

# Chromosomal-level reference genome of a wild North American mallard (*Anas platyrhynchos*)

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## Abstract

The mallard (*Anas platyrhynchos*) is one of the most common, economically, and socially important birds around the world. Mallards were not only an important food source for early humans but eventually becoming intimately linked with people as they were domesticated over the last 2,000 years. To date, mallard genomes are largely reconstructed from samples of domestic or unknown genetic heritage. Here, we report the first high-quality genome assembly and annotation of a genetically vetted wild mallard from North America (NAwild\_v1.0). The genome was assembled using a combination of shotgun libraries, proximity ligation Chicago, and Dovetail Hi-C libraries. The final assembly is ~1.04 Gb in size, with 98.3% of the sequence located in 30 full or nearly full chromosome-level scaffolds, and with a N50/L50 of 79.1 Mb/4 scaffolds. We used a combination of gene prediction and similarity approaches to annotate a total of 23,584 functional genes, of which 19,242 were associated to GO terms. The genome assembly and the set of annotated genes yielded a 95.4% completeness score when compared with the BUSCO aves\_odb10 dataset. Next, we aligned 3 previously published mallard genomes to ours, and demonstrate how runs of homozygosity and nucleotide diversity are substantially higher and lower, respectively, to ours and how these artificially changed genomes resulted in profoundly different and biased demographic histories. Our wild mallard assembly not only provides a valuable resource to shed light onto genome evolution, speciation, and other adaptive processes, but also helping with identifying functional genes that have been significantly altered during the domestication process.

**Keywords:** *Anas platyrhynchos*, wild mallard, chromosome-scale, genome assembly

## Introduction

Even before becoming forever linked with humans as one of the more recently successful domestication events occurring in Eurasia over 2,000 years ago (Larson and Fuller 2014), mallards (*Anas platyrhynchos*) were an important food source dating back to ancient peoples (Jensen et al. 2019). This long-standing interlink is exemplified by the myriad of efforts to ensure that mallards thrive in the wild, including their relocation and establishment well outside their native range, as well as increasing the number and types of domestic variants; all of which resulting in these birds having significant socio-economic importance worldwide. In fact, the intentional or accidental release of mallards has increased their range to include the entire world outside the Poles (Baldassarre 2014). Attempts to understand evolutionary mechanisms, consequences of contemporary anthropogenic hybridization, or looking for gene-trait associations to optimize agricultural practices require a contiguous and ancestrally wild reference genome. Although several mallard genomes exist, they are either of specific domestic breeds, un-vetted wild samples (i.e. samples determined to be ancestrally wild prior to full genome sequencing), and/or sequences of pooled individuals. Given the history of captive-reared mallard releases worldwide (Guay and Tracey 2009; Söderquist et al. 2017; Lavretsky et al.

2020), it is no longer easily assumed that a mallard from the wild is genetically so.

To date, there are 9 mallard genomes published on NCBI (Huang et al. 2013; Zhou et al. 2018; Liu et al. 2020; Li et al. 2021; Zhu et al. 2021), with all of these being from Eurasian mallards that are either of known domestic origins, and with the 2 “wild” mallard genomes being the result of multiple sample poolings (i.e. CAU-Wild GenBank Accession ID GCA\_008746955.1; Zhou et al. 2018) and/or of un-vetted origins (i.e. ASM222489V1 GenBank Accession ID GCA\_002224895.1; Liu et al. 2020; Xi et al. 2021). Understandably, genomes have focused on domestic over wild mallards due to their agricultural and medical importance, including understanding gene linkage of favorable agricultural traits (Zhang et al. 2018; Zhou et al. 2018) and disease resistance in the poultry industry more broadly (Munster et al. 2006; Skinner et al. 2009). However, these genomes are not appropriate for studies understanding more natural processes or even how wild genomes are transformed through the domestication process due to the constant constraint of artificial selection imposed on domestic lineages (Larson and Fuller 2014). Consequently, we used partial-genome sequences to first establish ancestry of the sample, resulting in the generation of a de novo assembly of a wild mallard collected in New Mexico, United States of America with >98% assignment probability to wild ancestry. In addition, we overlay complementary Topologically Associated Domain

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(TAD) information, bioinformatically annotate the genome, and demonstrate how the domestication process results in accentuated demographic results that can substantially bias inferences.

## Materials and methods

### Sample collection and DNA extraction

One wild male mallard was collected at Sierra County, New Mexico (32.953 N, -107.295 W). Breast tissue was sent to Dovetail Genomics, LLC (a.k.a Cantata Bio; Scotts Valley, CA) where high molecular weight (HMW) DNA was extracted using the Blood and Cell Culture Midi Kit (Qiagen, GmbH) following the manufacturer's protocol. The bird is curated in the University of Texas at El Paso's Biodiversity Collection (Catalog Number: UTEP-Bird 3056).

### Sequencing and assembly

Extracted DNA was sheared for Illumina library preparation using Bioruptor Pico. Two short-insert libraries with insert length of approximately 400 and 500 base pair (bp) were prepared (Dovetail Genomics) following the Illumina TruSeq DNA PCR-free protocol. Shotgun libraries were sequenced on an Illumina HiSeq with paired-end 150-bp chemistry. Raw sequences were filtered for sequencing adapters and low-quality bases using Trimmomatic (Bolger et al. 2014). Short insert reads were profiled at a variety of k-mer values (19, 31, 49, 75, 109), with a negative binomial model fit to k-mer distribution to optimize coverage and achieve a balance between repetitive and heterozygous fractions during assembly. De novo assembly was generated using paired-end libraries with Meraculous v2 (Chapman et al. 2011) with a k-mer size of 55 and minimum k-mer frequencies of 15, and the diploid nonredundant haplotig mode.

Using the HMW DNA, 3 Chicago libraries were prepared (Cantata Bio) following the methods described in Putnam et al. (2016). In short, ~500 ng of HMW genomic DNA was reconstituted in vitro into chromatin and fixed with formaldehyde. Fixed chromatin was digested with DpnII, and with the 5' overhangs filled in with biotinylated nucleotides, and free blunt ends were then ligated. After ligation, crosslinks were reversed to remove protein from DNA. Purified DNA was treated to remove biotin that was not internal to ligated fragments and sheared to ~350-bp mean fragment size. Preparation of sequencing libraries was generated from these sheared DNA using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. Libraries were sequenced for a total of 71 Gb (i.e. ~70× genomic coverage assuming a ~1.1-Gbp genome) on a single Illumina HiSeqX lane and using PE 150-bp chemistry.

Next, Omni-C libraries were prepared by Cantata Bio following a modified Hi-C protocol described in Lieberman-Aiden et al. (2009). Briefly, chromatin was fixed in place with formaldehyde in the nucleus and then extracted. Fixed chromatin was digested with DNase I, followed by chromatin end repair and ligation to a biotinylated bridge adapter, and then by proximity ligation of adapter containing ends. After proximity ligation, crosslinks were reversed, and the DNA purified. Purified DNA was treated to remove biotin that was not internal to ligated fragments. Sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The library was sequenced for a total of 68 Gb (i.e. ~62× genomic coverage assuming a ~1.1-Gbp genome) on a single Illumina HiSeqX lane and using PE 150-bp chemistry.

The initial de novo assembly from Meraculous, along with shotgun reads, and Chicago library reads were used as input for

the HiRise bioinformatics pipeline (Putnam et al. 2016). Following, Omni-C library sequences were aligned to the draft input assembly using Burrows Wheeler Aligner v07.15 (bwa; Li and Durbin 2009). Then, the separations of Cantata Bio Omni-C read pairs mapped within draft scaffolds were analyzed by HiRise to produce a likelihood model for genomic distance between read pairs. In addition, the model was used to identify and break putative misjoins to score prospective joins and make join(s) above the default threshold that is automated in the HiRise algorithm. Finally, after aligning and scaffolding the draft assembly using the Chicago data, the Chicago assembly and Omni-C reads were used to improve scaffolding and the mallard assembly using the above method.

Finally, for the wild mallard mitogenome, wild mallard raw Illumina reads were assembled using the mitogenome of the ZJU1.0 pekin duck genome [NCBI BioProject PRJNA20199, version NC\_009684.1; (Zhu et al. 2021)] as a reference in Geneious 10.0.5 (Biomatters Ltd.). We ran the internal Geneious mapper with medium sensitivity and up to 5 iterations for fine-tuning. We retained read and base-position with mean quality PHRED Q scores > 30, and positions were genotyped based on a minimum coverage depth of 10 sequences.

### TAD identification

Hi-C contact matrices were generated using the BAM file with read pairs filtered for mapping quality of 60. Topologically Associated Domains (TADs) were identified using Arrowhead within the Juicer tool package (Durand et al. 2016) with 3 different resolutions, 10 kbp, 25 kbp, and 50 kbp. Clodius was used to create multiplies files, and HiGlass was used to visualize the results (Kerpedjiev et al. 2018). CTCF sites were predicted using the *cread* program (Schones et al. 2007) utilizing the position weight matrix from CTCFBSDB 2.0 (Ziebarth et al. 2012). Genes were isolated, and significant GO terms with more than expected gene content were identified using in-house python scripts.

### Assembly statistics

Contiguity statistics of scaffolded assembly was computed using Quast (Gurevich et al. 2013). BUSCO analysis was performed using BUSCO v3.1.0 (Waterhouse et al. 2018) with the associated aves\_odb10 dataset. Scaffolds were assigned chromosomal numbers based on alignments to previously published duck genomes (Huang et al. 2013; Zhou et al. 2018; Li et al. 2021; Zhu et al. 2021).

### Genome annotation

Chromosomal sequences were passed through Omics-box (<https://www.biobam.com>) to identify protein-coding regions using Augustus (Hoff and Stanke 2019). Functional annotation of predicted proteins was performed using Omics-Box Functional Genomics package (<https://www.biobam.com>). In short, a blastp (Altschul et al. 1990) search was performed against the nr database, along with InterProScan (Jones et al. 2014) run for each of the protein sequences. Within Omics-Box, sequences with significant hits for either the blast or Interpro search were then mapped to a Gene Ontology annotation database (Ashburner et al. 2000). The resulting functional annotation table and a wego formatted file with a gene and GO term were exported for use in further analysis. Predicted protein sequences of *Gallus gallus* (chicken, RefSeq: GCF\_016699485.2; Warren et al. unpublished) and *Anser cygnoides* (goose, RefSeq: GCF\_002166845.1; Gao et al. 2016) genome assemblies were downloaded from NCBI to compare with the predicted mallard proteins. OrthoVenn2 (Xu et al. 2019) was used to cluster the homologous proteins and create a Venn diagram across the different animals. Note

that all compared genomes were re-annotated using the same parameters within Omics-box for more direct evaluation.

## Variant calling, genomic diversity, genomic differentiation, and demographic analyses

Variant calling was performed across raw fastq files representing 2 domestic breeds (i.e. CAU-Pekin and CAU-Laying ducks), a mallard of un-vetted wild ancestry (Zhu *et al.* 2021), and our wild mallard using the *process\_sequences* script (Python scripts available at <https://github.com/jonmohl/PopGen>; Lavretsky *et al.* 2020). In short, the *process\_sequences.py* performs the following bioinformatics steps in which poor quality sequences were trimmed and/or discarded with Trimmomatic v0.38 (Bolger *et al.* 2014). Next, quality sequence reads were then aligned to our wild mallard assembly using bwa v07.15 (Li and Durbin 2009). Samples were then sorted and indexed in bcftools v1.14 (Danecek *et al.* 2021) and combined using the bcftools “mpileup” function with the following parameters “-c -A -Q 30 -q 30,” which set a base pair and an overall sequence PHRED score of  $\geq 30$  to ensure that only high-quality sequences are retained. The resulting VCF file was then filtered using VCFtools v0.1.17 (Danecek *et al.* 2021) with a minimum quality of 30 (-minQ30), a minimum depth of 10 (-minDP 10), and removing all sites with a minimum allele depth of 5 (-remove-filtered ‘AD<5’). Runs of homozygosity and nucleotide diversity was calculated for each genome using VCFtools v0.1.17 (Danecek *et al.* 2021). Whereas all possible base-pairs were used when calculating runs of homozygosity, nucleotide diversity was calculated using a window size of 50 kbp and a sliding window of 5 kbp.

Next, demographic histories were estimated for the same 3 previously published genomes and ours using a Pairwise Sequentially Markovian Coalescent (PSMC) method (Li and Durbin 2011). Genomic data was first filtered for variants having a minimum read sequencing depth of 10, and then followed PSMC parameters optimized for birds as outlined in Nadachowska-Brzyska *et al.* (2015), which included a maximum number of iteration (i.e.  $N = 30$ ), maximum  $2N_0$  coalescent time (i.e.  $t = 5$ ), initial theta/rho ratio (i.e.  $r = 5$ ), and a pattern of parameters (i.e.  $p = “4+30*2+4+6+10”$ ). Each analysis was run with 100 bootstrap replicates. Finally, PSMC parameter estimates were converted into biologically informative values based on a generation time ( $G$ ) that was calculated as  $G = \alpha + (s / (1 - s))$ , where  $\alpha$  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 1 year (i.e.  $\alpha = 1$ ; Alerstam and Högstedt 1982), and the average survival rate of wild mallards is  $\sim 0.57$  (i.e. range: 0.46–0.68; Smith and Reynolds 1992; Arnold and Clark 1996; Drilling *et al.* 2020); resulting in an estimated generation time of 2.32 years. The nuclear mutation rate was set to  $1 \times 10^{-9}$  (Lavretsky *et al.* 2020).

## Results and discussion

### Genome sequencing and assembly results

We constructed a chromosome-level assembly for a genetically vetted wild mallard (NAwild\_v1.0) using a multi-level approach

**Table 1.** Sequencing results.

| Library type | Bases (Gb) | Coverage ( $\sim 1.04$ -Gb genome) |
|--------------|------------|------------------------------------|
| Shotgun      | 247.3      | 237.8                              |
| Chicago      | 153        | 147.1                              |
| Omni-C       | 62.1       | 59.7                               |

**Table 2.** Sequencing and assembly results of our wild mallard genome and other publicly available mallard assemblies.

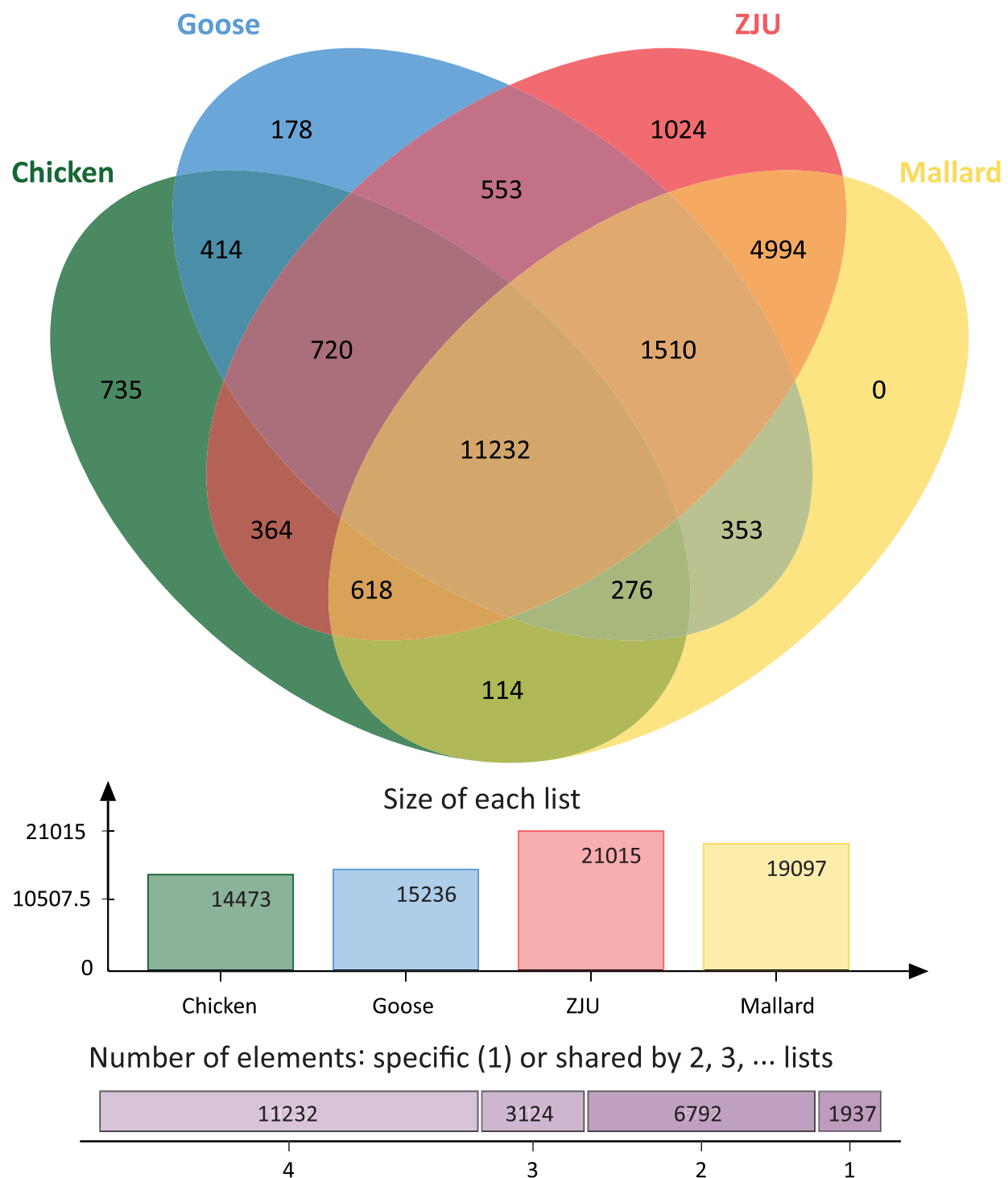
| Name            | Mallard <sup>a</sup> | Mallard <sup>b</sup> | BGI_duck_1.0  | CAU_duck1.0   | ASM222489V1   | IASCAAS       | ZJU1.0        | CAU-Wild      | CAU-Pekin     | CAU-Laying    |
|-----------------|----------------------|----------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Year            | 2023                 | 2023                 | 2013          | 2017          | 2017          | 2018          | 2020          | 2021          | 2021          | 2021          |
| Assembly level  | Chr. + scaffold      | Chr.                 | Chr.          | Scaffold      | Scaffold      | Chr.          | Chr.          | Chr.          | Chr.          | Chr.          |
| Genome coverage | 444x                 | 444x                 | 60x           | 150x          | 208x          | 50x           | 143x          | 100x          | 92.6x         | 93.1x         |
| Total length    | 1,045,577,032        | 1,038,030,224        | 1,105,035,747 | 1,136,415,614 | 1,265,073,014 | 1,126,159,488 | 1,188,533,289 | 1,211,992,756 | 1,186,367,508 | 1,217,695,176 |
| Largest contig  | 195,430,658          | 195,430,658          | 88,405        | 52,057,279    | 17,658,973    | 202,842,836   | 207,238,429   | 208,326,429   | 207,246,783   | 212,526,513   |
| # of contigs    | 2,466                | 31                   | 227,448       | 44,791        | 120,214       | 73,852        | 1,661         | 1,974         | 618           | 834           |
| GC%             | 41.01                | 40.94                | 40.5          | 41.0          | 41.5          | 41.5          | 42.0          | 42.0          | 42.0          | 42.5          |
| N50             | 79,203,680           | 79,203,680           | 1,233,631     | 74,988,519    | 2,490,036     | 76,129,154    | 76,269,206    | 77,626,585    | 76,279,691    | 76,919,215    |
| L50             | 4                    | 4                    | 268           | 5             | 124           | 5             | 5             | 5             | 5             | 5             |
| # N's           | 5,851,869            | 5,243,420            | 35,079,597    | 56,309,843    | 95,192,265    | 2,915,825     | 4,230,438     | 1,235,381     | 1,228,040     | 7,102,323     |

Note that waterfowl generally have a consensus diploid (2N) number of 80 (Wójcik and Smalec 2007; Basyouny Shahin *et al.* 2014), with autosomal chromosomes 1–29 autosomes and sex chromosomes considered as macro-chromosomes, and the remaining being micro-chromosomes.

<sup>a</sup> Assembly results for our genome.

<sup>b</sup> Assembly results of our genome for the top 31 chromosome-level scaffolds that accounted for 99.3% of the data.

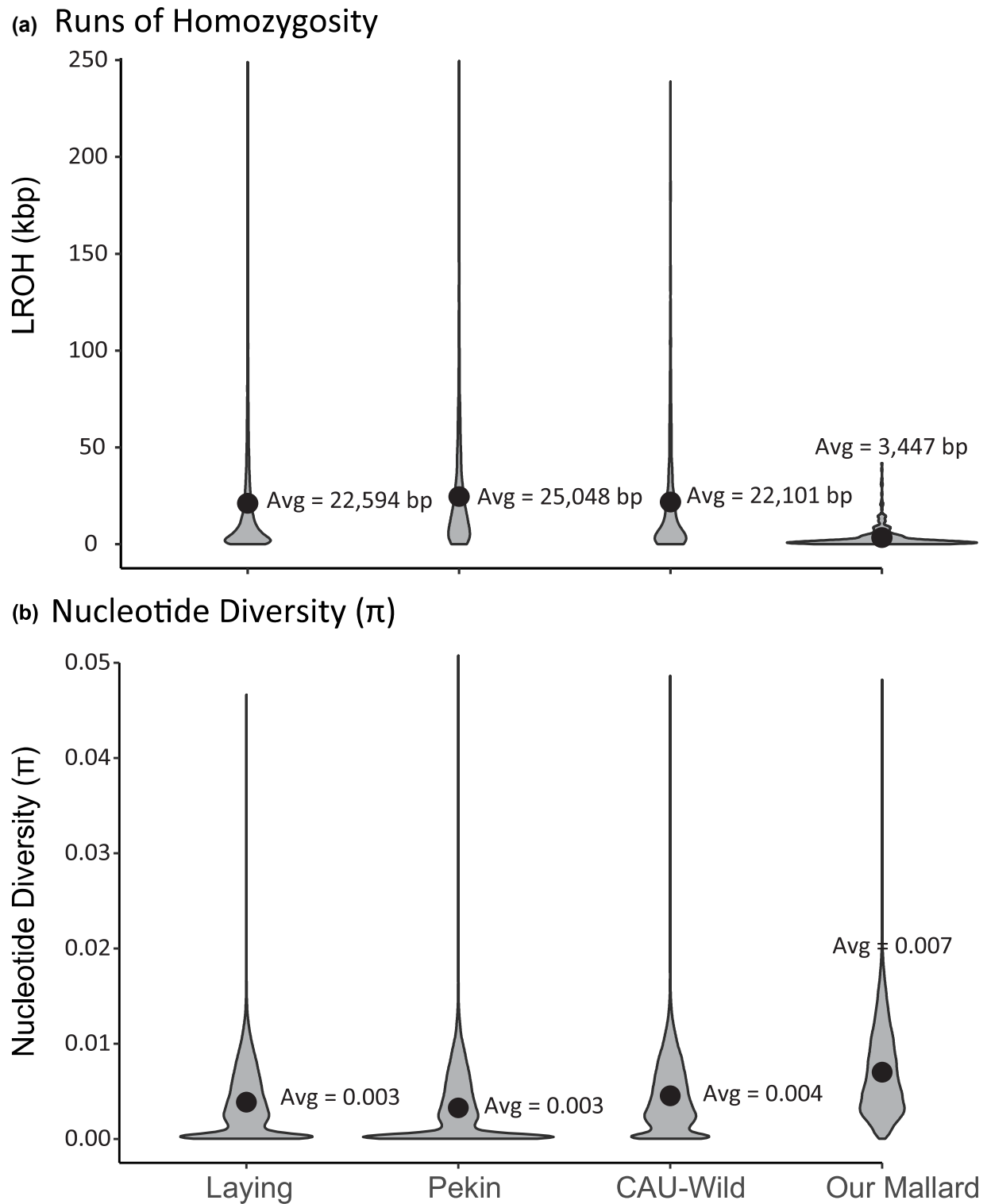




**Fig. 2.** (top) OrthVenn2 Venn diagram highlighting the overlap of the protein clusters of each of the avian species. Singletons (i.e. proteins that did not form a cluster and were not counted within the figure) numbered 11,174 for chicken, 10,380 for goose, 9,153 for ZJU mallard, and 3,888 for our mallard. (middle) Protein cluster counts among the different genomes. The size of the list of proteins in each cluster was balanced suggesting that assembly did not have many duplicated regions. (bottom) Majority of the protein clusters overlapped among all the different taxa (11,232). For the protein clusters between two lists, an increased amount between the ZJU Pekin duck and mallard were found (4,994 of the 6792, or 74%). Furthermore, there was a greater overlap with the 2 ducks and the goose (1,510) then with the chicken (618) as expected based on evolutionary history.

**Table 3.** Topologically Associated Domains (TADs) results called by Arrowhead (Durand et al. 2016).

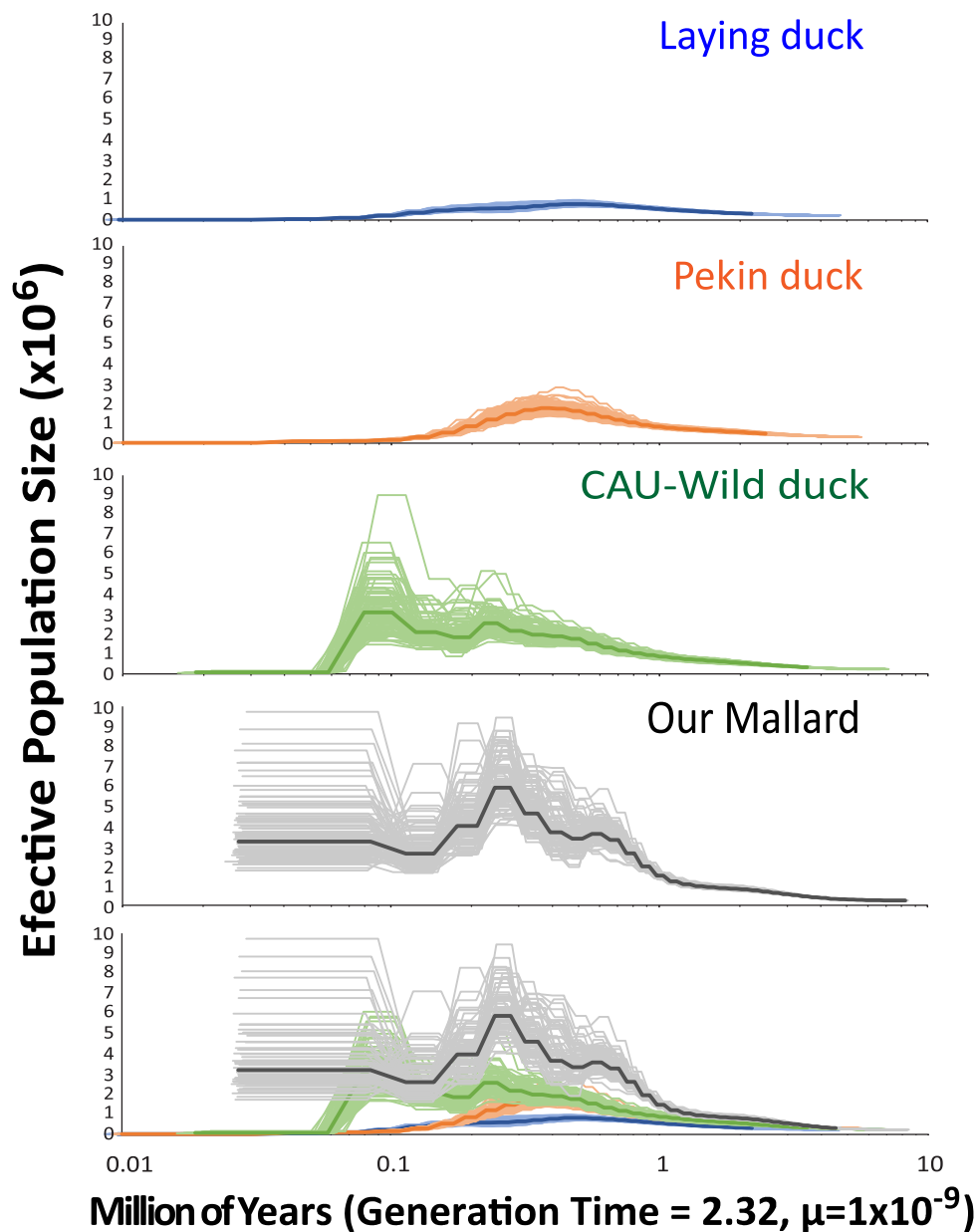
| Resolution (kbp) | Number of TADs | Mean TAD size (bp) | Base pair TADs (kbp) | % of genome in TADs |
|------------------|----------------|--------------------|----------------------|---------------------|
| 10               | 558            | 235,017            | 1,308                | 12.58%              |
| 25               | 619            | 592,164            | 3,483                | 33.49%              |
| 50               | 358            | 1,139,106          | 3,742                | 35.98%              |



**Fig. 3.** Violin plots of a) long runs of homozygosity (LROH) and b) nucleotide diversity ( $\pi$ ) calculated across 2 domestic breeds (CAU-Laying and CAU-Pekin duck), an un-vetted wild mallard (CAU-Wild), and our wild mallard genomes. Note that black dots along with their values denote average LROH and  $\pi$  in each plot.

have been summarized in Table 3. In short, a total of 619 TADs were identified at a resolution of 25 kbp, with a mean size of 592,164 bases that encompassed 33.49% of the genome. The strictest resolution had the lowest number at 558 with a mean size of 235,017 and only 12.58% of the assembly. The 50-kbp resolution had 358 TADs with a mean size of 1,139,106 bases and covered 35.98% of the genome. At the 25-kbp resolution, 6,722 genes

were identified within the TADs that are associated with 8 significant GO terms as follows: 4 biological processes (GO:0034765, GO:0006278, GO:0035335, and GO:0050911), 3 molecular functions (GO:0003964, GO:0004725, and GO:0004984), and 1 cellular component (GO:0005634). Of note, genes within the regulation of monoatomic ion transmembrane transport (GO: 0034765) term were shown to be differentially expressed in a feeding study in



**Fig. 4.** PSMC results for 3 published mallards and our mallard. For each analysis, the dark and lighter lines denote the average and bootstraps, respectively. Deviations in demographic histories suggest a divergence of 1 million years before present, with significant bottlenecks for all but our mallard; showcasing how using domesticated (i.e. CAU-Laying and CAU-Pekin) or unknown origin (i.e. CAU-Wild) breeds can result in substantial deviations in demographic reconstructions, including accentuating divergence times (Brown *et al.* 2022; Lu *et al.* 2022). Most extreme loss in effective population size estimates is among known domesticated breeds, showcasing how sequential bottlenecks with/without artificial selection during the domestication process severely impacts genetic diversity.

chickens, which the authors suggest is related to muscle contraction (Juanchich *et al.* 2018). Additionally, genes within the regulation of monoatomic ion transmembrane transport (GO:0034765) were also associated with high altitude adaptations as demarcated through haplotype-based scanning in Rhesus macaques (*Macaca mulatta*; Szpiech *et al.* 2021).

### Genetic diversity, runs of homozygosity, and demographic histories

A total of 1,030,125,353 quality base pair sites were retained across the 4 genomes: the CAU-Laying, CAU-Pekin, CAU-Wild, and NAWild\_v1.0. First, we recovered near similarly low levels of nucleotide diversity (avg.  $\pi \sim 0.003$ ) and long runs of homozygosity (LROH  $\sim 23$  kbp) for CAU-Laying and CAU-Pekin ducks, followed

by the CAU-Wild mallard (i.e. avg.  $\pi \sim 0.004$ , LROH  $\sim 22$  kbp), and with our mallard having the highest nucleotide diversity (avg.  $\pi \sim 0.007$ ) and shortest long runs of homozygosity (LROH  $\sim 3.5$  kbp) (Fig. 3). These calculated parameters suggest that the domesticated ducks have nearly 6.5-times longer runs of homozygosity that translates to 2.4-times less genetic diversity. These differences in genetic diversity translated to substantially differing demographic histories that were recovered across the 4 analyzed mallard genomes, with the greatest exaggerations in effective population size ( $N_E$ ) and time corresponding with source's respective domestication history, and thus, extent of lost heterozygosity (Figs. 3 and 4). First, the most extreme differences in demographic histories were recovered for the CAU-Laying and CAU-Pekin duck genomes, where both of them

show much lower overall peaks of effective population size—CAU-Laying duck  $N_E = 81,473$  at time 400,000 years before present (YBP) and CAU-Pekin duck  $N_E = 168,084$  at time 334,650 YBP—after which, both reaching and remaining at near 0 since 45,000 years ago. Next, whereas the general trend between our wild mallard and the CAU-Wild mallard appears similar, they too greatly differ. Specifically, while both genomes show increasing demographic histories starting ~1 million YBP, the CAU-Wild genome eventually declines to near 0 between 40 and 50,000 YBP, whereas our wild mallard reaches and remains at an effective population size of ~3 million since 100,000 YBP. Note that the exponential-like increase and contemporary estimates of effective population size are highly concordant with previous estimates using partial genome data for hundreds of vetted wild mallards (Brown et al. 2022). Generally, the most extreme loss in effective population size estimates is among known domesticated breeds (i.e. CAU-Laying and CAU-Pekin ducks), showcasing how sequential bottlenecks with/without artificial selection during the domestication process severely impacts genetic diversity (Fig. 3b).

Finally, deviations in demographic histories suggest that the domesticated breeds diverged from their wild ancestor in deep time at nearly ~1 million YBP. However, animal husbandry and domestication among human civilizations generally occurred between 15,000 and 36,000 years ago with domestication of fowl being one of the last ventures happening over the last 5,000 years (Sossinka 1982; Grayson 2001; Vigne 2011; DeMello 2021). Thus, we argue that inferences made thus far using domesticated (e.g. CAU-Laying and CAU-Pekin) or unknown origin (e.g. CAU-Wild) samples could be misleading, and that our genome provides a less biased demographic history of the species. Together, sample origin is clearly important and caution is required when attempting to infer species' demographic histories when using genomes of naturally inbred, domestic, or highly admixed individuals (Brown et al. 2022; Lu et al. 2022).

## Data availability

The assembled and annotated Wild North American mallard genome (NAwild\_v1.0) is available at the National Center for Biotechnology Information (NCBI) Genome Archive under BioProject accession no. PRJNA991977 and sample accession number JAUKTP000000000. All raw sequences associated with NAwild\_v1.0 are available from NCBI Sequence Read Archive under BioProject accession no. PRJNA991977 and sample accession number SAMN36329575. Finally, variant calling (VCF) files, and other data used in the analyses are available in UTEP's Bioinformatics Data Repository (<https://datarepo.bioinformatics.utep.edu/getdata?acc=JQSHW81NVCY96OE>).

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## Conflicts of interest

The authors declare no conflict of interest.

## Author contributions

PL conceived and funded the project; sample acquisition was done by PL. TS obtained molecular data; FH, TS, and JEM performed bio-informatics and molecular analyses; PL, FH, TS, and JEM equally contributed to the writing of the manuscript.

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