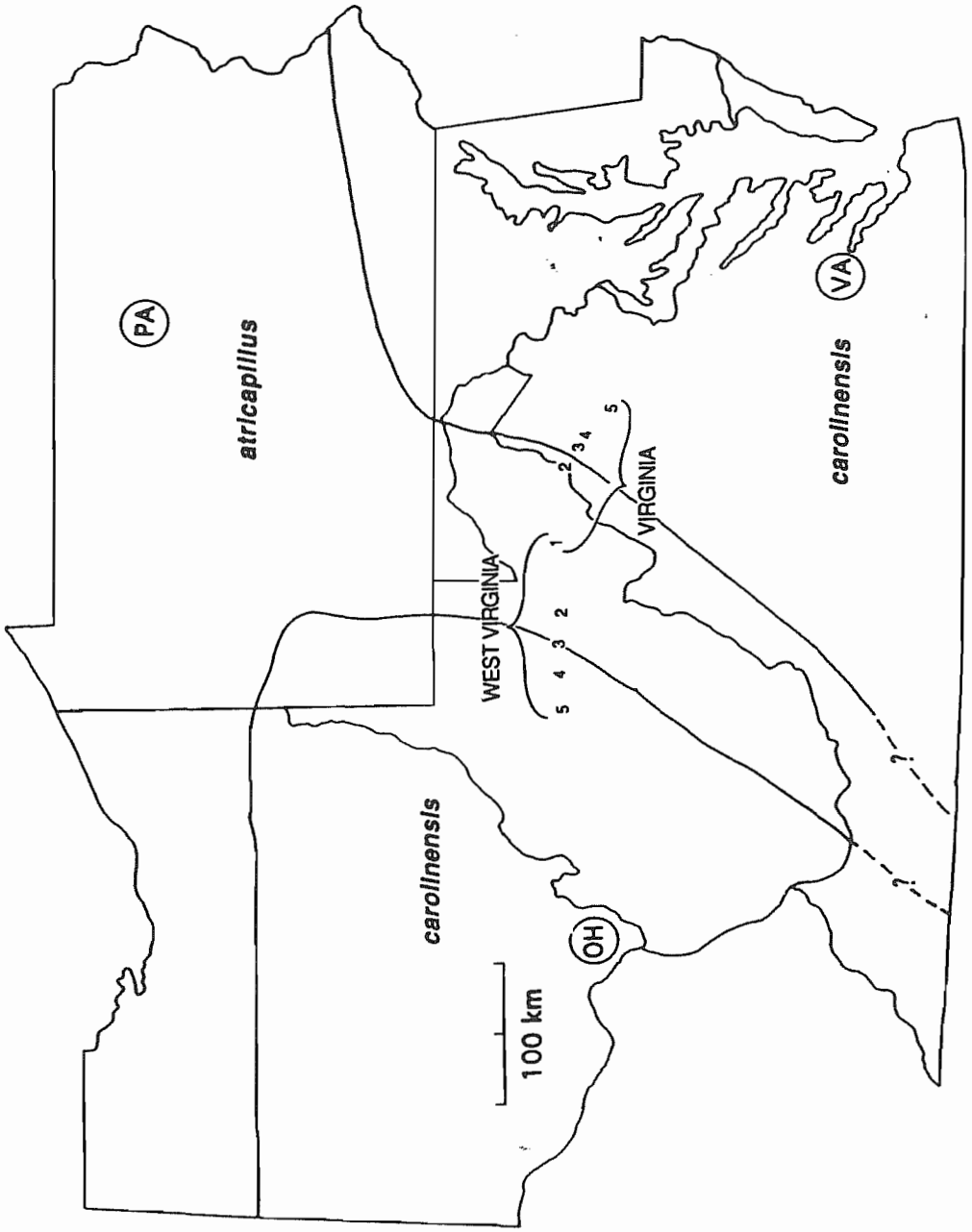
PA
atricapillus
WEST VIRGINIA
VIRGINIA
carolinensis
VA
carolinensis
OH
100 km
1
2
3
4
5
1
2
3
4
5

Figure 1

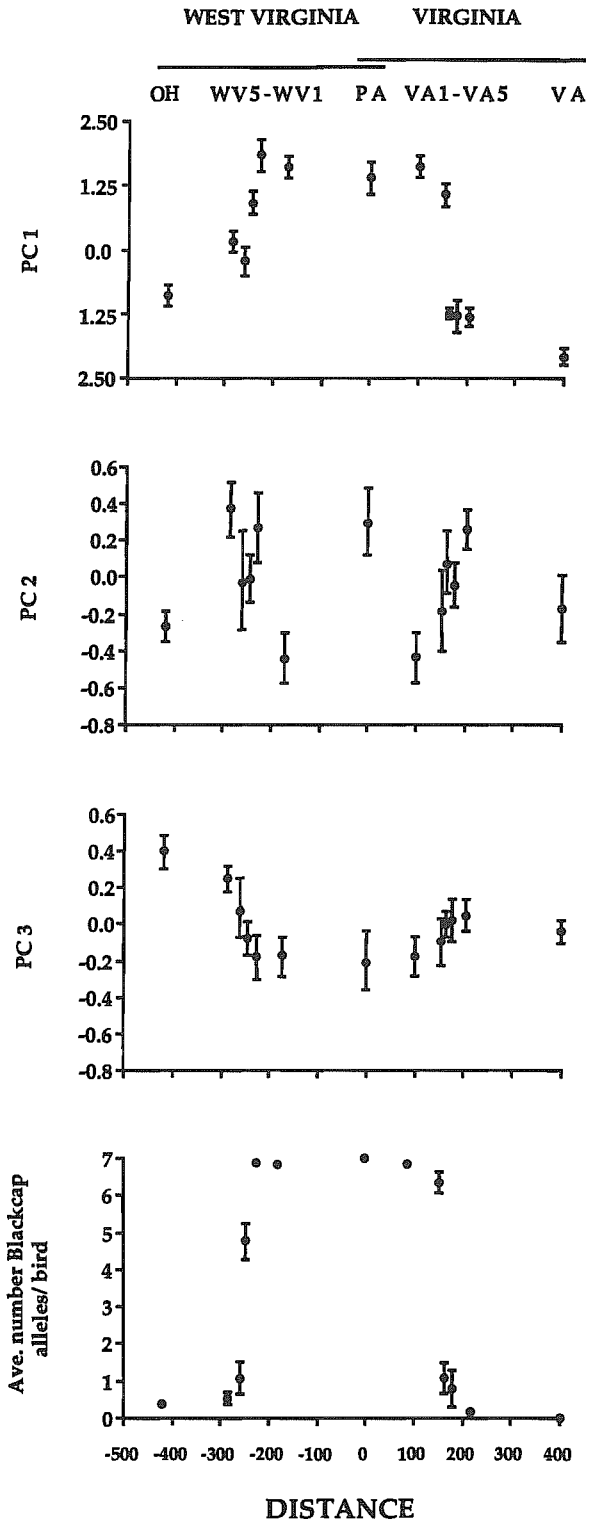
WEST VIRGINIA
VIRGINIA
OH
WV5-WV1
PA
VA1-VA5
VA
PC 1
2.50
1.25
0.0
1.25
2.50
PC 2
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
PC 3
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Ave. number Blackcap alleles/ bird
7
6
5
4
3
2
1
0
-500 -400 -300 -200 -100 0 100 200 300 400
DISTANCE

Figure 2

PARENTAL POPULATIONS

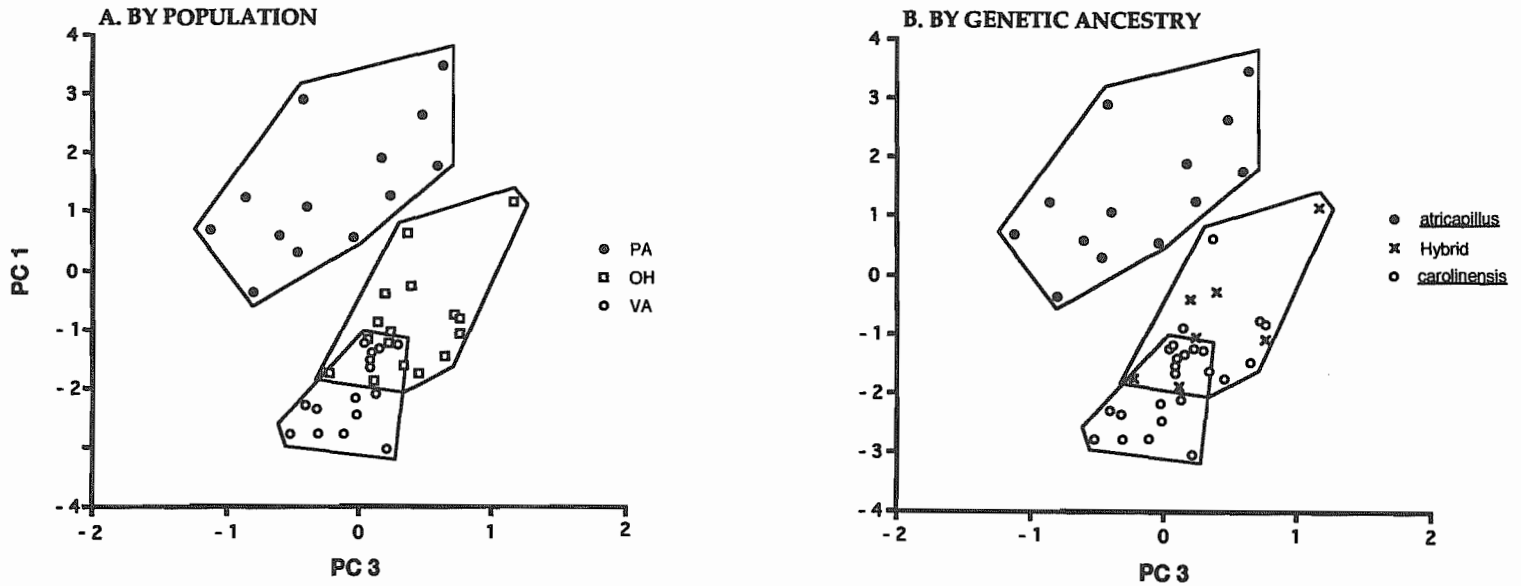

Figure 3

VIRGINIA TRANSECT

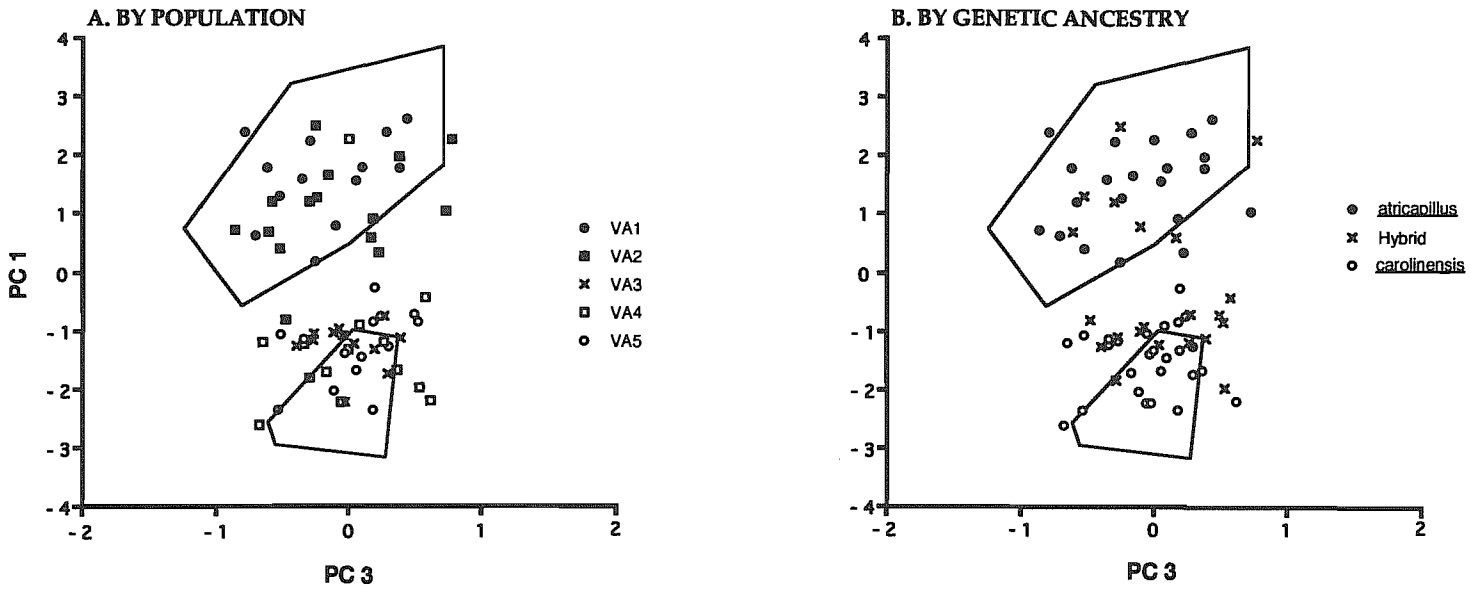

Figure 4

WEST VIRGINIA TRANSECT

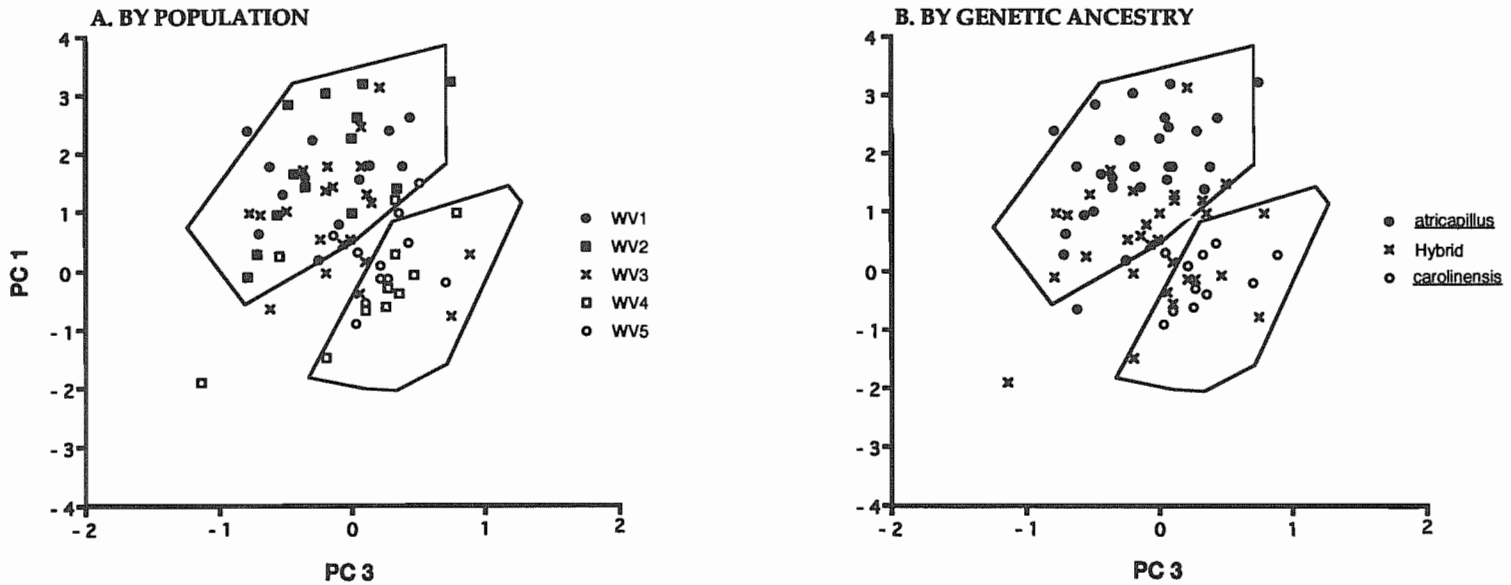

Figure 5

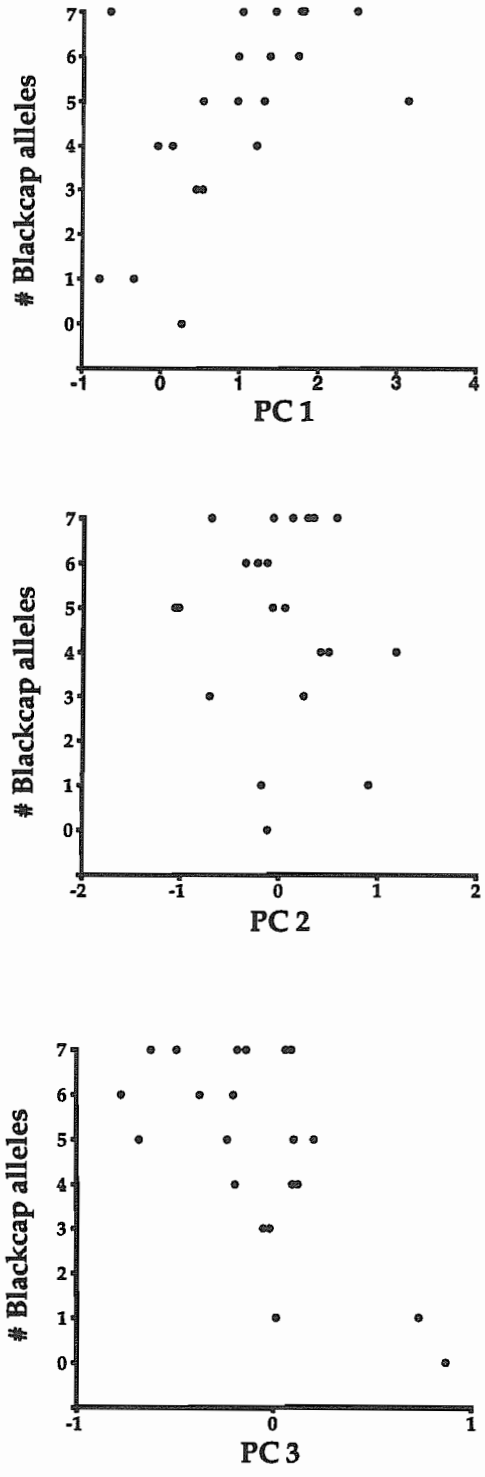
Blackcap alleles
PC 1
Blackcap alleles
PC 2
Blackcap alleles
PC 3

Figure 6

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3. AN ASSESSMENT OF MIXED SINGING AND ITS RELIABILITY AS AN INDICATOR OF GENETIC ANCESTRY IN BLACK-CAPPED AND CAROLINA CHICKADEES AT THEIR CONTACT ZONE IN THE APPALACHIAN MOUNTAINS

Introduction

Black-capped and Carolina chickadees (Parus atricapillus and P. carolinensis) meet and form a contact zone along their parapatric range boundary across the eastern half of North America (Brewer 1963, Rising 1968, Johnston 1971, Ward and Ward 1974). The two forms are morphologically similar, but sing different songs. Along their range interface, individuals can often sing both species' songs, and many songs are intermediate or abnormal in nature (Brewer 1963, Johnston 1971, Ward and Ward 1974, Simpson 1977, Robbins et al. 1986, Ballard 1988).

Birds some distance from the contact zone exhibit mensural and plumage differences that distinguish the two, and morphometric intermediacy is found at the range interface (Rising 1968, Robbins et al. 1986). This morphological intermediacy, in conjunction with mixed singing, suggests that hybridization commonly occurs along this range interface. At other locations along the atricapillus/carolinensis range boundary, such as portions of the Midwest and in the Smoky Mountains, mixed singing and

morphological intermediacy is minimal, suggesting that hybridization at these locations is rare or absent (Tanner 1952, Brewer 1963, Merritt 1978, 1981).

Vocal and morphological intermediacy might not accurately reflect levels of hybridization or introgression in this complex, however. Learning is an important component in the song ontogeny of both atricapillus and carolinensis (Shackleton and Ratcliffe 1993, Kroodsma et al. 1995), bringing into question the reliability of mixed singing as evidence of hybridization. And both mensural and plumage differences between atricapillus and carolinensis, while substantial at the extremes of their ranges, are less where the two meet, due to clinal variation and subspecies differences in each (Duvall 1945, Lunk 1952, James 1970).

Advances in molecular methods have provided new tools for studying patterns of variation in natural populations, offering the advantage of having an established genetic basis and the possibility of greater resolution. Protein electrophoresis was first used in the search for genetic markers in these chickadees, but only one diagnostic difference was identified (Braun and Robbins 1986, Gill et al. 1989). Surveys of restriction fragment length variation in these chickadees' DNA at both the mitochondrial and nuclear level revealed several additional diagnostic markers (Mack et al. 1986, Sawaya 1990) that now allow more

comprehensive investigations to be made of genetic interactions between atricapillus and carolinensis. An investigation of the atricapillus/ carolinensis contact zone in southwestern Missouri using these markers recently confirmed the presence of a high proportion of hybrids at the range interface, and suggested that genetic introgression between these chickadees is relatively limited at that location (Sawaya and Braun in prep.).

Using these genetic markers, we have undertaken a survey of genetic interactions between atricapillus and carolinensis in the Appalachian Mountains of Virginia and West Virginia. Here, we assess the nature of mixed singing between atricapillus and carolinensis, and its reliability as evidence for hybridization and introgression between these chickadees in the Appalachian Mountains.

Materials and Methods

Study sites and population samples.

Nine populations comprising two transects crossing the contact zone on the east and west side of the Appalachian Mountains were sampled in Virginia and West Virginia (Fig. 1, Table 5). Allopatric populations of carolinensis (VA, OH) were also collected on either side of the Appalachian Mountains as parental populations of that species. A population collected in the central Appalachians served as

the terminal atricapillus population for both transects. This population was within 60 km of the nearest carolinensis population, however, and could potentially experience gene flow from both sides of the Appalachian Mountains. Therefore an allopatric population of atricapillus (PA) was collected in north-central Pennsylvania as a parental population for this species. Locality information for each Appalachian population is provided in Chapter 2.

Data from these Appalachian transects were also compared with the song data of Robbins et al. (1986) for a transect crossing the contact zone in Missouri (Fig. 14). Populations referred to here as MO1-MO4, comprising the Missouri transect, correspond to Populations 1-4 respectively of Robbins et al. (1986). Birds from all transects were collected with shotguns, frozen within a few hours on either dry ice or in liquid nitrogen, and later transferred to a -80°C freezer. All collecting was done during the breeding season. Mated pairs of birds were collected when possible; all birds from which song was recorded were males, and only the songs of adult males were analyzed. The sex of each bird was confirmed by examination of gonads, and age was determined by examining skull pneumatization. Some juveniles were collected in three populations (N= 9, 12 and 9 in VA2, VA3 and VA4 respectively), and included in estimating the proportion of

hybrids. Specimens collected for the Appalachian transects were deposited at the U.S. National Museum of Natural History, and catalog numbers are provided in Table 1.

Song and playback analysis.

Birds were located by their calls and spontaneous song, or by their response to a playback tape. Prior to collection, the response of males to playback of both atricapillus and carolinensis song was noted, and their songs were tape recorded. In populations where atricapillus song predominated (Table 10), two min of carolinensis song was broadcast first and any response noted. We then waited two min if there was no response, or waited two min following the cessation of any song response. Two min of atricapillus song was then broadcast, and any response again noted. This order of song presentation was reversed in populations where carolinensis song predominated (Table 10). In WV3 where both species' songs were common, we alternated which species' song was broadcast first. Both atricapillus and carolinensis playback tapes were produced from the Peterson Field Guide to Eastern Bird Song (Peterson 1983) (Fig. 7).

Response to each broadcast was ranked on a scale from zero to two. A score of zero denoted no song response and no approach to the broadcast source. A score of one was given if a) a bird responded with song but did not approach,

b) did not respond with song but did approach, or c) responded with song and approached, but to no closer than 12 m. A score of two was given if a bird responded with song and approached to less than 12 m. Playback trials were sometimes initiated prior to the detection of chickadees in the vicinity, and always without knowledge of a given pair's territorial boundaries. These facts might lower the response score for trials in which we were outside a male's territorial bounds, but should not bias results for a given bird toward either conspecific or heterospecific response.

During and following playback experiments, we attempted to record representative samples of any song types a male sang using a Sony TCM-5000EV cassette recorder with a Sennheiser ME-80 shotgun microphone. Spectral analysis of songs was performed using Canary software (version 1.1) from Cornell Laboratory of Ornithology (Bioacoustics Research Program) run on a Macintosh Quadra 700 computer. A 176 Hz filter bandwidth setting was used in most cases to measure song parameters, unless greater resolution was needed in measuring note duration, in which case a 1400 Hz bandwidth setting was employed. Each song's waveform was also used to measure note duration. Following Robbins et al. (1986), eight measurements were taken from the spectrogram of each song: duration of the first note; duration of the second note; onset, midpoint, and offset frequency of the first

note; and onset, midpoint, and offset frequency of the second note.

Robbins et al. (1986) classified songs into song types based solely upon the number of notes in a song. While easy to implement operationally, this method sometimes results in lumping of rather distinct songs into a single song type when songs differ in frequency or patterning, but not in number of notes. As in many songbirds, these chickadees sing multiple renditions of one song type before switching to a bout incorporating a new song type (pers. obs.). We therefore recognized a song type as a group of songs that were unified by similarity in note frequency, duration, and syntax when compared with other songs from the same and other chickadees (Kroodsma 1982, Nowicki et al. 1994). In Appalachian populations, occasional songs deviating from one of the common carolinensis song types (see below) occurred interspersed in bouts of typical song. It appeared that these variants were formed by the addition or deletion of notes from the end of otherwise typical four note songs. We ignored such variants, analyzing the predominant four note songs unless such a variant was the predominant song of an individual.

We measured the eight song variables for up to five renditions of each song type a bird sang. A principal component analysis (PCA)(SAS/PROC PRINCOMP; version 6.04;

SAS 1987) was performed on the matrix of correlations among averages for the eight untransformed song variables, combining all 12 Appalachian populations in the analysis. A similar PCA was performed on the data of Robbins et al. (1986) using the present definition of song types, to allow comparison of the Missouri data with our Appalachian data.

A direct (standard) discriminant analysis using averages for all eight untransformed song variables was also performed on each of the Appalachian transects and on the Missouri transect (DISCRIMINANT; Norusis 1988). Each analysis yielded a discriminant function maximally separating the pair of parental populations for each transect. Unweighted discriminant coefficients for each variable were used to produce a discriminant score for every individual in the analysis.

DNA extraction and restriction fragment analysis.

DNA extraction and restriction fragment analysis followed the protocol outlined in Chapter 2. Three probes were used to detect restriction fragment length variants diagnostic for atricapillus and carolinensis (Sawaya 1990, Sawaya and Braun in prep.). The first was a 1.2 kb fragment of the chicken oncogene ski (Li et al. 1986) used to probe Eco RI digests. The second, designated DPAC121, was a 4.0 kb fragment of Tufted Titmouse (Parus bicolor) DNA used to probe Pst I digests. The third was Carolina Chickadee

mitochondrial DNA (mtDNA) purified by ultracentrifugation in a cesium chloride gradient and dialysed, following Dowling et al. (1990). Three restriction enzymes (Pst I, Pvu II and Ava II) were used to identify species-specific mitochondrial restriction fragment patterns. Some intraspecific polymorphisms in restriction fragment pattern occurred in both atricapillus and carolinensis for DPAC121 and mtDNA haplotypes, but all fragment patterns could be unambiguously assigned to one or the other species (Sattler and Braun in prep). DPAC121 is located on the sex chromosome (Z) in these chickadees, while ski is autosomal.

Isozyme electrophoresis.

Liver tissue was thawed and 0.05-0.2 g homogenized in deionized water with a pestle. Samples were centrifuged for two min and supernatant aliquoted and stored at -80°C until use. Cellulose acetate electrophoresis was performed on liver tissue homogenate following Sattler and Braun (in prep), and the plates stained for guanine deaminase (GDA) following methods of Richardson et al. (1986). GDA exhibits variation in these chickadees that is consistent with its being sex-linked; only male hybrids display a heterozygous pattern for this marker (Sawaya 1990, Sawaya and Braun in prep, Sattler and Braun in prep).

Results

Genetic analysis.

The four genetic markers used to identify hybrids were shown previously to be diagnostic for atricapillus and carolinensis (Sawaya 1990, Sawaya and Braun in prep.). Three of the markers (DPAC121, GDA and MtDNA haplotypes) were also fixed in our Appalachian transect parental populations, while ski was fixed in PA and VA but not in OH (see below).

Any individual was defined as a hybrid that possessed a mixture of atricapillus and carolinensis alleles for these four markers. Estimates of hybrid frequency are likely to be conservative, as individuals resulting from backcross or hybrid matings could potentially be classified as parentals because of our limited sample of their genome. Potential linkage of DPAC121 and GDA on the Z chromosome (see above) could result in non-independence of these markers, further increasing the chance of misclassifying later-generation hybrids as parentals. Hybrids made up well over 50% of some populations sampled at the range interface in both Virginia and West Virginia, compared with at least 44% found by Sawaya and Braun (in prep.) in Missouri (Table 5). The proportion of hybrids declined rapidly away from the range interface, except in genetically predominately carolinensis populations west of the Appalachians. Populations WV4, WV5

and OH retained a high proportion of hybrids (Table 5). All hybrids found at distances greater than 20 km from the contact zone, however, were identified as hybrids on the basis of only a single foreign allele, and always for the autosomal marker ski.

Song types.

All birds that responded to song playback and whose song we analyzed were males. The song of Parus atricapillus (song type "B") typically consists of a two note whistle (Fig. 7A), with the first note higher in frequency than the second note by several hundred Hz (Weisman et al. 1990). Frequencies of both notes are usually less than 4.3 kHz (Ward and Ward 1974), and audible frequency shifts are commonly heard in certain behavioral contexts in this species (Ratcliffe and Weisman 1985, Horn et al. 1992). The song of atricapillus is highly stereotypic throughout its range, with one and three note songs and other variants rarely reported away from the range interface with carolinensis (Ficken et al. 1978, Weisman et al. 1990). We heard no deviations from this song in our parental population (PA) of this species.

The song considered most typical of P. carolinensis consists of four whistled notes alternating high and low in frequency (HLHL), in which the first and third notes have frequencies above 6.0 kHz and the second and fourth notes

have frequencies below 4.2 kHz (Ward 1966). In the predominant carolinensis song of our samples, the frequency of notes one and three were similar to one another, while note four was lower than note two. Hereafter, we refer to this song type as "C" (Fig. 7B). It predominated in several populations (Fig. 8). P. carolinensis displays extensive individual and geographic variation in its song, however (Ward 1966), and we recognized a number of carolinensis song variants other than the "C" song type in our samples.

Song type "D" differed from song type "C" in that notes two and four were of comparable frequencies (Fig. 7C). This song type was detected in the majority of populations containing song type "C" (Fig. 8). In song type "E" the frequency of note one was significantly lower than note three, and was in the frequency range typical of notes two and four (Fig. 7D). Like song type "D", song type "E" was heard in a majority of populations containing song type "C" (Fig. 8).

Three other song types designated as carolinensis variants had distributions limited to only one or two of our populations. Song type "F" was found only in parental population VA and in VA5. It was characterized by the presence of two low frequency notes following note one and sometimes note three (HLLHL or HLLHLL). The first of these paired low frequency notes was always much shorter than the

second, and of the same frequency as the note it preceded (Fig. 7E; see also Lohr and Nowicki 1991, Fig. 1). Song type "G" was found only in WV4, and was similar to song type "F", except that the abbreviated note(s) following note one and often note three was at a slightly higher frequency than the note it preceded (Fig. 7F). In song type "F" there was often not a clear break on the spectrogram between the abbreviated note(s) and the following note, although some type of break or attenuation was clearly audible to us. This made it somewhat arbitrary to designate the abbreviated note as an additional note in the song. For song type "F" we therefore included the abbreviated syllable as part of the note it preceded in our measurements. In song type "G" on the other hand, the abbreviated notes were distinct and appeared to be "extra" notes inserted into song type "C". These notes did not appear to be homologous to the corresponding second note in the song of atricapillus to which comparisons were being made, so we ignored them and used the second full note in song type "G" in our analysis.

Finally, song type "H" was found in VA5, MO3 and MO4. Several variants actually comprised this song type (e.g. Fig. 7G), all characterized by a departure from the HL pitch alternation typical of the "C" song type. The three primary variants encountered can be represented as HHLL, HLLH, and LLHL, with additional variation on these patterns produced

by the frequent inclusion of additional notes at the end of a song. As in all song types, only notes one and two of song type "H" were analyzed in spite of the deviation from the normal HLHL frequency alternation of carolinensis song.

Song types "C" through "H" have been reported in other carolinensis populations distant from the range interface with atricapillus (Ward 1966, Crock 1975), and do not appear to be attributable to the influence of contact with atricapillus. Three additional song types were only encountered at the range boundary of atricapillus and carolinensis, and were not easily attributable to either species. Song type "X" resembled the song of atricapillus, but with note two repeated one or more times (Fig. 7H). This song type was encountered uncommonly at both the Virginia and West Virginia range interface (Fig. 8), but has been encountered more commonly at the contact zone in southeastern Pennsylvania (Ward and Ward 1974) and in southern Virginia (Sattler and Braun unpubl. data). Song type "Y" resembled two renditions of atricapillus song sung consecutively (Fig. 7I). Only one example was heard in the Appalachian transects, but it also occurred in the Missouri contact zone population MO3 (Fig. 8). Finally, song type "Z" resembled song type "D" with note three deleted (Fig. 7J). Song type "Z" thus bore some resemblance to carolinensis song, but in frequency alternation pattern

(HLL) it resembled song type "X". Only one bird was heard to sing this song type (Fig. 8).

Quantitative analysis of song.

P. atricapillus and P. carolinensis song in parental populations PA, OH and VA was distinct in each of the eight variables measured, with the first two notes of carolinensis song having a shorter duration and higher frequency than in atricapillus (Table 6). Almost all songs analyzed from Appalachian populations sounded to our ears like typical songs attributable to either atricapillus or carolinensis, and the bulk of population averages for the eight song variables were within the expected range for one of the two species (Table 6).

A principal component analysis confirmed the impression that intermediate songs were rare in our sample. Songs sorted into two clusters well resolved along the PC 1 axis, with only a few songs intermediate (Fig. 9A-D). PC 1 and PC 2 together explained 90.1% of the total variation. PC 1 was positively correlated with frequency variables and negatively correlated with duration variables, while PC 2 was positively correlated with duration of notes one and two and frequency of note two, and weakly negatively correlated with frequency of note one (Table 7).

In our PCA, song type "E" appears intermediate or atricapillus-like (Fig. 9A, C, E), but this could have

resulted from analysis of only the first two notes, both of which were of low frequency. Only a few other songs fell well between the two main song clusters, and these were of song type "D" with low frequency values for notes 1 and 3 (Fig. 9C).

Birds that sang both atricapillus-like and carolinensis-like songs were rare in the Virginia transect (two birds from VA2; Fig. 9B), but more common in the West Virginia transect (eight from WV3, one from WV4; Fig. 9D). Songs of such bilingual birds fell within the two main clusters, indicating that these songs were accurate renditions in terms of the frequency and duration characteristics of the first two notes. The apparent rarity of bilingual singers at the range interface of the Virginia transect in our original sample (1989-1990) was confirmed by a more intensive survey of songs in this area in 1994 and 1995. Bilingual singers (one additional bird) and the geographical mixing of atricapillus and carolinensis song were confined to a narrow region 1-3 km wide on the flank of Little North Mountain where it meets the Shenandoah Valley (Fig. 10). In contrast, at the range interface along the West Virginia transect, bilingual singers and the mixing of birds singing either atricapillus or carolinensis song occurred over an area at least 8.6 km wide (Fig. 11). This is probably an underestimate, as we did not determine the western limit of

this area of song mixing.

An individual collected at 680 m on Massanutten Mountain above the Shenandoah Valley was one of two birds from VA4 heard singing atricapillus song in an area where carolinensis song predominated (Fig. 10). This bird was genetically characterized as an atricapillus, and its song fell within the cluster of atricapillus songs (Fig. 9B). Other reports of atricapillus-singing individuals have been made during the breeding season at the highest elevations of the Blue Ridge Mountains east of the Shenandoah Valley, approximately 25 km SSE of VA4 (Stevens 1965, Abbott 1986).

The finding that vocal intermediacy at these two Appalachian transects of the atricapillus/carolinensis hybrid zone is reflected primarily in bilingual singing by some birds and not in intermediacy of individual songs contrasts with the findings of Robbins et al. (1986). They not only encountered bilingual singing, but their discriminant analysis indicated that 37% of songs from the hybrid zone in Missouri were intermediate in duration and frequency characteristics of the first two notes. To address this apparent contradiction, we performed a PCA on their data according to the criteria used for our Appalachian data. Songs were reclassified according to the song type definition used here. Songs that varied from one of the standard song types ("B" through "H") only in the

number of notes were excluded from analysis. The only exception to this in all analyses were cases in which these variants were the only songs recorded in the individual's repertoire, in which case they were included. We also extended the discriminant analysis used by Robbins et al. (1986) to our Appalachian data.

Univariate measures for each major song type from Missouri are given in Table 8. Values for atricapillus and carolinensis parental population song are comparable to our Appalachian parental populations (Table 6) except that note duration is longer for atricapillus in Missouri. This could be due both to technical differences in spectrographic equipment used and to observer differences in judging the termination point of notes in spectrograms.

A PCA of the Missouri data produced eigenvalues and component loadings comparable to the analysis of Appalachian populations (Table 7). A larger degree of intermediacy is evident from a scatterplot of PC 1 and PC 2 scores in Missouri than was present in either Appalachian transect (Fig. 9E, F). Part of this is due to the larger number of birds (nine) singing "E" song types in the Missouri transect analysis relative to either the Virginia or West Virginia transects (five and four birds respectively). When "E" song types are discounted, however, there is still a higher proportion of songs in Missouri falling between atricapillus

and carolinensis parental populations. Including the ten songs in the PCA that were deleted from the Missouri data set because they varied from the recognized song types in number of notes did not affect the degree of song intermediacy appreciably; only one additional song of obviously intermediate nature was revealed (data not shown).

Discriminant analyses were also performed on each Appalachian transect, and on the Missouri transect data reclassified by song type with variants in number of notes deleted (Table 9, Fig. 12). For the Missouri data, MO1 and MO2 combined served as the atricapillus reference population, while MO4 served as the carolinensis reference population. For all three analyses, "E" song types were removed because of the potentially artifactual nature of their intermediacy resulting from the analysis of only notes one and two. In addition, one "H" song type (LLLH) was removed from the MO4 reference population for this analysis. The discriminant analysis showed a smaller degree of separation of the two species' songs and more intermediacy in Missouri (Fig. 12E, F) relative to the Appalachians (Fig. 12A-D). Only 6% and 10% of songs in the Virginia and West Virginia transects respectively had discriminant scores intermediate between those of atricapillus and carolinensis parental populations, in contrast to 28.6% of songs in MO3. To investigate whether this increased intermediacy might be

due to the proximity of the Missouri reference populations to the contact zone, we included them in a PCA with our three Appalachian parental populations. Both atricapillus and carolinensis reference populations for Missouri clustered with the respective Appalachian parental populations, showing no evidence of intermediacy (data not shown).

The relationship of an individual's genetic ancestry and their song was assessed for range interface populations with Spearman's rank correlation tests. Each bird's ancestry was quantified according to the number of atricapillus alleles they possessed for the four diagnostic markers. "E" song types were removed to avoid a possible artifactual bias against a correlation that could arise if this song type is a carolinensis one that looks atricapillus-like in our PCA because only notes one and two were analyzed. The correlation of an individual's PC 1 score for song with their genetic ancestry was significant only in WV3 (r_s = -0.36; one-tailed P=0.025; n = 30), and even here, PC 1 score was poor predictor of the singer's genetic ancestry (Fig. 13).

Playback analysis.

Responsiveness of birds to playback of both species' songs was nearly absent in allopatric populations VA and VA5, as expected (Table 10). Also expected was the fact

that dual responsiveness was high in WV3 and VA2/VA3 where both atricapillus and carolinensis song was present at the range interface. Surprisingly, dual responsiveness was also high outside the contact zone in carolinensis populations of the West Virginia transect (WV4, WV5, OH), and also relatively high in Appalachian atricapillus populations VA1/WV1 and WV2, and in atricapillus parental population PA. The representative carolinensis song in our original playback experiments and in those of Robbins et al. (1986), taken from the Peterson Field Guide to Eastern Bird Songs (Peterson 1983), was of song type "E". It seemed plausible that apparent heterospecific responsiveness of atricapillus populations was due to the structural similarity that the first two notes of song type "E" bear to typical atricapillus song (song type "B"). To test this possibility, playback analysis was repeated in parental population PA in 1995 using song type "C". Dual responsiveness was again strong, with an average response score of 1.3 to playback of "C" song type, compared with 1.6 in response to playback of atricapillus song (Table 10).

Discussion

Hybridization and introgression.

The high proportion of hybrids identified genetically at the range interface in both Virginia and West Virginia is

consistent with a genetic analysis by Sawaya and Braun (in prep.) of the contact zone in southwestern Missouri, where at least 44% of their sample at the range interface were of hybrid ancestry. It also agrees with the conclusion that hybridization is commonplace along much of the atricapillus/carolinensis range interface, based on observations of mixed singing and morphological intermediacy there (Brewer 1963, Rising 1968, Johnston 1971, Ward and Ward 1974, Robbins et al. 1986, Ballard 1988).

Significant introgression across the hybrid zone was detected, with a small proportion of hybrids found in the center of the Appalachian Mountains in VA1/WV1, and a relatively high proportion present in WV4, WV5, and parental population OH. Introgression appeared more limited on the east side of the Appalachian Mountains and in the more northerly Appalachians, with no hybrids detected in either parental population VA or PA. Issues pertaining to patterns of hybridization and introgression are taken up in greater detail in Chapter 4.

Nature and extent of mixed singing.

Two types of "mixed singing" by songbirds occur, in which individuals show the influence of another species in its song (Helb et al. 1985). The first is duality of song, in which individuals are fluently bilingual, and the second is intermediacy of individual songs. Both bilingual singing

and song intermediacy have been reported in these chickadees, and have also been documented together in a variety of other avian hybrid zones (Baptista 1977, Gelter 1987, Lille 1988, Martens and Nazarenko 1993, Martens et al. 1994).

Bilinguality.- We found bilingual singing to be common among individuals in WV3 in contrast to its limited extent at the interface of populations VA2 and VA3. Ward and Ward (1974), Robbins et al. (1986) and Ballard 1988) also reported frequent bilingual individuals at the range interface in southeastern Pennsylvania, southwestern Missouri, and southwestern Virginia respectively. Given the demonstration that both atricapillus and carolinensis can learn most if not all elements of the other's song (Kroodsma et al. 1995), it seems probable that bilingual singers will be present wherever there is sufficient contact between the two for juveniles to hear both songs during song development (but see below).

In a parallel case, while two subspecies of the Willow Tit (P. montanus montanus and P. m. salicarius) were until recently known to show bilinguality only occasionally at their parapatric range interface, Martens and Nazarenko (1993) have reported that montanus exhibits a bivalent repertoire of both song forms throughout much of its range in Asia. Nestlings of both subspecies raised in acoustical

isolation also have a tendency to develop both song forms (G. Heckershopp unpubl. data).

Song intermediacy.-Previous studies have recognized the difficulty of evaluating vocal intermediacy at the atricapillus/carolinensis range interface because of the extensive variation present in the song of carolinensis across its range (Ward 1966, Ward and Ward 1974). Caution must therefore be exercised in attributing song variation in the contact zone to the influence of atricapillus until normal variation in the song of carolinensis has been studied more extensively in allopatry. Our studies were focused on evaluating genetic interactions between these species, so our observations on carolinensis song in allopatry and sympatry with atricapillus are necessarily incomplete. In addition, our analyses have looked at only a subset of the song variables that might be examined. Other components of song might show intermediacy as well. Nonetheless, some insights are provided.

While intermediacy of song has commonly been noted between atricapillus and carolinensis (Brewer 1963, Ward and Ward 1974, Robbins et al. 1986), we found minimal evidence for it at these two Appalachian locations on the basis of the analysis of frequency and duration variables of the first two notes. We looked for evidence of such intermediacy both in the form of species-specific song types

that showed evidence of intermediacy, and in the form of unique song types not found outside the contact zone that mixed elements of atricapillus and carolinensis song.

Song types recognized as characteristic of atricapillus ("B") and carolinensis ("C" through "H") provided little evidence of song intermediacy, showing minimal intermediacy in either frequency or duration variables. A few individuals of the West Virginia transect sang "D" songs that were intermediate in frequency of notes 1 and 3 (Fig. 9C). However the majority of Appalachian songs were clearly species-typical, including songs of bilingual singers.

Three apparently unique song types ("X", "Y", and "Z") were recognized at these Appalachian contact locations, but each was rare. In contrast, song type "X" is common at the contact zone in southeastern Pennsylvania (Ward and Ward 1974), and both "X" and "Z" song types are common at another contact zone site we are studying in east-central Virginia (Sattler and Braun unpubl. data).

Song type "E".-While we have treated song type "E" as a song characteristic of carolinensis in our discussion thus far, it is in fact intermediate or atricapillus-like in the duration and frequency of its first two notes, as demonstrated by the PCA. It also occurred in the Missouri sample of Robbins et al. (1986) at a higher frequency in the contact zone (MO3) than outside it (MO4). However the

apparent intermediacy of song type "E" can also be interpreted as being due to an uncommon syntactical arrangement of the notes of carolinensis song (LLHL), and to the restriction of our analysis to the first two notes of a song. Thus, this song type might represent a variant carolinensis song not influenced by atricapillus.

Song type "E" has been recorded at two locations in southwestern Ohio, 120 km and 200 km south of the contact zone (S. Gaunt pers. comm. and Peterson 1983 respectively). However, these carolinensis populations may have experienced some influence from atricapillus, given the high frequency of atricapillus ski alleles we found in parental population OH, which lies 250 km south of the range interface. Furthermore, there is anecdotal evidence that the hybrid zone in Ohio has moved northward in this century (Wheaton 1882). And Smith (1972) noted song type "E" in Kansas, but only 40 km south of the contact zone. On the other hand, one of our populations using this song type was our carolinensis parental population VA. We found no evidence of genetic introgression from atricapillus here, and contact with that species during periodic winter irruptions of atricapillus southward is rare or absent at this location (Virginia Society of Ornithology 1987). Resolving the question of whether song type "E" represents intermediacy in song between atricapillus and carolinensis would be aided by

a better understanding of the distribution of this song type within the range of carolinensis.

Geographic variation in song intermediacy.-Some song types showed more intermediacy between these chickadees in the Missouri contact zone than they did in the Appalachian contact zones. Robbins et al. (1986) found that 37% of songs in MO3 had intermediate scores in their discriminant analysis. Nearly 30% still had intermediate discriminant scores after we redefined song types in a way compatible with our Appalachian analysis, and discounted the "E" song type, which might provide an artifactual picture of song intermediacy. This compares with only 6% and 10% for the Virginia and West Virginia interfaces respectively. The PCA analysis likewise reflected greater intermediacy in Missouri relative to the Appalachians. Ecological, temporal and genetic factors are all plausible explanations for these differences, given the latitudinal versus altitudinal nature of the Missouri transect, its distance from the Appalachian transects and thus potential for differences in age of contact between atricapillus and carolinensis, and the genetic differences at the mtDNA level known to exist within carolinensis between these two areas (Gill et al. 1989, Sawaya 1990).

Differences were also noted between our two Appalachian transects in the extent of song intermediacy present. While

we found intermediate songs to be equally rare at both Appalachian transect interfaces, bilingual singing and the co-occurrence of atricapillus and carolinensis song spanned only 1-3 km at the Virginia contact zone, while the region of bilingual and mixed song spanned a minimum of 8.6 km in West Virginia. Other studies of this contact zone have established vocal admixture to span from 8-32 km (Brewer 1963, Ward and Ward 1974, Robbins et al. 1986). We interpret the limited extent of vocal mixing at the Virginia interface as resulting from ecological factors influencing the distribution of these chickadees. The elevational transition at the Virginia transect where the Appalachian Mountains meet the Shenandoah Valley is abrupt, producing a tight interface where the two species meet (Fig. 11). In contrast, considerable interdigitation of ridge and lowland occurs at the range interface in West Virginia (Fig. 10). Ecological transitions are also not great along other portions of this contact zone where vocal intermediacy is relatively broad, such as in Missouri and southeastern Pennsylvania. These conditions appear to result in a broader zone of mixing between atricapillus and carolinensis, leading to more extensive bilingual singing at the contact zone. These results suggest that caution should be exercised in interpreting reported cases of reproductive isolation between these species on the basis of vocal

behavior. Narrow gaps where no chickadees breed have been reported at the range interface both latitudinally from central Illinois to Ohio (Brewer 1963, Merritt 1981) and elevationally in the Smoky Mountains (Tanner 1952, Tove 1980). Partly on the basis of limited vocal intermediacy, hybridization has been thought to be rare or absent at these locations. However our vocal and genetic data, especially from the Virginia transect, show that extensive hybridization and introgression can exist in spite of limited vocal intermediacy.

Reliability of vocal intermediacy as an indicator of hybridization.-While obvious intergrade song types such as "X", "Y", and "Z" are common at some Appalachian contact zone locations not analyzed here, and bilingual singing appears to occur at most locations where hybridization is present, we conclude that song is an unreliable criterion to use in identifying atricapillus and carolinensis at their contact zone. A high proportion of hybrids were present at the range interface of both Appalachian transects, yet few songs in these populations showed intermediacy. In addition, correlation between a bird's PC 1 score for song and its number of atricapillus alleles for the diagnostic genetic markers scored was significant only in WV3, and this association was not strong. Numerous individuals were very carolinensis-like in ancestry and yet sang "normal"

atricapillus songs, and vice versa (Fig. 13). Finally, genetic introgression in both species extended far beyond the limits of any intermediate songs or bilingual singing.

The use of song playback to quantify responsiveness to conspecific and heterospecific song could potentially bias observation away from the detection of songs showing intermediacy if song matching to the playback tape occurred (Krebs et al. 1981). We think song matching is unlikely to have had a major effect on the level of song intermediacy we detected, and would not alter our conclusion that song is an unreliable genetic marker at these species' contact zone. As noted earlier, we detected a high proportion of "X" and "Z" intergrade song types at another portion of the atricapillus/carolinensis Appalachian contact zone in west-central Virginia (Sattler and Braun unpubl. data). We used song playback at that location in the same manner as described for these two Appalachian transects, so these differing levels of song intermediacy do not appear attributable to song matching. In addition, a large proportion of songs were also recorded and more heard that were sung spontaneously, yet our song analysis detected virtually no songs in either Appalachian transect showing intermediacy, and our impression of songs heard but not analyzed was the same. Thus, any effect of the playback on song intermediacy is likely to have been subtle, and not

alter the conclusion that songs poorly reflected the hybrid ancestry of many birds at the range interface.

Song has been found to be an unreliable marker of hybridization in other songbird hybrid zones (Ficken and Ficken 1967, Gill and Murray 1972, Morrison and Hardy 1983, Gelter 1987, Lein and Corbin 1990), as well as in zones of contact where two bird species meet and song intermediacy occurs but plumage traits indicate that hybridization is rare (Emlen et al. 1975, Sorjonen 1986). In such cases, a typical species' song may be transmitted by learning in spite of genetic hybridization, or mixed singing may develop as a result of learning in spite of a lack of genetic intermediacy. This non-genetic component of song contrasts with the presumed polygenic basis for most plumage differences. Learning has been found to be important in the development of song in all oscine songbirds that have been studied (reviewed by Kroodsma and Baylis 1982).

Evidence exists for vocal learning in several members of the genus Parus (reviewed by Kroodsma and Baylis 1982). More significantly, atricapillus nestlings tutored with a tape of carolinensis song learned most elements of the heterospecific song, and carolinensis nestlings developed songs nearly identical to an atricapillus tutor tape (Kroodsma et al. 1995). Thus, bilingual singing and intermediate songs present at the range interface between

atricapillus and carolinensis can be explained as the result of nestlings and/or fledglings being exposed to both species' song during their song development period.

Dual responsiveness.

Males in populations within the contact zone consistently responded to song playback of both atricapillus and carolinensis song regardless of their own song repertoire, while allopatric populations of carolinensis in Virginia (VA, VA5) showed little or no heterospecific responsiveness. Both observations are in accordance with previous results (Ward and Ward 1974, Robbins et al. 1986, Ballard 1988). However, allopatric populations of carolinensis in the West Virginia transect (OH, WV5) and allopatric populations of atricapillus (PA, VA1/WV1) showed a strong tendency to respond to the other species' song in addition to their own. S. Gaunt (pers. comm.) has also noted a heterospecific response to song playback by carolinensis in southern Ohio, and by atricapillus in Michigan. Some of these populations are hundreds of kilometers away from the contact zone. There are several possible explanations for this result.

One is the presence of genetic introgression among some of these populations. WV5 and carolinensis parental population OH both had a high proportion of atricapillus alleles at one of our diagnostic loci (ski). Levels of genetic introgression from atricapillus were low in MO4

where Robbins et al. (1986) found no responsiveness to atricapillus song (Sawaya and Braun in prep.), consistent with this hypothesis. However parental population PA shows no evidence of introgression from carolinensis despite strong dual responsiveness. Also, both hybrids and individuals classed as pure atricapillus or carolinensis at the Appalachian and Missouri range interfaces showed dual responsiveness, further weakening a genetic explanation. The current analysis shows introgression at only a limited portion of the genome, and evidence to date shows learning to play a prominent role in song responsiveness, suggesting that an alternative explanation is needed.

A second potential explanation for dual responsiveness in allopatric populations is prior experience of individuals with both songs. Such prior experience with a competitor appears capable of inducing a heterospecific song response (Emlen et al. 1975, Catchpole and Leisler 1986, Prescott 1987). Periodic winter irruptions occur in atricapillus as birds move south into the range of carolinensis temporarily (Lawrence 1958, Bagg 1969). These invasions take atricapillus as far as southern Ohio (Peterjohn 1989), and because song in both atricapillus and carolinensis occurs throughout the year (Dixon and Stefanski 1970, Smith 1972), these invasions provide an opportunity for exposure of both species to the other's song. However, this explanation

would seem to require that a high proportion of individuals in PA, VA1/WV1 and OH have engaged in or been exposed to such winter irruptions of atricapillus into carolinensis territory. In addition, while we found a high level of dual responsiveness in OH, 250 km south of the range interface in Ohio, Ward and Ward (1974) found no dual responsiveness in a carolinensis population just 32 km south of the contact zone in southeastern Pennsylvania. Another possible factor that could have provided carolinensis individuals in Ohio with prior exposure to atricapillus is the hybrid zone's movement northward in Ohio within historical times (Wheaton 1882). Earliest available records place the two chickadees in contact in central Ohio in the mid 1800's, raising the possibility that the contact zone was positioned in southern Ohio in the recent past.

Finally, we considered the possibility that atricapillus and carolinensis might show heterospecific responsiveness in allopatry because their songs are sufficiently similar to release an aggressive response in both. Several cases of such "mistaken identity" have been proposed in which heterospecific responsiveness is viewed as nonadaptive (Gill and Murray 1972, Morrison 1982, Nuechterlein 1981, Lynch and Baker 1990). Such cases are supported by the fact that, in sympatry, discrimination improves, presumably as experience with heterospecific song increases. In the present case,

however, discrimination does not improve where atricapillus and carolinensis are sympatric; heterospecific response actually increases. Further, this explanation for dual responsiveness requires that geographic variation exist in the factors promoting mistaken identity, as dual responsiveness did not occur in VA and VA5.

One factor that might promote dual responsiveness in atricapillus populations through mistaken identity was the fact that, on the test tape used for our original playback experiments, carolinensis was represented by song type "E". As discussed above, notes 1 and 2 of song type "E" resemble atricapillus song, and might elicit an unusually high rate of responsiveness in atricapillus. The frequency change between notes 1 and 2 has been reported to be constant in atricapillus relative to the absolute frequency of notes (Weisman et al. 1990), and much more variable in carolinensis (Lohr and Nowicki 1991). This frequency ratio might be important to atricapillus in species recognition, producing a response to song type "E" as a result of mistaken identity. However a second set of playback trials in population PA using typical carolinensis song (type "C") confirmed a strong heterospecific response here. Many other factors can potentially influence the results of playback experiments conducted in the field, and more work is needed to resolve the significance of differences found in

heterospecific responsiveness among allopatric populations of both atricapillus and carolinensis.

Could this dual responsiveness between atricapillus and carolinensis in sympatry be a factor promoting hybridization and genetic introgression between them? Brewer (1961) believed that song was an important cue preceding copulation in carolinensis. However Smith (1972) and Ficken et al. (1978) observed song in P. atricapillus and P. carolinensis to be atypical of many passerines in seeming to play an insignificant role in mate choice. Females may play a deciding role in mate choice among many passerines, and in some instances use a number of cues other than song in choosing a mate (Baker and Baker 1990). So the occurrence of dual responsiveness by males may have little significance to the question of reproductive isolation between these chickadees.

Many factors can play a role in promoting or limiting introgression between hybridizing species. Post-mating reproductive barriers have the potential to limit rates of gene flow even in the absence of pre-mating reproductive barriers. The number of genetic differences between two hybridizing taxa and the interaction of these differences can play an important role in determining levels and patterns of genetic introgression (Barton and Hewitt 1983, 1989). The availability of the diagnostic genetic markers

employed here, in addition to others we are developing, hold the promise of providing some of the first detailed information on both the fitness of various genetic classes of avian hybrids in nature, and levels of genetic introgression between these chickadees.

Table 5. Sample size, year (s) collected, distance from range interface, and percentage of hybrids for populations comprising three transects crossing the atricapillus/carolinensis hybrid zone.

Population	No. birds[a]	Year(s)	Distance (km)[b]	% Hybrids[a]
Missouri transect[c]				
MO	20 (19)	1978	-260.0	0 (0)
MO1	17 (9)	1980	-41.0	0 (0)
MO2	14 (8)	1980	-8.5	28.6 (12.5)
MO3	36 (25)	1978, 1980	0	44.4 (56.0)
MO4	21 (11)	1980	37.0	4.8 (0)
LA	21 (12)	1979	950.0	0 (0)
West Virginia transect				
PA	20 (14)	1991	-245.0[d]	0 (0)
WV1[e]	20 (13)	1990	-72.7	15.0 (15.4)
WV2	20 (13)	1990	-17.3	15.0 (15.4)
WV3	31 (21)	1990, 1992	0	58.1 (66.7)
WV4	19 (11)	1990	16.0	57.9 (54.5)
WV5	19 (11)	1990	39.8	47.4 (54.5)
OH	20 (17)	1991	172.2	40.0 (41.2)

Table 5 continued

Virginia transect

PA	20 (14)	1991	-159.0[d]	0 (0)
VA1	20 (13)	1990	-59.0	15.0 (15.4)
VA2	33 (17)	1989, 1991	-5.4	45.5 (35.3)
VA3	24 (17)	1989	5.4	62.5 (64.7)
VA4	21 (14)	1989	12.3	28.6 (28.6)
VA5	20 (14)	1990	46.4	10.0 (14.3)
VA	21 (16)	1991	240.4	0 (0)

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[a] Sample sizes and % hybrids given are for males and females combined, followed by males only in parentheses. Figures for males only include birds not analyzed vocally.

[b]Distances are from population centroids estimated by eye. Population diameters sometimes spanned a few tens of kilometers owing to the density of birds, and the spacing of collecting sites to ensure that song playback trials were independent. Some distances in the West Virginia and Virginia transects are adjusted because transects were not perpendicular to the hybrid zone interface, and reflect straight line distances to the nearest portion of the range interface as determined from breeding Bird Atlas data.

[c] Data from Sawaya and Braun (in prep).

Table 5 continued

[d] Distance of this population from the hybrid zone measured to the closest portion of the range interface as determined from Gill (1992). For the West Virginia transect the distance was measured to the range interface in southwestern Pennsylvania, while for the Virginia transect the distance was measured to the range interface in southeastern Pennsylvania.

[e] Constitutes same population as VA1.

Table 6. Sample size of individuals and song bouts, univariate measurements, and the first two principal components of song for populations comprising the West Virginia and Virginia transects. PCA performed on all populations combined. Values are x ± 1 SE.

Popu-lation	No. indiv-iduals	No. Bouts	Note 1[a] Dura-tion	Note 1[a] Onset freq.	Note 1[a] Midset freq.	Note 1[a] Offset freq.	Note 2 Dura-tion	Note 2 Onset freq.	Note 2 Midset freq.	Note 2 Offset freq.	PC1	PC2
OH	16	16	357 ± 7	6.85 ± 0.27	5.83 ± 0.25	5.78 ± 0.25	329 ± 6	3.76 ± 0.13	3.77 ± 0.11	3.77 ± 0.11	0.70 ± 0.46	-0.23 ± 0.20
OH (-E)	12[b]	12	360 ± 9	7.42 ± 0.09	6.34 ± 0.11	6.30 ± 0.11	322 ± 5	4.03 ± 0.05	4.00 ± 0.06	4.00 ± 0.06	1.66 ± 0.21	0.13 ± 0.13
WV5	7	8	308 ± 9	7.70 ± 0.27	6.41 ± 0.19	6.37 ± 0.19	287 ± 9	3.85 ± 0.11	3.88 ± 0.10	3.88 ± 0.10	1.86 ± 0.39	-0.74 ± 0.20
WV4	10[c]	15	239 ± 8	7.80 ± 0.25	6.87 ± 0.10	6.66 ± 0.07	282 ± 6	3.69 ± 0.07	3.76 ± 0.06	3.76 ± 0.06	2.16 ± 0.22	-1.61 ± 0.14
WV3 (C)	10[d]	13	335 ± 8	7.71 ± 0.29	6.51 ± 0.08	6.44 ± 0.08	286 ± 7	3.78 ± 0.10	3.78 ± 0.08	3.78 ± 0.08	1.61 ± 0.27	-0.88 ± 0.27

Table 6 continued

WV3 (B)	17[e]	17	390 ± 9	4.10 ± 0.06	3.92 ± 0.05	3.91 ± 0.05	394 ± 10	3.33 ± 0.05	3.43 ± 0.05	3.44 ± 0.04	-2.23 ± 0.17	0.32 ± 0.13
WV2	7	7	388 ± 13	3.93 ± 0.10	3.77 ± 0.08	3.74 ± 0.07	406 ± 12	3.41 ± 0.11	3.31 ± 0.06	3.31 ± 0.06	-2.56 ± 0.20	0.29 ± 0.06
WV1/VA1	6	6	391 ± 13	4.34 ± 0.06	4.08 ± 0.07	3.98 ± 0.06	420 ± 7	3.45 ± 0.05	3.54 ± 0.04	3.54 ± 0.04	-2.00 ± 0.15	0.68 ± 0.12
PA	14	14	446 ± 12	4.04 ± 0.08	3.84 ± 0.07	3.78 ± 0.06	434 ± 5	3.20 ± 0.06	3.30 ± 0.05	3.31 ± 0.05	-3.03 ± 0.18	0.49 ± 0.13
VA2	16[f]	16	376 ± 12	4.02 ± 0.05	3.83 ± 0.05	3.78 ± 0.05	405 ± 11	3.21 ± 0.04	3.28 ± 0.04	3.30 ± 0.04	-2.55 ± 0.17	0.00 ± 0.12
VA3	12	12	340 ± 10	6.23 ± 0.37	5.76 ± 0.28	5.72 ± 0.27	296 ± 6	3.75 ± 0.07	3.79 ± 0.05	3.79 ± 0.05	0.85 ± 0.31	-0.34 ± 0.17
VA3 (-E)	9[g]	9	336 ± 13	6.67 ± 0.39	6.11 ± 0.28	6.06 ± 0.27	292 ± 7	3.85 ± 0.07	3.84 ± 0.06	3.84 ± 0.06	1.30 ± 0.25	-0.39 ± 0.21
VA4	5[h]	5	297 ± 32	7.66 ± 0.40	6.56 ± 0.13	6.52 ± 0.12	286 ± 11	3.95 ± 0.15	3.94 ± 0.14	3.94 ± 0.14	2.16 ± 0.34	-0.64 ± 0.39

Table 6 continued

VA5	8	8	285 ± 10	5.88 ± 0.31	5.68 ± 0.37	5.59 ± 0.36	334 ± 28	4.66 ± 0.46	4.39 ± 0.28	4.37 ± 0.28	2.17 ± 0.61	1.53 ± 0.93
VA5 (-H)	5[i]	5	302 ± 10	6.56 ± 0.05	6.44 ± 0.03	6.31 ± 0.06	389 ± 10	4.03 ± 0.04	3.99 ± 0.04	3.99 ± 0.04	1.43 ± 0.14	0.28 ± 0.11
VA	16	16	335 ± 7	7.13 ± 0.22	6.33 ± 0.15	6.16 ± 0.19	388 ± 11	4.36 ± 0.10	4.27 ± 0.09	4.26 ± 0.09	1.96 ± 0.37	1.20 ± 0.16
VA (-E)	14[j]	14	337 ± 7	7.41 ± 0.11	6.53 ± 0.08	6.34 ± 0.17	382 ± 13	4.49 ± 0.06	4.39 ± 0.06	4.37 ± 0.06	2.40 ± 0.26	1.40 ± 0.10

[a] Note duration measured in msec, and onset, midpoint and offset frequencies in kHz.

[b] Omits four individuals singing "E" song type.

[c] Omits atricapillus song from one bilingual individual.

[d] carolinensis song.

[e] atricapillus song.

[f] Omits carolinensis song from two bilingual individuals.

Table 6 continued

[g] Omits three individuals singing "E" song type.

[h] Omits one individual singing only atricapillus song.

[i] Omits three individuals singing "H" song type.

[j] Omits two individuals singing "E" song type.

Table 7. Eigenvectors generated by principal component analyses of eight song variables for all individuals of the Virginia and West Virginia transects combined, and of the Missouri transect.

	Virginia and West Virginia		Missouri	
Character	PC1	PC2	PC1	PC2
Duration 1	-0.29	0.33	-0.26	0.62
Onset 1	0.38	-0.23	0.39	0.08
Midpoint 1	0.39	-0.23	0.39	0.01
Offset 1	0.39	-0.24	0.39	0.02
Duration 2	-0.30	0.35	-0.22	0.66
Onset 2	0.34	0.48	0.37	0.25
Midpoint 2	0.36	0.43	0.38	0.23
Offset 2	0.36	0.43	0.38	0.22
Eigenvalue	6.01	1.19	6.00	1.36
Variation explained	.699	.171	.750	.170

Table 8. Song type, sample size, univariate measurements, and the first two principal components of song for populations comprising the Missouri transect. Values are x ± SE.

Population	Song Type	No. individuals	No. Bouts	Note 1[a] Duration	Note 1 Onset freq.	Note 1 Midset freq.	Note 1 Offset freq.	Note 2 Duration	Note 2 Onset freq.	Note 2 Midset freq.	Note 2 Offset freq.	PC1	PC2
MO1&2	B	14	14	519 ± 14	4.19 ± 0.12	3.90 ± 0.08	3.86 ± 0.08	558 ± 17	3.26 ± 0.08	3.45 ± 0.05	3.46 ± 0.04	-2.62 ± 0.22	0.81 ± 0.22
MO3	B	18	18	452 ± 15	4.24 ± 0.09	4.01 ± 0.08	3.94 ± 0.08	490 ± 23	3.45 ± 0.09	3.58 ± 0.06	3.57 ± 0.06	-1.89 ± 0.22	0.17 ± 0.29
MO3	C	10	10	320 ± 20	7.10 ± 0.19	6.73 ± 0.14	6.68 ± 0.13	383 ± 24	4.41 ± 0.11	4.24 ± 0.09	4.20 ± 0.08	3.08 ± 0.37	-0.05 ± 0.28
MO3	E	7	13	402 ± 23	4.56 ± 0.08	4.30 ± 0.05	4.28 ± 0.05	400 ± 24	3.50 ± 0.03	3.68 ± 0.03	3.69 ± 0.03	-1.05 ± 0.14	-0.57 ± 0.29
MO3	Y	2	3	425 ± 23	4.57 ± 0.19	4.27 ± 0.09	4.20 ± 0.10	429 ± 29	3.70 ± 0.10	3.80 ± 0.10	3.80 ± 0.10	-0.84 ± 0.27	-0.01 ± 0.48

Table 8 continued

MO4	D	5	5	218 ± 32	6.88 ± 0.33	6.54 ± 0.17	6.44 ± 0.12	332 ± 27	3.54 ± 0.04	3.72 ± 0.06	3.74 ± 0.05	1.67 ± 0.24	-1.99 ± 0.33
MO4	C	9	9	355 ± 16	7.47 ± 0.19	6.41 ± 0.19	6.27 ± 0.16	376 ± 9	4.50 ± 0.07	4.41 ± 0.08	4.40 ± 0.08	3.31 ± 0.35	0.39 ± 0.18

[a] Note duration measured in msec, and onset, midpoint and offset frequencies in kHz.

Table 9. Standardized coefficients of eight song variables from discriminant analyses of three transects crossing the atricapillus/carolinensis hybrid zone.

	Standardized discriminant weighting coefficient		
Character	VA	WV	MO
Duration 1	0.2487	0.3305	0.6107
Onset 1	1.4471	0.7896	-1.7560
Midset 1	1.6296	-1.9736	2.1066
Offset 1	0.3282	2.6692	-1.4573
Duration 2	-0.1788	-0.6566	0.4825
Onset 2	0.9604	0.3470	0.3334
Midset 2	4.6210	4.1870	0.9317
Offset 2	-8.1868	-5.2361	-0.9928

Table 10. Sample size, song heard, responsiveness, and percent hybrids for populations comprising the West Virginia and Virginia transects in playback trials.

Popu-lation	No. birds	Song Present	Average Score (Number responding) B	C	% Hybrids [a]
OH	9	C	1.0 (7)	1.6 (9)	40.0 (43.8)
WV5	8	C	0.9 (6)	1.6 (8)	47.4 (54.5)
WV4	11	C[b]	1.0 (6)	1.5 (11)	57.9 (54.5)
WV3	11	B,C	1.4 (11)	1.5 (10)	58.1 (66.7)
WV2	13	B	1.3 (12)	0.7 (8)	15.0 (7.7)
WV1/VA1	11	B	1.4 (10)	1.2 (9)	15.0 (15.4)
PA	10	B	1.7 (9)	0.5 (5)	0 (0)
PA[c]	16	B	1.6 (14)	1.3 (12)	0 (0)
VA2	11	B[d]	1.5 (11)	0.6 (4)	45.5 (40.0)
VA3	5	C[e]	1.2 (4)	1.8 (5)	62.5 (58.3)
VA4	8	C[e]	1.1 (5)	1.6 (8)	28.6 (28.6)
VA5	7	C	0.1 (1)	1.7 (7)	10.0 (14.3)
VA	8	C	0.1 (1)	1.8 (8)	0 (0)

[a] Percent hybrids given are for males and females combined, followed by males only in parentheses.

[b] One bilingual individual heard.

Table 10 continued

[c] Playback trial repeated in 1995 at same location using "C" song type in place of "E" song type for carolinensis vocalization broadcast.

[d] Three bilingual individuals and one carolinensis-singing individual heard.

[e] Two individuals singing atricapillus song heard.

FIGURE LEGENDS

Fig. 7. Representative spectrograms of playback songs and designated song types. Population and location (or source) of songs is as follows: A. New York (Peterson 1983), B. VA (Charles City Co.), C. WV4 (Upshur Co.), D. Adams Co., Ohio (Peterson 1983), E. VA (Charles City Co.), F. WV4 (Upshur Co.), G. VA5 (Rappahannock Co.), H. VA2 (Shenandoah Co.), I. VA2 (Shenandoah Co.), J. VA3 (Shenandoah Co.).

Fig. 8. Frequency of individuals singing atricapillus ("B"), carolinensis ("C" through "H"), and abnormal ("X", "Y", "Z") song types within populations comprising the Virginia, West Virginia and Missouri transects. Includes individuals that were not collected and whose songs were not quantitatively analyzed. Missouri individuals singing variations of song types differing in number of notes were only counted once.

Fig. 9. Scatterplots of the first two principal component scores of individuals from PCA of eight song variables. Ten songs were deleted from MO3 that varied in number of notes from typical song types of four notes sung by the same individual. One individual of song type "H" from VA5 omitted in figures A and B (PC 1 = 6.6, PC 2 =

7.9). Song of bilingual singers is indicated in Figure B (J, K), Figure D (N-V), and Figure F (V-Z). Other individuals in Figure F (P-U) not detected as bilingual singers also sang multiple song types, and some bilingual singers in Figures D and F sang more than two song types. One individual in Figure B (L) from VA4 where carolinensis song predominated sang typical atricapillus song.

Fig. 10. Distribution of song types sung by individuals at the range interface of the Virginia transect in Shenandoah Co., Virginia, with VA2, VA3, and VA4 encompassed by polygons. Shaded region represents terrain above 394 m. VA3 encompasses all birds found in the Shenandoah Valley, while VA2 and VA4 encompass birds found above the valley floor on portions of the adjoining ridges. Upper case song types represent songs analyzed quantitatively, while small case song types represent songs heard but not recorded. Lower case "c's" are also underlined for emphasis. Song types with an asterisk represent birds collected for genetic analysis, while song types lacking an asterisk represent uncollected birds. Non-singing birds collected for genetic analysis not included.

Fig. 11. Distribution of song types sung by individuals at the range interface of the West Virginia transect in

Upshur Co., West Virginia, with WV3 encompassed by polygon. Shaded region represents terrain above 545 m. Symbols as identified in Figure 5.

Fig. 12. Distribution of scores from discriminant analyses of the Virginia (A,B), West Virginia (C,D), and Missouri transects (E,F). Parental populations of each species used in deriving discriminant functions are in A, C and E. Only scores for populations at the range interface of each transect are shown (B, D, F). Three "E" songs from VA3 and 13 "E" songs from MO3 are excluded, but had discriminant scores of intermediate value between means of reference populations.

Fig. 13. Scatterplots of first principal component scores and number of atricapillus alleles for singing individuals genetically characterized in populations at the range interface of the Virginia (VA2, VA3), West Virginia (WV3) and Missouri (MO3) transects. "E" songs identified by open circles. All other song types identified by closed circles.

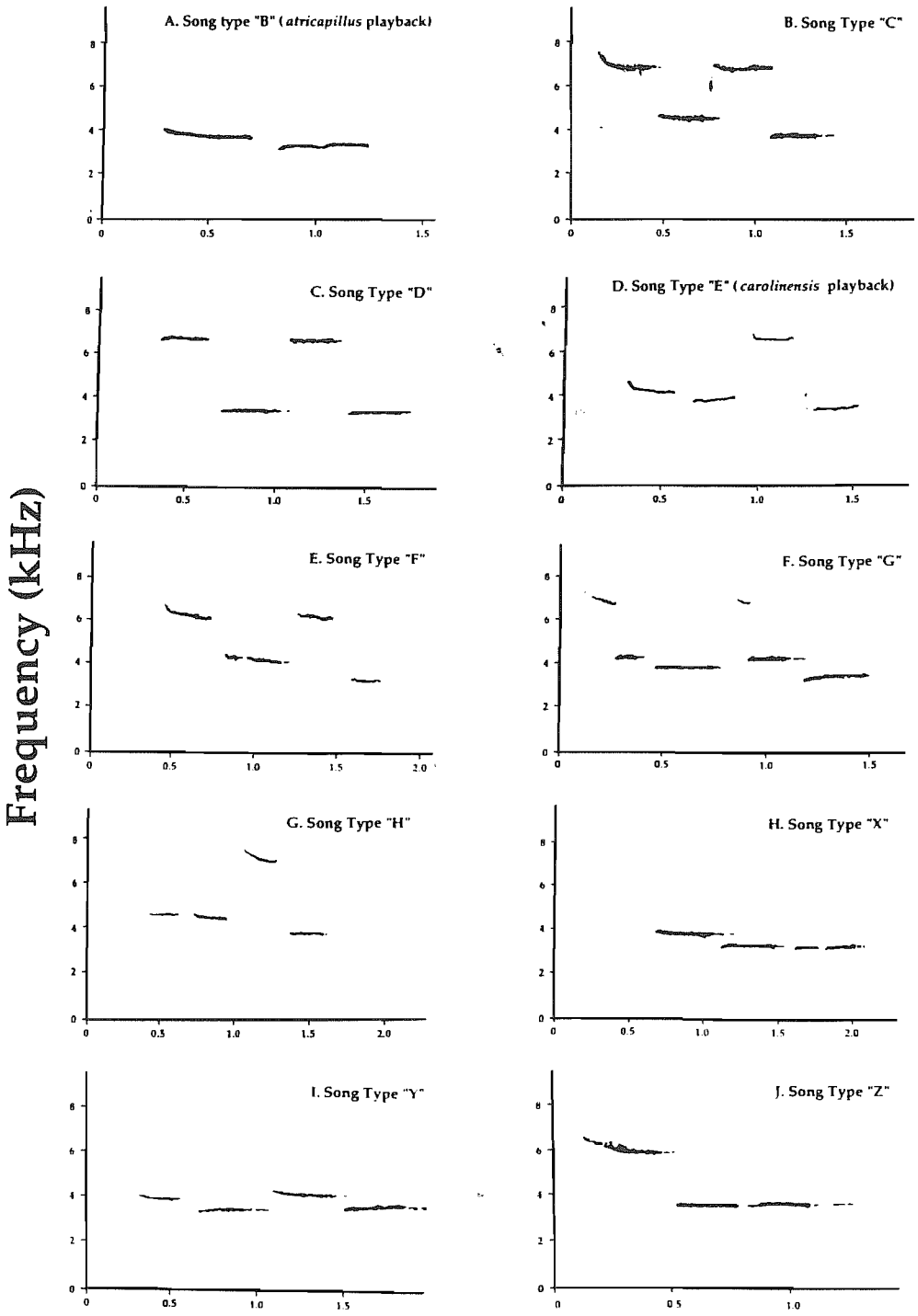
A. Song type "B" (atricapillus playback)
B. Song Type "C"
C. Song Type "D"
D. Song Type "E" (carolinensis playback)
E. Song Type "F"
F. Song Type "G"
G. Song Type "H"
H. Song Type "X"
I. Song Type "Y"
J. Song Type "Z"
Frequency (kHz)
Time (sec)

Figure 7

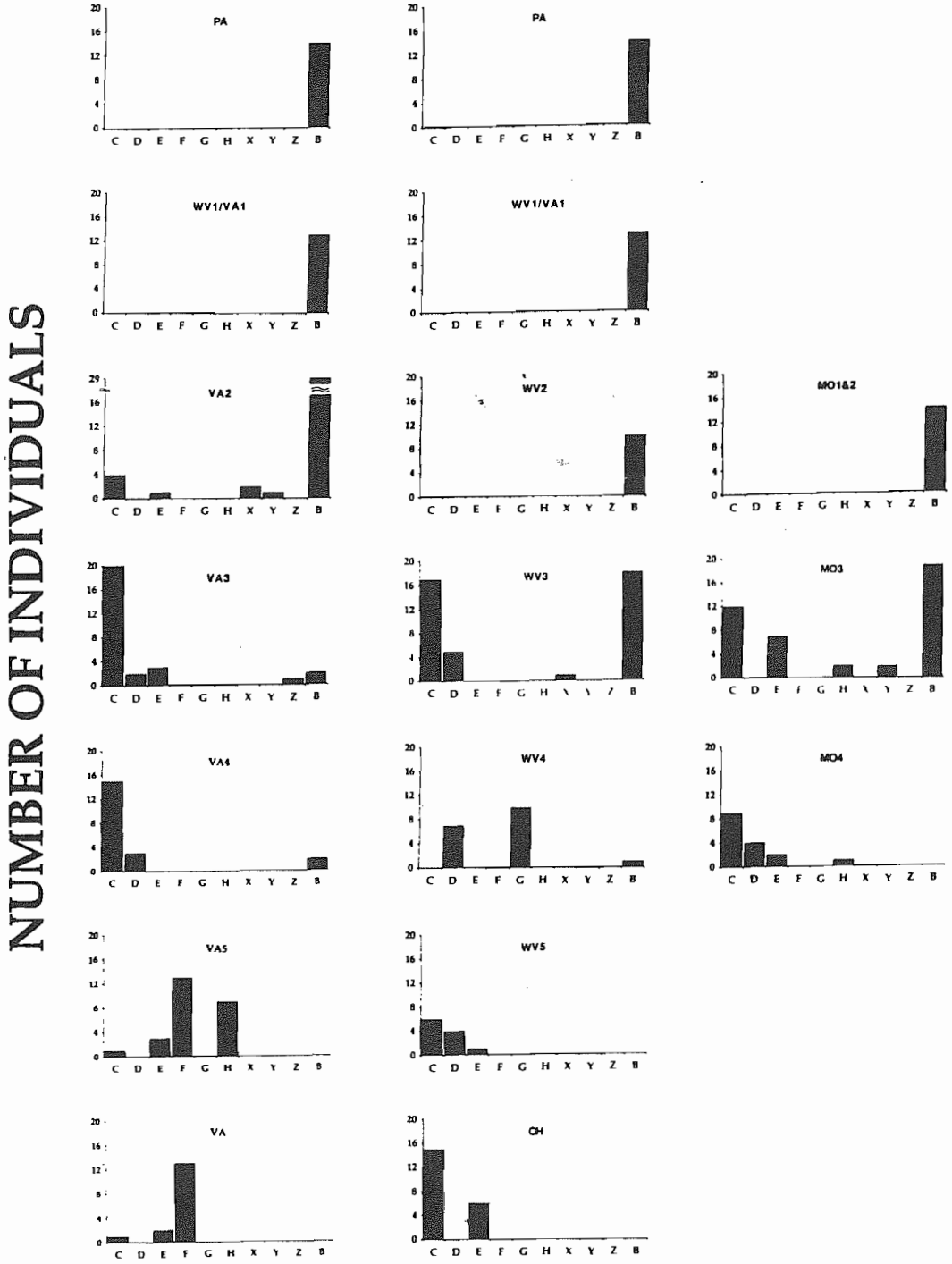
NUMBER OF INDIVIDUALS
PA
PA
WV1/VA1
WV1/VA1
VA2
WV2
MO1&2
VA3
WV3
MO3
VA4
WV4
MO4
VA5
WV5
VA
OH
C D E F G H X Y Z B
SONG TYPE

Figure 8

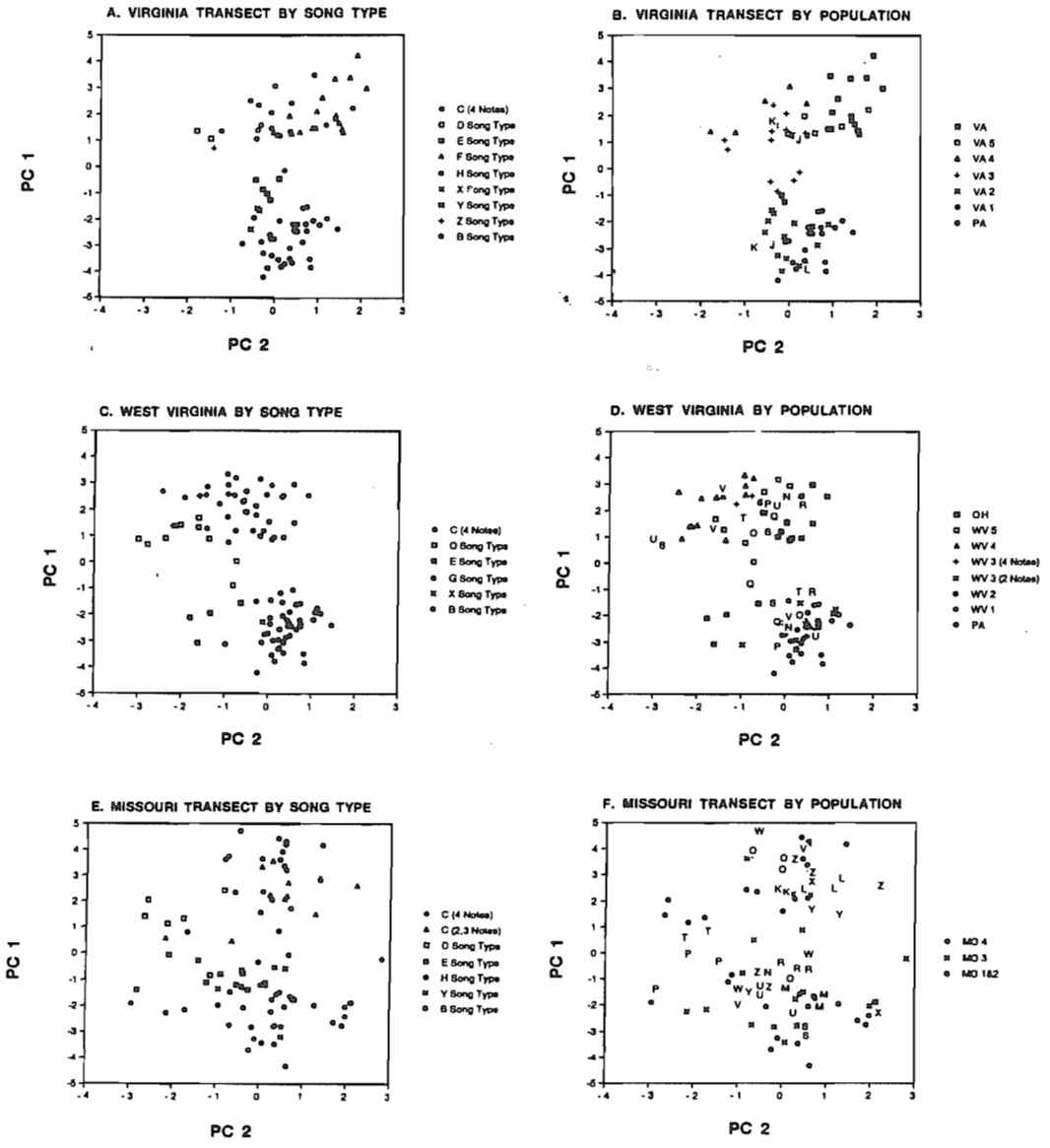
A. VIRGINIA TRANSECT BY SONG TYPE
PC 1
PC 2
C (4 Notes)
D Song Type
E Song Type
F Song Type
H Song Type
X Song Type
Y Song Type
Z Song Type
B Song Type
B. VIRGINIA TRANSECT BY POPULATION
PC 1
PC 2
VA
VA 5
VA 4
VA 3
VA 2
VA 1
PA
C. WEST VIRGINIA BY SONG TYPE
PC 1
PC 2
C (4 Notes)
D Song Type
E Song Type
G Song Type
X Song Type
B Song Type
D. WEST VIRGINIA BY POPULATION
PC 1
PC 2
OH
WV 5
WV 4
WV 3 (4 Notes)
WV 3 (2 Notes)
WV 2
WV 1
PA
E. MISSOURI TRANSECT BY SONG TYPE
PC 1
PC 2
C (4 Notes)
C (2,3 Notes)
D Song Type
E Song Type
H Song Type
Y Song Type
B Song Type
F. MISSOURI TRANSECT BY POPULATION
PC 1
PC 2
MO 4
MO 3
MO 1&2

Figure 9

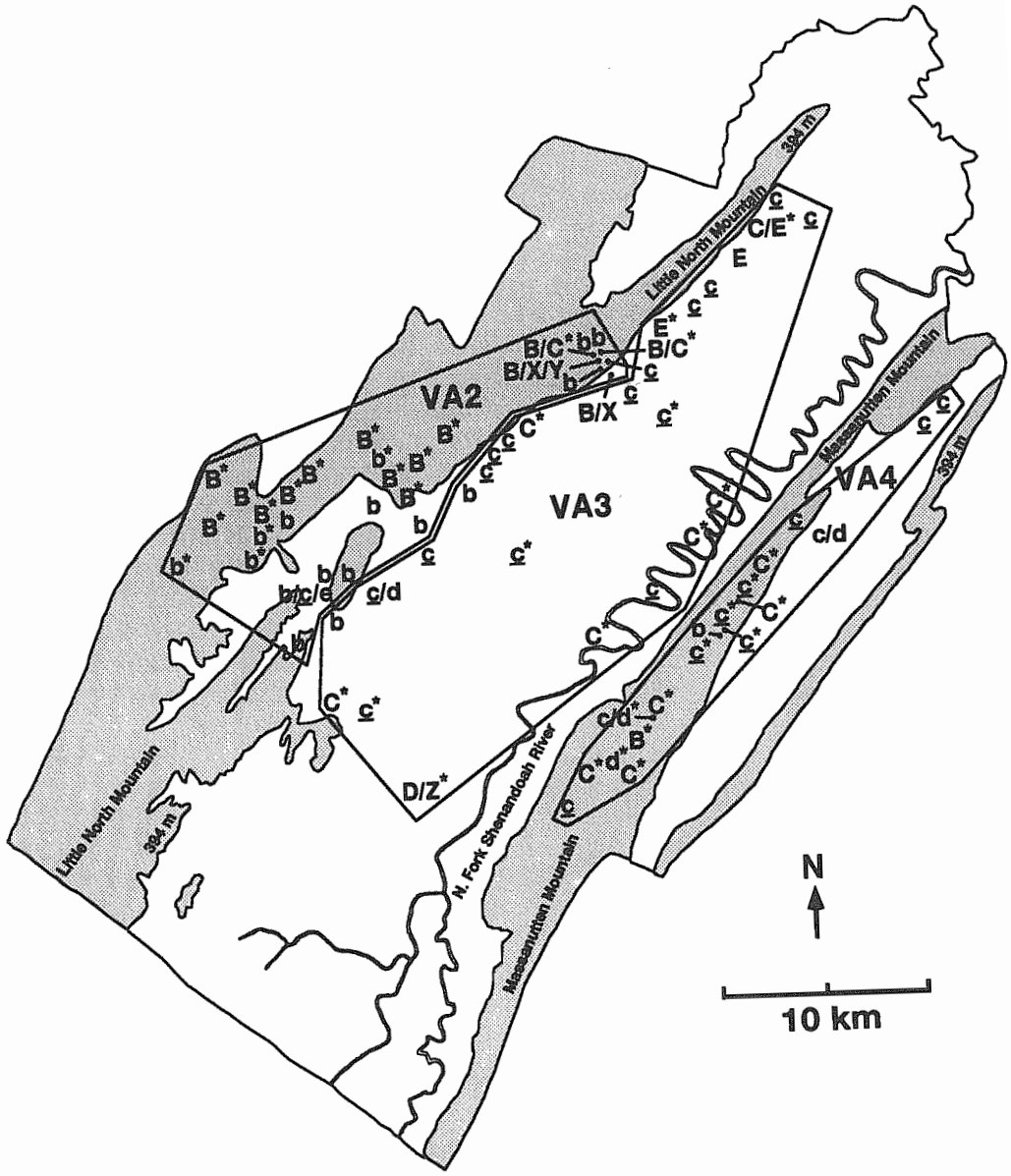
VA2
VA3
VA4
Little North Mountain
394 m
Massanutten Mountain
N. Fork Shenandoah River
D/Z*
B/X
B/C*
B/X/Y
C/E*
E
E*
C*
c/d
b/c/e
N
10 km

Figure 10

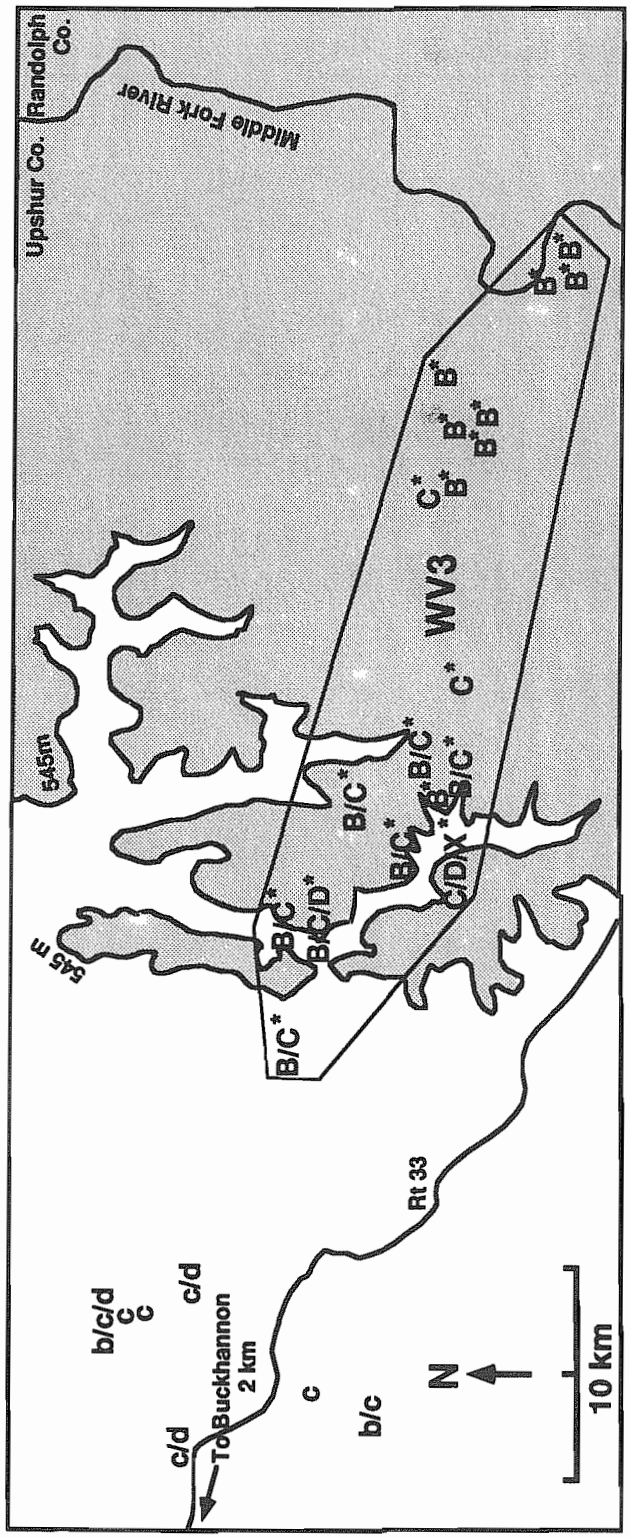
Upshur Co.
Randolph Co.
Middle Fork River
WV3
B*
B*B*
C*
B*
B*B*
B*B*
C*
B/C*
B/C*
B/C*
B/C*
B/C*
B/C*
C/D*
B/C*
B/C*
B/C*
545m
545m
Rt 33
To Buckhannon
2 km
b/c/d
c
c/d
c/d
c
b/c
N
10 km

Figure 11

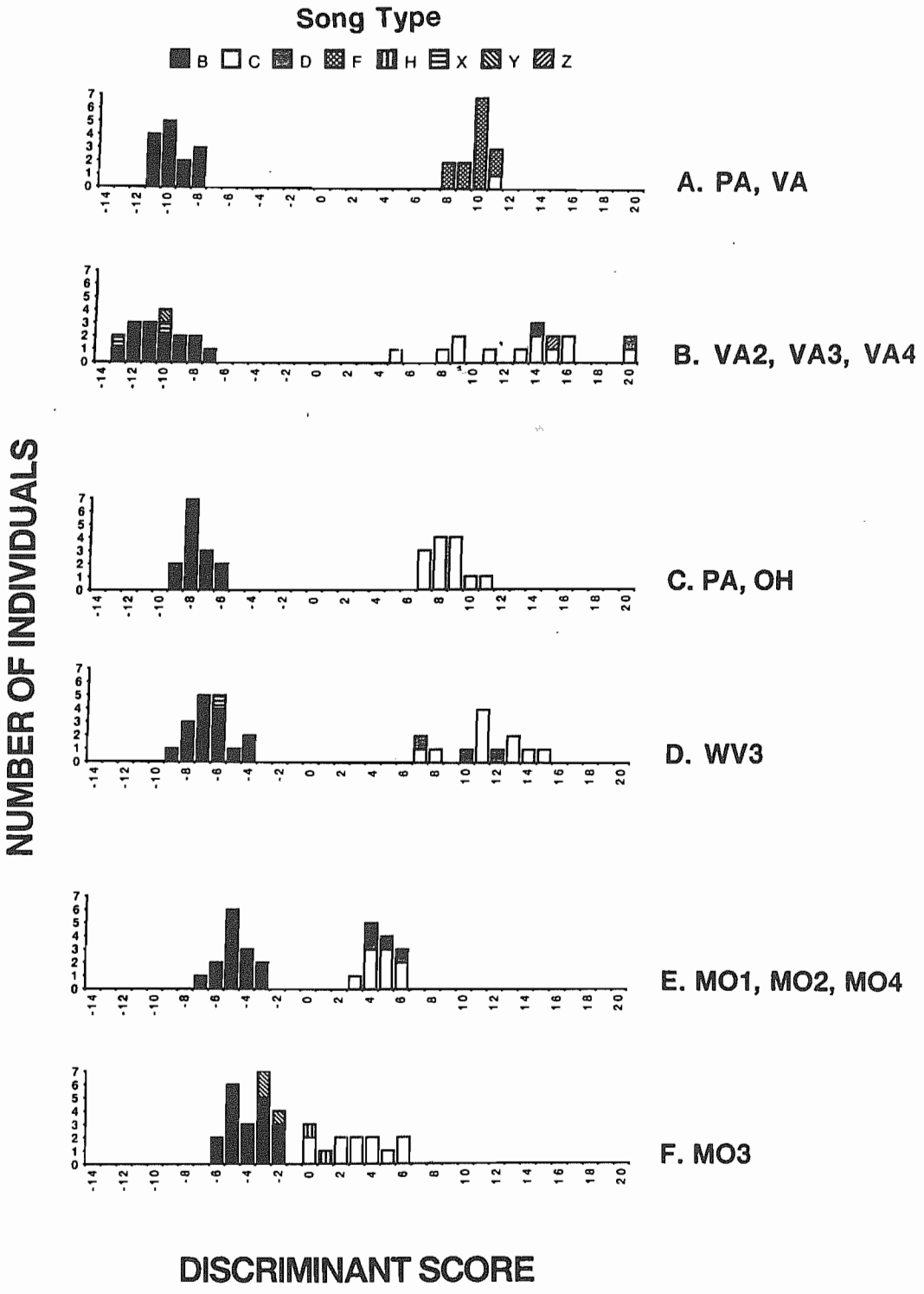
Song Type
B C D F H X Y Z
A. PA, VA
B. VA2, VA3, VA4
C. PA, OH
D. WV3
E. MO1, MO2, MO4
F. MO3
NUMBER OF INDIVIDUALS
DISCRIMINANT SCORE

Figure 12

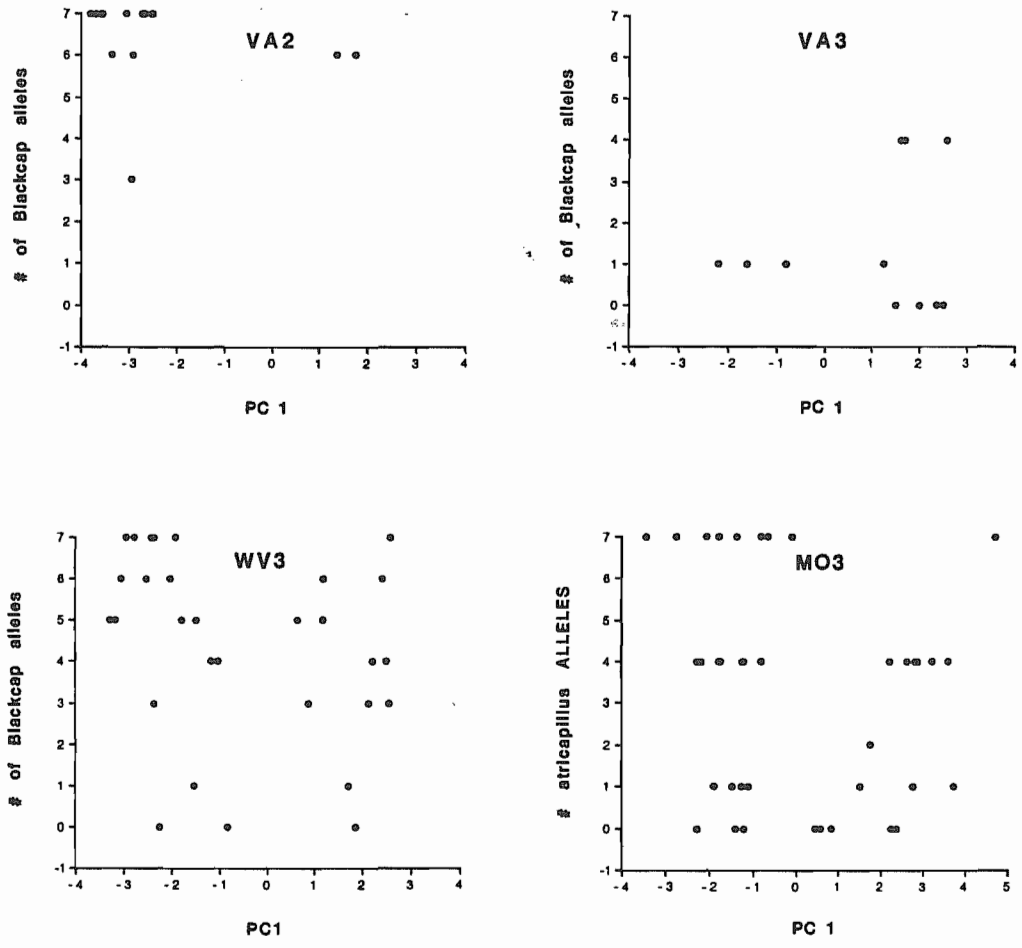
VA2
# of Blackcap alleles
PC 1
VA3
# of Blackcap alleles
PC 1
WV3
# of Blackcap alleles
PC1
MO3
atricapillus ALLELES
PC 1

Figure 13

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4. PATTERNS OF MOLECULAR INTROGRESSION AT A CHICKADEE HYBRID ZONE: COMPARISON OF ALLOZYMES, MITOCHONDRIAL DNA AND NUCLEAR DNA

Introduction

Current interest in the study of hybrid zones is high, because they can offer insight into evolutionary issues such as the determination of which genetic differences might be important in the speciation process, and the evolutionary significance of hybridization between species (Barton and Hewitt 1985; 1989; Hewitt 1988; Harrison 1990; Arnold 1992). They are also a rich source of variation for studying such microevolutionary processes as the development and maintenance of genetic differentiation, gene flow, selection, assortative mating, and linkage relationships (Harrison 1986; Szymura and Barton 1986; 1991; Gale and Barton 1993; Sites et al. 1995). Advances in these areas have come as new techniques and theory have been developed for hybrid zone analysis, and as new taxa have been studied that vary in such characteristics as dispersal ability, population structure, level of genetic differentiation, and degree and nature of reproductive isolation.

One issue receiving much attention is the degree of congruence in cline structure at different molecular loci. Multiple markers sometimes show congruent and narrow widths,

suggesting that genetic incompatibilities may be widespread throughout the genome (Szymura and Barton 1986, 1991). In other cases markers differ in the extent to which they have introgressed across the hybrid zone, providing clues as to where differentiation contributing to reproductive isolation might be located (Tucker et al. 1992, Dod et al. 1993). Another important issue concerns the fate of a hybrid zone. While the evidence is usually circumstantial, many hybrid zones are presumed to be relatively old, and to be maintained stably through time by selection (Barton and Hewitt 1985, Harrison 1990). Models to explain the maintenance of stable hybrid zones fall into two general classes (Moore and Price 1993). Endogenous selection models encompass those in which a hybrid zone is maintained by a balance between selection against hybrids and dispersal of parental genotypes into the hybrid zone (Bazykin 1969, Barton 1979a, b, 1983; Barton and Hewitt 1981, 1985, 1989). Selection in these models is dependent only on genetic interactions between disharmonious combinations of the two taxa's alleles in hybrids. The width of such hybrid zones is therefore dependent only on the strength of this endogenous selection and on the magnitude of dispersal. In contrast, exogenous selection models incorporate an ecological selection gradient that influences the relative fitness of various genotypes (Slatkin 1973, 1975, May et al.

1975, Endler 1977, Moore 1977). The width of hybrid zones maintained by exogenous selection is determined both by the magnitude of dispersal and the steepness of the ecological selection gradient influencing the fitness of each genotype. Exogenous selection models therefore predict a closer association of a hybrid zone's width and position with ecological gradients.

Birds were one of the first groups of animals in which hybridization and hybrid zones were studied, by virtue of their conspicuousness, frequently obvious plumage differences, aesthetic appeal, and propensity to hybridize (e.g. Meise 1928a, Sutton 1938, Cockrum 1952, Mayr and Gilliard 1952, references in Mayr 1963). Some of the earliest insights into the dynamics of hybridization came out of these early investigations, such as the role of man-induced habitat disturbance in promoting hybridization (Chapin 1948, Sibley 1954), the positioning of many hybrid zones where taxa expanding from glacial refuges met and the concentration of multiple hybrid zones at such "suture zones" (Meise 1928b, Remington 1968), and variation in width along a hybrid zone in congruence with ecological variables (Huntington 1952, Yang and Selander 1968). Studies of avian hybridization have the potential to yield unique insights because birds possess several traits that might play important roles in their genetic interactions. Their

dispersal capabilities are generally high, which promotes a low degree of population structuring, they typically have lower levels of protein and mitochondrial differentiation than other vertebrates of comparable taxonomic rank (Avise et al. 1980a, b, c, Kessler and Avise 1985), which might facilitate genetic exchange between different taxa, and in birds as in butterflies, females are the heterogametic sex.

The conservative nature of avian genetic differentiation at the protein level has hindered the analysis of avian hybrid zones, limiting the availability of differentiated allozyme loci that are typically abundant sources of genetic markers to investigate the structure and dynamics of hybrid zones in other taxa (e.g. Hunt and Selander 1973, Moran et al. 1980, Lamb and Avise 1986, Szymura and Barton 1986). Inferences into patterns of genetic introgression across avian hybrid zone have thus been limited to those that can be made from morphological traits, which may be polygenic in nature, and which are more likely to be subject to the effects of selection.

Restriction fragment length polymorphism (RFLP) variation at single-copy nuclear loci now provides an alternative source of genetic markers for investigation of hybrid zone pattern and process (Arnold et al. 1987; Baker et al. 1989; Keim et al. 1989; Arnold et al. 1990; Hall 1990). In the case of avian hybrid zones, this technique is beginning to

provide some of the first detailed information on levels and patterns of nuclear introgression (Parsons et al. 1993).

Black-capped and Carolina chickadees (Parus atricapillus and P. carolinensis) meet parapatrically across the eastern United States from Kansas to New Jersey, with the range of the more northerly distributed P. atricapillus extending in a peninsular fashion through the Appalachian Mountains to North Carolina (Fig. 1). Both plumage and morphometric differences distinguish these chickadees at the extremes of their ranges; however, these phenotypic differences are minimal at their range interface because of clinal subspecific variation in both P. atricapillus and P. carolinensis (Duvall 1945; Lunk 1952; James 1970). Therefore, morphological analyses, while providing evidence that hybrids are present where the two meet, have not resolved the extent to which hybridization occurs, or the degree to which introgression, if present, occurs across their hybrid zone (Brewer 1963; Rising 1968; Johnston 1971; Robbins et al. 1986; Ballard 1988). The songs of P. atricapillus and P. carolinensis are distinctive both in number of notes and in pattern of frequency variation between notes, providing the most certain means of diagnosing the two forms in the field. Bilingual individuals and songs of intermediate nature are frequent along much of the range interface (Brewer 1963, Johnston

1971, Ward and Ward 1974, Robbins et al. 1986), suggesting that hybridization is commonplace. However, song is probably not a reliable indicator of a bird's ancestry near the range interface (Kroodsma et al. 1995). While their mtDNA haplotypes are strongly divergent (Mack et al. 1986; Sawaya 1990), searches for allozyme differentiation between P. atricapillus and P. carolinensis have revealed only one diagnostic difference, with no other loci showing appreciable differentiation (Braun and Robbins 1986; Gill et al. 1989; Sawaya and Braun in prep).

A recent search for nuclear RFLP differences between P. atricapillus and P. carolinensis produced two such markers (Sawaya 1990, Sawaya and Braun in prep). These markers were used in conjunction with allozyme and mtDNA markers to examine the structure of this hybrid zone in southwestern Missouri. Clines for diagnostic molecular markers were steep and congruent with song and morphological characters. While hybrids were common at the range interface and there was no evidence of assortative mating, genetic introgression at these loci into surrounding populations was limited. There was a trend towards under-representation of the heterogametic sex (females) among F_1's, in accordance with Haldane's rule (Haldane 1922). P. atricapillus mtDNA was significantly under-represented among non-F_1 hybrids, suggesting a possible mating asymmetry. Finally, linkage

disequilibrium was strong among the three nuclear loci at the center of the hybrid zone, and, in combination with evidence for selection against some classes of female hybrids, indicated that this hybrid zone may be maintained by a combination of selection against hybrids and dispersal of parentals into the zone (Key 1968; Barton and Hewitt 1985).

We have now expanded our genetic analysis of this hybrid zone in two ways. First, we have identified additional nuclear RFLP's that are either fixed or partially differentiated between P. atricapillus and P. carolinensis. Second, we have sampled two new transects of the zone in the Appalachian Mountains. With this expanded analysis we address three primary questions. First, how representative is the preliminary analysis of the hybrid zone in Missouri of genetic interactions between these taxa as a whole? A wider sampling of genetic loci and of geographic locations will provide more accurate estimates of the proportion of hybrids. Surveys of the new Appalachian transects will also provide additional data with which to test some of the tentative results of the earlier analysis regarding selection against female hybrids, levels of assortative mating, and mating asymmetries. Second, is there coincidence in position and congruence in shape of clines among molecular markers, or are there marked differences in

these parameters that can provide insight into the dynamics of this hybrid zone? Finally, is there variation in cline structure among transects indicative of an ecological component to this hybrid zone's dynamics? Elevation changes dramatically along the two Appalachian transects, but changes little along the Missouri transect. If an elevational gradient or associated environmental variables influence this hybrid zone's structure, then both Appalachian transects are predicted to exhibit narrower cline widths than the Missouri transect.

METHODS

Study sites and populations.

Six populations comprised the Missouri transect (Table 11, Fig. 14). Populations referred to here as MO1-MO4 correspond to Populations 1-4 respectively of Robbins et al. (1986), while populations MO and LA correspond to allopatric populations studied by Braun and Robbins (1986). The West Virginia and Virginia transects each consisted of 7 populations (Fig. 1; Table 11). Population WV1/VA1 served as a common central Appalachian population for both transects. Because WV1/VA1 was less than 60 km from the nearest *P. carolinensis* population on either side of the Appalachian Mountains, an allopatric population of *P. atricapillus* (PA) in north-central Pennsylvania served as

the terminal population of this species for both Appalachian transects. Allopatric populations OH and VA served as terminal populations on the P. carolinensis end of the West Virginia and Virginia transects respectively. An additional allopatric population of P. carolinensis (MD) collected in central Maryland is not associated with any of these linear transects. Birds were collected with shotguns during the breeding season except in MD, where collecting occurred year-round. Birds were frozen within a few hours on either dry ice or in liquid nitrogen, and later transferred to a -80°C freezer. Mated pairs of birds were collected when possible for all populations. Appalachian transect skins and tissue specimens were deposited at the U.S. National Museum of Natural History. Precise localities for each population are given in Table 1.

Protein electrophoresis.

Tissue homogenization, cellulose acetate electrophoresis, and staining for guanine deaminase (GDA, E.C. Number 3.5.4.3) in all individuals followed the protocol in Chapter 2. In addition, aconitase (ACON, E.C. Number 4.2.1.3) was scored from liver homogenates electrophoresed at 200 V for 1.5 to 4.5 h using a 50 mM Tris, 1 mM Na_2EDTA, 1mM $MgCl_2$ buffer (pH. 7.8), and stained by agar overlay using the recipe of Richardson et al. (1986). Isozyme variation at ACON has previously shown some evidence of differentiation

in P. atricapillus or P. carolinensis, but was not fully scorable (F. Gill pers comm).

Isolation and restriction endonuclease analysis of DNA. Total DNA was isolated from pectoral muscle tissue of each individual by phenol chloroform extraction followed by ethanol precipitation, DNAs were digested with restriction endonucleases, electrophoresed in horizontal agarose gels, and transferred to nylon membranes using Southern transfer (Southern 1975). Membranes were then hybridized with probes labelled by random priming (Feinberg and Vogelstein 1983), and restriction fragments were visualized by autoradiography. Detailed methods are given in Chapter 2. A total of 27 probes were used. These included 24 randomly cloned fragments of chickadee nuclear DNA (see below) and three probes shown previously to detect species specific RFLP's in these birds (Mack et al. 1986, Sawaya 1990, Gill et al. 1993). The three probes used previously were: 1) mtDNA isolated from muscle, liver, heart and kidney tissue of three P. carolinensis collected in Prince Georges County, Maryland; 2) a 1.2 kb fragment of the chicken oncogene ski (Li et al. 1986); and 3) a 4.0 kb randomly cloned fragment of Parus bicolor DNA designated DPAC121, designated as C7 in previous use to diagnose these chickadees (Sawaya 1990; Sawaya and Braun in prep). MtDNA isolation followed the protocol of Chapter 2. DPAC121 is sex-linked in P.

atricapillus and P. carolinensis, while ski is autosomal (Sawaya 1990; Sawaya and Braun in prep).

Cloning and screening of random single-copy nuclear probes.

A search was conducted for additional nuclear RFLP markers fixed or partially differentiated between P. atricapillus and P. carolinensis. Total DNA from three P. carolinensis collected in Rappahannock County, Virginia was combined and digested to completion overnight using the restriction endonuclease Eco RI. The digested DNA was reextracted following the protocol outlined in Chapter 2, except that the DNA was ethanol precipitated by centrifugation, the recovered pellet dried, and resuspended in 600 µl of 1X TE. The digested DNA was next size-selected in a sucrose gradient following Sambrook et al. (1989; pp. 2.85-2.87), except that ultracentrifugation was at 21,500 rpm. Following dialysis of aliquots containing fragments in a size range from 16-23 kb, the DNA was concentrated with a Centricon 30 microconcentrator filter according to the manufacturer's recommendations (Amicon Division), then ethanol precipitated and resuspended in 10 µl of TLE (1 mM Tris, 0.1 mM EDTA).

The size-selected DNA was next ligated and packaged in the bacteriophage lambda vector EMBL4 following manufacturer's specifications (Stratagene), and plated on the E. coli host strain SRB(P2). Single plaques were picked

at random, and a second round of plaque purification performed to ensure that plaques representing single clones were obtained. A modified protocol of Sambrook et al. (1989; pp. 2.67-2.81) was then followed to obtain DNA from each clone. Stocks of each clone were prepared from plaques with small-scale liquid cultures using NZY medium, and large-scale preparations of each clone obtained using a high multiplicity infection of the bacterial host strain LE392. DNA was purified from lysed cultures by precipitating bacteriophage with polyethylene glycol, extracting twice with chloroform, twice with phenol/chloroform/isoamyl alcohol, and three additional times with chloroform. DNA was ethanol precipitated, and further purified by electrophoresis in a 0.3% agarose gel. The high molecular weight lambda DNA band was then excised from the gel and recovered following binding and elution from a silica matrix following the manufacturer's recommended protocol (Geneclean II; Bio101, Inc.). High copy number probes were detected and eliminated from analysis by either dot blot analysis or by examining autoradiographs for repetitive fragments.

DNA from each random clone was used as probe in Southern transfer analyses to screen allopatric populations of P. atricapillus and P. carolinensis for differentiation between the two species. A total of 24 probes were tested in two levels of screening. In the first level, each probe was

tested against either five or six different restriction endonucleases (Bam HI, Bgl II, Pst I, Pvu II, Taq I, sometimes Xba I), screening 4 or 5 individuals each from PA, OH, VA and LA, as well as one individual from MO. If a restriction fragment pattern was found in which all P. carolinensis individuals (OH, VA, LA) differed from all P. atricapillus individuals (PA, MO), then the remaining individuals from all three transects were screened. In a second level of screening, 15 of these 24 probes were also tested against either two or three enzymes (Ava II, Bcl I, Bgl I, Dra I, Hae II), screening three individuals each from PA and VA. If the three P. carolinensis restriction profiles differed from the three P. atricapillus profiles, then all remaining individuals were screened.

While the mtDNA genome can be considered as one linked locus in RFLP analysis, two options are available in the designation of loci in RFLP analysis of the nuclear genome (Quinn and White 1987). In the site method, each restriction site is considered a locus, and alleles correspond to the presence or absence of that site. In the region method, a region of DNA with adjacent varying sites is considered a locus, and alleles correspond to particular fragment patterns produced by this region. Each region or locus is separated from others by one or more invariant sites, and while variation in the size of fragments within

one region affects the size of other fragments within the same region, the size of fragments in one region is independent of fragment size in another region. We use the region method here in designating loci detected by our nuclear probes, and all alleles detected were interpretable as resulting from base substitutions in enzyme recognition sequence. Each probe in an RFLP analysis typically detects multiple loci, regardless of whether the site or region method is employed. In the study of population differentiation and gene flow, variation at each locus can provide useful information, although loci detected by a particular probe are often physically linked to one another. For the purposes of this study, however, only variation reflecting strong differentiation between P. atricapillus and P. carolinensis is of interest, and variation at all other loci/regions is ignored. As recommended by Quinn and White (1987), we use the conventional nomenclatural system for naming human RFLP probes; each probe's name begins with DPAC denoting "DNA, Parus atricapillus/ carolinensis", and is then numbered (Table 12).

Statistical analysis.

Individuals were assigned a genotype for each locus, numbering sequentially P. atricapillus alleles A1, A2, A3, etc and P. carolinensis alleles C1, C2, C3, etc (Table 12). Criteria for the species designation of alleles included

their distribution in allopatric populations, their distribution across transects, and for RFLP's only, similarity of fragment pattern to a pattern of known species origin, using band intensities to help infer the origin of novel fragments. In a few cases the species origin of a rare allele could not be confidently assigned, in which case the allele was deleted from analysis. For the present analyses, all P. atricapillus (=A) alleles for a marker were pooled, as were all P. carolinensis (=C) alleles. Frequencies of A and C alleles in each population were then estimated for each marker (Table 12), and the fit of genotypes to Hardy-Weinberg expectations tested using BIOSYS (Swofford and Selander 1981). Genotypic disequilibrium was calculated for all pairwise comparisons of markers in each population using a modified version of the program of Weir 1990 (Lewis and Zaykin in press), and chi-square tests performed for deviation from zero.

Cline analysis.

Data were collected for n_i individuals at location d_i, with a linear set of populations at d_i constituting a transect. $A_i = 1$ signifies the possession of an A allele by the i_{th} individual, and $A_i = 0$ signifies the lack of such an allele. Frequency of the A allele for each marker was plotted as a function of distance, starting with the P. atricapillus terminus of each transect. Such plots exhibit

a sigmoidal distribution that has traditionally been modeled using logistic regression. In this approach, frequency of the A allele at distance d, $\pi(d)$ is modeled as:

$$\pi(d) = e^{\alpha + \beta x}/(1 + e^{\alpha + \beta x})$$

Solving for α and β is accomplished with the logit transformation:

$$g(d) = \log (\pi(d)/1-\pi(d)) = \alpha + \beta x$$

The logit g(d) has several desirable statistical properties (Hosmer and Lemeshow 1989). It is linear, may be continuous, and can range from $-\infty$ to $+\infty$, depending on the range of x. It is solved using the method of maximum likelihood in an iterative manner (McCullagh and Nelder 1989). Limitations of this approach are that it assumes a symmetric distribution, and that the distribution ranges from 0 to 1. We obtained good fits to the data in which markers were fixed or nearly so at both ends. Fit was poor, however, for markers that were not close to fixation at both ends, or that were fixed at only one end, and so asymmetrical.

An alternative approach is to use smoothing splines, which are free of these two assumptions (Hastie and Tibshirani 1990). The approach is similar to logistic

regression, but a general smoothing function [f(d)] replaces α and ßx, so that:

$$f(d) = \log \pi(d)/1-\pi(d)$$

This approach has been used in a variety of biological settings (Schluter 1988, Culver et al. 1994, Smith et al. 1995). The smoothing function is estimated using the maximum likelihood approach, and following Schluter (1988), the log likelihood is represented by:

$$l(f) = \Sigma l(A_i;d_i,f)$$

where $l(A_i;d_i,f)$ is the natural logarithm of the probability that frequency of the A allele = 1 at distance d_i. This likelihood will be maximized by any function that connects all data points, but such a function will have low predictive value, and may not conform to biological expectations that the function will be smooth and simple (Schluter 1988). A modified technique of penalized maximum likelihood is therefore used, minimizing the negative penalized log likelihood function

$$l(f) = -\Sigma l(A_i;d_i,f) + n\lambda[f''(d)]^2dd$$

The second term of the function penalizes for lack of smoothness, with the integral measuring how "rough", or of rapidly changing slope, the chosen function is. The parameter imposes a larger penalty as it increases and the resulting function is smoother. When $\lambda = \infty$, a straight line results, while at $\lambda = 0$, the minimized function can be rough, connecting all data points.

Our choice of an optimal smoothing parameter was based on the strong monotonic pattern each marker exhibited, and our belief that a smooth monotonic function is a logical biological expectation in this case. Consequently, for each marker we began with no penalty for roughness ($\lambda = 0$), and incrementally increased λ, enforcing a progressively greater degree of smoothness. The final λ chosen was that which resulted in a monotonic fit to the data with the least penalty for roughness.

Functions fit the data well in cases when markers were fixed. When markers were not fixed, however, a poor fit sometimes resulted, because it is assumed that $\beta = 1$ at $d = 0$, and that A approaches 0 as d approaches ∞. A modification of the model was therefore necessary to constrain it to lie within the observed range of A allele frequency. The model chosen was:

$$\pi(d) = a + b[e^{f(d)}/1 + e^{f(d)}]$$

where a is the maximum observed frequency of the A allele and a + b approaches the minimum observed frequency of the A allele as d approaches ∞. $\pi(d)$ can thus still be interpreted as frequency of the A allele at distance d.

Fit of a function to the data was still poor under one circumstance, in which the minimum A allele frequency did not correspond to the greatest d. Under the general logistic regression model, each data point has a variance associated with it. The variance is close to zero near $\pi(d) = 0$ and 1, and so has little weight in determining the function's structure. This is to be expected, since the logistic curve naturally approaches 0 and 1. When allele frequency is not at a minimum or maximum in the tails, however, the increased variance of these points affects the function's structure greatly. In these instances, the resulting splines appeared too shallow to provide accurate estimates of the parameters. We therefore excluded a marker from statistical comparisons when there was a chance that spline fit to the data would not accurately estimate parameters.

Two parameters were estimated from a spline. Cline width was estimated as the inverse slope of a resulting spline. This estimate assumes that allele frequency is distributed between 0 and 1. A correction to cline width was thus necessary in those cases in which markers were not fixed.

For instance, if allele frequency only spanned 90% and 20%, cline width was reduced 30%, because the actual frequency range was 70%, not 100%. The second parameter, cline position, was estimated as the position at which a spine's steepest slope occurred.

Theoretical statistical properties of these estimates of cline width and cline position are not known. We have therefore used a bootstrap procedure to allow statistical comparison of these estimates among markers. Replicate data sets were constructed for each marker that conformed to the real data set's underlying probability structure. For example, if in a given population we had sampled 20 individuals, and frequency of the A allele in this sample was 30%, a replicate data set was created with the same number of individuals. Two A alleles (in the case of a diploid marker) were then assigned to each of these individuals with a probability of 30%. Two hundred such replicate data sets were constructed in each transect for every marker, and an estimate of cline width and cline position was obtained from each replicate data set. We then used ANOVA and Tukey tests (SAS 1990) to make comparisons both among different markers within a transect (for both width and position), and among different transects for the same marker (for width only).

Results

Random single-copy nuclear probes.

Twenty-four random DNA clones were chosen for screening as probes. Of these, five were eliminated due to high copy number or poor resolution of fragment pattern. Insert size for five of the remaining 19 clones averaged 13.7 kb. Hence, these clones represent about 260 kb of the chickadee genome. A total of 134 probe/enzyme combinations were screened, resulting in 802 restriction fragments representing 963 restriction sites, or about 5200 base pairs. Ten of these clones passed the initial stage of screening (see above) for differentiation between P. atricapillus and P. carolinensis, but two were dropped due to unreliable scoring. One of the remaining eight random probes (DPAC96) detected differentiation in two separate regions (as defined above), and a second of these probes (DPAC1) detected RFLP's using two separate restriction enzymes. These eight probes therefore provide information on ten distinct loci. The two loci detected by DPAC96 (DPAC96A and DPAC96B) and the two loci detected by DPAC1 (DPAC1A and DPAC1B) are likely to be physically linked, however, and linkage relationships among the remaining nuclear markers are unknown.

Intraspecific variation.

Of the two loci screened for isozyme variation, one

allele each in P. atricapillus and P. carolinensis was detected at GDA, while two and three alleles respectively were present at ACON in P. atricapillus and P. carolinensis. The three restriction enzymes used to screen mtDNA haplotypes (Pvu I, Pvu II, and Ava II) are all diagnostic for the three major haplotypes associated with P. atricapillus, eastern populations of P. carolinensis (Appalachian transects), and western populations of P. carolinensis (Missouri transect). These major haplotypes differ from one another at many restriction sites (Gill et al. 1993, Sayawa 1990, Sawaya and Braun in prep). One or more minor haplotypes differing by a small number of inferred restriction site losses or gains were also associated with each major haplotype. Fragment profiles for each of the three restriction enzymes were congruent in all individuals with respect to species designation, and there was no indication of heteroplasmy or length variation in the mitochondrial genome of these birds. An analysis of population structure in these chickadees based on both mitochondrial and nuclear DNA markers will be presented elsewhere.

One of the ten RFLP markers developed (DPAC4) revealed strong differentiation between western P. carolinensis (LA) and eastern P. carolinensis (VA), but not between P. atricapillus and eastern P. carolinensis (Table 13).

Therefore, transect populations were not screened with DPAC4, and it is excluded from further analysis. The number of P. atricapillus alleles among the remaining nine nuclear RFLP loci ranged from one to five, and averaged 1.6 per marker, while the number of P. carolinensis alleles ranged from one to three, and averaged 1.7 per marker.

Sex-linkage of marker loci.

DPAC121 is sex-linked in these chickadees; only males, the homogametic sex in birds, display a heterozygous pattern (see below), and band intensities of restriction fragments on autoradiographs controlled for DNA concentration are twice as strong for homozygous males as for females (Sawaya 1990, Sawaya and Braun in prep). Female heterozygotes for both GDA and ACON are likewise lacking, consistent with sex-linkage of these loci (Sawaya 1990, Sawaya and Braun in prep, present study). Combining data from all three transects, at both GDA and DPAC121 there were 25 male and no female heterozygotes among 273 males and 130 females scored ($X^2 = 13.2$, $P < 0.001$). For ACON the data were similar; there were 31 male and no female heterozygotes among 273 males and 130 females scored ($X^2 = 16.07$, $P < 0.001$). ACON has been reported to be sex-linked in three avian orders, including Passeriformes (Baverstock et al. 1982, Lacson and Morizot 1988), while GDA has been found to be autosomal in one passerine species (Baker 1990). The nine randomly-

cloned loci and *ski* all displayed heterozygous RFLP profiles for some females, indicating that they represent autosomal loci.

Differentiation of marker loci.

Four marker loci (MtDNA haplotypes, GDA, DPAC121, and DPAC7) were each fixed for either A or C alleles in all parental populations of the three transects (Tables 12, 13). All parental reference populations were also fixed for either A or C alleles at ACON, with the exception of PA, in which 6.2% C alleles were found. *Ski* was fixed in *P. atricapillus* parental populations (PA, MO) and in *P. carolinensis* parental populations LA and VA, but not OH or MD (Table 12, 13). Two other markers, DPAC102 and DPAC104, were fixed only in parental *P. atricapillus* populations (PA, MO) but not in any parental *P. carolinensis* populations. None of the remaining seven markers were fixed in all reference populations of either species. Of these seven markers, however, five (DPAC1A, DPAC96A, DPAC96B, DPAC97, and DPAC98) showed a stronger tendency towards fixation in parental *P. atricapillus* populations than in parental *P. carolinensis* populations (Tables 12, 13).

Frequency of hybridization.

Five markers (mtDNA, GDA, DPAC121, DPAC7, and *ski*), were considered diagnostic in assessing genetic ancestry. Although A alleles for *ski* were present in OH and MD, the

two parental P. carolinensis populations closest to the range interface, we believe they result from introgression of P. atricapillus alleles from the north (See Discussion). Individuals possessing all A or all C alleles for each of these five markers were classified as potentially pure P. atricapillus and P. carolinensis respectively. Individuals that possessed any mixture of A and C alleles at these five loci were classified as hybrids. Among hybrids, individuals potentially of the F_1 generation were distinguished by the possession of a heterozygous genotype at each diagnostic diploid locus (males: GDA, DPAC121, ski, DPAC7; females: ski, DPAC7). Potential F_1 females must also have alternate species alleles for the mtDNA haplotype, which they receive from their mothers, and the sex-linked loci GDA and DPAC121, the allele of which lies on their single Z chromosome that they receive from their fathers. Estimates of hybrid frequency are conservative, as backcross or later-generation hybrid matings can produce some parental and F_1 genotypes for these loci. Potential linkage of GDA and DPAC121 on the sex chromosome could result in non-independence of these markers, further increasing the chances of misclassifying non-F_1 hybrids as parentals.

Hybrids made up from 44% to 62% of populations at the range interface along all three transects (Table 11). These populations have been screened previously with MtDNA, ski,

DPAC121 and GDA (Sawaya and Braun in prep), and the addition of DPAC7 to determine genetic ancestry revealed no additional hybrids among these populations. Backcross and later generation hybrids predominated among individuals of mixed genetic ancestry, with potential F_1's comprising less than 25% of any population, and being confined to near the hybrid zone's center (Table 11). The frequency of hybrids declined rapidly away from the range interface, except on the P. carolinensis side of the West Virginia transect. Here, hybrids remained at frequencies near 50% through WV4 and WV5 to OH, 170 km from the contact zone. However, virtually all hybrids found greater than 20 km from the range interface were identified as being of mixed genetic ancestry on the basis of a single foreign ski allele. The single exception to this was one bird in WV5, 40 km from the range interface, with both introgressed ski and DPAC7 alleles. Mitochondrial haplotypes and alleles of the three sex-linked loci were not detected introgressing beyond 20 km of the range interface.

Deviations from panmixia.

Significant deviations from Hardy-Weinberg expectations occurred in only seven of 175 comparisons ($P < 0.05$, exact probability tests), a rate (4.0%) not significantly different from random expectations. For the sex-linked markers, these tests were performed only on males. Three of

the seven significant deviations were in VA4, and occurred for the three sex-linked markers. VA4 was fixed for the C allele at these three loci with the exception of one individual that was homozygous for the A allele at each of these loci, and at all other diagnostic loci. Heterozygote deficiency is therefore not detectable for these three loci in VA4 when this individual is removed from the analysis, resulting in only 4 of 173 (2.3%) of all comparisons deviating significantly from Hardy-Weinberg equilibrium. However, of the remaining four significant heterozygote deficiencies, all occurred in populations near the center of the molecular clines. Three of the four occurred in MO3, in which A and C alleles frequencies were relatively balanced for each marker (Table 12). One also occurred in WV3 where A alleles predominated somewhat, while none occurred in VA2 or VA3, in which A and C alleles predominated respectively. Both small sample sizes and skewed allele frequencies greatly reduce the power to detect such deviations, and we believe that heterozygote deficiency is more prevalent at the hybrid zone's center than reflected by our tests. For instance, we noted that among the 173 comparisons for Hardy-Weinberg equilibrium, those in which fewer heterozygotes than expected were observed predominated only in these four central populations (38 of 52), while the number of comparisons among the other populations, in which there was

a heterozygote deficiency were in the minority (31 of 121).

Previous analysis of this hybrid zone in Missouri (Sawaya and Braun in prep) suggested a deficiency of females among F_1 hybrids in the center of the hybrid zone, in accordance with Haldane's rule (Haldane 1922). None of six potential F_1's in MO3 were females (P = 0.07; Fisher's one-tailed exact test; Table 14). The same trend was observed in the West Virginia transect but not significantly so (P = 0.32), while in VA2 there was a deficiency of males among F_1's (P = 0.035) (but see Discussion). We detected no F_1's in VA3, and males and females were equally represented among non-F_1 hybrids in all three transects.

Previous analysis of the Missouri transect by Sawaya and Braun (in prep) also detected a deficiency of the A mtDNA haplotypes among non-F_1 hybrids but not among potential F_1's in MO3 (P = 0.05 and 0.65, respectively; two-tailed Fisher's exact test; Table 15). There was no evidence of a deficiency of either species' haplotype among non-F_1 or potential F_1 hybrids in the hybrid zone in West Virginia (P = 1.00 in each case, Fisher's two-tailed exact test; Table 15). In VA2, there was a significant deficiency of the A haplotype among potential F_1's (P = 0.0001, Fisher's two-tailed exact test), while the A haplotype was well represented among non-F_1 hybrids. No potential F_1's were detected in VA3, and neither haplotype was deficient among

non-F_1's there.

Sawaya and Braun (in prep) found little or no evidence for assortative mating in this hybrid zone in Missouri; the correlation of the proportion of P. atricapillus alleles in males versus females for mated pairs of birds, utilizing only diagnostic loci, did not provide strong evidence (r_s = 0.48, one tailed P = 0.10). Only WV3 of the Appalachian populations had an adequate representation of both species' alleles to allow a test of this hypothesis, and the correlation among mated pairs of birds in this population provided no evidence of assortative mating (r_s = -0.09).

Linkage disequilibrium estimates averaged highest along the Missouri and West Virginia transects in populations at the range interface between P. atricapillus and P. carolinensis, and were highest in those interface populations in which A and C allele frequencies were most evenly balanced (Fig. 15). The largest number of significant disequilibrium estimates were also found in these populations. Along the Virginia transect, disequilibrium averaged highest in VA4, but this was primarily due to the presence of one individual of predominantly B allele type. When this individual was removed, linkage disequilibrium still averaged higher here than in the two populations nearest the range interface (VA2 and VA3). However, the number of significant disequilibrium

estimates in VA2 and VA3 was now twice as great as those in VA4. All significant estimates of disequilibrium reflected a deficiency of repulsion gametes, with one exception in VA4 and one in MD.

Cline structure and genetic introgression.

The frequency of A and C type alleles for each marker changed abruptly in a stepped pattern along all three transects (Table 12, Figs. 16-18). Cline position, as defined by the steepest slope of the smoothing spline fit to the data, differed significantly among marker loci in all three transects (Table 16; ANOVA $F = 128.7$, 152.7, and 197.8 respectively for Missouri, West Virginia and Virginia, $df = 9$ and 1990, $P < 0.0001$). In the Missouri transect, only one marker differed significantly in cline position from the others (Fig. 19, Tukey test, $P < 0.05$). In the West Virginia and Virginia transects, many of the pairwise multiple comparison tests revealed significant differences in cline position (Fig. 19, Tukey test, $P < 0.05$). However, cline positions within a transect never differed by more than 20 km, and often differed by only a few kilometers, along a scale measured in several hundreds of kilometers. Thus, molecular clines were strongly coincident in position among markers within all three transects, and were also coincident with the range interface as judged by vocal and morphological characters (Sawaya and Braun in prep, this

study).

Estimates of cline width varied more than did estimates of cline position. Some clines were steep and narrow; others were broad and flat. Width estimates ranged from 11.0 to 143.7 km. Cline width differed significantly among marker loci within each transect (ANOVA $F = 212.4$, 240.1, and 271.1 respectively for Missouri, West Virginia, and Virginia, $df = 9$ and 1990, $P < 0.00001$). Numerous significant differences in cline width between markers occurred within each transect (Fig. 20, Tukey test, $P < 0.0001$). Furthermore, certain classes of markers showed consistent patterns in cline width (Table 17, Tukey test, $P < 0.05$). Cline widths for mitochondrial haplotypes were significantly narrower than for autosomal loci in most cases (Table 17). The same was true in comparing the sex-linked loci GDA and DPAC121 to autosomal loci. The third sex-linked locus, ACON, showed a weaker trend towards narrower cline width relative to autosomal loci, principally in West Virginia (Table 17). Cline width was relatively homogeneous among the mtDNA and sex-linked loci, except for several cases in which ACON was significantly wider than the others (Table 17, Fig. 20). Among the autosomal markers, there was greater heterogeneity in cline width (Table 16). One trend evident, however, was for the two fixed markers (ski and DPAC7) to exhibit narrower cline widths than the non-fixed

autosomal loci (Table 17).

Several of the above trends are also evident from examining the maximum extent of introgression detected for markers (Table 18). Foreign alleles for mtDNA and the three sex-linked loci were not detected beyond 18 km of the range interface. In contrast, introgression of autosomal alleles was detected from 40 to 170 km from the range interface in several cases.

Consistent trends were evident in cline widths across markers among the three transects (Table 19, Tukey test, $P < 0.05$). For each of nine markers compared between the two Appalachian transects, the West Virginia cline width estimate was significantly wider than the Virginia cline width estimate. In comparing the Missouri transect with the two Appalachian transects, cline width estimates in Missouri were significantly wider than in Virginia in most cases. No consistent pattern was seen in comparison of Missouri and West Virginia transects. The topographic landscape of each transect was examined to see if there is an association between cline width and elevational gradient. Elevation changes abruptly along the Virginia transect, with an increase from 450 m to 600 m occurring over about 1 km (Fig. 21). The border of this interface is also relatively linear. In contrast, this same elevational change from 450 m to 600 m at the West Virginia interface occurs over a

minimum of 5 km, and the border of the interface is substantially more interdigitated (Fig. 22). The elevational change across the interface of the Missouri transect is not appreciable.

To further explore the association of cline width with ecological factors, we examined the correlation of the proportion of diagnostic P. atricapillus alleles in each population with the average elevation at which individuals in these populations were collected. For the 12 populations of the Virginia and West Virginia transects, $r_s = 0.77$. Elevation is related to geographic distance from the range interface, however, as is allele frequency, so such a correlation may or may not represent a cause-and-effect relationship. A more meaningful correlation might be one in which allele frequency is associated with elevation independent of distance from the range interface. Elevation varied sufficiently and A and C alleles were each sufficiently common within WV3 and VA2 to test for an association between the proportion of A type alleles in an individual and both their proximity to the range interface and the elevation at which they were collected (Table 20). In VA2, immediately east of the range interface, the subpopulation closest to the interface did not differ in proportion of A alleles from the subpopulation further from the range interface. In contrast, the VA2 subpopulation

collected at a higher elevation had a significantly higher proportion of type A alleles than did the subpopulation collected at lower elevation (Table 20, $t = -4.26$, $df = 13$, $P < 0.001$). In WV3 which straddles the range interface, high and low elevation subpopulations did not differ in allele frequency. However, the subpopulation closer to P. atricapillus populations in the Appalachian Mountains had a significantly higher proportion of A alleles (Table 20, $t = -6.36$, $df = 13$, $P < 0.001$). Allele frequency was not significantly correlated with distance relative to the range interface for individuals in either VA2 or WV3.

Discussion

Hybridization and introgression.

Hybridization at this contact zone is ongoing and consistently high at all three study sites, with little evidence to support the occurrence of assortative mating. Most hybrids represented backcross or later-generation hybrids, but introgression at the loci studied was limited geographically to a relatively narrow region. All markers exhibited sharp step clines in allele frequency, and of the diagnostic markers, only ski displayed substantial introgression beyond 20 km of the range interface. In addition to four markers found fixed for either A or C alleles in all allopatric populations of P. atricapillus and

P. carolinensis, the RFLP ski was also considered to be diagnostic in assessing the genetic ancestry of these two species, in spite of A alleles detected in allopatric populations OH and MD. For several reasons, we believe these alleles are the result of introgression from P. atricapillus and not merely an ancestral polymorphism retained in P. carolinensis. First, the frequency of A alleles in OH and MD was significantly higher or nearly so ($P = 0.005$ and $P = 0.07$ respectively; Fisher's exact text) relative to their absence in VA and LA, suggesting that sampling error is not the cause of these differences. This frequency difference suggests the possibility that introgression led to their presence in OH and MD. Second, the occurrence of long-distance introgression at ski, which is autosomal, is consistent with the greater cline width among autosomal loci in this hybrid zone relative to sex-linked loci and mitochondrial haplotypes (see above). Finally, the presence of A alleles in OH and MD, the two allopatric P. carolinensis populations closest to the range interface, is consistent with the possibility of past introgressive hybridization from the north. Historical records indicate that this hybrid zone has been advancing north in Ohio since at least the mid-1800's (Peterjohn 1989) and in Pennsylvania in recent decades and perhaps much earlier (P. Hess in prep). This historical advance makes it

likely that the range interface was closer to OH and MD in the past, increasing the possibility for introgression of P. atricapillus alleles into those populations.

Three of the "non-diagnostic" markers were fixed at one end of the transects and provide additional information on introgression levels. Allopatric P. carolinensis populations were all fixed for the C ACON allele. The presence of the A ACON allele in individuals characterized as pure P. carolinensis by the five diagnostic markers therefore likely represents introgression from P. atricapillus. No such birds were identified, and A ACON alleles were found no further than 16 km on the P. carolinensis side of the hybrid zone.

Allopatric P. atricapillus populations were likewise fixed for the B allele at DPAC102 and DPAC104. At DPAC102, no birds characterized as pure P. atricapillus by the diagnostic markers possessed a C allele, and such DPAC102 C alleles were found no further than 8.5 km on the P. atricapillus side of the hybrid zone. In contrast, at DPAC104, 14 birds characterized as pure P. atricapillus by the diagnostic markers possessed a C allele, and C alleles were found in every population on the P. atricapillus side of the hybrid zone, save the parentals, to a distance of 73 km in WV1. While rampant introgression is not occurring across this hybrid zone at many surveyed loci, both ski and

DPAC104 reveal a moderate level of relatively long-distance introgression between these species.

The other six autosomal RFLP's are not fixed in either species. This might result from the retention of ancestral variation at these loci. However, long-distance introgression is another potential explanation. For five of these six, "foreign" alleles are found as far as 950 km from the hybrid zone, in LA. Rates of gene flow for birds are relatively unknown, but are likely high relative to most vertebrates. Even for relatively sedentary species such as chickadees, it seems plausible that alleles could travel hundreds of kilometers, given sufficient time. Once neutral markers recombine and segregate away from any loci under negative selection in hybrids, their introgression will be unhindered by hybrid unfitness, and alleles experiencing positive selection could introgress more rapidly yet (Barton and Gale 1993). The finding of a large number of strongly differentiated RFLP's in these chickadees, in contrast with the paucity of completely fixed differences found, might be the result of a low degree of long-distance introgression operating over time.

The absence of the A haplotype among F_1's in VA2 might indicate an asymmetry in the direction or success of matings that produce F_1's, as is sometimes observed (e.g. Kaneshiro 1990, Paige et al. 1991, Konkle and Philipp 1992, Patton and

Smith 1993). More likely, it represents a spurious result due to the small sample sizes analyzed. If this deficiency reflected a mating asymmetry favoring crosses between P. carolinensis females and P. atricapillus males, the A haplotype should also be deficient among backcross hybrids. It was not. The deficiency of A type mtDNA in later-generation hybrids in MO3 is also probably due to sampling error. The alternatives are that there is selection against P. atricapillus backcross hybrids only in MO3, or that there is an asymmetry in the direction of backcrossing here, but not in the other transects. The disequilibrium (D) values of Asmussen et al. (1987) detected strong cytonuclear disequilibria in both MO3 and WV3, where A and C alleles were each sufficiently represented to test for such association (data not shown). These disequilibria suggest the presence of strong assortative mating or selection against hybrids. Such disequilibrium statistics, however, assume that populations are closed to immigration (Asmussen et al. 1989), an assumption almost certainly violated here. The cytonuclear disequilibria observed, like the nuclear disequilibria seen in the center of each transect, are probably generated by continuing immigration of parental genotypes into the center of the hybrid zone.

Variation in cline width among markers.

MtDNA.- Mitochondrial haplotypes exhibited a narrower

cline along all three transects than most autosomal loci and showed minimal levels of introgression, a trend counter to that established from many other observed cases of mitochondrial introgression. The first recorded instances of mtDNA crossing species' boundaries were surprising because of the scale on which they occurred. At the Mus musculus/M. domesticus hybrid zone in Denmark, one mitochondrial genome completely replaces the other (Ferris et al. 1983). In the case of hybridization between Drosophila pseudoobscura and D. persimilis, Powell (1983) found only limited mitochondrial divergence in areas of sympatry, in contrast to extensive mitochondrial divergence in allopatry, and proposed mitochondrial introgression as the mechanism producing this pattern. Other instances of apparently wide scale exchange of mitochondrial genomes between taxa due to hybridization have since been reported in frogs (Spolsky and Uzzell 1985), Drosophila (Solignac and Monnerot 1986), deer (Carr et al. 1986), and voles (Tegelström 1987). This phenomenon has led to the speculation that the mitochondrial genome may be more susceptible to crossing species barriers, possibly because it is not physically linked to the nuclear genome (Barton and Jones 1983, Takahata and Slatkin 1984). The frequent observation of interspecific mitochondrial transfer might also be a function in part, however, of its ease of

detection (Avise et al. 1984, Barton and Hewitt 1989, Degnan 1993). Because the mitochondrial genome is essentially non-recombining, evidence of its interspecific transfer will be preserved, whereas recombination can quickly erase the effects of nuclear introgression. Such might especially be the case where the introgressive event occurred in the past, as appears true in many cases (Aubert and Solignac 1986, Gyllensten and Wilson 1987, Tegelström 1987, Marchant et al. 1988, Dowling and Hoeh 1991, Ballinger et al. 1992, Degnan and Moritz 1992, Dowling and DeMarais 1993). Wilson et al. (1985) have also suggested that widespread replacement of one species' mitochondrial haplotype by another is often a result of founder events related to the fact that the effective population size for mitochondrial genes is one quarter that of autosomal genes. They predicted that rates of introgression for mitochondrial genes, once across a hybrid zone, would not differ appreciably from those of autosomal genes.

A relatively small number of hybrid zones have been analyzed so as to allow comparison of mitochondrial and autosomal introgression rates away from the immediate zone of hybridization. One hybrid zone in which long-distance mitochondrial introgression has been found occurs between the field crickets Gryllus firmus and G. pennsylvanicus (Harrison et al. 1987). They emphasized, however, that

rates of introgression for many autosomal loci might be comparable to that for mtDNA in hybrid zones where relatively few autosomal loci contribute to genetic isolation and influence the rate of introgression for autosomal loci to which they are physically linked (Barton 1979, 1983). Several other hybrid zone analyses have found comparable levels of mitochondrial and autosomal introgression (Szymura and Barton 1986, Nelson et al. 1987, Baker et al. 1989, Parsons et al. 1993). In the Mus hybrid zone, while domesticus mtDNA is found throughout populations both south and north of the hybrid zone, different domesticus haplotypes exist on either side of the hybrid zone,with a steep cline between them (Vanlerberge et al. 1988). This favors the hypothesis that the domesticus mtDNA introgression is a result of a past founder event, and does not indicate that the mtDNA introgression across the contact zone is free. Vanlerberge et al. (1988) favored sex-related differences in dispersal rate as the cause, although evidence for lower dispersal rate of females relative to males in Mus is ambiguous (reviewed in Vanlerberge et al. 1988). Mitochondrial introgression between M. mus and M. domesticus at another portion of the hybrid zone in Bulgaria is predominantly into domesticus, the direction opposite that found in Denmark (Vanlerberge et al. 1988). This was taken as further evidence that the smaller effective

population size of mitochondrial loci relative to autosomal loci can influence introgression patterns, increasing the possibility that genetic drift will affect allele frequencies in populations.

Genetic drift is an unlikely explanation for the narrow mitochondrial clines observed in this chickadee hybrid zone. Cline structure is consistent among the three independent transects, and effective population size in songbirds is typically large enough to rule out stochastic processes (Barrowclough 1980). Differential dispersal between the sexes can also be ruled out as producing the differential introgression; dispersal in P atricapillus, in other members of the genus Parus, and in birds in general, is greater for females than for males (Greenwood et al. 1978, Weise and Meyer 1979, Greenwood 1980, Nilsson 1989), which would favor greater mitochondrial introgression. Differences between mitochondrial and autosomal genes in their transmission genetics is a third factor that can be eliminated as producing the observed differences in their cline structure. Due to the maternal inheritance of the mitochondrial genome in animals and its haploid nature, only half an allele per individual on average is transmitted, compared to an average of two alleles per individual for autosomal genes. In itself, this has the potential to result in a four-fold greater flow of autosomal alleles across the hybrid zone

relative to mitochondrial alleles, and might produce a narrow mitochondrial cline relative to autosomal loci. This is offset, however, by the four-fold smaller population size of mitochondrial genes relative to autosomal genes. Thus, the effective contribution per dispersing individual for the two types of genes is the same.

There is another significance to the maternal inheritance of mtDNA that we believe is relevant to levels of mitochondrial introgression in this hybrid zone. Because females are the heterogametic sex in birds, they are the sex whose fitness is more likely to be adversely affected by hybridization (Haldane 1922). Known as Haldane's rule, this phenomenon provides an obvious mechanism for limiting introgression of maternally-transmitted traits such as the mitochondrial genome. Evidence of hybrid unfitness for either sex in these chickadees is currently anecdotal (Brewer 1963, C. Bronson unpubl data). An indirect test for Haldane's rule revealed a nearly significant deficiency of females among potential F_1's in MO3 and a non-significant trend in this direction for the West Virginia transect. However, a significant deficiency of males, not females, among potential F_1's occurred in the Virginia transect. Sample sizes are small in each case, so the power of our tests is low. In VA2 where a trend counter to Haldane's rule was observed, type A alleles predominate; one might

question if putative F_1's here are more likely to be misclassified later-generation hybrids than in WV3 and MO3 .

Haldane's rule has considerable support, including in birds and butterflies, in which females are the heterogametic sex (reviewed in Coyne and Orr 1989; but see Read and Nee 1991, 1993, Brookfield 1993). A recent example of Haldane's rule and support for its role in limiting mitochondrial introgression in a bird hybrid zone comes from pied and collared flycatchers (Ficedula hypoleuca and F. albicollis) (Gelter et al. 1992). Here, mitochondrial divergence is the highest that has been reported for intrageneric avian comparisons, while their nuclear differentiation is low. Thus, it is proposed that sex-biased gene flow resulting from Haldane's rule has produced this differential level of mitochondrial and nuclear divergence (Tegelström and Gelter 1990). In the Ficedula case, clinal variation across the hybrid zone has not been examined to determine whether levels of mitochondrial and nuclear introgression differ. Alternative explanations of this pattern include variation in assumed rates of molecular divergence and mitochondrial transfer from a third species.

While we believe that sex-biased gene flow resulting from Haldane's rule best explains the observed noncongruence in mitochondrial and nuclear clines, we cannot eliminate variation in selection level among loci as the mechanism.

The lack of physical linkage between the mitochondrial and nuclear genomes could allow them to introgress more freely than nuclear genes (Barton and Jones 1983, Gyllensten et al. 1985). The intensity of selection the mitochondrial genome experiences in a foreign background, however, should depend on levels of divergence between the mitochondrial haplotypes and the nuclear backgrounds with which they must interact. Such interactions have been shown to be adversely affected when taxa are sufficiently divergent (reviewed in Moritz et al. 1987). Birds typically exhibit smaller protein and mitochondrial genetic distances than most non-avian vertebrates of comparable taxonomic rank (Avise et al. 1980, Kessler and Avise 1985), however, and neither mitochondrial nor nuclear divergence between these chickadees is particularly high (see Results); if mitochondrial/nuclear interactions were a significant factor influencing levels of mitochondrial introgression in general, we would expect to see many more cases of reduced mitochondrial introgression between hybridizing non-avian taxa that are significantly more differentiated than these chickadees.

Sex-linked loci.- Like mitochondrial haplotypes, Z-linked loci exhibited narrower clines and reduced levels of introgression relative to autosomal markers along all three transects across this hybrid zone. Limited sex-linked introgression has also been reported in hybrid zones for

mice (Vanlerberge et al. 1986, Tucker et al. 1992, Dod et al. 1993), grasshoppers (Moran 1979, Ferris et al. 1993), butterflies (Hagan and Scriber 1989, Hagan 1990), and water striders (Sperling and Spence 1991), but is detected here for the first time in an avian hybrid zone. These observations imply that there is strong selection against the introgression of sex-linked genes, and that they play an important role in maintaining the genetic integrity of the hybridizing taxa. Also supportive of the importance of sex chromosome divergence to reproductive isolation between P. atricapillus and P. carolinensis is the greater representation of differentiation on their sex chromosomes, relative to the autosomes. Of the 13 identified nuclear markers strongly differentiating P. atricapillus and P. carolinensis, three, or 23.1% appear to be sex-linked. The Z chromosome represents roughly 5-6% of the genome in the genus Parus (Hammar 1970), so detected differentiation between these taxa on the Z chromosome is four to five times higher than expected by chance. This is probably a conservative estimate; our cloned library of chickadee DNA represented equal proportions of DNA from one male and one female individual, so the Z chromosome was under-represented with respect to autosomes by 25%. The importance of sex chromosome differentiation between P. atricapillus and P. carolinensis specifically is also apparent from a

consideration of patterns of allozyme differentiation. Two loci, ACON and CK, have been reported previously to be sex-linked in birds (Baverstock et al. 1982, Morizot et al. 1987, Lacson and Morizot 1988), while we have also found evidence for the sex-linkage of GDA in these chickadees (Sawaya 1990, present study, Sawaya and Braun in prep). Thus, 67% (2/3) of surveyed sex-linked loci show strong differentiation, compared with 4.6% (2/43) of all loci screened in these birds (Braun and Robbins 1986, Gill et al. 1989). The general prominence of sex-linked genes in maintaining species boundaries is also suggested by the susceptibility of the heterogametic sex to hybrid sterility and inviability (Haldane's rule), and by laboratory crosses that introgress chromosomes or portions of chromosomes into foreign genetic backgrounds to assess their affects on fitness (both reviewed by Coyne and Orr 1989).

Two primary mechanisms have been offered to explain how sex-linked loci contribute disproportionately to hybrid unfitness. In the first, sex chromosome divergence proceeds more rapidly than autosomal divergence due to lower population size for sex-linked loci and greater exposure to selection of recessive alleles in the hemizygous state (Charlesworth et al. 1987). In hybrids, these divergent loci are then postulated to pleiotropically reduce fitness as they interact with each other and with autosomal loci.

The second model proposes that divergence of meiotic drive elements of sex ratio distorters results in disadvantageous X/Y (Z/W in birds and butterflies) interactions that reduce fitness of hybrids (Frank 1991, Hurst and Pomiankowski 1991). We propose that either one of these mechanisms could be producing the selection limiting the movement of sex-linked markers across the chickadee hybrid zone. Disruption of dosage compensation has been offered as another mechanism whereby sex-linked genes might contribute to hybrid dysfunction, but the apparent lack of dosage compensation in birds is one of a number of reasons making it an unlikely cause (Baverstock et al. 1982, Coyne and Orr 1989).

Selection need not be acting directly on these sex-linked markers; they may be responding to selection on loci they are tightly linked to physically. The probability of such hitchhiking is enhanced by the lack of recombination on the sex chromosome in the heterogametic sex. Dod et al. (1993), while finding that all X- and Y-linked loci introgressed to a more limited extent than mitochondrial haplotypes and autosomal loci, also observed variation in the extent of introgression among the three X-linked markers studied, which spanned a 45 cM portion of the X chromosome. We, too, observed variation in extent of introgression among our three sex-linked loci, with ACON exhibiting greater introgression in two of the three transects. Linkage

relationships for these loci are unknown. However, we observed no recombinant genotypes for GDA and DPAC121, in contrast to ten recombinant genotypes between ACON and these two loci, supporting the potential for ACON to behave independently of GDA and DPAC121.

Autosomal loci.- We likewise observed variation in the extent of introgression among the ten autosomal markers. For instance, the two fixed markers (ski and DPAC7) tended to have narrower cline widths then non-fixed markers. Loci under selection are more likely to become fixed, so markers that are not completely differentiated between two taxa should have the potential to introgress to a greater extent then diagnostic markers. This underscores the caution that should accompany the use of fixed differences in assessing levels of introgression across a hybrid zone. Such estimates may not accurately portray the potential of two taxa to mix genetically. Neutral markers will be hindered from crossing a species' boundary only until they recombine and segregate away from loci experiencing negative selection in hybrids, and alleles experiencing positive selection should be able to sweep across even a strong selective barrier (Barton 1979a, b, 1983, Barton and Hewitt 1983). Many other differences were also noted in cline width among autosomal markers. Comparison of introgression levels among multiple autosomal markers in both the Mus and Bombina

hybrid zones has revealed a marked uniformity in cline width, in contrast to our results (Szymura and Barton 1986, 1991, Vanlerberge et al. 1988). This congruence in autosomal cline widths has been attributed in both cases to physical linkage and genetic hitchhiking among a large number of genes under selection against introgression and those loci in close proximity to selected genes. Extensive morphological and behavioral differentiation between hybridizing Bombina species, and congruence of clines for these traits with molecular clines, has been offered as additional evidence for the involvement of many genes (N = 55) in contributing to species differences in Bombina (Szymura and Barton 1991). The marked noncongruence among autosomal markers across this chickadee hybrid zone, in conjunction with the small level of genetic, morphological, behavioral, and ecological differentiation between P. atricapillus and P. carolinensis (this study, Brewer 1963) may suggest that a smaller number of genes contribute to their reproductive isolation.

Hybrid zone origin and dynamics.

We found congruence in cline position along each of the three transects among the 14 markers, which represent mitochondrial, sex-linked and autosomal loci, allozymes, cloned genes and random RFLP's. Clinal variation in morphological and vocal characters also exhibited sharp

steps along each transect in positions congruent with the molecular clines. Such extensive congruence among a suite of different characters is most readily explained as a result of secondary contact along a common boundary. Alternative explanations must invoke either common selection regimes (Endler 1977) or a clumping of these clines by selection as a result of linkage disequilibrium between these loci (Barton 1983).

The fate of a hybrid zone has an important bearing on its evolutionary significance. Many hybrid zones possess narrow widths relative to the potential dispersal ability of the hybridizing taxa, and some stabilizing force is implicated. Under a neutral diffusion model following secondary contact, the number of generations since contact can be estimated as $T = 0.35\ (w/l)^2$, where w is the hybrid zone's width and l is root-mean-square dispersal distance (Endler 1977). Taking an average width of the autosomal RFLP clines as 60 km, and a dispersal estimate for P. atricapillus at about 1 km per generation, based on mark/recapture data (Weise and Meyer 1979), the age of this hybrid zone is estimated as 1260 years old. Dispersal estimates for birds based on mark/recapture data may underestimate actual dispersal distances by an order of magnitude or more, because of the bias against long-distance recoveries, which greatly influence estimates (Moore and Dolbeer 1989, Baker et al.

1995). If chickadee dispersal rates are only twice that currently estimated, neutral diffusion of autosomal RFLP's would predict an age for this hybrid zone of about 300 years. We have no reliable means of dating the age of this hybrid zone beyond the 150 years or so of recorded history on their distributions. But given the paleoecological history of eastern North America (Delcourt and Delcourt 1987, Pielou 1991, Prentice et al. 1991), it is reasonable to suppose that P. atricapillus and P. carolinensis differentiated following isolation brought on by changes is forest distribution during cycles of glaciation, and that their hybrid zone formed no earlier than about 10,000 years ago following the last glacial maximum. Some form of selection is therefore implicated in restricting the width of this hybrid zone. Without long-term detailed information on its structure, we cannot rule out the possibilities that it is either widening or narrowing very slowly. Reinforcement of premating reproductive isolation as a mechanism for restriction of the hybrid zone's width appears improbable, given the lack of evidence for assortative mating despite the likely length of contact between P. atricapillus and P. carolinensis. Thus, given a relatively old contact between these chickadees and their dispersal potential, a reasonable supposition is that this hybrid zone is stable in its structure, despite its recorded changes in

position.

Limited observations of nesting success suggest that substantial hybrid unfitness occurs in this hybrid zone (Brewer 1963, C. Bronson unpubl. data), but this conclusion needs verification. Our indirect evidence for the operation of Haldane's rule was inconclusive (see above). Populations did not deviate significantly from Hardy-Weinberg equilibrium, but our sample sizes limited our ability to detect such differences. All significant deviations reflected a deficiency of heterozygotes, and among non-significant cases, heterozygote deficiencies exceeded heterozygote excesses only in central populations MO3, WV3, VA2 and VA3. These trends are consistent with the operation of selection against hybrids. Dispersal of parental genotypes into the hybrid zone's center, which is the likely explanation for the strong linkage disequilibria there, could also be producing a Wahlund effect, creating a heterozygote deficiency. Given a lack of evidence for assortative mating, there should be a homogenizing effect on allele frequencies in the hybrid zone. The observance of strong linkage disequilibrium thus suggests that selection against hybrids balanced by dispersal of parentals into the hybrid zone helps to maintain this hybrid zone. More direct evidence on the fitness of hybrids between P. atricapillus and P. carolinensis is necessary, however, to verify that

this hybrid zone exhibits characteristics of a tension zone.

The operation of endogenous selection in this hybrid zone would not exclude an exogenous component to selection; ecological selection might lead to coadaptation of gene complexes within each taxon, the disruption of which could result in endogenous selection against hybrids (Hewitt 1988). We observed variation in the width of this hybrid zone that showed some correlation with the steepness of the elevational transition across transects. Narrow cline widths in Virginia were associated with a steep elevational change there. Variation in hybrid zone width associated with ecological variables is not uncommon (Yang and Selander 1968, Hunt and Selander 1973, Cook 1975, Bert and Harrison 1988, Búno et al. 1994), and selection along an environmental gradient is typically inferred from this association. While suggestive, such a correlation does not formally demonstrate that an ecological selection gradient must be shaping the hybrid zone's structure, and the correlation was incomplete. Cline widths were similar between West Virginia and Missouri in spite of a much steeper altitudinal change across the West Virginia transect. Differences in dispersal rate associated with the steepness of an elevational/ecological gradient could also produce such a correlation. However, such differences in dispersal rate would imply that habitat selection is

occurring. Habitat selection itself suggests that an exogenous selection factor has produced the habitat preference, although a current habitat preference need not mean that the selection responsible for producing it currently operates. But a possible correlation between hybrid zone structure and an ecological gradient would seem at a minimum to implicate habitat selection as a factor influencing the hybrid zone's dynamics, and the operation of exogenous selection itself as a force contributing to the hybrid zone's maintenance might be a reasonable inference.

A correlation in VA2 between A allele frequency and elevation lent strong support to the supposition that altitude itself or related variables play a role in structuring this hybrid zone. This correlation suggests that on a relatively small scale, this chickadee hybrid zone exhibits characteristics of a mosaic hybrid zone (Rand and Harrison 1989, Howard and Waring 1991). Such mosaicism can result either from the direct effect of selection, from habitat selection, or a combination of the two. It is not expected to result from endogenous selection, however, as in a strictly tension zone model.

Also supportive of habitat selection and/or exogenous selection in this hybrid zone were fine-grained details we noted in the distribution of genotypes in the Virginia transect. VA2, collected on top of and on the eastern flank

of the eastern-most ridge of the Appalachian Mountains, contained only P. atricapillus and hybrid individuals. VA3, confined to the floor of the Shenandoah Valley, was nearly continuous with VA2, with only a gap of a few kilometers separating their borders. Only P. carolinensis and hybrid individuals were collected in VA3. VA4 was collected on top of a ridge system in the eastern side of the Shenandoah Valley, and immediately west of the Blue Ridge. VA4 contained P. carolinensis and hybrid individuals, with the exception of one individual that is pure P. atricapillus on the basis of our markers. A second bird singing only P. atricapillus song was also heard in VA4, and breeding of P. atricapillus at the higher elevations of the Blue Ridge in Shenandoah National Park is suspected on the basis of occasional individuals singing P. atricapillus song during the breeding season (Abbott 1986). While of an anecdotal nature, these observations suggest that P. atricapillus is choosing to occupy high elevation locations and/or is fitter than P. carolinensis in these environments. Other isolated populations of P. atricapillus within the range of P. carolinensis are established in two locations in southwest Virginia (Scott 1982, Peake 1987), and a disjunct population of P. atricapillus occurs in the Smoky Mountains (Tanner 1952). These isolated populations are found on especially high mountain tops in the region. Tanner (1952) also noted

that when P. atricapillus is not present at other such high altitude locations, P. carolinensis typically ranges to the top of these mountains. This pattern of replacement suggests that it is not merely habitat selection that governs these chickadees's distributions, but that competition in tandem with exogenous selection occurs.

Finally, the large-scale distributional pattern of P. atricapillus and P. carolinensis in the eastern U.S. is more readily explained as being the result of exogenous selection. The range of P. atricapillus extends southward in a peninsular fashion through the Appalachian Mountains, bisecting the range of P. carolinensis, which occupies the lower elevations on either side of the mountains. Modelling of tension zones predicts that such zones will strengthen and minimize their length. Such pronounced curvature to a hybrid zone therefore suggests that the environment is playing a more direct role in its dynamics (W. Moore pers comm). Only a density trough trapping the hybrid zone along the entire foot of the Appalachian Mountains and not elsewhere could provide another explanation for this striking range distribution.

Taxonomic considerations.

Despite vocal and morphological evidence of intermediacy between P. atricapillus and P. carolinensis along much of their range interface, their specific status has not

traditionally been questioned seriously for a number of reasons. First, it has been suspected that song is not a reliable indicator of species status in these birds at their range interface because of their ability to learn elements of each others song in the laboratory (Kroodsma et al. 1995), a hypothesis we have subsequently confirmed (Sawaya 1990, Sawaya and Braun in prep, Chapter 3). In addition, morphological analyses have not detected evidence of substantial genetic introgression between them (Rising 1968, Robbins et al. 1986). Finally, narrow gaps have been reported between their ranges in two locations (Tanner 1952, Brewer 1963, Merritt 1978, 1981) that suggest reproductive isolation. The limitations of these data have been recognized, however, and only direct measures of introgression levels can address this taxonomic question.

We have now examined the structure of this hybrid zone at three locations with 14 strongly differentiated genetic markers. Introgression at some of these loci is quite limited, encompassing a small fraction of the species' total range. The extent of introgression at other loci, however, is more substantial, and indicates that these alleles are not experiencing strong selection against their incorporation into a heterospecific genetic background. Hybrid zone theory predicts such semipermeability at different loci as a function of the strength of selection

against their introgression (Bazykin 1969, Barton 1979, 1983). Neutral alleles, as they recombine or segregate away from loci experiencing negative selection against introgression, should be able to pass relatively unhindered into a foreign genome. Yet other alleles, experiencing positive selection, should sweep unimpeded from one species to the other. In this manner, a hybrid zone might function as an evolutionary conduit for globally adaptive gene exchange between two taxa (Parsons et al. 1993). Introgression at such neutral or positively selected loci will be difficult to detect, however. These alleles, if detected at an intermediate frequency, will be difficult to distinguish from ancestral polymorphisms, whereas if they become fixed in the foreign genetic background, they will not be detected because of their monomorphic state.

Given the confirmation of substantial introgression between these chickadees at some loci, and the potential for more rampant, undetected introgression at others, what taxonomic status should be accorded to taxa in such cases? One's operational definition of a species will necessarily influenced the answer. The biological species concept (BSC) can either be interpreted strictly as requiring absolute reproductive isolation (Barton and Hewitt 1983), or can allow for incomplete reproductive isolation, such as occurs across a stable hybrid zone (Mayr 1982). A recognized

weakness of the BSC, therefore, is its subjective nature in dealing with such cases of hybridization. A second criticism of the BSC is that when two taxa are given conspecific status on the basis of incomplete reproductive isolation, non-sister taxa can be combined in this label, resulting in a paraphyletic assemblage whose component histories are obscured (Zink and McKitrick 1995). Proposed alternatives for defining species include the phylogentic species concept (PSC; Cracraft 1983, Nixon and Wheeler 1990) and the evolutionary species concept (ESC; Wiley 1981). Both emphasize the definition of entities that are logically consistent with the recovered history of evolution, though they differ operationally in how such entities are recognized (Frost and Hillis 1990).

In theory, these two types of species concepts will produce different results. But whether they would give concordant results or not in practice is not clear (Zink and McKitrick 1995). In the case of P. atricapillus and P. carolinensis, it could be argued on the basis of the detected diagnostic differences they are separate evolutionary units. The PSC would therefore recognize them as distinct species. Application of the BSC is not as straightforward. Our results reveal the existence of a general barrier to gene flow, but with the property of semipermeability, and at least the potential for some

alleles to cross the hybrid zone relatively freely. The issue of how to apply the BSC in such cases has not been fully addressed. A proposed solution has been offered by Avise and Ball (1990), who suggested that species be recognized on the basis of intrinsic reproductive barriers that signal essentially irreversible evolutionary isolation. Subspecific status only would be accorded to taxa that exhibit phylogentic distinction, but which are reproductively compatible because the barriers to gene flow between them are extrinsic, and so not as firmly established as intrinsic reproductive barriers between species.

Applying the criterion of Avise and Ball (1990), P. atricapillus and P. carolinensis would be maintained as distinct species, because the structure of their hybrid zone indicates the existence of a substantial intrinsic component to the reproductive barrier between them. The BSC and the PSC would agree in this case. Such would not be true in every application, however. Avise and Ball (1990) cited as an example of subspecific status under their criterion the bluegill sunfish (Lepomis macrochirus). Two phylogenetically distinct forms are largely allopatric, but come together and hybridize extensively in a secondary contact zone. The genetic evidence indicates that the two genomes are mixing freely here, suggesting that their reproductive isolation is extrinsically based only. Under

this proposed application of the BSC then, these sunfish would be ranked as subspecies, while the PSC would rank them as separate species.

Avise and Ball (1990) offered their solution as a good compromise that retains the philosophical framework of the BSC, while addressing concerns of the PSC that taxonomic practice might obscure the historicity of taxonomic units. Principles of the PSC would be used to designate subspecies in those cases where multiple phylogenetic units were included under a BSC label. Criticisms have been made of such a suggested synthesis, however (McKitrick and Zink 1988, Zink and McKitrick 1995). One objection is that under such a species concept, species would not be comparable evolutionary units, and so would be of little use to comparative biologists. Another criticism is the potential creation of paraphyletic groups not consistent with the recorded history of evolution.

The PSC holds that species should consistently be defined so as to be single evolutionary units, while the BSC maintains that reproductive isolation should be the hallmark that characterizes what is called a species. This marks a fundamental philosophical difference that has yet to be resolved.

Table 11. Sample size, year(s) collected, distance from P. atricapillus terminus of transect, and frequency of genotypic classes for populations comprising three transects. A, H, F_1, and C are the frequencies of apparently pure P. atricapillus, hybrid, putative F_1, and apparently pure P. carolinensis genotypes, respectively. F_1's are a subset of all hybrids.

Popu-lation	No. birds	Year(s)	Distance (km)[a]	A	H	F_1	C
West Virginia transect							
PA	20	1991	0	1.000	---	---	---
WV1[b]	20	1990	172.3	0.850	0.150	---	---
WV2	20	1990	227.7	0.850	0.150	---	---
WV3	31	1990, 1992	245.0	0.290	0.581	0.161	0.129
WV4	19	1990	261.0	---	0.579	0.053	0.421
WV5	19	1990	284.8	---	0.474	---	0.526
OH	20	1991	417.2	---	0.400	---	0.600
Virginia transect							
PA	20	1991	0	1.000	---	---	---
VA1[b]	20	1990	100.0	0.850	0.150	---	---
VA2	33	1989, 1991	153.6	0.545	0.455	0.121	---
VA3	24	1989	164.5	---	0.625	---	0.375
VA4	21	1989	171.4	0.047	0.286	0.095	0.667
VA5	20	1990	205.5	---	0.100	---	0.900
VA	21	1991	399.5	---	---	---	1.000
MD[c]	14	1990, 1993	289.0	---	0.214	---	0.786

Table 11 continued

Missouri transect

MO	20	1978	0	1.000	---	---	---
MO1	17	1980	218.0	1.000	---	---	---
MO2	14	1980	251.0	0.714	0.286	0.214	---
MO3	36	1978, 1980	259.5	0.278	0.444	0.167	0.278
MO4	21	1980	296.5	---	0.048	---	0.952
LA	21	1979	1209.0	---	---	---	1.000

[a] Distances are from population centroids estimated by eye. Population diameters spanned from a few kilometers to a few tens of kilometers. Because PA is displaced from the east/west orientation of the West Virginia and Virginia transects, the distnce from PA to WV1 was calculated by measuring the distance from PA to the closest point of the range interface in southwestern Pennsylvania, and subtracting the distance between WV1 and the range interface in West Virginia. All subsequent distances along this transect were measured relative to WV1. The same procedure was employed for the Virginia transect, except that distances were measured to the range interface in southeastern Pennsylvania and Virginia. Some distances in West Virginia and Virginia are adjusted because the transects were not completely perpendicular to the hybrid zone interface, and reflect straight line distances to the nearest portion of the range interface as determined from Breeding Bird Atlas data.

[b] WV1 and VA1 represent the same population.

[c] MD is not included as part of the Virginia transect in analysis of molecular clines, but its distance is calculated as for other populations of this transect.

Table 12. Allele frequencies with sample size for 14 markers in populations comprising three transects. A dash indicates the allele was not detected. Multiple alleles for P. atricapillus and P. carolinensis are numbered sequentially (A1, A2, A3, etc and C1, C2, C3, etc respectively), followed by a composite frequency for both type A and type C alleles. Sample size precedes allele frequency, including separate numbers for each sex (male/female) in the case of sex-linked markers (GDA, ACON, DPAC121). For RFLP markers, restriction enzyme (s) used and fragment sizes produced are also given.

Marker	Enzyme/ Fragment sizes (kb)	Population												
		OH	WV5	WV4	WV3	WV2	WV1/ VA1	PA	VA2	VA3	VA4	VA5	VA	MD[a]
MtDNA[b]		20	19	19	31	20	20	20	33	24	21	20	21	14
A		---	---	0.053	0.645	0.950	1.000	1.000	0.879	0.042	0.048	---	---	---
C[c]		1.000	1.000	0.947	0.355	0.050	---	---	0.121	0.958	0.952	1.000	1.000	1.000
GDA		17/3	11/8	11/8	21/10	13/7	13/7	14/5	17/16	17/7	14/7	14/6	16/5	4/3
A		---	---	0.067	0.596	1.000	1.000	1.000	0.940	0.049	0.114	---	---	---
C		1.000	1.000	0.933	0.404	---	---	---	0.060	0.951	0.886	1.000	1.000	1.000

Table 12 continued

ACON		17/3	11/8	11/8	21/10	13/7	13/7	14/4	17/16	17/7	14/7	14/6	16/5	4/3
A1		---	---	0.067	0.577	0.969	0.875	0.938	0.920	0.024	0.114	---	---	---
A2		---	---	---	---	---	---	---	---	0.024	---	---	---	---
C1		0.946	0.900	0.800	0.404	0.031	0.125	0.031	0.080	0.952	0.857	0.971	0.946	1.000
C2		0.054	0.067	0.100	0.019	---	---	---	---	---	---	---	0.027	---
C3		---	0.033	0.033	---	---	---	0.031	---	---	0.029	0.029	0.027	---
A		---	---	0.067	0.577	0.969	0.875	0.938	0.920	0.048	0.114	---	---	---
C		1.000	1.000	0.933	0.423	0.031	0.125	0.062	0.080	0.952	0.886	1.000	1.000	1.000
DPAC121	Pst I	17/3	11/8	11/8	21/10	13/7	13/7	14/6	17/16	17/7	14/7	14/6	16/5	10/5
A	3.2, 1.7, 1.2	---	---	0.067	0.596	1.000	1.000	1.000	0.940	0.049	0.114	---	---	---
C	5.3, 1.2	1.000	1.000	0.933	0.404	---	---	---	0.060	0.951	0.886	1.000	1.000	1.000
ski	Eco RI	20	19	19	31	20	20	20	33	24	21	20	21	15
A	8.0, 3.6, 2.6, 2.1	0.200	0.237	0.316	0.629	0.950	0.925	1.000	0.773	0.375	0.190	0.050	---	0.100
C	8.0, 3.6, 2.4, 2.1	0.800	0.763	0.684	0.371	0.050	0.075	---	0.227	0.625	0.810	0.950	1.000	0.900

Table 12 continued

DPAC1A	Bgl II	20	18	19	31	19	20	20	33	24	21	20	21	14
A1	11.0, 8.6, 4.5, 3.7	0.103	0.057	0.194	0.436	0.764	0.550	0.750	0.531	0.167	0.195	0.075	0.122	0.071
A2	9.4, 8.6, 4.5, 3.7	0.205	0.171	0.194	0.113	0.184	0.325	0.225	0.172	0.062	0.122	---	0.073	---
A3	8.6, 5.1, 4.5, 4.2, 3.7	---	---	---	0.016	0.026	0.025	---	0.016	---	---	---	---	---
A4	8.6, 5.9, 4.5, 3.7	---	---	---	---	---	---	---	---	---	---	---	---	---
A5	11.8, 8.6, 4.5, 3.7	---	---	---	---	---	---	0.025	---	---	---	---	---	---
C1	13.8, 8.6, 4.5, 3.7	0.692	0.772	0.612	0.419	0.026	0.100	---	0.265	0.771	0.683	0.925	0.805	0.929
C2	15.1, 8.6, 4.5, 3.7	---	---	---	0.016	---	---	---	0.016	---	---	---	---	---
A		0.308	0.228	0.388	0.565	0.974	0.900	1.000	0.719	0.229	0.317	0.075	0.195	0.071
C		0.692	0.772	0.612	0.435	0.026	0.100	---	0.281	0.771	0.683	0.925	0.805	0.929
DPAC1B	Pvu II	20	19	19	31	20	20	20	33	24	21	19	21	11
A1	9.8, 5.7, 2.6, 1.7, 1.4, 0.8	0.075	0.079	0.184	0.420	0.775	0.525	0.750	0.576	0.167	0.190	0.105	0.143	0.091
A2	8.1, 5.7, 2.6, 1.7, 1.4, 0.8	---	---	---	0.032	---	---	0.050	0.030	---	---	---	---	---

Table 12 continued

C1	12.4, 5.7, 2.6, 1.7, 1.4, 0.8	0.900	0.921	0.790	0.516	0.200	0.425	0.175	0.394	0.833	0.810	0.895	0.857	0.909
C2	11.0, 5.7, 2.6, 1.7, 1.4, 0.8	0.025	---	0.026	0.032	0.025	0.050	0.025	---	---	---	---	---	---
A		0.075	0.079	0.184	0.452	0.775	0.525	0.800	0.606	0.167	0.190	0.105	0.143	0.091
C		0.925	0.921	0.816	0.548	0.225	0.475	0.200	0.394	0.833	0.810	0.895	0.857	0.909
DPAC7	Ava II	20	19	19	31	20	20	19	32	24	21	20	12	13
A1	10.5, 3.2, 2.9, 1.8, 1.7, 1.5, 1.4	---	0.029	---	0.516	1.000	1.000	1.000	0.800	0.062	0.071	---	---	---
A2	10.5, 3.2, 2.3, 1.8, 1.7, 1.5, 1.4	---	---	0.028	0.049	---	---	---	0.092	---	0.048	---	---	---
C1	10.5, 7.2, 3.2, 1.8, 1.7, 1.5, 1.4	0.974	0.853	0.805	0.403	---	---	---	0.077	0.646	0.762	0.875	0.962	0.808
C2	10.5, 7.0, 3.2, 1.8, 1.7, 1.5, 1.4	0.026	0.118	0.139	0.032	---	---	---	0.031	0.271	0.119	0.125	0.038	0.192

Table 12 continued

C3	10.5, 5.8, 3.2, 1.8, 1.7, 1.5, 1.4	---	---	0.028	---	---	---	---	---	0.021	---	---	---	---
A		---	0.029	0.028	0.565	1.000	1.000	1.000	0.892	0.062	0.119	---	---	---
C		1.000	0.971	0.972	0.435	---	---	---	0.108	0.938	0.881	1.000	1.000	1.000
DPAC96A	Bgl II	20	19	19	31	20	20	20	33	24	21	20	21	14
A1	9.8, 4.0, 2.4, 1.5,1.2	0.050	0.079	0.158	0.597	0.925	0.975	0.975	0.862	0.250	0.238	0.100	0.050	0.036
A2	9.8, 4.0, 2.4, 1.5,1.2	---	---	---	---	---	---	---	---	0.021	---	---	---	---
A		0.050	0.079	0.158	0.597	0.925	0.975	0.975	0.862	0.271	0.238	0.100	0.050	0.036
C	9.8, 6.4, 1.5, 1.2	0.950	0.921	0.842	0.403	0.075	0.022	0.025	0.138	0.729	0.762	0.900	0.950	0.964
DPAC96B	Bgl II	20	19	19	31	20	20	20	33	24	21	20	21	14
C1	9.8, 5.1, 1.5, 1.2	0.375	0.395	0.395	0.098	0.050	---	---	0.106	0.333	0.333	0.282	0.405	0.536
C2	9.8, 5.3, 1.5, 1.2	0.050	0.053	---	---	---	---	0.025	---	0.042	0.048	0.077	---	0.107
A	9.8, 3.7, 1.5, 1.2	0.575	0.552	0.605	0.902	0.950	1.000	0.975	0.894	0.625	0.619	0.614	0.595	0.357
C		0.425	0.448	0.395	0.098	0.050	---	0.025	0.106	0.375	0.381	0.359	0.405	0.643

Table 12 continued

DPAC97	Pvu II	20	19	19	31	20	20	20	33	24	21	20	21	8
A	4.9, 2.0, 1.8, 1.6, 1.2, 1.0, 0.8, 0.7	0.225	0.237	0.184	0.661	0.900	0.975	0.975	0.828	0.312	0.262	0.175	0.095	0.188
C	5.2, 2.0, 1.8, 1.6, 1.2, 1.0, 0.8, 0.7	0.775	0.763	0.816	0.339	0.100	0.025	0.025	0.172	0.688	0.738	0.825	0.905	0.812
DPAC98	Pvu II	20	19	19	31	20	20	20	33	24	21	20	21	8
C1	3.3, 2.7, 2.2, 1.8, 1.4, 1.0	0.550	0.632	0.605	0.210	0.100	0.050	0.025	0.121	0.562	0.429	0.500	0.524	0.688
C2	3.3, 2.7, 2.2, 1.8, 1.4, 0.6, 0.4	---	---	---	---	---	---	---	---	---	---	---	---	---
C3	3.3, 2.8, 2.7, 2.2, 1.4	0.025	0.026	---	0.016	---	---	---	---	---	---	---	---	---
A	3.6, 3.3, 2.2, 1.8, 1.4	0.425	0.342	0.395	0.774	0.900	0.950	0.975	0.879	0.438	0.571	0.500	0.476	0.312
C		0.575	0.658	0.605	0.226	0.100	0.050	0.025	0.121	0.562	0.429	0.500	0.524	0.688

Table 12 continued

DPAC102	Bgl II	20	19	19	31	20	20	20	33	24	21	20	21	12
C1	7.9, 3.3, 1.7, 1.4	0.700	0.553	0.579	0.290	---	---	---	0.106	0.458	0.575	0.667	0.595	0.583
C2	7.9, 3.3, 1.7, 1.1	0.100	0.026	0.079	0.049	---	---	---	0.030	0.167	0.100	0.077	0.095	0.042
A	7.9, 3.3, 1.7, 1.5	0.200	0.421	0.342	0.331	1.000	1.000	1.000	0.864	0.375	0.325	0.256	0.310	0.375
C		0.800	0.579	0.658	0.339	---	---	---	0.136	0.625	0.675	0.744	0.690	0.625
DPAC104	Bgl II	20	19	19	31	16	20	20	33	24	21	19	21	14
A	8.0, 4.6, 4.0, 1.5, 1.3	0.125	0.211	0.211	0.597	0.906	0.975	1.000	0.848	0.188	0.262	0.184	0.071	0.037
C	8.0, 5.0, 4.0, 1.5, 1.3	0.875	0.789	0.789	0.403	0.094	0.025	---	0.152	0.812	0.738	0.816	0.929	0.963

Table 12 continued

Marker	Enzyme/ Fragment sizes (kb)	Population MO	MO1	MO2	MO3	MO4	LA
MtDNA[b]		20	17	14	36	21	21
A		1.000	1.000	0.929	0.417	---	---
C[c]		---	---	0.071	0.583	1.000	1.000
GDA		19/1	9/8	8/6	25/11	11/10	14/7
A		1.000	1.000	0.909	0.475	---	---
C		---	---	0.091	0.525	1.000	1.000
ACON		15/1	9/8	8/6	26/10	11/10	12/6
A1		1.000	0.962	0.864	0.484	---	---
A2		---	0.038	0.045	---	---	---
C1		---	---	0.091	0.500	1.000	1.000
C2		---	---	---	0.016	---	---
C3		---	---	---	---	---	---

Table 12 continued

A		1.000	1.000	0.909	0.484	---	---
C		---	---	0.091	0.516	1.000	1.000
DPAC121	Pst I	19/1	9/8	8/6	25/11	11/10	14/7
A	3.2, 1.7, 1.2	1.000	1.000	0.909	0.475	---	---
C	5.3, 1.2	---	---	0.091	0.525	1.000	1.000
ski	Eco RI	20	17	14	36	21	21
A	8.0, 3.6, 2.6, 2.1	1.000	1.000	0.821	0.500	0.024	---
C	8.0, 3.6, 2.4, 2.1	---	---	0.179	0.500	0.976	1.000
DPAC1A	Bgl II	20	17	14	36	21	21
A1	11.0, 8.6, 4.5, 3.7	0.575	0.441	0.464	0.206	0.024	0.024
A2	9.4, 8.6, 4.5, 3.7	0.375	0.324	0.357	0.338	0.143	0.167
A3	8.6, 5.1, 4.5, 4.2, 3.7	---	---	0.036	---	---	---
A4	8.6, 5.9, 4.5, 3.7	0.025	0.147	---	0.015	---	---
A5	11.8, 8.6, 4.5, 3.7	---	---	---	---	---	---

Table 12 continued

C1	13.8, 8.6, 4.5, 3.7	0.025	0.088	0.143	0.441	0.833	0.809
C2	15.1, 8.6, 4.5, 3.7	---	---	---	---	---	---
A		0.975	0.912	0.857	0.559	0.167	0.191
C		0.025	0.088	0.143	0.441	0.833	0.809
DPAC1B	Pvu II	20	17	14	36	21	21
A1	9.8, 5.7, 2.6, 1.7, 1.4, 0.8	0.575	0.441	0.464	0.222	0.024	---
A2	8.1, 5.7, 2.6, 1.7, 1.4, 0.8	---	0.029	---	---	---	---
C1	12.4, 5.7, 2.6, 1.7, 1.4, 0.8	0.425	0.530	0.536	0.764	0.952	0.976
C2	11.0, 5.7, 2.6, 1.7, 1.4, 0.8	---	---	---	0.014	0.024	0.024
A		0.575	0.470	0.464	0.222	0.024	---
C		0.425	0.530	0.536	0.778	0.976	1.000

Table 12 continued

DPAC7	Ava II	20	17	14	36	21	15
A1	10.5, 3.2, 2.9, 1.8, 1.7, 1.5, 1.4	1.000	1.000	0.893	0.435	---	---
A2	10.5, 3.2, 2.3, 1.8, 1.7, 1.5, 1.4	---	---	---	---	---	---
C1	10.5, 7.2, 3.2, 1.8, 1.7, 1.5, 1.4	---	---	0.107	0.551	0.917	1.000
C2	10.5, 7.0, 3.2. 1.8, 1.7, 1.5, 1.4	---	---	---	0.014	0.083	---
C3	10.5, 5.8, 3.2, 1.8, 1.7, 1.5, 1.4	---	---	---	---	---	---
A		1.000	1.000	0.893	0.435	---	---
C		---	---	0.107	0.565	1.000	1.000

Table 12 continued

DPAC96A	Bgl II	20	17	14	36	21	21
A1	9.8, 4.0, 2.4, 1.5, 1.2	1.000	1.000	0.821	0.500	0.143	0.167
A2	9.8, 4.2, 2.4, 1.5, 1.2	---	---	---	---	---	---
A		1.000	1.000	0.821	0.500	0.143	0.167
C	9.8, 6.4, 1.5, 1.2	---	---	0.179	0.500	0.857	0.833
DPAC96B	Bgl II	20	17	14	36	21	21
C1	9.8, 5.1, 1.5, 1.2	---	---	0.071	0.097	0.238	0.262
C2	9.8, 5.3, 1.5, 1.2	---	---	---	---	0.071	0.024
A	9.8, 3.7, 1.5, 1.2	1.000	1.000	0.929	0.903	0.691	0.714
C		---	---	0.071	0.097	-.309	0.286
DPAC97	Pvu II	20	17	14	36	21	21
A	4.9, 2.0, 1.8, 1.6, 1.2, 1.0, 0.8, 0.7	0.975	0.853	0.857	0.431	0.024	0.025

Table 12 continued

C	5.2, 2.0, 1.8, 1.6, 1.2, 1.0, 0.8, 0.7	0.025	0.147	0.143	0.569	0.976	0.975
DPAC98	Pvu II	20	17	14	36	21	21
C1	3.3, 2.7, 2.2, 1.8, 1.4, 1.0	0.050	0.118	0.143	0.278	0.429	0.738
C2	3.3, 2.7, 2.2, 1.8, 1.4, 0.6, 0.4	---	---	---	0.014	---	---
C3	3.3, 2.8, 2.7, 2.2, 1.4	---	---	---	---	---	---
A	3.6, 3.3, 2.2, 1.8, 1.4	0.950	0.882	0.857	0.708	0.571	0.262
C		0.050	0.118	0.143	0.292	0.429	0.738
DPAC102	Bgl II	20	17	14	36	20	21
C1	7.9, 3.3, 1.7, 1.4	---	---	---	0.278	0.600	0.500
C2	7.9, 3.3, 1.7, 1.1	---	---	0.036	0.069	0.175	0.143
A	7.9, 3.3, 1.7, 1.5	1.000	1.000	0.964	0.653	0.225	0.357

Table 12 continued

C		---	---	0.036	0.347	0.775	0.643
DPAC104	Bgl II	20	17	14	36	21	21
A	8.0, 4.6, 4.0, 1.5, 1.3	1.000	0.941	0.857	0.542	0.286	0.073
C	8.0, 5.0, 4.0, 1.5, 1.3	---	0.059	0.143	0.458	0.714	0.927

[a] MD is not included as part of any transect in analysis of molecular clines.

[b] Restriction fragment sizes in kilobases of major type A and major eastern and western type C haplotypes (C_e and C_w respectively) as follows: Pst I A = 16.4, C_e = 14.9,1.5, C_w = 8.8, 6.3, 1.5; Pvu II A = 11.9, 6.0, C_e = 13.1, 4.8, C_w = 10.6, 6.0, 0.9; Ava II A = 3.6, 2.1, 1.6, 1.3, 1.0, 0. 0.9, 0.6, 0.5, C_e = 3.6, 2.7, 2.5, 1.6, 1.4, 1.2, 0.9, 0.6, 0.5, C_w = 3.6, 3.4, 2.7, 1.7, 1.6, 1.1, 0.9, 0.6, 0.5.

[c] Frequencies in populations MO to MO4 and LA are for western type C haplotype. Frequencies in all other populations are for eastern type C haplotype.

Table 13. Frequency of type A alleles in allopatric populations of P. atricapillus and P. carolinensis for fifteen markers surveyed in this study. Sample sizes are in parentheses.

	P. atricapillus		P. carolinensis			
Marker	MO (20)	PA (20)	VA (21)	LA (21)	OH (20)	MD (14)
MtDNA	1.000	1.000	0.000	0.000	0.000	0.000
GDA[a]	1.000	1.000	0.000	0.000	0.000	0.000
ACON[a]	1.000	0.938	0.000	0.000	0.000	0.000
DPAC121[a]	1.000	1.000	0.000	0.000	0.000	0.000
ski	1.000	1.000	0.000	0.000	0.200	0.090
DPAC1A	0.975	1.000	0.195	0.191	0.308	0.071
DPAC1B	0.575	0.800	0.143	0.000	0.075	0.091
DPAC4	0.882	0.889	0.762	0.103	0.725	---[b]
DPAC7	1.000	1.000	0.000	0.000	0.000	0.000
DPAC96A	1.000	0.975	0.050	0.167	0.050	0.036
DPAC96B	1.000	0.975	0.595	0.714	0.575	0.357
DPAC97	0.975	0.975	0.095	0.025	0.225	0.188
DPAC98	0.950	0.975	0.476	0.262	0.425	0.312
DPAC102	1.000	1.000	0.310	0.357	0.200	0.375
DPAC104	1.000	1.000	0.071	0.073	0.125	0.037

[a] Known or strongly suspected to be sex-linked.

[b] Not screened in this population.

Table 14. Contingency table showing the number of males and females for three genotypic classes collected in central populations of three transects. P values are from Fisher's one-tailed exact tests; Ho: females are not under-represented among F_1's. A dash indicates a P value could not be calculated because of empty cells.

	Parental		Non-F_1 hybrid			F_1		
Population	Male	Female	Male	Female	P	Male	Female	P
MO3	11	8	8	2	0.22	6	0	0.07
WV3	7	6	10	3	0.20	4	1	0.32
VA2	11	6	6	4	0.74	0	1	1.00[a]
VA3	6	3	11	4	0.54	0	0	---

[a] For H_o: males are not under-represented among F_1's, P = 0.035.

Table 15. Contingency table showing number of A and C mtDNAhaplotype individuals for three genotypic classes collected in central populations of three transects. P values are from Fisher's two-tailed exact tests; H_0: the proportion of A and C haplotypes is the same among F_1 or non-F_1 hybrids as among parentals. A dash indicates a P value could not be calculated for that comparison because of empty cells.

	Parental		Non-F_1 hybrid			F_1		
Popu-lation	A	C	A	C	P	A	C	P
MO3	10	10	1	9	0.05	4	2	0.65
WV3	11	6	6	3	1.00	3	2	1.00
VA2	18	0	11	0	---	0	4	0.0001
VA3	0	9	1	14	1.00	0	0	---

Table 16. Cline width and position estimates (± 1 SE) for 14 genetic markers in the three transects. Each estimate is based on 200 bootstrap replicates in which P. atricapillus allele frequency in each population of the transect was reestimated by resampling with replacement, and cubic splines fit to the sigmoidally-distributed population estimates of allele frequencies. Cline width was estimated as the inverse of the spline's steepest slope, and cline position was estimated according to the location at which each spline's steepest slope occurred.

	Cline width (km)			Cline midpoint (km)		
Marker	WV	VA	MO	WV	VA	MO
MtDNA	28.8 ± 0.5	12.0 ± 0.2	25.0± 0.6	248.2 ± 0.1	157.6 ± 0.1	257.0 ± 0.1
GDA[a]	21.6 ± 0.2	12.0. ± 0.1	21.2 ± 0.5	245.9 ± 0.1	158.3 ± 0.0	257.6 ± 0.1
ACON[a]	51.2 ± 1.8	25.2 ± 1.2	20.7 ± 0.5	248.9 ± 0.2	159.3 ± 0.1	257.6 ± 0.1
DPAC121[a]	21.1 ± 0.2	11.0 ± 0.2	20.2 ± 0.5	246.0 ± 0.1	158.3 ± 0.1	257.5 ± 0.1
ski	62.8 ± 1.6	46.9 ± 0.9	30.2 ± 0.5	247.2 ± 0.3	161.8 ± 0.1	257.4 ± 0.2
DPAC1A	110.4 ± 2.6[b]	136.9± 2.7[b]	243.7 ± 16.2[b]	240.6 ± 0.5[b]	144.9 ± 0.5[b]	253.4 ± 0.9[b]
DPAC1B	118.6 ± 2.9[b]	161.3 ± 2.7[b]	82.2 ± 3.2	249.3 ± 0.6[b]	114.8 ± 2.2[b]	258.6 ± 0.5
DPAC7	21.6 ± 0.3	12.7 ± 0.2	18.0 ± 0.4	245.2 ± 0.1	157.8 ± 0.1	257.0 ± 0.1

Table 16 continued

DPAC96A	38.1 ± 0.9	32.8 ± 1.2	215.3 ± 15.9[b]	247.4 ± 0.2	160.1 ± 0.1	249.0 ± 0.7[b]
DPAC96B	98.7 ± 3.7	116.1 ± 4.4[b]	409.4 ± 28.1[b]	254.8 ± 0.5	150.1 ± 0.7[b]	265.2 ± 1.4[b]
DPAC97	72.4 ± 2.1	43.1 ± 1.5	87.9 ± 6.5	244.4 ± 0.2	159.1 ± 0.2	258.1 ± 0.4
DPAC98	100.8 ± 3.1[b]	112.9 ± 4.4[b]	143.7 ± 5.4	246.2 ± 0.4[b]	151.1 ± 0.6[b]	277.3 ± 0.5
DPAC102	49.1 ± 0.9	83.2 ± 4.2[b]	548.4 ± 18.8[b]	244.0 ± 0.2	153.4 ± 0.4[b]	257.4 ± 0.8[b]
DPAC104	57.5 ± 1.2	40.2 ± 1.2	69.9 ± 1.5	244.8 ± 0.3	158.2 ± 0.1	258.4 ± 0.4

[a] Evidence supports sex-linkage of these markers.

[b] Estimate not used in comparisons among markers or among transects because of poor spline fit to the data.

Table 17. Summary of Tukey tests (alpha = 0.05) comparing cubic spline inverse slope width (km) estimates among certain classes of genetic markers for each of three transects. Figures under the greater than (>) column represent the number of comparisons for which the first marker or class of marker had a significantly greater width than the second marker or class of marker. Figures under the equal (=) column represent the number of comparisons for which the two markers or class of markers did not differ significantly. Figures under the less than (<) column represent the number of comparisons for which the first marker or class of marker had a significantly smaller width than the second marker or class of marker. A dash indicates zero observations.

	Missouri			West Virginia			Virginia			Total		
	>	=	<	>	=	<	>	=	<	>	=	<
MtDNA vs autosomal	---	2	4	1	---	6	---	1	4	1	3	14
Sex-linked vs autosomal:												
GDA	---	2	4	---	1	6	---	1	4	---	4	14
DPAC121	---	2	4	---	1	6	---	1	4	---	4	14
ACON	---	2	4	3	2	2	1	---	4	4	3	11
Total	---	6	12	3	4	14	1	2	12	4	11	39

Table 17 continued

MtDNA vs sex-linked	---	3	---	1	1	1	2	---	1	1	6	2
Sex-linked vs sex-linked:												
GDA vs DPAC121	---	1	---	---	1	---	---	1	---	---	3	---
ACON vs GDA	---	1	---	1	---	---	1	---	---	2	1	---
ACON vs DPAC121	---	1	---	1	---	---	1	---	---	2	1	---
Total:	---	3	---	2	1	---	2	1	---	4	5	---
Fixed autosomal vs non-fixed autosomal	---	---	8	2	2	6	2	1	3	4	3	17

Table 18. Maximum extent of introgression detected for markers considered diagnostic.

Marker	Distance introgressed (km)	Population[a]
MtDNA	17.3	WV2
Sex-linked:		
GDA[b]	16.0	WV4
ACON[b,c]	16.0	WV4
DPAC121[b]	16.0	WV4
Autosomal:		
ski	172.2	OH
DPAC7	39.8	WV5
DPAC102[d]	5.4	VA2
DPAC104[d]	72.7/59.1[e]	WV1/VA1

[a] Population most distant from the range interface where an introgressed allele was detected.

[b] Evidence supports sex-linkage of these markers.

[c] Considered diagnostic only in populations on the P. carolinensis side of the range interface.

Table 18 continued

[d] Considered diagnostic only in populations on the P. atricapillus side of the range interface.

[e] Distance introgressed calculated separately from the range interface in West Virginia and Virginia respectively.

Table 19. Summary of Tukey tests comparing inverse slope cline width estimates among three transects for 14 genetic markers. A greater than sign (>) indicates that cline width of the marker for the first transect was significantly wider than for the second transect, while a less than sign (<) indicates the opposite relationship. An equal (=) sign indicates no difference between the two transects at the 5% level. A question mark indicates that this comparison was not made because of the unreliability of a marker's cline width estimate for one or both transects, due to poor spline fit to the data.

Marker	Missouri versus Virginia	Missouri versus West Virginia	West Virginia versus Virginia
MtDNA	>	<	>
GDA	>	=	>
ACON	<	<	>
DPAC121	>	=	>
ski	<	<	>
DPAC1A	?	?	?
DPAC1B	?	?	?
DPAC7	>	<	>
DPAC96A	?	?	>
DPAC96B	?	?	?
DPAC97	>	>	>
DPAC98	?	?	?
DPAC102	?	?	?
DPAC104	>	>	>

Table 19 continued

Total:			
>	6	2	9
=	0	2	0
<	2	4	0
?	6	6	5

Table 20. Relationship of allele frequency to distance from the range interface and to elevation within two subpopulations each of VA2 and WV3. Both VA2 and WV3 were divided evenly into two subpopulations based on distance relative to the range interface (West and East) and based on elevation (Low and High).

Distance					Elevation			
	No. birds	Average distance (km ± 1 SE)[a]	Allele frequency change[b] (± 1 SE)	r_s[c]		No. birds	Elevation (m ± 1 SE)	Allele frequency change[b]	r_s[d]
VA2:					VA2:				
West	17	6.08 ± 0.34	0.00 ± 0.025	0.19	Low	17	623.1 ± 29.7	+0.099 ± 0.023***	0.42**
East	16	2.46 ± 0.34			High	16	482.2 ± 13.3		
WV3:					WV3:				
West	16	1.42 ± 0.25	0.16 ± 0.024***	0.20	Low	15	613.8 ± 7.4	-0.030 ± 0.025	0.02
East	15	5.45 ± 0.45			High	16	522.0 ± 11.8		

Table 20 continued

**P < 0.01 ***P < 0.001

[a] Measured in VA2 subpopulations from each individual to the range interface, and measured in WV3 subpopulations from each individual to the western boundary of WV3.

[b] An average of type A allele frequency change between subpopulations for the 14 genetic markers analyzed. A positive number reflects an increase in type A alleles from west to east subpopulations or from low to high elevation subpopulations. P values reflect t-tests performed on arcsine-transformed data, testing H_0: $\mu = 0$.

[c] Spearman correlation of type A allele frequency and distance from the range interface for individuals of VA2 and WV3. Allele frequency for each individual was an average of the number of diagnostic (mtDNA, GDA, ski, DPAC7, DPAC121) A alleles present out of all alleles scored for these five loci.

[d] Spearman correlation of type A allele frequency and elevation for individuals of VA2 and WV3. Allele frequency for each individual was calculated as for correlations between allele frequency and distance.

FIGURE LEGENDS

Fig. 14. Position of the range interface in Missouri, with locations of populations comprising the Missouri transect, including parental populations.

Fig. 15. Mean genotypic disequilibria (± 1 SE) for populations comprising three transects. Populations are identified along the top of each graph. Means are calculated only from disequilibria significantly different from zero, with sample size of each estimate given. Estimates are given for VA4 both with and without the single P. atricapillus individual included (N = 41 and 11 respectively).

Fig. 16. Monotonic smoothing splines for each marker locus across the Missouri transect.

Fig. 17. Monotonic smoothing splines for each marker locus across the Virginia transect.

Fig. 18. Monotonic smoothing splines for each marker locus across the West Virginia transect.

Fig. 19. Summary of Tukey tests comparing cline position (km) estimates among genetic markers for each of three transects. Cline position is judged according to the location at which each spline's steepest slope occurs. Markers are ranked in geographic order of position, which appears next to the name of each marker; 0 would represent the P. atricapillus terminus of each transect. Adjacent vertical lines connect population means found not to differ at the 5% significance level. Some markers are omitted from each transect comparison because of the unreliability of these markers' position estimates, due to poor spline fit to the data.

Fig. 20. Summary of Tukey tests comparing inverse slope cline width estimates (km) among genetic markers for each of three transects. Markers are ranked in order of magnitude of cline width estimate, which appears next to the name of each marker. Adjacent vertical lines connect population means found not to differ at the 5% significance level. Some markers are omitted from each transect comparison because of the unreliability of these markers' cline width estimates, due to poor spline fit to the data.

Fig. 21. Topography along the Virginia transect at the range interface between VA2 and VA3. The 450 m and 600 m contour lines are shown, with elevation above 600 m shaded.

Fig. 22. Topography at the range interface of the West Virginia transect, with the location of VA3 indicated. The 450 m and 600 m contour lines are shown, with elevation above 600 m shaded.

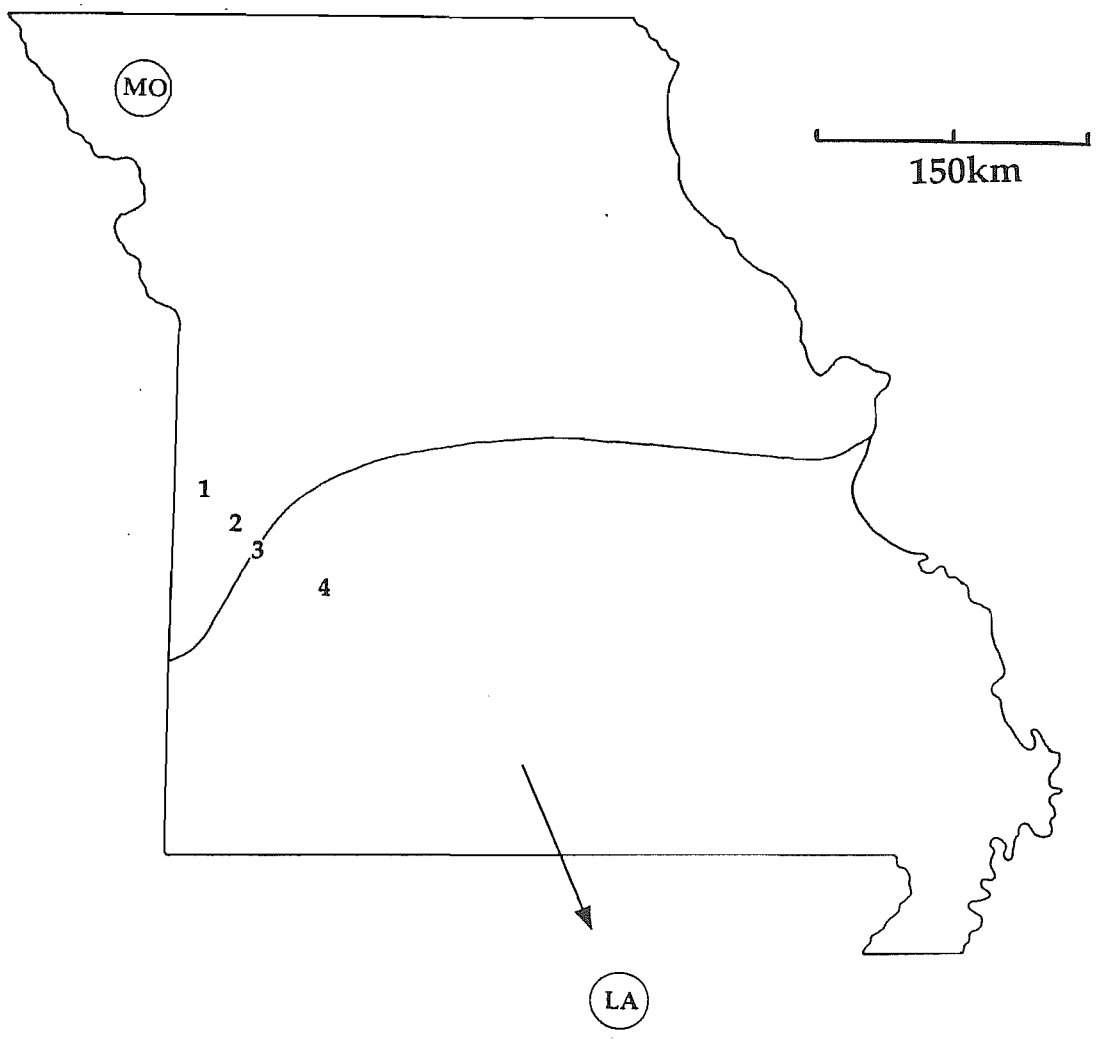
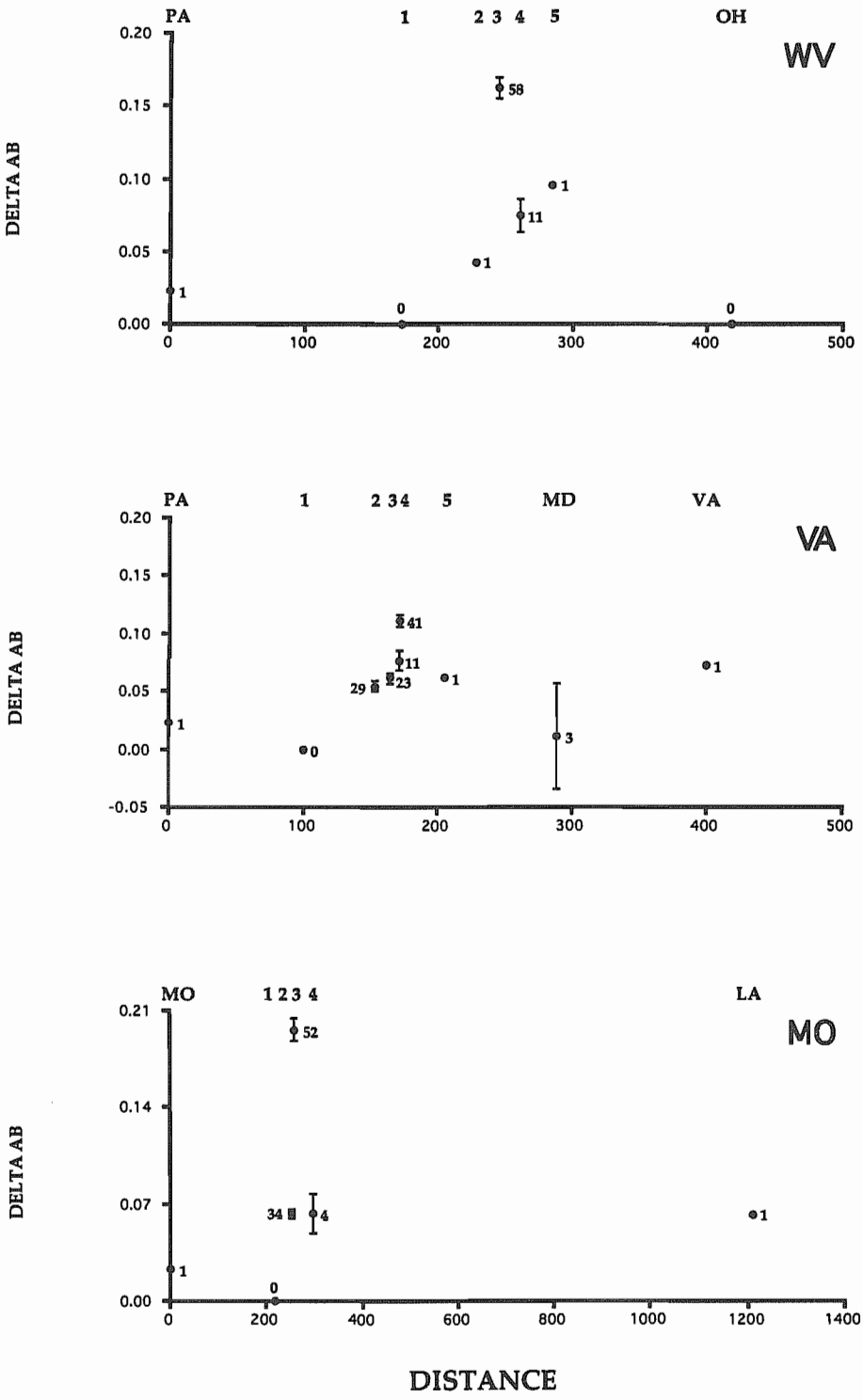


Figure 14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5
OH
WV
DELTA AB
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0.10
0.05
0.00
58
1
11
1
1
0
0
0
100
200
300
400
500
PA
1
2 3 4
5
MD
VA
VA
DELTA AB
0.20
0.15
0.10
0.05
0.00
-0.05
41
11
29
23
1
1
1
0
3
0
100
200
300
400
500
MO
1 2 3 4
LA
MO
DELTA AB
0.21
0.14
0.07
0.00
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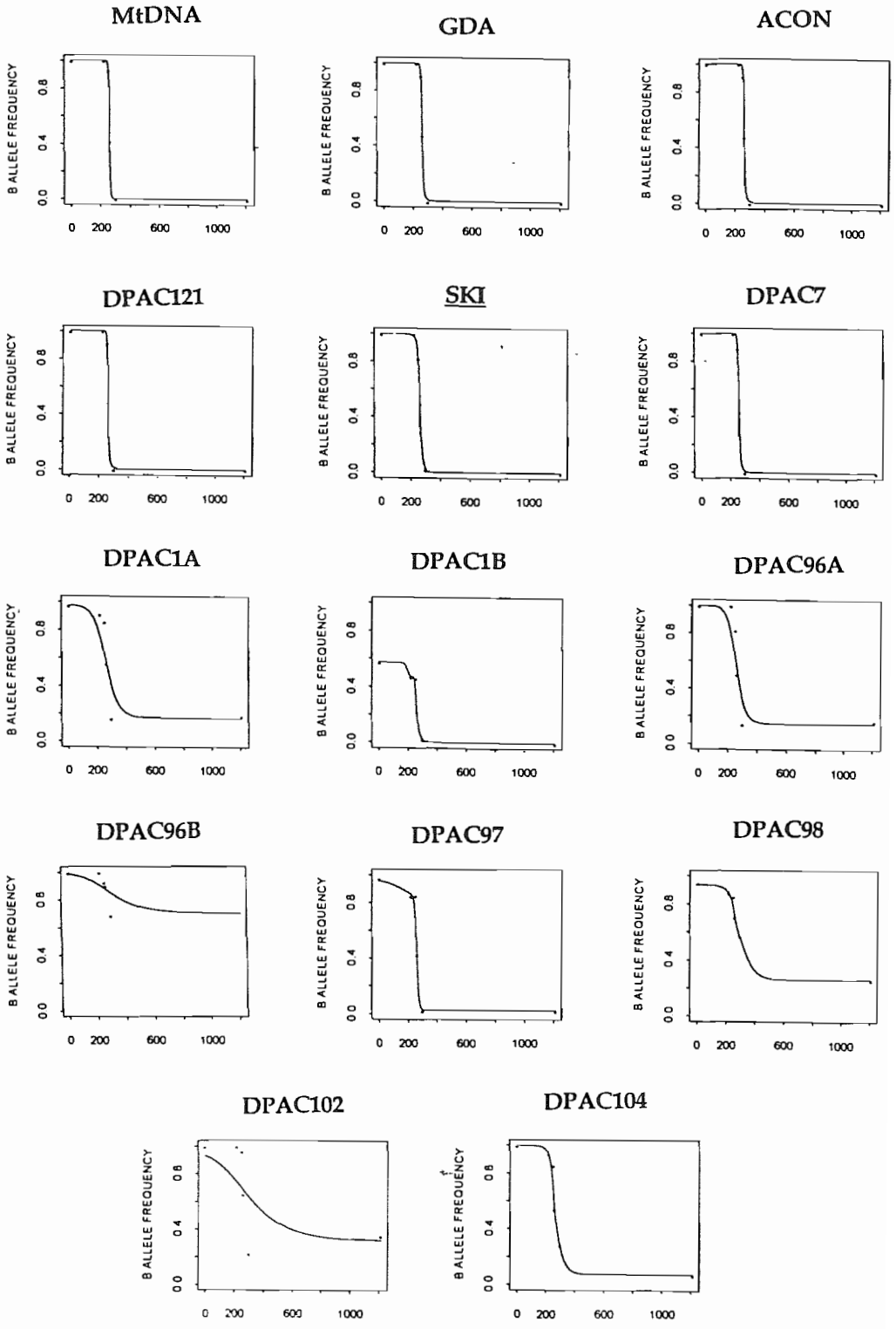
34
4
1
1
0
0
200
400
600
800
1000
1200
1400
DISTANCE

FIGURE 15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DPAC121
SKI
DPAC7
DPAC1A
DPAC1B
DPAC96A
DPAC96B
DPAC97
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DPAC102
DPAC104
B ALLELE FREQUENCY
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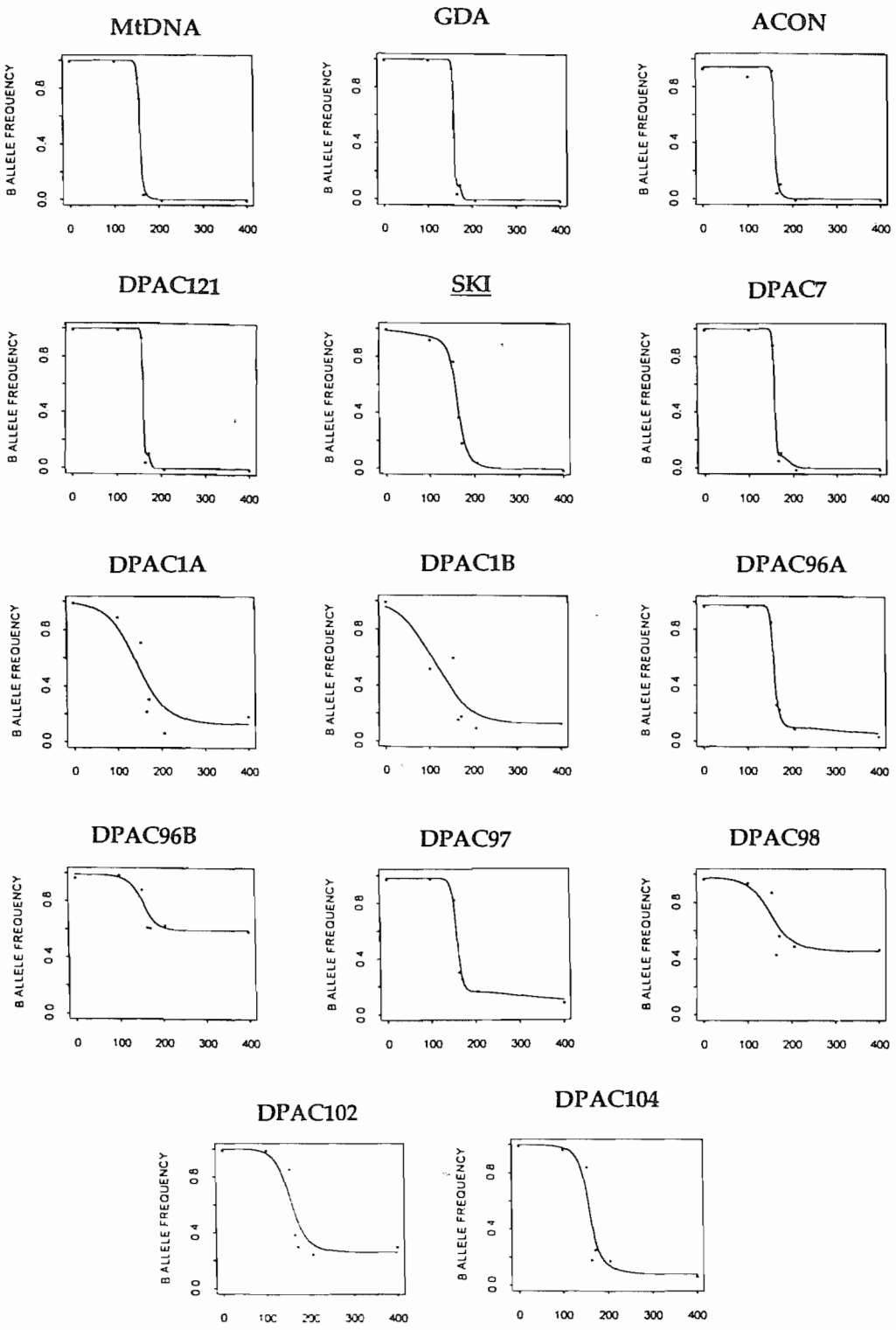
0.8
0
200
600
1000

Figure 16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GDA
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DPAC121
SKI
DPAC7
DPAC1A
DPAC1B
DPAC96A
DPAC96B
DPAC97
DPAC98
DPAC102
DPAC104
B ALLELE FREQUENCY
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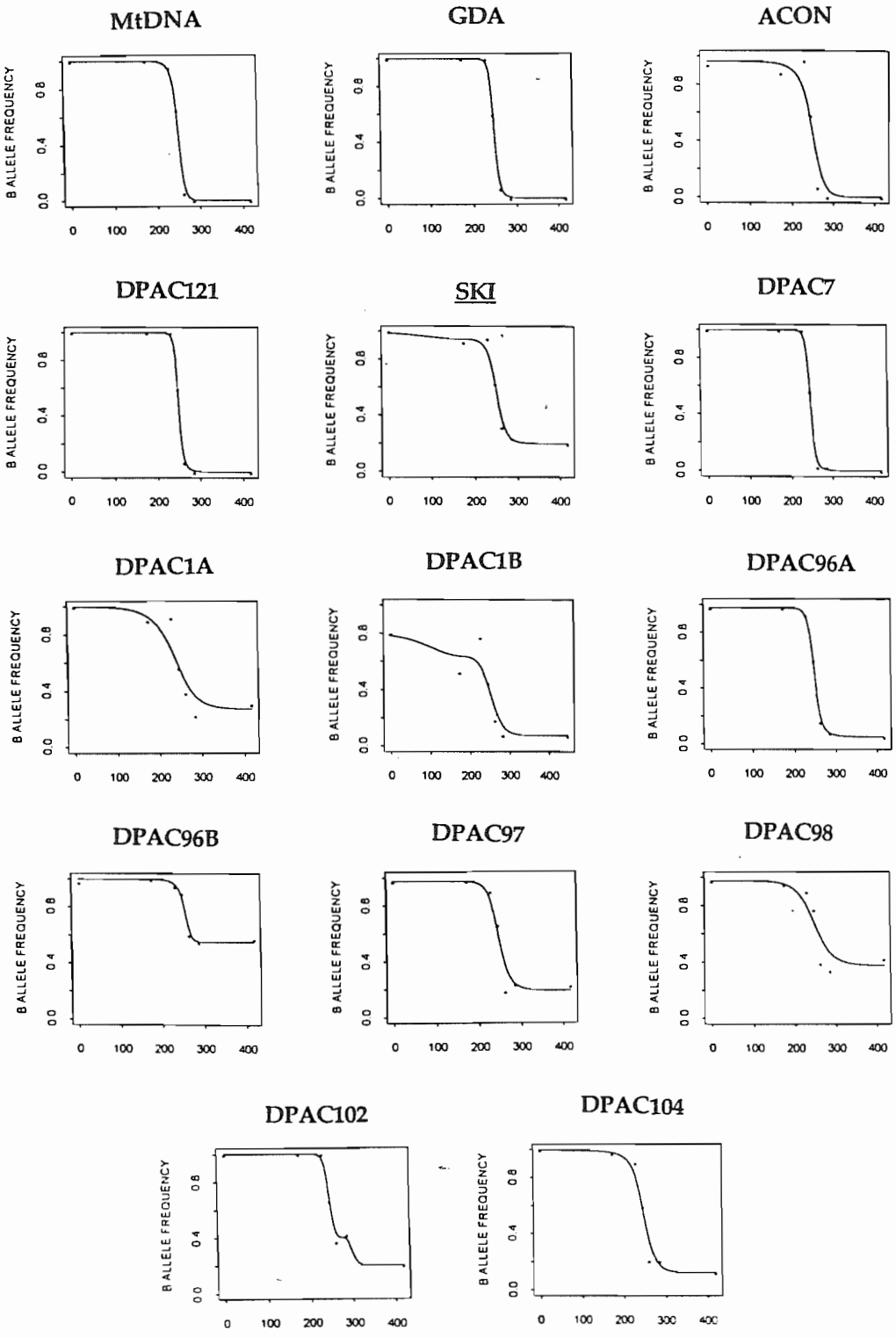
0.4
0.8
0
100
200
300
400

Figure 17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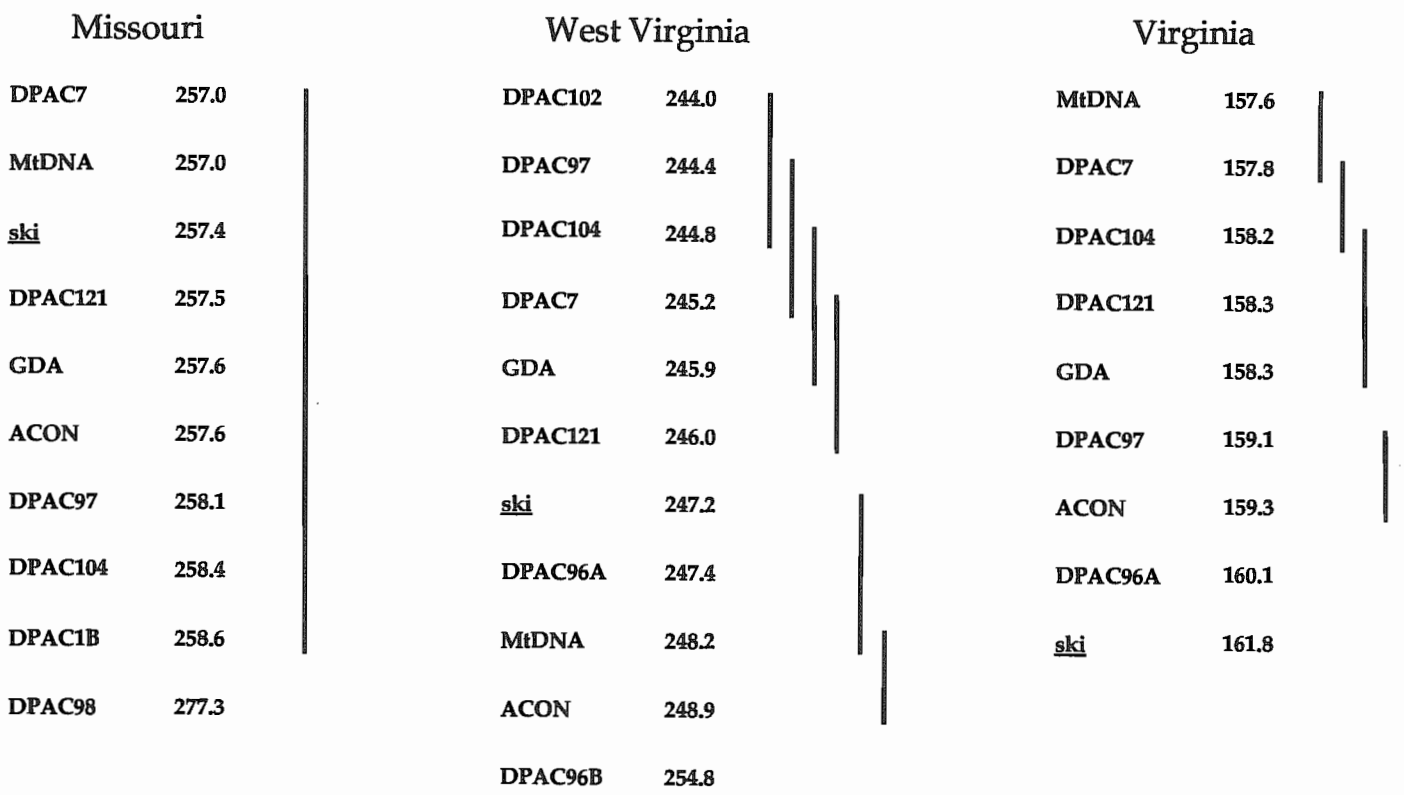
GDA
ACON
DPAC121
SKI
DPAC7
DPAC1A
DPAC1B
DPAC96A
DPAC96B
DPAC97
DPAC98
DPAC102
DPAC104
B ALLELE FREQUENCY
0.0
0.4
0.8
0
100
200
300
400

Figure 18


Missouri
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MtDNA 257.0
ski 257.4
DPAC121 257.5
GDA 257.6
ACON 257.6
DPAC97 258.1
DPAC104 258.4
DPAC1B 258.6
DPAC98 277.3
West Virginia
DPAC102 244.0
DPAC97 244.4
DPAC104 244.8
DPAC7 245.2
GDA 245.9
DPAC121 246.0
ski 247.2
DPAC96A 247.4
MtDNA 248.2
ACON 248.9
DPAC96B 254.8
Virginia
MtDNA 157.6
DPAC7 157.8
DPAC104 158.2
DPAC121 158.3
GDA 158.3
DPAC97 159.1
ACON 159.3
DPAC96A 160.1
ski 161.8

Figure 19

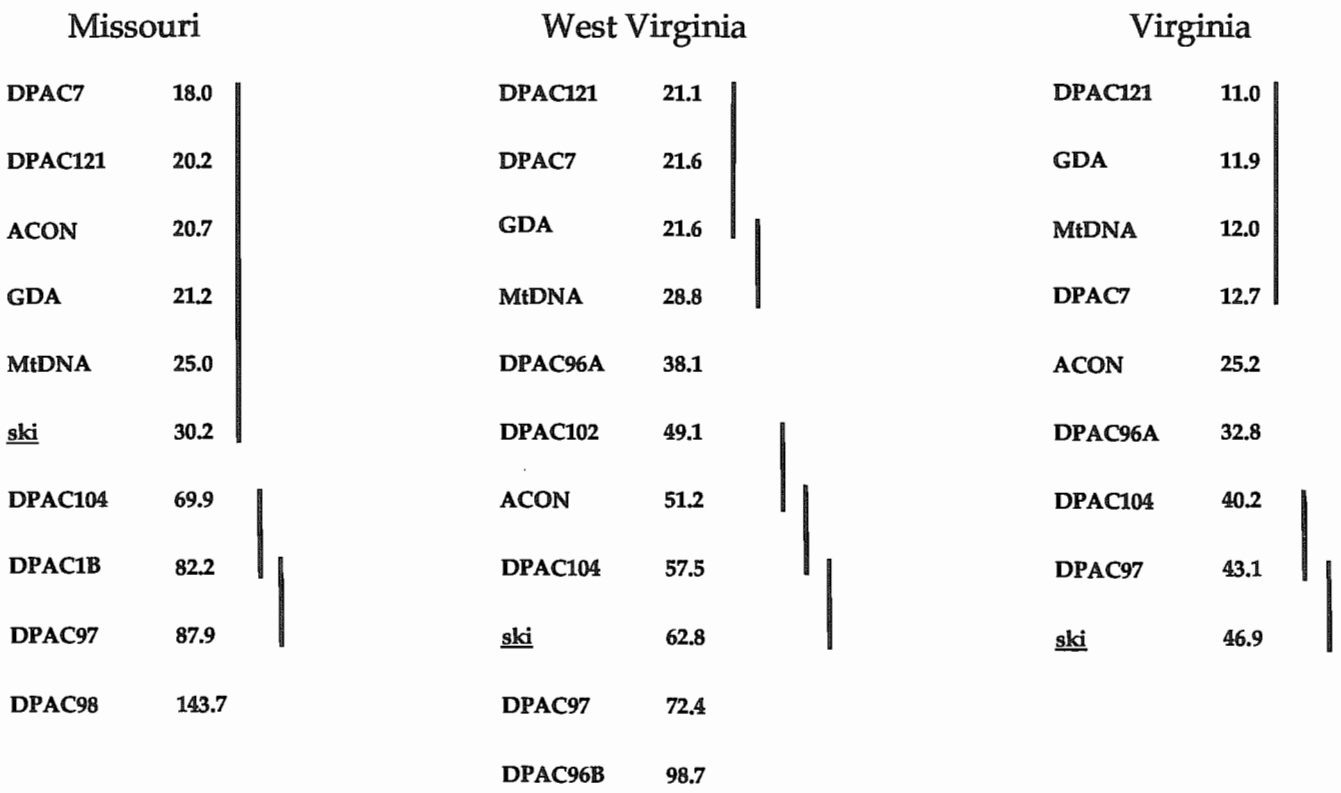
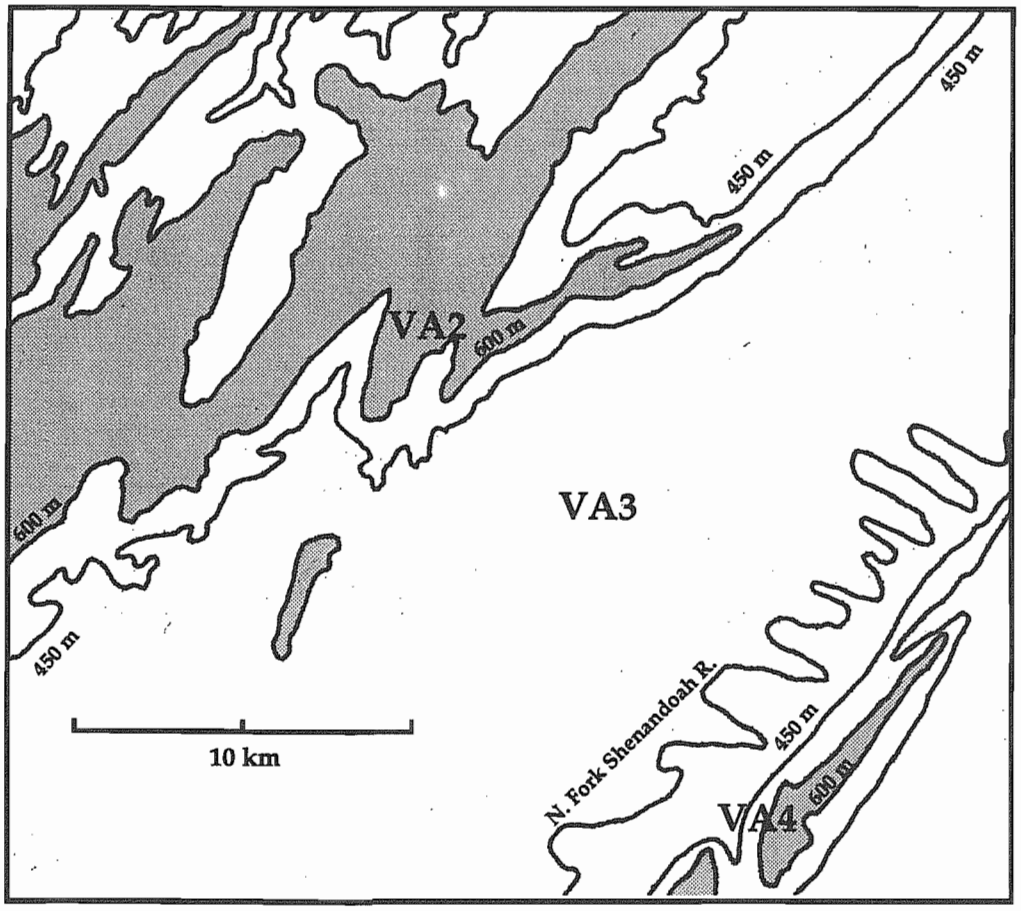

Missouri
DPAC7 18.0
DPAC121 20.2
ACON 20.7
GDA 21.2
MtDNA 25.0
ski 30.2
DPAC104 69.9
DPAC1B 82.2
DPAC97 87.9
DPAC98 143.7
West Virginia
DPAC121 21.1
DPAC7 21.6
GDA 21.6
MtDNA 28.8
DPAC96A 38.1
DPAC102 49.1
ACON 51.2
DPAC104 57.5
ski 62.8
DPAC97 72.4
DPAC96B 98.7
Virginia
DPAC121 11.0
GDA 11.9
MtDNA 12.0
DPAC7 12.7
ACON 25.2
DPAC96A 32.8
DPAC104 40.2
DPAC97 43.1
ski 46.9

Figure 20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450 m
VA2
600 m
VA3
600 m
450 m
10 km
N. Fork Shenandoah R.
450 m
VA4
600 m

Figure 21

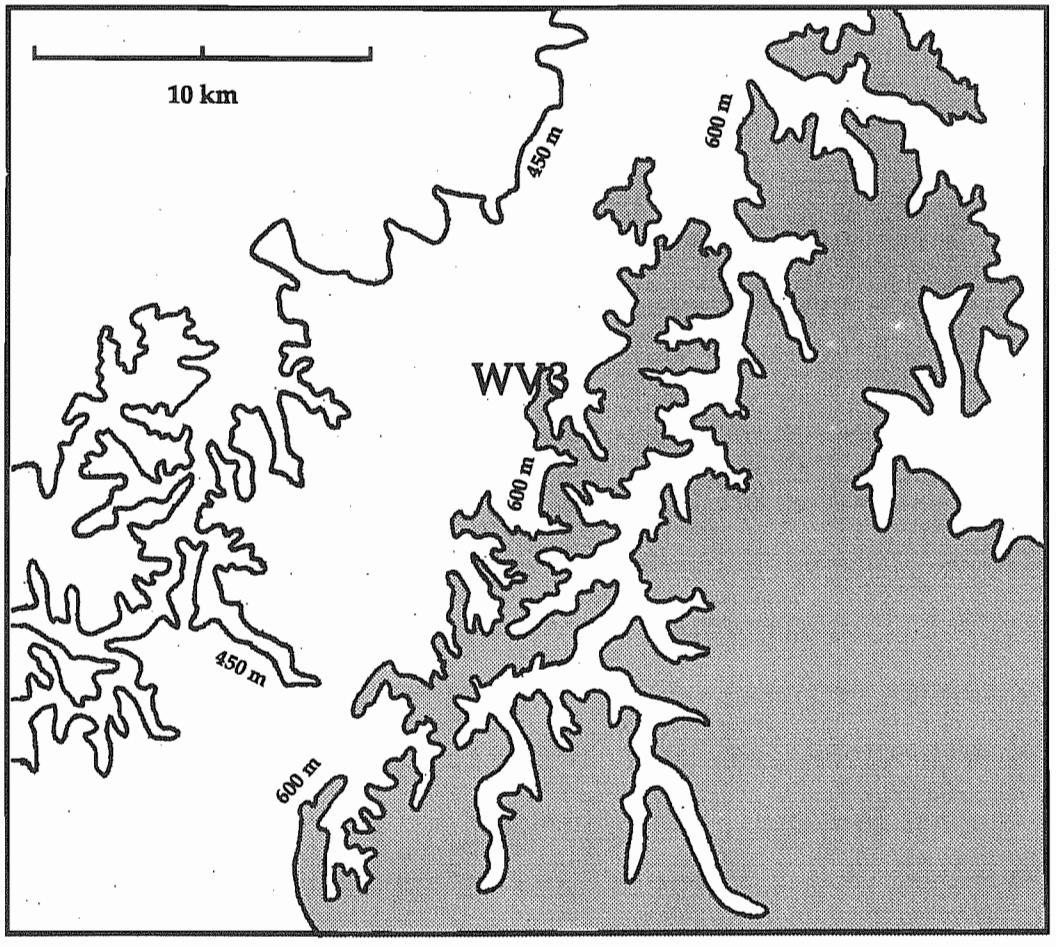

Figure 22

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5. CONCLUSION

The complexity of the processes of genetic divergence and the development of reproductive isolation makes it important to utilize a variety of markers and characters in studying hybrid zone dynamics (Nelson et al. 1987, Baker et al. 1989). This investigation has revealed that different characters vary in their ability to detect hybrids, in the degree to which they introgress, and in the extent to which they are influenced by factors shaping hybrid zones, such as selection, genetic drift, recombination, dispersal patterns, mating structure, and transmission genetics.

Song.-Song was shown here to be an unreliable marker of genetic interactions between P. atricapillus and P. carolinensis. Many hybrids sang good renditions of one or both of the two species' songs, there was a poor correlation between a song's characteristics and the individual's genetic ancestry singing it, and genetic introgression occurred far beyond the narrow region in which mixed song was found. In addition, the degree to which mixed song was detected varied among localities without regard to levels of hybridization. Few intermediate songs were found in either Appalachian transect, while about 10% of songs in the Missouri contact zone had intermediate discriminant scores. Yet each location had a high proportion of hybrids.

Another complicating factor in attempting to infer hybridization from song intermediacy is that the results obtained can vary with the criteria used to measure song intermediacy. Gelter (1987) found that some songs of the pied flycatcher (Ficedula hypoleuca) sounded like the collared flycatcher (F. albicollis) and were classified as such by a discriminant analysis, but clustered with conspecific F. hypoleuca songs in a UPGMA analysis. The songs apparently consisted of F. albicollis notes sung in a F. hypoleuca manner. Likewise, the "E" song type described here appears to be a P. carolinensis variant, but it grouped in an intermediate position in the PCA, and there is still some uncertainty as to its association with the hybrid zone. Bilingual song was more common than intermediate song, but its frequency also varied with location, probably as a result of ecological differences at the contact zone (see below). It was usually solicited as a response to song playback trials and may occur more infrequently as a spontaneous song behavior, so that its occurrence may be underestimated unless it is actively sought.

The unreliable nature of song in reflecting genetic interaction between P. atricapillus and P. carolinensis is consistent with the result of hybrid zone studies in other birds (Ficken and Ficken 1967, Gill and Murray 1972, Morrison and Hardy 1983, Gelter 1987, Lein and Corbin 1990),

and with laboratory work showing the importance of learning in the ontogeny of song in both of these chickadees (Kroodsma et al. 1995). Because of their morphological similarity at the range interface, much reliance is put on vocal criteria in identifying them and evaluating their genetic interactions. These results amply demonstrate, however, that assessment of chickadee hybridization cannot rely on song data.

Morphology.-Morphology proved to be moderately reliable as a criterion for judging hybridization levels in P. atricapillus and P. carolinensis. Principal component analysis reflected some intermediacy in both Appalachian transects, in accord with genetic data. There was also a significant correlation in WV3 between both PC1 and PC3 morphological axes and number of P. atricapillus alleles an individual possessed. Moreover, the high proportion of P. atricapillus alleles present in WV3 was paralleled by more P. atricapillus-like PC1 and PC3 scores here. There were limits to its utility, however, because of the similarity of P. atricapillus and P. carolinensis, and because levels of morphological differentiation between parental populations in the Virginia and West Virginia transects led to differing levels of intermediacy. Morphology also proved to have a limited ability to detect the presence of later-generation hybrids, which predominated among hybrids, and which will be

more parental-like in appearance than F_1's and F_2's (Anderson 1949). As with song, then, the presence of intermediacy in morphology was a reasonable indication of hybrid ancestry, but its absence or limited manifestation was not a reliable indication of nonhybridity of individuals or populations. Evidence of morphological intermediacy between P. atricapillus and P. carolinensis near their contact zone also needs to be evaluated carefully because of clinal variation in these chickadees' morphology that leads to convergence in their appearance at the hybrid zone. Of course this clinal variation itself could be viewed as evidence of genetic introgression or connectedness.

Genetics.-The availability of discrete, diagnostic genetic markers exhibiting Mendelian inheritance provided a means of unambiguously identifying hybrids, thus allowing an evaluation of the reliability of song and morphology in detecting hybrids. The limited degree of protein differentiation in birds restricted the availability of diagnostic allozyme markers. Even after intensive searches for differentiated single-copy nuclear RFLP's in this and a previous study, only three nuclear loci and the mitochondrial genome were found to be completely fixed for different alleles in both P. atricapillus and P. carolinensis.

Comparisons among classes of characters.-Vocal, morphological, and molecular characters displayed varying levels of introgressive hybridization related to their different characteristics. Some of these differing patterns of introgression revealed important clues about the hybrid zone's dynamics. Clines for song, morphology, and a few representative molecular markers across the Appalachian Mountains along the West Virginia and Virginia transects are illustrated in Fig. 23 for comparative purposes. The cline for mtDNA is characteristic of the narrowest molecular clines such as for DPAC7 and the sex-linked markers. Clines for ski, DPAC97 and DPAC104 were moderate to wide in width. While splines could be fit to vocal and morphological clines in PC1 to obtain cline width and position estimates, such estimates are difficult to compare to allele frequency cline estimates, because PCA axes have no minimum and maximum limits that establish comparable scales with allele frequency. So only gross comparisons will be made.

Song exhibited the steepest change across the range interface, visibly steeper even than mtDNA. This pattern has been seen for vocal characters in other avian hybrid zones (e.g. Braun 1983, Fleischer and Rothstein 1988, Lein and Corbin 1990; but see Emlen et al. 1975). A possible explanation relates to both song's selective significance and transmission characteristics. Both songs and calls in

birds are likely to be under strong selection for conspecific recognition, because of their role in mate attraction and communication, territorial defense, etc. This should lead to steep transitions at species boundaries. In addition, the cultural transmission of song can contribute to producing a steep gradient in this trait. The sensitive period of song learning in passerines can extend months beyond fledging (reviewed in Baptista and Gaunt 1994). Given the capacity of both P. atricapillus and P. carolinensis to learn the other's song (Kroodsma et al. 1995), learning of the predominant species' song in an area could occur following dispersal of an individual across the contact zone. A steep song cline would therefore be established. Lower dispersal rate in males relative to females (Weise and Meyer 1979), with subsequent limited introgression of song which is essentially a male-transmitted trait, has also been suggested to limit vocal introgression (Baker 1987), and would contribute to the steepening effect.

The PC 1 cline for morphology was substantially wider than for song. The morphological cline is appreciably wider than for mtDNA and the sex-linked markers, but is more comparable to the autosomal RFLP clines; specific conclusions beyond this are difficult. I have suggested that strong selection on sex-linked loci and against

transmission of the mitochondrial genome has produced steep clines for these characters. Allozyme introgression has regularly been observed to exceed morphological introgression in hybrid zones (Harrison 1990 and references therein), prompting the inference that such molecular characters may often be relatively neutral. All of these autosomal markers except ski are anonymous DNA fragments that may be linked to loci under selection rather than experiencing direct selection themselves. Greater autosomal introgression relative to morphology, then, is expected. However, given the clinal variation in both P. atricapillus and P. carolinensis, which produces convergence towards their range interface (Duvall 1945, Lunk 1952, James 1970), a morphological cline might be relatively broad, and not substantially narrower than neutral introgression.

Finally, variation in levels of introgression among the molecular markers was observed which probably reflects differing levels of selection on them (or on loci to which they are linked). I propose that the limited introgression of sex-linked markers observed in this chickadee hybrid zone is due to greater selection on them directly, including that which might result from such proposed mechanisms as Z chromosome/autosome interactions or meiotic drive between the sex chromosomes (Coyne and Orr 1989, Frank 1991, Hurst and Pomiankowski 1991). I also propose that the limited

mitochondrial introgression results indirectly from selection on the sex chromosomes, which produces a Haldane's rule phenomenon, thus limiting the transmission of maternally-inherited traits such as mtDNA because females in birds are the heterogametic sex. Limited sex-linked introgression has been observed in several hybrid zones (Moran 1979, Vanlerberge et al. 1986, Hagan and Scriber 1989, Hagan 1990, Sperling and Spence 1991, Tucker et al. 1992, Ferris et al. 1993, Dod et al. 1993), and is consistent with the hypothesis of greater selection on sex-linked genes. Evidence supportive of indirect selection operating on sex-linked loci in this hybrid zone must come from 1) confirmation of the operation of Haldane's rule in hybrids of P. atricapillus and P. carolinensis, and 2) the observance of a similar relationship between Haldane's rule and limited mitochondrial introgression for hybrid zones in birds, butterflies, or other systems in which females are the heterogametic sex and mitochondrial inheritance is maternal.

Introgression of the autosomal marker DPAC7 was limited to an extent comparable with the sex-linked markers and mtDNA. It presumably is linked to a marker that contributes to species differences between P. atricapillus and P. carolinensis. Both it and ski, the two fixed autosomal markers, had overall lower introgression levels relative to

non-fixed autosomal markers. This is consistent with a scenario of stronger levels of selection on particular marker loci that can ultimately lead to their fixation, while weakly selected or neutral marker loci become fixed less often. Sample sizes are low to base such a conclusion on. However, the potential for such differential patterns of introgression highlights the utility of using a variety of markers in analyzing hybrid zone structure. Reliance on a restricted number could lead to a limited or even misleading picture of the genetic interactions occurring.

Ecological correlates.-Several lines of evidence lent support to the hypothesis that ecological gradients are important in shaping this hybrid zone's structure. The initial prediction that Appalachian clines would be narrower than clines along the Missouri transect was generally upheld in the comparison of the Missouri and Virginia transects, but not in comparison of the Missouri and West Virginia transects. In seeking a plausible explanation for these results, I looked at ecological (elevational) differences between the two Appalachian transects, and noted a more abrupt and less interdigitated altitudinal transition along the Virginia transect. Consistent with the influence of ecology on introgression levels, all molecular cline widths along the Virginia transect were significantly narrower than along the West Virginia transect. The similarity in cline

widths between the Missouri and West Virginia transects appears to contradict the hypothesized relationship between elevationally-associated variables and introgression levels. An unknown factor, however, is what variable(s) chickadee distribution might hypothetically be tracking, and how this variable(s) would change across a latitudinal versus an elevational gradient.

Other data supports the hypothesized influence of elevation on hybrid zone structure. One of the strongest corroborative observations was a strong correlation between elevation and allele type but not between distance from the interface and allele type in VA2. I observed a similar correlation in the hybrid zone in southern Virginia, where atricapillus allele frequency on a ridgetop averages 85-90%m but only 15-20% one kilometer away (towards the range of P. atricapillus) along a low elevation stream valley (Sattler and Braun unpubl. data). These correlations rule out dispersal rate as a possible cause, although they could be due to direct selection on phenotypes or habitat selection with an elevational component. Additional support for the role of ecology on this hybrid zone's structure was also noted from the occurrence of isolated populations of P. atricapillus within the range of P. carolinensis at high elevation, and from the unusual correlation of this range interface with the boundary of the Appalachian Mountains.

Finally, a sharp interface between P. atricapillus and P. carolinensis song in the Virginia transect contrasted with that in West Virginia, and is also consistent with the influence of the environment on the hybrid zone's dynamics. Taken together, these observations offer strong support to the geographical selection gradient model for the maintenance of the P. atricapillus/P. carolinensis hybrid zone. They do not, however, rule out the tension zone model; rather, it is likely that both will be found to be applicable here, as has been proposed on theoretical grounds (Hewitt 1988), and as has been seen in a clam hybrid zone (Bert and Arnold 1995).

Taxonomy.-The taxonomic significance of these results are open to interpretation, depending on the species concept that is being applied. I primarily contrasted the biological species concept (BSC) and the phylogenetic species concept (PSC), because of their prominence, especially in ornithology Zink and McKitrick 1995). A PSC would recognize P. atricapillus and P. carolinensis as distinct species, because they are separate evolutionary units. If the criteria of Avise and Ball (1990) are applied, they would also be maintained as distinct "biological species", because the structure of their hybrid zone revealed here clearly indicates the existence of a substantial intrinsic component to the reproductive barrier

between them. This perspective on the influence of hybridization in making taxonomic decisions is not necessarily widespread, however; the frequency of hybridization is often equated with the magnitude of genetic exchange. Insights from hybrid zone analysis on the dynamics of gene flow between taxa need to be more widely appreciated.

Future directions.-This study has illustrated that much can be learned from the use of molecular markers in hybrid zone analysis. Characters with the potential to be influenced by nongenetic factors can be assessed, to determine if this pattern of variation does in fact reflect genetic variation across the hybrid zone. Clues can be found relevant to the hybrid zone's possible origins and fate, including the forces that might be operating to maintain it if it is stable. Inferences can be drawn regarding the relative importance of different characters in contributing to reproductive isolation, and issues relating to taxonomic status can be addressed within the framework of different species concepts. However, not all questions relating to process can be answered by looking at pattern. Time often erases the evidence necessary to infer process, and multiple processes can sometimes produce the same pattern (Endler 1977, Harrison 1986). For instance, even if the current chickadee hybrid zone formed from a secondary

contact, the initial divergence of the taxa could have arisen via primary differentiation, followed by disjunction and a subsequent reestablishment of contact (Endler 1977, 1982). The fitness of hybrids relative to each parental taxon must be examined directly, either in the laboratory or in the field. I am currently participating in a collaboration with other investigators to address this question for P. atricapillus and P. carolinensis. Even when the role of certain classes of marker are implicated in contributing to reproductive isolation, the issue remains as to which were important to the speciation process, and which arose subsequent to it (Coyne 1992). And it remains for detailed crossing experiments in the laboratory to dissect out details as to the number, location and nature of genes contributing to reproductive isolation (reviewed by Wu and Palopi 1993). No organism is ideally suited for addressing all of these issues. Birds, however, provide a unique perspective on the process of differentiation, reproductive isolation, and speciation, and analysis of avian hybrid zone structure, such as offered here, is an important step in this process.

FIGURE LEGEND

Fig. 23. Clines in frequency of A alleles across the West Virginia and Virginia transects for PC 1 scores of song, PC 1 scores of morphology, and for four molecular markers.

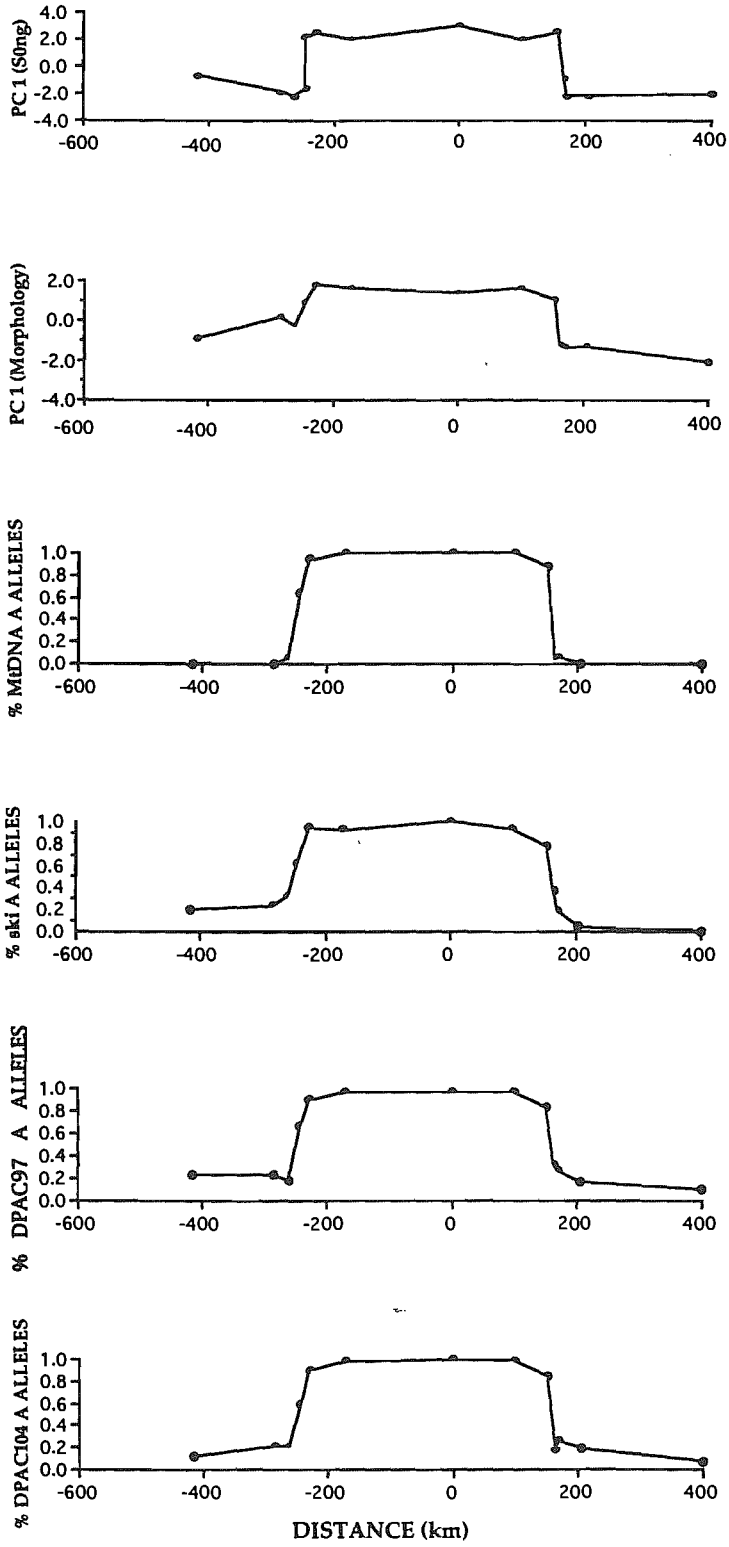
PC 1 (50ng)
4.0
2.0
0.0
-2.0
-4.0
PC 1 (Morphology)
2.0
0.0
-2.0
-4.0
% MtDNA A ALLELES
% ski A ALLELES
% DPAC97 A ALLELES
% DPAC104 A ALLELES
1.0
0.8
0.6
0.4
0.2
0.0
-600
-400
-200
0
200
400
DISTANCE (km)

Figure 23

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