57th Annual Meeting of the European Association for Animal Production
Antalya (Turkey), 17th to 20th September 2006 – Session G17.4 – Abstract n. 479
marica.soattin@unipd.it

Chicken DNA fingerprinting with molecular markers designed on interspersed repeats

Soattin M.[1], G. Barcaccia.[2], M. Cassandro [1] and G. Bittante [1]

[1]Department of Animal Science; [2]Department of Agronomy and Crop Science, Agriculture Faculty of the University of Padova. Campus of Agripolis, 35020 Legnaro, Padova (Italy).
E-mail: marica.soattin@unipd.it

Summary
The chicken genome contains 15% of repetitive DNA sequences organized as short tandem repeats and several families of longer interspersed elements. This research deals with the development of a forensic genomics assay for the chicken DNA fingerprinting based on the analysis of micro- and minisatellite polymorphisms. The identification of breed-specific markers was based on the S-SAP and M-AFLP systems derived from the AFLP technology. Genomic DNA fingerprints were generated in 84 individuals belonging to six local breeds and one commercial line. A number of variation statistics were computed: the effective number of alleles per locus (n_e=1.570), total and single-breed genetic diversity (H_T=0.365 and H_S=0.208, respectively) and the fixation index (G_{ST}=0.433). The mean genetic similarity coefficients within and between local breeds were 0.769 and 0.581, respectively. Markers useful for the genetic traceability of breeds revealed significant sequence similarities with either genic or intergenic regions of known chromosome position. Sequence tagged site primers were designed for the most discriminant markers in order to develop multiplex non-radioactive genomic PCR assays. The identification of single local breeds according to multilocus genomic haplotypes is currently under evaluation. In conclusion, the setting up of a molecular reference system seems to be feasible for the precise identification in a genetic traceability system of the chicken breeds considered.

Introduction
The relatively small genome of chicken (1.200 Mpb) has been shown to contain around 15% of repetitive DNA organized as short tandem repeats (e.g. centromeric and telomeric tandem repeats) as well as numerous families of interspersed repeats, mainly derived from transposable elements (both transposons and retrotransposons) and located over all chromosomes (autosomes, macro-, intermediate, and micro-chromosomes, and sex chromosomes) even though not uniformly (Wicker *et al*., 2005). It has also been proved that micro-chromosomes contain more single-copy sequences and less repeated sequences than macro-chromosomes, and that sex chromosomes are very rich of highly repetitive DNA. The most abundant repeated sequence is that known as CR1 (Chicken Repeat One): the chicken genome contains over 90.000 copies of this interspersed element belonging to the class LINE (Long Interspersed Nuclear Element). Each element is about 4,5 kb long and includes two genes, one encoding a reverse transcriptase, responsible for the replication of the element itself, and another encoding for an unknown protein likely involved in the transposition process. Additional repeated elements, very abundant in the chicken genome, are those containing tandem repeats of short nucleotide sequence motifs or microsatellites, also known as SSR (Simple Sequence Repeat). Usually they are less than 200 bp long and are very useful for population genetic studies because of their repeatability and high degree of polymorphism.

Genomic DNA sequences of the interspersed hypervariable repetitive elements are considered very useful to set up analysis systems of forensic genetics for a reliable identification of species, breeds and also single individuals on the basis of the banding patterns generated or haplotypes recovered. The generation of multi-locus DNA fingerprints and the detection of sequence polymorphisms in the LINE and SSR regions is usually based on the S-SAP (Sequence-Specific Amplification Polymorphism) and M-AFLP (Microsatellite-Amplified Fragment Length Polymorphism) systems, derived from the more widely known AFLP (Amplified Fragment Length Polymorphism) technology. The approach includes the amplification of genomic cleaved fragments, ligated to specific adaptors and pre-amplified with selective primers, using an AFLP primer in combination with a primer that hybridize to a repeated sequence conserved region or, alternatively, to a microsatellite anchored motif. M-AFLP and S-SAP markers derived, respectively, from

simple repetitive sequences and transposable elements or minisatellites are also suitable for phylogenetic analyses, since they provide information on genome evolution. Moreover, *in situ* chromosome hybridization experiments and *in silico* bioinformatic investigations have revealed that DNA repeats and retrotransposons are often localized in euchromatic regions, into or close to functional genes. This finding suggests that repetitive and/or transposable elements may be involved in the evolution of animal gene structure and expression, supplying genes with regulatory sequences and facilitating gene duplication and/or exon shuffling (Coullin *et al.*, 2005).

In Italy several poultry breeds are known to be present. Recently, more than 90 distinct breeds were identified, of which 53 belonging to the chicken. The overall situation of these breeds is nevertheless critical since as much as 61% are becoming extinct, 13% threaten and only 7% are object of conservation plans (Zanon *et al*., 2001). Nowadays, it is generally accepted that the high number of still existing breeds is attributable to the breeding activity based on controlled crosses and selection programs in order to bred very productive lines able to perform and adapt themselves better in a specific territory. To such activity the origin of a consistent biodiversity of the species is mainly owed (Fumihito *et al*., 1996). With the ending of the agriculture in marginal areas and the beginning of chicken breeding at the industrial level, highly specialized lines have been developed in the near past and are currently commercialized, covering the vast majority of the chicken meat and egg market value. This big change is putting to risk of extinction most of the locally spread breeds, particularly those characterized by double purpose which are low productive and not enough competitive compared to commercial broilers. Local breeds of chicken are the populations with the highest genetic variation as well as with the best adaptation to the natural and anthropological environment where they have originated and/or evolved. They contain locally adapted alleles and represent an irreplaceable bank of highly co-adapted genotypes. As a consequence, local breeds are known to posses a good rusticity and many resistance traits to both environmental and biotic stresses, all characteristics that make them of particular interest for the use in biological farms, the recovery of marginal lands and the raising of niche productions. For all the above mentioned reasons, they have recently been object of schemes of conservation and animal genetic variability preservation, as well as of studies aimed at the characterization of their gene pools.

Molecular markers are known as a particularly effective and reliable tool for the characterization of genomes and the investigation of gene polymorphisms not only of poultry species, but of all organisms in general (Soattin *et al.*, 2005). In particular, the use of molecular markers allows to measure the genetic variation within single populations and to evaluate the genetic relatedness among populations so that the formulation and implementation of germplasm maintenance programs can be optimized (Cassandro *et al*., 2005). The AFLP and SSR markers along with STS (Sequence Tagged Site) and SNP (Single Nucleotide Polymorphism) markers are the most powerful and robust molecular marker systems for the analysis of genomes and genes, and hence for the molecular characterization of chicken individuals and populations by means of DNA fingerprinting, genotyping or haplotyping (Wimmers *et al*., 2000, Vanhala *et al*., 1998, Zhang *et al*., 2002).

The present research deals with the development of an innovative system of forensic genomics for the chicken DNA fingerprinting based on the M-AFLP and S-SAP analyses of repetitive sequence families (both microsatellites and minisatellites) with the aim of cloning breed-discriminant STS markers and identifying breed-specific SNP haplotypes.

Materials and methods

Animal populations

Twelve individuals for each of the six indigenous chicken breeds under investigation were used for genomic DNA fingerprinting together with a commercial broiler (Golden Comet line) selected for meat production and adopted as reference population, for a total of 84 animals. The indigenous populations at risk of genetic erosion analyzed in this study are the following: Ermellinata, Padovana, Pépoi, Polverara, Robusta lionata and Robusta maculata. The animals were reared in three conservation nucleus located throughout the Veneto region, Italy, and their morphological characteristics were previously described by De Marchi *et al*. (2005). The population size for the indigenous breeds have been estimated at 1,500 individuals for Ermellinata, Pépoi, Robusta lionata and at 2,000 individuals for Padovana and Polverara. The conservation scheme is based, for each breed and nuclues, on size group of 34 pure females and 20 males with a breeding scheme based, for each breed, with a males rotation among conservation units (Cassandro *et al.*, 2004).

Molecular markers

Nucleic acids were extracted from whole blood through cell lyses. After purification from RNA residuals and proteins using, respectively, RNase and ammonium acetate, each sample of genomic DNA was precipitated with isopropanol and washed twice with 70% ethanol. Then, all DNA pellets were vacuum dried and redissolved in Tris-EDTA buffer. The concentration of DNA samples was determined by optical density readings at 260 nm (1 optical density (OD) = 50 µg/ml) and their purity calculated by the OD_{260}/OD_{280} ratio and by the $OD_{210}–OD_{310}$ pattern (Sambrook *et al*., 1989). An aliquot of each genomic DNA was also assayed by electrophoresis on 1% agarose gels.

The detection of polymorphisms on repetitive sequences, such as SSR and CR1 elements, was based on the S-SAP (*Sequence-Specific Amplification Polymorphism*) and M-AFLP (*Microsatellite-Amplified Fragment Length Polymorphism*) systems, derived from the more widely known AFLP technology. The approach included the amplification of genomic cleaved fragments, ligated to specific adaptors and pre-amplified with selective primers, using an AFLP primer (i.e., *Eco*RI or *Taq*I rare and frequent cutter-associated primers) in combination with a primer that specifically anneal to the CR1 element or, alternatively, with a primer anchored to a given SSR motif (**Table 1**).

Table 1: List of conventional AFLP, CR1-specific and ISSR-anchored primers used and information on their sequences.

Primer	Sequence 5'-3'
EcoRI+A	GACTGCGTACCAATTCA
TaqI+A	GATGAGTCCTGACCGAA
CR1Dx1F	TAGTAAATGGGGATGTTGGT
CR1Dx2F	TGATCCTCGAGGTCCCTTCC
CR1Sx1R	AGCAGCCTTCTGGACCTCTT
CR1Sx2R	CAGCAACACTTCACCTCTGG
CR1 Int For	AGTTCATGATCTCAAGGGATGTGGGCC
CR1 Int Rev	CAGCCCCCTGATCATCTTTGTGGCCCT
I-SSR 6	$(CA)_8GC$
I-SSR 13	$CAG(CA)_8$
I-SSR 33	$(AGC)_4T$
I-SSR 37	$(AGC)_4GT$

M-AFLP and S-SAP fingerprints were generated using the AFLP technology according to Vos *et al*. (1995) as modified by Barcaccia *et al*. (1999). A total of 500 ng of genomic DNA from 84 individuals was digested with a combination of *Eco*RI/*Taq*I restriction enzymes, and ligated to the correspondent adapters with T4 DNA ligase. An aliquot of the restricted-ligated DNA samples was pre-amplified using *Eco*RI and *Taq*I restriction site-specific primers with one selective base each. A radiolabelled specific primer (CR1 or I-SSR-anchored) were used for the final amplification along with an AFLP primer (EcoRI+A o TaqI+A). Each 20 µl PCR reaction contained 5 µl of the pre-amplified DNA, 0.2 mM of labelled specific primer and of unlabelled AFLP primer, 2 µl of 10x PCR buffer, 0.2 mM dNTPs and 0.4 U of *Taq* DNA polymerase. The following cycling conditions ensured optimal primer selectivity: 1 cycle of 45 s at 94°C, 30 s at 65°C, 1 min at 72°C followed by 13 cycles of 0.7°C lower annealing temperature each cycle and 18 cycles of 30 s at 94°C, 30 s at 55.9°C, 1 min at 72°C and a final step of 5 min at 72°C. AFLP-derived markers were loaded onto a 6% polyacrylamide gel and electrophoresis was performed at 1.500 V, 40 mA and 40 W. Markers were visualized on autoradiograms after 18 hr exposure at –80°C with intensifying screens.

Genetic diversity analysis

A preliminarily investigation of diversity was performed computing descriptive statistics such as the observed and effective number of polymorphic loci (n_o and n_e parameters, respectively). The amount of heterozygosity was assessed at two different levels of complexity: single populations or local breeds (H_S) and species as a whole (H_T) according to the formula of Nei (1973) based on marker allele frequency estimates. These statistics of genetic diversity were used to define the genetic structure of populations belonging to single breeds, to estimate the degree of genetic differentiation among breeds or, equivalently, the fixation index (F_{ST}) as well as the rate of gene flow (Nm). Then, the allele frequency over all marker loci assayed was used to calculate the genetic distance among breeds in all pair-wise comparisons according to Nei (1978). It was also possible to estimate genetic similarities between individuals within single breeds and between different breeds on the basis of genetic fingerprints, adopting the similarity index of Jaccard (1980).

Subcloning and sequencing of AFLP-derived products

Single discriminant molecular markers that proved to be useful for the traceability of chicken breeds were excised and eluted from the blotted gels, subcloned into plasmid vectors and re-amplified with the same primer combination that yielded the specific genomic DNA fragment. An aliquot of the re-amplified template was sticky-end ligated into a pBluscript II Phagemide. The plasmid DNA was purified from 5 ml of an over-night culture on LB medium of *E. coli* using Plasmid mini prep kit (Sigma Aldrich) following the kit instruction. Plasmid sequences of both strands were performed by the dideoxynucleotide chain reaction termination method using either the M13 forward or reverse primer.

SCAR marker analysis

The sequence of the most discriminant M-AFLP and S-SAP markers was used for designing primers on their upstream and downstream terminal ends using PerlPrimer program and hence converted into SCAR (Sequence Characterized Amplified Region) markers. PCR of genomic DNA with pairs of sequence-tagged site primers was done using various annealing temperatures (56–66°C) in order to optimize amplification profiles for each selected clone and to visualize polymorphisms for the identification of breeds. The 50 µl reaction volume contained 1× PCR buffer (50 mM EDTA, 1.5 mM MgCl2, 10 mM Tris-HCl), 0.2 mM dNTPs, 0.2 µM of each primer, 200 ng of genomic DNA and 1 U Taq DNA polymerase (Sigma Aldrich Red Taq). PCR was carried out with an initial denaturation step of 94°C for 3 min, followed by 35 cycles of 94°C for

30 s, primers optimal annealing temperature for 30 s, 72°C for 1 min, and a final extension step of 72°C for 10 min. Amplification products were separated by electrophoresis in 2% agarose gels.

Bioinformatic analysis

The sequence of all discriminant molecular markers was used as query for bioinformatic analyses of the chicken genome database and the major transcript and protein databases. Gene homologues were also searched in public databases by BLASTN and BLASTX applications (Altschul *et al*., 1990) to compare, respectively, nucleotide and translated sequences. Retrievals enabled to attribute given sequences to specific chromosomes and to eventually acquire information on their putative function. All nucleic acid sequences were recorded in a database, including molecular markers conserved within breeds and polymorphic between breeds as well as molecular markers useful to discriminate local breeds from commercial broilers.

For each DNA clone, both strands from at least three distinct animals were aligned to test the veracity of each sequence and to recover their consensus sequence by using the Vector NTI program. Sequences of SCAR markers from all chicken breeds were used for multiple sequence alignments in the CLUSTALW program (Higgins *et al*., 1992) to find out SNP (Single Nucleotide Polymorphisms) and eventually IN/DEL (Insertions/Deletions), and to identify breed-specific haplotypes.

Results and discussion

The detection of sequence repeats for the fingerprinting of the chicken genome was based on the S-SAP and M-AFLP systems, derived from the more widely known AFLP technology. The approach provided for the amplification of genomic cleaved fragments, ligated to specific adaptors and pre-amplified with selective primers, using an AFLP primer in combination with a primer that anneal to a repeated element (i.e., CR1) or with a primer anchored to a microsatellite (e.g., $(AGC)_n$ and $(CA)_n$) motif. In particular, the S-SAP and M-AFLP marker systems based on the use of either individual or bulked DNA samples allowed reproducible and informative fingerprints and polymorphisms to be obtained within as well as between chicken breeds (Ermellinata di Rovigo, Padovana, Pépoi, Polverara, Robusta lionata and Robusta maculata) and the commercial broiler (Golden Comet line), as shown in **Figure 1**.

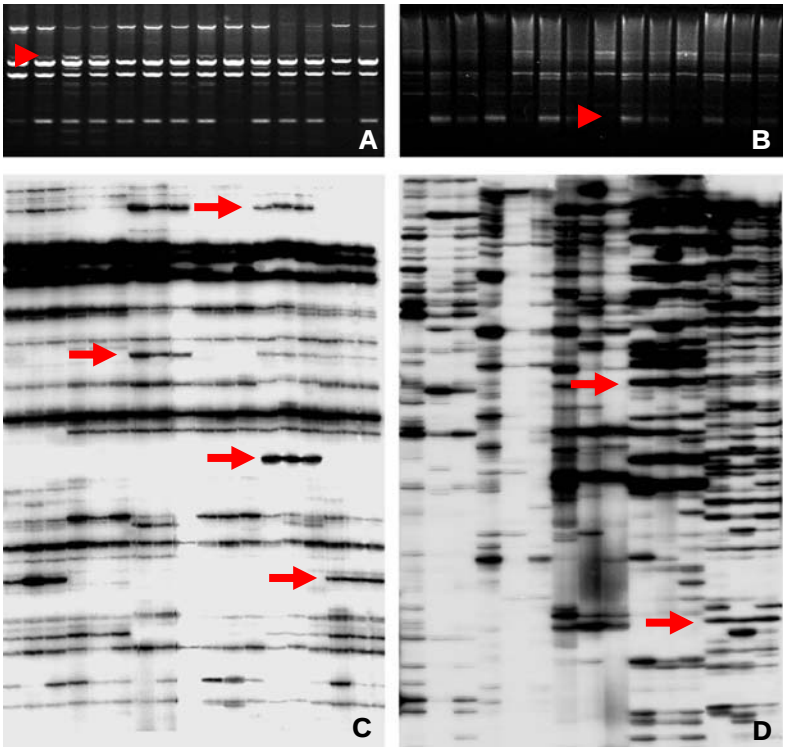

Figure 1: Example of fingerprints generated by primers I-SSR 33 (A), CR1int nested-PCR (B), I-SSR 33/EcoRI+A (C) and S-SAP primers testing (D) using a random sample of genomic DNA of animals belonging to different local breeds. Arrows show polymorphic markers.

Each selected primer combination generated DNA fingerprints showing, on average, from a minimum of 40 to a maximum of 80 markers. In terms of polymorphism information content, the dinucleotide CA repeat-anchored primers produced the highest number of M-AFLP markers, whereas among the CR1 element-specific primers the highest number of S-SAP markers was yielded by forward ones designed in the most conserved region of the chicken repeats. In particular, highly informative and discriminant fingerprints based on microsatellite DNA motifs and repetitive CR1 elements were scored using the primer combinations $CAG(CA)_8$/EcoRI+A and CR1–Dx2F/TaqI+A, respectively. Polymorphic molecular markers that proved to be useful for the traceability of chicken breeds were recovered from the blotted gels, subcloned into plasmid vectors and sequenced. The sequence analysis of all selected polymorphic M-AFLP and S-SAP markers enabled to verify the presence of the specific SSR motif or the partial CR1 element (**Figure 2**), thus demonstrating the specificity of the amplification products and, hence, the reliability of the fingerprinting techniques used.

The sequence of the most among-breed discriminant markers as well as the sequence of the breed-specific markers were also used as queries for public database interrogations. Chicken genome retrievals revealed significant similarities with genic and intergenic sequences of known chromosome position and primary structure homologies with known gene products (**Table 2**).

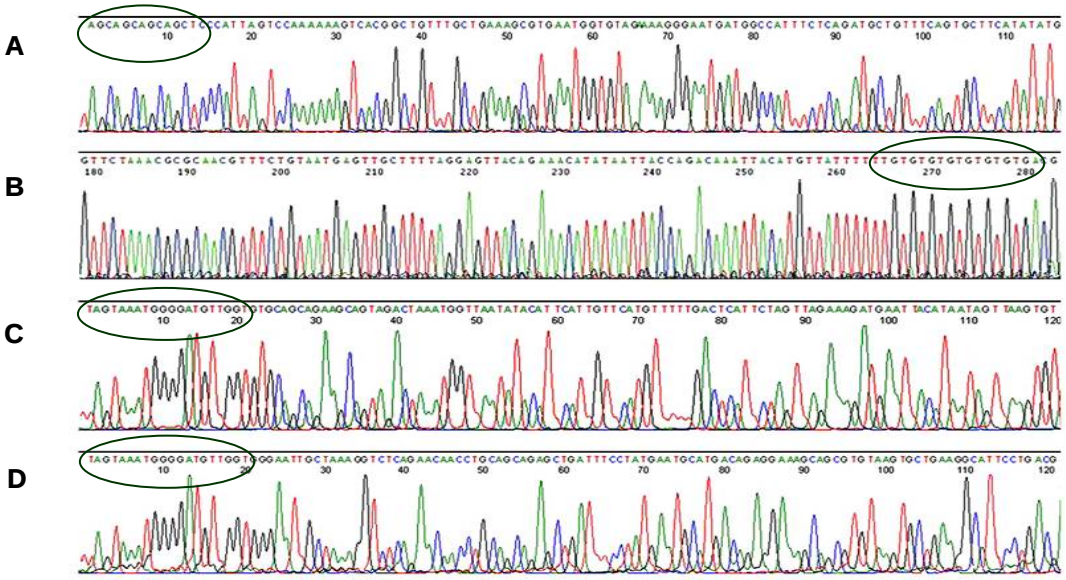

Figure 2: Example of chromatograms obtained by sequencing of two M-AFLP (A and B) and two S-SAP primer combinations (C and D). The circles point out the repetitive motifs microsatellite (GT and AGC) and CR1 used as primers.

Tabella 2: List of breed-specific clones with relative length, position on chicken chromosom, accession number, sequence type and amminoacidic omology.

Clone	bp	Chr.	Locus	Sequence type	Genic product
#38	320	15	NW 060535	genic	KIAA1944 protein
#39	290	14	NW 060533	genic	Acetyl-CoA Synthetase 2
#42	282	3	NW 060336	genic	SNX14 similar protein
#44	200	2	NW 060277	genic	Neuropilin- and tolloid-like protein 1 isoform 3 precursor
#48	650	4	NW 060359	Intergenic	unknown
#50	540	1	NW 060216	genic	Mitochondrial carrier protein
#52	400	1	NW 060224	genic	Interferon alpha/beta receptor 1
#55	280	9	NW 060420	Intergenic	Alpha-1,4-N-acetylglucosaminyltransferase-Ephrin type-B receptor 1
#56	200	17	NW 060632	genic	nasal embryonic LHRH factor

Both monomorphic and polymorphic DNA markers were scored as present or absent over all chicken DNA fingerprints and used to summarize the M-AFLP and S-SAP data by computing genetic diversity statistics and assessing population relationships. The effective number of alleles per locus was equal to n_e=1.570. Total Nei's genetic diversity was quite similar between M-AFLP markers (H_T=0.334) and S-SAP (H_T=0.381) markers, whereas the mean genetic diversity of single breeds was higher for M-AFLP than S-SAP, measuring H_S=0.162 and 0.232, respectively. Fixation index was around 51% fo M-AFLP and 39% for S-SAP, suggesting that the local breeds conserved well-separated their gene pools over time. The mean genetic similarity coefficients within and between local breeds were 0.769 and 0.581, respectively.

The construction of UPGMA dendrograms and the definition of centroids according to the principal coordinate analysis were also performed using total and mean Dice's genetic similarity matrices. **Figure**

3 shows centroids plotted using the two principal coordinates separately for M-AFLP markers and S-SAP markers which overall accounted for about 37% of the total genetic variance.

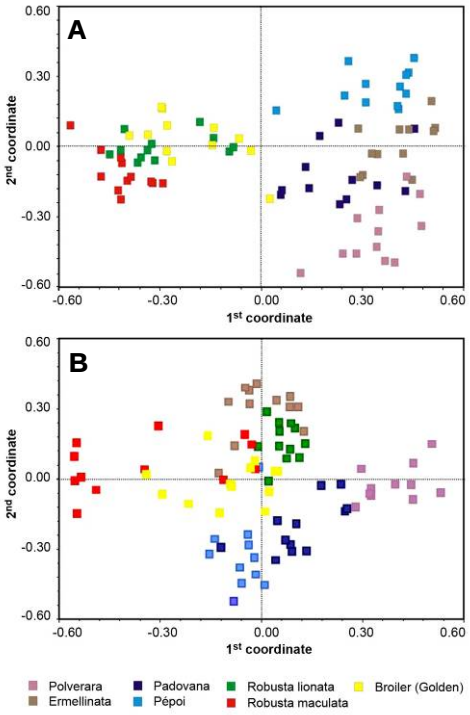

Figure 3: Centroids identified from total and mean Dice's genetic similarity matrices using polymorphisms obtained by M-AFLP (A) and S-SAP (B) molecular markers.

A number of individuals of each breed overlapped the distribution of individuals of other breeds, even though distinct subgroups were clearly discriminated by one or both coordinates. With M-AFLP markers, Robusta lionata and Robusta maculata breeds along with the commercial Broiler were subgrouped separately from the rest of breeds. Moreover, Pépoi and Polverara breeds were clearly distinguishable each other, while Padovana and Ermellinata di Rovigo were clustered very closely. The main distribution difference observed with S-SAP markers concerns the Ermellinata di Rovigo which was clustered apart, together with some individuals of Robusta lionata and Robusta maculata breeds (**Figure 3**). Such a result can be explained by considering the different chromosomic regions assayed by M-AFLP and S-SAP markers, since the former was applied with different dinucleotide and trinucleotide repeat-anchored primers whereas the latter was mainly based on the genome-wide spread CR1 elements.

To set up of a reliable PCR-based molecular reference system suitable for the precise identification of the single breeds, sequence-tagged site primers were designed on the 9 most discriminant clones in order to convert the breed-specific M-AFLP and S-SAP derived markers into easily detectable non-radioactive SCAR markers. The sequence of the forward and reverse primers is reported in **Table 3**. When these primers were used in PCR experiments using chicken genomic DNA as templates, in most cases amplification products showed to be shared among individuals over all breeds and thus not useful for discriminating single breeds. Certain polymorphisms could be generated using very stringent PCR conditions but they proved to be not fully reliable. This result is most likely attributable to the origin of AFLP polymorphisms usually based on single nucleotides on the restriction endonuclease action site and/or oligonucleotide primer annealing region.

Table 3: List of primers designed on 9 most discriminant breed-specific clones and their annealing temperature.

Clone	Primer sequence 5'-3'	Ta (°C)
#38	For GGATGTTGGTCACCAGAAAGTAGGAAAAATG Rev GTACCAATTCACATCAATATAAAGCAAAACT	56
#39	For GATGTTGGTGGGAATTGCTAAAGGTCTCA Rev AATTCAGCACGTTTCACTACAAAAACAACTGC	64
#42	For AATTCAGTAAGAAAGACCAGAAGTAACAGG Rev GATGTTGGTCACTTTCCTATAATAGCTAT	58
#44	For ATGGGGATGTTGGTGTGCAGCAGAAG Rev TTCACCTGCCTATCAAATTATCACCATC	64
#48	For AATTCAGCTATGGGACCATTAAAAACTCCA Rev AGCAGCAGCTCACACTAATCCACAGC	60
#50	For AGCAGCAGCTCCCATTAGTCCAAAAAG Rev AATTCAGGGAGCTTGCAGATCATCAACTTC	60
#52	For CAGAAGGAATAGCTTTAGTAACAGGCCCA Rev AGCAGCTGGCAGTGGAACAGGAAAGAAA	66
#55	For AGCAGCTCAGCACAGGGACGACAACTTC Rev GGTTATCATTTCCCATTAATCCCAACT	58
#56	For AATTCACACAGAAACGTCTCCAAGAG Rev AGCAGCTCAGTATGGGGGGTTATGGA	60

To verify the occurrence of single nucleotide polymorphisms in the selected clones, amplification products of all SCAR markers were recovered from the gels and sequenced. A multiple alignment of the consensus sequences of each of the 9 clones from all local breeds and the commercial broiler allowed us to discover SNPs and IN/DELs, not only in the terminal ends of the SCAR markers as expected but also in

their internal regions. The sequence information was used to define haplotypes to be adopted for the recognition of the single populations (**Figure 4**).

A

Breeds	Clone #38		Clone #39			Clone #50			Clone #56			
	62	226	175	177	282-285	11	207	286	50	69	95	200-201
Ermellinata (ERM)	A	A	C	T	AAAT	–	–	–	A	C	T	TA
Padovana (PAD)	C/T	A	C	T	AATA	C	G	G	A	C	T	AT
Pépoi (PEP)	C/T	A/C	T	C	AATA	T	A	A	C	G	C	T
Polverara (POL)	C/T	A/C	–	–	––––	C	A	A	A	C	T	AT
Robusta lionata (ROL)	C or C/T	A	C	T	AATT	C	A	A	A	C	T	AT
Robusta maculata (ROM)	C or C/T	A	C	T	AAAT	C	A	A	C	C	T	T
Broiler (GCL)	A	A	C	T	AATT	C	G	A	C	C	T	T

B

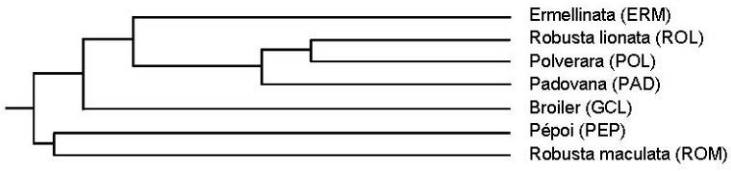

Figure 4: Main haplotypes defined by sequencing of the breed-specific clones #38, #39, #50, #56 (A); phylogenetic relationship among six local breeds and broiler defined on the basis of haplotypes detected (B).

For the identification of the Pépoi individuals several breed-specific SNPs were detected, for instance, in the clones #39, #50 and #56. The haplotypes of all clones were also adopted for the reconstruction of the phylogenetic relationships among breeds (**Figure 4**). It is worth mentioning that in the clone #38 two different SNPs showed either a homozygote or heterozygote state in some of the analyzed individuals. For instance, at position 62 individuals with CC, TT or CT were observed (**Figure 5**).

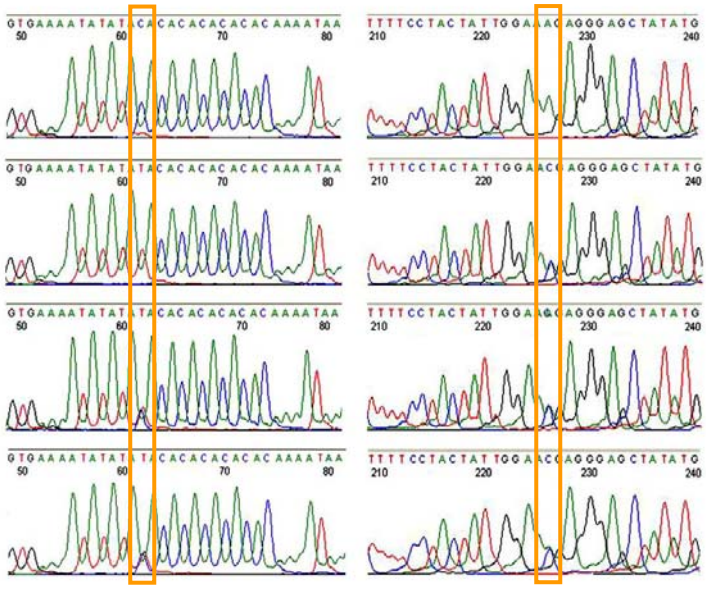

Figure 5: Chromatograms of clone #38 showing individuals at omozigote (CC or TT, AA or CC) and eterozygote state (CT or AC) at positions 62 and 226, respectively.

Conclusions and perspectives

On the basis of gained results, the S-SAP and M-AFLP marker systems were shown to be suitable to produce reproducible and informative fingerprints and polymorphisms within and between chicken breeds and the broiler; they can be exploited for investigating genetic variation within and assessing genetic relatedness among populations. Highly informative and discriminant fingerprints were obtained assaying the variation for short tandem repeats and longer unique repeats by CAG$(CA)_8$/EcoRI+A and CR1-Dx2F/TaqI+A primer combinations. These kinds of molecular marker systems can be used in genetic characterization studies including the determination of the main genetic variability statistics, such as marker allele frequency, degree of expected heterozygosity of single breeds, genetic distance and gene flow among breeds. For instance, the genetic variation of the considered populations was measured to be around 45% meaning that 55% of genetic variation was due to differences within populations at the considered loci. Overall, the combination of M-AFLP and S-SAP data and their comparison with previously obtained SSR data (unpublished results) confirmed the high genetic variation detectable within breeds and the clear genetic differentiation still present among their gene pools, but overall results also revealed substantial differences concerning the genetic distance estimates among local breeds and the commercial broiler. This finding is most likely due to the fact that the three molecular marker systems were applied to different individual sample sizes and the analysis was based upon different genomic loci numbers. Additional molecular analyses are needed to corroborate the preliminary findings and to confirm the statistics related to the genetic diversity within and the phylogenetic relationships among breeds.

The polymorphic markers isolated from genomic interspersed repeats can be useful to set up a genetic traceability system allowing the identification of the different Italian chicken breeds and the commercial valorization of their meat products. One of the final aims of our research is to develop a chicken DNA barcoding method based on simple PCR-detectable markers designed on repetitive regions and transposable elements. SCAR analysis seems not reliable for genetic traceability since a given discriminant marker isolated by AFLP-based systems proved to be not reproducible. This can be due to

the presence of single nucleotide polymorphisms at the restriction site level not detectable using clone-specific primers. Moreover, the sequencing of SCAR markers of the different breeds underscored the presence of SNPs and IN/DELs both in terminal and internal regions of the clones. The sequence information was used to define breed-specific haplotypes to discriminate individuals belonging to a given autochthonous population. Results on breed-specific haplotypes obtained for each clone, although very promising, need to be deeply investigated and finally validated increasing the number of animals for single breed.

It is interesting to note that breed-specific markers were shown to be highly similar to genic regions of known chromosome position. Most of the breed-specific clones isolated has shown homology with genes implicated in metabolic processes. For instance, the clone #39 scored high similarity with an acetyl-CoA synthetase whereas clone #50 with a mitochondrial carrier protein. Both proteins are molecules utilized in various metabolic pathways, including fatty acid and cholesterol synthesis. The implementation of a genomic database for Italian chicken local breeds, including many more molecular markers conserved within breeds and polymorphic among breeds as well as molecular markers useful to discriminate local breeds from commercial broilers will be one of the future goals of our project. An increasing number of agro-alimentary companies provides for an internal traceability system, though it is necessary to document the whole food product chain from the producers to the consumers. The European Union, with the approval of the Reg. CE no. 178, 28.01.2002, makes obligatory from the January 1st, 2005 the traceability of any food item, as the possibility to reconstruct and recover the pathway followed by a given product across all the steps of production, transformation and distribution. The traceability must be referred to every single product portion. As a consequence, for specific products like, for instance, the avian carcasses which reach the consumer as disjuncted parts, the setting of a molecular marker-based genetic traceability system is extremely useful. Such a molecular system is the only one that can offer, at any time of the food product chain, the possibility to assess the origin and reveal the nature of meat products, with a very low possibility of errors, as instead can happen with the traditional labelling system.

Acknowledgements

This work was supported by grant from Veneto Agricoltura with regional project CO.VA. titled “Piano regionale di intervento per la conservazione e la valorizzazione di razze avicole locali”. The authors wish to thank Chiara Targetta e Chiara Dalvit for providing genomic DNA samples of local chicken breeds.

An extended version of the paper will be published elsewhere.

References

Altschul S.F., W. Gish, W. Miller, E.W. Myers, D.J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Barcaccia G., Albertini E., Tavoletti S., Falcinelli M. and Veronesi F. (1999). AFLP fingerprinting in *Medicago* spp.: its development and application in linkage mapping. Plant Breed., 118: 335-340.

Barcaccia G., Albertini E. and Falcinelli M. (1999). AFLP fingerprinting in *Pelargonium peltatum*: its development and potential in cultivar identification. J. Hort. Sci. Biotech., 74(2): 243-250.

Cassandro M., De Marchi M., Targhetta C., Dalvit C., Ramanzin M., Baruchello M. (2004). An in situ marker-assisted conservation scheme of 11 Italian avian breeds. 55th Anual meeting of the European Association for Animal Production (EAAP), Bled. 5-9 September. (vol. vol. Book of abstracts No. 10, pp. 30). ISBN/ISSN: 9076998450/1382-6077. Wageningen Academic, Publishers.

Cassandro M., Targhetta C., De Marchi M., Dalvit C., Barcaccia G. and Bittante G. (2005). DNA fingerprinting characterization of four italian poultry species. Proc. of Plant and Animal Genome XIII Conference. January 15-19, 2005, Sherago Intl., San Diego, CA (USA), p. 210.

Coullin P., B. Bed'Hom, J. J. Candelier, D. Vettese, S. Maucolin, S. Moulin, S. A. Galkina, A. Bernheim & V. Volobouev (2005). Cytogenetic repartition of chicken CR1 sequences evidenced by PRINS in Galliformes and some other birds. Chromosome Research 13:665–673.

De Marchi M., Cassandro M., Targhetta C., Baruchello M. and Notter D.R. (2005). Conservation of poultry genetic resource in the Veneto region of Italy. Animal Genetic Resources (in press).

Fumihito A., T. Miyake, M. Takada, R. Shingu, T. Endo, T. Gojobori, N. Kondo., S. Ohno (1996). Monophyletic origin and unique dispersal patterns of domestic fowls. Proc. Natl. Acad. Sci USA, 93:6792-6795.

Higgins D., J. Thompson, T. Gibson, J.D. Thompson, D.G. Higgins, T.J. Gibson (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

Jaccard P. (1908). Nouvelles recherches sur la distribution florale. Bull. Soc. Vaudoise Sci. Nat. 44:223-270.

Nei M. (1973). Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA, 70:3321-3323.

Nei M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89:583-590.

Sambrook J., E. F. Fritsch, T. Maniatis (1989). Molecular cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Soattin M., Barcaccia G., Cassandro M., Targhetta C., Dal Vit C. and Bittante G. (2005). Chicken DNA fingerprinting with M-AFLP and S-SAP markers designed on satellite genomic regions. Atti del 49° Convegno Società Italiana di Genetica Agraria, Potenza, 12-15 Settembre 2005. Oral Pres. 2.05.

Vanhala T., M. Tuiskula-Haavisto, K. Elo, J. Vilkki, A. Maki-Tanila (1998). Evaluation of genetic variability and genetic distances between eight chicken lines using microsatellite markers. Poultry Sciences, 77:783-790.

Vos P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, , M. Kuiper, M. Zabeau (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407 - 4414

Wicker T., J. S. Robertson, S. R. Schulze, F. A. Feltus, V. Magrini, J. A. Morrison, E. R. Mardis, R. K. Wison, D. G. Peterson, A. H. Paterson, R. Ivarie (2005). The repetitive landscape of the chicken genome. Genome Research 15:126-136.

Wimmers K., S. Ponsuksili, T. Hardge, A. Valle-Zarate, P. K. Mathur, P. Horst (2000). Genetic distinctness of African, Asian and South American local chickens. Animal Genetics, 31: 159-165.

Zanon A., A. Sabbioni (2001). Identificatione e salvaguardia genetica delle razze avicole italiane. Annali Facoltà di Veterinaria, Università di Parma, 21:117-134.

Zhang X., F. C. Leung, D. K. O. Chan, G. Yang, C. Wu (2002). Genetic diversity of Chinese native chickens breeds based on protein polymorphism, randomly amplified polymorphic DNA, and microsatellite polymorphism. Poultry Science, 81:1463-1472.