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Sequencing and characterization of complete mitogenome DNA of worldwide turkey (*Meleagris gallopavo*) populations

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ABSTRACT

The history of turkey (*Meleagris gallopavo*) domestication can be traced back to the period between 700 and 200 BC in Mexico. This process involved multiple contributors and resulted in the development of modern local turkey breeds. This research investigates the complete mitochondrial diversity across a diverse range of local turkeys. Seventy-three turkeys were sampled from various populations, including autochthonous Italian breeds, an American breed (Narragansett), as well as wild turkeys from the USA and Mexico. The mitochondrial DNA (mtDNA) was employed as a powerful tool for biodiversity and breed phylogeny investigation. An analysis of the entire mtDNA was conducted to identify breed-specific unique traits, mitochondrial-specific characteristics, and the phylogenetic relationship among turkey populations. A total of 44 polymorphic sites were identified. Brianzolo and Narragansett birds were characterized as genetically homogeneous populations. Thirty-two different haplotypes were identified when our samples were compared with mtDNA D-loop of 96 online available turkeys from various geographical countries. H1 and H2, differing for one mutation, were the most abundant, comprising 132 of the 185 sequences. H1 included samples coming from every region, while H2 was predominantly characterized by Italian samples. USA and Mexican samples appear to be more variable in their mtDNA than the other populations.

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Introduction

The domestication of turkeys can be traced back to between 700 and 200 BC in Mexico:[1] when Spanish arrived in Central America, they introduced these birds to various countries in Central and South America, as well as to Europe, during the 16th century.[2] From this moment on, the complex interactions between people and cultures, trade and traffic routes, high adaptability to specific environments and the historical events, caused the differentiation of turkey population in two subunits: the commercial highly productive strains, which selection in based on hybrid vigor and the traditional heritage populations reared in small scale production systems in different ecological conditions and with low economic investments.[3–5]

The widespread replacement of local turkey populations with highly productive commercial hybrids, selected for their ability to produce large quantities of meat at lower costs, has significantly reduced the prevalence of traditional low-input breeds.[6,7] These traditional breeds, which were historically raised in diverse environmental conditions, have been supplanted by commercial strains optimized through quantitative genetic strategies to enhance production efficiency and reproductive capabilities. In contrast, local populations were developed through phenotypic selection, resulting in a wide diversity of breeds within domestic species. This selection process has led to the development of distinct morphologies, behaviors, and adaptive traits specific to each breed.[8] Poultry species, including turkeys, are particularly vulnerable to genetic variability erosion and biodiversity loss.[9] Biodiversity is represented by the genetic variability within species, breeds, populations, and genes, as well as their interactions with the ecosystems in which they evolved.[10] Specifically, Italian local turkey breeds exhibit a notable variety of morphological traits and adaptability to diverse environmental conditions, reflecting the

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cultural and historical processes that have shaped these breeds across the Italian peninsula.[11]

As with all domesticated species, it is essential to establish conservation programs to monitor genetic variability among populations. Genomic analysis should be regarded as an effective tool for assessing biodiversity and informing conservation strategies.[9,12]

The constant evolution of genomic analysis protocols, with particular reference to mitogenome, along with the reduction in analysis costs and the availability of a huge amount of data, provides valuable information for biodiversity conservation studies in these birds.[11,13–17] In this context, the mitochondrial D-loop region has been proven to be an effective tool in genetic diversity investigation due to its high variability and high substitution rate.

Previous research has concentrated only on this genomic region in an attempt to trace the lineage of the extinct wild subspecies, *Meleagris gallopavo gallopavo*.[18]

Comprehensive mitogenome analysis also enables the study of the differentiation between regional domestic breeds and other heritage shedding light on their genetic relationships.[19]

This research aims to investigate the complete mitochondrial diversity across Italian breeds, a North American breed, and Mexican and American wild turkeys. The study utilizes extranuclear and maternally inherited mitochondrial DNA (mtDNA) as a source of information for analyzing biodiversity and elucidating breed phylogeny.

Materials and methods

Sampling and whole genome sequencing of our dataset

A total of 89 samples have been analyzed in this study (S1 Table, S2 Table—Sheet 1). These sequences were sampled from 11 distinct genetic breeds of turkeys. Samples have been collected within the activity of the TuBavI project (S1 Table). DNA was extracted from the collected turkey blood and feather samples using the ZR Genomic DNATM Tissue MiniPrep kit (Zymo, Irvine, CA). Specifically, blood samples were used for DNA extraction from Italian turkeys, feather samples were used for Mexican turkeys, and muscle tissue was used for American turkeys. Subsequently, the DNA samples were quantified using the NanoQuant Infinite®m200 instrument (Tecan, Männedorf, Switzerland), with a dilution to a concentration of 40 ng/μL.

Samples have been sequenced externally by Novogene Corporation. The sequencing library was prepared using the NEBNext® UltraTM DNA Library Prep Kit for Illumina. Following polymerase chain reaction (PCR), the resulting products were purified using the AMPure XP system (Beverly, MA). Subsequently, the library's quality was evaluated on the Agilent 5400 system (Agilent Technologies, Inc. Santa Clara, CA) and quantified by quantitative PCR at a concentration of 1.5 nM. The qualified libraries were pooled and sequenced on Illumina platforms using the 150 bp paired-end strategy, determined based on the effective library concentration and the required data amount.

Among these samples, 74 were collected from eight Italian different breeds: 8 samples of Brianzolo (Br), 7 of Bronzato Comune (BrCo), 8 of Castano Precoce (CaPr), 5 of Colli Euganei (CoEu), 11 of Ermellinato di Rovigo (ErRo), 11 of Nero Italiano (NI), 15 of Parma e Piacenza (PrPc), 9 of Romagnolo (Rom). Additionally, 2 samples were obtained from the USA turkeys, 10 from Mexico (Mex), and 3 from the Narragansett breed (Narr).

The Italian turkey samples were obtained from individuals originating in different regions of Northern Italy (Veneto, Lombardia, and Emilia Romagna), specifically from 15 farms, each typically breeding one or three specific breeds. The Mexican samples were collected from 12 different states across Mexico, encompassing a range of climatic and geographical conditions. These birds were part of backyard flocks, maintained on small farms where, to the best of our knowledge, no intentional selection was applied by the owners; the birds reproduced naturally through random mating. The Narragansett turkeys were sourced from two family farms located in Northern Italy.

Statistical analysis of the entire mitogenome within our dataset

After checking the quality of sequencing, the whole mtDNA consisting of 16,719 bp has been extracted from each sample sequence (.bam files) using Samtools package.[20] Additionally, the read depth of the mtDNA sequencing was evaluated using the Samtools package.

To perform multiple sequence alignment (MSA), consensus sequences were obtained starting from forward and reverse strands using Bcftools package.[20]

The consensus sequences were generated using the 'mpileup' package within Bcftools. The inputs comprised individual bam files for each sample and the mtDNA reference sequence (NC_010195.2). For each

position, under default parameters, the algorithm identifies the most prevalent base among the aligned reads for each sample.

All the mitochondrial consensus sequences, consisting of 16,719 bp, were aligned with the *M. gallopavo* mtDNA reference sequence (NC_010195.2) using ClustalW MSA algorithm implemented in Mega v.11.0.13 software.[21]

After undergoing quality assessment, it has been decided to remove a region of mtDNA due to its low level of mapping depth. The region spanning nucleotides 9462 to 12,828, encompassing 3366 base pairs, contains four variable sites considered unreliable. These four positions are located within the COX3, ND3, and ND5 genes. Previous studies focused principally on cytochrome B and D-loop regions, due to their high variability. However, in our samples, twelve regions, consisting of genes together with Cytochrome B and D-loop regions, exhibited variability, justifying the decision to keep the entire mitochondrial sequence.

The maximum composite likelihood estimate of nucleotides substitution pattern was calculated using Mega v.11.0.13 software. Parameters including the number of polymorphic sites (S), parsimony informative (S_{PI}) and singleton sites (S_S), number of haplotypes (NH), haplotype diversity (hd), nucleotide diversity (π), and average number of nucleotide difference (k) were computed according to Tajima (1983) and Nei (1987) using DnaSP6 v.6.12.03 software.[22]

An Analysis of Molecular Variance (AMOVA) was performed using Arlequin version v.3.5.2.2 software.[23] The analysis hypothesis asserted that genetic variation was correlated with geographical origin. Samples were categorized into two distinct groups: the first group comprises Italian samples, the second includes the USA, Mex, and Narr populations.

Furthermore, the F_{ST} values representing genetic differentiation between populations were calculated through pairwise comparisons with Arlequin version 3.5.2.2, computing 1000 permutations.

To elucidate the genetic relationships among haplotypes and their frequencies, a haplotype network was constructed using the Median-Joining Network (MJN) method implemented in PopART using default parameters.[24,25]

A Neighbor-Joining (NJ) tree[26] was constructed based on the Kimura-2-parameter model using Mega version 11.0.13 software,[27] to infer the genetic relationships among the samples. To ensure the reliability of the dendrogram, a bootstrap analysis with 1000 replicates was conducted.

Additionally, pairwise Nei's genetic distances were calculated in MEGA to categorize haplotypes into haplogroups.

Comparison of mitochondrial D-loop with literature

In addition to the samples collected for this study, a total of 96 turkey mtDNA sequences were acquired from publicly available samples obtained from GenBank (S3 Table). These sequences represent various turkey originating from different geographical regions (USA n. 34, Italy n. 22, Egypt n. 5, Israel n. 5, Spain n. 10, Brazil n. 5, Mexico n. 15).

To facilitate the alignment and comparison of all samples, DNA sequences of our dataset were trimmed to a selected region corresponding to 280 base pairs of the mitochondrial D-loop of *M. gallopavo* to overlap downloaded sequences.

That segment, which represents the shared region among all the samples obtained from previous studies, is considered the variable portion of the D-loop. Before conducting the comparison, we verified that, within our samples, there were no variants outside of that 280 bp region within the D-loop. Therefore, truncating the sequences did not result in the loss of any potentially valuable phylogenetic information. In addition, we checked that samples from previous studies longer than 280 bp and so truncated did not exhibit variability outside the portion selected for the analysis.

The alignment process was executed using ClustalW MSA algorithm, implemented in MEGA version 11.0.13 software.[21] Haplotypes were derived from the aligned sequences using DnaSP6 version 6.12.03 software.[22]

To establish genetic relationships among populations included in the analysis, a MJN was constructed using PopART with default parameters.[24,25] All Italian breeds were considered as a unique group as for USA and Narr turkeys that were grouped together. Furthermore, a NJ tree was constructed based on the Kimura-2-parameter model using MEGA version 11.0.13 software. This tree was employed to visualize the genetic relationships among the studied populations.

To assess the distribution of genetic variation both within and between populations examined in this study, an AMOVA was conducted using Arlequin version 3.5.2.2 software.[23] The AMOVA analysis has been performed grouping the samples depending on their geographical origin. The first group is composed of

Italian, Spanish, Iranian, and Egyptian samples, while the second from USA Narr, Mexican, and Brazilian turkeys.

Results and discussion

Whole mitochondrial genome analyses of our samples

Samples were sequenced with a genomic average depth of 31.03 and an average mapping rate of 98.55. The averaged mitochondrial read depth values ranged from 50 to 5000. The sequencing results are reported in S2 Table—Sheet 2. The entire mtDNA sequences were acquired for all samples and subsequently deposited in GenBank (accession numbers are listed in S2 Table—Sheet 1).

Twenty-three haplotypes have been identified and all polymorphic sites resulted substitutions, with the majority being transitions. The maximum composite likelihood estimates of the nucleotide substitution pattern revealed the following percentages: 30.89% (A), 25.41% (T), 29.74% (C), and 13.96% (G). As from Table 1, the total number of polymorphisms found within populations is 44 over 89 samples. Polymorphisms are split in singleton sites (S_S), defined in literature as non-informative sites, and parsimony informative sites (S_{PI}), which are defined as polymorphisms occurring with a minimum frequency of two within the population. All the breeds showed one or more singleton sites except for Br, ErRo, PrPc, and Narr. The highest number of non-informative sites (i.e., S_S) has been registered in Mex population with a value of 12.

Regarding the polymorphic sites reported in Table 2, the smallest number has been documented in Narr population, where all the three individuals of this breed shared an identical haplotype, that is, H23.

As well known, haplotype diversity is defined as the probability that two randomly sampled alleles are different, whereas nucleotide diversity represents the average number of nucleotide differences per site in pairwise comparisons among DNA sequences.[28] Our results display a generally high overall haplotype diversity, but many breeds showed one or more parsimony informative sites.

The number of haplotypes varied among populations, ranging from 1 in Br and Narr and breeds to 6 in Mex. Genetic diversity within breeds, measured by nucleotide diversity (π), ranged from 0.00002 (CaPr) to 0.00038 (CoEu), with an average value of 0.000114. Haplotype diversity (hd) ranged from 0.250 (CaPr) to 1 (USA), with an average value of 0.572. The average number of nucleotide differences (*k*) ranged from 0 (Br, Narr) to 11 (USA), with an average value of 2.392.

Both Br and Narr populations have a value of zero for haplotype and nucleotide diversity. This could be attributed to the very reduced registered population size of Brianzolo birds.[29] mtDNA, which is maternally inherited, shows no variation because all individuals in these populations may share a common maternal ancestor. This is also evident in Narr turkeys and could be attributed to the limited number of samples available. This lack of genetic diversity suggests that the breeding population is descended from a limited number of female ancestors, resulting in uniform mitochondrial haplotypes and nucleotide sequences.

The Mexican and USA populations demonstrated a high haplotype diversity (0.844 for Mexican and 1 for American) and low nucleotide diversity.

This finding aligns with the results observed in wild turkey populations in the United States.[15,17,30] As reported by Grant and Bowen,[31] this could be to a secondary encounter between previously distinct allopatric lineages or to a prolonged evolutionary history within a large, stable population. In other words, the turkey population believed to be the site of domestication should display greater mtDNA variability. When making this assumption, it is important to consider that only two wild American samples have these characteristics, and they represent a restricted sample size. Furthermore, the remaining samples from the United States, specifically the Narr population, exhibit homogeneity, with both nucleotide and haplotype diversity values equal to zero.

In the Mexican population, the elevated degree of haplotype diversity may be attributed to the presence of a stable population native to this geographical region,

Table 1. Mitochondrial nucleotide polymorphisms and molecular diversity indices of turkey breeds.

Breed	*n*	S	S_{PI}	S_S	NH	hd ± s.d.	π ± s.d.	*k*
Br	8	0	0	0	1	0	0	0
BrCo	7	2	1	1	3	0.724 ± 0.127	0.00007 ± 0.00005	0.857
CaPr	8	1	0	1	2	0.250 ± 0.180	0.00002 ± 0.00002	0.25
CoEu	5	11	3	8	4	0.900 ± 0.161	0.00038 ± 0.00023	5
ErRo	11	3	3	0	3	0.564 ± 0.134	0.00011 ± 0.00005	1.455
NI	11	3	1	2	3	0.673 ± 0.123	0.00006 ± 0.00005	0.8
PrPc	15	2	2	0	2	0.533 ± 0.126	0.00006 ± 0.00004	0.819
Rom	9	4	3	1	4	0.806 ± 0.089	0.00014 ± 0.00007	1.778
USA	2	11	0	11	2	1.000 ± 0.500	0.000084 ± 0.00062	11
Mex	10	16	4	12	6	0.844 ± 0.103	0.00033 ± 0.00020	4.356
Narr	3	0	0	0	1	0	0	0
Total	89	53	17	36	31			

Sample size (n), total polymorphic sites (S), parsimony informative (S_{PI}) and singleton site (S_S), number of haplotypes (NH), haplotype diversity (hd), nucleotide diversity (π) with their standard deviations (s.d.) and average number of nucleotide differences (*k*) within and across the populations, not available data (n.a.).

Table 2. Polymorphic sites of the turkey mtDNA according to the reference NC_010195.2 (REF) along with the corresponding genic region.

	Positions and corresponding genes																																												Number of samples/breed										
	12S						16S	ND1				ND2				COX1			Asp	COX2			ATP6			ND5		CYTB						ND6		D-loop																			
Haplotypes	106	258	387	423	433	833	1465	2859	3558	3610	3783	4214	4358	4499	4629	6141	6168	6415	7042	7230	7317	7659	8302	8411	8597	13484	13613	13717	13871	13912	14007	14489	14687	14996	15114	15686	15708	15725	15785	15808	15863	15886	15953	16159	Br	BrCo	CaPr	CoEu	ErRo	NI	PrPc	Rom	USA	Mex	Narr
REF	G	G	A	G	C	G	G	T	T	A	A	A	T	T	G	C	A	A	C	T	T	T	C	G	G	G	T	A	G	A	A	A	A	G	A	C	A	T	A	T	T	C	C	G											
H1	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	G	.	.	G	.	G	.	G	.	.	.	.	.	.	.	8										
H2	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	.	.	.	.		3	1			6		2		4	
H3	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	C	.	.	.	.	.	.		3	7	1	7	3	3	3			
H4	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	C	.	.	.	.	.	.		1									
H5	A	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	G	.	.	.	C	.	.	.	.	.	.	G	.	G	.	.	.	.	G	.	G	.	.	.	.	.	T	.				1							
H6	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	G	.	.	G	.	G	.	G	.	.	.	.	.	.	.				2							
H7	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	C	C	.	.	.	.	.	.	.	G	.	.	G	.	G	.	G	.	G	.	.	.	.	.				1							
H8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	C	.	.	.	.	.	.					1						
H9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	.	.	.	.					3						
H10	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	.	.	.	.						1					
H11	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	.	.	.	.						1					
H12	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	G	.	.	.	G	.	G	.	.	.	.	.	.	.							10				
H13	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	G	.	.	.	G	.	G	C	.	.	.	.	.	.							2				
H14	.	A	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	.	.	.	.								3			
H15	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	G	.	.	.	.	G	.	G	C	.	.	.	.	.	.								1			
H16	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	G	.	T	.	C	.	.	.	A	.	C	.	A	G	.	.	.	.	G	.	G	.	.	C	.	T	.	.									1		
H17	.	.	G	.	.	.	.	C	.	.	G	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	A	G	.	G	.	.	C	.	.	.	.									1		
H18	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	C	.	.	.										2	
H19	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	C	.	.	A										1	
H20	.	.	.	.	.	.	.	.	.	G	G	.	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	C	.	.	.										1	
H21	A	.	.	.	.	.	.	.	.	G	G	.	C	.	.	.	G	.	.	.	C	.	.	.	.	.	.	G	.	G	.	.	.	.	G	.	G	.	.	.	.	.	T	.										1	
H22	.	.	.	C	.	.	.	.	.	.	G	G	.	.	.	T	.	.	.	C	C	.	.	.	.	.	.	.	.	G	.	G	.	.	G	T	G	.	.	.	.	.	.	.										1	
H23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.	.	.	.	.											3

characterized by a long evolutionary history.[31] Among the populations, CoEu exhibited one of the highest numbers of polymorphic sites with a value of 11, but low number of parsimony informative sites and low number of informative sites. The Mex population displayed the highest number of polymorphisms with a value of 16 but only had 4 parsimony informative sites.

The study conducted by Padilla-Jacobo et al. identified a high level of haplotype and nucleotide diversity.[15] This contrasts with our findings, as we did not observe a high level of nucleotide diversity between populations, instead, we observed a high level of haplotype diversity. In some subspecies, particularly M. g. silvestris, Osceola, and intermedia, they noted the highest level of hd and a low π, attributing this pattern to a bottleneck event followed by rapid expansion. Interestingly, this observation aligns with our own findings.

The high haplotype diversity, combined with low nucleotide diversity, indicates that while there are many distinct maternal lineages (high hd), there is relatively little sequence variation within these lineages (low π). This can be interpreted as evidence of a historical population bottleneck, where the population size was drastically reduced, followed by a rapid population growth that allowed new haplotypes to develop without accumulating significant nucleotide differences. This pattern is consistent with the concept of genetic drift acting on small populations, where a few maternal lineages become predominant and diversify quickly as the population expands.[31]

The 23 haplotypes identified through comparison of our 89 turkey sequences with the turkey reference sequence are presented in Table 2.

Polymorphisms are distributed throughout the entire mitochondrial sequence, particularly in 12 distinct genic regions as shown in Table 2. H3 is the most present haplotype, with a frequency of 30.3%, and it is shared among twenty-seven different samples representing all Italian turkey breeds except Br. Following closely there is H2, with a frequency of 18.0%. H23, which exhibits the closest similarity to the reference sequence, is exclusively found in the Narr breed, differing from the reference by only three nucleotide positions across the entire mitochondrial sequence. Almost half of the haplotypes (13 out of 23) are unique to individual samples, but 10 haplotypes are shared among two or more samples representing 85.4% of the turkeys.

With the exception of the haplotype H2, which is shared between Italian and Mexican populations, there is a distinct separation between the haplotypes of Italian breeds and those of USA and Mexican turkeys. The presence of H2 in both Italian and Mexican populations suggests a shared historical lineage, reflecting the origins of Italian turkey populations as descendants of Spanish turkeys, which were among the earliest domesticated from wild turkeys in Central America. Turkeys were likely introduced from the Americas to Spain around 1511 and subsequently spread to Italy.[32] The observed geographic clustering of Spanish and Italian turkeys can be attributed to the birds' high adaptability to various environmental conditions.[11,16]

All the samples exhibit polymorphisms at three specific genomic positions compared to the reference sequence (positions 13,912, 15,114, and 15,708). These mutations were identified through alignment with the current version of the reference genome. In the previous reference genome version, the nucleotide at the corresponding position was guanine (G), which is consistent across all the analyzed samples. Additionally, two other positions are consistent across all samples except for the ErRo and Narr breeds (positions 3783 and 7317). In this instance, the variation is not attributed to the reference version.

Some polymorphisms are exclusive to samples within specific breeds; for example, at position 7,659, all Br samples present a C in contrast to the T found in the reference sequence.

This type of polymorphism can be significant in understanding genetic diversity and lineage tracing within breeds. The same polymorphism is shared by three out of five CoEu breed samples. Additionally, the same samples at position 14,687 possess a G rather than the A observed in the reference sequence. In the past both breeds were reared for their brooding ability and high maternal attitude, in addition, both breeds are particularly adapted to *en plen air* rearing system. Historically Lombardy (where Brianza are is located: Brianzolo = from Brianza) and Veneto were part of the same Empire: the Austro-Hungarian one and both these breeds were selected in hilly areas.[33]

To assess the distribution of genetic variability, we conducted an AMOVA under the hypothesis of variation attributed to geographical origin. The outcomes of the AMOVA are presented in Table 3. The findings suggest that a substantial amount of genetic variability

Table 3. AMOVA analysis among the 11 breeds of Turkey.

Source of variation	d.f.	Sum of squares	Variance components	Proportion of variation	Fixation Indices
Among groups	1	9	0.11901 Va	7.41	FSC: 0.43808
Among population within groups	9	54.015	0.65176 Vb	40.56	FST: 0.47970
Within populations	78	65.209	0.83601 Vc	52.03	FCT: 0.07407
Total	88	128.224	1.60678		

(52.03%) is present within individual populations, underscoring the high level of genetic diversity at this level. The considerable variation among populations within the major groups (40.56%) further emphasizes the significant genetic differentiation among populations within each group. In contrast, the relatively small proportion of variation between the major groups (7.41%) indicates that the broad geographic or cultural distinctions are not as pronounced in terms of genetic differentiation.

These results highlight that the hypothesis attributing significant genetic variation to geographic origin is not consistent with the observed data.

The variation could instead be primarily attributable to the diversity of birds within the groups Italy and USA-Mexico-Brazil. The obtained results underscore the complex effects of the phylogeny of turkey breeds and populations in combination with selection targets, adaptation, and human history.

When comparing selection practices among ornamental breeds, characterized by specific plumage colors and patterns, to traditional 'low-input' productive breeds and commercial hybrids, a complex interplay of phylogeny, historical context, cultural traditions, and selective breeding for performance traits emerges. This intricate combination shapes the genetic makeup of each breed, contributing to their unique characteristics and genetic distinctiveness.[33]

The Br and Narr result outputs, throughout the different performed analyses, to be very homogeneous in their mtDNA sequence, it is possible to suppose a strong selection for morphological traits based on high standard quality inbred breeders for Narr birds (fancy breed) and a very reduced population size (local breeds) with high inbreeding levels for both the breeds.[11]

The matrix of pairwise fixation indexes (F_{ST}) is presented in Table 4. The highest value observed results between Italian breeds and others, specifically between Br and Narr being 1.000. Br birds have been selected in the hilly area of Brianza in the very North of Italy: they are characterized by high grazing ability, fast growth, and resistance, these traits make them excellent birds to be reared in marginal areas and their body proportions and weight clearly define these attitudes. The Br individuals should be mainly considered, on the contrary of Narr, as productive birds more than fancy turkeys selected for conformation exhibitions. Anyway, as in Narr birds, plumage color is a distinct trait in Br breed too: the partridge color which characterizes Br birds is a distinctive trait, the same color can be find in Belgian Ronquiere turkey underlining a shared phylogenetic origin strictly linked to Spanish conquests throughout Europe.[33] In addition, when compared with USA turkey population, the Narr breed shows a high genetic differentiation value (F_{ST} value of 0.629), suggesting a notable genetic distinction between these two groups. These results could be oriented to characterize the original Narr mitochondrial sequence which could be interpreted as the result of strong phenotype-oriented selection; on the contrary, in wild turkeys natural selection preserved high variability in mtDNA.

From Table 4, it can be observed that the Narr breed is the more differentiated from all others: it exhibits, in facts, the highest pairwise fixation indices in comparison to each of the other examined breeds.

Among the Italian breeds, the lowest observable indexes in the table are 0.035 and 0.065 between BrCo and NI and BrCo and Rom respectively. The pairwise genetic differentiation of the considered breeds underlines the different target traits in the selection: ErRo belongs to a breed characterized by a single plumage color defined by recessive alleles described in the standard, the area of origin is the same (Northern Italy) and common ancestors could be present. Furthermore, these results are in accordance with the historical development and productive orientation of the two others considered breeds: Rom and BrCo which are traditional breeds

Table 4. Pairwise genetic differentiation index (F_{ST}—permuting haplotypes among population among groups) of populations.

Pop	Br	BrCo	CaPr	CoEu	ErRo	NI	PrPc	Rom	USA	Mex	Narr
Br	0										
BrCo	0.853	0									
CaPr	0.957	0.073	0								
CoEu	0.217	0.261	0.396	0							
ErRo	0.708	0.144	0.183	0.334	0						
NI	0.813	0.035	0.356	0.299	0.263	0					
PrPc	0.832	0.431	0.581	0.475	0.457	0.424	0				
Rom	0.710	0.065	0.217	0.262	0.217	0.112	0.401	0			
USA	0.821	0.654	0.759	0.353	0.635	0.715	0.772	0.607	0		
Mex	0.491	0.175	0.304	0.174	0.291	0.161	0.354	0.191	0.423	0	
Narr	1.000	0.832	0.950	0.498	0.565	0.816	0.834	0.687	0.629	0.446	0

Brianzolo, Br; Bronzato Comune, BrCo; Castano Precoce, CaPr; Colli Euganei, CoEu; Ermellinato di Rovigo, ErRo; Nero Italiano, NI; Parma Piacenza, PrPc; Romagnolo, Rom; United States of America, USA; Mexican, Mex; Narragansett, Narr.

originated in northern Italy with a shared diffusion areal: both are characterized by high adaptability to low input extensive production systems and show high natural reproductive ability and strong maternal instinct. Additionally, all of them were raised for meat production in rural contexts. An interesting observation is that the name of BrCo turkeys is in accordance with the coloration of their plumage, which is bronze.

The MJN in Figure 1 visualizes the genealogical relationships between haplotypes and their frequencies and support the evidences hereinbefore presented based on F_{ST} values. The size of the circles in the dendrogram is directly proportional to the number of samples.

The Narr breed is prominently represented by haplotype H23, shown in dark red at the bottom of the MJN. These three samples are clustered within haplogroup 8 (HG8), underscoring their distinct genetic divergence from other samples. Specifically, these samples exhibit a divergence of one mutation from haplogroup 4 (HG4) and four mutations from the reference haplotype, further illustrating their unique genetic position within the network nd distinguishes them clearly from other haplotypes.

Notably, H2 and H3 are the most abundant haplotypes, consisting of 16 and 27 samples, respectively. These two haplotypes are grouped together within haplogroup 2 (HG2). From the network is possible also to observe that these two haplotypes are separated only by a mutation in position 15,725, in the region of mitochondrial D-loop. Specifically, in that position H2 exhibits a T, consistent with the reference, whereas H3 displays a C. HG2 is the most prevalent haplogroup, encompassing seven distinct haplotypes. It predominantly includes Italian breeds, with the exception of four Mexican turkey.

Haplotypes ranging from 16 to 23 belong to non-Italian samples. H23 which represents Narr samples, is separated by ErRo haplotype (H9) by only one mutation. In both the breeds the color of the plumage is a distinguish feature and the main characterizing trait.[34–36]

The Mexican samples were distributed among 6 different haplotypes, a relatively high number considering that the Mexican population consists of only 10

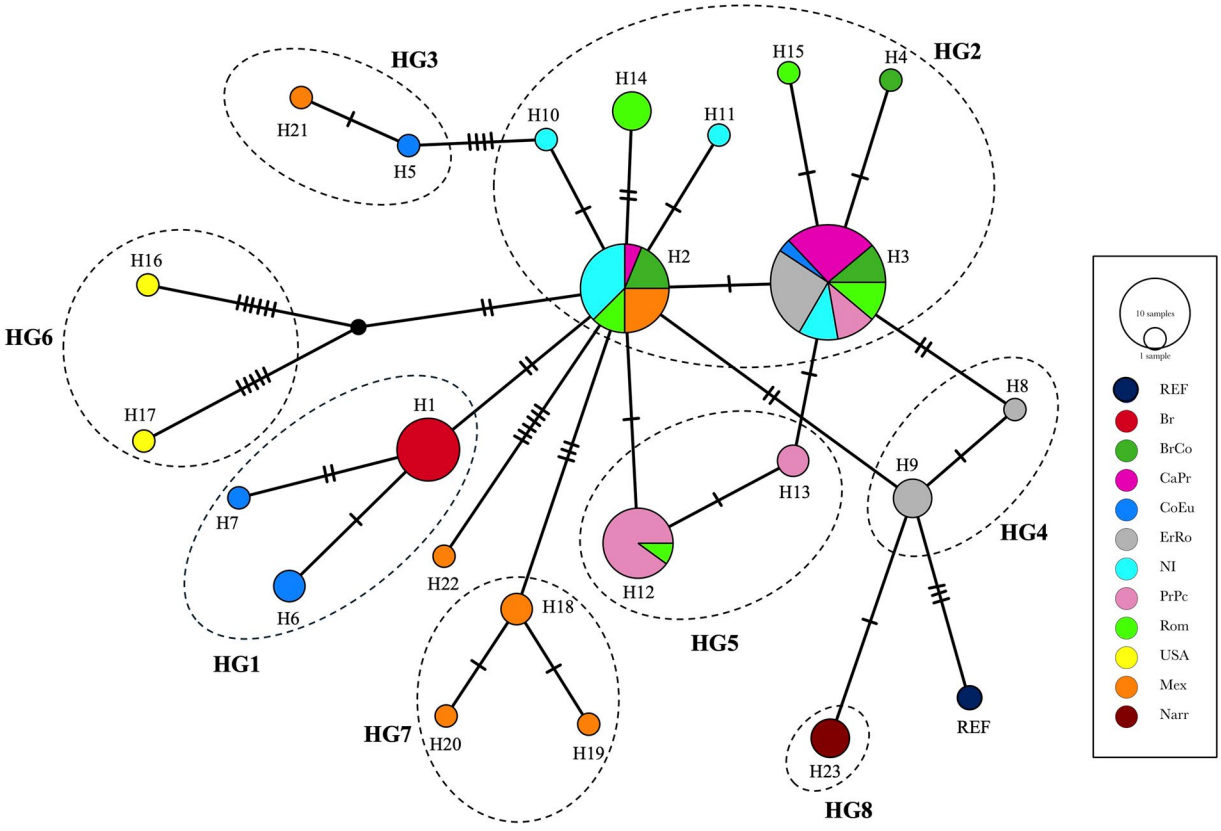

Figure 1. The Median-Joining network (MJN) based on the 73 Turkey mtDNA sequences and the reference sequence NC_010195.2. Circles are proportional to the numerosity of the samples. Thick marks represent point mutations. Dashed circles represent haplogroups (HG).

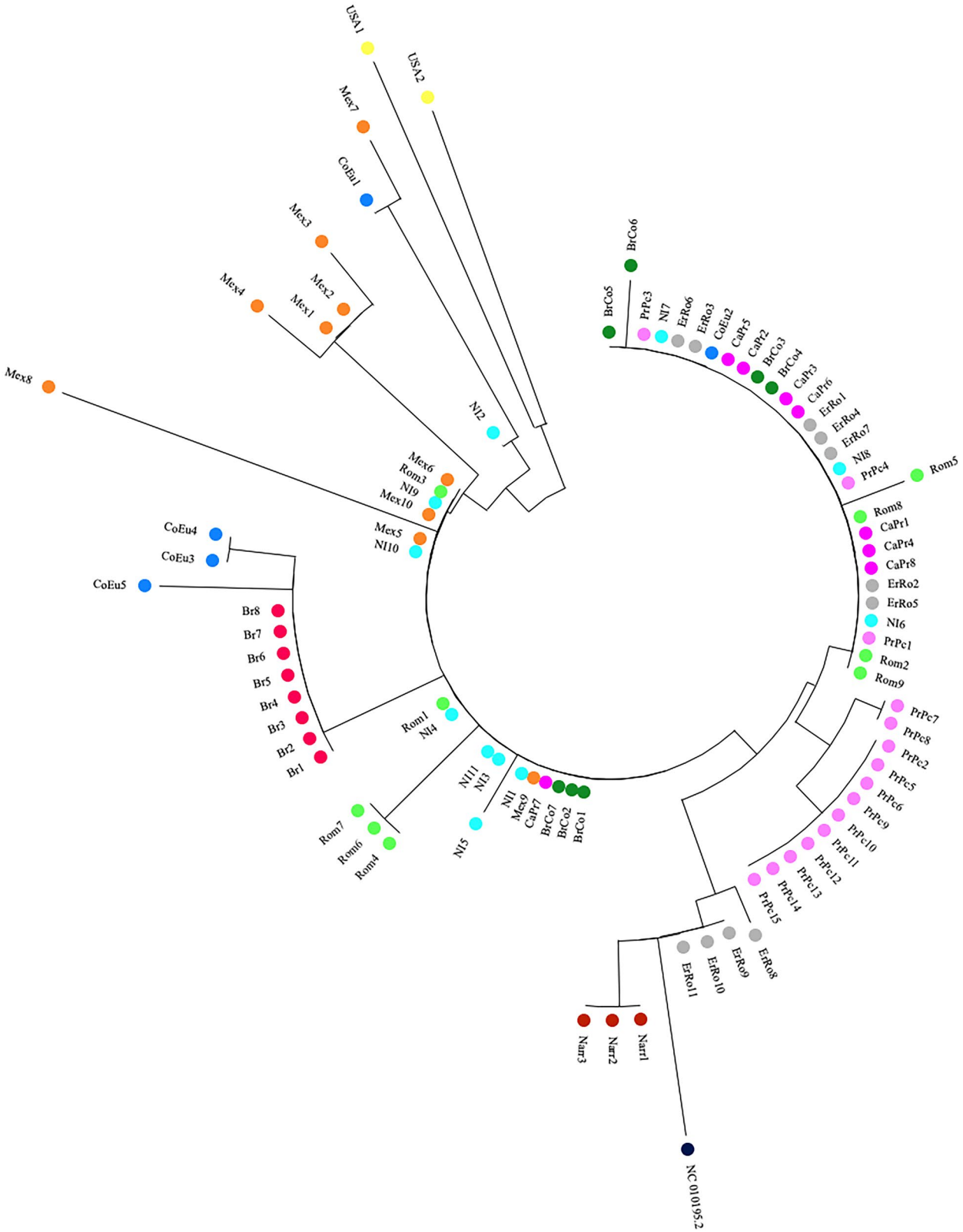

Figure 2. Neighbor-joining (NJ) tree of 89 samples belonging to 11 breeds of Turkey. NJ tree based on Kimura-2 parameter model distances. The numbers represent the robustness of the dendrogram.

individuals. However, this observation is consistent with the results reported by Padilla-Jacobo et al., who identified five different haplotypes in a population of nine Mexican turkeys.[15] They suggest that increasing the sample size will likely result in a proportional increase in the number of haplotypes.

Table 5. Polymorphisms of the 280 bp region of the D-loop found in our samples together with 96 publicly available sequences. Samples have been grouped on the base of their geographical origin.

	POSITIONS																																			NUMBER OF SAMPLES/ BREED						
	15575	15577	15625	15646	15668	15677	15679	15686	15698	15708	15719	15725	15727	15735	15743	15746	15771	15776	15782	15785	15787	15791	15793	15794	15796	15808	15824	15838	15844	15845	15863	15886	15893	15953	15965	15966	15999	Italy	Mexico	USA	Brazil	Spain	Iran	Egypt
REF	C	C	C	A	G	T	C	C	A	A	C	T	A	T	A	A	A	C	C	A	C	C	A	C	T	T	A	G	C	C	T	C	G	C	C	A	C							
H1	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	56	14	7	3	9		2
H2	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	37			2			2
H3	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	1	1	2			4	1
H4	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1						
H5	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.			2				
H6	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			5				
H7	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.		4					
H8	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		2					
H9	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A	T	.	.	.			1				
H10	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	T	.	.	.	.	.			1				
H11	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	C	.	1						
H12	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1					
H13	.	.	.	C	.	.	.	.	T	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.					1		
H14	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.						1	
H15	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H16	.	.	.	.	.	.	T	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H17	.	.	T	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H18	.	.	.	.	.	.	.	T	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H19	T	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	T	.	.	A	.	T	.	C	.	.	.	T	.	.	.	.	.	.	.			3				
H20	T	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	T	.	.	A	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.			1				
H21	T	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H22	T	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.	C	G	.	.	.	.	.	.	.	.	.	.			1				
H23	.	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.	.	T		2	1				
H24	T	.	.	.	.	C	T	.	.	G	T	.	.	.	.	.	.	.	T	.	.	A	.	T	.	C	.	.	.	T	.	.	.	.	.	.	.			1				
H25	.	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.	C	.	.	.	T	.	.	.	.	.	.	.			1				
H26	.	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	T	T	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H27	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H28	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T			1				
H29	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			3				
H30	.	.	.	.	.	C	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	T	C	C	.	.	.	.	.	.	.	.	.	.	T		1					
H31	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.			1				
H32	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	.	.	.			1				

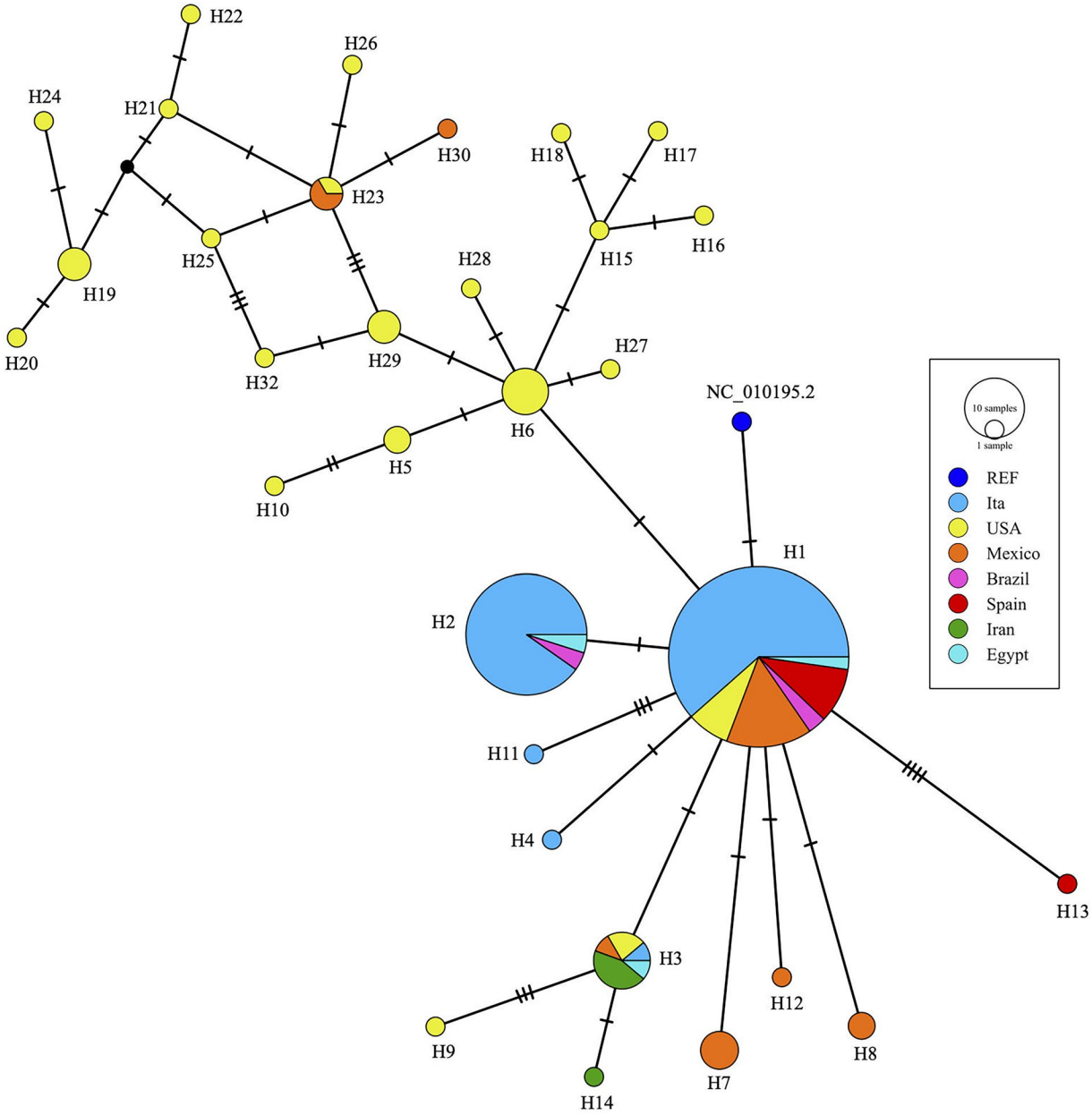

Figure 3. Median joining network of the 185 D-loop sequences.

Table 6. Hierarchical AMOVA between three groups, Italy-Spain-Iran-Egypt, USA-Mexico-Brazil.

Source of variation	d.f.	Sum of squares	Variance components	Proportion of variation	Fixation indeces
Among groups	1	21.869	0.12224 Va	11.39	FSC: 0.22252
Among populations within groups	5	19.11	0.21171 Vb	19.72	FST: 0.31104
Within populations	178	131.669	0.73971 Vc	68.9	FCT: 0.11385
Total	184	172.649	1.07367		

Groups: 'Italy-Spain-Egypt-Iran vs. USA-Mexico-Brazil'. Variance for group, Va; variance for population, Vb; variance for haplotypes within a population within a group, Vc; permuting haplotypes among population among groups, FST; permuting haplotypes among populations within groups, FSC; permuting populations among groups, FC.

Mexican turkeys from our study, as well as those investigated by Padilla-Jacobo et al., were collected from various locations in Mexico.[15] This suggests considerable genetic variability within the Mexican turkey population, as evidenced by the presence of distinct maternal lineages among the individuals.

Analysis of the network reveals that USA samples (highlighted in yellow), represented by haplotypes H16 and H17, exhibit some of the greatest divergence, with 7 and 8 mutations, respectively, from H2.

Except for Mexican samples (in orange) all the samples belonging to the same breed are separated

by few mutations. The 15 PrPc samples (in pink), for example, are represented mostly by H12 and H13, the two most different haplotypes are separated by only two polymorphisms and clustered together in HG5.

PrPc birds are a traditional breed adapted to extensive rearing systems in marginal areas. As a result of this traditional farming strategy, the breeds show a specific structure maintaining genetic variability in accordance with previous literature.[5,11]

The NJ tree illustrated in Figure 2 was built using 89 sequences of the dataset along with the reference sequence. Distribution of samples in the dendrogram supports the findings obtained and showed in the MJ network (Figure 2). It is evident that the two sequences of USA turkeys exhibit a substantial phylogenetic distance not only from each other but also from other samples, as demonstrated by the significant number of mutations relative to other haplotypes. From the tree, it is possible to observe the similarity between NI and Mex samples represented in different cluster of the graph together. The majority of Mexican samples and USA samples are quite separated from the rest of the birds. These results suggest that the 500 years selection of the European and Italian birds acted diversly from the one occurring on the Mexican and USA birds.

Figure 2 illustrates that the three animals of the Narr breed exhibit the highest phylogenetic similarity to the reference sequence, with only three nucleotide variations compared to it. Of these three mutations, two are present in all 89 animals included in the dataset, while the third is specific to the Narr breed and located within the ATP6 gene.

Comparison of mitochondrial D-loop with literature

Our mtDNA sequences, properly trimmed, were compared to 96 publicly sequences of turkeys from various countries, available for only a mtDNA D-loop portion of 280 bp long (S2 Table). The 280 bp region was adequate to include all the polymorphic sites of the D-loop previously identified in our samples, with the exception of position 16,159, which exhibited variability in one of our samples.

Table 5 presents the results of the alignment and haplotype generation process. It is possible to notice that out of the 185 sequences analyzed, 32 different haplotypes have been identified.

For what concern our samples, the number of haplotypes decrease to 8, when we focus the analysis only on mitochondrial D-loop region.

H1 and H2 were the most abundant, comprising 132 of the 185 sequences (71.35%). H1 included samples coming from every region examined the analysis, except for the samples from Iran, while H2 was predominantly characterized by Italian samples (37 out of 41).

Among the haplotypes generated, 21 out of 32 were representative of single samples, only 11 haplotypes were shared between two or more samples.

The MJN in Figure 3 was obtained including only the mtDNA D-loop portion of the mtDNA sequences and grouping all Italian birds in a unique group. From Figure 3, it's possible to observe that H1 and H2, the most prevalent ones (as also shown in the NJ tree in S1 Figure) are separated by just one mutated position (n. 15,725), highlighting their close similarity.

H1 is shared among 91 distinct samples, originating from both our dataset and sequences retrieved from Genbank. This particular haplotype corresponds to the one identified by Canales Vergara et al. as haplotype MGDH2, which is shared by 62.67% of the samples they analyzed.[14] These samples belong to turkeys from various geographical regions, including Brazil, Egypt, Spain, Mexico, USA, Italy (Parma and Romagnolo breeds). A subset of the sequences analyzed by the same authors has been integrated into our study. This observation supports the hypothesis of the presence of a shared maternal lineage among samples originating from diverse breeds.[14]

The same haplotype is described by Speller et al. as haplotype mHap2, observed in eight samples belonging to *Meleagris gallopavo gallopavo*, that is reported as closely related to domestic turkeys without breed specification.[18]

When comparing our Italian samples with the ones analyzed by Canales Vergara et al.,[14] it's possible to notice the presence of two different maternal lineages (H1 and H2) for the Parma and Romagnolo breeds.

In contrast to Canales Vergara et al.,[14] who reported a moderate value for hd in the Romagnolo breed population, our samples from the same breed exhibited a high hd value of 0.806; however, the value of π here identified is low, consistent with the findings in the study by Ref. 14.

Additionally, the network illustrates that a significant separation exists between the majority of USA-Narr and Mex samples (in yellow and orange, respectively) and the samples from European and Asian regions.

Even though most haplotypes correspond to single samples, the network clearly demonstrates that the

vast majority of them differ by only a single mutated position, emphasizing their underlying similarity.

H4 corresponds to a CoEu sample, which is represented as H7 in Figure 1. It differs by only one nucleotide position from H1, which includes the majority of the Italian samples. H11, as depicted in Figure 3, corresponds to a PrPc turkey, with data obtained from NCBI. The distinction of H4 and H11 from the main cluster of Italian haplogroups, separated by one and three mutations respectively, suggests the presence of distinct maternal lineages or unique evolutionary events.

AMOVA analysis was conducted by grouping samples according to their geographical origin, which included groupings for samples from Europe and Northern Africa, as well as North, Central, and South America. The results are illustrated in Table 6. The percentage of variation among groups, which stands at 11.54, is lower than the value among populations, which is 11.38. This finding doesn't support the hypothesis of diversity dependent on the geographical origin. However, the value of variation among groups evaluating samples coming from other studies is higher than the one found in the analysis of our samples.

Conclusion

Our study provides a comprehensive analysis of mitochondrial haplotype and nucleotide diversity across Italian turkeys and the ones coming from other countries (wild turkeys). This is the first extensive examination of the entire mitochondrial genome in turkeys, as previous research has primarily focused on the mitochondrial D-loop region. Our findings reveal a high overall haplotype diversity, with notable differences among breeds. Specifically, Br and Narr turkeys are characterized by relatively low genetic variability, reflecting their homogeneous breeding histories and selection practices. In contrast, traditional Italian 'low input' breeds exhibit considerable genetic diversity, differentiating them from more uniformly selected breeds like Narr. We identified 32 distinct haplotypes from a comparison of mtDNA D-loop sequences across various geographical regions, highlighting breed-specific genetic diversity levels. This underscores the importance of comprehensive mtDNA genome sequencing, as it provides a more detailed understanding of genetic variation compared to studies limited to the D-loop region. Our results illustrate the positive impact of conserving genetic diversity within Italian breeds and emphasize the necessity of protecting local breeds to maintain overall genetic health and diversity.

The limitations of previous studies, which often involved restricted sample sizes and focused solely on the mitochondrial D-loop, have made it challenging to draw broad conclusions about interpopulation relationships. Our analysis, encompassing 12 distinct mitochondrial regions, offers new insights and reinforces the need for holistic approaches in mitochondrial research. These patterns of genetic homogeneity and distinctiveness can be important for conservation, breeding programs, and understanding the evolutionary history of these breeds.

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Authors' contribution

Conceptualization: MGS.; Methodology: CF, MGS; Formal analysis: CF; Writing original draft: CF, SPM, MGS; Manuscript editing: all authors; Review and supervision: AB; Funding acquisition: SC, SPM. All authors have read and agreed to the published version of the manuscript.

Consent for publication

Not applicable

Data availability

The accession numbers of the complete MTDNA sequences of all samples used in this study are listed in S1 Table.

Disclosure statement

The authors declare no competing interests.

Ethics approval and consent to participate

The animal study was reviewed and approved by Università degli Studi di Milano: OPBA_103_2020 and OPBA_101_2023

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