GENETIC VARIATION AND DIFFERENTIATION IN THE PARULIDAE

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ABSTRACT.—We used electrophoretic methods to investigate genetic heterozygosity and the degree of differentiation in 15 species of birds of the family Parulidae. The observed heterozygosity is similar to that found in other vertebrates. The pattern of genotypes observed suggests that population subdivision exists for some of these birds. The genetic distances between species and genera of birds examined are considerably less than are those of non-avian taxa. *Received 11 January 1978, accepted 6 April 1978.*

ELECTROPHORESIS of avian proteins was first reported in the late 1930's (Landsteiner et al. 1938). During the 1960's and early 1970's, Sibley and his colleagues examined the egg white proteins of more than 1,000 avian species (Sibley 1970, Sibley and Ahlquist 1972). The principal objective of these studies was the elucidation of the taxonomic relationships among species. The pattern of genetic variation itself was not of primary concern. In the last decade, however, it was recognized that electrophoretic techniques could be used to obtain a quantitative estimate of the amount of genetic variation in natural populations (Lewontin 1974, Selander 1976). Additional theoretical developments made possible the quantitative analysis of variation among species as well (Nei 1975). Since genetic variation is essential for evolutionary change and genetic differentiation is a result of the evolutionary process, the characterization of these quantities is of prime importance to evolutionary biologists.

The analysis of patterns of genetic variation in birds has not received a great deal of attention. Besides the study of two species of starlings of the genus *Aplonis* (Corbin et al. 1974), much of the work to date has been a by-product of research associated with other aspects of avian biology (e.g. Nottebohm and Selander 1972, Baker 1975). In this study we examine the pattern of genetic variation within a family of birds containing several common congeners as well as common species of different genera. We have chosen the new world family Parulidae, the wood warblers, for this purpose.

METHODS

Migrating wood warblers were collected using mist nets during the spring of 1975 and 1976 in Minnesota, and during the autumn of 1976 in Wisconsin. In addition, a small number of resident birds were collected during the summer of 1976 in Minnesota and British Columbia. The species and number of individuals examined were: Black-and-white Warbler (*Mniotilta varia*), 8; Tennessee Warbler (*Vermivora peregrina*), 13; Orange-crowned Warbler (*V. celata*), 8; Nashville Warbler (*V. ruficapilla*), 22; Northern Parula (*Parula americana*), 2; Magnolia Warbler (*Dendroica magnolia*), 14; Yellow-rumped Warbler (*D. coronata*), 35; Blackpoll Warbler (*D. striata*), 6; Palm Warbler (*D. palmarum*), 12; Ovenbird (*Seiurus aurocapillus*), 10; Northern Waterthrush (*S. noveboracensis*), 24; Mourning Warbler (*Oporonis philadelphia*), 8; Common Yellowthroat (*Geothlypis trichas*), 16; Canada Warbler (*Wilsonia canadensis*), 2; and American Redstart (*Setophaga ruticilla*), 12. Blood samples were taken by cardiac puncture using heparin as an anticoagulant, and later centrifuged to separate plasma and red cells. These tissues, along with heart, liver, and pectoral muscle samples, were stored in liquid nitrogen or dry ice until used. Sixteen hours prior to electrophoresis, the proteins from the non-blood tissues were extracted at 4°C in a solution of 1.5% phenoxyethanol, 0.25 *M* sucrose, and 0.1 *M* phosphate buffer, pH 7.5 (Nakanishi et al. 1969).

TABLE 1. Electrophoresis and enzyme staining conditions.

Protein assayed	Abbreviation	Tissue used	Electrophoresis system	Stain pH	Reference	Notes
Acid Phosphatase	AcP	liver	8.6/9.1	5.0	Shaw & Prasad 1970	add 1 m*M* $MnCl_2$, use Fast Garnet GBC
Albumin	Alb	serum	8.6/9.1	7.0	Brewer 1970	protein stain
Creatine Kinase	CK	muscle	6.1	7.5	Shaw & Prasad 1970	use agar overlay
Esterase	Est	serum, liver	8.6/9.1	6.0	Corbin et al. 1974	
Glutamate Dehydrogenase	GDH	muscle	7.5	8.0	Shaw & Prasad 1970	use Tris buffer, add 1 m*M* ADP
Glutamate-oxaloacetate Transaminase	GOT	muscle	7.5	7.0	Shaw & Prasad 1970	.1 m*M* Pyridoxal-PO_4, use agar overlay
α-Glycerophosphate Dehydrogenase	GPDH	muscle	9.1	9.5	Brewer 1970	use 1 m*M* $MgCl_2$, add PMS after 1 h
Isocitrate Dehydrogenase	IDH	liver	7.5	8.0	Shaw & Prasad 1970	use 1 m*M* $MnCl_2$
Lactate Dehydrogenase	LDH	muscle	6.1	9.0	Brewer 1970	
Malate Dehydrogenase	MDH	heart	6.1	9.8	Brewer 1970	use l-malic acid
Malic Enzyme	ME	heart	6.1	9.8		as MDH, but use NADP
Mannose Phosphate Isomerase	MPI	heart	6.1	8.0	Nichols et al. 1973	use 1 m*M* $MgCl_2$, use agar overlay
Myoglobin	Mgb	muscle	9.1	7.0		protein stain
Peptidase A	Pep A	liver	7.5	7.5	Lewis & Harris 1967, Baker 1974	.1 *M* PO_4 buffer, add 1 m*M* $MnCl_2$, .2 m*M* l-valyl-l-leucine, use agar overlay
Peptidase B	Pep B	liver	7.5	7.5	see above	as above, use .1 m*M* l-leucyl-glycyl-glycine
Peptidase C	Pep C	liver	7.5	7.5	see above	as above, use .2 m*M* l-leucyl-l-alanine
6-Phosphogluconate Dehydrogenase	6-PGDH	liver	9.1	7.0	Shaw & Prasad 1970	add 1.5 m*M* $MnCl_2$, add 1 m*M* NAD to gel during preparation
Phosphoglucomutase	PGM	muscle, liver	7.5	8.0	Shaw & Prasad 1970	use 2 m*M* $MgCl_2$
Sorbitol Dehydrogenase	SDH	muscle	6.1	9.5	Shaw & Prasad 1970	use 3 m*M* $MgCl_2$
Superoxide Dismutase	SOD	muscle	6.1	9.5	Brewer 1970 (= IPO)	appears on SDH gel
Transferrin	Trf	serum	8.6/9.1	7.0		protein stain, appears on Alb gel

TABLE 2. Allelic frequencies at variable loci. In all cases allele A designates the product with greatest mobility, and consecutive alphabetical letters indicate successively slower allelic products. Isozymes are numbered in an analogous fashion. For instance, mannose phosphate isomerase-1 has a greater mobility than does mannose phosphate isomerase-2, etc. As not all loci were scored for each species, blanks for all alleles indicate that a species-locus combination was not examined.

		Species[a]														
Locus	Allele	vari	pere	cela	rufi	amer	magn	coro	stri	palm	auro	nove	phil	tric	cana	ruti
AcP	A	.833			1.0		.917	1.0	1.0	.833	1.0	1.0	1.0	1.0	1.0	1.0
	B	.167					.083			.167						
	C		1.0													
Alb	A							.625						1.0		
	B	1.0	1.0	1.0	1.0		1.0	.375	1.0	1.0	1.0	1.0				1.0
CK	A														.500	.083
	B	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.500	.917
Est-1	A								1.0			.056				
	B		1.0			.500		.967		.889	.333	.167	.250	.750		1.0
	C				.500	.500	1.0	.033		.111	.333	.611	.750	.250		
	D			.429	.500						.333	.167				
	E	1.0														
	F			.571												
Est-2	A	.500				.500				.056				.063		
	B	.500		.429	.125	.500	.100	.033		.500	.333	.150	.375	.125	1.0	
	C		.400	.429	.833		.500	.733	1.0	.389	.667	.500	.625	.625		.900
	D		.600	.143	.042		.400	.217		.056		.350		.188		.100
	E							.017								
GOT-M	A	1.0														
	B		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.969	1.0	1.0	1.0	1.0
	C											.031				
GOT-S	A				.042						.167					
	B							.031	1.0						.500	
	C	1.0	1.0	1.0	.958	1.0	1.0	.969		1.0	.833	.875	1.0	1.0	.500	1.0
	D											.125				
GPDH	A		1.0	1.0	1.0		1.0	1.0	1.0	1.0	.250	1.0		1.0	1.0	1.0
	B										.750					
IDH	A										.200		.286			
	B	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.800	1.0	.714	1.0	1.0	1.0
LDH-H	A	1.0	1.0	1.0	.909	1.0	.875	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	B						.125									
	C				.091											

TABLE 2. (Continued)

Locus	Allele	Species[a]														
		vari	pere	cela	rufi	amer	magn	coro	stri	palm	auro	nove	phil	tric	cana	ruti
ME	A	1.0	1.0	1.0	.958	1.0	.900	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	B						.100									
	C				.042											
MPI-1	A														.500	
	B		.300									.100		.125		
	C	1.0	.700	.833	.917	1.0	1.0	1.0	1.0	1.0	1.0	.850	.875	.875		1.0
	D			.083											.500	
	E			.083	.083							.050				
	F												.125			
MPI-2	A						.900	.842	.500	1.0		.750		1.0		1.0
	B		.750		.091		.100	.105	.500							
	C			.250	.182			.053								
	D		.250	.750	.727						1.0	.250	1.0			
MPI-3	A		.500	.625	.750		1.0	.875	1.0	.800						
	B		.500	.375	.250			.125		.200	1.0	1.0				
PEP-A	A				.042							.111				
	B	1.0			.042						.667	.500		.571		
	C		.800	1.0	.542	1.0	1.0	1.0	1.0	.889	.333	.389	.875	.429	1.0	.800
	D		.200		.375					.111			.125			.200
PEP-B	A		.200													
	B	1.0	.500	.857	1.0			.056		.222		.150				
	C		.200	.143		1.0	.900	.833	1.0	.667	1.0	.800	1.0	1.0	.500	1.0
	D		.100				1.00	.111		.111		.050			.500	
PEP-C	A				.091											
	B				.045	1.0				.100			.125			
	C	.250		.429	.727		.250	.214		.700	.750	.600	.750	.750	.500	.833
	D	.750		.571	.136		.750	.786	1.0	.200	.250	.400	.125	.250	.500	.167
6-PGDH	A					.500		.341		.111	.500					.250
	B		.200				.500	.659	1.0	.556		.556			1.0	.500
	C		.600				.500			.222	.500	.167				.250
	D		.200	.571	.333	.500				.111		.111	1.0	.667		
	E			.429	.667							.167		.333		

TABLE 2. (Continued)

Locus	Allele	Species[a]														
		vari	pere	cela	rufi	amer	magn	coro	stri	palm	auro	nove	phil	tric	cana	ruti
PGM-1	A													.063		
	B			.143				.017								
	C		.100												1.0	
	D	1.0	.900	.857	1.0	1.0	1.0	.950	1.0	1.0	1.0	.944	1.0	.938		1.0
	E							.033				.056				
PGM-2	A	.500														
	B						.200									
	C	.500	.500	.800	.833	1.0	.800	.813	1.0	.571		.889	1.0	1.0		1.0
	D		.250	.200							.500					
	E		.250		.167			.125			.500	.111				
	F							.063		.429						
PGM-3	A					.500										
	B			1.0				.079				.111				
	C		1.0		1.0	.500	1.0	.842	1.0	1.0	1.0	.833	1.0	1.0	1.0	1.0
	D							.079								
	E											.056				
Trf	A	1.0	.250	.400			.200	.563	1.0	.444	1.0	.900		1.0		.500
	B		.250									.100				
	C		.500	.600	1.0		.800	.438		.556						.500

[a] Species coded by first four letters of species name; listed in same sequence as in Methods

TABLE 3. Observed and expected heterozygosities of some Parulids.

Taxon	Heterozygosity	
	Observed	Expected ± SE
Vermivora ruficapilla	0.042	0.123 ± .034
Dendroica coronata	0.039	0.121 ± .031
Dendroica palmarum	0.047	0.134 ± .039
Seiurus noveboracensis	0.054	0.158 ± .039

Four electrophoretic buffer systems were used; these included the pH 6.1 and pH 7.5 continuous systems of Clayton and Tretiak (1972) and the pH 9.1 continuous system of Turner (1973). In addition the discontinuous pH 8.6/9.1 buffer system of Corbin et al. (1974) was used, but was modified to make the concentration of lithium ion 0.0087 *M* in the gel buffer and 0.175 *M* in the electrode buffer. Gels were composed of 11% electrostarch (Electrostarch Co. Lot No. 146).

Horizontal electrophoresis was carried out overnight at 4°C. Voltage and current were adjusted to yield approximately 6–10 cm of protein migration. Enzymes and other proteins coded for by 31 gene loci were assayed after slicing the gel into three horizontal slabs. Standard recipes for specific stains frequently were found to give weak activity for these birds. Consequently, pH and cofactor/coenzyme concentrations were varied to obtain optimal in vitro activity using a Heath 707 EU spectrophotometer. Improved staining was then obtained by using these optimal conditions in gels after electrophoresis. These electrophoretic and staining conditions are described in Table 1. Not all species were examined for all 31 loci.

Allelic frequencies were converted to genetic distances using the methods of Rogers (1972) and Nei (1971, 1972, 1973). Approximations to minimum spanning trees were found using the algorithms developed by Farris (1972).

RESULTS

Heterozygosity.—Eight of the proteins examined in this study appear to be monomorphic, given sample size limitations, within and between all 15 species; these loci included three glutamate dehydrogenases, the muscle form of lactate dehydrogenase, the mitochondrial and supernatant forms of malate dehydrogenase, sorbital dehydrogenase, and superoxide dismutase. At the myoglobin locus of *Setophaga ruticilla* and *Wilsonia canadensis* an allele, which had a product with a greater mobility than that of the remaining 13 species, was fixed. The remaining 22 loci were variable either within or between species

The degree of heterozygosity has been calculated for the four species of wood warblers for which the product of the number of loci assayed times the number of individuals examined is equal to 600 or more (Table 3). For these species all 31 loci have been examined, in agreement with the suggestion of about 30 loci and 20 individuals that Nei and Roychoudhury (1974) thought were desirable for such studies. Heterozygosity was calculated in two ways. First, the actual heterozygosity was calculated as the ratio of the total number of heterozygotes divided by the product of the number of loci and individuals. Second, the theoretically expected heterozygosity was calculated as the number of heterozygotes expected on the basis of Hardy-Weinberg equilibria for the allelic frequencies listed in Table 2, divided by the product of the number of loci and individuals.

Expected heterozygosities were considerably larger than the observed values. As the sample sizes for the four species for which heterozygosities were calculated were relatively small, it was not possible to compute Chi-squared values for departure from Hardy-Weinberg proportions for some species-locus combinations. This was because small expected values can result in misleadingly large Chi-squared values. Nevertheless, these values, summed over those loci with adequate samples, were

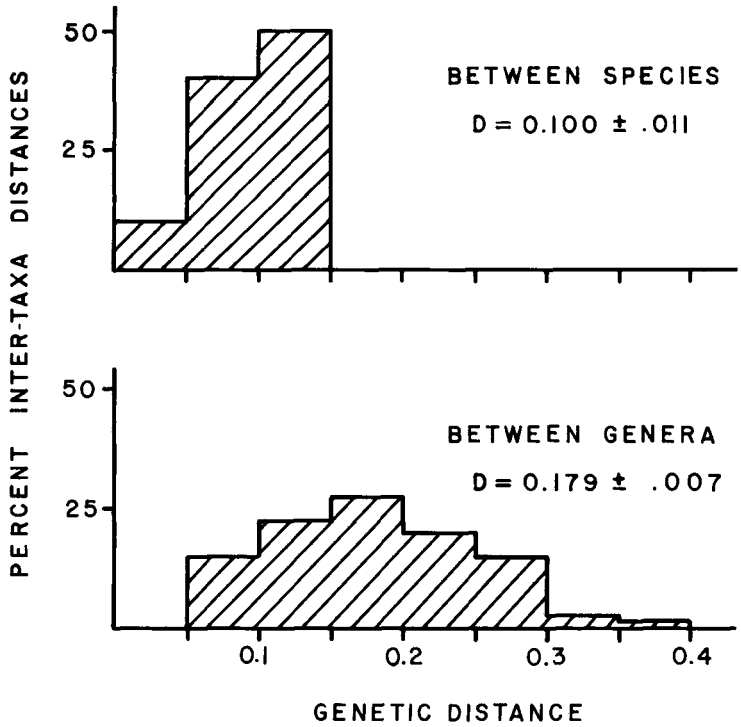

Fig. 1. Histogram of the distribution of genetic distances among wood warblers. Comparisons between congeners (above), and between species in different genera (below). Mean distance (D) and one standard error are indicated.

significant at the 0.005 level for all four species (*Vermivora ruficapilla*, $\chi_2^2 = 15.674$; *Dendroica coronata*, $\chi_3^2 = 28.062$; *Dendroica palmarum*, $\chi_3^2 = 19.552$; *Seiurus noveboracensis*, $\chi_3^2 = 15.604$). In each case the predominant contribution to the Chi-squared value comes from the paucity of the heterozygote classes.

Differentiation between species.—The degree of genetic differentiation between species and among higher taxa is a measure of the amount of divergent evolution between species since the time of the speciation event separating the lineages. Two measures of genetic distance have been used in recent years. These were proposed by Nei (1971, 1972, 1973) and Rogers (1972). Nei's measure, D, has some biological appeal as, unless electrophoretically detectable substitutions are selected against *per se*, there are theoretical reasons to believe it approximates the average number of nucleotide substitutions per gene between two species (Nei 1972). While Rogers' index does not have such a clear biological meaning, it does have the convenient mathematical property of satisfying the triangle inequality, which is necessary for inferring some types of evolutionary trees (Barrowclough and Corbin in prep.).

Genetic distances have been computed using both measures for all pairs of taxa examined in this study. When organized according to taxonomic level, the data appear as shown in Fig. 1. The between species distribution is not symmetrical, possibly due to the relatively small sample sizes. In spite of the sample size limitations, significant differences exist at the 0.001 level between the means of the between species and between genera distributions ($t = 6.41$, 23 df).

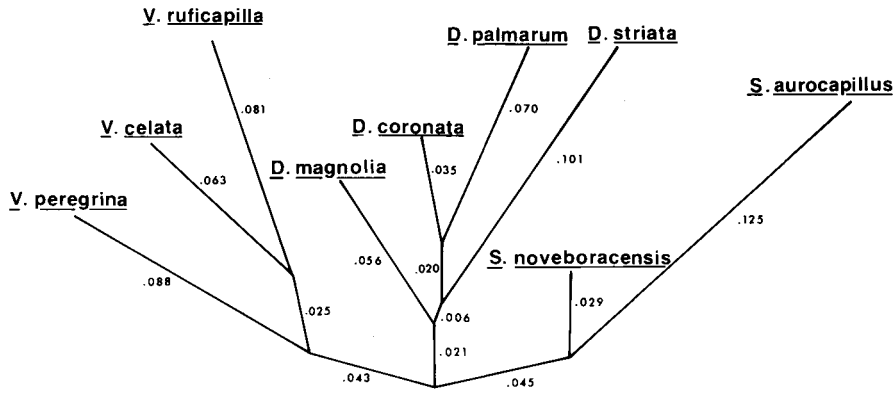

Fig. 2. Maximum parsimony phylogenetic network for some Parulids. Branch lengths are in units of Rogers' distance.

Phylogenetic inference.—Farris (1972) developed algorithms that provide efficient approximations to most-parsimonious trees, given a matrix of inter-taxa distances as a data source. The distance metric used, however, must satisfy the triangle inequality; Rogers' measure has this property while the proposed by Nei does not. Using the Farris algorithms and Rogers' distances, we have inferred a phylogenetic network for the nine taxa of wood warblers for which we have examined 30 or more loci. The network, drawn so as to make evolutionary distances approximately equal among branches, is shown in Fig 2.

DISCUSSION

Since genetic variation is necessary for natural selection to operate, the determination of the amount of such variation in natural populations is of interest to students of evolution. The use of electrophoresis to measure heterozygosity within a species has now become a widely established method of estimating the magnitude of this variation (Powell 1975).

The observed heterozygosity found in the four species of parulids reported on here is in quantitative agreement with other studies of genetic variation in birds (Table 4). Moreover, the overall average heterozygosity for all avian genetic surveys, 0.043 $\pm$ 0.005, is in the same range as that of other vertebrates (e.g. Powell 1975). This quantity is considerably less than the amount of variability observed in invertebrates.

There exist several possible explanations for the discrepancy in heterozygosity as calculated by the observed and expected methods. We believe the most likely cause of the observed deficiencies of heterozygotes is an absence of panmixia in these species. This is equivalent to asserting that a significant portion of the total genetic variation is maintained between rather than within demes, as a Wahlund component of variance. That is, if local populations of a species have different allelic frequencies, then sampling a mixture of individuals from the various populations, as would occur in a collection of migrants, will result in an apparent overall excess of homozygotes even if each local population is in Hardy-Weinberg equilibrium. Population structure of this sort also was found in the White-crowned Sparrow (*Zonotrichia leucophrys*) (Baker 1975). As Selander (1970) pointed out, however, the inference of

TABLE 4. Observed heterozygosity in birds.

Species	Number of loci examined	Number of individuals examined	Heterozygosity (H)	Reference
Zonotrichia capensis	24	154	0.035	Nottebohm & Selander 1972
Aplonis metallica	18	354	0.047	Corbin et al. 1974
Aplonis cantoroides	18	108	0.012	Corbin et al. 1974
Icterus g. galbula	19	240	0.055	Corbin et al. 1978
Icterus g. bullockii	19	132	0.058	Corbin et al. 1978
Vermivora ruficapilla	31	22	0.042	this study
Dendroica coronata	31	35	0.039	this study
Dendroica palmarum	31	12	0.047	this study
Seiurus noveboracensis	31	24	0.054	this study
Mean heterozygosity in birds (±SE)			0.043 ± .005	

population subdivision based on a shortage of heterozygous genotypes is somewhat tenuous. Selection against heterozygotes or the existence of null alleles could result in a similar pattern of genotypic frequencies. Nevertheless. we do not think these effects likely in this case. Strong selection against heterozygotes would be required at several loci in each of the four species to obtain the observed result; this seems unlikely since selection against heterozygotes does not lead to a stable polymorphism. The existence of null alleles at intermediate frequencies at many loci seems equally unlikely for two reasons. First, null alleles have not been found in avian studies. Second, in the four cases examined here a significant contribution to the Chi-squared value comes from enzymes known to be polymeric. For such enzymes, individuals heterozygous for a null allele are often easily recognizable by an electrophoretic pattern resembling the heterozygote pattern, but with a single band missing (e.g. Parr 1966, Corbin 1977). Such patterns were not observed in this study. In spite of these arguments, though, the evidence for population subdivision remains indirect, and needs to be tested. Current research on genetic differentiation in the *Dendroica coronata* complex (Barrowclough pers obs.) may provide additional insight into this situation.

In Table 5 we compare the within-taxa differentiation of these parulids, along with some values from other avian studies, to the emerging pattern of differentiation in other groups of organisms. The level of genetic divergence in birds appears to be

TABLE 5. Genetic distances (Nei) between different taxonomic levels.

Taxa	Local populations	Subspecies	Semispecies	Sibling species	Species	Genera	Reference
Drosophila	.028	.230	.226	.740	1.056	—	Ayala 1975
Sunfish	.020	.174	—	—	.616	—	Avise & Smith 1974a, b
Minnows	.031	—	—	—	—	.528	Avise 1976
Salamanders	.051	.174	—	—	.462	1.170	Hedgecock & Ayala 1974
Mammals[a]	.056	.219	—	—	.302	.651	Ayala 1975
Birds							
Aplonis	.003	.007	—	.035	—	—	Corbin et al. 1974
Zonotrichia	.004	.009	—	—	—	—	Baker 1975, Handford & Nottebohm 1976
Icteridae	.002	—	.003	.012	—	.248	Corbin et al. 1978, Smith & Zimmerman 1976
Parulidae	—	—	—	—	.100	.179	this study

[a] Distances based on the method of Rogers

considerably less than that of other taxa, including both vertebrates and invertebrates. For instance, the degree of differentiation between genera of birds is of the same order of magnitude as that between subspecies and semispecies of *Drosophila*. It is conceivable that there is simply less divergence between taxa of birds, at the structural gene level, than between comparable taxa of other organisms. This possibility has been suggested previously on the basis of morphological data (Bock 1969, Mayr 1976), and immunological data (Prager et al. 1974, Prager and Wilson 1975). A second possibility, however, is that for some reason electrophoretically detectable substitutions in birds are resisted by natural selection. It is not obvious why this should be the case; nevertheless, the two hypotheses could be distinguished easily by comparing nonelectrophoretic differentiation in these same birds. Such techniques as micro-complement fixation and DNA-DNA hybridization could be used for this purpose.

If the finding that the degree of genetic differentiation between the various levels of avian taxa is less than that in other organisms proves to be generally true, then we must distinguish between two further possibilities: 1) genetic divergence over time is slower in birds than in other organisms, or 2) avian taxa are more recent in origin than are comparable taxa of other organisms. In either case, the concept of an electrophoretic clock (Nei 1972, Smith and Zimmerman 1976, Sarich 1977) will not be useful for avian studies without extensive analysis and calibration.

Some authors (Farris et al. 1970, Farris 1973) have suggested that maximum parsimony trees represent our best estimates of evolutionary history, as they invoke the smallest number of events to explain an existing pattern. Hence, they may be less likely to be incorrect than are hypotheses invoking more complex histories. Since the maximum parsimony algorithms require that no assumptions be made concerning evolutionary rates, the tree illustrated in Fig. 2 represents an estimate of the evolutionary distance along branches (anagenesis) as well as an estimate of the branching pattern (cladogenesis). However, for the reasons discussed above many more data will be required before a reliable time scale can be assigned to these distances. Thus the indicated branch lengths should be taken to represent only relative amounts of evolutionary change.

Finally, it should be emphasized that all the estimates of genetic heterozygosity and distance reported on here are based entirely on structural genes. It remains a matter of conjecture as to whether regulatory genes will tell the same story.

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