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EVOLUTION OF BROWN TOWHEES: MITOCHONDRIAL DNA EVIDENCE[1]

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Abstract. Mitochondrial DNA (mtDNA) differentiation was studied among members of the Brown Towhee complex, *Pipilo albicollis, P. aberti, P. fuscus,* and *P. crissalis.* Using 16 restriction endonucleases, 196 fragments and 119 sites were observed, and an average interspecific sequence divergence of 6.4% was estimated. There was little geographic differentiation between Baja California and California samples of *crissalis* or between California and Arizona samples of *aberti.* Using phylogenetic procedures, it was shown that *aberti* and *crissalis* are sister taxa, as predicted by Davis (1951). Allozyme and mtDNA data clearly support the species distinction of *crissalis* and *fuscus.* The relationship between *albicollis* and *fuscus,* viewed as sister taxa by Zink (1988) based on allozymes, is supported albeit not strongly by the mtDNA data. MtDNA and allozyme data reveal similar patterns of evolutionary history within this group.

Key words: Brown towhees; allozymes; mitochondrial DNA; phylogeny inference; species limits; biogeography.

INTRODUCTION

The evolution of taxa in the Brown Towhee complex poses interesting problems in historical biogeography, speciation, and species limits (Davis 1951, Hubbard 1973, Zink 1988, Cracraft 1989). Currently, four species are recognized (*Pipilo albicollis, P. aberti, P. fuscus,* and *P. crissalis*; AOU 1989), which inhabit the aridlands and Mediterranean habitats of the southwestern United States, from Texas to Oregon to California, and southward into Baja California and the Mexican Plateau (see Hubbard [1973] and Zink [1988] for distribution maps). The patterns of endemism reflect those observed in other avian taxa, especially thrashers (*Toxostoma*), and provide examples of apparent vicariant events and subsequent isolation and speciation (Zink 1988).

Species limits in brown towhees have been controversial. Davis (1951) concluded that the Western and Eastern Brown Towhees (*Pipilo fuscus crissalis* and *P. f. fuscus,* respectively) were conspecific, in spite of differences in ecological preferences, vocal traits and plumage patterning. Davis further postulated that the Abert's Towhee (*P. aberti*) was derived from *P. f. crissalis* and that the White-throated Towhee (*P. albicollis*) was derived from *P. f. fuscus.* Davis and authors of classifications (e.g., AOU 1957) were not concerned that this could make the "brown towhee" paraphyletic (in modern systematics terms), because it was presumed that if sympatric, *fuscus* and *crissalis* would interbreed, but that the other taxa would (or did) not. Also, because Davis (1951) did not believe that *fuscus* and *crissalis* were full species, he essentially postulated a trichotomy of *aberti, albicollis,* and "*fuscus*" (including *crissalis*); hence, if the ancestor had not speciated (i.e., if *crissalis* and *fuscus* were not species), recognizing only three species was deemed an acceptable classification.

Zink (1988) studied variation in morphometrics and allozymes and concluded that *fuscus* and *crissalis* were genetically differentiated, and that *fuscus* was the sister taxon of *albicollis.* Therefore, the "brown towhee" was demonstrably paraphyletic and not a monophyletic evolutionary unit (sensu Cracraft 1983). The AOU Checklist Committee (1989) followed Zink and recognized *fuscus* and *crissalis* as separate species. Zink further suggested that *P. aberti* was the most basal taxon of the brown towhees. Cracraft (1989) reanalyzed Zink's allozyme distance data and concluded that a tree that depicted *P. aberti* and Western Brown Towhees (*crissalis*) as sister taxa was equally consistent with the data as the tree presented by Zink.

We used restriction enzyme analysis of mitochondrial DNA (mtDNA) to test the phylogenies proposed by Zink (1988) and Cracraft (1989), and to gather data on variation in this organellar

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genomic region to complement data from protein electrophoretic studies (allozymes). MtDNA is generally more rapidly evolving than allozymes in birds (Avise and Zink 1988). Zink and Avise (1990) found that mtDNA and allozymes yielded generally congruent patterns of phylogeny in the genus *Ammodramus*. In that the allozyme evidence for towhees yielded different trees, it seemed appropriate to use a sensitive genetic marker such as mtDNA to test alternative trees.

METHODS

The following specimens (general locality, n) were used: *P. aberti* (California, 4; Arizona, 4), *P. albicollis* (Mexico: Oaxaca, 3), *P. fuscus* (Arizona, 5), *P. crissalis* (Mexico: Baja California Sur, 2; Mexico: Baja California Norte, 2; California, 6); precise locality information is available from the senior author. The specimens of *P. albicollis*, and several other specimens, were those also used by Zink (1988), which reveals that intact mtDNA can be recovered from specimens frozen at ultracold temperatures for up to seven years. To conform to Zink's (1988) study, the samples of *P. aberti* were combined for data analysis, and data for *P. crissalis* from Baja California Sur and Norte were combined and referred to as "BA." Samples of *crissalis* from California are denoted as such by the suffix "CA." A single individual of the Green-tailed Towhee (*P. chlorurus*) was used as an outgroup to root trees. MtDNA was isolated from frozen tissue and purified in cesium chloride density equilibrium gradients following established protocols (Lansman et al. 1981, Avise and Zink 1988). MtDNA from each individual was digested with 16 restriction endonucleases and fragments were end-labeled with ^{32}P or ^{35}S, separated in 0.8% to 1.4% agarose gels, and visualized with autoradiography. A molecular size standard, a 1 kb ladder purchased from Bethesda Research Laboratories, was used to determine the sizes of mtDNA fragments. For each individual the fragment pattern for each enzyme was scored, and the scores tallied into a multi-enzyme code. In addition, each individual was scored for the presence or absence of each fragment. Although restriction site maps are more informative than fragment analyses (Swofford and Olsen 1990), restriction fragments do qualify as characters for phylogenetic analysis (Zink and Avise 1990), especially for testing a priori hypotheses of phylogenetic relationships. Using estimates of fragment sizes, restriction sites were inferred from the fragment profiles, and individuals were also scored for the presence and absence of sites. Some fragment profiles were too complicated for complete inference of sites and minimum estimates of site differences were made; thus fragments and sites were analyzed separately. The presence/absence matrix of fragments was used to compute the percent nucleotide divergence (p) following Nei and Li (1979). The fragment and site data were entered into the Boot and DolBoot programs in PHYLIP, which are bootstrap analyses (Felsenstein 1985) using Wagner and Dollo parsimony, respectively; we present the results of 1,000 bootstrap replicates. These analyses reveal the nature of character support for particular phylogenetic hypotheses (Sanderson 1989). For bootstrap analysis, we coded each fragment and site as to the restriction endonuclease that produced it, and resampled, or "bootstrapped," binary characters by endonuclease, rather than treating each fragment or site as if it were independent. This reduces bias owing to the fact that some enzymes produce more fragments than others, and bias due to the fact that restriction fragments are not independent. Because statistical interpretation of bootstrapping results requires independence of characters, which restriction fragments often are not, we consider the bootstrap analyses of fragments as descriptions of patterns in our data, and not valid statistical tests of phylogenetic patterns. It is safer to assume that restriction sites are independent (Moritz et al. 1987) and bootstrapping results can be interpreted statistically. We used Dollo parsimony because it favors gains over losses, and it is easier to lose a restriction site (or fragment) than to gain one. We entered the fragment and site data into HENNIG86 (option ie) written by J. S. Farris to find the most parsimonious tree using Wagner parsimony.

RESULTS

Each restriction endonuclease produced a diagnostic fragment profile for at least one of the taxa examined (Table 1). For seven enzymes, each species exhibited a distinct restriction profile, and for the remaining nine, there was considerable interspecific variation (Table 1; Fig. 1). A total of 196 fragments (Appendix) was scored, representing about 7% of the mitochondrial genome. A total of 119 sites was scored, which is an underestimate owing to the minimum estimates of site differences for some endonucleases; there-

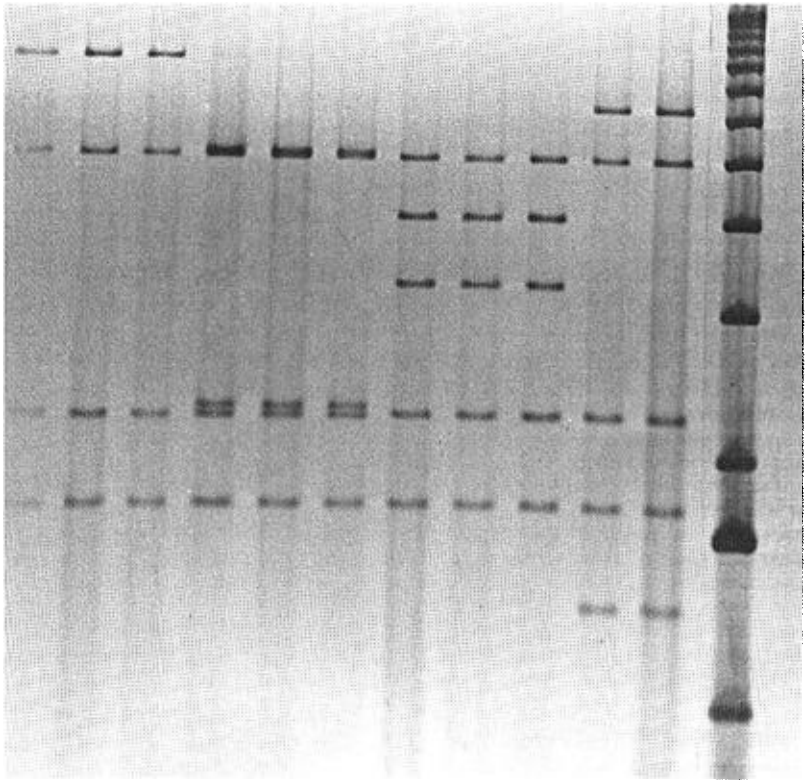


FIGURE 1. Autoradiograph of restriction fragments produced by digestion of towhee mtDNA with *Ava* I. From left to right: three *P. albicollis,* three *P. aberti,* three *P. crissalis,* two *P. fuscus,* and a molecular size standard, with bands (from bottom to top; in kb) of 1.0, 1.6, and 2.0 to 12.0 in 1-kb intervals.

fore we do not report estimates of levels of intertaxon divergence based on sites. For each species, the size of the mtDNA molecule was estimated at either 16.7 (*chlorurus* and *aberti*) or 16.8 (*albicollis, fuscus,* and *crissalis* CA and BA); each estimate was based on sizes of fragments produced by between five and eight enzymes (all standard deviations were between 0.16 and 0.28). The *p*-values among taxa (exluding comparisons involving the outgroup, and BA and CA samples of *P. crissalis*) averaged 6.4% ± 2.3% (SD) (Table 2). The single individual of *P. chlorurus* differed from the other taxa at an average *p* of 9.1% ± 4.9%; the range of values, 8.3% to 9.7% indicates some rate heterogeneity, although this is due primarily to the lower value to *P. albicollis* (Table 2). The two samples (BA and CA) of *P. crissalis* were an order of magnitude more similar than the other samples ($p = 0.12\%$).

The trees derived according to Wagner parsimony from both fragments (Fig. 2A) and sites (not shown, length = 98, ci = 0.91) show *P. aberti* and *P. crissalis* (CA plus BA) as sister taxa as well as *P. fuscus* and *P. albicollis.* These trees were also obtained in the bootstrap analyses (Fig. 2A), although the support for the latter relationship is relatively weak because it occurred in only 60.8% (fragment data) or 78% (site data) of the bootstrapped replicates. The trees derived according to Dollo parsimony and the bootstrapped Dollo analysis of both fragments and sites (Fig. 2B) showed that *P. albicollis* was an outgroup to the other three brown towhees, with *P. crissalis* (CA and BA) and *P. aberti* as sister taxa.

DISCUSSION

LEVELS AND DATES OF GENETIC DIFFERENTIATION

Although there are relatively few data for comparison, the towhee taxa examined here are differentiated ($p = 6.4\%$) at a level similar to or greater than that observed among other temperate-breeding congeneric birds (Kessler and Avise

TABLE 1. Clonal designations for common mtDNA genotypes observed in towhee taxa. Letters, from left to right, refer to multi-fragment mtDNA profiles produced by digestion with: *Ava* I, *Ava* II, *Bam*H I, *Ban* II, *Bgl* I, *Bgl* II, *Eco*R I, *Hin*d III, *Hin*c II, *Hin*f I, *Nci* I, *Nde* I, *Pst* I, *Pvu* II, *Sst* II, and *Xba* I.

MtDNA clone	Designation																No. of birds
P. chlorurus	E	G	A	E	E	C	D	D	E	E	F	C	C	D	A	D	1
P. albicollis	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	2
	A	B	A	A	A	A	A	A	A	A	A	A	A	A	B	A	1
P. aberti	B	C	B	B	B	A	B	B	B	B	B	A	A	B	A	B	7
	B	C	B	B	B	D	B	B	B	B	B	A	A	B	A	B	1
P. crissalis CA	C	D	C	C	C	A	B	B	C	C	C	B	A	B	A	B	5
	C	E	C	C	C	A	B	B	C	C	C	B	A	B	A	B	1
P. crissalis BA	C	D	C	C	C	A	B	B	C	C	C	B	A	E	A	B	1
	C	D	C	C	C	A	B	E	C	C	H	B	A	B	A	B	3
P. fuscus	D	F	D	D	D	B	C	C	D	D	D	A	A	C	A	C	2
	D	F	D	D	D	B	C	C	D	D	D	A	B	C	A	C	3

1985, Avise and Zink 1988; Shields and Helm-Bychowski 1988; Zink and Avise 1990). In fact, the two "brown towhees" are differentiated at a level, $p = 4.3\%$, equivalent to most well-differentiated avian congeners, although they were considered separate species until 1989 (AOU 1989). As discussed elsewhere (e.g., Zink 1988), levels of genetic differentiation, irrespective of the genetic marker surveyed, cannot be used as absolute taxonomic yardsticks. It is the pattern of relationships as well as the nature of distinctness that arbitrate species limits (McKitrick and Zink 1988).

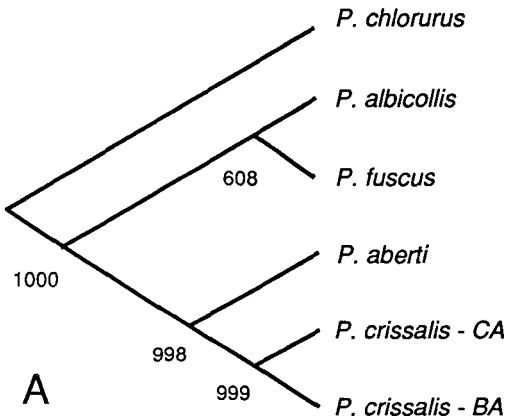
If one converts estimates of genetic differentiation into time since common ancestry, the allozyme estimate (assuming 26.3 MY for each unit of Nei's [1978] genetic distance; see Zink and Avise 1990) gives an average divergence date of 1.8 MYBP for the average timing of speciation events. The average mtDNA distance value, 6.4%, gives an estimate of 3 MYBP, assuming a rate of 2% per million years (Shields and Wilson 1987). In contrast, in Zink and Avise's (1990) study of *Ammodramus,* the allozymes gave an estimated divergence date older than that derived from mtDNA data. Average values can misrepresent congruence in timing of speciation events. We converted to a divergence date each element in the allozyme and mtDNA distance matrices using the conversion factors noted above. These are rough approximations, and other authors have used different calibrations (see Zink and Avise 1990). Note, however, that the correlation coefficient (but not the slope) between the two data sets will remain the same no matter what calibration is used because each genetic distance value is multiplied by a constant. In the plot (Fig. 3) of mtDNA versus allozyme estimates of divergence, the first plot of its kind for birds, there are some outliers, but a generally high correspondence. A point of disagreement between the two data sets concerns the value for *fuscus* versus *albicollis* (shown with an arrow in Fig. 3), where mtDNA differentiation is relatively much greater. Additional such comparisons are required to establish calibration factors and determine covariation patterns for rates of evolution of different molecular regions.

GEOGRAPHIC VARIATION

As in Zink's (1988) study, the purpose here was not a survey of variation with any species. However, because allozyme data indicate little geographic structure in temperate birds in general

TABLE 2. Matrix of *p*-values calculated according to Nei and Li (1979). For *P. crissalis,* CA refers to California samples and BA to those from Baja California Sur and Norte combined.

P. chlorurus	0.0000					
P. albicollis	0.0833	0.0000				
P. aberti	0.0977	0.0496	0.0000			
P. crissalis CA	0.0932	0.0488	0.0245	0.0000		
P. fuscus	0.0886	0.0357	0.0465	0.0419	0.0000	
P. crissalis BA	0.0935	0.0491	0.0259	0.0012	0.0438	0.0000



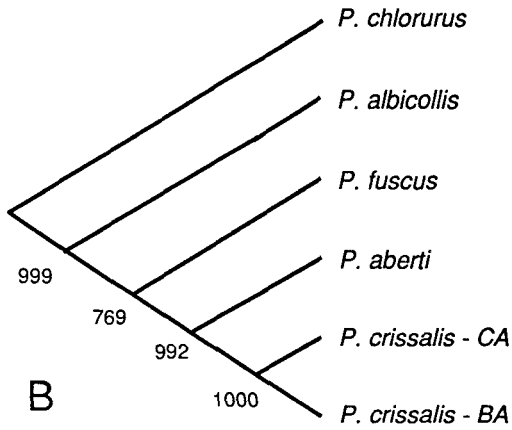


FIGURE 2. A. Cladogram derived from the presence/absence matrix of fragments (Appendix) using the program HENNIG86 and the principle of maximum parsimony (length = 189, consistency index = 0.92). The same tree structure resulted from the bootstrapped analysis (1,000 replicates using the program Boot in PHYLIP), and the numbers at nodes refer to the number of times out of 1,000 that the node occurred. B. Cladogram derived from the presence/absence matrix of fragments (Appendix) using the program Dollop in PHYLIP, which uses the principle of Dollo parsimony. The same tree structure resulted from 1,000 bootstrap replicates also using Dollo parsimony and the numbers at nodes represent the number of times that node occurred out of 1,000.

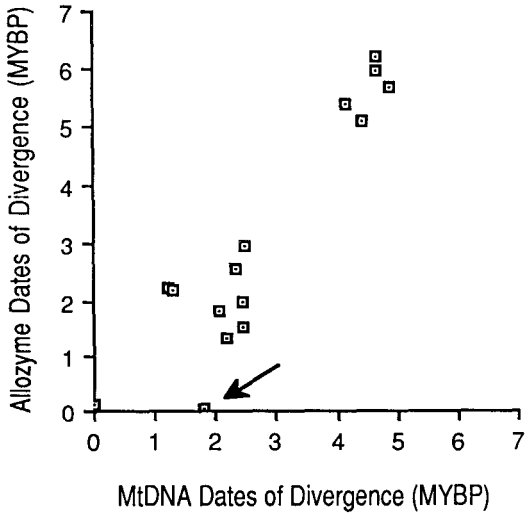


FIGURE 3. Plot of pairwise comparisons of dates of divergence derived from mtDNA and allozymes. Although the two data sets are genetically independent, values within each are not; hence, we do not present statistical analysis. Scales are in millions of years. The arrow refers to the comparison of *fuscus* and *albicollis*.

(e.g., Barrowclough 1983), it is of interest to examine mtDNA for geographic variability. Some mtDNA surveys reveal considerable geographic differentiation (Avise and Nelson 1989; Zink, in press), whereas others do not (Ball et al. 1988). Although analyses of mtDNA seem more likely than allozymes to reveal geographic patterns of variation, there are too few data to generalize.

In towhees, *crissalis* samples from Baja California and California differ in frequencies of fragment profiles at *Hin*d III, although sample sizes are small, and the sequence divergence across all endonucleases is only 0.12%; there was little allozyme divergence as well (Nei's [1978] D = 0.004). Thus, the mtDNA and allozyme data are consistent with Zink's (1988) opinion that *crissalis* towhees dispersed into Baja California after its connection with California in the Miocene. Towhees in Baja California either have not had sufficient time to evolve mtDNA differences if isolated, or current gene flow mediates the effect of geographic distance. Our two small samples of *P. aberti* did not show any geographic differentiation, which is perhaps not surprising considering the small distance separating them (Salton Sea and near Tuscon, Arizona, a distance of about 500 km). However, our samples of *crissalis* and *fuscus* are from approximately the same geographic sites and show extensive differentiation. Thus, *aberti* probably dispersed westward from its Sonoran site of origin subsequent to the differentiation of *crissalis* and *fuscus*, because *aberti*

samples do not exhibit mtDNA divergence across the geographic gap that presently separates *crissalis* and *fuscus*.

ALLOZYMES VS. MTDNA

In the avian genus *Ammodramus*, both allozymes and mtDNA indicated significant genetic differentiation, and phylogenetic patterns were significantly congruent (Zink and Avise 1990). In a similar study of *Zonotrichia*, Zink et al. (unpubl. data) found congruent patterns of allozyme and mtDNA variation among species. In this study, a rank order correlation coefficient between allozymic and mtDNA distance matrices of 0.80 indicates substantial concordance of distance values. Because mtDNA is inherited as a single linkage group, it provides a single "gene tree" (Neigel and Avise 1986), whereas allozyme estimates are based on multiple loci. Therefore, the congruence of allozymes and mtDNA is satisfying.

SPECIES LIMITS, PHYLOGENY, AND BIOGEOGRAPHY

Phylogenies derived from both allozymes and mtDNA document that *crissalis* and *fuscus* are not sister taxa, and in our opinion they therefore cannot be considered conspecific (McKitrick and Zink 1988). Even if *crissalis* and *fuscus* did exist sympatrically with interbreeding, considering them as conspecific (as viewed until 1989, AOU [1989]) would obfuscate the pattern of evolutionary history revealed by both allozymes and mtDNA. That is, because reproductive compatibility is an ancestral condition, uniting *crissalis* and *fuscus* would create a paraphyletic group given the relationships in Figure 2. Therefore, *crissalis* and *fuscus* are phylogenetic species (sensu Cracraft 1983); whether or not they are biological species is unknown without a test of sympatry.

Davis (1951) concluded that *crissalis* and *fuscus* had not reached species-level differentiation in spite of six million years of independent evolution, whereas *albicollis* and *aberti* had. MtDNA evidence conflicts with Davis' opinion about level of divergence between *crissalis* and *fuscus*. The samples of *fuscus* and *crissalis* exhibited different mtDNA fragment profiles at 14 of 16 enzymes, and a frequency difference at *Pst* I (Table 1). In fact, the only enzyme that generated similar digestion profiles for the two taxa was *Sst* II, which exhibits the same fragment profile in most birds (Zink, pers obs.). Hence, external morphology, and mtDNA and allozymes have evolved at seemingly different rates in *crissalis* and *fuscus*.

Zink's (1988) allozyme phylogeny portrayed a sister-taxon relationship between *fuscus* and *albicollis*, and this relationship is supported by the analysis of mtDNA data using Wagner (Fig. 2A) but not Dollo (Fig. 2B) parsimony. However, it is not clear that Dollo parsimony is appropriate. Because closely related species likely inherit similar ancestral sequences, parallel gains might not be unlikely, and Dollo parsimony would be too conservative because it allows only a single site gain. The tree topology from Zink's (1988) study ([*chlorurus* [*aberti* [[*albicollis* + *fuscus*] [*crissalis* CA + BA]]]]) was input into HENNIG86 and found to be 203 steps for the fragment data and 111 steps for the site data, relative to 189 and 98 steps for the most parsimonious trees, respectively. Thus, the mtDNA data reject the tree shown by Zink (1988) in which *aberti* is an outgroup to the other taxa. The position of *albicollis* was not clearly resolved by the mtDNA data. The tree topology derived using Dollo parsimony (Fig. 2B), in which *albicollis* is outside the other brown towhees, requires only 190 steps for the fragment data and 100 steps for the site data using Wagner parsimony. In our opinion, a difference of one (fragments) or two (sites) steps does not warrant confidence in the shortest tree. We note, however, that the shortest allozyme tree (Cracraft 1989) is topologically equivalent to the most parsimonious trees for fragments (Fig. 2A) and sites. The use of *chlorurus* to root the Dollo tree could be problematic if it were too divergent; *chlorurus* was only recently put in *Pipilo*. With a different outgroup, perhaps a more typical *Pipilo*, the root might be indicated between *albicollis/fuscus* and the remaining taxa, restoring the sister-species relationship of the former pair. In summary, we suggest that a sister group relationship between *fuscus* and *albicollis* is most consistent with the data. Phylogenetic studies of other co-distributed lineages (e.g., *Toxostoma*) would provide a test of the sister-taxon relationship of *fuscus* and *albicollis* (Cracraft 1983), as would additional data sets.

It is clear, however, that the sister-taxon relationship between *P. aberti* and *P. crissalis* is extremely well supported (99% of the bootstrapped replicates in both the Wagner and Dollo

parsimony analyses) by the mtDNA analysis, whereas it is equivocal in the allozyme analysis (Zink 1988, Cracraft 1989). Davis (1951) hypothesized that the eastern and western lineages of "brown towhees" became isolated in the Pliocene (6 MYBP), and from the western lineage (modern *crissalis*) evolved *aberti* within the last million years, and *albicollis* evolved from the eastern lineage at an earlier time. Davis appears correct in hypothesizing that *aberti* evolved from *crissalis* and probably that *albicollis* evolved from *fuscus* (Fig. 2A), and that the former happened more recently than the latter. Interestingly, Davis' estimate of one million years for the evolution of *aberti* is entirely consistent with the calibration of mtDNA, in which the *p* value of 2.45% (Table 2) indicates a divergence of 1.25 million years. The divergence time for *albicollis* and *fuscus,* based on a *p* value of 3.57%, is estimated at 1.75 MYBP.

mtDNA RESTRICTION FRAGMENTS IN PHYLOGENETIC STUDIES

Restriction fragments are less preferable than restriction sites because fragments are not independent (one site produces two fragments), and clones can share sites but not fragments (Swofford and Olsen 1990). However, analyses of sites and fragments yield congruent results in several studies (Zink, in press, unpubl. data) including this one. Futhermore, the agreement between allozymes and restriction fragments in *Ammodramus* suggest that fragment analyses do recover phylogenetic relationships (Zink and Avise 1990). Analysis of restriction fragments is likely to be less informative than analysis of sites, but patterns of restriction fragment variation probably do not contradict true phylogenetic patterns.

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APPENDIX.

Presence and absence of restriction fragments in towhees.

P. aberti 000111111100000000110000011001000001101111000010000011111000001111000010011111000000000011100000000000000000011000111000000000000001110000000011101111100001010111111000000000011010001100010000010 1

P. chlorurus 000101000011111000000000010100001100100000001100100000000001111110000110000010000001100000000000000011111000010000010000011000000000000001111000000010000000000111111000100000100111100110000000001100100000000001111000100010000110000100011010 1

P. crissalis CA 000111111100000000110000011011000001101110100000000001111100000101111001101100111000000000001111100000000000000010000000111000000110100111100001100010100100010101111011100000000110100011000100000010 1

P. fuscus 010111110000000000001111000110100001111100000000110011100100001111000010011100001100000100010011000000011000110000101000000000000010001011000110000000000110111011000000011000100111000110000101 1

P. crissalis BA 000111101100001100110000011011000001101110100000000001111100000101111001101100111000000000001111100000000000000010000000111000000110100111100001100010100100010101111011100000000110100011000100000010 1

P. albicollis 111111100000000011000000001110000111110000000000000111110000000111100001011110000000011100000010000000000111111100000000000000001100000000001100110000001111111110000000011100001100010000111 0