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Determining evolutionary origin and phylogenetic relationships of mallard-like ducks of Oceania, greater Indonesia, and the Philippines with ddRAD-seq data

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ABSTRACT

Aim: We aim to determine the evolutionary origins and population genetics of mallard-like ducks of Oceania, greater Indonesia, and the Philippines.
Location: Oceania, greater Indonesia, and the Philippines.
Taxon: Mallard (*Anas platyrhynchos*), Pacific black duck (*A. superciliosa* spp.), and Philippine duck (*A. luzonica*)
Methods: Thousands of nuclear ddRAD-seq loci and the mitochondrial DNA control region were assayed across individuals representative of each species' range. We assessed population structure and phylogenetic relationships, as well as estimated demographic histories to reconstruct the biogeographical history of each species.
Results: Philippine and Pacific black ducks represent unique genetic lineages that diverged from the mallard 1–2 million years ago. We find no support for the Philippine duck representing a hybrid species as once posited; however, their low levels of genetic diversity requires further attention. We find a lack of substructure among Philippine ducks. However, we found pronounced differentiation between subspecies of Pacific black ducks, especially between *A. s. superciliosa* from New Zealand and *A. s. rogersi* from Australia, Papua New Guinea, and Timor-Leste, Indonesia. *Anas superciliosa pelewensis* gave mixed results; individuals from the Solomon Islands were differentiated from the other subspecies, but those from the island of Aunu'u, American Samoa, were genetically more similar to *A. s. rogersi* than *A. s. pelewensis* samples from the Solomon Islands. Finally, we find limited evidence of interspecific gene flow at evolutionary scales, and mallard introgression among contemporary samples.
Main conclusions: Mallard-like ducks radiated across Oceania, greater Indonesia, and the Philippines within the last 2 million years. Only the Pacific black duck showed unique sub-structuring that largely followed known subspecies ranges, except for *A. s. pelewensis.* We posit that the high interrelatedness among Solomon Island samples suggests that their genetic distinctiveness may simply be the result of high levels of genetic drift. In contrast, we conclude that mainland Australian Pacific black ducks were the most likely source for the recent colonization of American Samoa. As a result, our findings suggest that either the *A. s. pelewensis* subspecies designations and/or its geographical range may require re-evaluation. Continued re-evaluation of evolutionary and taxonomic relationships is necessary when attempting to reconstruct and understand biogeographical histories, with important implications towards any attempts to implement conservation strategies.

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1. Introduction

Accurate taxonomic identification has important implications for any attempts to implement conservation strategies (Supple and Shapiro, 2018), and thus, demarcating accurate conservation units is a critical first step (Brown et al., 2021; Peters et al., 2016). The criteria for defining a species are highly debated and largely subjective (e.g., evolutionary, biological, phylogenetic, genic, differential fitness species concepts, etc.; Cracraft, 1983; Eldredge, 1980; Frankham et al., 2012; Mayr, 1999, 1942; Wu, 2001), and identifying lower levels of partitioning (i.e., subspecies) can be equally complicated (Patten, 2015).

Advances in molecular methods provide tools for the conservation community to examine the extent of genetic differentiation among populations, and thus, where they fit along the speciation continuum (Brown et al., 2021; Crandall et al., 2000; Delaney et al., 2008; Oswald et al., 2016; Peters et al., 2016; Ralls et al., 2018). Such efforts have been especially important for the conservation of locally adapted and cryptic species (Esterhuizen et al., 2013; Johnson et al., 2010; Kon et al., 2007; Wada et al., 2003; Watanabe et al., 2010). Here, we apply a landscape genomics approach to examine the biogeographic and evolutionary histories of several mallard-like ducks endemic to Oceania, Indonesia, and the Philippines, and relate these to their island biogeography.

The Mallard Complex is comprised of 14 closely related species of ducks with populations now found on almost all major landmasses and islands (Lavretsky et al., 2014b, 2014a). Lavretsky et al. (2014b) hypothesized that an ancestral African mallard-like species likely simultaneously colonized Oceania and the Holarctic, with divergence beginning approximately 1 million years ago. Currently, there are two extant species of mallard-like ducks endemic to Oceania, Greater Indonesia, and the Philippines: the Philippine duck (*Anas luzonica*) and Pacific black duck (*A. superciliosa*). Despite current classifications, the history of island colonization and adaptation remains unclear. For example, the Philippine duck has been hypothesized to be of hybrid origin resulting from interbreeding between ancestral Pacific black duck and Holarctic mallard (*A. platyrhynchos*) populations (Lavretsky et al., 2014b). The same history was proposed for another geographically close but extinct species, the Mariana mallard (*A. oustaleti*; Yamashina, 1948), and empirical evidence exists supporting a hybrid origin for the Hawaiian duck (*A. wyvilliana*) – i.e., Laysan duck (*A. laysanensis*) x mallard (Lavretsky et al., 2015). Thus, it is evident that interbreeding among these mallard-like ducks can result in the evolution of unique taxa.

Whereas the Philippine duck is monotypic and endemic to the Philippines, three Pacific black duck subspecies are currently recognized; these range widely across Oceania and Indonesia (Fig. 1B). Specifically, *A. s. rogersi* is found throughout Australia, southern New Guinea, and Timor-Leste, Indonesia, where it is commonly known as the Pacific black duck. In New Zealand, *A. s. superciliosa* is commonly known as the grey duck. Finally, *A. s. pelewensis* ranges across the Solomon Islands, northern New Guinea, and other Pacific Islands and is commonly known as the lesser grey duck (Beehler and Pratt, 2016; Kear, 2005). Most taxonomic and range classifications for Pacific black ducks are based on morphological differences (Williams, 2019), and although some genetic studies of individual subspecies exist, they were based on only a few molecular markers (Brown et al., 2021; Rhymer et al., 2004; Seibert et al., 2023). Therefore, a comprehensive genomic analysis of all three subspecies is needed to test whether morphological, molecular, and taxonomic associations are consistent across their range.

Here, we take a landscape genomics approach, examining the

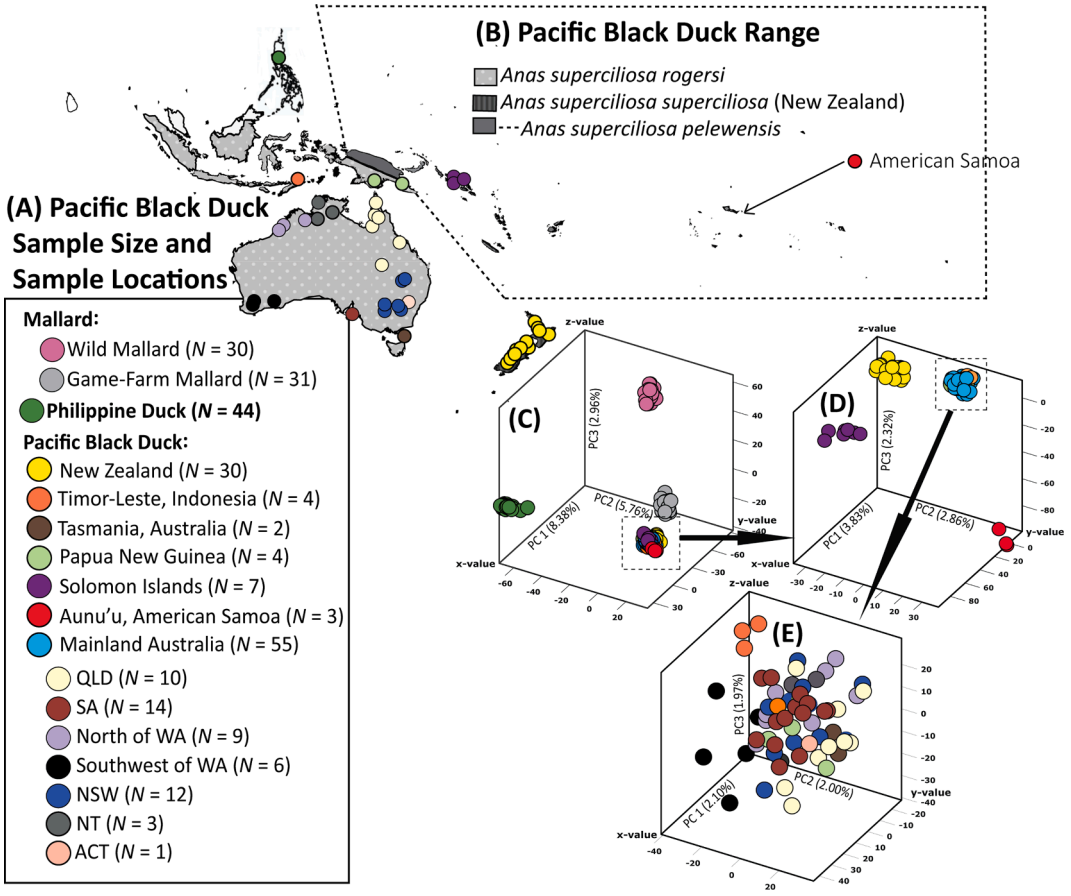

Fig. 1. (A) Sampling locations and ranges of Philippine ducks and Pacific black duck subspecies. Principal Component Analysis (PCA) of nuclear ddRAD-seq bi-allelic SNPs for (B) mallards, Philippine ducks, and Pacific black ducks, as well as partitioned datasets of Pacific black ducks by (D) all sampled location or (E) those of known *A. s. rogersi* subspecies status. Sample sizes across analyzed groups are provided.

mitochondrial control region and thousands of nuclear loci for samples across the ranges of the Philippine and Pacific black ducks (Fig. 1A, B). Notably, our sampling included Pacific black ducks from Aunu'u, American Samoa; these represent one of the easternmost locations and recent colonization events for the species (~1970s; U.S. Fish and Wildlife Service employees, *personal communication,* 2019). Given the geographic proximity of American Samoa and the Solomon Islands, we expect these individuals to be genetically similar to *A. s. pelewensis*. In addition to assessing population structure within both species, we test whether the Philippine duck is an example of a hybrid species, while also determining whether contemporary secondary contact with mallards is increasing hybridization for the Philippine and/or Pacific black duck. Both the Philippine and Pacific black ducks have come into secondary contact with introduced mallards (wild or domestic) and are potentially threatened by introgressive hybridization, although studies of this issue are limited (Brown et al., 2021; Rhymer et al., 2004; Rhymer and Braun, 1994). Most current work has solely focused on hybridization of Pacific black ducks in mainland Australia (Guay et al., 2014; Taysom, 2015) and New Zealand (Dryer and Williams, 2010; Gillespie, 1985; Guay et al., 2014; Guay and Tracey, 2009; Tracey et al., 2008; Williams and Basse, 2006), and no studies have examined potential hybridization between Philippine ducks and mallards. Moreover, given that the Philippine duck and many populations of Pacific black duck are considered vulnerable and are of high conservation concern, establishing measures of standing genetic diversity and population interconnectedness will be critical in future conservation planning (Brown et al., 2021).

2. Materials and methods

2.1. Sampling and DNA isolation

Sampling consisted of 44 Philippine ducks and 105 Pacific black ducks across their respective ranges (Fig. 1A, B; Supporting Information Table S1). Philippine ducks were sampled from wild birds that were confiscated from poachers in 2014. For Pacific black ducks, *A. s. rogersi* was sampled from mainland Australia ($N = 55$), Timor-Leste, Indonesia ($N = 4$), Tasmania, Australia ($N = 2$), and Papua New Guinea ($N = 4$); *A. s. superciliosa* from New Zealand ($N = 30$); and *A. s. pelewensis* from Aunu'u, American Samoa ($N = 3$), and the Solomon Islands ($N = 7$). Previously published comparable ddRAD-seq and mtDNA sequences for game-farm ($N = 31$) and wild mallard ($N = 30$) samples were included in analyses (Lavretsky et al., 2020, 2019a), permitting us to test for evidence of ancestral (i.e., hybrid species hypothesis for the Philippine duck; Lavretsky et al., 2014b) or contemporary gene flow between wild and/or domestic mallards, Philippine ducks, and Pacific black ducks. Note recent molecular studies recovered wild mallards being weakly structured across their Holarctic range but providing identical demographic histories regardless of sampling location, as well as that the same game-farm mallard lineage propagated world-wide, including New Zealand (Lavretsky et al. 2023). Thus, despite these mallards not being sampled from the same countries as our Philippine and Pacific black ducks, they remain good reference sets to test for signatures of gene flow. Finally, New Zealand grey duck samples were specifically chosen based on preliminary ancestry analyses showing that they were not admixed with New Zealand mallards (Brown, 2021); thus, our analyses only focus on ancestral gene flow for this group.

DNA was extracted from blood or muscle tissue for Pacific black ducks, and liver from confiscated illegally harvested wild ducks (Philippine ducks; Licuanan et al., 2017) using Qiagen's DNeasy Blood and Tissue kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA). We ensured DNA quality based on the presence of high molecular weight bands visualized using gel electrophoresis with a 1% agarose gel, and DNA was quantified using a Qubit 3 Fluorometer (Invitrogen, Carlsbad, CA).

2.2. Mitochondrial DNA

Primers L78 and H774 were used to amplify the mtDNA control region (Sorenson et al., 1999; Sorenson and Fleischer, 1996) following PCR reaction concentrations and thermocycler conditions described in (Lavretsky et al., 2014a). The PCR products were visualized via agarose electrophoresis and then purified using ExoSAP-IT (ThermoFisher). Clean PCR products were then sent for Sanger sequencing using the L78 primer on a 3130XL Genetic Analyzer at either the Core Laboratories of Arizona State University School of Life Sciences or the University of Texas at El Paso, Border Biomedical Research Center's Genomic Analysis Core Facility. Sequences were aligned and edited using Sequencher version 4.8 (Gene Codes) and all sequences were subsequently submitted to GenBank (accession numbers PP746861 - PP756430; see Supporting Information Table S1 for sample specifics). Comparable mtDNA control region sequences of mallards and Pacific black ducks from previous studies were included in the alignments and analyses (Lavretsky et al., 2020, 2014b, 2014a; Wells et al., 2019). We visualized relationships among mtDNA haplotypes sequenced for Pacific black ducks, mallards, and Philippine ducks by reconstructing a median-joining haplotype network in the program NETWORK v. 4.6.1.6 (Bandelt et al., 1999). Finally, pairwise estimates of relative divergence (Φ_{ST}) and nucleotide diversity (π) of the mtDNA control region were calculated in the R package PopGenome v. 2.7.5 (Pfeifer et al., 2014).

2.3. Double digest restriction-site associated DNA (ddRAD-seq) library preparation and bioinformatics

For each sample, we constructed ddRAD-seq libraries following procedures outlined in DaCosta and Sorenson (2014), but with fragment size selection following Hernández et al., (2021). Briefly, genomic DNA was enzymatically fragmented using SbfI and EcoRI restriction enzymes, Illumina TruSeq compatible barcodes were then ligated, which permitted future de-multiplexing. Next, 0.8x double-sided bead clean-up using QuantaBio sparQ PureMag Beads resulted in a fragment size range of 100–400 base pairs (bp), with an average size fragment of 350 bp. Size-selected libraries were then PCR amplified before being cleaned using a 1.8x solution of sparQ PureMag Beads. Clean libraries were then pooled in equimolar concentrations, and 150 base pair (bp), single-end (SE) sequencing was completed on an Illumina HiSeq X at Novogenetics LTD (Sacramento, CA). Illumina reads were deposited in NCBI's Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra; SRA BioProject ID PRJNA1106373; see Supporting Information Table S1 for sample specifics).

First, we used the *ddRADparser.py* script of the BU ddRAD-seq pipeline (DaCosta and Sorenson, 2014) to de-multiplex raw Illumina reads based on perfect barcode/index matches. Illumina reads were then processed and de-multiplexed via the computational pipeline from DaCosta and Sorenson (2014; https://github.com/BU-RAD-seq/ddRAD-seq-Pipeline). Reads were condensed by similarity (i.e., 90% sequence similarity) and filtered by PHRED score (20) using the *CondenseSequences.py* and *FilterSequences.py* scripts, respectively.

Before proceeding with genotyping, comparable molecular ddRAD-seq data from wild and game-farm mallards and Pacific black ducks from northern mainland Australia were included in the dataset (Lavretsky et al., 2020, 2019a; Seibert et al., 2023). Samples were then concatenated and clustered into putative loci using USEARCH v. 5 (Edgar, 2010) with an ID threshold of 0.85. High quality reads were then mapped to the wild mallard reference genome (Lavretsky et al. 2023) using BLASTN v. 2 (Altschul et al., 1990). Nearly identical BLAST hits were then combined to minimize error of an arbitrary similarity threshold (Harvey et al., 2015). Reads were then aligned using MUSCLE v. 3 (Edgar, 2004) and genotyped using the *RADGenotypes.py* script. Genotypes were then categorized as homozygous if $\geq$93% of reads were identical, heterozygous if the second allele was present in 29% of the reads, and ambiguous if genotypes were outside these thresholds.

Therefore, genotypes were placed into four categories, 'missing', 'good', 'low depth', and 'flagged' by the pipeline. Loci with <10% 'missing' and ≤6% 'flagged' genotypes were scored for downstream analysis. Any locus that was flagged were then manually assessed in Geneious v. 10.2.6 (Biomaters Inc., San Francisco, CA, USA). Briefly, loci "flagged" due to excessive SNPs, indels, or heterozygosity were either excluded or manually corrected. Finally, autosomal and Z-sex chromosome linked loci were each identified based on perfect blast hits to the reference mallard genome. Final output files were created (i.e., FASTA, nexus, etc.) using scripts within the DaCosta and Sorenson pipeline (2014; http://github.com/BU-RAD-seq/ddRAD-seq-Pipeline). To eliminate biases associated with RAD-seq protocols, we excluded any sequences with overall PHRED scores <30, and alleles with less than 5x sequence coverage (i.e., 10x coverage per locus) were considered missing (Lavretsky et al., 2016).

2.4. Nuclear population structure

A dataset consisting of independent bi-allelic ddRAD-seq autosomal SNPs, and without *a priori* subspecies or population identity were used across analyses of population structure. PLINK v. 1.90 (Purcell et al., 2007) was used to filter SNP datasets for singletons (−-maf 0.0055), missingness (−-geno 0.15), and linkage disequilibrium (LD) (−-indep-pairwise 2 1 0.5) where one of two SNPs would be randomly excluded if an LD correlation factor (r^2) > 0.5 was found between them. In addition to analyzing all possible samples, more fine scale evaluations were done with datasets including Pacific black ducks only.

First, the program fineRADstructure, which includes RADpainter v. 0.1 and finestructure (Lawson et al., 2012; Malinsky et al., 2018) was used to estimate pairwise-sample co-ancestry and visualize relationships among Pacific black ducks only. The analysis included a burn-in of 100,000 iterations and 100,000 MCMC steps, with default settings for remaining parameters. Results were visualized using R scripts fineradstructureplot.r and finestructurelibrary.r (available at: http://cichlid.gurdon.cam.ac.uk/fineRADstructure.html).

Next, we used the R package "adegenet" with the function *dudi.pca* (Dray and Dufour, 2007; Jombart, 2008) to run a Principle Component Analysis (PCA). Relationships were visualized by plotting the first three principal components. Additionally, the program ADMIXTURE v. 1.3.0 (Alexander et al., 2009; Alexander and Lange, 2011) was used to calculate maximum-likelihood-based individual assignments. SNP datasets were formatted following steps outlined in Alexander and Lange (2015). Each ADMIXTURE analysis was run with a 10-fold cross validation (CV) and with a quasi-Newton algorithm employed to accelerate convergence (Zhou et al., 2011). ADMIXTURE uses a block relaxation algorithm for point estimation and terminated once the change in the log-likelihood of the point estimations increased by < 0.0001. We ran ADMIXTURE for K populations of 1 through 10, with 100 iterations per each value of K. The optimum K was based on the average of CV-errors across the iterations per K value; however, higher values of K were examined to test for additional resolution. The R program PopHelper (Francis, 2016) was then used to convert files from each evaluated K from ADMIXTURE into CLUMPP files. A Greedy algorithm and 1,000 permutations was used in the program CLUMPP v. 1.1 (Jakobsson and Rosenberg, 2007) to determine the soundness of each assigned K value.

Finally, composite pairwise-species estimates of relative divergence (Φ_{ST}) and nucleotide diversity (π) for concatenated FASTA files of autosomal and Z-sex chromosome linked ddRAD-seq loci were calculated in the R package PopGenome v. 2.7.5 (Pfeifer et al., 2014).

2.5. Phylogenetic analyses

Phylogenetic relationships across all sampled groups were assessed independently for nuclear loci and mtDNA. Note that we treated Philippine ducks, game-farm mallards, and wild mallards as their own respective lineages, while Pacific black ducks were analyzed by sampled population to determine how well the three subspecies lineages are recapitulated. Although we did not include an outgroup in analyses, we were able to still understand general relationships among groups as the Philippine duck acted as a natural outgroup to root trees on. First, the bi-allelic ddRAD-seq autosomal SNP dataset described above was analyzed in the program TreeMix v. 1.12 (Pickrell and Pritchard, 2012). TreeMix uses a maximum likelihood (ML) framework to simultaneously estimate phylogenetic relationships, while also testing species trees with and without gene flow. In short, TreeMix analyses are optimized by adding migration edges until ≥ 98% of the variance within the data was explained (Pickrell and Pritchard, 2012). Additionally, statistical significance was assessed by estimating standard errors (−se) and associated p-values for each migration edge. Analyses were run with global rearrangements occurring during tree building (−global) and based on 1,000 bootstraps and a burn-in of 10%. Finally, trees were summarized with TreeAnnotator v. 2.5.2 (Rambaut et al., 2018) and viewed in FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

For mtDNA, phylogenetic relationships were analyzed in the program *BEAST v. 2.5.2 (Bouckaert et al., 2014). BEAST implements a Bayesian tree reconstruction method that estimates a posterior distributions of species trees (Bouckaert et al., 2014). The mtDNA species tree was based on a strict mutation clock and a Hasegawa-Kishino-Yano (HKY) substitution model (Hasegawa et al., 1985) with gamma distribution and invariable sites as previously found to be optimum for mtDNA (Lavretsky et al., 2014a, 2014b). Analyses were run for 500,000,000 MCMC generations and sampled every 5,000 steps until all effective sample size (ESS) values across parameters were >200. Next, the first 10% of trees were discarded in TreeAnnotator (Rambaut and Drummond, 2009) as burn-in and visualized in FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

2.6. Effective population size, divergence time, and migration rates

For nuclear DNA, we used the program ∂a∂i (Diffusion Approximations for Demographic Inference; Gutenkunst et al., 2010) to simultaneously estimate rates of gene flow, time since divergence, and effective population sizes within and between each species. In short, ∂a∂i uses site frequency spectrum (SFS) data and implements a diffusion approximation-based approach to fit specified evolutionary models against empirical data (Gutenkunst et al., 2010, 2009). While ∂a∂i can be quite powerful given full genome data, results from ddRADseq can still posit important information regarding the evolutionary histories of a species. Given that ∂a∂i is most accurate and efficient when working with only two populations (Noskova et al., 2018), we calculated the best fit model for each species-by-species comparison. Moreover, comparisons were only done between wild mallards, Philippine ducks, and Pacific black ducks (i.e., all Pacific black duck subspecies were treated as a single species), as we were interested in understanding the evolutionary histories between species.

Briefly, optimum evolutionary models were based on the multinomial estimate of log-likelihood for optimized parameters that best fit the model SFS to the empirical SFS; Likelihood values were averaged across the five replicates run for each model. After concatenating all ddRAD-seq autosomal loci in a Nexus file format, we used custom python scripts (Hernández et al., 2021) to derive an empirical SFS using ∂a∂i. Each SFS was based on only minor alleles and was folded because we lacked outgroup information (Gutenkunst et al., 2010). Additionally, nucleotides that were invariable were ignored ('masked') by ∂a∂i. Finally, to account for missing data and the lack of shared variants between species, each SFS was projected down to create the most complete SFS, while maintaining the most alleles possible (Gutenkunst et al., 2009). Given that relatedness between each species varies, projections for each species-by-species comparison differ to account for the low number of shared variants. We used the following datasets: (1) wild mallard (N = 50 alleles) x Philippine duck (N = 80 alleles), (2) wild

mallard ($N = 50$ alleles) x Pacific black duck ($N = 120$ alleles), and (3) Philippine duck ($N = 80$ alleles) x Pacific black duck ($N = 120$ alleles). For each dataset, we tested the empirical data against four evolutionary models that are included in the program ∂a∂i: (1) Neutral-No-divergence, (2) Isolation-With-Migration, (3) Split-With-Migration, and (4) Split-No-Migration model where we forced zero migration in the standard models. Note that whereas IM assumes divergence proceeding with ongoing gene flow, split-with-migration models a case of divergence and secondary contact where species divergence proceeds in allopatry but with instances or bout(s) of secondary contact. We tested for statistical significance between models using a Likelihood Ratio Test at an alpha of 0.01. We determined which model best fit the empirical SFS based on the highest log-likelihood of the optimal parameters for each model. We then performed 50 independent parameter optimizations of the best fit model for each species comparison. Different demographic parameters were estimated for each model, including a scaling factor ($\theta = 4N_{ANC}$ x μ; N_{ANC} = Ancestral effective population size), effective population sizes ($N_i = v_i$ x N_{ANC}), migration rates ($m_{i \leftarrow j} = M_{i \leftarrow j}/(2N_{ANC})$; $m_{i \leftarrow j}$ = proportion of migrants/generation in population i from population j), and time since divergence ($t = T$ x $2N_{ANC}$; t = time since divergence in generations; Gutenkunst et al., 2009).

To convert parameter estimates from ∂a∂i into biologically informative values, we estimated generation time (G) and mutation rates per locus (μ). First, generation time (G) is calculated as $G = \alpha + (s/(1-s))$, where α is the age of maturity and s is the expected adult survival rate (Sæther et al., 2005). The age of maturity for mallard-like ducks generally is one year (i.e., α = 1; Alerstam and Hogstedt, 1982), and the average adult survival rate is 0.54 (range: 0.34–0.74; Nichols et al., 1987) and 0.63 (range: 0.59–0.67; Halse et al., 1993) for wild mallards and Pacific black ducks, respectively; no survival data currently exists for Philippine ducks. Thus, we used an overall adult survival rate average of 0.585, which results in an estimated generation time of 2.4 years for all pairwise comparisons. Finally, to attain a mutation rate for nuclear genes, we multiplied a 1.2×10^{-9} substitutions/site/year mutation rate – previously calculated for nuclear genes in other ducks (Peters et al., 2008) – by generation time to attain a 2.89 x 10^{-9} substitutions/site/generation (s/s/g) mutation rate. A final mutation rate was calculated as the product of the above mutation rate and the total number of base pairs.

3. Results

We attained 610 base-pairs (bp) of overlapping sequences for the mtDNA control region across samples (Supporting Information Table S1) and recovered 3,154 autosomal loci (397,114 base-pairs) and 177 designated Z-sex chromosome linked loci (21,819 bp) that met filtering and missing data criteria.

3.1. Population structure of mitochondrial DNA

Five distinct haplogroups were identified across samples in the haplotype network (Fig. 2). First, we recovered expected haplogroups among mallards (i.e., mallard haplogroups I and II; Ankney et al., 1986; Avise et al., 1990; Johnson & Sorenson, 1999), and Pacific black ducks (i.e., PBDU haplogroups I and II; Brown et al., 2021; Rhymer et al., 2004). Whereas the mallard Haplogroup II only had wild mallards ($N =$

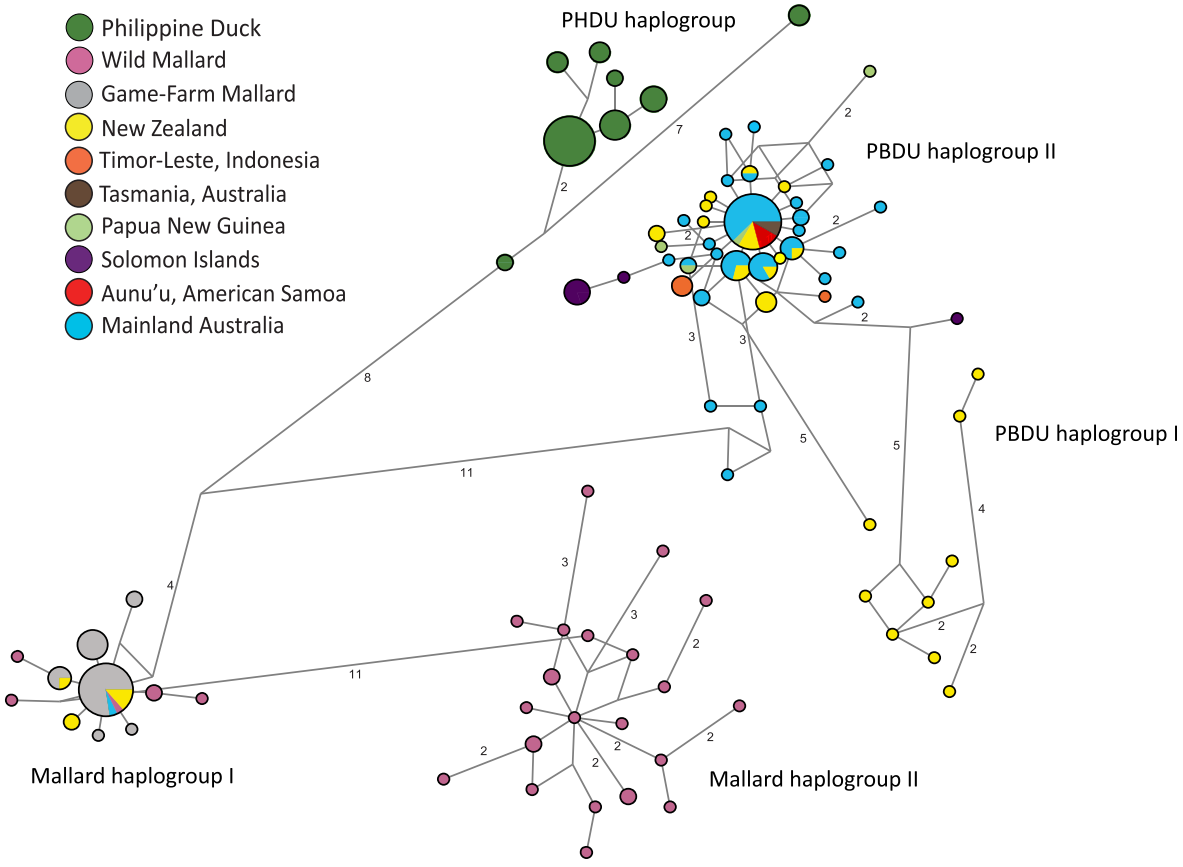

Fig. 2. Haplotype network of mtDNA control region sequences of game-farm ($N = 31$) and wild ($N = 30$) mallards, Philippine ducks ($N = 44$), and Pacific black ducks ($N = 105$); each group is color coded. Note that Pacific black ducks are further distinguished by sampling location. Circle sizes correspond to the total number of individuals within each haplotype, and the number of mutations between haplotypes depicted by branch lengths; however, all branch lengths separated by more than one mutation are also denoted.

25), mallard haplogroup I contained wild mallards ($N = 5$), game-farm mallards ($N = 31$), and Pacific black ducks from New Zealand ($N = 6$) and mainland Australia ($N = 1$; Fig. 2). Only Pacific black ducks comprised PBDU haplogroups I and II, with PBDU haplotype I only recovered from New Zealand ($N = 8$), and PBDU haplotype II from mainland Australia ($N = 54$), New Zealand ($N = 16$), Papua New Guinea ($N = 4$), Tasmania, Australia ($N = 2$), Timor-Leste, Indonesia ($N = 4$), Solomon Islands ($N = 7$), and the island of Aunu'u ($N = 3$; Supporting Information Figure S2). Individuals from Timor-Leste, Indonesia, and Solomon Islands possessed two and three haplotypes, respectively, that were not shared with any other group. Note that a single major haplotype within Haplogroup II was recovered for Pacific black ducks sampled in mainland Australia ($N = 15$), New Zealand ($N = 3$), Papua New Guinea ($N = 1$), and Aunu'u ($N = 3$). Finally, Philippine ducks made up a unique haplogroup comprising eight haplotypes (Fig. 2).

Pairwise composite Φ_{ST} estimates of mitochondrial DNA varied widely between Pacific black ducks, as well as in mallards and Philippine ducks (Supporting Information Fig. S1A). As expected, based on the haplotype network (Fig. 2), the Philippine duck was strongly differentiated from all other sampled groups (i.e., $\Phi_{ST} = 0.70–0.95$; Supporting Information Fig. S1A). Among Pacific black ducks, we recovered no differentiation among mtDNA for samples from mainland Australia; New Guinea; Tasmania, Australia; and the island of Aunu'u (i.e., Φ_{ST} range = 0.00; Supporting Information Fig. S1A). Samples from New Zealand were moderately differentiated from all other Pacific black ducks (average composite $\Phi_{ST} = 0.25 \pm 0.03$ Standard Error, i.e., SE; Supporting Information Fig. S1A). Finally, Pacific black ducks from the Solomon Islands were the most differentiated from all other Pacific black ducks (average composite $\Phi_{ST} = 0.55 \pm 0.06$ SE; Supporting Information Fig. S1A).

3.2. Nuclear population structure

All nuclear analyses were based on 11,961 (of 13,166) independent bi-allelic autosomal SNPs that met filtering criteria, and with >98% of samples present across loci.

Population structure was first visualized based on pairwise sample co-ancestry estimated in fineRADstructure in which three major species' lineages were recovered, and included mallards, Philippine ducks, and Pacific black ducks (Fig. 3). As expected, further partitioning was observed within mallards, distinguishing between game-farm and wild mallards. Co-ancestry matrices also recovered three Pacific black duck subgroups, which largely corresponded to subspecies: *A. s. rogersi* (i.e., Australia; Timor-Leste, Indonesia; Papua New Guinea; and the island of Aunu'u), *A. s. superciliosa* (i.e., New Zealand grey ducks), and *A. s. pelewensis* (i.e., lesser grey Ducks of the Solomon Islands). We note that samples from Aunu'u clustered within the *A. s. rogersi* and *A. s. pelewensis* as expected; though the higher co-ancestry recovered within this group supports recent intra-population structure. Finally, a group of samples from mainland Australia were identified to have substantially higher co-ancestry as compared to other samples across mainland Australia, suggesting these to have high kinship; and thus, we excluded all but one of these in PCA and ADMIXTURE analyses.

Both PCA clustering (Fig. 1C-D) and ADMIXTURE (Supporting Information Figure S2) analyses were highly concordant with co-ancestry results. Again, samples were expectedly partitioned as game-farm mallards, wild mallards, Philippine ducks, or Pacific black ducks when analyzing the full dataset (Fig. 1C; Supporting Information Fig. S2A). Additional resolution was found when restricting PCA and ADMIXTURE analysis of samples to either all Pacific black ducks (Fig. 1D; Supporting Information Fig. S2B) or Pacific black ducks from mainland Australia only (Fig. 1E; Supporting Information Fig. S2C). Note that while

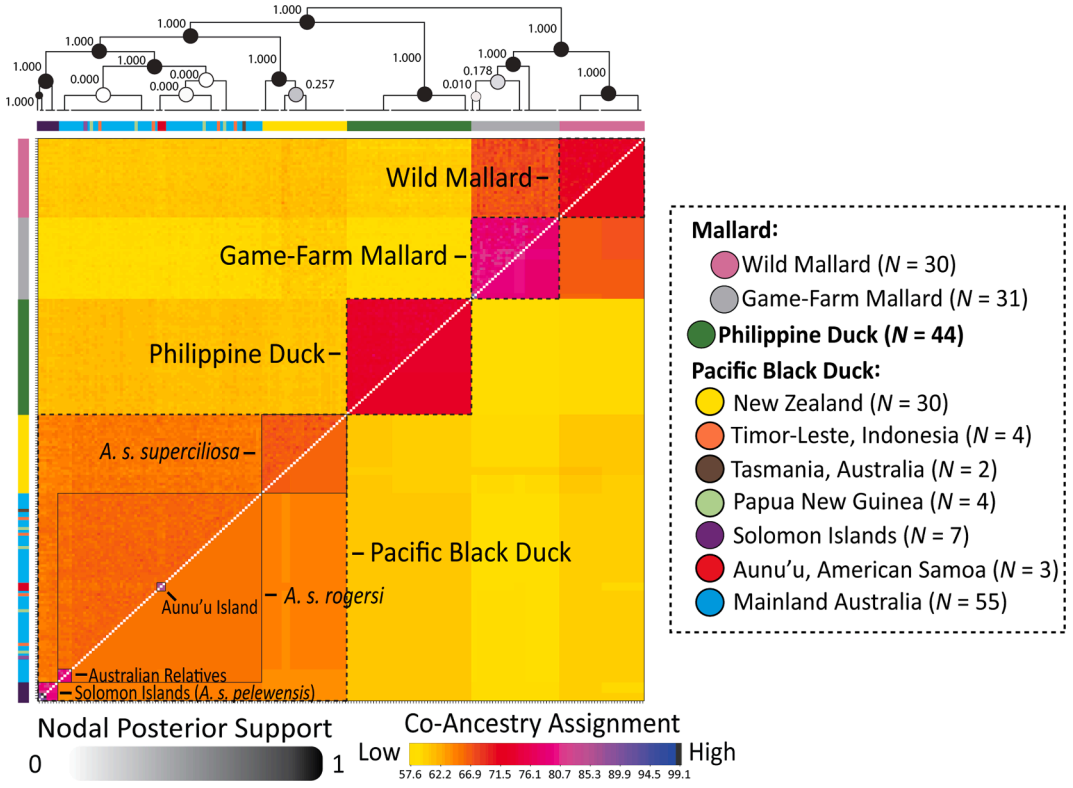

Fig. 3. Pairwise fineRADstructure matrix of individual (above diagonal dashed line) and average (below diagonal dashed line) co-ancestry of Pacific black ducks sampled from various locations and based on independent autosomal ddRAD-seq SNPs. Pairwise coefficients of co-ancestry are color coded from low (yellow) to high (blue). The dendrogram depicts a clustering of individual samples based on the pairwise matrix of co-ancestry coefficients and are denoted by boxes within the matrix. Finally, "Australian Relatives" labeled on the fineRADstructure matrix depicts a group of waterfowl harvested in the same location that were likely all related, as seen by the co-ancestry assignment.

ADMIXTURE recovered an optimum *K* value in each of the analyses, further resolution was obtained by examining additional *K* values (Supporting Information Figure S2). Specifically, using PCA or ADMIXTURE to evaluate Pacific black ducks independently provided clear differentiation among the three subspecies (Fig. 1; Supporting Information Figure S2). Both analyses included recovering Pacific black ducks from Aunu'u, American Samoa, as distinct clusters (Fig. 1D; Supporting Information Fig. S2B), and samples collected from the southwestern region of Western Australia (WA) as another cluster (Fig. 1E; Supporting Information Fig. S2C).

Finally, composite Φ_{ST} for ddRAD-seq autosomal and Z-sex chromosome linked loci were consistent (Supporting Information Fig. S1A). First, the highest differentiation was recovered in comparisons including Philippine ducks (i.e., $\Phi_{ST(A)} \geq 0.20$ & $\Phi_{ST(Z)} \geq 0.14$). Second, among sampling areas of Pacific black ducks, *A. s. pelewensis* from the Solomon Islands was the most differentiated from the other groups (i.e., $\Phi_{ST(A)} = 0.173–0.219$ and $\Phi_{ST(Z)} = 0.175–0.196$), and *A. s. superciliosa* was 1–5% different from *A. s. rogersi* (i.e., $\Phi_{ST(A)} = 0.007–0.056$ and $\Phi_{ST(Z)} = 0.012–0.028$; Supporting Information Fig. S1A). Interestingly, despite Pacific black ducks from American Samoa and the Solomon Islands both occurring within the geographical range for *A. s. pelewensis* (i.e., islands in the Pacific Ocean; Fig. 1B), Aunu'u samples were more similar to other *A. s. rogersi* than to samples from the Solomon Islands (Supporting Information Fig. S1A). Finally, we recovered little to no differentiation among *A. s. rogersi* (i.e., composite $\Phi_{ST(A)} = 0.007$ and $\Phi_{ST(Z)} = 0.012$; Supporting Information Fig. S1A).

3.3. Phylogenetic relationships from species trees

Phylogenetic analysis of the mtDNA control region (Fig. 4B) and nuclear ddRAD-seq autosomal SNPs (Fig. 4A) identified the same three main clades that include mallards, Pacific black ducks, and Philippine ducks. Among Pacific black ducks, phylogenetic relationships recovered three main clades that corresponded to subspecies, including (1) Solomon Islands (i.e., *A. s. pelewensis*), (2) New Zealand (i.e., *A. s. superciliosa*), and (3) Australia, Papua New Guinea, and Timor-Leste, Indonesia (i.e., *A. s. rogersi*). However, relationships within Pacific black ducks were not as well supported across analyses (see bootstrap support in Fig. 4). Whereas Pacific black ducks from Australia, Papua New Guinea; and Timor-Leste, Indonesia, were consistently closely related, those from Aunu'u, American Samoa, were recovered as either sister to Tasmania, Australia (mtDNA), or Solomon Islands (nuclear); however, these relationships were once again not well supported. Regardless of the relatedness between Pacific black ducks of Aunu'u, American Samoa, or the Solomon Islands, both were nested within the primary *A. s. rogersi* clade in both phylogenies. Finally, testing for evidence of gene flow in a phylogenetic context recovered a species tree without migration already explaining 99% of the variation (Fig. 4), suggesting that any addition of gene flow to the model would likely result in overfitting.

3.4. Nucleotide diversity

Highest levels of nucleotide diversity (π) for ddRAD-seq autosomal and Z-sex chromosome loci were recovered in wild ($\pi_Z = 0.0029$; $\pi_A = 0.0068$) and game-farm ($\pi_Z = 0.0028$; $\pi_A = 0.0057$) mallards, followed

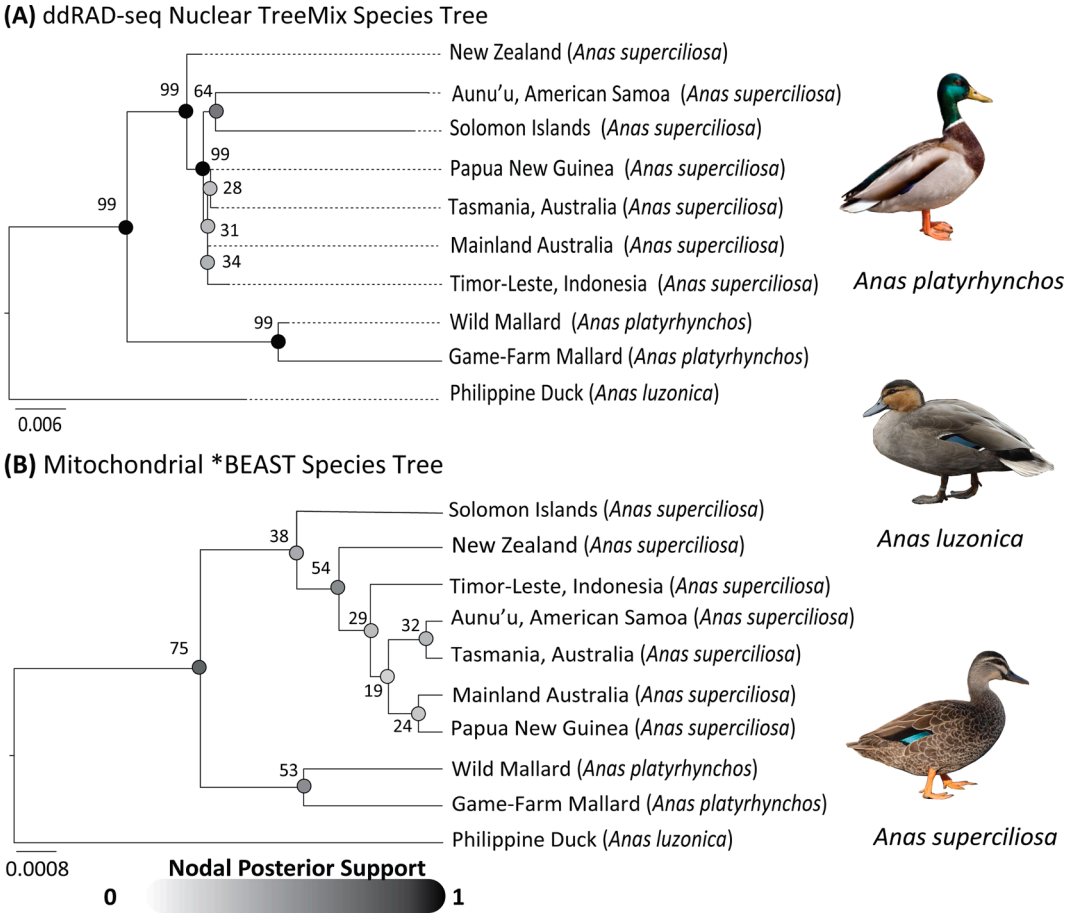

Fig. 4. Phylogenetic relationships as determined from species tree reconstructions from (A) independent ddRAD-seq autosomal SNPs analyzed in the program TreeMix with proportion of explained variance of migration edges, and (B) mitochondrial DNA analyzed in the program *BEAST.

by comparable levels for Pacific black ducks from Timor-Leste, Indonesia; Papua New Guinea; Tasmania, Australia; New Zealand; and mainland Australia (range π_Z = 0.0015–0.0018; range π_A= 0.0047–0.0051; Supporting Information Fig. S1B). Philippine ducks (π_Z = 0.0012; π_A= 0.0034), and Pacific black ducks from the Solomon Islands (π_Z = 0.0013; π_A= 0.0034) and Aunu'u, American Samoa (π_Z = 0.0016; π_A= 0.0032), had the lowest levels of diversity (Supporting Information Fig. S1B).

3.5. Effective population size, divergence time, and migration rates

Based on log-likelihoods and Likelihood Ratio Test, "Split-with-Migration" was statistically (p-value < 0.01) the best-fit ∂a∂i model for all three pairwise species comparisons (Supporting Information Table S2). To transform ∂a∂i results into biologically meaningful values, we used an average mutation rate of 2.89 x 10^{-9} substitutions/site/generation multiplied by the total 397,114 base-pairs to obtain a scalable mutation rate of 1.15 x 10^{-3} substitutions/site/generation (Fig. 5).

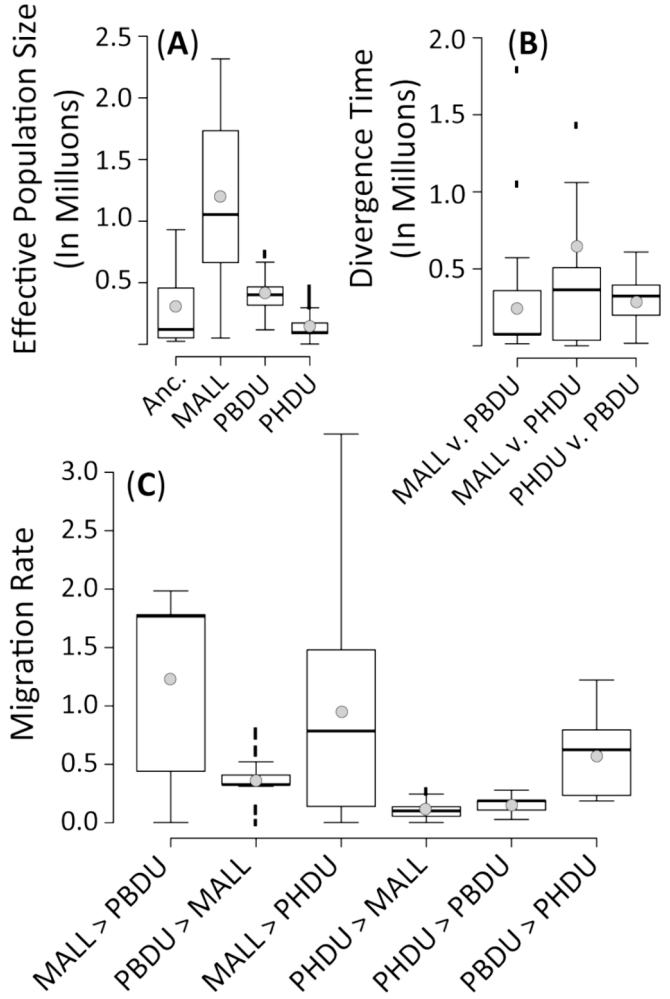

Fig. 5. Box plots are of values across pairwise species ∂a∂i analyses of ddRAD-seq autosomal loci, and estimates of (A) effective population size of the ancestral (Anc.), and contemporary populations of wild mallards (MALL), Pacific black ducks (PBDU), and Philippine ducks (PHDU), (B) divergence time, and (C) migration rates (directionality is denoted as "from > to"). Note that effective population sizes were all values calculated for the ancestor or each of the species across pairwise ∂a∂i analyses. Center lines in each box plot represent the medians, while the box limits are represented by the 25th and 75th percentiles. Grey dots denote the average of each respective estimate (also see Supporting Information Table S2). Outliers are represented by a bolded vertical dash.

As expected, wild mallards had the highest effective population size (N_E ~ 1,000,000), followed by Pacific black ducks (N_E ~ 400,000), whereas Philippine ducks had the smallest size (N_E ~ 100,000; Fig. 5A; Supporting Information Table S2). An estimated time since divergence between the three species ranged from 1 to 2 million years before present (Fig. 5B), which is concordant with previous estimates for the radiation of the mallard complex occurring about one million years ago (Lavretsky et al., 2014a). Finally, average migration rates across most pairwise comparisons were all ≪ 1 migrant per secondary contact event with most overlapping zero (Supporting Information Table S2); however, migration between wild mallards and either Pacific black ducks or Philippine ducks ranged from 0 to 3 migrants per secondary contact event (Fig. 5C).

4. Discussion

We present the most complete molecular assessment of mallard-like ducks in Oceania, Greater Indonesia, and the Philippines, and provide evidence for at least four distinct evolutionary units. Philippine and three subspecies of Pacific black duck are strongly structured without any evidence of ancestral or contemporary gene flow between them. Importantly, whereas Philippine ducks were hypothesized to be a candidate hybrid species (Lavretsky et al., 2014a, 2014b), we find no evidence in support of this scenario. Inconsistent with a hybrid origin, Philippine ducks have significant levels of differentiation across pairwise comparisons in both mtDNA and nuclear markers (Supporting Information Fig. S1A) and contain a fixed mtDNA haplogroup with significant divergence from other groups (Fig. 2). Moreover, Philippine ducks do not show shared co-ancestry (Fig. 3) or evidence of ancestral gene flow in phylogenetic analyses (Fig. 4) with mallards and Pacific black ducks that would be indicative of a hybrid origin. Thus, we conclude that *A. luzonica* is a valid species-group taxon not of hybrid origin.

Concordant with previous research based on geographic (Kear, 2005; Marchant and Higgins, 1990), intronic (Brown et al., 2021), mtDNA (Rhymer et al., 2004), and phenotypic (Fullagar, 2005) differentiation, intraspecific population structure for Pacific black ducks largely followed subspecies designations (Figs. 1 & 3; Supporting Information Figure S2). However, we present genomic data supporting additional structure within *A. s. rogersi* across mainland Australia. Specifically, we find subtle genomic divergence between *A. s. rogersi* from western Australia and populations from the remainder of mainland Australia (Fig. 1E; Supporting Information Fig. S1A & S2C), which corroborates morphological data showing unique facial plumage traits in western Australia (Williams, 2019). Whether this population structure is the result of local adaptation, genetic drift (i.e., bottlenecking or founder events; Clegg et al., 2002) or biogeographic differentiation that is well known in the region's biota remains unknown (Rix et al., 2015). Similarly, whereas Timor-Leste was previously known to possess unique mtDNA haplotypes (Fig. 2; Brown et al., 2021; Rhymer et al., 2004), we show for the first time, subtle population structure within nuclear markers (Supporting Information Fig. S2B). Thus, further research on life history and ecology is needed to better understand whether the subtle population differences constitute independent conservation units. In general, future research into establishing conservation units of *A. s. rogersi* across its range will benefit from expanded population and genomic sampling to better demarcate intrapopulation structure, including full genome analyses to determine potential adaptive differences among them.

Finally, on reconstructing the evolutionary histories of these species based on an optimal model of divergence followed by periods of secondary contact, we find that the three species diverged over the last 1–2 million years, the Philippine duck having the deepest divergence time (Fig. 5B; Supporting Information Table S2). Moreover, we find that the effective population size of the wild mallard is three and 10 times that of Pacific black and Philippine ducks, respectively (Fig. 5A; Supporting

Information Table S2). This is concordant with differences in census population sizes. Importantly, despite finding an optimal evolutionary history involving secondary contact, estimates were generally ≪ 1 migrant per contact event (Supporting Information Table S2). Moreover, while the highest rates of gene flow were from mallards into the island species, these estimates remained relatively low, averaging ~ 0.5 migrants per contact event (95% confidence of 0–3 migrants per contact event; Fig. 5C; Supporting Information Table S2). Thus, we conclude that Oceania, Greater Indonesia, and the Philippines were colonized by mallard-like ducks nearly two million years ago, and that they have since been diverging largely in allopatry.

4.1. Origin of American Samoa and Solomon Islands Pacific black ducks

Although most samples assigned to *A. s. rogersi* and *A. s. superciliosa* were recovered within their expected geographic ranges, the two putative populations of *A. s. pelewensis* did not show a close genetic affinity to each other (Fig. 3). In particular, we acknowledge discordance in the phylogenetic placement of Aunu'u Pacific black ducks across analyses (Figs. 2, 3, 4A; Supporting Information Figure S2). While Pacific black ducks from Aunu'u show genetic differentiation from Solomon Island Pacific black ducks and some distinction from *A. s. rogersi* (Fig. 1D and 4A; Supplementary Information Fig. S2B), these Pacific black ducks generally cluster genetically closer with *A. s. rogersi* (Fig. 3). Although such a discordance could be a result of unrecognized gene flow with Pacific black ducks from the Solomon Islands, we would expect even closer co-ancestry clustering given the higher sensitivity of such analyses for such events (Lavretsky et al., 2019b). Rather, we posit that genetic drift acting on these small island populations results in higher-than expected relatedness (Fig. 3) and may be biasing results. In the case of the Aunu'u population, our data suggests a founder event from *A. s. rogersi*, as evidenced by measures of co-ancestry (Fig. 3) and genomic differentiation (Supporting Information Fig. S1A), as well as that they share a major Australian mtDNA haplotype with *A. s. rogersi*, suggesting a single major founder event (Fig. 2). We also note that while our sample size from Aunu'u was limited (i.e., $N = 3$), this sample set likely makes up ~5–10% of the total population, which is currently thought to be ~30–50 individuals (USFWS, *personal communication, 2019*). In fact, we posit that the genetic uniqueness of the Solomon Islands may similarly be due to an older *A. s. rogersi* founder event. Like those birds from Aunu'u, we not only find high co-ancestry among Solomon Island Pacific black ducks (Fig. 3), but that they appear nested within the primary *A. s. rogersi* clade of the nuclear phylogeny (Fig. 4A). Together, we conclude that mainland Australian Pacific black ducks may be the most likely source for Solomon Island, as well as the recent colonization of American Samoa. As a result, our findings suggest that either the *A. s. pelewensis* subspecies designations and/or its range extent may require re-evaluation. Regardless, future research will require increased sampling of both populations to more formally test whether genetic drift is obscuring phylogenetic signals or that *A. s. pelewensis* may not be an appropriate classification for one or both of the populations.

4.2. Conservation considerations for mallard-like ducks in Oceania, greater Indonesia, and the Philippines

The often-smaller population sizes of island taxa make them especially susceptible to extinction events (Bouzat, 2010; Frankham et al., 2002; Furlan et al., 2012). Not only does low genetic diversity in small island populations naturally cause stronger genetic drift and higher genetic load (Frankham et al., 2012), it also makes them susceptible to genetic swamping from introduced congeners (Wells et al., 2019). Although we find no evidence of significant hybridization with mallards or other mallard-like ducks, Philippine ducks have the lowest levels of nucleotide diversity of the assayed species (Supporting Information Fig. S1B), which is in line with earlier estimates of genetic diversity (Licuanan et al., 2017). Furthermore, diversity for Philippine ducks was comparable to the extremely small population of Pacific black ducks from American Samoa and the Solomon Islands. While we conclude that the Philippine duck as well as island populations of Pacific black ducks are not currently under serious threat due to hybridization and genetic swamping, understanding how their limited genetic diversity might translate into adaptive potential requires careful consideration as the climate continues changing. Although we have a strong baseline for contemporary standing diversity, understanding genotype-environment interactions through a landscape genomic approach will be critical for predicting future conservation needs (Bay et al., 2017; Kawecki and Ebert, 2004; Razgour et al., 2019; Savolainen et al., 2013).

Unlike Philippine ducks, Pacific black ducks do not appear to be threatened by low genetic diversity (Supporting Information Fig. S1B), but hybridization with introduced mallards has been a growing conservation concern for decades (Marchant and Higgins, 1990). This existential threat is exemplified on Lord Howe Island where *A. s. rogersi* were known to have existed in isolation until mallards were introduced to the island in 1963 (Tracey et al., 2008). In as little as 15 years, rampant hybridization resulted in a hybrid swarm which essentially rendered the Pacific black duck extinct on the island (Guay et al., 2014; Taysom, 2015; Tracey et al., 2008). Similarly, since mallards were introduced in the mid-1800s, hybridization with *A. s. superciliosa* in New Zealand has exponentially increased to the point where only ~5% of the population is thought to comprise genetically pure *A. s. superciliosa* (Brown et al., 2021; Dryer and Williams, 2010; Gillespie, 1985; Guay et al., 2014; Guay and Tracey, 2009; Tracey et al., 2008; Williams and Basse, 2006). Like New Zealand, mallards introduced to mainland Australia now pose a threat to local *A. s. rogersi* (Guay et al., 2014; Taysom, 2015), although hybridization rates in mainland Australia appear to be low (~1.5%; Taysom, 2015) and largely confined to urban areas (Guay et al., 2014; Taysom, 2015). Although nuclear-based co-ancestry or assignment probabilities did not find interspecific or inter-mixed clustering with mallards (wild or game-farm) for any of the sampled Pacific black ducks (Figs. 1 & 5), we did find several mallard haplogroup I haplotypes (Fig. 2), which likely result from introgression of game-farm mallards that carry mallard haplogroup I haplotypes (Lavretsky et al., 2023b, 2020). We also acknowledge that by using *a priori* knowledge regarding ancestry of New Zealand's Pacific black ducks, our results for this subspecies should be taken with caution and should not be considered to suggest low mallard hybridization rates (see Brown et al., 2021; Dryer and Williams, 2010; Gillespie, 1985; Guay et al., 2014; Guay and Tracey, 2009; Tracey et al., 2008; Williams and Basse, 2006). Specifically, the threat of Pacific black ducks hybridizing with mallards should not be overlooked in any location where these two species overlap (Williams and Basse, 2006), particularly as the potential for increased contact is expected to increase with rapidly expanding urbanization and climate change. To do so will require genetic monitoring at geographically fine scales for each of the subspecies that now co-occur with mallards.

Declaration of competing interest

The authors have no competing financial interest or personal interests to report that would affect this manuscript.

CRediT authorship contribution statement

Marissa Kaminski: Writing – original draft, Formal analysis, Visualization, Investigation. **Joshua I. Brown:** Writing – review & editing, Formal analysis, Investigation. **Sara R. Seibert:** Writing – review & editing, Resources, Conceptualization, Investigation. **Flor Hernández:** Writing – review & editing, Investigation. **Melizar V. Duya:** Writing – review & editing, Resources. **Ian Kendrich C. Fontanilla:** Writing – review & editing, Resources. **David Roshier:** Writing – review & editing, Resources. **Adam Miles:** Writing – review & editing, Resources, Funding acquisition. **Leo Joseph:** Writing – review & editing,

Resources, Conceptualization. **Jeffrey L. Peters:** Writing – review & editing, Resources, Conceptualization, Investigation. **Philip Lavretsky:** Conceptualization, Writing – review & editing, Resources, Funding acquisition, Methodology, Formal analysis, Investigation, Project administration, Visualization.

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Data availability statement

Mitochondrial DNA sequences: GenBank accessions PP746861 - PP756430.

Illumina ddRAD-Seq Reads: NCBI's Sequence Read Archive data PRJNA1106373.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108085.

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