INVESTIGATING THE ORIGIN OF THE AMERICAN MINK *(NEOVISON VISON)* IN POLAND, INCLUDING A STUDY ON MINK MITOCHONDRIAL DNA FROM FARM, FERAL AND WILD NORTH AMERICAN POPULATIONS

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The American mink (*Neovison vison*), native to North America, is a controversial invasive species in many European countries, including Poland. Mitochondrial DNA data (concatenated MT-CYTB and MT-COI sequences) were used to investigate the genetic diversity of native, introduced, and farm populations of this species. Phylogenetic analysis revealed two major clades including individuals from the farm, feral, and wild populations. This supports the origin of Polish minks from the two native lineages existing in North America. The genetic structure in the invasive range may result from the diverse origins of the released founder individuals and the rapid expansion and contact zones of the introduced populations. Spatial mixing of both North American lineages in Polish feral populations may lead to higher levels of genetic diversity in introduced populations, despite some evidence of a founder effect as one of the main factors influencing their diversity.

Key words: American mink, *Neovison vison*, phylogenetics, invasive species, mink farming.

**INTRODUCTION**

Studies of introduced species indicate an important role of genetics in the success of invasion, pointing the role of the quality of the genetic variation introduced rather than the total quantity of genetic variation (Dlugosch et al. 2015). The genetic diversity of founding populations, in combination with potential increased genetic diversity from multiple introductions, play an important role in the successful establishment and spread of introduced species (Suarez & Sutsui 2008, Sol et al. 2012). However, it is not claimed that invaders coming from multiple native origins are more successful than those from single populations or that demographic bottlenecks limit the invasion success of a species (Edelaar et al. 2015, Estoup et al. 2016). A general rule says that introduced populations lose genetic variation in relation to their source populations (Dlugosch & Parker 2008). However, numerous introduction events and a greater number of source populations may buffer such genetic losses (Kolbe et al. 2004, Dlugosch & Parker 2008, Uller & Leimu 2011). In some cases, successful invaders show large increases in genetic diversity in the in-
introduced range, apparently caused by mixing of populations from different source regions (Novak & Mack 1993, Genton et al. 2005). Therefore, it is crucial to obtain information on the phylogeographic structure and genetic diversity throughout the native and introduced ranges to understand the factors affecting genetic diversity during invasion (e.g. multiple introduction events or admixture levels among populations) and the interaction between genetic diversity and invasive potential (Edelaar et al. 2015).

Besides the effect of founding population diversity, an important factor affecting the level of genetic diversity of introduced species might be the coexistence of its farm and feral populations. American mink (Neovison vison) was originally endemic only to North America. It has been introduced into Asia and South America. In Europe, this species was introduced for commercial fur farming, which started in the 1920s. Farms were established in many countries and mink stocks were composed of individuals of different native origin. Together with large-scale farming, farm animal escapees were noticed. These individuals started to breed in the wild and became the founders of feral populations (Bonesi & Palazon 2007). However, feral populations in Eastern Europe originate not only from farm escapees, but also from thousands of individuals that were deliberately released into the wild in the former Soviet Union since the 1930s (Heptner et al. 2001). In Poland, it has been documented that a migration wave from the east crossed the Polish–Belarusian border at the end of the 1970s and spread west over subsequent years (Ruprecht et al. 1983, Brzeziński & Marzec 2003). In Poland, mink farming developed much later than in other European countries. The first farm was opened in 1953 (Lisiecki & Sławoń 1980). Since that time, observations of single animals, considered to be escapees, have been reported from various sites. Although, there is no evidence that local feral populations originating from farm mink were established in any region of Poland until the beginning of the 1980s. At that time, feral mink reaching Poland from the Belarus border side, were already present in the north-east of the country. Due to the high adaptive potential, in the following years, there was an increasing number of reports of feral mink living in the west (Bartoszewicz & Zalewski 2003, Brzeziński & Marzec 2003). It is still under discussion whether the invasion from the east was the only process that has led to the colonization of Poland or whether it was supported by populations originating from farms.

Earlier studies comparing farm and feral populations showed that American mink in Poland exhibit high genetic diversity and originate from different source populations (Zalewski et al. 2010, 2011). A conclusion of the study was that the process of colonization was triggered mainly by immigrants from Belarus but there is also some evidence for contribution of escapees from various farms. The genetic structure of local Polish feral mink populations was shaped by the founder effect and multiple introductions
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(Zalewski et al. 2010). Mixing of gene pools of different populations might have increased the fitness of individuals and improved the invasive potential of this species (Zalewski et al. 2011). It is one of the most important factors influencing the level of genetic diversity of feral mink populations, not only in Poland. Another equally important factor, is the level of genetic diversity of wild populations in North America. Sequence data from populations in the native range can help to explain whether invasive populations in Poland have single or multiple origins from the North American sources. Determination of the phylogeographic structure of native and invasive populations can be used to evaluate whether mitochondrial genetic diversity observed in established invasive populations can also be explained by genetic characteristics of native populations. In the studies carried out by García et al. (2017), a wild population of American mink in North America was analyzed in relation to a population introduced from the Iberian Peninsula. The phylogenetic analysis of American mink in the native range revealed two major lineages, and thus showed a clear separation between western and eastern wild populations. Spatial mixing of both North American lineages was detected in several sampling localities of the Iberian Peninsula. This genomic mixture contributed to high levels of genetic diversity in some invaded areas compared to North American populations. This may result in higher fitness of these animals and thus increase the viability of feral populations of this species and their invasive potential (García et al. 2017).

Wild North American individuals are the progenitors for all other mink populations all over the world. Some animals were trapped and served as a source for farm populations. In turn, farm escapees became founders for many feral populations, together with animals that had been deliberately released from fur farms (Lisiecki & Sławoń 1980). The key factor is the time of transformation from one population type to another. The aim of the study was to clarify the origin of feral American mink in Poland measured by their relatedness to farm and native populations.

MATERIAL AND METHODS

The research was carried out with the approval of the Local Ethical Committee for animal experiments in Lublin (Resolution No. 83/2009). The studies included farm mink of the standard color type, both males and females, in the first year of their life. Samples were taken from unrelated animals (the relatedness of individuals was determined according to mating history in breeding books) from one farm (100 individuals). Second group included 39 feral animals, representing both sexes and belonging to two subpopulations - from the area of north-eastern (NE) Poland (near Olsztyn – 24 individuals) and north-western (NW) Poland (around Drawsko Pomorskie – 15 individuals). Two individuals came from road traffic accidents while the rest were obtained in cooperation with Polish Hunting Association. Obtained material was immediately put in –4°C. Third group included 50 wild liv-
ing animals from North America, whose skins were purchased on an auction in Canada in 2011. Unfortunately, specific region of their origin was not known (Fig. 1). Conserved skins were kept in dry conditions and biological samples were taken right after receiving in the laboratory. In the case of the farm animals, whole peripheral blood was collected to vacuum tubes containing EDTA. Fragments of skin with hair were the material from the wild living animals from Poland and North America. DNA extraction was performed using a DNeasy Blood and Tissue Kit (QIAGEN). The purity and concentration of the isolated DNA was assessed by spectrophotometry (BioPhotometer, Eppendorf) and electrophoretic separation on a 1% agarose gel containing ethidium bromide in 1XTBE buffer, with voltage 70 V for 40 minutes. The samples were visualized under UV light and archived using the ScionImage program.

The PCR for the investigated fragment of the MT-CYTB gene was performed using universal primers F: 5’-CCATCCAACTCAGCATGATGAAA-3’; R: 5’-CCCCTCAGAATGATATTTGTCCTCA-3’, from Alonso et al. (2006). Primers for MT-COI: (F: 5’-TGGGGGCTTTTGGAACGTGAC-3’; R: 5’-CCGGCTGGTCAAAGAAAGT-3’) were designed according to the sequence of the American mink MT-COI gene from the NCBI database – GenBank accession number AY377347 – using the Primer-Blast tool at the NCBI website. Amplification of gene fragments was carried out using AmpliTaq Gold DNA Polymerase 360 (Life Technologies). PCR reactions were performed in a volume of 25 µl. A single sample contained 1 x AmpliTaq Gold 360 buffer, 2.5 mM MgCl₂, 1U of AmpliTaq Gold 360 DNA Polymerase and 0.4 mM of each primer, 0.2 mM of each DNTP (Fermentas), and 3 µl of genomic DNA. PCR was performed on a Labcycler (SensoQuest) using the following thermal profile: 10 min at 95°C prior to 35 cycles of 30 s at 95°C, 60 s at 56°C – MT-CYTB/ 52°C – MT-COI, and 60 S at 72°C, followed by an extension step of 10 min at 72°C.

Spectrophotometric evaluation of the PCR product was then performed on BioPhotometer (Eppendorf AG). Electrophoresis of samples to check the length of the obtained DNA fragments was performed on a 2% concentration agarose gel with the presence of 1 x TBE buffer and ethidium bromide, using Gene Ruler 100 bp DNA Ladder from Fermentas. The reaction was performed at a constant voltage of 70 V for the time of 180 min. The gels were obtained using ScionImage. Sequencing PCR for both genes was performed using a BigDye® Terminator v 3.1 Cycle Sequencing Kit from Life Technologies, with forward primers used previously in the preparative PCR. The quantitative composition of the mixture and the temperature-time profile were used as recommended by the manufacturer (final sample volume of 20 µl). Sequencing PCR products were then purified from the reactants with a DyeEx 2.0 Spin Kit (QIAGEN) using Qiacube and separated by electrophoresis in a 50-cm capillary in 6% polyacrylamide gel – POP-6. Sequencing was performed on a molecular analyzer 3100-Avant Genetic Analyzer.

Preliminary elaboration of the sequencing results was carried out with a DNA Baser (DNA Baser Sequence Assembler v. 3) and MEGA 6 (Tamura et al. 2013) software. It consisted in localization of SNP mutations for all animals in the analyzed gene fragments. On the basis of SNP changes identified in the sequences of MT-CYTB and MT-COI, the haplotypes were defined. Maximum-likelihood phylogenetic trees were obtained separately for MT-CYTB and MT-COI with MEGA 6 using a general time reversible (GTR) substitution model. In the second step, concatenated sequences of MT-CYTB and MT-COI were used. The frequencies of concatenated haplotypes for each population individually were calculated using SAS v. 9.1.3. (SAS Institute, Cary NC). The information about the type and frequency of haplotypes occurring in different populations was used to calculate the haplotype diversity (h), the nucleotide diversity (π), the average number of nucleotide differences between haplotypes (k), and the pairwise fixation index (φST) with the program.
Arlequin v. 3.5 (Excoffier & Lischer 2010). For assessment of mtDNA genetic diversity at the population level, basic diversity measures were also used, based on the number and nucleotide sequence of the haplotypes (Halldórsson et al. 2004, Goodall-Copestake et al. 2012). The most commonly used coefficients are haplotype diversity (h), which is the probability that two haplotypes randomly selected from the population are not the same (Nei 1987), and nucleotide diversity (π) determining the average number of nucleotide differences per position between two randomly selected sequences (DNA haplotypes) (Nei & Li 1979). An important factor that describes the level of genetic diversity between populations is the parameter $\phi_{ST}$ (Excoffier et al. 1992), which is an equivalent of $\text{FST}$ statistics. Although $\phi_{ST}$ provides more detailed information on the genetic structure of the population, at the same time it is more adequate for mtDNA, as it is estimated on the basis of haplotype sequences and nucleotide variation between them. It determines the proportion of nucleotide diversity between subpopulations in relation to the total diversity.

The relationships among haplotypes were represented as a median-joining haplotype network, because of its recognized role in inferring phylogenetic relationship within species (Bandelt et al. 1999), and a UPGMA tree. Both analyses were carried out with SplitsTree 4.13.1 software (Huson & Bryant 2006).

RESULTS

In the three studied groups, eight MT-CYTB haplotypes were identified and their sequences were deposited in GenBank under accession no. MH047256–MH047263. Only one of these haplotypes was found in all three investigated populations. Also one haplotype was shared by both wild-living

![Fig. 1. Sources of biological samples used in this study. Samples from farm individuals (n = 100) were taken from farm in SE Poland; samples from feral animals were obtained from two subpopulations from NE (n = 24) and NW (n = 15) Poland; samples from wild animals (n = 50) were taken from skins bought on auction in Canada and their specific locality of origin in North America was not known.](image)
populations (Polish and North American). Other identified haplotypes were private for specific populations. The greatest number of private MT-CYTB haplotypes (four) was identified for the wild mink from North America. In the case of MT-COI, only four different haplotypes were found (GenBank accession numbers MH047264–MH047267), including one present in all the studied populations. The haplotypes identified in this study, as well as the reference sequences from GenBank, were used to construct ML phylogenetic trees, separately for MT-CYTB and MT-COI. In both cases, the phylogenetic analysis revealed the occurrence of two major clades (clade I and clade II) (Figs 2 & 3). Each of the clades consisted of mixed Polish (both farm and feral) and North American haplotypes. The North American haplotypes mined from GenBank were also present in both clades.

Based on concatenated MT-CYTB and MT-COI sequences (719 bp), a total number of 13 haplotypes was detected in the investigated three populations. Detailed information concerning their sequences can be found in Table 2. Haplotype HP1 was found in all three populations of mink. Neighboring HP5, differentiated by two mutations, was identified in the Polish feral and North American wild mink populations. Interesting was the HP2, which was private for the Polish feral mink and was recorded for over 60% of representatives of this group. The relations between the haplotypes are presented as a haplotype network (Fig. 4).

![Fig. 2. Maximum-likelihood phylogenetic tree derived from the partial MT-CYTB sequences from this study (in bold) and mined from GenBank](image-url)
Fig. 3. Maximum-likelihood phylogenetic tree derived from the partial MT-COI sequences from this study (in bold) and mined from GenBank.

Fig. 4. Neighbor-joining haplotype network based on haplotype frequencies showing relationships between concatenated MT-CYTB and MT-COI sequences of farm, feral, and wild mink. The sizes of the circles are proportional to the haplotype frequency.
The concatenated haplotype genealogy (Fig. 5) also revealed two groups of mixed origin. All three haplotypes identified for the Polish feral mink (HP1, HP2, HP5) were placed within clade I, while clade II mainly consisted of haplotypes private for the wild North American mink.

The wild North American population was the most diverse, with the haplotype diversity of 0.77±0.11, nucleotide diversity of 0.12±0.08, and average number of nucleotide differences between haplotypes of 2.46±1.40. The lowest values of these coefficients were found for the population of the feral mink from Poland (h = 0.54±0.10, π = 0.03±0.02, k = 0.62±0.52). The total value of $\phi_{ST}$ calculated for the three populations was 0.26 (Table 1). In the case of the pairwise $\phi_{ST}$, the highest value (0.36) was recorded between the Polish feral and wild individuals from North America, and the lowest (0.14) was found between the group of farm animals and the wild population from North America. The pairwise $\phi_{ST}$ value between both Polish populations (farm and feral) was 0.29.
DISCUSSION

The coexistence at the common area of farm and wild living populations of the same species is usually controversial in terms of the risk of their potential hybridization. Fur farming is one of fastest developing branches of animal breeding in Poland. Two leading species are American mink (*Neovison vison*) and red fox (*Vulpes vulpes*). Red fox is one of the species native in Europe, and it has quite clear phylogeographic status. Polish wild red fox populations show high genetic distinctiveness in relation to farm individuals (*Jeżewska-Witkowska et al.* 2012, *Horecka et al.* 2017). It reflects their separate evolutionary pathways as farm foxes have their source of origin in a North American lineage. The situation of American mink is more complex, as it is invasive species in Europe and feral populations are quite common in many European countries. This raises the need for further research in order to better characterize these populations, also on the genetic level. The choice of a gene, which is the basis for characterizing populations, significantly affects the assessment of their differentiation. According to *Zardoya* and *Meyer* (1996), mitochondrial genes encoding proteins are characterized by different levels of usefulness in the reconstruction of phylogenetic relationships, although both MT-CYTB and MT-COI genes analyzed in this study are most commonly used and should then provide reliable information. The sequence of the control region (cr) is a specific mtDNA region often used in phylogenetic studies. This part of the mitochondrial genome was used in the studies by *Zalewski et al.* (2011) in order to assess the diversity of farm and feral American mink populations in Poland. The length of the analyzed fragment was 373 bp, and the total number of cr haplotypes found was 31, 19 of which were found in feral

![Fig. 5. UPGMA phylogenetic tree derived from the concatenated MT-CYTB and MT-COI haplotypes](image-url)
mink, while there were 20 haplotypes in farm animals. In the present studies, the number of haplotypes identified for all the studied populations was 13. It was therefore lower than in the studies of Zalewski et al. (2011), although this difference probably results from the number of individuals analyzed by these authors, which was higher than the total number of farm and feral animals included in this study. It may be also a result of the higher level of polymorphism generally observed in the control region, in relation to other parts of the mitochondrial genome. Zalewski et al. (2011) noted the presence of eight haplotypes shared between farm and feral American mink populations, and their frequency varied in both groups. Also in the present research, there was a haplotype common to both populations from Poland – HP1. However, it was also reported in the wild North American population. This means that it might be one of the most common haplotypes for the species and reflects their source of origin in North America. Genetic diversity indices reported by Zalewski et al. (2011) for feral and farm mink showed significantly higher values of nucleotide diversity; however, the haplotype diversity values were similar for both groups of animals. The comparison between feral and farm mink carried out using pairwise $\phi_{ST}$ revealed great genetic divergence, with a value of approximately 0.3 in the present study and in the study of Zalewski et al. (2011). Each of these populations was shaped by different factors. The highest values of genetic diversity indices found for the wild mink population reflects the coexistence of many subspecies as well as the presence of genetically distinct mitochondrial lineages in the native range of American mink. Similarly, the farm animals have their origin in both North American lineages, which can be supported by the results of the phylogenetic analyses (ml trees). A different situation is observed in the feral population. This is well seen in the presence of haplotype HP2, which was private for this population and was characterized by a very high frequency of 60%. This is most likely a result of the founder effect that might be connected with farm animal escapes in the past and led to the fixation of this variant in subsequent generations.

Based on mitochondrial DNA sequence data, the results from this study provide evidence that the American mink populations introduced in Poland have overall lower genetic diversity than native mink populations in North America. This supports the theory of the ‘genetic paradox’ (Estoup et al. 2016), which holds that low genetic variability can be expected in invasive species because of the loss of rare alleles, population bottlenecks and, in the case of the investigated Polish feral populations, especially the founder effect (Allendorf & Lundquist 2003). However, previous studies have demonstrated that the genetic diversity of invasive populations in their new range may be increased through the admixture of lineages from multiple native populations (Keller & Taylor 2010). It is also possible that cross-breeding of different North American subspecies or populations took place within farms.
High values of genetic diversity have already been registered in some mink populations in Poland (Zalewski et al. 2011).

Phylogenetic analysis carried out by García et al. (2017) revealed two large native phylogeographic lineages, which indicated a clear separation between the western and eastern populations in North America. Therefore, the relationship between the mtDNA haplotypes and the location from which they were sampled provides evidence for the phylogeographic structure among populations of American mink in their native range. The results of this study are consistent with those obtained by García et al. (2017) for a feral population in the Iberian Peninsula and show that the establishment of the invasive American mink population is most likely a product of the introduction of individuals from the two main genetic lineages found in western and eastern North America. In the invasive range, these lineages were characterized by a complete lack of phylogeographic structure. Unfortunately, the lack of information about the sampling sites of the American mink in North America and the different regions of mtDNA analyzed in this study do not allow direct comparison of the results from both studies. However, the pattern of the spatial structure recorded in North America by García et al. (2017) allowed more precise identification of the origin of invasive populations in the Iberian Peninsula and, based on that, the results from this study on the Polish populations evidence that there were introductions from both western and eastern regions in North America. Two possible haplotype groups of mink in Poland, for which the net divergence was 0.9%, were already distinguished in the study of Zalewski et al. (2011). These two mtDNA groups were present in both the feral and farm mink. This most likely occurred as a result of the additive effect of the multiple origins of mink and the fast range expansion of the occupied area since their introduction in the 1960s–1970s. It is also possible that farms received founder individuals from different source populations in the native range. It is therefore likely that the same processes suggested by García et al. (2017) for the Iberian Peninsula (i.e. multiple introductions, rapid expansion, and encounters of individuals of different lineages) can be applied to other areas with invasive populations of American mink, including Poland.

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