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MITOCHONDRIAL DNA POLYMORPHISMS AND FERTILITY IN BEEF CATTLE. *(INTERNASIONAL JOURNAL OF THERIOGENOLOGY 57*: 1603-1610, 2002)

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ABSTRACT

Two regions of mitochondrial DNA, D-loop and ND-5 were characterized using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) involving 422 beef cattle of Hereford and composite breeds from Wokalup's research station. ANOVA models (model I, II) were used to estimate associations between molecular haplotypes and quantitative traits. The phenotypic data used were records on calving rate, defined as the mean number of live calves born over four years, while the genotypic data used were the result of PCR-RFLP analysis in both regions of mitochondrial DNA using 7 restriction enzymes. The results of the present study have provided evidence that mitochondrial polymorphisms in the D-loop and ND-5 regions are associated significantly with fertility. This is the first report of a correlation between mitochondrial polymorphism in D-loop and ND-5 on fertility in beef cattle.

Key words: PCR-RFLP, bovine mitochondrial DNA, D-loop, ND-5.

Introduction

There have been many recent attempts to evaluate cytoplasmic genetic effects as a source of variation in quantitative traits related to animal production. In dairy cattle, extensive mtDNA diversity has been found (Freeman, 1990; Hauswirth & Laipis, 1982; Koehler *et al*., 1991; Ron *et al*., 1990), and significant cytoplasmic effects have been reported for milk yield traits (Faust, Robison & McDaniel, 1990; Ron *et al*., 1992; Schutz *et al*., 1992; 1993; 1994). In beef cattle, however, mitochondrial DNA diversity has been less commonly reported and no significant effects on growth traits have been found (Tess & Macneil, 1994; Tess & Robison, 1990). Hiendleder *et al*. (1995a), however, suggested that the lack of evidence for cytoplasmic genetic effects on growth traits in beef cattle could be due to lack of mtDNA variation among the animal studied.

Mitochondrial DNA appears to evolve more rapidly than nuclear DNA in most species (Brown, 1980; Hutchison *et al*., 1974). Even though thousands of copies of the mitochondrial genome are present in each cell (Michaels, Hauswirth & Laipis, 1982), nucleotide substitutions accumulate approximately five to ten times faster than similar mutations in nuclear DNA (Brown, George & Wilson, 1979; Brown *et al*., 1982). Modification in mitochondrial DNA can have profound effects on the phenotype. Studies of a variety of chronic degenerative diseases of humans, involving the brain, heart, muscle and endocrine glands, indicated that the cause of the diseases is mutations in mtDNA (Holt, Harding &

Morgan-Hughes, 1988; Wallace *et al*., 1988). The first pathogenic mtDNA mutations identified were associated with Leber's hereditary optic neuropathy (LHON) disease (Wallace *et al*., 1988), myoclonic epilepsy and ragged-red fiber (MERRF) disease (Shoffner & Wallace, 1990) and the Kearns-Sayre syndrome (Holt *et al*., 1988).

Many studies have suggested that cytoplasmic genetic effects influence growth, reproduction and production traits of livestock (Bell, McDaniel & Robison, 1985; Huizinga *et al*., 1986; Schutz *et al*., 1992; 1994; Tess, Reodecha & Robison, 1987). Maternal inheritance studies have indicated that 2-10% of the variation in milk and fat production in dairy cattle can be explained by maternal effects (Bell *et al*., 1985; Freeman, 1990; Huizinga *et al*., 1986; Ron *et al*., 1990). More direct associations between milk production traits and sequence variation in mitochondrial DNA have also been reported in dairy cattle (Ron *et al*., 1992; Schutz *et al*., 1993; 1994). Schutz *et al*. (1993) found a significant effect on fat percentage of milk of a substitution at base pair (bp) 169 of the D-loop sequence region. More recently, Schutz *et al*. (1994) found that nucleotide substitutions, especially at bp 169 and 16074 of the D-loop, have significant effects on milk, fat and solids-non-fat (SNF) yield, while substitution at bp 16085 of the D-loop has the largest impact on reproduction traits.

Brown (1985) indicated that the D-loop is the most variable region of mtDNA. The sequence variability is observed within species (Aquadro & Greenberg, 1983) and between species (Saccone, Attimonelli & Sbisa, 1987). Furthermore, D-loop variability is also observed within maternal lineages of cattle (Laipis, Van de Walle & Hauswirth, 1988; Olivo *et al*., 1983). Lindberg (1989) found 51 sequence differences, generally due to a single bp substitution, in 36 distinct registered maternal lineages.

The ND-5 region of mtDNA is one of the 7 subunits of the NADH-dehydrogenase complex (Anderson *et al*., 1982; Cantatore & Saccone, 1987; Smith & Alcivar, 1993) involved in oxidative phosphorylation. PCR-RFLP analyses in the ND-5 region have shown variability (Suzuki, Kemp & Teale, 1993). Recent studies indicate that mitochondrial genes which contribute subunits to the enzymes involved in respiratory-chain activities could influence growth in lambs via mitochondrial respiratory metabolism (Hiendleder *et al*., 1995a; Hiendleder, Herrmann & Wassmuth, 1995b).

The aim of this study was to evaluate the effects of mitochondrial D-loop and ND-5 polymorphisms on fertility trait in beef cattle.

Experimental animals

A total of 422 of purebred Hereford and composite breed (comprising approximately 1/4 Brahman, Charolais and Friesian, and 1/8 Angus and Hereford) cattle were used for the study. They were part of a selection experiment described in detail by Meyer *et al*. (1993) and maintained at Agriculture Western Australia's Wokalup Research Station.

Phenotypic Data

The trait used in association analyses was a calving rate (defined as the mean number of live calves born over four years).

Extraction of mitochondrial DNA

Mitochondrial DNA was extracted from white blood cells using the Wizard Minipreps DNA Purification System (Promega, Madison, USA). Mitochondrial pellets were prepared according to published methods (Welter *et al*., l989). 250µl white blood cells were homogenized in a clean microcentrifuge tube containing 1ml cold homogenization buffer (100mM Tris-HCl, pH 7.4; 250mM sucrose; 10mM EDTA). Nuclei and cellular debris were removed by centrifugation at 1500g for 10 minutes at 4°C. The supernatant was transferred to a clean microcentrifuge tube and a crude mitochondrial pellet was prepared by centrifugation in a microcentrifuge at 11,000g for 20 minutes at 4°C. The mitochondrial pellet was resuspended in 1ml TE buffer (10mM Tris HCl pH 7.5, 1mM EDTA), placed on ice for 10 minutes and repelleted at 11,000g for 20 minutes at 4°C. MtDNA was then purified from the pellet using the Wizard Miniprep protocol.

PCR-RFLP

All PCR amplification reactions were performed in an Omnigene thermocycler machine. The reactions were performed in a 50 ml reaction mix consisting of 200 ng of template DNA, 0.15 µM each of the oligonucleotide primers, 200 µM each dNTPs, 2 mM $MgCl_2$, 10x buffer and 1.5 units Taq DNA polymerase (Biotech, Australia) in 0.6µl PCR reaction tube.

PCR products were used directly in the restriction endonuclease digestion. A master mix of each restriction enzyme, its buffer and water was made, and then aliquoted into each tube containing 7 µl of PCR products of the GH gene or 5 µl of amplified mtDNA fragments, and incubated as directed by the manufacturer. BSA at a final concentration of 100µg/ml was used for many enzymes as directed by the manufacturer.

Agarose gel electrophoresis was carried out using 1-2% of agarose (Promega) in TAE buffer (40mM Tris-HCl; 20mM Acetate; 2mM EDTA, pH adjusted to 7.9). Electrophoresis was performed using horizontal gels, in electrophoretic cells (Bio-Rad, Richmond, U.S.A). Ethidium bromide was included in the gel at a final concentration of 0.5µg/ml (Sambrook *et al*., 1989). After electrophoresis, DNA was visualized under UV-illumination and photographed using Polaroid type 57 film with a red filter.

Comparisons Between Selected and Control Lines

Genetic diversity, estimated by Nei's gene diversity or expected heterozygosity (Nei, 1978), was compared between selected and control

lines of each breed, using the method of Archie (Archie, 1985). Allelic frequencies for each locus were compared using X^2.

Association Analyses

Associations between molecular haplotypes and quantitative traits were estimated from 2 ANOVA models:

Model I: $Y_{ijklmn} = \mu + B_i + S_j + L_k + D_l + Y_m + G_n + e_{ijklmn}$

Where μ is the least square mean value, B_i is the effect of breed, S_j is the effect of sex, L_k is the effect of line (selected or control), D_l is the effect of the age of the individuals dam, Y_m is the effect of year of birth, G_n is the effect of haplotype and e_{ijklmn} is the residual error.

Model II: $Y_{ijklmno} = \mu + B_i + S_j + L_k + D_l + Y_m + G_n + A_{ijklmno} + e_{ijklmno}$

Where μ is the least square mean value, B_i is the effect of breed, S_j is the effect of sex, L_k is the effect of line (selected or control), D_l is the effect of the age of the individuals dam, Y_m is the effect of year of birth, G_n is the effect of haplotype, $A_{ijklmno}$ is a random animal effect, determined from the additive relationship matrix, and $e_{ijklmno}$ is the residual error.

Differences in least square means between haplotype classes were determined by the F-test, with a comparison error rate of 0.05. Model I was implemented using the program JMP (SAS, 1989) and model II using PEST (Groeneveld, 1990; Groeneveld & Kovac, 1990). The major differences between the models was the addition of the relationship matrix in model II, which eliminates confounding between the effects of marker and non-marker genes among relatives. Preliminary analyses using the models identified no significant interaction effects between haplotype and other factors, and interactions were therefore not included in final analyses.

Results

Products resulting from amplification of the mitochondrial D-loop (1142bp) by PCR using primers D-L and D-R, and the ND-5 (453bp) region, using primers ND-L and ND-R, are shown in Figure 1 and 2. Figure 3 shown an example of PCR-RFLP using HindIII.

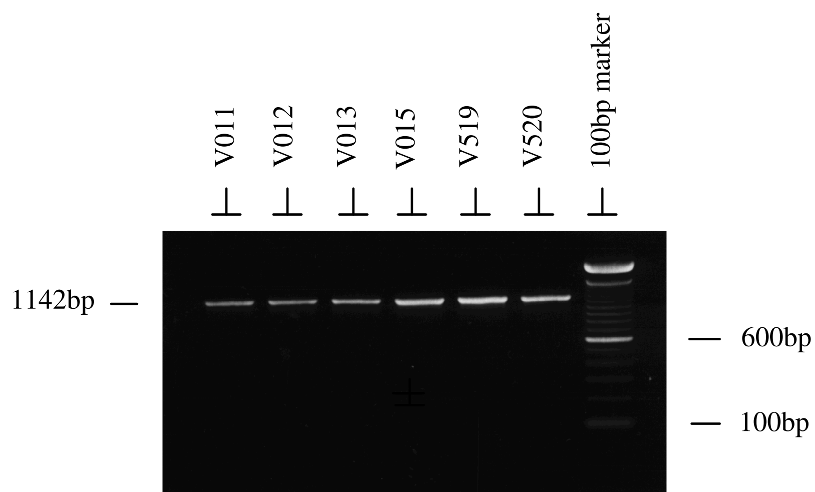

Figure 1. Photograph of an ethidium bromide stained agarose gel showing the specificity of the PCR products (1142 bp) representing the whole mitochondrial D-loop and flanking sequence at both ends amplified using primers D-L and D-R.

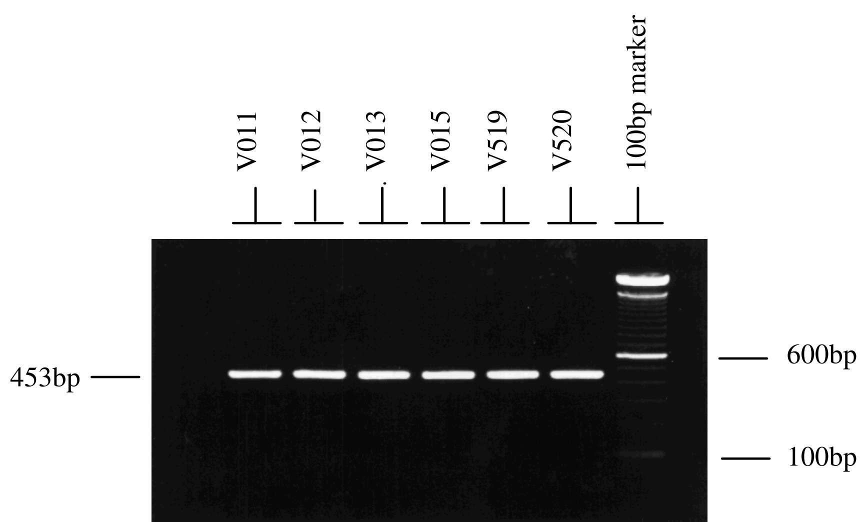

Figure 4.5. Photograph of an ethidium bromide stained agarose gel showing the specificity of the PCR products (453 bp) of mitochondrial ND-5 between positions 12058 and 12510, amplified using primers ND-L and ND-R.

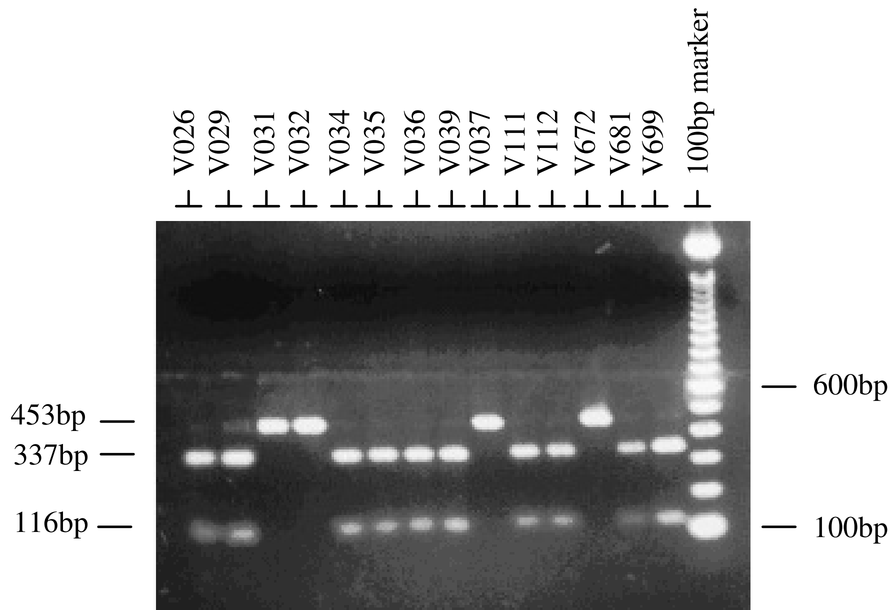

Figure 3. Gel photographs of an ethidium bromide stained agarose gel showing mitochondrial ND-5 polymorphism detected by PCR-RFLP using HindIII.

Comparison of Selected and Control lines

Comparisons of the genetic diversity between selected and control groups of composite and Hereford cattle are shown in Table 1. Table 2 shows the comparison of the allelic frequencies of selected and control groups between composite and Hereford cattle. There were no significant differences between groups at any locus.

Table 1. Comparisons of the genetic diversity of selected and control groups of composite and Hereford breeds. d is nucleotide diversity (average number of nucleotide substitutions per site within breeds).

Breeds	Groups	N	d (Nei and Li)
Composite	Selected	177	0.038908 ± 0.015761
	Control	58	0.049068 ± 0.020182
Hereford	Selected	138	0.055031 ± 0.028593
	Control	56	0.054549 ± 0.028048

Table 2. Comparisons of the allelic frequency of selected and control groups between composite and Hereford breeds based on based on PCR-RFLP analysis in the mitochondrial D-loop and ND-5.

Breeds	Groups	D-loop										ND-5			
		TaqI		PstI		SspI		ApaI		AvaII		HindIII		SpeI	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B
Composite	selected	0.69	0.31	0.69	0.31	0.69	0.31	0.99	0.01	1.00	0.00	0.69	0.31	0.69	0.31
	control	0.58	0.42	0.58	0.42	0.58	0.42	1.00	0.00	1.00	0.00	0.58	0.42	0.58	0.42
Hereford	selected	0.59	0.41	0.59	0.41	0.59	0.41	1.00	0.00	0.89	0.11	0.59	0.41	0.59	0.41
	control	0.63	0.37	0.63	0.37	0.63	0.37	1.00	0.00	0.92	0.08	0.63	0.37	0.63	0.37

Effect of D-loop Polymorphisms

Table 3 shows the least square mean values of each haplotype in the two D-loop polymorphisms for 4-year calving rate. Table 4 shows the probability of observing the differences in means between haplotypes under each ANOVA model. Differences in calving rate are significant under model I for the TaqI / PstI/ SspI polymorphism and almost significant under model II for both polymorphisms.

Table 3. Least square mean ± S.E. of each haplotype in the two D-loop polymorphisms for calving rate (CR) in Hereford and composite cattle with different haplotypes.

Traits	TaqI / PstI / SspI		AvaII	
	A	B	A	B
Calving rate (CR)	0.70 ± 0.04	0.61 ± 0.05	0.66 ± 0.04	0.89 ± 0.16

Table 4. Probability of observing the differences in means between haplotypes under each ANOVA model for calving rate (CR).

Trait	TaqI/PstI/SspI		AvaII	
	Model I	Model II	Model I	Model II
Calving Rate (CR)	0.04	0.06	0.16	0.08

Effect of ND-5 Polymorphisms

Table 5 shows the least square mean values of each haplotype in the ND-5

polymorphisms for 4-year calving rate. Table 6 shows the probability of observing the differences in means between haplotypes under each ANOVA model. Differences in calving rate are significant under model I and almost significant under model II.

Table 5. Least square mean ± S.E. of each haplotype in ND-5 polymorphism for calving rate (CR) in Hereford and composite cattle with different haplotypes.

Traits	HindIII/ SpeI	
	A	B
Calving Rate (CR)	0.70 ± 0.04	0.61 ± 0.05

Table 6 Probability of observing the differences in means between genotypes under each ANOVA model. CR = calving rate.

Trait	HindIII/ SpeI	
	Model I	Model II
Calving Rate (CR)	0.04	0.06

Discussion and conclusion

The analyses of the total data from both breeds of beef cattle in this study indicate that the female fertility is only trait (other traits nor shown) affected by variation in mitochondrial DNA, as measured by the mean number of calves born over a four year period. This trait is significantly affected by the TaqI/ PstI/ SspI polymorphisms in the D-loop region and the HindIII/ SpeI polymorphism in ND-5. The effect was present in both breeds and in both selected and control lines, although the strongest effect was found in Hereford selected animals (Table 7 & 8). This study failed to show any effect of mitochondrial polymorphism on any growth or milk production traits. Selection for increased daily gain in each breed had no influence on mtDNA diversity or on allelic frequencies at any of the polymorphic restriction sites.

Table 7. Least square mean ± S.E. of calving rate in each haplotype of selected and control lines of Hereford and composite cattle.

Breed	Group	Least Square Mean			
		TaqI/ PstI/ SspI		HindIII/SpeI	
		A	B	A	B
Composite	Selected	0.58 ± 0.07	0.42 ± 0.08	0.58 ± 0.07	0.42 ± 0.08

	Control	0.83 ± 0.08	0.81 ± 0.09	0.83 ± 0.08	0.81 ± 0.09
Hereford	Selected	0.73 ± 0.07	0.48 ± 0.07	0.73 ± 0.07	0.48 ± 0.07
	Control	0.76 ± 0.05	0.68 ± 0.07	0.76 ± 0.05	0.68 ± 0.07

Table 8. Probability of observing the differences in mean calving rate between genotypes under each ANOVA model.

Breed	Group	Probability	
		TaqI/ PstI/ SspI	HindIII/ SpeI
Composite	Selected	0.09	0.09
	Control	0.85	0.85
Hereford	Selected	0.01	0.01
	Control	0.24	0.24

In dairy cattle, the effect of cytoplasmic inheritance on measures of production and reproduction have been demonstrated (Bell *et al*., 1985; Huizinga *et al*., 1986; Schutz *et al*., 1992). Recent studies conducted by Schutz *et al.* (1994) found that nucleotide substitutions in D-loop region have significant effects on milk, fat and solids-non-fat (SNF) yield and reproduction traits in dairy cattle. However, studies using simulated data (Kennedy, 1986; Southwood *et al*., 1989), and other studies using the same database but different approaches, have given contradictory results (Kirkpatrick & Dentine, 1989; Reed & Van Vleck, 1987).

In beef cattle, very view studies have evaluated the effects of cytoplasmic inheritance on production traits. Tess *et al*. (1987) reported cytoplasmic genetic effects on preweaning growth in two herds of Hereford cattle, and suggested that cytoplasmic effects were mediated through milk production. The results were further evaluated by Tess and Robison (1990) and Tess and MacNeil (1994) using a more statistically valid mixed model analysis, and the results failed to show that cytoplasmic genetic effects were important sources of variation for growth traits in beef cattle. Northcutt *et al*. (1991) also reported that preweaning performance was not affected by cytoplasmic variance.

The effects of D-loop and ND-5 polymorphisms on female fertility may be direct or due to linkage with other mitochondrial genes. Bell *et al*. (1985) demonstrated small effects of maternal lineages on days open and pointed out the role of mitochondria in the biosynthesis of steroids. The significant correlation may be due to mitochondrial effects in relation to energy needs on oocyte maturation, since elevated concentrations of adenosine triphosphate (ATP) for localized activities in the ooplasm has been suggested (Van Blerkom & Runner, 1984). In cattle, mitochondria are increased proportionally to the increase in cytoplasmic volume, at which stage oocytes require a fixed amount of mitochondria per unit volume of cytoplasm to remain viable (Smith & Alcivar, 1993). Mitochondrial DNA copy number appears to correlate with oocyte volume

since the amount of mtDNA per cell increases from about 0.1 pg in primordial cells to 4.5 pg in the preovulatory oocyte (Hauswirth & Laipis, 1985), and this distribution of mitochondria in the bovine oocyte has been suggested to be correlated with the hormonal patterns of both gonadotrophins and steroids (Hyttel, Callensen & Greve, 1986; Kruip *et al*., 1983). The D-loop is the site of transcriptional and replicational control (Anderson *et al*., 1982). Schutz *et al.* (1994) suggested that differences in production associated with sequence polymorphism in the D-loop region of mtDNA may relate to the control of mtDNA function. Recent studies have also suggested that D-loop polymorphisms may serve as indirect markers for differences elsewhere on the mtDNA genome in coding regions of genes directly affecting phenotypic expression of traits (Schutz *et al*., 1993; 1994).

In conclusion, the results of the present study have provided evidence that mitochondrial polymorphisms in the D-loop and ND-5 regions are associated significantly with fertility. This is the first report of a correlation between mitochondrial polymorphism in D-loop and ND-5 on fertility in beef cattle. Fertility is a lowly heritable trait and therefore difficult to improve through traditional phenotypic selection. The presence of a DNA marker may enable the rate of genetic improvement in fertility to be greatly increased.

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