Folia biologica (Kraków), vol. **58** (2010), No 3-4
doi:10.3409/fb58_3-4.257-263

Identification of the Rate of Chimerism of Different Tissues with Microsatellite Markers in Chicken Chimeras*

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Accepted May 25, 2010

SIWEK M., SŁAWIŃSKA A., ŁAKOTA P., GRAJEWSKI B., WAWRZYŃSKA M., WIŚNIEWSKA E., PŁAWSKI A., SŁOMSKI R., BEDNARCZYK M. 2010. Identification of the rate of chimerism of different tissues with microsatellite markers in chicken chimeras. Folia biol. (Kraków) **58**: 257-263.

The goal of our study was to evaluate whether private alleles can be defined in microsatellite markers for the breeds under investigation; to evaluate if these private alleles distinguish chicken chimera when using different tissues; to trace them back to the donor: Green-Legged Partridgelike and recipient: White Leghorn chicken breeds, and further on, to estimate the level of chimerism in each tissue. Private and common alleles were defined for donor and recipient chicken breeds in 3 loci. The rate of chimerism was defined based on private alleles present in liver, heart, breast muscle, femoral muscle and gonads. The highest rate of chimerism was observed in liver. A lower rate of chimersim was observed in gonads, and femoral muscle, and finally the lowest rate of chimerism was observed in breast muscle and heart.

Key words: Chimera, chicken, microsatellite marker, private allele.

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Blastodermal cells (BC) from stage X (EYAL-GILADI & KOCHAV 1976) of a chicken embryo offer an excellent opportunity to create chicken chimera. These pluripotent cells can be removed from the chicken embryo, dispersed and injected into the subgerminal cavity of the recipient embryo at the same stage of development (MARZULLO 1970; BEDNARCZYK *et al*. 2000). Further, these cells can colonize somatic tissues of treated embryos and can contribute to the germline because stage X blastodermal cells obtained from freshly laid eggs contain primordial germ cells (PGC) or their precursor cells (NIEUWKOOP *at al*. 1979).

In our research, the production of chicken chimeras has been established by transfer of stage X blastodermal cells from pigmented breed embryo (Green-Legged Partridgelike) (BEDNARCZYK *et al*. 2000; BEDNARCZYK *et al*. 2002; BEDNARCZYK *et al*. 2003) into the germinal cavity of the non-pigmented breed embryo (White Leghorn) (PETITTE *et al*. 1990). Alternative to the BC transfer is the use of primordial germ cells (PGC) isolated from the vascular system or from gonads, reported by TAJIMA *et al*. (1993).

The establishment of these robust techniques to produce high level chicken chimeras has been a prerequisite for transgenesis in the chicken. Recently efforts have been directed into improving *in vitro* culture and transfection methods of avian embryonic stem cells (ESCs) originating from blastodermal cells (PAIN *et al*. 1996; MOZDZIAK & PETITTE 2004), as well as PGC-derived embryonic germ cells (EGCs) (HAN *et al*. 2002) in order to facilitate stable modification and selection of

*Partially supported by the State Committee for Scientific Research (KBN) Project No. PBZ-KBN-084/P06/2002/4.3, and Project No. 2P06D 029 26.

the donor cells. Despite obstacles in the long-term culture of undifferentiated embryonic cells, both approaches towards generating transgenic birds have prospects for future studies, according to some preliminary reports on first detection of transgene expression in the chimeric chick (BRAZOLOT *et al*. 1991; VICK *et al*. 1993; VAN DE LAVOIR *et al*. 2006).

Once the new potential chimera has hatched, its chimerism has to be confirmed using one or more indicators, such as feather pigmentation markers, inter se mating or DNA screening. The first method concerns somatic chimeras, in which injected BC differentiate into the somatic skin cells, providing a chimerism indicator that is visible at hatching (BEDNARCZYK *et al*. 2002). Inter se mating of hatched chicks demonstrates that the injected cells incorporate into gonads and give rise to functional gametes in the germline chimera. DNA screening with molecular markers enables quick identification of both somatic and germline chimerism in every tissue. For this purpose, different molecular tools have been developed, such as White Leghorn-specific SCAR markers (PARK & HAN 2000; AFLP markers (HU *et al.* 2005), W chromosome-specific markers (PETITTE *et al.* 2004), breed specific DNA polymorphism (CHOI *et al.* 2007). Quantitative estimation of chimerism with microsatellite markers has been already performed by DELHAISE *et. al*. (1993) but this experiment focused on mouse embryonic stem cells. However, mouse stem cells are isolated from the cell mass of preimplantation blastocyst (MARTIN 1991), they are rather comparable to chicken preblastodermal cells (stages V-VII EG&K) than to blastodermal cells which are the major tool in our experiment.

In a previous study (BEDNARCZYK *et al*. 2002), we have already presented that both RFLP markers and microsatellite markers are powerful enough to distinguish chicken germline chimeras when using DNA obtained from peripheral blood samples.

The goal of the current study was to evaluate whether we can define private alleles in microsatellite markers typical for the breeds under investigation; to evaluate if these private alleles distinguish chicken chimera when using different tissues (gonads, heart, liver, femoral muscle and breast muscle); to trace them back to the donor: Green-Legged Partridgelike and recipient: White Leghorn chicken breeds, and moreover, to estimate the level of chimerism in each tissue.

Material and Methods

Experimental Populations

Individuals from the Green-Legged Partidgelike (GP) and White Leghorn (WL) breed were used in this study. The GP is a native Polish breed of layers, created in 1870 and may have ancestors from Italy, imported in the 16th century. The GP birds have characteristic green legs and a partridge-like plumage, the cocks have dark-yellow manes and red saddle (WITKOWSKI *et al.* 2009). The GP population has been closed for conservation since more than 50 generations.

The White Leghorn (WL) is a commercial egg layer type of domestic fowl. The commercial breeding line of WL used in this study was extensively selected for quantitative traits, especially for egg production ability.

Construction of the Chicken chimeras

WL chickens, being homozygous at the dominant white locus (*II*) were used as recipients, while donor blastodermal cells (BC) were obtained from GP, being homozygous recessive (*ii*) at the white locus. Given such an experimental setup, donors and recipient breeds provide convenient feather pigmentation markers that allow an immediate indicator at hatching. Blastodermal cell isolation and production of the chicken chimeras were performed as described elsewhere (BEDNARCZYK *et al*. 2000). In brief, stage X (E-G&K) blastoderms taken from 20 freshly laid donor eggs were isolated, using filter paper rings, from the yolk and washed several times with calcium- and magnesium-free PBS (CMF-PBS) to remove as much yolk as possible. In the next step, the blastodermal cells were dispersed for 7 min at 20^{o}C in CMF-PBS supplemented with 0.05% (wt/vol) trypsin and 0.04% (wt/vol) EDTA and by repeated aspirations of the medium into a Pasteur pipette. After dispersion, the cells were centrifuged for 5 min at a relative centrifugal force (RCF) of 300 x g and then washed three times with CMF-PBS supplemented with 10% (vol/vol) fetal bovine serum. Each time the supernatant was removed and the cells from several blastoderms were resuspended in Dulbecco's Modified Eagle medium prior to injection.

In our experiment a total of 26 potential chicken chimeras were used for microsatellite screening in order to establish the level of chimerism in the tissues. The animals used were fully developed embryos at the 20th day of incubation.

Generating Genotypic Data

The DNA was isolated from peripheral blood samples of donor (GP/48 individuals) and recipient (WL/56 individuals) chicken breeds using the guanidinum isothiocyanate (GTC) method. Blood was collected from the wing vein on 0.2% EDTA, diluted with lysis buffer and incubated at 0^{o}C for

30 min. After centrifugation at 2500 rpm at 4°C for 15 min, the cell pellet was suspended in 500 μl in lysis buffer, mixed with 2 ml GTC and incubated at 0°C for 15 min. Lysate was extracted with 2.5 mL of equilibrated phenol: chloroform: isoamyl alcohol and centrifuged at 3000 rpm. The aqueous phase was re-extracted with 2.5 ml of chloroform and centrifuged. DNA was precipitated with an equal volume of isopropanol, collected and dissolved in 300 μl of water.

Tissue DNA was isolated from: gonads (22 samples), heart (23 samples), liver (23 samples), femoral muscle (24 samples) and breast muscle (25 samples), using MasterPure™ DNA Purification Kit, cat no MCD85201 (Epicentre Biotechnologies, Wisconsin USA).

Nine microsatellite markers: ADL0102, ADL0136, ADL0158, ADL0171, ADL0172, ADL0176, ADL0181, ADL0210, and ADL0267 were chosen for analysis. These microsatellite markers have been included in the Microsatellite Locus Population Tester Kit (http: // poultry.mph.msu.edu /resources/ poptest1.htm), and have been recommended by the FAO / MoDAD Advisory Group (http://www.fao.org/ dad-is) for characterizing breeds.

The current experiment consisted of two parts. The first stage focused on characterizing the donor (GP) and recipient (WL) chicken breeds with microsatellite markers and defining common and private alleles for each locus for these two breeds. The second part aimed at detecting donor and recipient genotypes in each tissue with microsatellite markers preselected at the first stage.

In the first part of the analysis 100 ng of DNA from blood or tissue (donor and recipient) was used in the PCR reaction. The reaction was performed in a mixture containing 50 pmol of each primer and 0.5 units of Taq polymerase (Fermentas) in a total volume of 25 μl at the following cycling conditions: initial denaturation, 94°C, 300 s; 25 cycles of denaturation, 92°C, 30 s, annealing, 55°C, 45 s and elongation, 72°C, 300 s; final extension, 72°C, 300 s 2 μl of PCR products were separated in a 6% polyacrylamide gel using ALFExpress DNA sequencer (1500 V, 60 mA, 25 W) and analyzed using Fragment Manager software. The forward primer was labeled with indodicarbocyanine (Cy5) phosphoramidite.

In the second part of the analysis (chimera tissue) PCR was performed as follows: the PCR amplifications were performed in a volume of 10 μl, containing 20 ng of the template DNA, 0.5 pmol of the forward primer, 0.5 pmol of the reverse primer, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 1.5 mM $MgCl_2$, 2mM dNTP mixture, and 1 unit of Taq Polymerase (Fermentas). Each forward primer was 5'– labeled with fluorescent dye IRD800 (MWG), which enabled accurate sizing of alleles on a Li-Cor DNA Analyzer. The amplification was carried out in a thermocycler (Eppendorf Mastercycler Personal) using the following conditions: an initial denaturation step at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55-65°C for 30 s, 72°C for 30 s, and final extension step at 72 °C for 2 min. PCR products were separated in a 6% polyarcylamide gel using a LICOR DNA sequencer (1500 V, 60 mA, 25W) and analyzed using SAGA software. Allele frequencies were calculated by direct counting.

Results

The presented study consists of two parts. The first part aimed at the characterization of two chicken breeds: GP and WL based on microsatellite markers (seven microsatellite markers) and the detection of private alleles for each marker and each studied chicken breed. For this part nine microsatellite markers from the Microsatellite Locus Population Tester Kit were used, as recommended by FAO as the most suitable for a population differentiation study. The second part aimed at performing a DNA assay and defining the level of chimerism in tissue samples based on molecular markers (three microsatellite markers) for which private alleles were detected.

Initial tests of nine microsatellites using the Microsatellite Locus Population Tester Kit showed that two out of nine markers were not polymorphic for the populations under the study. For the remaining 7 microsatellite loci, the total number of alleles for both donor and recipient breeds was 56, on average 8.0 alleles per locus (Table 1). All loci,

Table 1

Microsatellite allele numbers in recipient (White Leghorn) and donor (Green-Legged Partridgelike) populations

Locus	Private alleles number WL	GP	Common alleles number	Total alleles number
ADL0136	1	3	5	9
ADL0158	4	0	3	7
ADL0171	0	4	1	5
ADL0176	1	5	5	11
ADL0181	0	3	3	6
ADL0210	1	0	6	7
ADL0267	1	4	6	11
Total	8	19	29	56

except ADL0171 (for WL), were polymorphic. The number of alleles per locus ranged from 5 (ADL0171) to 11 (ADL0176 and ADL0267). For each of the 7 markers, all together, 27 breed specific private alleles for WL or GP breed were observed. The private alleles were generally less frequent in WL (8) than in GP (19). In the latter case, in 5 loci three or more alleles were observed which did not occur in the WL population. The size and frequency of private alleles are presented in Table 2. Among 27 private alleles, two alleles in WL and five alleles in GP had frequencies higher than 10%. In the GP population the marker ADL 0176 had four private alleles which were absent in the WL gene pool. Two of these alleles (112 and 114) were extremely frequent: 45.8 and 33.3, respectively. The maximum size difference between the alleles observed within the loci ranged from 0 (ADL0158) to 30 (ADL0158) and from 12 (ADL0158, ADL0181, and ADL0210) to 44 (ADL0136) in WL and GP populations, respectively. Average heterozygosity was higher for the GP (62.2) population compared to the WL population (43.3). In general, GP was a very polymorphic population with the gene diversity value $H_o = 0.53$, while an average H_o within the 52 populations across all 22 loci was 0.47.

Based on the information obtained from analysis of donor (GP) and recipient (WL) breeds, three microsatellites: ADL 0136, ADL0176, ADL0267 were selected as good candidates to perform an assay for genotype identification in chimera tissues. All three markers were characterized by a high heterozygosity and a high number of private alleles specific for the GP (donor) chicken breed. Private alleles detected in chimera tissues are presented in Table 3. In all studied chimera tissues private alleles specific for a donor breed were detected for all microsatellite markers. In more details: allele 131 for marker ADL0136, alleles 181 and 197 for marker ADL0176, and alleles 100 and 102 for marker ADL0267.

Table 2

Size and frequency of private alleles in recipient (White Leghorn) and donor (Green-Legged Partridgelike) populations

Locus	Size (bp)	Frequency (%) WL	GP
ADL0136	121	0.0	10.4
	133	30.4	0.0
	163	0.0	12.5
	165	0.0	3.1
ADL0158	189	6.3	0.0
	196	27.7	0.0
	204	9.8	0.0
	218	1.8	0.0
ADL0171	106	0.0	4.2
	112	0.0	45.8
	114	0.0	33.3
	136	0.0	7.3
ADL0176	181	0.0	1.1
	183	0.9	0.0
	189	0.0	2.1
	197	0.0	23.4
	199	0.0	7.4
	203	0.0	6.4
ADL0181	183	0.0	1.0
	185	0.0	9.4
	189	0.0	2.1
ADL0210	117	1.8	0.0
ADL0267	100	0.0	6.3
	102	0.0	1.0
	112	1.8	0.0
	122	0.0	1.0
	134	0.0	2.1

Table 3

Size and frequencies of private donor (Green-Legged Partridgelike) and private recipient (White Leghorn) alleles in chimeric tissues

Locus	Size (bp)	Frequencies (%) GP	WL
ADL0136	121	27.0	0.0
	133	0.0	9.0
	163	0.0	0.0
	165	0.0	0.0
ADL0176	181	24.0	0.0
	183	0.0	9.5
	189	7.5	0.0
	197	24.0	0.0
	199	0.0	0.0
	203	0.0	0.0
ADL0267	100	10.0	0.0
	102	15.0	0.0
	112	0.0	8.2
	122	0.0	0.0
	134	0.0	0.0

Discussion

The goal of this study was to evaluate whether microsatellite markers would distinguish a chimeric genotype in different tissues (gonads, heart, liver, femoral muscle and breast muscle) and trace them back to the donor: Green-Legged Partridge-like and recipient: White Leghorn chicken breeds, and moreover, to estimate the level of chimerism of each tissue. In other words, to examine if donor blastodermal cells migrate into gonads of the recipient (potential chimera) with satisfying frequency. This argument is of special interest in relation to the possibility of exploiting the presented method for obtaining chicken chimeras and further transgenic birds producing biofarmaceutics in an oviduct. So far, the identification of germline was possible only as a result of progeny tested the putative chimeras. The GP population was included in the study of genetic diversity and relationships among various (52) chicken populations (HILLEL *et al.* 2003).

In our investigation the GP population showed higher heterozygosity compared to the WL population. This might be explained by the genetic background of these two breeds: GP is a native breed, whereas WL is a commercial breed selected for production traits. This is also in agreement with the number of private alleles which were less frequent in the selected breed (WL) compared to the unselected breed (GP). The key marker in the current experiment for estimating the rate of chimerism were private alleles defined for the Green-Legged partidge-like breed. These unique alleles were amplified by PCR reaction and their presence was traced back to each tissue sample. The rate of chimerism in each of the analyzed tissues was defined in two ways. First, a tissue was recognized as 'chimeric' if two or more alleles were detected, including at least one private allele (Fig. 1). Based on this analysis, the highest rate of chimerism was observed in gonads, the lowest rate was observed in heart, with femoral muscle, liver and breast muscle showing an intermediate level. These results were in agreement with expectations, especially the high rate of gonad chimerism observed in progeny tested chimeras produced using the same method (BEDNARCZYK *et al*. 2002; SECHMAN *et al*. 2006). Gonads are the final destination for PG. Precursors of PG, Vasa-positive cells were identified among BC isolated at X stage of embryogenesis (KARAGENC *et al.* 1996). Second, each private allele was counted, therefore the number of private alleles in tissues exceeds the number of tissues recognized as chimeric (Fig. 2). Based on this analysis, the highest rate of chimerism was observed in liver (23 donor private alleles). A lower rate of chimersim was observed in gonads, and femoral muscle, and finally the lowest rate of chimerism was observed in breast muscle and heart. A very high rate of chimersism in the liver has been already reported by MA *et al.* (2001) using a W chromosome specific DNA probe. Results of a high ratio of donor alleles detected in live tissue might explain the usefulness of egg shell color as a phenotypic marker (CZEKALSKI 2004). Czekalski reported that egg shell color is the best phenotypic marker of chicken chimerism. The dark pigment ooporphyrin or protoporphyrin originates from blood hemin and is responsible for the dark color of an egg shell. These pigments are synthesized in the liver. Therefore liver metabolic function is indirectly related with egg shell color. Our results suggest the existence of a mechanism(s) responsible for preferential migration of donor cells to the liver than to others. Results reported by CZEKALSKI (2004) and our own results indicate that donor cells, by colonizing the recipient's liver, may influence pigment production and indirectly the dark color of egg shells of chicken chimeras.

Formation of chicken chimera involves blastodermal cell injection into the blastodermal cavity at stage X and later regular procedure of embryo development. A crucial moment in embryo development is gastrulation. At that moment a massive reorganization of the embryo from a simple spherical ball of cells, the blastula, into a multi-layered organism occurs. During gastrulation, many of the cells at or near the surface of the embryo move to a new, more interior location. The primary germ layers (endoderm, mesoderm, and ectoderm) are formed and organized in their proper locations. Endoderm, the most internal germ layer, forms the lining of the gut and other internal organs such as e.g. liver, spleen. Ectoderm, the most exterior germ layer, forms skin, brain, the nervous system, and other external tissues. Mesoderm, the middle germ layer, forms muscle, the skeletal system, and the circulatory system. Our current results show that injected blastodermal cells are present in the formation of at least two out of the three primary germ layers: mesoderm (gonads, breast muscle and femoral muscle, heart) and endoderm (liver). Concluding from our previous results (BEDNARCZYK *et al.* 2002) in which the level of chimerism was detected based on feather pigmentation, we suggest that blastodermal cells are also present in the third germ layer, the ectoderm. This suggests that embryonic cells isolated from the stage X (E-G&K) embryo are not only pluripotent (PETITTE *et al*. 2004) but are totipotent, meaning they are able to contribute to all tissues.

In summary, we presented an experiment aiming at detecting and estimating the rate of chimerism in different tissues obtained from GPxWL chicken

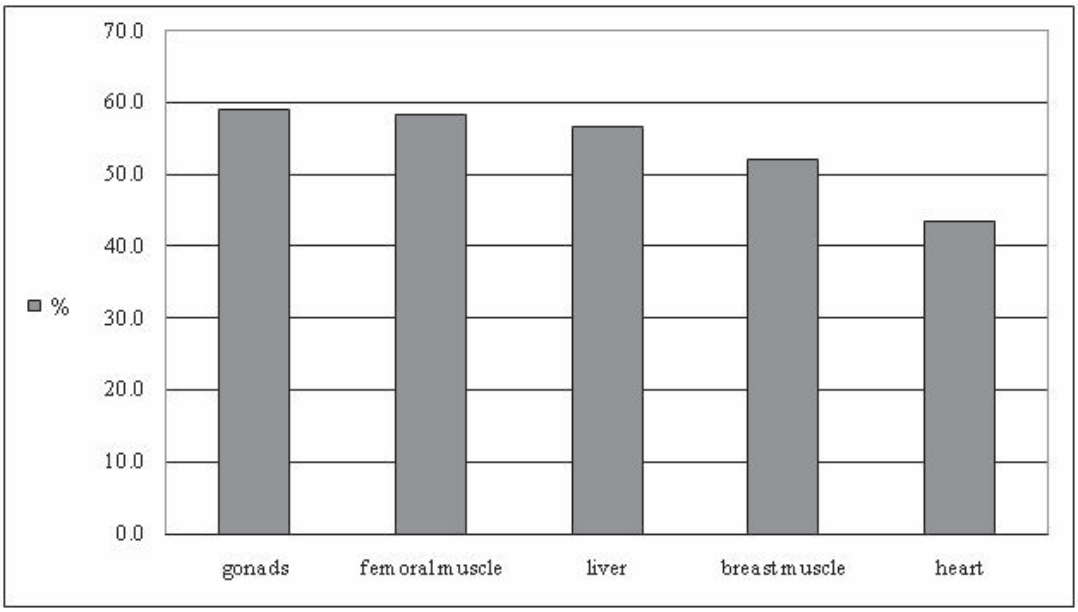

Fig. 1. Percentage of chimerism in analyzed tissues obtained from Green-Legged Partidge-like / White Leghorn chicken chimeras.

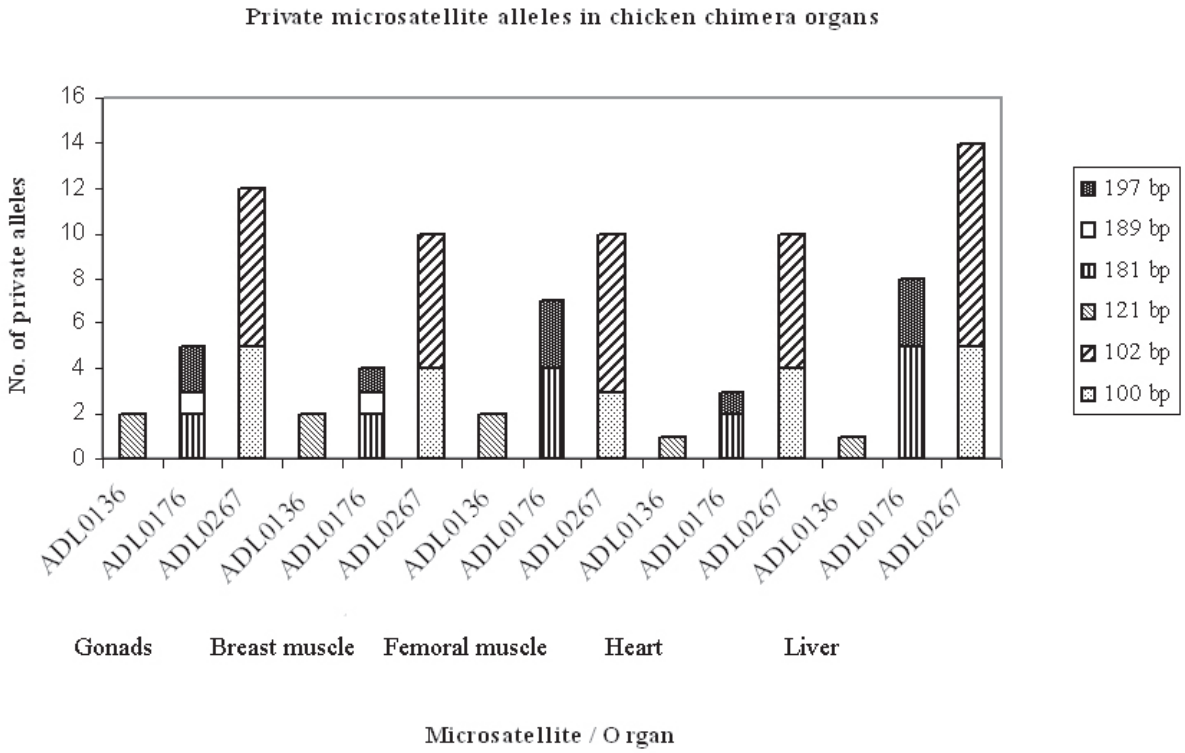

Fig. 2. Private microsatellite alleles in chicken chimera organs.

chimeras. To estimate the level of chimerism, we used private alleles defined for polymorphic microsatellite markers in previously analyzed donor (GP) and recipient (WL) breeds. It can be concluded that microstatellite markers and especially carefully defined private breed alleles are a useful tool for tracing the origin of tissue, and estimation of the rate of chimerism in chicken Green-Legged Partridge-like /White Leghorn chimeras. It might be also concluded that each hatched individual obtained after BC injection is a true chimera. It's a matter of careful identification its level of chimerism with properly defined tool. Therefore private alleles defined for microsatellite markers seem to be the method of choice for the identification of the rate of chimerism.

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