Romanian Biotechnological Letters
Copyright © 2009 Bucharest University
Romanian Society of Biological Sciences

Vol. 14, No. 6, 2009, pp. 4827-4834

ORIGINAL PAPER

Genetic diversity analyses of seven Romanian pig populations based on 10 microsatellites

Received for publication, October 20, 2009
Accepted, December 20, 2009

MARIA ADINA MANEA, S. E. GEORGESCU, STELIANA KEVORKIAN, MARIETA COSTACHE
University of Bucharest, Molecular Biology Center, 91-95 Splaiul Independentei, 5 Bucharest, Romania, E-mail: adina_manea@yahoo.com, marietacostache@yahoo.com

Abstract

Genotype data from 10 microsatellites were used to assess genetic diversity and relationships among 7 swine breeds: Synthetic Line-345 Peris, Synthetic Line LSP-2000, Pietrain, Large White, Landrace, Mangalitsa and Wild boar. Hardy-Weinberg equilibrium was tested for all breed-combinations and the exact P-value over all loci and breeds was not significant.

Estimates of average observed and expected heterozygosities, and mean number of alleles per locus/population were obtained. A total of 112 alleles were detected, the mean number of alleles ranged from 4.6 to 7.5. The highest observed heterozygosities were found in the Large White breed (0.699) and the lowest in Wild Boar (0.5). The global population differentiation tests showed highly significant ($p<0.01$) results for all 10 loci. Estimation of population subdivision using Wright's F_{ST} index showed that the average proportion of genetic variation explained by breed differences was 9.5%. Neighbour-joining phylogenetic trees based on D_C distances showed that the genetic relationships of the seven breeds studied are consistent with their historical origins: the two Synthetic Lines alongside the Pietrain breed form a cluster, the Large White and Landrace breed are located between Mangalitsa and the Synthetic Lines, meanwhile the Mangalitsa breed is closest to the Wild Boar.

Keywords: Romanian swine breed, microsatellite, genetic diversity, clustering.

Introduction

Modern animal breeding is considered to have begun in the late 18th century and is associated with Robert Bakewell who declared the secret of the "art" to be "in chusing [sic] the best Males to the best Females" (PAWSON [1] 1957). This process was developed extensively across Europe over the next 200 years and is well illustrated in pigs where many breeds were developed to satisfy local requirements, whether on physical characteristics (colour or size) or to meet specific market needs. For these, about 40 different species have been used, and humans have produced some 4 500 breeds. But a high selection pressure also implies a reduction in the effective size of the selected population as the number of mating animals is reduced. Therefore, the price paid for selection efficiency can be a reduction in the genetic variance of selected populations. Today, more than 30% of the breeds used in these selections (known as the world's animal genetic resources, (BARKER J.S.F. [2]) are in danger of extinction.

Mangalitsa is one of the swine breeds in Romania included on the FAO list of endangered species. This breed has a similar origin to other Mediterranean breeds produced at the same time, but originates from the Balkan region where there was less crossing with Asian pigs. The Sumadija pig from the Morava and Sava Valleys and the Syrmia pig from Slavonia are considered to be possible ancestors of the Mangalitsa (MOLDOVEANU G & al., 1944,

[3]). Mangalitsa was introduced into Romania from Serbia in the 19th century (Transylvania - 1833; Oltenia, 1860) (GLIGOR V. & al., 1969 [4]).

A number of studies have already analyzed the genetic diversity of European (LAVAL & al. 2000 [5], FABUEL & al. 2004 [6]) and Chinese (LI & al. 2000 [7], ZHANG & al. 2003 [8]) pig breeds as well as other swine breeds from other countries; however, there is no microsatellite-based study regarding the genetic diversity of swine breeds in Romania. Ciobanu & al. 2001 [9] have undertaken a study regarding the genetic variation of two swine breeds in Romania, based on type I DNA markers, without including microsatellite-based methods in their research. However, microsatellite markers provide a powerful tool to analyze genetic diversity within and between breeds and have been used widely to investigate domestic animals.

In this study we investigate the genetic divergence between seven swine populations: Wild boar (the European ancestor of domestic pigs), Mangalitsa breed (a pig breed that descended directly from wild boar populations), Large White, Landrace and Pietrain pig breeds (the most common breeds used in the European Union) and two Synthetic Lines formed between 1980-2000 in Romania.

This analysis included the distribution within and between breeds of the observed genetic variation, phylogenetic analysis of breed assignment from microsatellite allele frequencies.

Materials and Methods

Sampling and DNA extraction

Fresh blood from the following swine populations was collected: Synthetic Line-345 Peris (LS-345 Peris), Synthetic Line LSP-2000 (LSP-2000), Pietrain (P), Large White (LW), Landrace (L), Mangalitsa (M) and Wild boar (WB). The individuals were chosen at random and we avoided closely related animals. The isolation of genomic DNA from fresh blood was performed with Wizard Genomic DNA Extraction Kit (Promega).

Microsatellite analyses

The animals were genotyped for 11 microsatellite markers (Table 1). These were chosen for their reproducibility, position on the chromosome, polymorphism and absence of null alleles. All the markers belong to the panel recommended by the ISAG-FAO Advisory Committee for genetic distance studies (FAO, 1998). Two multiplex polymerase chain reactions (PCRs) using fluorescently labelled primers were developed: 8plex: SW936, SO228, SO155, SW911, SO355, SW240, SW857, SO10; and 3plex: SW24, SO386, SO005.

The polymerase chain reaction was performed in a 25µl final volume with 50 ng template DNA and 2.5 µl PCR Buffer, 1.5mM MgCl2, 0.8mM dNTP, 0.23-0.48µM from each primer, 1.5-2.5U AmpliTaqGold DNA Polymerase (Applied BioSystems). Thermal cycling conditions in GeneAmp PCR System 9700 (Applied Biosystems) included an initial denaturation for 10 min at 95°C, followed by 34 cycles of 30s at 95°C, 30s at 60°C 1 min at 72°C, and a final extension step of 72°C for 60 min.

For genotyping the samples, PCR products were combined and detected by capillary electrophoresis using an ABI Prism 310 DNA Genetic Analyzer (AppliedBiosystems). The size of alleles was determined by using GeneScan-500 LIZ Size Standard and the results were processed with the GeneScan® 3.1.2 and Genotyper® 2.5.2 Softwares (AppliedBiosystems). The French PigMap reference DNAs F9110010 and F9119912 (INRA online: http://www.toulouse.inra.fr/ lgc/pig/panel/controlgeno.htm) were used to calibrate the fragment sizes. Since one of the markers (SW24) was not amplified in all the animals tested,

we preferred to remove it from the assays, thus only 10 microsatellites were used for the subsequent analyses.

Table 1. The panel of microsatellites used in the analysis (ROHRER G.A. & al. 1996 [10]).

Microsatellite loci	Chromosome	Size obtained (bp)						
		LS-345 Peris	LSP-2000	P	LW	L	M	WB
SW936	15q2.5	94-114	90-114	94-114	90-110	88-114	92-110	90-108
SO155	9	148-164	148-164	148-162	148-164	148-164	148-160	148-164
SO228	6	244-270	248-270	248-270	248-270	248-270	248-258	252-258
SW911	9p2.2	152-164	154-166	146-166	150-166	152-164	154-166	162-166
SO355	15	244-270	244-270	244-272	244-272	244-270	244-272	244
SW240	2	90-110	90-116	90-116	90-112	88-112	92-112	94-110
SW857	14q2.1-q2.2	138-156	138-156	138-156	138-156	138-158	142-156	148-154
SO101	7	206-212	206-212	208-212	194-212	194-210	190-212	202-212
S0386	11	154-188	172-186	176-180	154-188	158-180	176-190	170-188
S0005	5	204-250	202-244	222-244	202-246	202-244	208-238	206-240

Statistical analysis

The allelic frequencies, observed and expected heterozygosities (Ho and He) were estimated using the CERVUS 2.0 program (MARSHALL & al. 1998 [11]).

The probability test approach described by Guo and Tomson (1992 [12]) and implemented in the GENEPOP software (ROUSSET & al. 2007 [13]) was employed to test for HW equilibrium. The HW test for each locus in each population and the hypothesis that all four swine breeds are significantly distinguishable on the basis of genic and genotypic differentiation was also tested. The exact P-values were calculated either by the complete enumeration method [LOUIS & al. 1987 [14])] (loci with fewer than five alleles) or otherwise by the Markov-Chain algorithm (with 1000 dememorization steps for 100 batches and 5000 iteration per batch).

To determine the genetic variation within and between breeds, we used the fixation indices of Wright (F_{ST}, F_{IS}, and F_{IT}). We calculate also the pairwise F_{ST} using the program FSTAT (GOUDET & al. 1995 [15]).

Phylogenetic analysis was performed using the PHYLIP software package version 3.5 (FELSENSTEIN & al. 1989 [16]). Cavalli-Sforza's chord distance D_C (Cavalli- Sforza and Edwards, 1967 [17]) was calculated from allele frequencies, and a dendrogram was constructed using the Neighbor-Joining method. 1000 bootstrap samples were generated with the Seqboot program in order to evaluate the robustness of the tree topology.

Results and discussions

Genetic variance and HWE

A total of 112 different alleles were detected for all the 10 analyzed microsatellites.

Table 2. The number of alleles per locus in each population.

Locus	LS-345 Peris	LSP-2000	P	LW	L	M	WB	TOTAL
SW936	8	9	7	8	6	7	4	12
SO155	7	7	6	6	5	4	4	8
SO228	6	5	6	6	5	6	4	9
SW911	4	4	6	5	3	5	3	9
SO355	6	6	6	7	6	6	1	9
SW240	8	7	6	8	7	9	5	14

SW857	8	9	7	7	7	7	4	10
SO101	4	4	3	5	4	4	5	7
S0386	9	7	3	8	5	7	8	13
S0005	11	13	6	14	8	8	8	21

The most polymorphic marker was SO005 with 21 alleles in total while the SO101 microsatellite was the least polymorphic with only 7 alleles in total (Table 2). Considering the polymorphism of the microsatellites per population, in the case of the wild boar population all 10 microsatellite markers display a low level of polymorphism, and one of them SO355 is actually monomorphic.

Table 3. Observed (H_O) and expected (H_E) heterozygosities and the mean number of alleles (MNA) over 10 microsatellites.

Population	**H_O**	**H_E**	**MNA**
Synthetic Line-345 Peris (LS-345 Peris)	0.68±0.115	0.723±0.115	7.2±2.25
Synthetic Line LSP-2000 (LSP-2000)	0.674±0.122	0.728±0.122	7.1±2.726
Pietrain (P)	0.65±0.283	0.765±0.121	5.6±1.43
Large White (LW)	0.699±0.141	0.746±0.083	7.5±2.592
Landrace (L)	0.626±0.159	0.706±0.083	5.7±1.337
Mangalitsa (M)	0.651±0.138	0.616±0.138	6.3±1.636
Wild boar (WB)	0.5±0.286	0.591±0.267	4.6±2.117

The observed and expected hetergozygosities and the mean number of alleles (MNA) together with their standard deviations are displayed in table 3. Observed and expected heterozygosities per breed ranged from 0.5 and 0.591 (WB) to 0.699 (LW) and 0.765 (P) respectively. Markers to be useful for measuring genetic variation, they should have an average heterozygosity between 0.3 and 0.8 in the population (Takezaki and Nei (1996)[18]). The range of heterozygosity of the markers in the 7 populations analyzed in this study was between 0.5 and 0.699, and therefore the markers were appropriate for measuring genetic variation. Although it varies among populations, the observed mean heterozygosity was lower than the expected mean heterozygosity for all the populations.

HWE was tested for all breed-combinations. The exact P-values over all loci and breeds were not significant.

F-statistics

Breed differentiation was shown by fixation indices (F_{IT}, F_{IS}, and F_{ST}) (Table 4). The F_{ST} per locus varied from 0.051 (SW857) to 0.141 (SO101) and the average F_{ST} of all loci was 0.095. These implied that 90.5% of the genetic variation lay within breeds, and only 9.5% between breeds. On average, each of the seven breeds had a 6.2% deficit of heterozygotes, whereas the entirety of individuals had a 15.3% deficit of heterozygotes. This 9.5% genetic variation observed among the swine breeds in Romania is higher than the one observed in the case of indigenous swine breeds in China (7%, S-L YANG & al., 2003 [19]) but lower than the genetic variations observed in the case of swine breeds in Europe (27%, G. LAVAL & al., 2000 [5]). By comparison to other farm animal breeds, this value is higher than the variation observed in the case of some bovine breeds (7%, GEORGESCU & al., 2009 [20], CANON & al. 2001 [21]), and for Spanish donkey breeds (3.6%) (ARANGUREN-MENDEZ & al. 2001 [22] and it is nearly equal to that observed in the case of dogs (9.9%) (JORDANA & al. 1992 [23]; but lower than the 12% value obtained for Norwegian horse breeds, (BJØRNSTAD G &

al., 2000 [24]), 17% for goats (SAITBEKOVA N. & al.,1999 [25]) and 10-20% observed for humans [CAVALLI-SFORZA L. L & al., 1994 [26]

Table 4. F statistical estimates and their significances by locus.

Locus	**F_{ST}**	**F_{IS}**	**F_{IT}**
SW936	0.073	0.15	0.213
SO155	0.132	-0.072	0.07
SO228	0.076	0.03	0.104
SW911	0.135	-0.028	0.111
SO355	0.078	0.045	0.119
SW240	0.116	0.157	0.255
SW857	0.051	0.044	0.092
SO101	0.141	-0.003	0.139
S0386	0.064	0.279	0.326
S0005	0.083	0.023	0.104
Mean	**0.095**	**0.062**	**0.153**

The global population differentiation tests (genic and genotypic) showed highly significant ($p < 0.01$) results for all 10 loci. Table 5 shows the F_{ST} values for pairs of breeds (above the diagonal). Genic differentiation values among breeds range from 1.86% for LSP-2000- LS-345 pair to 20.3% for the WB-L pair (table 5). Highly significant ($p < 0.01$) genetic differences for all breeds combinations were shown through pairwise tests.

Breed relationships

A Neighbor-Joining tree (Fig. 1) of the seven pig breeds was constructed using Cavalli-Sforza's chord distances, D_C (1978) (Table5, below the diagonal) based on the 10 microsatellite loci data. The node numbers are bootstrapping values for 1000 replicates of the 10 loci genotyped.

Table 5. Fst estimates compared in pairs (above diagonal) and Cavalli-Sforza's chord distances D_C (below diagonal).

	LS-345	**LSP-2000**	**P**	**LW**	**L**	**M**	**WB**
LS-345		*0.018*	0.058	0.056	0.057	0.094	0.192
LSP-2000	*0.034*		0.025	0.057	0.055	0.113	0.169
P	0.067	0.046		0.067	0.067	0.133	0.203
LW	0.056	0.056	0.084		0.036	0.105	0.176
L	0.083	0.085	0.097	0.069		0.123	**0.203**
M	0.107	0.111	0.133	0.094	0.130		0.155
WB	0.198	0.173	**0.219**	0.170	0.199	0.146	

The D_C distance ranged from 0.034 (pair LS-345-LSP-2000) to 0.219 (pair P-WB) (Table 5).

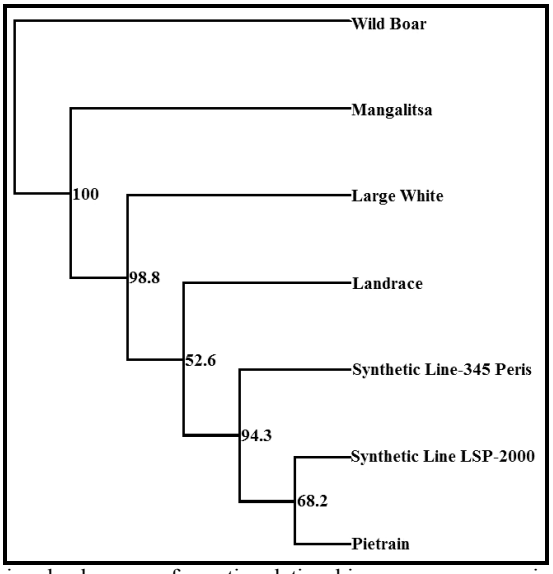

Figure 1. Neighbour-joining dendrogram of genetic relationships among seven swine breeds using D_C genetic distances based on 10 microsatellite loci. The numbers on the nodes are percentage bootstrap values in 1000 replications.

Takezaki and Nei [18] compared various measures of genetic distance used for the reconstruction of phylogenetic trees from microsatellite frequency data and showed that the accuracy of the Cavalli-Sforza and Edwards chord distances (D_C) and Nei D_A (1978) distance were generally higher than the other distances whether a bottleneck effect existed or not.

The dendrogram obtained by the Neighbour-joining method based on the Cavalli-Sforza distances illustrate bootstrap values between 52.6 and 100% (Figure 1). This dendrogram confirms the phylogenetic relationships among the analyzed swine breeds. Thus, the two Synthetic Lines together with the Pietrain breed form a distinct cluster with a 94.3 bootstrap value. The formation of the Synthetic Line-345 Peris breed included the participation of three different swine breeds: Landrace Belgian, Duroc and Hampshire; and Synthetic Line LSP-2000 was formed by crossing swines from the Synthetic Line-345 Peris with swines of the Pietran breed, and it is genetically closer to the Pietran than to Synthetic Line-345 Peris, according to our study. Thus, these breeds are very close to each other from a genetic point of view, which has been confirmed by our study, since they form a distinct cluster in the dendrogram obtained.

The fact that the Great White and Landrace breeds were formed before the Synthetic Lines but after the Mangalitsa breed can be clearly confirmed by the dendrogram. The Great White breed was formed in Yorkshire County, England, but the history is difficult to trace; the first records regarding this breed date back to 1831. In the case of the Landrace breed, its development began around 1895 and it resulted from crossing the Large White hog from England with the native Danish swine. Thus, the dendrogram obtained confirms the origin of these two breeds: the Great White is closer to the wild boar than the Landrace breed.

With a bootstrap value of 100%, the Mangalitsa breed is the closest breed among the studied breeds of swine to the wild variety. The Mangalitsa is one of the old type breeds, originating several centuries ago as a result of crossing between European and Asian primitive pigs.

The Mangalitsa was much favored as a bacon and lard producer. Our eating habits have changed; now hams and cutlets are in demand; thus, currently this old breed is endangered and it is included on the FAO list.

Conclusions

This study is the first to apply a panel of microsatellite markers for the genetic characterization of pig breeds from Romania. The markers used were selected according to their genome wide spanning, polymorphism information content values, and precise genotyping.

It was possible to describe genetic differentiation and establish a clear cut genetic structuring among the populations studied, including commercial breeds (Large White, Landrace, Pietrain), old breeds (Mangalitsa), Synthetic Lines (Synthetic Line-345 Peris, Synthetic Line LSP-2000) and the Wild Boar. The data obtained clearly confirm the ancestral origin of the Mangalitsa breed as well as the phylogenetic relationships existing amond the swine breeds analyzed.

This study contributes to the knowledge of the genetic structure and molecular characterization of pig population from Romania, one of them potentially threatened by extinction (Mangalitsa). It also shows how microsatellites can be used to establish the genetic relationships between populations providing reasonable statistical power for breed assignment, regardless of whether they are closely related or not, allowing their future management to be based on a greater knowledge of genetic structuring and relationships between populations.

Acknowledgments

The authors would like to thank Denis Milan, INRA, France, for the French PigMap reference DNAs disposal.

References

1. H.C. PAWSON Robert Bakewell (Pioneer Livestock Breeder). Crosby Lockwood, London. (1958).
2. J.S.F. BARKER Conservation of livestock breed diversity, Anim. Genet. Resource Inform., **25**, 33-43, (1999).
3. MOLDOVEANU G., FISTEAG I., Suicultura, Faculty of Veterinary Medicine Press,
Bucharest, 1944.
4. GLIGOR V., RADU A., STANCIULESCU M., Zootehnia Romaniei - porcine, Editura Academiei, Bucuresti, 1969.
5.LAVAL, N. IANNUCCELLI, C. LEGAULT, D. MILAN, M. A. GROENEN, E. GIUFFRA, L. ANDERSSON, P. H. NISSEN, C. B. JORGENSEN, P. BEECKMANN, H. GELDERMANN, J. L. FOULLEY, C. CHEVALET, and L. OLLIVIER. Genetic diversity of eleven European pig breeds**.** Genet. Sel. Evol., **32**, 187–203, (2000).
6.E. FABUEL, C. BARRAGAN, L. SILIO, M. C. RODRIGUEZ, and M. A. TORO, Analysis of genetic diversity and conservation priorities in Iberian pigs based on microsatellite markers. Heredity, **93**, 104-113, (2004).
7.K. LI, Y. CHEN, C. MORAN, B. FAN, S. ZHAO, and Z. PENG, Analysis of diversity and genetic relationships between four Chinese indigenous pig breeds and one Australian commercial pig breed. Anim. Genet., **31**, 322-325, (2000).
8.ZHANG G. X., Z. G. WANG, F. Z. SUN, W. S. CHEN, G. Y. YANG, S. J. GUO, Y. J. LI, X. L. ZHAO, Y. ZHANG, J. SUN, B. FAN, S. L. YANG, and K. LI. Genetic diversity of microsatellite loci in fifty-six Chinese native pig breeds. Yi Chuan Xue Bao, **30**, 225-233 (2003).

9.CIOBANU D. C., DAY A. E., NAGY A., WALES R., ROTHSCHILD M. F., PLASTOW G. S. Genetic variation in two conserved local Romanian pig breeds using type 1 DNA markers. Genet. Sel. Evol., **33**, 417-432, (2001).
10.ROHRER G.A., ALEXANDER L.J., HU Z., SMITH T.P.L., KEELE J.W.; BEATTIE C.W. A comprehensive map of the porcine genome, Genome Res., **6**, 371-391, (1996).
11.MARSHALL T. C., SLATE KRUUK L., PEMBERTON J. M., Statistical Confidence For Likelihood-Based Paternity Inference In Natural Populations. Mol. Ecol., **7,** 639-655, (1998).
12.GUO, S. W. and THOMPSON E. A. Performing the Exact Test Of Hardy-Weinberg Proportion for Multiple Alleles. Biometrics, **48**, 361-372, (1992).
13.ROUSSET F., GenePop007: A Complete Reimplementetion of the GENEPOP Software for Windows and Linux. Mol. Ecol., **8**, 103-106, (2007).
14.LOUIS E. J., DEMPSTER E. R., An Exact Test for Hardy-Weinberg and Multiple Alleles. Biometrics, **43,** 805-811, (1987).
15.GOUDET, J., FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. J. Hered., **86,** 485-486, (1995).
16.FELSENSTEIN, J., PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics, **5**, 164-166, (1989).
17. CAVALLI-SFORZA L.L. & EDWARDS A.W.F. (Phylogenetic analysis: models and estimation procedures. American Journal of Human Genetics **19**, 233–257, 1967).
18. TAKEZAKI N, NEI M. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA, Genetics., **144(1)**, 389-99, 1996.
19. S-L YANG, Z-G WANG, B LIU, G-X ZHANG, S-H ZHAO, M YU, B FAN, M-H LI, T-A XIONG and K LI Genetic variation and relationships of eighteen Chinese indigenous pig breeds, Genet. Sel. Evol. **35**, 657-671, (2003).
20. S. E. GEORGESCU, MANEA M A, ZAULET M, COSTACHE M Genetic diversity among Romanian cattle breeds with a special focus on the Romanian Grey Steppe Breed, Romanian Biotechnological Letters **14**, 4194-4200, (2009).
21. CAÑÓN J., ALEXANDRINO P., BESSA I., CARLEOS C., CARRETERO Y., DUNNER S., FERRAN N., GARCIA D., JORDANA J., LALOË D., PEREIRA A., SANCHEZ A., MOAZAMI-GOUDARZI K., Genetic diversity measures of local European beef cattle breeds for conservation purposes. Genet. Sel. Evol., **33**, 311-332, (2001).
22. ARANGUREN-MENDEZ J., JORDANA J., GOMEZ M., Genetic Diversity in Spanish Donkey Breeds Using Microsatellite DNA Markers. Genet. Sel. Evol. **33,** 433-442, (2001).
23. JORDANA J., PIEDRAFITA J., SANCHEZ A., PUIG P., Comparative F Statistics Analysis of the Genetic Structure of Ten Spanish Dog Breeds. J. Hered., **83,** 367-374, (1992).
24. BJØRNSTAD G, GUNBY. E., RØED K. H. Genetic Structure of Norwegian Horse Breeds. J. Anim. Breed. Genet., **117**, 307-317 (2000)
25. SAITBEKOVA N., GAILLARD C., OBEXER-RUFF G., DOLF G. Genetic Diversity in Swiss Goat Breeds Based on Microsatellite Analysis. Anim. Genet., **30,** 36-41, (1999)
26. CAVALLI-SFORZA L. L, MENOZZI P., PIAZZA A. The History And Geography Of Human Genes. Princeton University Press, Princeton, NJ. USA. (1994)