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Microsatellite DNA Loci for Population Studies in Brazilian Chicken Ecotypes

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Abstract: In poultry, the reduction in genetic variability of native chicken populations has led to the use of microsatellites in many genetic studies of chicken ecotypes. To be of maximum usefulness as a genetic marker, microsatellite primers should be amplifying the same locus other than the source of the primer sequence in different populations. Even in closely related lines or breeds microsatellite genotyping errors may be introduced from primer mismatches as a result of mutations in the primer binding sites. Therefore, the selection, use and optimization of microsatellites are considered to be a fundamental step towards full success in genetic studies. Herein, 20 microsatellite loci are presented with great potential for diversity studies in Brazilian chicken ecotypes. The analyses of these ecotypes revealed a total of 191 robust alleles, ranging from three to 18, with an average of 9.6 alleles per locus. The average observed heterozygosity was 0.785, while the mean expected heterozygosity was 0.688. Additionally, the mean polymorphic information content value (0.731) further reflected high level of polymorphism across all microsatellite loci. The topology of the dendrogram constructed with the neighbour-joining method showed probable patterns of relationship and genetic differentiation among the individual ecotypes. Overall, microsatellite loci have proven to be highly useful for studying the variability of chicken ecotypes in the Mid-North region of Brazil.

Key words: Molecular markers, genetic variability, SSR, local chicken

INTRODUCTION

Microsatellites are short DNA sequences consisting of tandemly repeated units of 2-6 nucleotides distributed throughout the eukaryotic genome (Diniz *et al*., 2007). Due to their multiallelic nature, high level of polymorphism, ease of detection by PCR, reproducibility and codominant inheritance, microsatellites are now considered the most used markers for population studies (Zane *et al*., 2002).

In poultry, the reduction in genetic variability of native chicken populations, with consequent risk of extinction, has led to the use of microsatellites in many genetic studies of chicken ecotypes (FAO, 2000; Mariante and Cavalcante, 2006). The loss of these ecotypes would eliminate unique genetic traits that could be used for future improvement programs and commercial exploitation and therefore, their preservation should be treated as of the highest priority. The Brazilian chicken ecotypes consist of different phenotypes of almost 500 years of natural selection raised by smallholder farmers across distinct agro-ecological regions. To date, however, no investigation has yet been directed towards the use of microsatellite markers to study the genetic variability of these ecotypes, despite the large number of microsatellite primers designed from commercial chicken or database sequences (FAO, 2000).

To be of maximum usefulness as a genetic marker, microsatellite primers should be amplifying the same loci other than the source of the primer sequence in different populations (Kaiser *et al*., 2000). Even in closely related lines or breeds microsatellite genotyping problems (null alleles, homoplasy, allele dropout) may be introduced from primer mismatches as a result of mutations in the primer binding sites (Pompanon *et al*., 2005; Soulsbury *et al*., 2009). Large genetic changes from commercial chicken breeds have been accumulated due to artificial

selection and a wide diversity is known to exist between local and commercial poultry (Tadano *et al*., 2008). Therefore, the selection, use and optimization of

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microsatellites are considered to be a fundamental step towards full success in genetic studies.

The aim of our study was to select and optimize a set of DNA microsatellites, originally developed from commercial chicken breeds and evaluate their efficacy and informativeness within Brazilian chicken ecotypes inhabiting the Mid-North region of Brazil.

MATERIALS AND METHODS

Sampling and DNA extraction: Skin tissue samples were collected from Brazilian chicken ecotypes, characterized by high morphological variation, inhabiting the States of Maranhão and Piauí. A tissue fragment of approximately 3 mm^3 was removed from the lower part of the chicken wing and stored at -20°C in 100% ethanol for further analysis. Genomic DNA was extracted using the proteinase K and phenol/chloroform/isoamyl alcohol method as described in the literature (Sambrook and Russell, 2001). The purified genomic DNA was used as a template for PCR amplification of microsatellite loci as described below.

Microsatellite amplification: A total of 25 microsatellite loci isolated from commercial chicken lines were initially selected based on the degree of polymorphism and genome coverage (Cheng and Crittenden, 1994; Gibbs *et al*., 1997; McConnell *et al*., 1999; Crooijmans *et al*., 1996, 1997). Each microsatellite primer pair was analyzed against a panel of 20 different individuals to verify amplification and optimization of PCR reactions.

PCR conditions: The PCR reaction was carried out in a total volume of 10 µL containing 15-30 ng of genomic DNA, 1-1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.3 µM for each microsatellite primer (forward and reverse), 1.0 µL of 10x reaction buffer (40 mM Tris-HCl; 100 mM KCl), 1 U Taq DNA polymerase (Invitrogen) and deionized water. The total mixture of PCR reaction was subjected to the gradient thermocycler model Veriti®96-well thermalcycler (Applied Biosystems) under the following conditions: Initial denaturation at 94°C for 1 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at annealing temperature of each primer pair and an extension of 50 sec at 72°C. The final extension was at 72°C for 2 min. A two-stage touchdown amplification (Don *et al*., 1991) profile was designed where specific PCR products were not found. Reactions were conducted with a 1°C reduction at each cycle from either 65°C (63°C) to 55°C (53°C), followed by 27 cycles at an annealing temperature of 55°C (53°C) as shown in Table 1.

PAGE electrophoresis: Three to five microliters of the optimized PCR product were mixed with an equal volume of formamide loading dye and pre-heated to 95°C for 2 min. Four microliters were separated on a 6% polyacrylamide gel and silver-stained according to the protocol developed by Benbouza *et al*. (2006), with the following modifications: fixation solution (20% ethanol, 0.5% glacial acetic acid) for 5 min; silver nitrate solution (9 mM) with formaldehyde (1.5 mL/L) for 7 min and developer (0.37 M NaOH) with formaldehyde (2.0 mL/L), or until bands started to appear. The allele size was determined by direct comparison with a 10 bp ladder DNA (Invitrogen). Gel images were scanned for subsequent scoring of allele size.

Data analysis: Allele frequency, number of alleles per locus, allelic richness (a measure of the number of alleles per locus corrected by the sample size), expected (H_e) and observed heterozygosity (H_o) were computed using the program FSTAT v. 2.9.3 (Goudet, 2001). Deviations from Hardy-Weinberg Equilibrium (HWE) were determined using GENEPOP (Raymond and Rousset, 1995). The Polymorphic Information Content (PIC) was estimated by Cervus version 3.0 (Kalinowski *et al*., 2007). Distance estimates across individuals were measured using proportion of shared alleles (Dps) calculated on the web-based Genetic Distance Calculator (http://www2.biology.ualberta.ca/jbrzusto/sharedst.php). The neighbor-joining tree on Cavalli-Sforza and Edward chord genetic distance was constructed using the software PAST v1.34 (Hammer *et al*., 2001).

RESULTS AND DISCUSSION

The amplification of microsatellite loci developed from commercial chicken lines was highly successful on the Brazilian chicken ecotypes, which is consistent with the knowledge that microsatellite amplification is inversely related to the genetic distance between species/breeds/ecotypes (Kuleung *et al*., 2004). The closer the evolutionary distance is between taxa, the higher the chance of successful amplification.

Of 25 microsatellite primer pairs tested, 80% successfully amplified the samples collected in the Brazilian Mid-North region, with robust and reproducible bands and easily visualized alleles. The remaining 5 primer pairs (20%) failed to amplify alleles under all PCR conditions tested. The touchdown PCR technique (Don *et al*., 1991) was necessary for the optimization of 55% of microsatellite loci. The annealing temperatures and concentrations of magnesium chloride varied among primer pairs at different loci, as shown in Table 1. At the end, the concentration of each primer, dNTP and Taq polymerase DNA were fixed at 0.33 mM, 200 mM and 0.1 U/µL, respectively.

Table 1: Primer sequences, characteristics and variation across 20 microsatellite loci successfully amplified in Brazilian chicken ecotypes from the Mid-North region

					PCR conditions										
Loci	Primers (5'→3')	Motif	Chrom.	Reference	RP	Op-Ta (°C)	[$MgCl_2$] mM	EA‡	OA	A	A_R	Ho	He	PIC	*p*HWE
ADL0278	CCAGCAGTCTACCTTCCTAT TGTCATCCAAGAACAGTGTG	$(TG)_{13}$	8	Cheng and Crittenden 1994	TCD¶	65→55	1.5	100-130	110-122	6	6.000	0.734	0.882	0.672	0.053
LEI0166	CTCCTGCCCTTAGCTACGCA TATCCCCTGGCTGGGAGTTT	$(CA)_4TA(CA)_{14}$	3	Gibbs *et al*., 1997	STD§	60	1.5	250-280	248-364	8	7.511	0.723	0.650	0.665	0.167
LEI0192	TGCCAGAGCTTCAGTCTGT GTCATTACTGTTATGTTTATTGC	$T_8N_6(CTTT)_{11}$	6	McConnel *et al*., 1999	STD	60	1.5	244-370	254-370	17	15.734	0.922	0.950	0.891	0.021
LEI0194	TCCTTGGCATGTACATATGA ACTGCATGTTCTTTGATAGGC	$(TTTC)_{15}(T)_{10}$	1	McConnel *et al*., 1999	TCD	65→55	1.5	163	129-179	8	8.000	0.839	0.118	0.791	0.001*
LEI0209	AATTTGGTGTCATACCTCTCC GACTTTCCAGTGTCTCGTTTAG	$(CTTT)_{22}$	1	McConnel *et al*., 1999	TCD	63→53	1.0	169	129-181	13	11.910	0.864	0.800	0.825	0.411
LEI0214	TGCCTCGTCTTACTGAGTGA GATCAAGCACTGTATTTTATTC	$(CTGT)_7(CTTT)_8$	11	McConnel *et al*., 1999	TCD	65→55	1.5	159	179-187	3	3.000	0.608	0.222	0.504	0.003*
LEI0217	GATGACTGAGAGAAATAACTTG AAATTACTGAGGCACAGGAG	$(CTTT)_{31}$	1	McConnel *et al*., 1999	STD	56	1.0	198	182-274	15	14.244	0.888	0.750	0.857	0.003*
LEI0221	CCTTTATCCACTCTTCATGCAC TGCATAAATTCCATGGGTAAGC	$(CTTT)_{21}$	1	McConnel *et al*., 1999	STD	60	1.5	203	184-224	9	8.807	0.860	0.990	0.820	0.734
LEI0234	ATGCATCAGATTGGTATTCAA CGTGGCTGTGAACAAATATG	$(TTTC)_{18}$	2	McConnel *et al*., 1999	STD	60	1.0	216-364	212-360	14	13.057	0.909	0.700	0.876	0.005*
LEI0237	GTTAAGTGTTCTCTGATGTAGC CTTCAACTATAAAGCATAGCTG	$(CTTT)_{17}$	2	McConnel *et al*., 1999	STD	60	1.0	224	272-384	17	15.736	0.931	0.750	0.901	0.016
LEI0246	TTGCACTGAGACCAAATGTC CATAGATTTTCCTTAGTAGGTAACTTG	$(CTTT)_{28}$	1	McConnel *et al*., 1999	STD	60	1.0	250	190-262	13	12.338	0.901	0.650	0.868	0.017
LEI0258	CACGCAGCAGAACTTGGTAAGG AGCTGTGCTCAGTCCTCAGTGC	$(TTCTTTCTTTCC)_5$	16	McConnel *et al*., 1999	STD	66	1.5	205	197-275	18	16.569	0.936	0.800	0.906	0.001*
MCW0020	TCTTCTTTGACATGAATTGGCA GCAAGGAAGATTTTGTACAAAATC	$(TG)_{13}(TTTATT)_2$	1	Crooijmans *et al*., 1996	TCD	65→55	1.5	179-185	192-200	5	5.000	0.651	0.824	0.579	0.178
MCW0098	GGCTGCTTTGTGCTCTTCTCG CGATGGTCGTAATTCTCACGT	$(TTTA)_5(TG)_6(TG)_7$	4	Crooijmans *et al*., 1996	TCD	65→55	1.5	261-265	192-264	6	5.772	0.654	0.947	0.570	0.001*
MCW0104	TAGCACAACTCAAGCTGTGAG AGACTTGCACAGCTGTGTACC	$(AGC)_5(GT)_{17}$	13	Crooijmans *et al*., 1996	TCD	65→55	1.5	190-134	196-210	5	5.000	0.595	0.647	0.508	0.067
MCW0183	ATCCCAGTGTCGAGTATCCGA TGAGATTTACTGGAGCCTGCC	$(AC)_{11}$	7	Crooijmans *et al*., 1997	TCD	65→55	1.5	290-311	338-358	4	4.000	0.717	0.500	0.642	0.045
MCW0213	CTGTTCACTTTAAGGACATGG GACAAGTCAACAACTTGCCAG	$(AC)_{25}$	13	Crooijmans *et al*., 1997	STD	55	1.0	293-311	281-321	13	12.636	0.929	0.800	0.899	0.022
MCW0222	GCAGTTACATTGAAATGATTCC TTCTCAAAACACCTAGAAGAC	$(CATTAG)_2(GT)_8$	3	Crooijmans *et al*., 1997	TCD	65→55	1.5	221-225	218-226	5	4.998	0.684	0.444	0.620	0.008*
MCW0248	GTTGTTCAAAAGAAGATGCATG TTGCATTAACTGGGCACTTTC	$(AC)_9$	1	Crooijmans *et al*., 1997	TCD	65→55	1.5	205-225	212-220	3	2.981	0.478	0.550	0.395	1.000
MCW0305	TCAGAAACAAAGCAGGAGCTG TGACATCTTTCAAACGAGACC	$(GT)_{13}$	8	Crooijmans *et al*., 1997	TCD	65→55	1.5	258-268	258-282	9	8.887	0.873	0.778	0.831	0.001*

¶Touchcdown PCR; §Standard PCR; ‡According to references on the table. p-value indicates significance of deviation from Hardy-Weinberg equilibrium. $p \leq 0.01$. Chrom. = Chromossome; RP = Reaction Profile; EA = Expected allele size range (bp)‡; OA = Observed allele size range (bp)

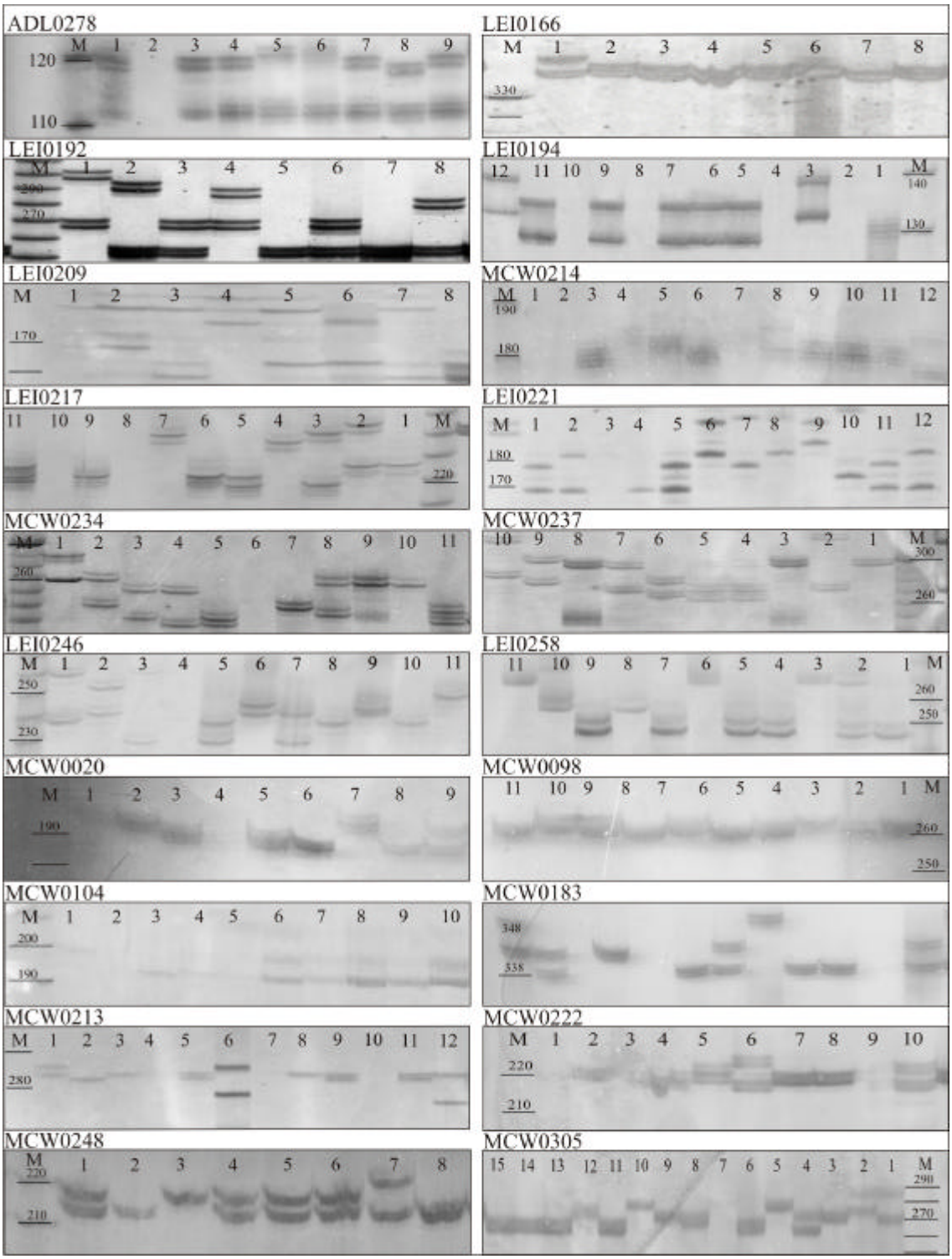

Fig. 1: Silver-stained denaturing polyacrylamide gel electrophoresis of PCR products of 20 SSR markers on ten Brazilian chicken ecotypes. M: 10-bp ladder (Invitrogen)

A total number of 191 alleles were detected in the Brazilian chicken ecotypes. The number of alleles ranged from three (MCW0248 and LEI0214) to 18 (LEI0258), with an average of 9.6 alleles per locus. The

mean number of alleles in the Brazilian ecotypes was higher than what has been reported for local chickens in Ghana (7.8), Iran (5.4) and China (3.8) (Osei-Amponsah *et al*., 2010; Mohammadabadi *et al*., 2010; Liu *et al*., 2008).

The observed heterozygosity (H_O) was higher than the expected heterozygosity (H_E). The observed heterozygosity for all polymorphic loci varied from 0.478 (MCW0248) to 0.936 (LEI0258) with an average of 0.785. The expected heterozygosity varied between loci from 0.118 (LEI0194) to 0.990 (LEI0221), with an average of 0.688. These values were comparable to the heterozygosity found in other native breeds (Ethiopian, Iranian, Indian and Chinese) in earlier reports (Hassen *et al*., 2009; Nasiri *et al*., 2007; Rajkumar *et al*., 2008; Ya-Bo *et al*., 2006).

The mean Polymorphic Information Content (PIC) value (0.731) further reflected high level of polymorphism across all microsatellite loci. PIC values of higher magnitude (0.840) have been reported for Indian native chicken using microsatellite markers, 8 of them similar to those used in our study (Ding *et al*., 2010). Microsatellites showing PIC values higher than 0.5 indicate that more genetic information can be provided by SSR loci (Botstein *et al*., 1980). A positive correlation for $PIC x A_R$ with an R^2 value of 0.826 shows the close relationship between the two variables.

From all polymorphic loci 40% showed significant departure from Hardy-Weinberg Equilibrium (HWE), which might be due to the unwitting pooling of populations (Wahlund effect), inbreeding, the presence of null alleles or even an artifact of small sampling size (Lessios, 1992; De La Rua *et al*., 2001).

The topology of the dendrogram constructed with the neighbour-joining method showed probable patterns of relationship and genetic differentiation among the individuals (Fig. 2). These were grouped in four clusters formed by ecotypes from distinct site collections (i.e. N: Regeneração and T: Teresina in the State of Piauí; B: Brejo and G: Itapecuru-Mirim in the State of Maranhão). A more extensive survey is necessary to obtain a precise picture of the population structure and genetic diversity of these ecotypes inhabiting the Brazil's Mid-North region. No such studies have yet been performed for these local chickens.

Overall, results from this microsatellite survey clearly indicate that all 20 microsatellite markers listed on Table 1 are suitable tools to investigate questions of genetic variability, gene flow and the mating system in Brazilian chicken ecotypes. Further research is being carried out by our group aimed at evaluating genetic variations within this important resource, yet lacking an efficient strategy for its conservation and management.

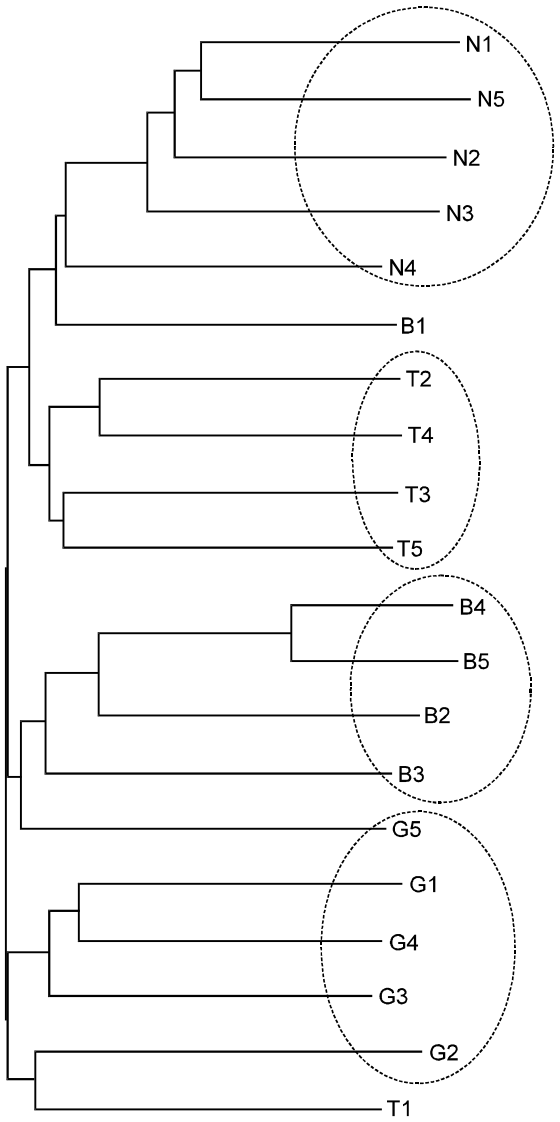

Fig. 2: Neighbour-joining dendrogram of Cavalli-Sforza and Edwards chord genetic distances for microsatellite loci

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