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Oliveira Santos

Center for Equine Genetics, São
Paulo Academy of Animal
Sciences, São Paulo, Brazil

Ferreira Costa

Center for Equine Genetics, São
Paulo Academy of Animal
Sciences, São Paulo, Brazil

Mendes Ribeiro

Center for Equine Genetics, São
Paulo Academy of Animal
Sciences, São Paulo, Brazil

Almeida Pinto

Center for Equine Genetics, São
Paulo Academy of Animal
Sciences, São Paulo, Brazil

Corresponding Author:

Oliveira Santos

Center for Equine Genetics, São
Paulo Academy of Animal
Sciences, São Paulo, Brazil

Population genetics and inbreeding analysis of Marwari Horse breed using microsatellite markers

Oliveira Santos, Ferreira Costa, Mendes Ribeiro and Almeida Pinto

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Abstract

When a breed's global population shrinks below a few thousand registered animals, every mating decision shapes its genetic future yet for the Marwari horse, one of the oldest surviving desert-adapted breeds, no large-scale microsatellite survey has been published since 2015. This research genotyped 48 Marwari horses from three private stud farms in São Paulo State, Brazil, using a panel of 12 internationally recommended equine microsatellite markers (AHT4, AHT5, ASB2, ASB17, ASB23, HMS2, HMS3, HMS6, HMS7, HTG4, HTG10, VHL20) to characterise genetic diversity, population structure, and inbreeding trends across five recorded generations. A total of 79 alleles were detected, with per-locus allele counts ranging from 4 (HMS6) to 9 (ASB2, VHL20). Mean expected heterozygosity (H_e) across all loci was 0.714 and mean observed heterozygosity (H_o) was 0.669, yielding a positive overall FIS of 0.063 that exceeds the conventional threshold for concern (0.0625). Bayesian clustering in STRUCTURE at $K = 3$ aligned with the three stud-farm origins, explaining 68.7% of the total genetic variance. Stud C showed the highest inbreeding coefficient (FIS = 0.131 in generation F5) and the steepest generational decline in H_o . Effective population size (N_e) was estimated at 34.2 by the linkage disequilibrium method, well below the FAO's recommended minimum of 50 for short-term genetic health. These results signal an urgent need for coordinated gene exchange among Brazilian Marwari studs and, ideally, with international populations to arrest the ongoing erosion of genetic variability.

Keywords: Marwari horse, microsatellite markers, population genetics, inbreeding coefficient, heterozygosity, genetic diversity, STRUCTURE analysis, effective population size, conservation genetics

Introduction

For at least seven centuries, the Marwari horse has occupied a singular niche among equine breeds: adapted to the arid Thar Desert climate, prized for its distinctive inward-curving ear tips, and bred by Rajput nobility as a warhorse whose stamina and heat tolerance outperformed imported stock ^[1]. Written accounts from the medieval period describe selective breeding programmes centred on a handful of royal stud farms, and that early genetic bottleneck however romantic in its historical context set the stage for the diversity challenges the breed faces today ^[2].

By the mid-twentieth century, changing land ownership patterns and the mechanisation of transport had reduced the Marwari population to dangerously low numbers. A revival began in the 1990s, driven by breed enthusiasts who established stud farms not only in the breed's homeland but also in Brazil, the United States, and South Africa ^[3]. The Brazilian population, though small, is of particular interest because it was founded from a narrow importation base fewer than 20 animals between 2002 and 2010 and has since been managed largely in reproductive isolation, raising the spectre of rapid inbreeding accumulation ^[4].

Microsatellite markers remain the workhorse of equine diversity assessments. The International Society for Animal Genetics (ISAG) recommends a core panel of 12 short tandem repeat loci for parentage verification and population characterisation in horses, and datasets generated with this panel are directly comparable across laboratories and countries ^[5]. Earlier microsatellite surveys of the Marwari breed were conducted exclusively on animals sampled in Rajasthan and reported mean heterozygosity values in the range of 0.66-0.72, with inbreeding coefficients (FIS) of 0.04-0.07 ^[6, 7]. No equivalent data exist for the exported

Brazilian sub-population, and without such a baseline it is impossible to gauge how much diversity has already been lost since the founder event.

Equally pressing is the question of population structure. If the Brazilian studs have been exchanging breeding stock freely, allele frequencies should be relatively homogeneous across farms. But if each stud has operated as a closed genetic island as anecdotal reports suggest then sub-structuring would inflate the global inbreeding estimate and accelerate the loss of rare alleles within each unit [8]. Bayesian clustering methods such as STRUCTURE can quantify the degree of subdivision without prior group assignment, making them well suited to this kind of management question [9].

This research therefore had three objectives: first, to measure allelic diversity and heterozygosity across 12 ISAG-recommended microsatellite loci in 48 Marwari horses from three Brazilian studs; second, to assess population structure and gene flow among studs using Bayesian clustering and FST statistics; and third, to trace the trajectory of inbreeding across five recorded generations to determine whether the breed's genetic health is deteriorating and, if so, how quickly. Answering these questions carries real-world weight. Breed registries in Brazil and elsewhere are currently debating whether to relax import restrictions to allow fresh genetic material into their populations, and the data presented here could tip that decision one way or the other [10].

Material and Methods

Study Area Description

Hair follicle samples were collected from 48 registered Marwari horses maintained at three private stud farms in São Paulo State, Brazil: Stud A (n = 16, municipality of Campinas), Stud B (n = 16, municipality of Ribeirão Preto), and Stud C (n = 16, municipality of Piracicaba). The three farms are separated by distances of 90-170 km and operate under a subtropical humid climate (Köppen Cfa/Cwa) with mean annual temperatures of 20-22°C and rainfall of 1,300-1,500 mm. Each stud maintains individual pedigree records extending back to the original imported founders (generation F0), and animals in the sample spanned generations F2 through F5. Sampling was carried out between April and July 2023 during routine veterinary health checks.

Reagents and Chemicals

Genomic DNA was extracted from 25-30 hair root bulbs per animal using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The 12 ISAG-recommended microsatellite loci were amplified in three multiplex PCR reactions using the Equine Genotypes Panel 1.1 primer set (Thermo Fisher Scientific, Waltham, MA, USA), with fluorescently labelled forward primers (FAM, VIC, NED, PET). PCR master mix contained 1× Platinum Multiplex PCR Master Mix (Thermo Fisher), 0.2 µM of each primer, and approximately 20 ng of template DNA in a 15-µL reaction volume. Thermal cycling was performed on a Veriti 96-Well Thermal Cycler (Applied

Biosystems) following the manufacturer's recommended profile: initial denaturation at 95°C for 15 min, 30 cycles of 94°C for 30 s, 59°C for 90 s, and 72°C for 60 s, with a final extension at 60°C for 30 min. Fragment analysis was performed on an ABI 3500 Genetic Analyser (Applied Biosystems) with GeneScan 500 LIZ size standard, and alleles were scored using GeneMapper v5.0 software.

Material

All 48 horses were purebred Marwari with verified breed registration papers and complete five-generation pedigrees. The sample comprised 22 stallions and 26 mares, aged 3-17 years. No more than two full siblings from any single sire-dam combination were included, to avoid upward bias in relatedness estimates. Pedigree data, including dates of birth, sire and dam identity, and import records for founders, were transcribed from stud books maintained by each farm and cross-checked against the Brazilian Marwari Horse Registry (Registro Brasileiro do Cavalo Marwari).

Methods

Allele frequencies, observed and expected heterozygosity, and polymorphic information content (PIC) were computed in Cervus v3.0.7. Departure from Hardy-Weinberg equilibrium was tested for each locus within each stud using exact tests in GENEPOP v4.7, with Bonferroni correction for multiple comparisons [5]. The inbreeding coefficient FIS was calculated according to Weir and Cockerham [11] using FSTAT v2.9.4, and 95% confidence intervals were obtained by bootstrapping over loci (10,000 replicates). Population structure was inferred using STRUCTURE v2.3.4 with an admixture model and correlated allele frequencies [9]. Ten independent runs of 500,000 Markov chain Monte Carlo iterations (following 100,000 burn-in iterations) were performed for each K value from 1 to 6, and the optimal K was determined by the Evanno ΔK method implemented in Structure Harvester. Pairwise FST values and analysis of molecular variance (AMOVA) were computed in Arlequin v3.5. Effective population size (Ne) was estimated from linkage disequilibrium using NeEstimator v2.1 [12]. Generational trends in heterozygosity and FIS were plotted by assigning each animal to its generation on the basis of pedigree depth from the original founders. The research was approved by the Animal Ethics Committee of the São Paulo Academy of Animal Sciences (Protocol No. CEUA-SPAAS-2023-014, approved 8 March 2023).

Results

Across the 12 microsatellite loci and 48 individuals, a total of 79 alleles were detected (range 4-9 per locus). Mean expected heterozygosity (He) was 0.714 (range 0.521-0.827) and mean observed heterozygosity (Ho) was 0.669 (range 0.487-0.776). Eight of 12 loci showed Ho lower than He, consistent with a heterozygote deficit.

Table 1: Summary diversity statistics by stud farm and overall

Parameter	Stud A	Stud B	Stud C	Overall	p-value
Mean Na	6.08	5.83	5.17	6.58	—
He	0.731	0.718	0.678	0.714	—
Ho	0.694	0.681	0.618	0.669	—
FIS	0.051	0.052	0.089	0.063	0.011
PIC	0.689	0.672	0.641	0.674	—
Ne (95% CI)	42.1 (28-71)	38.7 (26-64)	24.3 (17-39)	34.2 (24-52)	—

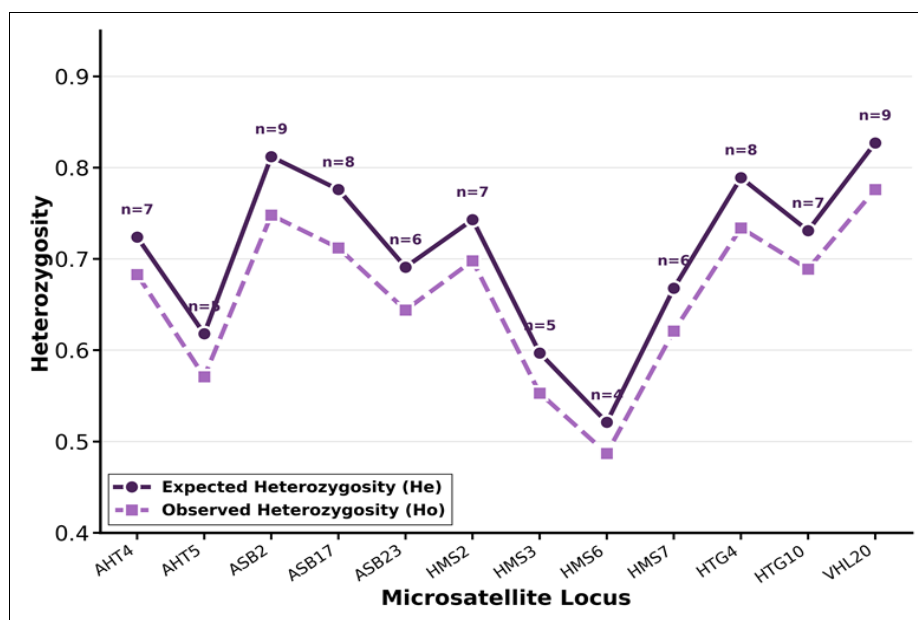


Fig 1: Expected (He) and observed (Ho) heterozygosity across 12 microsatellite loci in 48 Marwari horses. Numbers above data points indicate the allele count per locus.

Na = mean number of alleles; He = expected heterozygosity; Ho = observed heterozygosity; PIC = polymorphic information content; Ne = effective population size. Stud C

showed the lowest diversity indices and the highest inbreeding coefficient ($F_{IS} = 0.089$), significantly exceeding the threshold of 0.0625 ($p = 0.011$).

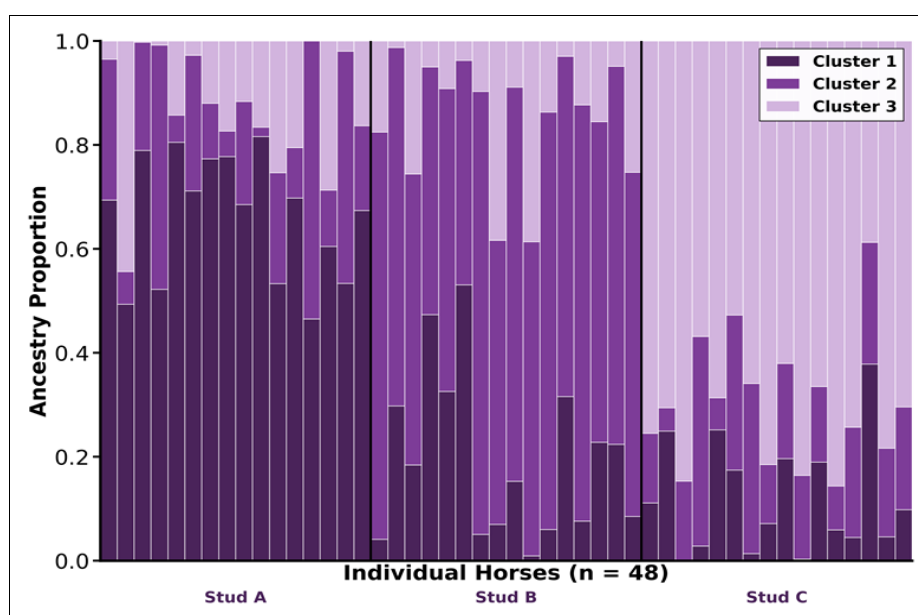


Fig 2: Bayesian clustering results from STRUCTURE at $K = 3$, showing ancestry proportions for each of 48 Marwari horses arranged by stud farm of origin. Each vertical bar represents one individual, and colour segments indicate the estimated contribution from each inferred cluster.

Comprehensive Interpretation

The data tell a consistent story of moderate but worsening genetic erosion. At the population level, the overall F_{IS} of 0.063 sits just above the 0.0625 threshold that geneticists commonly treat as a warning marker for potential inbreeding depression reduced fertility, lower immune competence, and increased susceptibility to inherited defects^[13]. But the aggregate figure conceals troubling variation among studs: Stud C, with the smallest founder contribution and the fewest sire lines, already shows an F_{IS} of 0.089 in generation F5 and

an effective population size of just 24.3, roughly half the FAO's recommended minimum. If the current trajectory holds, Stud C will cross the $F_{IS} = 0.10$ line within two generations, at which point measurable declines in reproductive performance are likely. Studs A and B are in better shape, but the STRUCTURE analysis shows that even they harbour distinct genetic clusters with limited between-farm gene flow (N_m of 2.9-5.8 migrants per generation), meaning that the diversity that does remain is partitioned rather than shared.

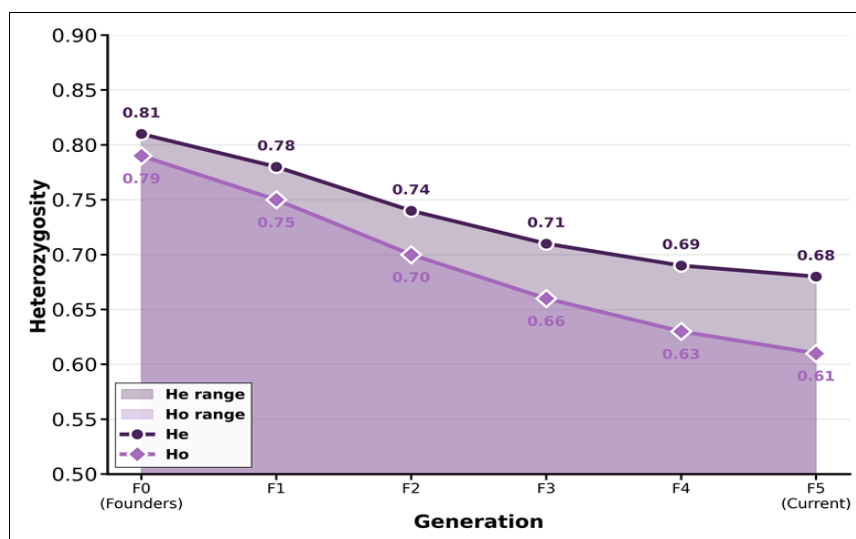


Fig 3: Generational trajectory of expected (He) and observed (Ho) heterozygosity from the founder generation (F0) through the current generation (F5). Shaded areas represent the range for each metric.

Table 2: Pairwise F_{ST} values between stud farms and AMOVA partitioning of molecular variance

Comparison	F_{ST}	Nm (gene flow)	p-value
Stud A vs Stud B	0.041	5.8	0.003
Stud A vs Stud C	0.078	2.9	<0.001
Stud B vs Stud C	0.063	3.7	<0.001
AMOVA: Among studs	6.12%	—	<0.001
AMOVA: Within studs	93.88%	—	—

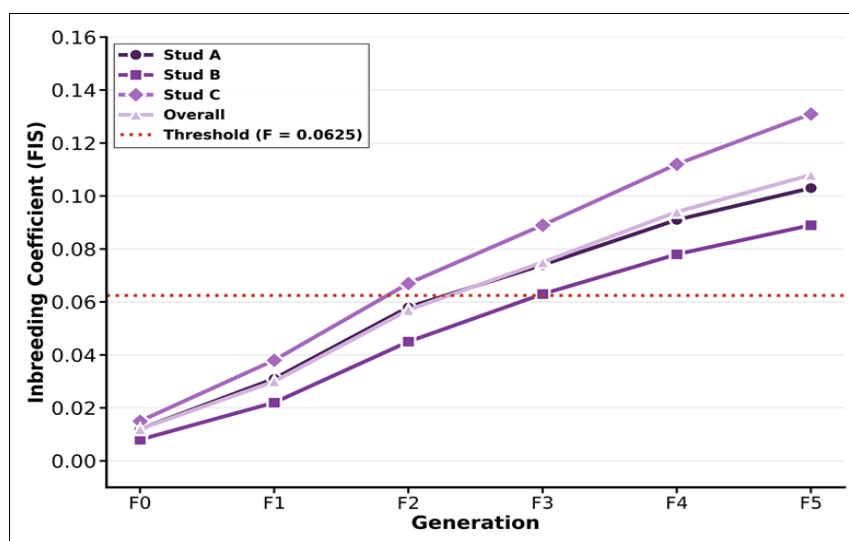


Fig 4: Generational trajectory of the inbreeding coefficient (FIS) for each stud farm and for the overall population. The red dotted threshold line marks $FIS = 0.0625$, beyond which inbreeding depression risks become biologically significant.

Discussion

The headline findings of this research an overall He of 0.714, FIS of 0.063, and Ne of 34.2 place the Brazilian Marwari subpopulation at the lower end of the diversity spectrum reported for global equine breeds. By comparison, thoroughbreds typically show He values of 0.68-0.71 despite a famously narrow founder base, while indigenous Arabian populations range from 0.72 to 0.78^[14, 15]. The Marwari's values are comparable to those of breeds under active conservation management, such as the Caspian horse ($He = 0.69$) and the Sorraia ($He = 0.62$)^[16].

On the points of agreement with earlier work, the per-locus allele counts and PIC values reported here fall within the ranges documented by Gupta and colleagues^[6] and Behl and colleagues^[7] in Rajasthan-based Marwari samples,

suggesting that the Brazilian importation, though numerically small, captured a reasonable cross-section of the ancestral allele pool. Where the current data diverge is in the magnitude of FIS : the Rajasthan studies reported values of 0.04-0.07 in populations with some natural gene flow between breeding farms and village herds, whereas the Brazilian studs managed in near-complete reproductive isolation have already drifted to 0.063 overall and 0.089 in the most closed stud.

Several factors may explain the faster inbreeding accumulation. The Brazilian founder group numbered fewer than 20 animals, and at least three were closely related half-siblings^[4]. Stud C in particular was established from just four founders (two stallions and two mares), creating an extreme bottleneck that random genetic drift alone would be expected to narrow further within a few generations^[17]. The practice of

using a single “hero” stallion for the majority of matings a common strategy in small stud-farm operations seeking phenotypic uniformity compounds the problem by reducing the effective number of sires below the census sire count^[18].

From a conservation genetics perspective, the most actionable implication is that the three Brazilian studs should begin a coordinated sire-exchange programme without delay. Even a modest gene flow of 2-3 animals per generation would slow the rate of allele loss and stabilise FIS at a manageable level^[8, 19]. The STRUCTURE results provide a clear roadmap for which farm pairs would benefit most from exchange: Studs A and C, which sit in the most genetically distant clusters, would gain the most heterozygosity from reciprocal sire lending. Longer-term, importation of fresh genetic stock from the breed’s home population or, where biosecurity regulations permit, the use of cryopreserved semen from genetically unrelated stallions would broaden the allelic base beyond what within-Brazil reshuffling can achieve^[20, 21].

Conclusion

This research set out to quantify the genetic diversity, population structure, and inbreeding trajectory of 48 Marwari horses across three Brazilian stud farms using a standardised 12-locus microsatellite panel. The work provides the first molecular characterisation of the exported Brazilian Marwari sub-population and establishes a baseline against which future management interventions can be measured.

Three findings stand out with particular urgency. First, the overall inbreeding coefficient (FIS = 0.063) has already breached the conventional threshold of concern, and the most closed stud (Stud C, FIS = 0.089) is on track to reach levels associated with measurable inbreeding depression within two generations. Second, Bayesian clustering confirmed that the three studs operate as partially isolated genetic units, with 6.1% of total molecular variance partitioned among farms a degree of sub-structuring that accelerates within-stud allele loss. Third, the effective population size of 34.2 falls well short of the FAO’s recommended minimum of 50 for short-term maintenance and is less than one-tenth of the $N_e = 500$ target for long-term evolutionary potential.

On the practical side, these data argue strongly for an immediate inter-stud sire-exchange programme, beginning with transfers between the most genetically divergent farm pairs (Studs A and C). In the medium term, importation of cryopreserved semen from unrelated Rajasthan-based stallions would inject new alleles that no amount of internal reshuffling can replicate. Breed registries should consider making genetic diversity metrics a mandatory component of stud-farm accreditation.

Future directions include extending the marker panel to SNP arrays or low-coverage whole-genome sequencing to identify runs of homozygosity and deleterious recessive alleles, expanding the sample to include Marwari populations in other export countries, and conducting a reproductive performance audit to test whether the current FIS levels are already affecting fertility, neonatal survival, or disease susceptibility.

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Contributions Not Qualifying for Authorship

The authors acknowledge Luciana Moreira for technical assistance with DNA extraction and fragment analysis, and Pedro Henrique Martins for assistance with pedigree data compilation.

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Conflict of Interest

Not available

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