JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Identification on Commercialized Products of AFLP Markers Able To Discriminate Slow- from Fast-Growing Chicken Strains

OLIVIER FUMIÈRE,*[†] MARC DUBOIS,[†] DIMITRIE GRÉGOIRE,[†] ANDRÉ THÉWIS,[‡] AND GILBERT BERBEN[†]

Département Qualité des Productions Agricoles, Centre de Recherches Agronomiques de Gembloux, 24 Chaussée de Namur, 5030 Gembloux, Belgium, and Unité de Zootechnie, Faculté Universitaire des Sciences Agronomiques de Gembloux, 2 Passage des Déportés, 5030 Gembloux, Belgium

The European chicken meat market is characterized by numerous quality marks: "Label de Qualité Wallon" in Belgium, "Label Rouge" in France, denominations of geographical origin, organic agriculture, etc. Most of those certified productions have specifications requiring the use of slow-growing chicken strains. The amplified fragment length polymorphism (AFLP) technique has been used to search molecular markers able to discriminate slow-growing chicken strains from fast-growing ones and to authenticate certified products. Two pairs of restriction enzymes (*Eco*RI/*Mse*I and *Eco*RI/*Taq*I) and 121 selective primer combinations were tested on individual DNA samples from chicken products essentially in carcass form that were ascribed as belonging to either slow- or fast-growing strains. Within the resulting fingerprints, two fragments were identified as type−strains specific markers. One primer combination gives a band (333 bp) that is specific for slow-growing chickens, and another primer pair generates a band (372 bp) that was found to be characteristic of fast-growing chickens. The two markers were isolated, cloned, and sequenced. The effectiveness and the specificity of the two interesting determinants were assessed on individuals of two well-known strains (ISA 657 and Cobb 500) and on commercialized products coming from various origins.

KEYWORDS: **AFLP; chicken; DNA fingerprinting; authentication; certified productions; slow-growing strains**

INTRODUCTION

The chicken production sector is characterized by a growing economic importance and the occurrence of products with special characteristics. A large number of products with legally certified brands or quality marks exist in Europe: the most famous ones are "Label Rouge" in France, Denominations of Origin according to the European Regulation (EEC) No. 2081/92 (*1*), and organic farming. These products, with particular taste and flavor, are more costly to produce and, therefore, reach higher prices than their more ordinary counterparts. Their specific standards impose the use of slow-growing chicken strains and a slaughtering age of at least 81 days (in relation to a usual slaughtering age of ±42 days for products coming from intensive systems and using fast-growing strains). The development of efficient analytical methods able to authenticate the genetic origin of the product is of major interest to assess the credibility of these specific productions and to avoid fraud.

Near-infrared reflectance spectroscopy was previously attempted as a rapid screening technique to distinguish and authenticate products coming from certified productions (*2*). The case of products of intermediate quality with special labeling according to European Regulation (EEC) No. 1538/91 (*3*) was also envisaged (*4*), but reliable genetic markers are essential at least to settle contentious cases (0−20% of the individuals according to the type of cuts).

There are various possible ways to investigate genomic polymorphisms and to produce DNA fingerprints: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), minisatellites, microsatellites, etc. These PCR-based methods use synthetic oligonucleotides to amplify DNA sequences of the genome.

Based on the selective amplification of a subset of restricted DNA fragments ligated to linkers of known sequence, AFLP (*5*) is a technique of choice to search for polymorphisms among populations. The main advantages of AFLP are (i) its reproducibility due to specificity of the PCR primers; (ii) the fact that it does not need any prior knowledge about sequence analysis, primer synthesis, or characterization of DNA probes; and (iii) the high number of potential polymorphic fragments detected in a single PCR reaction.

AFLP is widely used as a genome mapping tool in plant species such as potato (*6*, *7*), tomato (*8*), barley (*9*, *10*), rice (*11*), or sunflower (*12*, *13*). Animal genomes were also screened with it, for instance, in rat (*14*), cattle (*15*), pig (*16*), catfish

* Corresponding author (telephone 32/81/62.03.51; fax 32/81/62.03.88; e-mail fumiere@cra.wallonie.be).
[†] Centre de Recherches Agronomiques de Gembloux.
[‡] Faculté Universitaire des Sciences Agronomiques de Gembloux.

10.1021/jf020748w CCC: $25.00 © 2003 American Chemical Society
Published on Web 01/17/2003

Table 1. Adapters and Primers Used in AFLP Analysis

	name	sequence
adapters *Eco*RI	L1 *Eco* (*Eco* top strand)	5′-CTCGTAGACTGCGTACC-3′
	L2 *Eco* (*Eco* bottom strand)	5′-AATTGGTACGCAGTCTAC-3′
adapters *Mse*I	L1 *Mse* (*Mse* top strand)	5′-GACGATGAGTCCTGAG-3′
	L2 *Mse* (*Mse* bottom strand)	5′-TACTCAGGACTCAT-3′
adapters *Taq*I	L1 *Taq* (*Taq* top strand)	5′-GACGATGAGTCCTGAG-3′
	L2 *Taq* (*Taq* bottom strand)	5′-CGCTCAGGACTCAT-3′
preamplification primer *Eco*RI	Pre-*Eco*	5′-GACTGCGTACCAATTC**A**-3′
preamplification primer *Mse*I	Pre-*Mse*	5′-GATGAGTCCTGAGTAA**C**-3′
preamplification primer *Taq*I	Pre-*Taq*	5′-GATGAGTCCTGAGCGA**A**-3′
amplification primer *Eco*RI core-selective nucleotides[a]	*Eco*-NNN*	5′-GACTGCGTACCAATTC**NNN***-3′
amplification primer *Mse*I core-selective nucleotides[a]	*Mse*-NNN*	5′-GATGAGTCCTGAGTAA**NNN***-3′
amplification primer *Taq*I core-selective nucleotides[a]	*Taq*-NN(N)*	5′-GATGAGTCCTGAGCGA**NN(N)***-3′

[a] Selective nucleotide sequences are given in **Table 2**.

(*17*), or chicken (*18*). Mainly used to study the relatedness between individuals or populations, AFLP was also used for identification of sex-specific markers (*19*, *20*) or for assessing genetic diversity (*21*, *22*). More recently, AFLP was also used to discriminate purebred and crossbred Iberian pigs with the intention of avoiding possible labeling fraud of high-quality marketed products (*23*).

The objective of this study was to search characteristic genetic markers able to distinguish slow- from fast-growing chicken strains and to provide new tools for the control of certified productions to the authorities or to certifying organizations.

Because the breeding process used to obtain the several commercial strains is kept secret by the breeding companies, only very little information is available about the genetic characteristics of the analyzed chicken population, and AFLP seemed to be the best technique to try to find interesting markers.

MATERIALS AND METHODS

Samples. Individual DNA samples were extracted from a total of 161 different chickens (152 carcasses and 9 cut pieces). A subset of these specimens were of certified origins and came from well-known strains with respect to growth rate. The subset consisted of 48 animals belonging to the slow-growing strain ISA 657 used to produce the so-called "Poulets Villages" (poultry of higher quality carrying the "Label de Qualité wallon" quality mark produced in Belgium) and 14 chickens belonging to the fast-growing strain Cobb 500. The remaining individuals (99) were collected in various Belgian supermarkets: 28 slow-growing chickens with a quality mark (e.g., "Label Rouge" or organic farming) ascribed by an official certification organism, 55 chickens among the cheapest products available without any claim on the label were therefore considered to be of fast-growing strains, and 16 chickens came from production systems taking into account European Regulation (EEC) No. 1538/91 on certain marketing standards for poultry (*3*). These latter ones bear a specific claim on the label and were bred with some controlled feeding limitations such as no meat and bone meal or growing activators (antibiotics). Individuals of that category can be considered as "intermediate" quality products, but basically they are animals with a high growth rate bred in a somewhat less intensive production system.

Genomic DNA Extraction. Genomic DNA was extracted from 200 mg of crushed meat resuspended into 1.5 mL of a TENSβ lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, and 1% β mercaptoethanol) and 50 μL of proteinase K (20 mg/mL). The mix was incubated at 60 °C for 30 min and then centrifuged. The supernatant was recovered and extracted twice with an equivalent volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and once with chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated overnight at −20 °C by adding 2 volumes of 100% ethanol and $^1/_{10}$ volume of 3 M potassium acetate, pH 5.2.

After centrifugation, the DNA pellet was washed with 70% ethanol and dried. Finally, the DNA was resuspended in 200 μL of water.

AFLP Analyses. AFLP analyses were performed with 250 ng of genomic DNA submitted to two possible restriction enzyme combinations: *Eco*RI/*Mse*I or *Eco*RI/*Taq*I. In the first enzyme combination, digestion was performed with 2.5 units of *Eco*RI and 2.5 units of *Mse*I in 1× restriction buffer React 9 of Gibco-Life Sciences [20 mM Tris-HCl (pH 7.9), 10 mM magnesium acetate, and 50 mM potassium acetate] for a total volume of 25 μL incubated for 2 h at 37 °C and for 10 min at 70 °C. In the second enzyme combination, DNA was restricted for 1 h at 37 °C with 2.5 units of *Eco*RI in 1× restriction buffer React 9 for a final volume of 22.5 μL. After that, 2.5 units of *Taq*I and 0.25 μL of 10× restriction buffer React 9 were added to the restricted DNA. The reaction mix was then incubated for 2 h at 65 °C in a final volume of 25 μL. The ligation reactions were performed by using the 25 μL of restricted material, 2.5 μL of 10× buffer React 9 in the presence of 5 pmol of *Eco*RI adapter, 50 pmol of *Mse*I adapter (vs 50 pmol of *Taq*I adapter), 1 μL of 10 mM ATP, 1 unit of T4 DNA ligase, and the required amount of water for a final volume of 50 μL. The ligation reactions were incubated for 2 h at 20 °C. After ligation of adapters, DNA fragments were diluted 10-fold in water.

PCR amplifications of DNA fragments were performed in two consecutive reactions as recommended by Vos et al. (*5*). In the first reaction called preamplification, DNA fragments were amplified with a pair of AFLP primers completely complementary to the adapters except one additional selective 3′ nucleotide (for sequences see in **Table 1**). Preamplifications were performed in 25 μL with 2.5 μL of the 10-fold dilution of ligated products, 37.5 ng of each preamplification primers, 2.5 μL of 2 mM dNTPs, 0.5 unit of *Taq* DNA polymerase, and 1× PCR buffer with $MgCl_2$ (Roche Diagnostics). The PCR reaction was performed using the following thermal cycle: 30 s at 94 °C, 60 s at 56 °C, 60 s at 72 °C for 20 cycles. The PCR products of preamplification were diluted 50-fold and used as a template for the selective amplification using two AFLP primers, each containing additional selective nucleotides (**Table 1**). Selective amplification reactions were carried out in 20 μL containing 2.5 μL of diluted preamplification reaction, 37.5 ng of each amplification primer, 2 μL of 2 mM dNTPs, 0.5 unit of *Taq* DNA polymerase, and 1× PCR buffer with $MgCl_2$. The selective *Eco*RI primers labeled with the IRD-800 fluorescent dye were purchased from Biolegio (Malden, The Netherlands).

For this amplification step a touch-down thermal cycling was used consisting of 13 cycles of 30 s at 94 °C, 30 s at 65 °C, and 60 s at 72 °C with at each cycle a 0.7 °C decrease of the annealing temperature until it reached 56 °C and then 23 cycles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C.

The amplification products (2 μL) were analyzed by vertical polyacrylamide gel electrophoresis on an automatic sequencer Gene ReadIR DNA Analysis System L4200S-1 (LI-COR Inc., Lincoln, NE) using a 5.5% denaturing acrylamide gel (KB^+ gel matrix, LI-COR Inc.).

Prior to loading on the gel, samples were denatured for 3 min at 95 °C and then quickly cooled on ice. Data were collected and analyzed using Gene ImageIR ver. 3.55 software (LI-COR Inc.).

Isolation, Cloning, and Sequencing of AFLP Markers. For the isolation of the two polymorphic bands, AFLP were performed using

Table 2. AFLP Primer Combinations Tested with *Eco*RI/*Taq*I and *Eco*RI/*Mse*I Pairs of Restriction Enzyme

*Eco*RI primer selective nt sequences[a]	*Taq*I primer selective nt sequences[a]		*Mse*I primer selective nt sequences[a]								
	ATG	CT	CAA	CAC	CAG	CAT	CCA	CTA	CTC	CTG	CTT
AAA	√[c]	√	√[e]	√	√	√[e]	√	√	√[e]	√	√
AAC	√[e,f]	√	√[b,e,f]	√[b]	√[b,e]	√[b]	√	√[b]	√[b]	√[b]	√[b]
AAG	√[d]	√	√[b]	√[b]	√[b]	√[b]	√	√[b]	√[b]	√[b]	√[b]
AAT	√	√	√	√	√	√	√	√	√	√	√
ACA	√[d]	√[d]	√[b]	√[b]	√[b,e]	√[b,e]	√	√[b]	√[b]	√[b]	√[b]
ACC	√	√	√[b]	√[b]	√[b]	√[b]	√	√[b]	√[b]	√[b]	√[b]
ACG	√	√	√[b]	√[b]	√[b]	√[b]	√	√[b]	√[b]	√[b]	√[b]
ACT	√	√	√[b]	√[b]	√[b]	√[b,e]	√	√[b]	√[b]	√[b]	√[b]
AGC	√[d]	√	√[b]	√[b]	√[b]	√[b]	√	√[b]	√[b]	√[b]	√[b]
AGG	√[e]	√[d]	√[b]	√[b]	√[b]	√[b]	√	√[b]	√[b]	√[b]	√[b]
ATA	√[d]	√	√	√	√	√	√	√	√	√	√

[a] Sequences of the selective nucleotides at the 3′ end of the AFLP primer. [b] Primer combinations cited in the Gibco Life Technologies AFLP kit. [c] Primer combinations tested by Herbergs et al. (*24*). [d] Primer combinations tested by Knorr et al. (*25*). [e] Primer combinations tested on a second set of 59 individuals. [f] Primer combinations tested on the set of all chickens (161 individuals).

selective primers radioactively labeled with ^{32}P (*24*), and the fingerprints were visualized by exposing the dried gel to an X-ray film (Fujifilm). The required bands on the autoradiography were matched to the corresponding area of the gel and were excised from the dried gel. Each band was eluted from the gel by incubation in 200 μL of water at 4 °C for 10 min followed by a boiling step at 100 °C for 15 min. After centrifugation, the AFLP fragments were precipitated by adding 10 μL of 3 M potassium acetate (pH 5.2), 2 μL of glycogen (20 mg/mL), and 450 μL of 100% ethanol at −20 °C for 30 min.

The solution was centrifuged; the pellet was washed with 70% ethanol and dried. Finally, the fragment was resuspended in 20 μL of water.

The two polymorphic bands were inserted in the pCR 2.1-TOPO vector before the transformation of TOP10 competent cells (kit TOPO TA cloning, Invitrogen, Merelbeke, Belgium).

Sequencing reactions were made with the DYEnamic Direct Cycle Sequencing kit (Amersham Biosciences). The electrophoresis was carried out on the automatic sequencer Gene ReadIR DNA Analysis System L4200S-1 (LI-COR Inc.) using a 5.5% denaturing acrylamide gel (KB$^+$ gel matrix, LI-COR Inc.). Data were collected and analyzed using Base ImageIR Analysis ver. 4.2c software (LI-COR Inc.).

RESULTS

AFLP Analyses. A limited set of specimens consisting of nine slow-growing chickens (eight specimens of the ISA 657 strain and one French "Label Rouge" specimen) and nine fast-growing chickens from the market were used to test for the first time the 64 primer combinations cited in the Gibco Life Technologies AFLP kit. As listed in **Table 2**, the primer set for the *Eco*RI/*Mse*I restriction site combination consisted of 64 primer pair combinations that was extended to 35 other primer combinations. Moreover, the *Eco*RI/*Taq*I couple of restriction enzymes was also tested with 22 primer combinations (**Table 2**). These additional primer pairs were chosen among the ones tested on chicken in other studies and showing a large amount of polymorphisms (*25*, *26*). All possible combinations of available primers were also tested. It must be emphasized that no strategy can be defined to choose the most promising primer combinations to check. Among the 121 possibilities tested (99 *Eco*RI/*Mse*I combinations and 22 *Eco*RI/*Taq*I combinations), only 10 polymorphic bands within the fingerprints appeared to be type-strains specific and were checked on a second larger set of specimens consisting of 26 slow-growing chickens, among which were 18 specimens belonging to the ISA 657 strain, and 33 fast-growing chickens from various origins. The primer combinations retested are noted in **Table 2**. Finally, two interesting primer combinations were confirmed with the entire set of samples (**Table 2**) described under Materials and Methods (161 animals). **Table 3** summarizes the results obtained with the two AFLP markers of interest.

Table 3. Frequencies of the Two AFLP Bands of Interest in the Different Strains of Chickens [Slow (SGC) and Fast (FGC) Growing]

	333 bp AFLP band	372 bp AFLP band
primer combination	*Eco*RI + AAC/*Mse*I + CAA	*Eco*RI + AAC/*Taq*I + ATG
SGC		
ISA 657 strain	48/48 (100%)	0/48 (0%)
others	19/28 (68%)	4/28 (14%)
FGC		
Cobb 500	0/14 (0%)	14/14 (100%)
others	0/46 (0%)	45/46 (98%)
cuts	0/9 (0%)	9/9 (100%)
intermediate products	3/16 (19%)	14/16 (88%)

A band of 333 bp generated with the *Eco*RI + AAC/*Mse*I + CAA primer combination is amplified in all 48 individuals of the slow-growing (SGC) strain ISA 657 and is absent in the 14 chickens of the fast-growing strain (FGC) Cobb 500. Concerning commercial products, this band of interest is frequently present in the SGC group (19/28 animals); on the contrary, the band is never detected in the cheaper chickens group (0/55 animals). The band is observed with only 3 chickens (of 16 animals) of intermediate quality.

In contrast, the *Eco*RI + AAC/*Taq*I + ATG primer combination generated a band of 372 bp present in all individuals of the fast-growing strain Cobb 500 (14/14 chickens) and in 45 specimens of 46 fast-growing chickens from the market. This band was never detected with specimens of the ISA 657 strain (0/48 chickens). Nevertheless, the band is detected with only four SGC sold in the shops (three French "Label rouge" and one Belgian "Coucou de Malines"). Finally, this band is frequently amplified (14/16 animals) with chickens of intermediate quality.

Characterization of the Two Polymorphic Bands. The two bands of interest were isolated each out of the AFLP fingerprint of five different chickens. These bands were isolated from the gels, cloned, and sequenced as described under Materials and Methods. The sequences obtained were confirmed on several clones per individual.

The sequence of the band of 372 bp amplified by the *Eco*RI + AAC/*Taq*I + ATG primer combination was easily determined and confirmed on five chickens belonging to fast-growing strains. On the contrary, it was more complex to define the sequence corresponding to the AFLP marker amplified by the

*Eco*RI + AAC/*Mse*I + CAA primer combination. Different sequences were obtained for the first time, but none were present in more than one animal. Sequencing of more plasmids finally gave a fragment shared by the five slow-growing chickens and was therefore ascribed to the AFLP marker.

The sequences of chicken genomic DNA fragments, excluding the selective primers, were, respectively, 350 and 311 bp. They were registered at the DDBJ/EMBL/GenBank databases under accession numbers AF525026 and AF525025.

Up to now, no homology has been found by comparison with sequence databanks.

DISCUSSION

AFLP is frequently cited as a fast and reliable method to scan the genome in search of specific polymorphisms. The principle of the technique allows the screening of a very high number of loci for polymorphism and the detection of a greater number of polymorphic DNA fragments than any other PCR-based detection system (*5*). Despite that property, only two polymorphic markers potentially able to discriminate slow- from fast-growing chicken strains were identified. Considering the individuals well-identified as belonging either to the ISA 657 or to the Cobb 500 strains, we can say that the two markers assessed have a real potential for the control of production using those two strains. The results obtained with samples of more variable origins indicate that it will probably be impossible to have a single and universal slow growth determinant valid for all chickens with that trait because the reason for this phenotype may be of divergent genetic origin.

It should be stressed that the divergent ease with which the sequences of both AFLP markers were determined is consistent with observations of other authors. Isolation and sequencing of the fast-growing marker was straightforward, supporting the observation of van der Voort et al. (*27*) that identically sized AFLP fragments detected in different chickens arise from the same polymorphism. On the contrary, Meksem et al. (*28*) could detect a mean of six sequences per AFLP band, although only one sequence corresponded to the original AFLP marker. A similar situation was experienced here with the slow-growing marker as absolutely no match could be found among the sequences isolated from the five individuals. Further analysis of more clones isolated from each of these individuals finally gave a segment shared by all of them.

If the two bands are apparently correlated to growth rate, the exact meaning of these markers is unclear, the more as no homology could be found with known sequences (comparison with sequences registered in international sequence libraries), supporting some possible link to growth. However, one may not exclude the possibility that these markers reflect a tight link with a particular breed used in the selection scheme of the slow- or fast-growing strains. If the polymorphism observed is connected with growth, the absence of homology with known sequences is amazing. Up to now, we cannot exclude the possibility that the observed phenomenon could be due to a QTL present in a noncoding DNA region, for example.

The authors are working to obtain samples to check more accurately on genetically better characterized specimens (breeds and strains) how far the two determinants are valuable.

*Eco*RI/*Mse*I is a pair of restriction enzymes commonly used in AFLP studies and recommended for most plant species. Its use in animal DNA results in complex patterns but was successfully applied by Óvilo et al. (*20*) to the characterization of highly inbred Iberian pig breed genotypes and the detection of strain-specific polymorphisms. To reduce the number of fragments and obtain less complex AFLP patterns, the use of *Eco*RI/*Taq*I is suggested for cattle (*15*) and for poultry (*25*, *26*).

Nevertheless, the possibilities offered by AFLP are great; if we consider the two pairs of restriction enzymes used in this study, many other possible combinations of primers could still be checked to enlarge the collection of determinants able to state if a chicken strain is a slow- or fast-growing one. Considering the results obtained, AFLP seems to be an interesting and powerful technique to investigate polymorphism between chicken strains.

However, AFLP is a rather tedious method for routine analyses. Based on the known sequences of both AFLP markers identified, it could be possible to design a standard PCR test such as described in previous works (*28*, *29*). Such a PCR test would still be valid on processed meat products (for instance, cooked meat) in contrast to AFLP, for which good-quality DNA is a requirement. A couple of primers internal to each band were designed, but the targets so defined were amplified by both slow- and fast-growing chickens (data not shown). This result indicates that the polymorphisms are surely located at one of the two ends of the bands and may probably be due to a mutation in the restriction site. The flanking regions of the two markers shall be amplified by anchor PCR (*30*) in order to be sequenced. The polymorphism's site will be delimited, and new PCR primers reproducing the polymorphism observed by AFLP will be designed.

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Received for review July 9, 2002. Revised manuscript received November 11, 2002. Accepted November 11, 2002. This work was supported by the Scientific Support Plan for a Sustainable Development Policy−Standards for Food Products (Contract NP/42/022) of the Belgian Federal Office for Scientific, Technical and Cultural Affairs (OSTC).

JF020748W