Article Type: Original Article

Evolutionary dynamics of hybridization and introgression following the recent colonization of Glossy Ibis (Aves: *Plegadis falcinellus*) into the New World

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Keywords: Ultraconserved elements, Range expansion, Secondary contact, Natural colonization, Introgression

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mec.15008

Running title: *Plegadis* Hybridization

Abstract

Geographic range shifts can cause secondary contact and hybridization between closely related species, revealing mechanisms of species formation and integrity. These dynamics typically play out in restricted geographic regions, but highly vagile species may experience major distributional changes resulting in broad areas of contact. The Glossy Ibis (*Plegadis falcinellus*) is a dispersive waterbird of the Old World and Australia that colonized eastern North America in the early 19th Century and came into contact with the native White-faced Ibis (*P. chihi*). Putative hybrids between the two species have been observed across North America. To examine the population genomic consequences of this natural invasion, we sequenced 4,616 ultraconserved elements from 66 individuals sampled across the distributions of *falcinellus, chihi,* and the Puna Ibis (*P. ridgwayi*) of South America. We found genomic differentiation among the three species. Loci with high sequence divergence were often shared across all pairwise species comparisons, were associated with regions of high nucleotide diversity, and were concentrated on the Z chromosome. We detected signals of genetic admixture between *chihi* and *falcinellus* in individuals both near and far from their core area of sympatry. Genomic cline analyses revealed evidence of greater introgression into *falcinellus* from *chihi,* but we found little evidence for selection against hybrids. We also found signals of admixture between *ridgwayi* and South American populations of *chihi*. Our results indicate vagile species can experience pervasive introgression upon secondary contact, although we suggest these dynamics may be more ephemeral than the stable hybrid zones often observed in less dispersive organisms.

INTRODUCTION

Isolation in allopatry is a primary driver of population divergence and speciation. Divergent allopatric taxa, however, can be reunited as a result of anthropogenic habitat changes (Lehman et al. 1991, Seehausen et al. 2008, Grabenstein & Taylor 2018), species introductions (Perry et al. 2002, Fitzpatrick et al. 2010), or natural range dynamics (Bernatchez & Wilson 1998). In secondary contact, any differences that confer reproductive isolation accrued in the allopatric populations may lead to reinforcement of taxon boundaries (Coyne & Orr 1989). If these barriers are lacking or incomplete, hybridization can lead to introgression if hybrids are fertile and viable. Introgression may result in beneficial genetic novelty (Hedrik 2013, Racimo et al. 2015), the production of new hybrid species (Mallet 2007, Gompert et al. 2006, Schumer et al. 2014), or the loss of genetic diversity and ultimately the extinction of one of the divergent forms (Rhymer & Simberloff 1996, Brumfield 2010).

The dynamics of secondary contact and hybridization may vary depending on the dispersal ability of the organism and the geography of the interaction. A narrow hybrid zone is expected to form along the advancing front of the colonizer if dispersal distance of the organisms is low and the expansion of the colonizer stems from a limited geographical region (Brumfield 2010). When dispersal distances are high, the zone of hybridization is expected to be broader and more diffuse, with long-distance migrants producing additional pockets of hybridization far from the advancing front (Nichols & Hewitt 1994).

An extreme case of recent trans-oceanic colonization followed by hybridization and introgression involves ibis in the genus *Plegadis*. The three species of *Plegadis* are all ecologically and phenotypically similar colonial-nesting waterbirds that inhabit freshwater wetlands, irrigated pastureland, and fields (Burger & Miller 1977, Matheu et al. 2017a, b, c).

The species differ subtly in plumage and bare parts coloration, particularly the color of the eye and facial skin (additional information in Methods). Like many waterbirds *Plegadis* are exceptional dispersers, with regular migratory movements and cases of long-distance vagrancy recorded routinely throughout history (e.g. Brewster 1886, Stejneger 1887).

Two species of *Plegadis* ibis are native to the New World. White-faced Ibis (*P. chihi*; hereafter *chihi*) occur in western North America east to the Florida panhandle, north to Alberta, Canada, and south to Nicaragua, with a disjunct population in southern South America (Fig. 1). North American *chihi* underwent a population decline in the 20th century due to drought and conversion of wetlands (Ryder 1967), but have since increased dramatically with implementation of practices to create, manage, and preserve freshwater wetlands (Ivey et al. 1988). During the last few decades, *chihi* have expanded their range into the Great Plains (Jorgensen & Dinsmore 2005, Shaffer et al. 2007), and have exhibited a concomitant increase in extralimital records in eastern North America (Patten & Lasley 2000, Jorgensen & Dinsmore 2005). Puna Ibis (*P. ridgwayi*; hereafter *ridgwayi*) is endemic to South America, where it occurs in the high Andes (3500-4800 m) from southern Peru to northern Argentina (Schulenberg et al. 2010, Matheu et al. 2017b). Since the 1960's, *ridgwayi* has also expanded, appearing with increasing frequency in Pacific coastal Peru where breeding was documented as early as 1993 (Pearson 1974, Hughes 1984, Gonzalez et al. 1999).

The third species, Glossy Ibis (*P. falcinellus*; hereafter *falcinellus*), historically occurred in the Old World (Europe, Africa, southern Asia) and Australia, but colonized North America in the early 1800s. The first documented New World record of *falcinellus* was a specimen collected in New Jersey in May 1817 (Baynard 1913, Peterson & Peterson 1981). Breeding was established in Florida by 1880 (Palmer 1962) and the first evidence of breeding in Louisiana was of hundreds of Glossy Ibis at a rookery in the southwestern part of the state

(Holt 1933). During the late 1950s and 1960s *falcinellus* was recorded in northern South America and Trinidad (Gochfeld 1973), and in the late 1970s and 1980s colonized Mexico to Costa Rica (Stiles & Skutch 1989, Davis Jr & Kricher 2000), and the West Indies (Raffaele et al. 1998). From their initial colonization up until the 1980s, *falcinellus* were rare through much of their New World distribution. Since then, a large-scale expansion of *falcinellus* has been underway, presumably due in part to the creation of artificial wetlands (Patten and Lasley 2000).

Even though *chihi* and *falcinellus* are capable of hybridizing in captivity (citations in McCarthy 2006) and have had at least some overlap in their breeding distributions since the 1930s, including mixed colonies in Louisiana and Alabama (Holt 1933, Duncan & Johnson 1977), the first documentation of putative hybrids was of birds found in Oklahoma in 2002 (Arterburn & Grzybowski 2003). Since then, putative hybrid individuals have been frequently observed along the Gulf Coast (pers. obs.) and throughout much of North America, with published records from Wyoming, (Faulkner 2005), Oklahoma (Arterburn & Grzybowski 2003), Colorado (Leukering 2008), Michigan (Chu 2011), and New Jersey (Boyle 2011) and observations from an even wider range of locations in the eBird citizen science project database (Sullivan et al. 2009: http://www.ebird.org). The 70-year delay between the onset of sympatry and detection of hybridization is thought to reflect the difficulty of detecting hybrids between these phenotypically similar species, and to their breeding biology. It is not entirely clear how breeding colonies form or when pair-bonding occurs in *Plegadis*, though Belknap (1957) noted that pair-bonding in *chihi* occurs prior to arrival at colonies. Moreover, colony formation may often occur by large, homospecific groups of dispersing individuals. For example, the first *falcinellus* colony in Louisiana (Holt 1933) was composed entirely of *falcinellus*, even though the colony was embedded within the distribution of *chihi*.

Here we use range-wide sampling and markers associated with genomic ultraconserved elements (UCEs) and mitochondrial sequence data to investigate patterns of genetic differentiation and hybridization in the three *Plegadis* species, focusing in particular on the area of sympatry between *falcinellus* and *chihi*. We conduct a suite of analyses to evaluate (i) the extent to which the three *Plegadis* species are genetically distinct, (ii) whether genetic divergence is concentrated in the same loci among the three species, (iii) if genomic signatures of admixture support phenotypic evidence of hybridization between *chihi* and *falcinellus*, (iv) the geographic and genomic extent of introgression, and (v) whether there is evidence natural selection or the demography of expansion is influencing patterns of introgression. We discuss the significance and implications of divergence and introgression in *Plegadis*, and contrast inferences from this group with those from other systems involving similar patterns of hybridization.

METHODS

We sampled 66 *Plegadis* individuals (36 *chihi*, 17 *falcinellus*, 5 *chihi-falcinellus* hybrids, 5 unidentified immature individuals collected in Louisiana, and 3 *ridgwayi*; Table S1). We focused sampling on adults collected in breeding condition and in the vicinity of breeding localities. Both pure and hybrid individuals were initially identified based on phenotype at the time of collection. *Plegadis chihi* and *falcinellus* adults in breeding plumage were differentiated as follows: *chihi* have a red iris surrounded by pinkish-red facial skin bordered by white feathers, and brighter reddish legs, whereas *falcinellus* have a brown iris and dark gray facial skin bordered by bluish-white or white skin (not feathers), and duller gray-green legs with reddish joints. Non-breeding adults are similar except that *chihi* loses the white feather border post-breeding and facial coloration is less intense. Juveniles of both

species have a brown iris color; the red iris of *chihi* is attained during the late fall through the first winter. *Plegadis ridgwayi* are most similar to *chihi* in facial coloration and shares a red iris and pinkish-red facial skin, but are stockier with shorter legs and a thicker bill at the base relative to the other species (see Fig. 1 for illustrations). Non-breeding and juvenile individuals of all species are similar and difficult to separate. Presumed hybrids between *falcinellus* and *chihi* show a spectrum of intermediate phenotypes (Arterburn & Grzybowski 2003, Leukering 2008, pers. obs.). For example, they may possess the red eye of *chihi* but grayish facial skin similar to *falcinellus* or they may have *falcinellus* bare parts colors with some white feathering around the face. We are unaware of records of hybrids between *ridgwayi* and *chihi* or between *ridgwayi* and *falcinellus* and did not identify any phenotypic hybrids between these taxa.

We extracted total DNA from pectoral muscle of vouchered specimens using a DNeasy tissue extraction kit (Qiagen, Valencia, CA, USA). After quantification using a Qubit 2.0, we sent 2 μg of DNA at a concentration of 40-100 ng/μl to Rapid Genomics, LLC (Gainesville, FL, USA) for in-solution sequence capture targeting 5,060 ultraconserved elements (UCEs) using the Tetrapods-UCE-K5v1 probe set (ultraconserved.org) and sequencing following the protocol in Faircloth et al. (2012). UCEs are genomic regions that are highly conserved across amniote genomes (Faircloth et al. 2012) and can therefore be easily aligned for the purpose of comparing adjacent, variable markers in their flanks. Although the core regions of UCEs are under purifying selection (Katzman et al. 2007), recombination in the flanking regions results in a rapid restoration of higher levels of variability useful for population genetic inference (Smith et al. 2014, Oswald et al. 2016, Harvey et al. 2016). Samples were multiplexed at 160 samples per lane on a 100 bp paired-end Illumina HiSeq 2500 run.

Bioinformatics

We demultiplexed raw reads using Casava 1.8 (Illumina, Inc.) and cleaned reads with Illumiprocessor 2.0.6 (Faircloth 2013) with trimmomatic 0.32 (Bolger et al. 2014). We used the seqcap_pop pipeline (Harvey et al. 2016, https://github.com/mgharvey/seqcap_pop) to assemble data. This involved initial contig assembly with Velvet 1.2.10 (Zerbino & Birney 2008) and the wrapper program Velvet Optimiser 2.2.5 (Gladman 2009) to build a reference using reads from one randomly selected *falcinellus* and one randomly selected *chihi* individual (Plegadis_falcinellus_17030 and Plegadis_chihi_63627). We then mapped reads from each individual to the reference using bwa 0.7.4 (Li & Durbin 2009), allowing mismatches of up to 4 nucleotides to alleviate the loss of divergent alleles (Harvey et al. 2015). We converted sam files to bam format using samtools 0.1.19 (Li et al. 2009), and cleaned bam files by soft-clipping reads outside the reference contigs with PICARD 1.106 (http://broadinstitute.github.io/picard/). We then added read groups for each individual using PICARD and merged the bam files across individuals with samtools. We realigned reads to minimize mismatched bases using the RealignerTargetCreator and realigned indels using IndelRealigner in the GATK 3.3 (McKenna et al. 2010), identified single nucleotide polymorphisms (SNPs) and insertions or deletions (indels) using the GATK UnifiedGenotyper, annotated SNPs with the GATK VariantAnnotator, masked indels using the GATK VariantFiltration, removed SNPs with a quality score below Q30, and conducted read-backed phasing, also with GATK. We output SNPs in vcf format and used the program add_phased_snps_to_seqs_filter.py from seqcap_pop to insert SNPs into reference sequences and to produce alignments for each locus across individuals (SNP and alignment files available as Supplementary Information). SNPs on the same locus for which phasing failed were inserted using the appropriate IUPAC ambiguity codes. We collated sequences and

produced final alignments using MAFFT (Katoh et al. 2005). Python scripts from seqcap_pop were used to make input files for analyses programs.

Mitochondrial data collection and gene tree estimation

We sequenced mitochondrial DNA to compare mitochondrial divergence, species assignments, and gene tree topology with those from nuclear data. We sequenced 1041 bp of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2; L05216 and H06313; Sorenson et al. 1999) from a subset of presumed hybrid and pure individuals (n = 24). In two samples (Plegadis_chihi_25018 and Plegadis_falcinellus_51600) only the forward sequencing reaction was successful (see Table S1 for GenBank accession numbers). ND2 was amplified via the following polymerase chain reaction (PCR) protocol for 25 µl reactions: a denaturation stage at 94°C for 5 minutes followed by 34 cycles of 94°C for 30 seconds, 60 seconds of annealing at 50°C, and 60 seconds at 72°C and terminating with a 10 minute 72°C elongation. PCR products were purified and Sanger sequenced at Beckman-Coulter (Danvers, MA, USA). Geneious 9.0.2 (http://www.geneious.com, Kearse et al. 2012) was used to evaluate and trim chromatograms and MUSCLE (Edgar 2004) to align consensus sequences.

We used ND2 sequence data from GenBank and the sequence data generated for this project to build a phylogeny of the genus *Plegadis* and close relatives with available sequence data from prior work (Ramirez et al. 2013; see Table S2 for GenBank accession numbers). To determine the best-fit finite-sites substitution model for the dataset, we used jModelTest 2.1.6 (Darriba et al. 2012). The jModelTest 2.1.6 likelihood settings included three substitution schemes, base frequencies, gamma rate variation, and base tree search. We then used BEAST2 to estimate a phylogeny using a relaxed lognormal clock with a mean of 2.5% per

million years (Smith & Klicka 2010), and the HKY+Γ substitution model for calibration. We ran the Bayesian analysis for 100 million iterations, checked convergence in Tracer (Rambaut et al. 2014), and estimated a maximum clade credibility tree after removing the first 10% of iterations as burnin. We used the R package Pegas (Paradis 2010) and the ND2 sequence data to calculate raw pairwise distances between individuals.

Quantifying population structure and identifying admixed individuals

We identified clusters of genetically similar individuals in the UCE data using a discriminant analysis of principal components (DAPC; Jombart et al. 2010). DAPC is a multivariate method designed for large genomic datasets. It maximizes the genetic differences between groups and minimizes the variation within groups while assigning individuals to genetic clusters probabilistically. DAPC was run using one randomly selected SNP from each locus.

We estimated the number of populations, population assignments of individuals, and admixture using a Bayesian genetic modeling approach in Structure 2.3.4 (Pritchard et al. 2000). Structure assigns individuals in the dataset to a user-defined number of populations (K) by maximizing Hardy-Weinberg and linkage equilibrium within groups. We did not *a priori* assign individuals to populations or provide geographic information. After an initial burn-in of 10,000 generations, we used 500,000 Markov chain Monte Carlo (MCMC) generations in analyses. We performed 10 replicates for each value of K ranging from 1 to 6. We ran Structure with all SNPs, but used the linkage model to account for linkage between sites on the same locus. Structure Harvester (Earl & vonHoldt 2012) was used to summarize the Structure output, evaluate support for different values of K, and produce input files for CLUMPP (Jakobsson & Rosenberg 2007). Using the CLUMPP *FullSearch* algorithm, we found the optimal alignment of all individual assignments to each population across replicates in the Structure analyses. We used R (R Core Team 2015) and the CLUMPP output file with

individual assignments to visualize results. Unequal sample sizes in Structure analyses have been shown to lead to downward-biased K values (Puechmaille 2016). Accordingly, we ran a second round of Structure analyses on populations randomly subsampled to the size of the smallest population sample (n = 3 individuals; K:1-5; Table S3).

For subsequent analyses requiring discrete populations, we use two sets of population assignments - one without and one with admixed individuals. In the assignments without admixed individuals, only "pure" individuals supported by a combination of plumage, DAPC assignment, and Structure assignment probabilities of 95% or greater were examined. In the assignments with admixed individuals, we included both the "pure" individuals and admixed individuals, assigning the latter to species where necessary based on DAPC results.

Examining introgression between *chihi* and *falcinellus*

We further explored introgression between *chihi* and *falcinellus* both among individuals and across the genome. We first compared observed heterozygosity within admixed individuals to their hybrid index, or the proportion of ancestry from either parental species. We used the R package HIest (Fitzpatrick 2012) and examined only SNPs fixed between pure *chihi* and *falcinellus*. Hybrid index varies from 0 to 1, with 1 representing entirely *chihi* ancestry. For admixed individuals with intermediate hybrid indices, heterozygosity at fixed SNPs should be 1 in F_1 individuals, but lower in later generation hybrids.

The individuals showing admixture between *chihi* and *falcinellus* were too widely dispersed (see Results) for spatial hybrid zone analyses such as the estimation of geographic clines. In such cases, genomic cline analysis permits the estimation of cline parameters with respect to the genome-wide admixture gradient (Szymura & Barton 1986). We used Bayesian

genomic cline estimation in the program bgc v1.03 (Gompert & Buerkle 2012) to estimate genomic clines for each locus with data from pure *chihi*, pure *falcinellus*, and admixed *chihi*/*falcinellus* individuals. We ran bgc using two MCMC chains of 150,000 iterations printing every 20, and combined the chains after removing the first third as burn-in. The Bayesian genomic cline model estimates two parameters: **α** reflects the probability of ancestry from one of the two parent species (akin to cline displacement from the genomic cline center), whereas **β** reflects the rate of change in the probability of ancestry from one of the two parent species (akin to the steepness of the cline across admixed individuals). Extreme values of **α** may reflect differential success of homozygous genotypes from one of the parental species as a result of directional selection or underdominance, whereas extreme values of **β** reflect sharp transitions in genotype frequencies, which in situations with high dispersal may be driven by underdominance (Gompert & Buerkle 2011). Cline shape in general reflects a balance between selection on loci undergoing introgression and recombination across the genome (Barton & Bengtsson 1986). The degree of linkage disequilibrium across sites can help to distinguish between the importance of selection and recombination in driving extreme cline parameters. The impact of linkage disequilibrium can be assessed by calculating Moran's I for each parameter (Gompert et al. 2012, Parchman et al. 2013, Singhal & Bi 2017). We calculated Moran's I for **α** and **β** within loci.

Assessing patterns of genomic divergence among *Plegadis* species

We estimated the overall divergence based on all loci between each pair of *Plegadis* species using Reich et al.'s (2009) F_{ST} estimator for multiple SNPs, which is robust to small sample sizes. We estimated variation in differentiation across the genome using locus-specific measures of d_{XY} and F_{ST}. We measured d_{XY} as the per-nucleotide Jukes-Cantor corrected

sequence divergence between all haplotypes between species at each locus. For F_{ST}, we examined Hudson et al.'s (1992) sequence-based estimator for each locus. All statistics were calculated with and without admixed individuals. We examined whether the same loci were responsible for divergence in comparisons involving all three possible pairwise combinations of *Plegadis* species. We examined patterns of divergence relative to genomic position after mapping loci to the Crested Ibis (*Nipponia nippon*) genome (Zhang et al. 2014), which is assembled in 59,555 scaffolds, and to the chromosome assemblies of the Zebra Finch (*Taeniopygia guttata*; Warren et al. 2010) using Blastn (Altschul et al. 1990).

Species tree estimation and species delimitation

We inferred a species tree for the three *Plegadis* species using SNAPP (Bryant et al. 2012) included in BEAST2 (Bouckaert et al. 2014). As an outgroup, we used UCE sequences from the *Nipponia nippon* genome, extracted with Blastn and aligned to *Plegadis* sequences using MAFFT. We selected a single SNP without missing data from each UCE locus for analysis to maximize marker independence, and ran SNAPP for at least 1,000,000 generations, sampling every 1,000 to estimate a species tree. Log-normal distributions were used for the prior on the parameter (lambda) governing species divergence rate and for the rate priors. Convergence was evaluated based on ESS values above 200 for all parameters in Tracer (Rambaut et al. 2014). We ran species tree analyses with and without the admixed individuals.

We then assessed statistical support for treating the *Plegadis* as three separate species using SNAPP in combination with Bayes factor species delimitation (BFD*; Grummer et al. 2014, Leaché et al. 2014). We compared support for a tree in which the current three-species *Plegadis* taxonomy was maintained, *falcinellus* and *chihi* were lumped as one species, *chihi* and *ridgwayi* were lumped as one species, or all three *Plegadis* species were considered a

single species. Bayes factor species delimitation is computationally intensive. To streamline computation, we examined a subset of four randomly selected alleles for each species at each SNP. We conducted BFD* analyses both with and without sampling from the admixed individuals. Path sampling analyses for species delimitation were run for 24 steps with 100,000 MCMC generations following 10,000 pre-burnin generations using the same prior distributions as in the SNAPP runs. We compared corrected likelihood values using Bayes Factors (Kass & Raftery 1995). To ensure SNAPP and BFD* were not sensitive to the inclusion of a distant outgroup (*Nipponia nippon*), we also ran both analyses without an outgroup.

Modeling historical demography

We used historical demographic modeling to estimate population sizes in each species and their ancestral populations and rates of historical gene flow in each direction between species. We used the program G-PhoCS 1.2.1 (Gronau et al. 2011), which is a Bayesian demographic modeling program for large datasets. We used a model in which *falcinellus* diverged first, followed by a split between *chihi* and *ridgwayi*, consistent with both UCE (SNAPP) and mitochondrial phylogenies (see Results). We ran analyses both with and without admixed individuals. We randomly selected 1,000 loci to streamline computation. We used 2,000,000 MCMC iterations, removed 10% of the iterations as burn-in, and evaluated convergence using Tracer (Rambaut et al. 2014) to ensure all ESS values were greater than 200. Following the supplementary material in Gronau et al. (2011), effective population size (N_e) was calculated using $\theta = 4N_e\mu$, where μ is mutation rate per nucleotide site per generation. Migration rates are based on the migration rate per generation parameter ($m_{sx} \times \theta_x/4 = M_{sx}$), which is the proportion of individuals in population x that arrived by migration from

population *s* per generation. We used gamma priors of (1, 5000) for mutation-scaled effective population size (θ) and divergence time ($\boldsymbol{\tau}$) and a gamma prior of (1, 0.001) for migration rate. Standardized substitution rates are not available for UCE loci, so we determined the substitution rate by setting the UCE divergence time to the divergence time inferred from the BEAST2 analysis of mitochondrial data (see below). This substitution rate was used for all parameter calibrations.

RESULTS

Population genetic structure and admixture

We recovered 4,616 UCE loci. Of these, 199 were invariant. Variable loci contained 25,422 SNPs within *Plegadis*. Simple population genetic diversity statistics are presented in Table S4. Structure analysis using all SNPs recovered K=3 as the best-fit model based on likelihood values, and K=2 as the best-fit model based on delta K values (Table S5). The assignments with K=3 corresponded well overall with current species limits and the phenotypic species assignments (Fig. 2a), whereas K=2 combined *falcinellus*, *ridgwayi*, and the majority of the phenotypic *falcinellus-chihi* hybrids in a cluster separate from phenotypically pure *chihi* (Fig. S1). The dataset with normalized sample sizes across species recovered K=3 as the best model based on both ΔK values and likelihood scores (Table S6). DAPC analysis also indicated three clusters best fit the distribution of *Plegadis* genotypes in multivariate space (Fig. S2). Groupings from DAPC and based on the highest assignment probabilities from Structure with K=3 were very similar, differing only in the assignments of three *chihi* or *falcinellus* individuals (Table S7).

Eighteen individuals showed varying levels of mixed genetic ancestry between *chihi* and *falcinellus*. Fifteen individuals had <95% ancestry probability from one of the two parental species in Structure, including five individuals with nearly equal ancestry from *chihi* and *falcinellus*. An additional 11 or more admixed individuals had greater ancestry assigned either to *chihi* or *falcinellus*, which suggests these individuals were backcrosses rather than F1 hybrids. The admixed *falcinellus* and *chihi* individuals included birds identified phenotypically as hybrids, as pure *chihi*, and as pure *falcinellus*. All four birds identified phenotypically as hybrids had admixed genotypes. An additional three individuals without high levels of admixture (>95% assignment to a parental cluster) in Structure results had a phenotype or DAPC assignment at odds with the Structure assignment and were also considered admixed for subsequent analyses. Genetically admixed individuals were not only from the area of sympatry in Louisiana, but also from New York, Florida, and Idaho (Fig. 2b, Table S7). Unexpectedly, at least two South American individuals: the single representative of the South American population of *chihi* (Plegadis_chihi_16899) and one of the *ridgwayi* individuals (Plegadis_ridgwayi_82891), showed evidence of admixed ancestry involving *chihi* and *ridgwayi* (≤ 95% assignment to the parental species).

We observed four instances of discordance between ND2 haplotypes and species assignments using either or both phenotype and UCE genotypes (Table S7). One individual (Plegadis_chihi_77245) identified as a pure *chihi* based on phenotype, DAPC, and Structure (albeit with a probability of 0.91) had a mitochondrial haplotype clustering with pure *falcinellus* individuals. Similarly, one individual (Plegadis_falcinellus_85048) identified as *falcinellus* using phenotype, DAPC, and Structure (with a low probability of 0.54) had a *chihi* ND2 haplotype. One individual with a hybrid phenotype (Plegadis_hybrid_77536) was assigned to *chihi* by Structure, but had a *falcinellus* ND2 haplotype, while another phenotypic hybrid (Plegadis_hybrid_77525) was assigned to *falcinellus* in Structure results but had a

chihi haplotype. All four of these cases of mismatched mitochondrial haplotypes showed some evidence of admixture in the nuclear genome (either some admixture in Structure analysis or Structure and DAPC assignments that disagreed).

Introgression between *chihi* and *falcinellus*

We found that most birds with intermediate hybrid indices had low to intermediate levels of heterozygosity at SNPs fixed between parental populations (Fig 3a). This suggests that these were later generation hybrids rather than F_1 individuals. Across the genome, 173 loci displayed excess *chihi* ancestry (lower bound of 95% CI for $\boldsymbol{\alpha} > 0$), whereas only 6 loci had excess *falcinellus* ancestry (upper bound of 95% CI for $\boldsymbol{\alpha} > 0$). Rates were shifted such that, at some loci, individuals with an intermediate hybrid index (0.5) had 80% or greater probability of showing *chihi* alleles (Fig. 3b). However, there was not a clear separation between the curves with outlier $\boldsymbol{\alpha}$ values (particularly positive outliers) relative to non-outliers. No loci had estimates of cline rate ($\boldsymbol{\beta}$) with bounds that did not overlap zero. The association between F_{ST} and both cline parameters was significant, but relatively weak ($\boldsymbol{\alpha}$: $R^2 = 0.030$, $p < 0.001$; $\boldsymbol{\beta}$: $R^2 = 0.001$, $p = 0.013$) in simple linear models. As with regions of high divergence, regions with the most extreme cline parameters were widely scattered across the genome, although some higher values of $\boldsymbol{\alpha}$ were concentrated on the Z chromosome (Fig 3c). Both cline parameters were autocorrelated only at very short distances (<20 bp) across the genome based on Moran's *I* (Fig. 3d).

Patterns of genomic divergence among *Plegadis* species

In this section, we focus on results from the pure individuals to examine patterns of genomic divergence among the three *Plegadis* species without the signature of recent admixture. Results including the admixed individuals were similar aside from reduced divergence between species exhibiting admixture and are presented in the Supplement. Average sequence divergence (d_{XY}) across loci was lowest between *chihi* and *ridgwayi* (0.00127 ± 0.00170) and higher between *chihi* and *falcinellus* (0.00137 ± 0.00177) and *falcinellus* and *ridgwayi* (0.00140 ± 0.00164). F_{ST} estimators, however, showed elevated divergence in comparisons involving *ridgwayi*, presumably due to the small sample size for that species (Table S8).

Seventy-eight loci contained 92 SNPs that were fixed between pure *chihi* and *falcinellus* (of 24,792 SNPs that were variable and had >50% complete data). When admixed individuals were included, no fixed differences were observed between *chihi* and *falcinellus* (based on DAPC assignments). Many more SNPs were fixed in comparisons of pure birds that involved *ridgwayi* (733 of 24,813 SNPs were fixed between *chihi* and *ridgwayi* and 838 of 24,558 between *falcinellus* and *ridgwayi*), again reflecting the small sample of unadmixed individuals (n = 4 alleles) from that species. The 78 loci with fixed SNPs between pure *chihi* and *falcinellus* individuals were distributed across 43 of 59,555 scaffolds (378 of which contained UCEs) in the *Nipponia nippon* genome and across 14 of the 36 chromosomes (plus one unplaced scaffold) in the *Taeniopygia guttata* genome (Table S9). Elevated F_{ST} values were widely scattered across the genome (Fig. S3). d_{XY} between species was higher on the Z chromosome than on autosomes (Fig. S4).

Hudson's F_{ST} estimator at a locus in any one pairwise comparison between *Plegadis* species tended to predict that in the other two possible pairwise comparisons (Fig. 4a). 6.5% of F_{ST} outliers (those in the top 5%) were shared among all three pairwise species comparisons. d_{XY} was even more strongly correlated among the three possible pairwise species comparisons (Fig. 4b). 42.4% of d_{XY} outliers (those in the top 5%) were shared among the three comparisons. d_{XY} in all pairwise comparisons was correlated with the average nucleotide diversity of pure birds in the two species involved in each comparison (Fig. S5), although F_{ST} was not (Fig. S6). Together, these results indicate that the correlated sequence divergence across *Plegadis* species at particular loci is linked to genetic diversity at those loci.

Species tree and tree-based support for species limits

The SNAPP phylogeny (Fig. 5a) recovered a sister relationship between *chihi* and *ridgwayi* with high support (posterior probability [PP] = 1.0 including admixed individuals, PP = 0.96 with only pure individuals). This clade was sister to *falcinellus* with complete support (PP = 1.0) in both analyses. SNAPP analyses without the outgroup (*Nipponia nippon*) recovered the same branching structure with complete support. Similarly, the BEAST2 phylogeny based on ND2 sequence data (Fig. 5b) recovered a sister relationship between primarily *chihi* and primarily *ridgwayi* (PP = 0.83) haplotypes (see exceptions above), which in turn were sister to primarily *falcinellus* (PP = 0.94) haplotypes. The BEAST2 divergence estimate between *falcinellus* and (*chihi* + *ridgwayi*) groups was 3.5 Mya (HPD = 0.6 – 8.0 Mya) and between *chihi* and *ridgwayi* groups was 2.7 Mya (HPD = 0.4 – 6.5 Mya). ND2 average pairwise distance between individuals was 1.9% between the *chihi* and *falcinellus* haplotype groups, 1.3% between *chihi* and *ridgwayi*, and 1.4% between *falcinellus* and *ridgwayi*. There was

negligible haplotype divergence within species, for example between *falcinellus* samples from Australia and North America or between *chihi* samples from North and South America (Table S10).

Bayes factor species delimitation provided mixed support for treatment of the *Plegadis* ibis as separate species. In the analysis including the outgroup alleles from *Nipponia nippon*, a model in which the species were lumped had the highest marginal likelihood (Table S11). Bayes factors supported this model marginally over lumping just *chihi+ridgwayi* or *chihi+falcinellus* and strongly over treating all three as separate species (Bayes Factors >10 are generally considered decisive; Kass and Raftery 1995). Results including the admixed individuals were similar (Table S12). However, because *Nipponia nippon* is very divergent from *Plegadis*, inclusion of the outgroup results in most biallelic SNPs representing fixed differences between the outgroup and all *Plegadis* species. When the outgroup was removed, we were able to examine many more SNPs that were variable within *Plegadis* (192 SNPs versus 26 SNPs variable within *Plegadis* in the outgroup dataset). This resulted in strong support for splitting the three species rather than combining either the sister species *chihi* and *ridgwayi*, or the widely hybridizing *chihi* and *falcinellus* (Table 1). Therefore, although the BFD* results are sensitive to the use of datasets with and without the outgroup, they do appear to provide support for the separation of the three *Plegadis* ibis as separate species.

Demographic history

By calibrating the divergence time of (*chihi + ridgwayi*) and *falcinellus* from G-PhoCS using the date their haplotype groups diverged in the time-calibrated tree of ND2 (see above), we were able to examine absolute estimates of migration rates, effective population sizes, the divergence time between *chihi* and *ridgwayi*, and substitution rate parameters. Analyses

including both pure and admixed individuals (Fig. 5c; Table 2) indicated migration rates are slightly higher from *chihi* into invading *falcinellus* than the reverse, and also that rates from *chihi* to *ridgwayi* were higher than from *ridgwayi* to *chihi*. This is consistent with evidence of movement of *chihi* alleles into *falcinellus* based on genomic cline parameters. In the analysis of pure individuals only, however, migration was about 3.63 times higher from *falcinellus* (invader) to *chihi* than the reverse (Table S13). Because this analysis ignored admixed individuals, it may reflect demographic dynamics prior to recent admixture events and provide information complementary to the results of analysis including admixed birds. In the analysis without admixed individuals, the divergence time between *chihi* and *ridgwayi* was 2.9 Mya (HPD = 2.5 - 3.2 Mya), roughly consistent with the divergence of *chihi* and *ridgwayi* ND2 haplotype groups. Ancestral effective population size (N_e) in the same analysis was 2.0 million (HPD =1.8 - 2.1 million; Fig. 5c) and remained similar in *chihi* at 2.0 million (HPD = 1.9 - 2.2 million) but increased in *falcinellus* to 4.4 (HPD = 4.1 - 4.7 million). The UCE substitution rate was 1.03×10^{-4} substitutions per site per million years (95% highest posterior density [HPD] = 4.48×10^{-5} to 6.30×10^{-4} substitutions per site per million years).

DISCUSSION

Our study investigated a dramatic instance of transoceanic colonization and revealed extensive hybridization and introgression between congeners. The incumbent White-faced Ibis (*Plegadis chihi*) of North America is hybridizing with the colonist from the Old World, Glossy Ibis (*P. falcinellus*), and admixed individuals occur widely across the continent far from the core area of sympatry in the western Gulf Coast of the United States. Our results support prior evidence for hybridization and the wide dispersion of hybrid individuals based on field observations and phenotypic data (Patten & Lasley 2000, Aterburn & Gryzbowski

2003, Leukering 2008). Surprisingly, we also found evidence for admixture between South American *chihi* and Puna Ibis (*P. ridgwayi*), which may reflect either recent contact between the two species or older admixture events. Although birds are highly vagile, there are few other instances of recent cross-continent colonizations. Wading birds are exceptional in this respect. Cattle Egret (*Bubulcus ibis*), a Eurasian and African species, was first found in Venezuela in 1943 (Phelps 1944) and has subsequently expanded across the Americas in a similar fashion to *falcinellus* (Rice 1956). Cattle Egrets have also colonized Australia from Asia (Rice 1956). Little Egret (*Egretta garzetta*) colonized the West Indies in 1954, and the Western Reef-Heron (*Egretta gularis*) may well be following suit (Mlodinow et al. 2004). However, few of these colonizations have resulted in secondary contact with a closely related congener or in significant hybridization (hybridization is rare between Little Egret and its close relative the Snowy Egret [*E. thula*] in the West Indies). The case of *Plegadis* is therefore remarkable in that a transoceanic colonization event has resulted in secondary sympatry and extensive hybridization among species that are closely related, ecologically similar, previously allopatric, and highly mobile.

Divergence among species and across the genome

Consistent with phenotypic differences and current taxonomic treatment, the three *Plegadis* species exhibit sufficient divergence across the genome for separation using population genetic clustering methods. Moreover, Bayesian species delimitation based on the multispecies coalescent supported treatment of the three *Plegadis* as separate species. Divergence between the species was widely distributed across the genome based on the distribution of fixed SNPs and d_{XY} and F_{ST} values. This contrasts with many recent studies in birds that have found isolated regions of elevated divergence, and often support for the

importance of those regions in reproductive isolation (e.g., Poelstra et al. 2014, Küpper et al. 2016, Tuttle et al. 2016, Campagna et al. 2015, Toews et al. 2016, Oswald et al. 2016, Stryjewski & Sorenson 2017). Many of those studies, however, involved taxa that likely experienced episodes of contact and introgression during the history of species formation. Hybridization contributes to heterogeneous divergence across the genome because some genomic regions have a higher probability of introgression (Harrison & Larson 2014). In contrast, prolonged long-distance allopatry in *Plegadis* may have suppressed genome-wide heterogeneity in divergence. We did find that divergence, based on d_{XY}, was higher on the Z chromosome relative to autosomes, consistent with proliferating evidence for the importance of the Z chromosome in avian divergence and speciation (Irwin 2018).

Despite the lack of sharp peaks of differentiation among *Plegadis*, sufficient variation in divergence across the genome was present to conduct a preliminary examination of signatures of parallel divergence across independent lineages. We did find that loci of elevated d_{XY} between species tended to be shared across all three pairwise species comparisons, indicative of correlated divergence. However, d_{XY} in all comparisons was positively correlated with genetic diversity (based on observed heterozygosity) at a given locus, similar to results from recent studies in Muscicapid flycatchers (Burri et al. 2015, van Doren et al. 2017, Vijay et al. 2017). Recent analyses of whole genome datasets from birds suggest the concordance between divergence and diversity may be driven by linked selection combined with a conserved recombination landscape across the avian genome, rather than parallel selection on the same loci across lineages (Burri et al. 2015, Singhal et al. 2015, van Doren et al. 2017, Dutoit et al. 2017). Given the association between divergence and genetic diversity we observed, a similar situation is likely the cause of shared regions of divergence in pairwise comparisons across the three *Plegadis* species.

Admixture and introgression across space and the genome

Between *chihi* and *falcinellus*, for which our sampling of hybrids was sufficient for more detailed analyses, we found that many hybrid individuals exhibited low to moderate levels of heterozygosity at SNPs fixed between the parental species. This signature is indicative of later-generation hybrids rather than F_1 crosses and suggests that reproductive isolation between the two species is weak. We also failed to find loci with particularly narrow genomic clines relative to the genome-wide admixture gradient. This differs from the heterogeneous patterns of introgression across the genome often observed in hybrid zone studies (Gompert et al. 2017). Selection against hybrids, which is implicated in maintaining hybrid zones in the context of the equilibrium tension zone model, is expected to produce heterogeneity in cline width across the genome (Barton 1983, Payseur 2010). As a result, we failed to find support for selection against hybrids between *chihi* and *falcinellus*. There may be little fitness disadvantage to interspecific breeding in these species, but further confirmation from data on mating preferences and outcomes in the field is desirable. The high vagility of *Plegadis* ibis may also facilitate wide clines through the regular dispersal of individuals across populations with different genomic backgrounds.

Contrary to cline width, cline centers did exhibit heterogeneity across loci, with many loci exhibiting greater *chihi* ancestry in individuals than expected based on their genome-wide admixture gradient. Many previous hybrid zones studies have found similar asymmetries in introgression at a subset of markers (e.g., Brumfield et al. 2001, Devitt et al. 2011). The weak correlation between F_{ST} and the cline center parameter might suggest that directional selection is involved in asymmetrical introgression in some loci (Gompert et al. 2012, Taylor et al. 2014). However, neutral processes may also be at work. Migration rates from our demographic model suggest that gene flow from *chihi* to *falcinellus* is similar to or higher than gene flow in the other direction (in the analysis containing admixed individuals).

This is consistent with expectations in cases of hybridization resulting from colonization of one species into the range of another. In such cases, the movement of alleles generally flows from the native species to the invader as invaders move into areas lacking conspecifics with which to mate (Moran 1981, Barton & Hewitt 1985, Allendorf et al. 2001, Buggs 2007, Currat et al. 2008). This also fits with our observation that in the area of sympatry between the two species in the Gulf States, where many of our admixed individuals were collected, there is a lower density of birds with *falcinellus* than *chihi* phenotypes. It seems likely that asymmetric introgression resulting from recent invasion dynamics, combined with stochasticity and recombination rate variation across the genome, is at least partly responsible for the clines with elevated introgression from *chihi* relative to the genome-wide background.

It is important to note that our inferences pertaining to patterns of differentiation and cline parameters across the genome may be influenced by the marker set we examined. Specifically, markers flanking UCEs may fail to capture genomic regions of elevated divergence or reduced gene flow. However, the rapid decay of Moran's *I* in cline parameters suggests that the variable loci used for cline analyses may not be impacted by processes responsible for conservation in the nearby UCEs. The non-random dispersion of UCEs across the genome (Bejerano et al. 2004, Kim & Pritchard 2007, Polychronopoulos et al. 2014), on the other hand, may be more problematic. If regions of elevated divergence or reduced introgression tend to occur in areas without UCEs, they may not be represented in our dataset. Of course, similar concerns exist with any reduced representation marker set non-randomly distributed across the genome, including those obtained with genotyping by sequencing or RAD-Seq (Lowry et al. 2017). Whole genome coverage will be required to fully resolve the distribution of divergence and introgression in *Plegadis*.

Our sampling of individuals may also have affected observed patterns of admixture across space and the genome. Although most samples were from the breeding season and in known breeding areas (Table S1), few were from birds collected on nests in actual colonies. As a result, some admixed individuals observed away from the contact zone may have been passing through rather than breeding locally. However, most analyses should not be affected by sampling of some non-breeding individuals. For example, Structure and genomic cline analyses do not require the *a priori* identification of pure individuals (Szymura & Barton 1986, Pritchard et al. 2000, Gompert & Buerkle 2012), and thus should be valid provided we sampled at least some individuals with primarily parental genomic backgrounds. The presence of distinct *falcinellus* and *chihi* groups made up primarily of individuals far from the zone of contact and each containing little Hardy-Weinberg or linkage disequilibrium based on Structure results suggests that we were able to sufficiently sample parental genomic backgrounds.

The biogeographic history and future of Plegadis

We found that the deepest divergence within *Plegadis* was the split between the Old World and Australian *falcinellus* and the two New World species, which diverged more recently. Widespread human colonization and deforestation associated with European settlement may have led to an increase of open habitats and agricultural fields that resulted in the ability of *falcinellus* to colonize other continents, following either natural or perhaps boat-assisted long-distance travel across water. Our estimates of effective population size suggest populations of all three species, and particularly *chihi* and *falcinellus*, are large and have increased over their history, and population surveys and vagrancy patterns also support expansions in the last century (Patten & Lasley 2000, Jorgensen & Dinsmore 2005, Shaffer et

al. 2007). Large-scale irrigation practices rapidly expanded worldwide in the mid-1900s (Sokja et al. 2002), and irrigation practices likely facilitated recent expansions (Patten & Lasley 2000). Also, during the last century, *ridgwayi* has expanded its range and colonized the coastal marshes and agricultural areas in Peru (Pearson 1974, Hughes 1984, Gonzalez et al. 1999), potentially also as a result of human-mediated habitat modification.

The migratory and nomadic nature of *Plegadis* ibis, combined with additional anthropogenic habitat changes, will likely result in further range expansion, sympatry, hybridization, and introgression across the New World. In *chihi* and *falcinellus*, the wide area of admixture, evidence that introgression can occur across the genome, and lack of obvious selection against hybrids suggest that significant gene flow and potentially homogenization of parental genetic backgrounds are possible. In addition to field studies to assess the frequency and outcome of interspecific mating and finer-resolution genomic scans for signatures of reproductive isolation, monitoring of introgression dynamics through time will help reveal the fate of genetic diversity in *chihi* and *falcinellus*. More work and larger samples are also needed from South American *chihi* and *ridgwayi* to assess whether admixture in that region is due to recent hybridization and, if so, to understand the causes and impacts.

ACKNOWLEDGMENTS

Tissues were collected by the Louisiana State University Museum of Natural Science or were donated to LSUMNS by the Florida Museum of Natural History (David Steadman and Andrew Kratter), San Bernardino County Museum (Eugene Cardiff), and Kevin Johnson (Illinois Natural History Survey). For loans of additional tissues we thank the American Museum of Natural History (Paul Sweet and Peter Capainolo) and CSIRO (Leo Joseph). Sonal Singhal provided advice with analyses. Van Remsen, Ryan Terrill, Matthew Brady,

Oscar Johnson, Daniel Lane, Rachael Herman, Jason Sardell, the David Blackburn lab, and anonymous reviewers provided comments that improved the manuscript. Louisiana State University High Performance Computing, the University of Michigan Advanced Research Computing Technology Services, and Daniel Rabosky provided access to computational resources.

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Data Accessibility Statement

Sequence data can be found NCBI Sequence Read Archive (SRA) PRJNA506077; SAMN10442556- SAMN10442621 and GenBank (ND2 data; Accession MK208900-MK208923). Files containing UCE SNPs and UCE alignments can be found in the Supplementary Material.

Author Contributions

J.A.O. designed the project, collected and analyzed data, and wrote the manuscript; M.G.H. analyzed data and wrote the manuscript; R.C.R. and D.U.F. data collected data; D.L.D. and S.W.C. designed the project and collected specimens; R.T.B. designed the project, contributed materials and resources, and assisted in writing the manuscript. All authors contributed to the final manuscript. This work was supported in part by the US National Science Foundation (grant DBI-1523893 to M.G.H.). Funding was provided by Research Experiences for Undergraduates (REU) and Research Assistantships for High School Students (RAHSS) supplements to National Science Foundation grant DEB-1146255 (to R.T.B.).

FIGURES

Fig. 1. Range maps for the three species of *Plegadis* ibis. The species names are colored according to the range map color for that species. Within *falcinellus* and *chihi*, lighter shades depict breeding-only distributions, intermediate shades depict resident distributions, and dark shades depict winter-only distributions. A blue arrow depicts the hypothetical route of colonization by of the New World by *falcinellus.* A black circle outlines the core region of current sympatry between *falcinellus* and native *chihi*. Ibis illustrations are courtesy the Handbook of the Birds of the World Alive/Lynx Edicions (del Hoyo et al. 2018).

Fig. 2. Assignments of individuals and populations based on STRUCTURE results with K=3. **(A)** The probability of ancestry of each individual (horizontal axis) to each of three populations (vertical axis). The three clusters correspond closely with phenotypic species assignments with green corresponding largely with *falcinellus* phenotypes, orange with *chihi*, and red with *ridgwayi*. **(B)** Pie charts indicate the proportion of ancestry at a locality assigned to each of the three genetic clusters from STRUCTURE with K=3, plotted on the breeding range distributions for the three species, colored as in figure 1. The sizes of the pie charts represent the relative square-root transformed population sample sizes for those areas and are on the same scale between the detailed map and the inset.

Fig. 3. Patterns of introgression across the genome between *chihi* and *falcinellus*. (**A**) Heterozygosity of each admixed individual relative to its hybrid index at SNPs fixed between parental forms (1 is pure *chihi* ancestry). The triangle outlines possible parameter values. (**B**) Bayesian genomic clines for all SNPs. Clines with outlier values of cline center (**α**) are colored black, whereas the remainder are colored gray. (**C**) The distributions of F_{ST} between *chihi* and *falcinellus* and both cline center and cline width (**β**) parameters across the genome. (**D**) Both cline parameters show a rapid decay in Moran's I with genomic distance.

Fig. 4. Pairwise species comparisons reveal correlations in the loci exhibiting elevated divergence. **(A)** F_{ST} is weakly correlated across loci in all three possible pairwise species comparisons, whereas **(B)** d_{XY} shows a stronger correlation, particularly in the outliers with highest divergence. Correlations are based on ordinary least squares regression.

Fig. 5. *Plegadis* species histories. **(A)** The SNAPP tree of "pure" individuals with *Nipponia nippon* included as an outgroup. **(B)** The ND2 gene tree based on BEAST analysis. Colors to the right represent the Structure assignments of each individual based on UCE data and correspond to the colors in Figure 2 (hatching indicates an individual exhibiting admixture between two clusters). **(C)** A graphical depiction of the G-PhoCS results using only "pure" individuals. The height of each rectangle represents the relative effective population size (N_e), and the size of the arrows represents the relative rate of migration between populations (m_{sx}).

Table 1. Bayes factor species delimitation (BFD*) results with pure individuals and no outgroup.

	marginal likelihood	Bayes Factor (relative to best model)
All separate	-383.1453771	
chihi and *ridgwayi* lumped	-422.5165547	-39.37117759
chihi and *falcinellus* lumped	-433.5622228	-50.41684571

Table 2. Demographic parameter estimates from G-PhoCS with pure and admixed individuals.

	Posterior Mean	95% HPD lower	95% HPD upper
N_e *chihi*	3,390,152	3,137,155	3,654,714
N_e *falcinellus*	5,905,369	5,576,330	6,239,614
N_e *ridgwayi*	408,553	339,449	483,440
N_e (*chihi*+*ridgwayi*)	1,902,533	1,668,330	2,150,036
N_e ancestral	2,461,438	2,262,511	2,661,233
Divergence Time *chihi* vs. *ridgwayi*	1,015,455	894,017	1,155,398
Divergence Time root	NA	NA	NA
Migration *chihi* to *falcinellus*	0.858	0.701	1.020
Migration falcinellus to *chihi*	0.678	0.577	0.787
Migration *chihi* to *ridgwayi*	0.015	0.000	0.048
Migration *ridgwayi* to *chihi*	0.007	0.001	0.017
Migration *falcinellus* to *ridgwayi*	0.440	0.229	0.675
Migration *ridgwayi* to *falcinellus*	0.005	0.000	0.015
Substitution Rate (s/s/My)	8.65×10^{-5}	5.28×10^{-4}	3.75×10^{-5}