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INTROGRESSION OF COYOTE MITOCHONDRIAL DNA INTO SYMPATRIC NORTH AMERICAN GRAY WOLF POPULATIONS

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Abstract.—Mitochondrial DNA (mtDNA) genotypes of gray wolves and coyotes from localities throughout North America were determined using restriction fragment length polymorphisms. Of the 13 genotypes found among the wolves, 7 are clearly of coyote origin, indicating that genetic transfer of coyote mtDNA into wolf populations has occurred through hybridization. The transfer of mtDNA appears unidirectional from coyotes into wolves because no coyotes sampled have a wolf-derived mtDNA genotype. Wolves possessing coyote-derived genotypes are confined to a contiguous geographic region in Minnesota, Ontario, and Quebec, and the frequency of coyote-type mtDNA in these wolf populations is high (>50%). The ecological history of the hybrid zone suggests that hybridization is taking place in regions where coyotes have only recently become abundant following conversion of forests to farmlands. Dispersing male wolves unable to find conspecific mates may be pairing with female coyotes in deforested areas bordering wolf territories. Our results demonstrate that closely related species of mobile terrestrial vertebrates have the potential for extensive genetic exchange when ecological conditions change suddenly.

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In the mammalian genus *Canis*, the issue of hybridization has long been debated. The existence of fertile hybrids and of apparent intermediate forms both in the wild and in the fossil record have not only created classification problems but also have been given as examples of where the classical biological species concept breaks down (Templeton, 1989). Hybrids between dogs (*C. familiaris*) and gray wolves (*C. lupus*) are common and produce fertile offspring in captivity and sometimes in the wild (Mech, 1970; Bibikov, 1982; Boitani, 1982). Hybrids between dogs and coyotes (*C. latrans*) are also fertile and are occasionally found in the wild, being recognized by morphological and behavioral traits (Mengel, 1971). These types of crosses are expected given the ubiquitous presence of dogs in areas occupied by man. Yet hybridization in natural populations of gray wolves and coyotes is less expected because these two species coexist as ecological competitors (Bekoff and Wells, 1986). Nonetheless, the potential for hybridization exists, as fertile offspring can be raised under experimental conditions (Kolenosky, 1971). Wolf-coyote interbreeding has been invoked to explain both the coyote-like characteristics of the nearly extinct red wolf (*C. rufus*) (Elder and Hayden, 1977; Ferrell et al., 1980) and the large size of the coyotes of New England and southeastern Canada (Silver and Silver, 1969; Mengel, 1971; Kolenosky and Standfield, 1975; Hilton, 1978; Schmitz and Kolenosky, 1985).

The demographic dynamics of gray wolves and coyotes have changed dramatically in North America over the last two centuries. During the late Pleistocene, gray wolves once inhabited all of North America except for coastal areas of Mexico, and ranged widely across several habitats including forests, plains, warm deserts, and tundra (Nowak, 1979; Kurtén and Anderson, 1980). With the advance of agriculture westward and northward, wolf numbers declined rapidly through habitat destruction and direct extermination (Young, 1944). As large, highly mobile predators, wolves require extensive tracts of relatively undisturbed land to hunt ungulates. The coyote is a more flexible predator, using smaller prey that are abundant in disturbed habitats and adapting its social behavior to accommodate agricul-

tural and even urban environments (Vaughan, 1983). Coyote distributions, once confined primarily to plains and deserts, recently have expanded greatly following the spread of civilization and the reduction of gray and red wolf ranges (Gier, 1975; Bekoff and Wells, 1986). Perturbation of habitats historically occupied by gray wolves may have led to increased interactions between coyotes and wolves. If so, one would predict hybridization to be more frequent in wolf ranges where coyotes have become abundant only recently.

In this study, we assess the prevalence of hybridization through a geographic survey of mitochondrial DNA (mtDNA). The mitochondrial DNA genome of mammals is inherited maternally and clonally (cf. Brown, 1985). Thus, unlike nuclear alleles, whose persistence will be damped by recombination through the generations subsequent to hybridization, a female's mtDNA genotype can be inherited without disruption, and can increase in populational frequency in future generations without additional hybridization. Evidence of hybridization will remain in a population as long as the mtDNA matriline survives; an mtDNA analysis can reveal vestiges of hybridization even after one of the two species has gone extinct in the hybrid zone.

We present here an examination of mtDNA genotypes found in a wide geographical survey of both gray wolves and coyotes. Our sampling design includes most of the present North American geographic ranges of these species. We surveyed individuals from areas of sympatry as well as from highly isolated areas of allopatry, to determine if any mitochondrial types of either species have become established in populations of the other as a consequence of hybridization. If substantial hybridization has occurred, we can test the specific hypothesis that only in areas of recent ecological change will hybridization be common. Our results provide insights into the determinants of reproductive isolation in highly mobile terrestrial vertebrates.

Materials and Methods

Tissue samples for genetic analyses were obtained from 276 gray wolves and 240 coyotes. DNA was extracted by standard methods (Maniatis et al., 1982) from either frozen organ samples (heart, liver, kidney, or skeletal muscle) or from white blood cells obtained by venipuncture of individuals live-trapped and released (Wayne et al., 1989). Wolf samples include one captive Chinese wolf and two captive Iranian wolves, plus 273 wolves from wild North American populations (Table 1; Fig. 1). The latter sample includes individuals from known packs in Alaska, Isle Royale National Park (in Lake Superior), Minnesota, Montana, Alberta, Manitoba, Ontario, Quebec, and the Northwest and Yukon Territories. Much of the gray wolf's current North American range has thus been represented along with two distinct Asian populations. Coyote samples include individuals from Alaska, California, Florida, Maine, Michigan, Minnesota, Nebraska, Texas, Utah, Washington, Alberta, Manitoba, and Ontario (Table 1; Fig. 1). This sampling spans most of the coyote's geographic range except Mexico. Areas have been sampled where today only wolves exist (e.g., Asia and northern Canada), where only coyotes exist (e.g., California and Florida), and where the species are currently sympatric (e.g., Kenai Peninsula, Alaska; Riding Mountain National Park, Manitoba; and Minnesota). Prior to settlement by Europeans, the gray wolf's range covered most of the United States including California, Utah, and Washington, where now only coyotes survive.

Approximately 10 μg of genomic DNA from each of the coyote samples and from 239 of the wolf samples were digested with an excess of each of the following 21 restriction enzymes: *Apa* I, *Bam* HI, *Bcl* I, *Bgl* I, *Bgl* II, *Bst* EII, *Cla* I, *Dra* I, *Eco* RI, *Eco* RV, *Hind* III, *Nco* I, *Sca* I, *Sst* I, *Stu* I, *Xba* I, and *Xmn* I, all of which recognize unambiguous six base sequences, *Acc* I and *Hinc* II, which recognize ambiguous six base sequences, and *Bst* UI and *Hha* I, which recognize four base sequences. These enzymes were selected to minimize recognition sequence overlap, with the exception of the four base enzymes whose recognition sequences overlap by three bases. The remaining 37 wolf samples were digested with only two of the enzymes, *Eco* RV and *Bgl* II (see Results).

TABLE 1. Collection locations of canid samples.

	Sample size	Region	Locality	Source	Location in figures
Wolves	20	Alaska	Anaktuvik Pass	L. Adams	a
	7	Alaska	Kenai Peninsula	T. Bailey	b
	9	Alaska	Nome	W. Ballard	c
	9	Alaska	Denali National Park	T. Meier	d
	1	Alaska	Brooks Range	P. Kinnis	e
	6	Yukon Territory	Exact location unknown	P. Marchant	f
	11	Northwest Territories	Fort Reliance	F. Jackson	g
	32	Northwest Territories	MacKenzie River Delta	P. Clarkson	h
	6	Northwest Territories	Keewatin District	F. Mallory	i
	6	Montana	Kalispell	L. Boyd	j
	1	Alberta	Banff National Park	P. Paquet	k
	3	Alberta	Swan Hills	L. Carbyn	l
	2	Manitoba	Riding Mountain National Park	L. Carbyn	m
	46	Minnesota	Northeastern counties	L. D. Mech	n
	2	Minnesota	Northeastern counties	R. Peterson	n
	18	Minnesota	Northern counties	B. Paul	n
	22	Minnesota	Voyageurs National Park	P. Gogan	q
	7	Michigan	Isle Royale National Park	R. Peterson	r
	48	Ontario	Western districts	R. Peterson	t
	3	Ontario	Algonquin Provincial Park	G. Forbes	v
	1	Quebec	La Verendrye Provincial Park	F. Potvin	w
	4	Quebec	Laurentides Provincial Park	F. Potvin	x
	9	Quebec	Papineau-Labelle Prov. Park	F. Potvin	y
	2	Iran	Exact location unknown	V. O'Toole	—
	1	China	Exact location unknown	O. Ryder	—
	276	Total			
Coyotes	30	California	Northern counties	R. Thompson	A
	30	California	Southern counties	P. Butchko	B
	20	California	Los Angeles and Ventura counties	R. Plantrich	B
	32	California	Los Angeles county	C. P. Ryan	C
	9	Alaska	Kenai Peninsula	T. Bailey	D
	25	Washington	King and Thurston counties	T. Quinn	E
	16	Maine	Penobscott and Hancock counties	S. McKenzie	F
	10	Nebraska	Lancaster county	S. McKenzie	G
	17	Michigan	Ogemaw and Oscoda counties	S. McKenzie	H
	2	Michigan	Houghton Co. (Upper Peninsula)	R. Peterson	I
	21	Minnesota	St. Louis and Itasca counties	L. D. Mech	n
	3	Texas	Webb county	M. Allard	J
	2	Utah	Cache county	J. Patton	K
	1	Florida	Northwest counties	M. Roelke	L
	2	Alberta	Southern portion	A. Eisenhawer	M
	19	Manitoba	Near Riding Mountain Natl. Park	H. D. Cluff	N
	1	Ontario	Fort Frances	R. Peterson	P
	240	Total			

The digested DNA was electrophoresed into 20 × 22 cm, 1% agarose gels for 19 hours at 25 volts, transferred by capillary action to Nytran nylon membranes (Schleicher & Schuell) for 12–48 hours in 10× SSC, and immobilized by baking at 80°C under vacuum for 2–8 hours. In vitro hybridization to a probe of cloned domestic dog mtDNA was carried out in heat-sealed bags at 65°C for 12–16 hours in 7% SDS, 1% BSA, and 0.5 M phosphate buffer. The probe was first radiolabelled with ^{32}P-dCTP by oligonucleotide primer extension (Boeringer-Mannheim kit #1004 760). Nonspecific radioactivity was washed off the membranes by several SSC/SDS washes including a high stringency wash of 0.1× SSC/0.25% SDS for 30 minutes at room tem-

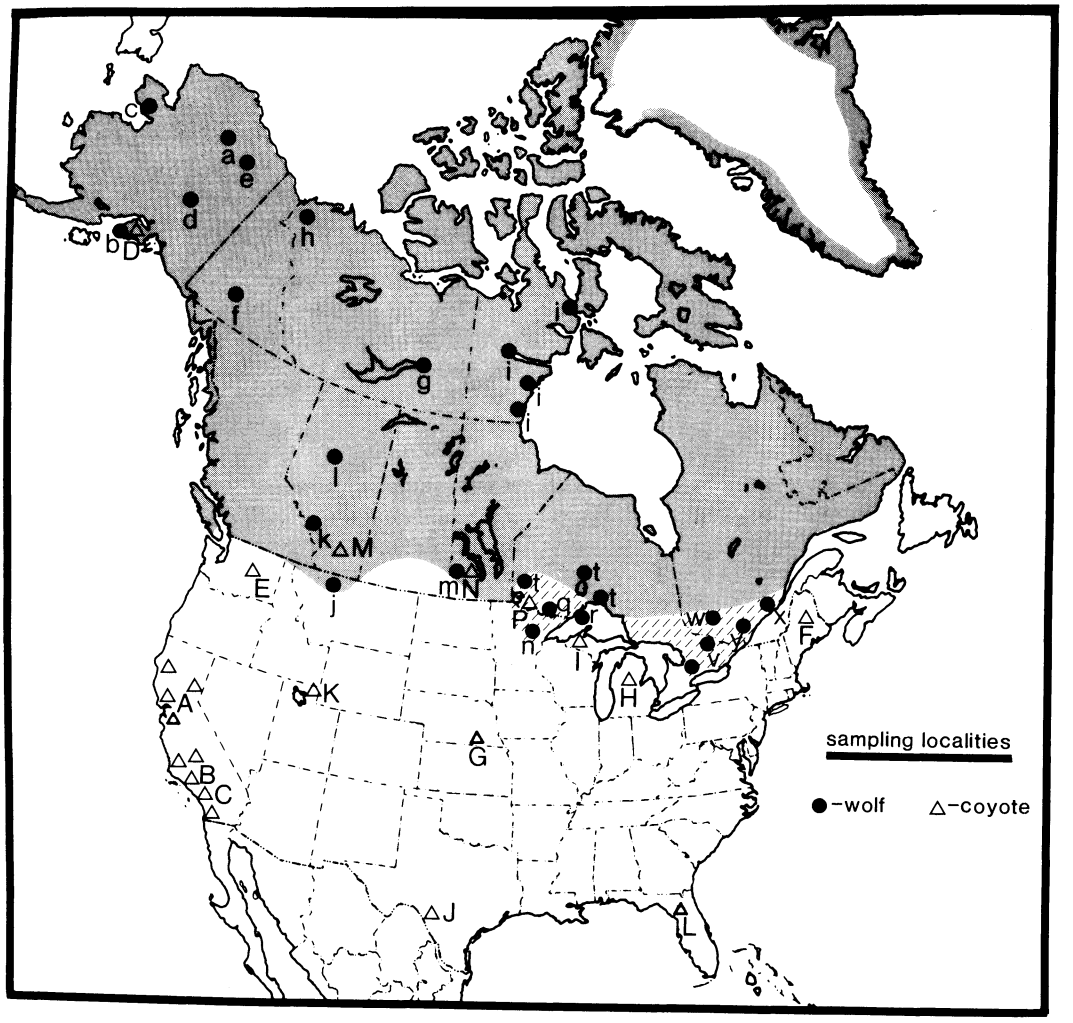


FIG. 1. Geographic distribution of gray wolf and coyote samples in North America. Shading indicates region where only pure wolf mtDNA genotypes have been found. Striping indicates the observed hybrid zone where we have found wolves with coyote-type mtDNA. Wolf and coyote sampling localities are described with the same letter designations as in Table 1.

perature. Mitochondrial DNA fragments were visualized by autoradiography with Kodak XAR film for 1–7 days at −70°C with one intensifying screen.

The restriction fragment patterns for each individual from all 21 restriction enzymes were used to define composite mtDNA genotypes (Lansman et al., 1983; Ball et al., 1988). Restriction site differences were readily estimated from fragment patterns because for any one enzyme, with the exception of *Hinc* II, the genotypes differed by the inferred loss or gain of only one or two restriction sites. Even though a network of *Hinc* II site differences among genotypes could not be constructed with confidence, this enzyme differentiated between several genotypes, which were otherwise indistinguishable. Thus, it was included in the analysis by assuming that a minimum number of *Hinc* II restriction sites, as reflected by fragment patterns, differentiated each pair of genotypes (Wayne et al., 1990).

A presence-absence matrix of restriction sites for each genotype was used to generate a maximum parsimony tree relating wolf

and coyote genotypes. This tree was produced using the global-branch-swapping option in the PAUP program of David Swofford, version 2.4 (1985). It was rooted at the midpoint of the longest patristic distance. An estimate of the percent nucleotide sequence divergence between selected genotypes was obtained using the proportion of shared restriction sites (Nei and Li, 1979). When possible, restriction site data from restriction enzymes having different numbers of nucleotides in their recognition sequences were treated separately and then combined in a weighted average for the final estimate. This could not be done when no variation existed between all patterns in a particular class of enzymes; in these cases enzymes were lumped into fewer classes.

Results

Thirteen gray wolf and 24 coyote genotypes were defined by the panel of 21 restriction enzymes (Table 2: wolves are W1 through W13 and coyotes are C1 through C24). Four of the genotypes found in wolves are also found in coyotes (W10 is identical to C14, W11 is identical to C17, W12 is identical to C18, and W13 is identical to C24). Moreover, three other genotypes found in wolves bear a strong similarity to coyote genotypes: W7 differs by only three restriction sites from C24, and W8 differs from W7 by a single site, and W9 differs from C17 by two sites. These data also indicate that the wolf genotypes W1 through W6 are very distinct from the remaining wolf and coyote genotypes (Table 2). Fourteen of the 21 enzymes show restriction fragment patterns specific to either genotype group (for example *Bgl* I; Fig. 2) whereas the remaining 7 enzymes produce patterns found in both groups. A minimum of 26 restriction sites differ between the W1 through W6 group and the group containing the genotypes W7 through W13 and C1 through C24. This is in contrast to the maximum within-group difference of 16 restriction sites.

These restriction site differences are illustrated in a phylogenetic tree relating genotypes (Fig. 3). Wolf genotypes W1 through W6 are a monophyletic group well distinguished from both the coyote genotypes and the wolf genotypes W7 through W13. The phylogenetic tree clearly suggests that the "coywolf" genotypes (W7 through W13) are derived from hybridization with coyotes. Also, despite samples from coyotes in areas where wolves were historically or are currently abundant, no "pure" wolf genotypes W1 through W6 are found in coyotes. Therefore, introgression of mtDNA appears to be unidirectional from coyotes into wolves.

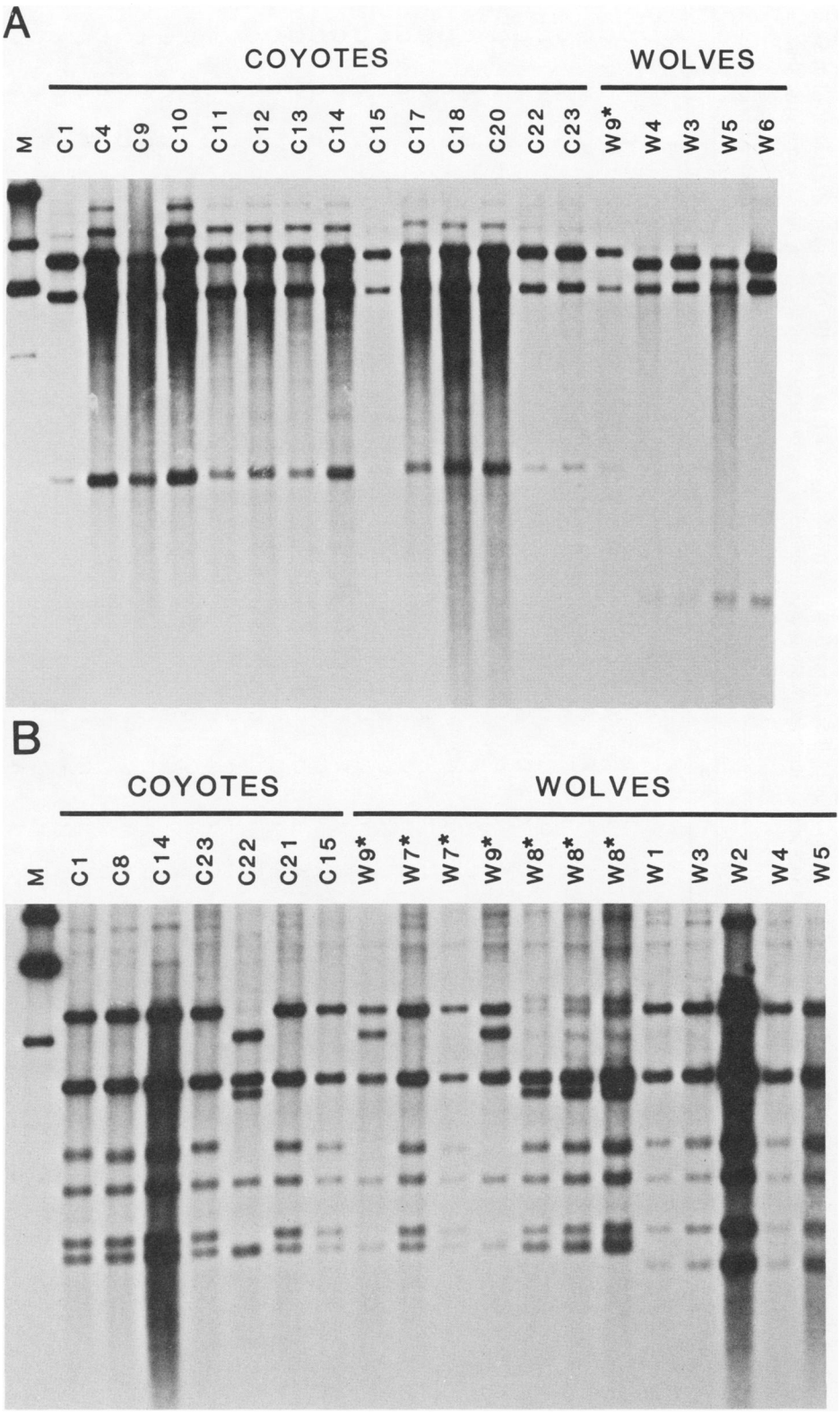
With the availability of 14 enzymes that will distinguish between an individual having the pure wolf mtDNA type or the coyote-like mtDNA type, an additional 37 wolves could be assayed quickly with only two enzymes (*Eco* RV and *Bgl* II) to determine their general genotypic affiliations. This allowed us to include highly degraded organ samples in our survey because the coyote-type fragment pattern generated by these enzymes is quite distinct from the wolf-type pattern. Among these wolf samples, most of which were from Alaska and the Northwest Territories, a pure wolf type was found in all (Table 3).

The range of sequence divergence within and among coyote and gray wolf genotypes can be estimated by calculation of the average number of shared sites between genotypes (Nei and Li, 1979). The estimates of divergence between the eight most distinct genotypes are given in Table 4. The sequence divergence between any pair of coyote and pure wolf genotypes ranges between approximately 2.7–4.2%. The maximum intraspecific divergence between wolf, coyote, and coywolf genotypes is 0.63%, 2.0%, and 0.92%, respectively. Thus, the interspecific divergence between pure wolf and coyote types is 1.4–6.7 times greater than within each genotype group.

The geographic distribution of the wolf genotypes delineates a potential hybrid zone. Coywolf genotypes are restricted to northern Minnesota, southern Ontario and Quebec, and Isle Royale (Figs. 1, 4; Table 3), areas where coyotes have become abundant only since 1900 (Nowak, 1979; Voigt and Berg, 1987). The northern limit of coywolf genotypes coincides with the northern extent of coyotes in Ontario, as described by Kolenosky and Standfield (1975) and in Quebec, as described by Georges (1976). With the exception of two individuals in

TABLE 2. A description of restriction enzyme fragment patterns in gray wolves and coyotes. Distinct patterns are given different letters. The most common pattern in coyotes is given the designation C and the others are designated A, B, D, etc. The enzymes used are the following: a) *Bgl* II; b) *Bam* HI; c) *Bcl* I; d) *Bgl* I; e) *Bst* EII; f) *Bst* UI; g) *Stu* I; h) *Cla* I; i) *Dra* I; j) *Eco* RV; k) *Hinc* II; l) *Sca* I; m) *Hha* I; n) *Nco* I; o) *Eco* RI; p) *Hind* III; q) *Xba* I; r) *Acc* I; s) *Apa* I; t) *Sst* I; u) *Xmn* I. Asterisks denote coyote-type genotypes found in wolves. Genotype W3 was also detected in two domestic dogs tested in our lab, indicating that the hybrid genotypes were unlikely to have originated from dogs.

		Enzymes																				
	Genotype	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u
Wolves	W1	A	C	F	A	A	E	F	A	A	B	J	D	F	B	C	C	B	D	C	A	C
	W2	A	C	F	A	A	E	F	A	A	D	J	D	F	C	C	C	B	D	C	A	C
	W3	A	C	F	A	A	E	F	A	A	B	J	D	F	C	C	C	B	D	C	A	C
	W4	A	A	F	A	A	E	F	A	A	B	J	D	F	C	C	C	B	D	C	A	C
	W5	A	C	F	A	A	E	F	A	A	B	K	D	F	C	C	C	B	D	C	A	C
	W6	A	C	G	A	A	E	F	A	A	B	L	D	F	C	C	C	B	D	C	A	C
	W7*	C	C	H	C	C	C	C	C	C	C	A	C	C	C	C	C	C	F	C	C	C
	W8*	C	C	H	C	C	C	D	C	C	C	A	C	C	C	C	C	C	F	C	C	C
	W9*	C	C	B	C	A	D	E	C	C	C	E	C	C	C	C	C	C	E	C	C	C
Coyotes	C1	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
	C2	C	C	C	C	C	C	C	C	C	C	A	C	B	C	C	C	C	C	C	C	C
	C3	C	C	A	C	C	C	A	C	C	C	B	C	C	A	A	C	C	A	C	C	C
	C4	C	C	C	C	C	B	C	C	C	C	A	C	B	C	C	C	C	C	C	C	C
	C5	C	C	C	C	C	C	C	C	C	A	A	B	C	C	C	C	C	C	C	C	C
	C6	C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	C	C
	C7	C	C	A	C	C	C	B	C	C	C	B	A	C	C	B	C	A	A	C	C	C
	C8	C	C	B	C	A	A	C	C	C	C	I	C	A	C	C	C	C	C	A	C	C
	C9	C	C	A	C	A	C	C	C	C	C	I	C	C	C	C	C	C	C	C	C	C
	C10	C	C	B	C	A	C	C	C	C	C	I	C	C	C	C	C	C	C	C	C	C
	C11	C	C	C	C	C	A	C	C	C	A	A	C	A	C	C	C	C	C	C	C	C
	C12	C	C	D	C	C	C	A	C	C	C	B	C	C	C	A	C	C	A	C	C	C
	C13	C	C	C	C	C	C	C	C	C	A	H	C	C	C	C	C	C	C	C	C	C
	C14-W10*	C	C	C	C	C	C	C	C	C	C	D	C	C	C	C	C	C	C	C	C	C
	C15	C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	C	C	B	C	C	C
	C16	C	C	C	C	C	C	C	C	C	C	F	C	B	C	C	C	C	C	C	C	A
	C17-W11*	C	C	B	C	A	C	C	C	C	C	E	C	C	C	C	C	C	E	C	C	C
	C18-W12*	C	C	C	C	C	C	C	C	C	C	G	C	C	C	C	C	C	C	A	C	C
	C19	C	C	B	C	C	C	C	C	C	C	A	C	C	C	C	C	C	C	C	C	C
	C20	C	C	A	C	C	C	A	C	C	C	B	C	E	C	D	C	C	A	C	C	B
	C21	C	C	E	B	C	C	C	C	C	A	A	C	C	C	C	C	C	C	C	C	C
	C22	C	C	A	C	C	C	A	C	C	C	B	C	C	C	A	C	C	A	C	C	C
	C23	C	C	C	C	C	C	C	C	C	C	A	C	D	C	C	C	C	C	A	C	C
	C24-W13*	C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	C	C	C	C	C	A


A
COYOTES
WOLVES
M
C1
C4
C9
C10
C11
C12
C13
C14
C15
C17
C18
C20
C22
C23
W9*
W4
W3
W5
W6
B
COYOTES
WOLVES
M
C1
C8
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C15
W9*
W7*
W7*
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W8*
W8*
W1
W3
W2
W4
W5

central Ontario (one each at localities t5 and t8), to the north and west of this line only pure wolf mtDNA genotypes are found in wolves. Unfortunately our sample size of wolves in Montana, Alberta, and Manitoba is small, leaving open the possibility that coyote-type genotypes exist among wolves in these areas with low frequency. Nevertheless, hybridization is common only where the coyote range has recently expanded into the wolf's distribution.

It is difficult to determine from our data the frequency of hybridization between gray wolves and coyotes. The percentage of wolves with a coyote-type mtDNA genotype varies by region from zero in Alaska to 100% in Quebec (Table 5). However, the phylogenetic relationships of the coyote and coywolf genotypes provide an indication that the minimum number of successful hybridizations has been six. The genotypes W10, W11, W12, and W13 are identical to coyote types and consequently are the direct result of four hybridization events. In contrast, the genotypes W7, W8, and W9 have not been found in our coyote sample, and we cannot distinguish between the possibility that they are actual coyote genotypes, which have not been sampled, or that they have each evolved after hybridization from observed coyote types. However, the W7 and W9 types differ in sequence by an estimated 0.92%, reflecting 10 restriction sites. For one of these types to have evolved from the other since coyotes invaded this region would require an improbably high evolutionary rate. Thus, these genotypes likely diverged during the Pleistocene evolution of coyotes and probably represent two additional hybridization events.

The most likely candidate genotype for in situ evolution is W8, which has been found only in the seven wolves sampled from Isle Royale plus in one wolf from the Ontario mainland near the island. The Isle Royale population was founded by a single pair of wolves 40 years ago (Mech, 1966). Genotype W8 differs from W7 by the gain of a single *Stu* I restriction site (see Fig. 2), suggesting that all individuals on the island

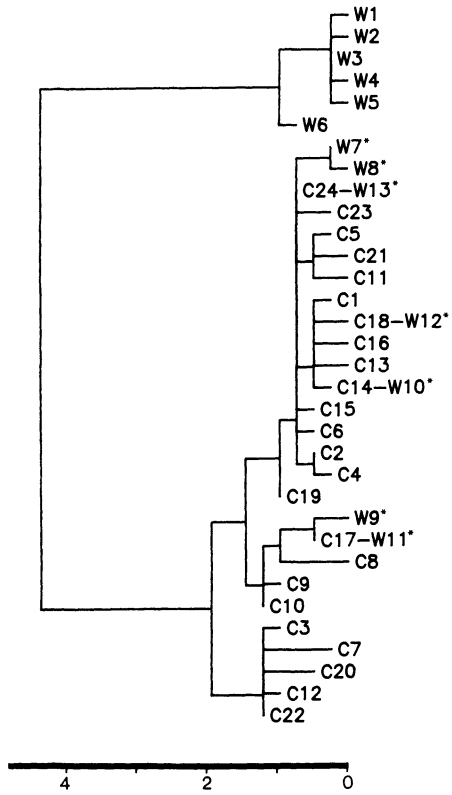

FIG. 3. A phylogenetic tree relating the gray wolf and coyote mtDNA genotypes. The tree was generated using the global-branch-swapping option of PAUP. Note the tight clustering of the true wolf genotypes (W1 through W6) and their dissimilarity to the other wolf genotypes (W7 through W13; with asterisks) found in the coyote-type clade. Genotype W10, found in wolves, is identical to coyote genotype C14, W11 to C17, W12 to C18, and W13 to C24. Scale is percent sequence divergence using the shared site estimate of Nei and Li (1979).

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FIG. 2. Sample autoradiograms of gray wolf and coyote mitochondrial DNA restriction fragment length polymorphisms. A) Coyote and wolf DNA digested with the restriction enzyme *Bgl* I. The true wolf genotypes (W1 through W6) are distinguishable from the coyote genotypes (C1 through C24) and the coyote-derived wolf genotypes (W7 through W13; with asterisks). In the marker lane (M), molecular weight bands appear at 23.1, 9.4, 6.6, and 4.3 kilobases, from top to bottom. B) Coyote and wolf DNA digested with the restriction enzyme *Stu* I. This enzyme reveals extensive variation only within the coyote-type genotypes, and distinguishes the W8 genotype found in the wolves of Isle Royale from all other genotypes. Visible here in the marker lane are bands at 9.4, 6.6, and 4.3 kb.

TABLE 3. Frequencies and distribution of canid genotypes. Asterisks denote coyote-type genotypes found in wolves.

	Genotype	Frequency	Locations found
Wolves	W1	73	NE Minnesota; Montana; Alberta; Northwest Territories; W Ontario
	W2	2	Riding Mountain National Park, Manitoba
	W3	38	All Alaska localities; Montana; Northwest Territories; Yukon Territory
	W4	16	Alaska; NE Minnesota; Montana; Banff N.P., Alberta; Northwest Territories; W Ontario
	W5	2	Iran
	W6	1	China
	W7*	39	NE Minnesota; western Ontario
	W8*	8	Isle Royale, Lake Superior; Nipigon, Ontario
	W9*	42	NE Minnesota; western Ontario
	W10*	2	Algonquin Provincial Park, Ontario; Laurentides Provincial Park, Quebec
	W11*	1	La Verendrye Provincial Park, Quebec
	W12*	7	Algonquin Provincial Park, Ontario; Papineau-Labelle Provincial Park, Quebec
	W13*	8	Manitouwadge, Ontario; Laurentides Provincial Park, Quebec
Unspecified wolf type		37	Several locations, including District of Keewatin, Northwest territories
Coyotes	C1	28	California
	C2	5	California
	C3	16	California
	C4	6	California
	C5	2	California
	C6	32	California; Minnesota; Utah; Washington; Alberta
	C7	4	California
	C8	4	California
	C9	2	California
	C10	1	California
	C11	1	Nebraska
	C12	2	Nebraska
	C13	3	Nebraska
	C14	39	Florida; Maine; Central Michigan; Upper Peninsula, Michigan; Minnesota; Nebraska; Texas
	C15	5	Nebraska; Texas; Manitoba
	C16	3	California; Upper Peninsula, Michigan
	C17	2	Maine
	C18	6	Maine; Central Michigan; Minnesota; Fort Frances, Ontario
	C19	2	California
	C20	9	Washington
	C21	1	Manitoba
	C22	34	California; Manitoba
	C23	6	Manitoba
	C24	27	Alaska; California; Minnesota; Nebraska; Utah

share the same mutation inherited from the ancestral W7 type from the mainland. Even with this mutation being very recent, a minimum of six coyote genotypes has apparently introgressed into the gray wolf species. The actual number of hybridization *events* leading to the transfer of these genotypes is likely to have been much higher. Both repeated introgression of the same coyote-type genotype and the existence of other coyote-type genotypes in wolves not yet sampled are strong possibilities.

DISCUSSION

The Ecology and Geography of Hybridization

Substantial interbreeding between individuals of two distinct species presents difficulties for several areas of evolutionary analysis (Templeton, 1989). Reproductive isolating mechanisms are generally believed to be strong enough to preclude the broad-scale existence of hybrid individuals whose presence can confound interspecific com-

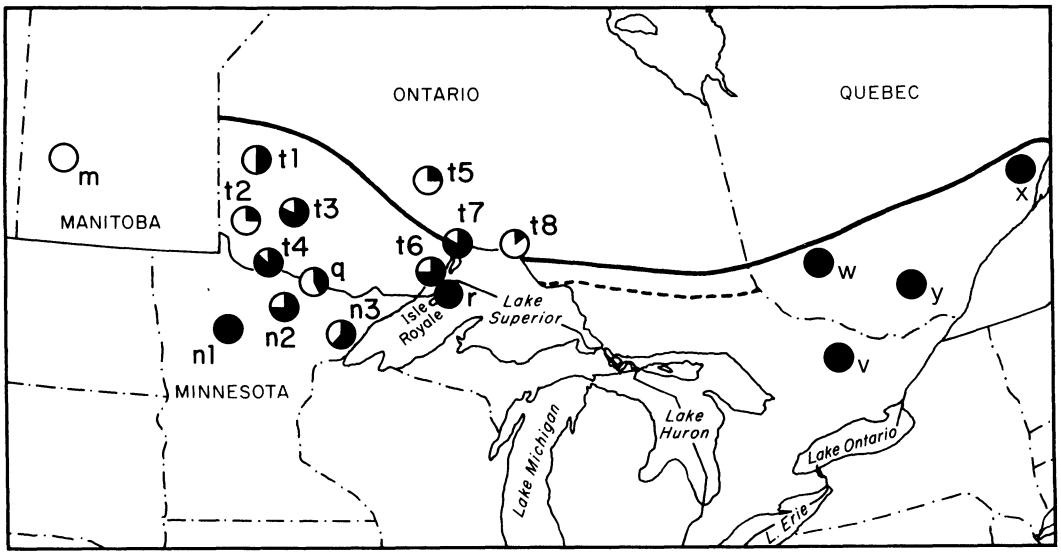

FIG. 4. Observed percentages of wolves with coyote-type mtDNA from localities in and near the hybrid zone. Filled circles indicate 100% coyote-type mtDNA; open circles indicate 0% coyote-type mtDNA. Localities described by the same letter designations as in Table 1. Detail of wolf sample localities (sample sizes in parentheses): n1, Becker County (2); n2, Beltrami and Koochiching counties (10); n3, Lake, St. Louis, and Carlton counties (54); q, Voyaguers National Park (22); r, Isle Royale National Park (7); t1, area near Red Lake (6); t2, area near Kenora (4); t3, area near Dryden (6); t4, Rainy River District (8); t5, Armstrong Station (4); t6, area near Thunder Bay (8); t7, area near Nipigon (6); t8, area near Manitouwadge (6); v, Algonquin Provincial Park (3); w, La Verendrye Provincial Park (1); x, Laurentides Provincial Park (4); y, Papineau-Labelle Provincial Park (9). Solid line describes the northern extent of coyotes in Ontario (Kolenosky and Standfield, 1975) and in Quebec (Georges, 1976). Dotted line describes the southern extent of *C. lupus lycaon* (Boreal type) as determined by Kolenosky and Standfield (1975), wolves which presumably have not hybridized with coyotes.

parisons of morphology, physiology, and genetics. Recently however, an increasing number of examples of genetic exchange between species have been reported. These species include wild mice (Ferris et al., 1983), water frogs (Spolsky and Uzzell, 1984), sunfish (Avise and Saunders, 1984), tree frogs (Lamb and Avise, 1986), deer (Carr et al., 1986), and voles (Tegelström, 1987). In each case, mitochondrial DNA was observed to have been transferred across species boundaries either in one or both directions. The clonal and uniparental inheritance of vertebrate mtDNA allows for a relatively easy assessment of the geographic extent and direction of horizontal genetic transfer (Avise and Saunders, 1984; Avise et al., 1987).

Our data indicate that repeated hybrid-

TABLE 4. Estimated sequence divergence between selected mtDNA genotypes. Above the diagonal: sequence divergence between two genotypes, weighted by classes of restriction enzymes (Nei and Li, 1979). Below the diagonal: number of restriction site differences between two genotypes. Sequence divergence values with two significant figures reflect the inaccuracy incurred when two or three classes of enzymes were lumped together due to a lack of variation in one or two of the classes. Asterisks denote coyote-type genotypes found in wolves.

	W1	W4	W6	W7*	W9*	C7	C12	C21
W1	—	0.34	0.63	3.61	3.51	4.21	3.34	4.05
W4	3	—	0.63	3.61	3.51	4.21	3.34	4.05
W6	5	5	—	3.25	3.19	3.45	2.72	3.72
W7	31	31	27	—	0.917	1.7	0.92	1.3
W9	30	30	28	10	—	2.01	1.18	1.84
C7	33	33	29	14	13	—	0.83	2.0
C12	30	30	26	11	10	5	—	1.7
C21	33	33	31	6	11	16	13	—

TABLE 5. Distribution of coyote-type mtDNA genotypes in wolves.

Region	Sample size	Percentage of wolves with coyote-type mtDNA
Asia	3	0
Alaska	46	0
Yukon Territories	6	0
Northwest Territories	49	0
Alberta	4	0
Montana	6	0
Manitoba	2	0
Minnesota	88	62
Western Ontario	48	58
Southeast Ontario	3	100
Isle Royale	7	100
Quebec	14	100
Total	276	38.8

ization between gray wolves and coyotes has led to the introgression of several coyote mtDNA genotypes into wolf populations. No coyotes have been found with wolf genotypes despite the fact that the sampling of coyotes included areas of current and past sympatry. Although relatively few coyotes have been assayed from localities where the two species coexist, all the coyotes originated from regions occupied historically by wolves. Consequently if substantial introgression of wolf mtDNA into coyote populations had occurred in the past, "true" wolf genotypes (W1 through W6) likely would have appeared in our coyote survey as surviving matrilines.

The distance that adult gray wolves disperse from their natal territories (σ) is quite variable, but studies on radio collared Minnesota wolves show that a conservative estimate of the average dispersal distance in wolves is 50 km (Mech, 1987, and unpubl. data). Barton and Hewitt (1989) have surveyed over 170 hybrid zones and conclude that most have a width of less than 50-σ. Although we feel that the zone described in the current study is quite dynamic and subject to rapid expansion or contraction depending on human intervention (see below), 50 σ would span 2,500 km, which exceeds the zone's present width of no more than 500 km (e.g., Armstrong, Ontario to Duluth, Minnesota).

Dispersing wolves may breed with coyotes if the latter are abundant, and the two species come into frequent contact. In the observed hybrid zone of northern Minnesota, southern Ontario, and southern Quebec, coyote densities are increasing (Carbyn, 1987; Voigt and Berg, 1987), and have become substantial only in the last few decades (cf. Georges, 1976). Though wolf numbers here are not particularly low, there are many local regions where wolves are rare in comparison to coyotes, such as near human settlements (L. D. Mech, unpubl. data). In addition, heavy predator control programs against both species have had a drastic effect on wolves but can actually promote coyote population growth (Connolly and Longhurst, 1975). Thus, while wolf densities are subject to reduction through conflict with humans, coyotes seem to thrive under such conditions.

The habitat in the hybrid zone is being altered from forest to agriculture by an escalating human population. With the spread of deforestation westward and northward across North America, coyote numbers have risen steadily since the 1800s concomitant with an extirpation of wolves (Nowak, 1979). As more forested areas are converted to farmland in the wolf's range, opportunistic coyotes invade and increase their contact with wolves (Kolenosky and Standfield, 1975; Berg and Chesness, 1978).The idea that human-induced environmental alteration may lead to interspecific hybridization is not new (Anderson, 1948). For coyotes and wolves, the condition of successful hybridization seems to be the existence of a region where coyote densities are increasing, and frequent interspecific contacts are made.

In other areas of sympatry, where conversion to agriculture is slow or nonexistent, such as in Alaska, Montana, and in Riding Mountain National Park, no wolves appear to possess coyote genotypes (Table 5). Interspecific partitioning, either spatial or behavioral, may well be sufficient to prevent hybridization between wolves and coyotes. In northeastern Alberta, for instance, coyotes generally avoid wolves by occupying areas at the periphery of wolf pack territories, even when wolf densities are low (Fuller and Keith, 1981). Also, though coyotes in Riding Mountain National Park are known to follow wolf packs, perhaps to scavenge food (Paquet, 1989), reports of

coyotes being killed by the packs are common (Carbyn, 1982). In fact, Mech (1966) suggested that coyotes were extirpated from Isle Royale by wolves. If true, then the coyote-like mtDNA genotype probably entered the wolf population before wolves colonized the island.

The distribution of coywolf genotypes in Minnesota, Ontario, and Quebec (Fig. 4) matches well with the distributions of morphologically defined subspecific wolf types as described by Kolenosky and Standfield (1975). The larger *C. lupus lycaon* (Boreal type) may represent pure wolf lines in northern regions where coyotes have not yet advanced. *Canis lupus lycaon* (Algonquin type) are smaller and may reflect a low, yet steady infusion of coyote nuclear alleles into southern wolf populations. A third type, *C. lupus lycaon* (Tweed type), is even closer to coyotes in appearance and perhaps are wolves only two or three generations removed from a hybridization, sporadically distributed throughout southeastern Ontario and Quebec.

Interestingly, in later morphological examinations of Ontario *Canis* samples, it was concluded that the size cline in wolves was a function of prey size and abundance rather than differential frequencies of coyote hybridization as suggested here (Schmitz and Kolenosky, 1985; Schmitz and Lavigne, 1987). These authors also tentatively concluded that coyotes in Ontario were larger than typical western coyotes as a result of hybridization with wolves. For this to be true, the offspring of a wolf-coyote mating would have to backcross into the coyote population. Our sample of 16 Maine coyotes reveals no pure wolf genotypes, but as in Ontario, coyotes in this region could be descendants from crosses between male wolves and female coyotes.

The fact that the two most abundant coywolf genotypes, W7 and W9, have not been found in coyotes could mean that hybridization has occurred also in the distant past, and subsequently the progenitors of these two coyote-type lineages have gone extinct through mutation and drift in coyotes. Alternatively, the types W7 and W9 could now be rare in coyotes, having declined in frequency at our sampling localities over the last century.

However, the history of coyote range expansion implicates a definite pattern of recent hybridization events. As summarized by Nowak (1979), historical records show coyotes were rare in the Great Lakes region until approximately 1890. Immigrating from the south and the west, they first appeared in central Minnesota around 1875, in the Rainy River District of southwestern Ontario around 1890, on Isle Royale around 1910, and in southeastern Ontario around 1920. From there, coyotes reached into southern Quebec by 1945, and crossed the St. Lawrence River to colonize New Brunswick and Maine, becoming common in these regions by 1970.

Accordingly, a noticeable dichotomy exists in wolf mtDNA genotype frequencies between the newer and older wolf territories invaded by coyotes. In Quebec and southeastern Ontario, all of the sampled wolves ($N = 17$) possess one of the genotypes found identically among coyotes, and three of these genotypes (C14-W10, C17-W11, and C18-W12) are found in Maine coyotes. By contrast, the wolves of Minnesota contain exclusively the unique coywolf genotypes W7 and W9, along with the pure wolf genotypes W1 and W4, suggesting that hybridization occurred earlier in Minnesota than in the East; this is corroborated by the historical data.

The Directionality of Hybridization

Because mammalian mtDNA is strictly maternally inherited (Giles et al., 1980; Brown, 1985), it appears that coyote mtDNA is transferred into gray wolves through matings of male wolves with female coyotes, their offspring backcrossing into the wolf population to generate wolves with coyote mtDNA. Of course, if crosses of this type bred back into the coyote population, we would not be able to detect it with an mtDNA analysis because the hybrids would have coyote mtDNA. Thus, it is still conceivable that the populations of larger coyotes in central Ontario (Schmitz and Lavigne, 1987) and New England (Silver and Silver, 1969; Richens and Hugie, 1974; Hilton, 1978) have genetic contributions derived from male wolves. Yet sterility of male F1 hybrids, known to deter introgression across species of mice (Forejt and Iványi,

1975), may be inhibiting the introgression of wolf nuclear genes into coyote populations.

The purity of coyote mtDNA lines continent-wide suggests that the reverse cross of male coyotes with female wolves is not prevalent or that the female offspring of such crosses do not breed further. The observed type of cross is expected; size differences alone may preclude successful breeding between male coyotes and the larger female wolves. Male coyotes range between 8–20 kg, and female wolves range between 18–55 kg (Nowak and Paradiso, 1983).

Both Mengel (1971) and Hilton (1978) have addressed the subject of hybridization in canids. These authors have proposed that wolf-coyote hybrids are more likely to be responsible for the observed morphological extremes in natural populations than are hybrids between these species and dogs. A phase shift in the breeding cycle of offspring of coyote-dog matings has been invoked to explain the inability of the hybrids to backcross into the coyote population (Mengel, 1971). Moreover, coydog hybrids, along with wolf-dog hybrids, presumably would not be as well suited to surviving under natural conditions as wild canid individuals whose competitiveness has not been dulled by the influence of domestication (Hilton, 1978). Compounding the problems of such hybrids is the fact that their fathers, if dogs, would provide little parental care for their young, again lowering the chances that the hybrids would survive and reproduce (Mengel, 1971). Nevertheless, there are reports of scattered wolf-dog hybrids surviving near cities in Italy (Boitani, 1982) and in the Soviet Union (Bibikov, 1982).

While hybrids of gray wolves and coyotes would not be expected to suffer from these handicaps, it is challenging to provide a scenario in which offspring of male wolves and female coyotes successfully integrate into wolf populations, whereas hybridization between male coyotes and female wolves, if occurring, does not result in introgression of wolf mtDNA into coyote populations. Table 6 describes the possible crosses. We hypothesize that the most probable sequence of events is the following. First, in areas of recent sympatry, young dispersing male wolves will encounter sexually mature female coyotes. If female wolves are rare in the locality, the male wolf may mate with the female coyote. Under more stable ecological conditions, such as in areas of long-term sympatry, the most common interaction between gray wolves and coyotes is that lone coyotes are killed by wolf packs, as discussed above. However, in agriculturally developed areas bordering wolf habitat, the more abundant coyote may be tolerated and even courted by dispersing male wolves.

Second, the wolf-coyote pair raise their young in these regions not occupied by resident wolf packs. The hybrids would presumably have the benefits of biparental care. Last, the female hybrids eventually become breeding adults, and new wolf-like packs are established when additional dispersing male wolves are encountered by the hybrids. They and their descendants develop into "legitimate" wolf packs with only a coyote mtDNA to betray their ancestry.

We believe this scenario to be more likely than one in which female coyotes (or the female hybrids of wolf-coyote matings) are directly accepted into pre-existing wolf packs. Even if they were not killed by the pack, these females would seem to stand little chance of becoming dominant and having the degree of reproductive success that is documented in our mtDNA study.

From our present data, we cannot deduce the frequency with which coyote mtDNA has introgressed into wolf populations. Even though 83 of 136 wolves assayed in Minnesota and western Ontario have coyote-type mtDNA (Table 5), this may represent the proliferation of only a very few coyote matrilines. A survey of nuclear loci would be needed to estimate the percentage of coyote genome currently present in wolves with a coyote-type mtDNA. However, successful hybridizations must have occurred at least six times in the wild to explain the existing coyote-type mtDNA genotypes in wolves (allowing in situ evolution). Additional coyote genotypes may be discovered in a larger sample of wolves.

Genetic Divergence and Diversity

Restriction site differences indicate approximately 2.7–4.2% sequence divergence between the mtDNA of gray wolves and coyotes (Table 4). Using an estimate of a constant 2% mtDNA sequence evolution per million years (Shields and Wilson, 1987),

TABLE 6. Scenarios of hybridization between gray wolves and coyotes. Bold crosses are those suggested in this study.

Cross	Likelihood	Phenotype of offspring	Mitochondrial DNA type of offspring
I. First hybridization event (F_1 hybrid):			
1. wolf (f) × coyote (m)	unlikely because male is smaller	intermediate in size between coyote and wolf	wolf
2. wolf (m) × coyote (f)	**likely because male is larger**	**intermediate in size between coyote and wolf**	**coyote**
II. Subsequent generations (F_2 hybrids and backcrosses), assuming cross #2 has taken place:			
3. hybrid × hybrid	unlikely if male hybrids are sterile; possible if not	much variability (a hybrid swarm)	coyote
4. wolf (m) × hybrid (f)	**likely because male is larger**	**increasingly wolf-like in later generations**	**coyote**
5. wolf (f) × hybrid (m)	unlikely if male hybrids are sterile; possible if not	increasingly wolf-like in later generations	wolf (hybridization not detectable)
6. coyote (m) × hybrid (f)	possible but increasingly unlikely in later generations	mainly coyote-like	coyote (hybridization not detectable)
7. coyote (f) × hybrid (m)	likely because male is larger	mainly coyote-like	coyote (hybridization not detectable)

we would conclude that the two species diverged 1.4–2.1 million years ago. This age is more recent than the date of 3 million years ago, which has been estimated from allozyme genetic distances (Wayne and O'Brien, 1987). On the other hand, paleontological data place the divergence during the later Pleistocene, 600,000 to 800,000 years ago (Kurtén and Anderson, 1980). Thus, our data confirm the notion that the fossil record may not have accurately timed the split of these species, even allowing substantial error in the estimations of sequence variation from site data or in the constancy of the molecular clock. An interesting alternative, however, is that the genealogy of mtDNA may not reflect the genealogy of the species (cf. Takahata and Nei, 1985; Takahata, 1989). In this case, the mtDNA lineage giving rise to the pure gray wolf types may have diverged from lines ancestral to existing coyote types significantly prior to the coyote-wolf species split. If true, then one would not necessarily expect agreement between molecular and fossil data.

The differences in intraspecific variation within the species also present alternative explanations. There is maximally about 2% sequence divergence among coyote-type genotypes as compared to 0.63%, or about one-third as much, among the pure wolf genotypes. Assuming no significantly distinct lineage has been missed in our survey of both species, there are at least two hypotheses that explain the difference. First, in accord with the fossil record, the coyote lineage may be three times older than the gray wolf lineage, such that more sequence variation has been able to accumulate. Coyote-like fossil forms are thought to extend further back in time, 2–3 million years, such that the gray wolf is a more recent offshoot of the *Canis* line, being one-third as old (Kurtén, 1974). Second, the gray wolf may have undergone a sharp population bottleneck in the recent past, with the loss of most mtDNA lineages. Undergoing 2% sequence evolution per million years, the pure wolf mtDNA types would have coalesced roughly 300,000 years ago to a single common ancestor.

Finally, from the phylogenetic tree (Fig. 3) and Table 3, it can be seen that phylogeographic partitioning in coyotes is not particularly strong. This is not surprising given the good dispersal capabilities of large canids (see Wayne et al., 1990). It is notable, however, that the coywolf genotypes W7

through W13 are all derived from the more diverse coyote-type clade in Figure 3, the clade that contains all, but not exclusively, the easternmost coyote genotypes.

Conclusions

Our results suggest that in disturbed areas, previously ecologically distinct species may interbreed if one is rare and the other abundant. In large, highly mobile carnivores such as coyotes and gray wolves, introgression can be rapid and occur over broad areas. This study in particular reveals a unidirectional introgression of genes resulting from matings between male wolves and female coyotes. Such an event has taken place a minimum of six times, and there is evidence for sequence evolution within the hybrid matrilines. As areas historically occupied by wolves become more agricultural, the genetic integrity of wolves may be increasingly threatened by interbreeding with coyotes. Thus, in addition to the direct effects of habitat destruction and depredation programs on wolves, there is a need for biologists to be concerned with the insidious effects of interspecific hybridization.

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