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Molecular detection and association of 12.1 kb deletion within the high mobility AT-hook 2 gene in the Netherlands dwarf rabbit (*Oryctolagus Cuniculus*)

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Abstract

Rabbits are mainly bred for human consumption and medical research. However, it has been recently showed that several rabbit breeds are also kept as pets for human leisure. The Netherlands dwarf rabbit is currently in the immense interest of many Vietnamese customers due to its personality and miniature stature. However, 12.1 kb deletion from position 44,709,089 to 44,721,236 bp in the high mobility AT-hook 2 (HMGA2) gene on chromosome 4 was identified as the structural variant causing dwarfism and altered craniofacial development in this breed. It has been documented that HMGA2 plays an important role in regulating growth and individuals with genotype HMGA2 *del/del* are fatal several days after birth. Despite the economically high value of the Netherlands dwarf rabbit, there has been no study on the genetic survey of lethal alleles in this breed in Vietnam. The aim of this study is to develop a fast and reliable method to screen the frequency of lethal alleles of HMGA2 in the South of Vietnam. Rabbit saliva was collected, and DNA extraction was followed. Multiplex polymerase chain reaction (PCR) with three primers was optimized and performed to detect the presence of 12.1 kb deletion within the HMGA2 sequence. Our data showed that the 12.1 kb deletion in the Netherlands dwarf rabbit population was detected by our optimized multiplex PCR. In 100 rabbit animals, 34 and 16 individuals were homozygous wild type (+/+) and homozygous mutant (del/del), respectively, while 50 rabbits were heterozygous. The frequency of HMGA2 lethal allele carrier was 66% (66/100 individuals). Our results indicated that we successfully developed a fast, accurate multiplex PCR to detect carrier individuals. Verification of the genotypes was followed by sequencing. We recommend implementing our multiplex PCR procedure in genetic selection for carrier and homozygous wild-type animals in the mating scheme to prevent the lethality of the rabbit offspring. Additionally, awareness should be raised among rabbit breeders to monitor the genetic makeup of the Netherlands dwarf rabbit populations. However,

List of Abbreviations: DNA, deoxynucleotide acid; HMGA2, high mobility AT-hook 2; LOF, loss of function; PCR, polymerase chain reaction.

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due to the limitation of the sample size, more samples should be taken in future studies to obtain the genetic frequency of the HMGA2 lethal allele more accurately.

KEYWORDS
dwarfism, high mobility AT-hook 2, multiplex PCR, recessive lethal gene

1 | INTRODUCTION

The rabbit (*Oryctolagus cuniculus*) was likely domesticated in France 1500 years ago.[1,2] Traditionally, rabbits were primarily bred for meat production due to their higher calcium and phosphorus content, and lower fat and cholesterol compared to other types of meat.[3] However, since the development of several dwarf breeds including Netherlands dwarf, Holland Hop and long-haired dwarf rabbits, humans began to use them as pets. Dwarf rabbits were first described in 1934[4] and later genetically analysed in 1958.[5] Since then, the selection of dwarf breeds has mainly relied on the dwarf (*dw*) allele (OMIA 000299-9986, https://omia.org/OMIA000299/9986/).

High mobility AT-hook 2 (HMGA2) protein (Gene ID: 103348319) is a member of the non-histone chromosomal high mobility group (HMG) protein family. As one of the essential components in the enhanceosome complex, HMGA2 indirectly regulates gene expression by interacting with other proteins through its structural DNA-binding domains. It has been well-established that HMGA2 plays a crucial role in cancer,[6] body sizes of various species including human,[7,8] mouse,[9] horse,[10] and dog.[11] More importantly, recessive lethal HMGA2 loss of function (LOF) alleles were also reported in humans. HMGA2 LOF could be lethal since intragenic deletion within the HMGA2 sequence has been associated with short stature and homozygotes for the HMGA2 LOF have not been documented due to early fatality before birth.

It was not until 2017 that the Next Gen Sequencing approach enabled researchers to identify the structural variant responsible for the dwarf phenotype in rabbits.[12] Studies suggested that 12.1 kb deletion (position 44,709,089–44,721,236 bp) spanning from the promoter and first three exons of the HMGA2 gene sequence produced a truncated and unfunctional protein. As a result, rabbit individuals with two copies of the mutant allele (*del/del*), also called "peanut", are smaller than their siblings in the same litter, and display an indistinguishably expanded head, and minute ears. Peanut rabbits usually die shortly after birth. On the other hand, heterozygotes (*+/del*), also called "true dwarf", are viable. The size and weight of the "true dwarf" are smaller than that of the wild-type homozygotes (+/+), which are also known as "false dwarf". However, *+/del* animals exhibit small ears, and larger heads due to flat, brachycephalic faces when compared to the rest of the body[12] (Figure 1). This observation was in accordance with another study, asserting that HMGA2 is a major locus in the head development of the animal.[13]

Netherlands dwarf rabbits have widely gained immense popularity among Vietnamese pet breeders because of their personality, neotenic appearance, and plenty of human interaction. Since the market value of one Netherlands dwarf rabbit individual is from 50 to 100 US dollars, the fatality reduction of offspring in one litter by monitoring the mating scheme is of high importance to the rabbit breeders. In this study, we aimed to develop a fast and accurate three-primer multiplex polymerase chain reaction (PCR) to survey the genetic frequency of the recessive lethal allele of HMGA2 in the Netherlands dwarf rabbit population.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All clinical examinations were performed in accordance with the Guidelines for Institutional Laboratory Animal Care and Use of Nippon Veterinary and Life Science University in Vietnam. Sample collection and data recording were conducted strictly according to the Vietnamese law on animal protection and welfare.

2.2 | Animals

Only the animals that fit the description of the Netherlands dwarf rabbits from the previous study[4] were chosen for this study. Of 100 Netherlands dwarf rabbits, 50 were sampled in Ben Tre province, Vietnam, and 50 were sampled in Ho Chi Minh City, Vietnam. Each animal was reared in a single cage under a closed-house system, with an ambient temperature of 28–30°C and relative humidity of 60%–70%. Eighty-four animals were healthy and in their reproductive age and sixteen animals were neonates (Figure 1). The procedure of obtaining saliva samples for DNA extraction was carried out as previously described by Mitsouras et al.[14] and saliva samples were stored at minus 20°C until use.

2.3 | DNA extraction

The genomic DNA was extracted using MagMAX Saliva gDNA Isolation Kit (Thermo Fisher Scientific- Catalog number: A39059) according to the manufacturer's manuals. The integrity of extracted DNA was analysed by electrophoresis. The concentration of the DNA was determined on a Nanodrop 2000 spectrophotometer (Thermo Scientific). Samples with a 260/280 ratio between 1.8 and 2 were diluted to a final concentration of 50 ng/μL and were used for PCR amplification.

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FIGURE 1 Two Netherlands dwarf rabbits in this study. The "false" Netherlands dwarf individual (A) and the "true" Netherlands dwarf individual (B).

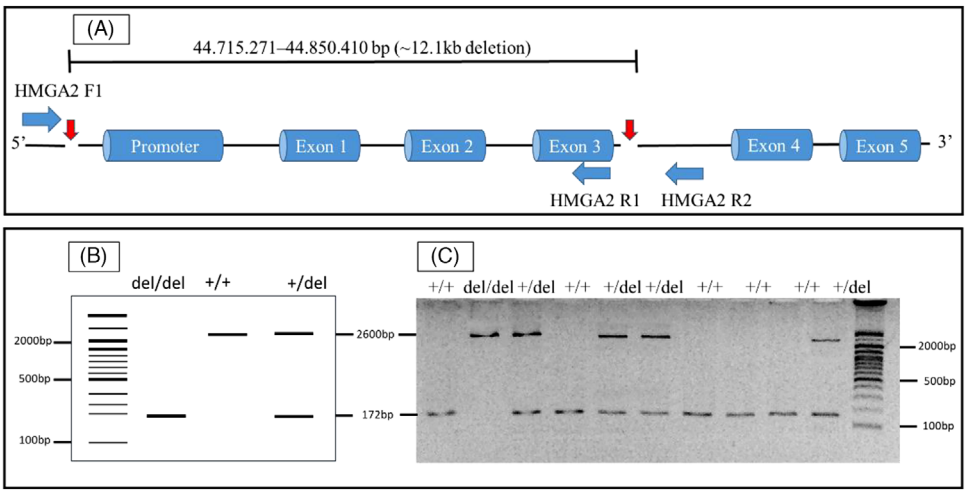

FIGURE 2 In silico design and graphical representation of genotyping high mobility AT-hook 2 (HMGA2) mutation. (A) Polymerase chain reaction (PCR) amplification in detecting 12.1 kb deletion within the HMGA2 genomic sequence. (B and C) In silico representation and gel electrophoresis of PCR amplification of three genotypes (++, +/del, del/del) of HMGA2 in the Netherlands dwarf rabbit. PCR amplification of structural variant of HMGA2 in the Netherlands dwarf rabbits. Agarose gel picture of the three genotypes for the control group, three +/del genotypes, and four wild-type genotypes. (L) 100–4000 bp molecular marker.

2.4 | Genotyping

The primers were designed as shown in Figure 2. The sequences and expected size of the PCR product are shown in Table 1. The HMGA2 F1- HMGA2 R1 combination primers amplify a region of 2600 bp, corresponding to the HMGA2 + allele (wild-type allele). The HMGA2 F1- HMGA2 R2 combination primers amplify a region of 172 bp, corresponding to the *del* allele (mutant allele) (Figure 2). After tests of optimization, three primers multiplex PCR was performed in a 10 µL mixture containing 1 µL DNA (10 ng/µL), 5 µL CoolDry Premix PCR Mastermix (Salagene), 0.4 µL 10 pM for each primer and 3.2 µL ultrapure water. The reaction conditions for Multiplex PCR were as follows: pre-incubation at 94°C for 5 min; 35 cycles including denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 3 min; 1 cycle of final extension at 72°C for 7 min. The PCR reaction was carried out in the thermal cycler Gene Amp PCR System (Thermo Fisher Scientific). After thermal cycling, 5 µL of the amplified products were analysed by horizontal electrophoresis on 1.5% agarose gel. The electrophoresis conditions were 150 V for 30 min using 0.5X Tris/Borate/EDTA solution along with a 100 bp molecular weight marker (Salagene) to determine the size of the amplified fragments. Gel images were captured by GelDoc-It2 (UVP).

TABLE 1 High mobility AT-hook 2 (HMGA2) multiplex polymerase chain reaction (PCR) primer sequences.[12]

Primer	Primer sequence (5′ – 3′)	Tm (°C)	Amplicon (bp)
HMGA2 R1	GGGGTGCCATTAGGAACTTT	57	2600 bp
HMGA2 F1	CAAAGGGAACAAGCCAATGT	65	
HMGA2 R2	GCTCACAGCTCCCTCCTAAA	60	172 bp

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3 | RESULTS AND DISCUSSION

3.1 | Optimization of multiplex PCR method

Because of the difference in conditions of the conventional PCR methods used in two sets of primers, tests of optimization were carried out to determine the final concentrations and T_m of each primer set to get successful PCR amplification. In this study, we showed that the optimal concentration and T_m oligonucleotide were 10 pM/μL and 62°C, respectively. As expected, the wildtype homozygote HMGA2 +/+ and the recessive homozygote *del/del* were observed as a single band of 2600 bp, and a single band of 172 bp, respectively. On the other hand, the heterozygote *+/del* was seen with both bands.

Owing to the need for quick identification of HMGA2 carriers in the studied rabbit populations, we developed a rapid, simple, and cost-effective multiplex PCR to screen the *+/del* individuals. After our optimization procedure, we were able to obtain the clear amplification products for each allele in a single reaction. PCR method was previously documented in detecting large genomic deletion in cattle and pigs.[15,16] For instance, in cows, the multiplex PCR was successfully applied in screening 50 kb deletion within the RSPO2 gene in Holstein Friesian cattle.[17] In pigs, numerous large deletions were discovered and genotyped. However, the conventional PCR method used in this study could not differentiate the deletion carrier and the wild type when the deletion is more than 10 kb.[15] In our study, we developed a multiplex PCR method with three primers that can distinguish 12.1 kb deletion in the HMGA2 gene and provide an efficient genomic screening approach for rabbit breeders to select the appropriate mating scheme. Compared to other time-consuming and laborious methods including whole genome sequencing, real-time PCR and multiplex ligation-dependent probe amplification, multiplex PCR can circumvent these limitations due to its ability to amplify multiple alleles in a single reaction with minimal economic set-up and professional training.

3.2 | Validation of Multiplex PCR amplification

3.2.1 | Frequency of the recessive lethal HMGA2 allele

We performed a screening test by utilizing multiplex PCR on 100 animals. While the 12.1 kb deletion was detected in 66 individuals, 34 animals of the Netherlands dwarf rabbit breed were wild-type homozygote +/+. The frequency of +/del individuals in Netherlands dwarf rabbits was 50%. This observation was in agreement with the previous study which indicated the 12.1 kb deletion was present only in the Netherlands dwarf and the Netherland-originated breeds. Additionally, our study illustrated the frequency of the carrier was lower than that in the study conducted by Carneiro et al. (75%–80%).[12] It is suggested that more samples should be analyzed to obtain a more accurate frequency of HMHA2 carriers in Netherlands dwarf rabbit populations in the South of Vietnam (Table 2).

TABLE 2 Genotype and allele frequency of high mobility AT-hook 2 (HMGA2) polymorphism.

Genotype N = 100 (%)	
++	34 (34%)
+/del	50 (50%)
del/del	16 (16%)
HWE	0.958 Chi-square 0.086 df 2
Alleles	
+	84 (84%)
del	16 (16%)
HWE	$P = 0.256$

Abbreviation: HWE, Hardy-Weinberg Equilibrium.

From our genetic data, we recommend that the mating scheme should be the heterozygotes +/del individual (true dwarf) with homozygous +/+ wild type (false dwarf). The +/+ individual has a genetic background of Netherlands dwarf rabbit and when mating with a *+/del* rabbit, the resulting offspring will not produce the lethal *del/del* animals and more importantly, the *del* allele is still maintained in the "true dwarf" Netherlands dwarf rabbit.

4 | CONCLUSION

In conclusion, this study estimates the frequency of HMGA2 polymorphism in the Netherlands dwarf rabbit population in Vietnam. Our multiplex PCR method not only provides a reliable, cost-effective, and practical tool for HGMA2 carrier detection. We hope that this approach will help rabbit breeders promote a genetic monitoring system for rabbit lethality in Netherlands dwarf rabbit populations.

AUTHOR CONTRIBUTIONS

Tai Duc Nguyen, Lam Van Dang, Phuong Nhu Nguyen Tran and Dai Van Nguyen: sample collection, experimenting and data collection.

Anh Phu Nam Bui: conceptualization, data curation, writing and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw data were generated at the Animal Genetics Laboratory, Faculty of Biotechnology, Ho Chi Minh City Open University, Vietnam. Derived data supporting the findings of this study are available from the corresponding author [APNB] on request.

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