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MICROSATELLITE GENETIC DIFFERENTIATION ANALYSIS AND ORGANIC MATRIX OF EGGSHELL IN THE 16th GENERATION OF CHICKENS SELECTED FOR EGG PRODUCTION TRAITS

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SUMMARY

Two lines of Alexandria chicken (selected L1 and control L2) were characterized for their genetic diversity and identified population priorities for egg traits. Eight microsatellite markers linked to QTLs associated with the studied egg traits were used. The selected Alexandria line L_1 showed higher frequency, than the control lineL_2, of loci associated with MCW241 and MCW0145 markers that might be associated with body weight (BW), age at first egg (AFE) and Shell thickness at 53 weeks of age (ST53) traits. Meanwhile the tested markers, ADL188, MCW 246 and MCW0170 that are associated to Haugh units (Hu), egg shell strength (ESS) and albumin weight at 33 weeks of age (AW33), didn't show frequency differences between the control and the selected lines. No significant variation was observed between the selected lines and the control line in eggshell soluble protein.

In conclusion, our current study indicates that, selection for early age of sexual maturity; the body weight, and shell thickness were improved.

Keywords: Microsatellite, organic matrix, selection

INTRODUCTION

The internal and external egg quality of the local Egyptian breeds are very good, while their egg production traits are inferior than the commercial strains (Galal *et al.*, 2012; Radwan *et al.*, 2010 and Radwan, 2013). Genetic selection programs need to monitor a range of characteristics to ensure that improvement of one characteristics is not at the expense of other equally important traits (Roberts, 2010).This process is being assisted by increased knowledge of the genetic basis of egg shell quality. Dunn *et al.*, 2005, research investigated candidate genes for egg shell quality parameters. The selection programs have interested improvement in economic traits whether production or quality traits. Recent advances in the availability of genomic information have made the dissection of the hereditary variation behind these traits possible. The first genome scans to identify loci affecting egg quality traits have been based on medium-density microsatellite maps (Vilkki, 2012).

Among the genetic markers which are currently employed, microsatellites have been found to be abundant, evenly distributed and highly polymorphic in all resource populations. Moreover, most of the economic traits displayed a wide variation in the expression of genes at distinct loci, referred to as quantitative trait loci (QTLs) (Cheng *et al.*, 1995). Large number of genetic markers that facilitate QTL analysis has been generated and mapped in experimental populations. The genetic linkage maps of chicken contain over 1900 loci, out of which nearly, 800 are highly polymorphic microsatellite markers (Groenen *et al.*, 2000). A comprehensive characterization of chicken markers is needed to monitor and conserve genetic diversity in chicken. DNA-based molecular markers have been used as efficient tools for a large number of applications, including phylogenetic analysis, the assessment of genetic diversity for accelerated breeding, the selection of hybrid parents, studying population structure, marker-assisted selection (MAS) and mapping and tagging genes and quantitative trait loci (QTLs) (Collard *et al.*, 2005). Moreover, the usefulness of the microsatellite system has been verified; it is capable of effectively improve genetic diversity and has beneficial applications in breeding in many species. This approach is also effective for detecting polymorphisms associated with a low level of intraspecific diversity (Mittal and Dubey, 2009).

The Egyptian local breeds, which are well-adapted to extensive husbandry systems and suitable for resource-poor poultry farmers endowed with very limited means, but these breeds were low production so, should be thoroughly studied as a basis for enhancing their use and conservation. The program selection was play role important to improved production of Egyptian local breeds. However, these breeds cannot compete with highly selected commercial hybrids. Thus, a breeding programme involving local breeds should identify alternative breeding goals, and capitalize on the breeds' specific attributes. (Zatter, 1994; Ghanem, 1995; Abd El-Halim, 1999; El-Tahawy, 2000; Ghanem, 2003; El-Dlebshany, 2004 and Khalil, 2010) crossing three strains of local breeds (Alexandria, Norfa and Matroh) and selection hybrids were for parameter egg production to 16 generation. Mahrous *et al.*, 2013 were estimated and comparison quality and ultrastructure of eggshell between selection and control lines, they found selection line had benefit

good eggshell quality and good ultrastructure eggshell than control line. Radwan (2010) found relationship between ultrastructure organic matrix of eggshell. Genetic variability and relatedness among the native and improved breeds/lines of chicken are necessary information required because the genetic variation is considered as the primary biological resource that can be exploited in selective breeding program. Moreover, Microsatellite marker has been widely used to evaluate genetic structure, variation and relationship in various organisms. The advantage of this technique includes its ability to detect polymorphisms in many loci and the codominan nature of generated markers.

This study aims to assess organic matrix of eggshell in the two selected local lines and to define the microsatellite markers associated with egg traits in the two selected lines of Alexandria chicken (selected L1 and control L2).

MATERIAL AND METHODS

Two Alexandria chicken lines (selected LI and control L2) were used in this study. The individual selection program was applied for 16th consecutive generations from 1995 to 2011. The base selection line was initiated from crossing three strains of local chickens, i.e. Alexandria, Norfa and Matrouh. The two-way crosses and their reciprocals among them were produced which was followed by three-way crosses. were produced during season 1992/1993 (Zatter, 1994). A control line (males and females) were random selected from the base population, while egg line was selected by earlier age at sexual maturity comparing to the population mean of this trait (Ghanem, 1995). The field work was done at the Poultry Research Center, Faculty of Agriculture, Alexandria University. However, the lab work, including organic matrix of eggshell and microsatellites was fulfilled at the Dept. of Poultry Production, Faculty of Agriculture, Ain Shams University.

Genomic DNA isolation:

Twenty four blood samples were randomly collected from females of each line into vacuum tubes containing EDTA and stored at -20°C. Genomic DNA was isolated from their blood samples using AXYGEN kit (Axyprep TM) from Axygen Scientific, inc. USA Cat. No. AP-MN-BL-GDNA-50. DNA concentration was determined using spectrophotometer and the final concentration was adjusted up to 50 ng/µl for PCR analysis.

Microsatellite markers:

A total of 8 informative microsatellite markers were selected from Roslin Institute database (http://www.thearkdb.org.) according with the association of QTL loci with the studied egg traits. Microsatellite loci were chosen from MCW and ADL markers these where: MCW241 is situated in the chicken GGVAYY-gene of the chicken ovalbumin family, MCW258 is located in GGCALBO4 chicken gene associated with vitamin D-induced cal binding D28K gene; MCW0145 associated with eggshell thickness; MCW246 is located in high-mobility group protein 14 A1 gene and ADL273 associated egg number. (Cheng *et al.*, 1995; Crooijmans *et al.*, 1996; Groenen *et al.*, 1997, Miksic, *et al.*, 2003 and Mann *et al.*, 2008). Microsatellite markers names, sequences, annealing temperatures and chromosomal locations are present in Table (1).

Table 1. Microsatellite Markers Names, sequences, Annealing temperatures and Chromosomal locations

Marker Name	Primer sequence (5'-3')	Chromosomal Location	Annealing temperature(°C)
MCW241	AACCAGTTTGTTAACATCAGC ATTGGAGTTGGTACCATACTC	Z -72cM	50
ADL273	GCCATACATGACAATAGAGG TGGTAGATGCTGAGAGGTGT	Z -65cM	55
MCW246	TCATAAGGCAGAGAATTCATC TTTCCATTCAGACAACAAGGC	Z -104cM	55
MCW258	TTCTTAGTCCTTGCCAGAGGC CTGCAGGAGGATGTGTCCTAG	Z -63cM	55
ADL188	CACTTCCAGTATTAACGTGA GTGGACACAATGAGTTCC	1- 107cM	50
MCW0145	ACTTTATTCTCCAAATTTGGCT	Locus 5 (cM)	55
MCW0170	AAACACAATGGCAACGGAAAC TTGTGAAACTCACAGCAGCTG	Chromosome 1 Locus 2(cM)	55
MCW0068	TTATAGCAGGCTGGCCTGAAG CCTCACTGTGTAGTGTGGTAGTCA GAGAAGCTTGAACCTACCAGTCTT	Chromosome 4 Locus 3(cM) Chromosome 1	55

PCR conditions:

Polymerase chain reaction (PCR) was performed in 20 µl volumes containing 4 µl of PCR Master mix 5x (Bio Basic inc. Canada), 2 µl of each forward and reverse primer (10 pmol/ µl), 1 µl genomic DNA (50 ng/ µl) and 11 µl sterile deionized water. Amplification was performed in a thermo cycler (LongGene - MG96G / china) with the following temperature profiles: initial denaturation 94 °C for 4 min, 35 cycles (denaturation 94°C for 1 min/ annealing temp. (50-55 °C) for 1 min / extension 72°C 1

min and final extention 72°C for 4 min. The reaction was hold at 4°C.

Microsatellite-PCR products were resolved by electrophoresis on 3% agarose gel containing ethidium bromide for 90 min. at 60 volt, visualized via UV illuminator and then photographed. Molecular size of the amplified fragments, separated on gels were measured by analyzing gel images with GelAnalyzer software package version 2010a (freeware) with 100 bp DNA ladder (Larova GmbH-Germany) as DNA size marker.

Microsatellite data analysis:

The amplified bands were scored, for each microsatellite marker, based on the presence or absence of bands, generating a binary data matrix of 1 and 0 for each marker system. Effective alleles per locus (Aep) were calculated according to Weir *et al.*, (1989). Matrix was then analyzed using the PAST, ver. 1.90 (Hammer *et al.*, 2001). The data matrix was used to calculate genetic similarity based on Jaccard's similarity coefficients.

Extraction of eggshell matrix proteins:

Eight eggshell samples randomly collected from each line at 30 weeks of age. The eggshell matrix proteins were extracted as described by Gautron *et al.*, 2001 with some modifications.

Eggshells - collected from fresh eggs – were filled (the interior of the eggshell) with EDTA (5%) for 2 h and then rinsed with distilled water, then the eggshell membrane was removed. After removing the eggshell membrane, eggshells were rinsed with saline. The eggshells were then air dried and ground into fine powder. The powder (5 g) was demineralized with 50% acetic acid for 2h, centrifuged at 10000 rpm and the pellet was washed with distilled water. The pellet was then continuously stirred at room temperature in 20 ml of 4 mol l-1 guanidine-HCl (pH 7.4) for 4 hours. The mixture was then centrifuged at 12,000 rpm to separate the supernatant. The protein was precipitated from the extracted supernatant by 13% final concentration TCA. The protein pellets were dried and resuspended in 200 µl laemmli sample buffer.

Protein analysis and Electrophoresis:

Sodium dodecyl sulfate-PAGE was performed on 4% and 12% PAG (for stacking and separating gels, respectively). Aliquots of samples (resuspended protein in laemmli sample buffer) were heated in boiling water for 5 min. thirty µl of each sample were applied and constant 20 mA was adapted for about 6 h. After separation on the gel, protein bands were visualized by staining with coomassie blue according to Laemmli (1970).

Statistical analysis:

Data of eggshell components were statistical analysis by to one-way analysis of variance, with the Lines as the main effect using the General Linear Models (GLM) procedure of SAS User's Guide, Ver.8.2, 2001. Duncan's multiple range tests was used to separate means when differences existed. Molecular weight of proteins measured and scored manually and by GelAnalyzer program.

All scored microsatellite data was firstly corrected to estimate each allele size according to its number of repeats for each marker GelAnalyzer software package was adopted for this purpose. Then, a spread sheet program (Microsoft Excel) was used to arrange the included data for each breed regarding each locus. All possible extracted population figures were carried out employing a GENEPOP software package after data
conversion using CON.
The statistical models used in this study were as follows;

$Y_{ijk} = \mu + B_i + e_{ij}$

Where; μ= overall mean, B_i = line effect and e_{ij} = experimental error.

RESULT AND DISCUSSION

Organic matrix of eggshell:

Data presented in Table.2. Shows components of eggshell, it could be observed that insignificant difference for organic matrix and total proteins when compared between control and selected lines. However, the eggshell weight and the mineral weight of eggshell significantly were higher in the select line than the control line ($P<0.05$).

The eggshell soluble protein patterns (Figure 1) indicates no significant variations between the selected and the control lines. Genetic changes in eggshell quality, however, depend not only on having a measurement that contains a substantial genetic component but also it must relate to the incidence of breakages in the field.

The eggshell matrix is mainly composed of proteins that are thought to influence shell formation and calcification and, thus, modify the resulting properties of the shell. Although the concentrations of these proteins were higher in eggshell extracts from the selected line compared to those from the control line for ovotransferrin, ovoalbumin, and ovocledin-17 these result agree with Panheleux, *et al.*, (2000), however they studied effect age. The quantification of specific eggshell matrix proteins in different quality shells, is therefore, a promising tool for analyzing the origin of eggshell faults and may provide useful information for breeding programs. They also reported that the Ovotransferrin was negatively correlated with shape index, thickness, braking strength and stiffness. While, ovocleidin 17 was positively correlatied with braking strength and stiffness (Radwan 2010 and Ahmed *et al.*, 2005).

Mann *et al.* (2006; 2007 and 2008) and Miksic, *et al.* (2003 and 2007) identified 528 different proteins as constituents of the eggshell matrix. These proteins, that were found in eggshells were divided into specific proteins (proteins found only in eggshell) and non specific proteins. Radwan 2010, stated that there are 4 eggshell matrix proteins (ovocleidins-116 and -17, ovocalyins-36 and -32) that play important role in the ultrastructure of eggshells. Mahrous *et al.*, 2013; stated that the 15th generation of this selected line had better ultrastructure than control line.

Table 2. Eggshell components from the selected line for 16 generation and and control line

Traits	Line		
	Selection line	Control line	P value
Egg shell weight, gm	5.35±0.32	4.91±0.40	0.02
Total protein, gm	0.157±0.06	0.161±0.08	NS
Organic matrix, gm	0.45±0.03	0.50±0.02	NS
Mineral weight, gm	4.90±0.29	4.41±0.32	0.05

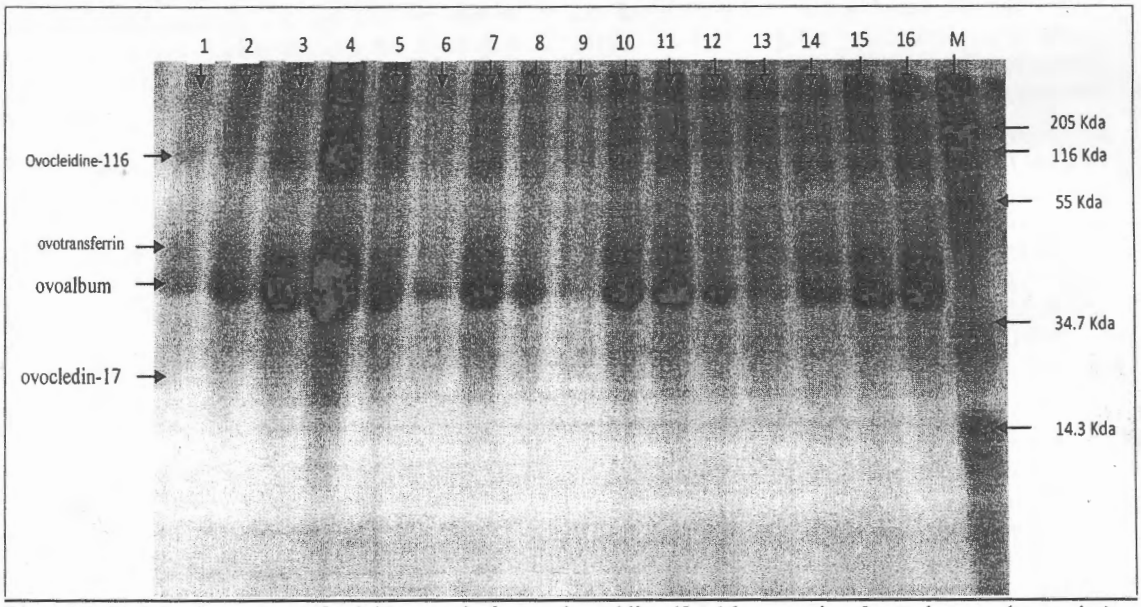

Figure 1. SDS-PAGE pattern of soluble protein from selected line (for 16 generation for early sexual maturity) and control line. Lanes 1-8 are random selected individuals from L1, lanes 9-16 are random selected individuals from control L2 and M is wide range protein marker.

Microsatellite Analysis:

Eight highly polymorphic microsatellites markers were used in the present investigation. Four out of them, are located on the Z-chromosome linkage group and cover approximately 41 cM (centi Morgan) of the Z chromosome map. The Z-chromosome four markers are associated with four egg traits; MCW241 is associated with BW and AFE (age at first egg; 72 cM), ADL273 is associated with EN (egg number, 65 cM), MCW246 is associated with ESS (egg shell strength, 104 cM) and MCW 258 is associated with EW (egg weight; 63 cM).

Chromosome 1 has two markers associated with three egg trait; MCW0145 (shell thickness at 53 weeks of age and shell weight at 53 weeks of age). Also (ADL188) is associated with HU. It is located on 1st chromosome linage group at the 107 cM site. The remaining marker MCW0170 is associated with albumin weight at 33 weeks of age is located on chromosomal four.

The chicken genome consists of 38 pairs of autosomes and a sex chromosomes Z. these chromosomes can be classified into two size groups, nine macro chromosomes and 5 micro chromosomes. For both used populations, the overall mean numbers of alleles detected per locus were 5.38 for the selected line and 5.0 for the control line (Table 3). The observed variability, of average number of alleles, seemed to reflect different potentialities of these genetic markers to detect genetic variability between such genetic groups.

The average number of alleles, per locus, can be divided into three groups. The first is that of the highest estimate (7.5 at locus MCW241 and 6.5 at locus MCW0145). The second group had moderate average (4.5 at both loci ADL273, MCW0068 and 5 at loci MCW246, ADL 188 and MCW0170). The third group is associated to locus MCW258 (3.5).

The 8 microsatellite markers, used in this study, were applied to both the control and the selected lines. The associations between their frequencies and the studied quantitative traits were investigated. In this respect, the selected line had higher frequencies of the loci MCW241 and MCW0145 than control line. This might be associated to BW, AFE and ST53 trait. These results reflect to improved body weight and shell thickness when selection to early age sexual maturity. Not differences in the frequencies of alleles were observed at the loci ADL188, MCW 246, and MCW0170 between control and the selected lines. These loci might be associated with Hu, ESS and AW33 trait. Thus the different in performance of both lines in their Hu, ESS and AW33 trait its might be due to management factors and not genetic effects. Our results, based on microsatellite genetic markers, proved the usefulness of this type of markers in chickens genome analysis. Soller *et al.* (2006) reported that breeding for egg quality traits by

traditional methods is difficult because the phenotypic measurements are time consuming. Also, their use in breeding programs is complicated due to unfavorable negative correlations with other relevant traits. Thus, genetic diversity measures, using microsatellites, yield reliable estimates of variability within the genetic relationships among chicken populations, as demonstrated in many studies (Delany, 2003). The QTL region on the Z chromosome is a large area including QTL for sexual maturity, egg weight, and number of eggs during the laying periods, as well as eggshell strength (Tuiskula-Haavisto *et al.*, 2002). Allen *et al.*, (1995), STRs have proven to be useful in the assessment of the overall genetic variation estimate, for most population's parameters, as well as, to gain insight into the degree of population substructure. Also, Zhang *et al.* (2002a, b) illustrated that microsatellite polymorphisms enable a clearer differentiation, even between closely related breed, and increase the accuracy of the predicted divergence. The eight microsatellite genetic markers applied in the present study succeeded to reveal high degree of polymorphism between the two lines (the selectied and control).

Table 3. Number of detected alleles, range of frequencies, both lowest & highest allele(s) and its frequency corresponding from line selected 16th and line control for each locus. (Selected L1 and control L2)

No	Trait	Locus	Line	No. allele	Frequencies range	Highest	Lowest
1	AFE	MCW241	L1	8	0.047 – 0.48	310	160
	BW		L2	7	0.039 – 0.42	330	115
			Average	(7.5)			
2	EN	ADL273	L1	5	0.082 – 0.72	154	142
			L2	4	0.071 – 0.57	140	110
			Average	(4.5)			
3	ESS	MCW246	L1	5	0.082 – 0.031	250	200
			L2	5	0.063 – 0.51	230	210
			Average	(5)			
4	EW	MCW258	L1	3	0.125 – 0.22	130	112
			L2	4	0.026 – 0.32	150	112
			Average	(3.5)			
5	HU	ADL188	L1	5	0.25 – 0.53	120	95
			L2	5	0.17 – 0.47	140	126
			Average	(5)			
6	YW33	MCW0068	L1	5	0.364 – 0.636	109	95
			L2	4	0.072 – 0.571	138	126
			Average	(4.5)			
7	SW53	MCW0145	L1	7	0.236 – 0.318	110	142
	ST53		L2	6	0.237 – 0.419	99,	131
			Average	(6.5)			
8	AW33	MCW0170	L1	5	0.464 – 0.636	119	99
			L2	5	0.272 – 0.571	108	106
			Average	(5)			
Total average			L1	5.38	Overall mean of alleles = 5.19		
			L2	5			

AFE= age at first egg; BW= Body weight; EN=Egg number; ESS= Egg shell strength; EW= egg weight (g); HU= Haugh units; YW33=Yolk weight (g) at 33 weeks of age; SW53=Shell weight (g) at 53 weeks of age; ST53=Shell thickness (mm) at 53 weeks of age; AW33= Albumin weight at (g) 33 weeks of age.

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تحليل الاختلافات الوراثيه لواسمات الميكروساتيللايت والتركيب العضوى لقشرة البيض فى دجاج منتخب ١٦ جيل لانتاج البيض

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تم دراسة الاختلافات الوراثية فى خطى الدجاج الاسكندرانى (الخط المنتخبL1 والخط الكنترولL2) للتعرف على التنوع الوراثى والخواص العشيره لصفات المتعلقة بالبيض وذلك باستخدم ٨ واسمات ميكروساتيللايت. اظهر الخط الاسكندرانى١ المنتخب ارتفاع للتكرار الجينى عن الخط المنتخب للماركر MCW241 وMCW0145 المرتبط بالموقع الذى يتحكم فى صفات وزن الجسم والعمر عند اول بيضه وسمك قشرة البيض عند عمر ٥٣ اسبوع . فى الوقت نفسه الماركر ADL188 و MCW 246 وMCW0170 المرتبطة بمواقع صفات وحدات هوه , متانة قسرة البيض و وزن البياض عند عمر ٣٣ اسبوع اظهر انه لا توجد اختلافات بين الخط المنتخب والخط الكنترول. لوحظ ايضا ان التباين غير معنوى بين الخط المنتخب ١٦ جيل وخط الكنترول لصفة البروتينات الذائب لقشرة البيض .نستنتج من هذه الدراسه ان الانتخاب للنضج الجنسى المبكر فان وزن الجسم وسمك القشره يتحسن .