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GENETICS OF A HIGH-LATITUDE CRYPTIC SPECIATION EVENT: AMERICAN AND PACIFIC GOLDEN-PLOVERS

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ABSTRACT.—Diversification during the Pleistocene is thought to have contributed significantly to taxonomic diversity at high latitudes. In some cases this diversity is cryptic, in that speciation has occurred with little change in phenotype. We examined the genetic signatures of one such case, between American and Pacific golden-plovers (*Pluvialis dominica* and *P. fulva,* respectively). This high-latitude species pair is morphologically very similar, and they are obligate long-distance migrants. They were only relatively recently recognized as separate species. We used 1,041 bp of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) from 20 *dominica* and 22 *fulva* and 242 amplified fragment length polymorphisms (AFLPs) from 29 individuals of each species sampled from sympatric and allopatric breeding populations to assess the levels of divergence, divergence date, and gene flow. A divergence date of ~1.8 mya was estimated, and although we detected a seemingly old hybridization event, very little gene flow was detected (effectively zero). Significant genetic divergence was found between species (4.7% uncorrected sequence divergence in mtDNA; F_{ST} = 0.21 in AFLPs). We suggest that ecological factors and possibly sexual selection acted to limit gene flow during the divergence of these cryptic species during the Pleistocene, but given the age of the split we could not determine the mode of speciation that occurred.
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The repeated cycles of glaciation at high latitudes during the Quaternary are thought to have caused diversification and/or speciation in many taxa (Hewitt 1996, 2000). The genetic effects of this history have been studied in diverse life forms using several different molecular markers (e.g., Taberlet et al. 1998, Shafer et al. 2010). Many of these studies have found evidence of isolation during Pleistocene glacial cycles, and allopatric divergence is often thought to be the main route to speciation in birds (Mayr 1963, Coyne and Orr 2004). However, in some cases inference of historical allopatry may be open to alternative interpretations or refinements of the geographic context of divergence (e.g., heteropatry, or cyclical allopatry and sympatry during an annual cycle), and the strictly allopatric model may not be the best fit for migratory species (Winker 2010). Studying obligate migrants at high latitudes may help us better understand how and when speciation occurred in these regions that were so strongly affected by the dynamic climatic cycling of the Pleistocene.

Here we examine the American and Pacific golden-plovers (*Pluvialis dominica* and *P. fulva* respectively), a recently recognized species pair with a broad arctic and subarctic breeding distribution. Because of their extreme similarity in appearance and habits and their largely parapatric distribution, they appear to be an excellent candidate system in which to study the genomics of a high-latitude cryptic (Bickford et al. 2007) speciation event. These birds breed at high latitudes and are obligate long-distance migrants whose ancestor was also likely migratory (Sauer 1963, Baker et al. 2012). Long considered subspecies of the same biological species (e.g., Peters 1934, Gabrielson and Lincoln 1959, Mayr and Short 1970, American Ornithologists' Union 1983), they were elevated to full species status in 1993 (American Ornithologists' Union 1993). They breed across a large geographic area of arctic and subarctic tundra, with *fulva* occupying a largely Palearctic range from the Yamal Peninsula, Russia, to western Alaska (Vaurie 1965, American Ornithologists' Union 1998), and *dominica* occupying a Nearctic breeding range from western Alaska to Baffin Island, Canada (American Ornithologists Union 1998; Fig. 1a, b). These birds migrate long distances to their wintering grounds on islands in the Pacific Ocean and Australia (*fulva*) and the pampas of South America (*dominica*), often covering thousands of kilometers nonstop over open ocean. The largely parapatric nature of this distribution includes areas of sympatry, which enabled researchers to determine that reproductive isolation appears to be complete between these two phenotypically very similar species (Connors et al. 1993).

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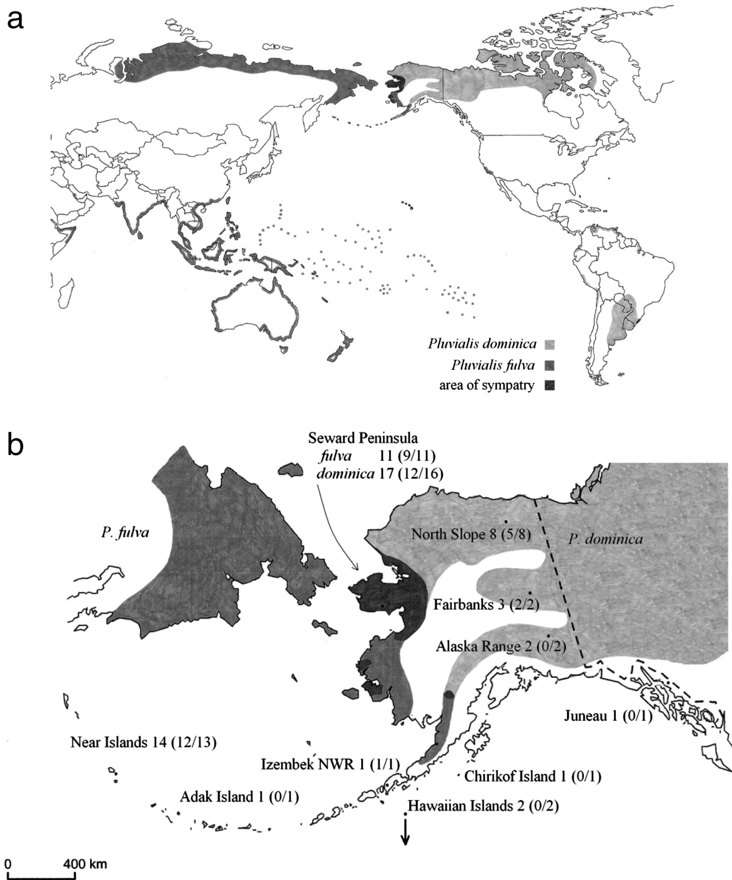

FIG. 1. a,b. Global ranges of *Pluvialis dominica* (Nearctic) and *P. fulva* (Palearctic) showing breeding grounds and temperate and tropical wintering grounds (upper), and Beringian breeding ranges showing areas of sympatry (black) and sampling sizes and locations, with sample sizes for NADH dehydrogenase subunit 2 and amplified fragment length polymorphisms, respectively, given parenthetically. Sampling spans 40° of longitude and includes samples removed by hundreds of kilometers from the area of sympatry.

This species pair breeds in a region that underwent dramatic cyclical changes during the Pleistocene. We ask several questions designed to quantify and assess the genetic divergence between them. First, what is the level and nature of genetic divergence between the two lineages and does it include gene flow? Secondly, we wished to estimate the timing of their divergence and how this may have been correlated with past geographic and climatic events. Thirdly, we explore the roles that migratory direction, pair bonding, and wintering ground adaptations may have had in causing divergent selection between the lineages, and contrast these factors with other high-latitude avian taxa. Finally, we consider the modes of speciation that might apply to this case.

MATERIALS AND METHODS

We used two types of molecular data: DNA sequence from the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) and amplified fragment length polymorphisms (AFLPs; Vos et al. 1995). ND2 is a well-known mtDNA marker in birds and has been shown to be particularly informative and approximately neutrally evolving (Zink et al. 2006), hence providing confidence when used to estimate population parameters (Lovette 2004). AFLPs have several benefits, including a sampling of the entire genome and inclusion of many unlinked loci. They have also been used successfully to study the genetics of closely related species (e.g., Parchman et al. 2006, Toews and Irwin 2008, Maley and Winker 2010,

TABLE 1. Species, locations, University of Alaska Museum (UAM) specimen voucher numbers, and GenBank accession numbers for NADH dehydrogenase subunit 2 sequences of American and Pacific golden-plovers.

Species	Location	Voucher numbers (UAM)	GenBank numbers by species
P. dominica	North Slope, AK	13341, 13536, 13537, 13883, 13884, 18930[a] 18931[a], 19412[a]	KC628677–KC628696
	Seward Pen., AK	8550, 8783, 8784, 8938, 8939, 8941, 8995, 9510, 9511, 13181, 13576, 8551[a], 8940[a], 11758[a], 12573[a], 12572[b]	
	Nulato Hills, AK	24584	
	Alaska Range, AK	26934[a], 26935[a]	
	Fairbanks, AK	19498, 19497[a], 14590[b]	
	Juneau, AK	17751[a]	
P. fulva	Aleutian Is., AK	11110, 12442, 13370, 13545, 14175, 14671, 15177, 19275, 20111, 21830, 22577, 10492[a], 11579[a], 26906[a], 15066[b]	KC628697–KC628718
	Seward Pen., AK	8555, 8785, 9509, 9512, 11307, 11308, 11392, 11756, 11757, 8798[a], 9513[a]	
	Alaska Pen., AK	20178	
	Chirikof I., AK	26907[a]	
	Hawaiian Is., HA	8786[a], 14602[a]	

[a] Denotes individuals for which AFLP but not ND2 data were generated.
[b] Denotes individuals for which only ND2 data were generated.

Brelsford et al. 2011). All birds are vouchered by study skins at the University of Alaska Museum, and identifications were confirmed by measurements following Connors (1983) and plumage characteristics (e.g., Dunn et al. 1987, Golley and Stoddart 1991, Johnson and Johnson 2004).

Mitochondrial Sequence Data and Sampling.— We amplified 1,041 bp of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) for 20 *dominica* and 22 *fulva*. We employed a sampling scheme that allowed us to compare sympatric and allopatric breeding segments. To achieve this, samples were obtained across 40° of longitude, and allopatric samples were taken hundreds of kilometers from the nearest area of sympatry. Approximately half of each species' samples came from the Seward Peninsula. In *fulva*, 12 individuals came from the Near Islands in the Aleutian Islands, where many migrant birds are from Asia (Gibson and Byrd 2007); although the circular migratory pathway of *fulva* suggests that some of these birds may be Alaska breeders, four of them were collected in the fall when they are likely to have come from Russia (Johnson et al. 2012). Nine more were from the Seward Peninsula, and one was from the Alaska Peninsula (Fig. 1b; Table 1). In *dominica*, 13 birds came from the Seward Peninsula area, five came from the North Slope, and two were from Fairbanks (Fig. 1b; Table 1). DNA was extracted from frozen tissues of museum specimens using a Qiagen (Valencia, CA) Dneasy tissue kit following the manufacturer's protocol. PCR amplification was conducted using 2.5 μL each of 10 μM primers H6313 (Johnson and Sorenson 1998) and L5215 (Hackett 1996), 3 μL of a 10 μM solution of dNTPs, 0.2 μL (1 unit) *Taq* DNA polymerase, 6 μL of 25 mM MgCl2, 5 μL of 5× *Taq* buffer (Promega Corp., Madison, WI), 2.5 μL of 1 mg/mL BSA, and 23.3 μL water for a total reaction volume of 50 μL. PCR thermal regime began with 2 mins at 94 °C followed by 29 cycles of 94 °C for 1 min, 48 °C for 2 mins, 72 °C for 2 mins, with a final elongation step at 72 °C for 5 mins. Cycle sequencing was done using Big Dye (Applied Biosystems Inc. [ABI], Foster City, CA), and the same primers from the initial PCR reaction. Product was cleaned by passage through a 0.067 g/mL Sephadex (G-50, Sigma Aldrich, St. Louis, MO) column and run on an ABI 3,100 automated sequencer (ABI). Sequences were aligned and edited using Sequencher 4.7 (Gene Codes, Ann Arbor, MI).

Amplified Fragment Length Polymorphisms Data and Sampling.—To make comparisons between sympatric and allopatric population samples, and to broadly encompass any geographic genetic variation, we chose roughly half the sampled birds of each species from the Seward Peninsula (where they occur in sympatry) and half

TABLE 2. Amplified fragment length polymorphism (AFLP) amplification and scoring results for each primer pair and distribution of the total 242 putative loci. (T) indicates total peaks, (P) represents the number of polymorphic peaks, and (%P) gives the percentage of peaks that were polymorphic.

Primer pair extensions and dye			Both species			Within *P. fulva*			Within *P. dominica*		
*Eco*R1	*Mse*I	dye	T	P	%P	T	P	%P	T	P	%P
-ACT	-CAA	FAM	32	19	59.4	31	16	51.6	31	13	41.9
-ACT	-CAC	FAM	38	20	55.6	30	13	43.3	34	15	44.1
-ACT	-CAG	FAM	45	15	33.3	44	11	25.0	44	14	31.8
-ACT	-CAT	FAM	34	23	67.7	33	14	42.4	33	18	56.3
-ACA	-CAG	FAM	23	13	56.5	23	10	43.5	20	8	40.0
-ACA	-CAT	FAM	40	24	63.2	36	16	44.4	38	17	44.7
-ACA	-CTA	FAM	30	23	76.7	27	14	51.9	27	16	59.3
Totals			242	137	58.9	224	94	43.2	227	101	45.4

from areas away from the Seward Peninsula (Fig. 1b). In *fulva*, 13 birds came from the Near Islands (see above regarding Near Island samples). Of the remaining 16 birds, 11 were from the Seward Peninsula and were grouped with five birds taken in migration south of there (Fig. 1b). Sixteen of the *dominica* specimens came from the Seward Peninsula area and 13 came from areas away from breeding *fulva*.

AFLP data were generated using a protocol modified from Vos et al. (1995) for 29 *dominica* and 29 *fulva*. Sample DNA concentration was quantified on a spectrophotometer prior to subsequent experiments; all had concentrations of 30–70 ng/μL. Restriction and ligation steps of adapter pairs were performed together using a reaction mixture consisting of 0.28 μL water, 1.1 μL 10× T4 buffer (New England BioLabs, Inc. [NEB], Ipswich, MA), 0.55 μL 1 M NaCl, 0.55 μL 1 mg/mL BSA (NEB), 0.1 μL at 10,000 units/mL *Mse*I (NEB), and 0.25 μl at 20,000 units/ml *Eco*RI (NEB) per sample. The reaction mixture was held at 37 °C for approximately 12 hrs and then diluted with 94.5 μL of 0.1 M TE buffer and frozen until pre-selective amplification.

Pre-selective amplification followed normal PCR protocols. A reaction mixture containing 5.68 μL dH_2O, 1.0 μL 5× GoTaq (Promega Corp., Madison, WI), 0.6 μL 25 mM MgCl, 0.1 μL 10 μM dNTPs, and 0.125 μL (0.625 units) *Taq* per sample was added to 2.0 μL of diluted template. The thermal-cycler regime consisted of 1 min at 72 °C followed by 19 cycles of 94 °C for 20 secs, 56 °C for 30 secs, and 72 °C for 2 mins followed by 30 mins at 60 °C. After pre-selective amplification, reactions were diluted with 80 μL of 0.1× TE buffer and frozen until selective amplification.

Selective amplifications were performed using the same PCR mixture and methods as in the pre-selective amplification, but extended, dye-labeled *Mse*I and *Eco*RI primers were used to selectively amplify a subset of DNA. The thermal cycler regime consisted of 2 mins at 94 °C followed by 11 cycles of 94 °C for 20 secs, 66 °C for 30 secs, and 72 °C for 2 mins. During each cycle the annealing temperature was dropped by one degree, and at 56 °C, 19 additional cycles were run at that annealing temperature followed by 30 mins at 60 °C. Samples were selectively amplified and 1.0 μL of each sample was loaded into a 96-well plate. 8.5 μL deionized formamide, and 0.5 μL GeneScan 500 LIZ size standard (ABI) were added to each sample. Samples were run on an ABI 3100 automated sequencer (ABI).

We used seven primer pair combinations for selective amplification (Table 2). GeneMapper ver. 3.7 (ABI) was used to score the chromatograms. Only unambiguous loci with clean, well-defined peaks were scored. We used a minimum peak width of 1.5 bp and a minimum peak height of 75 as a starting point, but then we examined each peak individually to maximize the useful phylogenetic signal (Holland et al. 2008). We discarded two samples that did not amplify properly, likely because of tissue degradation related to specimen care in the field. Data were transformed into a binary state matrix using a Microsoft Excel® macro, which also transformed the matrix into nexus format (Rinehart 2004). Although not specifically tested, all bands were considered independent, orthologous loci (Meudt and Clarke 2007).

Genetic Differentiation and Population Structure.—A median-joining network that illustrated haplotype frequencies was generated for mtDNA data using NETWORK 4.6.0.0 (Bandelt et al.

1999). We used Arlequin 3.11 (Excoffier et al. 1992) to calculate pairwise Φ_{ST} values between populations. Genotypes were permuted 1,000× to obtain *P*–values to determine whether Φ_{ST} values were significant.

For AFLP data, we calculated F_{ST} (and *P*-values) following Lynch and Milligan (1994) using AFLP-SURV 1.0 (Vekemans et al. 2002), using the Bayesian method with non-uniform priors, 10,000 random permutations, and 1,000 bootstraps for genetic distances. To assess intra- and inter-species divergence and divergence related to geography, we made five F_{ST} comparisons: between species, between Seward Peninsula (i.e., sympatric populations) and non-Seward Peninsula birds within a species, between Seward Peninsula birds across species, and between non-Seward Peninsula birds across species.

We also analyzed AFLP data in STRUCTURE 2.2 (Pritchard et al. 2000, Falush et al. 2007) to determine the most likely number of populations and to identify any admixed individuals. STRUCTURE uses MCMC simulations in a Bayesian framework to assign individuals to populations based on Hardy-Weinberg/linkage equilibrium and can be used to determine the most likely number of populations (Pritchard et al. 2000). The program's model-based clustering method effectively deals with the genotypic ambiguity present in dominant markers such as AFLPs (Falush et al. 2007). Preliminary runs indicated that a burn-in of 20,000 iterations was sufficient. We then ran three independent simulations under the admixture model for 100,000 iterations with the number of populations (*K*) varying from 1–5, then calculated the likelihood of *K* given the data as $P(K|X)$. To avoid biasing the inference of population structure, we did not use population origin information, although some individuals were phenotypically identifiable. We used the program Distruct (Rosenberg et al. 2002) to transform and apply information from STRUCTURE.

A mismatch between mtDNA and AFLP markers in one individual (a chick) resulted in follow up work in which the hybrid individual was re-extracted and re-sequenced for ND2, and all AFLP primer pairs were rerun to verify that the original data were correct. This second independently scored AFLP profile was nearly identical to the original profile, which was nearly identical to the putative male parent. Unfortunately, no female was present. As far as we know no other individuals in the study were relatives.

Divergence Time, Effective Population Size, and Gene Flow.—The program Isolation with Migration (IM; Nielsen and Wakeley 2001) was used with mtDNA data to estimate divergence time (*t*) and a population parameter ($\theta = 4N_e\mu$; where N_e is effective female population size and μ is the mutation rate) and also to assess the likely number of migrants ($m = 2M/\theta$, where *M* is the effective number of migrants moving into a population per year) between populations. Initial runs using a six parameter model where θ was estimated for both current populations and the ancestral population and two migration rates showed poor convergence, possibly because mtDNA had sorted to reciprocal monophyly and therefore did not contain enough information to estimate a full set of parameters (Nielsen and Wakeley 2001). A simpler model was used in which we constrained the analysis so that all three effective population sizes and both migration rates were constant. We ran three independent runs, treating the hybrid individual as *P. dominica*, using the initial starting maxima of $\theta = 100$, $m = 2$, and $t = 50$, with a burn-in of 500,000 steps and a different random number seed for each run. An estimated generation time of 5 years was used based on Johnson and Connors (2010). The HKY model of molecular evolution (Hasegawa et al. 1985), which takes into account multiple nucleotide substitutions at the same position, nucleotide frequency differences, and any transition/transversion bias was used in all runs. We let each run proceed for more than 10 million updates to achieve a minimum effective sample size (ESS) of 100 for any given parameter estimate (Hey and Nielsen 2004); most ESS values were several orders of magnitude higher than this. Results from the three runs were similar, and we report here only the parameters estimated in the longest run of 87,479,393 updates after burn-in. The parameters estimated by IM are dependent on the mutation rate, which is an uncertain quantity (Lovette 2004; Ho et al. 2005, 2011). A mutation rate of 2.61% divergence per myr (0.0000135 per year per 1,041 bp of ND2) was used (following Weir and Schluter 2008), together with a range to incorporate uncertainties. We set this range at 0.48–4.31% divergence per myr (0.00000249–0.00002243 per year per 1,041 bp), the lower bound was based on Pacheco et al. (2011) for ND2 in Charadriiformes and the upper bound was based on Weir and Schluter (2008) for cytochrome *b* in Charadriiformes. Following calculations outlined in Hey (2005),

we estimated the effective population sizes of *dominica* and *fulva* (N), the number of individuals coming into a population from the other population per year (Nm), and the time since divergence (t).

Genetic Diversity and Selection.—We used DnaSP (Rozas et al. 2003) to calculate nucleotide diversity (π) and haplotype diversity (H_d) in ND2 following Nei and Chesser (1983). We conducted χ^2 tests of genetic differentiation between populations based on haplotype frequencies and nucleotide diversity indices. The χ^2 tests with Yates continuity corrections were conducted in PopTools 2.6.9 (Hood 2005), an add-in for Microsoft Excel.

We counted the number of AFLP loci that were fixed in one species but polymorphic in the other, as well as the number of loci that were present in one species but absent in the other. We used χ^2 tests as implemented in PopTools 2.6.9 (Hood 2005) to test for significant population differences in these values.

To determine whether the genes sampled through AFLPs diverged via genetic drift or selection, we compared F_{ST} and heterozygosity estimates for each locus in our dataset with a simulated dataset acting under drift alone using an infinite-alleles model. To produce these simulated data we used the program dfdist (Beaumont and Nichols 1996, Beaumont 2000). This program uses an average divergence of F_{ST} and expected heterozygosity (H_S) calculated from the data to simulate the expected distribution of differentiation across loci (Campbell and Bernatchez 2004, Bonin et al. 2006). It uses an F_{ST} calculated by the method of Nei (1977) as modified by Nei and Chesser (1983) and generates a uniform distribution of heterozygosities in place of a specified mutation rate. This distribution is then used to calculate quantiles of the median and upper and lower 99% confidence intervals of the distribution of loci for the population diverging under drift alone. It also calculates F_{ST} and H_S for all polymorphic loci in the dataset, which are then plotted against the confidence intervals. Loci falling outside this distribution in excess of expected false positives are assumed to be under selection or closely linked to loci under selection (Beaumont and Nichols 1996, Nosil et al. 2009). The data were also analyzed to obtain an estimate of the average F_{ST} across all loci. The model was then fit to this F_{ST} for simulation. We ran the simulation for 50,000 realizations, with two demes total, sampling the two populations of *Pluvialis* with an expected $F_{ST} = 0.11$ and an average sample size per population of 29 individuals (i.e., all of them).

RESULTS

Genetic Differentiation and Population Structure.—Our data clearly showed that *dominica* and *fulva* are genetically distinct and have likely been so for a considerable period of time. There were 49 fixed differences (4.7%) in ND2 between *dominica* and *fulva*. Mitochondrial DNA was highly structured between species ($\Phi_{ST} = 0.65$, $P < 0.001$). The haplotype network showed that 17 of 22 *fulva* shared a common haplotype, while 16 of 20 *dominica* shared a common haplotype (Fig. 2). Other haplotypes were composed of single individuals, differing by one or two bases from the common haplotypes within a species (Fig. 2).

Genomic measures of differentiation mirrored those seen in mtDNA. The full interspecies comparison resulted in an $F_{ST} = 0.21$ ($P < 0.001$). Both intraspecific AFLP comparisons between sympatric and nonsympatric populations showed low but significant levels of differentiation (*fulva* $F_{ST} = 0.038$; $P = 0.033$ and *dominica* $F_{ST} = 0.030$; $P = 0.016$). Comparisons between Seward Peninsula *dominica* and *fulva* ($F_{ST} = 0.24$; $P < 0.001$) were similar to those between non-Seward Peninsula *dominica* and *fulva* ($F_{ST} = 0.22$; $P < 0.001$).

Three independent 100,000-step Bayesian clustering algorithms run on AFLP markers gave similar results and clearly separated the two species (Fig. 3). These runs estimated that the most likely number of populations involved in the samples was two ($\ln \Pr(K|X) = -2323.8$; $P(X|K) = \sim 1$). No individuals were misassigned to population. Most individuals (81%) were estimated to contain greater than 99% genomic material originating from their putative population of origin. The lowest estimate was 91.7% (Fig. 3).

One individual exhibited a mismatch between mitochondrial and nuclear DNA markers (Figs. 2, 3). This individual had the common *fulva* ND2 haplotype but exhibited the AFLP banding pattern of a *dominica*. STRUCTURE estimated that the hybrid individual had essentially all (99.6%) of its nuclear alleles originating from the *dominica* population (Fig. 3). Although previous reports of hybrids exist, we could find no convincing

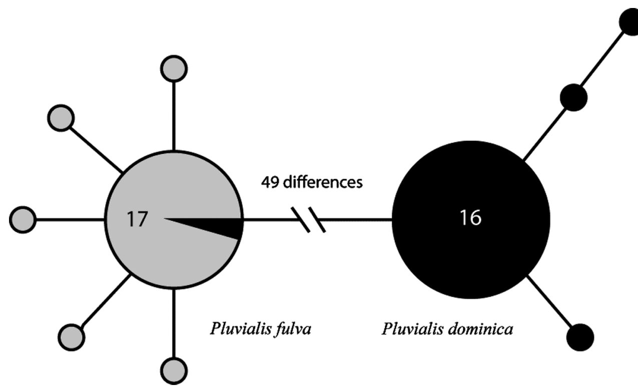

FIG. 2. Haplotype network for 1,041 bp of NADH dehydrogenase subunit 2, depicting the number and relationships among haplotypes of both species. Small circles represent one haplotype each. The single hybrid individual is indicated by a wedge within the most common *P. fulva* haplotype.

physical evidence (i.e., museum specimens) to support them (contra Gray 1958, McCarthy 2006, and references therein).

Divergence Time, Effective Population Size, and Gene Flow.—Coalescent analysis of mtDNA estimated the high point and 95% confidence interval (in parentheses) of the following population parameters: t = 23.98 (18.0–48.7), θ = 4.6 (2.2–11.7), and m = 0.001 (0.001–0.12). Using our best estimate of μ (1.3%) this resulted in an estimated divergence time of 1.8 mya and a long-term effective population size of 16,800 individuals (females). Ranges of these values based on different values of μ appear in Table 3. The estimated effective number of migrants per year was 0.0023 or two every 1,000 years, effectively zero (Table 3). IM's estimated long-term effective female population size of 16,800 (lineages constrained to be equal) is lower than current estimated census population levels in both *dominica* (134,000–200,000 breeding individuals) and *fulva* (90,000–250,000; Morrison et al. 2000, Delany and Scott 2006).

Genetic Diversity and Selection.—Nucleotide diversity was lower in *fulva* (π = 0.00040) than *dominica* (π = 0.00048; χ^2 = 1177.9, df = 1, $P <$ 0.001), but haplotype diversity was similar between taxa (*fulva* H_d = 0.35, *dominica* H_d = 0.29; χ^2 = 2.9, df = 8, P = 0.089).

A total of 242 bands were produced by the seven AFLP primer pairs. Of these, 137 (58.9%) were polymorphic (Table 2) when both species were included. There were 23 AFLP loci that were fixed in *fulva* but polymorphic in *dominica*, and there were 19 alleles fixed in *dominica* but polymorphic in *fulva*; these differences were not significantly different from one another (χ^2 = 0.381, df = 2, P = 0.899). There were nine AFLP loci present in *fulva* that were absent in *dominica*, and 11 loci present in *dominica* that were absent in *fulva*, and these differences were also not significant (χ^2 = 0.200, df = 2, P = 0.726).

Five loci fell outside the simulated data set's 99% confidence interval (Fig. 4). The five loci had unusually high F_{ST}. This result includes more loci than would be expected by chance (expected

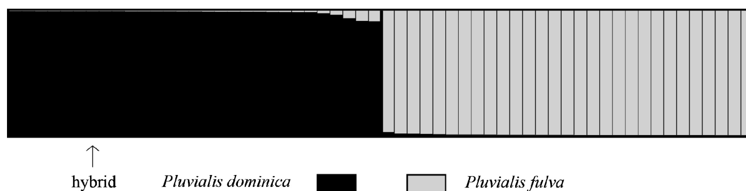

FIG. 3. The genotypic makeup of 29 *P. dominica* and 29 *P. fulva* inferred using amplified fragment length polymorphisms and STRUCTURE (Pritchard et al. 2000). Each bar represents a phenotypically identified individual. The hybrid is indicated by an arrow.

TABLE 3. Demographic parameter estimates calculated using mtDNA gene ND2 between American and Pacific golden-plovers with an Isolation with Migration coalescent analysis (Hey 2005). 95% highest posterior densities are given in parentheses.

Parameter	Substitution rate (μ)			
	$\mu_{0.24\%}$	$\mu_{0.5\%}$	$\mu_{1.3\%}$	$\mu_{2.16\%}$
Population size[a]	91 (43–233)	43 (20–111)	16 (7.9–43)	10 (4.7–24)
Migration rate[a,b]	0.0023 (0.001–0.12)			
Divergence time[a]	9.6 (7.2–19.6)	4.6 (2.5–9.4)	1.8 (1.3–3.6)	1.0 (0.8–2.2)

[a] Estimates of population size are of the long term effective female population size in thousands of individuals. Migration rate is individuals per generation. Divergence time is given in millions of years.
[b] This parameter is independent of mutation rate.

N = 2.4; 1%), but this could be a result of drift operating to cause an increasing number of loci to go to fixation over an extended period following speciation or as a result of the uncertainty with which F_{ST} is calculated in a heterogeneously evolving genome ('n = 1 constraint'; Nosil et al. 2009, Buerkle et al. 2011).

DISCUSSION

Genetically, American and Pacific golden-plovers are very distinct in both mtDNA and genomic AFLP markers (Figs. 2, 3). Coalescent analyses of mtDNA estimated a divergence date of ~1.8 mya. This level of divergence was deeper than we expected given such phenotypically similar lineages so recently treated as subspecies, and it suggests that this cryptic species pair has existed through many of the glacial cycles of the Pleistocene. Nuclear genotype also clearly separated the two species, with every individual having >90% assignment probability to its putative population of origin (most were much higher; Fig. 3).

Despite our empirical evidence of hybridization, gene flow rates between these plover

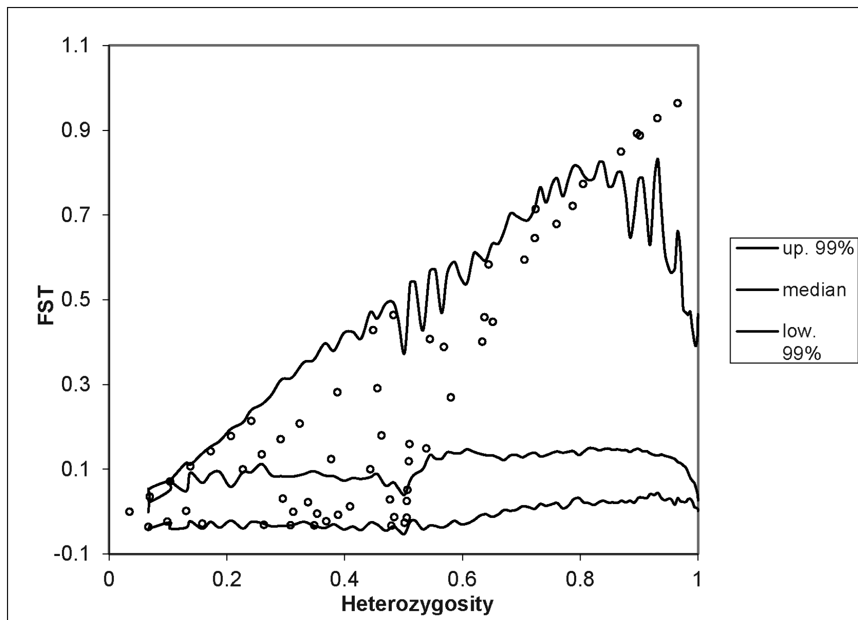

FIG. 4. Distribution of amplified fragment length polymorphism data and quantiles. Upper and lower 99% and median quantiles were calculated using simulated data diverging via drift alone using the program dfdist (Beaumont and Nichols 1996).

lineages are effectively zero (Table 3), a rate suggesting that isolating mechanisms are very strong despite sympatry in western Alaska. We found that 2% of AFLP loci may have been under selection, a value higher than expected by chance (1% at $\alpha = 0.01$) but lower than that seen in other studies (Nosil et al. 2009). However, the linear distribution of loci once a heterozygosity of 0.5 was reached (Fig. 4) is consistent with a process of genetic drift acting over an extended period of time, and we do not infer evidence of selection at these loci from these results. While it is clear that these two species diverged in the Pleistocene, it is not possible to correlate a specific glacial-interglacial cycle with this speciation event. Paleo-temperature records suggest that there were more than 40 glacial-interglacial cycles in the Quaternary (Ruddiman et al. 1986). In addition, the complex refugial history of Beringia (Hopkins 1967, Kaufman and Manley 2004), coupled with uncertainties in mutation rates (Lovette 2004, Weir and Schluter 2008), make precise correlation of these species' divergence with a particular glacial cycle unlikely. Further, our data suggest they probably speciated during a period when the cycles were occurring at a higher frequency (approximately every 40,000 years) than later in the Pleistocene (approximately every 100,000 years; Ruddiman et al. 1986), and the confidence interval on the estimate was broad (Table 3).

Although it is possible that Near Islands specimens of *fulva* included some Alaska-breeding birds, significant intraspecific population structure similar to that found in *dominica* suggests that separate populations were sampled, even though STRUCTURE analyses suggest all of the *fulva* and all of the *dominica* samples each effectively represent a single genetic population (Fig. 3). Green-winged Teal (*Anas crecca*) from the Near Islands are different in this respect, in that two populations appear to occur together there during migration (Winker et al. 2013).

Modes of Speciation.—The historic breeding range(s) of the ancestor of this *Pluvialis* species pair is unknown. However, we can assume that this ancestor possessed key phenotypic attributes that both species share today: long-distance migration to breed on high-latitude tundra and winter at tropical and/or south-temperate latitudes. These factors suggest an alternative to the traditional allopatric speciation hypothesis proposed in this system (Larsen 1957, Connors 1983). During the glacial-interglacial cycles of the Pleistocene, the breeding ranges of these two lineages would have fluctuated dramatically (Bartlein and Prentice 1989), with a likelihood that they were often connected. At the height of many of these glacial cycles, the current area of sympatry (Beringia) would have been much larger than it is today (Hopkins 1967, Kaufman and Manley 2004), suggesting that parapatric speciation could have occurred. Parapatric speciation and other types of speciation with gene flow are proving to be more common than previously thought (Nosil 2008a, Papadopulos et al. 2011, Cristescu et al. 2012, Galligan et al. 2012), and although we were unable to assess whether speciation with gene flow occurred, acknowledgment of its possibility is warranted.

Many recent discussions of speciation have moved past distributional conditions (e.g., allopatry, sympatry) to instead consider that geographic context is but one attribute of the divergence process (Fitzpatrick et al. 2009, Butlin et al. 2012). Increasingly, ecological, environmental, and behavioral factors are also being considered as important contributors to speciation (Schluter 2001, Gavrilets 2003, McKinnon et al. 2004, Verzijden et al. 2012). These factors may in some cases complement and act in conjunction with allopatry or parapatry to bring speciation to completion (Howard and Berlocher 1998, Nagel and Schluter 1998, Schluter 2000, Nosil 2008b). Migratory birds in particular can cover large distances in their semiannual movements, increasing opportunities for gene flow and making allopatry difficult. These seasonal, cyclic movements of birds can reduce differentiation within species by increasing dispersal distances and promoting gene flow (Montgomery 1896, Mayr 1963), but these movements can also accompany divergence and thus have been proposed as sometimes being a driver of speciation (Winker 2010). Thus, migratory birds exhibiting divergence may help us better understand speciation with gene flow (e.g., Peters et al. 2012). In this case, while consideration of all the evidence suggests that speciation with gene flow may have occurred, our data cannot resolve whether in fact it did.

Isolating Mechanisms.—With respect to gene flow, we found a pattern different from several other Beringian taxa. For example, *Anas crecca* (Green-winged Teal), a migratory species whose geographic distribution is similar to the plovers, with Old and New World forms meeting in

Beringia, is experiencing ongoing parapatric speciation, with distinct Eurasian (*A. c. crecca*) and North American (*A. c. carolinensis*) forms (Peters et al. 2012). However, these teal fall short of achieving full speciation (because of ongoing gene flow at substantial levels), even though they may have been diverging for a longer period of time (~2.6 mya) than *dominica* and *fulva*. In another case, a highly mobile group of Arctic-breeding gull species (*Larus* spp.) show limited genetic structuring, with a high number of shared alleles (Sonsthagen et al. 2012). This complex of migratory gulls apparently lacks the isolating mechanisms needed to prevent lineages from reticulating during interglacial periods of sympatry. The plovers successfully diverged long enough ago, and with such apparently effective isolating mechanisms, that shared alleles are rare and gene flow is very low.

The reasons for *dominica* and *fulva* having effectively ceased interbreeding while *A. crecca* and Arctic *Larus* species have not are likely partially because of differences in the breeding systems of these different avian groups. For example, most ducks, which have female-biased philopatry, form pair bonds on the wintering grounds (Carboneras 1992). Wintering males are therefore more likely to pair-bond with a member of a different population and follow a mate back to a different breeding ground, resulting in introgression. Plovers, however, form pair bonds on their breeding grounds, diminishing the chances of an individual pair-bonding with a member of a different population (in this case another species), because these individuals have the opportunity to return to a breeding ground where members of the same population (species) are present. The high rates of interspecific hybridization in ducks (Johnsgard 1960, Grant and Grant 1992, Tubaro and Lijtmaer 2002, Gonzalez et al. 2009) also suggest that isolating mechanisms may be weaker in general in waterfowl. Similarly, in *Larus* species, interspecific hybrids are common (Good et al. 2000, Crochet et al. 2003, Malling Olsen and Larsson 2004), suggesting that here, too, isolating mechanisms are weak. Gulls also tend to be colonial nesters (Malling Olsen and Larsson 2004), which may increase chances for non-assortative mating. Although breeding systems vary in *Pluvialis*, *Anas*, and *Larus*, the use of courtship displays to form pair bonds, and whether these bonds are formed on breeding or wintering grounds, may affect the likelihood of introgression in each group. Differences in mating calls and displays between plover species are likely to be determined genetically (Connors et al. 1993, Miller 1996) and tend to be conserved evolutionarily (Miller 1996), suggesting that they may have contributed to speciation in this case, a common occurrence generally in birds (Price 2008). But such mechanisms are also present to some degree in *Anas* and *Larus* species, too (e.g., McKinney et al. 1990, Tinbergen 1960).

There are several plausible ecological sources for divergent selection between these *Pluvialis* species. The most obvious difference between the two forms is migratory direction: the Pacific Ocean divides their wintering grounds (Fig. 1a). It has been suggested that ''requirements of juvenile migration might exert severe selection pressures against hybrid[s]'' (Connors 1983:618). However, because golden-plovers can modify their genetically determined pattern of migration by learning (Sauer 1963) and they often migrate in flocks (Johnson and Connors 2010), it is conceivable that migratory orientation is not an impervious isolating barrier causing strong selection against hybrid individuals. Our single backcrossed hybrid individual is additional evidence of this. Ultimately, our data do not provide any insights into whether divergent selection has occurred between these lineages or whether neutral divergence in allopatry led to incompatibility on secondary contact. Given current life histories, however (e.g., long migrations to breeding grounds in areas without extensive glaciation, such as eastern Asia and Beringia), long periods of allopatry may be unlikely, leading us to infer that some divergent selection has occurred.

Our discovery of a hybrid individual shows that it does occur, albeit rarely, despite previous unsubstantiated reports (Gray 1958, McCarthy 2006). Given the degree of haplotype divergence between the two species, we view the mismatch in mtDNA and AFLP data in this individual as a case of hybridization and not incomplete lineage sorting (Peters et al. 2007). AFLPs have been used before to assess hybrids (Vallender et al. 2007, Rush et al. 2009, Irwin et al. 2009), and given that this individual showed a nearly identical banding pattern to its presumed male parent it is unlikely that it was an F1 hybrid. A first-generation hybrid would be expected to show an AFLP profile intermediate between the two populations and would show a more even

distribution of nuclear alleles between the two genomic groups in the STRUCTURE analysis. Although we did not anticipate finding a hybrid, detection of one is not altogether surprising, given that reproductive isolation is often incomplete after speciation in birds (Price 2008), although it is less common in shorebirds (McCarthy 2006).

If migratory orientation alone failed to provide a definitive isolating barrier, flyway-specific ecological attributes of a particular migratory pattern might do so instead. Prevailing winds, weather, the distances involved, and potential stopover sites are all factors likely to be unique to a particular migratory route. These differences would in turn lead to differences in phenology. For example, *dominica* arrive in western Alaska in mid-May, a time when winter prevails on the west side of the Bering Strait (Kessel 1989). Even if hybrids were able to survive a migratory cycle, a strong tendency to migrate in one direction or the other would facilitate differentiation in phenology related to a migratory route. Similarly, differences in migration destinations could lead to differing ecological selection pressures on wintering ground adaptations (Connors 1983, Byrkjedal and Thompson 1998). It is likely that these ecological factors (migratory patterns and the subsequent differences in phenology, wintering ground adaptations, and navigational requirements), combined with sexual selection on the breeding grounds, provide important isolating mechanisms in these plovers (Connors 1983, Connors et al. 1993). This might explain why there is one species of golden-plover in each major flyway (*Pluvialis apricaria* occupies the Palearctic–African flyway; Cramp 1983). These selective pressures stemming from different migratory patterns would have similar effects under allopatric or parapatric speciation scenarios, either reinforcing differences acquired largely in allopatry or mitigating the effects of low levels of gene flow.

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