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GENETIC VARIATION IN SYMPATRIC WILLOW FLYCATCHERS (*EMPIDONAX TRAILLII*) AND ALDER FLYCATCHERS (*E. ALNORUM*)

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ABSTRACT.—We used allozyme electrophoresis to estimate the level of genetic variability and differentiation of allopatric and sympatric populations of the Willow (*Empidonax traillii*) and Alder (*E. alnorum*) flycatchers from southeastern Canada. We reasoned that, if hybridization occurs, sympatric populations should be more variable and interspecifically less differentiated than allopatric populations. These predictions were not supported by our results. Therefore, we concluded that in the surveyed region the two species are reproductively isolated. This conclusion is weakened by the fact that sample sizes were relatively small and that genetic differentiation of the two species is low. These conditions render interbreeding difficult to detect if it occurs at a low frequency. *Received 3 June 1987, accepted 16 November 1987.*

THE Tyrannidae form the largest and most diverse family of birds in the New World (Traylor and Fitzpatrick 1982). Many genera comprise groups of similar species, a number of which are considered superspecies (e.g. Mayr and Short 1970, A.O.U. 1983). Reproductive isolation between sympatric members of these species groups is often achieved through habitat isolation (e.g. Johnson 1963, 1980). If ecological preferences of two species are similar, however, their isolation depends either on the existence of a postmating isolating mechanism or on the ability of individual birds to discriminate between heterospecifics and conspecifics before mating (Mayr 1963).

In birds, song differences are thought to be of prime importance to ensure reproductive isolation in many pairs of sympatric sibling species (Becker 1982), particularly in the tyrannids (e.g. Lanyon 1963, Johnson 1980). Reproductive isolation of two taxa, however, cannot be inferred from the mere presence of differences in their vocalizations. For instance, in *Myiarchus swainsoni* and in *M. tuberculifer* some conspecific populations have different vocalizations, and allopatric birds do not recognize foreign songs as being conspecific. Nevertheless, they hybridize freely when sympatric (Lanyon 1978).

Ornithologists usually have relied on analysis of morphometric and plumage characters to determine if potentially hybridizing taxa are genetically isolated (e.g. Rising 1983). It is difficult to study hybridization between sibling species using phenotypic characteristics, however. Electrophoretic techniques provide ornithologists with a potentially powerful tool for the investigation of such cases (Barrowclough 1983, Matson 1984). In a few instances they were used to survey contact zones between taxa that are known or suspected to hybridize (Martin and Selander 1975, Corbin et al. 1979, Barrowclough 1980, Braun 1981, Corbin 1981, Johnson and Zink 1983, Braun et al. 1984, Braun and Robbins 1986, Grudzien and Moore 1986). Al-

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lozyme traits are of great interest for the analysis of hybridization because they are discrete characters, generally inherited in a simple Mendelian fashion, and are usually thought to be selectively neutral (Kimura 1983, Barrowclough et al. 1985). Alleles of different frequencies in allopatric populations of two taxa may be good markers to assess gene flow across a contact zone. Moreover, simple models make it possible to predict the behavior of some genetic parameters through a hybrid zone (Workman 1969, Endler 1977, Corbin 1981). These predictions can then be used to detect hybridization between sympatric sibling species.

We used this approach to determine if Willow (*Empidonax traillii*) and Alder (*E. alnorum*) flycatchers have hybridized in areas of sympatry in southeastern Canada. These are closely related sibling species (Stein 1958, 1963; Zink and Johnson 1984; Shields et al. 1987) that can be separated almost exclusively by advertising songs. Their phenotypic resemblance and the close similarity of their ecological preferences in southeastern Canada (Barlow and McGillivray 1983) make them good candidates for hybridization.

Materials and Methods

We analyzed the genetic variability of 94 specimens from 6 Willow or Alder flycatcher populations. Birds were collected during the summers of 1985 and 1986. Geographic location of the population samples, their status (sympatric or allopatric), and sample sizes are given in Table 1. Most specimens from sympatric populations were collected at locations where syntopy occurred. Specific identity was established by a playback experiment before collection (Seutin 1987a). Procedures for collection and storage of tissue samples followed those described by Johnson et al. (1984). Samples were transported from the field on dry ice and stored at −80°C in the laboratory. Extracts of breast and heart muscles were prepared separately; liver and kidney samples were combined to produce a third extract. Tissue samples were homogenized with an equal volume of the buffer solution (Karig and Wilson 1971) and centrifuged at 23,000 *g* for 20 min at 4°C.

Electrophoreses were carried out on horizontal 10% starch gels or on vertical polyacrylamide slab gels (Chapel et al. 1974) that varied in acrylamide concentration from 7% to 10% (Seutin 1987b). A discontinuous buffer system (Ornstein 1964) was used for electrophoreses conducted in acrylamide gels, and a 25–30-mA current was applied for 4–7 h. Four buffer systems were used for starch gels (Selander et al. 1971, Braun and Parker 1985). To maximize the reliability of our estimates of genetic variability and differentiation, a specific effort was made to detect hidden alleles (Coyne et al. 1979). For most loci assayed, a set of individual samples of both species was run in different gels and buffer systems. The gel-buffer combination that provided the clearest banding pattern and highest variability was selected for the analysis of the remaining individuals (Table 2).

Staining techniques were similar to those described by Harris and Hopkinson (1976) and Barrowclough and Corbin (1978). GLUD was stained by the method of Brewer (1970). Two esterases (Es-1 and Es-D) were characterized using 4-methylumbelliferyl acetate (4-Mμ) as substrate; Es-3 and Es-4 were stained using α-naphthyl acetate. On the basis of heterozygote banding patterns, Es-D and Es-4 were dimeric, and the other two were monomeric. Three globins (Gb) were stained using the benzidine test (Gordon 1969), and three general proteins (Ptm) were characterized with Coomassie R-250 (Righetti and Drysdale 1974). Thirty-six presumptive genetic loci were scored. We also attempted to analyze GDH (E.C no. 1.1.1.47), GR (1.6.4.2), NP (2.4.2.1), HK (2.7.1.1), ACP (3.1.3.2), HEX (3.2.1.3.), ALD (4.1.2.13), and FH (4.2.1.2.), but they proved to be unscorable. When more than one locus was observed for a particular enzyme, they were numbered sequentially, beginning with the most anodal. Alleles at variable loci were coded by letters, beginning with "a" for the most anodal.

Presumptive individual genotypes were deduced from banding patterns on stained gels. From these we derived a table of allelic frequencies. Observed heterozygosity (H_{obs}) was determined by direct count for each specimen and then averaged for each sample (Corbin 1983). The BIOSYS-1 program (Swofford and Selander 1981) was used to compute the proportion of polymorphic loci, the mean number of alleles per locus, the expected heterozygosity (H_{exp}), *F*-statistics (Wright 1965, 1978), and Nei's (1978) and Rogers' (1972) genetic distances. For each sample genotypic distribution at each locus was examined for departure from Hardy-Weinberg expectations, using a χ^2 test (Sokal and Rohlf 1981). When more than two alleles occurred at a locus, the actual genotypes were grouped into three classes, according to the procedure proposed by Swofford and Selander (1981). This reduced the number of classes with low frequency and lessened the probability of generating artificially inflated test values. Among-sample heterogeneity in allelic frequency distributions was analyzed using the *G* test. This test was used because it is less sensitive than the χ^2 test to the presence of rare classes in the distributions, and it usually better approximates the theoretical χ^2 distribution (Sokal and Rohlf 1981). The SPSS (Nie et al. 1975) and BMDP (Dixon 1981) computer packages were used to compute these and other statistics.

We used nonmetric multidimensional scaling (MDS)

TABLE 1. Genetic variability at 36 loci in 6 flycatcher populations. Acronyms in parentheses correspond to those used in other tables and in Fig. 1.

Population[a]	Status	n	% of polymorphic loci[b]	Mean no. of alleles/locus	$H_{obs} \pm SE$	$H_{exp} \pm SE$[c]	$\bar{F}_{is}$[d]
Empidonax alnorum							
St.-Jean-Vianney (Ea-SJV)	Allopatric	19	22.2	1.56	0.082 ± 0.009	0.083 ± 0.030	−0.025
Mont-Tremblant (Ea-MtT)	Allopatric	16	16.7	1.44	0.063 ± 0.007	0.073 ± 0.030	0.048
Montreal (Ea-Mtl)	Sympatric	16	19.4	1.50	0.081 ± 0.010	0.084 ± 0.030	−0.001
Brighton (Ea-Br)	Sympatric	14	19.4	1.44	0.070 ± 0.009	0.075 ± 0.028	0.021
Empidonax traillii							
Montreal (Et-Mtl)	Sympatric	16	27.8	1.44	0.082 ± 0.013	0.090 ± 0.029	0.006
Brighton (Et-Br)	Sympatric	13	22.2	1.42	0.069 ± 0.009	0.076 ± 0.026	0.063

[a] Coordinates of locations are given by Seutin (1987a).
[b] Frequency of most common allele ≤ 0.95.
[c] Unbiased estimate (Nei 1978).
[d] Mean F_{is} over all polymorphic loci.

and correspondence analysis (CA) ordination techniques to summarize genetic relationships among populations. The matrix of Rogers' distance values was subjected to a principal coordinate analysis (Legendre and Legendre 1983) to produce an initial configuration of samples for MDS. MDS was then performed to optimize the fit of data in the reduced space (Kruskal 1964, Legendre and Legendre 1983), using the MDSCALE routine in the NT-SYS computer package (Rohlf et al. 1982).

Correspondence analysis (CA) directly produces an ordination from a contingency table. CA preserves, in the reduced space, the χ^2 distances between rows or columns of the contingency table (Legendre and Legendre 1983, Greenacre 1984). The ordination was produced by the ACOR computer program (Cléroux undated), using the table of actual (not relative) allele frequencies at polymorphic loci.

RESULTS

Genetic variation within population samples.—Of the 36 loci scored, 16 (44.4%) were variable in at least one sample (Table 2). The percentage of polymorphic loci, average number of alleles per locus, and mean observed heterozygosity of the population samples are given in Table 1. Values resembled those reported by Zink and Johnson (1984) for the same species. There were no significant differences among populations in the percentage of polymorphic loci (G test; $G_{adjusted} = 0.740$, $P > 0.975$), average number of alleles per locus (Kruskal-Wallis test; $H_{corrected} = 0.684$, $P = 0.984$), and mean observed heterozygosity (Kruskal-Wallis test; $H_{corrected} = 4.450$, $P = 0.487$).

The inbreeding coefficient, F_{is} (Wright 1965), was calculated for each polymorphic locus in each population. Values ranged from −0.524 to 1.000. However, Chi-square tests for conformance to Hardy-Weinberg equilibrium revealed only 4 cases, from a total of 69, for which observed and expected distributions of genotypes were significantly different ($P < 0.05$). For Es-4 in Montreal Alder Flycatchers, Lgg-2 in Brighton Alder Flycatchers, and Lgg-1 in Brighton Willow Flycatchers, F_{is} was positive, indicating a deficiency of heterozygotes; only for Gb-1 in Montreal Alder Flycatchers was F_{is} negative. The average F_{is} of the samples, calculated over all polymorphic loci, ranged from -0.025 to 0.063 (Table 1). These values imply that all populations are close to Hardy-Weinberg equilibrium and, therefore, are panmictic.

Genetic variation among population samples.—The 6 samples were similar in allelic frequencies at the 36 loci studied. No fixed allelic differences were found between the two species. All alleles that were limited to one species were rare alleles and, owing to small sample sizes, cannot be considered good species markers (Gregorius 1980). Contingency analyses revealed that populations differed significantly in allelic frequencies at 4 loci: Pgm-2, Es-D, Es-3, and Gb-1 (Table 3). Interspecific differences seem to be responsible for the results observed at Pgm-2, Es-D, and Es-3. No significant heterogeneities were observed at these loci when groups of conspecific samples were analyzed separately (Table 3).

F_{st} values, a measure of genetic differentiation

TABLE 2. Allelic frequencies and electrophoretic conditions for polymorphic protein loci.[a]

Locus[b] (E.C. no.)		Population sample						Tissue, buffer[c]
		Ea-SJV	Ea-MtT	Ea-Mtl	Ea-Br	Et-Mtl	Et-Br	
Adh	a	0.026	0.031					L, TC I
(1.1.1.1)	b	0.974	0.969	1.000	1.000	1.000	1.000	
Gpd	a	0.789	0.844	0.688	0.750	0.844	0.885	M, TC II
(1.1.1.8)	b	0.211	0.156	0.312	0.250	0.156	0.115	
Icd-1	a	0.139	0.187	0.063		0.031	0.115	L, TC 7.5
(1.1.1.42)	b	0.416	0.344	0.375	0.429	0.406	0.423	
	c	0.056	0.125	0.031	0.107	0.157	0.077	
	d	0.389	0.344	0.531	0.464	0.406	0.385	
Pgd	a	0.026		0.031				L, TC I
(1.1.1.44)	b	0.974	1.000	0.969	1.000	1.000	1.000	
Pgm-1	a	1.000	1.000	0.969	0.964	1.000	1.000	L, TC 7.5
(2.7.5.1)	b			0.031	0.036			
Pgm-2	a			0.031		0.219	0.115	L, TC 7.5
	b	0.974	1.000	0.969	1.000	0.781	0.885	
	c	0.026						
Es-D	a	0.079		0.031	0.071	0.187	0.231	L, LiOH
(3.1.1.X)	b	0.921	1.000	0.969	0.929	0.813	0.769	
Es-3	a	0.079	0.031					H, TGly
	b	0.158	0.094	0.250	0.179	0.031		
	c	0.053	0.125	0.125	0.036	0.063	0.038	
	d	0.263	0.125	0.125	0.321	0.281	0.308	
	e	0.447	0.562	0.469	0.393	0.625	0.654	
	f		0.063	0.031	0.071			
Es-4	a	0.028	0.063	0.100	0.038	0.067		H, TGly
	b	0.972	0.937	0.900	0.962	0.933	1.000	
Lgg-1	a	0.026						L, LiOH
(3.4.11.X)	b	0.053	0.063	0.067	0.071	0.031	0.077	
	c	0.921	0.937	0.933	0.929	0.969	0.923	
Lgg-2	a	0.053	0.031	0.033	0.071		0.038	L, LiOH
	b	0.921	0.969	0.967	0.893	0.906	0.962	
	c	0.026			0.036	0.094		
La-1	a	0.105		0.063	0.036			L, TC 7.5
(3.4.11.X)	b	0.895	1.000	0.937	0.964	1.000	1.000	
Ada	a		0.031				0.038	M, TC II
(3.5.4.4)	b	1.000	0.969	1.000	1.000	0.937	0.962	
	c					0.063		
Gpi	a				0.036	0.063	0.077	H, TGly
(5.3.1.9)	b	1.000	0.969	0.969	0.964	0.937	0.923	
	c		0.031	0.031				
Gb-1	a					0.100	0.042	H, TGly
	b	0.763	0.375	0.656	0.893	0.700	0.875	
	c	0.026						
	d	0.211	0.625	0.344	0.107	0.200	0.083	
Ptm-3	a	1.000	1.000	1.000	1.000	1.000	0.962	M, TGly
	b						0.038	

[a] The following loci were monomorphic in all individuals assayed (E.C. no., tissue, and buffer system in parentheses): Sdh (1.1.1.14, L, TC I), Ldh-1 and -2 (1.1.1.27, L, TGly), Mdh-1 and -2 (1.1.1.37, L, TGly), Me-1 (1.1.1.40, L, TC I), Me-2 (H, TC I), Icd-2 (L, TC 7.5), Gd (1.1.1.47, L, TGly), Glud (1.4.1.3, M, TC II), Sod-1 and -2 (1.15.1.1, H, TGly), Got (2.6.1.1, H, TGly), Es-1 (L, LiOH), La-2 (L, TC 7.5), V1 (3.4.11.X, L, TGly), Gb-2 and -3 (H, TGly), Ptm-1 and -2 (M, TGly).

[b] Abbreviations follow Harris and Hopkinson (1976); lowercase letters indicate alleles.

[c] M = breast muscle, H = heart, L = liver and kidney; TGly = discontinuous tris-HCl and tris-glycine buffer, from Ornstein (1964),TC I = tris-citrate pH 6.3, from Selander et al. (1971); TC II = tris-citrate pH 8.1, from Selander et al. (1971); TC 7.5 = tris-citrate pH 7.5, from Braun and Parker (1985); LiOH = lithium-hydroxide, from Selander et al. (1971).

TABLE 3. F_{st} for polymorphic loci (Wright 1978). Total population is formed either by the 6 samples or by conspecific samples. Loci showing significant among-sample heterogeneities in allelic frequency distribution are indicated by asterisks (*G* tests).[a]

Locus	6 samples	*E. alnorum*	*E. traillii*
Adh	0.020	0.015	—
Gpd	0.027	0.018	0.004
Icd-1	0.016	0.020	0.005
Pgd	0.020	0.015	—
Pgm-1	0.023	0.017	—
Pgm-2	0.106**	0.018	0.019
Es-D	0.075**	0.023	0.003
Es-3	0.041*	0.025	0.002
Es-4	0.022	0.014	0.034
Lgg-1	0.005	0.002	0.010
Lgg-2	0.021	0.013	0.027
La-1	0.048	0.031	—
Ada	0.032	0.024	0.015
Gpi	0.024	0.014	0.001
Gb-1	0.152***	0.167***	0.034
Ptm-3	0.032	—	0.020
Mean	0.051***	0.043	0.011

[a] * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

among populations, were calculated for each polymorphic locus, considering the total population to be either the six samples or each group of conspecific samples (Table 3). All values were low, indicating that most of the genetic variance is found within populations. At most loci, and on average, values were slightly higher when the heterospecific group was analyzed, than when only conspecific populations were studied. A hierarchical analysis with *F*-statistics (Wright 1978), with localities and species as the levels, revealed that only 12.9% of the genetic variance found among the six samples was distributed between the species.

Mean values of Nei's (D_N) and Rogers' (D_R) genetic distances among Alder Flycatcher samples were $\bar{D}_N = 0.002$ and $\bar{D}_R = 0.027$, and those between Willow Flycatcher samples were $D_N = 0.000$ and $D_R = 0.021$. Mean interspecific distances ($\bar{D}_N = 0.004$, $\bar{D}_R = 0.035$) were similar to those obtained by Zink and Johnson (1984; $D_N = 0.009$, $D_R = 0.041$).

Because all distance values were extremely small, we recomputed genetic distances using only polymorphic loci (Table 4). These values are overestimated, but they are easier to interpret and to process. The matrices of D_N and D_R values were highly correlated ($r = 0.769$), implying that both provide a similar picture of the relationships among populations. All pairwise comparisons among Alder Flycatcher samples revealed similar levels of genetic differentiation, with no tendency for the sympatric populations to have diverged significantly from allopatric ones. Similarly, both allopatric and sympatric populations of the Alder Flycatcher were equally differentiated from Willow Flycatcher populations ($\bar{D}_R = 0.078$ vs. 0.080).

Principal coordinate analysis of the matrix of Rogers' distances explained 78.71% (axis 1: 49.42%, axis 2: 29.29%) of the total variation among samples in two dimensions. Using the resulting coordinates as an initial configuration, nonmetric multidimensional scaling (MDS) achieved a final stress of 0.000 (perfect fit) (Fig. 1). Matrix correlation with the original distance matrix was 0.9901, suggesting a nearly perfect representation of the original relationships in the reduced space.

The ordination produced by the correspondence analysis (CA) method was almost identical to that generated by MDS. Only the position of the Montreal and Mont-Tremblant samples of the Alder Flycatcher along the first axis were interchanged (Seutin 1987b). The first two axes of the CA ordination explained 70.81% (axis 1: 46.80% axis 2: 24.01%) of the total variance of the table of allelic frequencies.

The close resemblance of both ordinations was verified by the strong correlation between the matrices of euclidean distances between samples in each of the reduced spaces ($r = 0.8466$). In both ordinations populations are dis-

TABLE 4. Genetic distances based on 16 polymorphic loci between 6 flycatcher populations. Above diagonal: Nei's (1978) distances; below diagonal: Rogers' (1972) distances.

	Ea-SJV	Ea-MtT	Ea-Mtl	Ea-Br	Et-Mtl	Et-Br
Ea-SJV	—	0.011	0.000	0.000	0.003	0.002
Ea-MtT	0.069	—	0.006	0.021	0.014	0.023
Ea-Mtl	0.052	0.065	—	0.001	0.007	0.012
Ea-Br	0.041	0.083	0.054	—	0.004	0.002
Et-Mtl	0.072	0.083	0.086	0.072	—	0.000
Et-Br	0.068	0.088	0.093	0.067	0.048	—

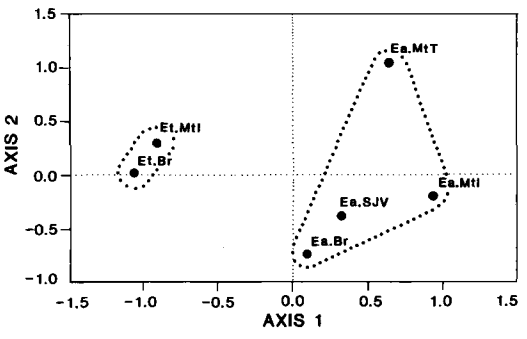

Fig. 1. Projection of 6 flycatcher populations along the first two axes of a nonmetric multidimensional scaling analysis of the matrix of Rogers' distances. The initial configuration consisted of the population coordinates derived from a principal coordinate analysis of the distance matrix. Final stress = 0.000; matrix correlation = 0.9901.

tributed along the first axis according to their taxonomic status. The large dispersion of Alder Flycatcher samples along axis 2 of both ordinations seems to be due to the heterogeneity of their allelic frequency distributions at the Gb-1 locus (Seutin unpubl. data).

Discussion

Genetic variability of the population samples.—The amount of genetic variability within samples of both species was within the normal range for avian breeding populations (Barrowclough 1983, Corbin 1983). All populations sampled exhibited nearly the same numbers of polymorphic loci and levels of heterozygosity. Because founder events usually result in reduced polymorphism and heterozygosity (Nei et al. 1975, Selander 1976), we conclude that this was not a factor in the spread of the Willow Flycatcher to northern latitudes in northeastern America (Stein 1963, Barlow and McGillivray 1983).

Hybrid populations are formed by the admixture of differentiated gene pools and should show increased genetic variability. The percentage of polymorphic loci (p) will increase if the parental forms are fixed for alternative alleles, or if one form is fixed at some loci and the other is not. As a result of the increase in p and in the number of alleles showing intermediate frequencies, observed heterozygosity should also be higher in hybrid populations than in pure parental stocks (Corbin 1981). We found no locus that was fixed at alternative alleles in the two species. Only for three loci were there significant differences in allelic frequency distributions that could be attributed to interspecific differences (Table 3). This number of differentiated loci is probably not sufficient for a significant increase in p and H_{obs} to be seen if hybridization is rare. That we found no significant differences in the level of genetic variability of the populations does not indicate the complete absence of hybridization between the two species, but this suggests that interbreeding does not occur at a high frequency in the surveyed areas.

In hybrid populations increased heterozygosity will be particularly noticeable at loci that show substantial interspecific differences in allelic frequencies. This may result in deviations of the observed genotypic distributions at these loci from those expected under Hardy-Weinberg equilibrium (Workman 1969). In our sympatric samples F_{is} values for the three loci with significant interspecific differences were close to zero and usually were slightly positive. This reflects a deficiency in heterozygotes, which is opposite to what we expected to observe if the two taxa were hybridizing. Chi-square tests further revealed that differences between the observed and expected genotypic distributions at these loci were not significant.

Genetic differentiation of the population samples.—The calculated genetic distances between Willow and Alder flycatchers are among the lowest reported for avian species. The mean Nei's distance between heterospecific samples ($\bar{D}_N = 0.004$) is an order of magnitude lower than the average distance calculated by Barrowclough (1980) for congeners ($\bar{D}_N = 0.044$, based on 71 comparisons). The close genetic resemblance of the two species is also reflected by the small F_{st} values found at most loci. The mean F_{st} over all polymorphic loci was 0.051 when the six samples were pooled to form the total population. This is only slightly higher than values for conspecific populations of other passerines (Barrowclough 1983), and it implies that only 5.1% of the total genetic variation is found among populations.

Because care was taken in choosing electrophoretic conditions that yielded the highest protein variation, our estimates of interspecific differentiation should be viewed as representative of the actual differentiation of the two taxa at the protein level. This conclusion is supported indirectly by the fact that genetic dis-

tances calculated for conspecific samples are similar to those reported for populations of other bird species (Barrowclough 1980). We did not include allopatric samples of the Willow Flycatcher in the analyses, however, so our estimates of the genetic differentiation between the two species should be viewed as minimal because of the possibility that the sympatric Willow Flycatcher samples are of partly hybrid origin.

Extremely small interspecific genetic distances ($D_N < 0.01$ or $D_R < 0.05$) seem not to be unusual in birds (e.g. Avise and Aquadro 1982). Possible reasons for this were discussed by Sibley and Ahlquist (1982) and Avise (1983). The small genetic differentiation of Willow and Alder flycatchers at the protein level should not be taken as evidence for conspecificity.

We found no single allele whose presence or frequency distribution among samples could be used to detect hybrids in sympatric samples of the Alder Flycatchers. Therefore, we used multilocus statistics that summarize differences at all loci. The ordination produced by a nonmetric multidimensional scaling analysis of the matrix of Rogers' distances (Fig. 1) showed that the two species were genetically distinct. Of the two sympatric Alder Flycatcher samples, only the one from Brighton showed signs of intermediacy, and was closer to Willow Flycatchers than any other Alder Flycatcher sample. The confidence intervals of our genetic distance estimates are relatively large, however (Nei and Roychoudhury 1974), and, therefore, the position of the samples in the reduced space may imperfectly reflect their actual relationships. It would be premature to infer from the only slightly intermediate position of the Brighton Alder Flycatcher sample that hybridization occurs, especially because the other sympatric Alder Flycatcher population (Montreal) is clearly not intermediate. A low level of hybridization will have very limited influence on genetic distances, especially if hybrids exhibit reduced viability of fertility, and our results cannot be taken as indicative of the complete absence of interspecific gene flow. Moreover, because we included no allopatric Willow Flycatcher populations in our analysis, it is possible that unidirectional introgression, toward the Willow Flycatcher, occurs.

Willow and Alder flycatchers have long been treated as subspecies of the Traill's Flycatcher (*E. traillii*), but Stein's (1958, 1963) extensive behavioral and ecological studies showed that they probably are reproductively isolated. Our results support that contention. Our sample sizes were relatively small and genetic differentiation between the two species is very low, however, making it difficult to detect hybridization if it occurs at a low frequency. Much larger samples and, probably, other types of genetic markers will be needed to assess the extent of gene flow, if any, between the two species.

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