EVOLUTIONARY GENETICS OF FLYCATCHERS. II. DIFFERENTIATION IN THE *EMPIDONAX DIFFICILIS* COMPLEX

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ABSTRACT.—We used starch-gel electrophoresis to assess variability at 41 genetic loci in 208 individuals from 11 breeding populations of the Western Flycatcher (*Empidonax difficilis*) complex. Genic variability was substantial in most populations and equivalent to levels found in other avian taxa. A sample of *E. d. insulicola* from Santa Catalina Island, however, showed reduced heterozygosity and an unusually low percentage of polymorphic loci. We attribute this to a bottleneck at the time of the original colonization. Nei's genetic distances among populations of one taxon ranged from $\bar{D} = 0.0003$ (in *E. d. difficilis*) to $\bar{D} = 0.0033$ (in *E. d. hellmayri*). Intertaxon Nei's $\bar{D}$ ranged from 0.009 (*E. d. insulicola* vs. *E. d. difficilis*) and 0.0149 (*E. d. difficilis* vs. *E. d. hellmayri*) to 0.0228 (*E. d. insulicola* vs. *E. d. hellmayri*). F_{st} statistics revealed significant population subdivision within the complex. With Slatkin's rare-allele method we estimated the gene-flow parameter, *Nm*. Mainland populations experience moderately high gene flow (9.62 immigrants/generation). In contrast, Santa Catalina Island receives an estimated 0.093 immigrants/generation, pointing to very low gene flow and essential genetic isolation.

Genetic distances yielded phenograms and distance Wagner trees that provide hypotheses for the relationships and phylogenesis of populations in western North America. The lineage leading to modern *E. d. difficilis* split from that leading to *E. flavescens* in the mid-Pleistocene at 866,800 yr BP; the ancestors of modern *E. d. difficilis* diverged from those of present-day *E. d. hellmayri* at 248,700 yr BP; and the stock leading to modern *E. d. insulicola* budded from the lineage that became *E. d. difficilis* in the late Pleistocene, approximately 187,000 yr BP.

Empidonax d. difficilis and *E. d. hellmayri* nest sympatrically and mate assortatively in the Siskiyou region of northern California. Interbreeding has not been demonstrated conclusively, and we regard these taxa as biologic species. In the absence of a test of sympatry, the well-differentiated form *E. d. insulicola* of the California Channel Islands cannot be proved to be a biologic species. It is clearly a phylogenetic species, however, in the sense of Cracraft.

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THE American Ornithologists' Union (A.O.U. 1983) includes the various forms of the *Empidonax difficilis* complex under one species, the Western Flycatcher. Insular, coastal, and interior populations of the western United States, however, are strongly differentiated in size, color, voice, and preferred habitat (Johnson 1980). In this paper we analyze allozyme data as related to the evolutionary status and taxonomic rank of *E. difficilis*.

It is also of interest to compare allozyme variation in the Western Flycatcher complex with that shown by its more distantly related congeners (Zink and Johnson 1984). Levels of genetic variation in restricted lineages comprised of related clusters of near-species and full species provide a valuable perspective on phylogenetic history, one that is not always clear from study of morphology (Wake 1981). Furthermore, because of their great morphologic resemblance, these tyrannids are aptly termed sibling species (Dobzhansky 1951), a category often suspected in the past of being more similar genetically than "ordinary" species (Dobzhansky 1978: 101). Although the evidence available for sibling species of flycatchers (in *Empidonax* and *Mionectes*) does not support this view (Zink and Johnson 1984, Capparella and Lanyon 1985), the degree to which molecular and morphologic features are either congruent or decoupled in both sibling species and non-sibling species continues to merit attention (Lewin 1985).

Other general goals encourage additional avi-

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TABLE 1. Genetic variability measures for 11 samples representing 3 taxa of the *Empidonax difficilis* complex.

Taxon	*n*	Sample area name[a]	$H_{obs.} \pm SE$	$H_{est.} \pm SE$	Percentage polymorphic loci[b]	Mean no. alleles[c]
E. d. insulicola	27	Santa Catalina Isl.	0.037 ± 0.005	0.034 ± 0.018	9.76	1.12
E. d. difficilis	16	Monterey	0.049 ± 0.009	0.053 ± 0.021	19.51	1.24
E. d. difficilis	15	Lake Co.	0.054 ± 0.007	0.057 ± 0.021	24.39	1.29
E. d. difficilis	18	Shasta	0.051 ± 0.007	0.054 ± 0.021	26.83	1.34
E. d. difficilis	10	Rogue River	0.061 ± 0.013	0.058 ± 0.023	19.51	1.29
E. d. hellmayri + *E. d. difficilis*	17	Siskiyou	0.066 ± 0.004	0.065 ± 0.025	19.51	1.32
E. d. hellmayri	23	Warner	0.053 ± 0.007	0.057 ± 0.021	24.39	1.39
E. d. hellmayri	20	Snake	0.052 ± 0.006	0.055 ± 0.021	21.95	1.34
E. d. hellmayri	20	Black Hills	0.045 ± 0.005	0.039 ± 0.017	17.07	1.22
E. d. hellmayri	28	Wet Mtns.	0.063 ± 0.006	0.066 ± 0.021	29.27	1.49
E. d. hellmayri	14	SE Arizona	0.064 ± 0.014	0.065 ± 0.021	26.83	1.34
Total	208	Mean	0.054	0.055	21.73	1.31

[a] Breeding specimens only. Exact localities are available from authors.
[b] Frequency of most common allele ≤ 0.99.
[c] Unweighted by sample size.

an electrophoretic studies. Although beyond infancy, avian allozyme analysis has not completed adolescence (Barrowclough 1983, Corbin 1983, Matson 1984, Barrowclough et al. 1985). Taxonomic representation and geographic coverage of investigations are still spotty. For the huge family Tyrannidae, only two allozyme studies have appeared (Zink and Johnson 1984, Capparella and Lanyon 1985); together these cover comparatively limited samples of only 5% of the existing species. Geographic analyses of breeding populations of single species are also scarce and often based on few widely spaced, regional samples. A notable exception is Zink's (1986) study of 31 populations of the Fox Sparrow (*Passerella iliaca*). Because severe difficulties accompany collection of even common bird species (Johnson 1982), we can never expect many comprehensive avian studies of intraspecific genetic variation across geography.

Finally, good techniques for estimating relative levels of gene flow are now available. One of these, Slatkin's (1981, 1985a) rare-allele method, can be applied directly to electrophoretic results, and we make such an attempt in this paper. Few estimates of gene flow in birds have been offered previously (Barrowclough 1980, Zink 1986, Zink et al. 1987).

METHODS AND MATERIALS

A total of 208 specimens of the Western Flycatcher complex was collected in the western United States during the spring and summer from 1977 through 1982. These specimens were segregated into 11 breeding populations (Table 1). Liver, muscle, kidney, and heart tissue was taken from each individual in the field and placed in liquid nitrogen (−196°C) until transport to Berkeley, California, for permanent storage. Tissue from 10 individuals of the Acadian Flycatcher (*E. virescens*) from Oklahoma was analyzed for outgroup comparisons. Tissue homogenates (a combination of a single tissue type and an equal volume of de-ionized water) were centrifuged at 4°C and 15,000 rpm for 40 min. The aqueous protein extracts were stored at −76°C for later electrophoretic analysis.

Forty-one presumptive genetic loci were examined by horizontal starch-gel electrophoresis using standard procedures described by Selander et al. (1971) and Yang and Patton (1981), with the slight modifications of Johnson et al. (1984). Protein assays were prepared according to Harris and Hopkinson (1976) and Selander et al. (1971). Alleles (electromorphs) at each locus were designated alphabetically in decreasing order of mobility. The most frequent allele at a locus was designated 100; other alleles were assigned higher or lower numbers, on a percentage basis, depending on their position on the gel relative to the most frequent allele. For example, at the malic enzyme locus (ME), allele "a" (105%) moved 5% farther from the origin than did the most common allele, "b" (100%). For multiple isozymes of proteins, the most anodal locus was designated "1," and more cathodal loci were indicated by progressively higher numbers. Hemoglobin was the only cathodal protein detected on LiOH gels.

Levels of heterozygosity were determined by direct count ($H_{obs.}$) and by estimation based on the expectation of Hardy-Weinberg equilibrium (Nei 1975). The

program BIOSYS-1 (Swofford and Selander 1981) was used to calculate expected heterozygosity per sample ($H_{exp.}$) and heterogeneity Chi-square statistics. Allelic frequencies were converted to genetic distances using the methods of Nei (1978) and Rogers (1972). To compare patterns of population relatedness, phenograms (UPGMA and WPGMA; Sneath and Sokal 1973) and a phylogenetic tree (distance Wagner network [Farris 1972], optimized according to Swofford 1981) were constructed from Rogers' distance values. Wright's (1965) F_{st}, with the modifications of Wright (1978) for small sample size and of Nei (1975) for multiple alleles, was computed overall for the 11 populations in the complex and for various subsets of populations.

To estimate levels of gene flow we used Slatkin's (1985a) formula, $\ln[p(1)] = a \ln(Nm) + b$, where $p(1)$ is the average frequency of private alleles, Nm is the product of the population size and immigration rate, a is a constant, -0.505, and b is a constant, -2.44. This formula is suitable for the analysis of samples of 25 or greater. Because our sample sizes differed among populations, we applied Slatkin's (1985a) recommended correction in which Nm is divided by the ratio of the actual average sample size to 25. Thus, $Nm_c = Nm(\bar{N}_i/25)^{-1}$, where $\bar{N}_i = (\Sigma N_i/11)$, the average sample size. N_i is the number of specimens in a given sample. For our average sample size of 18.9, the correction factor is therefore 0.756.

Results

Variation at loci and heterozygosity.—Of the 41 loci scored, 21 (51%) were variable either within or between members of the *E. difficilis* complex and *E. virescens* (Table 2). Of these variable loci, 17 (41.4% of total) showed at least a single heterozygote in the complex; the remaining 4 loci were monomorphic. The outgroup, *E. virescens*, was fixed at 2 loci (αGPD and GPT) at alleles that did not occur in any form of the *E. difficilis* complex and was polymorphic at 3 other loci (EST-4, SDH, ADH) at which all members of the complex were fixed. The 24 monomorphic loci in the *E. difficilis* complex were: ACON, ALD, ICD-2, EST-1, EST-4, CK-3, SOD-1, SOD-2, PT-1, PT-2, PT-3, HG, MDH-1, MDH-2, GOT-1, GOT-2, GLUD, GDA, GPT, LAP, LDH-2, ACP, ADH, and SDH. Three loci, GLO, GAPDH, and G-6-PDH, were unscorable.

Levels of genetic variation among populations of the *E. difficilis* complex are listed in Table 1. $H_{obs.}$ ranged from 0.037 in *E. d. insulicola* to 0.066 in the sympatric Siskiyou sample, which is comprised about equally of *E. d. hellmayri* and *E. d. difficilis*. The average H over all populations was 0.054. The percentage of polymorphic loci ranged from 9.76% (on Santa Catalina Island) to 29.27% (Wet Mountains, Colorado), with a mean of 21.73%. The mean number of alleles per locus ranged from 1.12 in *E. d. insulicola* on Santa Catalina Island to 1.49 (*E. d. hellmayri* of Wet Mountains, Colorado), with an average of 1.31.

In simple regressions, $H_{obs.}$ showed no relationship to sample size ($r = 0.391$), a weak relationship to percentage of polymorphic loci ($r = 0.527$), and a weak relationship to mean number of alleles per locus ($r = 0.647$). As expected, percentage of polymorphic loci correlated strongly with mean number of alleles per locus ($r = 0.893$). No significant relationship was found between sample size and either percentage of polymorphic loci ($r = -0.106$) or mean number of alleles per locus ($r = 0.149$). Furthermore, in a multiple regression analysis, with sample size as the dependent variable and (1) total number of alleles at polymorphic loci, (2) observed heterozygosity, and (3) percentage of polymorphic loci as independent variables, $R^2 = 0.0124$, an insignificant value ($P = 0.744$). The lack of dependence of mean levels of H (or of number of alleles) on sample size, when the number of loci surveyed is relatively large, agrees with both the theoretical predictions of Nei (1978) and the empirical findings of Gorman and Renzi (1979).

Geographic trends in allelic frequency.—Five loci exhibited clear geographic patterns in the frequency of alleles. Patterns in four of these loci are illustrated (Fig. 1). At ICD-1 (Fig. 1A) all mainland populations were monomorphic for the M allele. A unique fast allele (F) occurred only on Santa Catalina Island, where it was found with fairly high frequency (33.3%). A slightly more complicated pattern was shown at the EST-2 locus (Fig. 1B) at which two alleles (F, M) were widespread and showed weak clines in their geographic frequency. The F allele was common in the interior of the southeastern portion of the region but declined in frequency toward the north and northwest. Two other, rare alleles (F+, S) occurred only in the interior, at low frequencies (2.5%), in the populations of the Snake Range and Black Hills, respectively. Five alleles were recorded at 6-PGD (Fig. 1C). Again, only two of these turned up in coastal populations, one of which (M) was shared with all other populations; the other (S) occurred only at low levels in the northern three coastal populations. The Monterey and Santa Catalina Island populations were monomorphic for M. In

TABLE 2. Allelic frequencies at variable loci in 3 taxa of the *Empidonax difficilis* complex and *E. virescens*.

Locus (EC no.)	Allele	Santa Catalina	Monterey	Lake Co.	Shasta	Rogue River	Siskiyou	Warner	Snake	Black Hills	Wet Mtns.	SE Arizona	*Empidonax virescens*
ME	a (105)												0.050
(1.1.1.40)	b (100)	0.185		0.033	0.176	0.100	0.500	0.739	0.750	0.750	0.839	0.857	0.750
	c (90)	0.130	0.313	0.300	0.118	0.100	0.059	0.043	0.075	0.075	0.107	0.036	0.050
	d (80)	0.685	0.688	0.667	0.706	0.800	0.441	0.217	0.175	0.175	0.054	0.107	0.150
6-PGD	a (115)								0.025		0.036		
(1.1.1.44)	b (110)						0.059	0.109	0.175	0.050	0.214	0.143	
	c (105)						0.029	0.043					
	d (100)	1.000	1.000	0.933	0.972	0.950	0.912	0.848	0.800	0.950	0.750	0.857	1.000
	e (95)			0.067	0.028	0.050							
EST-2	a (115)								0.025				0.200
(3.1.1.1)	b (110)	0.204	0.250	0.167	0.111	0.250	0.176	0.109	0.200	0.075	0.357	0.321	0.700
	c (100)	0.796	0.750	0.833	0.889	0.750	0.824	0.891	0.775	0.900	0.643	0.679	0.100
	d (95)									0.025			
LA-1	a (120)					0.050							
(3.4.11)	b (115)		0.031				0.118	0.022	0.025			0.036	
	c (110)		0.313	0.367	0.472	0.350	0.382	0.283	0.375	0.150	0.214	0.143	0.050
	d (100)	1.000	0.656	0.633	0.528	0.550	0.500	0.696	0.600	0.850	0.786	0.821	0.500
	e (97)					0.050							
	f (95)												0.450
LA-2	a (100)	1.000	1.000	0.967	1.000	1.000	0.971	0.978	1.000	1.000	0.982	0.964	1.000
(3.4.11)	b (97)			0.033			0.029	0.022			0.018		
	c (95)											0.036	
LGG	a (115)												0.100
(3.4.11)	b (110)		0.063	0.033				0.043			0.071	0.036	0.500
	c (100)	1.000	0.937	0.967	0.972	1.000	1.000	0.914	1.000	1.000	0.929	0.964	0.400
	d (90)				0.028			0.043					
MPI	a (115)										0.054	0.107	
(5.3.1.8)	b (110)		0.094	0.033	0.028	0.050	0.118	0.022	0.050	0.050			0.100
	c (100)	1.000	0.906	0.933	0.972	0.950	0.882	0.978	0.950	0.950	0.929	0.893	0.850
	d (95)										0.018		0.050
	e (90)			0.033									
NP	a (120)										0.018		
(2.4.2.1)	b (115)			0.067		0.050	0.059	0.065	0.025		0.214	0.214	0.050
	c (107)					0.050							
	d (103)										0.018		0.050
	e (100)	1.000	1.000	0.933	1.000	0.900	0.912	0.913	0.975	1.000	0.732	0.786	0.850
	f (95)										0.018		0.050
	g (90)						0.029	0.022					

TABLE 2. Continued.

Locus (EC no.)	Allele	Santa Catalina	Monterey	Lake Co.	Shasta	Rogue River	Siskiyou	Warner	Snake	Black Hills	Wet Mtns.	SE Arizona	*Empidonax virescens*
αGPD (1.1.1.8)	a (200)												1.000
	b (150)				0.028					0.025			
	c (100)	1.000	1.000	1.000	0.972	1.000	1.000	1.000	1.000	0.975	1.000	1.000	
GPI (5.3.1.9)	a (150)				0.028							0.036	
	b (100)	1.000	0.969	1.000	0.917	1.000	1.000	1.000	0.975	1.000	0.982	0.964	1.000
	c (50)		0.031		0.056				0.025		0.018		
EAP (3.1.3.2)	a (140)											0.036	
	b (120)					0.050							
	c (100)	1.000	1.000	1.000	1.000	0.950	1.000	1.000	1.000	1.000	1.000	0.964	1.000
PGM (2.7.5.1)	a (125)							0.022	0.025		0.018		
	b (110)				0.028								
	c (100)	1.000	1.000	0.967	0.972	1.000	1.000	0.978	0.975	1.000	0.964	1.000	0.950
	d (90)			0.033							0.018		0.050
ADA (3.5.4.4)	a (110)										0.036		
	b (100)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.964	1.000	1.000
CK-2 (2.7.3.2)	a (115)				0.028								
	b (100)	1.000	1.000	1.000	0.972	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GR (1.6.4.2)	a (150)		0.031		0.028								
	b (130)	0.944	0.719	0.600	0.583	0.500	0.471	0.500	0.200	0.625	0.125	0.429	0.050
	c (100)	0.056	0.250	0.400	0.389	0.500	0.471	0.477	0.775	0.375	0.839	0.536	0.950
	d (85)						0.059	0.023	0.025		0.036	0.036	
ICD-1 (1.1.1.42)	a (200)	0.333											0.100
	b (175)												0.100
	c (100)	0.667	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.800
EST-4 (3.1.1.1)	a (120)												0.050
	b (100)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.950
SDH (1.1.1.14)	a (100)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.950
	b (75)												0.050
LDH-1 (1.1.1.27)	a (300)		0.031										
	b (100)	1.000	0.969	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GPT (2.6.1.2)	a (110)												1.000
	b (100)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
ADH (1.1.1.1)	a (100)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.900
	b (75)												0.100

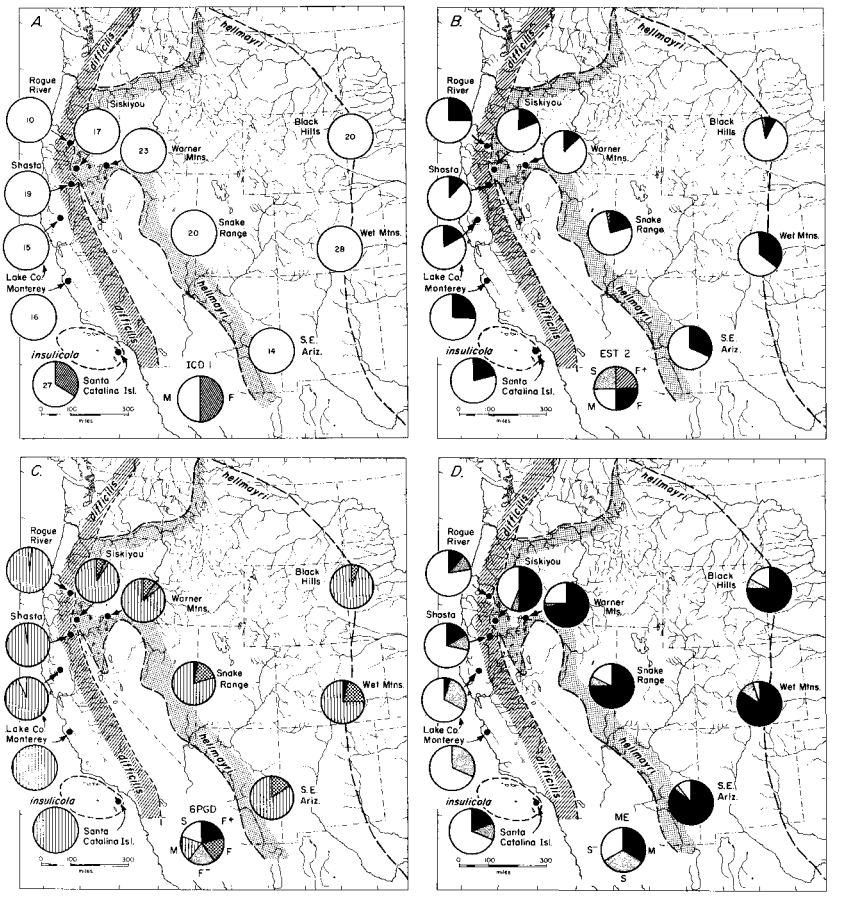

Fig. 1. Geographic occurrence and percentage frequency of alleles at four loci in the *Empidonax difficilis* complex. The 11 samples represent three taxa: *E. d. insulicola* (1 population), *E. d. difficilis* (4), and *E. d. hellmayri* (6). The key to alleles present is located in the lower center of the quadrant for each locus. Sample sizes are given within the symbol for each population in quadrant A.

the interior, a fairly common (5.9–21.4%) allele (F) was found that was missing along the west coast. Furthermore, two scarce (2.9–4.3%) alleles (F+, F−) were shared by two populations each of the interior group. The most striking pattern occurred at the malic enzyme (ME) locus (Fig. 1D). There, coastal and interior populations contrasted dramatically in the proportions of M and S− alleles. In *E. d. difficilis* the M allele was found at levels of zero (Monterey) to 17.6% (Shasta). In *E. d. insulicola* this allele occurred at 18.5%. In contrast, in all populations of "pure" *E. d. hellmayri* (those populations exclusive of the mixed sample from Siskiyou), the M allele was very common, occurring at 73.9% (Warner) to 85.7% (SE Arizona). In the Siskiyou population the M allele occurred at 50%. At the ME locus, the M allele apparently is heading toward fixation in the interior samples but is disappearing in the coastal samples. Finally, at the glutathione reductase (GR) locus (not illustrated), a clear clinal pattern of allelic frequencies occurred (Table 2). Allele b was very common (94.4%) and allele c was scarce (5.6%) on Santa Catalina Island. On the mainland, allele b gradually diminished as allele c increased in frequency from along the coast northward, and into the interior, until allele c was more common than allele b. The population from the Black Hills was slightly deviant from this trend; there, b was at 62.5% and c at 37.5%.

A heterogeneity Chi-square analysis at each locus (Swofford and Selander 1981) demonstrated significant geographic variation among populations.

Genetic distances.—Nei's genetic distances

TABLE 3. Matrix of genetic distances between 11 samples of 3 taxa of the *Empidonax difficilis* complex and *E. virescens*. Nei's (1978) *D*-values are above the diagonal, and Rogers' (1972) *D*-values are below the diagonal.

	1	2	3	4	5	6	7	8	9	10	11	12
1. Santa Catalina Isl.	—	0.007	0.009	0.011	0.011	0.015	0.017	0.028	0.013	0.034	0.021	0.110
2. Monterey	0.032	—	0.0	0.001	0.001	0.006	0.012	0.018	0.012	0.027	0.018	0.099
3. Lake Co.	0.037	0.016	—	0.0	0.0	0.003	0.009	0.014	0.011	0.021	0.015	0.097
4. Shasta	0.037	0.022	0.018	—	0.0	0.002	0.008	0.012	0.010	0.022	0.015	0.097
5. Rogue River	0.038	0.021	0.017	0.019	—	0.002	0.009	0.012	0.012	0.019	0.014	0.092
6. Siskiyou	0.045	0.030	0.022	0.025	0.021	—	0.001	0.003	0.004	0.009	0.005	0.085
7. Warner	0.049	0.036	0.027	0.030	0.031	0.019	—	0.002	0.0	0.006	0.001	0.084
8. Snake	0.057	0.041	0.036	0.037	0.033	0.025	0.020	—	0.006	0.002	0.003	0.078
9. Black Hills	0.039	0.033	0.030	0.029	0.034	0.025	0.016	0.025	—	0.010	0.002	0.089
10. Wet Mtns.	0.069	0.056	0.050	0.057	0.049	0.042	0.031	0.023	0.038	—	0.001	0.074
11. SE Arizona	0.057	0.047	0.043	0.049	0.040	0.032	0.024	0.028	0.027	0.020	—	0.077
12. *E. virescens*	0.142	0.134	0.133	0.136	0.129	0.121	0.119	0.112	0.122	0.111	0.118	—

(Table 3) between pairs of samples representing the same subspecies were low: $\bar{D} = 0.0003$ in *E. d. difficilis* (excluding Rogue River, a sample that is near the boundary with *E. d. hellmayri*), and $\bar{D} = 0.0033$ in *E. d. hellmayri* (excluding the mixed Siskiyou sample). The lesser value for *E. d. difficilis* vs. *E. d. hellmayri* suggests that the geographically more far-flung populations of the latter are more strongly differentiated genetically.

Empidonax d. insulicola is clearly allied to *E. d. difficilis* ($\bar{D} = 0.009$ for Santa Catalina Island vs. Shasta, Lake County, and Monterey) rather than to *E. d. hellmayri* ($\bar{D} = 0.0228$ for Santa Catalina Island vs. Warner, Snake, SE Arizona, Wet Mountains, and Black Hills). The value 0.009 is similar to those commonly seen between subspecies; the value 0.0228 is a level of *D* commonly seen between avian species (Barrowclough 1980: 661).

Comparison of all populations of *E. d. difficilis* (except Rogue River) with all populations of *E. d. hellmayri* (except Siskiyou) gave a $\bar{D}$ of 0.0149 for the 15 values. This value is intermediate between those given by Barrowclough (1980: 661) for subspecies (0.0048) and species (0.0440) of other birds. On the basis of genetic distance alone, *E. d. difficilis* and *E. d. hellmayri* qualify as megasubspecies (Amadon and Short 1976), a conclusion reached earlier on the basis of morphology and song (Johnson 1980: 111–113).

Analysis of genetic population structure.— Wright's (1951) F_{st} statistic measures the degree of genetic fragmentation of populations showing varying degrees of interbreeding (Barrowclough 1980: 657, Corbin 1983). At a given locus, an F_{st} value of 1 points to fixation of alternative alleles among populations, whereas a value of 0 indicates total panmixis. In 11 populations of the Western Flycatcher complex, the average F_{st} across loci was 0.153, indicating definite genetic subdivision among populations. Loci that contributed heavily to F_{st} values were ME (total $F_{st} = 0.324$), GR ($F_{st} = 0.173$), and ICD-1 ($F_{st} = 0.313$). Among the loci surveyed these three, therefore, have significantly differentiated geographically. Computation of F_{st} for different subsets of populations is also instructive, for in this manner geographic unevenness in amount of differentiation can be identified. For example, F_{st} for *E. d. insulicola* plus *E. d. difficilis* was 0.079. But when *E. d. insulicola* was excluded and the F_{st} calculated for the four populations of *E. d. difficilis* alone, a value of 0.026 resulted. Similarly, for the six populations of *E. d. hellmayri*, F_{st} equaled 0.069. Removal of Siskiyou from the latter analysis of *E. d. hellmayri* gave an F_{st} of 0.062. From these analyses we conclude that *E. d. difficilis* is more homogeneous genetically than is *E. d. hellmayri*.

Slatkin (1981) introduced a method for qualitatively estimating gene flow in subdivided populations. His approach was based on the average frequency of alleles in relation to the number of populations in which they occur (the "conditional average frequency"). Figure 2 presents the results of such an analysis for the Western Flycatcher complex. The shape of the curve indicates that gene flow is relatively high among the populations analyzed. That is, in general, alleles found only in one or a few populations occur with very low frequencies; alleles with high frequencies occur in all or nearly all populations.

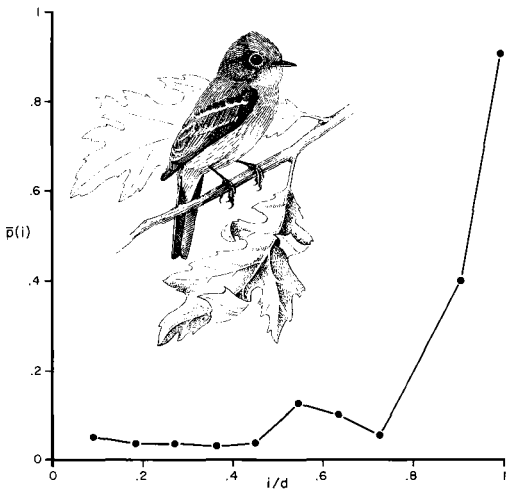

Fig. 2. Relationship between the conditional average frequency of a protein variant, $p(i)$ (the average frequency of a protein variant in the populations in which it occurs), and its incidence (the number of populations in which the variant is observed [i] divided by the total number of populations sampled [d]). The plotted points represent the average value of $p(i)$ for all variants having the same value of i/d. This curve is roughly congruent with an island model simulation when $Nm = 0.25$ (Slatkin 1981). Sample calculations of the coordinates for a single data point (e.g. the third data point from the left) are as follows: Allele e of the 6-PGD locus occurs in three populations (at a frequency of 0.067 in Lake County, 0.028 in Shasta, and 0.050 in Rogue River; Table 2). The other allele occurring only in three populations, allele a of the PGM locus, was found at a frequency of 0.022 in Warner, 0.025 in Snake, and 0.018 in the Wet Mountains. Thus, these two alleles occur at an average frequency of 0.048 and 0.022, respectively. Therefore, $p(3) = (0.048 + 0.022)/2 = 0.035$, and $i/d = 3/11 = 0.273$.

Recently, Slatkin (1985a) refined this technique to enable quantitative estimation of gene flow using the average frequency of alleles found only in single populations (private alleles). Slatkin's new method derives from his discovery that the logarithm of the average frequency of private alleles, $p(1)$, is approximately linearly related to the logarithm of the average number of migrants exchanged between local populations, Nm. The method assumes that the populations are in genetic and demographic equilibrium, which is usually uncertain. The average frequency of private alleles (Table 4) in the Western Flycatcher complex was very low in all continental populations in which they occurred at all, from 0.022 (Wet Mountains) to 0.050 (Rogue River). Two populations (Siskiyou and Warner) lacked private alleles. In contrast, the single private allele in the sample from Santa Catalina Island, ICD-1, allele a, occurred at a substantial frequency, 0.333. These allelic frequency values translate into moderate or high estimates of Nm_e for continental populations (3.97–20.22) but into an extremely low Nm_e, 0.093, for Santa Catalina Island (Table 4, left column).

As Slatkin (1985a) suggested, additional information on population structure can be obtained by computing $p(1)$ for different subsamples of the overall data set. Among other possibilities, such an approach can identify the influence of a single, isolated, or otherwise deviant population on the estimate of Nm_e. The right column of Table 4 shows the estimated values of Nm_e in 10 of the 11 total samples after successive removal of different single samples. Considering all locations, $Nm_e = 4.14$, a value similar to that seen when any continental sample was excluded ($Nm_e = 2.86$–4.69). When the isolated sample from Santa Catalina Island was removed, however, the estimate of $Nm_e = 9.62$, a value 2–3 times greater. Again, this result points to a substantial probable difference in the magnitude of gene flow, as estimated by Nm_e, among continental populations vs. that between the mainland and insular populations.

Inheritance of electromorphs.—Seven partial or complete family groups totaling 32 specimens were collected. These ranged in composition from one family consisting of 1 parent and 3 nestlings to an example of both parents and 4 nestlings. In every example the parents and their offspring had electromorphic patterns at all polymorphic loci that were expected according to a system of Mendelian inheritance.

Phenograms and phylogenetic trees.—To allow visual assessment of relationships of populations, three schemes were applied. UPGMA and WPGMA clustering algorithms (Sneath and Sokal 1973; Fig. 3) are phenetic approaches that assume constant rates of allozymic change in accordance with a molecular-clock hypothesis (Wilson et al. 1977, Thorpe 1982). The third distance approach, a Wagner network (Fig. 4), provided a phylogenetic perspective; it does not assume homogeneity of rates of allelic substitution. The three approaches in essence offer different hypotheses on patterns of relationship, patterns developed from a somewhat dif-

TABLE 4. Number and average frequency of private alleles and estimates of gene flow in populations of the *Empidonax difficilis* complex. See Table 1 for sample size of each population. Average sample size was 18.9.

	Single populations			Combined populations (one population excluded[a])		
Sample location	No. private alleles	$p(1)$	Nm_c[b]	No. private alleles	$p(1)$	Nm_c
Santa Catalina Isl.	1	0.333	0.093	17	0.032	9.62
Monterey	1	0.031	10.25	18	0.049	4.14
Lake Co.	1	0.033	9.05	18	0.048	4.31
Shasta	2	0.028	12.54	20	0.048	4.31
Rogue River	4	0.050	3.97	15	0.048	4.31
Siskiyou	0	0	0	20	0.047	4.50
Warner	0	0	0	21[c]	0.046	4.69
Snake	1	0.025	15.69	18	0.050	3.98
Black Hills	1	0.025	15.69	18	0.049	4.14
Wet Mtns.	5	0.022	20.22	16	0.059	2.86
SE Arizona	2	0.036	7.62	18	0.050	3.98
All samples				18	0.049	4.14

[a] For example, 17 private alleles occur at an average frequency of 0.032 in the 10 populations excluding Santa Catalina.

[b] $Nm_c = Nm$ corrected for sample size.

[c] More private alleles can occur in a subsample (e.g. 21 in all populations exclusive of Warner) than in all samples combined (18) because alleles found in two different populations will be private alleles in some subsamples.

ferent mathematical treatment of Rogers' *D*. We assumed that topologic similarity among trees served as evidence for true relationships of the populations involved. Conversely, when differing clustering approaches yielded ambiguous or conflicting patterns of branchwork, we judged relationships among the populations to be unresolved by the genetic distance data.

We are aware that some current workers advocate the general use of "consensus trees" to resolve conflicting branching topologies. However appropriate such trees may be for the reconciliation of a series of equally parsimonious but different trees, *when each is derived by the same method* (see Swofford 1985 for examples), the construction of a consensus tree is unwarranted when the trees on which it is based were derived through different methodologies. If each approach (e.g. UPGMA vs. Wagner) is based on different assumptions, then the trees that result cannot be blended logically without destroying the integrity of either set of underlying assumptions. Furthermore, significant information on branch lengths can be lost in consensus trees because they identify only the position of nodes and not the distances between nodes. Finally, we feel that one or more plausible hypotheses of relationship that are by definition uncertain are better than either no hypothesis or an illogical hypothesis. For example, if the conflict in the relationship of three taxa as expressed by two different trees, ((a,b)c) and (a(b,c)), each produced by a different branching algorithm, is "resolved" by the consensus method, the resulting tree, (a,b,c), is an unresolved trichotomy. In this exercise we have lost two potentially valid hypotheses, one of which may be true, and gained the unlikely hypothesis that all three taxa sprang simultaneously from the same ancestor. For all of the above reasons we did not apply the consensus approach in our analysis of branching patterns.

The results of the phenetic and phylogenetic analyses were consistent in all important respects. Resolution was especially clear at the level of fine branch tips. For example, the UPGMA and WPGMA phenograms each revealed four clusters, two of interior samples, one of coastal samples, and one of the single insular sample. The coastal series, Shasta, Rogue River, Lake County, and Monterey, formed one tight clade; the interior series of populations, Warner, Black Hills, Siskiyou, and Snake, formed another. In the UPGMA a third clade, that linking SE Arizona with Wet Mountains, formed a sister group with the previous eight populations. In the WPGMA, SE Arizona and Wet Mountains joined as a sister group to the clade consisting of the remaining four interior populations. The fourth "cluster," Santa Catalina Island, linked with the four geographically proximal coastal populations in the WPGMA. In the UPGMA, in contrast, the island sample formed a sister group to all the remaining 10

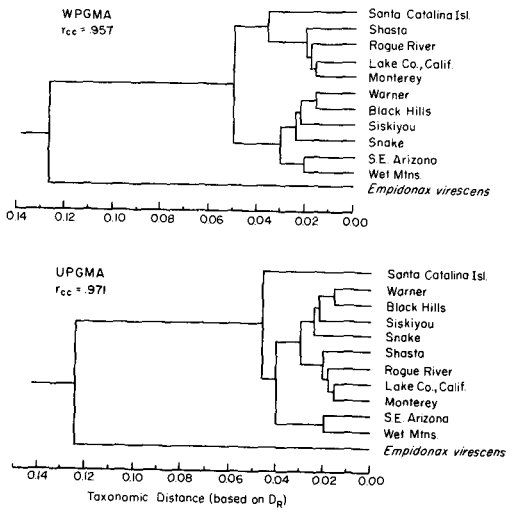

Fig. 3. Phenograms based on Rogers' *D*-values and derived by the WPGMA and UPGMA methods. The high cophenetic correlation coefficients (r_{cc}) indicate excellent agreement between the distances shown in each phenogram and the original data matrix.

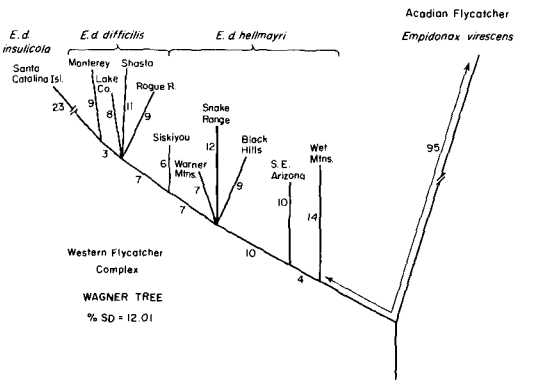

Fig. 4. Optimized distance Wagner network rooted at the outgroup, *Empidonax virescens*. This analysis produced no negative branches. In additional distance Wagner analyses, jumbling the order in which data were entered produced trees essentially identical to the one shown.

populations of the complex. The shortness of the branch involved in this linkage, however, indicates that this implied relationship is not definitive.

Although the distance Wagner network revealed somewhat less clumping, the fundamental pattern expressed was similar to that of the two phenetic analyses. In the Wagner results, the series of populations began with a distantly located interior sample, Wet Mountains, and ended with the most remote western sample, Santa Catalina Island. Between these two extremes, the five interior samples and, in turn, the four coastal samples, split off in orderly progression in a sequence that rather closely paralleled their geographic arrangement. Appropriately, the mixed Siskiyou sample was placed between the interior series and the coastal group of populations. Finally, we note that the great length of the branch leading from the *E. d. difficilis* clade to the single sample of *E. d. insulicola* underscores the basic distinctiveness of the latter taxon.

Sympatry in the Siskiyou region.—Coastal and interior representatives of the complex contact and interact in the Siskiyou region of north-central California (Johnson 1980: 87–94). Because of the enormously expanded range of discriminant function scores shown by a sample from that region taken in 1964 and 1968, the Siskiyou population was assumed to represent either a hybrid swarm or a situation of secondary sympatry with either limited or no hybridization (Johnson 1980). Reinterpretation of the earlier sample and analysis of 17 new specimens taken on their breeding territories in 1981 clearly supported the second alternative. The new sample consisted entirely of pure parental types: 8 certain or probable *E. d. hellmayri* and 9 definite or probable *E. d. difficilis*. The discriminant scores of adult specimens from both the old and new samples are plotted in Fig. 5. Two specimens from the older sample, a male at 0.5146 and a female at 2.739, had low scores for typical *E. d. difficilis* (half-shaded squares in Fig. 5). Although identified as *E. d. difficilis* by the discriminant analysis, because of their apparently intermediate scores the possibility cannot be ruled out that they represent hybrids. Another individual, from the recent sample and hence with genetic data (NKJ No. 4616), although possessing a discriminant score of 0.7025 (typical of male *E. d. difficilis*), had an "interior" genotype (FM) at the 6-PGD locus. The F allele at this locus is unknown from other examples of coastal *E. d. difficilis*. This bird may have been of recent backcross origin. Alternatively, the F allele at 6-PGD may occur in the Siskiyou population at low frequency even in typical *E. d. difficilis* because of infrequent, ancient interbreeding with *E. d. hellmayri*.

In 1981 three mated pairs were taken, two of *E. d. difficilis* and one of *E. d. hellmayri*. A single pair, both mates representing *E. d. difficilis*, had

been reported earlier from Siskiyou (Johnson 1980). Thus, the four known breeding pairs were each mated in a positively assortative manner.

Except for the male mentioned above, individuals with either interior genotypes or vocalizations, or both, were consistently large and represented *E. d. hellmayri.* The discriminant analysis showed clearly the sympatric character of the Siskiyou population. Sympatry of significant populations, with little or no interbreeding and assortative mating of pure parental types, argues for the biologic species status of *E. d. difficilis* and *E. d. hellmayri.*

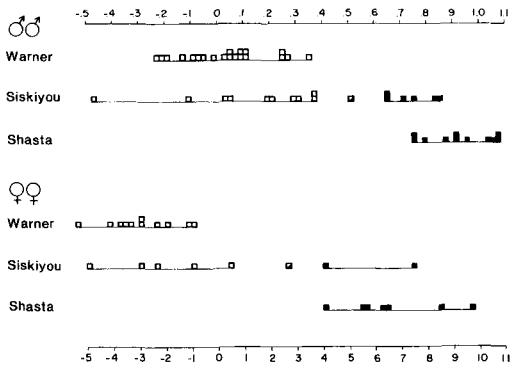

Fig. 5. Discriminant scores of adult specimens of Western Flycatchers, with Shasta serving as a reference standard for *E. d. difficilis* and Warner representing the standard for *E. d. hellmayri.* Birds from Siskiyou represent unknowns, as described by Johnson (1980: 87–92). *Empidonax d. difficilis* and *E. d. hellmayri* are clearly sympatric in the Siskiyou region. The two half-shaded symbols represent either hybrids or extreme values for either form. The scores are based on lengths of primaries 10, 7, and 4; length of tail; length, depth, and width of bill; length of tarsus plus middle toe; and body mass. Because color measurements were unavailable for the 1981 sample from Siskiyou, color characters were excluded from the present analysis. Thus, the discriminant scores of reference specimens from Warner and Shasta differ from those presented by Johnson (1980), although they are computed from the same specimens. Note that the scale for males is in tenths whereas that for females is in whole numbers.

Discussion

These genetic results add qualitatively different information to that already published on geographic variation in the *Empidonax difficilis* complex (Johnson 1980). Such analyses of protein-coding loci elucidate patterns of genetic isolation, gene flow, and dispersal with respect to environmental barriers. In turn, these patterns permit inferences on important historical events such as founding (colonization), bottlenecks, and population fluctuations (Lewontin 1974, Nei et al. 1975, Barton and Charlesworth 1984, Brussard 1984, Carson and Templeton 1984). Knowledge of patterns of population structure, considered in concert with geographic variation in morphology, behavior, and habitat selection, can illuminate the processes responsible for speciation in particular groups and can provide evidence for rational decisions on their taxonomic status. Finally, because these allozyme data were obtained from moderately large breeding samples, they contribute generally to our as yet superficial understanding of genetic structuring of natural populations of birds (Barrowclough 1980, 1983).

Levels of genetic variation.—Although levels of H ranged from 3.7 to 6.6% and are therefore typical of other birds ($\bar{H}$ = 5.3%; Barrowclough 1983), it is noteworthy that the two lowest values of observed heterozygosity were from isolated populations, Santa Catalina Island (H = 3.7%) and Black Hills (H = 4.5%). The value for the insular sample is especially interesting because on the basis of another important measure of genetic variation, percentage of polymorphic loci, the Santa Catalina Island birds, at 9.8%, also fell far below the range (17.1–29.3%) and mean (21.7%) of the other 10 samples of the complex. Low values of H are expected if the total population of a species has passed through a bottleneck or exists currently at low densities (Nei et al. 1975). Because the species is common on Santa Catalina Island, we assume that the diminished genetic variability cannot be related to low present density. Instead, the low genetic variability of the Insular Flycatcher, compared with most continental populations, probably resulted from a bottleneck at the time the original group of founders colonized the California Channel Islands. The population of California Quail (*Callipepla californica catalinensis*) on Santa Catalina Island also has the lowest value for observed heterozygosity when compared with six mainland populations (Zink et al. 1987). Low observed heterozygosities have been reported previously for other insular forms (Selander 1976).

Genetic population structure.—As Barrowclough (1983: 231) described, "If most of the genic variability present in natural populations

is neutral or near-neutral . . . it could then be used as an indicator of population structure and relative divergence times." An explicit test of the infinite allele–constant mutation rate model, using the most thorough studies available of genic variation within avian populations (Barrowclough et al. 1985), found good agreement with the neutral theory. Because one of the populations examined in that study was from the *Empidonax difficilis* complex, we feel justified in using F_{st} statistics (Wright 1951, 1978) as a measure of relative population fragmentation.

The average F_{st} of 0.153 over the entire complex indicates that 15.3% of the total genetic variance was distributed among populations, a clear departure from panmixia. Computation of F_{st} for subsets of the 11 total populations was also revealing. For example, although $F_{st} = 0.079$ among all populations of *E. d. difficilis* plus *E. d. insulicola,* when the latter was removed F_{st} dropped to 0.026. This suggests substantial interchange of birds among the coastal mainland populations but much less dispersal between the continent and Santa Catalina Island. $F_{st} = 0.069$ for the six populations of *E. d. hellmayri,* a value almost three times larger than in *E. d. difficilis.* This reflects the known greater geographic interruption of interior vs. coastal mainland populations.

Gene flow.—Although application of Slatkin's (1981, 1985a) technique to the allozyme data for all populations suggests moderately high gene flow generally, the analyses of subsets of populations showed that it is far from uniform geographically. In particular, an estimated 0.093 immigrants/generation enter the Santa Catalina Island population. In contrast, mainland populations receive an estimated average of 9.62 immigrants/generation. The island value is exceptionally low, whereas the mainland average falls at the upper end of estimates of Nm (range, 1.8–9.5) for the few other species of birds that have been examined (Zink and Remsen 1986: 28). We conclude that the miniscule value of Nm_e for the Santa Catalina Island sample provides strong evidence for its essential genetic independence. We hope these indirect estimates of gene flow will be tested directly in the future through recapture studies of marked individuals.

Larson et al. (1984), working with salamander data, expressed concern that Slatkin's method cannot distinguish present from past gene flow. Slatkin (1985b: 424) responded by arguing that, in his simulations, "patterns in both rare alleles and F_{st} when there is no gene flow are too sensitive to weak selection to be of use in understanding the history of populations." We thus assume that the geographic distribution of rare alleles serves best to illuminate current, not past, levels of gene flow. This is not to say that these estimates of present, "average" rates of gene flow cannot be misleading. As Slatkin (1985b) emphasized, because of changing demographic or environmental conditions, gene flow may be unpredictable and episodic, resulting irregularly in levels far higher than are average for a given species. Therefore, even for *E. d. insulicola,* which at present seems to be characterized by very low gene flow, we should not rule out infrequent periods of higher gene flow, perhaps resulting from a dramatic flush in mainland populations. Episodic gene flow in the reverse direction could also occur.

Broad congruence of genetic and other patterns across geography.—Gene flow is assumed to influence all loci in the same manner. In contrast, because both selection and mutation affect each genetic locus and each phenotypic character separately, loci can vary greatly in their degree of geographic variation (Slatkin 1985b). Such variability in genetic differentiation is shown clearly by allozymes in the *E. difficilis* complex (Table 2, Fig. 1). The geographic patterns of allelic frequency at several loci are not chaotic; rather, they illustrate differentiation in insular vs. mainland populations (at ICD-1), coastal vs. interior populations (EST-2, 6-PGD, ME), or, in one instance (GR), a north–south cline. Furthermore, definite geographic concordance is seen between the genetic patterns and features of morphology, voice, coloration, and habitat described previously (Johnson 1980). Such integration of diverse character suites into three regional groups of populations, representing the California Channel Islands, the Pacific coast, and the interior of the western United States, respectively, strongly supports the view that these groups comprise distinctive but cohesive evolutionary units.

Phylogenesis and historical biogeography.—In addition to portraying the genetic relationships of populations, the distance Wagner network (Fig. 4) can serve as a hypothesis of phylogenesis in western North America. Earlier, Johnson (1980: 120) speculated on the origin, spread, and diversification of the *E. difficilis* complex as an example of a general model for speciation in

the genus. That model envisioned the stepwise budding of pioneer populations from the periphery of the breeding ranges of existing taxa. These pioneers would cross barriers, colonize, expand their distributions, speciate, and repeat the process. For the *E. difficilis* complex specifically, Johnson proposed that the lineage arose somewhere within the range of modern *E. d. occidentalis* in central or southern Mexico. Very early, the ancestors of *E. flavescens* split off in Central America. Sometime later, the lineage leading to modern *E. d. hellmayri* spread from Mexico northward into the southern Rocky Mountains and Great Basin. Eventually, pioneers from the stock that became present-day *E. d. hellmayri* crossed the Cascade Mountains and evolved into *E. d. difficilis* along the Pacific coast. After the lineage that developed into *E. d. difficilis* expanded into southern California, colonists spread to the California Channel Islands. These individuals eventually evolved into *E. d. insulicola*. Thus, speciation by the founder effect (Mayr 1942: 237, Bush 1975) accounted for the present diversity of the complex (Johnson 1980).

The genetic distance data completely support the above scenario. Furthermore, by using Marten and Johnson's (1986) modification of the calibration of Gutiérrez et al. (1983), we can offer estimated dates for three important cladogenetic events. We apply the formula $t = 19.7 \times 10^6 D$, where t is the time since divergence and D is Nei's (1978) genetic distance. This exercise assumes a molecular clock (Wilson et al. 1977, Thorpe 1982), in which allelic substitutions among populations accumulate more or less steadily over time, and neutrality of patterns of genetic divergence (Kimura 1979, 1982; Barrowclough et al. 1985). Because of problems associated with the calibration figure of 19.7, we caution that divergence times calculated by this formula are gross approximations at best (Marten and Johnson 1986).

Based on a Nei's D of 0.044 (Zink and Johnson 1984), the lineage that eventually led to present-day *E. d. difficilis* diverged from that which became modern *E. f. flavescens* at 866,800 yr BP (years before present). At 248,700 yr BP (based on $D = 0.0126$), the ancestors of modern *E. d. difficilis* split from the stock that became *E. d. hellmayri*. In turn, the lineage leading to present-day *E. d. insulicola* divided from that which eventually became modern *E. d. difficilis* at 187,150 yr BP (based on $D = 0.0095$). Therefore, this complex of the genus *Empidonax* diversified from the middle to late Pleistocene.

Taxonomic status of populations.—If significant populations of two forms can maintain their integrity in local sympatry, they are reproductively isolated and typically are regarded as biologic species. *Empidonax d. difficilis* and *E. d. hellmayri* satisfy this criterion in the Siskiyou region of northern California. Although the possibility of infrequent hybridization cannot be excluded, most individuals in the region of overlap clearly represent pure parental types and, insofar as is known, mating is positively assortative. Thus, we do not hesitate to regard *E. d. difficilis* and *E. "d." hellmayri* as full species and formally recommend that they be so considered by the Committee on Classification and Nomenclature of the American Ornithologists' Union. If this proposal is accepted, we would recommend further that the vernacular names "Coastal Flycatcher" and "Interior Flycatcher" would be appropriate for these taxa.

Although originally described as a species, the taxonomic status of *E. d. insulicola* is less easily determined. This taxon breeds only on five of the California Channel Islands and has never reinvaded the adjacent mainland coast, as have two other island endemics, to permit a natural test of sympatry with *E. d. difficilis*. Nonetheless, insular and coastal birds differ substantially in morphology, coloration, voice, and habitat preference (Johnson 1980). The genetic differences and extremely reduced gene exchange described here further argue for the evolutionary independence of these forms. Despite the fact that its biologic species status cannot be proved in the absence of a test of sympatry, we conclude that *E. d. insulicola* is at least a "basal evolutionary unit" or phylogenetic species in the sense of Cracraft (1983).

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