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Divergence in the Mitochondrial DNA of *Empidonax traillii* and *E. alnorum*, with Notes on Hybridization

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The *Empidonax* flycatchers represent a morphologically conservative species assemblage. This conservatism has made recognition of species limits difficult, and there has been a recent trend toward recognizing separate species among populations formerly considered conspecific (e.g. Stein 1958, 1963, Johnson 1980, Johnson and Marten 1988). *Empidonax* flycatchers represent a group where molecular systematics can address a variety of heretofore unanswered questions, especially those regarding degrees of distinctiveness and gene flow between populations or species (see also Hewitt 1988, Avise and Ball 1991). Sibling species such as the Willow Flycatcher (*E. traillii*) and Alder Flycatcher (*E. alnorum*) are particularly interesting with regard to the evolution of intrinsic isolating mechanisms, an important component of the speciation process. The degree to which intrinsic reproductive isolating mechanisms (i.e. ability to discriminate between con- and heterospecifics, resulting in assortative mating) have arisen between two allopatric populations will affect how distinct these gene pools will remain following secondary contact.

A scarcity of recognized hybrids among *Empidonax* flycatchers suggests that, although they are morphologically conservative, their intrinsic reproductive isolating mechanisms (or cohesion mechanisms; Templeton 1989) are remarkably well developed. Perhaps, however, it is our inability to recognize *Empidonax* hybrids, rather than their true rarity, that leads to their apparent scarcity. Two of the five recognized tyrannid hybrids (see Short and Burleigh 1965, Phillips 1966, Phillips and Short 1968) are intergeneric, suggesting that congeneric hybrids are being overlooked. Further, because individuals of the genus *Empidonax* (including *E. traillii*) have produced hybrids with heterospecifics (see Short and Burleigh 1965, Phillips 1966), hybridization might be predicted between the closely related *E. traillii* and *E. alnorum*. Seutin and Simon (1988) arrived at the same conclusion in a different manner, suggesting that the close phenotypic similarity and habitat preferences of *E. alnorum* and *E. traillii*, together with the extensive zone of sympatry, makes hybridization between them likely. These authors failed to find evidence of hybridization, however, and concluded that these species were reproductively isolated in southeastern Canada. They sought evidence of hybridization using allozyme electrophoresis, although this technique had already revealed no fixed allelic differences between the two species in Minnesota (Zink and Johnson 1984). Without a fixed-allelic difference, the ability to detect hybrids is compromised. Another problem with the study was that it did not include *traillii* from allopatric populations.

My study consisted of three parts: (a) estimating the level of divergence in the mitochondrial DNA (mtDNA) of *E. traillii* and *E. alnorum*; (b) finding spe-

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cies-specific genetic markers for their separation; and (c) determining whether evidence of hybridization between the two species can be found in a small sample from Minnesota (i.e. whether hybridization is widespread there).

Methods.—Thirty specimens of *E. traillii* and *E. alnorum* were collected in Minnesota: ca. 150 km from a zone of sympatry (six *traillii* from Goodhue Co. and Wabasha Co., three *alnorum* from Mille Lacs Co.); within 50 km of the sympatric zone (see Zink and Fall 1981; one *traillii* from Wilkin Co., nine *alnorum* from Anoka Co. and Stearns Co.); and from sites of syntopy (five of each species from Clay Co. and Anoka Co. and one unknown [song equivocal] from Anoka Co.). The two species are difficult to distinguish morphologically (Seutin 1991, Winker 1991, unpubl. data), but song can be used to separate them and is genetically determined (Kroodsma 1984). Song was used to confirm specific identification in most males, and was tape recorded when possible (18 of 28 males). Following song-based identification, individuals were collected and stored on dry ice. Two birds collected were nonsinging females (one *traillii* from a syntopic site in Clay Co. and one *alnorum* from Stearns Co.; identifications based on morphology and plumage color). Tissue samples (heart, kidney, liver, brain, and muscle) were taken in the laboratory when the specimens were prepared; tissues were stored at −29°C until analyzed (ca. six months). Skins and skeletons of these birds are in the Bell Museum of Natural History, University of Minnesota, Saint Paul, Minnesota.

Extraction and purification of mtDNA from the tissue samples followed standard procedures (Hillis and Moritz 1990, Gill and Slikas 1992). Two birds of each species collected from sites about 150 km from the sympatric zone were used to estimate genetic distance and identify genetic markers for the two species. Samples (ca. 5 ng) of the purified mtDNA of these four individuals were digested with 18 restriction endonucleases (Gibco BRL) that recognize 6-base-pair (bp) nucleotide sequences. The enzymes used were *Apa* I, *Ava* I, *Bam*H I, *Bcl* I, *Bgl* II, *Eco*R V, *Hae* II, *Hinc* II, *Hind* III, *Hpa* I, *Kpn* I, *Nci* I, *Pst* I, *Pvu* II, *Sal* I, *Sma* I, *Xba* I, and *Xho* I. Resultant fragments were end-labeled with alpha-^{32}P-deoxynucleoside triphosphates, separated electrophoretically on horizontal 1% agarose gels alongside standard DNA markers (1 Kb Ladder, Gibco BRL), and visualized by autoradiography. Fragments larger than about 400 bp were scored. An estimate of nucleotide sequence divergence and its standard error was made from a matrix of shared fragments using REAP (McElroy et al. 1992), which employs the formulae of Nei and Li (1979) and Upholt (1977).

To determine whether there was evidence of hybridization in the sample, the remaining 26 individuals were screened for mtDNA type using two (of the many) enzymes that were found to be species diagnostic: *Bgl* II (one site difference between the two species) and *Nci* I (two sites different). A mismatch between song and mtDNA types would constitute evidence of hybridization.

TABLE 1. Presence (1) and absence (0) of fragments from restriction-enzyme digests of *Empidonax alnorum* and *E. traillii* mtDNA. Enzymes were *Ava* I, *Bam*H I, *Bgl* II, *Eco*R V, *Hae* II, *Hinc* II, *Hind* III, *Hpa* I, *Nci* I, *Pst* I, *Pvu* II, *Sma* I, *Xba* I, and *Xho* I. Fragment profiles from each enzyme are separated by a space.

E. traillii
110111110 111 1011 10001 0111000 1001111
10010110 0110 1001010 011111
0111 100 1001 100
E. alnorum
101111111 111 1100 01110 1000111 0111100
01101111 1001 0111111 101100
1010 011 0111 011

Results.—Four of the restriction enzymes (*Apa* I, *Bcl* I, *Kpn* I, *Sal* I) either failed to cut the mtDNA molecules or did not cut well and produced equivocal results. The remaining 14 enzymes produced one to seven fragments per species, with a total of 74 fragments for both species (Table 1); the two species shared 20 fragments (27%; Table 1). Thirteen of the 14 enzymes produced fragment profiles that could be used to separate the two species. The total mitochondrial genome size was 16.6 ± SE of 0.3 kb (estimated by adding fragment sizes), which appears typical of passerines (Shields and Helm-Bychowski 1988), but little is known of the mitochondrial genome size of suboscines (cf. Tegelström and Gelter 1990).

The proportions of shared fragments (Table 1) produced an estimate of nucleotide sequence divergence between the two species of 5.5% ± 1.3%. This value is similar to other estimates of mtDNA sequence divergence between species of the same genus (Tegelström and Gelter 1990, Avise and Ball 1991), but is higher than the average of the few sibling species that have been compared (see Avise and Zink 1988, Tegelström and Gelter 1990, Avise and Ball 1991, Bermingham et al. 1992).

In screening the remaining sample for possible hybrids, no mismatches between mtDNA and song type were found. Thus, there was no conclusive evidence for hybridization in this sample; widespread hybridization does not appear to be occurring. However, this methodology was not satisfactory for determining the possible hybrid origin of the two females and of the male whose song was incomplete and the one whose song was equivocal. Four males which did not sing (Stearns Co.) were thought to be *alnorum* based on plumage color, morphology, and other singing males in the same population (an allopatric population of the species). All four had *alnorum* mtDNA.

Discussion.—Based on their examination of allozyme differences among *Empidonax* flycatchers, Zink

and Johnson (1984) concluded that the *Empidonax* radiation occurred rather rapidly, and suggested that this radiation occurred in the late Pliocene or early Pleistocene, approximately 1.8 mya. Based on an mtDNA sequence divergence rate for birds of about 2% per million years (Shields and Wilson 1987), my data suggest that the sibling species *E. traillii* and *E. alnorum* last shared a common ancestor about 2.7 mya. Because sibling species would represent "twigs" in the generic phylogeny, an *Empidonax* radiation would presumably have begun much earlier. Considering the great phenotypic similarity and relatively high genetic divergence between these two sibling species, together with genetic evidence in other avian congeners (e.g. Avise and Zink 1988, Tegelström and Gelter 1990, Avise and Ball 1991, Bermingham et al. 1992), *Empidonax* flycatchers exhibit rather severe developmental canalization for passerines (although this may be found to be more typical of the poorly known suboscines).

Stein (1963) suggested that Willow Flycatchers were advancing their range northward at the expense of Alder Flycatchers. Such advancement could occur through ecological and/or behavioral displacement, perhaps together with introgressive hybridization. The latter is occurring in Blue- and Golden-winged warblers (*Vermivora pinus* and *V. chrysoptera*; see Gill 1987). Although there are no data about the stability of the zone of sympatry between *E. traillii* and *E. alnorum* in Minnesota, my data suggest that the hypothesized movement of the range of *E. traillii* has not been accompanied by widespread hybridization and introgression with *alnorum*. The two species clearly showed interspecific territoriality at sites of syntopy in southern Ontario (Prescott 1987) and Minnesota (pers. obs.). Of the two species, *traillii* is more aggressive (pers. obs., Stein 1963, Prescott 1987), and Prescott (1987) observed several cases of behavioral displacement, all in favor of *traillii*.

Because aggressive responses to heterospecific song occurred more frequently at sites of syntopy, Prescott (1987) concluded that the development of response to heterospecific song was learned. This is a result, presumably, of interspecific competition due to broad ecological niche overlap (see Barlow and McGillivray 1983). Response to heterospecific song in allopatric populations is uncommon (pers. obs., Prescott 1987). Templeton (1989) pointed out that reproductive isolating mechanisms, as products of speciation, should not be confused with the process of speciation itself. They nevertheless become important during secondary contact of formerly isolated gene pools. Templeton advanced the useful concept of "cohesion mechanisms," which produce genetic cohesion over evolutionary time. Conspecific song recognition in these two species may act as the cohesive force which has maintained their apparent genetic isolation in areas of secondary contact.

My results suggest that further examination is warranted, both of the nuclear DNA of *E. traillii* and *E. alnorum*, and of mtDNA within the genus *Empidonax*. In a recent review of avian hybridization, Grant and Grant (1992) noted that approximately 1 in 10 species shows evidence of hybridization, and that this number is likely to grow. Studies of this sibling species pair (Stein 1958, 1963, Zink and Johnson 1984, Seutin and Simon 1988, this study) suggest that the determination of hybridization even where it is likely can be difficult.

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