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Research article

Polymorphisms in INHA, IGF1, and MSTN genes show no significant association with morphometric traits in Madura cattle

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Abstract

This research aimed to investigate the association of Polymorphisms in Inhibin Alpha (*INHA*), Insulin-like Growth Factor 1 (*IGF1*), and Myostatin (*MSTN*) genes as well as morphometric traits in local cattle. A total of 57 Madura bulls traditionally reared (under the supervision of the Department of Food Security and Agriculture of Pamekasan Regency) Pamekasan, Indonesia, were use. The morphometric traits analyzed were body length (BL), chest girth (CG), chest width (CW), wither height (WH), hip height (HH), and hip length (HL). Furthermore, DNA of the animals was extracted, amplified, and analyzed using Sanger sequencing to identify Single Nucleotide Polymorphisms (SNPs). Statistical association analyses were also performed through linear mixed models based on Restricted Maximum Likelihood (REML) method. The six SNPs identified were 1) one SNPs in *IGF1* located in the 5′ upstream region of the *IGF1* gene (ARS-UCD2.0: chr5:g.66191099G>T), 2) four SNPs in *INHA* in Exon 1, including two missense mutations (*INHA*, chr2:g.107501905T>C and g.107502030G>A) and two synonymous mutations (g.107502020G>A and g.107502062C>G), and 3) one synonymous variant in *MSTN* (*MSTN*, chr2: g. 6279263G>A). The results showed that *INHA* SNP4 was established as a novel variant, without preceding reports. In addition, no significant associations were reported between SNPs and morphometric traits ($p > 0.05$). Although no significant associations were found with the studied morphometric traits, the genetic variability observed in MSTN, INHA, and IGF1 may hold potential relevance for other economically important characteristics. This baseline information could serve as a starting point for future studies aiming to evaluate their possible role in genetic improvement programs of Madura cattle.

Keywords: Madura cattle, Morphometric traits, SNP polymorphism.

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INTRODUCTION

The improvement of beef cattle production is an important goal in breeding programs. This can be achieved through an effective method such as Marker-Assisted Selection (MAS), which uses genetic information to identify and select superior animals more accurately. MAS has the potential to increase the process of breeding in addition to productivity and profitability. In addition, selection of individuals based on favorable genetic variations for growth and phenotypic traits improves meat yield and performance (Liang et al., 2014; Han et al., 2017). The relationship between genetic markers and economically important traits should be analyzed for MAS to be successful.

Several candidate genes, such as Inhibin A (*INHA*), Insulin-like Growth Factor 1 *(IGF1*), and Myostatin (*MSTN*), are included in cattle growth and development. *INHA* encodes a member of the transforming growth factor-beta (TGF-β) superfamily, which regulates follicular development and gonadal function (Han et al., 2023). Polymorphisms in the *INHA* gene have been associated with body weight and average daily gain in beef and dairy cattle, indicating its potential as a candidate marker for growth traits in genetic selection programs (Bayraktar and Özdemir, 2022). The morphometric characteristics in cattle are complicated and under the control of a group of genes with pleiotropic effects. Candidate genes, including *MYC, PPARG,* and *GSK3B*, have been implicated in the regulation of growth, feed efficiency, and reproductive function (Fonseca et al., 2018). The genes can interact with INHA in larger gene networks that influence physical characteristics. *IGF1* is a key gene in mammalian growth and developmental processes. Polymorphisms in the gene, including *IGF1*-SnaBI SNPs, have shown a considerable effect on growth traits in beef cattle, especially prepubescent to puberty (Rogberg-Muñoz et al., 2013). *MSTN* gene that inhibits muscle growth has been under extensive research regarding the effect on growth in livestock. Single Nucleotide Polymorphisms (SNPs) of *MSTN* gene have been associated with significant phenotypic characteristics, such as wither height (WH), chest girth (CG), and hip height (HH), characterized in Sumba Ongole cattle (Gaina and Amalo, 2022; Martínez-Castillero et al., 2023). Mutations or alterations in the gene sequences can affect the size of the body and muscle development in animals.

An investigation is required to determine the association of SNPs with morphometric traits in indigenous cattle populations, given the functional roles of the genes and the potential of MAS in breeding programs. This can guide the development of genomic-based selection methods specific to native breeds such as Madura cattle. The analysis will be conducted with Restricted Maximum Likelihood (REML) method in a linear mixed model framework that incorporates genomic relationship matrix (GRM) derived using the VanRaden method to capture genetic relatedness. The VanRaden method is widely applied to build GRM for genomic selection to predict breeding values of non-phenotyped animals and identify associations between genetic markers and economically important traits (Tiezzi and Maltecca, 2015; Zepeda-Batista et al., 2021). Even though REML is most commonly used to estimate variance components in mixed models, the combination with GRM has also been adopted in the estimation of heritability and genetic parameters in genomic selection (Fragomeni et al., 2015; Raidan et al., 2018). This research aims to evaluate the association between SNPs in *INHA*, *IGF1*, and *MSTN* genes as well as key morphometric traits in Madura cattle, including body length (BL), CG, body height, HH, hip width (HW), and chest width (CW). A scientific foundation is provided for the application of MAS strategies in Madura cattle by identifying genetic variants significantly associated with the traits. The results are expected to support the genetic improvement and sustainable productivity of valuable indigenous breeds through the integration of molecular information into breeding programs.

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MATERIALS AND METHODS

Ethical Approval

The experiments were conducted under the guidelines and regulations for the care and use of animals approved by the Animal Care and Use Committee, Universitas Brawijaya, Malang, Indonesia (approval no. 197-KEP-UB-2024).

Material and Location

This research was conducted on Madura Island, Indonesia, and the samples consisted of Madura cattle traditionally raised at Pamekasan, under the supervision of the Department of Food Security and Agriculture of Pamekasan Regency. The morphometric measurements were obtained in 2021 during the field phase of the study, whereas molecular (DNA) analyses were performed in 2024 as part of the laboratory-based follow-up. A total sampling method was applied to obtain 57 male Madura Sonok cattle. The age of the cattle was determined based on the eruption of permanent incisors (PI), which provides a reliable biological indicator of chronological age. The animals were classified into four age groups: PI1 (n = 16), PI2 (n = 24), PI3 (n = 8), and PI4 (n = 9). development continue until the animals reach physical maturity, typically around 18–24 months of age. As cattle grow older, the diameter and circumference of muscle fibres increase, leading to the enlargement of muscle fibre bundles and greater cross-sectional muscle areas, which in turn contribute to larger body dimensions (Gibson et al., 2021). In parallel, bone morphology and strength also change with age; for example, in beef-cross-dairy cattle, the relationship between live weight and bone strength is influenced by age, with older animals showing a reduced responsiveness of bone strength to live weight. Altogether, these physiological changes in muscle and bone development explain why older cattle generally exhibit greater body size and altered morphometric characteristics compared to younger ones. Madura cattle are one of Indonesia's indigenous cattle breeds, resulting from the hybridization of *Bos indicus* (zebu) and *Bos javanicus* (banteng) (Nijman et al., 2003). They are well adapted to tropical environments, resistant to poor-quality feed and parasites, and valued for cultural purposes such as bull racing (*karapan*), beauty contests (*sonok*), and meat production. Their multifunctionality and adaptability make them a key local genetic resource for conservation and breeding improvement (Perwitasari-Farajallah et al., 2019; Hartati et al., 2021).

Morphometric characteristics investigated were BL, CG, CW, WH, HH, and HW. These characteristics were selected to portray Madura cattle structural growth and body conformation, which were the most important determinants of growth performance and genetic potential of local cattle populations. The measurements were taken following standard morphometric protocols to provide uniformity and precision of all individuals sampled.

Sample Collection and DNA Extraction

Blood samples were drawn from the jugular vein into EDTA-coated vacutainer tubes. Genomic DNA was isolated with a commercially available DNA extraction kit according to the protocol recommended by the manufacturer. Furthermore, the desired fragments were amplified by polymerase chain reaction (PCR), and the resulting products were purified and characterized by Sanger sequencing. The results were verified with BioEdit software, and the balance was conducted by subjecting sequences to BLAST searches against NCBI database for verification.

Primer Design

Primers used for *INHA* and *IGF1* genes were procured from earlier research conducted by Ge et al. (2001) and Sang et al. (2011), respectively. However,

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primers for *MSTN* gene were newly designed with the aid of a reference sequence in the ensamble.org database. The primers targeted exon 1 of the *INHA* and *MSTN* genes, and the promoter region of the *IGF1* gene, as shown in Table 1. These regions were selected to cover both coding and regulatory elements, allowing the identification of SNPs with potential relevance to gene function or expression. For novel or unregistered variants, annotation and prediction of molecular effects were performed using the Ensembl Variant Effect Predictor (VEP) online tool (https://www.ensembl.org/Tools/VEP), referencing the ARS-UCD1.2 bovine genome assembly.

Table 1 Primer sequences used for amplification of target regions in the *INHA, IGF1*, and *MSTN* genes.

Gene	Location	Forward	Reverse
INHA	Exon1	5′- CTATGTGGCTTCAGCTGCTC -3′	5′- GGTCTGGGATTCAACCCAAC -3′
IGF1	Promoter Region	5′- GAGATCATTCCCCTCATTG -3′	5′- CTGCTAATACACCTTACCG -3′
MSTN	Exon 1	5'-CAAGTTGTCTCTCAGACTGG-3'	5'-CTAGAACAGCAGTCAGCAGA-3'

Genotype Frequency Analysis and Hardy-Weinberg Equilibrium (HWE) Test

Genotype and allele frequencies were determined by hand calculation and confirmed using R software. Hardy-Weinberg equilibrium (HWE) was assessed using the chi-square (χ^2) test at 5% significance to check the genetic equilibrium of the population.

Genetic Diversity Analysis (Ho, He, and PIC)

The calculated observed heterozygosity (Ho) was the percentage of heterozygous individuals observed at each locus. The expected heterozygosity (Ho) was estimated using allele frequencies by the formula:

$$H_0 = \sum_{i \neq j} \frac{N_{1ij}}{N} \quad (1)$$

The expected heterozygosity (He) was calculated based on allele frequencies using the following formula:

$$H_e = 1 - \sum_{i=1}^{q} {x_i}^2 \quad (2)$$

Where H_0 is Observed heterozygosity; N_{1ij} is Number of heterozygous individuals at the ith locus; *N* is Total number of individuals analyzed; H_e is Expected heterozygosity; x_i is Frequency of the ith allele; *q* is Total number of alleles at the locus.

Polymorphic Information Content (PIC) was estimated to assess the informativeness of each SNPs marker. PIC is defined as the probability that two individuals, selected without bias, will exhibit distinct genotypes at a particular genetic locus. This value can be frequently be adopted as a measure of marker diversity using the following formula:

$$PIC = 1 - (p^2 + q^2) \quad (3)$$

where p^2 and q^2 are the frequencies of the two alleles. Higher PIC values show greater polymorphism and utility of the marker for genetic analysis and selection purposes.

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Association Analysis between SNPs Genotypes and Morphometric Traits

The relationship between SNPs genotypes and morphometric characteristics was examined using a linear mixed model (LMM) with fixed and random effects of individual animals according to the genomic membership. GRM (G matrix) showing the similarity among individuals was developed from SNPs data following the method proposed by VanRaden (2008). SNPs genotypes were coded 0, 1, or 2 based on the number of minor alleles present (0 = major allele homozygote, 1 = heterozygote, 2 = minor allele homozygote). Additive coding enabled the incorporation of SNPs as fixed covariates in the model. Only SNPs with a minor allele frequency (MAF) > 5% were considered in the analysis to ensure adequate genetic diversity and statistical power. The formula used to compute G matrix is:

$$G = \frac{ZZ'}{2\sum p_j(1-p_j)} \quad (4)$$

In this case, Z is the centered genotype matrix, with genotypes coded and scaled by subtracting twice the allele frequency, and p_j is the reference allele frequency at the ith SNPs. The use of G matrix simplifies the calculation of additive genetic variance with accuracy by including true genomic relationships, improving the predictability of genomic selection models in livestock breeding programs.
The model was evaluated using REML method as implemented in the sommer package of R software. The method allowed for credible variance component estimation under unbalanced data and genomic relationships (Covarrubias-Pazaran, 2016).

$$y_{ijklmn} = \mu + X_1S_i + X_2L_j + X_3T_k + X_4U_l + X_5O_m + X_6M_n + Z_a + e \quad (5)$$

where y_{ijklmn} is a matrix of observations for the studied traits with dimension (n x p). X_1 to X_6 represent matrices fixed effect, $S_i, L_j, T_k, U_l, O_m,$ and M_n represent age, *INHA* SNP1, *INHA* SNP3, *INHA* SNP4, SNPs of *IGF1*, and *MSTN*. Z_a is the random additive genetic effect of individual animals. Z is the incidence matrix linking records to individuals and serves as the vector of additive genetic effects assumed to follow a normal distribution with mean zero. Covariance structure a ~ N (0, $G\sigma_a^2$) is the random effect of individual animals assumed to follow a normal distribution with a mean of zero and variance σ_a^2. The covariance structure is defined by GRM G. The residual error term e ~ N (0, $I\sigma_e^2$) is assumed to be normally distributed with a mean of zero and homogeneous variance.

P-values for fixed effects, including SNPs genotypes and age, were obtained from Analysis of Variance (ANOVA) tables generated by the `anova.mmer()` function applied to the linear mixed model using the `mmer()` in the `sommer` package (Covarrubias-Pazaran, 2016). The fixed effects were evaluated using approximate F-tests based on the fitted REML model.

RESULTS

DNA polymorphisms in candidate genes potentially associated with production traits were successfully identified through targeted sequencing of specific gene regions, as guided by previous research. A total of six DNA variants were detected and classified as SNPs. Additionally, there were no insertion or deletion (indel) variants observed in the analyzed regions.

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Table 2 Summary of identified single nucleotide polymorphisms (SNPs) in the *IGF1*, *INHA*, and *MSTN* genes, including their sequence context, genomic location, and variant type.

Gene	DNA variant name	Sequence Context	HGVS g. notation	Variant Type
IGF1	*IGF1* SNP1	TAGAGTA(G/T)GCTT	g.66191099G>T (chr5)	Upstream variant
INHA	*INHA* SNP1	GGGC(T/C)GGAG	g.107501905T>C (chr2)	Missense variant
	INHA SNP2	CACCG(G/A)AGGCA	g.107502020G>A (chr2)	Synonymous variant
	INHA SNP3	ATGCC(G/A)TGGG	g.107502030G>A (chr2)	Missense variant
	INHA SNP4	AGCC(C/G)GAGGA	g.107502062C>G (chr2)	Synonymous variant
MSTN	*MSTN* SNP1	AAACA(G/A)CTCCT	g. 6279263G>A (chr2)	Synonymous variant

A single SNPs was identified in *IGF1* gene, located in the 5' upstream region (ARS-UCD2.0:chr5:g.66191099G>T), which differed from the variant previously reported by Lirón et al. (2012). Approximately four SNPs were detected in *INHA* gene (inhibin α-subunit) in Exon 1. These genes were classified as missense (*INHA* SNP1: ARS-UCD2.0:chr2:g.107501905T>C and *INHA* SNP3: ARS-UCD2.0:chr2:g.107502030G>A) and synonymous mutations (*INHA* SNP2: ARS-UCD2.0:chr2:g.107502020G>A and *INHA* SNP4: ARS-UCD2.0:chr2:g.107502062C>G). *INHA* SNP1 resulted in a leucine-to-proline substitution at position 21, while *INHA* SNP3 led to a valine-to-methionine substitution at position 63. *INHA* SNP2 was previously described by Sang et al. (2011) in relation to semen quality, unlike the other three SNPs. According to the Ensembl database and the Bovine Genome Variation Database (BGVD), *INHA* SNP4 was classified as a novel variant. This SNV (chr2:107502062C>G) is located within exon 1/2 of the *INHA* gene. Ensembl Variant Effect Predictor (VEP) annotated this substitution as a missense mutation with LOW predicted impact. However, the codon change from CCC to CCG did not alter the amino acid (proline at position 73), thus constituting a synonymous substitution at the protein level. Functionally, *INHA* is associated with growth factor activity (GO:0008083) and is localized to the extracellular region (GO:0005576), indicating its role in intercellular signaling despite the synonymous nature of this variant. In *MSTN* gene, one SNPs was identified and designated as *MSTN* SNP1: ARS-UCD2.0: chr2:g. 6279263G>A in Exon 1 with a nucleotide substitution from G to A. A synonymous mutation in the *MSTN* gene, located in Exon 1, was identified as a Glutamat amino acid at position 89 (*ENSBTAP00000015674.4:p.Glu89=*), which does not alter the Myostatin protein sequence.

Table 3 shows genotype, observed genotype, and expected Hardy-Weinberg frequencies for each of SNPs loci in *INHA*, *IGF1*, and *MSTN* genes. The observed values were simply obtained from the population sample, while the expectations were calculated based on allele frequencies under Hardy-Weinberg proportions. These data were used to determine the genetic variability and equilibrium status of each locus.

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Table 3 Genotype distribution, observed and expected frequencies of SNP markers in *INHA*, *IGF1*, and *MSTN* genes

SNP	Genotype	Observed	Freq_Observed	Expected	Freq_Expected
***INHA* SNP1**	CC	0	0.00	1.75	0.03
	CT	20	0.35	16.49	0.29
	TT	37	0.65	38.75	0.68
***INHA* SNP2**	AA	55	0.96	55.02	0.97
	AG	2	0.04	1.96	0.03
	GG	0	0.00	0.02	0.00
***INHA* SNP3**	AA	2	0.04	0.63	0.01
	AG	8	0.14	10.74	0.19
	GG	47	0.82	45.63	0.80
***INHA* SNP4**	CC	36	0.63	37.93	0.67
	CG	21	0.37	17.13	0.30
	GG	0	0.00	1.93	0.03
IGF1	GG	33	0.58	32.44	0.57
	GT	20	0.35	21.12	0.37
	TT	4	0.07	3.44	0.06
MSTN	AA	36	0.63	34.74	0.61
	AG	17	0.30	19.52	0.34
	GG	4	0.07	2.74	0.05

Genetic characterization of six SNPs in *INHA*, *IGF1*, and *MSTN* genes reported a heterogeneous makeup of genotypes in the population of Madura cattle. Genotypic frequencies and expected values were also calculated to infer genetic structure and deviation from HWE.

There were four distinct SNPs discovered in *INHA* gene. *INHA* SNP1 was found to have a 65% frequency of TT genotype, 35% of CT, with no subjects having CC. The frequency observed was in close adherence to the expected frequencies (TT: 0.68, CT: 0.29, CC: 0.03) since the population would be in HWE.

The second SNPs had extremely high dominance of AA genotype (96%) with very few AG (4%) and no GG individuals, reflecting potential genetic disequilibrium caused by selection pressure, small population size, or non-random mating. The third and fourth SNPs in *INHA* exhibited dominance of a single genotype, where GG and CC accounted for 82% and 63%, respectively. The very low frequency or absence of minor genotypes at the loci showed potential allelic imbalance and deviation from equilibrium.

For *IGF1* gene, GG genotype was most prevalent (58%), followed by GT (35%) and TT (7%). These frequencies were relatively consistent with the expected values, but slight deviations suggested the influence of selection or other population-level factors. *MSTN* gene possessed a predominant AA genotype frequency of 63%, with 30% and 7% of AG and GG, respectively. Observed frequencies were in close agreement with expected values (AA: 0.61, AG: 0.34, GG: 0.05) since the genetic locus was in HWE.

Table 4 presents genetic diversity parameters of six SNPs in *INHA*, *IGF1*, and *MSTN* genes. These comprised the major allele, MAF, Ho, He, PIC, and p-value of HWE test. The table values represented the degree of genetic variation and equilibrium condition of each locus in Madura cattle population.

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Table 4 Genetic diversity parameters for six SNPs in *INHA*, *IGF1*, and *MSTN* genes, including MAF, Ho, He, PIC, and Hardy-Weinberg Equilibrium (HWE) p-values

SNP	Major Allele	MAF	HO	HE	PIC	HWE p-value
***INHA* SNP1**	T	0.18	0.35	0.29	0.25	0.2752
***INHA* SNP2**	A	0.02	0.04	0.03	0.03	0.991
***INHA* SNP3**	G	0.11	0.14	0.19	0.17	0.157
***INHA* SNP4**	C	0.18	0.37	0.30	0.26	0.2338
IGF1	G	0.25	0.35	0.37	0.30	0.9226
MSTN	A	0.22	0.30	0.34	0.28	0.6224

Note: MAF = minor allele frequency; HO = observed heterozygosity; HE = expected heterozygosity; PIC = polymorphism information content; HWE = Hardy-Weinberg equilibrium

The results showed that five out of the six SNPs exhibited a minor allele frequency (MAF) greater than 0.05. Therefore, the markers were genetically and statistically informative and could be used for relatedness and association research. Observed (Ho) and expected heterozygosity (He) were dependent on the majority of the loci, implying a fairly stable population structure with no indication of rampant inbreeding.

PIC was low to moderate, and the highest value was observed for *IGF1* SNPs (0.30), followed by *MSTN* (0.28). Most of SNPs fell in the moderately informative range and were used as molecular markers in genetic selection. There was no significant deviation from HWE with p-values more than 0.05. This result confirmed that the population was in genetic equilibrium and there was no segregation distortion in alleles. The utilization of the tested SNPs is validated for future genetic research and MAS in Madura cattle.

Table 5 summarizes the estimated effects, standard errors (SE), and P values for age and genotypes of six SNPs markers (*INHA* SNP1, *INHA* SNP3, *INHA* SNP4, *IGF1*, and *MSTN*) on CG, WH, BL, HW, HH, and CW morphometric traits in Madura cattle. The analysis was performed using an LMM that incorporated fixed effects of age and genotype, as well as a random animal effect based on GRM.

The parameter estimates from LMM reported that animal age (AGE) had a significant effect on several morphometric traits. For CG, AGE exhibited a strong positive effect, with a slope estimate of 5.492 and a standard error of 1.638, statistically significant at the 1% level ($p = 0.001$). Similarly, the variable showed a significant positive effect ($p = 0.040$) for BL, with an estimate of 2.728 and a standard error of 1.272. A significant effect was also observed for HW, where AGE had an estimate of 2.079 ($p = 0.016$). The variable did not significantly affect WH, HH, or CW, even though the estimated coefficients were positive, showing a consistent directional trend.

The fixed effects of SNPs markers coded additively as 0, 1, or 2 to represent the number of minor alleles did not show statistically significant associations with any of the morphometric traits analyzed. The estimated slopes for *INHA* SNP1 ranged from 2.432 to 10.587, with all p-values above 0.05. In HH, *INHA* SNP1 had a slope estimate of 8.855 and a standard error of 5.386 ($p = 0.102$), which approached significance. Similarly, *INHA* SNP3 and *INHA* SNP4 were not significant, with p-values reaching 0.946 and 0.860, respectively. The estimated effects of *IGF1* were positive but small in BL (estimate = 0.621; SE = 4.002; $p = 0.876$) and WH (estimate = 3.649; SE = 3.043; $p = 0.237$). *MSTN* tended to show negative slopes for most traits, but none were statistically significant in CG (estimate = -2.609; SE = 9.027; $p = 0.701$) and CW (estimate = -1.441; SE = 2.899; $p = 0.622$). The results suggested that animal age consistently contributed to phenotypic variation in several body measurements. Even though SNPs markers did not show significant effects, some trends in the direction of the estimates indicated potential associations with specific traits.

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Table 5 Estimated effects of age and SNP genotypes on morphometric traits in Madura cattle based on linear mixed model analysis

Trait	Effect	Estimate	SE	CI		p-value
CG	(Intercept)	156.099	3.706	148.835	163.363	—
	AGE	5.492	1.638	2.282	8.702	0.001
	INHA SNP1	10.587	13.996	-16.845	38.019	0.451
	INHA SNP2	3.474	5.697	-7.692	14.640	0.544
	INHA SNP3	-6.817	6.292	-19.149	5.515	0.627
	IGF1	1.169	5.177	-8.978	11.316	0.821
	MSTN	-2.609	9.029	-20.306	15.088	0.772
WH	(Intercept)	122.801	2.758	117.395	128.207	—
	AGE	0.728	0.971	-1.175	2.631	0.456
	INHA SNP1	10.025	8.269	-6.182	26.232	0.23
	INHA SNP2	2.108	3.355	-4.468	8.684	0.531
	INHA SNP3	-7.95	5.288	-18.314	2.414	0.339
	IGF1	3.649	3.043	-2.315	9.613	0.237
	MSTN	1.201	3.128	-4.930	7.332	0.701
BL	(Intercept)	134.222	2.75	128.832	139.612	—
	AGE	2.728	1.27	0.239	5.217	0.035
	INHA SNP1	2.432	10.751	-18.640	23.504	0.823
	INHA SNP2	2.419	4.411	-6.227	11.065	0.585
	INHA SNP3	-1.957	6.781	-15.248	11.334	0.856
	IGF1	0.621	4.078	-7.372	8.614	0.878
	MSTN	-4.196	4.09	-12.212	3.820	0.308
HW	(Intercept)	43.229	1.012	41.245	45.213	—
	AGE	2.079	0.851	0.411	3.747	0.018
	INHA SNP1	5.303	7.295	-8.995	19.601	0.469
	INHA SNP2	1.047	2.944	-4.723	6.817	0.724
	INHA SNP3	-5.513	7.244	-19.711	8.685	0.451
	IGF1	0.617	2.679	-4.634	5.868	0.819
	MSTN	-1.193	2.732	-6.548	4.162	0.664
HH	(Intercept)	118.694	2.582	113.633	123.755	—
	AGE	0.553	0.632	-0.686	1.792	0.385
	INHA SNP1	8.855	5.386	-1.702	19.412	0.107
	INHA SNP2	2.561	2.19	-1.731	6.853	0.248
	INHA SNP3	-5.802	5.371	-16.329	4.725	0.287
	IGF1	2.692	1.989	-1.206	6.590	0.186
	MSTN	0.116	2.134	-4.067	4.299	0.956
CW	(Intercept)	38.61	0.821	37.001	40.219	—
	AGE	1.311	0.9	-0.453	3.075	0.154
	INHA SNP1	8.525	7.688	-6.543	23.593	0.275
	INHA SNP2	3.163	3.126	-2.964	9.290	0.317
	INHA SNP3	-8.158	7.677	-23.205	6.889	0.291
	IGF1	0.061	2.663	-5.158	5.280	0.978
	MSTN	-1.441	2.898	-7.121	4.239	0.62

Note: CG = Chest Girth, WH = Wither Height, BL = Body Length, HW = Hip Width, HH = Hip Height, CW = Chest Width. The estimates were obtained using linear mixed model analysis with animal effect modeled as a random factor.

Table 6 presents the variance components estimated from the linear mixed model, including the additive genetic variance attributable to individual animals (σ_a^2), residual variance, and the proportion of total variance explained by the animal effect for each morphometric trait. The results showed relatively consistent proportions across traits since individual animal genetic variation contributed uniformly to phenotypic variation.

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Table 6 SNP-based additive genetic variance, residual variance, heritability estimates, and standard errors for morphometric traits in Madura cattle

Trait	σ_a^2	σ_e^2	h^2	SE
CG	29.43	147.17	16.66%	0.02
WH	10.28	51.4	16.67%	0.02
BL	17.67	88.34	16.67%	0.02
HW	7.92	39.6	16.67%	0.02
HH	4.36	21.79	16.67%	0.02
CW	8.87	44.36	16.66%	0.02

Note: CG = Chest Girth, WH = Wither Height, BL = Body Length, HW = Hip Width, HH = Hip Height, CW = Chest Width. The estimates were obtained using linear mixed model analysis with animal effect modeled as a random factor.

The variance component estimates for seven morphometric traits in Madura cattle reported that the additive genetic variance among individuals, modeled as a random effect, consistently contributed to the total phenotypic variance, ranging from 16.66% to 16.67%. For CG, the additive genetic variance was estimated at 29.43, while the residual variance was 147.17. WH showed an additive genetic and a residual variance of 10.28 and 51.4, respectively. Furthermore, BL presented a genetic variance of 17.67 with a residual of 88.34. Other traits, such as HW, HH, and CW, reported comparable patterns, each with approximately 16.67% of the phenotypic variance explained by additive genetic effects.

The uniformity of the heritability estimates across different traits suggested that SNPs-based GRM captured a stable portion of additive genetic variation. This result supported the applicability of targeted SNPs panels for estimating genetic parameters in indigenous cattle populations and reported the potential utility in MAS programs aimed at improving economically important traits.

DISCUSSION

The discovery of six SNPs in *IGF1*, *INHA*, and *MSTN* genes is an important foundation for conducting genetic association research on morphometric and reproductive traits in Madura cattle. SNPs found in the 5' upstream region of *IGF1* gene imply a role in the regulation of transcription. The variants differ from the description of Lirón et al. (2012), which points to the existence of population-specific variations. A total of four SNPs in exon 1 of *INHA* gene, comprising two missense mutations (SNP1 and SNP3), have the potential to influence the function of inhibin protein, a key regulator of reproductive hormones (Novianti et al., 2021). The identification of a novel SNP (*INHA* SNP4) indicates the significance of local populations as valuable sources of untapped genetic diversity. Using variant Effect Predictor (VEP) analysis provided by Ensemble database (www. Ensemble.org), the potential function for the identified SNP was analysed. It is indicated this SNP has function in growth factor activity. It stimulates a cell to grow and proliferate although it only has low impact. The identification of a novel SNPs (*INHA* SNP4) shows the significance of local populations as valuable sources of untapped genetic diversity. Some loci, such as *INHA* SNP3, exhibited the presence of three genotypes in Madura to provide greater opportunities for association research, even though the majority of the genotype distributions were in biallelic patterns.

Identification of a missense SNPs in exon 1 of *MSTN* gene implies great functional implications, considering the established role as a negative regulator of muscular growth (Liu et al., 2023). *MSTN* SNP1 variant has the potential to change the biological function of the protein and is expected to be related to growth and carcass characteristics (Khasanah et al., 2016). The results are in line with other livestock breeds and validate the potential use of *MSTN* SNP1 as a marker for MAS programs (Thepa and Tyasi, 2024). The results of genetic variants provide a solid basis for continued investigation into the inheritance effects on economically valuable traits in indigenous cattle breeds.

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The genotyping of six SNPs in *INHA*, *IGF1*, and *MSTN* genes reported different distributions in the population of Madura cattle. The uncovered genetic variability shows the existence of adequate variability to facilitate MAS programs (Eusebi et al., 2019; Deb et al., 2020). HWE tests report that the majority of loci are in equilibrium ($p > 0.05$), pointing toward the lack of intense selection pressures or substantial genetic drift acting on the population. The highly significant deviations from equilibrium at some loci may indicate possible effects of selection, finite population size, or non-random mating practices (Bortoluzzi et al., 2020; Sunde et al., 2024). The extreme allelic imbalance observed in *INHA* SNP2 (96% AA) despite HWE ($p = 0.991$) may be influenced by selective breeding practices specific to Madura cattle, which are maintained primarily for contest purposes. Such directed selection may reduce genotypic diversity and cause skewed genotype frequencies even when HWE is statistically met. Moreover, the overall low to moderate PIC values may reflect the narrow genetic base of this subpopulation, potentially limiting the informativeness of the SNPs tested.

A total of five MAF above 0.05 are genetically and statistically informative (Gorlov et al., 2008) as well as suitable for association with traits and kinship analyses (Ali et al., 2020). The observed (Ho) and expected heterozygosity (He) values were largely in equilibrium, indicating limited inbreeding (Nietlisbach et al., 2016). The results confirm that the population is homogeneous and genetically diverse, which is essential for efficient MAS breeding.

PIC values vary from low to moderate, with the largest found in *IGF1* and *MSTN* genes at the values of 0.30 and 0.28, respectively. These results show that most of SNPs are moderately polymorphic markers with great potential for use in MAS programs (Koenig and Simianer, 2008; Thomas and Anilkumar, 2008; Rezende, 2013). Despite the absence of significant deviations from HWE, the generally low PIC values and genotype imbalances, as observed in *INHA* SNP2 ($p = 0.991$), suggest limited allelic diversity, possibly resulting from artificial selection or a narrow breeding base in Madura cattle. These conditions reduce marker informativeness and imply that conclusions regarding their suitability for molecular selection should be drawn with caution. It is also important to note that the Madura cattle used in this study were Sonok-type animals, which are highly selected and culturally maintained for traditional performances. As a result, the population is naturally limited in number. Although this limits statistical power, the uniqueness and genetic uniformity of the Sonok cattle provide valuable insights into population-specific variants. Therefore, the findings from this elite group serve as an important reference for future validation studies using broader or more diverse populations.

The mixed linear model analysis indicated the influence of age on most of the morphometric traits. Positive significant effects on CG, BL, and HW show that increasing age contributes to body growth size in the breed (Prihandini et al., 2018; Widyas et al., 2018). SNPs markers in *INHA*, *IGF1*, and *MSTN* genes do not exhibit significant associations with the analyzed morphometric traits. Even though certain SNPs, such as *INHA* SNP1, report relatively large estimated coefficients, the corresponding p-values remain above 0.05, indicating statistical non-significance. The effects of *IGF1* SNPs are positive and small, while *MSTN* SNPs generally report negative and non-significant effects across most traits. These results may be attributed to the limited sample size, weak association strength, or the polygenic nature of morphometric traits regulated by multiple genes with minor individual effects (Fosgate, 2009; Sivasamy, 2023). *INHA* gene, encoding *INHA* subunit, is primarily part of reproductive processes by regulating follicle-stimulating hormone (FSH) secretion and ovulation frequency (Liu et al., 2017; Cui et al., 2019; Abuzahra et al., 2024), rather than directly influencing body size. However, members of the TGF-β superfamily, including inhibin, contribute to skeletal muscle and bone development by regulating cell proliferation, differentiation, and tissue homeostasis (Itoh and ten Dijke, 2007; Sartori et al., 2014; Hata and Chen, 2016). Even though *IGF1* affects livestock performance directly, the absence of significant associations may similarly reflect insufficient sample size, which limits statistical power and the

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detection of true genetic effects (Fosgate, 2009; Sivasamy, 2023). Given the small sample size and low phenotypic variation in cattle, these results may reflect a Type II error, where real associations remain undetected. Therefore, the findings remain relevant and should be further explored using larger samples and more diverse phenotypic data.

MSTN (growth differentiation factor 8; GDF8) functions as a negative regulator of muscle growth and development (Deng et al., 2017). Mutations in this gene can lead to the double-muscle phenotype, characterized by increased muscle mass due to fiber hyperplasia and hypertrophy (Stinckens et al., 2008; Liu et al., 2023). However, *MSTN* did not show a significant association with performance traits in this research since the evaluated performance parameters were more related to bone growth than muscle development (Madula et al., 2024). While the limited sample size likely reduced the power to detect significant associations, other factors may have also played a role. These include low minor allele frequencies, narrow phenotypic variation, potential mismatch between the targeted SNPs and the traits examined, and limited SNP coverage within the candidate genes. Larger sample sizes are particularly important when traits are influenced by multiple genes with small effects (Oexle and Meitinger, 2011).

The variance component estimates showed that individual additive genetic variation consistently accounted for 16.67% of the total phenotypic variance across all evaluated traits. Although the genomic relationship matrix (G-matrix) was based on only six candidate SNPs, it offered a preliminary approximation of genetic relatedness among individuals. However, the low marker density reduces its accuracy, and future studies should consider using genome-wide SNP panels to improve the resolution and reliability of such models. The genetic contribution homogeneity addresses the possible usefulness of separate SNPs panels to assess parameters in regional animal populations. Previous research showed that SNPs panels were widely used across many livestock species to map genes associated with economically important traits such as growth, reproduction, and production performance. In addition, the panels significantly improve the accuracy of genetic selection even in cases of reduced substantial associations (Meuwissen et al., 2001; Suchocki et al., 2017). The moderate size heritability estimates obtained in the current research agree with previous reports, reporting that genetic enhancement by selection is possible, especially for morphometric traits with moderate to high heritability (Frizzas et al., 2009; Pantelić et al., 2011). The results justify the use of MAS, which includes the adoption of molecular markers to increase the accuracy and efficiency of traditional breeding programs (Hudcovicová et al., 2008; Babar et al., 2010). Even though some of SNPs are devoid of association significance, the uncovered genetic variation provides immense opportunities for the enhancement of economically important morphometric traits in indigenous cattle populations.

CONCLUSIONS

In conclusion, six DNA variations were successfully identified in *IGF1, INHA*, and *MSTN* genes, including a novel C/G missense variant in Exon 1 of *INHA*. The genotype distribution reported a balanced genetic diversity in Madura cattle. There was no significant association between SNPs and body measurements, but the animal age consistently influenced several growth traits. Generally, the observed genetic variation in the gene markers indicates their potential value as resources for future breeding programs in Madura cattle.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Irida Novianti : Methodology, Investigation, Data curation, Writing –original draft.
Rizal Rinaldi : Validation, Formal analysis, Writing— review and editing.
Habib Asshidiq Syah : Validation, Formal analysis, Writing— review and editing.
Emmanuela Jennifer Sharon Dolorosa Purwanto Junior : Investigation, Writing –original draft
Veronica Margareta Ani Nurgiartiningsih : Conceptualization, Funding acquisition, Project administration, Data curation
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