UDC636

Print ISSN 1450-9156
Online ISSN 2217-7140

BIOTECHNOLOGY IN ANIMAL HUSBANDRY

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VOL 33, 1
Founder and publisher
INSTITUTE FOR ANIMAL HUSBANDRY
11080 Belgrade-Zemun
Belgrade 2017

Journal for the Improvement of Animal Husbandry

UDC636

Print ISSN 1450-9156
Online ISSN 2217-7140

BIOTECHNOLOGY IN ANIMAL HUSBANDRY

Belgrade - Zemun 2017

Biotechnology in Animal Husbandry 33 (1), p 1-133, 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636

Address of the Editor's office
Institute for Animal Husbandry, Autoput 16, P. Box 23, 11080 Belgrade-Zemun, Republic of Serbia Tel. 381 11 2691 611, 2670 121; Fax 381 11 2670 164;
e-mail: biotechnology.izs@gmail.com; www.istocar.bg.ac.rs

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According to CEON bibliometrical analysis citation in SCI index 212, in ISI 9, impact factor (2 and 5) of journal in 2012: 0,667 and 0,467, - M51 category

Annual subscription: for individuals -500 RSD, for organizations 1200 RSD, -foreign subscriptions 20 EUR. Bank account Institut za stočarstvo, Beograd-Zemun 105-1073-11 Aik banka Niš Filijala Beograd.

Journal is published in four issues annually, circulation 100 copies.

The publication of this journal is sponsored by the Ministry of Education and Science of the Republic of Serbia.
Printed: "Mladost birošped", Novi Beograd, St. Bulevar AVNOJ-a 12, tel. 381 11 2601-506

Biotechnology in Animal Husbandry 33 (1), p 1-11 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.09
DOI: 10.2298/BAH1701001M

OVERVIEW OF CURRENT ADVANCES IN THE DEVELOPMENT OF SUBUNIT AND RECOMBINANT VACCINES AGAINST NEWCASTLE DISEASE VIRUS

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Review paper

Abstract: Newcastle disease virus (NDV) is one of the most important viral pathogens of avian species and the causative agent of atypical fowl plague, a highly contagious and economically important disease characterized by high mortality rates and reduction of egg production. The HN and F proteins are the main targets for immune response to NDV. Vaccination of poultry with live and inactivated NDV vaccines is the most effective method of control and prevention of Newcastle disease, however due to their disadvantages, efforts are being invested into developing subunit vaccines. To this end, the NDV HN and/or F protein have been expressed using different viruses as vectors, but have also been expressed using transgenic plant systems, yeast and lactic acid bacteria in order to produce the NDV subunit vaccine. Many authors have investigated the possibility of preparation of vaccines from purified and biologically active NDV subunits with HN and F glycoproteins, purified from nucleocapsids, viral ribonucleic acid (RNA) and pyrogens. The above mentioned viral glycoproteins with preserved antigenic structure and biological activities can be used as subunit vaccinal antigens due to their immunogenic properties.

Key words: NDV, HN, F, subunit vaccines, recombinant vaccines

Introduction

Newcastle disease virus (NDV) is one of the most important viral pathogens of avian species and the causative agent of atypical fowl plague, a highly contagious and economically important disease characterized by high mortality rates and reduction of egg production (*Westbury, 2001; Ganar et al., 2014*). This is an enveloped virus with negative-sense single-stranded RNA and is classified in the genus *Avulavirus* of the subfamily *Paramyxovirinae* in the family

Paramyxoviridae (*Mayo, 2002; Kapczynski et al., 2013*). The viral genome contains six open reading frames (ORF) which encode the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin-neuraminidase (HN) and the large protein (L) (*Steward et al., 1993*). However, the F gene and the HN gene encodes essential proteins for virulence determination. The fusion (F) protein is responsible for mediating fusion of the viral envelope with cellular membranes while the HN protein is involved in cell attachment and release (*Milić et al., 2001; Milić et al., 2003; Nišavić et al., 2007; Heiden et al., 2014; Qiu et al., 2014; Jaganathan et al., 2015*). The HN and F proteins are the main targets for immune response to NDV (*Morgan et al., 1992; Arora et al., 2010; Chaturvedi et al., 2011; Kumar et al., 2011*). Newcastle disease virus strains were classified according to pathotyping assays to three classes: virulent - velogenic, moderately virulent - mesogenic, and non-virulent – lentogenic virus strains (*Dortmans et al., 2011; Susta et al., 2015*). Lentogenic NDV strains sometimes cause subclinical infections with mild respiratory or enteric disease and are considered as low-virulent. Mesogenic NDV strains are of intermediate virulence causing respiratory infection with moderate mortality (< 10%), while velogenic NDV strains are highly virulent causing mortality rates up to 100% (*Beard and Hanson, 1981*). The ND virus spreads horizontally between healthy and infected birds through direct contact with bodily secretions from infected birds (*Alexander, 2009*). The diagnosis of Newcastle disease virus infection is performed by the application of standard methods including virus isolation in chicken embryos, hemagglutination (HA test) and hemagglutination inhibition tests (HI test) as well as molecular methods based on Reverse transcription polymerase chain reaction - RT-PCR (*Nišavić et al., 2007; Milić et al., 2012*).

Vaccination of poultry with live and inactivated NDV vaccines is the most effective method of control and prevention of Newcastle disease (*Senne et al, 2004*). However, previous experience in immunoprophylaxis of atypical fowl plague has shown that vaccination with both mentioned vaccine types has both advantages, as well as disadvantages and that routine vaccinations are insufficient to control this disease given the increasing number of outbreaks in commercial poultry flocks worldwide (*Arora et al., 2010; Kang et al., 2016*). Live NDV vaccines generally induce protective immunity in vaccinated poultry, but circulating live vaccine viruses present additional risks such as reversion of virulence and recombination with wild-type strains. Furthermore, the immune response of wild birds induced by infection with vaccinal strains may provide selective pressures resulting in viral antigenic drift or increased virulence (*Lee et al., 2012; Palya et al., 2012; Read et al., 2015; Devlin et al., 2016*). Also, vaccines prepared from inactivated NDV strains often have a weaker immunogenic effect in immunized poultry, compared to live vaccines, and can cause local inflammation after the application of oil-emulsion inactivated vaccines (*Homhuan et al., 2004*).

Other types of vaccines that have been developed include the subunit and recombinant vaccines (*Boursnell et al., 1990; Nagy et al., 1991; Peeters et al., 2001*). Another major drawback of all currently used whole-virus-based live and inactivated NDV vaccines is that vaccinated animals cannot be distinguished from infected animals with standard serological tests, such as hemagglutination inhibition (HI test) or virus neutralization (VN test). A different concept for the development of a marker vaccine is based on the use of subunit vaccines and it has been achieved for many antigens involved in inducing protective immunity, including the two glycoproteins F and HN of NDV (Morgan et al., 1992). The above mentioned viral glycoproteins with preserved antigenic structure and biological activities can be used as subunit vaccinal antigens (*Milić et al., 1996; Tanabayashi and Compans, 1996; Milić et al., 2001; Arora et al., 2010; Milić et al., 2015)*. Furthermore, birds vaccinated with the recombinant fowlpox-NDV HN subunit vaccine can now be distinguished from the naturally infected ones by their antibody responses to the subunit vaccine on ELISA plates coated with recombinant baculovirus-NP protein as the coating antigen. However, such tests can only be useful if the current live or inactivated vaccines are replaced by recombinant subunit vaccines (*Yusoff and Tan, 2001*).

Theoretically, the genes encoding any protein can be cloned and expressed in bacteria, yeasts or mammalian cells. A number of genes encoding surface antigens from viruses, bacteria and other single celled pathogens have been cloned in expression systems and the expressed antigens have been used as vaccines (*Arntzen and Mason, 1995*). Efforts are being invested into developing subunit vaccines because of the disadvantages presented by the existing traditional vaccines. To this end, the NDV HN and/or F protein have been expressed using different viruses as vectors, but have also been expressed using transgenic plant systems, yeast and lactic acid bacteria in order to produce the NDV subunit vaccine.

As an initial approach to the development of novel anti-NDV vaccines, *Berinstein et al. (2005)* demonstrated that NDV F and HN proteins can be correctly expressed in transgenic potato plants. Specific anti-NDV antibodies recognize them and they are immunogenic in mice after parenteral administration or as edible vaccines, stimulating, in the latter case, the production of specific IgA in the gut. *Shahriari et al. (2015)* studied the application of tobacco hairy roots for expression of the F and HN epitopes of Newcastle disease virus. The authors have suggested that since plant-based systems possess a number of drawbacks in recombinant vaccine production, these might be overcome by using transient expression systems like tobacco hairy roots as they have proved to be an efficient tool for expression of these viral antigens.

Kang et al. (2016) demonstrated the potential of F protein of NDV expressed by the methylotrophic yeast *Pichia pastoris* (*P. pastoris*) as a subunit vaccine candidate when administered with flagellin as the adjuvant. The

aforementioned protein was efficiently expressed in the *P. pastoris* system and verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting. The F protein induced strong humoral and cell-mediated immune response in experimental mice when administered i.p. with *Salmonella* flagellin as adjuvant. *Khulape et al. (2015)* attempted to express the HN protein of NDV in a yeast expression system. The authors found that *Saccharomyces cerevisiae* was a better expression system for HN protein than *Pichia pastoris* as determined by codon usage analysis. The yeast cells were able to generate glycosylated HN protein with proper folding and antigenicity. The recombinant HN (rHN) protein was characterized by western blot and purified by affinity column purification and it was concluded that it could be further used as subunit vaccine. Since lactic acid bacteria are naturally associated with mucosal surfaces, particularly the gastrointestinal tract, they have also been considered as promising mucosal delivery vesicles to produce protective antigens (Shaw et al., 2000). Jiang et al. (2015) constructed a recombinant *Lactobacillus plantarum* (RLP) expressing HN protein of NDV. Oral administration of RLP significantly increased the production of secretory immunoglobulin A (SIgA) and the percentages of CD3+CD4+ T cells in chickens, providing at least partial protection in the NDV challenge experiment. The immunization with HN resulted in 40% survival rates in experimentally infected chicken. One of the possible explanations of only partial protection is that the selected HN protein performs less effective with regard to protection results compared to another glycoprotein, the fusion protein F, according to reports of *Meulemans et al. (1986), Kumar et al., (2011)* and *Kim et al., (2013).*

Recombinant vaccines based on viral coat protein subunits represent an efficient tool as a substitute for conventional, attenuated virus based vaccines (*Makela, 2000). Boursnell et al. (1990)* have investigated the expression of hemagglutinin-neuraminidase (HN) gene from the Beaudette C strain of NDV in a recombinant fowlpox virus vector. When the recombinant fowlpox virus was inoculated into chickens by intravenous or wing-web routes, specific antibodies against HN antigen from purified NDV virions were produced. Protective immunity to NDV was generated all experimental chickens and at the highest dose of vaccine 100% of the tested chickens were protected against challenge with a virulent strain of NDV. Recombinant baculoviruses containing the fusion (F) and hemagglutinin-neuraminidase (HN) glycoprotein gene of the viscerotropic velogenic (vv) NDV isolate, Kr-005/00, and a lentogenic La Sota NDV strain were constructed in an attempt to develop an effective subunit vaccine to the recent epizootic of vvNDV in Korea (*Lee et al., 2008*). The authors evaluated the protective effect of individual recombinant glycoproteins derived from velogenic and lentogenic NDV strains. The recombinant glycoproteins from the virulent strain produced complete protection after the second immunization, whereas those from the lentogenic strain had a slightly lower protective effect. A synergistic effect of the combined F and HN glycoprotein was noted and it was concluded that

the use of a subunit vaccine composed of the two glycoproteins can offer good protection against NDV. *Ge et al. (2016)* designed novel recombinant baculovirus vaccines expressing the NDV F or HN genes. The F-series of vaccines provided a greater degree of protection (87.5–100%) than the HN series (62.5–87.5%). The authors concluded that the baculovirus system is a promising platform for NDV vaccine development that combines the immunostimulatory benefits of a recombinant virus vector with the non-replicating benefits of a DNA vaccine. Kumar et al. (2011) achieved a 100% rate of protection by immunizing chickens using a recombinant NDV vaccine containing the F and HN gene using the avian paramyxovirus type III virus (APMV 3) as the vector. These vaccines were used to immunize 2-week-old chickens by the oculonasal route in order to evaluate the contribution of each protein to the induction of NDV-specific neutralizing antibodies and protective immunity. Protective immunity was evaluated by challenging the immunized birds 21 days later with virulent NDV and the obtained results indicated that F and HN proteins are independent neutralization and protective antigens, but that the contribution of F antigen in protection is greater. *Palya et al. (2014)* investigated the onset and long-term duration of immunity provided by a single vaccination with a turkey herpesvirus vector Newcastle disease (rHVT-ND) vaccine in commercial layers up to 72 weeks of age. Assessment of protection was done based on the prevention of clinical signs and reduction of challenge virus shedding via the oronasal and cloacal routes. Single vaccination with the rHVT-ND vaccine at one day of age provided complete or almost complete (95–100%) clinical protection against NDV challenges from 4 weeks of age up to 72 weeks of age when the latest challenge was done. Shedding of challenge virus both by the oronasal and cloacal route was significantly reduced compared to the controls.

Many authors have investigated the possibility of preparation of vaccines from purified and biologically active NDV subunits with HN and F glycoproteins, purified from nucleocapsids, viral ribonucleic acid (RNA) and pyrogens. The objective of the work of *Milić et al. (2015)* was to investigate some biological characteristics of purified glycoprotein subunits of PHY-LMV.42 Newcastle disease virus strain isolated from pigeons for the purpose of vaccine production. Testing for the immunogenicity of the viral subunits was carried out in a biological experiment on 75 Tetra SSL laying hens and 25 chickens Isa Brown after an artificial infection with Hertz 33 strain of NDV. The subunit vaccines of 256 and 128 HAU/0.5 ml induced a protective immune response in all vaccinated animals. Based on the obtained results it was concluded that the examined purified viral subunits of the PHY-LMV.42 strain of NDV, separated from nucleocapsids (NP proteins with viral RNA), large polymerase protein (L) and smaller fragment of F protein (F_2) can be used for a new potential vaccine. The study of *Arora et al. (2010)* concerned the immunization potential of purified HN and F glycoproteins of the Indian vaccinal NDV strain R_2B. This investigation indicates the role of

these glycoprotein subunits in the elicitation of protective immune response against NDV. Similarly, Meulemans et al. (1986) reported higher protective response of the F glycoprotein which could be explained by the fact that specific anti-F antibodies block cell-fusion activity thus preventing the spread of infection.

Conclusion

Nowadays, the development of subunit vaccines is based on the expression of HN and F proteins of NDV using viruses, plant-based systems, yeast and lactic acid bacteria as vectors in order to prepare recombinant immunogens. Some of the developed vaccines stimulate a satisfactory immunological response against NDV and have proved to be successful in protection of vaccinated animals in challenge experiments. The advantage of NDV subunit vaccines comparing to live and inactivated vaccines is in their safety for vaccinated animals and the fact that there are no unwanted postvaccinal effects. The subunit vaccine production procedure enables the recovery of a larger concentration of vaccinal antigens, thus a greater number of doses of the vaccine compared to live or inactivated vaccines. Some of the abovementioned vaccines like VectorVax FP-N, Trovac-NDV and Innovax-ND have been licensed in certain countries. However, vaccinated animals may have acquired immunity to certain vaccinal vectors which could be unfavourable regarding the development of the immune response to HN and F antigens contained in the vaccine. Additionally, most vectors used for the preparation of subunit recombinant vaccines are potential pathogens for the population of vaccinated animals which raises the question of vaccine application in field conditions. A large number of NDV subunit vaccines are prepared form genetically modified live viruses which must pass rigorous testing before vaccine registration. Aside from that, the use of other expression systems like transgenic plants may cause a biological safety problem. Subunit NDV vaccines can also be prepared from purified and biologically active NDV subunits with HN and F glycoproteins, purified from nucleocapsids with viral ribonucleic acid (RNA) and pyrogens which elicit a strong immunological response in vaccinated animals with no unwanted postvaccinal effects.

Pregled savremenih saznanja o razvoju subjediničnih i rekombinantnih vakcina protiv virusa Newcastle bolesti živine

Nenad Milić, Jakov Nišavić, Andrea Zorić, Dejan Krnjaić, Marina Radojičić, Aleksandar Stanojković

Rezime

Virus Newcastle bolesti je jedan od najznačajnijih patogena u populaciji ptica i domaće živine koji izaziva atipičnu kugu živine, kontagiozno oboljenje koje prati visoka stopa morbiditeta i mortaliteta, što ima za posledicu i velike ekonomske gubitke u živinarstvu. Glikoproteinski HN i F antigeni virusa atipične kuge živine su najznačajniji prilikom razvoja imunološkog odgovora prijemčivih jedinki. Vakcinacija živine živim i inaktivisanim vakcinama protiv virusa Newcastle bolesti predstavlja najefikasniji metod kontrole i prevencije navedenog oboljenja, međutim klasične vakcine imaju izvesne nedostatke i iz tog razloga se sve više istraživanja se usmerava na razvoj subjediničnih vakcina. U cilju razvoja subjediničnih vakcina u današnje vreme se za ekspresiju HN i F proteina virusa Newcastle bolesti koriste različiti vektori kao što su virusi, transgene biljke, kvasci i mlečnokiselinske bakterije. Pored toga, mnogi autori su ispitivali mogućnosti pripremanja subjediničnih vakcina od prečišćenih i biološki aktivnih subjedinica, odnosno HN i F glikoproteina pomenutog virusa, oslobođenih od nukleokapsida sa virusnom ribonukleinskom kiselinom (RNK) i pirogena. Virusni glikoproteini sa očuvanom antigenskom strukturom i biološkim aktivnostima se zbog svojih imunogenih svojstava mogu koristiti kao subjedinični vakcinalni antigeni.

Ključne reči: NDV, HN, F, subjedinične vakcine, rekombinantne vakcine

Acknowledgment

This work was realized within the Project TR 31008 under the title: “Development and application of molecular methods based on polymerase chain reaction (PCR) in rapid and direct identification of Newcastle disease virus strains and examination of immunogenicity of subunit vaccine prepared of their antigens” financed by The Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Received 25 October 2016; accepted for publication 28 December 2016

Biotechnology in Animal Husbandry 33 (1), p 13-25 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.291
DOI: 10.2298/BAH1701013M

GENOME-WIDE SNPs ANALYSIS OF INDIGENOUS ZEBU BREEDS IN PAKISTAN

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Original scientific paper

Abstract: Prospects of high throughput technology in animal genetics makes easy to investigate hidden genetic variation in farm animal's genetic resources. However, many SNPs technologies are currently practicing in animal genetics. In this study, we investigated genome wide SNPs variations and its distribution across the indigenous cattle population in Pakistan using Illumina Bovine HD (777K) SNPs bead chip. A total of 136 individuals from ten different breeds were genotyped and after filtration 500, 939 SNPs markers were used for further analysis. The mean minor allele frequency (MAF) was 0.23, 0.20, 0.22, 0.22, 0.20, 0.18, 0.20, 0.22, 0.21 and 0.18 observed for Achi, Bhagnari, Cholistani, Dhanni, Dajal, Kankraj, Lohani, Red sindi, Sahiwal and Tharparkar cattle, respectively. Significant difference ($P<0.001$) of MAFs were observed in selected population. A common variants minor allele frequency (≥ 0.10 and ≤ 0.5) was estimated (64%). Across all sampled populations 64% SNPs markers were observed polymorphic ($MAF>0.05$) within breeds and remaining 36% were considered as monomorphic markers. Average observed (H_o) and expected (H_E) heterozygosity values 0.662 and 0.640 were estimated among these breeds. In conclusion, this preliminary study results revealed that these SNPs variation level could potentially be used for genetic characterization of zebu cattle breeds and could also be used to estimate genetic potential of these cattle breeds for livestock improvement in country.

Keywords: minor allele frequency, SNPs, variation, distribution, cattle

Introduction

Bovine high density (HD) SNPs assay is a most comprehensive genotyping tool to explore genome variation with high coverage resolution across cattle breeds (*Howard et al., 2015*). This features more than 777,962 SNPs probes that are equally distributed across entire bovine genome (*Leroy, 2014*). This array was first time introduced in 2009 (*Mbole-Kariuki et al., 2014*). Applications of this array include genome wide association studies, quantitative trait loci identification, prediction of genetic merit, linkage disequilibrium and breed characterization (*Pryce at el., 2014*). The potential of this array has been proven in several studies that identified genomic regions which have strong contribution in phenotypic variation. These genomic regions that are related with feed efficiency and intake traits (*Lin at el., 2010; Edea et al., 2014*) milk production traits and meat type traits (*Howard at el., 2015; Kim at el., 2015*).

In addition, genomics values prediction in breeding programme based on genomic data have been extensively used for cattle selection (*Edea et al., 2014*). The genomic selection tools reliability is based mainly on linkage disequilibrium (LD) existence and their association between SNPs and QTL that affects the traits of interest (*Caruthers et al., 2011; Curik at el., 2014; Kim at el., 2015*). In U. S. A and other developed countries genomic information is widely used for genetic evaluation of farm animals (dairy and beef) (*Howard at el., 2015*). The Bovine HD SNP assay has also been used to identify copy number variations (CNV) that are used for QTL association with phenotypes (*Bickhart at el., 2016*). In addition, Bovine high density (HD) genotyping assay has also been used to detect genetic relationships among and within cattle breeds and also been applied to detect signature of selection in different dairy and beef breeds (*Kim at el., 2015*).

In Pakistan, all genetic improvement programmes for dairy and beef cattle breeds are based on conventional quantitative genetics methods. There is also limited availability of phenotypic and pedigree data information for estimation of breeding values in these breeds (*Mustafa at el., 2014*). Conventionally, the genetic structure of economically important traits was considered to be a black box with little information of the genes variations affecting phenotypic expression of these traits, gene interactions, and the location of these genes in the genome (*Decker et al., 2014; Hussain at el., 2016*). Meanwhile, it has been found that genetic selection has a high probability to increase genetic gain in cattle and also permits more accurate genetic predictions for traits of low heritability in farm animals than conventional phenotypic selection (*Groeneveld at el., 2010; Curik at el., 2014; Leroy, 2014; Kim et al., 2015*). Currently, indigenous cattle breeds in Pakistan still lack the opportunity for high throughput evaluation. To better understand complex evolutionary process and breeding improvement programmes. To date, no indigenous Pakistani cattle breed has been included either in training or a validation population using the Bovine HD SNP BeadChip (*Mustafa at el., 2014*).

Therefore, it is necessary to assess the usefulness of the Bovine HD SNP BeadChip in indigenous Pakistani cattle breeds. The evaluation of this high throughput technique would be help to improve the cattle farming and establish a reference population. Therefore, the aim of this analysis was to find the level of informativeness of Bovine HD SNP BeadChip by measuring loci polymorphism in indigenous cattle population in Pakistan.

Materials and Methods

Animals sampling, genomic DNA extraction and Genotyping

A 10ml Jugular blood samples were obtained from ten different breeds from potential agro-geographical area of these breeds using EDTA containing tubes (Table 1 & Figure 1-2). The gDNA extraction and quality control of data was described in a previous study (*Mustafa at el., 2014*). Genotyping of selected samples was performed at USDA platform using Illumina Bovine high density (HD) SNPs bead chip (version 2) spanning 777, 962 SNPs markers across all bovine genome. 200 ng gDNA quantities were used to genotyped these samples according to manufacture protocol.

Table 1. Animals sampling and geographical details

Population	Code	N	Agro-ecology*	Purpose	Province
Achi	AC	18	West Mountains	Milk and Meat	Khyber Phaktunpkhua
Bhagnari	BH	14	Sulaiman Piedmont	Work	Balochistan
Cholistani	CL	13	Sandy Desert	Milk and Meat	Punjab
Dhanni	DH	10	Barani Lands	Work and Milk	Punjab
Dajal	DJ	10	Sulaiman Piedmont	Work and Meat	Punjab
Kankraj	KK	12	Sandy desert	Work and Meat	Sindh
Lohanni	LH	19	Western Dry Mountains	Work and Milk	Balochistan
Red Sindhi	RH	13	Southern Irrigated	Milk	Sindh
Sahiwal	SH	14	Northen Irrigated Plains	Mlik	Punjab
Tharparkar	TH	13	Sandy desert	Mlik	Sindh

*Figure 1 showed complete agro-geographic location

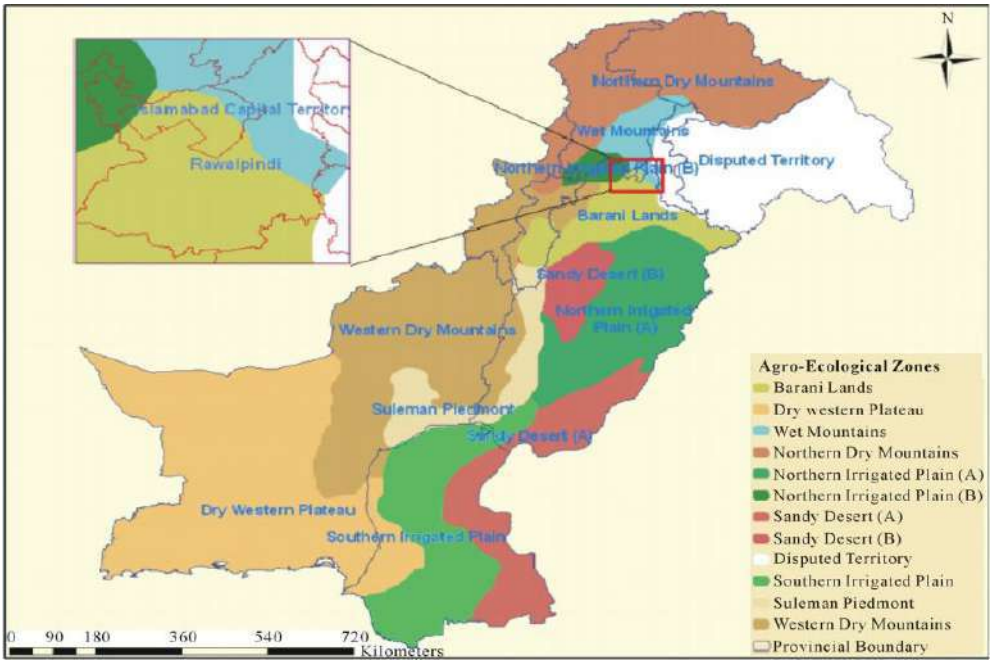

Figure 1. Agro-ecological zones of Pakistan (Kazmi & Rasul, 2012).

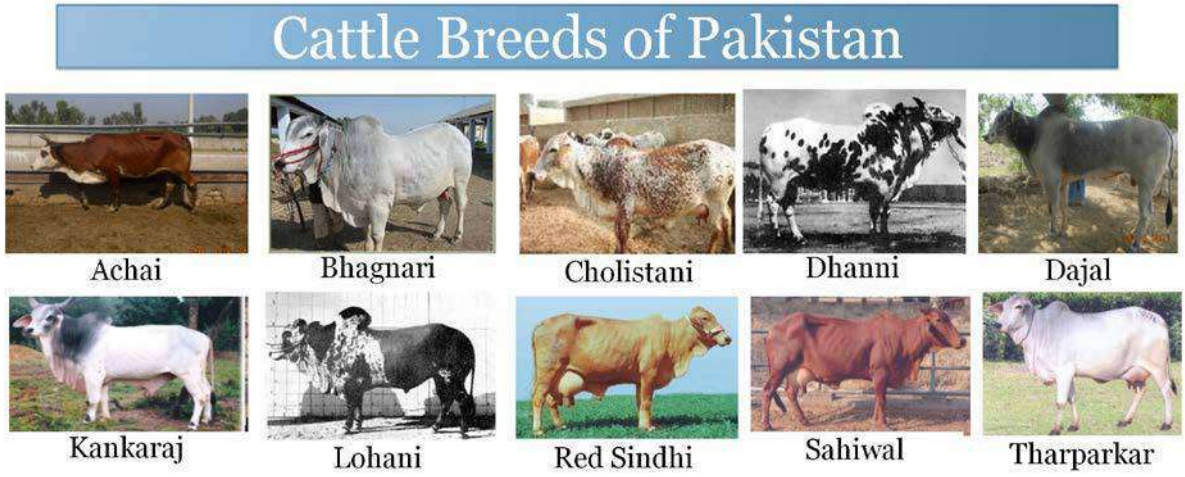

Figure 2. Cattle Breeds of Pakistan (Mustafa *et al.*, 2012)

Data analysis

Genotypic data were generated from the iScan system. The raw data analysis including genotyping calling, clustering and data normalization was performed by using genome studio version 1.9.0 software (*Edea at el., 2015*) .Pad and map. file was created for downstream analyses from the genome studio using PLINK (version 1. 9). Quality assurance module were used form SVS (version 8; Golden Helix Inc., USA) for genotypic statistics each markers were analyzed for call rate, Hardy- Weinberg equilibrium (HWE), minor allele frequency (MAF) and genotypes count. Quality control (QC) criteria for further analysis were < 95% call rate and <0.05 minor allele frequency (MAF). Hardy Weinberg equilibrium (P<0.001) was tested to help identify genotyping errors (*Kim at el., 2015*).

Results and Discussion

Minor Allele Frequency (MAF) Distribution

The minor allele frequency (MAF) was calculated and presented in Table 2 & figure 3 for each SNP from the generated data set. The analysis of 500,939 SNP markers indicate an average minor allele frequency (MAF) that is 0.23, 0.20, 0.22, 0.22, 0.20, 0.18, 0.20, 0.22, 0.21 and 0.18 for Achi, Bhagnari, Cholistani, Dhanni, Dajal, Kankraj, Lohani, Red sindi, Sahiwal and Tharparkar cattle, respectively. There was a significant difference observed among these selected breeds (p<0.001). The overall minor allele frequency (MAF) was observed in this study was higher than previous reported studies in *indicine* breeds (*McKay at el., 2008; Edea et al., 2015; Kim at el., 2015*) and lower than the average value reported for Red Chittagong that was 0.28 (*Uzzaman at el., 2014*). The lower average minor allele frequency (MAF) value is as expected than most of the Bos *taurus* cattle breeds (*Mckay et al., 2008; Mustafa at el., 2014*). The minor allele frequency (MAF) found in this study revealed that these attributes to different markers density (Illumina Bovine 8K, 10K, 50K, 80K and 700K) used in previous studies in different cattle breeds around the world and most of these breeds samples were not used before or during designing of these chips (*Chen at el., 2010; Lin at el., 2010; Melka at el., 2011; Edea at el., 2014; Uzzaman at el., 2014*).

Table 2. Minor Allele Frequency (MAF) values of indigenous cattle breeds in Pakistan.

	Breed	Minor Allele Frequency (MAF)
1	Achi	0.23
2	Bhagnari	0.20
3	Cholistani	0.22
4	Dhanni	0.22
5	Dajal	0.20
6	Kankraj	0.18
7	Red Sindhi	0.20
8	Lohani	0.22
9	Sahiwal	0.21
10	Tharparkar	0.18
		0.21

The SNP variation across all Pakistani cattle breeds was also examined. The SNPs minor allele frequency (MAFs) distribution at common variants (≥ 0.10 and ≤ 0.5) accounts is 64% (Table 3). Among these selected breeds, Dhanni cattle displayed high proportions of common variants (69%). The minor allele frequency (MAFs) variation at rare variant (>0 and <0.05) were observed 11% in overall breed samples. The higher proportion of alleles (fixed) in selected cattle populations indicate inbreeding that is due to uncontrolled breeding management in country (*Groeneveld at el., 2010; Lin at el., 2010; Leroy, 2014*). The high proportions of common variants were also reports in sheep that was 83 % (*Kijas et al., 2009*). The average minor allele frequency (MAF) distributions at ≥0.30 and ≤0.5 were displayed 32 % that is higher than previous reported polymorphism in cattle breeds (*McKay at el., 2008; Kim at el., 2015*). It is an established fact that higher proportions of minor allele frequency (MAFs) were observed in Bos *taurus* rather than Bos *indicus* using different Illumina bovine Bead chips due to limited numbers of indicus breeds were used during chip developments (*Decker at el., 2014; Mustafa at el., 2014; Bickhart at el., 2016*). The SNPs distribution at fixed level (0) was also examined and average 8% was observed among all these breeds. The highest SNPs proportion at fixed level was observed in Dhanni and Tharparkar (10%) and lower level in Bhagnari and Lohani (6%) cattle breeds, respectively.

Across all sampled populations 64% SNPs markers were observed polymorphic (MAF>0.05) within breeds and remaining 36% were considered as monomorphic markers (Figure 5). The higher proportion of polymorphism among these breeds was showed in Dhanni breed (69%). the high proportion of SNP variation in this study was higher than previous reported SNP variation in different

cattle breeds (*Curik at el., 2014; Howard at el., 2015; Kim at el., 2015*). Although, the results of SNP variations in this study revealed close similarity with the some previously reported variation in other farm animals including sheep and goat using genome wide SNP array (*Kijas et al., 2009*). The observed polymorphism in these selected breeds could explain that maximum bovine sequence data were available in the development of bead chip were from European cattle breeds (Bos *taurus)* (*Gautier at el., 2010; Melka at el., 2011; Edea at el., 2014; Decker at el., 2014*).

Genetic Diversity among Pakistani cattle breeds

The genomic variability within these cattle breeds were also examined and compare heterozygosity level between these breeds (Table 4). Across all these cattle breeds, the average observed (H_o) and expected (H_E) heterozygosity were 0.662 and 0.640, respectively. The average heterozygosity level was observed higher than the previous reported microsatellite markers analysis in some *indicine* cattle breeds (*Hussain et al., 2016*). Meanwhile, there is close agreement with previous reported values using SNPs in Brahman, Gir cattle and Nellore cattle (*Dadi at el., 2012; Leroy, 2014; Pryce at el., 2014; Decker at el., 2014; Bickhart at el., 2016*).

The F-statistics were also estimated within these selected breeds. Overall inbreeding within population (F_{IS}) value was estimated (0.073), where total inbreeding (F_{IT}) was 0.082. Genetic differentiation (F_{st}) was estimated at 0.076 (*Mbole-Kariuki at el., 2014; Edea at el., 2015; Kim at el., 2015*). Previously, in a study of Genetic characterization in Pakistani cattle population reported (inbreeding within population (F_{IS}) of 0.2819, F_{IT} (total inbreeding) of 0.3864 and F_{st} of 0.1456) using microsatellite makers (Hussain at el., 2016). The F_{st} of cattle breeds in Pakistan was observed low as reported in some previous zebu cattle studies (*Gautier at el., 2010; Groeneveld at el., 2010; Lin at el., 2010; Leroy at el., 2014*) that may be due to common origin.

The overall Hardy-Weinberg equilibrium (HWE) deviation ($p<0.05$) were significantly observed for 840 markers in these cattle breeds. Including Achi, 789; Bhagnari, 813; Cholistani, 744; Dhanni, 821; Dajal, 799; Kankraj, 811; Lohani, 787; Red sindi, 852; Sahiwal, 818; and Tharparkar, 911. Achi cattle showed lower proportion of markers deviating from Hardy-Weinberg equilibrium (HWE) similarly described in a previous study of African zebu cattle breeds (*Decker at el., 2014; Edea at el., 2015*). The proportion of SNPs variation displaying deviating from Hardy-Weinberg equilibrium (HWE) among the selected breeds could be expounded by population structure (admixture) and selection pressure.

Table 3. Distribution of minor allele frequency (MAF) of high density SNP (777, 962K) BeadChip in indigenous Pakistani cattle breeds.

		Fixed (0)		Rare (> 0 & < 0.05)		Intermediate (≥ 0.05 & ≤ 0.10)		Common (≥ 0.10 & ≤ 0.50)			≥ 0.30 & ≤ 0.5	
Breed	N	SNP	Prop.	SNP	Prop.	SNP	Prop.	SNP	Prop.		SNP	Prop.
Achi	18	54,329	0.070	84,317	0.108	98,421	0.127	431,897	0.56		215,949	0.28
Bhagnari	14	50,203	0.065	88,213	0.113	98,341	0.126	508,995	0.65		222,341	0.28
Cholistani	13	68,900	0.089	87,973	0.113	96,774	0.124	510,898	0.66		255,449	0.32
Dhanni	10	78,790	0.101	89,645	0.115	98,771	0.127	533,996	0.69		266,998	0.34
Dajal	10	69,471	0.089	89,763	0.115	98,721	0.127	498,898	0.64		256,631	0.33
Kankraj	12	55,431	0.071	89,789	0.115	97,631	0.125	499,399	0.64		243,421	0.31
Red Sindhi	13	58,991	0.076	88,976	0.114	98,984	0.127	521,999	0.67		261,000	0.34
Lohani	19	52,381	0.067	86,881	0.112	97,423	0.125	499,798	0.64		253,451	0.33
Sahiwal	14	68,360	0.088	84,953	0.109	93,946	0.121	500,968	0.64		250,484	0.32
Tharparkar	13	79,432	0.102	86,977	0.112	99,781	0.128	502,538	0.65		251,269	0.32
Overall	136	63,629	0.082	87,749	0.113	97,879	0.126	500,939	0.64		250,469	0.32

Table 4. Observed (H_o), expected (H_e) heterozygosity (F_{IT}) Total inbreeding, (F_{IS}) within population inbreeding of indigenous cattle breeds in Pakistan

Population	N	H_O	H_E	F_{IT}	F_{IS}
AC	18	0.663	0.628	0.034	0.089
BH	14	0.666	0.645	0.068	0.039
CL	13	0.657	0.645	0.039	0.017
DH	10	0.602	0.628	0.062	0.045
DJ	10	0.672	0.645	0.086	0.059
KK	12	0.628	0.619	0.028	0.040
LH	19	0.679	0.645	0.109	0.083
RH	13	0.651	0.645	0.021	0.043
SW	14	0.66	0.635	0.095	0.086
TH	13	0.701	0.645	0.179	0.164
Total	136	0.662	0.64	0.082	0.073

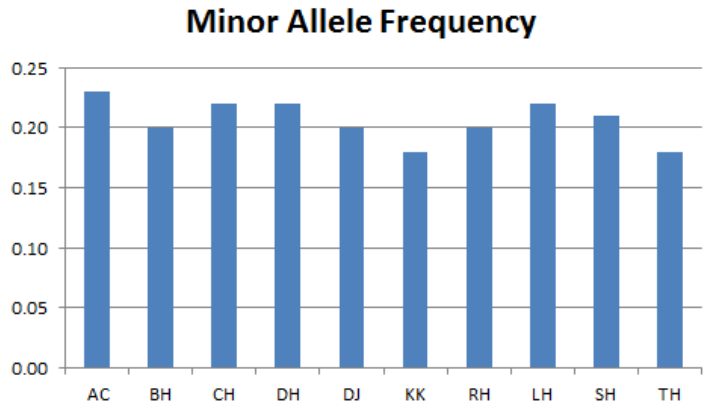

Figure 3. Minor Allele Frequency

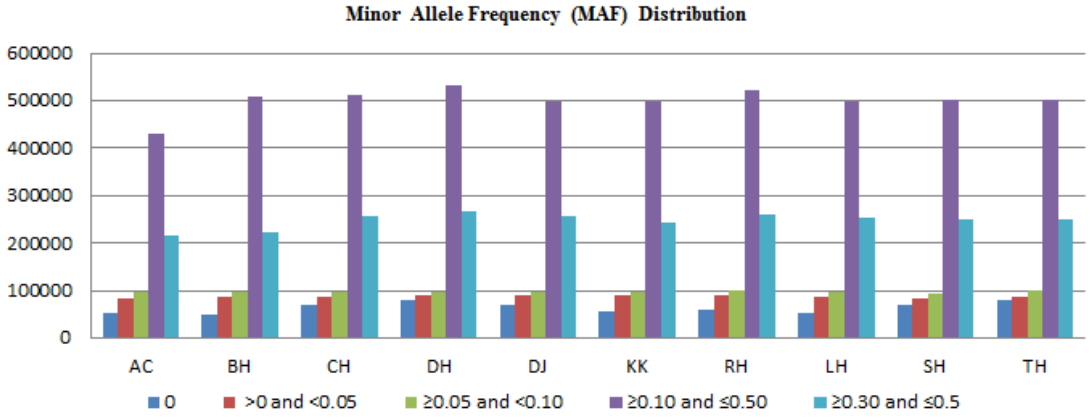

Figure 4. Minor Allele Frequency Distribution across all ten indigenous cattle Breeds in Pakistan

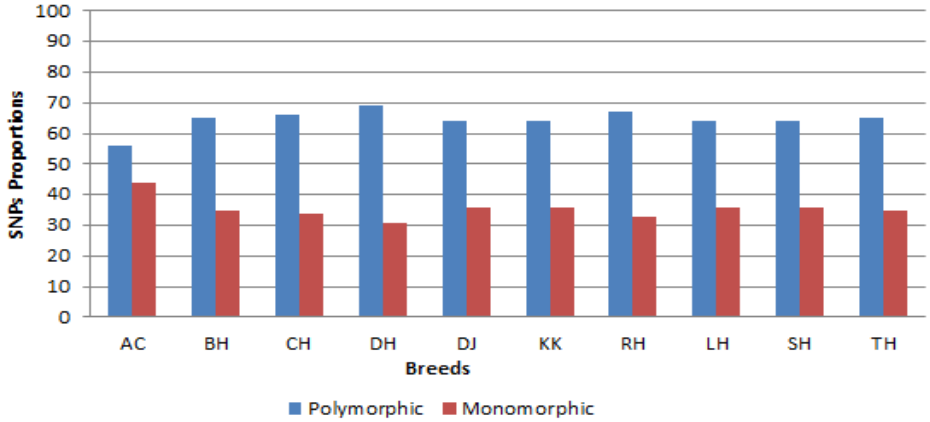

Figure 5. Polymorphic and Monomorphic SNPs distributions across all sampled breeds

Conclusion

The results of this preliminary study of Bovine high density SNPs revealed that the distribution of SNPs markers across the bovine genome of native zebu breeds in Pakistan was significantly different and identified some level of polymorphism and minor allele frequency (MAF) rate among these breeds. The levels of SNPs variation in this study encourage future use of Bovine High Density SNP assay with great extent for genetic studies in these breeds. These results could be effectively used to understand breed composition and within breed diversity, which could be an attractive opportunity to allow this important genetic resource improvements through effective population selection strategy.

SNPs analiza na nivou genoma autohtonih zebu rasa u Pakistanu

Hamid Mustafa, Kim Eiusoo, Huson J. Heather, Adeela Ajmal, David Riley, Talat Nasser Pasha, Afzal Ali, Khalid Javed, Tad S. Sonstegard

Rezime

Genetički resursi domaćih životinja (AnGR) u Pakistanu imaju jedinstven identitet širom sveta. Postoji petnaest različitih rasa goveda. Ispitali smo SNP varijacije i distribuciju među deset rasa goveda koristeći Bovine high density (777k) Bead čip. Ukupno 136 individualnih grla deset različitih rasa su genotipizirani i posle filtracije 500, 939 SNP markera je korišćeno za dalju analizu. Srednja niže frekvencija alela (MAF) je bila 0,23, 0,20, 0,22, 0,22, 0,20, 0,18, 0,20, 0,22, 0,21 i 0,18 za Achi, Bhagnari, Cholistani, Dhanni, Dajal, Kankraj, Lohani, Red Sindi, Sahival i Tharparkar goveda, respektivno. Značajna razlika ($p<0,001$) MAF je uočena u odabranoj populaciji. Zajednička varijanta niže frekvenciji alela ($\geq 0,10$ i $\leq 0,5$) je procenjena (64%). U uzorkovanoj populaciji, 64% SNP markera su polimorfni (MAF> 0,05) u okviru rasa i preostalih 36% su smatrani monomorfnim markerima. Prosečno registrovane (H_o) i očekivane (H_E) vrednosti heterozigotnosti od 0,662 i 0,640 su dobijene kod ovih rasa. Ovaj rezultat ukazuje na značajnu razliku između ovih rasa i ukazuje na to da SNP varijacije imaju potencijal koji bi mogao da se koristi u budućnosti za efikasnu selekciju i odgajivačke programe u poboljšanju stočarske proizvodnje i studijama konzervacije ovih rasa u zemlji.

Ključne reči: frekvencija minor alela, SNP, varijacija, distribucija, goveda

Acknowledgments

The authors would like to thanks USDA (BFGL) for laboratory facilities. HEC is greatly acknowledged for financial assistance for this study. All public Livestock Experiment Stations are also appreciated for providing blood samples.

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Received 7 November 2016; accepted for publication 22 January 2017

Biotechnology in Animal Husbandry 33 (1), p 27-36 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.082.21'62
DOI: 10.2298/BAH1701027P

THE EFFECT OF BULL SIRE PROVENANCE ON PRODUCTION TRAITS OF SIMMENTAL COWS

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Original scientific paper

Abstract: The aim of the present study was to obtain relevant results related to the basic indicators of fertility and milk yield of Simmental cows, in production conditions on farms of agricultural producers, using appropriate mathematical and statistical procedures, i.e. to determine the influence of bull sires originating from Serbia, Austria and Germany on the implementation of the main breeding program and improvement of production traits of Simmental cows on the territory of Šumadija district. The study of the effect of bull sires who are originally from Serbia, Austria and Germany on performance traits of Simmental cows included a total of 303 cows in first three lactations. Milk production of cows descendents of bulls from the German population was higher compared with the production of cows originating from Austria in the first lactation by 58.29 kg and in the third by 67.72 kg, but in the second it was lower by 12.31 kg. The variability of age at first calving ranged from 766.93 (cows progeny of domestic bulls) to 813.06 days (cows progeny Austrian bulls). Average duration of service period had the interval of variation of 86.80 in cows from domestic bulls in the third lactation to 109.88 days in cows originating from Austrian bulls in the first lactation.

Key words: milk yield, fertility, Simmental breed, bull sires.

Introduction

Genetic improvement of Simmental cattle in our country is realized through selection, i.e. rearing in pure breed. The breeding selection work included attempts to introduce genes of Red Holstein Friesian breed , to improve milk production traits and milking traits. Improvement of the genetic base population of Simmental cattle in our conditions is mainly done through the implementation of

high quality bull sires originating from Austria and Germany, and imports of high-quality heifers.

Romčević et al. (1990) have studied the milk performance traits of the progeny of the same Simmental bulls used, and in Germany and in Serbia. In the rearing conditions that exist in Germany, based on the absolute difference of 417 kg milk, 0.15% of milk fat content and 21.98 kg of milk fat quantity, the highly significant difference in relation to the production of the Serbian population is established.

In regard to Simmental cattle imported from Germany and Austria to Slovakia, *Strapak and Strapákova (1997)* have come to the result that in the first lactation, average yield was 3636 kg of milk with 4.76% milk fat, i.e. 171 kg. Imported cows, in terms of average milk yield, have exceeded the population of the Slovak spotted breed by 626 kg of milk and 48 kg of milk fat. In 1995, the average milk production in Slovakia amounted to 3010 kg with 4.19% fat.

Perišić (1998) has examined the production and reproduction traits of different genotypes of Simmental cows (cows of Domestic Spotted breed and Simmental cows imported from Germany and Slovenia) in the region of upper course of the river Kolubara. The average milk production throughout lactation of all examined cattle amounted to 4311.1 kg. The lowest value is recorded for the cows of Domestic Spotted breed with production in the first three lactations of 3738.8 kg, 4033.4 kg and 4384.3 kg, respectively, and the highest value for German Simmental cows, 4,120.4 kg, 4669.2 kg and 5153.2 kg, respectively. The average milk fat content throughout lactation for all investigated animals is 3.83% with an average quantity of milk fat of 165.06 kg. The production of 4% FCM for all investigated animals is 4191.17 kg.

Perkovic et al. (2003) have examined the impact of Montbeliard bulls on the improvement of the properties of milk and meat performance in Domestic Spotted cattle. Daughters of Montbeliard bulls, compared with the daughters of Domestic Spotted bulls, have had by 299 kg more milk and 0.54% less milk fat.

Comparative examination of the results obtained in 2 groups of F1 generation daughters, *Kučević et al. (2005)* have concluded that the first calvers tested in Germany have achieved a significantly higher yield of milk, milk fat and milk fat content (1057 kg, 41 kg, 0.22%, respectively). Based on the results of RBV (relative breeding value), German bulls show superiority to the average of our population, but also the daughters of bulls in Germany have much better results.

The results of comparative study of first calving heifers of Simmental breed, of domestic and Austrian provenance, in the same rearing conditions (*Medić et al.2006*) show that in the imported animals realize higher production of milk by significant 1171 kg and 0.49% milk fat.

Examining the impact of genetic and non-genetic factors on performance traits of Simmental cows, *Pantelić et al. (2014)* have established the production of

3701.67 kg of milk, i.e. corrected to 4% FCM, of 3644.58 kg. The average production of milk fat was 144.26 kg and milk fat content 3.88%. The interval from calving to first insemination lasted an average of 124.19 days, and animals calved for the first time at the age of 789.95 days.

Material and Methods

In the last two decades there has been a reduction in the number of cattle of Simmental breed in Serbia and the trend is still present. In order to increase the number of cattle and of high-quality breeding animals, and to improve the genetic composition and increase milk production in the Šumadija district, 150 Simmental heifers were imported from Germany. Import of Simmental heifers had a significant impact on the implementation of the main breeding program in the Šumadija region.

Imported animals are under control and included in the implementation of the basic breeding program carried out by the Association of Simmental cattle breeders "Šumadija" Kragujevac. Simmental cattle in Šumadija region are grown mainly in semi-intensive rearing conditions, i.e. on the small private farms with only several cows. However, there are a small number of milk producers who rear on their farms over 10 quality breeding Simmental cows.

The study of the effect of bulls sires who are originally from Serbia, Austria and Germany, on performance traits of Simmental cows, were analyzed on a total of 303 cows in first three lactations. Heifers and cows are grown on a variety of individual farms, but we can say mainly in very similar housing and feeding conditions. Cows are mostly kept tied in stalls with, with long and medium bedss with straw bedding. Nutrition was based on hay and alfalfa haylage, rarely grass silage, whole maize silage and mainly concentrate finished mixtures. Control of productivity was conducted by breeding organizations according to the AT4 milk recording principles. The following milk production traits in the first three standard lactation were studied:

- Milk yield, kg;
- Milk fat content, %;
- Milk fat yield, kg
- Yield of 4% FCM, kg.

In addition to milk performance traits, the following was determined for each cow:

- age at first calving
- service period after first thre parities.

Average values and variability of all studied traits was determined using standard mathematical and statistical methods, and the significance of the impact of

lactation, heifers origin and provenance of their bull sires by applying the *t* and *F* test. For all investigated traits the basic variational-statistical parameters were first calculated:

- arithmetic mean (X)
- standard deviation (SD)
- coefficient of variation (CV)
- variation interval (Min.-Max.)

Results and Discussion

The use of bulls, i.e. of their frozen semen, is one of the oldest and most accessible methods for genetic improvement in dairy farming. Today, due to the development of information resources and possibilities of using large quantities of information about every individual animal and its siblings and half-siblings, as well as development of methods for molecular genetic analysis, the intensity of selection of bulls was raised to a higher level. By applying genomic testing of bulls, extremely reliable results are obtained on the ability of bulls to improveo milk performance traits in their offspring, immediately after the birth of male calf - future bull. The results of the impact of the country of origin (provenance) of bull sires on the tested milk performance traits of their daughters are shown in Tables 1-3.

Table 1. Average values and variability of tested milk performance traits of cows in the first standard lactation observed relevant to the origin of bulls-sires

Indicator	$\bar{X}$	SD	CV	Min,	Max,
Daughters of domestic bull sires (n=109)					
Milk yield, kg	4386,19	339,18	7,73	3497	5468
Milk fat content, %	3,91	0,08	1,95	3,71	4,18
Milk fat yield, kg	171,65	14,24	8,30	132,59	228,56
Yield of 4%FCM, kg	4329,27	346,95	8,01	3395	5615
Daughters of German bull sires (n=178)					
Milk yield, kg	4524,92	479,13	10,59	3166	5844
Milk fat content, %	3,93	0,08	1,93	3,37	4,11
Milk fat yield, kg	177,65	19,60	11,03	116,83	233,60
Yield of 4%FCM, kg	4474,68	483,74	10,81	3019	5831
Daughters of Austrian bull sires (n=16)					
Milk yield, kg	4466,63	361,10	8,08	3780	4994
Milk fat content, %	3,96	0,04	0,97	3,90	4,02
Milk fat yield, kg	176,88	14,03	7,93	151,20	197,26
Yield of 4%FCM, kg	4439,84	354,32	7,98	3780	4957

Table 2. Average values and variability of tested milk performance traits of cows in the second standard lactation observed relevant to the origin of bulls-sires

Indicator	$\bar{X}$	SD	CV	Min,	Max,
Daughters of domestic bull sires (n=109)					
Milk yield, kg	4968,17	301,64	6,07	4241	5659
Milk fat content, %	3,93	0,07	1,69	3,71	4,08
Milk fat yield, kg	195,33	12,34	6,32	165,79	225,23
Yield of 4%FCM, kg	4917,24	303,06	6,16	4187	5642
Daughters of German bull sires (n=178)					
Milk yield, kg	5141,38	541,55	10,53	3836	7430
Milk fat content, %	3,94	0,06	1,55	3,75	4,13
Milk fat yield, kg	202,39	21,69	10,72	148,07	294,97
Yield of 4%FCM, kg	5092,44	540,66	10,62	3755	7397
Daughters of Austrian bull sires (n=16)					
Milk yield, kg	5153,69	378,87	7,35	4416	5818
Milk fat content, %	3,93	0,11	2,69	3,72	4,10
Milk fat yield, kg	202,32	16,67	8,24	177,08	238,54
Yield of 4%FCM, kg	5096,33	396,29	7,78	4423	5905

Table 3. Average values and variability of tested milk performance traits of cows in the third standard lactation observed relevant to the origin of bulls-sires

Indicator	$\bar{X}$	SD	CV	Min.	Max.
Daughters of domestic bull sires (n=109)					
Milk yield, kg	5336.88	332.48	6.23	4247	6174
Milk fat content, %	3.95	0.07	1.72	3.53	4.14
Milk fat yield, kg	210.97	13.48	6.39	169.88	245.11
Yield of 4%FCM, kg	5299.32	332.32	6.27	4247	6146
Daughters of German bull sires (n=178)					
Milk yield, kg	5512.16	553.16	10.04	4129	8480
Milk fat content, %	3.94	0.08	1.95	3.35	4.20
Milk fat yield, kg	217.20	21.87	10.07	165.16	336.66
Yield of 4%FCM, kg	5462.80	546.80	10.01	4129	8442
Daughters of Austrian bull sires (n=16)					
Milk yield, kg	5444.44	415.78	7.64	4942	6441
Milk fat content, %	3.97	0.06	1.59	3.84	4.13
Milk fat yield, kg	216.15	16.89	7.82	195.70	257.64
Yield of 4%FCM, kg	5419.98	417.72	7.71	4912	6441

In regard to milk performance traits in the first three standard lactations, it can be concluded that cows imported from Germany and Austria had much better

results compared to domestic animals. A similar conclusion can be made in the comparison of daughters which originate from domestic and foreign bull sires. The results fully correspond to those published by *Perišić (1998), Medić et al. (2006).*

In all three lactations, daughters sired by bulls of foreign provenance achieved higher yields of milk, milk fat and 4% fat corrected milk. Milk yield of cows originating from domestic bulls increased gradually from the first (4386.19 kg), to the second (4968.17 kg) lactation, reaching its peak in the third lactation (5336.88 kg). Milk yield of cows originating from bulls from the German population was higher compared with the production of cows, originating from Austrian bulls, in the first lactation – by 58.29 kg, in the third by 67.72 kg, but in the second it was lower by 12.31 kg. Daughters of domestic bulls had lower milk fat content in the first standard lactation (3.91%) compared to first calvers whose sires originate from Germany (3.93%) and Austria (3.96%), but this rule was not observed in the second and third lactation in which the differences were minor. Approximately the same results in their studies are reported by *Romčević et al.(1990), Strapak and Strapakova (1997), Perković et al.(2003), Kučević et al.(2005),* and lower by *Pantelić et al.(2014).*

Table 4. Average values and variability of tested fertility traits of cows in the first parity observed relevant to the origin of bulls-sires

Indicator	$\bar{X}$	SD	CV	Min.	**Max.**
Daughters of domestic bull sires (n=109)					
Age at calving, days	766,93	87,93	11,46	652	1129
Duration of service period, days	94,82	46,38	48,91	35	308
Daughters of German bull sires (n=178)					
Age at calving, days	785,33	85,39	10,87	656	1311
Duration of service period, days	103,52	54,79	52,92	27	320
Daughters of Austrian bull sires (n=16)					
Age at calving, days	813,06	124,40	15,30	684	1175
Duration of service period, days	109,88	61,80	56,24	41	240

Table 5. Average values and variability of tested fertility traits of cows in the second parity observed relevant to the origin of bulls-sires

Indicator	$\bar{x}$	SD	CV	Min,	Max,
Daughters of domestic bull sires (n=109)					
Age at calving, days	1151,73	105,14	9,13	979	1478
Duration of service period, days	91,11	52,33	57,44	31	341
Daughters of German bull sires (n=178)					
Age at calving, days	1181,83	107,36	9,08	1017	1701
Duration of service period, days	97,89	52,19	53,31	30	317
Daughters of Austrian bull sires (n=16)					
Age at calving, days	1198,13	147,62	12,32	1023	1571
Duration of service period, days	97,50	38,68	39,67	58	198

Table 6. Average values and variability of tested fertility traits of cows in the third parity observed relevant to the origin of bulls-sires

Indicator	$\bar{x}$	SD	CV	Min.	Max.
Daughters of domestic bull sires (n=109)					
Age at calving, days	1529,46	115,17	7,53	1333	1871
Duration of service period, days	86,80	31,62	36,42	39	198
Daughters of German bull sires (n=178)					
Age at calving, days	1564,92	126,41	8,08	1230	2062
Duration of service period, days	92,25	35,23	38,19	39	244
Daughters of Austrian bull sires (n=16)					
Age at calving, days	1567,69	185,33	11,82	1194	1973
Duration of service period, days	108,13	38,06	35,20	41	170

The analysis of fertility indicators of cows according to the origin of bull sires (tables 4-6) shows differences in age at first calving of 766.93 (cows of domestic bulls) to 813.06 days (cows of Austrian bulls). Also, the average duration of service period ranged from 86.80 in cows progeny of domestic bulls in the third lactation to 109.88 days in cows originating from Austrian bulls in the first lactation. Significant deviation in the duration of service period was observed in the third lactation, where the difference between cows originating from domestic bulls and cows sired by Austrian bulls was an entire oestrus cycle (21.33 days).

Croatian Livestock Selection Center (2003), in its annual report, cites certain reproduction indicators for the population of Simmental cows. The average age of registered cows in the first lactation was 28 months, in the second lactation 39, and the third - 53 months. The average duration of service period was 120 days. By analyzing the production and reproductive performance of Simmental bull dams

in our country, *Pantelić et al. (2005)* have found the average age at first conception of 517.61 days, as well as the service period of 108.98 days.

Reproductive traits gain increasing importance in the implementation of the breeding program of the Republic of Serbia, since they have significant impact on the economic efficiency of milk production. Poor reproductive performance influences the economic losses that result from extended service period, increased insemination index, higher coefficient of culling of cows and increased production costs due to the use of veterinary services.

The results also indicate that although there are significant differences in age at calving and duration of service period between cows sired by domestic bulls and imported cows originating from Austrian and German bulls in the first lactation, these differences were not significantly increased during the second and third lactation. This indicates that the imported heifers were fertilized somewhat later, and it took them some time to adapt and prepare for fertilization for the second calving.

Conclusion

Improving of phenotypes of dairy animals requires continuous work, which includes systematic improvement of quantitative genetic traits and permanent work on their improved expression. The high yields of milk, milk fat and protein, in addition to the selection, require optimal provision and ensuring of non-genetic factors such as diet, housing, health care, etc.

The milk yield of cows sired by bulls from the German population was higher compared with the production of cows sired by Austrian bulls in the first lactation by 58.29 kg, in the third by 67.72 kg, but in the second it was lower by 12.31 kg. Daughters of domestic bulls had lower milk fat content in the first standard lactation (3.91%) compared to first calvers whose ancestors originated from Germany (3.93%) and Austria (3.96%), but this rule was not observed in the second and third lactation, in which the differences were minor.

Based on the obtained results of fertility traits of cows according to the provenance of bull sires, the differences were established in age at first calving of 766.93 (cows from domestic bulls) to 813.06 days (cows fromAustrian bulls). Also, the average duration of service period ranged from 86.80 in cows from domestic bulls after the third calving, to 109.88 days in cows originating from Austrian bulls after the first calving.

Based on these findings it can be concluded that the import of high-quality heifers of Simmental breed sired by bulls from Germany and Austria significantly influenced the improvement of milk and fertility traits of cows in the Šumadija district.

Uticaj provenijence bikova očeva na proizvodne osobine krava simentalske rase

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Rezime

Unapređenje fenotipova mlečnosti krava zahteva kontinuiran rad koji obuhvata sistematsko poboljšanje kvantitavnih genetskih osobina i permanentni rad na njihovom poboljšanom ispoljavanju. Visok prinos mleka, mlečne masti i proteina, pored selekcije zahteva i optimalno obezbeđenje paragenetskih faktora kao što su ishrana, držanje, odgoj, nega i dr.

Proizvodnja mleka kod krava poreklom od bikova iz Nemačke populacije, bila je veća u poređenju sa proizvodnjom krava poreklom od bikova iz Austrije u prvoj lataciji za 58, 29 kg, u trećoj za 67,72 kg, ali je u drugoj bila manja za 12,31 kg. Kćeri domaćih bikova su imale niži sadržaj mlečne masti u I standardnoj laktaciji (3,91%) u odnosu na prvotelke čiji očevi potiču iz Nemačke (3,93%) i Austrije (3,96%), ali to pravilo nije uočeno u II i III laktaciji u kojima su razlike bile neznatne.

Na osnovu dobijenih rezultata osobina plodnosti krava prema poreklu bikova-očeva ustanovljene su razlike u uzrastu pri prvom telenju od 766,93 (krave od domaćih bikova) do 813,06 dana (krave od austrijskih bikova). Takođe, prosečno trajanje servis perioda kretalo se od 86,80 kod krava od domaćih bikova posle trećeg teljenja, do 109,88 dana kod krava poreklom od austrijskih bikova posle prvog teljenja.

Na osnovu iznetog u zaključku može se konstatovati da je uvoz kvalitetnih priplodnih junica simentalske rase koje vode poreklo od bikova iz Nemačke i Austrije značajno uticao na poboljšanje osobina mlečnosti i plodnosti populacije krava u Šumadijskom okrugu.

Ključne reči: prinos mleka, plodnost, simentalska rasa, bikovi očevi

Ackonwledgement

This research is part of the Project EVB: TR-31053 financial supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Received 7 December 2016; accepted for publication 18 January 2017

Biotechnology in Animal Husbandry 33 (1), p 37-53 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.082'63
DOI: 10.2298/BAH1701037E

GENETIC POLYMORPHISM DETECTION IN BONE MORPHOGENETIC PROTEIN 15 (BMP15) GENE RELATED TO FECUNDITY IN TWO EGYPTIAN SHEEP BREEDS

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Original scientific paper

Abstract: This study was intended to detect the polymorphism of bone morphogenetic protein 15 (BMP15) gene that can act as marker influencing fertility for increasing litter size in Egyptian sheep breeds (191 Saidi and 145 Ossimi females). In this study, the mean litter size, showed highly significant between Saidi and Ossimi sheep breads, however, litter size of Saidi white sheep was significantly decreased compared to black and brown. Blood samples were collected from 19 Saidi and 13 Ossimi female and then genomic DNA was extracted. A portion of bone morphogenetic protein 15 (BMP15) gene, 310 bp was amplified using specific primers, and was sequenced and analyzed to clarify the phylogenetic relationship of Egyptian breed sheep. The data suggested that the gene shared a similarity in sequence compared to 9 accession numbers of *Ovis aries* found in GenBank. Molecular phylogenetic analyses were performed based on nucleotide sequences in order to examine the position of the Egyptian breeds among many other sheep breeds. The results indicate that 5 accession numbers of *Ovis aries* are closely related with Ossimi and Saidi female that produce single or twins lamb in UPGMA analysis. In addition, PCR-RFLP method using *Pst1* and *Msp1* restriction enzymes was used to mask polymorphisms of partial exon 2 in 18 female sheep. Results showed that FecX gene was monomorphic and disagreement with litter size, therefore, it is indispensable to survey other gene in order to establish marker assisted selection technique.

Key words: Sheep, Litter size, BMP15 gene, PCR-RFLP, Phylogenetic tree

Introduction

The main objective of sheep breed in the world is one or more of the following: Meat, milk, and wool production, where in Egypt the sheep meat production is more important than fiber production, and the sheep contribute 6% of the total red meet produced (*Abulyazid et al., 2011*). Sheep occupy a special niche in the Egyptian agricultural production system and are important for the rural economy, where the total sheep population in Egypt is 5,488,000 heads (*FAO, 2014*). There are three major breeds in Egypt; Rahmani, Ossimi, and Barki. Rahmani is distributed mainly in north of the Nile delta, Ossimi in mid Egypt and Barki in western Mediterranean coastal region. Minor breeds like Saidi, Sohagi, located in south Egypt, (*ICARDA, 2006*). In Egypt efforts are being made to intensify production systems, primarily through changing reproductive management and crossing native breeds with introduced breeds (*Ibrahim et al., 2010*).

The profitability of sheep farming mainly depends on lamb production per ewe and litter size. Both are important economical traits in sheep breeding and genetics, which mainly depend on breed. Also prolificacy refers to the ability of the female to produce multiple lambs through high ovulation rates and embryo survival. The ovulation rate and litter size are dependent on the interactions of endocrine and paracrine mediators in mammals (*Zhu et al., 2013*). Reproduction is a complex process and fecundity traits such as ovulation rate and litter size can be genetically regulated by many genes with small effects, and sometimes also by single genes with major effects, called fecundity (Fec) genes (*Drouilhet et al., 2009*). Various major genes have been reported to affect prolificacy in sheep, include three related oocyte derived components, namely, bone morphogenetic protein receptor type 1B (BMPR1B), known as FecB on chromosome 6 (*Souza et al., 2001*); growth differentiation factor 9 (GDF9), known as FecG on chromosome 5 (*Hanrahan et al., 2004*) and bone morphogenetic protein 15 (BMP15), known as FecX on chromosome x (*Galloway et al., 2000; Harahan et al., 2004*).

The mutations in the BMP15 gene increase ovulation rate in heterozygous individuals. Heterozygous ewes show multiple ovulations, earlier maturation of granulosa cells and reduced follicle size (*Bodin et al., 2007*). There are several point mutations in BMP15 gene, identified in different sheep breeds (*Galloway et al., 2000; Demars et al., 2013; Shabir et al., 2013; Zamani et al., 2015*). The BMP15 gene significantly affects prolificacy and ewes with two inactive copies of the BMP15 gene (homozygous animals) are sterile and exhibit a similar ovarian phenotype (*Galloway et al., 2000; Hanrahan et al., 2004*). Ewes with a single inactive BMP15 gene (heterozygous animals) are fertile and exhibit an increased ovulation rate and an increased incidence of twin or triplet births (*Davis, 2004, Kasiriyan et al., 2009; Monteagudo et al., 2009*). In Egypt, the genetic diversity of indigenous sheep in respect to these important economic genes has not been

sufficiently studied. Therefore, it becomes essential to make fingerprinting of some genes related to economic traits such as litter size, in order to determine the polymorphism pattern of these genes in the Egyptian sheep breeds. This investigation was carried out to explore the presence of polymorphism in BMP15 gene (exon 2) using DNA sequencing and PCR-RFLP methods in two Egyptian sheep breeds, that can act as marker influencing fertility and helpful in breed selection for genetic improvement programs.

Materials and Methods

Experimental animals

The Saidi and Ossimi sheep breeds used in this study were selected based on their single/twins production in three repetitive production cycles. The data of 191 Saidi and 145 Ossimi female sheep were collected from different farms belonging to the Ministry of Agriculture in Fayoum, Bani-Suef and Minia Governorates. These data were used to study the reproduction traits *i.e.* lambing rate (%), fecundity rate (%), twinning rate (%), triplet rate (%) and litter size. Nineteen Saidi individuals (12 ewes which producing twins and 7 which producing single) and 13 Ossimi individuals (7 ewes which producing twins and 6 which producing single) were used to study the polymorphism.

Statistical analysis

Data were statistically analyzed using the SPSS program, version 16.0 (*SPSS, 2007*). Means were compared for main effects and their interaction by Duncan's multiple range test (*Duncan, 1955*), when significant F values were obtained ($P<0.05$).

Blood sampling

Whole blood samples (5 ml) were collected from Jugular vein for each ewe of 19 Saidi and 13 Ossimi female in vacutainer glass tubes containing EDTA (1 mg/ml). Blood samples were transferred to the laboratory in an ice box kept at 4°C until used. The experimental procedures were performed according to protocols approved by the Biological Studies Animal Care and Use Committee of Egypt. All efforts were made to minimize any discomfort during blood collection.

DNA extraction

Genomic DNA was extracted from whole blood samples using Xanthogenate protocol described by *Tillett and Neilan* (*2000*) with some modification. The quantified DNA was stored at -20°C until further processing of PCR amplification of BMP15 gene.

PCR Amplification of BMP15 gene

Specific primers 5- -GCAGGCAGTATTGCATCGGAAG-3- and reveres 5- -CCTCAATCAGAAGGATGCTAATGG-3- were used to amplify one region of BMP15 gene (Exon 2) which corresponded to the GenBank accession number AH009593 (*Gholibeikifard et al., 2014*). The primer was synthesized by Invitrogen, Biotechnology Co. Ltd. (USA). The PCR amplification was performed in 25 µl total volume, each PCR reaction mixture containing 12.5 µl Master Mix (onePCR^{TM}), 1 µl of each primer, 2 µl of genomic DNA (50 ng/ µl) and 8.5 µl of sterile deionized water. PCR conditions were as follows an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min using thermal cycler 2720 (Applied Biosystems, USA). PCR products were checked by electrophoresis using 1.8% agarose gel in 1X TAE buffer. The products were then purified using QIAquick Gel extraction kit # 28706 (QIAGEN) following manufacturer instructions and sequenced by automated DNA sequencing reactions, which were performed using a sequencing ready reaction kit (Life Technologies) in conjunction with ABI-PRISM and ABI-PRISM big dye terminator cycler.

DNA Sequence and Phylogenetic analysis

A consensus sequence of BMP15 fragments from both Saidi and Ossimi ewes which producing twins and single was constructed by using the SeqMan^{TM} II 4.05 package for windows 32. These sequences were subjected to alignment with BMP15 sequences of the GenBank, EMBL, DDBJ and PDB from breeds of *Ovis aries* using the BLASTN 2.2.18 and BLASTP 2.2.18 (Basic Local Alignment Search Tool) algorithm at http://www.ncbi.npm. The MEGA version 5.2 programs (*Tamura et al., 2011*) was used to generate a phylogenetic tree using the UPGMA method according to *Sneath and Sokal* (*1973*). The evolutionary distances were computed using the Maximum Composite Likelihood method (*Tamura et al., 2004*).

Restriction Fragment Length Polymorphism (RFLP) analysis

Nineteen PCR products of partial BMP15 gene (exon 2) were digested using *Pst1 Msp1* restriction enzymes (Fermentas, Germany, #ER0611) according to the manufacturer instructions. A final reaction volume of 32 µl containing 10 µl PCR product, 18 µl H_2O free of nuclease, 2 µl of 10X buffer and 2 µl (5 units) of each restriction enzymes. The final volume of mixture was mixed gently and spins down for few seconds then incubated for 18 hours at 37 °C in water path and stopped at 65 °C for10 min. Restriction digestion products were checked by electrophoresis using 3% agarose gel in 1X TAE buffer and staining with ethidium bromide. The 100-bp ladder was used as molecular size marker.

Results and discussion

Reproduction traits

Reproductive ability has an important role in profitability of sheep production. The production and fertility traits of 191 and 145 individuals of sheep breeds with different families from Saidi (black, brown and white) and Ossimi sheep, respectively are summarized in Table (1). The data showed that, there is no significant in the average number of ewes mated between Saidi and Ossimi breed sheep. Also no significant between Saidi black and brown in the average number of ewes lambing, ewes lambing twin, ewes lambing triplet and live lambs born, while it's a significantly when compared with Saidi white and Ossimi sheep. The average number of dead lambs born and the average number of total lambs birth for Saidi black, Saidi brown and Ossimi sheep were significantly higher compared with Saidi white sheep. The higher average number of dead lambs born (10) was found in Saidi black sheep and the fewer average number (4.33) was found in saidi white sheep. The Saidi sheep (black, brown and white) had average number of ewes lambing triplet (0.67, 0.33 and 0.17), respectively, where no ewes lambing triplet in Ossimi sheep.

Fertility traits have a major impact on efficiency and profitability in lamb meat production. In this respect twinning rates and litter size are reflected ovulation rate, which they important economic value. As shown in Table (1), the litter size, twining rate and triplet rate showed highly significant between Saidi and Ossimi sheep breads, while lambing rate and fecundity rate showed no significant. However, the mean litter size of Saidi white sheep was significantly decreased compared to black and brown. The genetics of sheep litter size has been investigated (*Hanrahan et al., 2004; Mishra, 2014; Wan Samarny et al., 2013; Zamani et al., 2015*). Multiple genes were identified having substantial effects on reproduction traits and some of them are most important being affecting prolificacy in animals. High litter size or twinning is an economically important trait that enhances sheep productivity in terms of producing a higher number of lambs, meat and wool (*Mishra, 2014*).

Table 1. Mean traits of Saidi breeds (Black, Brown and White) and Ossimi breeds sheep

Traits	Saidi sheep			Ossimi sheep	± SEM
	Black	Brown	White		
Av. number of ewes mated	28.75	27.08	25.42	28.75	0.64
Av. number of ewes lambing	25.50^{a}	23.08^{a}	11.50^{b}	24.00^{a}	0.99
Av. number of ewes lambing twins	8.17^{a}	7.08^{a}	3.08^{b}	4.75^{b}	0.43
Av. number of ewes lambing triplet	0.67^{a}	0.33^{ab}	0.17^{ab}	0.00^{b}	0.09
Av. number of live lambs born	25.00^{a}	23.00^{a}	13.08^{b}	24.33^{a}	1.05
Av. number of dead lambs born	10.00^{a}	7.83^{b}	1.58^{d}	4.33^{c}	0.57
Av. number of total lambs birth	35.00^{a}	30.83^{ab}	14.67^{c}	28.75^{b}	1.39
Lambing rate (%)[1]	88.85^{a}	85.63^{a}	44.31^{b}	82.75^{a}	2.83
Litter size[2]	1.38^{a}	1.28^{b}	1.25^{b}	1.13^{c}	0.02
Fecundity rate (%)[3]	86.12^{a}	84.32^{a}	49.30^{b}	84.47^{a}	2.91
Twinning rate (%)[4]	34.53^{a}	32.81^{ab}	25.91^{bc}	19.55^{c}	1.57
Triplet rate (%)[5]	2.68^{a}	1.22^{ab}	1.20^{ab}	0.00^{b}	0.38

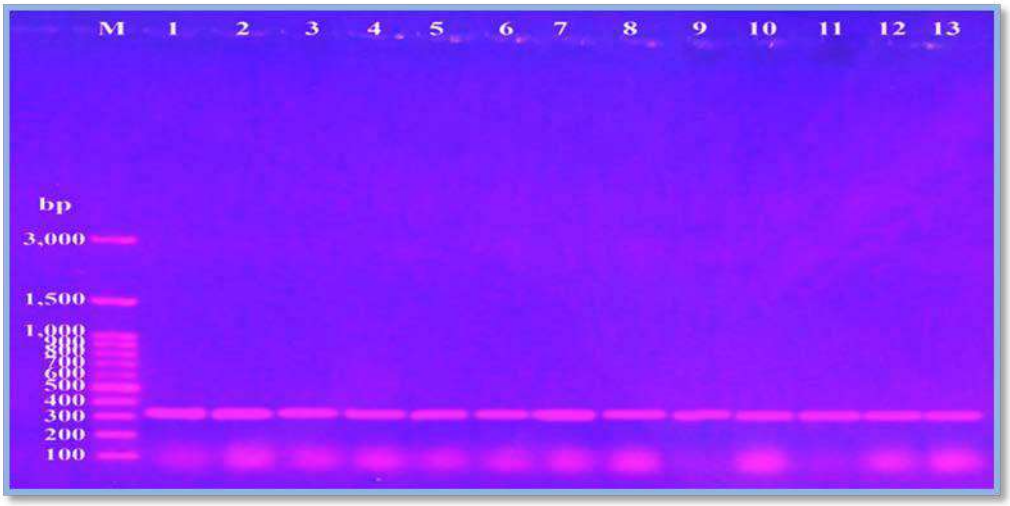
1. Lambing rate (%) = (Number of ewes lambing / Number of ewes mated) X 100.
2. Litter size = Total number of lambs birth / Number of ewes lambing.
3. Fecundity rate (%) = (Number of live lambs born / Number of ewes mated) X 100.
4. Twinning rate (%) = (Number of ewes lambing twins / Number of ewes lambing) X 100.
5. Triplet rate (%) = (Number of ewes lambing triplet / Number of ewes lambing) X 100.

Properties of BMP15 gene (exon 2) sequence

A single fragment of approximately 310 bp nucleotide sequences was amplified from each ewe individual (19 Saidi and 13 Ossimi) sheep breads (Figures 1 and 2). Alignments of four sequences from Saidi and Ossimi female that produce single or twins lamb revealed 100% similarity between them. The DNA sequence compositions are 54 (A), 70 (C), 66 (T) and 74 (G). The nucleotide frequencies were 23% (A), 28% (T), 29% (C) and 20% (G).

Lanes 3-7, 9, 12, 15, and 17-18: Saidi black color, Lanes 1-2, 8, 11, 14, 16 and 19: Saidi brown color Lane 10 and 13: Saidi white color, M: 100 bp DNA ladder.

Figure 1. PCR amplification of partial exon 2 fragments of BMP15 gene from Saidi sheep breed



Lanes 1-13: Ossimi white color, M: 100 bp DNA ladder.

Figure 2. PCR amplification of partial exon 2 fragments of BMP15 gene from Ossimi sheep breed

Phylogenetic analysis

The topology of UPGMA tree of Saidi and Ossimi sheep breads with 9 accession numbers of *Ovis aries* in the GenBank database represented a

monophyletic group (Figure 3). The DNA sequences of BMP 15 gene successfully grouped Egyptian breeds and *Ovis aries* sheep into two main cluster. The first cluster is extremely diverse and consisted of two accession numbers (JN655671 and JN655672). The second cluster had Egyptian sheep breeds and closely related with 5 accession numbers (HM583335, AH009593, KT853038, KT013294 and NM1114767 whereas, two accession numbers (HM583333 and HM583334) were the most distant. The genetic relationship of BMP15 based on nucleotide sequence using UPGMA revealed that accession numbers were closer while accession numbers were farther apart. This is in accordance with *Misra et al*., (*2011*); *Bibinu et al*., (*2016*).

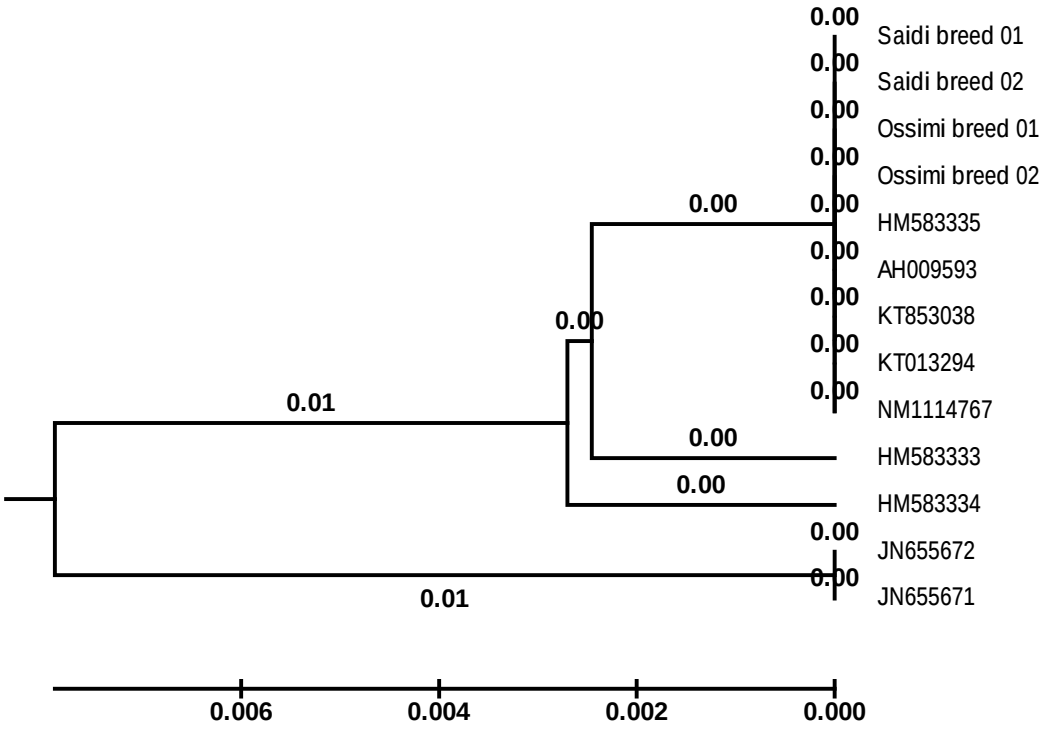
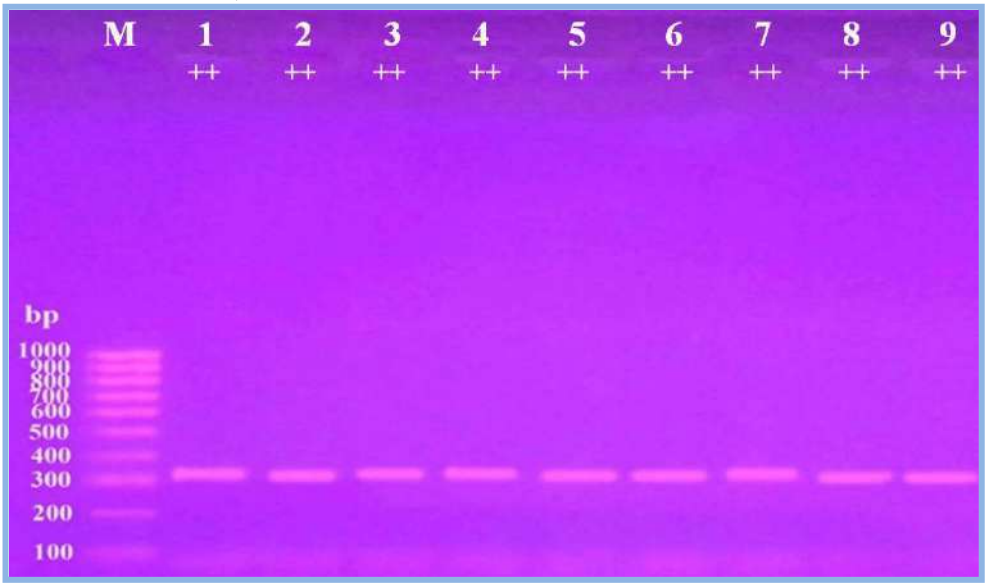


Figure 3. UPGMA dendrogram of 13 *Ovis aries* sheep generated based on Sneath and Sokal distances. Branch lengths are shown above the branches of clades

Saidi breed 01: Black color female which producing twins lamb, Saidi breed 02: Brown color female which producing single lamb, Ossimi breed 01: White color female which producing twins lamb, Ossimi breed 02: White color female which producing single lamb

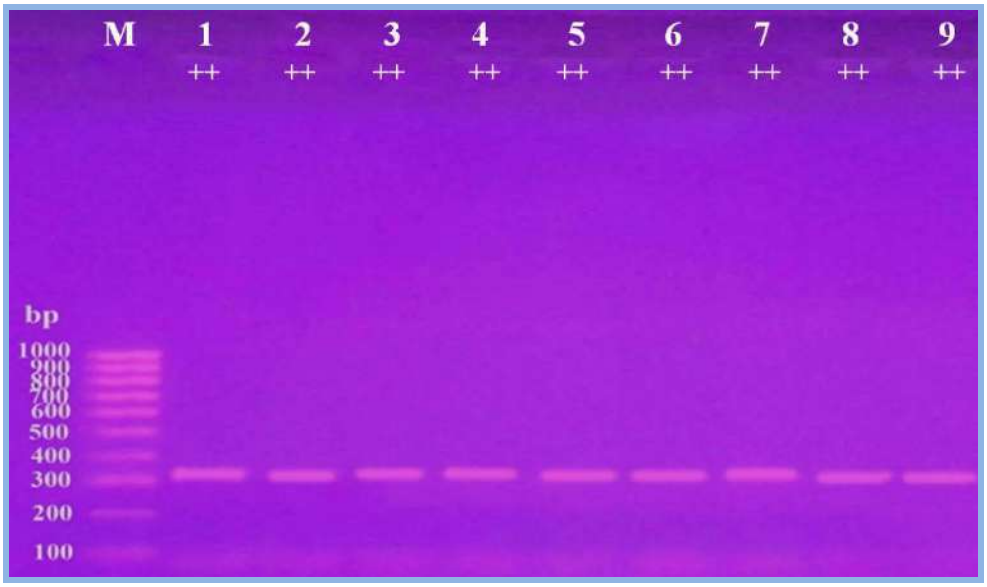
Restriction Fragment Length Polymorphism (RFLP) analysis

PCR-RFLP is a rapid, simple and exact technique for single nucleotide polymorphism (SNP) genotyping. The forced PCR-RFLP approach has been used previously to genotype prolific sheep (*Wilson et al., 2001*). The PCR products of BMP15 gene (exon 2) digested by *Pst1* and *Msp1* restriction enzymes to survey existence of mutations. Digested products were run on 3% agarose gel electrophoresis, which bands with 310 bp in length were observed, in all 9 Saidi ewes (Figures 4 and 6) and also in all 9 Ossimi ewes (Figures 5 and 7). The results showed that Saidi and Ossimi ewes producing single and twins lamb had a single band at 310 bp position indicating absence of mutation in the FecX gene ($FecX^{++}$), Figures (4, 5, 6 and 7). It can be assume that the cause of twinning in Saidi and Ossimi sheep breed might be due to the effect of other fecundity genes that segregate in other prolific sheep breed, or may be a combination of gene products that stimulate/alter the ovulatory cycle. The results found in the present study are in accordance with those obtained for Madras Red, Deccani, Bunnur breeds (*Wilson et al., 2001*; *Davis et al., 2006*) and Egyptian sheep breeds (*Amr and El-Saadani, 2009*).



Lanes 1, 2, 5, 6 and 9: Saidi black color with genotype ++, Lanes 3, 7 and 8: Saidi brown color with genotype ++, Lane 4: Saidi white color with genotype ++, Lanes 1-3: single producing female , Lanes 4-9: twins producing female , M: 100 bp DNA ladder.

Figure 4. Digestion product of partial exon 2 fragments of BMP15 gene with *Pst1* restriction enzyme from Saidi breed



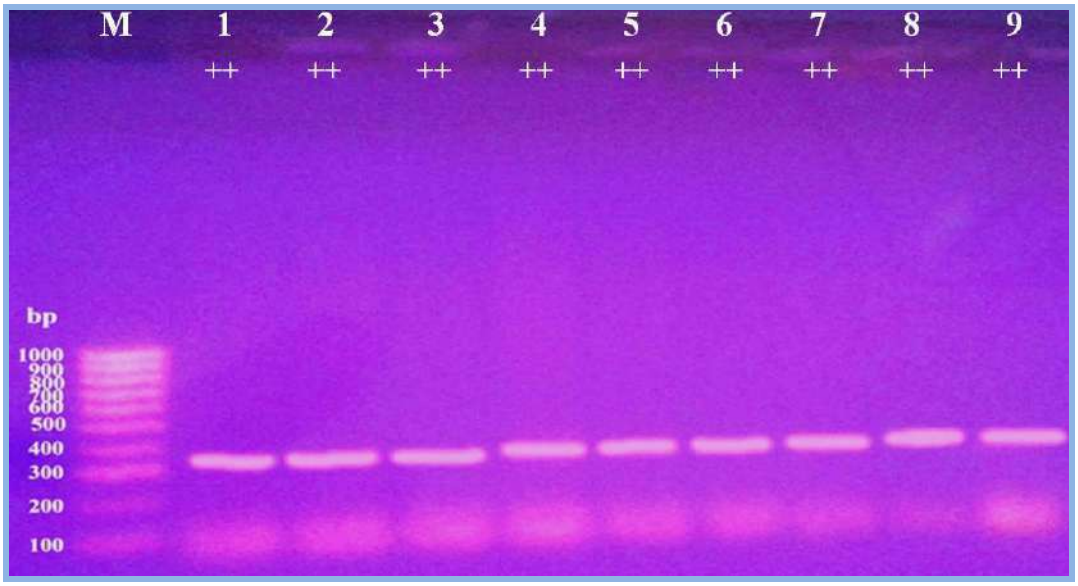
Lanes 1-9: Ossimi white color with genotype ++, Lanes 1-3: single producing female, Lanes 4-9: twins producing female, M: 100 bp DNA ladder.

Figure 5. Digestion product of partial exon 2 fragments of BMP15 gene with *Pst1* restriction enzyme from Ossimi breed

At least six different mutations have been identified in the BMP15 gene (*Galloway et al., 2000*; *Hanrahan et al., 2004*; *Bodin et al., 2007*; *Martinez-Royo et al., 2008*; *Monteagudo et al., 2009*; *Lahoz et al., 2011*) wherein ewes heterozygous for the mutations have increased ovulation rates between 0.8 and 2.4 above that of the respective non carrier flocks (*Hanrahan et al., 2004*). Animals homozygous for each of these mutations are an ovulatory and thus infertile. While all the mutations have similar general effects on fertility, there are subtle differences, as the increases in ovulation rate observed in the heterozygous animals vary from 35 to 100% (*McNatty et al., 2004*). Heterozygous ewes with mutations in both *FecB* and *FecX* exhibited increased fertility compared with ewes harboring a mutation in only one of these genes (*Mishra, 2014*).

Davis et al. (*2006*) reported that none of mutation in BMP15 gene isolated from Hu breed which high prolific sheep in China. *Gursel et al.* (*2011*) showed that none of Chios, Kivircik, Awassi and Imrose sheep breeds carries $FecX^{H}$, $FecX^{I}$ and $FecX^{B}$ mutations. In the other hand, *Wang et al.* (*2011*) and *Abdel-Rahman et al.* (*2013*) found two polymorphisms in exon 2 of BMP15 in different goat breeds. These findings show that there are differences in prolificacy inheritance patterns between sheep and goat and other species and even among different breeds, possibly. Mutations in *FecB* and *FecX* genes were not the only factors responsible

for high prolificacy (*Guan et al., 2007*) as Malin sheep which lacked mutations in these genes were highly prolific. The highly prolific sheep amongst the Egyptian breeds may indicate a presence of a genomic influence. While this could be related to differences in the background genetics of the various sheep breed.

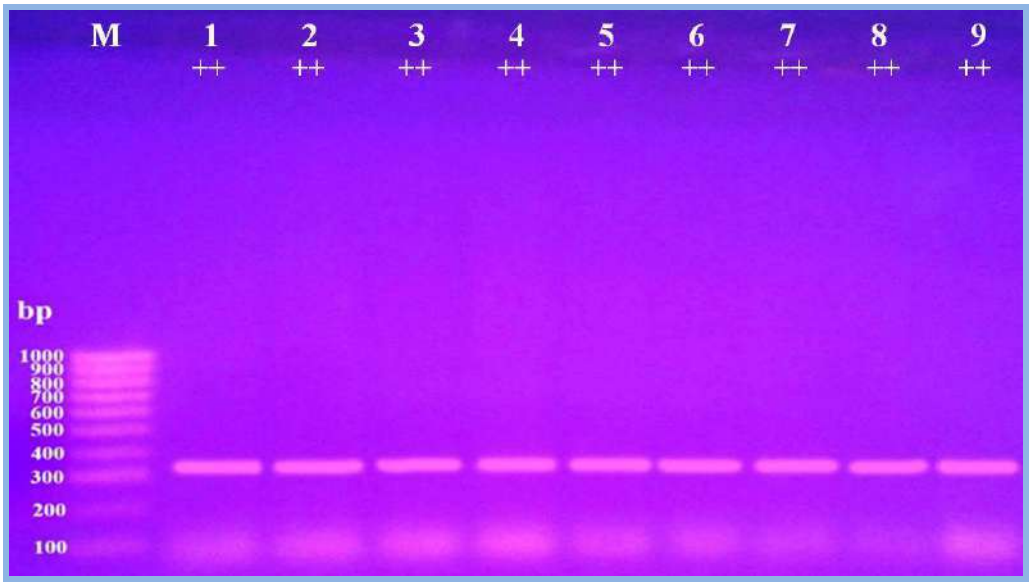


Lanes 1, 2, 5, 6 and 9: Saidi black color with genotype ++ , Lanes 3, 7 and 8: Saidi brown color with genotype ++, Lane 4: Saidi white color with genotype ++, Lanes 1-3: single producing female , Lanes 4-9: twins producing female , M: 100 bp DNA ladder.

Figure 6. Digestion product of partial exon 2 fragments of BMP15 gene with *Msp1* restriction enzyme from Saidi breed

Conclusion

The sequence analysis and diversity of polymorphism of the isolated BMP15 gene (exon 2) has been studied. It can be concluded that 100% similarity between Saidi and Ossimi female that produce single or twins lamb. PCR-RFLP used for detection of FecX mutations showed that there are not any polymorphisms in tested Saidi and Ossimi sheep. The genetic factor affecting fecundity should be investigated further by other candidate gene due to its higher litter size.



Lanes 1-9: Ossimi white color with genotype ++ , Lanes 1-3: single producing female, Lanes 4-9: twins producing female, M: 100 bp DNA ladder.

Figure 7. Digestion product of partial exon 2 fragments of BMP15 gene with *Msp1* restriction enzyme from Ossimi breed

Detekcija genetskog polimorfizma u genu BMP15 u vezi sa plodnošću dve egipatske rase ovaca

Zaki A. El Fiky, Gamal M. Hassan, Mohamed I. Nassar

Rezime

Svrha ovog istraživanja je bila da se otkrije polimorfizam koštanog morfogenetskog proteina 15 (BMP15) gena koji može da deluje kao marker koji utiče na plodnost u smislu povećanja veličine legla egipatskih rasa ovaca (191 ženka rase Saidi i 145 ženki rase Ossimi). U ovoj studiji, srednja veličina legla, je bila veoma značajno različita između rasa Saidi i Ossimi, međutim, veličina legla Saidi bele ovce je značajno manje u odnosu na crnu i braon. Uzorci krvi su

sakupljeni od 19 ovaca rase Saidi i 13 ovaca rase Ossimi i zatim ekstrahovana genomska DNK. Deo morfogenetski proteinskog 15 (BMP15) gena, 310 bp je pojačan upotrebom specifičnih prajmera, zatim sekvencioniran i analiziran kako bi se razjasnio filogenetski odnos egipatske rase ovaca. Podaci ukazuju da gen deli sličnost u nizu u odnosu na 9 brojeva pristupanja u *Ovis aries* koji se nalaze u GenBank. Molekularne filogenetske analize su izvedene na osnovu sekvence nukleotida u cilju ispitivanja pozicije egipatskih rasa među mnogim drugim rasama ovaca. Rezultati pokazuju da je 5 brojeva pristupanja *Ovis aries* blisko povezano sa ovcama rasa Ossimi i Saidi koje rađaju jedno jagnje ili blizance, kako pokazuje UPGMA analiza. Pored toga, PCR-RFLP metoda, koja koristi *Pst1* i *Msp1* restrikcione enzime, je korišćena da prikrije polimorfizam delimičnog eksona 2 u 18 ovaca. Rezultati su pokazali da FecX gen je monomorfan i u neslaganju sa veličinom legla, stoga, neophodno je da se pronađe drugi gen u cilju uspostavljanja tehnike MAS.

Ključne reči: ovce, veličina legla, BMP15 gen, PCR-RFLP, filogenetsko stablo

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Received 13 October 2016; accepted for publication 5 February 2017

Biotechnology in Animal Husbandry 33 (1), p 55-64 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.06'63
DOI: 10.2298/BAH1701055V

MORPHOMETRIC MEASUREMENTS AS PART OF THE GENETIC CHARACTERIZATION OF INDIGENOUS STRAIN KUPREŠKA PRAMENKA

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Original scientific paper

Abstract: For the purpose of genetic characterization of strains of sheep in Bosnia and Herzegovina, a morphometric characterization of Kupreška Pramenka has been performed. A total of 62 heads were measured, 56 ewes and 6 rams. The average height of the withers of ewes was 69.71 cm, the height of the hook was 70.57 cm, the body length was 72.57 cm, the chest width behind the shoulders was 21.12 cm, chest depth 31.98 cm, width of the hips was 20.28 cm, the chest volume was 90.95 cm and the circumference volume was 7.91 cm. The rams had an average height of 75.33 cm at the withers, the hook height of 76.33 cm, the body length 78.83 cm, the chest width behind the shoulders was 24.33 cm, chest depth 34.50 cm, width of the hips was 22.00 cm, chest volume was 98.50 cm and the circumference volume was 9.33 cm. In sheep and rams measures that have been shown the highest correlation and statistically highly significant difference were those related to the process of growth and development of the animal, and these are: the height of the withers and hook ($r = 0.841$ for ewes and $r = 1.00$ for rams), while the other hand, there are measures that do not show correlative relationship as hook height and chest volume ($r = 0.155$ for ewes and $r = 0.533$ for rams).

Keywords: genetic characterization, Kupreška Pramenka, morphometric measures, correlation

Introduction

Domestic sheep (*Ovis aries*) have played a significant role in the economy of small and marginal farmers, especially in developing countries, as they are a potential source of meat, wool, milk, hide and manure *(Gorkhali et al., 2015).* According to *Chessa et al. (2013)* local breeds represent an important component of the overall farm animal diversity to be maintained and exploited. Kupreška pramenka is autochthonus sheep breed from Republic of Srpska (Bosnia and Herzegovina), she inhabits Kupres plateau, which is located at an altitude of 1,100

to 1,200 m above sea level. The plateau length is 24 km, width is 10 km and the surface is about 93 km^2 *(Džaja and Draganović, 1994)*. Except Kupres plateau this strain of Pramenka can be found in the municipalities of Tomislavgrad (Duvno), Livno and Glamoč. This strain of the Pramenka has also two types, which differ among themselves by morphometry. The larger type inhabits areas of the northern part of the plateau, and the smaller southern part. Exterior differences arising under the influence of the environment and by mixing with other strains of Pramenka sheep. Kupreška Pramenka from the northern part of the plateau is located in a somewhat better living conditions and was under the influence of Dubska strain of Pramenka with quite bigger body frame, in relation to the type which is bred in southern part of Kupres plateau, where there are a somewhat worse environmental conditions, and sheep were under the influence of smaller Pramenka strain from Hercegovina region. In addition, the exterior of Kupreška Pramenka was under the impact of wealthy pastures, the long, cold and windy winters that have shaped the sheep of medium body frame, strong, with solid constitution, and sheep which is resistant, adaptable and robust *(Mioč et al., 2007)*. Accurate data about the origin of the Kupreška Pramenka are not available. The main phenotype characteristics of Kupreška Pramenka are white head with black or brown differently placed patches of irregular shapes ("grašaste" and "zrnaste") but totally white animals (with white head, legs and fleece) are also not rare. In population of Kupreška Pramenka there can be also found animals with extremely short ears (čule) or even without earlobes (sofe). The body of the sheep is covered with open fleece composed of sharp and long strands of fibers having an average diameter of 35 to 40 μm *(Mitic, 1984)*. Sheep breeding at the Kupres plateau is mostly extensive with the young lamb (lamb carcasses of quality) as a main product. Before 1991 most of the production was delivered to buyers at the Dalmatian market *(Ivankovic et al., 2009)*. During the twentieth century, at the area of Kupres plateau there were imported number of different races, with different production aims, and those were crossed with Kupreška Pramenka. There were karakul, Corriedale, Ile de France, Württemberg, Hampshire, Merino and Precos *(Palian et al., 1960)*. In addition to these sheep breeds at the Kupres plateau there was imported and Texel race *(Antonović et al., 1979)*. All these races were imported in order to improve production and morphometric characteristics of Kupreška Pramenka.

Nowadays Kupreška Pramenka is a sheep with three-purpose production: milk, meat and wool. Along with the Kupreška Pramenka, number of Dubska Pramenka sheep is bred at the Kupres area, and the last one threatens the survival of Kupreška Pramenka. The aim of this study was to determine the morphometric characteristics of Kupreška Pramenka and to compare the obtained data are with previous studies in order to determine whether there has been a change in morphometry of this strain of Pramenka sheep. Morphometric characterization is the part of total breed characterization, as part of animal genetic resources. Characterization of animal genetic resources includes all activities associated with

the identification of qualitative and quantitative traits, documentation of populations and breeds, their homeland, and production systems that are customized. Morphometric, productive, phenotypic or genetic characterization of indigenous sheep breeds is the part of many study *(Nsoso et al., 2004, Muigai et al., 2009, Gebretsadik and Anal, 2014, Pacinovski et.al, 2015).* The aim of this study is to gain more knowledge about resources, their current and potential future use in food production in defined environments, and on their current status in terms of threat *(FAO, 1984; FAO 2007; Rege, 1992; Caput et al., 2010).* In addition, the aim is to determine the correlation coefficients between the obtained measures of the body of ewes and rams Kupreška sheep.

Materials and Methods

Morphometric measures of Kupreška Pramenka were taken from three herds, and two of those were from the village Blagaj, and the third one from village Vukovsko, both from Kupres. Ewes and rams that were measured randomly. Totally there were measured 56 sheep and 6 rams. By Lydtin stick there were determined the following measures: height of the withers, the height of the hook, the body length, the chest width behind the shoulders, chest depth, width of the hips, while the chest and circumference volume measured by ribbon. All measured individuals have been completed their growth and development. The results were analyzed according to the principles of normal statistical analysis where was calculated mean value, standard deviation, standard error of arithmetical mean and coefficient of variation and minimum and maximum values. In addition, there were calculated the correlation coefficients between the taken measures of Kupreška Pramenka. The strength of the correlation relationship is defined by the scale of size of the correlation coefficient: 0.0 to 0.10 no correlation; 0.10 to 0.25 very weak correlation; 0.25 to 0.40 weak correlation; 0.40 to 0.50 medium correlation; 0.50 to 0.75 strong correlation; 0.75 to 0.90 very strong correlation and 0.90 to 0.999 complete correlation.

Results and Discussion

Kupreška pramenka belongs to the group of Prameka sheep with rough wool and three ways of production: meat, milk and wool. Kupreška Pramenka for centuries inhabits Kupres plateau, which with its specific climatic and other external influences created sheep with special morphometric and production characteristics. Table 1 provides information on the morphometric characteristics of Kupreška Pramenka.

Table 1. Descriptive statistics of morphometric characteristics of ewes of Kupreška Pramenka, cm

Morphometric measures in cm	$\bar{x}$	S	$S_{\bar{x}}$	V	Min.	Max.
height at the withers	69,71	2,39	0,32	3,43	64	75
height of the hook	70,57	2,52	0,34	3,57	63	76
the body length	72,57	1,44	0,19	1,97	70	76
chest width behind the shoulders	21,12	1,65	0,22	7,81	17	23
chest depth	31,98	1,45	0,19	4,53	28	35
width of the hips	20,28	0,95	0,13	4,68	18	22
chest volume	90,75	4,70	0,63	5,18	81	103
circumference volume	7,91	0,50	0,07	6,68	7	9

Kupreška Pramenka has a somewhat greater height of the hook (70.57 cm) comparing to the height withers (69.71 cm). The body length (72.57 cm) in ewes is greater than height of the withers for 2.86 cm, which leads to the conclusion that Kupreška Pramenka has almost square shape of the body. The chest width behind the shoulders is not emphasized (21.12 cm), what is characteristic of all strains of ewes of Pramenka sheep with a slightly greater chest depth (31.98 cm). The width of the hips (20.28 cm) of ewes is less than the width of the chest behind the shoulder. Chest volume (90.75 cm) is determined by the chest width and their depth and moves on an average of other strains of Pramenka sheep. According to research of *Telalbašić et al. (1979)* morphometric measures Kupreška Pramenka were: height at the withers 65.00 cm, the body length 68.50 cm, the chest depth 29.30 cm, the chest width 18.60 cm, the chest volume 85.50 cm and circumference volume 7.80 cm. For the same morphometric measures of Kupreška Pramenka *Antunovic et al. (1979)* presented following values: height at the withers 66.86 cm, the body length 68.06 cm, the chest depth 25.80 cm, the chest width 17.56 cm, chest volume 87.74 cm and circumference volume 8.67 cm. *Ivankovic et al., (2009)* examined the exterior characteristics of Kupreška Pramenka and come to the next results: height at the withers of ewes was 65.30 cm, body length 68.82 cm, chest depth 32.26 cm, chest width 21.67 cm, chest volume 94.57 cm and circumference volume 8.44 cm. Comparing the results obtained in this study with the results of the cited authors we can conclude that today's Kupreška Pramenka's is morphometrically more developed, which can be attributed to better nutrition and aspirations shepherd from this area to larger scale types of sheep. Comparing the results of other authors who have measured other strains of ewes of Pramenka sheep, it can be concluded, when it is about height at the withers, Kupreška Pramenka in this study were somewhat lower than Dubska Pramenka from Slavonia whose height at the withers was to 69.80 cm *(Antunovic al., 2013).* According to research of *Pavic et al. (1999)* the height at the withers of ewes of Dubska Pramenka was 66.76 cm, what is lower than height at the withers of Kupreška Pramenka in our study. The measured ewes of Dubska Pramenka which were located in Vrhovine, and were kept in a rather unfavorable conditions, which

maybe reflected on their exterior. When comparing Kupreška Pramenka ewes with ewes of Lička Pramenka with it height at the withers of 60.75 cm *(Mioč et al., 1998)*, it can be concluded that Kupreška Pramenka is larger. Ewe of Istrian sheep breed, whose origin is not known exactly, and it is assumed to be created by crossing of native Pramenka with different races imported mainly from Italia, had the height at the withers of 73.51 cm *(Mikulec et al., 2007)*, which is a higher value comparing to our findings for same characteristic in Kupreška Pramenka. Ewes of Kupreška Pramenka are larger than some ewes of Croatian indigenous breeds such as Dubrovačka ruda sheep (60.12 cm) *(Mioč et al., 2003)*, Krčka sheep (54.64 cm) *(Mioč et al., 2004)* Paška sheep (56.14 cm) *(Pavic et al., 2005)* and Creska sheep (59.97 cm) (*Pavić et al., 2006*).The growth and development outside the uterus takes place according to priority and functional significance of specific tissues and organs, what is reflected on development of specific body parts and whole animal. The correlation coefficients between the measures determined on the body of the sheep indicate the certain degree of connection of different measures during process of growth and development. Table 2 shows the correlation coefficients significant measures of Kupreška ewes.

Table 2. Correlation coefficients between the morphometric measures of rams of Kupreška Pramenka

Measures	Height at the withers	Height of the hook	Body length	Chest width	Chest depth	Width of the hips	Chest volume	Circumference volume
Height at the withers	1	0,841[b]	0,659[b]	0,426[b]	0,383[b]	0,285[a]	0,189	0,448[b]
Height of the hook	-	1	0,468[b]	0,364[b]	0,303[a]	0,279[a]	0,155	0,386[b]
Body length	-	-	1	0,431	0,510[b]	0,410[b]	0,292[a]	0,159
Chest width	-	-	-	1	0,246	0,362[b]	0,277[a]	0,369[b]
Chest depth	-	-	-	-	1	0,349[b]	0,196	0,272[a]
Width of the hips	-	-	-	-	-	1	-0,011	0,272[a]
Chest volume	-	-	-	-	-	-	1	0,133
Circumference volume	-	-	-	-	-	-	-	1

[a] level significant 0,05, [b] level significant 0,01

The height at the withers of ewes of Kupreška Pramenka has a very strong correlation with the height of the hook, and it is statistically highly significant. The height at the withers and the length of the body are in strong correlative cohesion, which is statistically highly significant. There was found a medium correlation of the height at the withers on one side and the width of the chest and the circumference volume on the other side. Determined correlation value is statistically significant. Poor correlation was found between height at the withers

and depth of chest, which was statistically significant, as it was also for height at the withers and the width of the hips, whose association is statistically significant. Only the correlation between height at the withers and the chest volume was weak, and not statistically significant. Height of the hook had medium correlation to the body length, whose correlation is a statistically highly significant. Poor correlation was found between the height of the hook with the chest width, chest depth, width of the hips and circumference volume. Correlation of height with of the hook with chest width and circumference volume is statistically highly significant, and with chest depth and chest with statistically significant. Body length in ewes has a strong correlation with the depth of the chest and this correlative relationship is highly significant. Medium correlative relationship was found between the body length and hook width and it is statistically highly significant. Body length and chest volume are in a weak correlation, but statistically significant. In the low correlation is chest width with the hook width, chest volume and circumference volume. The correlation of chest width is highly statistically significant with the hook width and circumference volume, while the correlation with chest volume is statistically significant. The correlation of chest depth with the hook width and circumference volume is weak, but with hook width is statistically highly significant, and with the circumference volume is statistically significant. The hook width in ewes of Kupreška Pramenka is poorly correlated with the circumference volume, and this relationship is statistically significant. Other correlative relationships between measures of the body of ewes of Kupreška Pramenka are not statistically significant. Rams of Kupreška Pramenka are strong animals, as it is shown by morphometric measures shown in Table 3.

Table 3. Descriptive statistics, morphometric characteristics of rams of Kupreška Pramenka, cm

Morphometric measures in cm	$\bar{x}$	S	$S_{\bar{x}}$	V	Min.	Max.
Height at the withers	75,33	4,64	2,07	6,16	69	80
Height of the hook	76,33	4,63	2,07	6,06	70	81
Body length	77,83	4,37	1,96	5,61	73	84
Chest width	24,33	3,29	1,47	13,52	20	28
Chest depth	34,50	2,50	1,12	7,25	30	38
Width of the hips	22,00	2,83	1,26	12,86	19	28
Chest volume	98,50	6,26	2,80	6,35	88	108
Circumference volume	9,33	0,94	0,42	10,07	8	10

Rams Kupreška Pramenka has pronounced morphometric characteristics comparing to ewes of same breed, which was confirmed in this study. The height of the withers (75.33 cm) is slightly lower for the rams in relation to hook height (76.33 cm), while the body length (77.83 cm) was more pronounced than the withers height and hook height. Body length of the rams is higher by 2.5 cm from the height at the withers, and for these reasons we conclude that the rams of Kupreška Pramenka have almost square shape of the body. As with all natural

breeds, also in rams of Kupreška Pramenka, chest width (24.33 cm) is not satisfactory, and the depth of the chest has a mean value 34.50 cm). The front part of the body is more developed than the hind, as evidented by the width of the hips (22.00 cm), what is lower than chest width (24.33). Rams of Kupreška Pramenka have chest volume of 98.50 cm and circumference volume of 9.33 cm. The obtained results for measures of the height at the withers, the length of the body, chest depth and circumference volume in this study are higher compared to studies of *Ivankovic et al. (2009)* who found that the height of the withers of rams of Kupreška Pramenka was 70.88 cm, the body length 75.88 cm, the chest depth 34.44 cm and circumference volume 8.55 cm, while the width of the chest (24.88 cm) and chest volume (103.88 cm) were higher compared to the same measures in our research. Rams of Kupreška Pramenka in this study had a lower withers height than the rams of Istrian Pramenka 78.06 cm *(Mikulec et al., 2007)*, and greater than rams of Lička Pramenka 67.60 cm *(Mioč et al., 1998)*, Rabska sheep rams 66.44 cm *(Mioč et al., 2006)*, Paška sheep rams 63.20 cm *(Pavic et al., 2005)* and Creska sheep rams 64.83 cm (*Pavic et al., 2006)*.

Correlation coefficients through which are presented the connections between morphometric measures of rams of Kupreška Pramenka are shown in Table 4.

Table 4. Correlation coefficients between the morphometric measures of rams of Kupreška Pramenka

Measures	Height at the withers	Height of the hook	Body length	Chest width	Chest depth	Width of the hips	Chest volume	Circumference volume
Height at the withers	1	1,00	0,774	0,580	0,862 [a]	0,533	0,871 [a]	0,432
Height of the hook	-	1	0,774	0,580	0,862 [a]	0,533	0,871 [a]	0,432
Body length	-	-	1	0,662	0,861 [a]	0,673	0867 [a]	0,216
Chest width	-	-	-	1	0,768	0,750	0,766	0,071
Chest depth	-	-	-	-	1	0,801	0,995[b]	0,566
Width of the hips	-	-	-	-	-	1	0,873 [a]	0,500
Chest volume	-	-	-	-	-	-	1	0,564
Circumference volume	-	-	-	-	-	-	-	1

[a] level significant 0,05, [b] level significant 0,01

In rams of Kupreška Pramenka it was determined the absolute correlation between height at withers and hook height, as well as very strong and statistically significant correlation between the height of the withers on one side and the chest depth and chest volume. Height of hook had a very strong correlation with the chest depth and chest volume, and said correlation is statistically significant. Depth of the chest of rams of Kupreška Pramenka is in complete correlation with the

chest volume, whose relationship is highly significant. Other correlative relationships between of measures determined at the body of rams of Kupreška Pramenka are not statistically significant.

Conclusion

Kupreška Pramenka belongs to a group of indigenous strains sheep and is a mirror of the environment in which it is located. Ewes and rams have a square shape of the body, and those have hook height slightly greater than height of the withers. Both sexes have a medium-developed chests, whose width is not emphasized with a slightly larger depth, which gives flat body shape. The morphometric measures of present animals of Kupreška Pramenka are higher in comparison to previous researches. The reason for this phenomenon can be explained by more complete feeding system of sheep on the Kupres Plateau and the desire of shepherd to have animals of higher frame. In both sexes of Kupreška Pramenka the highest correlation was found between the height at the withers and height of the hook, height at withers and body length, body length and chest depth. On the basis of determined correlations between the important morphometric measures it is easier to carry out the selection of sheep on several exterior qualities.

Morfometrijska merenja kao deo genetičke karakterizacije autohtonog soja kupreške pramenke

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Rezime

U cilju genetičke karakterizacije sojeva pramenki u Bosni i Hercegovini urađena je morfometrijska karakterizacija kupreške pramenke. Ukupno je izmereno 62 jedinke, od kojih je 56 ovaca i 6 ovnova. Prosečna visina grebena ovaca kupreške pramenke iznosila je 69,71 cm, visina krsta 70,57 cm, dužina trupa 72,57 cm, širina grudi iza lopatica 21,12 cm, dubina grudi 31,98 cm, širina kukova 20,28 cm, obim grudi 90,95 cm i obim cevanice 7,91 cm. Ovnovi su imali prosečnu visinu grebena 75,33 cm, visina krsta 76,33 cm, dužina trupa 78,83 cm, širina grudi iza lopatica 24,33 cm, dubina grudi 34,50 cm, širina kukova 22,00 cm, obim grudi 98,50 cm i obim cevanice 9,33 cm. Kod ovaca i ovnova najveću korelaciju i statistički visoko značajnu razliku pokazale su mere koje se u procesu rasta i razvoja uzajamno razvijaju, a to su: visina grebena i visina krsta (r = 0,841 za ovce i r = 1,00 za ovnove), dok na drugoj strani imamo mere koje ne pokazuju

korelativni odnos kao visina krsta i obim grudi (r = 0,155 za ovce i r = 0,533 za ovnove).

Ključne reči: genetska karakterizacija, Kupreška Pramenka, morfometrijske mere, korelacija

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Received 11 October 2016; accepted for publication 10 January 2017

Biotechnology in Animal Husbandry 33 (1), p 65-71 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.06'658
DOI: 10.2298/BAH1701065U

GENETIC PARAMETERS FOR SOME GROWTH TRAITS OF NIGERIAN LOCAL CHICKENS

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Abstract: Genetic parameters were estimated for bodyweight (BWT), shank length (SHL), and wing length (WL) of Nigerian local chicken (NLC) from 4 to 20 weeks of age by fitting dyadic mixed model (dmm) equations which yield estimates of variance components equivalent to minimum norm quadratic unbiased estimator (MINQUE). Data obtained from 600 chicks, progenies of 300 hens and 30 cocks were used for the analysis. The heritability estimates range from 0.08 to 0.80 for BWT, 0.03 to 0.69 for SHL and 0.22 to 0.47 for WL. The genetic correlations among BWT, SHL and WL at different ages were high and positive and range from 0.18 to 0.96 with the exemption of SHL and WL at 16 weeks (-0.06). The phenotypic correlations were positive and range from 0.10 to 0.91. The results imply that NLC could be improved on any of the studied traits through mass selection and that improvement in one trait will result to correlated improvement in the others.

Keywords: Correlations, dyadic mix model, heritability, Nigerian local chickens.

Introduction

The population of chicken in Nigeria has been estimated at approximately 166 million (*FAOSTAT, 2007*). The local chicken constitutes about 80% of this number (*Dana et al., 2011*). The Nigerian local chicken is characterized by poor growth, small body size, small egg size and egg number which is not desirable in a competitive economy (*Ebangi and Ibe, 1994*). Despite these undesirable characteristics, the NLC still plays important role in the rural economy of Nigeria by providing meat, egg and house hold income to the rural people. Studies have shown that the local chicken in Africa exhibit high genetic variability within their populations indicating their potential for genetic improvement through selective breeding (*Osei-Amponsah et al., 2010, Dana et al., 2011*). Knowledge of genetic parameters is necessary for designing an appropriate breeding plan for genetic

improvement of NLC. Estimates of genetic parameters for growth traits in NLC have been reported by many workers. *Asuquo and Nwosu (1987)* reported average h^2 estimates of 0.35 to 0.74, 0.31 to 0.89 and 0.27 to 0.49 for bodyweight of two 3 way crosses (YA x LC x GL, YA x GL x LC) and the local crosses (LC2 x LC1 x LC1) respectively. *Ndofor-Foleng et al. (2006)* reported h^2 estimates of 0.40 and 0.37 for bodyweight of light and heavy ecotypes of NLC. *Udeh and Isikwenu (2013)* reported h^2 estimates of 0.25-0.51 and 0.24-0.40 from sire-son and dam-daughter regressions respectively for bodyweight of NLC from 8 to 20 weeks of age. *Osei-Amponsah et al. (2013)* reported average h^2 estimates of 0.54 and 0.42 for bodyweight and shank length of local chicken of Ghana. The objective of this study was to estimate heritabilities, genetic and phenotypic correlations of bodyweight, shank length and wing length of NLC using dyadic mix model analysis.

Materials and Methods

The experiment was conducted at the poultry breeding unit of the Department of Animal Science, Enugu State University of Technology Enugu, Nigeria. The foundation stock comprised 300 hens and 50 cocks of NLC. These birds were the surviving population of NLC chicks housed intensively at day old and raised to sexual maturity. At sexual maturity, the 300 hens were grouped into 30 mating groups of 1 cock to 10 hens. Each group was randomly assigned into deep litter floor pens partitioned to hold 10 hens and 1 cock. Eggs collected from each mating group were sire and dam identified. Eggs were incubated and hatched according to pedigree (sire and its group of hens) using very efficient locally made incubators. A total of 600 chicks were produced at 2 hatches. The chicks from each mating group were tagged according to sires and reared on deep litter pens. The chicks were brooded for six weeks during which they were fed with chick mash diet with 20 % cp and 2685 kcal ME/kg. They were provided with growers mash with 16 % cp and 2642 kcal ME/kg from 8-18 weeks and layer mash containing 2676 kcal ME/kg, 17 % cp and 3 % calcium from 18 weeks to end of lay. Feed and water were provided *ad libitum*. The chicks were separated into sexes at 7 weeks of age. All necessary vaccinations were administered. The bodyweight of the birds were recorded at 4 weekly interval starting from week 4. Shank length and wing length were taken using a tape as described by *Adeleke et al. (2013)*. Estimates of variance components, heritability and correlations as well as their standard error were obtained by fitting dyadic mixed model (dmm) equations. DMM is an r package used for modelling pairs of observations or dyads and yields estimates that are equivalent to minimum norm quadratic unbiased estimator (MINQUE) if the fixed effect is adjusted using ordinary least square (OLS) or biased corrected maximum likelihood estimates if the fixed effect was adjusted using generalized least square (GLS). In this case the fixed effect of sex and hatch were adjusted

using OLS. Prior to the analysis, the data frame which include pedigree and parameter files was converted into a suitable format using modify data frame function (MDF) in the dmm package (*Jackson, 2016*).

Results and Discussion

The correlation between variance environmental individual (VarE(I)) and variance genetic individual additive (VarG(Ia)) was used to check if the dyadic equations have collinearity problem. As shown in Table 1, the correlation between VarE(I) and VarG(Ia) were 0.56. This shows that collinearity was not a serious problem in this analysis and that the two components of interest were well separated. It also implies that the data used for the analysis was adequate (*Jackson, 2016*).

Table 1. Correlation between VarE(I) and VarG(Ia)

	VarE(I)	VarG(Ia)
VarE(I)	1.00	0.56
VarG(Ia)	0.56	1.00

Note: VarE(I)= Variance environmental individual, VarG(Ia)= Variance genetic individual additive.

The heritability of bodyweight, shank length and wing length are presented in Table 2. The h^2 estimates were high and range from 0.50 to 0.80 for BWT and 0.52 to 0.69 for SHL. The only exemption was BWT and SHL at 4 weeks of age which had very low h^2 estimates of 0.08 and 0.03 respectively. The h^2 estimates of WL were moderate to high in magnitude and range from 0.22 to 0.47. The high h^2 estimates for BWT and SHL as well as the moderate to high h^2 estimates of WL imply that additive genetic variance made a greater contribution to the total phenotypic variance compared to environmental and gene combination variance. This implies that mass selection for any of the aforementioned trait could result to rapid improvement. Generally fitness traits such as BWT and SHL tend to have higher h^2 estimates compared to traits that are not connected to reproductive efficiency of animals. *Kinney* (*1969*) summarized the h^2 estimates of BWT, SHL and other body parameters of chicken published in literature and concluded that BWT of chicken was a highly heritable trait with h^2 range of 0.25 to 0.75. Similar observation was reported by *Adeyinka et al. (2006)*. The h^2 estimates of BWT and SHL observed in this study were in line with estimates of 0.41 (BWT) and 0.58 (SHL) reported for the local chicken at 12 weeks by *Ebangi and Ibe (1994)*. *Adeleke et al. (2011)* reported h^2 range of 0.15 to 0.29 for WL of pure and crossbred progenies of NIC which was less than the range of 0.22 to 0.47 observed in this study. In a recent study, *Rotimi et al. (2016)* estimated h^2 of BWT of three genotypes of NLC at 0-16 weeks from sire component of variance that range from 0.17 to 0.65 which was within the range reported in this study. *Osei-Amponsah et*

al. (2013) reported an average h^2 estimate of 0.54 for bodyweight of Ghanaian local chicken from 0 to 40 weeks which fall within the range reported in this study. Differences in h^2 estimates reported by different researchers could be attributed to method of estimation, breed, environmental effects and sampling error due to sample size (*Prado-Gonzalez et al., 2003*). The genetic correlations among BWT, SHL and WL were high and positive and range from 0.18 to 0.96. The only exemption was SHL and WL at 16 weeks of age with a genetic correlation of -0.06. This indicates pleiotrophic action of genes governing the three traits and implies that selection of any of the three traits will give positive correlated response to the other traits. *Ebangi and Ibe (1994)* reported genetic correlation between growth traits that range from 0.99 to 1.51 which is higher than the range observed in this study. Similarly, *Adeleke et al. (2011)* reported genetic correlation among growth traits in pure and crossbred progenies of NIC that range from 0.43 to 0.99 which was slightly higher than the range reported in this study. The phenotypic correlations among BWT, SHL and WL were positive and range from 0.10 to 0.91 implying high predictability among the three variables. Similar results were reported by *Momoh and Kershima (2008)* and *Ukwu et al. (2014)* in Nigerian local chickens.

Table 2. Heritabilities and genetic and phenotypic correlations of bodyweight, shank length and wing length of Nigerian local chickens at 4, 8, 12, 16 and 20 weeks of age.

Weeks	Bodyweight	Shank length	Wing length
4			
Bodyweight	**0.08(0.11)**	0.57(0.09)	0.10(0.09)
Shank length	0.36(0.35)	**0.03(0.11)**	0.42(0.07
Wing length	0.37(0.40)	0.56(0.34)	**0.31(0.08)**
8			
Bodyweight	**0.67(0.15)**	0.91(0.03)	0.86(0.03)
Shank length	0.94(0.05)	**0.69(0.15)**	0.80(0.04)
Wing length	0.96(0.05)	0.91(0.07)	**0.31(0.09)**
12			
Bodyweight	**0.80(0.17)**	0.79(0.05)	0.75(0.06)
Shank length	0.93(0.07)	**0.52(0.13)**	0.43(0.08)
Wing length	0.92(0.09)	0.74(0.13)	**0.47(0.11)**
16			
Bodyweight	**0.52(0.15)**	0.39(0.09)	0.41(0.10)
Shank length	0.56(0.15)	**0.59(0.10)**	0.21(0.10)
Wing length	0.18(0.24)	-0.06(0.07)	**0.22(0.10)**
20			
Bodyweight	**0.50(0.17)**	0.72(0.07)	0.59(0.08)
Shank length	0.83(0.11)	**0.57(0.13)**	0.73(0.05)
Wing length	0.53(0.16)	0.31(0.08)	**0.45(0.12)**

Heritabilities in bold, genetic correlations on lower diagonal and phenotypic correlations on upper diagonal. Standard errors are in parentheses.

Conclusion

From the high h^2 estimates for BWT and SHL at 8-20 weeks of age and the moderate to high h^2 estimates for WL from 4 to 20 weeks of age, it is inferred that these traits could be improved through mass selection. The high genetic correlations among the traits suggest that improvement in one trait will give correlated improvement in the others.

Genetski parametri za neke osobine porasta nigerijskih lokalnih rasa pilića

I. Udeh

Rezime

Genetski parametri su određivani za osobine telesne mase (BWT), dužinu tarzusa (SHL), kao i dužinu krila (WL) Nigerijskih lokalnih pilića (NLC) od 4 do 20 nedelje starosti postavljanjem jednačine diadičnog mešovitog modela (dmm) koji daju procenu varijanse komponenti ekvivalentne jednačini mešovitog modela MINQUE (Minimum Norm Quadratic Unbiased Estimator - MINQUE). Podaci dobijeni za 600 pilića, potomaka 300 kokošaka i 30 petlića, su korišćeni u analizi. Procene heritabilnosti su u rasponu od 0,08 do 0,80 za BWT, od 0,03 do 0,69 za SHL i 0,22 do 0,47 za WL. Genetske korelacije između BWT, SHL i WL u različitim uzrastima su bile visoke i pozitivne, i kreću se od 0,18 do 0,96 izuzimajući SHL i WL u 16. nedelji (-0,06). Fenotipske korelacije su bile pozitivne i kretale su se u rasponu od 0,10 do 0,91. Rezultati ukazuju da bi NLC mogle da budu poboljšane u pogledu bilo kojeg od ispitivanih svojstava, kroz selekciju i da će poboljšanje u jednoj osobini dovesti do korelativnog poboljšanja ostalih.

Ključne reči: korelacije, dijadički mešoviti model, heritabilitet, nigerijske lokalne rase pilića

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Received 9 November 2016; accepted for publication 20 February 2017

Biotechnology in Animal Husbandry 33 (1), p 73-79 , 2017
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 636.082.45'64
DOI: 10.2298/BAH1701073Z

INFLUENCE OF THE AGE OF THE FIRST INSEMINATION ON SOME REPRODUCTIVE INDEXES IN SOWS

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Original scientific paper

Abstract: The aim of the study was to establish the influence of the month and the year of birth of gilts from the hybrid combination 'Y' ((Tai Zumu x Landrace) x Large White), as well as of the age of the first insemination on the age of the first mating, the first farrowing, the duration of pregnancy, and the total number of born piglets. The experimental animals were divided into three groups depending on the age of the first insemination (by the 249th day (n=71), from the 250th to the 271st (n=42), and after the 272nd one (n=20). The average age of the first insemination and the first mating was respectively 258.8±0.78 and 276.71±3.54 days, on average. The age of the first farrowing was 391.56±3.54 on average, and the duration of pregnancy – 115.03±0.2 days. The total number of born piglets in a litter was 11.87±0.32 pigs on average. The age of the first insemination has a reliable influence on the age of the first mating and the first farrowing ($p<0.001$), as well as on the number of born piglets ($p<0.05$). The month ($p<0.001$) and the year of birth of the gilt pigs ($p<0.05$) have reliable influence on the age of the first insemination.

Key words: sows, insemination, fertilization, gestation, number of piglets, reproduction

Introduction

The increased worldwide demand for meat gives advantage of the fast-growing species such as pigs, and turns pig-breeding into one of the leading sub-branches of stock-breeding. At the same time, the population on the planet is expected to increase with 2 to 4 billion by 2050 (*Cohen, 2003*). This gives grounds of the scientific researches and the producers of pig-breeding production to adapt to these tendencies in order to satisfy the needs of the population (*Miclea et al. 2009*).

The improvement of the reproductive abilities of the pigs is of significant importance since it also has an influence on the born piglets and their productive

abilities (*Miclea et al. 2007, 2009*). According to the same authors, the highly specialized hybrid parental forms allow them to be used for breeding from younger age, but at the same time, the indications of estrus of these animals become weaker.

The lifelong productivity and longevity of breeding stock are of significant importance for the successful management of pig farming. The increased duration of use and the high productivity of sows reduce the expenses for the purchase of gilt pigs and increase the effectiveness and profitability of the group of pigs (*Sasaki and Koketsu, 2008, Saito et al., 2011*). In this regard, the age of the first insemination can be defined as a key factor, defining the productivity and longevity of the sows.

All this gave us a reason to analyze the influence of the age of the first insemination on the age of the first mating and first farrowing, the duration of the pregnancy and the total number of born piglets.

Materials and Methods

The study included a total of 133 gilt pigs of the hybrid 'Y' ((Tai Zumu x Landrace) x Large White), born in December 2012 and in January and February 2013, and bred in a pig farm located in the region around the town of Plovdiv, Bulgaria.

The stock was inseminated twice during the estrus, with sperm of imported terminal boars 'D' (Large White x Pietrain). The first insemination was not done earlier than the time of the first registered estrus after reaching sexual maturity. The duration of pregnancy was registered from the first day of artificial insemination to the day of labor.

The experimental animals were divided in three groups depending on the age of the first insemination (by the 249th day (n=71), from the 250th to 271st one (n=42), and after the 272nd day (n=20).

The following indications were studied:

- The age of the first insemination, days
- The age of the first mating, days
- The age of the first farrowing, days
- Duration of the pregnancy, days
- Total number of born piglets.

In the course of the study, the influence of the age group on first insemination as well as the month and the year of birth of the gilt pigs on the studied indications was analyzed.

In data processing, we used a multi-factor dispersion analysis of software product SPSS 19.

Results and Discussion

Table 1 shows the average value (LS) of the indicators, characterizing the reproductive ability of gilt pigs, as well as the total number of born piglets in a litter.

Table 1 Reproduction traits of hybrid sows (n=133)

Traits	LS	±SE	Cv, %
Age of 1-st insemination	258.81	0.78	7.37
Age of 1-st fertilization	276.71	3.54	14.35
Age of 1-st farrowing	391.56	3.54	10.03
Gestation duration	115.03	0.2	1.73
Number of piglets at birth	11.87	0.32	28.68

The productivity of pigs in a long-term plan depends largely on the age, in which gilt pigs start their reproductive life (*Babot et al., 2003)*. According to a number of authors (*Schukken et al., 1994; Xue et al., 1996*) the optimal age of the first insemination is between 200 and 260 days. According to the latter, the recommended age range is too big since the ability of pigs to express estrus in this time frame is too variable.

The average age of the first insemination of the gilt pigs in our experiment was 258.81±0.78 days. The age of the first mating and the first farrowing was 276.71±3.54 and 391.56±3.54 days, respectively. The average duration of the period of pregnancy of the gilt pigs was 115.03±0.2 days, which is within the limits of the norm for the age. The coefficient of variation under this indication was 1.73%, which corresponds to the one established by *Miclea et al., (*2009). The authors define limits of duration of pregnancy in gilt pigs of the Camborough hybrid from 114.42 to 116.70 days, with variation under this indication – around 2%. A number of live born piglets in the litter is one of the most studied genetic traits *(Popovac et al. 2012). Vidovic et al. (2012)* reported for 11,4 average number alive born piglets from purebred female Landrace. In the conditions of this study, the average number of born piglets was 11.87±0.32. For similar coefficient of variation (28.16%) for number of born alive piglets reported *Radojković et al. (2007)* in experiments with Swedish Landrace sow on three farms in Serbia.

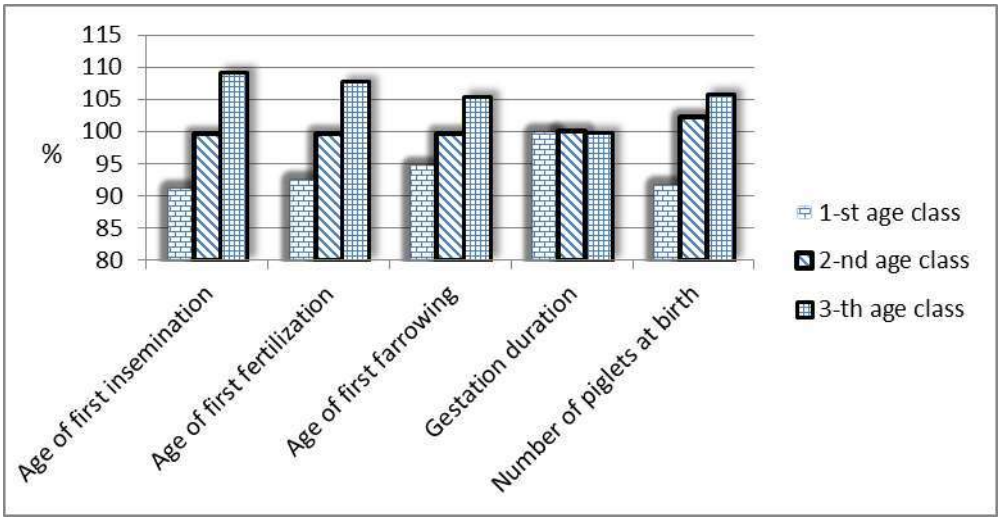
The factors studied by us have a reliable influence on the reproductive abilities except the duration of pregnancy (Table 2). The age of the first insemination has a reliable influence on the age of the first mating and the first farrowing ($p<0.001$), as well as on the number of the born piglets ($p<0.05$). The month ($p<0.001$) and the year of birth of the gilt pigs ($p<0.05$) have reliable influence on the age of the first insemination.

Table 2. The effect of the month and the year of birth, and the age class on the reproductive traits of sows

Model	Factor	F- criterion and degree of reability				
		Traits				
		Age of 1-st insemination	Age of 1-st fertilization	Age of 1-st farrowing	Gestation duration	Number of piglets at birth
1.	Year of birth	4.61*	0.001	0.002	1.91	0.02
2.	Month of birth	8.30***	0.66	0.68	1.02	0.98
3.	Age class	293.17***	11.57***	11.26***	0.37	3.04*
4.	-Year -Age class	2.01 284.2***	0.5 11.76***	0.6 11.5***	1.84 0.35	0.1 3.06*
***P<0.001, **P<0.01, *P<0.05						

The changes of the indicators characterizing the reproductive ability of pigs depending on the group they refer to according to the age of the first insemination are presented in Figure 1.

Figure 1. Variability of reproductive indices of sows depending on the age class of first insemination (like deviation of mean, %)



The average age of the first insemination in the first group was 236.32±0.94, 257.62±1.22 days in the second one, and 282.5±1.77 days in the third one. All three groups were homogeneous which is also proved by the coefficient of variation – 7.37%. There was also a similar distribution of the relative values in the other two studied indicators – the age of the first mating and first farrowing. They were lowest in the first group and highest – with the pigs in the third age class.

The duration of pregnancy in the first and second age class was practically the same, and with the animals inseminated for the first time after the 272nd day it was 0.42 days shorter.

The influence of the age of the first insemination plays an important role for the reproductive qualities of pigs in their first farrowing, especially on the number of live born piglets (*Babot et al., 2003*).

A number of authors state of an increase of multiple pregnancy with the advancing of age on insemination (*Archibong et al., 1987; Beltranena et al., 1991*), which they explain with the fact that the increase of the age of the first insemination probably increases the number of estruses, and consequently the level of ovulation.

In our experiment, the age of the first insemination had a significant effect on the number of born piglets ($p<0.05$). *Radojković et al. (2007)* also established a significant effect ($p<0.01$) on the same trait on the age of first farrowing. Figure 2 shows that the gilt pigs of the first class had the least number of pigs – 10.9±0.38 pigs, which is around 8% lower than the average for all the animals. The sows from the third age group gave birth to an average of 12.55±0.73 pigs, which is nearly 6% more compared to the average value of this indication. Gilt pigs, inseminated within the limits from the 250th to the 271st day after their birth, take an average position in the number of born piglets.

Conclusion

The average age of the first insemination and first mating in the conditions of our experiment was 258.8±0.78 and 276.71±3.54 days on average, respectively. The age of the first farrowing was 391.56±3.54 on average, and the duration of pregnancy – 115.03±0.2 days. The total number of born piglets in a litter was 11.87±0.32 pigs on average.

The age of the first insemination had a reliable influence on the age of the first mating and first farrowing ($p<0.001$), as well as on the number of born piglets ($p<0.05$).

The month ($p<0.001$) and the year of birth of the gilt pigs ($p<0.05$) have reliable influence on the age of the first insemination.

Uticaj uzrasta pri prvom osemenjavanju na neke reproduktivne indekse krmača

Ivelina Zapryanova

Rezime

Cilj istraživanja je bio da se utvrdi uticaj meseca i godine rođenja nazimica iz hibridne kombinacije 'Y' ((Tai Zumu x landras) x velika bela), kao i uzrasta pri prvom osemenjavanju, na uzrast pri prvom parenja, prvom prašenju, trajanju steonosti, i ukupni broj rođene prasadi. Ogledne životinje su bile podeljene u tri grupe u zavisnosti od starosti pri prvom osemenjavanju (do 249. dana (n = 71), od 250. do 271. (n = 42), i nakon 272. dana starosti (n = 20). Prosečan uzrast pri prvoj inseminaciji i prvom parenju je bio 258,8 ± 0,78, odnosno 276,71 ± 3,54 dana. Uzrast pri prvom prašenju je bio 391,56 ± 3,54 u proseku, i trajanje gestacije - 115,03 ± 0,2 dana. Ukupan broj rođenih prasadi u leglu je bio 11,87 ± 0,32 u proseku. Uzrast pri prvom osemenjavanju ima pouzdan uticaj na uzrast pri prvom parenju i prvom prašenju ($p < 0,001$), kao i na broja rođenih prasadi ($p<0,05$). Mesec ($p < 0,001$) i godina rođenja nazimica ($p < 0,05$) ima pouzdan uticaj na uzrast pri prvom osemenjavanju.

Ključne reči: krmače, osemenjavanje, fertilizacija, gestacija, broj prasadi, reprodukcija

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Received 28 September 2016; accepted for publication 12 November 2016

Biotechnology in Animal Husbandry 33 (1), p 81-90 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.06'61
DOI: 10.2298/BAH1701081L

GENETIC DIVERSITY ASSESSMENT OF AN INDIGENOUS HORSE POPULATION OF GREECE

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Original scientific paper

Abstract: Highly endangered local breeds are considered important not only for the maintenance of their genetic diversity for future survival but also because they regarded as part of the cultural heritage of the local and national communities. Using pedigree data and an analysis of 18 microsatellite loci we investigated the genetic diversity of a private (commercial) indigenous Skyros horse population, reared in an insular region of North-Western of Greece. The overall average animal inbreeding value reached 24%. Concerning average inbreeding value over non founding animals, it was estimated to 0.013, while the corresponding value over inbred animals were 0.13.The mean number of alleles per locus amounted to 3.72, ranging between 1 and 7 alleles. The average observed heterozygosity was 0.57. Taking into account the inbreeding estimated index, an average heterozygote deficit (F_{is}) of -0.09 was noted ($P<0.05$). Although the population maintained reasonable levels of genetic diversity, well studied inbreeding strategies should be implemented, in order to reduce the loss of genetic variability, to avoid extinction and further genetic drift of the population.

Keywords: Skyros Horse, Inbreeding, Conservation, STRs, genetic markers.

Introduction

Many local indigenous breeds of domestic animals (i.e. horse, sheep) are considered highly endangered due to their small population number. The proper genetic management of such populations is crucial for their survival and it involves two major steps. The first step involves the selection of the individuals who will be permitted to leave descendants and the second the design of the proper mating scheme. However, in order the aforementioned steps to be implemented, the

genetic diversity (inbreeding and heterozygosity levels) has primary to been considered.

High inbreeding and low heterozygosity levels are associated with undesired characteristics i.e. reduce fitness, reproductive capacity and survival (Falconer MacKay, 1996). Such characteristics are often observed in small populations of endangered breeds. The conservation of these breeds is justifiable due to their importance in their local environmental conditions.

Skyros horse, a small-sized pony originated from the homonymous Greek island, belongs to the highly endangered breeds in Greece rendering its population under evaluation regularly. Currently, less than 140 purebred Skyros horses have been recorded throughout the country and phenotypic characteristics have been well documented (SAVE, 2015). The fact that the majority of these horses roam freely and well-designed breeding schemes are missing renders the future of the breed under severe challenge.

The implementation of novel DNA techniques, such as microsatellite molecular markers (STRs), in combination with data retrieved from pedigree analysis render the identification of genetic diversity a very useful and precise tool for further breeding conservation strategies (*Marleta et al.,2006; Criscione et al., 2015*). Previous analysis of an experimental population of Skyros pony showed low levels of genetic diversity (*Avdi and Banos, 2008*). As inbreeding leads to undesired performance characteristics investigating the genetic background of Skyros horses is always of great importance as it would increase the chances for survival. The aim of the present study was to examine the genetic diversity of a small population of Skyros horses, reared in an insular region of North-Western Greece, away from its natural origin habitat. To the best of our knowledge this is the first time that the certain population is thoroughly analyzed presenting the most recent assessment of the genetic diversity levels, which may assist the future survival of the breed.

Materials and Methods

Population data

Data were retrieved from a private commercial farm of Skyros horses reared in an insular region of North-Western Greece (Corfu). A total number of 25 purebred animals (18 females and 7 males) with pedigree data were taken into account. Mating was at random.

A genealogical tree was developed using animal pedigrees and thereafter inbreeding coefficients for each animal were calculated based on the genetic relationships among their parents. Inbreeding levels of common ancestors were also taken into account. Concerning founder animals (without own pedigree), a total of 9 animals (6 females and 3 males) did not have any ancestor information. However, these were considered to be among the population founding animals that

were unrelated to each other. Inbreeding levels in the studied population were assessed based on the calculated genetic relationships among animals and according to F-statistic (*Wright, 1978*) as computed by *Caballero and Toro (2002).*

STRs marker genotyping

A set of 18 microsatellite markers were genotyped in the present study. Genomic DNA was firstly isolated from blood and then the desired STR sequence was amplified using the Polymerase Chain Reaction (PCR) and analysed at the premises of Labogena in France as previously described (*Avdi and Banos, 2008*). Allelic discrimination was conducted using GeneMapper Software (Applied Biosystems). The guidelines proposed by the International Society of Animal Genetics (*Solis et al., 2005*) were followed for the nomenclature of each marker.

Statistical analysis

The following parameters were determined for each marker: i) number and frequency of alleles, ii) observed heterozygosity (Ho), iii) expected heterozygosity (He), iv) heterozygote deficit (Fis), v) Hardy-Weinberg deviation vi) Polymorphism Information Content (PIC), vii) probability of identity (Pi), and viii) probability of parental exclusion (Pe) (considering 1 or 2 parents). Allelic frequency was estimated by direct counting.

The parameters concerning heterozygosity (observed/expected) and heterozygote deficit were calculated using POPGENE software (*Yeh at al., 1999*). Deviations from Hardy-Weinberg equilibrium were calculated using GENEPOP software (*Raymond and Rousset, 1995*), while the on-line platform at http://w3.georgikon.hu/pic/english/kezi.aspx was used to calculate Polymorphism Information Content (PIC) on the basis of observed allele frequencies (*Botstein et al., 1980*). IDENTITY software was used in order to calculate the probability of identity for each marker (Pi) and the probability of parental exclusion (Pe) for each marker separately and across all 18 markers respectively. Within inbreeding population estimates (F_{is},) was determined using FSTAT software (*Goudet, 1995*).

Results and Discussion

Genetic diversity in farmed animals is of utmost importance in order: a) to meet current production needs in various environments, b) to allow sustained genetic improvement and c) to facilitate rapid adaptation to changing (*Notter, 1999*). In endangered populations (i.e. Skyros pony), genetic characterization and avoidance of inbreeding becomes the first crucial and necessary step for breed conservation and application of future breeding strategies.

Pedigrees data: Intra-breed diversity

According to our–data, an 18.8% (3 animals) of the 16 non-founding animals were found to be inbred in the population. The overall average animal inbreeding value reached the 24%. Concerning the average inbreeding value over non founding animals, it was estimated to 0.013, while the corresponding value over inbred animals were 0.13. Inbreed coefficients frequency across all inbred animals was ranged between 0.0234 and 0.30 (Fig.1). No statistical significant differences were noted between males and females (P>0.05). Similar inbreeding profiles have been reported by previous author concerning other horses' small populations (*Curik et al., 2003; Aberle et al., 2004; Valera et al., 2005; van Eldik et al., 2006*). Interestingly, according to *Avdi and Banos (2008)* who analysed using pedigree data the genetic structure of a Skyros pony population, reared in an experimental university farm, revealed higher levels of inbreeding. Specifically, an extra 13% of the animals (32%) were found to be inbred in the total population and the average inbreeding value over non founding animals estimated to 0.03, revealing by means of genetic drift a narrow genetic status of the experimental population.

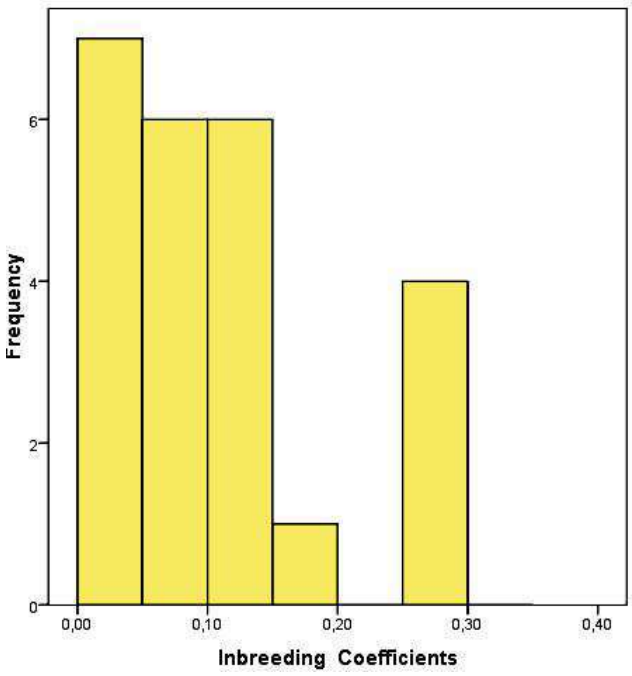

Figure 1. Frequency of inbreeding coefficients in the examined population populations

Genetic variability of molecular markers

Almost all the analysed microsatellite loci were found to be polymorphic apart from HTG4. The number of alleles ranged from 2 (HMS1) to 7 (ASB17) with a mean of 3.72 per locus, respectively (Table 1). The average allele frequency was estimated to 0.27.

Polymorphism Information Content (PIC) refers to the value of a marker for detecting polymorphism within a population. It depends on the number of detectable alleles and the distribution of their frequency. According to Botstein et al., 1980, when PIC>0.5 the marker is considered as highly informative while if (0.5>PIC>0.25) or (PIC<0.25) the marker is considered as reasonably informative or slightly informative, respectively. In regard to the PIC in the examined population, the majority of loci were highly informative (PIC>0.5), while the rest were reasonable informative (0.5>PIC>0.25) The observed mean number of alleles per microsatellite loci was closer to the estimates reported for inbreed Basques horses (4.33 per locus) (*Solis et al., 2005*) or other European horses (4.50) (*Luis et al., 2007*) and lower to that noted for South American (5.67-7.67), North American (6-7.25), Caspian (8.69), an Italian (9.58) and a local Brazilian (14.36) horses breed (*Luis et al., 2007; Pieragostini et al., 2005; Shasavarani and Rahimi-Mianji, 2010; Silva et al., 2012*). The differences in average number of observed alleles may be attributed to different set of microsatellite markers, number of markers, population structure (i.e. divided into folks) and different horse breed (i.e. local Brazilian horses, Caspian horses, Pindos pony etc.).

Concerning the observed heterozygosity, it ranged from 0.19 to 0.90 with a mean of 0.57 (Table 1). Similar levels of heterozygosity have been reported in many other international horses' breeds i.e. North American horses (*Colling and Kelly, 1996*), Kladruber horse (*Horin et al., 1998*), Lipizzan horses (*Curik et al., 2003*), Norwegian horse's breeds (*Bjørnstad et al., 2000*), Sorraia horse (*Luis et al., 2007*) and other European horses (*Solis et al., 2005*). Further, taking into account the expected heterozygosite (Table 1), an average heterozygote deficit (F_{is}) of -0.09 was estimated (P<0.05). Moreover, almost all loci were found in a Hardy-Weinberg equilibrium (Table 1) apart from loci LEX3 (P<0.05). The observed negative heterozygote deficit is indicative of a heterozygote surplus, meaning that more heterozygotes were observed than expected from allelic frequencies. It, also, suggests a potential outbreeding (i.e. occasional importation of external breeding animals into each population). Moreover, the lower average H_o compared to the H_e may reflect the narrow genetic base of the examined populations (Silva et al., 2012).

Table 1. Measures of genetic diversity per analysed locus

Marker	Number of alleles	Range of allele frequency	Theoretical Heterozygosity (He)	Observed Heterozygosity (Ho)	Heterozygote deficit (F_{is})	Polymorphism Information Content (PIC)	Hardy Weinberg Deviation (*P value*)
HTG6	3	0.19-0.57	0.5805	0.7143	-0.2305	0.52	0.4819
VHL20	4	0.14-0.43	0.70	0.81	-0.15	0.65	0.69
HTG10	3	0.10-0.74	0.42	0.52	-0.25	0.39	0.78
HTG4	1	-	-	-	-	-	
AHT5	5	0.07-0.62	0.66	0.71	-0.08	0.60	0.42
AHT4	3	0.17-0.62	0.54	0.67	-0.23	0.48	0.74
HMS3	5	0.07-0.60	0.60	0.67	-0.11	0.58	0.95
HMS6	2	0.21-0.79	0.34	0.33	0.01	0.27	1.00
HMS7	5	0.05-0.36	0.74	0.90	-0.23	0.70	0.167
HMS1	2	0.10-0.90	0.17	0.19	-0.11	0.16	1.000
ASB2	4	0.10-0.50	0.64	0.71	-0.11	0.60	0.917
ASB17	7	0.03-0.25	0.82	0.94	-0.15	0.79	0.537
ASB23	4	0.07-0.50	0.61	0.33	0.45	0.55	0.877
CA425	4	0.04-0.46	0.56	0.64	-0.13	0.48	0.214
HTG3	6	0.05-0.45	0.71	0.76	-0.07	0.69	0.860
HTG7	3	0.10-0.52	0.57	0.61	-0.08	0.55	0.313
LEX3	3	0.02-0.67	0.46	0.33	0.27	0.37	0.017*
LEX33	3	0.19-0.50	0.62	0.77	-0.24	0.55	0.720
Mean	3.72	0.27	0.57	0.63	-0.09	0.53	-
S.E.	0.12	0.03	0.04	0.05	0.04	0.48	-

*P<0.05

The probability of identity (P_i) across all analysed markers was estimated at 7.55×10^{-12} reaching an extremely low level. This reflects to the fact that the probability of randomly selecting two animals with exactly the same genotypes in all markers is practically zero. Moreover, the overall probability of parental exclusion (P_e) across all markers with 1 and 2 parents unconfirmed was 0.9798 and 0.9999, respectively, suggesting that the analyzed markers can be a useful tool for parentage verification in the population.

Table 2. Comparison of genetic parameters in previously reported populations of Skyros horses.

Population	N	He	Ho	Fis	TNA	PIC
Present Study (whole reared examined population)	25	0.57	0.63	-0.06	3.72	0.53
Avdi and Banos (2008) (experimental population)	77	0.66	0.66	-0.09	4.11	0.58
Bomche et al. (2010) (joint sample analysis from different populations)	99	0.64	0.65	-0.005	5.93	0.59

*N=population number; He: Expected Heterozygosity; Ho Observed Heterozygosity; Fis: Heterozygosity deficit; TNA=average number of alleles; PIC: polymorphism information content.

A few studies using STRs data have previously been reported in regard with the genetic structure of Skyros horse's population (*Criscione et al., 2015; Bomke et al., 2010*). In all reported populations a high heterozygosity excesses (Ho) was observed (Table 2) reaching similar levels to our results. In addition, an increase heterozygote deficit (F_{is}) was noted for the population analysed by *Avdi and Banos (2008)* compared to the population analysed herein. Although the population analysed by *Bomke et al. (2010)* appeared to have lower inbreeding index (F_{is}), it should be noted that this outcome results from a joint analysis of samples random chosen from three different subpopulations.

Conclusions

The genetic structure of a whole Skyros horse population reared in an insular region of North-Western Greece was analysed presenting recent levels of genetic diversity. Our results showed that the population maintained considerably levels of genetic diversity despite its small census breeding environment compared to previous experimental population. A heterozygote excess from that expected as a result, probably, of outbreeding strategies were noted in the analysed population. All analysed genetic variability measures are comparable to results from other breeds. Taking into account that Skyros horse belongs to the endangered breeds, well studied inbreeding population strategies (i.e. avoid mating between relatives, equal participation of all parents to the next generation) should be implemented, in order to reduce the loss of genetic variability, to avoid extinction and further genetic drift of the whole population. Correlation between important traits (i.e. reproduction performance) and genetic diversity of the studied populations would be a further step aiming to assist the chances of breed's survival. The

simultaneously determination of the effective size of the whole population of Skyros horse, as a mean of genetic stabilization it would be undoubtedly a future challenge.

Procena genetičkog diverziteta autohtone populacije konja u Grčkoj

George P. Laliotis, Meni Avdi

Rezime

Visoko ugrožene lokalne rasa se smatraju važnim ne samo za održavanje njihove genetske raznolikosti za budući opstanak, već i zbog toga što se smatraju delom kulturne baštine lokalnih i nacionalnih zajednica. Koristeći podatke iz pedigre i analizu položaja 18 mikrosatelita, ispitivali smo genetsku raznolikost komercijalne autohtone populacije konja - Skyros, odgajane u ostrvskom regionu severozapadne Grčke. Ukupna prosečna inbriding vrednost dostigla je 24%. Srednji broj alela po lokusu iznosio je 3,72, u rasponu između 1 i 7 alela. Prosečna utvrđena heterozigotnost je bila 0,57. Uzimajući u obzir inbreeding indeks, prosečni heterozigotni deficit (F F_{is}) od -0,09 je utvrđen ($P<0,05$). Iako je populacija održala razumni nivoe genetske raznovrsnosti, dobro proučena inbriding strategija bi trebalo da se sprovodi, kako bi se smanjio gubitak genetske varijabilnosti, i izbegao nestanak i dalje genetski drift populacije.

Ključne reči: Skyros konji, inbriding, konzervacija, STR, genetski markeri

Acknowledgements

The authors would like to thank Mrs Steen Sylvia, owner of the commercial farm, for her valuable cooperation. We would also thank Jean-Claude Meriaux and Jean-Michel Allamellou (Labogena, France) for their support in animal genotyping. Moreover, Professor George Banos is acknowledged for his useful comments on the manuscript and for the support on the statistical analysis. The Greek Ministry of Environment and the Greek Equestrian Club “Filippos Enois” partially funded the present study.

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Received 29 December 2016; accepted for publication 18 February 2017

Biotechnology in Animal Husbandry 33 (1), p 91-102 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 637.04'639.211
DOI: 10.2298/BAH1701091B

QUALITATIVE PROPERTIES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM, 1792) FROM AQUACULTURE FACILITY IN BITOLA REGION (MACEDONIA)

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Original scientific paper

Abstract: The main goal of this research was to determine the qualitative properties of the rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) from aquaculture facility – salmonid fishpond Strezhevo which is situated in Bitola region (Republic of Macedonia). The qualitative properties of the rainbow trout are established by determination of the chemical and the fatty acid composition of the fish meat, the energy value of the meat and the microbiological analysis for the total number of microorganisms on fish skin and presence of *Salmonella* sp. and *Listeria monocytogenes*. The main purpose of the research produced additional analyzes that determine the physical - chemical properties and also a microbiological analysis of the water in which the rainbow trout resides, the chemical composition of feed used for feeding of the rainbow trout, the condition factor (CF) and the feed conversion. The results obtained during the examination of the chemical composition of the rainbow trout meat from the fishpond Strezhevo determined the mean value of 74.533% water, 20.600% protein, 3.366% fat and 1.38% ash. The energy value of the meat was 484.635 kJ/100 g. Considering the results of the fatty acid composition of the rainbow trout from the fishpond Strezhevo, it can be concluded that the content of the saturated fatty acids (SFA) is 20.303%, the monounsaturated fatty acids (MUFA) is 52.359% and the polyunsaturated fatty acids (PUFA) is 27.268%. In terms of the amount of n-6 fatty acids, it is 20.180%, while the amount of n-3 is 7.088%.

Keywords: rainbow trout, chemical composition, fatty acid composition

Introduction

Rainbow trout (*Onchorhynchys mykiss* Walbaum, 1792) is one of the most consumed fish species in Macedonian kitchen. It is grown in many aquaculture facilities – salmonid fishponds in our country, due to its fast growth and

exceptional nutritive quality. According *Simonović (2001)* this fish species is farmed intensively for consumption and it is tolerant to environmental conditions.

The main goal of this research was to determine the qualitative properties of the rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) from aquaculture facility – salmonid fishpond Strezhevo which is situated near city of Bitola (Republic of Macedonia).

The qualitative properties of the rainbow trout are established by determination of the chemical and the fatty acid composition of the fish meat, the energy value of the meat and the microbiological analysis for the total number of microorganisms on fish skin and presence of *Salmonella* sp. and *Listeria monocytogenes*. The main purpose of the research produced additional analyzes that determine the physical - chemical properties and also a microbiological analysis of the water in which the rainbow trout resides, the chemical composition of feed used for feeding of the rainbow trout, the condition factor (CF) and the feed conversion.

Material and Methods

Examinations were performed on rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) with consumption size of 250-300 g from aquaculture facility – salmonid fishpond Strezhevo. During these examinations, 18 samples from rainbow trout were analyzed. The average mass of rainbow trout samples was 267 g, while the average length, 27.2 cm.

The fishpond Strezhevo is located near the city of Bitola (Macedonia) and it is supplied with water from the accumulation Strezhevo. During these examinations, three samples of fishpond water were analyzed for each microbiological and physical-chemical property, in the same time when the fish catching was organized, accordingly. The results below (Table 1) represent average value from these three repetitions.

In this fishpond pelleted feed Troco prime 18 (4.5 mm) from manufacturer Coppens International (Netherlands) is used, with following content (per specification): fish meal, wheat, fish oil, soy, wheat gluten, hemoglobin powder and oil rape. Besides the main components, the feed contain the following components (per specification): phosphorus (0.96%), calcium (1.6%), sodium (0.3%), vitamin A (10.000 ie/kg), vitamin C (150 mg/kg), vitamin E (200 mg/kg), vitamin D_3 (799 ie/kg), antioxidants E 324 ethoxyquin (100 mg/kg) and E321 butilat hidroxitoluen (40 mg/kg), as well as trace elements E1 iron (75 mg/kg), E2 iodine (5 mg/kg), E4 copper (5 mg/kg), E5 manganese (20 mg/kg) and E6 zinc (80 mg/kg).

Based on absolute indicators of mass and body length of the fish, the condition factor (CF) is calculated according to formula CF = (BW / L3) x 100 (*Ricker, 1975*), where BW is fish body weight and L is total fish length.

The feed conversion ratio (FCR) is calculated according to the formula FCR = F/G, where F is consumed feed (g) and G is fish growth (kg).

During examinations, the following methods for determination of chemical and fatty acid composition in fish meat were used:

- Determination of moisture content - ISO 712:2009;
- Spectrophotometric determination of total nitrogen according Kjeldahl - HACH DR 400 procedure method 2410;
- Determination of total fat by gravimetric method (Soxhlet extraction) - AOAC method 2003.6
- Determination of ash in an oven at 700°C - ISO 3593:1981;
- Determination of fatty acid composition by gas chromatography - AOAC method 996.06.

Methods for physical – chemical properties of water in fishpond that were used:

- pH determination - ISO 1052:1994;
- Chloride determination - ISO 9297:1989;
- Spectrophotometric determination of nitrates - HACH DR 400 procedure Method 8039;
- Spectrophotometric determination of iron - HACH DR 400 procedure Method 8365;
- Spectrophotometric determination of nitrites - HACH DR 400 procedure Method 8507;
- Turbidity determination of translucency - ISO 7027:1999;
- Spectrophotometric determination of ammonia - HACH DR 400 procedure Method 8038;
- Determination of chemical oxygen demand - Merck Method Spectroquant 1.18752.0001;
- Total nitrogen determination - Merck Method Spectroquant 1.14537.0001.

Methods for microbiological analyses of fish meat that were used:

- Horizontal method for detection and enumeration of *Listeria monocytogenes* - ISO 11290 - 1:2008;
- Horizontal method for detection and enumeration of *Salmonella* sp. - ISO 6579 - 2008;
- Horizontal method for the enumeration of microorganisms - ISO 4833:2003.

Methods for microbiological analyses of water that were used:

- Detection and enumeration of coliform bacteria and *Escherichia coli* - ISO 9308 -1:2000;

- Detection and enumeration of intestinal enterococci and *Streptococcus faecalis* - ISO 7899 - 2:2000

Energy value (EV) of rainbow trout meat was calculated according to formula: E.V. (κJ/100g) = proteins (%) x 17.16 + fats (%) x 38.96 *(Vitčenko et al., 1981).*

For data processing, standard statistical methods (Microsoft Office Excel 2010, Data Analysis ToolPak) were used.

Results and Discussion

Considering the results of the physical – chemical and microbiological analysis of water in fishpond Strezhevo, we`ve obtained the following results:

Table 1. Physical – chemical analysis of water in fishpond Strezhevo (Bitola, Macedonia)

Parameters	Amount
Represent of oxygen - saturation	78 %
5-day biochemical consumption of O_2 at 20 °C	1.30 mg/l
Chemical oxygen demand	3.00 mg/l
Dry residue of filtered water	39.0 mg/l
pH	7.15
Visible waste	No
Visible color	No
Noticeable odor	No
Fe	0.030 mg/l
Nitrites	0.0960 mg/l
Nitrates	0.00 mg/l
Ammonia	0.160 mg/l
Turbidity	1.0 NTU
Chlorides	6.80 mg/l
Total phosphorous	0.0070 mg/l
Total nitrogen	0.300 mg/l

Table 2. Microbiological analysis of water in fishpond Strezhevo (Bitola, Macedonia)

Parameters	Amount
The probable number of thermo-tolerant coliform bacteria in 100 ml	30
Streptococcus of faecal origin in 100 ml	0

Based on the physical - chemical and microbiological analysis of water from fishpond Strezhevo (Bitola, Macedonia), water is classified into class II (according to the Regulation on water classification Official Journal of RM 18/99), which is allowed for fish production.

Considering the results of the chemical composition of pelleted feed used in this fishpond, we`ve obtained the following results:

Table 3. Chemical composition of feed (Coppens International, Netherland) used in fishpond Strezhevo (Bitola, Macedonia)

Components	Amount (%)
Proteins	42
Fats	18
Carbohydrates	16
Ash	6.2

In this fishpond pelleted feed Troco prime 18 (4.5 mm) from the manufacturer Coppens International (Netherland) is used. By analyzing the chemical composition of the feed we received the following results: 42% proteins, 18% fats, 16% carbohydrates and 6.2% ash.

In practice, in conditions of intensive farmed fish production, it is found that the application of high quality feed is one of the most important factors affecting the fish growth, the feed conversion ratio and chemical composition of the fish meat. Today, complete forage mixtures in pelleted form are used for rainbow trout feeding.

In our examinations for the condition factor (CF) of rainbow trout, we established a value of 1.3267, which is in close correlation with the findings of *Çagiltay et al. (2015)* which in their research obtained the values of 1.24, 1.29 and 1.22, respectively. *Kiessling et al. (1991)* emphasize that under the influence of stable growing conditions, the fish growth is directly related to the feed utilization and fish age. Changes in the feed amount have impact on this ratio, so the increased amount of feed leads to greater deposition of fat and vice versa, reduced feed intake can requirements increased fat content in the fish tissues, compared to fish with the same size due to poor feed conversion ratio.

In our examinations we established a value of 1.18 for feed conversion ratio. From an economic point of view, it is very important to determine the most way of diet (continuous, intensive at the beginning or intensive feeding during the late phase of growth) that primarily reflects the chemical and fatty acid composition of fish meat.

Considering the results of the chemical composition of rainbow trout meat from fishpond Strezhevo, we`ve obtained the following results:

Table 4. Chemical composition of rainbow trout meat from fishpond Strezhevo (Bitola, Macedonia)

Chemical parameters	$\bar{X}$ ± SD	min	max
Water	74.533 ± 0.573	73.80	75.20
Proteins	20.600 ± 0.571	20.10	21.40
Fats	3.366 ± 0.880	2.60	4.60
Ash	1.380 ± 0.120	1.22	1.51

Legend: $\bar{X}$ - mean value; SD – standard deviation; min – minimum value; max – maximum value

The nutritional value of fish meat is determined by the amount of proteins, fats, minerals and vitamins, and it depends on the fish species, age, cultivation methods, the composition of the consumed feed, as well as the season.

The results obtained during the examination of the chemical composition of rainbow trout meat from the fishpond Strezhevo show the mean value of 74.533% water, 20.600% protein, 3.366% fat and 1.38% ash. The energy value of the meat was 484.635 kJ/100 g.

Variations in the chemical composition of fish meat are closely related to the proportion feed intake, so the percentage of proteins in muscle tissue increases slightly during the feed period, while the percentage of fat increases faster.

The protein content in salmonid fish is related to the fish size and it is controlled endogenous, while the fat content depends on many endogenous and exogenous factors. Protein content is stable during the growth period except in insufficient and unbalanced diet.

When the fat content in feed can cross most that fish can metabolize, fat will be deposited in muscle tissue. By increasing the fish weight and age, the metabolism is focused on increasing the percentage of dry matters, or water content reduction and fat accumulation in the fish muscle. Also, we conclude that the fat content in the rainbow trout meat is indirectly proportional to the water content.

According to the fat content, fish are classified in: lean fish (fat content of less than 5%), moderate fatty fish (5-10%) and fatty fish (more than 10% fat) *(Jabeen & Chaudhry, 2011).* According to the received amount of fat, rainbow trout from fishpond Strezhevo is being classified as lean fish (3.366% fat).

Bud et al. (2008) noticed that the total fat content in rainbow trout can range from 2.7 to 9%, depending on age, physiological condition, time of catch, individual differences, etc. The main factor of which the fat content in fish depends, is the content of fat in feed. According *Shimeno and Shikata (1993)*, the diet, dietary feed supplements, the amount of feed consumed as well as growth increase, generally influenced the increase of the fat content in fish meat. Other factors such as temperature, mobility and the addition of steroids, indirectly stimulate the diet and increase the fat content *(Viola et al., 1992).*

Considering the results of the fatty acid composition of the rainbow trout meat from fishpond Strezhevo, we`ve obtained the following results:

Table 5. The content of SFA (saturated fatty acid) in rainbow trout meat from fishpond Strezhevo (Bitola, Macedonia)

Lipid numbers	Name	Amount (%)
C12:0	Lauric acid	0.098
C14:0	Myristic acid	2.007
C15:0	Pentadecanoic acid	0.102
C16:0	Palmitic acid	14.335
C17:0	Heptadecanoic acid	0.118
C18:0	Stearic acid	3.389
C20:0	Arachidic acid	0.131
C21:0	Heneicosanoic acid	0.123
TOTAL SFA		**20.303**

Considering the results of the SFA (saturated fatty acids) composition of the rainbow trout from the fishpond Strezhevo, it can be concluded that from total fatty acid content, SFA participate with 20.303%. From those, the most dominant are palmitic (14.335%), stearic (3.389%) and myristic (2.007%) fatty acid.

Table 6. The content of MUFA (monounsaturated fatty acid) in rainbow trout meat from fishpond Strezhevo (Bitola, Macedonia)

Lipid numbers	Name	Amount (%)
C14:1	Myristoleic acid	0.027
C16:1	Palmitoleic acid	5.040
C17:1	Cis-10- Heptadecanoic acid	0.275
C18:1 n9 c	Oleic acid	43.317
C20:1	Cis-11- Eicosenoic acid	2.657
C22:1 n9 c	Cis - Erucid acid	0.694
C24:1	Nervonic acis	0.349
TOTAL MUFA		**52.359**

Monounsaturated fatty acids (MUFA) have the greatest participation in the fish meat from this fishpond, with 52.359%. From those, the most dominant is oleic (43.317%) and palmitoleic (5.040%) fatty acid.

Polyunsaturated fatty acids (PUFA) participate with 27.268% in total fatty acid content. From this type of acids, linoleic participate with the greatest percent (15.962%), followed by γ- linolenic (3.520%), docosahexaenoic DHA (4.536%) and eicosapentaenoic EPA (1.655%) fatty acid.

Table 7. The content of PUFA (polyunsaturated fatty acid) in rainbow trout meat from fishpond Strezhevo (Bitola, Macedonia)

Lipid numbers	Name	Amount (%)
C18:2 n6 t	Linoleic acid	0.100
C18:2 n6 c	Linoleic acid	15.962
C18:3 n6	γ- linolenic acid	3.520
C20:2 n6	Eicosadienoic acid	0.312
C20:3 n6	Eicosatrienoic acid	0.286
C18:3 n3	α- linolenic acid	0.596
C20:3 n3	Eicosatrienoic acid	0.301
C20:5 n3	Eicosapentaenoic acid	1.655
C22:6 n3	Docosahexaenoic acid	4.536
TOTAL PUFA		**27.268**

The fatty acid composition in different, but also within the same fish species shows some variations *(Valente et al., 2007; Robin and Skalli, 2007)*, and a number of factors such as temperature, water quality, type and availability of feed, season, age, gender, reproductive status, geographical location and individual differences are considered as significant factors that contribute to the occurrence of these variations.

Table 8. A review of fatty acid content in rainbow trout meat from fishpond Strezhevo (Bitola, Macedonia)

Total SFA	20.303
Total MUFA	52.359
Total PUFA	27.268
Total UFA	79.627
Total PUFA n-6	20.180
Total PUFA n-3	7.088
n-3/n-6	0.351
n-6/n-3	2.84
UFA/SFA	3.921
PUFA/SFA	1.343
PUFA/MUFA	0.520

N-6 fatty acids participate with 20.180 %, while n-3 with 7.088 %, so the n-3/n-6 ratio is 0.351. UFA/SFA ratio is 3.921, PUFA/SFA is 1.343, while PUFA/MUFA is 0.520.

The fat quality in fish is defined by the relationship between n-6/n-3 and PUFA/SFA ratio *(Ahlgren et al., 1996).*

According *HMSO (1994)* ideal ratio of n-6/n-3 fatty acids is up to 4. Values greater than the maximum value are harmful to health and can lead to cardiovascular diseases *(Moreira et al., 2001).* In our examinations, the ratio n-6/n-3 is 2.84, which is in correlation with provided recommendations.

According *HMSO (1994)*, the recommended minimum amount of PUFA/SFA ratio is 0.45 which is in correlation to our findings with value of 1.34.

The type and amount of fatty acids in fish muscle tissue is directly related to their diet, but also to other factors such as the fish size and age, reproductive status, season, geographical location, etc. which can influence the fatty acid profile of fish meat.

Considering the results of the microbiological analysis for the total number of microorganisms on fish skin and presence of *Salmonella* sp. and *Listeria monocytogenes*, we`ve obtained the following results:

Table 9. Microbiological analysis of rainbow trout from fishpond Strezhevo (Bitola, Macedonia)

Parameters	Total number of microorganisms (log cfu/cm^2)	*Salmonella* sp.	*Listeria monocytogenes*
$\bar{x}$	6.66	0	0

Legend: $\bar{x}$ - mean value

In terms of determining the total number of microorganisms on the skin of rainbow trout, in our examinations we obtained average value of 6.66 log cfu/cm^2, which is in correlation with the findings of *Adams and Moss (2008)* which concluded that the total number of microorganisms on the fish skin surface ranged from 2.00 - 7.00 log cfu/cm^2.

Conclusions

- The results obtained during the examination of the chemical composition of the rainbow trout meat from the fishpond Strezhevo (Bitola, Macedonia) show the mean value of 74.533% water, 20.600% protein, 3.366% fat and 1.38% ash.
- Energy value of rainbow trout meat from fishpond Strezhevo (Bitola, Macedonia) is determined as 484.635 кJ/100 g.
- Considering the results of the fatty acid composition of the rainbow trout meat from the fishpond Strezhevo (Bitola, Macedonia), it can be concluded that saturated fatty acids (SFA) participate with 20.303%, monounsaturated fatty acids (MUFA) with 52.359%, while the polyunsaturated fatty acids (PUFA) with 27.268% from total fatty acid content.
- N-6 fatty acids participate with 20.180%, while n-3 with 7.088%, so the n-3/n-6 ratio is 0.351, while the n-6/n-3 ratio is 2.84.
- UFA/SFA ratio is 3.921, PUFA/SFA is 1.343, while PUFA/MUFA is 0.520.

- The type and amount of fatty acids in fish muscle tissue is directly related to their diet, but also to other factors such as the fish size and age, reproductive status, season, geographical location, etc. which can influence the fatty acid profile of fish meat.
- In conditions of intensive farmed fish production, proper diet with adequate amount of high quality feed is the most important parameter that affects the fish growth, the feed conversion, as well as, chemical and fatty acid composition of fish meat.

Kvalitativne osobine kalifornijske pastrmke (*Oncorhynchus Mykiss* Walbaum, 1792) iz ribnjaka u regionu Bitola (Makedonija)

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Rezime

Glavni cilj ovog istraživanja bio je da se utvrde kvalitativne osobine pastrmke (*Oncorhynchus mykiss* Walbaum, 1792) iz ribnjaka za salmonidne vrste riba Strezhevo koji se nalazi u regionu Bitolja (Republika Makedonija).

Kvalitativna svojstva kalifornijske pastrmke se utvrđuje određivanjem hemijskog sastava i sastava masnih kiselina ribljeg mesa, energetske vrednosti mesa i mikrobiološkom analizom za ukupan broj mikroorganizama na koži ribe i prisustvo *Salmonella* sp. i *Listeria monocytogenes*. Glavni cilj istraživanja je uključivao i dodatne analize koje određuju fizičko - hemijske osobine, kao i mikrobiološku analizu vode u kojoj pastrmka boravi, hemijski sastav hrane koja se koristi za ishranu kalifornijske pastrmke, faktor stanja/kondicije (CF) i konverziju hrane.

Rezultati dobijeni tokom ispitivanja hemijskog sastava mesa kalifornijske pastrmke iz ribnjaka Strezhevo pokazuju srednju vrednost 74,533% sadržaja vode, 20,600% proteina, 3,366% masti i 1,38% pepela. Energetska vrednost mesa je 484,635 kJ/100 gr.

S obzirom na rezultate sastava masnih kiselina pastrmke iz ribnjaka Strezhevo, može se zaključiti da je sadržaj zasićenih masnih kiselina (SFA) bio 20,303%, mononezasićenih masnih kiselina (MUFA) 52,359% i polinezasićenih masnih kiselina (PUFA) 27.268%. U pogledu količine n-6 masnih kiselina, dobijena je vrednost od 20.180%, dok je količina n-3 bila 7.088%.

Ključne reči: kalifornijska pastrmka, hemijski sastav, sastav masnih kiselina

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Received 20 September 2016; accepted for publication 25 December 2016

Biotechnology in Animal Husbandry 33 (1), p 103-113 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 639.211
DOI: 10.2298/BAH1701103M

PROFITABILITY OF CARP PRODUCTION IN MACEDONIA AND SERBIA

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Original scientific paper

Abstract: Macedonia and Serbia are countries with long tradition in freshwater carp production. In this study, the aim is to assess the carp fish production economics, with particular focus on profitability. The findings revealed that carp production is profitable in both cases, though with better returns in the Macedonian case with the rate of profitability being 17.18%, in comparison to 8.10% at the Serbian farm. The full cost of production per kg is €2.56 and €2.25 in Macedonia and Serbia, respectively. The current profitability levels are highly sensitive to market price fluctuations, and there is considerable room for yield improvement and costs reductions.

Key words: carp production, profitability, Macedonia, Serbia

Introduction

Carp culture is the most widely practiced fish production system in Central and Eastern Europe *(Woynarovich et al., 2010)*. In Macedonia and Serbia, fish culture is mainly practiced in cold waters on trout farms and in warm waters on carp farms, with rainbow trout and common carp being the dominant species (*Spirkovski, 2007; Marković and Poleksić, 2008)*.

The consumption of fish in Macedonia and Serbia is very low, *i.e.* only 4.6 and 5 kg per capita on annual basis, respectively *(Milijašević et al., 2012; SSORM, 2016)*. This situation can be attributed to eating habits, low purchasing power of the population, relatively high price and limited and inadequate offer on the market *(Kostov, 2014; Kokot et al., 2015)*.

The development of any type of economic activity, including fish culture, needs to be supported with relevant economic analysis. Our aim is to investigate the profitability of carp production on Macedonian and Serbian carp enterprises. Although there is a long tradition of warm-water fish ponds culture in both

countries, as well as a large number of proven experts in practice, there is not enough research on the economics of fisheries in Serbia *(Čanak, 2012)* and this situation is even more pronounced in Macedonia, where no specific fish economics research has been conducted so far. This paper aims to fill part of this gap and to contribute to the freshwater fisheries economics literature in these two countries.

Material and Methods

The general trends in fish culture with focus on carp production are based on available data from Macedonian and Serbian state statistical offices *(SSORM, 2016; SSORS, 2016)*, FAO database *(FAO, 2016)*, as well as some national reports *(Kostov, 2014; MAFWE, 2014; Čanak, 2015)*.

This research additionally uses primary data collected from two case farms, in Macedonia (cage system, with 30 cages totalling 750 m^2 or 3,750 m^3) and in Serbia (pond system, with 215 ha of production area). A basis for calculation of the carp production enterprise performance is the analytical enterprise budget. The costs are allocated based on the relationship with the specific production line (enterprise) and therefore are further classified into direct costs and indirect costs.

In order to compare the carp enterprise performance between the two case farms, our analysis focuses on per unit derived indices, coefficients and ratios *(Milanov and Martinovska Stojcheska, 2002; Marković et al., 2014)*: gross and net profitability rate (as share of gross and net profit in the total income); income-to-cost ratio (total value of production as potential income in direct *i.e.* total costs); cost of production per kg of output (direct and total costs per quantity produced). The feed conversion ratio is also an important indicator of the efficiency of feeding referring to the quantity of feed necessary to produce one kilogram of fish.

Lastly, a sensitivity analysis is performed examining the change in profitability and costs of production on hypothetical shifts in yield, market price and costs. Potential increases or decreases on yield are set at a range from 1,000 to 2,500 kg per cage or hectare in the Macedonian and Serbian case, respectively. The market price and costs sensitivities are tested with 10 to 20 percent variation.

Results and Discussion

Fish and carp systems and production in Macedonia and Serbia

Different production systems are used worldwide for carp production *(Rahman et al., 1992)*: (i) extensive production (natural feed sources, low cost, low output); (ii) semi-intensive production (manure based, supplementary feeding is limited, moderate production cost and output); (iii) intensive production (pellet feed, high stocking density, high cost and high output).

Most of the carp production in both Serbia and Macedonia is characterized as semi-intensive. More concretely, in Macedonia carp production is carried out in classical and cage systems *(Kostov, 2015)*. The classical warm-water system is present in larger water areas, and functions without major costs and relatively low yields (900 to 1,500 kg ha^{-1}). The most intensive type of carp production in the country is in cages; the cages are set in some of the larger artificial lakes – accumulations and most of these are located in Lake Tikveš.

In Serbia, two main intensity levels of carp production are identified *(Čanak et al., 2015)*: (i) lower level, or classical semi-intensive production, where supplementary cereals, fertilization and liming are used; and (ii) higher level, or partly intensive production that was introduced since 2004, where instead of cereals, concentrated or pelleted feed is the main source of nutrition.

During the period 2005-2014, both Macedonian and Serbian fish productions show an increasing trend. The average fish production in Macedonia is 1,338 tons, out of which 228 tons of carp, since trout is the dominant *(SSORM, 2016)*. During the last decade, the average fish production in Serbia is 6,483 tonnes of fish, out of which 5,077 tons of carp *(SSORS, 2016)*.

Carp production in Serbia is more stable compared with the Macedonian carp production, with an average yield of 0.75 t ha^{-1}. The negative change rate per unit utilized area (-1.82%) indicates that there is still need for improvement of the production technologies in Serbia. The average annual carp production in Macedonia is 0.23 t ha^{-1}.

The area under carp ponds has significantly increased in Serbia during the last decade. The carp production was organized on a total area of 8,724 ha in 2014, which is almost doubled compared to the utilized area for carp production in 2005 (4,374 ha), the 2005-2014 average being 8,079 ha *(SSORS, 2016)*. On the other side, during the same period, the total area for fish production in Macedonia in the official statistics remains unchanged *i.e.* registered as being on1,000 ha, including ponds, reed beds and fish ponds for all fish species *(SSORM, 2016)*.

Both countries are importing fish to satisfy the domestic demand with 4,185 tons in Macedonia (SSORM, 2016) and 1,020 tons in Serbia *(SSORS, 2016)*. Export levels are comparatively low.

Comparative profitability analysis of carp production

In intensive culture systems such as cages, carp is usually bred as monoculture or dominant species. The analytical budget of carp enterprise in Macedonia is based on a farm that practices cage monoculture (Table 1). The budget is calculated on one cage basis (surface of 5m x 5m i.e. 25 m^2, with a depth of 5 m) and per hectare. The production value, given the yield of 1,500 kg per cage and the market price of €3.09 kg^{-1}, is estimated at €4,635 ha^{-1}.

The costs per cage amount to €3,838, confirming the high intensity of this production. The cage carp is fed exclusively by pelleted or extruded feed, which

represents the highest cost item and takes up almost two-thirds of the total costs structure. The farmer uses 2 kg of feed to produce 1 kg of carp, which leaves room for improvement. The producer breeds own fry and that is reflected in the low cost attributed to that segment (only €309 per cage, or 8% of the costs), which in other conditions could be rather significant. In terms of other costs, labour is in its minimal range, with only 10% share in the total cost. Other direct costs, such as veterinary services, are minor. Indirect costs are mainly derived from the depreciation of fixed assets (cages and nets). The producer pays concession fee in order to use the accumulation. The calculation of the interest on working capital is based on the assumption, in both Macedonian and Serbian carp budget, that one fourth of the variable investment is financed from borrowed sources of financing.

The cage production is profitable, leaving a €796 net result per cage. In addition to that, fish producers are entitled to use subsidy support, which in 2015 amounted to €0.16 kg^{-1} *(OG, 2013)*.

Table 1. Analytical budget calculation of carp production, Macedonia (case capacity 25 m^2)

1. Production value	**Quantity**	**Price (€)**	**Total (€)**	**Total (€ ha^{-1})**	**Share (%)**
Carp fish (kg)	1,500	3.09	4,635	1,853,921	100
Production value			**4,635**	**1,853,921**	**100**
2. Direct costs	**Quantity**	**Price (€)**	**Total (€)**	**Total (€ ha^{-1})**	**Share (%)**
Material costs					
Fish fry (fingerlings, kg)	58	5.37	309	123,432	8
Pelletted feed (kg)	3,000	0.81	2,439	975,748	64
Packaging (30 kg bags)	50	0.08	4	1,626	0
Total material costs			**2,752**	**1.100.806**	**72**
Labour (ratio per kg)	1,500	0.24	366	146,362	10
Transport (ratio per kg)	1,500	0.16	244	97,575	6
Veterinary services (visits)	3	10.84	33	13,010	1
Total direct costs			**3,394**	**1,357,753**	**88**
Contribution margin (1-2)			**1,240**	**496,168**	
Cost of production at direct costs (€/kg)				**2.26**	
3. Indirect costs			**Total (€)**	**Total (€ ha^{-1})**	**Share (%)**
Concession for accumulation			4.88	1,951	0
Fixed assets depreciation			362.42	144,968	9
Interest on working capital			34.40	13,760	1
Other costs			42.28	16,913	1
Total indirect costs			**444**	**177,593**	**12**
Total costs (2+3)			**3,838**	**1,535,346**	100
Profit (1-2-3)			**796**	**318,575**	
Cost of production at total costs (€/kg)				**2.56**	

For poly-culture in ponds, carp can be the major or a secondary species. In the Serbian case farm (Table 2), common carp is the major species with dominant share (94%). The total production value in the analysed pond is €4,767 ha^{-1}. Yields

in the fisheries sector in Serbia are modest in comparison with the yields which are realized in the world *(Marković et al., 2014)*, hence the low profitability.

The total costs per unit area are very high (€4,381ha^{-1}). Such high costs, place aquaculture in rank of highly intensive productions. The direct costs of this complex production include: the material costs (yearlings and fry, pellets, hydrant lime, fuel and lubricants, other materials), labour costs and direct services. Within the direct costs, pelleted feed has the largest share (€1,662 ha^{-1}, or 38%). Spawn represents a significant cost, which coupled with the feed costs takes up 73% of total costs. The remaining material costs have no significant share (less than 4%). Labour costs amount to €501 ha^{-1}, or 11% of total costs. In intensive production systems, labour costs could be lower, so their reduction significantly affects the level of the production economy. Direct services include pond maintenance and do not represent a significant element of the costs. The general or indirect costs are covered by the corresponding part of the depreciation of buildings and equipment, various overhead expenses, and interest on current assets.

Looking at the absolute performance indicators (contribution margin and profit), the achieved results are relatively modest. The realized contribution margin (€892 ha^{-1}) and profit (€386 ha^{-1}) cannot be considered as satisfactory for production with such intensity, which is characterized by high investments per unit of capacity.

The carp production analysis is done using performance indices (Table 3). Carp production is profitable in both cases, though with better returns in the Macedonian case (26.76% contribution margin and 17.18% rate of profitability), in comparison to the Serbian farm (18.71% and 8.10%, respectively). This is also reflected in the income-to-cost ratio, whereas there is €1.21 of return on €1 of related total costs in the Macedonian case, *i.e.* in the Serbian case only €1.09 of production value is achieved on €1 of the total costs.

Nevertheless, the cost of carp production calculated per unit of output is more elevated in Macedonia; the cost of production per kg calculated at direct costs is €2.26 and €1.99, and the full cost of production per kg €2.56 and €2.25, in Macedonia and Serbia, respectively. This indicates that the higher profitability previously discussed on the Macedonian farm is linked to the higher production value, as function of the achieved yield and market price of the product.

Table 2. Analytical budget calculation of carp production, Serbia (case capacity 215 ha)

1. Production value	**Quantity**	**Price (€)**	**Total (€)**	**Total (€ ha^{-1})**	**Share (%)**
Carp 1 (kg)	22,498	2.73	61,420	286	6
Carp 2 (kg)	74,594	2.73	203,642	947	20
Carp 3 (kg)	296,260	2.38	705,099	3,280	69
Grass carp (kg)	8,351	1.88	15,700	73	2
Silver carp (kg)	11,503	1.53	17,600	82	2
Catfish (kg)	6,312	3.41	21,524	100	2
Production value			**1,024,983**	**4,767**	**100**
2. Direct costs	**Quantity**	**Price (€)**	**Total (€)**	**Total (€ ha^{-1})**	**Share (%)**
Material costs					
Carp yearlings (units)	12,794	2.73	34,928	162	4
Two-year carp fry (units)	99,112	2.73	270,576	1,258	29
Two-year grass carp fry (units)	2,910	2.05	5,966	28	1
Two-year silver carp fry (units)	4,172	1.64	6,842	32	1
Two-year catfish fry (units)	2,690	4.09	11,002	51	1
Pelleted food 25/7 (kg)	533,562	0.47	250,774	1,166	27
Pelleted food 30/7 (kg)	208,841	0.51	106,509	495	11
Hydrant lime (kg)	131,729	0.07	9,221	43	1
Fuel and lubricants (total)			21,398	100	2
Other materials (total)			6,710	31	1
Total material costs			**723,925**	**3,367**	**77**
Labour (total)			107,615	501	11
Other direct costs			1,630	8	0
Total direct costs			**833,170**	**3,875**	**88**
Contribution margin (1-2)			**191,813**	**892**	
Cost of production at direct costs (€/kg)				**1.99**	
3. Indirect costs			**Total (€)**	**Total (€ ha^{-1})**	**Share (%)**
Fixed assets depreciation			101,226	471	11
Interest on working capital			7,601	35	1
Total indirect costs			**108,828**	**506**	**12**
Total costs (2+3)			**941,998**	**4,381**	100
Profit (1-2-3)			**82,986**	**386**	
Cost of production at total costs (€/kg)				**2.25**	

The overall structure of costs is very similar between the comparative budgets on the level of total direct and indirect costs shares. Namely, in both cases, total direct costs account for 88% and indirect costs for 12% in the total costs, which is an expected proportion. Looking more closely into the direct costs structure, major component is feed, the respective share in total costs being 64% in the Macedonian case and 38% in the Serbian case. The costs of carp production vary according to the culture practice and usually feed costs comprise the largest portion of production costs *(Weimin, 2004)*. The major feed in both cases are commercial feeds – pellets that ensure stable feeding patterns and higher intensity of production. Nutrition with pelleted complete feed allows higher yield in all the categories of analyzed cyprinid fish *(Ljubojević et al., 2012)*. However, many fish

farms both in Macedonia and Serbia, still use maize, wheat and barley as feed, which impacts the yield levels negatively, and therefore the quality of produced fish. Feed costs' share in the Serbian case is comparable with the literature whereas feed costs in carp production typically range from 30% to 50% in total costs *(Leopold, 1981)*. In the Macedonian case, this segment is higher, which results from the higher cost of feed, and also to the small contribution of other direct material costs such as fry.

The share of other direct costs, apart from labour, is higher in the Macedonian case. The labour share points to more effective use of this resource in the Macedonian case. This is related mainly to the fact that breeding and collecting fish in cage usually requires less labour input *(Weimin, 2004)*, but also may indicate more operative environment and management practices.

Table 3. Comparative carp production performance indices

	Macedonian case	***Serbian case***
Contribution margin rate (%)	26.76	18.71
Profitability rate (%)	17.18	8.10
Profit (€ per kg)	0.53	0.20
Income-to-cost ratio (at direct costs)	1.37	1.23
Income-to-cost ratio (at total costs)	1.21	1.09
Cost of production at direct costs (€ per kg)	2.26	1.99
Cost of production at total costs (€ per kg)	2.56	2.25
Feed conversion ratio	2.00	1.77

Feed intake and relative efficiency can be analysed through the feed conversion ratio. This ratio in carp production usually ranges from 1.5 to 2.5, depending on the type and quality of feed and feeding system *(Woynarovich et al., 2010)*. Similarly to other livestock productions, nutrition is of highest importance and in that respect increases in feed conversion efficiency contribute to improved farmer's profitability *(Petrovic et al., 2013)*. In our analysis, the Serbian case farm is more successful converting 1.77 kg feed to 1 kg of output, in line with the usual feed conversion ratio for such production in Serbia ranging from 1.4 to 1.8 *(Marković, 2010)*. In the Macedonian case, 2 kg of feed are needed in order to produce 1 kg of output.

Yield, market price and costs impact on profitability

The sensitivity analysis (Table 4) of hypothetical yield shifts takes into account the variable nature of most of the direct costs. Decreased outputs result into lower profitability or even potential loss in the Serbian case. We further calculated the threshold break-even yield levels at 536 kg in the Macedonian and 1,110 kg in the Serbian case, respectively, meaning that production volume beneath those levels would be unprofitable, *i.e.* the costs will out-weight the income.

Profitability levels are highly sensitive to market price reductions and total costs increases. The cost of production at full costs in this sense can be interpreted as a break-even threshold; hence, product sales below that price would result into negative financial result and unprofitability. It is interesting to note that if the market price for the Macedonian farmer is assumed to be at the Serbian market level, the costs would rise above the value of production and the farm will have negative result. In the case of the Serbian farm, even slightest 10% reduction in sales price would result in loss. The Serbian producer is more sensitive to cost changes; a 10% increase in cost, given the same production value, would already turn the net result under the break-even level; in the Macedonian case, a 20% increase in cost, would diminish the profits.

Table 4. Sensitivity analysis (Macedonia on 25 m^2 cage basis and Serbia on 1 ha basis)

	Macedonian case				***Serbian case***			
Yield level (kg)	**1,000**	**1,500***	**2,000**	**2,500**	**1,000**	**1,500**	**1,951***	**2,500**
Total costs (€)	2,706	3,838	4,970	6,103	2,494	3,486	4,381	5,470
Production value (€)	3,090	4,635	6,180	7,725	2,443	3,665	4,767	6,108
Profit (€)	384	796	1,209	1,622	-51	179	386	638
Profitability rate (%)	12.41	17.18	19.57	21.00	-2.08	4.88	8.10	10.44
Income-to-cost ratio	1.14	1.21	1.24	1.27	0.98	1.05	1.09	1.12
Cost of prod. (€ per kg)	2.71	2.56	2.49	2.44	2.49	2.32	2.25	2.19
Sales price (€/kg)	**2.47**	**2.78**	**3.09***	**3.40**	**1.95**	**2.20**	**2.44***	**2.69**
Total costs (€)	3,838	3,838	3,838	3,838	4,381	4,381	4,381	4,381
Production value (€)	3,708	4,171	4,635	5,098	3,814	4,291	4,767	5,244
Profit (€)	-131	333	796	1,260	-567	-91	386	863
Profitability rate (%)	-3.52	7.98	17.18	24.71	-14.88	-2.12	8.10	16.45
Income-to-cost ratio	0.97	1.09	1.21	1.33	0.87	0.98	1.09	1.20
Profit (€ per kg)	-0.09	0.22	0.53	0.84	-0.29	-0.05	0.20	0.44
Total costs (€)	**4,606**	**4,222**	**3,838***	**3,455**	**5,258**	**4,820**	**4,381***	**3,943**
Production value (€)	4,635	4,635	4,635	4,635	4,767	4,767	4,767	4,767
Profit (€)	29	413	796	1.180	-490	-52	386	824
Profitability rate (%)	0.62	8.90	17.18	25.47	-10.28	-1.09	8.10	17.29
Income-to-cost ratio	1.01	1.10	1.21	1.34	0.91	0.99	1.09	1.21
Cost of prod. (€ per kg)	3.07	2.81	2.56	2.30	2.69	2.47	2.25	2.02
Profit (€ per kg)	0.02	0.28	0.53	0.79	-0.25	-0.03	0.20	0.42

Note: *Actual levels.

Conclusions

Although the production systems illustrated though the country specific cases differ, the carp profitability comparative analysis provided new insights and grounds for further deeper investigation. The current performance of both Macedonian and Serbian farms reveals considerable room for interventions in yield

improvement and costs reductions, and accordingly increasing the profitability of carp production.

Overall, the fish production in both countries is still underdeveloped, considering that most of the production takes place at semi-intensive ponds, with outdated supporting infrastructure, while there are a small number of modern fish farms. The production is not cost-effective enough, relative to the volume of invested assets.

The applicability of economies of scale is evident through the sensitivity analysis. This concept applies primarily to the potential to decrease the average cost per unit; the increase of the volume of production, mainly attributable to the indirect/fixed costs segment, when allocated on per unit of output, triggers a decrease in the overall cost of production. Also, larger output volumes enable finding optimal operating levels, gains in productivity, and specialization in terms of more efficient use of the available production factors. Other reasons for enhancing economies of scale can be related to increased benefits for large-scale producers in discounts when procuring inputs *i.e.* getting lower input prices when purchasing higher quantities of input. Finally, producing larger volumes of output can boost the negotiating powers and market positioning of the producer.

Both Macedonia and Serbia have very favourable climatic and soil conditions for freshwater fish production, but the producers are faced with subordinate position compared to other branches of agriculture. As the consumption levels are low in both countries, there is realistic growth potential for changing consumer food patterns and increasing fish consumption on the domestic market. Increased production can additionally lead to intensified export in the case of Serbia (to the European Union and to Russia), and import substitution in the case of Macedonia. Nevertheless, in order to achieve that, it is necessary to emphasize the need for adequate fisheries' development strategy and stimulating support from the state *i.e.* policy for production, processing and marketing of fish.

Profitabilnost proizvodnje šarana u Makedoniji i Srbiji

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Rezime

Makedonija i Srbija su zemlje sa dugom tradicijom u proizvodnji slatkovodnog šarana. Cilj ove uporedne studije je procena ekonomskih obeležja proizvodnje šarana, sa posebnim naglaskom na profitabilnost. Rezultati su pokazali da je proizvodnja šarana profitabilna u oba slučaja, iako sa većom efikasnošću u slučaju Makedonije, gde je stopa profitabilnosti 17,18%, u poređenju sa 8,10%

koliko iznosi na ribnjaku u Srbiji. Ukupni troškovi proizvodnje po kg iznose 2,56 € u Makedoniji, odnosno 2,25 € u Srbiji. Trenutni nivoi profitabilnosti su veoma osetljivi na fluktuacije tržišnih cena, a postoji i značajan prostor za povećanje prinosa i smanjenje troškova.

Ključne reči: proizvodnja šarana, profitabilnost, Makedonija, Srbija

Acknowledgement

This study is partly supported by the project TR31011 titled "Effect of components quality in cyprinids feeding on meat quality, losses and economic efficiency" subsidized by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Received 19 September 2016; accepted for publication 20 January 2017

Biotechnology in Animal Husbandry 33 (1), p 115-125 , 2017
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 631.563'633.15
DOI: 10.2298/BAH1701115D

EFFECTS OF *LACTOBACILLUS PLANTARUM* INOCULANTS ON MAIZE SILAGE QUALITY

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Original scientific paper

Abstract: In the winter time in Serbia, maize silage is the main ruminant feed. Therefore, managing maize silage is an important contributor to maintain the silage quality for livestock feed. In the study were evaluated the chemical composition, energetic and fermentation characteristics in whole-crop maize silage inoculated with different bacterial inoculants under field conditions in the commercial dairy farm, during the 2015. Three treatments were tested: negative control (untreated silage), a positive control (competitor inoculant) and Silko treatment (contains a mixture of 4 strains of *Lactobacillus plantarum* (LP1, LP2, LP3 and LP4). Maize is ensiled in the milk-wax grain maturity. After 90 days of ensiling, the maize silages were analyzed. The application of bacterial inoculants improved the chemical composition and energetic characteristics of silage. The inoculant Silko was more effective at improving the fermentation characteristics than competitor inoculant. Ash, cellulose, soluble N/TN, NH_3-N/TN, ADF, NDF, acetic acid and pH were significantly lower in Silko treatment than positive control. There were no differences in crude fat, crude protein, ME, NEL, lactic acid and butyric acid between the treated silages. Generally, the new product bacterial inoculant Silko proved in field trials its ability to support the ensiling process in maize. The main action of the bacterial inoculant Silko is performed in two ways: the reduced degradation of protein in silage and the improvement of the aerobic stability due to the lower pH, higher content of acetic acid than negative control.

Key words: chemical composition, energetic characteristics, fermentation parameters, *Lactobacillus plantarum*, maize silage

Introduction

The maize is a standard component of livestock diets. It can be harvested for grain and used in feed mixes for livestock, or entire plants can be harvested, chopped, and fermented for silage. Silage is made in order to feed animals in periods when feed supply is inadequate, either in terms of quantity or quality. The method of making maize silage is the simplest and acceptable to all of our farms. In Serbia, maize silage is one of the most important livestock feed especially in the winter. The starch, energy and intake characteristics of maize silage, together with its high dry matter yield potential, make it a good feed for beef cattle and sheep. *Mandić et al. (2013)* stated that the maize is very convenient crop for forage production because it has high production of green mass, energy content of dry matter and quality of biomass. Especially, ensiling of maize should be practiced when the plants of maize are damaged by frost, rain or drought, when reduction of grain yields is expected. However, maize silage requires high yield and harvest management, and good ensiling practices (rapid filling, thorough packing, perfect sealing and compression). The maize silage can be fermented under anaerobic conditions due to the native bacteria on plants; however, microbial additives reduce aerobic spoilage and help to maintain quality. The addition of bacterial and bacterial-enzyme inoculants is necessary in the initial stages of fermentation in order to achieve a rapid reduction of the pH, to avoid the occurrence and growth of harmful microorganisms, to avoid losses of dry matter and increase aerobic stability of silage *(Jatkauskas et al. 2013)*. Many researches showed that the adding microbial additives, improves the aerobic stability of maize silage and increases level of acetic acid *(Tabacco et al., 2009; Nkosi et al. 2011; Basso et al. 2013)*. In aerobic conditions yeasts and molds are developing, resulting in utilization of soluble carbohydrates and reducing nutritional value of silage, especially in warm weather so use of bacterial inoculants is necessary *(Ashbell et al., 2002)*. *Đorđević et al. (2011)* reported that bacterial-enzyme additives reduce fiber content and increase the concentration of sugar and lactic acid and digestibility of silage. *Bijelić et al. (2015)* concluded that bacterial-inoculants reduced crude protein content, NH_3-N/total nitrogen, acetic acid and pH value and increased the proportion of lactic acid relative to the acetic acid. *Weinberg et al. (2003)* reported that bacterial inoculants had effect on animal performance by increasing the nutritional value of the silage, and that some strains of lactic acid bacteria can survive in the gastric juice and have the role of buffer thus maintaining the activity of a cellulase enzyme and thereby increasing the digestibility. Also, *Acosta Aragón et al. (2012)* concluded that bacterial inoculants had positive effect on whole-crop maize silage quality, intake, feed energy utilization and performance of beef cattle. In the current market there are various bacterial inoculants containing different species and strains of bacteria. In most

researches, their inclusion has provided positive effects on chemical or microbiological composition of the silage *(Wilkinson et al., 2003)* or on animal performance *(Contreras-Gouveia et al., 2010).* However, many researches showed that addition of homofermentative lactic acid bacteria (LAB) inoculants did not affect the fermentation parameter of maize silages *(Sucu and Filya, 2006; Sadeghi et al., 2012)*. So, observing inconsistent results about the impact of inoculants on the quality of silage, constant developing of new microbial inoculants from native LAB in world is not surprising.

This study was conducted to evaluate the effects of bacterial inoculants on ensiling characteristics and nutritive value of whole-crop maize silage. Also, this study is intended to evaluate effects of new product Silko (produced in Serbia) compared to competitor product (positive control) available at the market.

Materials and Methods

The maize hybrid ZP 684 was harvested in August at the milk-wax stage of growth at 10mm theoretical length of cut using maize forage combine harvester. The silage mass was subdivided into three equal parts (negative control (untreated silage), a positive control (a competitor product available on the market) and Silko treatment) and ensiled in trench silos. The liquid inoculants were sprayed using a plant sprayer over the course of filling the silos at the rate of 5g t^{-1} fresh maize material. The inoculant Silko contains a mixture of four strains of *Lactobacillus plantarum* (LP1, LP2, LP3 and LP4). The number of colony forming units in inoculant is $1x10^{10}$ CFU/ml. After 90 days of ensiling, nine composite samples, three from each treatment were analyzed in laboratory. Composite sample included twelve samples which are collected with different locations in trench silo, and were mixed in a clean plastic bucket to form a composite sample weighing about 1.5 kg.

The content of dry matter (DM), ash, crude fat (CF), cellulose (Cell), neutral detergent fiber (NDF), acid detergent fiber (ADF), NH_3-N, lactic acid (LA), acetic acid (AA) and butyric acid (BA) and pH value were determined following the method described by *AOAC (2000).*

Total digestible nutrients value (TDN) and estimate dry matter digestibility (EDDM) calculated according to *NRC (2001),* relative feed value (RFV) according to *Horrocks and Vallentine (1999),* metabolic energy (ME) according to *Nauman and Bassler (1993)* and net energy for lactation (NEL) according to *Baležentienė and Mikulionien (2006)*:

TDN (%) = 87.84 – (%ADF × 0.70);

RFV (%) = Digestible Dry Matter (DDM) × Dry Matter Intake (DMI) × 0.775,

DDM (%) = 88.9 - (0.779 × %ADF) and DMI (%) = 120/(%NDF);

ME (MJ kg^{-1}) = 14.07 + 0.0206 × crude fat (g kg^{-1}) – (0.0147 × crude fibre (g kg^{-1}) – 0.0114 × crude protein (g kg^{-1}) ± 4.5 %;

NEL (MJ kg^{-1}) = 9.10 + 0.0098 × crude fat (g kg^{-1}) – 0.0109 × crude fibre (g kg^{-1}) – 0.0073 × crude protein (g kg^{-1}).
EDDM (%) = 88.9 – (0.779 × %ADF).

The experimental data were analyzed as a one-way ANOVA using Statistica version 10 and Duncan Multiple Range ($P < 0.05$) was used to compare means.

Results

Chemical composition

Data of ANOVA in Table 1 shows that both inoculants have highly significant effect on content of ash, crude fat, crude protein and cellulose. Also, Silko inoculant has significant effect on ADF and NDF. Ash was significantly lower in negative control (21.67 kg^{-1} DM) than Silko treatment (24.94 g kg^{-1} DM) and positive control (25.75 g kg^{-1} DM). Crude fat (17.49 g kg^{-1} DM) and crude protein (72.09 g kg^{-1} DM) were significantly lower in negative control than positive control (21.55 g kg^{-1} DM and 76.21 g kg^{-1} DM) and Silko treatment (20.60 g kg^{-1} DM and 78.27 g kg^{-1} DM). However, values of these parameters did not differ between positive control and Silko treatment. Cellulose was significantly higher in negative control (84.76 g kg^{-1}) than positive control (79.02 g kg^{-1} DM) and Silko treatment (74.41 g kg^{-1} DM). Value of this parameter was significantly lower in Silko treatment than positive control. ADF and NDF were significantly lower in Silko treatment (225.11 g kg^{-1} DM and 372.14 g kg^{-1} DM) than negative control (237.41 g kg^{-1} DM and 390.49 g kg^{-1} DM) and positive control (234.18 g kg^{-1} DM and 386.65 g kg^{-1} DM).

Table 1 Chemical composition of untreated silage and silage treated with bacteria inoculants

Item	Control	Positive control	Silko	M	F test
Dry matter (DM) (g kg^{-1})	362.27	374.50	391.30	376.02	ns
Ash (g kg^{-1} DM)	21.67^{c}	25.75^{a}	24.94^{b}	24.12	**
Crude fat (CF) (g kg^{-1} DM)	17.49^{b}	21.55^{a}	20.60^{a}	19.88	**
Crude protein (CP) (g kg^{-1} DM)	72.09^{b}	76.21^{a}	78.27^{a}	75.52	**
Cellulose (CEL) (g kg^{-1} DM)	84.76^{a}	79.02^{b}	74.41^{c}	79.40	**
Acid detergent fiber (ADF) (g kg^{-1} DM)	237.41^{a}	234.18^{a}	225.11^{b}	232.23	*
Neutral detergent fiber (NDF) (g kg^{-1} DM)	390.49^{a}	386.65^{a}	372.14^{b}	383.09	*

Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability, * - significant at 5% level of probability and ns - not significant

Energetic characteristics

Results showed that addition of inoculants did not affect total digestible nutrients value (TDN), relative feed value (RFV) and estimate dry matter digestibility (EDDM), Table 2. Their values were the lowest in negative control

(71.22%, 167.68% and 70.41%) and highest in Silko treatment (72.08%, 179.02% and 71.36%). Values of ME and NEL were significantly higher in positive control (12.48 MJ kg^{-1} and 7.89 MJ kg^{-1}) and Silko treatment (12.51 MJ kg^{-1} and 7.92 MJ kg^{-1}) than negative control (12.36 MJ kg^{-1} and 7.82 MJ kg^{-1}).

Table 2 Energetic characteristics of untreated silage and silage treated with bacteria inoculants

Item	Control	Positive control	Silko	M	F test
Total digestible nutrients value (TDN) (%)	71.22	71.45	72.08	71.58	ns
Relative feed value (RFV) (%)	167.68	169.95	179.02	172.22	ns
Metabolic energy (ME) (MJ kg^{-1})	12.36^{b}	12.48^{a}	12.51^{a}	12.45	**
Net energy for lactation (NEL) (MJ kg^{-1})	7.82^{b}	7.89^{a}	7.92^{a}	7.88	**
Estimate dry matter digestibility (EDDM) (%)	70.41	70.66	71.36	70.81	ns

Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability and ns - not significant

Fermentation parameters

Data of ANOVA in Table 3 shows that silage inoculants have significant effect on all fermentation parameters. The content of soluble N/TN (370.96 g kg^{-1} TN) and NH_3-N/TN (38 g kg^{-1} TN) were lower in Silko treatment compared to negative control (381.63 g kg^{-1} and TN 45.16 g kg^{-1} TN) and positive control (494.98 g kg^{-1} TN and 51.45 g kg^{-1} TN). The lactic acid (70.08 g kg^{-1} DM) and acetic acid (5.23 g kg^{-1} DM) were significantly lower in negative control than silage treated with inoculants. Lactic acid was similar in positive control (82.57 g kg^{-1} DM) and Silko treatment (82.88 g kg^{-1} DM). Contrary, acetic acid was lower in Silko treatment (11.17 g kg^{-1} DM) than positive control (12.95 g kg^{-1} DM). The butyric acid (0.28 g kg^{-1} DM) and pH value (4.26) were significantly higher in negative control than positive control (0.11 g kg^{-1} DM and 3.97) and Silko treatment (0.09 g kg^{-1} DM and 3.82).

Table 3 Fermentation parameters of untreated silage and silage treated with bacterial inoculants

Item	Control	Positive control	Silko	M	F test
pH	4.26^{a}	3.97^{b}	3.82^{c}	4.02	**
Soluble N/TN (g kg^{-1} TN)	381.63^{a}	494.98^{b}	370.96^{a}	415.86	**
NH_3-N/TN (g kg^{-1} TN)	45.16^{b}	51.45^{a}	38.00^{c}	44.87	**
Lactic acid (LA) (g kg^{-1} DM)	70.08^{b}	82.57^{a}	82.88^{a}	78.51	**
Acetic acid (AA) (g kg^{-1} DM)	5.23^{c}	12.95^{a}	11.17^{b}	9.78	**
Butyric acid (BA) (g kg^{-1} DM)	0.28^{b}	0.11^{a}	0.09^{a}	0.16	**

Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability

Discussion

Inoculants did not cause significant changes of dry matter content. Similar results found *Bijelić et al. (2015)*. Dry matter content in investigated silages ranged from 362.27 g kg^{-1} (negative control) to 391.30 g kg^{-1} (Silko treatment). *Loučka (2010)* pointed that optimal content of DM for maize silage is 280-340 g kg^{-1}, but in our silages DM was higher. The higher dry matter content can be explained by the fact that is during the drying process of samples, there was a loss of moisture and volatile organic substances. During the drying process of silage samples, heat drying could result in losses of volatile substance, such as short-chain organic acids and alcohols *(McDonald et al., 1991)*. The content of ash in investigated silages ranged from 21.67 g kg^{-1} DM (negative control) to 25.75 g kg^{-1} DM (positive control). It is ideally because ash values were lower than 85 g kg^{-1} DM and indicate that silages are not contaminated with soil. The higher contents of ash in inoculated silages result is metabolism of inoculated strains bacteria. The inoculated silage had significantly higher crude fat and crude protein content. *Nkosi et al. (2011)* reported similar results. Contrary, *Bijelić et al. (2015)* found that crude protein content decreased with the addition of the bacterial inoculant. The studied inoculants significantly decreased cellulose content of maize silage compared to the untreated silage, especially Silko. This decrease in cellulose content can be due to ability of *Lactobacillus* species to produce cellulose enzyme, *Sadiya and Ibrahim (2015)*. Contrary, *Dinić et al. (2013)* concluded that bacterial inoculant did not affect cellulose content of maize silage, but *Konca et al. (2015)* found that LAB inoculation decreased cellulose content of sunflower silages. The silage inoculated with bacteria inoculants reducing fiber fractions (ADF and NDF) compared to negative control. Values of ADF and NDF are important because they relate to the ability of an animal to digest the forage. As ADF increases, digestibility of forage decreases, while NDF decreases, the dry matter intake increases. The ADF value refers to the cell wall portions of the forage that are made up of cellulose and lignin, while NDF value is the total cell (ADF fraction plus hemicellulose). In Silko treatment, content of ADF and NDF significantly reduced which indicates that part of the fiber was solubilized. NDF is reduced because of the increased degradation of hemicellulose. Generally, favorable anaerobic conditions reducing ADF and NDF. According to *NRC (2001)*, maize silage with over 400 g dry matter contains TDN 65.4%, NDF 445g kg^{-1} DM, ADF 275 g kg^{-1} DM and ash 40 g kg^{-1} DM. In our study, the values for NDF and ADF slightly lower.

Silages treated with inoculants have higher ME and NEL than untreated silage. However, TDN, RFV and EDDM values did not differ between treatments, although the highest values were in Silko treatment. *Dinić et al. (2013)* found that applying bacterial inoculant to maize silage increased the NEL and RFV values.

Maize silages inoculated with inoculants have a lower pH and content of butyric acid, and higher content of lactic acid and acetic acid compared to negative control. For whole-crop maize preservation, pH between 3.8 and 4.5 is considered to be beneficial. In our study the pH ranged from 3.82 (Silko treatment) to 4.26 (negative control) which is indicative of well-preserved silage. The content of soluble N/TN and NH_3-N was significantly lower in Silko treatment than positive control. These values were highest in positive control which can be associated with a slightly higher pH than Silko treatment. Also, this indicates that the proteins are extensively degraded. Studied silages have a less content of NH_3-N than the limit values 7-10% *(Đorđević and Dinić, 2003)*. Generally, treated silages with inoculant Silko contains more of the protein in an intact form that can be utilized directly by the animal. The studied silages had satisfactory content of lactic acid (>6.5%), indicating a good fermentation. Silages treated with inoculants have higher concentrations of acetic acid and lower concentrations of butyric acid than untreated silage. The high levels of acetic (> 3 - 4%) and butyric acid (> 0.5%) are undesirable because indicates poorly fermented silage. The studied silages have the lower content of acetic and butyric acid than these values and indicate good fermented silages. The significantly lower values of lactic acid and acetic acid and the higher value of butyric acid were recorded in negative control. Values of lactic acid and butyric acid did not differ between the inoculants tested, while content of acetic acid was significantly higher in positive control. Acetic acid has anti-fungal properties, reduces aerobic spoilage of silage and growth of molds and yeasts. Accordingly, application of the tested inoculant can have these effects, in a way of increasing acetic acid content. Bacterial inoculant Silko was effective to enhance aerobic stability of silages due to higher acetic acid production which have antimycotic properties. The decrease in the pH values of fermentation in treated silages may be justified by the increase in the concentration of lactic acid. Also, *Đorđević et al. (2016)* reported that Silko inoculant increases lactic acid and acetic acid, and decreases butyric acid production of alfalfa silage compared to control. The lower content of butyric acid indicates that investigated silages did not content Clostridia spores which degrade lactic acid to butyric acid, and are results of contamination of fresh plant material with soil. It can be concluded that field trials showed good ensiling practices (rapid filling, packing thorough, perfect sealing and compression). Generally, fermentation characteristics in treated silages with Silko indicate good silage quality.

Conclusion

This study showed that application of bacterial inoculants of whole-crop maize during ensiling may improve the silage quality compared to untreated silage. However, inoculant Silko was more effective at improving the fermentation

characteristics than competitor inoculant. The content of ash, cellulose, soluble N/TN, NH_3-N/TN, ADF, NDF, pH and acetic acid differences were found between the positive control (competitor inoculant) and Silko inoculant. These values were significantly lower in Silko treatment. Silage inoculated with Silko were lowest content of NH_3-N, which is indicative of well-preserved silage this effect arose as a result of the pH reduction with inoculation which inhibits protein degradation in silages. However, no differences in crude fat, crude protein, metabolic energy, net energy for lactation, lactic acid and butyric acid between inoculant treatments. Results showed that inoculant Silko is efficient to improve chemical composition and energetic characteristics and reduced fermentative losses of maize silage. New inoculant Silko is high-performance inoculant for maize silage.

Uticaj *Lactobacillus plantarum* inokulanata na kvalitet silaže kukuruza

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U zimskom periodu u Srbiji, silaža kukuruza je glavna hrana za preživare. Zbog toga je postupak proizvodnje silaže važan faktor u očuvanju kvaliteta silaže za ishranu zivotinja. U studiji su ocenjeni hemijski sastav, energetske i fermentacione karakteristike silaža od celih biljaka kukuruza inokulisanih različitim bakterijskim inokulantima u terenskim uslovima na komercijalnoj farmi goveda u 2015. godini. Tri tretmana su testirana: negativna kontrola (netretirana silaža), pozitivna kontrola (konkurentski proizvod) i Silko tretman (sadrži mešavinu 4 soja *Lactobacillus plantarum* (LP1 LP2, LP3 i LP4)). Kukuruz je siliran u fazi mlečno-voštane zrelosti zrna. Silaža je analizirana 90 dana nakon siliranja. Bakterijski inokulanti su poboljšali hemijski sastav i energetske karakteristike silaže. Inokulant Silko bio je efikasniji u poboljšanju fermentacionih karakteristika u odnosu na konkurentski proizvod. Sadržaj pepela, celuloze, rastvorljivog i amonijačnog azota u ukupnom azotu, ADF, NDF, sirćetne kiseline i pH značajno su niži u Silko tretmanu nego u pozitivnoj kontroli. Nije bilo razlike u sadržaju sirove masti, sirovih proteina, ME, NEL, mlečne i buterne kiseline između tretiranih silaža. Generalno, novi proizvod bakterijski inokulant Silko pokazao je da u poljskim ogledima ima sposobnost da podrži proces siliranja kukuruza. Delovanje bakterijskog inokulanta Silka vrši se na dva načina: smanjena degradacija proteina u silaži i poboljšana aerobna stabilnost zbog nižeg pH i većeg sadržaja sirćetne kiseline u poređenju sa negativnom kontrolom.

Ključne reči: hemijski sastav, energetske karakteristike, parametric fermentacije, *Lactobacillus plantarum*, silaža kukuruza

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Received 21 February 2017; accepted for publication 18 March 2017

Biotechnology in Animal Husbandry 33 (1), p 127-133 , 2017 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 633.32
DOI: 10.2298/BAH1701127N

COMPARATIVE EVALUTION OF DI- AND TETRAPLOID ACCESSIONS OF RED CLOVER (*TRIFOLIUM PRETENSE* L.) FOR RESISTANCE TO POWDERY MILDEW (*ERYSIPHE POLYGONI* DC)

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Original scientific paper

Abstract: The aim of this study was to evaluate the tolerance of twelve genetic accessions of red clover to Powdery mildew (Erysiphe polygoni DC). The trial was carried out during 2014-2015 at the Experimental station of soya-bean Pavlikeni at a natural infection. The plant type of reaction was evaluated by a four-grade scale depending on the % of leaves with typical symptoms. A significant effect ($P<0.001$) of the genotype, and hence the level of ploidy, on the type of reaction to the disease of powdery mildew was established. Average for the period of study, the tetraploid accessions of red clover were rated as moderately susceptible (score 3.1), and the diploid ones – as moderately resistant (score 2.7). In the selection (breeding) of red clover the diploid accessions P-3 and Sofia-52 variety can be used as genetic sources of tolerance to powdery mildew.

Key words: red clover, Powdery mildew

Introduction

Red clover (*Trifolium pratense* L.) is distinguished for very good summer productivity and after grass and in sown swards it has the function to increase forage productivity and quality in summer and autumn. Powdery mildew (with causal agent *Erysiphe polygoni* DC.) attacks red clover in the mentioned seasons and is the fungal disease having the greatest economic importance to this crop in Bulgaria. The disease symptoms are increasingly larger spots of powdery covering on the stems, adaxial and abaxial leaf surfaces. The leaves become yellow and wither, which decreases the quality of the fresh forage and hay, winter survival of the plants and hence the sward productivity (*Taylor, 2008*). Red clover is attacked by the pathogen considerably more severely, as compared to the other cultivated species of clovers – white, alsike and crimson clover (*Yarwood, 1936*). The development of resistant varieties is defined as the most efficient and ecological

way of controlling the diseases in legume forage crops (*Kimbeng et al., 2000; Taylor, 2008 a*). The resistance or tolerance to powdery mildew is reported to be characteristic of a number of new di- and tetraploid varieties of red clover (*Schubiger et al., 2004; Frick et al., 2008; Taylor, 2008; Rahjoo et al., 2012*). According to *Taylor and Quensenberry (1996)*, the resistance to the disease has a two-locus dominant-recessive model of inheritance. This resistance is defined as active inherent immunity and is manifested as necrotic reactions of the genotypes resistant to the causal agent of powdery mildew. In foreign and our studies the resistance to powdery mildew in genotypes of red clover is also associated with factors of the passive inherent immunity - micromorphological characteristics, such as synthesis and density of leaf wax covering (*Mika and Bumerl, 1984*) and a high degree of hairiness of the leaf surface by nonglandular trichomes (*Taylor, 2008; Naydenova and Georgiev, 2013*). According to *Hejduk and Knot (2010)*, the susceptibility also depends on the age of plants (sward), the young plants being affected more severely by the disease. The ecological factors have also a significant effect on the phenotypic reaction to the disease. The susceptibility of the varieties of red clover increases considerably when grown on soils of high content of calcium (neutral reaction) and available phosphorus (*Repsiene and Nekrosiene, 2006*).

The objective of this study was to investigate the presence of genotypic variance of the response to powdery mildew associated with the level of ploidy in accessions of red clover and to find out genetic sources of resistance to *Erysiphe polygoni* DC.

Material and Methods

The study was conducted in the Soybean Experimental Station of Pavlikeni (43^0 24’ N; 25^0 32’ E; 144 m above sea-level). The soil type is leached chernozem. The soil reaction is neutral. The attack by Powdery mildew with natural infection in July and August of the two experimental years 2014 and 2015 was observed in 12 accessions of red clover, including the two Bulgarian diploid varieties of the species - Nika 11 and Sofia 52, four local populations and six foreign tetraploid varieties. The accessions were sown in the spring of 2014 in randomized rows 1.5 m in length with two replications. Ten plants from each replication were observed. The plant type of reaction was evaluated by a four-grade scale depending on the % of leaves with typical symptoms: grade (1) resistant – leaves with no spots of powdery covering, (2) moderately resistant – up to 25 % of the leaves have symptoms of the disease, (3) moderately susceptible – up to 50 % of the leaves have symptoms and (4) susceptible – over 50 % of the leaves have symptoms.

A variance analysis (ANOVA model including the factors G = genotype and Y = year, cut, model 2) was used to determine the ecological and genotypic variance of type of reaction to powdery mildew in the studied group of genotypes.

Results and Discussion

According to the analysis of variance, the genotypic factorial influence had a significant effect (P<0.001) on the phenotypic variation in type of reaction to Powdery mildew – Table 1. The differences in the climatic conditions by years and cuts were also a significant source (P<0.001) of variability in the response of the studied genotypes to the pathogen. The disease develops in moderately dry conditions and according to the recorded average scores (Table 2) a more severe attack was observed in the periods with droughts – in 2014 it was in August, and in 2015 – in July (Figure 1).

Table 1. Analysis of variance by reaction to powdery mildew

Source of variance	SS	df	MS	F	P-value	F crit
Genotypes	17.19	11	1.56	10.93	0.000000	2.09
Year, cut	4.94	3	1.65	11.52	0.000025	2.89
Residual	4.72	33	0.14			
Total	26.84	47				

Table 2. Type of reaction to powdery mildew of red clover genotypes

Genotypes	Ploidy	Type of reaction to *Erysiphe polygoni* DC., Mean score from 20 plants			
		Jul 2014	Aug 2014	Jul 2015	Aug 2015
1. Nika 11(2x)	2x	3.00	2.55	2.75	2.50
2.Sofia 52 (2x)	2x	2.25	2.60	2.50	2.50
3.Kvarta (4x)	4x	3.15	3.35	4.00	3.60
4.Astur (4x)	4x	2.00	2.00	3.40	3.00
5.Carbo (4x)	4x	2.25	2.70	4.00	3.25
6.Elanus (4x)	4x	2.00	2.45	3.25	2.50
7.Fregata (4x)	4x	3.00	3.55	4.00	3.75
8.Larus (4x)	4x	2.60	3.25	4.00	3.50
9.P1 (2x)	2x	1.90	3.15	3.00	2.80
10.P2 (2x)	2x	1.55	3.40	2.75	2.00
11.P3 (2x)	2x	1.25	2.00	2.15	1.60
12.P4 (2x)	2x	4.00	4.00	4.00	4.00
Mean for diploids		2.3	2.9	2.9	2.6
Mean for tetraploids		2.5	2.9	3.8	3.1

* type of reaction – score: (1) - resistant, (2) - moderately resistant, (3) - moderately sensitive, (4) - sensitive

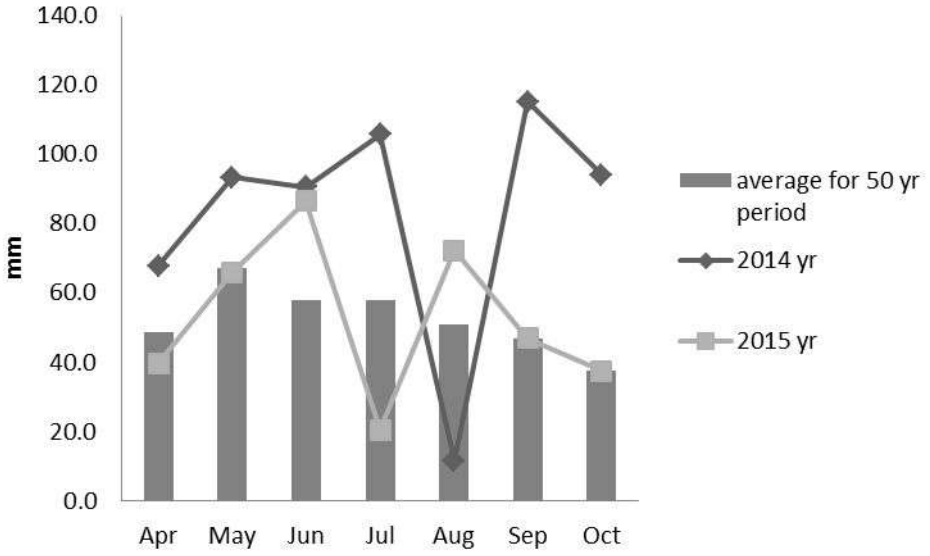

Figure 1. Monthly distribution of rainfall (mm) for investigation period

In some studies conducted with a large number of origins of red clover it was found that the tetraploid genotypes had lower susceptibility to fungal diseases (*Vleugels et al., 2012; Bukauskaite et al., 2014*), and in other studies they had higher susceptibility (*Jacob et al., 2010; Schubiger et al., 2003*). In this study the diploid Bulgarian varieties and populations, except for population 4, showed higher resistance to Powdery mildew, as compared to the foreign tetraploid germplasm – Figure 2. The lowest attack to the leaves was observed in the local population with number 3, for which the scores of the four records were within the range of 1.25 to 2.15 and therefore showed a resistant to moderately resistant type of reaction (Table 2). The reaction of the Bulgarian varieties Sofia 52 and Nika 11 to the disease remained constant when the conditions of regrowth and plant age changed. This characteristic can be associated with presence of genetic resistance to the local strains of the pathogen and therefore Sofia 52 variety, which is moderately resistant, can be used in plant breeding for tolerance to powdery mildew. In the group of tetraploid genotypes, variety Elanus, for which a predominately moderately resistant type of reaction was recorded, had lower susceptibility to the disease. This genotype also showed the best resistance to anthracnose, as compared to all tetraploid varieties of red clover registered in the European variety list (*Jacob et al., 2010*).

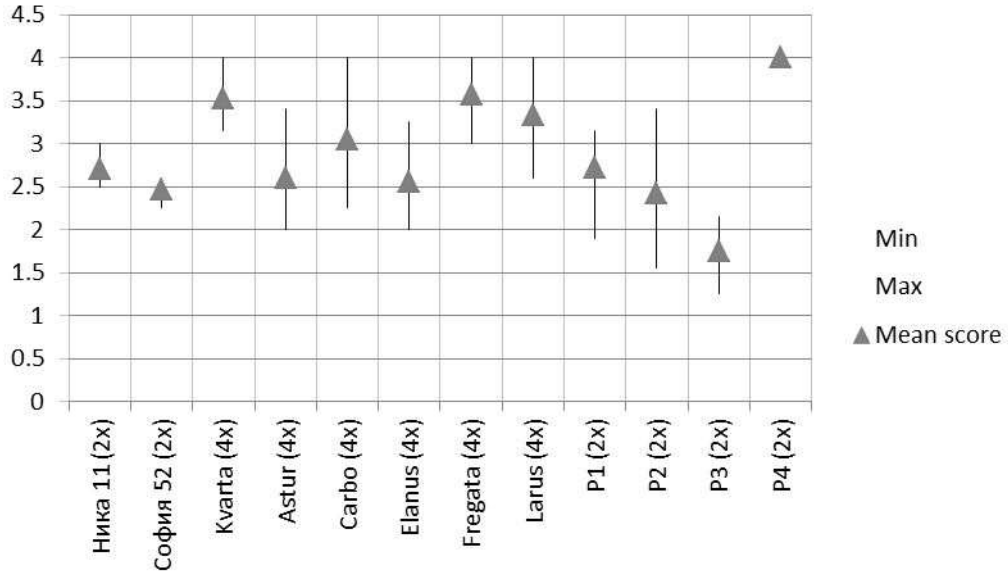

Figure 2. Marginal and mean scores by type of reaction to Powdery mildew at red clover accessions

Conclusion

A significant effect (P<0.001) of the genotype, and hence the level of ploidy, on the type of reaction to the disease of powdery mildew was established. Average for the period of study, the tetraploid accessions of red clover were rated as moderately susceptible (score 3.1), and the diploid ones – as moderately resistant (score 2.7). In the plant breeding of red clover the diploid accessions P-3 and Sofia-52 variety can be used as genetic sources of tolerance to powdery mildew.

Komparativna ocena di- i tetraploidnih formi crvene deteline (Trifolium pratense L.) na otpornost na pepelnicu (*Erysiphe polygoni* DC)

Galina Naydenova, Aksenia Aleksieva

Cilj ovog istraživanja je bio da se proceni tolerancija dvanaest genetskih formi crvene deteline na pepelnicu (*Erysiphe polygoni* DC). Ogled je sproveden

tokom 2014-2015 godine, u eksperimentalnu stanici Pavlikeni u uslovima prirodne infekcije. Tip reakcije biljke je procenjivan na skali četiri razreda zavisno od % lišća sa tipičnim simptomima. Utvrđen je značajan efekat (P<0,001) genotipa, a time i nivoa ploidnosti, na tip reakcije na bolest pepelnicu. Prosek za period istraživanja, tetraploidne forme crvene deteline su ocenjene kao umereno osetljive (rezultat 3.1), a diploidne forme - kao umereno otporne (rezultat 2.7). U selekciju (uzgoj) crvene deteline, diploidne forme P-3 i sorta Sofija-52 se mogu koristiti kao genetski izvori tolerancije na pepelnicu.

Ključne reči: crvena detelina, pepelnica

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Received 21 October 2016; accepted for publication 2 December 2016

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