



Lake Balaton as a geographical barrier for gene flow between wild boar (*Sus scrofa*) populations in Hungary

GEORGE WANJALA¹, BENDEGÚZ MIHALIK^{1,2}, VIKTOR STÉGER², SZILVIA KUSZA¹

¹ University of Debrecen, Animal Genetics Laboratory

² NARIC Agricultural Biotechnology Institute

ABSTRACT

Despite of the large number of literature on genetic diversity of *Sus scrofa* in Europe, little is known about the genetic differences between wild boar populations in South (Kecel-Imrehegy) and Northern parts of Lake Balaton and the effect of the Lake to gene flow. Therefore, the study aims to establish genetic variability of wild boar populations between sampling site in Veszprém and Bács-Kiskun counties and to investigate effects of Lake Balaton to gene flow.

Earlier developed thirteen STR microsatellite markers were used to amplify DNA isolated from 38 samples of wild boar populations between the hunting sites of Kecel-Imrehegy and North of Balaton (18 and 20, respectively). Data analysis detected a total of 12 loci, 10 polymorphic loci, low pairwise F_{ST} value of 0.066, low mean observed and expected heterozygosity ($H_{obs}=0.42\pm 0.23$ and $H_{exp}= 0.41\pm 0.23$), low within population Nei's gene diversity of 0.35 and 0.4 for North and south Balaton populations respectively. Analysis by Structure and PAST v2.17.c software's indicated very low genetic difference between populations.

From the results, the following conclusion could be made the two studied populations were genetically similar between and within populations. This point out significant level of gene flow between the populations despite being separated by Balaton and a long distance (150km apart) showing that Lake Balaton did not have effect on gene flow. Low pairwise F_{ST} value indicated that the geographical distance has not caused genetic difference.

Key- words: genetic diversity, gene flow, geographical barrier

INTRODUCTION

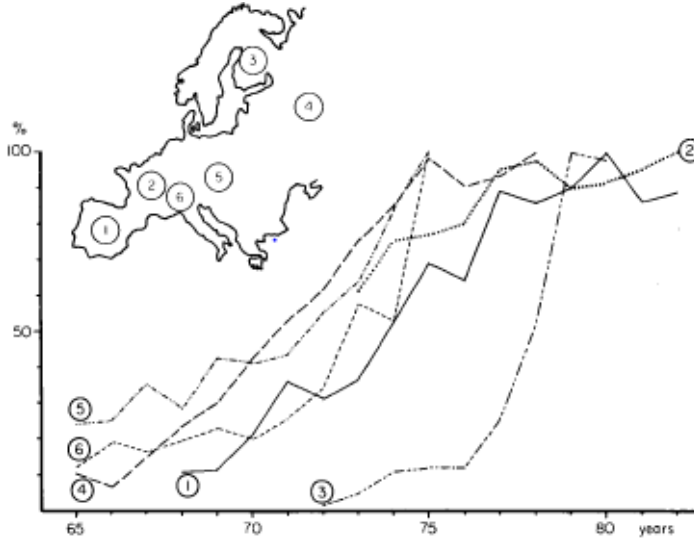
Wild boar (*Sus scrofa*) is a common ancestor of the recent domestic pig. Its distribution in all parts of the world makes it one of the widely spread ungulates especially in Europe, North-Africa, and much of Asia as far South as Indonesia (*Choi et al.* 2014). Wild boar attained prominence in ecological studies due to its recent rapid population increase which consequently affects its relationship with human hence creating a large human-wildlife conflict. Therefore, responsible bodies have been striving to put in place wild boar management plans in order to minimize or eradicate this human-wildlife conflict.

Several species of the genus *Sus* exists, but of prominence is Eurasian wild boar (*Sus scrofa*). The Eurasian wild boar has one of the widest geographic distributions of all terrestrial mammals with the expansion partially being aided by human. The species now occurs in pure wild or barely modified feral form on all continents except in Antarctica, and on many oceanic islands (*Oliver and Leus* 2008). Studies reveal that, it is the ancestor of most (but not all) ancient and modern domestic pig breeds, and there is evidence to suggest that it was independently domesticated in several different parts of its range, including Southeast Asia, the Far East. As a wild form, it has constituted a primary resource for subsistence for hunters since the earliest times, and it is one of the most important targets for recreational hunting wherever it remains sufficiently abundant. (*Oliver and Leus* 2008; *Larson et al.* 2005).

In Europe, studies show that wild boar numbers increased between 1960s and 1970s but stabilized in the 1980s (*Massei et al.* 2014) possibly due to a combination of factors, such as the depopulation of rural areas, changes in agricultural practices, reintroduction, lack of predators, reduced hunting pressure, and climatic changes (*Massei and Genov* 2004) above all their ability of high reproduction. In the 15-member states of European Union (EU), *Sus scrofa* population has been estimated to be approximately between 800,000 to 1,000,000 heads with demographic density varying from one state to another (*figure 1*) (*Laddomada* 2000).

This population increase has generated a big problem in many parts in Hungary, for example, the habituation of wild boar populations has created a very big problem in areas surrounding Lake Balaton. This lake is surrounded by reed on one side of the lake

shore and by the forest and Agriculture fields on the other side of the lake. Wild boars use both habitats and frequently attack or cause damage in Urban and agricultural areas situated between these two habitats (Tari et al. 2017).



(1) Spain, (2) France, (3) Finland, (4) European Russia, (5) Czechoslovakia, (6) Switzerland

(Source): Saez-Royuela and Telleria (1986).

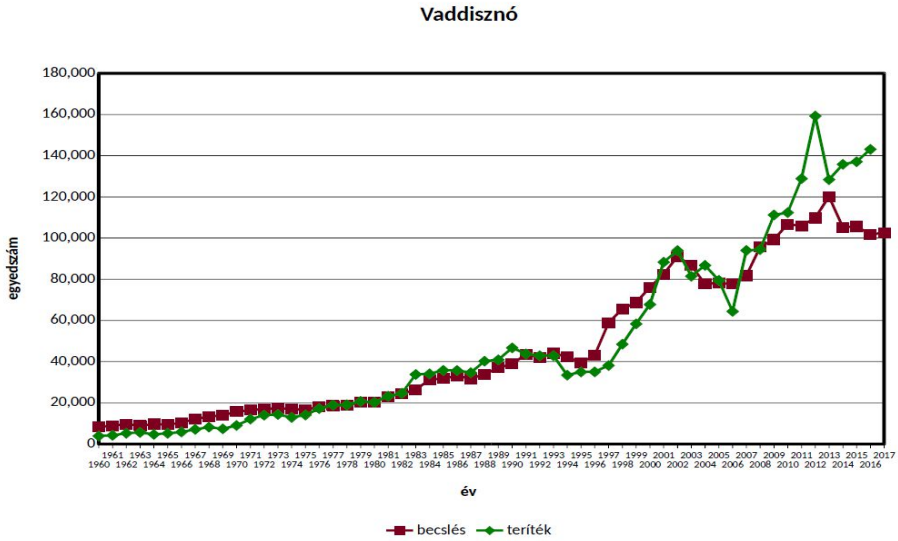
Figure 1 Evolution of wild boar populations in different European countries

According to Ministry of Agriculture (2017), wild boar population in Hungary is estimated to be approximately between 102,000 to 105000 (figure 2). Out of this 65% of the population is found in West Hungary, 31% in Northern mountainous areas and 4% in the plains to the East of the Danube River (Sevako Idegengorgalmi Kft).

This continuous wild boar population increase has led to increased shooting hence increased harvesting rate. Wild boars in Hungary have a high economic value and play a key role in hunting and venison production and therefore, their products attract high price premium. Most of the products are exported and sold within European Union. However, lack of routine methods for product control has encouraged product adulteration (Szemethy et al. 2016), (figure 3).

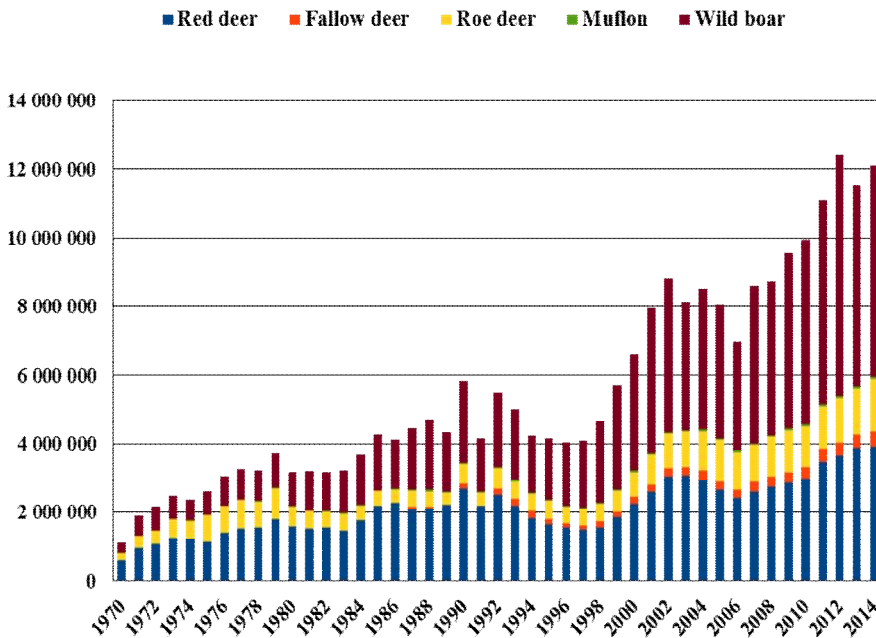
Ahmad and Khan (1989) in their study found out that the animal was an important vertebrate pest, especially to sugar cane, wheat and maize causing more damage than

rats and porcupines. In Hungary, a study by *Bleier et al. (2012)* revealed that the volume of damage is correlated with the population density of the game species.



(source): Csányi et al (2017).

Figure 2 Wild boar population dynamics in Hungary



Source: Szemethy et al. (2016).

Figure 3 Quantities of harvested game by species in Hungary (Kg/Year)

Several investigations suggest that wild boar (*Sus scrofa*) play a critical role in the spread of classical Swine Virus (CSF) with studies indicating that in Germany 46% of the primary outbreaks of CSF that occurred in domestic pigs between 1993 and 1997 were caused by direct or indirect contact with an infected wild boar (Laddomada 2000). Another zoonotic disease caused by the pathogenic serovars of the genus *Leptospira* called leptospirosis which is reserved in wild boar. Studies by Żmudzki et al. (2016) revealed the highest number of seropositive wild boars especially in wild boar populations bordering cities. The authors, therefore, concluded that relatively high prevalence of *Leptospira* infections in wild boars could constitute a threat to hunters and people having contact with forest lakes or marshlands. The results also indicate that an increasing population of wild boar living close to borders of cities could create additional risk for inhabitants in large urban areas.

African swine fever is a virus that can cause nearly 100% mortality in wild boars and domestic pigs. Studies reveal that this virus is spread by wild boar. The virus was

detected in several European countries like Ukraine, Romania, Czech Republic and Poland (*National Food Security Office 2017*).

Wild boar is adaptable and resistant to a variety of harsh and modified environments under hunting pressure (*Oliver and Leus 2008*). *Sus scrofa* habitat types range from semi-arid environments to marshes, rain forests and alpine grasslands, temperate woodlands and reed jungles where it feeds opportunistically on plant and animal species but often venturing onto Agricultural crops (*Massei et al. 2014*). In Europe, it prefers broadleaved forests and especially evergreen oak forests, but may also be found in more open habitats such as steppe, Mediterranean shrubland, and farmland, so long as there is water and tree cover nearby with a preferred altitude of 0- 2,400 m above the sea level (*Massei et al. 2014; Oliver and Leus 2008; Herrero and Garcia-Gonzales 2008*).

The aims of this study were:

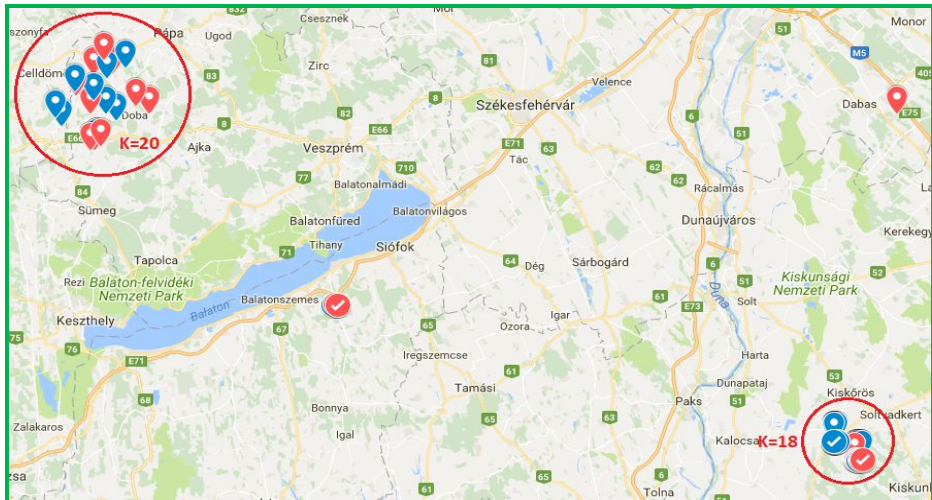
- To determine the genetic variability of wild boar samples between Southern and Northern Lake Balaton.
- To determine the correlation between genetics and geographical distance.
- To investigate the effects of Lake Balaton to gene flow between both populations.
- To use the results of the study to suggest ways of maintaining the wild boar population with genetic diversity.

MATERIALS AND METHODS

Samples and laboratory analyses

A total of 38 wild boar fresh muscle tissues were collected from hunting sites (N = 20 from Veszprem and N = 18 from Bacs-Kiskun) in 1,5 ml eppendorf tubes that contains 1ml ethanol. All samples were collected from dead animals which were shot for meat or trophy by licensed hunters. This study did not involve collection of samples from live animals and therefore, ethics statement was not required. Hunting was according to the provisions of the legal act amended in 2017 and currently in place in Hungary (Act LV of 1996 on the Protection of Wild Game, Wild Game Management and Hunting). The Act applies to the protection of species which may be legally hunted and the protection

of their habitat, however, it does not regulate protection of those wild species which may not be legally hunted and live in their natural habitats, or those kept in captivity in zoos (Jánoska 2010, *Magyar Közlöny* 70. szám 2017). Figure 4 shows the distribution of wild boar sample collection sites.



checked by gel electrophoresis (*Figure 5*) before sending to BIOMI for fragment-analyses.

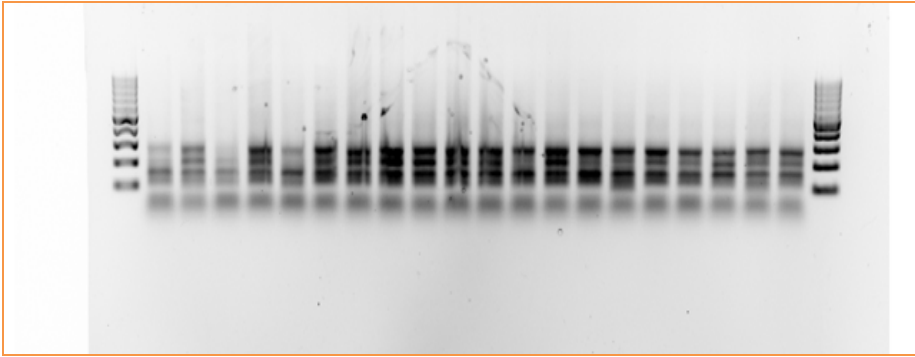


Figure 4: Image of gel electrophoresis for North Balaton samples

Statistical analysis

Analyzed fragments from the Biomi were further analyzed with PEAK SCANNER™ 1.0 software v1.0 (Applied Biosystems) for fragment sizing and peak identification. Genotypes were typed in a Microsoft Excel table. Levels of genetic variability in the sampled populations, observed (H_{obs}) and expected heterozygosity (H_{exp}), total number of alleles, number of alleles per locus (A), genetic differentiation between groups (F_{ST}) were calculated for all loci, based on overall set of samples using ARLEQUIN v.3 software (*Excoffier et al. 2005*). Population clustering was implemented following Bayesian Cluster analysis method by STRUCTURE v. 2.3.4. software (*Falush et al. 2012*). The value of K was first determined by exploring the best possible cluster. During the exploration of the value of K , 10 replicating runs were done for each value of K comprising between $K = 1-5$ at 250,000 Length of Burn the burn-in period and 750,000 as MCMC repetitions set at 750,000. All other setting parameters were put at default level. Bayesian Cluster analysis assumes that there is no prior knowledge of the population. This approach is also a powerful tool for analysing admixture when populations not included in the samples might have contributed to the genetic composition of the admixed individuals. To establish the genetic distance between population, PAST v.2.17c software (*Hammer, 2017*) was used for analysis.

RESULTS

Basic properties of populations

An average number of detected gene copies was 38 ± 2.8 , with population 1 (North Balaton) having higher number of 40 compared to population 2 (South Balaton). A total of 12 loci were detected with high level of polymorphism, on average 10 (83%) were polymorphic (*table 1*). Velićković et al. 2010 and 2012 also observed high levels of polymorphism in West Balkan Region and Podunavlje wild boars.

Table 1 Basic properties of the populations

Statistics	Population 1	Population 2	Mean	S.d
No of gene copies	40	36	38	2.8
No of loci	12	12	12	0
No of usable loci	10	11	10	0.71
No of Polymorphic Loci	9	11	10	1.41

Genetic variation at microsatellite loci

A total number of 51 alleles were found in the wild boar populations with an average number of alleles occurring per locus being 4.25 ± 2.2 . On average Locus PigSTR 15A had the highest number of alleles per locus ($A=7$) in both populations, while loci PigSTR 14A and PigSTR 1A had the lowest number with equal number of alleles in both populations ($A = 2$). Population wise, population 2 (South Balaton) had higher number of alleles per locus 3.8 ± 1.8 than population 1 (North Balaton), 3.0 ± 1.7 . Several studies observed higher mean alleles per locus than in present study populations. Studies by Velićković et al. 2010 observed $A= 14.7$ in wild boars in Podravlje (Danube basin between Serbia and Croatia), Vernesi et al. 2003 also observed $A= 12.1$ in Hungarian wild boars while Velićković et al. 2012 observed $A = 19$ in West Balkan Region wild boars. *Table 2* summarizes the Genetic variation at microsatellite loci.

Table 2 Population Microsatellite parameters

Locus No	Population 1	Population 2	Mean	Sd	Tot. number
PigSTR 14B	1	3	2.0	1.40	3
PigSTR 7B	4	8	6.0	2.80	8
PigSTR 4B	3	3	3.0	0.00	3
PigSTR 4C	4	4	4.0	0.00	4
PigSTR 17A	2	3	2.5	0.71	3
PigSTR 11A	2	3	2.5	0.71	3
PigSTR 14A	2	2	2.0	0.00	2
PigSTR 1B	1	4	2.5	2.12	5
PigSTR 15A	7	7	7.0	0.00	9
PigSTR 5C	3	3	3.0	0.00	3
PigSTR 13E	5	4	4.0	0.70	6
PigSTR 1A	2	2	2.0	0.00	2
Mean	3.00	3.80	3.40	0.59	4.25

Population 1 (Oroszi-Nemesszalók-Kemenespálfa-Veszprémgalsa, North Balaton)

Population 2 (Kecel-Imrehegy, South Balaton)

Genetic diversity within populations

Analysis for genetic diversity within population revealed varied low to medium levels of observed and expected heterozygosity at locus level ranging from 0.05 to 0.78. South Balaton had higher observed and expected heterozygosity ($H_{obs} = 0.48 \pm 2.6$, $H_{exp} = 0.46 \pm 2.1$) than North Balaton ($H_{obs} = 0.30 \pm 2.1$, $H_{exp} = 0.35 \pm 2.4$), showing low levels of inbreeding in South Balaton population than in North Balaton population.

North Balaton population had higher expected heterozygosity than observed heterozygosity, contrary to South Balaton population which had higher observed heterozygosity than expected heterozygosity, indicating higher levels of inbreeding in North Balaton population than it was expected. Both populations exhibited low mean Nei's gene diversity (1987) for the wild boar North Balaton (Pop 1) was 0.35 ± 0.2 lower than 0.48 ± 0.26 for South Balaton (pop 2), much lower than 0.872 observed by (Veličković *et al.* 2010) in Podunavlje wild boars. The mean genetic diversity within populations indicates that only 35 % and 48 % of genetic diversity emanates within

population 1 and 2 respectively. *Table 3* summarizes genetic diversity within populations.

Table 3 Summary of diversity indices for separate populations

Locus No	No of alleles	Obs Het	Exp Het	No of alleles	Obs Het	Exp Het
PigSTR 14B				3	0.11	0.11
PigSTR 7B	4	0.25	0.61	8	0.72	0.79
PigSTR 4B	3	0.45	0.38	3	0.27	0.34
PigSTR 4C	4	0.4	0.43	4	0.61	0.58
PigSTR 17A	2	0.05	0.05	3	0.44	0.36
PigSTR 11A	2	0.55	0.41	3	0.94	0.62
PigSTR 14A	2	0.2	0.18	2	0.27	0.25
PigSTR 1B				4	0.13	0.25
PigSTR 15A	7	0.61	0.72	7	0.78	0.78
PigSTR 5C	3	0.55	0.68	3	0.67	0.57
PigSTR 13E	5	0.25	0.51	4	0.56	0.59
PigSTR 1A	2	0.25	0.22	2	0.22	0.29
Mean	3.00	0.30	0.35	3.83	0.48	0.46
Sd	1.68	0.21	0.24	1.77	2.6	0.21

Mean genetic diversity of populations

There was observed deficiency in heterozygosity in both populations. This was confirmed by Hardy-Weinberg equilibrium (HWE) test for each locus. Varied levels of mean expected and total observed heterozygosity were registered at locus level ranging from 0.05 to 0.75. The populations exhibited low mean expected and observed heterozygosity of 0.41 and 0.42 respectively. The mean H_{exp} of these populations was lower than the one observed by Scandura et al. (2008) (0.57) in their study on European wild boars. Similar results were found in Podravlje wild boars by Veličković et al. (2010). Total Het was lower when compared with results from genetic diversity studies in wild boars' by Vernesi et al. (2003) (H_{exp} = 0.662) in Hungary (Budapest, Gemenc and Kab-Hegy), Veličković et al. (2010) (H_{exp} = 0.579) in Podravlje and Ferreira et al. (2009) (0.672) for wild boars in Portugal. The highest heterozygosity in the population was on locus PigSTR 15A (Tot Het=0.75) while least Tot Het was on locus

PigSTR 14B ($H_{obs} = 0.05$). *Table 4* gives a summary of genetic differences between populations.

Table 4 Summary of mean Expected and Total Heterozygosity populations

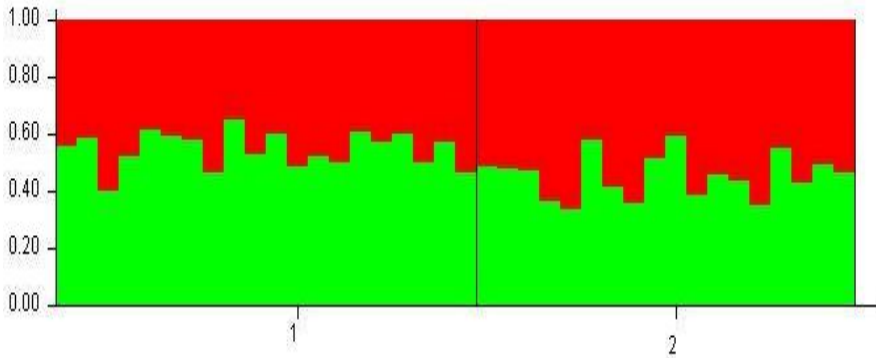
Locus no	Population 1	Population 2	Mean	Sd	Tot Het
PigSTR 14B	0.00	0.11	0.05	0.08	0.05
PigSTR 7B	0.61	0.79	0.70	0.12	0.71
PigSTR 4B	0.38	0.34	0.36	0.03	0.36
PigSTR 4C	0.43	0.58	0.50	0.11	0.53
PigSTR 17A	0.05	0.37	0.20	0.22	0.21
PigSTR 11A	0.41	0.62	0.51	0.15	0.57
PigSTR 14A	0.18	0.25	0.22	0.04	0.21
PigSTR 1B	0.00	0.25	0.13	0.18	0.13
PigSTR 15A	0.72	0.78	0.75	0.04	0.75
PigSTR 5C	0.98	0.56	0.63	0.08	0.64
PigSTR 13E	0.55	0.59	0.55	0.06	0.55
PigSTR 1A	0.22	0.29	0.26	0.04	0.25
Mean	0.35	0.46	0.41	0.08	0.42
Sd	0.25	0.21	0.23	0.4	0.23

Population 1 (Oroszi-Nemesszalók-Kemenespálfa-Veszprémgalsa, North Balaton)

Population 2 (KeceI-Imrehegy, South Balaton)

Genetically based partitions

Bayesian cluster analysis implemented in STRUCTURE software separated the populations into the two genetically meaningful clusters ($K=2$). The number of clusters and the equal possibility of all individual wild boars to belong on either cluster indicates insignificance genetic divergence between the samples and populations as illustrated on *Figure 6*.

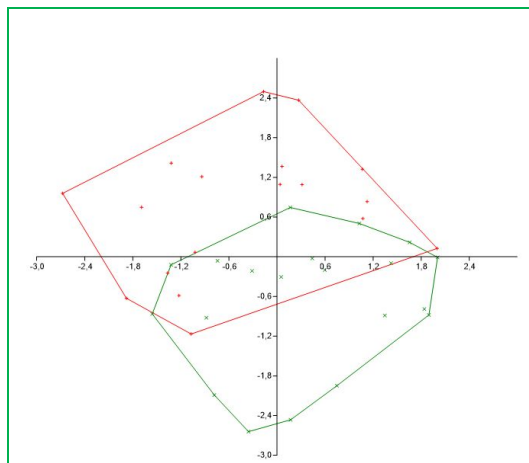


K=2. Population 1: Veszprem (North Balaton), population 2: Kecel-Imrehegy (South Balaton)

Figure 5 Genetic clusters by STRUCTURE software

Analysis of Genetic distance between populations by PAST software

Graphical analysis by PAST v2.17.c software provided an opportunity of analyzing the genetic distance between populations. The results of this analysis confirmed the notable overlap between both wild boar populations. They additionally, suggest that, notwithstanding the genetic similarity, there are some insignificant levels of genetic divergence. The results on *figure 7* should not be misinterpreted that there is a hybrid between the two populations but serves as an evidence of more than average genetic similarity between populations.



Red population: Kecel-Imrehegy (South Balaton), green population: Veszprém (North Balaton)

Figure 6 Illustrative Genetic distance between populations by PAST software

Correlation between genetic and geographical distance

Comparison of pairs of population samples revealed a low pairwise F_{ST} value of 0.066, ($p = 0.05$) indicating that the both populations have insignificant differences. This therefore confirms lack of correlation between genetic and geographical distance.

DISCUSSIONS AND CONCLUSIONS

Within population genetic differences

Both populations exhibited low levels on Observed and expected heterozygosity ($H_{obs} = 0.30$ and 0.48 and $H_{exp} = 0.35$ and 0.45) for North and South of Balaton. The mean Nei's gene diversity within populations was also low (0.35 and 0.48) showing insignificant genetic differences at population level.

Genetic differences between populations

Low Nei's gene diversity difference between populations, Low observed and expected heterozygosity between populations, low mean of total and expected Heterozygosity, structure and past analysis results all indicated insignificant genetic differences between populations.

The total heterozygosity of 0.42 in the present study was not a surprise since studies in several countries in Europe observed a close range of results for the same. For instance, Scandura et al. (2008) $H_{obs} = 0.57$ in European wildboars from several European countries excluding Italy Similarly, Vernesi et al. (2003) and Kusza et al.(2014) in their study grouped Hungarian wild boar samples in one cluster since they did not exhibit genetic differences. However, their observed and expected heterozygosity was higher than in the present study possibly because of the variabilities in sampling size and higher number of sampling sites included in the study.

Correlation between genetic and geographical distance

A low pairwise F_{ST} of 0.066 was detected implying lack of correlation genetic and geographical distances. According to (Scandura et al. 2008), the wild boar, pairwise F_{ST} values range between 0.00 and 0.31 across Europe (including Italy) and ,therefore, are comparable to those observed between wild boar in the present study.

Lake Balaton as a Geographical barrier for gene flow

It is evident, therefore, that Lake Balaton had an insignificant effect on gene flow between the two populations since the results showed that both populations are genetically similar and any differences between them are insignificant. Contrary studies by Velickovic et al. (2012) suggested that River Sava which separate Bosnia from Vojvodina in Serbia and Slavonija in Croatia regions possibly acted as a geographic barrier between wild boars in these two regions. Similarly, in Portugal, rivers Douro and Tejo acted as geographic barriers and divided Portuguese wild boar into three subpopulations: North, South and Central (Ferreira et al. 2009).

Suggestion

The present study results reveal that both populations are genetically similar and are homozygous. There is therefore need to improve and maintain genetic diversity within and between the populations. This could be done by reintroduction of new different genes in both populations. Levels of introgression in domestic pigs should also be studied in order to maintain required levels of diversity within and between these two species.

Since Lake Balaton as a perceived natural barrier did not have effect on gene flow between populations, construction of artificial barrier should be considered. Studies to establish ways in which these populations exchanged genes should also be conducted. According to Vernesi et al. (2003) analysis of microsatellite variation, even if the number of loci is relatively small, appears a very powerful tool to identify the genetic composition not only of populations, but also of single wild boar individuals. However, in future research, new approaches must be taken, to include more individuals and more markers in order to increase accuracy and also allow the assessment of genetic structure for wild boar. Comparison with pigs in study areas should also be considered to understand the levels of introgression and hybridization.

A Balaton, mint földrajzi barrier hatása a génáramlásra vaddisznó (*Sus scrofa*) állományok között Magyarországon

GEORGE WANJALA¹ - MIHALIK BENDEGÚZ^{1,2} - STÉGER VIKTOR² - KUSZA SZILVIA¹

¹ 2Debreceni Egyetem Állatgenetikai Laboratórium, Debrecen

² NAIK, Mezőgazdasági Biotechnológiai Kutatóintézet, Gödöllő

ÖSSZEFOGLALÁS

Annak ellenére, hogy Európában számos kutatás foglalkozik a vaddisznó (*Sus scrofa*) genetikai diverzitásával, a Balaton mint természetes választóvonal génáramlásra gyakorolt hatását eddig még nem vizsgálták. Jelen kutatás célja meghatározni a genetikai különbségek mértékét a Veszprém és Bács-Kiskun megyei állományok közt, valamint megállapítani a Balaton hatását a populációk diverzitására.

Irodalmi adatok alapján leírt és a helyi viszonyokra optimalizált 13 mikroszatellita STR markert használtunk összesen 38 Kecel-Imrehegyi (18), illetve a Balaton északi részén található vadászterületekről (20) származó vaddisznó minta izolálására. Az analízis során 12 lokusz bizonyult használhatónak, amelyből 10 volt polimorf, 0,066-os FST értékkel, valamint alacsony várt és kapott heterozigotizációs-értékkel ($H_{obs}=0.42\pm 0.23$ and $H_{exp}=0.41\pm 0.23$). A Nei-féle genetikai diverzitációs-érték Északon 0,35 Délen pedig 0,4 lett. Az adatok Structure és PAST v2.17.c szoftver segítségével történő elemzése nem mutatott ki szignifikáns különbséget a populációk között.

Az eredményekből megállapítható, hogy a két vizsgált állomány a területeken belül és területek között is megegyezik, tehát valójában egy populációról beszélhetünk. Ez nagyfokú génáramlást jelent a két csoport között annak ellenére, hogy a Balaton és kb. 150 km-es távolság is elválasztja őket egymástól, tehát a Balatonnak nincs érzékelhető hatása a génáramlásra. Az alacsony FST érték azt mutatta, hogy a földrajzi távolság ilyen léptékben nem okozza genetikai különbség kialakulását.

Kulcsszavak: genetikai diverzitás, génáramlás, földrajzi választóvonal

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A szerzők levélcíme - Addresses of the authors:

GEORGE WANJALA

University of Debrecen

4032 Debrecen, Böszörményi street 138.

e-mail: geog.wanjala@gmail.com

Debrecen, Hungary

BENDEGÚZ MIHALIK

NARIC Agricultural Biotechnology Institute

2100 Gödöllő, Szent-Györgyi Albert street 4

e-mail: mihalik.bendeguz@gmail.com

Gödöllő, Hungary

VIKTOR STÉGER

NARIC Agricultural Biotechnology Institute

2100 Gödöllő, Szent-Györgyi Albert street 4

e-mail: steger.viktor@abc.naik.hu

Gödöllő, Hungary

SZILVIA KUSZA

University of Debrecen, Laboratory of Animal Genetics

4032 Debrecen, Böszörményi Street 138.

e-mail: kusza@agr.unideb.hu

Debrecen, Hungary