ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF POULTRY SCIENCE

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International Journal of Poultry Science 4 (3): 133-137, 2005
ISSN 1682-8356
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Identification Of AFLP Markers Associated With Round Heart Syndrome In Turkeys

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Abstract: The Amplified Fragment Length Polymorphism (AFLP) method was used to identify 38 genetic markers that associate with either phenotypically normal or round heart turkeys, diagnosed by gross necropsy, from an inbred flock of Nicholas broad-breasted birds. Sixty-five polymorphisms were identified and analyzed by chi-square. A p-value of less than 0.05 determined markers that were kept and sequenced. Thirty-eight markers were identified, 19 that associated with the normal phenotype and 19 that associated with the round heart syndrome. The majority of the markers had a high A/T content, suggesting possible regulatory domains in which polymorphisms were occurring. Sequence Characterized Amplified Region (SCAR) primers were designed from marker sequences with the hopes of designing a simple Marker Assisted Selection (MAS) strategy. Work is ongoing to optimize these primers for SCAR analysis. With the development of a simple screening test, round heart may be greatly reduced or eliminated in commercial flocks.

Key words: Turkey, round heart, AFLP, SCAR, marker assisted selection

Introduction

Round heart syndrome (RHS), a spontaneous cardiomyopathy in avian species, was identified in turkeys (Meleagris gallopavo) in 1962. It is characterized by dilatation of the ventricles and hypertrophy of the cardiac muscles (Magwood and Bray, 1962; Jankus *et al.*, 1973). This syndrome is a widespread problem resulting in the loss of approximately $200,000 annually for just one commercial turkey producer (Olsen, 2005, personal communication). Many environmental factors have been implicated in contributing to this syndrome including age, stress, diet, and altitude (Czarnecki, 1984). Cardiomyopathy can also be induced in turkeys using alcohol and drugs such as furazolidone (Czarnecki, 1984). Though these factors play a role in the presence of round heart, they do not appear to be independent. There also appears to be a genetic component of this condition (Czarnecki, 1984; Genao *et al.*, 1996). The majority of the work done regarding round heart has involved characterizing the morphology and physiology of the syndrome. With the exception of breeding trials suggesting a familial inheritance of round heart in turkeys, little has been done to date to investigate the genetic basis of the syndrome (Jankus *et al.*, 1973).

Amplified fragment length polymorphism: Amplified Fragment-Length Polymorphism (AFLP) technology has been used to characterize polymorphisms of genomic DNA. The AFLP method was first described by Vos *et al.* (1995) and combines the strength of Restriction Fragment Length Polymorphisms (RFLP) with the sensitivity of Polymerase Chain Reaction (PCR). This technique enables the identification of rare polymorphisms that are too few in number for visualization by RFLP. AFLP technology has been successful in identifying genetic markers in various plant and animal species: including wheat (Barrett *et al.*, 1998); barley (Becker *et al.*, 1995); potato (Meksem *et al.*, 1995); and rats (Otsen *et al.*, 1996). It has also been useful in differentiating between highly related bacterial strains such as *Escherichia coli* (Lin *et al.*, 1996); *Agrobacterium tumefaciens* (Lin *et al.*, 1996); and varying *Xanthomonas* strains (Janssen *et al.*, 1996). In this project AFLP technology was used to characterize polymorphisms unique to either the normal or round heart phenotype in Nicholas broad-breasted turkeys.

Materials and Methods

DNA samples: DNA for 10 phenotypically normal and 10 round heart birds was obtained from an inbred flock of turkeys developed at the University of Minnesota (Staley *et al.*, 1981; Pierpont *et al.*, 1985). This strain exhibited a 70% incidence of round heart syndrome. Samples were taken from each individual of like phenotype and combined to form a bulk sample.

AFLP analysis: Genomic DNA (300 ng) was digested using 10 units each of EcoRI and HinP1I in a total volume of 15 µL at 37°C for 2 hours. The utility of these

restriction enzymes was previously demonstrated by Knorr *et al.*, (1999) in their work with genome mapping of the chicken (1999). AFLP primers and protocols were patterned after theirs.
Specific primers were obtained (Oligos etc., San Diego, CA) that, when annealed together, made double stranded adapters. The EcoRI-adapter (CTC GTA GAC TGC GTA CC plus AAT TGG TAC GCA GTC TAC) was added to a concentration of 0.1 µM, and the HinP1I-adapter (GAC GAT GAG TCC TGA G plus CGC TCA GGA CTC AT) was added to 1 µM. In a volume of 25 µL, these adapters were ligated to the fragments in the presence ligase buffer (0.5x) supplemented with ATP, and 1 Unit of T4 DNA ligase (Life Technologies, Gaithersburg, MD) at 15°C overnight. The reaction was diluted to a final volume of 200 µL with TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).
Six microliters of the digestion/ligation reaction was used for the initial amplification. The EcoRI-primer (5' GAC TGC GTA CCA ATT CA 3') and HinP1I-primer (5' GAT GAG TCC TGA TCG CA 3') were added to a final concentration of 5 µM each. The reaction mix was completed with dNTPs added to 200 µM each, PCR buffer including $MgCl_2$ to 1x and 1.5 units of Taq polymerase in a total volume of 20 µL. The resulting product was diluted with TE buffer to 200 µL; 5 µL was used for selective amplification.
In a total volume of 20 µL, the selective EcoRI-primer (5' GAC TGC GTA CCA ATT CA-WX 3') was added to 1 µM final concentration, the selective HinP1I-primer (5' GAT GAG TCC TGA TCG CA-YZ 3') was added to 5 µM. The remaining reagents were added in the same amounts and concentrations as the initial amplification reaction. The selective amplification used the profile: 94°C -30s, 66°C -30s decreased 2 C° every other cycle until annealing temperature reached 56°C, and 72°C -60s. Once the annealing temperature reached 56°C, 25 additional cycles were performed, for a total of 35 cycles, followed by a five-minute final extension at 72°C.
An equivalent volume of stop solution (98% Formamide dye, EDTA, 0.5% bromophenol blue, 0.5% Xylene Cyanol) was added to the reaction mix, The reaction was denatured at 90°C for 3 minutes and then immediately placed on ice. The product (6 µL) was resolved on a 6% polyacrylamide gel. The gel was mounted on filter paper and dried, and transferred to X-ray film. The film was stored at -80° C for two to three days and developed for analysis.

Statistical analysis of AFLP markers: Chi square analysis was used to determine if an amplified band observed in gel electrophoresis segregated significantly with a particular phenotype. The analysis compared the observed number of bands to the expected number of bands for a segregating marker.

Marker isolation and sequencing: The polymorphic bands, designated as markers by chi-square analysis, were cut out of the gel. The fragments were crushed, soaked in water for 20 minutes, then boiled in sealed microcentrifuge tubes for 15 minutes. The tubes were centrifuged at 14000xg for two minutes to collect the debris and the supernatant was transferred to a new tube. DNA was precipitated at -80°C for 30 minutes with 10 µL 3M Sodium Acetate, 5µL glycogen (10 mg/mL) and 450 µL ice-cold 100% ethanol. The DNA was pelleted by centrifugation at 10,000 X g, 4°C for 10 minutes. The supernatant was removed and the pellet washed with ice-cold 85% ethanol prior to resuspension in 20 µL of water.
Reamplification of the marker was carried out using the respective selective primers and 8 µL of the marker template in a total volume of 40 µL. The resulting product was ligated into the pCR 2.1 plasmid and transformed into INVαF' competent cells according to the manufacturer's protocol (Invitrogen, San Diego, CA). The cells were grown up overnight on agar plates containing ampicillin and BluoGal (Sigma, St. Louis, MO). White colonies were selected and the bacteria were cultured overnight in LB broth (Life Technologies, Gaithersburg, MD) containing ampicillin (Sigma, St. Louis, MO). Plasmid DNA was isolated using the boiling lysis method (adapted from Sambrook *et al.*, 1989). The DNA was pelleted and resuspended in water after removal of the supernatant. PCR was performed on the plasmid using M13 forward and reverse primers. These primers anneal at complementary sites approximately 100 bp on either side of the insertion site. The resulting PCR product was then sequenced for analysis.

Sequence characterized amplified regions: By locating the AFLP primer sequences in the fragments amplified from the plasmid we identified the polymorphic marker sequences. Sequence Characterized Amplified Region (SCAR) primers were designed, using the sequence for the endonuclease recognition site plus the 15-20 base pairs immediately following the AFLP primer sequences. Primers were obtained (Oligos etc., San Diego, CA) for 10 of the sequenced bands, and used to screen individual samples for the unique polymorphism. PCR conditions varied for individual SCARs depending on characteristics of the respective primers.

Results

We analyzed 19 primer combinations. Sixty-five polymorphic bands were identified and chi-square analyses were carried out on these fragments. Thirty-eight segregating bands with a p-value of less than 0.05 were identified as trait associated markers and isolated for sequencing. A number of markers had individual p-values of less than 0.01. From these primer sets we identified 19 markers associated with phenotypically normal and 19 associated with round heart birds diagnosed by gross necropsy.

Table 1: Of the thirty eight markers identified by AFLP analysis in this study, 19 segregate with the round heart phenotype. Primer combinations used to identify the individual markers are listed. For marker that were sequenced, marker length and A/T content is also listed

Polymorphic Marker	Selective Primer Sets		Sequence Obtained	Length%	A/T Content
	EcoRI	HinP1I			
Nm01	ACC	ACT	x	145	61.8
Nm02	ACC	ATC	x	109	48.6
Nm03	ATA	AGA	x	57	52.6
Nm04	ACC	AGC			
Nm06	ATA	ATG	x	240	70.0
Nm07	ATA	ATC	x	260	51.2
Nm08	ATA	AAG	x	134	64.2
Nm09	ATA	AAG	x	118	54.2
Nm10	ATA	ATC	x	273	59.3
Nm11	ATA	ATC	x	222	57.7
Nm12	ACC	ACA	x	180	64.2
Nm13	ACC	AGC	x	205	56.6
Nm14	ACC	AGC	x	204	46.4
Nm16	AGC	ATG	x	62	51.6
Nm17	AGC	ATC	x	109	49.6
Nm18	AAG	AAC			
Nm19	AAG	AAC	x	74	44.6
Nm20	AAG	AGC	x	94	63.8
Nm22	ACC	AAG	x	208	67.8
Rm01	ACC	AGC			
Rm02	ACC	AGC	x	57	70.2
Rm03	ACC	AGC	x	83	66.3
Rm04	ATA	ATG			
Rm05	ATA	ATG	x	218	70.1
Rm06	ATA	ATG	x	122	62.3
Rm07	ATA	ATG	x	58	44.8
Rm08	ATA	ATG	x	55	54.5
Rm09	ACC	ACA	x	301	64.4
Rm10	AGC	ATG			
Rm11	AAG	AAC	x	182	62.6
Rm12	AAG	AAC			
Rm13	AAG	AGC	x	217	43.3
Rm14	ATA	ATC	x	149	69.8
Rm15	ACC	ATG	x	301	62.6
Rm16	ACC	ATG	x	534	57.1
Rm17	ACC	ATG			
Rm18	ATA	AAG	x	52	46.2
Rm19	ATA	AAG	x	241	61.8

Sequences were obtained for 31 markers, 17 normal markers and 14 round heart markers. These fragments are relatively small compared to previously identified RAPD markers, which range from 700-1200 bp in size (unpublished data). Identified AFLP markers range in size up from 52-534 bp in length. The majority of these markers are between 100-300 bases in length (see Table 1).

Discussion

Round heart syndrome in turkeys is a significant economic problem for commercial turkey producers. Various environmental factors thought to be involved have been investigated, and refined to help control the disorder. Though genetic influences have been previously speculated, they have not been thoroughly investigated. Identification of genetic markers associated with both normal and round heart birds is significant because it further supports a genetic link to the syndrome. This link may pave the way to greater control of this syndrome in breeding flocks.

A commercial turkey producer located in south central

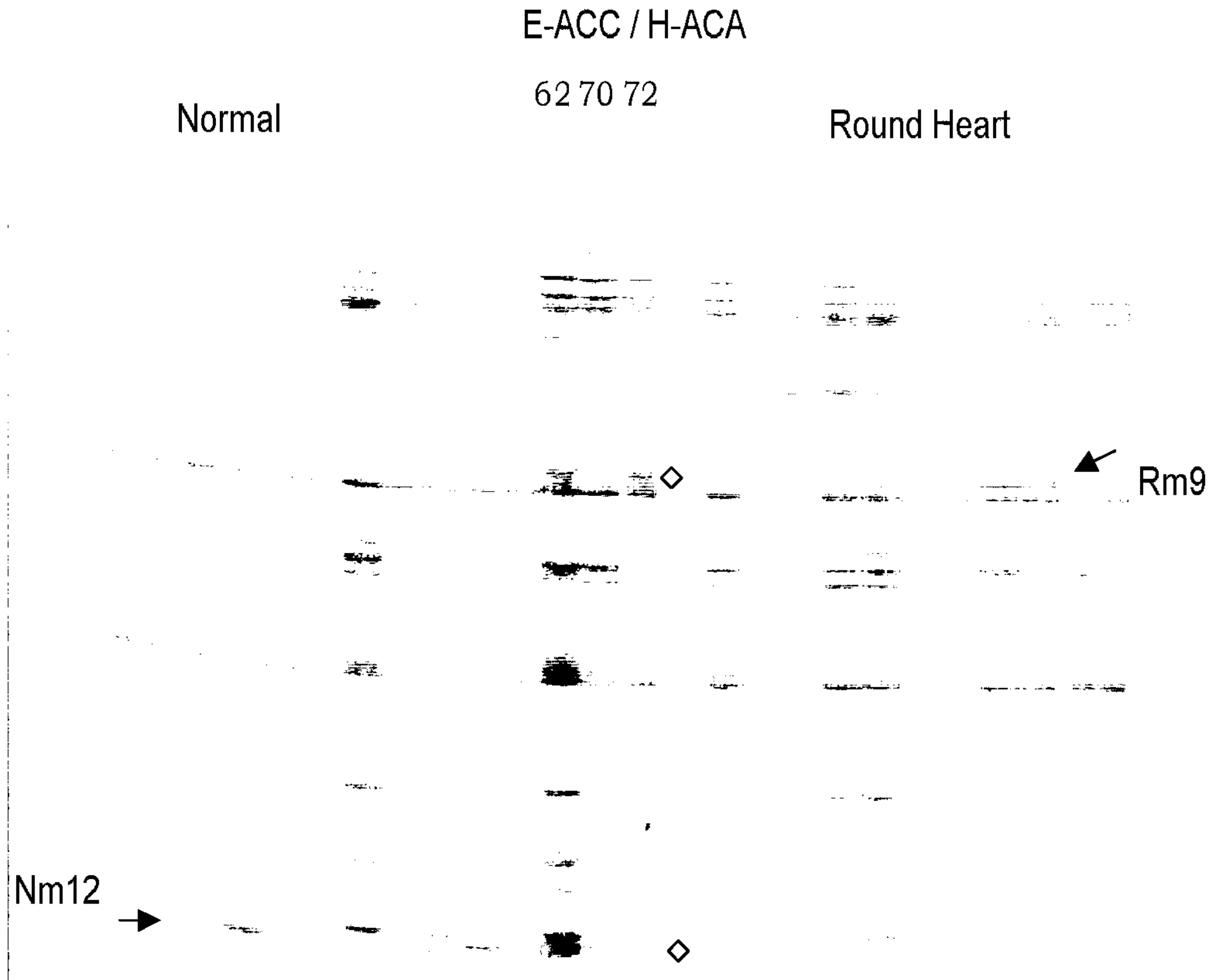

Fig. 1: Markers Nm12 and Rm9 are visualized from the E-ACC / H-ACA primer combination. E-AWX, and H-AYZ refer to the selective primers which anneal to the adapters ligated to the ends cut by EcoRI and HinP1I respectively. Three toms with unknown heart condition (62, 70, and 72) represent birds from a commercial flock. ◇-identifies AFLP markers observed in the three unknowns.

Utah observes a 2:1 female to male mortality ratio, due to acute RHS, at approximately two weeks of age (Olsen, personal communication). This observation opens up the possibility that the inheritance of RHS in turkeys, could be a sex-linked or sex-associated trait. Hens are the heterogametic sex of this species, which would account for a greater incidence of RHS in females. However, this observation conflicts with published reports that observed a greater frequency of RHS in males (Jankus *et al.*, 1973). There is presently no explanation available for these conflicting reports. Breeding trials are currently under way to further investigate inheritance patterns.

Of the markers identified, it is interesting to note that the majority contain a high percentage of A/T. The A/T content ranged from 44.8% to 70.2%, with many over 60%. This may be significant since regions of high A/T content tend to be regulatory domains in the DNA. Therefore, polymorphisms within these regions may alter gene regulation, with RHS being the end result. Further work is needed to investigate whether these markers are directly tied to a gene or multiple genes.

Genetic markers have been applied in a number of ways. One of the most common uses for these markers, at present, is the identification of undesirable traits to facilitate reduction or removal of the trait from the herd or flock. The implications of this work provide a basis for the implementation of a marker-assisted selection (MAS) strategy for reducing and possibly eliminating RHS from a commercial flock. These markers were identified in an experimental flock of turkeys. Preliminary data has suggested that at least some of these markers

are portable to a local commercial flock. For example, a number of the AFLP markers identified in the experimental flock have been observed in three toms of this local flock (Fig. 1).

The AFLP technique is very sensitive, as evidenced by the number of markers identified in this work. The strength of this procedure makes it a very effective tool for identifying genetic markers. However, production of AFLPs is expensive and time consuming. For these reasons attempts have been made to produce SCARs from the marker sequences obtained. This would allow for a simple, cost-effective screening procedure of large populations.

Polymorphisms produced by the AFLP technique may occur in one of two regions either in the restriction site sequence, or in one of the three nucleotides following this site where the selective nucleotides complementarily anneal. One of the potential problems with producing SCARs from AFLP markers is that many of the polymorphisms may result from a single nucleotide change in the restriction site sequence. Detection of a single base change with a 15-20 base SCAR primer would be extremely difficult. Numerous attempts to produce a SCAR from both these and previously identified RAPD markers have been unsuccessful. Failure to identify RAPD-SCARs might also suggest that RHS may be a complex polygenic trait.

Acknowledgements

This project was funded by grant #R0401007 from the Utah Department of Agriculture, and by Moroni Feed Company.

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