Genomic DNA fingerprinting of indigenous chicken breeds with molecular markers designed on interspersed repeats

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In Italy more than fifty different local breeds of chicken (*Gallus gallus* L.) are known to have been present in the past. The overall situation is now critical since most of these breeds are becoming extinct or threatened and only a few are subject of conservation plans. The use of molecular markers for the analysis of chicken populations could help in characterizing their genetic variation and preserving them from genetic erosion. Valuable and irreplaceable sources of chicken germplasm from indigenous populations of the Veneto Region were analyzed by means of DNA fingerprinting with molecular markers designed on interspersed mini- and microsatellite repeats. The identification of either among-breed discriminant or 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 (Ermellinata, Padovana, Pépoi, Polverara, Robusta Lionata and Robusta Maculata) and one commercial line used as reference standard. A number of variation statistics were computed to assess the genetic variability within and relatedness among breeds: the effective number of alleles per locus ($n_e = 1.570$), total and single-breed genetic diversity ($H_T = 0.366$ and $H_S = 0.209$, respectively) and the fixation index ($G_{ST} = 0.429$). The mean genetic similarity coefficients within and between local breeds were 0.769 and 0.628, 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. Analysis of the population structure along with individual assignment tests successfully identified all breed clusters and subclusters. The vast majority of animals were correctly allocated to their breed of origin, demonstrating the suitability and reliability of the chosen AFLP-derived marker systems for detecting population structure and tracing individual breeds. The local breeds have been preliminarily identified according to sequence-specific SNPs and haplotypes and the polymorphism information content of genomic AFLP-derived markers is reported and critically discussed.

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The domestic chicken (*Gallus gallus*) provides a main protein source for most human populations throughout the world. Its economic importance has made it the focus of numerous research projects, including a recent effort to sequence the entire chicken genome (<http://genome.wustl.edu/projects/chicken>). The vast majority of the sequence has been anchored to chromosomes, represented by 28 pairs of autosomes 1–24, 26–28 and 32, and two sex chromosomes W and Z (International Chicken Genome Sequencing Consortium, for details see HILLIER et al. 2004).

The relatively small genome of chicken (1200 Mbp) 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 if 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 in 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). The microsatellite elements are usually less than 200 bp long and are very useful for population genetics and evolution dynamics, as well as phylogenetic studies because of their high repeatability among laboratories, informativeness of sequences and degree of polymorphisms.

Genomic DNA sequences of the interspersed hypervariable repetitive elements are considered very useful to set up analysis systems of forensic genetics for a reliable

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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 (Vos et al. 1995; for a review on 10 years use of AFLP technology see Botton et al. 2008). 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 hybridizes 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, within or close to functional genes (Hillier et al. 2004; Wicker et al. 2005). 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).

Molecular markers are known as a particularly effective and reliable tool for the characterization of genomes and the investigation of gene polymorphisms in most crop and livestock species (Barcaccia et al. 1999a; Dekkers and Hospital 2002; Albertini et al. 2003; Lanteri and Barcaccia 2006). In particular, molecular markers can be used 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 programmes can be optimized (Barcaccia 2009). 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 (Vanhala et al. 1998; Wimmers et al. 2000; Porceddu et al. 2002; Zhang et al. 2002; Targhetta et al. 2003; Cassandro et al. 2005). The potentials of AFLP markers for the assessment of chicken diversity have been reported by several authors (De Marchi et al. 2003, 2006; Cassandro et al. 2004; Soattin et al. 2007). SSR and SNP markers have also been applied for investigating genetic variation within and differentiation among chicken breeds (Hillel et al. 2003; Twito et al. 2007). The analysis based on SSR markers resulted in highly discriminant banding patterns and significant clustering results due to their multi-allelic origin and polymorphism information content (Hillel et al. 2003). Nevertheless, SNP markers located in gene regions revealed advantages in terms of genome coverage, and proved to be an efficient molecular tool for estimating genetic distinctiveness and relatedness in chicken populations (Twito et al. 2007).

There are numerous known poultry breeds in Italy. More than 90 distinct breeds were recently identified, of which 53 are chickens. The overall situation of these breeds is nevertheless critical since as many as 61% are on the verge of extinction, 13% are threatened and only 7% are the subject of conservation plans (Zanon and Sabbioni 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 programmes in order to breed highly productive lines able to perform and adapt themselves better in a specific territory. The conservation of a consistent biodiversity of the species is mainly owed to such activity (Fumihito et al. 1996). With the abandoning of farming in marginal areas and the advent of chicken breeding at industrial level, highly specialized lines have been developed and commercialized, which now supply the vast majority of the chicken meat and egg market. This major change is placing most local breeds at risk of extinction, particularly those which have low productivity and so are less competitive than commercial broilers. Local breeds of chicken are usually considered to be 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 (Zanon and Sabbioni 2001; De Marchi et al. 2005). In addition, local breeds possess unique gene pools with private alleles generally not found in commercial lines (Granevitze et al. 2007). These locally-adapted alleles represent an irreplaceable bank of highly co-adapted genotypes. Furthermore, local breeds are known to be hardy and possess many resistance traits to environmental and biotic stresses, characteristics that make them of particular interest for raising on organic farms, the utilization of marginal lands and niche productions. Native breeds could be also exploited as an alternative to commercial broilers in the case of epidemics. For all these reasons, they have recently been the subject of protection and maintenance schemes, as well as of studies aimed at the characterization of their gene pools.

This paper deals with the development of innovative molecular systems of population genomics for chicken DNA fingerprinting based on the M-AFLP and S-SAP analyses of repetitive sequence families (both microsatellites and minisatellites) with the aim of genetically characterizing

local breeds and cloning breed-discriminant or breed-specific markers. The identification of novel SNPs is also reported and the polymorphism information content of genomic AFLP-derived markers critically discussed.

MATERIAL AND METHODS

Animal populations

Twelve morphologically representative individuals for each of the six indigenous chicken breeds being investigated were used for genomic DNA fingerprinting together with as many individuals of 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. Additional 62 and 86 animals belonging to the Polverara and Pépoi breeds, respectively, were also used for SNP analysis.

The animals were reared in three flocks in different parts of the Veneto Region, Italy, and their morphological characteristics were previously described by De Marchi et al. (2005). The population sizes of the indigenous breeds have been estimated as 1500 individuals for Ermellinata, Pépoi, Robusta Lionata and Maculata, and 2000 for Padovana and Polverara. For each breed, the conservation scheme is based on units of 34 pure females, with 20 males that rotate between the units (Cassandro et al. 2004).

Molecular markers

Nucleic acids were extracted from whole blood through cell lyses according to De Marchi et al. (2006) with minor changes. After purification from RNA residuals and proteins using RNase and ammonium acetate, respectively, each sample of genomic DNA was precipitated with isopropanol and washed twice with 70% ethanol. All DNA pellets were then vacuum dried and redissolved in TE buffer (Sambrook et al. 1989). The concentration of DNA samples was determined by optical density readings at 260 nm and their purity calculated by the OD_{260}/OD_{280} ratio and OD_{210}–OD_{310} pattern (Sambrook et al. 1989). An aliquot of each genomic DNA was also assayed by electrophoresis on 1% agarose gel.

The detection of polymorphisms on repetitive sequences, such as SSR and CR1 elements, was based on the S-SAP and M-AFLP systems, derived from the more widely known AFLP technology (Vos et al. 1995). 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 annealed 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 SSR-anchored primers.*

Primer	Sequence (5'–3')
*Eco*RI + A	GACTGCGTACCAATTCA
*Taq*I + A	GATGAGTCCTGACCGAA
CR1-D1F	TAGTAAATGGGGATGTTGGT
CR1-D2F	TGATCCTCGAGGTCCCTTCC
CR1-S1R	AGCAGCCTTCTGGACCTCTT
CR1-S2R	CAGCAACACTTCACCTCTGG
CR1-InF	AGTTCATGATCTCAAGGGATGTGGGCC
CR1-InR	CAGCCCCCTGATCATCTTTGTGGCCCT
ISSR-6	$(CA)_8GC$
ISSR-13	$CAG(CA)_8$
ISSR-33	$(AGC)_4T$
ISSR-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. (1999b) and De Marchi et al. (2003). 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 corresponding 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 SSR-anchored) was used for the final amplification along with an AFLP primer (*Eco*RI + A o *Taq*I + A). Each 20 µl PCR reaction contained 5 µl of the pre-amplified DNA, 0.2 mM of labelled specific primer and unlabelled AFLP primer, 2 µl of 10× PCR buffer, 0.2 mM dNTPs and 0.4 U of *Taq* DNA polymerase (GE Healthcare Life Sciences). 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 h exposure at −80°C with intensifying screens.

Statistical analysis: population structure and individual assignment tests

A preliminary 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 different breeds or, equivalently, the fixation index (G_{ST}) as well as the rate of gene flow (Nm). The allele frequency over all marker loci was then 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 Dice (1945). UPGMA dendrograms and centroids were constructed using the genetic similarity and diversity matrices. All calculations and analyses were conducted using the software POPGENE (Yeh et al. 1997) and NTSYS (Rohlf 1993).

The software STRUCTURE (Pritchard et al. 2000) was used to analyze the genetic structure of the population and to perform an assignment test on the studied individuals. This program implements a model-based clustering method for inferring population structure using genotype data of unlinked markers. Here it was also applied to assign individuals to each subpopulation or cluster. All AFLP-derived amplicons were treated as haploid markers as suggested by Negrini et al. (2007). Analyses were performed using the admixture model with correlated marker allele frequencies. To choose the appropriate number of inferred clusters to model the data, 2 to 12 inferred clusters were performed with 5 independent runs each as suggested by Pritchard et al. (2000) and by other authors (Álvarez et al. 2004; Glowatzki-Mullis et al. 2006). All computations used a burn-in period of 50 000 and 100 000 iterations for data collection.

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 instructions. Plasmid sequences of both strands were performed by the dideoxynucleotide chain reaction termination method using either the M13 forward or reverse primer.

Bioinformatics

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 nucleotide and translated sequences, respectively. A BLAST analysis of clone sequences against the chicken (*Gallus gallus*) genome sequences was also performed. Retrievals enabled given sequences to be attributed to specific chromosomes and to eventually acquire information on their putative function according to the best hits. All nucleic acid sequences were deposited in the NCBI databases and recorded in our DNA sequence collections, 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 different animals were aligned to test the veracity of each sequence and to recover their consensus sequence by using the Vector NTI program (Invitrogen). Sequences of SCAR (sequence characterized amplified region) markers from all chicken breeds were used for multiple sequence alignments in the CLUSTALW program (Higgins et al. 1992) to find SNP (single nucleotide polymorphisms) and any IN/DEL (insertions/deletions), and to attempt the identification of breed-specific haplotypes.

SCAR and SNP analyses

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 (Marshall 2004; freely available at <http://perlprimer.sourceforge.net/>) and hence converted into SCAR markers. For each sequence, the analysis of breed-specific SNPs was performed by designing primers with their 3 -end localized on the discriminant point mutation site. 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 $MgCl_2$, 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 in 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 gel and photographed (DC120 camera, Kodak) after staining with ethidium bromide.

The identification of individuals belonging to single local breeds using AFLP-derived SNP markers was based on

larger chicken populations. In particular, clones no. 38 and no. 56 selected as specific to the Polverara and Pépoi breeds were tested on 62 and 86 genomic DNA samples, respectively.

RESULTS

Genetic characterization of indigenous chicken breeds by S-SAP and M-AFLP markers

The detection of sequence repeats for fingerprinting 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 anneals 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 enabled 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 Fig. 1.

Each selected primer combination generated DNA fingerprints showing, on average, from a minimum of 40 to a maximum of 80 fragments. 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 internal 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–D2F/TaqI + A, respectively. Polymorphic molecular markers that proved to be useful for the traceability of chicken breeds (i.e. both among-breed discriminant and breed-specific markers)

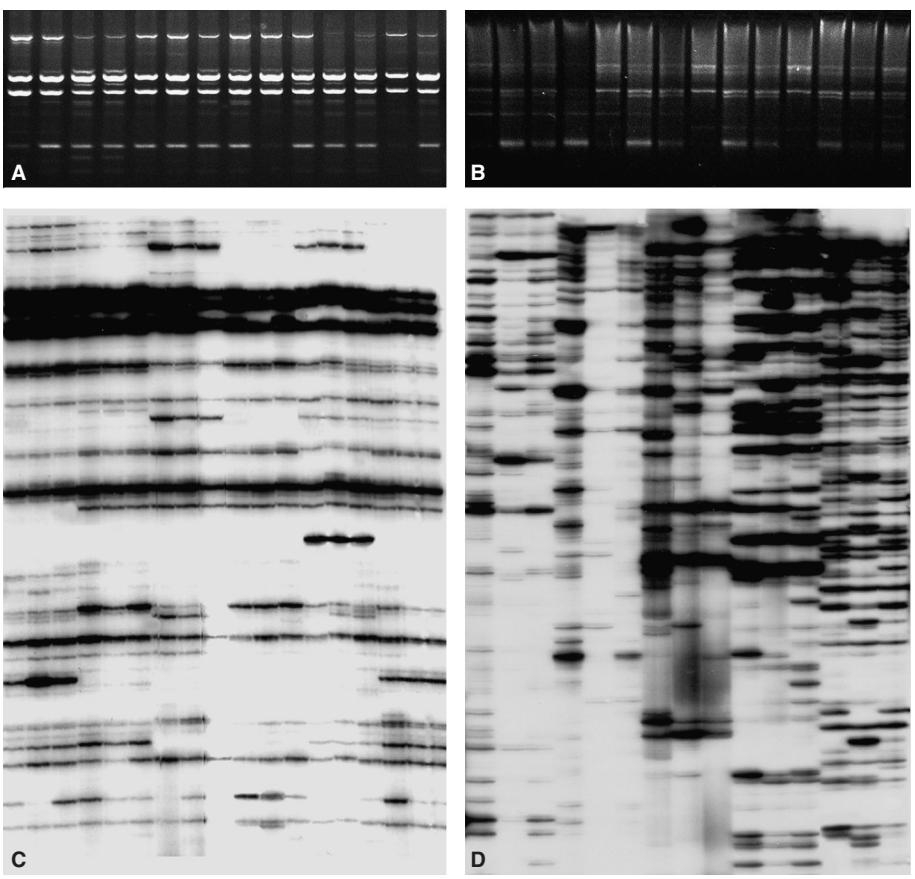

Fig. 1A–D. Results of primer testing by means of nested PCR experiments using AGC microsatellite-anchored (**A**) and CR1-internal core (**B**) primers. Example of genomic DNA fingerprints generated by M-AFLP (**C**), using three individual DNA samples from Ermellinata, Padovana, Pépoi, Polverara, Robusta Lionata and Robusta Maculata breeds (from left to right), and by S-SAP experiments (**D**), using five different primer combinations and a random sample of animals as genomic DNA template.

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 allowed us to verify the presence of the specific SSR motif or the partial CR1 element (Appendix 1 Fig. 1S), thus demonstrating the specificity of the amplification products and, hence, the reliability of the fingerprinting techniques used.

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 for M-AFLP markers ($H_T = 0.334$) and S-SAP ($H_T = 0.381$) markers, whereas the mean genetic diversity of single breeds was lower for M-AFLP than S-SAP, being $H_S = 0.162$ and $H_S = 0.232$, respectively. Considering all markers together, the total genetic diversity of the species as a whole was equal to $H_T = 0.366$, while the mean genetic diversity within local breeds was $H_S = 0.209$, ranging from 0.165 to 0.236 (Table 2). Fixation index was around 51% for M-AFLP and 39% for S-SAP, suggesting that the local breeds conserved their gene pools well separated over time. The overall value of $G_{ST} = 0.429$ revealed that about 43% of the total genetic variation found within the species is attributable to genetic polymorphisms among local breeds. Moreover, the estimate $Nm = 0.674$ is evidence of little gene flow among populations (for details see Table 2).

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. Variation measured by comparing DNA fingerprints resulted in mean genetic similarity coefficients of 0.769 and 0.628 within and between local breeds, respectively (for the coefficients related to single breeds and pair-wise comparisons of breeds see Appendix 1 Table 1S). Figure 2 shows centroids of single animals plotted using the two principal coordinates separately for M-AFLP and S-SAP markers, which overall accounted for about 37% of the total genetic variance.

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. On the basis of either M-AFLP or S-SAP markers, certain breeds were distinct from each other, whereas others were more similar. In particular, the broiler and Robusta Lionata and Robusta Maculata breeds clustered together separately from the other local breeds. Moreover, Pépoi and Polverara breeds were clearly separated from each other, while Padovana and Ermellinata di Rovigo were closely grouped. The Ermellinata di Rovigo individuals were plotted differently according to the set of molecular markers (Fig. 2). The main distribution differences observed with M-AFLP and S-SAP markers can be explained by considering the different chromosome regions assayed by the two molecular marker systems, 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. It is interesting to note that, on the basis of the whole set of markers, the commercial broiler showed the lowest genetic differentiation estimate (0.094).

The commercial broiler scored the lowest Nei's genetic distance with the Padovana breed (0.178), whereas the Polverara and Ermellinata breeds proved to be the most genetically distant from the other local germplasm resources (see also Appendix 1 Fig. 2S).

Analysis of molecular marker data performed by STRUCTURE software identified the minimum number of subgroups required to explain the total genetic diversity observed in the population as a whole. A total of eight clusters were identified in the total population, each one corresponding to a single breed, except for cluster 3 and 4 (Table 3). In fact, the Padovana breed proportions of membership were rather high for these two clusters reaching, altogether, more than 0.90. It is worth mentioning that the Padovana animals analyzed here belonged to two different varieties: Dorata and Camosciata, depending on the colour of their feathers. Actually, all

Table 2. *Mean Nei's genetic diversity and differentiation statistics, and gene flow estimates for single breeds and over all breeds.*

	N_{pl} (%)	n_e	H_T	H_S	D_{ST}	G_{ST}	Nm
Ermellinata	31 (52.5)	1.376		0.210	0.156	0.426	0.673
Padovana	38 (64.4)	1.402		0.236	0.130	0.355	0.908
Pépoi	27 (45.8)	1.304		0.172	0.194	0.530	0.443
Polverara	35 (59.3)	1.345		0.201	0.165	0.451	0.609
Robusta Lionata	24 (40.7)	1.299		0.165	0.201	0.549	0.410
Robusta Maculata	34 (57.3)	1.374		0.214	0.152	0.415	0.704
Broiler	41 (69.5)	1.474		0.272	0.094	0.257	1.447
Overall	59	1.637	0.366	0.209	0.157	0.429	0.674
Standard deviation	6	0.308	0.172	0.011	0.037	0.099	0.353

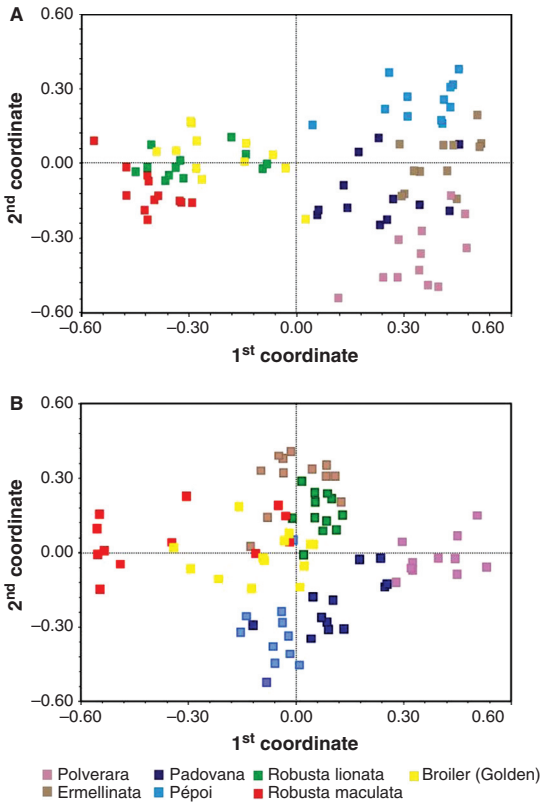

Fig. 2A–B. Centroids identified from total Dice's genetic similarity matrices using polymorphisms obtained by M-AFLP (**A**) and S-SAP (**B**) molecular markers.

Dorata variety animals were identified in cluster 3, whereas Camosciata ones were grouped in cluster 4, showing the ability of the chosen markers to detect this substructure. The average proportion of membership of each breed to the cluster it belongs to was always greater than 0.90, with the exception of Robusta Maculata (0.88).

Results of the individual assignment test, considering different thresholds, are shown in Table 4. Most of the animals were correctly assigned to their breed of origin. A total of about 79% of the samples were correctly allocated considering a threshold of 90%. Pépoi and Robusta Lionata evidenced the best results with more than 90% of individuals correctly assigned at 90% probability rate.

Polymorphism information content of discriminant AFLP-derived markers

The sequence of the most among-breed discriminant markers as well as the sequence of breed-specific markers were used as queries for public database interrogations. Chicken genome retrievals revealed significant similarities with either genic or intergenic sequences of known chromosome position and primary structure homologies with putative or known gene products (Table 5). All nucleotide sequences recovered from the indigenous chicken breeds as AFLP-derived markers were deposited in the NCBI databases (accession no. EF417921–EF417932).

In an attempt to set up a reliable PCR-based molecular reference system suitable for the precise identification of the single breeds, sequence-tagged site primers were designed on the 12 most discriminant clones in order to convert the among-breed discriminant and breed-specific M-AFLP and S-SAP markers into easily detectable non-radioactive SCAR markers. The sequence of forward and reverse primers is reported in Table 6. When these primers were used in PCR experiments with chicken genomic DNA as templates, in most cases amplification products were shown to be shared among individuals over all breeds and thus not useful for discriminating single breeds (data not shown). 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. Nevertheless, certain polymorphisms could be generated using very stringent PCR conditions, but with a few exceptions they proved to be not fully reliable. The most robust result with SCAR markers was obtained for the clone no. 38 preliminarily selected as specific to the Polverara breed. When the corresponding SCAR primers were used to analyze genomic DNA samples from all breeds, two distinct amplification products of 307 and 333 bp were detected. The upper marker proved to be shared among the

Table 3. *Proportion of membership of each of the seven chicken breeds in each of the eight inferred clusters.*

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Cluster 8
Ermellinata di Rovigo	0.016	0.014	0.009	0.009	0.008	0.923	0.015	0.007
Padovana	0.039	0.011	0.532	0.378	0.012	0.006	0.014	0.007
Pépoi	0.935	0.004	0.006	0.010	0.004	0.031	0.004	0.006
Polverara	0.010	0.006	0.014	0.019	0.004	0.006	0.934	0.007
Robusta Lionata	0.007	0.006	0.004	0.007	0.008	0.006	0.004	0.958
Robusta Maculata	0.005	0.010	0.005	0.006	0.879	0.005	0.006	0.083
Broiler	0.007	0.912	0.027	0.012	0.017	0.008	0.007	0.009

Table 4. *Percentage of individuals correctly assigned to their breed of origin considering different threshold in each chicken breed.*

	>99%	>95%	>90%	No threshold
Ermellinata di Rovigo	0	41.7	75.0	100
Padovana	0	25.0	75.0	100
Pépoi	0	66.7	91.7	100
Polverara	0	58.3	75.0	100
Robusta Lionata	0	83.3	91.7	100
Robusta Maculata	0	50.0	66.7	100
Broiler	0	50.0	75.0	100
Total	0	53.6	78.6	100

vast majority of individuals over all local breeds and the commercial broiler, whereas the lower marker was detected only in the Polverara individuals (Fig. 3), with a relative frequency of 52% (32 individuals out of 62). It is interesting to note that the two marker sequences differed only for an insertion/deletion of 26 bp in length found in the internal region. Owing to their high nucleotide sequence similarity (92%) and apparent co-segregational patterns in the population (i.e. the longer, the shorter or both amplification products scored over all assayed DNA samples), a total of 62 Polverara individuals were molecularly characterized to study the marker allele frequencies and to verify the allelic relationships between marker alleles. The three possible genotypes at the marker locus being tested were found in the following relative proportions: $M_{303}M_{303}=0.500$, $M_{303}M_{307}=0.403$ and $M_{307}M_{307}=0.097$. Since the observed proportions proved to be not statistically deviating from the proportions expected in the Hardy-Weinberg equilibrium ($^2=0.0919$), the two amplification products of 307 bp and 333 bp can be most likely considered as alleles of the same gene. On the basis of GeneBank retrievals, the core sequence of clone no. 38 confirmed a significant similarity with the gene encoding for a hypothetical protein of locus NW_001471459 located on chicken chromosome 15.

To verify the occurrence of single nucleotide polymorphisms in the selected clones, as main source of AFLP polymorphisms, several amplification products yielded with each of the SCAR primer combinations were recovered from the gels, subcloned into plasmid and sequenced. A multiple alignment of the consensus sequences of each of the 12 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 preliminarily adopted for the recognition of single breeds (Table 7).

In clone no. 38, the detection of a SNP at nucleotide position 67 was useful not only to test the reliability of the IN/DEL polymorphism found in the cosegregating sequence, but also to confirm its utility for the identification of individuals belonging to the Polverara breed. As expected, a forward SNP site-specific primer, designed with its discriminant 3 -end in the point mutation position (p#38_SNP_{67}: GCTATTAGATGAAGTGAAAATATATAC), yielded a doublet of amplicons when used in combination with a reverse SCAR marker-specific primer. The nucleotide sequences of the two markers confirmed complete identity except for the 26 bp insertion/deletion, and the segregation patterns proved to fully match the segregation ratios previously scored as SCAR analysis (Fig. 3). It is worth mentioning that in clone no. 38 two different SNPs showed either a homozygote or heterozygote state in some of the analyzed individuals. For instance, at nucleotide position 67 individuals with CC, TT or CT were observed (Fig. 4).

For the identification of the Pépoi individuals a number of breed-specific SNPs were discovered, for instance, in clones no. 39, 50 and 56. In particular, each of two different SNPs found in the sequence of clone no. 56 proved to be useful for the traceability of individuals belonging to this breed. For the detection of single nucleotide polymorphisms, a forward SNP site-specific primer was designed with its discriminant 3 -end in the point mutation position (p#56_SNP_{69}: GGAGGTTCCCAAGCCCG and p#56_SNP_{95}: CTGTAGAGTTTTCAGCC). When either of these two primers was used in combination with a common reverse SCAR marker-specific primer, a single amplicon was specifically detected in most of the individuals of the Pépoi breed (Fig. 3). However, the same primer combinations yielded a second amplification product, shorter in length and with fainter signal intensity, which was shared by all individuals tested of the Padovana and Polverara breeds. The upper marker of 157 bp was scored in 67 individuals (78%) out of the total 86 analyzed, whereas the remaining 19 (22%) showed the lower marker of 105 bp, as did all Padovana and Polverara individuals analyzed. This finding supports the occurrence of gene flow among Padovana, Polverara and Pépoi populations for an allele encoding for a hypothetical protein (locus NW_001471503).

DISCUSSION

Molecular markers have revolutionized our ability to characterize genetic variation and rationalize genetic selection, being effective and reliable tools for the analysis of genome architectures and gene polymorphisms in animals (Lanteri and Barcaccia 2006). Until now, the area of chicken genomics that has seen the greatest development with the use of molecular marker technology is that of population genetics. For instance, SSR and AFLP markers have been exploited for assessing genetic diversity in

Table 5. *List of breed-specific chicken genomic clones along with information on NCBI accession number, chromosome number, locus ID, physical map position and orientation (+ or – strand), sequence type and deduced amino acid homology (best BLAST hits).*

Sequence		Physical map				
Clone no.	Accession no.	Chr.	Locus ID	Position	Type	Gene product (best BLAST hit)
37	EF417921	1	NW 001471534	10290143 +	Intergenic	5' *Taeniopygia guttata* Norrie disease (pseudoglioma) isoform 2 – 3' *Gallus gallus* monoamine oxidase B
38	EF417922	15	NW 001471459	3943690 +	Genic	*Homo sapiens* transmembrane protein 132C (TMEM132C)
39	EF417923	3	NW 001471677	3659414 –	Genic	*Gallus gallus* acetyl-CoA synthetase 2
40	EF417924	2	NW 001471654	8491475 +	Intergenic	5' *Gallus gallus* syntrophin beta 1–3' *Gallus gallus* hyaluronan synthase 2
42	EF417925	3	NW 001471671	18742762 –	Genic	Sorting nexin 14
44	EF417926	2	NW 001471639	24522854 +	Intergenic	5' *Gallus gallus* hypothetical protein – 3' *Gallus gallus* neuropilin- and tolloid-like protein 1
48	EF417927	4	NW 001471687	17083920 –	Genic	*Gallus gallus* fibroblast growth factor receptor-like 1
50	EF417928	1	NW 001471525	1136390 +	Genic	*Taeniopygia guttata* mitochondrial carrier protein MGC4399
51	EF417929	26	NW 001471609	2035491 +	Genic	*Gallus gallus* potassium voltage-gated channel, Shal-related subfamily
52	EF417930	1	NW 001471534	4215433 –	Genic	*Gallus gallus* interferon alpha/beta/omega receptor 1
55	EF417931	9	NW 001471743	3544570 +	Intergenic	5' *Gallus gallus* Cek6 protein – 3' *Gallus gallus* alpha-1,4-N-acetylglucosaminyl-transferase
56	EF417932	17	NW 001471503	1639854 +	Genic	*Homo sapiens* nasal embryonic LHRH factor (NELF)

chicken breeds (Hillel et al. 2003; De Marchi et al. 2005). Although not yet investigated at population level, SNP markers should also be very useful for surveying genetic variation and differentiation in poultry breeds (Wong et al. 2004). One strategy for genetic variability and traceability studies could be based on the identification of informative AFLP-derived markers and exploitation of the SNPs contained in their sequences as source of among-breed discriminant or breed-specific polymorphisms.

Our results show that the S-SAP and M-AFLP marker systems are suitable to visualize reproducible multi-locus DNA fingerprints of chicken breeds: they can be used for investigating genetic variation within and assessing genetic relatedness among populations on the basis of polymorphic interspersed repeats. Highly informative and discriminant genetic fingerprints were obtained assaying the variation for short tandem repeats (e.g. CA/TG) and longer unique repeats (i.e. CR1) by using repeat-anchored primers in combination with *Eco*RI + A or *Taq*I + A primers. These two AFLP technology-derived 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 in single breeds, genetic distance and gene flow among breeds. In our study, the genetic variation among the indigenous populations was found to be around 43%, meaning that 57% was due to polymorphisms within single populations at the assayed marker loci. Overall, the combination of M-AFLP and S-SAP data and their comparison with previously obtained SSR data (unpubl.) confirmed the high genetic variation detectable within breeds of the Veneto Region and the clear genetic differentiation still present among their gene pools. Our results also revealed substantial differences in the genetic distance estimates among local breeds and the commercial broiler adopted as reference line. 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 on different genomic loci numbers. Additional molecular analyses are needed to corroborate these preliminary results and confirm the statistics related to the genetic diversity within and the phylogenetic relationships among indigenous breeds reared locally in northeastern Italy.

On the basis of our results, the polymorphic markers isolated from genomic interspersed repeats can be applied not only to assess genetic variability estimates of any chicken germplasm resource, but also to develop a genetic traceability system for the identification of the different regional chicken breeds and the promotion of their meat and eggs as niche market products. In fact, the analysis of the population structure along with individual assignment tests successfully identified all breed clusters. In particular,

Table 6. *Forward and reverse sequences of the SCAR primers designed for the most discriminant AFLP-derived clones.*

Clone	Expected size (bp)	Primer sequence (5'–3')
37	486	For TAGTAAATGGGGATGTTGGTGG TGA Rev GAATTCACCAACATCCCCATT
38	307	For TAGTAAATGGGGATGTTGGT CACCA RevGAATTCACATCAATATAAAGCAA
39	300	For TAGTAAATGGGGATGTTGG TGGGAA Rev GAATTCAGCACGTTTCACTACA
40	302	For GAATTCAAACAGACAAAA TAAATG Rev TAGTAAATGGGGATGTTGG TATAAA
42	272	For GAATTCAGTAAGAAAGACCA Rev TAGTAAATGGGGATGTTG GTCACT
44	201	For TAGTAAATGGGGATGTTGG TGTGCA Rev GAATTCACCTGCCTATCAAATT
48	630	For GAATTCAGCTATGGGACCAT Rev AGCAGCAGCAGCTCACACTAA
50	597	For AGCAGCAGCAGCTCCCATTAG Rev GAATTCAGGGAGCTTGCAGA
51	493	For GAATTCACAGGCCTTGGTTC Rev AGCAGCAGCAGCTAGGAAAG
52	422	For GAATTCAGAAGGAATAGCTTTA Rev AGCAGCAGCAGCTGGCAGT
55	295	For AGCAGCAGCAGCTCAGCACAG Rev GAATTCAGGGTTATCATTTCC
56	209	For GAATTCACACAGAAACGTCT Rev AGCAGCAGCAGCTCAGTATGG

six main clusters out of the eight totally inferred were found to correspond to as many individual breeds, with average membership proportions for each breed of around 90%. Two additional smaller clusters identified two varieties of the Padovana breed, with an overall proportion of membership higher than 90%. The vast majority of animals were correctly assigned to their breed of origin (about 79% were correctly allocated considering a probability threshold of 90%), thus demonstrating the suitability and reliability of the chosen AFLP-derived marker systems for detecting population substructure and tracing individual breeds.

The final aims of our research at regional scale are the following: 1) to characterize the gene pools of indigenous chicken breeds using multi-locus DNA-based assays; 2) to generate chicken DNA barcodes using single-locus unique marker tags. Knowledge of genetic variation within local breeds and genetic differentiation among breeds is expected to have a significant impact on the preservation and development of regional chicken germplasm resources. As a matter of fact, AFLP-derived markers anchored to interspersed mini- and microsatellite repeats can be used to find out multiple polymorphisms per assay and to investigate genetic variability levels. SCAR analysis does not seem to reliable for genetic traceability since a given discriminant marker isolated by AFLP-based systems only proved to be reproducible in some cases. This could be due to the presence of single nucleotide polymorphisms at the restriction site level not detectable using clone-specific primers. The sequencing of SCAR markers of the different breeds also underscored the presence of SNPs and IN/DELs both in terminal and internal regions of the clones. The sequence information was used in an attempt to define breed-specific haplotypes to discriminate individuals belonging to a given local population. Preliminary results on breed-specific SNPs and haplotypes obtained for each clone, although very promising, need to be further investigated and validated by increasing the number of animals per breed and by analyzing additional local breeds. Once informative AFLP-derived sequences and discriminant SNPs have been selected and tested, the use of high-throughput methods will be essential to develop a robust PCR-based and low cost genetic traceability system.

Table 7. *Main single nucleotide polymorphims detected by sequencing of the breed-specific clones no. 38, 39, 50 and 56 amplified using SCAR primers (for each clone, the numbers indicate the nucleotide position of the SNP).*

Breed	Clone 38		Clone 39		Clone 50			Clone 56		
	67	251	189	191	23	223	312	51	70	96
Ermellinata	C/T (A)	A	C	T	–	–	–	A	C	T
Padovana	C/T	A	C	T	C	G	G	A	C	T
Pépoi	C/T	A/C	T	C	T	A	A	C	G	C
Polverara	C/T	A/C	–	–	C	A	A	A	C	T
Robusta Lionata	C or C/T	A	C	T	C	A	A	A	C	T
Robusta Maculata	C or C/T	A	C	T	C	A	A	C	C	T
Broiler	C/T (A)	A	C	T	C	G	A	C	C	T

– not determined; ( ) indicates rare nucleotides.

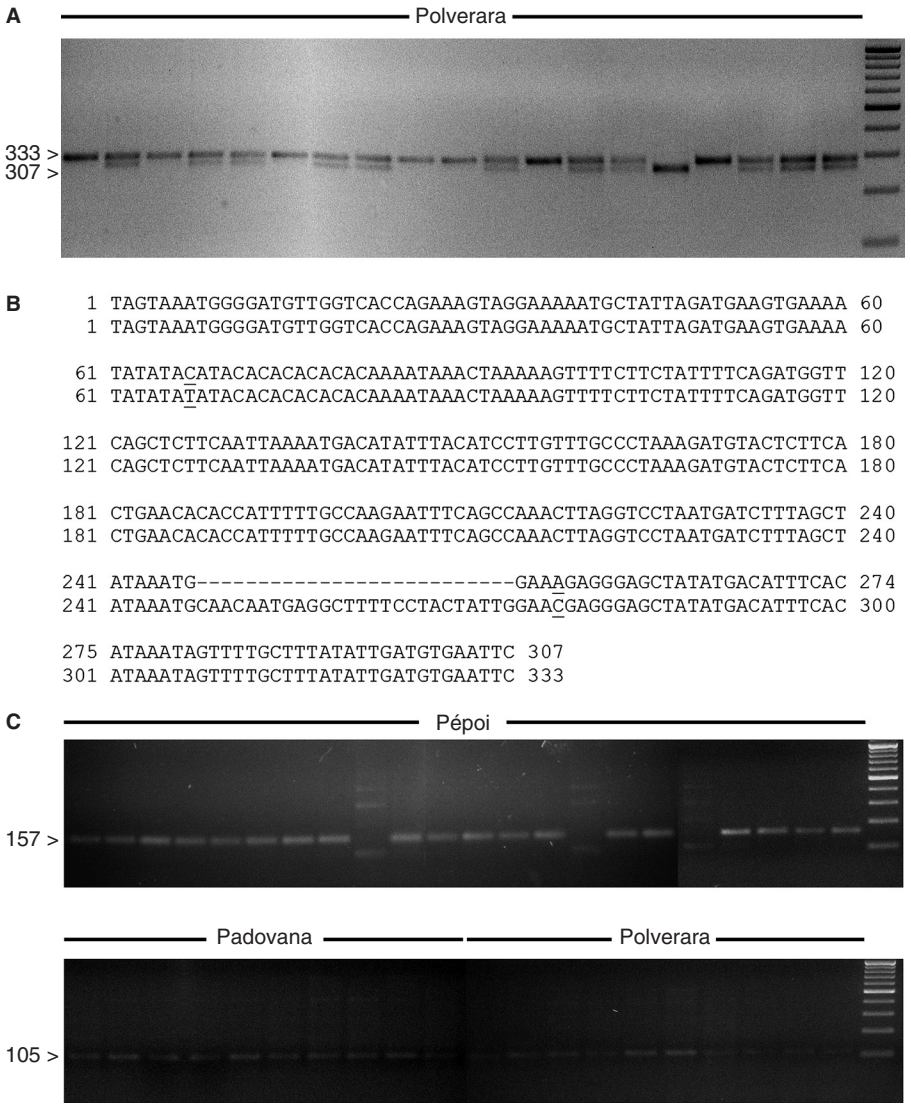

Fig. 3A–C. Amplification products of 333 bp and 307 bp generated with SCAR primers specific for clone no. 38: the lower band is specific to the Polverara breed and 52% of individuals share it (**A**). Simple alignment of the two nucleotide sequences showing a 26 bp-long insertion/deletion: SNP sites at nucleotide positions 67 and 251 are marked by underlined letters. (**B**). Amplification products of 157 bp and 105 bp generated with a forward SNP site-specific primer in combination with a reverse SCAR marker-specific primer: the upper band is specific to the Pépoi breed and is present in 78% of individuals (**C**).

Wong et al. (2004) reported that the majority of SNPs identified are common among most poultry breeds and are highly abundant, their average frequency being equal to 1/200 bp when two chromosomes are compared. The identification of polymorphisms due to single nucleotide substitutions in the AFLP-derived sequences is thus unsurprising if one considers that when a random fragment of genomic DNA is sequenced in a sample of 10 or more animals one SNP will be theoretically identified every 80–100 bp (M. Groenen, pers. comm.). However, our main goal was not to discover additional SNPs, but to assess the type and nature of polymorphisms in the most informative AFLPs visualized in our genomic DNA fingerprints. Indeed, if it is true that almost 3 million SNPs are now available for genetic studies in chicken (Wong et al. 2004), it is also true that no AFLP-derived sequences in chicken are retrievable from the NCBI databases (<http://www.ncbi.nlm.nih.gov/>). Furthermore, in our case-study, the detection of SNPs in among-breed polymorphic and breed-specific S-SAP and M-AFLP markers was preferred to a random investigation of publicly available SNPs: the former were expected to be useful for the genetic traceability of indigenous chicken breeds because preliminarily selected as shared within and polymorphic between breeds, whereas for the latter no information in terms of polymorphism information content was available for local chicken populations.

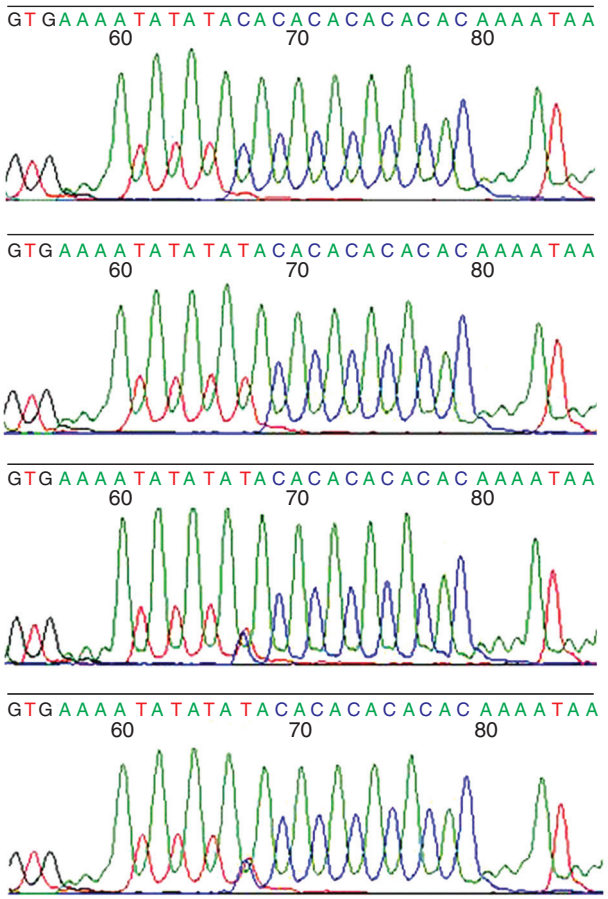

Fig. 4. Chromatograms of clone no. 38 showing individuals in a homozygote (CC or TT) and heterozygote state (CT) at nucleotide position 67.

To the best of our knowledge, this is the first survey of the polymorphism information content of AFLP-derived sequences belonging to chicken breeds. Despite the increasing number of publications based on the use of AFLP markers for genome fingerprinting and gene mapping purposes, as well as for quantitative genetics and population genetics studies, the number of AFLP-derived sequences deposited in the GenBank is very low for animals (less than 100 records!). This means that AFLP markers are exploited by researchers worldwide as anonymous polymorphisms without performing any sequence content analysis or without recording any sequence in public databases.

It is also 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 have shown homology with genes implicated in metabolic processes. For instance, clone no. 39 scored high similarity with an acetyl-CoA synthetase, whereas clone no. 50 with a putative 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 local chicken 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-food companies have an internal traceability system, though it is necessary to document the entire production chain from producer to consumer. The European Union, with Reg. EC no. 178, 28.01.2002, made the traceability of any food item obligatory from 1 January 2005. This is intended as the ability to follow and retrace the movements of any given product through all the steps of production, transformation and distribution, and applies to every single item. A sequence-tagged molecular marker-based genetic traceability system is therefore extremely useful for products such as avian carcasses, which reach the consumer as jointed pieces, being the only one that offers the possibility to access the origin and reveal the nature of meat products at any point in the production chain, thus improving the reliability of traditional labelling systems.

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REFERENCES

Albertini, E., Porceddu, A., Marconi, G. et al. 2003. Microsatellite-AFLP for genetic mapping of complex polyploids. – Genome 46: 824–832.

Altschul, S. F., Gish, W., Miller, W. et al. 1990. Basic local alignments tool. – J. Mol. Biol. 215: 403–410.

Àlvarez, I., Royo, L. J., Fernández, I. et al. 2004. Genetic relationships and admixture among sheep breeds from northern Spain assessed using microsatellites. – J. Anim. Sci. 82: 2246–2252.

Barcaccia, G. 2009. Using molecular markers for characterizing and preserving germplasm resources. – In: Jain, S. M. and Brar, D. S. (eds), Molecular techniques in crop improvement (2nd ed.). Springer, in press.

Barcaccia, G., Albertini, E., Tavoletti, S. et al. 1999a. AFLP fingerprinting in *Medicago* spp.: its development and application in linkage mapping. – Plant Breed. 118: 335–340.

Barcaccia, G., Albertini, E. and Falcinelli, M. 1999b. AFLP fingerprinting in *Pelargonium peltatum*: its development and potential in cultivar identification. – J. Horticult. Sci. Biotechnol. 74: 243–250.

Botton, A., Galla, G., Conesa, A. et al. 2008. Large-scale gene ontology analysis of plant transcriptome-derived sequences retrieved by AFLP technology. – BMC Genomics 9: 347.

Cassandro, M., De Marchi, M., Targhetta, C. et al. 2004. An *in situ* marker-assisted conservation scheme of 11 Italian avian breeds. – Proc. 55th Annu. Meeting Eur. Ass. Anim. Production, September 2004. Bled, Slovenia. p. 30.

Cassandro, M., Targhetta, C., De Marchi, M. et al. 2005. DNA fingerprinting characterization of four Italian poultry species. – Proc. Plant Anim. Genome XIII Conf., January 2005. San Diego, CA, USA, p. 210.

Coullin, P., Bed'Hom, B., Candelier, J. J. et al. 2005. Cytogenetic repartition of chicken CR1 sequences evidenced by PRINS in Galliformes and some other birds. – Chromosome Res. 13: 665–673.

Dekkers, J. C. M. and Hospital, F. 2002. The use of molecular genetics in the improvement of agricultural populations. – Nat. Rev. Genet. 3: 22–32.

De Marchi, M., Targhetta, C., Contiero, B. et al. 2003. Genetic traceability of chicken breeds. – Agric. Conspectus Sci. 68: 255-259.

De Marchi, M., Cassandro, M., Targhetta, C. et al. 2005. Conservation of poultry genetic resource in the Veneto region of Italy. – Anim. Genet. Resour. Inf. 37: 63–74.

De Marchi, M., Dalvit, C., Targhetta, C. et al. 2006. Assessing genetic diversity in indigenous Veneto chicken breeds using AFLP markers. – Anim. Genet. 37: 101–105.

Dice L. R. 1945. Measures of the amount of ecological association between species. – Ecology 26: 297–307.

Fumihito, A., Miyake, T., Takada, M. et al. 1996. Monophyletic origin and unique dispersal patterns of domestic fowls. – Proc. Natl Acad. Sci. USA 93: 6792–6795.

Glowatzki-Mullis, M. L., Muntwyler, J., Pfister, W. et al. 2006. Genetic diversity among horse populations with a special focus on the Franches-Montagnes breed. – Anim. Genet. 37: 33–39.

Granevitze, Z., Hillel, J., Chen, G. H. et al. 2007. Genetic diversity within chicken populations from different continents and management histories. – Anim. Genet. 38: 576–583.

Higgins, D. G., Bleasby, A. J. and Fuchs, R. 1992. ClustalW: a package for performing multiple sequence alignments on a micro-computer. – Comput. Appl. Biosci. 8: 189–191.

Hillel, J., Groenen, M. A. M., Tixier-Boichard, M. et al. 2003. Biodiversity of 52 chicken populations assessed by microsatellite typing of DNA pools. – Genet. Selection Evol. 35: 533–557.

Hillier, L. W., Miller, W., Birney, E. et al. 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. – Nature 432: 695–716.

Lanteri, S. and Barcaccia, G. 2006. Molecular markers based analysis for germplasm preservation. – In: Ruane, J. and Sonnino, A. (eds), The role of biotechnology in exploring and protecting agricultural genetic resources. FAO, Rome. pp. 105–120.

Marshall, O. J. 2004. PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. – Bioinformatics 20: 2471–2472.

Negrini, R., Nijman, I. J., Milanesi, E. et al. 2007. Differentiation of European cattle by AFLP fingerprinting. – Anim. Genet. 38: 60–66.

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.

Pritchard, J. K., Stephens, M. and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. – Genetics 155: 945–959.

Porceddu, A., Alberini, E., Barcaccia, G. et al. 2002 Development of an S-SAP technique based on a LTR-like sequence of *Medicago sativa* L. – Mol. Genet. Genomics 267: 107–114.

Rohlf F. J. 1993. NTSYS-pc numerical taxonomy and multi variate analysis system, ver. 1.8. – State Univ. New York, Stony Brook, NY.

Sambrook, J., Fritsch E. F. and Maniatis T. 1989. Molecular cloning: a laboratory manual (2nd ed.). – Cold Spring Harbor Laboratory Press.

Soattin, M., Barcaccia, G., Cassandro, M. et al. 2007. Development of sequence-specific DNA markers for the genetic traceability of autochthonous chicken breeds. – Plant Anim. Genome XV Conf., January 2007. San Diego, CA, p. 240.

Targhetta, C., De Marchi, M., Cassandro, M. et al. 2003. Use of AFLP markers in poultry genome characterisation. – Proc. 54th Annu. Meeting Eur. Ass. Anim. Production, August 2003. Rome, p. 12.

Twito, T., Weigend, S., Blum, S. et al. 2007. Biodiversity of 20 chicken breeds assessed by SNPs located in gene regions. – Cytogenet. Genome Res. 117: 319–326.

Vanhala, T., Tuiskula-Haavisto, M., Elo, K. et al. 1998. Evaluation of genetic variability and genetic distances between eight chicken lines using microsatellite markers. – Poultry Sci. 77: 783–790.

Vos, P., Hogers, R., Bleeker, M. et al. 1995. AFLP: a new technique for DNA fingerprinting. – Nucleic Acids Res. 12: 3396–3303.

Wicker, T., Robertson, J. S., Schulze, S. R. et al. 2005. The repetitive landscape of the chicken genome. – Genome Res. 15: 126–136.

Wimmers, K., Ponsuksili, S., Hardge, T. et al. 2000. Genetic distinctiveness of African, Asian and South American local chickens. – Anim. Genet. 31: 159–165.

Wong G. K. et al. (Int. Chicken Polymorphism Map Consortium) 2004. A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. – Nature 432: 717–722.

Yeh, F. C., Yang, R. C. and Boyle, T. 1997. POPGENE ver. 1.21. – CIFOR and Univ. of Alberta, Canada.

Zanon, A. and Sabbioni, A. 2001. Identification and protection of Italian poultry breeds. – Ann. Vet. Sci. Fac., Univ. of Parma, n. 21, pp. 117–134.

Zhang, X., Leung, F. C., Chan, D. K. O. et al. 2002. Genetic diversity of Chinese native chickens breeds based on protein polymorphism, randomly amplified polymorphic DAN and microsatellite polymorphism. – Poultry Sci. 81: 1463–1472.

APPENDIX 1

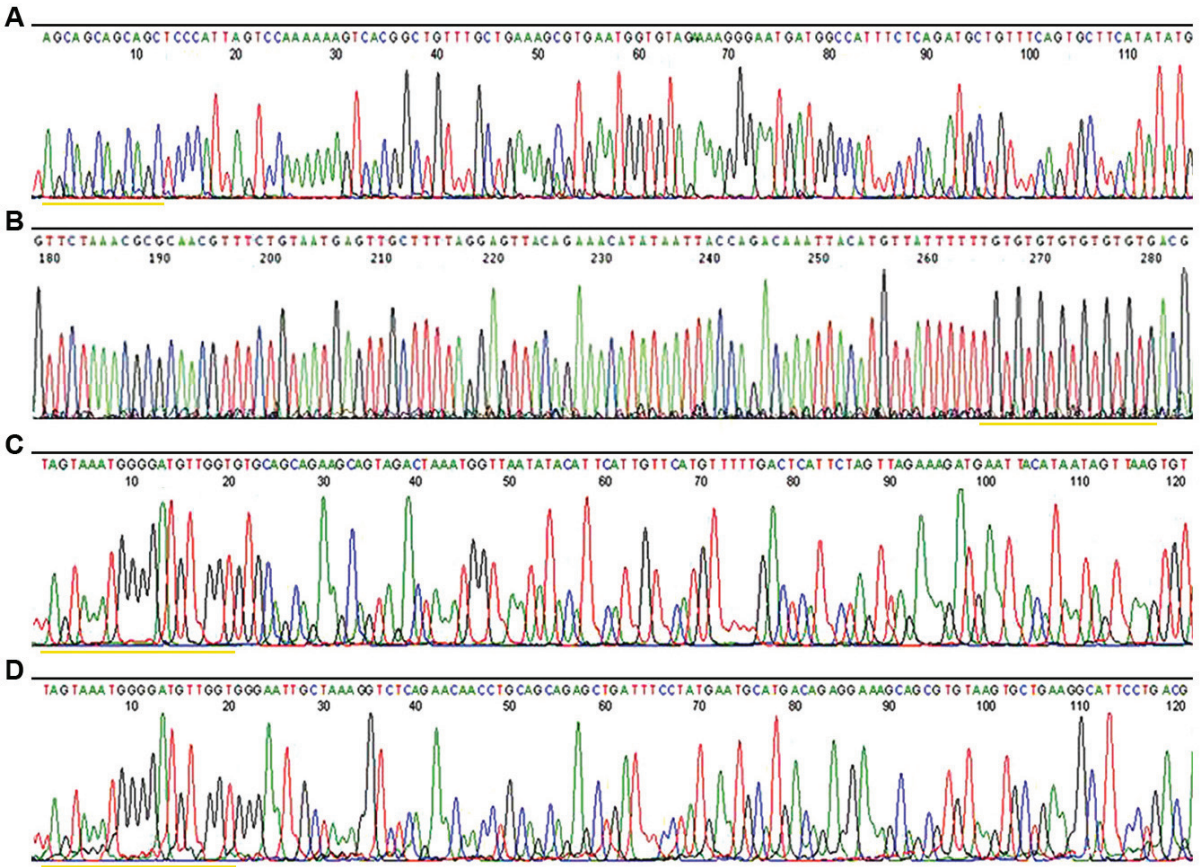

Fig. 1S. Example of chromatograms obtained by sequencing of two amplicons for M-AFLP (**A** and **B**) and two for S-SAP primer combinations (**C** and **D**). The upstream or downstream region of the sequences includes the repetitive motifs of the microsatellite (GT and AGC) or CR1 element in which the specific primers were designed.

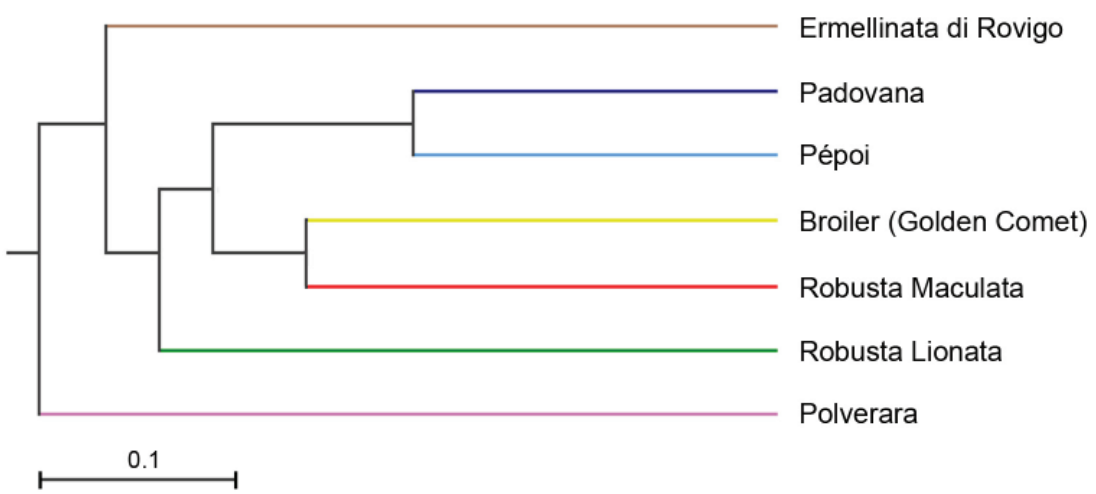

Fig. 2S. UPGMA dendrogram of chicken breeds based on Nei's genetic distances calculated using the whole set of M-AFLP and S-SAP markers.

Table 1S. *Mean Dice's genetic similarity estimates within and between breeds along with overall values and standard deviations for single breeds.*

	Ermellinata	Padovana	Pépoi	Polverara	Robusta Lionata	Robusta Maculata	Broiler
Ermellinata	0.816						
Padovana	0.595	0.691					
Pépoi	0.623	0.621	0.802				
Polverara	0.612	0.606	0.527	0.755			
Robusta Lionata	0.682	0.861	0.610	0.582	0.861		
Robusta Maculata	0.545	0.729	0.508	0.527	0.633	0.729	
Broiler	0.607	0.586	0.567	0.507	0.671	0.572	0.734
Overall	0.611	0.588	0.576	0.544	0.634	0.530	0.585
Standard deviation	0.058	0.087	0.066	0.057	0.039	0.089	0.092