

*Full Length Research Paper*

# Analysis of genetic variation of inducible nitric oxide synthase and natural resistance-associated macrophage protein 1 loci in Malaysian native chickens

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The genetic diversity of 100 Malaysian native chickens was investigated using polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) for two candidate genes: inducible nitric oxide synthase (INOS) and natural resistance-associated macrophage protein 1 (NRAMP1). The two genes were selected because of their important role in chicken's immune system. INOS and NRAMP1 PCR products were digested by *AfuI* and *SacI* restriction enzymes, respectively. The restriction digests produced fragment sizes of 322 and 173 bp for INOS and 722 and 79 bp for NRAMP1 as one allele and an undigested PCR product as the other allele. Both loci were polymorph, however only INOS gene showed Hardy-Weinberg equilibrium. Average heterozygosity and the Shannon information index (I) was 0.43 and 0.62 for INOS and 0.48 and 0.68 for NRAMP1 genes, respectively. The observed polymorphism in this study shows the ability of these candidate genes in marker assisted selection and introgression programs to increase resistance to diseases in both Malaysian native and commercial chickens.

**Key words:** Malaysian native chickens, polymorphism, inducible nitric oxide synthase (INOS), natural resistance-associated macrophage protein 1 (NRAMP1).

## INTRODUCTION

Many studies on the reaction of immune system to bacteria in chicken indicated that resistance to disease has strong genetic components (Bumstead and Barrow, 1988; Kramer et al., 2003). As genetic selection for productivity is performed in highly hygienic environments (Ye

et al., 2006), generally, the improvement of immune system simultaneously with production traits is ignored. A strong negative correlation between antibody production and body weight has been consistently observed across different species (Bayyari et al., 1997; Muir and Aggrey, 2003).

Recent advances in molecular technology offers a new approach to selection for resistance to diseases in chicken (Soller et al., 2006). Two methods, candidate gene analysis and genome-wide linkage disequilibrium scan, can be utilized to identify genetic regions related to resistance to diseases (Kaiser and Lamont, 2002). The candidate gene approach has been successfully used to detect genes responsible for *Salmonella enteritidis* (SE) resistance, such as major histocompatibility complex (MHC), Caspase1, inducible nitric oxide synthase (INOS) and toll-like receptor 4 (TLR4) genes (Weigend and Lamont, 1999; Liu and Lamont, 2003; Malek and Lamont,

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**Abbreviations:** SE, *Salmonella enteritidis*; MHC, major histocompatibility complex; INOS, inducible nitric oxide synthase; TLR4, toll-like receptor 4; SNPs, single nucleotide polymorphisms; TRAIL, tumor necrosis factor related apoptosis inducing ligand; TGF- $\beta$ 2, transforming growth factor; IgL, immunoglobulin G light chain; NO, nitric oxide; NOS, nitric oxide synthase; NRAMP1, natural resistance-associated macrophage protein 1; MAS, marker assisted selection.

**Table 1.** Primer sequences, PCR product size and annealing temperature for identification of polymorphism in INOS and NRAMP1 genes.

| Gene   | Accession Number | Primer Sequence   | PCR product           | Annealing temperature/ Time |
|--------|------------------|---|-----------------------|-----------------------------|
| INOS   | AF537190         | 5' CCAATAAAAGTAGAAGCGA 3'<br>5' CTCTTCCAGGACCTCCA 3'    | 495 bp, genomic       | 50°C/1 min                  |
| NRAMP1 | AY072001         | 5'GGCGTCATCCTGGGCTGCTAT 3'<br>5'AGACCGTTGGCGAAGTCATGC3' | 801 bp, exon 11 to 13 | 60 – 66°C/ 45 s             |

2003, and Malek et al., 2004). Single nucleotide polymorphisms (SNPs) identified for INOS, tumor necrosis factor related apoptosis inducing ligand (TRAIL), transforming growth factor  $\beta$ 2 (TGF- $\beta$ 2), transforming growth factor  $\beta$ 3 (TGF- $\beta$ 3) and immunoglobulin G light chain (IgL) have been utilized to develop polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques for genotyping these candidate genes in broiler chicken (Malek and Lamont, 2003).

Nitric oxide (NO) generated by NO synthase (NOS) has many biological functions. INOS modulate the production of large quantities of NO which can be cytotoxic after instigation by several immunological factors. The infected INOS mutant mice produced a significantly stronger Th1 type of immune response than wild type mice (Wei et al., 1995). Natural resistance-associated macrophage protein 1 (NRAMP1) gene belongs to a large gene family and is located on chicken chromosome 7 that has many pleiotropic effects such as L-arginine flux and INOS synthesis, antigen processing, MHC Class II molecule expression and influence on the intraphagosomal microbial replication by modulating divalent cation content in lysosomes. This supports the hypothesis that polymorphism in NRAMP1 locus can affect its expression or change the structure and performance of the protein encoded (Blackwell, 1996; Liu et al., 2003; Calenge et al., 2010). Association of INOS-*AluI* and NRAMP1-*SacI* allele with spleen SE bacterial burden was observed in previous studies (Malek and Lamont, 2003; Liu et al., 2003).

There is no report on polymorphism of candidate genes related to immune system in Malaysian native chickens. The objective of the present study is to analyze the variation in INOS and NRAMP1 genes in this genetic type.

## MATERIALS AND METHODS

One hundred village chickens from a farm belonging to Malaysia Agricultural Research and Development Institute (MARDI) were randomly chosen for this study. DNA was extracted from blood samples using the QIAGEN DNeasy blood and tissue kit.

Primers were used based on previous studies on INOS and NRAMP1 (Table 1) (Malek and Lamont, 2003; Liu et al., 2003). The PCR reactions were performed in 25  $\mu$ l reaction volumes containing 50 to 100 ng chicken genomic DNA, 0.1  $\mu$ M of each primer, 200  $\mu$ M of each dNTP, 1.5 unit of Taq DNA polymerase (Promega), 5  $\mu$ l of

5X PCR reaction buffer, and 1.5 mM MgCl<sub>2</sub>. The PCR protocol used to amplify the 495 bp fragment of INOS was according to that described by Malek and Lamont (2003). For amplification of the 801 bp fragment from exon 11 to 13 of NRAMP1, a touch down program with an initial denaturation at 95°C for 5 min, followed by 38 cycles of denaturation at 95°C for 45 s, annealing at 66 to 60°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min, was applied.

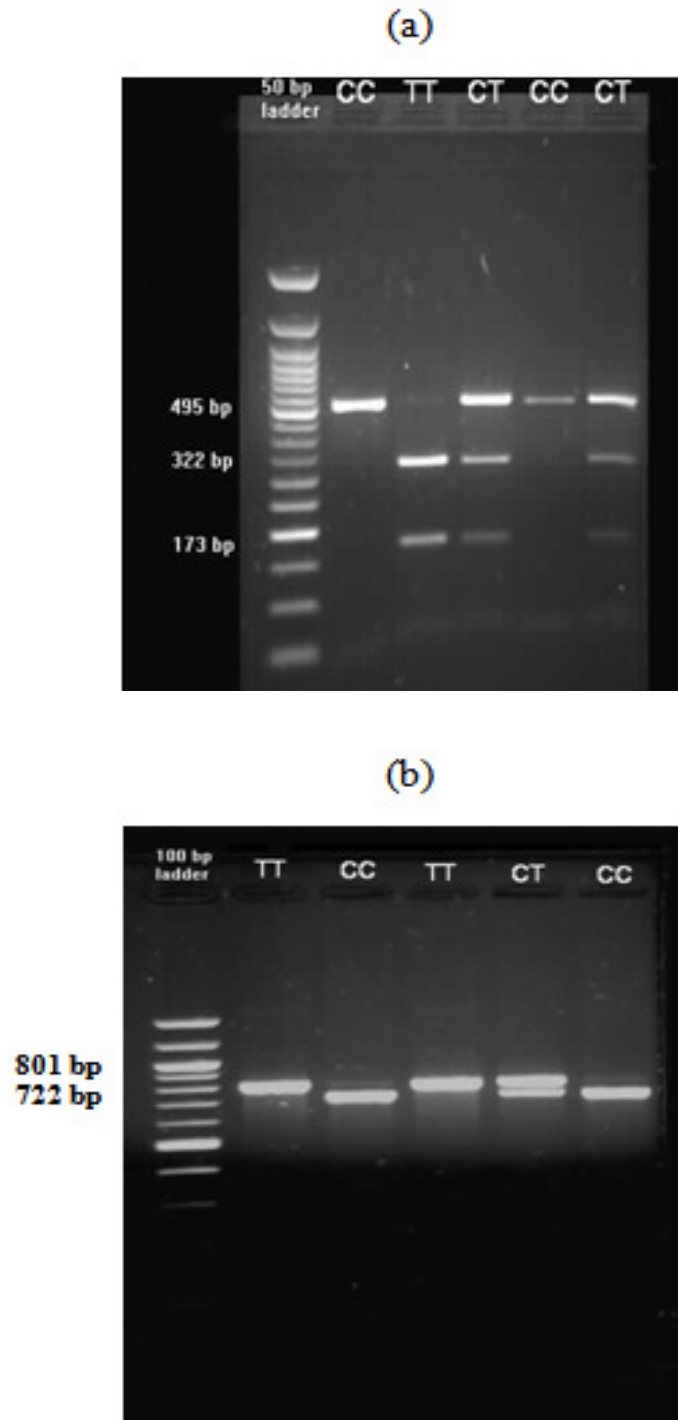
The restriction enzymes, *AluI* and *SacI* (5U), were used to digest INOS and NRAMP1 PCR products, respectively, at 37°C for overnight. Separation of the digested products was performed by electrophoresis through 2.5 and 1.5% agarose gel for INOS and NRAMP1, respectively.

The data were analyzed using POPGENE version 1.31 to estimate expected and observed heterozygosity (Levene, 1949),  $F_{IS}$  (Wright, 1978), average heterozygosity and Hardy-Weinberg equilibrium. The Shannon information index (Lewontin, 1972) was computed to estimate genetic diversity.

## RESULTS AND DISCUSSION

The results showed that the two loci were polymorphic. The observed SNPs were similar to that reported previously by Malek and Lamont (2003) for INOS and Liu et al. (2003) for NRAMP1 genes. Digestion of INOS PCR products by *AluI* produced two fragments of 322 and 173 bp as one allele (T), and the undigested PCR product (495 bp) as the other allele (C). The NRAMP1 *SacI* digested products included two fragments of 722 and 79 bp (invisible in gel) as one allele (C) and the undigested PCR product of 801 bp as the other allele (T) (Figure 1). The digested fragments are due to a T/C substitution at position 173 bp in intronic region for INOS and a C/T substitution in exonic region for NRAMP1.

Observed and expected genotypic and allele frequencies for the two candidate genes are displayed in Table 2. Chi-square test ( $\chi^2$ ) showed that the population followed Hardy-Weinberg equilibrium for INOS ( $p > 0.05$ ) but significant deviation was observed for NRAMP1 ( $p < 0.001$ ). The observed, expected and average heterozygosity and the Shannon information index for INOS and NRAMP1 was 0.51, 0.43, 0.43, 0.62 and 0.68, 0.49, 0.48 and 0.68, respectively (Table 3). There was excess heterozygosity for both loci (negative  $F_{IS}$ ). The measures of the Shannon information index as a diversity index (Weaver and Shannon, 1949) indicated that these two loci can show polymorphism in this population well. Chatterjee et al. (2010) reported a range of 0.25 to 1.5



**Figure 1.** PCR-RFLP electrophoresis pattern of (a) endonuclease *AfuI* digested PCR products from INOS gene with 2.5% agarose gel, and (b) endonuclease *SacI* digested PCR products from NRAMP1 gene with 1.5% agarose gel.

with an average of 1.0 for Shannon information index in their study. This measure in the study of Alatiyat (2010) was 0.58.

MARDI had established the village chicken flock in

2008 with birds from three different farms and these were allowed to mate randomly. The high level of heterozygosity found in this study is expected. However, mutation could make deviation from Hardy-Weinberg equilibrium

**Table 2.** The observed and expected genotypic and allele frequencies of INOS and NRAMP1 in Malaysian native chickens.

| Locus  | Observed (expected) Genotype Frequency |             |             | Allele Frequency |      | $\chi^2$ value for test of HWE |
|--------|--|-------------|-------------|------------------|------|--------------------------------|
|        | CC                                     | CT          | TT          | C                | T    |                                |
| INOS   | 0.06 (0.10)                            | 0.51 (0.43) | 0.43 (0.47) | 0.32             | 0.68 | 3.13 <sup>ns</sup>             |
| NRAMP1 | 0.25 (0.35)                            | 0.68 (0.49) | 0.07 (0.16) | 0.59             | 0.41 | 16.05 <sup>**</sup>            |

ns: Non significant; \*\* :  $p < 0.001$ ,  $\chi^2$ : Chi square, HWE: Hardy-Weinberg equilibrium.

**Table 3.** The observed, expected and average heterozygosity for INOS and NRAMP1 in Malaysian native chickens.

| Locus  | Observed heterozygosity | Expected heterozygosity | Average heterozygosity | I*   | Fis    |
|--------|-------------------------|-------------------------|------------------------|------|--------|
| INOS   | 0.51                    | 0.43                    | 0.43                   | 0.62 | - 0.18 |
| NRAMP1 | 0.68                    | 0.49                    | 0.48                   | 0.68 | - 0.41 |
| Mean   | 0.60                    | 0.46                    | 0.46                   | 0.65 |        |
| St.dev | 0.12                    | 0.04                    | 0.04                   | 0.04 |        |

I\*: Shannon information index.

for NRAMP1 gene. The two candidate genes analyzed in the present study were chosen because of the association with resistance to diseases, especially salmonellosis (Blackwell, 1996; Liu et al., 2003; Malek and Lamont, 2003; Ye et al., 2006).

$F_{is}$  measures the mean deficit or excess of heterozygotes within populations (Wright, 1978). Crossbreeding and random mating are some reasons of excess heterozygosity. The positive  $F_{is}$  reported by Vanhala et al. (1998) and Davila et al. (2009) for Spanish chicken breeds and eight lines of chickens in Finland respectively, showed a little decrease of heterozygosity in these populations. Previous study on polymorphism of twenty microsatellite markers in Italian native chicken by Zanetti et al. (2007) showed deficit of heterozygotes, but  $F_{is}$  was negative for commercial broiler. The probability of polymorphism in native chickens assumed to be higher than commercial chickens because of high selection intensity in the commercials.

As there is negative correlation between productive traits and immune system, susceptibility to diseases is a major threat to the poultry industry. Breeding for resistance to disease in addition to higher production performance is an option to be considered especially since it is cost effective. Marker assisted selection (MAS) has been considered in recent years as an efficient method that does not have the problems of direct selection such as requiring many samples, challenge to disease and increase of generation interval followed by progeny test. Introgression is another approach to increase resistance to disease. All of these methods need information about polymorphism of candidate genes related to immunity. The existence of polymorphism in the two candidate genes in the present study has a remarkable role in selection programs to increase resistance to diseases.

Therefore, further studies need to evaluate the association of INOS and NRAMP1 genes with resistance to diseases in Malaysian native chickens.

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