Molecular Population Genetics of Tufted and Black-crested Forms of *Parus bicolor*

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The ranges of the Tufted Titmouse (*Parus bicolor bicolor*) and the Black-crested Titmouse (*Parus bicolor atricristatus*) meet in central Texas (Oberholser 1974). *Bicolor* is common to the woodlands of eastern North America, while *atricristatus* inhabits the drier, scrubbier habitats of southcentral Texas and the east coast of Mexico (Fig. 1). The two forms are morphologically distinct and were originally treated as separate species. In the narrow zone of transition between their habitats, however, Dixon (1955) found that the two forms interbreed freely. Subsequently, the A.O.U. Check-list Committee (A.O.U. 1976, 1982) has upheld his view that these titmice are conspecific. Although four weakly differentiated subspecies of the black-crested form have been recognized (A.O.U. 1957), for convenience we will refer to the group by its original specific name, *atricristatus*.

Dixon (1978) constructed a possible evolutionary history of the group from distributional, paleontological, and archeological data. He postulated initial isolation coinciding with the end of the last glacial age, the Wisconsinan, 11,000–14,000 yr B.P. At that time the corridor of woodlands that extended into Mexico during the pluvial period was broken, thereby separating the titmouse populations. In Dixon's view, contact was re-established 4,000–6,000 yr B.P. along forested river courses that connected their ranges. Remington (1968), in a paper surveying hybridizing forms in central Texas, placed the onset of introgression even more recently, at about 2,000 yr B.P.

Modern biochemical methods are valuable in the study of hybridizing populations when there is sufficient molecular differentiation in allopatric populations to compare with that of populations in and near the contact zone (Corbin et al. 1979, Barrowclough 1980). This study utilizes electrophoretic techniques to make a preliminary estimate of molecular differentiation in allopatric populations of Black-crested and Tufted titmice.

Specimens from "pure" populations of *bicolor* and *atricristatus* were collected at localities well outside the hybrid zone (Fig. 1). Five specimens of *bicolor* were taken near Conroe, Texas, and 12 specimens of *atricristatus* were collected near Bandera, Texas. In addition, seven tissue samples of *bicolor* collected from localities in southeastern Louisiana were obtained from the frozen tissue collection of the Louisiana State University Museum of Zoology. Each specimen was dissected within 4 h of collection. Samples of skeletal muscle, brain, heart, liver, and kidney were frozen on dry ice until they were transferred to permanent

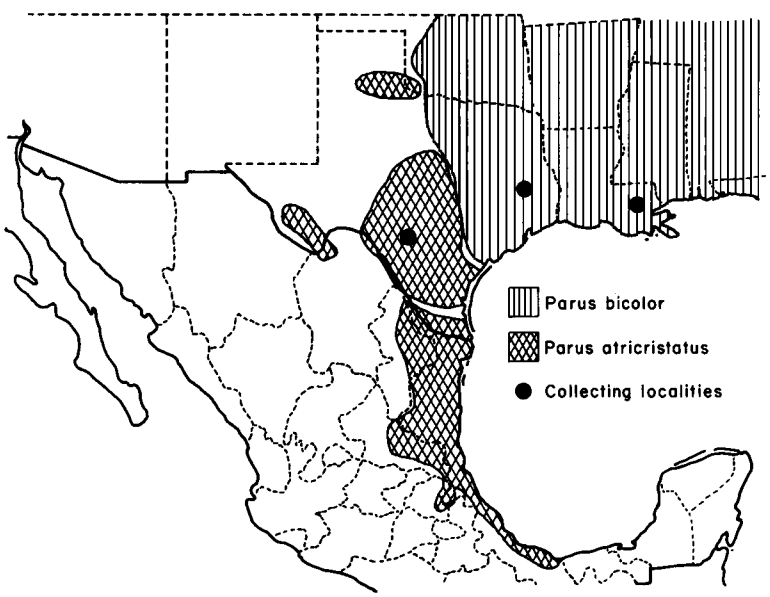

Fig. 1. Range map and collecting localities.

TABLE 1. Allelic frequencies of polymorphic and monomorphic loci.[a]

Locus	Allele	*P. bicolor* frequency ($2n = 24$)	*P. atricristatus* frequency ($2n = 24$)
I. Polymorphic loci			
1.1.1.1 Octanol dehydrogenase	A	1.000	0.917
	B	0.000	0.083
1.1.1.42 Isocitrate dehydrogenase-1	A	0.000	0.375
	B	1.000	0.625
1.1.1.42 Isocitrate dehydrogenase-2	A	0.000	0.042
	B	1.000	0.958
1.1.1.44 6-Phosphogluconate dehydrogenase	A	0.042	0.000
	B	0.917	1.000
	C	0.042	0.000
1.1.1 unknown dehydrogenase	A	0.000	0.792
	B	1.000	0.208
1.15.1.1 Superoxide dismutase-1	A	0.000	0.042
	B	1.000	0.958
2.6.1.1 Glutamic-oxaloacetic transaminase-1	A	0.708	1.000
	B	0.292	0.000
2.6.1.2 Glutamic-pyruvic transaminase-2	A	0.000	0.042
	B	1.000	0.958
2.7.5.1 Phosphoglucomutase	A	0.917	1.000
	B	0.083	0.000
3.1.1.1 Esterase-1 Esterase-2	A	0.125	0.000
	B	0.875	1.000
3.4.11 Leucylalanine peptidase-1	A	0.167	0.000
	B	0.708	0.087
	C	0.125	0.174
	D	0.000	0.739
3.4.11 Leucylalanine peptidase-2	A	0.125	0.000
	B	0.000	0.042
	C	0.875	0.958
3.4.11 Leucylglycylglycine peptidase-2	A	0.083	0.083
	B	0.917	0.917
3.4.11 Phenylalanylproline peptidase-1	A	0.000	0.042
	B	1.000	0.958
3.5.4.4 Adenosine deaminase	A	0.042	0.917
	B	0.958	0.083
5.3.1.8 Mannose phosphate isomerase	A	0.958	1.000
	B	0.042	0.000
5.3.1.9 Phosphogluconate isomerase	A	0.042	0.000
	B	0.958	1.000

II. Monomorphic loci

1.1.1.1 Ethanol dehydrogenase
1.1.1.8 α-Glycerophosphate dehydrogenase
1.1.1.14 Sorbitol dehydrogenase
1.1.1.27 Lactate dehydrogenase (heart)
1.1.1.27 Lactate dehydrogenase (muscle)
1.1.1.37 Malate dehydrogenase-1
1.1.1.37 Malate dehydrogenase-2
1.1.1.40 Malic enzyme
1.1.1.47 Glutamate dehydrogenase
1.2.1.12 Glyceraldehyde phosphate dehydrogenase
1.15.1.1 Superoxide dismutase-2
2.6.1.2 Glutamic-pyruvic transaminase-1
2.7.3.2 Creatine kinase-1
2.7.3.2 Creatine kinase-2
2.7.4.3 Adenylate kinase
3.1.1.1 Esterase-2
3.1.3.2 Acid phosphatase
3.4.11 Leucylglycylglycine peptidase-1

[a] Loci and alleles are numbered and lettered beginning with those traveling most anodally.

storage at −60°C. A museum skin was prepared from each specimen and scored using the morphological hybrid index of Dixon (1955). Scores obtained indicated morphologically pure populations. These skins are on deposit at the Texas Memorial Museum and Louisiana State University Museum of Zoology.

Homogenates of skeletal muscle and heart and liver tissue were prepared from each specimen and analyzed by starch gel electrophoresis. In total, 36 presumed genetic loci encoding proteins with 25 enzyme activities were examined. Similarities with other vertebrates in apparent subunit structures and staining specificities reinforce the presumed identity of the proteins in these birds. One dimeric dehydrogenase of unknown specificity consistently stained weakly on glutamate dehydrogenase gels. We report this unidentified locus because of its utility in separating the titmice. The general procedures for electrophoresis and localization of proteins are widely available and will not be described here (Dessauer 1966, Harris and Hopkinson 1976, Ferguson 1980). Details of stains and buffer systems are available from the first author. The determination of mean heterozygosity per locus, *H*, and genetic distance, *D*, were made directly from the locus-specific banding patterns on gels (Nei 1978).

Differentiation between Texas and Louisiana populations of *P. bicolor* was found to be insignificant and in the results that follow all *bicolor* specimens are treated as one population. Of the 36 genetic loci studied, 18 were polymorphic in at least one population and 18 were monomorphic. Allelic frequencies of all loci studied are reported in Table 1. Five polymorphic loci exhibited strong allelic frequency differences between *bicolor* and *atricristatus*: isocitrate dehydrogenase-1, glutamic oxaloacetic transaminase-1, leucylalanine peptidase-1, the dehydrogenase of unknown specificity that was described earlier, and adenosine deaminase. At the first four of these loci, alleles that were present at least 30% of the time in one population were not detected in the other. These five loci represent the main differentiation found between *bicolor* and *atricristatus*. They form the core of molecular characters that can be used to study the nature and extent of the contact zone.

Unbiased estimates of heterozygosity were equal for *bicolor* and *atricristatus*. *H* is 0.06 ± 0.02.

Genetic distance, *D*, is estimated to be 0.063. Intralocus standard error for *D* is 0.00005. Barrowclough (1980) in his summary of genetic distances reported for birds finds the average *D* to be 0.005 for subspecies and 0.044 for species. Clearly, *bicolor* and *atricristatus* have achieved differentiation more in line with species than subspecies. For comparison within the same genus, the Black-capped and Carolina chickadees (*P. atricapillus* and *P. carolinensis*) are currently treated as species, although a narrow hybrid zone is known to exist. A recent study (M. B. Robbins et al. unpubl.) has found *D* between allopatric populations of chickadees to be 0.001.

The time of divergence, estimated from *D* by the method of Nei (1975), is 240,000 yr B.P. There are many problems associated with calibration of "protein clocks" (Ferguson 1980), and this date should be used in a comparative way only. The data we have do suggest strongly, however, that the time of separation in these taxa was well before the figure of 14,000 yr B.P. suggested by Dixon (1978). In passing, we note that if Dixon's historical outline applies to the close of the Illinoian glacial age, approximately 225,000 yr B.P., rather than to the Wisconsinan age, good correlation with our data is obtained.

A more detailed electrophoretic study of the group near the hybrid zone could reveal the extent of introgression and thereby yield a clearer picture of the specific status of these *Parus bicolor* forms. Further, it might reveal the existence of selective forces at work in the hybrid zone. The facts that genetic distance is large (for birds) despite reproductive contact and that the hybrid zones are narrow suggest that selection against hybrids may be a factor in maintaining the integrity of the two groups. Clearly, as shown by Dixon (1955), the geographic isolation experienced by these groups did not result in complete reproductive isolation. The existence of hybrid zones, however, does not rule out continued genetic isolation (Hunt and Selander 1973, Moore 1977).

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Feather Pulp: a Non-destructive Sampling Technique for Electrophoretic Studies of Birds

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Samples of tissue for electrophoretic analysis of avian species are usually obtained from the muscle, heart, kidney, or liver of sacrificed individuals (e.g. Smith and Zimmerman 1976, Barrowclough and Corbin 1978, Avise et al. 1980). Egg-white proteins have also been used (e.g. Sibley 1961). Non-destructive collection of blood is possible but yields less information than that obtained from the muscle or internal organs, because fewer structural gene loci are expressed in blood (e.g. Barrowclough and Corbin 1978, Ryttman and Tegelstrom 1981). Moreover, the loss of comparatively minute quantities of blood in small birds, such as many passerines, or the stress of taking the sample may cause fatal shock. A skilled worker can perform a biopsy of pectoral muscle (Baker 1981), which expresses more loci than blood, but this, again, is not a benign technique.

Because of the need for non-destructive sampling of birds in studies of behavior, ecology, and evolution (e.g. Sherman 1981), we sought to identify another source of tissue that could be taken without harm to the birds. This paper examines the potential of an alternative non-destructive technique for obtaining avian tissue for electrophoresis—the use of the pulp inside the shafts of newly emerging feathers.

A single individual of each of six species of birds was used: turkey (*Meleagris gallopavo*), Red Junglefowl (*Gallus gallus*), Common Quail (*Coturnix coturnix*), domestic duck (derived from *Anas platyrhynchos*), Rock Dove (*Columba livia*), and Bobolink (*Dolichonyx oryzivorus*). All birds except the Bobolink, which was frozen intact for 2 weeks, were fully dissected within 15 min of sacrifice. A 0.5-cc sample was taken of kidney, liver, heart, and pectoral muscle; whole blood was collected from the jugular vein or heart. Developing feathers with pink, living tissue showing through the quill were collected from each bird, regardless of stage of development. For each gel sample, approximately 0.05 ml of homogenate permits an examination of up to seven enzyme systems, if a 0.95-cm-thick gel is used. In the larger birds (turkey, duck, Red Junglefowl and Rock Dove), even body feathers had useable quantities of tissue in their quills; we collected tissue from a variety of wing, tail, and body feathers. In the Bobolink, the 5th and 6th primaries on each wing were approximately half emerged, and all four were collected to obtain sufficient tissue. Fortunately, as molt of flight feathers is symmetrical and often overlaps tail molt, 2-4 large feathers are available throughout most of the molting period. The quail had only superficial head molt, and the 5 feathers that were collected were homogenized whole (see below). In addition, flight feathers in three distinct stages of molt—quill just emerged, feather half grown, and fully emerged—were collected from the turkey in order to assess possible changes in enzyme expression during feather development.

During sampling, care was taken to grasp the feather quill next to the skin and to pull straight outward in order to avoid breaking the quill. This is especially important in living birds, as a broken quill left in the skin inhibits the growth of a new feather and may cause infection. If a bird is not in molt, feather growth may be induced by plucking a feather