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AUTHENTICATION OF POULTRY PRODUCTS AT THE BREED LEVEL USING GENETIC MARKERS

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ABSTRACT

The Oravka tawny is a Slovak national breed of chicken. This breed has combined utility, which means it is valuable for both its meat and eggs. The Oravka tawny is linked to a specific region, Orava, and therefore these products could be protected by European geographical indication. The labeling and sale of chicken meat by the traditional breed of origin are widely used to promote quality and attract those products in the marketplace. For that use, we created the system and method of authentication that can reliably distinguish between the Oravka tawny, other chicken breeds, and other of Oravka's colorful characters. In our research, we analyzed 153 chicken feathers from the Oravka breed as well as from breeds used in the process of breeding the Oravka to their current state. They were divided into nine populations. To separate those populations, we used seven microsatellite markers recommended by FAO (Food and Agriculture Organization) and other authors. To create separate clusters of individual breeds, we used DAPC (discriminant analysis of principal components) analysis.

Keywords: Oravka; microsatellite; marker; DAPC; cluster

INTRODUCTION

By the term food authentication, we understand the process of verifying that the label description of the food is accurate and contains all the necessary information for customers. That information may include the origin (species, geographical or genetic), production method (conventional, organic, traditional procedures, free-range), or processing technologies (irradiation, freezing, microwave heating). The induction of select quality attributed to highly valuable products is an especially interesting topic. Those certain food products are often the target of falsification by deceptive labels. Proof of provenance is an essential topic for food safety, food quality, and consumer protection, as well as for compliance with national legislation, international standards, and guidelines **(Aung and Chang, 2014)**.

The information presented to consumers is also most important for them in the process of choosing specific foods over others. This choice could be driven by lifestyle. For example, vegetarianism, or religious practices such as those of Jews and Muslims, for whom pork meat should be absent. The occurrence of several food crises in recent years has emphasized food safety and protection of consumer's health as the primary goal for the food labeling legislation **(Cheftel, 2005)**. **Directive 2003/89/EC** of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs, Off J Eur Union L 308:15–18 says: "The increased awareness of consumers regarding the composition of foods has resulted in the need to verify the labeling statements. The incorrect labeling of foods represents commercial fraud, considering consumer acquisition. It is crucial to establish that species of high commercial value declared are not substituting, partial or entirely, by other lower-value species. The misleading labeling might also have negative implications concerning health, especially for sensitive consumers, to nondeclared potential allergens. Food allergies are considered a new public health problem, especially in developed countries."

The Oravka is a Slovak chicken named after the region of Orava, from where this breed originated. The Oravka was created in a crossbreeding process of the regional hens with Rhode Island, Wyandotte, and New Hampshire breeds. The breeding process started in the 1950s and continued with numerous individual stages. The Oravka was recognized as a breed in the year 1990. The main aim of the breeding process was to create dual-purpose poultry. That is, to create a breed with good egg production and growth ability. The ability to adapt to harsh outdoor rearing was requested as well. The agreement for the standard of the Oravka breed is yearly egg production of

180 to 200 eggs with a brownish eggshell; the minimum hatching egg weight is 58g **(Hrnčár et al., 2017)**. Authors **Kukučková et al., (2018)** published work of genetic diversity with aim to protect and preserve breed of Slovak Pinzgau cattle. We are aiming to protect national breed and preserve biodiversity of Oravka tawny chicken.

DNA-based analytical methods can provide much more information about processed food and foodstuff than methods based on proteins. This is due to the much higher thermostability of DNA in comparison to most proteins. Also, DNA is present in the majority of cells in organisms, potentially enabling identical information to be obtained from any appropriate sample from the same source, regardless of the tissue of origin. Furthermore, driven by the clinical arena, nucleic acid-based technologies are developing rapidly, and the informed adoption of suitable methods by the food industry has the potential to simplify methods of authentication **(Lockley and Bardsley, 2000)**.

Currently, microsatellite loci are the method of choice to study the genetic diversities within and between populations, because they are highly polymorphic, show co-dominant inheritance, and are found to be abundant and evenly distributed throughout the genome. So far, many studies have been conducted to assess chicken's genetic diversity using microsatellite markers, and the reported results are clear evidence of the usefulness of these panels for biodiversity studies. Understanding the genetic structure of native chickens is a vital step in setting up their conservation and genetic improvement programs **(Yacoub and Fathi, 2013; Khanyile, Dzomba and Muchadeyi, 2015)**.

In our study, 153 individual chickens were tested, and seven markers were used. We are aiming to create a useful method of authenticating products made from the Slovak national chicken breed – the Oravka tawny.

Scientific hypothesis

We are expecting that separation of the Oravka breed from other chicken breeds used in its breeding process will be possible with the usage of chosen microsatellite markers.

MATERIAL AND METHODOLOGY

In our study, we used 153 samples of hen feathers (Table 1). These samples were divided into nine populations. The most numerous population was the group of Oravka tawny (119 individuals). Other color variants of Oravka are genetically younger. All of them were bred later than the original autochthonous breed of Oravka tawny. We also included breeds used in the early stages of the breeding process of Oravka tawny and inbreeding processes, including the younger color variations. The Polymerase Chain Reaction (PCR) was performed as suggested **(Choi et al., 2015)** in total volume of 20 µL; 50 ng of genomic DNA, 10 pmol of labeled modified forward primer and standard reverse primer, 2.5 mM of each dNTPs, 10 X reaction buffer, 2.5 units of prime Taq DNA polymerase. The PCR was performed in an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 60 °C, 30 seconds of extension at 72 °C and final extension at 72 °C for 10 minutes. Seven microsatellite markers were used in our analysis: LEI0254, LEI0166, MCW0034, LEI0192, MCW0069, LEI0234 and LEI0228 (Table 2). Those markers were selected based on authors' **(Choi et al., 2015)** research and the recommendation of FAO. Genotyping was performed on ABI PRISM 310 Genetic Analyzer. The detection of genomic regions affected by natural selection was carried out based on the approach adopted in R package PCAdapt **(Duforet-Frebourg, Bazin a Blum, 2014; Moravíková et al., 2018)**.

Statistic analysis

To create clusters of selected chicken breeds, we used the DAPC analysis. For this analysis we used the software program Rstudio with additional packages installed.

RESULTS AND DISCUSSION

Seo et al. (2013) wrote: "In 1994, a program for the creation of lines of Korean national breeds began. The result was to document five breeds with nine chicken lines. A structured genetic analysis program based on microsatellite markers was used to investigate the genetic structure of the five Korean native chicken lines. Based on line specific clusters, the structure of the chicken line was estimated." Like the authors cited above, we have also sought to find microsatellite markers in order to improve the traceability of the national poultry breed.

Table 1 Populations of selected chicken breeds.

Breed	Number	Acronym
Oravka tawny	119	OTW
Oravka white	11	OWH
Oravka black	6	OBL
Oravka versicolor	6	OVC
Oravka tawny diminutive	1	OTD
Šumavka	4	SUM
New Hampshire	2	HAM
Rhode Island Red	2	RHI
Wyandotte	2	WYA

Table 2 Microsatellites used in analysis.

Microsatellite	Lenght (bp)	No.of alleles	Repetition
LEI0254	86 – 93	7	tetranucleotid
LEI0166	354 – 370	4	dinucleotid (CA)
MCW0034	220 – 240	9	dinucleotid (CA)
LEI0192	256 – 296	6	tetranucleotid
MCW0069	156 – 174	8	dinucleotid (CA)
LEI0234	219 – 315	8	tetranucleotid
LEI0228	165 - 255	14	tetranucleotid

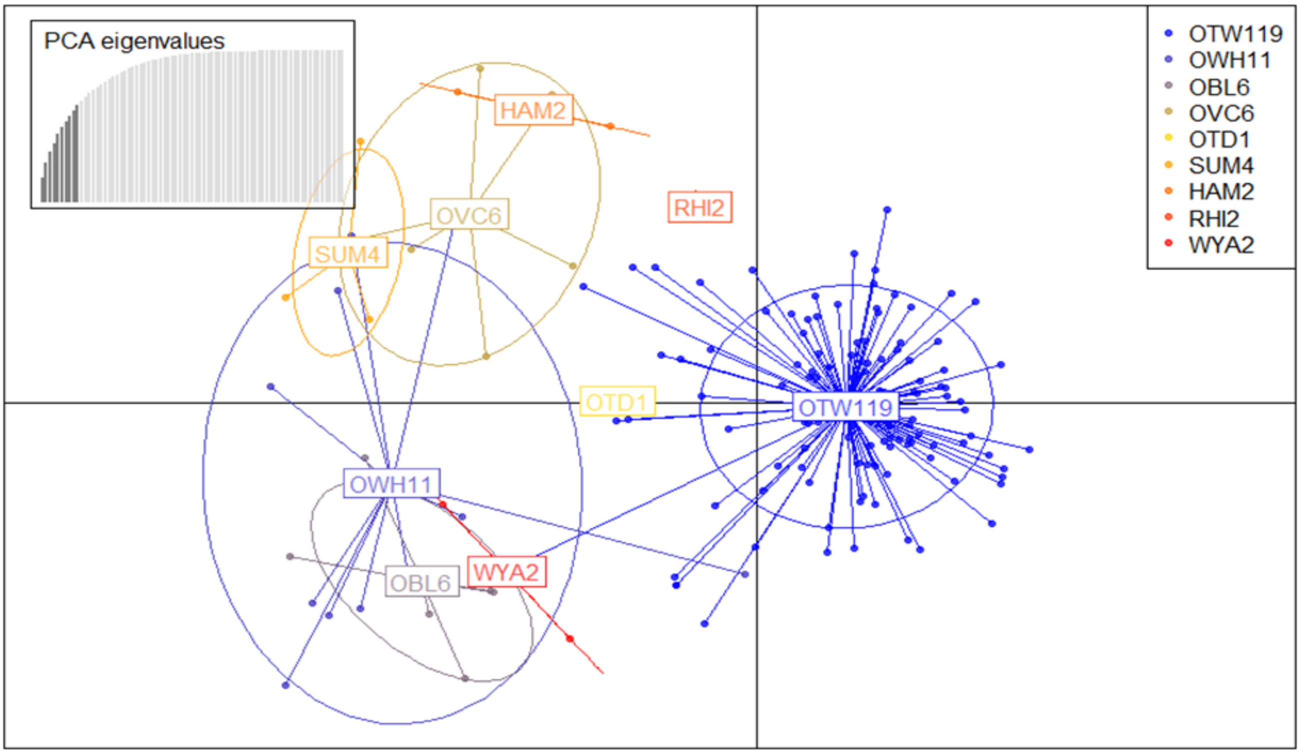

Figure 1 Clustes of selected chicken breeds after DAPC analysis.

In studies of human genetic variation, at the continental level, there is good agreement in the ordering by region of gene diversity measures among different kinds of markers, once ascertainment bias is removed **(Rogers a Jorde, 1996; Jorde et al., 2000)**. The same is true for the ordering of genetic distances among populations assessed for different markers **(Jorde et al., 2000)**. It is reasonable, therefore, to believe that the ordering among chicken breeds of diversity and distances seen here for microsatellites in DNA pools would not be very different than for other genetic marker systems. The microsatellite loci used here were selected to be polymorphic for use in gene mapping. However, **Rosenberg et al. (2002)** obtained diversity and genetic distance patterns for humans that largely agreed with those of **Bowcock et al. (1994)**. The recent study used microsatellites from a mapping set and the last used markers that were not selected. Ascertainment bias is, therefore, not expected to have had a significant effect on the ordering of statistics for our chicken data.

On the picture above (Figure 1) are visible clusters of populations used in our study. The most numerous cluster is that of our primary targeted population of Oravka tawny. We can see that this cluster is separated from others. Also, it is visible that the diminutive form of Oravka tawny is placed inside of the cluster. That means that with the usage of our chosen microsatellite markers, we cannot separate them from one another. Since the initial genotyping array has a large enough number of loci, even a low proportion of cross-amplifying SNPs may represent a useful set of markers for species which lack genomic resources. The distribution of SNPs over entire genomes of all chromosomes was not uniform and varied among the analysed groups **(Kasarda et al., 2015)**. The massive merging of breeding companies in recent years should call attention to the need for conservation of genetic variation among breeds and lines. Appropriate strategies for conservation of populations is out of the scope of the present report but is an important and controversial issue **(Fujihara, 1999)**.

Discriminant analysis of principal components (DAPC) uses Nei´s genetic distance to separate the observed populations of chickens. That means the longer the distance of the population on the created graph, the less they are related on the genetic level. With our analysis, we can differentiate the population of Oravka tawny from other color breeds as well as from breeds used in the early stages of its breeding process. Chosen microsatellite markers can be useful for the authentication of products made from this autochthonous chicken breed. With a functional system of authentication, products made from the breed of Oravka tawny could be eligible to be certified with European geographical indication (GI). Acquisition of this certification might help the preservation of this, the only Slovak national breed of chicken. Also, European certification would be able to support farmers and producers with increased credibility in the eyes of customers.

The European Union established measures to support breeds in danger of being lost to farming **(Commission Regulation (EC) No 1974/2006)**. The threshold under which a chicken breed is considered in danger is 25,000 breeding females; the number of Bionda and Bianca are below this value **(DeMarco et al., 2013)**. The AVIANDIV-FAO microsatellite tool meets the need to establish a standard approach to characterize animal genetic resources, and the number of loci ensures high differentiation power **(FAO, 2011; Gärke et al., 2012)**. The widespread use of these markers provides the most significant amount of data to perform comparisons among populations of different origins. Therefore, models for linking information are based mainly on this tool, and new data on indigenous poultry may be combined with available data sets of other breeds and commercial lines

(Granevitze et al., 2007; Zanetti et al., 2010). The markers of the present investigation are suitable to evaluate genetic relationships among populations and to assess whether a supported plan should be implemented. Although different markers were used, the number of alleles and the expected heterozygosity of the present analysis are in agreement with the investigation of **Guidobono Cavalchini et al. (2007)** on the same Piedmont breeds. Moreover, our results show that genetic variability has not changed in the short term.

Kumar et al. (2015) wrote: "In conclusion, this study clearly demonstrates the potential of locus-specific microsatellite markers in genetic diversity, phylogenetic relationships, and population structure analysis between wild and domestic chicken populations. The information generated by microsatellite marker analysis in the form of population-specific alleles may be used in the development of microsatellite assays for the identification of pure or admixed RJF in the wild chicken population. Apparent separate clustering of the four populations suggests pronounced population structure and minimal or no gene flow. High genetic diversity across the population and microsatellite panels with high levels of heterozygosity and PIC suggest the appropriateness of the methodological design."

As mentioned authors, we were able to prove the difference between the autochthonous breed of Oravka tawny and other color variants and other breeds.

CONCLUSION

There is a financial incentive for meat producers to substitute meat from chicken of cheaper breeds, thereby defrauding the consumer. There is a need for legal authorities to be able to authenticate the breed of origin of meat labeled by breed, in order to deter mislabeling fraud of breed of chicken. In our analysis, we proved that with the usage of seven selected microsatellite markers and discriminant analysis of principal components, we could separate clusters of Oravka tawny from other populations of chicken.

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