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Understanding the Genetic Variation and Structure of the Rustipollos Chicken Synthetic Population Locally Adapted to Paraguay: Opportunities for a Sustainable Chicken Productivity

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Abstract: The production of backyard chickens is an activity of great importance in the economy of rural families in Paraguay. The Rustipollos population was created through directed crosses between a commercial meat line and a local population belonging to non-specific breeds but phenotypically assimilated to Creole breeds. The aim of this study was to evaluate the genetic diversity, relationship, and structure of Rustipollos using 29 microsatellite markers. Analysis was performed on 50 Rustipollos animals and 926 other individuals as reference breeds/populations from Europe, Africa, South, and North America. A total of 318 alleles were detected, with a mean of 10.97 per *locus*. The polymorphic information content indicated that 80% of all *loci* were highly to moderately informative. Only two breeds/populations showed *loci* that did not deviate from the Hardy–Weinberg equilibrium. The results of genetic diversity indexes suggested moderate levels of genetic variability in Rustipollos population and low inbreeding level. The genetic differentiation index indicates a high genetic differentiation between populations. The results of the Neighbor-Net tree and STRUCTURE analyses indicate the existence of distinct gene pools, with some genetic relationships between Rustipollos, the commercial chicken strain, and south Spanish breeds. The Discriminant Analysis of Principal Components confirmed the observed genetic distances between breeds/populations. The results will be useful for sustainable use and official recognition of this population.

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1. Introduction

Of all livestock species, chickens are most distributed in rural and semi-urban regions in the tropics and semi-tropics characterized by free-range agricultural production systems [1]. Chickens farming has advantages over other livestock species, such as reduced body size [2], maternal skills [3,4], low cost of production, scavenging ability, high adaptability to harsh environmental conditions, source of renewal for rural families with limited resources [5,6], and meat quality due to lower levels of fat, cholesterol, and iron compared to red meats [7]. However, the local breeds have low productivity [8] which is insufficient to meet the requirements of the population and alleviate poverty among smallholder farmers [6]. This fact has prompted the creation of improved poultry in centers and universities capable of contributing to food generation and the rural economy [4,8–11]. The Rustipollos poultry population was created in 2001 at the Poultry Division of the Faculty of Veterinary Sciences of Paraguay in response to the demand from small producers to engage in extensive or semi-extensive poultry farming. The chicken population originated by direct crossing between a commercial meat line and a local population belonging to non-specific breeds but phenotypically assimilated to Creole in order to obtain a synthetic breed of dual-purpose birds that is well adapted to the environmental conditions of Paraguay [12]. Since 2006, the Rustipollos has been a well-defined population, that has undergone a management program including pedigree registration, use of mating plans, and selection scheme. Rustipollos chickens are characterized by their moderate growth, with males reaching an average weight of 2.32 kg at 10 weeks of age [13] and females starting to lay eggs at 16 weeks, with an average weight of 2.36 kg [14]. Their egg production is remarkable, with an average weight of 60.7 g between 30 and 40 weeks of age [15]. Beyond their productive performance, Rustipollos chickens stand out for their adaptability. A survey of 31 Rustipollos producers revealed that 71% agree with their productivity in both meat and eggs [16]. In addition, 94% of respondents mentioned their high capacity to adapt to free-range feeding, making them an attractive option for sustainable production. Currently, the experimental nucleus consists of 155 hens and 43 roosters.

Comprehensive knowledge of the population structure and distribution of genetic diversity in livestock are essential aspects to improve selection and breeding designs, support the safeguarding of biodiversity, enhance the efficient use of breeds, and implement conservation programs adapted to local conditions [17]. Regarding the conservation of livestock genetic resources, if there are cost or breeding site constraints, the population can be maintained as a core collection that has the highest possible genetic variability with an adequate population size. For the development of a proper core collection, accurate genetic evaluation for the population using molecular markers would be required [18]. It is widely accepted that microsatellite markers remain a useful molecular tool for the assessment of genetic diversity and population structure and differentiation [19] due to many advantages, such as being numerous and ubiquitous throughout the genome, thus showing a higher degree of polymorphisms and codominant inheritance [20]. In addition, microsatellites have been recommended by [21] and have been used in many studies to allow direct comparisons [22].

Several research studies have been conducted in indigenous creole or local chicken populations [5,6,17,22–25] and native and commercial lines [10,11,14,26,27]; however, the synthetic breed Rustipollos has never been investigated until today. Therefore, this study aimed to genetically characterize the Rustipollos population using microsatellite markers. More specifically, the genetic diversity and population structure of Rustipollos and the genetic relationship between the studied population and some neighboring and worldwide breeds were determined. This study should provide adequate baseline data for the characterization of the local Rustipollos chicken genetic resource with the purpose of maintaining

genetic variation and minimizing the inbreeding and consequently contributing to its improvement and sustainable use.

2. Materials and Methods

2.1. Chicken Population and Samples Collection

A total of 50 unrelated individuals of both sexes (27 females and 23 males) were sampled from the original nucleus of the Paraguayan Rustipollos chicken population at the experimental farm of the Poultry Division of the Faculty of Veterinary Sciences (Supplementary Material Figure S1). Blood samples (Rustipollos, Cobb broiler, White Plymouth Rock, and Brahma) were collected by brachial venipuncture and placed on Whatman FTA™ filter cards (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK), left to dry in a cool place for approximately one hour, and then stored in discrete envelopes at room temperature until the DNA extraction was made [6]. Blood sampling tasks were carried out by trained veterinarians who adhered to standard procedures and relevant national guidelines to ensure appropriate animal care. The research was carried out in adherence to the guidelines and regulations outlined in the ARRIVE guidelines (https://arriveguidelines.org, accessed on 20 September 2023). Therefore, no ethical approval was required for the sampling of biological material. Samples from individuals belonging to Paraguayan Creole populations were not available for this study.

However, a total of 804 samples from eighteen potentially related chicken breeds/populations were chosen from those available within the BIOCHICKEN research consortium (https://conbiand.site, accessed on 20 September 2023). These breeds had been genotyped at the same microsatellite *loci* and in the same laboratory (Department of Genetics, University of Cordoba, Spain). Some data had been published previously [5,25], while the remaining data were obtained upon request to the consortium.

The breed acronym, sample size, breed origin, and main production purposes for each breed/population are shown in Table 1.

Table 1. Summary information on the 22 domestic chicken breeds/populations.

Breed/Population Name	Breed Acronym	Sample Size	Breed Origin	Utility	Source
Rustipollos	RUP	50	Paraguay	Dual purpose	This study
Cobb broiler	COB	25	United States	Meat	This study
White Plymouth Rock	WPR	12	United States	Dual purpose	This study
Brahma	BRH	10	China via USA	Dual purpose	This study
Total		97			
Criolla Pilaraneña	ECU	70	Ecuador	Dual purpose	[5]
Caneluda do Catolé	CDC	30	Brazil	Dual purpose	[25]
Canela Preta	CAP	40	Brazil	Dual purpose	[25]
Lineage Pesadão	LPE	30	Brazil	Dual purpose	[25]
Peloco	PEL	30	Brazil	Dual purpose	[25]
Araucana	ARA	47	Chile	Dual purpose	[5]
Andaluza Azul	AAZ	50	Spain	Eggs	[25]
Combatiente Español	CES	50	Spain	Fighting	[25]
Sureña	SUR	30	Spain	Dual purpose	[25]
Pita Pinta Asturiana	PPA	50	Spain	Dual purpose	[25]

Table 1. *Cont.*

Breed/Population Name	Breed Acronym	Sample Size	Breed Origin	Utility	Source
Menorquina	MEN	41	Spain	Eggs	Unpublished
Mallorquina	MLL	50	Spain	Dual purpose	[25]
Ibicenca	IBI	50	Spain	Dual purpose	[25]
Castellana Negra	CAN	50	Spain	Eggs	[25]
Extremeña Azul	EAZ	50	Spain	Dual purpose	[25]
Cornish Dark	COR	26	United Kingdom	Meat	[25]
Leghorn	LEG	40	Italy	Eggs	[25]
Nigerian	NIG	70	Nigeria	Dual purpose	[25]
Total		804			

2.2. DNA Extraction and Microsatellites Genotyping

Three circles were cut in filter papers exposed to a flat surface using a 2 mm Harris Micro punch (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK), which was cleaned using a 1% bleach solution between each sample. The circles were placed in a Polymerase Chain Reaction (PCR) plate and incubated in 100 µL of a 5% Chelex® 100 resin solution (Bio-Rad, Hercules, CA, USA). Subsequently, the PCR plate was incubated in a thermocycler at 95 °C for 15 min, 60 °C for 15 min, and finally 99 °C for 3 min. The lysate was removed and frozen at −20 °C until use, as described by Araújo de Carvalho and collaborators [25].

To assess the DNA polymorphism, 29 microsatellite *loci* used by the AVIANDIV project (http://aviandiv.tzv.fal.de/, accessed on 20 September 2023) and recommended for biodiversity studies in chicken by ISAG/FAO [28] were also used here. Multiplex PCR was carried out according to FAO recommendations [28]. The PCR products were genotyped using an automated DNA sequencer (ABI 3130XL, Applied Biosystems, Foster City, CA, USA) and an internal size standard marker (GeneScan™ 500 LIZ™, Applied Biosystems, Foster City, CA, USA). The resultant fragment analysis data and sizes of alleles were interpreted using the GENEMAPPER software, version 5 (Applied Biosystems, Foster City, CA, USA). To facilitate direct comparison, the studied breed and the comparison breeds were all genotyped in the same laboratory.

2.3. Statistical and Genetic Analysis

The mean number of alleles (Na), polymorphic information content (PIC) for each microsatellite *loci*, and the expected (H_E) and observed heterozygosity (H_O) in the 22 populations were estimated using Cervus 3.01 [29].

The average allelic richness and the richness of private alleles for each breed/population were calculated by adopting a sample of six individuals [30], hence allowing comparisons among different sample sizes and using the rarefaction method [31] implemented in the HP-RARE software, version 1.0. The number of private alleles (Np) was counted.

The Hardy–Weinberg equilibrium (HWE) test was conducted using a Markov Chain Monte Carlo method (20 batches, 5000 iterations per batch, and a dememorization number of 10,000) implemented in the GENEPOP software, version 4.0 [32]. Levels of significance were adjusted using the Bonferroni procedure [33]. Fixation indices per *locus* (F_{IS}, F_{IT}, and F_{ST}) were calculated according to Weir and Cockerham [34] using the software GENETIX 4.05 [35].

The F_{IS} index for each breed/population was calculated via bootstrapping using 1000 replicates with the GENETIX software, version 4.05 [35]. The extent of population differentiation was investigated by calculating the global multi-locus F_{ST} value. The index

of pairwise F_{ST} of Weir and Cockerham [34] between populations was estimated using the GDA software [36].

The Reynolds weighted genetic distance [37] among the breeds/populations was calculated using the Populations software, version 1.2.31 [38]. A Neighbor-Net was constructed using the software package SplitsTree4, version 4.13.1 [39].

Analysis of molecular variance (AMOVA) was tested using Arlequin 3.5 [40]. The population structure was determined utilizing a Bayesian approach implemented in the STRUCTURE software, version 2.3.4 [41], to assess the most probable number of partitions in the dataset without the assumption of the breed identities. The assignment of individuals to populations considered an ancestry model with admixture, correlated allele frequencies, and defined sampling location for each individual. For each value of K, 100 independent runs with 600,000 MCMC (Markov Chain Monte Carlo) iterations and a burn-in of 300,000 steps were performed for $2 \leq K \leq 24$ (K = number of clusters) to estimate the most likely number of clusters present in the dataset. The optimal number of clusters was identified by using both the Evanno method (ΔK) [42] and the highest probability for K following [41] on the CLUMPAK online platform [43].

To further investigate the genetic structure of each breed when adopting an approach without assumptions about HWE or linkage disequilibrium, Discriminant Analysis of Principal Component (DAPC) was performed as an alternative method to the Bayesian clustering algorithm with the method implemented in the ADEGENET package [44] within the statistical package R, version 3.3.2. DAPC was conducted without a posteriori group assignments by inferring the most likely number of genetic clusters (K) using the *find.clusters* function. This procedure utilizes K-means clustering to compute a Bayesian Information Criterion (BIC) value for each potential value of K (the most likely K has the lowest BIC value) and delineates individual group assignments for DAPC.

3. Results

3.1. Polymorphism of Markers across the 22 Chicken Breeds/Populations

All microsatellite *loci* genotyped were polymorphic. A total of 318 alleles were detected across all analyzed breeds/populations with an average of 10.97 alleles per *locus* (Supplementary Material Table S1). The number of alleles by *locus* ranged from 4 (MCW165) to 33 (LEI0234). LEIO166 revealed the maximum sum of private alleles (5). The expected heterozygosity ranged from 0.273 (MCW014) to 0.778 (LEIO234), with a mean of 0.560. While the observed heterozygosity values ranged from 0.079 (MCW014) to 0.714 (MCW183), with a mean value of 0.511. The polymorphic information content mean was 0.500 and ranged from 0.219 (MCW248) to 0.737 (LEI0234).

The fixation indices (F_{IS}, F_{IT}, F_{ST}) for each *locus* across all breeds/populations are also shown in the Supplementary Material Table S1. The mean of the inbreeding coefficient within populations (F_{IS}) was 0.090 ($p < 0.01$) for all *loci*, ranging from −0.078 (MCW248) to 0.704 (MCW014). The global heterozygosity deficit of individuals within the total populations (F_{IT}) ranged from 0.133 (MCW098) to 0.751 (MCW014), with a mean of 0.245 ($p < 0.01$). The average fixation index of subpopulations in relation to the total population (F_{ST}) was 0.171 ($p < 0.01$) and varied from 0.077 (MCW098) to 0.258 (MCW330).

3.2. Genetic Diversity among the 22 Chicken Breeds/Populations Analyzed

The genetic diversity indexes are summarized in Table 2. In the Rustipollos population, the average number of observed alleles was 3.31 and the mean allelic richness was 2.80. The highest count of private alleles (9) was observed in ECU, while RUP harbored only one private allele. The mean expected heterozygosity and observed heterozygosity were 0.53 and 0.50, respectively. The F_{IS} index showed a low value (0.072) with two microsatellites deviating from the Hardy–Weinberg equilibrium.

Table 2. Genetic diversity indexes of the 22 chicken breeds/populations studied.

Breed/Population	MNA [1] (SD)	AR [2] (R [3])	Np [4]	H_E [5] (SD)	H_O [6] (SD)	dHWE [7]	F_{IS} [8]
RUP	3.31(1.04)	2.80 (0.05)	1	0.53 (0.03)	0.50 (0.01)	2	0.072 [a]
ECU	6.79 (3.43)	3.80 (0.15)	9	0.63 (0.03)	0.53 (0.01)	6	0.153 [a]
CDC	5.41 (2.61)	3.83 (0.12)	2	0.65 (0.03)	0.62 (0.02)	3	0.052
CAP	5.72 (2.90)	3.68 (0.15)	2	0.62 (0.03)	0.61 (0.01)	2	0.026
LPE	4.41 (2.01)	3.43 (0.08)	1	0.62 (0.02)	0.62 (0.02)	3	0.004
PEL	4.86 (1.88)	3.61 (0.14)	3	0.62 (0.03)	0.60 (0.02)	2	0.029
ARA	6.76 (3.77)	4.22 (0.37)	5	0.67 (0.03)	0.57 (0.01)	10	0.142 [a]
AAZ	4.72 (2.23	2.71 (0.05)	1	0.43 (0.05)	0.39 (0.01)	9	0.077 [a]
CES	5.24 (2.59)	2.88 (0.05)	0	0.45 (0.04)	0.40 (0.01)	7	0.108 [a]
SUR	5.14 (2.50)	3.62 (0.09)	2	0.60 (0.03)	0.54 (0.02)	5	0.099 [a]
PPA	5.07 (2.19)	3.41 (0.13)	2	0.59 (0.03)	0.48 (0.01)	12	0.196 [a]
MEN	3.69 (1.51)	2.76 (0.04)	0	0.47 (0.04)	0.43 (0.01)	3	0.099 [a]
MLL	3.55(2.01)	2.69 (0.06)	1	0.46 (0.05)	0.46 (0.01)	1	0.000 [a]
IBI	5.52 (3.60)	3.61 (0.10)	2	0.60 (0.04)	0.52 (0.01)	7	0.135 [a]
CAN	4.97 (2.67)	3.13 (0.07)	0	0.53 (0.04)	0.47 (0.01)	5	0.110 [a]
EAZ	5.38 (2.72)	3.56 (0.08)	0	0.60 (0.03)	0.52 (0.01)	6	0.138 [a]
COR	4.66 (2.11)	3.41 (0.18)	1	0.57 (0.03)	0.47 (0.02)	2	0.182 [a]
COB	3.90 (1.40)	3.10 (0.03)	0	0.55 (0.03)	0.55 (0.02)	0	0.002
WPR	2.69 (1.00)	2.34 (0.02)	0	0.39 (0.04)	0.37 (0.03)	0	0.041
BRH	4.34 (1.80)	3.98 (0.17)	2	0.69 (0.03)	0.63 (0.03)	1	0.093
LEG	3.17 (1.44)	2.29 (0.10)	0	0.39 (0.04)	0.43 (0.01)	10	−0.112
NIG	6.83 (3.78)	3.66 (0.20)	6	0.59 (0.03)	0.53 (0.01)	7	0.115 [a]
Mean		3.30 (0.11)		0.56 (0.03)	0.51 (0.02)		

[1] MNA: mean number of observed alleles; [2] AR: allelic richness; [3] R: richness of private alleles; [4] Np: Number of private alleles; [5] H_E: Expected heterozygosity; [6] H_O: Observed heterozygosity; [7] dHWE: number of *loci* that deviate from the Hardy–Weinberg equilibrium per breed/population (after Bonferroni correction $p < 0.0022$; [33]). [8] F_{IS}: inbreeding coefficient; [a] Significantly different from zero ($p < 0.05$). RUP: Rustipollos; ECU: Criolla Pilaraneña; CDC: Caneluda do Catolé; CAP: Canela Preta; LPE: L. Pesadão; PEL: Peloco; ARA: Araucana; AAZ: Andaluza Azul; CES: Combatiente Español; SUR: Sureña; PPA: Pita Pinta Asturiana; MEN: Menorquina; MLL: Mallorquina; IBI: Ibicenca; CAN: Castellana Negra; EAZ: Extremeña Azul; COR: Cornish Dark; COB: Cobb Broiler; WPR: White Plymouth Rock; BRH: Brahma; LEG: Leghorn; NIG: Nigerian.

The mean number of observed alleles per population ranged from 2.69 (WPR) to 6.83 (NIG). The average allelic richness per breed/population was 3.30 ± 1.11, rarefied to a sample size of six individuals, varied from 2.29 (LEG) to 4.22 (ARA). The expected heterozygosity ranged from 0.39 (WPR and LEG) to 0.69 (BRH), while the observed heterozygosity varied from 0.37 (WPR) to 0.63 (BRH). All populations exhibited *loci* deviating from HWE, except COB and WPR breeds. The inbreeding coefficient (F_{IS}) was significantly different from zero in several breeds/populations (RUP, ECU, ARA, AAZ, CES, SUR, PPA, MEN, MLL, IBI, CAN, EAZ, COR, and NIG) and ranged from −0.112 to 0.196.

The results of the analysis of molecular variance (AMOVA) are shown in Table 3. The AMOVA data highlighted that 76.38% of the total genetic variation was attributed to variation within individuals, while 17.25% of the genetic variation derived from variation among populations.

Table 3. Hierarchical analysis of molecular variance (AMOVA) based on 29 microsatellite markers for the entire dataset.

Source of Variation	df	Sum of Squares	Variance Component	Variance (%)
Among populations	21	3043.95	1.55 *	17.25
Among individuals within populations	954	7664.33	0.57 *	6.37
Within individuals	976	6719.50	6.88 *	76.38
Total	1273	17,427.78	9.01	100.00

* $p < 0.001$.

3.3. Genetic Differentiation, Genetic Distance, and Phylogenetic Relationships among Rustipollos Population and the 22 Reference Chicken Breeds/Populations

The relationships between the studied chickens were assessed by calculating a pairwise F_{ST} matrix (Supplementary Material Table S2) and represented with a gradient graphic as shown in Figure 1. F_{ST} values for all pairs of breeds/populations differed significantly from 0 ($p < 0.05$) and ranged from 0.036 to 0.404, with the closest pair-wise value (0.036) observed between ARA and BRH. Regarding the RUP population, it showed the closest genetic relationship with BRH (0.132) and the longest distance (0.331) from LEG.

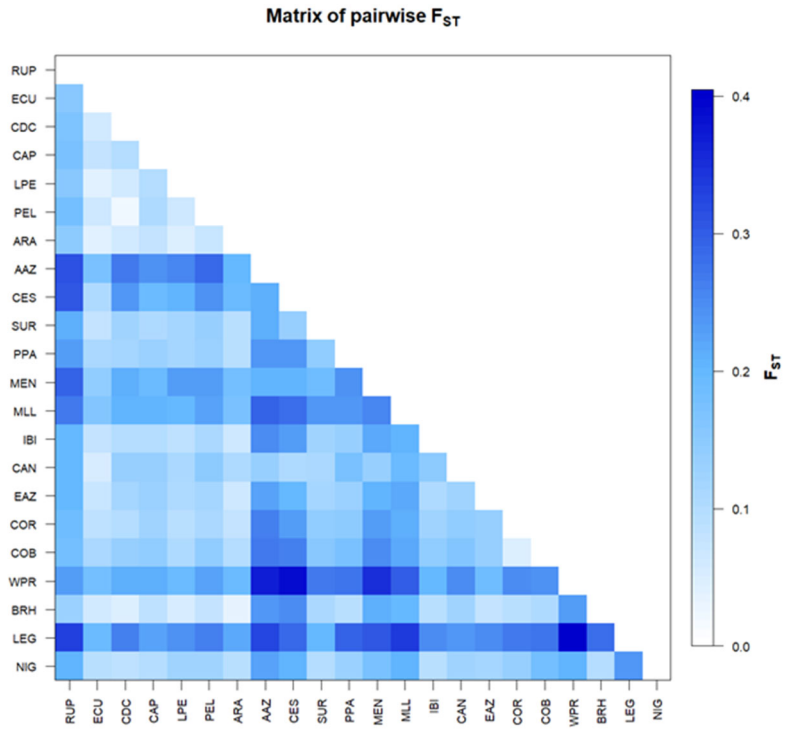

Figure 1. Pairwise fixation index of subpopulation total (F_{ST}) distance matrix. RUP: Rustipollos; ECU: Criolla Pilaraneña; CDC: Caneluda do Catolé; CAP: Canela Preta; LPE: L. Pesadão; PEL: Peloco; ARA: Araucana; AAZ: Andaluza Azul; CES: Combatiente Español; SUR: Sureña; PPA: Pita Pinta Asturiana; MEN: Menorquina; MLL: Mallorquina; IBI: Ibicenca; CAN: Castellana Negra; EAZ: Extremeña Azul; COR: Cornish Dark; COB: Cobb Broiler; WPR: White Plymouth Rock; BRH: Brahma; LEG: Leghorn; NIG: Nigerian.

To further investigate the genetic relationship among the studied chicken breeds/populations, a Neighbor-Net tree was constructed using the Reynolds' genetic distance (Figure 2).

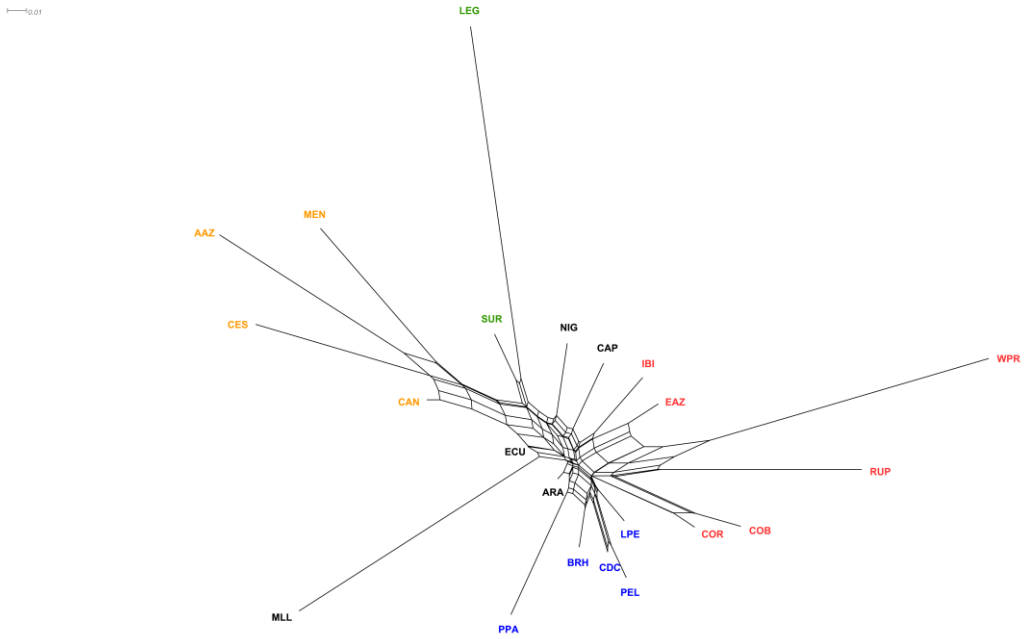

Figure 2. Neighbor-Net network of the Reynolds' genetic distance among the 22 chicken breeds/populations. Different colors identify the main clusters. RUP: Rustipollos; ECU: Criolla Pilaraneña; CDC: Caneluda do Catolé; CAP: Canela Preta; LPE: L. Pesadão; PEL: Peloco; ARA: Araucana; AAZ: Andaluza Azul; CES: Combatiente Español; SUR: Sureña; PPA: Pita Pinta Asturiana; MEN: Menorquina; MLL: Mallorquina; IBI: Ibicenca; CAN: Castellana Negra; EAZ: Extremeña Azul; COR: Cornish Dark; COB: Cobb Broiler; WPR: White Plymouth Rock; BRH: Brahma; LEG: Leghorn; NIG: Nigerian.

The phylogenetic tree confirmed the previous results and revealed four main clusters. The first cluster comprised the RUP in an intermediate position between WPR and the Spanish IBI and EAZ breeds and another branch formed by COR and COB. In a nearby position, a second cluster can be observed and includes the Creole breeds (LPE, PEL, and CDC), BRH, and the Spanish PPA breed. Another cluster is generated from the Spanish AAZ, CES, MEN, and CAN breeds. The Leghorn showed the longest branch and clustered with the SUR breed. Finally, ECU, NIG, CAP, MLL, and ARA breeds are positioned at the center of the network tree.

3.4. Genetic Structure and Admixture Analysis

A model-based clustering was performed to investigate the genetic structure using an increasing number of inferred populations (Figure 3).

According to Evanno et al. [42], the highest ΔK value was found at $K = 22$, thus identifying the most probable number of clusters in the dataset. Before reaching the plateau at $K = 22$, a further five peaks were observed at $K = 7$, $K = 11$, $K = 13$, and $K = 16$, respectively (Supplementary Material Figure S2a). For a K value equal to 2, individuals were clustered in two groups, one includes RUP, COB, COR, WPR, and all the Creole breeds. Whereas the second group comprised AAZ, CES, MEN, MLL, and LEG breeds. All the remaining breeds/populations showed different levels of admixture (Figure 3). From $K = 3$ to $K = 5$, the South American chicken populations showed a common genetic relationship with Iberian chicken strains, commercial lines, and the Nigerian chicken breed. Interestingly, the RUP chicken population from $K = 7$ to $K = 22$ was classified into the same group. At $K = 22$, each breed showed its own cluster, with some complex levels of admixtures in different breeds (e.g., Ecuadorian Creole chickens). The Evanno method of determining ΔK is recognized as the best way to identify the highest level of genetic structure and may not be particularly reliable under complex scenarios, especially when dealing with breeds likely shaped by the genetic drift process. Although it is a simple method from the

statistical point of view, it always produces a solution for the given data and the solution may not be the best one [45]. The alternative assessment of clusters performed by DAPC revealed K = 20 inferred clusters (Supplementary Material Figure S2b), producing two fewer clusters by DAPC than those generated by STRUCTURE. In this analysis, 150 PCs of the PCA were retained as input to the discriminant analysis, accounting for approximately 97.50% of the total genetic variability. The scatterplot of the first three components of the DA (Figure 4) showed that RUP and AAZ breeds/populations appeared separate from the other breeds/populations. However, a genetic proximity and a particular affinity can be identified between AAZ, CAN, and CES breeds/populations and breeds of Spanish origin. A certain gene flow can also be identified between RUP and WPR breeds/populations. The major cluster of the gene pool contained several breeds/populations, indicating an extensive sharing of genetic variation.

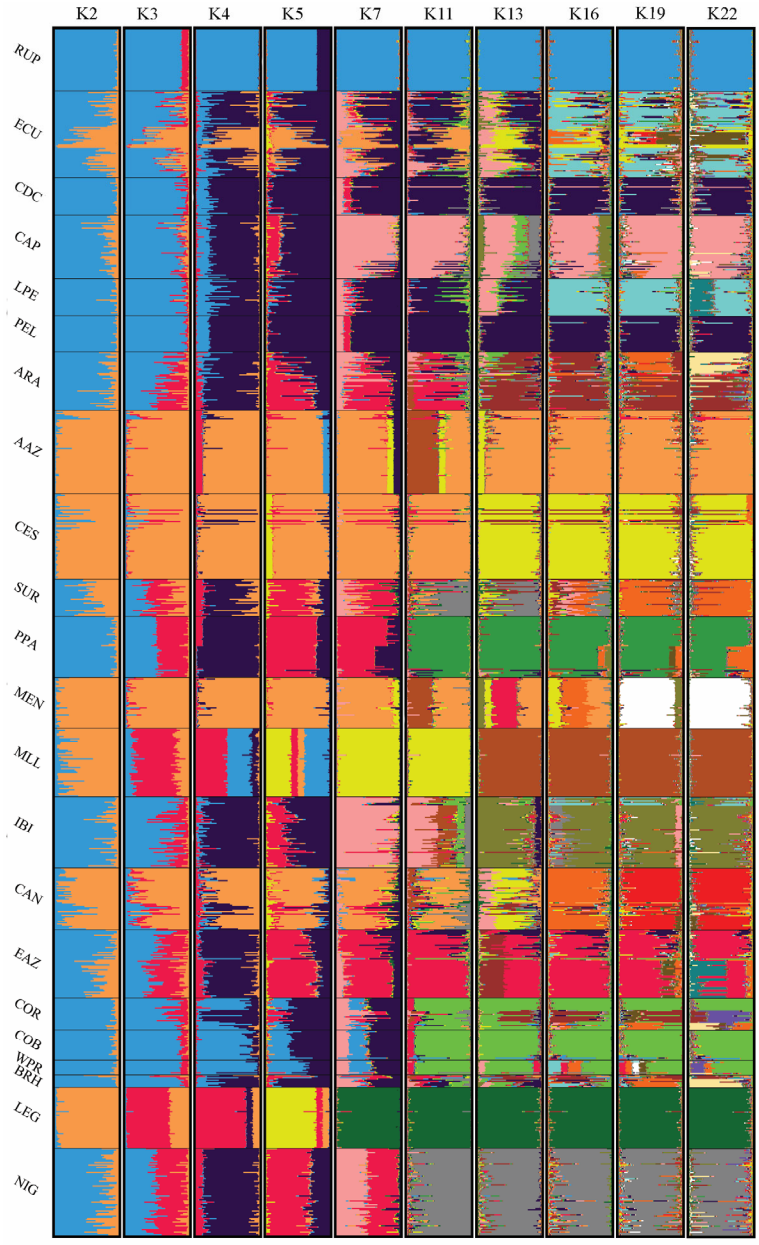

Figure 3. Clustering of the 22 chicken breeds/populations with STRUCTURE analysis. RUP: Rustipollos; ECU: Criolla Pilaraneña; CDC: Caneluda do Catolé; CAP: Canela Preta; LPE: L. Pesadão;

PEL: Peloco; ARA: Araucana; AAZ: Andaluza Azul; CES: Combatiente Español; SUR: Sureña; PPA: Pita Pinta Asturiana; MEN: Menorquina; MLL: Mallorquina; IBI: Ibicenca; CAN: Castellana Negra; EAZ: Extremeña Azul; COR: Cornish Dark; COB: Cobb Broiler; WPR: White Plymouth Rock; BRH: Brahma; LEG: Leghorn; NIG: Nigerian.

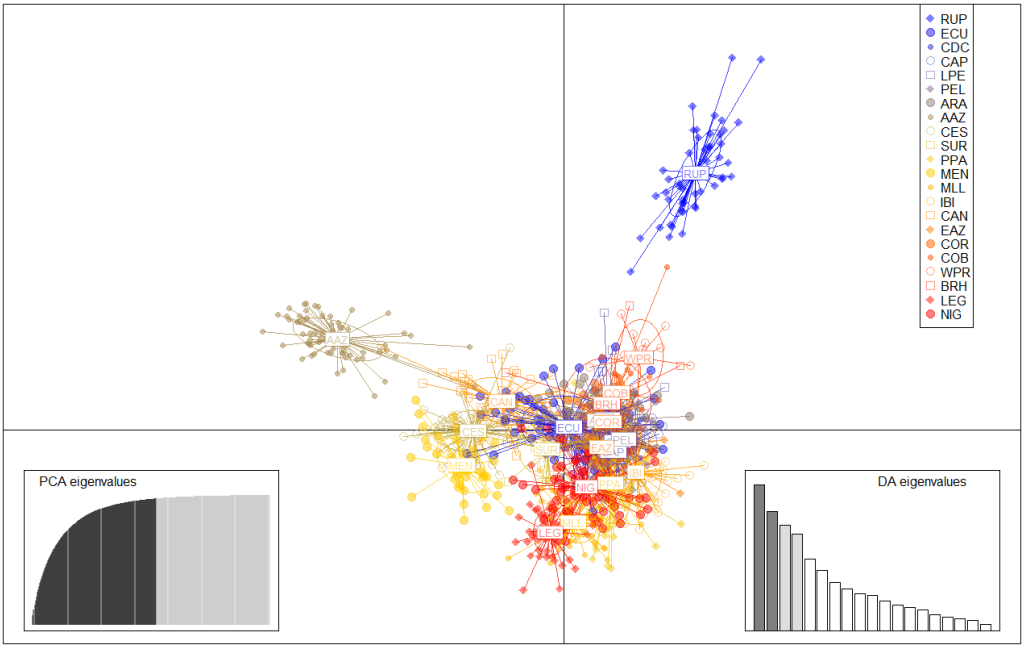

Figure 4. Scatterplot of the first two principal components of Discriminant Analysis of Principal Component (DAPC) using populations as *a posteriori* clusters. The individuals are assigned to populations *a posteriori*, that is, after determining the number of clusters by the software, instead of forcing them into known populations. RUP: Rustipollos; ECU: Criolla Pilaraneña; CDC: Caneluda do Catolé; CAP: Canela Preta; LPE: L. Pesadão; PEL: Peloco; ARA: Araucana; AAZ: Andaluza Azul; CES: Combatiente Español; SUR: Sureña; PPA: Pita Pinta Asturiana; MEN: Menorquina; MLL: Mallorquina; IBI: Ibicenca; CAN: Castellana Negra; EAZ: Extremeña Azul; COR: Cornish Dark; COB: Cobb Broiler; WPR: White Plymouth Rock; BRH: Brahma; LEG: Leghorn; NIG: Nigerian.

4. Discussion

All microsatellite markers were found to be polymorphic. PIC values were calculated according to the algorithm proposed by Botstein and collaborators [46]. The mean PIC among *loci* was 0.500, and almost all markers were moderately informative. These results confirm the usefulness of this set of microsatellite markers to determine the genetic diversity in the studied breeds/populations. PIC values reported in this study were similar to those described in Ecuadorian Creole chicken, Denizli chicken subpopulations, and local Swedish chicken and Vietnamese chicken [5,15,22,24], but lower than those found in Nigerian and Rwandan chicken [4,6].

The mean number of alleles per *locus* (10.97) was higher than those described in Colombian Creole chickens and Italian local chickens [47,48], but lower in comparison to values observed in the Nigerian chicken population and Thai indigenous chickens [4,49]. The number of private alleles distributed throughout the breeds/populations showed that there was moderate genetic diversity between populations. RUP population exhibited only one private allele. Despite the number of private alleles being a good indicator of population relationship and structure, further studies need to be carried out to elucidate the genetic background of RUP. The observed heterozygosity (0.511) was lower than the expected heterozygosity; however, the expected heterozygosity for all *loci* was higher than 0.50 further suggesting the usefulness of markers for this kind of study [49]. In general, an extensive level of genetic diversity is expected in a synthetic breed due to the gene segregation as already observed in Egyptian synthetic breeds by Eltanany et al. [50].

Wright's F-statistics provide important insights into the evolutionary processes that influence the structure of genetic variation within and among populations and they are among the most widely used descriptive statistics in population and evolutionary genetics [51]. The mean value of the F_{IS} index was significantly different from zero, showing heterozygous deficiency of individuals within the total population (F_{IT}: 0.245, $p < 0.01$). It follows that the mating in almost all the breeds/populations is not completely random, being directly related to the large number of markers that presented deviations in the Hardy–Weinberg equilibrium [48]. The F_{ST} value indicates that 17% of the total variation found in the populations is due to population differences, demonstrating a high degree of genetic differentiation among the 22 chicken breeds/populations.

The average number of alleles and expected and observed heterozygosis (MNA: 3.31; H_O: 0.50; H_E: 0.53) in the Rustipollos population were similar to those reported in Mediterranean chicken breeds, Swedish local chickens, and Spanish breeds [23,24,52]. However, they were lower than those reported in South African, Ecuadorian Creole, African, Paraguayan Creole, and Asian chickens [5,17,53,54].

The reported differences could be related to geographic origin and management practices; this was already reported by Lyimo and collaborators [54], who showed that chicken populations located in geographic areas close to domestication centers had higher genetic diversity values. In addition, management practices influence the levels of diversity; therefore local populations, or free-ranging chickens and in the absence of selection schemes, usually present an exchange of genetic material, which is reflected in the number of alleles and levels of heterozygosity compared to closed populations selected for phenotypic and/or productive traits [53].

Considering the value of allelic richness in the Rustipollos population (3.30), it was similar to values reported in South African chicken ecotypes [17], but lower than values reported in Mediterranean breeds [24]. Allelic richness is a solid measure of genetic diversity that is indicative of a population's long-term potential for adaptability and persistence [55], which are objectives pursued in the constitution of a synthetic breed.

The results of this study evidenced that the values of observed heterozygosity were lower or equal than expected in most of the examined breeds. The observed and expected heterozygosity variation can be attributed to differences in location, sample size, population structure, and microsatellite marker sources [56].

The ARA, AAZ, PPA, and LEG breeds/populations showed several markers in the Hardy–Weinberg disequilibrium. This probably indicates that these breeds/populations are under some systematic and random forces, such as migration, mutation, selection, or genetic drift which change the genotypic frequencies [57]. It follows that breeding strategies and non-random mating applied to maintain the morphological standard of some breeds may have caused an increase in F_{IS} values and also deviations from the Hardy–Weinberg equilibrium especially in low effective size populations [19]. On the other hand, LEG had a non-significant negative F_{IS} value (-0.112) and a high number of markers in the Hardy–Weinberg disequilibrium, which could be related to its productive aptitude, since this population has been genetically selected for high egg production [54].

High values registered in the AMOVA analysis demonstrated that a large proportion of the genetic variation is due to differences between individuals; a comparable trend was also reported in indigenous chickens from Rwanda and Creole chickens from Brazil [6,25]. These findings suggest the overall genetic diversity of the studied chicken dataset to be highly influenced by heterogeneities of individuals within and across populations, unlike differences between chicken breeds/populations. This fact is detectable in local and/or crossbreed populations not genetically stabilized and not included in any breeding or selection program [25].

The F_{ST} value determines the genetic differentiation degree, an F_{ST} value of 0.00 to 0.05 is considered a slight genetic differentiation, 0.05 to 0.25 a moderate to strong genetic differentiation, and more than 0.25 is considered a very strong genetic differentiation [58]. Based on these criteria, moderate to strong levels of differentiation were observed between

Rustipollos and the reference breeds/populations as expected. Pairwise F_{ST} values calculated between the Rustipollos population and reference breeds/populations were higher than reported in Ecuadorian Creole chickens [5]. The short generation interval and random genetic drift have contributed to the elevated levels of observed genetic differentiation [59].

Genetic relationships observed in the Neighbor-Net graph are mainly related to genetic origin, productive fitness, and geographic location. The RUP population shows a close relationship with the WPR breed (commercial meat lines), probably one of its founder breeds, together with COR, COB, and South Spanish chickens (EAZ and IBI breeds). In fact, the White Plymouth Rock and Cornish Dark were used as a breeder in crossbreeding for commercial meat lines. Furthermore, this cluster may be related to their productive aptitude, knowing that both have a dual-purpose [28]. In relation to the proximity with Spanish populations, this can be explained because the European chickens were introduced into the American continents by the Spanish after their arrival in the 15th century [60]. The last cluster consisting of Spanish populations (AAZ, CES, CAN, and MEN) could indicate gene flow between them considering their geographic proximity. The LEG breed had a large branch indicating that the population is distant from all chicken breeds/populations analyzed. This may be due to real differences and the lack of genetic diversity in the commercial egg layer populations based on both management histories and genetic background [23].

The results of the Bayesian and DAPC analyses further confirmed the findings from the previous analysis. Similar gene pool patterns were observed among most of the Creole and Spanish breeds. Whereas the RUP shares a genetic component with most of the reference breeds/populations used in the present work, although at very low *K*. This fact can be explained because the Rustipollos population originates from commercial lines and animals assimilated to Creole chickens. The origin of the Creole breed on the American continent, as the founder population of the Rustipollos, is still controversial. The current Creole chickens derive from animals imported to America from Spain, starting in the early years of discovery and colonization but it is likely that they are the result of multiple admixture events, including the introduction of industrial genetic types in recent times [61].

5. Conclusions

Our findings demonstrated that the synthetic origin of the Rustipollos population was clearly evidenced by all analyses applied. This population has shown moderate levels of genetic diversity and a low degree of inbreeding, according to the determined genetic parameters. In addition, a genetic differentiation of Rustipollos populations from the reference chicken breeds/populations was highlighted. However, further studies based on larger samples are needed to confirm our findings by focusing on the Creole component used in the past crossbreeding. Future strategies focused on the sustainable development of this valuable genetic resource are recommended with interventions appropriate to empower the existing operations of genetic fixation to ensure that genetic diversity is maintained over time. Therefore, our results could be implemented by policymakers to support a dissemination program with establishment schemes of mating including selection, multiplication, and production of this genetic resource in free-range, considering the adaptation to the environmental conditions in Paraguay and the excellent productive performance of the breed as a dual-purpose population.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/poultry3030018/s1, Figure S1: Phenotypical characteristics of male (a) and female (b) of Rustipollos synthetic chicken population in Paraguay; Figure S2: Distribution of ΔK (a) and log probability plot (b) for different values of K in STRUCTURE analysis; Bayesian Information Criterion (BIC) values (c) plotted for the number of clusters ranging from $K = 1$ to $K = 40$; Table S1: Microsatellite markers polymorphism and diversity parameters across the 22 chicken populations/breeds; Table S2: Pairwise F_{ST} values estimates among 22 chicken populations/breeds.

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S.C. and E.G.; investigation, L.A.C.R., A.M.M., E.G., N.M.M., J.Q., J.F.B. and M.T.Ñ.; resources, L.A.C.R. and A.M.M.; data curation, L.A.C.R., S.C., E.G., A.M.M., P.A.T.V., D.A.d.C., A.L.P.B. and E.L.; writing—original draft preparation, L.A.C.R.; writing—review and editing, S.C., S.M., A.M.M., M.E.C.V. and E.L.; visualization, L.A.C.R., S.C. and E.L.; supervision, S.C., A.M.M., M.E.C.V. and E.L.; project administration, L.A.C.R. and M.T.Ñ.; funding acquisition, L.A.C.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All relevant data are included in the manuscript and its additional files. The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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