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Allozymic and Morphometric Comparisons among Indigo and Lazuli buntings and their Hybrids

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Analyses of genetic variation, as inferred from morphological and biochemical traits, have contributed to our understanding of avian hybridization (Braun and Robbins 1986, Avise and Zink 1988, Gelter et al. 1989). Such analyses, together with behavioral studies in the field and laboratory (Emlen et al. 1975, Robbins et al. 1986, Baker and Baker 1990), provide an important component in understanding causes and consequences of interspecific hybridization. Conclusions about the degree of genetic isolation between two species and the geographic extent of introgression may depend upon the trait examined. Thus, by necessity, it is important to use all of the possible information available in attempting to reach a general conclusion about hybridization in any case study of a species pair. We examined allozymes, morphometric traits, and plumage patterns in Indigo Buntings (*Passerina cyanea*), Lazuli Buntings (*P. amoena*), and their hybrids to gain further understanding of events in a zone of overlap and hybridization.

Methods.—Morphological measurements and allozyme frequencies were obtained from adult males in allopatric populations of Indigo and Lazuli buntings and from a population exhibiting Lazuli, Indigo, and hybrid plumage characteristics in an area of sympatry in northeastern Wyoming (1 to 5 km south of Beulah, along Sand Creek). Samples for allozyme analysis and for morphological measurements were taken from different individuals; specimens were obtained or individuals measured during the course of a number of field behavioral studies and laboratory experiments spanning a period of four years. Enzyme electrophoresis was conducted initially with the objective of finding one or more electrophoretic markers that could be used to identify hybrid offspring in the nest or soon after they fledged. We analyzed five morphological traits to see how well they corresponded with plumage traits that have been used traditionally to describe Lazuli, Indigo, and hybrid phenotypes (Sibley and Short 1959, Emlen et al. 1975, Kroodsma 1975).

For electrophoretic analyses, buntings were obtained from allopatric and sympatric populations. Assignment of individuals from the hybridizing population to the three morphs (Indigo, Lazuli, hybrid) was based on plumage characteristics (Emlen et al. 1975). Allopatric Indigos (n = 12) were sampled near Vinton, Iowa, and allopatric Lazulis near Gateway, Colorado (n = 6); Pocatello, Idaho (n = 9); and Logan, Utah (n = 12). In none of the allopatric populations were birds of the alternative species observed during several years of field studies. Sympatric Indigos (n = 4), sympatric Lazulis (n = 12), and hybrids (n = 6) were from northeastern Wyoming where the morphological data were collected. In this mixed population, all three forms could be found in

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adjacent territories. Birds were killed, and liver tissue was frozen in liquid nitrogen and stored in a freezer (−80°C). Tissue samples were shipped on dry ice to the University of Western Australia and maintained at −80°C until February 1992, when electrophoresis was conducted.

Liver samples were examined electrophoretically following maceration in 10% sucrose containing 0.1% (v/v) mercaptoethanol and 0.1% (w/v) bromphenol blue. Twenty-three assays for enzyme variability were performed by horizontal starch-gel electrophoresis using the staining procedures of Richardson et al. (1986). These 23 assays revealed 29 presumptive gene loci, of which 21 were monomorphic and 8 were polymorphic (Appendix). Another assay, Aconitate Hydratase (ACON; E.C. no. 4.2.1.3), gave banding patterns identical to those found by the assay for Isocitrate Dehydrogenase (IDH). Therefore, a further assay was prepared with no substrate (nothing dehydrogenase); it produced the same pattern of bands as the IDH and ACON assays. Because the bands revealed with the IDH assay developed more rapidly and stained more intensely, we refer to this locus as IDH in this report. Statistical description of allozyme data was carried out with BIOSYS-1 (Swofford and Selander 1981).

For analyses of morphometric data, five traits were measured by MCB on each live bird. Wing length was measured from the bend of the wing to the tip of the longest primary, with the wing flattened on the ruler. The remaining measurements were taken with a dial caliper. Tarsus length was the measured diagonal distance from the joint between the tibia and metatarsus to the joint at the base of the middle toe in front. Bill length was measured from the anterior edge of the nares to the tip of the upper mandible. Bill depth was measured from the top of the upper mandible to the bottom of the lower mandible at the flattened area where the upper mandible joins to the skull. Bill width was measured as the greatest width of the upper mandible at the base of the bill. A discriminant function (Minitab 1988) was constructed from the five mensural traits measured on a sample of allopatric male Indigos (n = 30) from a population near Vinton, Iowa, and on a sample of allopatric male Lazulis (n = 32) from a population near Pocatello, Idaho, which served as the reference populations. The output from this analysis was used to calculate discriminant scores for the 29 birds from the hybridizing population.

Plumage characteristics were used to assign birds to categories of Indigo, Lazuli, or hybrid phenotypes. Plumage analysis was carried out according to the scoring system of Emlen et al. (1975), which examines color patterns of the plumage in six topographic regions. By this composite index, total scores of an individual male can range form 0 (pure Indigo) to 11 (pure Lazuli). Here, scores of 0 to 2 were judged as Indigo, 3 to 8 as hybrid, and 9 to 11 as Lazuli.

Results.—For the allozyme study, samples from three separate populations represented allopatric Lazuli Buntings (Colorado, Idaho, and Utah). Allozyme frequencies were compared among these three samples by G-test (Sokal and Rohlf 1981). No significant differences were found for any locus, and they were grouped for subsequent analyses.

Of the 29 loci sampled, 8 were polymorphic in at least one of the five samples (Table 1); only the hybrid sample at the Aspartate Amino Transferase (AAT) locus showed significant departure of allelic frequencies from Hardy-Weinberg expectations. The proportion of polymorphic loci ranged from 0.172 to 0.276 ($\bar{x}$ = 0.227; Table 2). Observed mean heterozygosities in these samples ranged from 0.061 to 0.120 ($\bar{x}$ = 0.084). Genetic distances among the five samples ranged from a low of zero to a high of 0.017 ($\bar{x}$ = 0.004; Table 3). Variation in allelic frequencies was minor except at the AAT locus, which showed large differences between Indigo and Lazuli samples, especially in allopatry (Table 1).

Means and standard deviations of the measurements of the five traits are given in Table 4. Discriminant function analysis performed on the five mensural traits resulted in a discriminant function that correctly classified all 62 birds from the allopatric reference populations into Lazuli or Indigo groups. The standardized dependent variable canonical coefficients (wing, −0.961; tarsus, 0.587; bill length, 0.289; bill depth, 0.390; bill width, −0.309) were applied to the measurements of both reference groups and the 29 birds from the hybridizing population in Wyoming. All Lazulis from the reference population near Pocatello had negative discriminant scores, all Indigos from the reference population near Vinton had positive scores, and the sympatric sample from Wyoming had both negative and positive scores (Fig. 1). Whereas assignment of individuals in the Wyoming sample to Lazuli, Indigo, and hybrid plumage morphs revealed that all 13 birds with negative discriminant scores had the expected Lazuli plumage patterns, 10 birds in Lazuli plumage also had positive discriminant scores, as did the 4 birds with hybrid plumage patterns and the 2 in Indigo plumage (Fig. 1).

Discussion.—In general, the genetic variability found in the bunting samples is similar in magnitude to average values observed in other avian species. Evans (1987) and Corbin (1983) tabulated the proportion of polymorphic loci among birds, which averaged 0.240 and 0.222, respectively, compared with the buntings' average value of 0.227. Mean heterozygosity in the buntings (0.084) is somewhat greater than the averages of values accumulated by Evans (0.065) and Corbin (0.067). The genetic distance between allopatric Lazuli and Indigo samples (0.017) is intermediate between that found for comparisons between other bird species ($\bar{x}$ = 0.044; Barrowclough 1980) and that for comparisons between bird sub-

TABLE 1. Observed allelic frequencies in each bunting population sample for eight enzyme loci. Allele no. 1 had the fastest mobility; higher numbers migrated more slowly. *G*-values were calculated for observed allelic frequencies versus Hardy-Weinberg expectations.

	Allopatric		Sympatric		
Allele	Indigo (24)[a]	Lazuli (54)	Indigo (8)	Lazuli (24)	Hybrid (12)
			AAT[b]		
1	0.167	0.815	0.375	0.750	0.500
2	0.833	0.185	0.625	0.250	0.500
G	0.79	0.03	0.88	2.04	8.32
			EST		
1	0.208	0.000	0.000	0.042	0.000
2	0.167	0.407	0.500	0.417	0.333
3	0.625	0.537	0.500	0.541	0.667
4	0.000	0.056	0.000	0.000	0.000
G	4.39	0.08	5.54	0.31	0.37
			GPI		
1	0.792	0.852	0.750	0.750	0.833
2	0.208	0.148	0.250	0.250	0.167
G	1.34	1.39	0.68	2.04	0.41
			IDH		
1	0.000	0.000	0.125	0.000	0.000
2	1.000	1.000	0.875	0.916	1.000
3	0.000	0.000	0.000	0.042	0.000
4	0.000	0.000	0.000	0.042	0.000
G	0.00	0.00	0.00	0.17	0.00
			MPI		
1	0.042	0.000	0.000	0.042	0.000
2	0.916	0.944	0.875	0.875	1.000
3	0.042	0.056	0.125	0.083	0.000
G	0.04	0.17	0.14	0.18	0.00
			PEP-LGG		
1	1.000	0.926	1.000	0.875	1.000
2	0.000	0.074	0.000	0.125	0.000
G	0.00	3.06	0.00	3.19	0.00
			PEP-LT		
1	0.708	0.852	0.625	0.875	0.667
2	0.292	0.130	0.375	0.125	0.333
3	0.000	0.018	0.000	0.000	0.000
G	1.77	1.39	1.91	0.41	2.07
			PEP-VL		
1	0.000	0.019	0.000	0.000	0.083
2	0.833	0.907	0.625	0.875	0.750
3	0.167	0.074	0.375	0.125	0.167
G	1.56	0.32	1.91	0.41	0.41

[a] Number of alleles.
[b] See Appendix for enzyme names.

species ($\bar{x}$ = 0.005; Barrowclough 1980). The largest values in our bunting comparisons are those between Lazuli and Indigo, whether the samples compared are from allopatry or sympatry. Slightly smaller values of genetic distance were found for comparisons with hybrids.

TABLE 2. Allozyme variability of 29 loci in five samples of buntings.

Population	% Polymorphic loci	Mean heterozygosity (SE)	
		Direct count	Hardy-Weinberg exp.
Allopatric Indigo	20.7	0.068 (0.029)	0.072 (0.029)
Allopatric Lazuli	24.1	0.061 (0.024)	0.062 (0.024)
Sympatric Indigo	24.1	0.120 (0.049)	0.107 (0.038)
Sympatric Lazuli	27.6	0.080 (0.028)	0.083 (0.028)
Hybrid	17.2	0.092 (0.043)	0.078 (0.033)

Examining the locus-specific contributions to divergence in this study, the significance of AAT stands out. This locus appears to provide substantial discrimination between allopatric Lazuli and Indigo buntings. Genotypes and their frequencies at this locus for the allopatric Indigo sample (n = 12) were: genotype 1-1 (0), 1-2 (0.33), and 2-2 (0.67). For the allopatric Lazuli sample (n = 27), the values were: 1-1 (0.67), 1-2 (0.30), and 2-2 (0.03). Thus, given an unknown individual with a particular homozygous genotype, these frequencies represent estimates of the probability that the individual is an Indigo or a Lazuli bunting. However, in the samples from sympatry, AAT genotype frequencies of Lazuli and Indigo buntings were not significantly different, although they were in the same direction as those from allopatry. Whereas the AAT locus contains a substantial amount of information about species identity, it proved to be inadequate for determining unequivocally the identity of nestlings in a sympatric population.

Analysis of the five mensural traits indicated that in sympatry, a substantial number of males with distinctly Lazuli plumage color patterns were morphometrically more similar to Indigo Buntings. This result may suggest discordant gene flow for those alleles contributing to the measured characters and those contributing to color patterns of the plumage.

Differential introgression of genes for metric traits and those for plumage color pattern has been observed in a study of manakins in Panama (Parsons et al. 1993). In that study, the case was made that the one-way introgression of plumage genes was driven by sexual selection via female preference. Manakin

TABLE 3. Genetic distances (Nei 1978) among five samples of buntings.

Population	AL	SI	SL	Hybrid
Allopatric Indigo	0.017	0.000	0.013	0.001
Allopatric Lazuli (AL)	—	0.006	0.000	0.002
Sympatric Indigo (SI)	—	—	0.002	0.000
Sympatric Lazuli (SL)	—	—	—	0.001

TABLE 4. Morphometric characters measured on Lazuli and Indigo buntings in allopatry and on Lazuli, hybrid, and Indigo buntings in sympatry. Values are $\bar{x} \pm$ SD in mm.

Population	Wing	Tarsus	Bill length	Bill depth	Bill width
Allopatric Lazuli[a]	72.0 ± 1.6	14.7 ± 0.6	7.8 ± 0.3	6.2 ± 0.2	6.9 ± 0.4
Allopatric Indigo[b]	67.1 ± 1.6	15.7 ± 0.6	8.1 ± 0.3	6.4 ± 0.2	6.9 ± 0.2
Sympatric Lazuli[c]	70.6 ± 2.1	15.4 ± 0.8	7.5 ± 0.4	6.2 ± 0.3	6.5 ± 0.5
Sympatric hybrid[c]	66.6 ± 0.9	15.3 ± 0.9	7.6 ± 0.1	6.3 ± 0.3	6.8 ± 0.4
Sympatric Indigo[c]	66.5 ± 0.7	15.5 ± 1.3	8.1 ± 0.2	6.2 ± 0.1	7.0 ± 0.3

[a] Pocatello, Idaho.
[b] Vinton, Iowa.
[c] Beulah, Wyoming.

nuclear genes, mitochondrial haplotypes, and morphometric traits all indicated that abrupt barriers to gene flow occurred some 40 km east of the extent of certain male plumage traits. Lacking the samples needed to establish overall clinal patterns in the bunting zone of overlap and hybridization, it is not possible to determine if genes for metric characters are introgressing from Indigo into Lazuli, if plumage genes are introgressing from Lazuli into Indigo, or if the introgression is bidirectional.

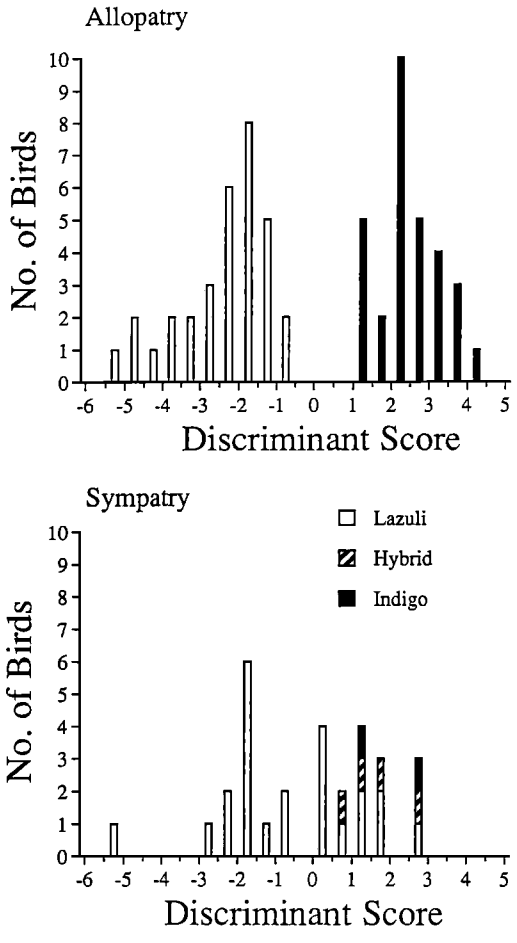

FIG. 1. Frequency histograms of discriminant scores for allopatric (upper) and sympatric (lower) Lazuli Bunting, Indigo Buntings, and their hybrids.

In conclusion, when an allopatric population of Indigo Buntings is compared with an allopatric population of Lazuli Buntings, we find striking differences in plumage patterns and concordant nonoverlapping distributions of discriminant scores derived from morphometric data on the two populations, but only weak differentiation judged by allozyme frequencies. In the sympatric population, morphometric and plumage traits become discordant, with evidence of introgression, whereas allozyme differentiation is even less than in allopatric comparisons. A possible explanation for these patterns is that the genes coding for the mensural traits and allozymes are relatively selectively neutral and undergoing diffusion, whereas those coding for plumage color and pattern are maintained by assortative mating. Although our results indicate that genetic mixing occurs between Indigo and Lazuli forms, field studies have found evidence of hybrid unfitness and positive assortative mating (Emlen et al. 1975, M. C. Baker and J. Boylan unpubl. data), arguing in favor of retaining Indigo and Lazuli buntings as separate species (AOU 1983).

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APPENDIX. Enzyme loci, E.C. numbers, and buffer systems used. All buffer systems are continuous except the discontinuous Lithium Hydroxide.

Enzyme locus	E.C. number	Buffer system
Monomorphic loci		
Acid Phosphatase	3.1.3.2	Lithium Hydroxide
Alcohol Dehydrogenase (2 loci; ethanol, octanol)	1.1.1.1	Tris Maleate
Aspartate Amino Transferase (cathodal)	2.6.1.1	Tris Maleate
Creatine Kinase (2 loci)	2.7.3.2	Tris Maleate
Esterase (beta napthylacetate)	3.1.1.1	Lithium Hydroxide
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	Tris EDTA Borate
Glutamate Dehydrogenase (2 loci)	1.4.1.3	Tris Citrate (pH 6)
Glycerol-3-Phosphate Dehydrogenase	1.1.1.8	Tris EDTA Borate
Lactate Dehydrogenase	1.1.1.27	Tris Citrate (pH 8)
Leucine Aminopeptidase	3.4.-.-	Tris EDTA Borate
L-Iditol Dehydrogenase (2 loci)	1.1.1.14	Tris EDTA Borate
L-Leucyl-Proline Peptidase	3.4.1.3	Tris EDTA Borate
Malate Dehydrogenase (2 loci)	1.1.1.37	Tris Maleate
Octopine Dehydrogenase	1.5.1.11	Tris EDTA Borate
Phosphoglucomutase	2.7.5.1	Tris Maleate
6-Phosphogluconate Dehydrogenase	1.1.1.44	Tris Citrate (pH 8)
Polymorphic loci		
Aspartate Amino Transferase (ATT)	2.6.1.1	Tris Maleate
Esterase (alpha-napthylacetate)(EST)	3.1.1.1	Lithium Hydroxide
Glucose-Phosphate Isomerase (GPI)	5.3.1.9	Tris Citrate (pH 6)
Isocitrate Dehydrogenase (IDH)	1.1.1.42	Tris Citrate (pH 8)
Mannose-Phosphate Isomerase (MPI)	5.3.1.8	Tris Citrate (pH 8)
L-Leucyl-Glycylglycine Peptidase (PEP-LGG)	3.4.1.3	Tris EDTA Borate
L-Leucyl-L-Tyrosine Peptidase (PEP-LT)	3.4.1.3	Tris EDTA Borate
L-Valyl-Leucine Peptidase (PEP-VL)	3.4.1.3	Lithium Hydroxide