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MOLECULAR GENETIC POLYMORPHISM OF AMERICAN MINK POPULATIONS (*NEOVISON VISON*) IN MODEL FUR FARMS AND ON THE ADJACENT TERRITORIES IN BELARUS

Abstract. American mink (*Neovison vison*) is both a problematic invader and an economically valuable fur breed species in Belarus. The increasing scale of mink breeding for fur and the growing impact of this invasive species on the local ecosystems requires sound, novel strategies for managing both farm and wild populations. Containment of wild American mink populations under the effects of possible migration from the fur farms could be especially problematic. With that issue in mind, we have used microsatellite analysis todetermine the genetic polymorphism in two color breeds of farm-bred American mink in the populations of several Belorussian model fur farms, as well as the same characteristics for the feral populations on the territories adjacent to said fur farms. We confirm the presence of effective influx of mink into the wild from fur farms, determined through the means of analyzing microsatellite genotype data of feral and farm populations.

Keywords: American mink, microsatellites, genetic structure, genetic polymorphism, invasive species, Belarus

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МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКИЙ ПОЛИМОРФИЗМ ПОПУЛЯЦИЙ АМЕРИКАНСКОЙ НОРКИ (*NEOVISON VISON*) В МОДЕЛЬНЫХ ЗВЕРОВОДЧЕСКИХ ХОЗЯЙСТВАХ И НА ПРИЛЕГАЮЩИХ К НИМ ТЕРРИТОРИЯХ В БЕЛАРУСИ

Аннотация. Американская норка (*Neovison vison*) в Беларуси является одновременно проблематичным инвазивным и экономически значимым хозяйственным видом. Растущие масштабы хозяйственного разведения на мех и увеличивающееся воздействие этого вида на локальные экосистемы требует принятия надежной и последовательной стратегии менеджмента его диких и хозяйственных популяций. Этот подход поднимает проблематику контроля диких популяций американской норки в условиях потенциальной миграции особей в дикую среду из звероводческих хозяйств – встает вопрос наличия факта побегов особей в дикую среду в Беларуси. С целью разрешения этого вопроса мы осуществили генетический анализ двух хозяйственных популяций американской норки, а также диких групп на сопряженной со зверохозяйствами территории, использовав в качестве генетических маркеров микросателлиты, чем подтвердили наличия эффективного потока особей в дикую среду из зверохозяйств, а также привели характеристики генетического полиморфизма для рассматриваемых популяций американской норки.

Ключевые слова: американская норка, микросателлиты, генетическая структурированность, генетический полиморфизм, инвазивные виды, Беларусь

Для цитирования. Молекулярно-генетический полиморфизм популяций американской норки (*Neovison vison*) в модельных звероводческих хозяйствах и на прилегающих к ним территориях в Беларуси / А. А. Волнистый [и др.] // Докл. Нац. акад. наук Беларуси. – 2020. – Т. 64, № 6. – С. 685–693 (in Russian). https://doi.org/10.29235/1561-8323-2020-64-6-685-693

Introduction. The American mink (*Neovison vison*, Schreber, 1777) is currently considered as both an economically-valuable and an invasive species in Belarus. Its invasion is sourced to multiple introduction pathways – including artificial releases of farm breeds into the wild in mid XX century, eastward spreading of feral populations from Poland, Ukraine and Baltic countries, and possible escapees from artificial fur farm populations [1].

Across Europe, the American mink (here and further on «*N. vison»)* is classified as invasive species threatening the biodiversity and stability of variouszoocenoses and preservation of multiple threatened species [2]. The most damning example being the role played by *N. vison* in the near extinction of the European mink *Mustela lutreola* (Linnaeus, 1761), the latter process being directly tied to uncontrolled spreading of *N. vison* [3–7]. It also acts as a carrier and natural reservoir for a number of diseases dangerous to the endemic wildlife. At the same time *N. vison* is an economically significant in Belarus, currently bred on multiple farms across the country for its valuable fur – a trade that shows no signs of declining locally in the foreseeable future.

This raises the concern of effective and ecologically-conscious breeding and management of this species in Belarus – presenting the perspective of controlling growth and expansion of *N. vison* populations in the wild for the sake of limiting its effects on the endemic fauna, all while carrying out structured, sustainable and effective mink fur breeding using novel approaches, such as genetic population management. A huge number of earlier studies on multiple other localities have, through the means of molecular genetic analysis (primarily STR (short tandem repeat) analysis), confirmed the source of feral American mink populations in fur farm escapees or their significant role in preventing efficient management of the wild populations [8; 9], but there is currently no clear consensus neither on the very existence of fur farm escapees among the feral populations of *N. vison* in Belarus in any significant numbers, nor on their capability to survive in localnatural environments and bolster the numbers of feral populations and serve as the origin of new wild populations.

In this context, we have established this study's goals as determining the extent of genetic polymorphism for several color breeds of *N. vison* in model fur farms, and using the method and data to check the feral American mink populations on the adjacent territories for presence of genetic influx from the fur farms to determine if there is a continuous source of escapees which effectively adapt to the natural environments and reproductively contribute to the feral populations.

The characteristics of genetic polymorphism in artificial populations can be effectively used for controlled and sustainable breeding [10]. Establishing a method for molecular genotyping and genetic population analysis that is valid in relation to the local populations of the particular species serves to develop foundations for perspective efforts and applied measures towards more controlled breed maintenance and specimen exchange in breeding, as well as rational and effective population management for the invasive species in the wild. Verifying the presence of genetic influx into the feral *N. vison* populations from the fur farms serves to determine the optimal strategies and measures for management and control of this invasive species in Belarus [7; 11].

Here, we report successfully using STR analysis for genotyping two model fur farm populations of two colour breeds, as well as the wild populations inhabiting the territory adjacent to one of the said fur farms. We have obtained the characteristic of genetic polymorphism for the populations in question, and confirmed the presence of a significant genetic input of fur farm escapees in the wild population bordering the abovementioned fur farm.

Materials and methods. The study was conducted using the SSPA «SPC NAS of Belarus on Bioresources»'s bank of genetic samples, including samples gathered over the course of this study. We have focused our efforts on sampling two model fur farms – the Grodno fur farm in Strievka village of the Grodno Region, and Kalinkovichi fur farm in the Homiel region. This choice of study populations was based on the geographic factor – one fur farm was located in close vicinity with the sources of our main bulk of current wild specimen samples, while the other one was significantly distant from it, while both fur farms use the same source of breeding stock from one fur farm in Denmark. We were also interested in including at least two different colour breeds in the study. During the sampling we have ensured that all samples were individual, that the used wild specimen samples were gathered from individuals that conformed to the wild-type coloration characteristic of *N. vison* to prevent mistake introduced

by possible rare original escapees that did not verifiably mix with the local wild population. We also ensured, to the best of our ability, that wild individuals were not used in the breeding process on farms to prevent the mistake on that end. The primary collection included 110 samples, majority of them being ethanol-preserved muscle tissues from hunted wild individuals and slaughtered fur farm individuals. A total of 37 samples, all of them fecal, were excluded from the study due to their insufficient DNA quality, which surfaced on the stages of DNA extraction, amplification and genotyping. Our final collection for the analysis included 73 individual samples: 18 individuals from the wild populations, designated as a single «Wild» groupin further analysis; 25 individuals from the Kalinkovichi fur farm of the «Brown» colour breed, designated as «Kalinkovichi Brown» group; 28 white individuals from Grodno fur farm, designated as «Strievka White» group, and 2 pearl individuals from Grodno fur farm, which were processed completely but largely excluded from the analysis results due to insufficient sample size. The samples are mapped in Fig. 1.

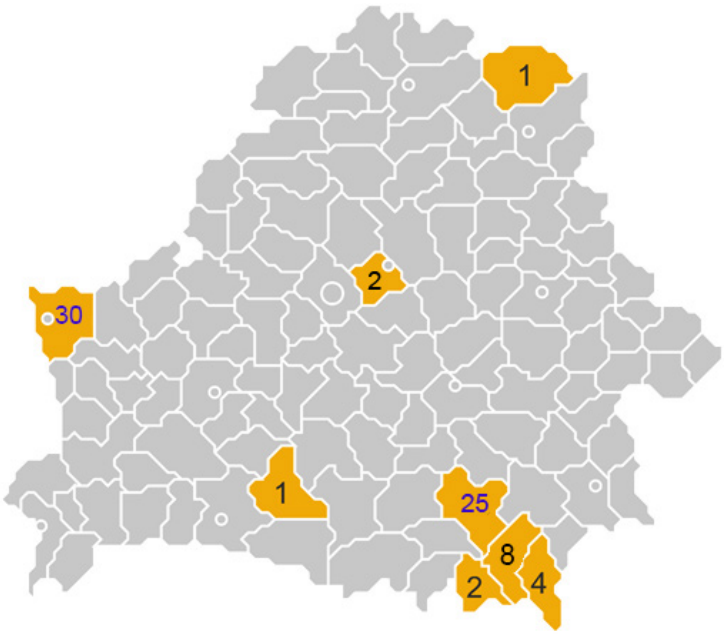

Fig. 1. Geographic distribution of *N. vison* samples used in this study across administrative districts of Belarus. Values reflect the number of used individual samples from that district. Values specifically in purple mark fur farm samples

While awaiting processing, the samples were preserved in 96 % ethanol, and stored in cryogenic conditions at ≈ –80 °C. Extracted DNA awaiting further processing was stored in Tris-EDTA elution buffer at ≈ –80 °C, with measures taken to minimize excessive freeze-thaw cycles.

DNA extraction from muscle tissue samples was performed using the «АртДНК» DNA extraction kit (ArtBioTekh, Belarus) according to manufacturer's instructions, with the lysis staged supplemented with additional 6 units of proteinase K (Primetech, Belarus), ending with a spectrophotometric DNA concentration measurement.

Fecal samples underwent DNA extraction with QIAmp DNA Stool Mini Kit (QIAGEN, Germany) following the manufacturer's protocol. Thermo Scientific IEC MicroCL 21R microcentrifuge, Biosan TDB-120 solid-body thermostatand Implen P330 nano spectrophotometer were employed in carrying out the procedures.

The microsatellite loci amplified for the analysis are listed in Table 1. Fifteen amplifications were joined into seven duplex reactions and a single uniplex reaction, carried out in a standard fashion for each of the 73 individual samples.The reaction arrangement was performed using the «Multiplex manager» software.

Primer panel was picked out of polymorphic loci successfully used in past studies, chosen according to the principles that would minimized introduced bias. Primers sequences were taken from the sources

T a b l e 1. **List of STR loci employed for the microsatellite analysis of *N. vison* individuals, and the loci characteristics: «MP #» – the designated multiplex reaction number, «Ta» – primer annealing temperature determined through individual gradient PCR reactions, in °C, weight range is specified in base pairs and includes literature data expanded by outlier cases encountered in the study**

MP #	Locus ID	Repeat motif	Range, b. p.	Ta, °C	Source
1	Mvis 099	CA	300–360	60	[12]
	Mvi 586	GT	134–154	60	[13]
2	Mvi 4060	ACAAA	200–302	60	[14]
	Mvis 075	CA	90–140	60	[12]
3	Mvi 1302	GT	210–234	64	[15]
	Mvi 1321	CA	91–107	63.5	[15]
4	Mvi 111	GT	90–115	55	[13]
	Mvi 219	GC	164–180	52	[13]
5	Mvi 4001	(GTTTTT)2(TG)	225–236	60	[14]
	Mvi 1341	CA	148–176	59	[15]
6	Mvi 87	GC	76–123	56	[13]
	Mvi 232	GC	139–164	58	[13]
7	Mvi 114	CA	77–92	55	[13]
	Mvis 020	CA	170–190	60	[12]
8	Mvi 192	CA	130–145	60	[15]

according to table 1. Primers were synthesized by «Primetech» company (Belarus). OPC-purified primers were used. Direct primers were labeled with a By5 (Cy5 analogue) fluorescent dye on the 5′ end. The optimal annealing temperatures and primer concentrations for each locus were determined experimentally in individual gradient PCR reactions, checked via ethidium bromide stained AGE. For the non-matching Ta's in a single duplex, the lowest Ta in a duplex was used. The final rection were performed in 25 µl reactions of: 1X ammonium sulphate buffer; 0,2 mM of dNTPs, 1 unit of Taq polymerase (Primetech); 3 mM of $MgCl_2$; 100 pmol of each primer; 5–40 ng of DNA template; and mQ water.

The protocol template for the amplification performed as follows: 5 min 95 °C; 40X cycles of 30 sec 95 °C, 30 sec Ta according to table 1 and 45 sec 72 °C; 15 min final extension at 72 °C; cooldown to 4 °C.

The amplifications were carried out using Biorad C1000 Touch amplifier.

The resulting PCR products were checked via ethidium bromide stained AGE in a 1.5 % agarose gel, employing 100 V current, a 15 cm horizontal phoresis chamber, pH 8,0 sodium borate buffer and Biorad MiniGel gel-documentation system.

Genotyping was performed via automatic laser fluorescence detection capillary PAGE using Beckman Coulter GeXP genetic analysis system employing reference size standard 600 and other standard consumables for the system. Raw data was analyzed using Fragment Analysis Tool from the GenomeLab GeXP Software Package v10.2 with manually optimized manufacturer settings and automatic peak detection to obtain individual allele sizes for each loci. Automatically detected peaks were checked manually. After the analysis was complete, 5 random individual samples were picked for a repeat genotyping to confirm reproducibility.

Data analysis. The individual allele size data was grouped into 4 population (n = 18; n = 25; n = 28; n = 2) units corresponding to their source as mentioned above.

Allele size binning was performed using TANDEM v 1.09 software. Binned data was converted for diverging analysis formats using CONVERT v 1.3.1 software and analyzed for null alleles and spurious peaks using Micro-Checker v 2.2.

Cluster analysis was performed using STRUCTURE 2.3.4 with the following setting: admixture model population, IDs used as geographic localities; infer α; prior α = 1,0; individual α for each population; equal prior α for every population; upper α threshold = 10,0; standard deviation for updated α values = 0,025; correlating allele frequencies between populations; infer varying Fst value for different subpopulations; prior median probability of Fst value for populations = 0,01; prior standard deviation of

T a b l e 2. **Genetic polymorphism characteristics of studied Belarussian *N. vison* populations according to microsatellite data**

Locus	Population																	
	Wild						Strievka White						Kalinkovichi Brown					
	HWE	Na	Ar	Fis	Ho	He	HWE	Na	Ar	Fis	Ho	He	HWE	Na	Ar	Fis	Ho	He
Mvi 1302	0.0000	6	4.6	0.4720	0.2941	0.5490	0.0000	12	8.3	0.2184	0.6296	0.8022	0.0960	10	7.5	0.2011	0.6364	0.7928
Mvi 219	0.0260	5	4.3	0.1375	0.5882	0.6791	0.9860	6	6.0	0.1318	0.7143	0.8208	0.9810	19	14.4	–0.0213	0.9600	0.9404
Mvi 1321	0.9200	7	6.5	–0.1223	0.9444	0.8444	0.1000	8	6.3	0.2486	0.5556	0.7359	0.0760	9	7.4	0.1472	0.6667	0.7793
Mvi 4060	1.0000	2	1.7	0.0000	0.0555	0.0556	NA	1	1.0	NA	NA	NA	NA	1	1.0	NA	NA	NA
Mvis 099	0.0000	11	8.2	0.4983	0.4615	0.9015	0.5630	10	8.1	0.0157	0.7857	0.7981	0.6860	10	7.5	–0.0939	0.8333	0.7633
Mvi 114	0.0240	9	7.0	–0.0193	0.7777	0.7635	0.0140	6	4.8	0.3994	0.4286	0.7084	0.2140	5	4.5	0.0417	0.7083	0.7385
Mvi 4001	0.0800	6	4.6	0.1429	0.3750	0.4355	0.0000	7	5.4	0.4841	0.3214	0.6175	0.7660	5	4.5	0.1570	0.6500	0.7680
Mvi 1341	0.0970	7	6.2	–0.0467	0.8235	0.7879	0.9960	9	7.3	–0.0649	0.8571	0.8058	0.0810	8	6.7	0.1709	0.6087	0.7314
Mvi 586	0.0100	7	6.1	0.3411	0.5555	0.8349	0.0170	9	7.0	–0.1015	0.8889	0.8085	0.8970	8	6.7	–0.1743	0.9583	0.8192
Mvi 192	0.2260	9	7.7	–0.0387	0.8333	0.8032	0.0000	9	7.0	0.2489	0.6296	0.8344	0.1450	8	6.5	0.2653	0.5455	0.7378
Mvi 111	0.0100	8	6.9	0.1299	0.7222	0.8270	0.9970	6	5.5	–0.1262	0.7143	0.6357	0.1180	7	4.6	0.1365	0.4583	0.5293
Mvis 020	0.0000	8	6.3	0.6958	0.2222	0.7159	0.0000	7	6.2	0.4343	0.3929	0.6890	0.0000	7	4.8	0.9338	0.0500	0.7372
Mvi 232	0.1610	6	5.1	0.0685	0.5555	0.5952	0.9030	6	5.6	–0.0847	0.8214	0.7584	1.0000	7	5.9	–0.1134	0.7917	0.7128
Mvis 075	0.3400	7	6.6	–0.0730	0.8888	0.8302	0.1660	10	7.0	0.0494	0.7407	0.7785	0.9910	8	7.4	–0.0813	0.9200	0.8522
Mvi 87	0.0000	7	5.8	0.7807	0.1666	0.7429	0.9950	4	4.0	0.1489	0.6429	0.7533	0.0000	5	4.4	0.9458	0.0417	0.7527
Mean	0.0000	7.0	5.8	0.1787	0.5510	0.6910	0.0000	7.8	6.0	0.1190	0.6516	0.7533	0.0020	8.3	6.3	0.1531	0.6306	0.7610

N o t e s: HWE – *p*-value for Hardy–Weinberg disequilibrium, values < 0.05 are highlighted with bold type; Na – number of alleles; Ar – allelic richness; Fis – Wright's inbreeding coefficient; Ho – observed heterozygosity; He – expected heterozygosity.

Fst value for populations = 0,05; Markov Chain dememorialization steps = 20000; MCMC iterations = 50000; K range from 1 to 6, with 20 iterations for every K value.

AMOVA and genetic polymorphism characteristics were calculated in Arlequin 3.5 with default settings. Determining of deviation from HWE (Hardy-Weinberg Equilibrium) and calculation of inbreeding coefficient were carried out with diveRsity 1.9.90 for R. Factorial correspondence analysis was carried out using Genetix v 4.05.2 with 4 factorson default settings. Migration network was calculated using divMigrate for Rusing model Nm with 9 5% confidence interval.

Results. A total of 73 individual American mink samples was genotyped using 15 microsatellite loci. All loci aside from Mvi 4060 were polymorphic across all population units. Out of 75 allele sizes obtained repeatedly for reproducibility confirmation, only 1 diverged meaningfully from the original result, which was deemed to be an acceptable error rate. Null allele checking using Micro-Checker showed possible null alleles, specifically for locus Mvis 099, but null allele probability did not exceed 0,15 so we decided not to exclude this locus from the analysis.

Summary results on the populations' genetic polymorphism are shown in Table 2. The «Strievka Pearl» population group was excluded from the summarydue to unreliable results caused by insufficient sample size ($n = 2$). Most loci displayed different HWE disequilibrium significance across different population units, but Fisher's method sum across all loci indicates clear HWE disequilibrium due to homozygote excess. The lowest molecular variety was characteristic of the «Wild» population group.

AMOVA results show that inter-population variance accounts for 9.21 % ($p = 0,000$) of overall variance in the sample. Most of the sample's variance (75.98 %) is caused by inter-individual variance.

Population structure analysis via STRUCTURE (Fig. 2) presented us with a clear population structure for the most likely $K = 3$ (optimal $\Delta K = 48.534$).

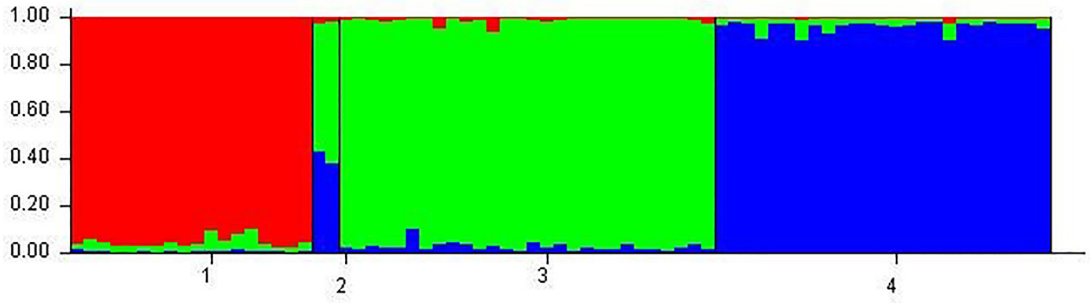

Fig. 2. Bar diagram of individual clustering for the studied sample of *N. vison* constructed in STRUCTURE for $K = 3$ using microsatellite data Individuals' origin among the population units displayed along the *x*-axis: *1* – «Wild»; *2* – «Strievka Pearl»; *3* – «Strievka White»; *4* – «Kalinkovichi Brown»

Factorial correspondence analysis performed in Genetix has shown a relatively smaller genetic distance between all fur farm individuals, but also a closer relation of «Wild» population unit members with both farm populations, rather than with other «Wild» individuals from the more distant regions (Fig. 3).

Migration network, assembled using divMigrate (Fig. 4) demonstrates a significant presence of migration from the population unit «Kalinkovichi Brown» into the «Wild» population unit, as well as possible various levels of migration from the unit «Strievka Pearl» into all other population units.

Discussion. The «Strievka Pearl» population unit was rejected from consideration due to severely lacking sample size, leading to unreliable results regarding this group in most analyses.

The observed deviation from HWE due to homozygote excess in all studied *N. vison* population units is consistent with the expectations due to inbreeding employed for breed maintenance regarding the farm populations, and due to Wahlund effect in the wild population. Genetic polymorphism characteristics display a lower heterozygosity, allele count, allele richness, and higher inbreeding coefficient compared to similar populations studied across Europe, including ones using highly similar microsatellite panels and sample sizes – which is likely caused by a smaller breeding stock and smaller population size across Belarus for farm and wild populations both. Significantly lower genetic polymorphism of

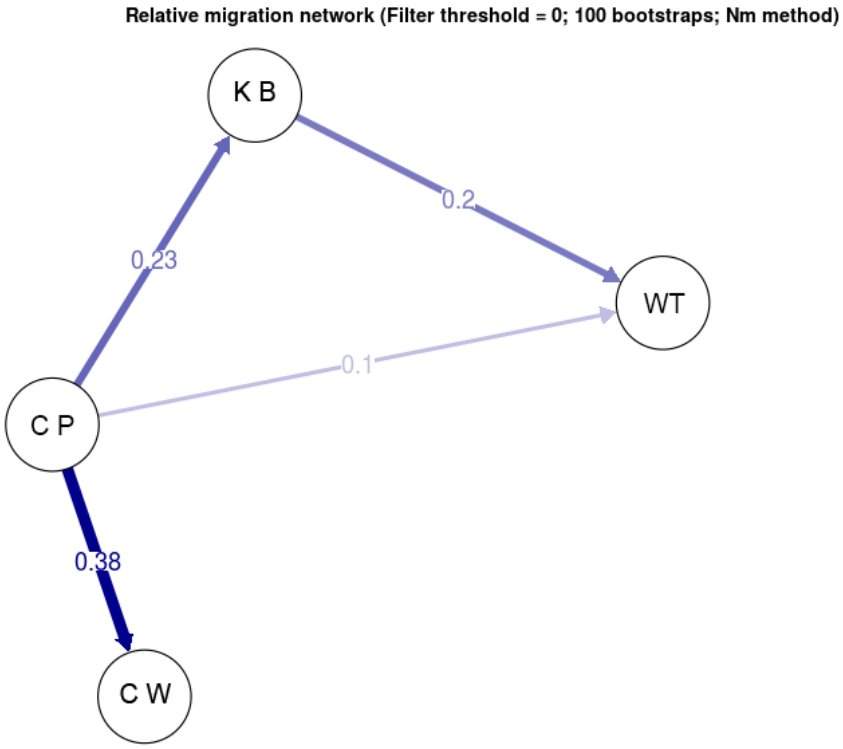

Fig. 3. 3D diagram of genetic relations between *N. vison* individuals according to factorial correspondence analysis utilizing microsatellite data. Yellow cubes correspond to «Wild» population unit members; blue cubes – to «Strievka Pearl»; white cubes – «Strievka White»; grey cubes – «Kalinkovichi Brown». Individuals marked as 2-1 and 16-1 for the «Wild» population unit are of a particular interest, as they correspond to individuals TH00295 (Vitebsk region) and TH00616 (Brest region)

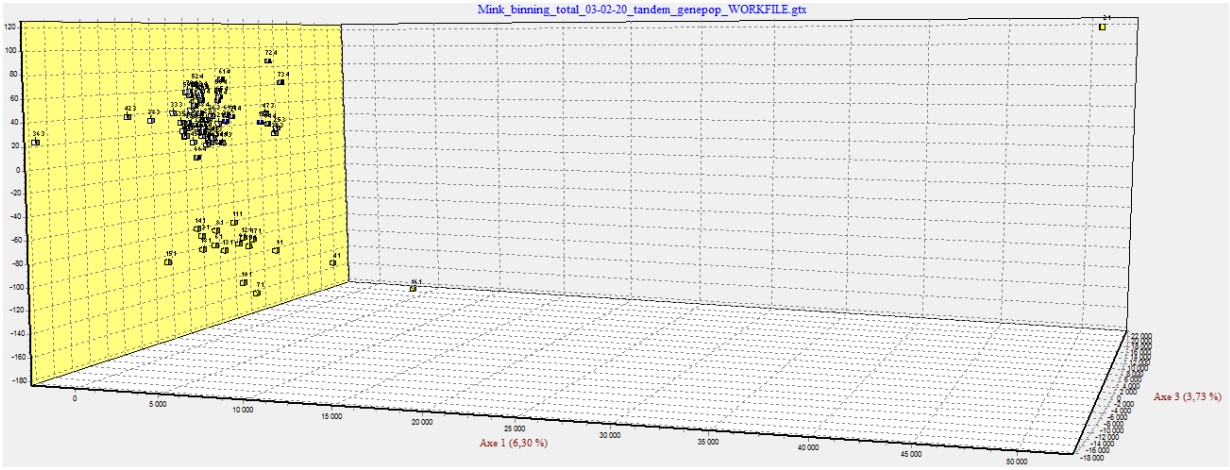

Fig. 4. Migration network of studied *N. vison* population units assembled with divMigrate according to the microsatellite data. The «WT» circle represents the «Wild» population unit; «CP» – «Strievka Pearl»; «CW» – «Strievka White»; «KB» – «Kalinkovichi Brown». Blue arrows and values on them represent migration flow

wild populations compared to farm populations reflects a nearly universal trend for *N. vison* populations beyond its historic range in North America [15]. The clustering of «Strievka White» and «Kalinkovichi Brown» units in factorial correspondence analysis originates in the closelyrelated sources of breeding stock used for both fur farms populations, procured from a certain mink farm in Denamark, differing only by their color breeds. The fact that populations displayed a perfectly clustered population structure serves to validate the used method and particularly the STR panel for determining the population affiliation of individuals. This shows method's potential application in mink breeding, particularly for controlling and certifying breeding stock for transactions, as long as a sufficiently large samples of relevant

populations are available – which should not be an issue considering regular slaughtering of the farmed animals. Noticeably, none of the studied farm populations show any indicators of significant inbreeding depression through their genetic polymorphism characteristics, indicating that the breeds should be stable in the foreseeable future, given the current breed management strategy is maintained.

A very likely presence of a stable gene flow from Kalinkovichi fur farm into the wild is indicated by migration and factorial analyses. A certain degree of relatedness can be inferred from the species' invasion history, but this explanation is not supported by isolation of wild individuals harvested in Homiel region from the wild individuals of other regions according tofactorial correspondence analysis. Population structure analysis indicated that all these individuals still belong to the same population unit, suggesting that differences among the wild individuals from different regions are unlikely to be due to isolation between wild subpopulations. It can be suggested that wild individuals could be related due to common ancestry from the invasion period [16], and later diverged due to migration from Lithuanian wild population, but this explanation is not supported by the results of migration analysis. Presence of accidental original escapees among wild individuals from Homiel is extremely unlikely due to all said individuals displaying wild type fur coloration, as well as due to the results of population structure analysis. Same results also suggest that, whatever is the extent of migration from the fur farm into the wild, escapees do not form the core of wild population unit in the Homiel region. Beyond that it's hard to determine the scale of migration from the fur farm due to lack of chronological depth in the analysis. What can be concluded is that individual minks almost certainly do escape from the Kalinkovichi fur farm, successfully adapt to the wild conditions and produce mixed offspring with local individuals that persists in the wild. Without a thorough survey, the actual scale of escapes can only be speculated on, since there can be an unknown number of escapees that fail to adapt and produce offspring due to various factors. Even migration of a minimal scale is troubling – past studies have shown that *N. vison* displays capacity to form rapidly expanding populations in the wild from a minimal pool of fur farm escapees in conditions quite similar to those present in most regions of Belarus [17]. Presence of a stable migration flow from the fur farms can drastically reduce the effect of measures that could be taken to control and manage growth and expansion of wild populations of American mink in Belarus, making expensive efforts futile as long as the flow from the fur farms is not taken into consideration and mitigated in one way or another. This is especially problematic as Belarus serves as the main land invasion corridor for American mink to expand eastward into the regions inhabited by the last surviving wild populations of European mink in Western Russia [18]. Continuous growth of wild American mink population with an uncontrolled conduit of individuals from various breeding farms across the globe through Belarussian fur farms and into the wild serves as a major threat to the local veterinarian security, as it threatens with establishment of a stable reservoir of numerous mink pathogens, dangerous to endemic wildlife, mink farm breeding operations, and humans. Any perspective strategy to manage and contain the rapidly growing *N. vison* population in Belarus must necessarily include element of genetic monitoring to control for fur farm escapee role in forming and maintaining the local wild populations, as well as timely and effective measures to restrict the flow of individuals from fur farms into the wild. A perspective genetic study focusing on a larger sample of wild *N. vison* individuals from more varied localities presents a valuable opportunity for an attempt to predict the future dynamic of *N. vison* population in Belarus according to it's genetic variance using such means as population viability analysis.

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