NEW WORLD SECTION

BIOCHEMICAL GENETIC STUDIES OF SHOREBIRDS : METHODS AND APPLICATIONS

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INTRODUCTION

A central premise of the synthetic theory of evolution is that natural populations of organisms contain stores of heritable variation upon which selection can act to produce adaptive shifts in response to environmental changes (Dobzhansky 1951, Mayr 1963, Lewontin 1974, Dobzhansky *et al.* 1975). Both genetic and morphologic variation are thus thought to be fundamental and important properties of successful populations. Although much work has been done in characterizing levels and patterns of morphologic variability in populations and taxonomic categories (Gould and Johnston 1972, Yablokov 1974, Sokal 1976, Thorpe 1976), only recently have molecular techniques been developed to investigate the amount and organization of genetic variation within and among species (Harris 1966, Hubby and Lewontin 1966, Lewontin and Hubby 1966).

Genetic variation in bird populations had been little studied until the mid-1970's; reviews of electrophoretically detectable variation by Powell (1975), Selander (1976) and Nevo (1978) include very few avian species out of several hundred surveyed. However, some pioneering researchers have now adapted and developed avian electrophoretic techniques to the point where a substantial number of loci can be examined. A solid core of data is accumulating for a range of species (Smith and Zimmerman 1976, Barrowclough and Corbin 1978, Corbin *et al.* 1976, Avise *et al.* 1980a, b, c, 1982, Barrowclough 1980a, 1983, Barrowclough *et al.* 1981, Cole and Parkin 1981, Yang and Patton 1981, Avise and Aquadro 1982, Aquadro and Avise 1982, Zink 1982, Gutierrez *et al.* 1983, Johnson and Zink 1983).

Two generalizations emerge from these studies, as follows:

(1) The genetic distances between lower taxa of birds are considerably smaller than those observed between many other vertebrates at equivalent levels of taxonomic distinction i.e. the pattern of protein evolution in birds is conservative relative to other vertebrates.

(2) Levels of genetic variation *within species*, on the other hand, are typical of those found in other vertebrates. Thus it does not follow that because bird taxa are less differentiated genetically than are equivalent taxa of other vertebrates, they should also be poor in within-species variation.

With these generalizations firmly in mind, we initiated biochemical genetic studies of shorebird populations. We were interested in addressing three questions of general theoretical and practical importance to shorebird biologists, as follows:

(1) What levels of genetic variation are maintained in shorebird populations i.e. are levels of within-species genetic variation in shorebirds comparable to those in other birds?

(2) Can we distinguish populations with electrophoretically detectable genetic markers, and what utility might these markers have in the conservation and management of migratory shorebirds?

(3) How much genetic differentiation has occurred among species of shorebirds, and what is the likely potential of biochemical genetic techniques in clarifying relationships of shorebirds at both lower and higher taxonomic levels?

In this report we provide some preliminary answers to these questions, and point up the need for international collaboration in securing more comprehensive data.

METHODS

Collection and preservation of tissue samples.

Although it is possible to perform biochemical genetic assays on blood (principally red cells), it is much easier to work with solid tissues (heart, liver and pectoral muscle) under field and laboratory conditions. Additionally, fewer loci are expressed in blood, they are often harder to resolve on gels, and their enzymatic products demonstrate lower activity (making staining procedures more difficult and gels harder to read accurately). Unfortunately, collection of solid tissues necessitates sacrifice of specimens, but the impact on populations can be lessened by autopsying specimens collected for other purposes, those damaged or killed in netting large samples for banding programmes, and from natural mortalities.

It is essential to cool and preferably freeze birds as soon after death as possible. We prefer to place specimens directly on dry ice taken into the field in a large 62 l cooler. Tissues can be removed and flash frozen in NUNC tubes if cryogenic freezing facilities are available, otherwise it is better to freeze

TABLE 1. Enzymes and proteins surveyed in shorebirds.

ENZYME OR PROTEIN	LOCUS SYMBOL	SOURCE OF GEL BUFFER RECIPE	BUFFER pH	POTENTIAL	TISSUE	SOURCE OF STAIN RECIPE	STAIN pH
Aconitase (EC 4.2.1.3)	ACON	1	6.1	9.5 V/cm	Liver	H&H	8.0
Acid Phosphatase (EC 3.1.3.2)[a]	ACP-1	1	5.7	9.5 V/cm	Liver	H&H	5.5
	ACP-2	1	5.7	9.5 V/cm	Liver	H&H	5.5
	ACP-3	1	5.7	9.5 V/cm	Liver	S&P	5.0
Adenosine deaminase (EC 3.5.4.4)[b]	ADA	1	7.5	7.5 V/cm	Muscle	H&H	8.0
Adenylate kinase (EC 2.7.4.3)	AK-1	1	5.8	7.5 V/cm	Heart	S&P, B&C	8.0
	AK-2	1	5.8	7.5 V/cm	Heart	S&P, B&C	8.0
Creatine kinase (EC 2.7.3.2)	CK-1	1	5.8	7.5 V/cm	Heart	S&P, B&C	8.0
	CK-2	1	5.8	7.5 V/cm	Heart	S&P, B&C	8.0
	CK-3	1	5.8	7.5 V/cm	Heart	S&P, B&C	8.0
Esterase (EC 3.1.1.1)[c]	ES-1	5	8.6/9.1	7.5 V/cm	Liver	B	7.0
	ES-2	1	8.6/9.1	7.5 V/cm	Liver	B	7.0
	ES-3	4	8.0	5.5 V/cm	Liver	B	7.0
	ES-4	1	7.5	7.5 V/cm	Liver	B	7.0
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)[d]	GD	3	9.1	11 V/cm	Liver	H&H	8.0
Guanine deaminase (EC 3.5.4.3)	GDA	4	8.0	5.5 V/cm	Liver	H&H	7.6
Glutamate dehydrogenase (EC 1.4.1.3)	GLUD	4	8.0	5.5 V/cm	Liver	H&H(suppl 1977)	8.0
Glutamate oxaloacetate transaminase (EC 2.6.1.1)	GOT-1	1	5.8	11 V/cm	Muscle	S, B&C	8.0
	GOT-2	1	7.5	7.5 V/cm	Muscle	B&C	8.0
Glycerophosphate dehydrogenase (EC 1.1.1.8)[e]	GPD-1	3	9.1	12 V/cm	Muscle	B, B&C	9.5
	GPD-2	3	9.1	12 V/cm	Muscle	B, B&C	9.5
Glucose phosphate isomerase (EC 5.3.1.9)	GPI	1	6.1	7.5 V/cm	Heart	S&P	8.0
Isocitrate dehydrogenase (EC 1.1.1.42)	ICD-1	4	8.0	5.5 V/cm	Liver	S	8.0
	ICD-2	2	7.5	7.5 V/cm	Liver	S	8.0
Lactate dehydrogenase (EC 1.1.1.27)	LDH-1	2	8.5	16.25 V/cm	Heart	S	8.0
	LDH-2	2	8.5	16.25 V/cm	Liver	S	8.0
Malate dehydrogenase (EC 1.1.1.37)	MDH-1	2	7.5	7.5 V/cm	Muscle	S	8.0
	MDH-2	2	7.5	7.5 V/cm	Muscle	S	8.0
Mannose phosphate isomerase (EC 5.3.1.8)	MPI	4	8.0	5.5 V/cm	Heart	H&H	8.0
Purine nucleoside phosphorylase (EC 2.4.2.1)	NP	1	6.1	11.25 V/cm	Liver	H&H	7.0
Peptidase (EC 3.4.11)[f]	PEPA	2	7.4	8 V/cm	Liver	H&H (method A)	
	PEPB	2	8.5	16.25 V/cm	Liver	H&H (method A)	
Phosphogluconate dehydrogenase (EC 1.1.1.44)[d]	PGD	1	7.6	7.5 V/cm	Liver	B&C	8.0
Phosphoglucomutase (EC 2.7.5.1)	PGM-1	2	8.5	16.25 V/cm	Liver	S	8.0
	PGM-2	2	7.5	7.5 V/cm	Liver	S	8.0
Non-enzymatic proteins[g]	Pt-1	1	6.1	9.5 V/cm	Muscle		
	Pt-2	1	6.1	9.5 V/cm	Muscle		
	Pt-3	1	6.1	9.5 V/cm	Muscle		
Superoxide dismutase (EC 1.15.1.1)	SOD-1	2	8.5	16.25 V/cm	Liver	H&H	8.0
	SOD-2	2	8.5	16.25 V/cm	Liver	H&H	8.0
Sorbitol dehydrogenase (EC 1.1.1.14)	SORDH	2	8.5	16.25 V/cm	Liver	H&H	8.0

[a] For ACP-1 and ACP-2 mix 10ml 0.1M acetic acid adjusted to pH 5.5 with sodium acetate with 5 mg 4-methylumbelliferyl phosphate, paint on gel and incubate in dark, view with UV light.
[b] Add 75 mg Na Arsenate to stain solution.
[c] ES-1, ES-2, ES-3: 50 mls phosphate buffer pH 7.0 containing α-naphthyl propionate (1% in acetone).
ES-4: 10 mg 4-methylumbelliferyl acetate dissolved in a few drops of acetone and then mixed with 20 mls phosphate buffer pH 7.0. View under long wave UV lamp.
[d] Add 1 mg NADP/40 ml gel after heating but before degassing, add 1 mg NADP/60 ml in cathodal chamber.
[e] Soak gel in substrate solution for 1 hr before adding PMS.
[f] After grinding in diothiothreitol, samples must be incubated at 39°C for 1/2 hr before application to wicks.
[g] 0.3 g Coomassie Brilliant Blue added to solution of 60 ml methanol, 115 ml distilled water and 20 g trichloroacetic acid. Soak gel in this for a couple of days.

Gel Buffer References

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2. Cole, S.R. and D.T. Parkin. 1981. Biol. J. Linn. Soc. 15: 13-22.
3. Turner, B.J. 1973. Comp. Biochem. Physiol. 44B: 89-92.
4. Shaw, C.R. and R. Prasad. 1970. Biochem. Genetics 4: 297-320.
5. Corbin, K.W., C.G. Sibley, A. Ferguson, A.C. Wilson, A.H. Brush & J.E. Ahlquist. 1974. Condor 76: 307-318.

Stain References

B Brewer, G.J. 1970. An introduction to isozyme techniques. New York, Academic Press.

B&C Barrowclough, G.F. and K.W. Corbin. 1978. Auk 95: 691-702.

H&H Harris, H. and D.A. Hopkinson. 1976. Handbook of enzyme electrophoresis in human genetics. Amsterdam North-Holland Publishing Company.

S Selander, R.K., M.H. Smith, S.Y. Yang, W.E. Johnson & J.B. Gentry. 1971. Studies in Genetics VI. Univ. Texas Publ. 7103.

S&P Shaw, C.R. and R. Prasad. 1970. Biochem. Genetics 4: 297-320.

whole specimens for transport to a preparation laboratory. In remote field locations we use liquid nitrogen dewars with a working capacity of 3-6 weeks (see Johnson *et al.* 1984 for further details).

Because most electrophoretically detectable loci are expressed in liver, we freeze as much of this organ as possible, and also extract the heart and a piece of pectoral muscle. For large birds we subsample these tissues, but maintain a preponderance of liver.

Electrophoresis

We routinely assay 40 loci from each specimen. Samples of all three tissues are ground in tris-HCl buffer solution (pH 7.0) for all loci except peptidases, the latter being ground in dithiothreital buffered to pH 6.0 in HCl (see Cole and Parkin 1981). We resolve all loci in 9% starch gels (Connaught Laboratories Limited, Willowdale, Ontario) using the combination of buffers, running conditions, tissues and stain recipes detailed in Table 1. Gels are run overnight for 16 hours at 4°C. Isozymes are numbered sequentially beginning with the most anodal form (*e.g.* ES-1) to the more cathodal ones (*e.g.* ES-2, ES-3).

LEVELS OF GENETIC VARIATION IN SHOREBIRDS

Allele frequency data for three species of Calidridine sandpipers are presented in Table 2. Note that 12 of 30 gene loci surveyed here are polymorphic for *Calidris maritima, C. fuscicollis* and *C. alpina*. Respective observed heterozygosities are 0.003, 0.025 and 0.035 (Table 3). The heterozygosities for the latter two species are typical of values of genic variability in other species of birds, whereas the heterozygosity estimate for *C. maritima* is extremely low. Parenthetically, the 36 birds in the *C. maritima* sample were all collected from a relatively isolated winter population of birds from New Brunswick. Long-distance band recoveries indicate that birds from this population breed in the Belcher Islands in Hudson Bay (Morrison 1984). Integration of the genetic and banding data provides new insights on the history of this population; the dramatic reduction in genetic variability in *C. maritima* relative to other calidris suggests that this New Brunswick population has undergone a severe bottleneck of population size in its recent history.

Genetic differentiation among populations of a species can be gauged using Wright's formulation of F_{ST} (Wright 1978), the among-population component of genetic variance. An example for *C. fuscicollis* based on only two populations is presented in Table 3. This is a very low value for F_{ST}, indicating that the populations of this species have not differentiated genetically. Because values of this parameter are heavily influenced by the population sampling regime employed, however, one must be cautious in making inferences about the genetic structure of species populations without comprehensive samples (see Barrowclough 1983 for further discussion).

The general conclusion that emerges from the survey of Calidridine sandpipers is that most possess levels of within-species genetic variation typical of other vertebrates. We can reasonably predict that shorebirds will be amenable to population genetic analyses based on electrophoretic data.

As noted by Barrowclough (1980b) the genetic structure of natural populations is of major importance in ecology and evolution because "theories of group and kinship selection, local adaptation and speciation all depend on the magnitude of an among-deme component of genetic variance". The effective size and degree of differentiation of populations may be influenced by parameters such as geographic structure, dispersal, presence or absence of overlapping generations, breeding sex ratio and the distribution of family sizes (Crow and Kimura 1970). Thus to understand the dynamics of shorebird populations beyond the level of banding and recapture studies, we need to investigate the genetics of species with different breeding systems.

Shorebirds are ideal for studying genetic structuring because they occur in discrete winter flocks that can be censused easily, most have strong breeding (and probably wintering) site fidelity, and they show a greater diversity of mating systems than any other order of birds (Johnsgard 1981).

UTILITY OF GENETIC MARKERS IN POPULATION STUDIES

The possible utility of electrophoretically detectable genetic markers in distinguishing different populations of shorebirds can be illustrated by comparing two populations of *C. fuscicollis* collected in North and South America (Table 2). In Fall migrants passing through James Bay in Canada, 10 of 30 loci assayed were polymorphic for two to five alleles ($\bar{x}$ = 2.40 alleles/locus). For the sample collected near Camarones in Argentina, 8 of 30 loci are polymorphic for two to four alleles ($\bar{x}$ = 2.37 alleles/locus). Eight of these loci are common to both samples (ES-1, ES-2, PGM-1, ICD-1, GOT-1, GPI, PGD, and PEP-A). Additionally, the Canadian sample reveals variation at the PGM-2 and MPI loci.

Although the results look qualitatively similar for both samples, closer inspection of the data shows that the Canadian sample contains six unique but relatively rare alleles (two at ES-2, one at MPI, one at GPI, one at GOT-1 and one at PGM-2), whereas the Argentinian sample has three unique alleles (two at PGD and one at GPI). Thus we can distinguish these two samples on the presence or absence of these nine alleles, though it is important to realise that this does not provide a means for allocating most birds to their respective populations.

For genetic data to be effective in the identification of significant numbers of birds from two or more populations, we probably need to detect about 15 loci segregating for alleles at intermediate frequencies in the range of 0.2 to 0.8. Empirical data for birds indicate that we would have to sample about 100 loci to be reasonably sure of locating the required number of intermediate frequency polymorphisms (Barrowclough *et al.* 1984), and this is currently beyond techniques in avian electrophoresis. Multilocus discriminant analysis (Smouse *et al.* 1982) might improve these odds somewhat, but more extensive surveys of loci will be required to test the effectiveness of this technique with bird populations.

GENETIC DIFFERENTIATION OF SHOREBIRD TAXA

Allele frequency data provide information on the differences among populations and taxonomic categories at each locus, but they do not provide a synthesis of genetic differentiation expressed across all loci simultaneously. For

Table 2. Allele frequencies at 12 variable loci in three species of Calidridine Sandpipers. Alleles at each locus are designated alphabetically.

Locus	Allele	*Calidris maritima* (n=36)	*Calidris alpina* (n=25)	*Calidris fuscicollis* Canada (n=32)	*Calidris fuscicollis* Argentina (n=32)
AK-2	A			1.000	1.000
	B	1.000	1.000		
ES-1	A			0.031	0.047
	B	1.000		0.969	0.953
	C		1.000		
ES-2	A	0.025			
	B	0.750			
	C		0.020		
	D		0.320		
	E		0.640	0.031	0.016
	F		0.020	0.781	0.906
	G			0.156	0.078
	H			0.016	
	I			0.016	
GOT-1	A		0.040	0.016	0.016
	B			0.016	
	C	1.000	0.960	0.968	0.984
GPI	A		0.020		
	B	1.000	0.980	0.984	0.953
	C				0.047
	D			0.016	
ICD-1	A	1.000	1.000	0.984	0.984
	B			0.016	0.016
LDH-1	A		1.000	1.000	1.000
	B	1.000			
MPI	A			0.020	
	B	1.000		0.980	1.000
	C		1.000		
PEPA	A	0.097			
	B		0.080	0.031	0.062
	C	0.903			
	D		0.920	0.969	0.938
PGD	A				0.047
	B	1.000	1.000	0.937	0.875
	C				0.031
	D			0.063	0.047
PGM-1	A	1.000	1.000	0.984	0.984
	B			0.016	0.016
PGM-2	A	1.000	1.000	0.984	1.000
	B			0.016	

Table 3. Genic heterozygosity and among-population genetic variance in some Calidridine Sandpipers.

A. HETEROZYGOSITY

Species	No. of loci	No. of Individuals	Heterozygosity
Calidris maritima	30	36	0.003
C. fuscollis	30	32	0.025
C. alpina	30	25	0.035

B. AMONG-POPULATION GENETIC VARIANCE

Species	No. of loci	No. of populations	
C. fuscicollis	10	2	0.002

this purpose we can employ Rogers (1972) index of genetic similarity (S_R), defined by the expression

$$S_R = 1 - [1/2 \sum_{i=1}^{m} (p_{ix} - p_{iy})^2]^{1/2}$$

where p_{ix} = frequency of allele i in population (or species) x,

p_{iy} = frequency of allele i in population (or species) y,

and m = number of alleles at each locus.

Cluster analysis of the matrix of Rogers genetic similarities among different populations and taxa of shorebirds assayed for genetic variation at 39 loci provides an overview of the usefulness of electrophoretic data at various hierarchical levels (Figure 1). Note that the multilocus genetic differences between Canadian and Argentinian samples of Red Knots on the one hand and White-rumped Sandpipers on the other, are less than half those between closely related species such as White-rumped Sandpipers and Least Sandpipers.

Red Knots are the most divergent of the sandpipers surveyed here, and the Calidridae and Scolopacidae (as represented by the Hudsonian Godwit) are well differentiated. It is clear that biochemical genetic analyses of shorebirds will provide a valuable new perspective on the relationships of shorebirds.

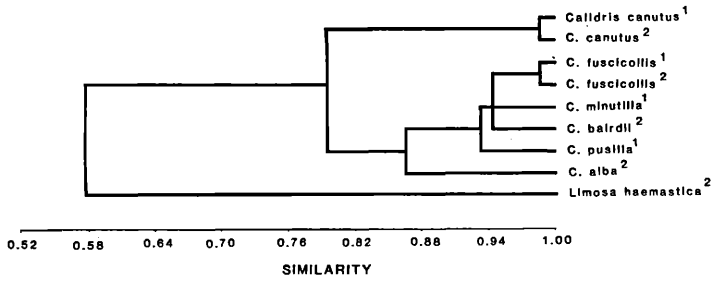

Figure 1. UPGMA cluster analysis of Rogers (1972) genetic distances among some population samples and taxa of shorebirds.

APPLICATIONS IN BIOCHEMICAL SYSTEMATICS

Some of the thorniest problems in shorebird systematics remain unresolved with traditional morphological approaches. For example, what are the relationships of 'oddball' taxa such as seedsnipe Thincoridae, the Crab Plover *Dromas ardeola*, Magellanic Plover, Ibis-bill *Ibidorhyncha struthersii* and sand grouse Pteroclidae? Are oystercatchers closely related to the plovers, or are they an aberrant group? What are the systematic affinities of the Surfbird *Aphriza virgata* and the turnstones, and how closely related are the phalaropes, curlews, godwits, dowitchers, snipes and woodcocks? Recent exciting results from DNA-DNA hybridization studies (Sibley and Ahlquist in press) suggest relationships among higher taxa which are radically different from those based on morphological data; oystercatchers, stilts and avocets are closely related to plovers and lapwings, this assemblage in turn is closer to the gulls, Crab Plover, pratincoles and coursers than to sandpipers, and the Sheathbill *Chionis alba* and Stone Curlew *Burhinus oedicnemus* are close to the plovers.

Electrophoretic surveys of the shorebirds will be invaluable in adding to this emerging pattern of relationships and in filling in details within and among closely related taxa which are beyond the resolution of the DNA hybridization data. Studies of mitochondrial DNA sequence variation will likely be profitable too because it evolves much faster than nuclear DNA (Brown 1983), and current results with birds have been extremely successful (Kessler and Avise 1984, 1985).

NEED FOR INTERNATIONAL COLLABORATION IN GENETIC STUDIES

Because of their worldwide distribution and the large number of species involved, a long-term programme of international collaboration is required to assay genetic variation in shorebirds. Agencies collecting specimens in different regions of the world are urged to save tissue samples, as are shorebird biologists confronted with natural or other mortalities of birds. Our goal should be to build up an international tissue collection for shorebirds similar to conventional anatomical collections.

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