Original Research

Journal of Advanced Veterinary Research
(2026) Volume 16, Issue 1, 141-144

Polymorphism of heat shock protein 70 (Hsp70) gene on local duck of West Sumatera

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ARTICLE INFO

Received: 06 October 2025

Accepted: 27 December 2025

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Keywords:

Enzymes, HSP70 gene, PCR-RFLP, Pitalah duck, Polymorphism

ABSTRACT

This research aimed to identify the polymorphism of Heat Shock Protein 70 (HSP70) gene on local duck of West Sumatra through PCR-RFLP. The samples used for this research were 45 blood samples of Pitalah ducks (37 females and 8 males) taken at UPT Faculty of Animal Husbandry, Andalas University. Blood samples were isolated with the (G-spinTM Total DNA Extraction Mini Kit protocol) from iNtRON Biotechnology genomic. The isolated DNA was amplified using a pair of primers F: 5'-CGC TGC TGA TTG GCT AGG A-3' and R: 5'-CCT CAC CGC ACG CTT ATT G-3' target of 1043 bp and F: 5'- TAACACCACCATTCCCACCA-3' and R: 5'-GCGTGGTTACTTTAGAA-GAGACA -3' target of 916 bp. The restriction enzymes *Hae*II step produced genotypes (+/-) 26 samples and (-/-) 19 samples with allele (+) 0,21 and allele (-) 0,79. Restriction enzymes HpyAV was produced genotypes (+/+) 17 samples, (+/-) 26 samples and (-/-) 2 samples with allele (+) 0.67 and allele (-) 0.33. The results of this research indicate that Pitalah duck population in West Sumatera was polymorphic and was in Hardy-Weinberg equilibrium.

Introduction

Indonesia is an agricultural country that has various types of germplasm spread throughout Nusantara. One of the small livestock that is a favorite for the people of Indonesia is ducks. Ducks are the second largest contributor of meat and eggs next to chickens (Purwantini *et al*., 2017). According to data from Badan Pusat Statistik (2022), the duck population in Indonesia reached 58,351,458 heads with an egg production of 355,187 eggs. This is an outstanding numerical opportunity to be continuously improved. Most of the regions in Indonesia have local ducks with potential and various names that originate from their respective regions. An example is West Sumatra. The duck nation is named based on the local area of origin such as Bayang ducks, Kamang ducks, Pitalah ducks and Sikumbang jonti ducks. According to Harahap *et al*. (1980), ducks raised in West Sumatra have similar phenotypes with ducks from Java of Indian Runner blood.

Pitalah ducks originate from Kanagarian Pitalah which is a plateau of 500-850 mdpl (Badan Pusat Statistik, 2022). Pitalah ducks have specific characteristics, such as high productivity, agile, easy to maintain and adaptive to unfavorable environments. Pitalah ducks have the advantage of not recognizing the term afkir like most Javanese ducks. To preserve and develop local ducks that have adapted to the surrounding environment, Pitalah ducks must be protected as one of Indonesia's livestock genetic resources (Ismoyowati, 2008). Efforts to develop and increase the efficiency of Pitalah ducks are currently experiencing problems in optimizing production and reproduction values. One of them is physiological conditions, where ducks raised in the tropics are very vulnerable to stress due to environmental temperature. Compared to other local ducks, Pitalah ducks are the most susceptible to heat stress characterized by rectal temperature and high panting frequency due to the impact of intensive maintenance in the lowlands (Subekti, 2019). Rami (2022) found slight stress in Pitalah ducks reared in the cages of the Faculty of Animal Husbandry, Andalas University. The maintenance temperature range in the cage was 23-28°C (Juwita, 2022). The lack of rice fields due to land conversion has made local duck rearing patterns in West Sumatra change from extensive to intensive systems. This rearing system has an impact on the ducks' limited access to water ponds for drinking and swimming as part of their waterfowl behavior (O'Driscoll and Broom, 2011). This has the effect of inhibiting the thermoregulation process in the duck's body.

The environmental temperature of West Sumatra which ranges from 23-32°C exceeds the comfortable temperature of ducks which is 18.3-25.5°C (Subekti, 2019). Raising ducks higher than the comfortable temperature has the potential for the animals to experience stress due to the difficulty in dissipating their body temperature to the environment (Cooper and Washburn, 1998). As waterfowl, ducks have a different physiology from other birds, making them more susceptible to heat stress (Ali *et al*. 2008). Physiologically, heat stress that occurs due to high temperatures affects the synthesis process, stability, and activity of enzymes (Tamzil, 2014) and the balance of biochemical reactions in weak bonds (Noor and Seminar, 2009).

Heat stress can cause several losses such as decreased performance and behavioral changes in livestock and even death (Daramola *et al*., 2012). To overcome the effect of heat stress on ducks, genetic approach selection efforts are needed to obtain ducks that are more tolerant in high temperature maintenance (Tamzil, 2014). During periods of heat stress, ducks will return their body temperature back into the physiological zone as it was before the stress. When failing to maintain homeostasis, ducks will use genetic pathways by activating Heat Shock Protein (HSP) genes that function only when experiencing heat stress (Noor and Seminar, 2009). One of the most studied types of HSP genes is the HSP70 gene. HSP70 is a group of heat shock proteins that work as chaperones, in charge of regulating protein folding to protect cells from heat stress damage (Tkacova and Angelovicova, 2012).

The HSP70 gene is an ideal biological marker of heat stress in livestock (Archana *et al*., 2017). Subekti (2019) reported the polymorphism of the HSP70 gene in local ducks by analyzing the end of the coding region

ISSN: 2090-6277/2090-6269/

using the SacII restriction enzyme. HSP70 gene diversity in ducks (white sansui) was first identified by Xia *et al.* (2013). Studies on HSP70 gene polymorphism were successfully analyzed using the PCR-SSCP method in native chickens, arabic chickens and broiler chickens (Tamzil *et al*., 2013).

Genetic analysis is carried out in the form of molecular markers through the MAS (Marker Assisted Selection) selection program. Molecular is a modern way of livestock selection with faster and more accurate results, one of which is Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Identification of genetic diversity is done through the multiplication of DNA sequences and then cut using enzymes as restriction sites to determine whether or not genetic mutations occur (Viljoen *et al*., 2005). Diversity in the RFLP process has a high level of recognition of nucleotide bases and is suitable for obtaining an overview of genetic populations that code for certain amino acids (Montaldo and Herrera, 1998).

Materials and methods

Time and location of research

This research was carried out at the Animal Biotechnology Laboratory, Faculty of Animal Husbandry, Andalas University, Biotechnology Laboratory, Faculty of Agriculture, Andalas University, and Biomedical Laboratory, Faculty of Medicine, Andalas University, from June to July 2023.

DNA samples and isolation

The material used in this research was blood samples from 45 Pitalah ducks (37 females and 8 males) which were reared intensively at the UPT Faculty of Animal Husbandry, Andalas University, Pauh District, Padang City, West Sumatra. Blood samples were taken from the brachial vein and collected in tubes containing K3EDTA. The DNA isolation method was carried out using the G-spin protocolTM. Total DNA Extraction Mini Kit dari iNtRON Biotechnology.

DNA amplification using the PCR method

Amplification of the HSP70 gene using the PCR (Polymerase Chain Reaction) method with a pair of primers using 0.5-2 µl DNA samples, 2 µl F and R primer mix, 10 µl master mix, and 6-7 µl nuclease free water. Tools used include a set of micro pipettes, gradient mastercycler Eppendorf machine, 10 µl micro tip, PCR tubes. The mixture was amplified using a PCR machine using Master Mix Solution (i-MAX II). Through 35 cycles with stages namely denaturation, annealing and extension. For the denaturation process at a temperature of 94ºC for 1 minute, at a temperature of 55ºC for 1 minute for the initial annealing and extension program at a temperature of 72ºC for 1 minute, and final extension at a temperature of 72ºC for 5 minutes.

Polymorphism detection using the PCR-RFLP method

Genotyping was performed to determine the genotype of the heat shock protein 70 (HSP70) gene part 5′ UTR until early coding sequnces with *Hae*II enzymes and part last coding sequence until 3′ UTR eith HpyAV enzymes. Tools and materials used in HSP70 gene restriction were micropipette, Eppendorf tube, buffer, nuclease free water, microtip, PCR product, *Hae*II enzyme and HpyAV enzyme. The visualization results of enzyme restriction products can be seen through electrophoresis procedures on 2% agarose gel by including markers (bench top). Furthermore, the process was observed using UV transilluminator and documented.

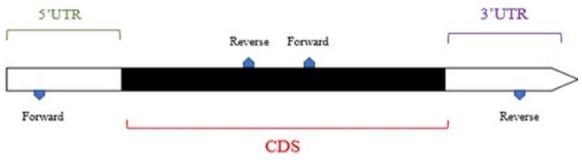

Fig. 1. HSP70 gene structure

Statistical analysis

The results of the data obtained will be analyzed using the following formula:

Genotype frequency obtained from calculations using the formula (Nei and Kumar, 2000)

$x_{ii}=n_{ii}/N$

Description:

x_{ii}= Frequency of the i-th genotype

n_{ii}= Number of individuals with genotype ii

N= Total number of individuals sampled

According to (Nei and Kumar, 2000) the allele frequency can be calculated by the following formula:

$$x_i = \left(2n_{ii} + \sum_{j \neq i} n_{ij}\right)/2n$$

Description:

X_i = i-th allele frequency

X_j = j- th allele frequency

n_{ii} = number of individuals with genotype ii (homozygous)

n_{ij} = number of individuals with genotype ij (heterozygous)

n = number of samples

Heterozygosity can be calculated using the formula of Yeh *et al*. (1999) as follows:

$$H_0 = \sum_{k}^{s} Wk \sum_{i \neq j}^{q} Xkij \;\; ; \;\; H_e = 1 - \sum_{k}^{s} |Wk \sum_{i}^{q} X_{ki}^2$$

Description:

H_o = observed heterozygosity

H_e = expected heterozygosity

W_k = effective population size

Xk_{ij} = frequency of genotype Aij, kth population

Results

DNA samples from Pitalah ducks as many as 45 were successfully isolated by following the procedure on the DNA Extraction Kit (Intron G-Spin TM Total DNA Extraction Kit).

Table 1. HSP70|genotype and allele frequencies *Hae*II on the Pitalah duck.

Genotype	Type	Number of Individuals	Genotype Frequency	Number of Alleles		Allele Frequency	
				+	-	+	-
(+/+)		0	0	0	0		
(+/-)		19	0,42	19	19	0,21	0,79
(-/-)		26	0,58	52	0		
Total		45	1	71	19		

DNA isolation from 45 blood samples of Pitalah ducks (40 females and 5 males) was amplified with a pair of primers forward 5'- CGCTGCT-GATTGGCTAGGA -3' and reverse 5′- CCTCACCGCACGCTTATTG -3′. DNA isolation from 45 blood samples of Pitalah ducks (37 females and 8 males) was amplified with a pair of primers F: 5′-TAACACCACCATTCCCACCA -3′ dan reverse R: 5′ - GCGTGGTTACTTTAGAAGAGACA -3′.

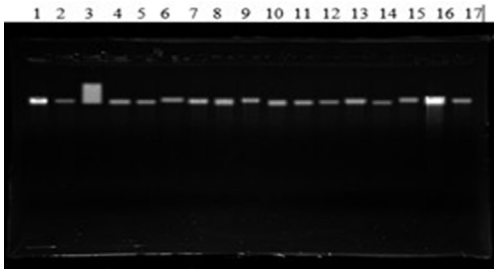

Fig. 2. Results of DNA isolation from Pitalah duck blood samples.

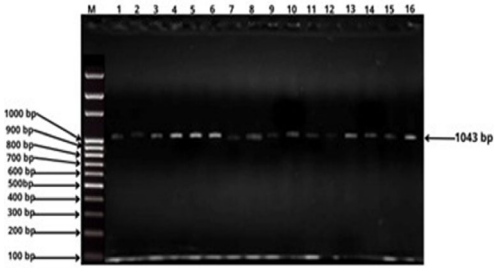

Fig. 3. Results of HSP70 gene amplification in Pitalah duck.
Note: M = marker; 1-16 = number of sample

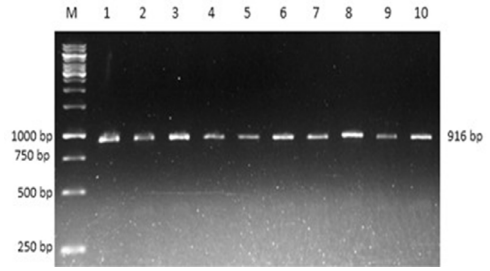

Fig. 4. Results of HSP70 gene amplification in Pitalah ducks
Note: M = marker; 1-10 = number of sample

Verification of HSP70 gene polymorphism was carried out using restriction enzymes *Hae*II of 45 blood samples of Pitalah ducks (40 females and 5 males). The part of DNA that is subjected to a cutting point by a restriction enzyme is called a recognition sequence. On cutting enzymes *Hae*II occurs in RGC↓GC. The result of the restriction obtained is the detection of HSP70|gene polymorphism. *Hae*II because 2 alleles were found, namely + and – with genotypes (+/-) and (-/-). In the results of genotyping the HSP70| gene *Hae*II After electrophoresis was carried out on a 1.5% agarose gel, 3 cut points were obtained with band lengths of 35 bp, 470 bp and 719 bp.

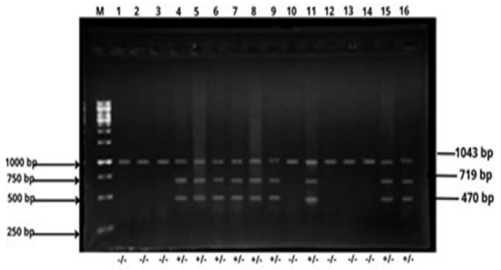

Fig. 5. Results of HSP70|*Hae*II gene restriction in Pitalah ducks
Note: M = marker; 1-10 = number of sample

HSP70|HpyAV gene polymorphism on Pitalah ducks can be detected through PCR-RFLP technique using HpyAV restriction enzyme. The HpyAV enzyme as much as 4 µl was mixed into the PCR product and incubated for 15 minutes at 37°C. The results of the cutting performed by the HpyAV enzyme can be visualized using a UV transilluminator with electrophoresis using 2% agarose.

The results obtained in Table 1. are for the HSP70|*Hae*II Two genotypes were found in Pitalah ducks, namely the homozygous uncut genotype (-/-) and the heterozygous (+/-). The homozygous uncut genotype (-/-) was found to be the highest, namely 0.58, while the heterozygous genotype was 0.42. Meanwhile, the truncated homozygous genotype (+/+) was not found in HSP70|*Hae*II on the Pitalah duck.

Based on the results of the study, the observed heterozygosity HSP70|*Hae*II value was 0.42 and the expected heterozygosity value was 0.33. The observed heterozygosity HSP70|HpyAV value (Ho) was 0.56 and the expected heterozygosity value (He) obtained was lower at 0.44.

Table 2. HSP70|genotype and allele frequencies HpyAV on the Pitalah duck.

Type Genotype	Number of Individuals	Genotype Frequency	Number of Alleles		Allele Frequency	
			+	-	+	-
(+/+)	17	0,378	34	0	-	-
(+/-)	26	0,578	26	26	0,67	0,33
(-/-)	2	0,045	0	4	-	-
Total	45	1	60	30	-	-

Table 3. Chi square test of Hardy-Weinberg equilibrium HSP70|*Hae*II.

Hardy-Weinberg balance	Genotype Frequency			Total	X^2h	$X^2t_{(0,05)}$
	(+/+)	(+/-)	(-/-)			
O	0	19	26	45		
E	1,98	14,93	28,09	45	3,24	5,99
$(O-E)^2/E$	1,98	1,10	0,15	3,24		

Table 4. Chi square test of Hardy-Weinberg equilibrium HSP70|HpyAV.

HW-Balance	Genotype Frequency			Total	X^2h	$X^2t(0,05)$
	(+/+)	(+/-)	(-/-)			
(O)	17	26	2	45	-	-
(E)	20,246	20,07	4,1	45	3,346	5,991
(O-E)2/E	0,52	1,75	1,076	3,346	-	-

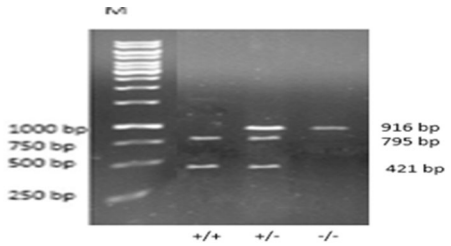

Fig. 6. Results of HSP70|HpyAV gene restriction in Pitalah ducks
Note: M = marker; (+/+; +/-; -/-) = type of genotype

Discussion

Based on Figure 3. and Figure 4, it is explained that the Pitalah duck sample DNA can be specifically amplified. There is only one DNA band in each well which indicates the size is in accordance with the designed primer. According to the opinion of Viljoen et. al. (2005) success in the PCR process is largely determined by the presence of appropriate concentrations such as annealing temperature, primers and DNA concentration.

From the data Table 2, the frequency of homozygous genotypes (+/+), heterozygote (+/-) and homozygote (-/-) in pitalah ducks obtained the highest frequency of heterozygote (+/-) compared to the other two types of genotypes. The frequency of heterozygous (+/-) genotypes obtained was 0.578. For homozygous genotype frequency (+/+) of 0.378 and homozygous (-/-) of 0.045. The results obtained for allele frequency (+) amounted to 0.67 and allele (-) amounted to 0.33.

HSP70 gene diversity in ducks was first reported by Xia et. al. (2013). One of them performed genotyping on S13 and S15 at the SNP point using SacII and HhaI enzymes.The frequency of genotypes that dominate in this study is different from that reported by Subekti *et al*. (2019). HSP70 gene diversity was also reported in native chickens, chickens and chickens by Tamzil *et al*. (2013). The results showed that the highest frequency of genotypes in native chickens was the AD genotype, in chickens represented by the AC genotype and in purebred chickens there was only one genotype, the DD genotype.

The results of the analysis obtained indicate that genetically, the Pitalah duck population studied is polymorphic. This is in accordance with the opinion of Nei and Kumar (2000) which states that a gene can be categorized as polymorphic if one of its alleles has a frequency of more than 1%.

Tambasco *et al*. (2003) stated that a high value of observed heterozygosity (Ho) in the population indicated that outbreeding and random mating had occurred in the rearing process. The results obtained in this study are different from the results of Subekti researched (2019) where the Ho value <He so that inbreeding is indicated. Rell *et al*. (2013) added that some things that can show differences in heterozygosity levels are mutation rates, migration, population size, selection and mating patterns.

Hardy-Weinberg equilibrium is closely related to genotype frequency and allele frequency. To test whether or not the data obtained deviates from the Hardy-Weinberg law, it is necessary to conduct a chi-square test.

Based on Table 3. It is known that there is a Hardy-Weinberg equilibrium where the value of 2count less than X^2 table (0,05) ($X^2h < X^2t(0,05)$) means that the genotype of the research results is not significantly different from the Hardy Weinberg genotype frequency. According to Allendorf and Luikat (2007), the population is said to be in Hardy-Weinberg equilibrium if the value of 2count is smaller than the value of X^2 table. Based on the Hardy-Weinberg equilibrium value, polymorphism in the HSP70|*Hae*II from the 5'UTR to the initial CDS in ribbon ducks it is in Hardy-Weinberg equilibrium.

Based on Table 4. It can be interpreted that the test results are in Hardy-Weinberg balance with the chi square (X^2) method test. This is evidenced by the Pitalah duck population studying has a calculated X^2 value smaller than the X^2 table. The results obtained in the form of the observed genotype frequency effect are not significantly different from the expected genotype frequency.

The population of Pitalah ducks in Hardy-Weinberg equilibrium is thought to be due to random mating and the absence of selection effects. According to Vasconcellos *et al*. (2003) that a population can be said to be in Hardy-Weinberg equilibrium if the frequency of alleles and genotypes is always constant due to the random merging of gametes.

Conclusion

Based on the results of the research that has been done, the conclusion is obtained two types of genotypes part 5'UTR until early coding sequence HSP70 gene with *Hae*II enzymes and three types of genotypes part last coding sequence until 3'UTR HSP70 with HpyAV enzymes on Pitalah ducks. Based on the genotype and allele frequencies obtained, the genetic population of Pitalah ducks was polymorphic and was in Hardy-Weinberg equilibrium. It's an honour to be part of this research and big thank you to Andalas University which plays a role in financing the entire research process that has been carried out.

Acknowledgments

The authors gratefully acknowledge the Research and Community Service Institute of Andalas University for providing funding for this research through the basic research grant in the 2023 fiscal year.

Conflict of interest

The authors have no conflict of interest to declare.

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