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Studies of mitochondrial DNA D-loop sequence variation may support the Polish Primitive Horse (Konik) conservation programme

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Original article

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The protection and balanced development of all existing maternal lines is one of the primary goals of the Polish Primitive Horse (PPH or Konik) conservation programme. However, previous studies have indicated that managing PPH conservative breeding may encounter challenges because of the numerous existing pedigree errors. We therefore attempted to check whether mtDNA markers may prove useful in correcting PPH pedigrees and improving the breed conservation programme. A 510 bp mtDNA D-loop fragment was sequenced for 396 samples representing all sixteen officially recognised maternal lines. These samples were derived from different time points in the PPH breeding history. Our analysis confirmed the presence of nineteen mtDNA haplotypes. A comparison of the molecular and pedigree data showed that the frequency of particular haplotypes was highly uneven. Although for the majority of the maternal lines we were able to identify a potential 'founder haplotype', only four of them (Dzina I, Popielica, Geneza and Bona) turned out to be 'genetically pure'. Our study confirmed that an mtDNA analysis is a useful method for assessing PPH maternal genetic diversity and illustrating the breed's history. Our findings suggest that PPH conservation programmes would benefit from revising the official pedigrees using molecular data alongside breeding records.

Key words: mtDNA, maternal line, genetic diversity, pedigree verification.

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Of the several horse breeds harboured by conservation programmes in Poland, special attention is given to the Polish Primitive Horse (PPH or Konik), which is considered to form an essential part of the genetic resources of the domestic horse (*Equus caballus*) (Pluta *et al*. 2016). In relation to its primitive body conformation (Fig.1) (a set of physical characteristics commonly found in primitive horse breeds that is often associated with natural selection and adaptation to harsh environments, rather than human-driven selective breeding), dun coat colour, good health and excellent adaptation to the natural environment, the PPH breed resembles the extinct Tarpan, which lived in Eurasia until the end of the 19th century (Slivinska *et al.* 2009). Even though the annual number of PPH registrations has increased significantly since the conservation programme began in the year 2000, the breed is still classified as 'endangered-maintained' by the UN Food and Agriculture Organisation (FAO). This is mainly related to

Fig. 1. Borówka PPH mare (Bona maternal line) with her offspring (Beroz PTOP colt). Phot. R. Kalski.

the fact that the current population of Polish Primitive Horses has a very limited number of ancestors, who were survivors of the Second World War. The official studbook has been closed since 1985, which means that from that date only those horses having at least four generations of ancestors present in the PPH studbook can be registered (no outside blood is permitted) (Lovász *et al.* 2021). The genetic diversity indices on the PPH breed thus need to be constantly monitored, and the selection of pairs for mating should be done carefully to avoid an excessive increase in inbreeding (Mackowski *et al.* 2015).

One of the most important goals of the PPH conservation programme is the protection and balanced development of all existing maternal lines. In tracing the history of the breed, it can be noted that, although 35 maternal PPH lines were recognised after the Second World War, improper breeding has led to nineteen of those lines becoming extinct. Thus, the official PPH registry currently distinguishes sixteen active dam lines: Liliputka I, Karolka, Zaza, Urszulka, Tarpanka I, Traszka, Tygryska, Tunguska, Wola, Geneza, Dzina I, Popielica, Białka, Misia II, Ponetna and Bona. Their representation in the overall population is not equal and some of these lines remain endangered. Moreover, in our previous study using mitochondrial DNA (mtDNA) sequence variation, it was revealed that only five of the PPH dam lines show the segregation of one specific mtDNA haplotype, while the remaining lines have a higher number of mtDNA haplotypes (Cieslak *et al.* 2017). Similar observations were made in the recent investigation by Musiał *et al.* (2024), which considered a longer mtDNA D-loop sequence fragment. Taking into account both of these studies showing uneven clustering and haplotype mixing between the dam lines, we might conclude that the official PPH pedigrees contain mistakes, which could be an effect of the incorrect identification of horses before the era of common genetic testing. Investigations based exclusively on breeding documentation may therefore be biased. In many cases, the pedigree might not reflect the real PPH maternal genetic diversity. In the study conducted by Fornal *et al.* (2021) using microsatellite markers data and the Bayesian algorithm, the results were not unambiguous and did not clearly answer the question of how many genetic clusters there were. In the cited study, as well as in the investigation by Szwaczkowski *et al.* (2016), we can also find conclusions suggesting that some of the PPH

dam lines are not noticeably genetically divergent.

Because our previous study was based on a limited number of samples and did not cover all the major branches of the PPH pedigree, we were not able to fully evaluate the level of pedigree inconsistencies and prove that the implementation of molecular data may significantly help in better protection of the PPH genetic diversity. Considering this, we decided to perform the current broad, comprehensive study including pedigree and molecular (mtDNA-based) data analyses, to assess potential inconsistencies between the two data sources and to better depict the PPH breeding history through the analysis of retrospective DNA samples. Our primary goal was to estimate the exact genetic diversity of the PPH maternal lines and to assess whether the results of molecular studies may help to better manage the PPH conservation programme. Moreover, we aimed to demonstrate how the combination of pedigree and molecular data may be practically applied by horse breeders in order to increase the maternal genetic diversity of their herds.

Materials and Methods

Research material

Altogether, a pedigree consisting of 9,310 PPH individuals was analysed, sourced from the Polish Horse Breeders Association (PHBA) database, official studbooks and other breeding documents stored in national and private horse studs archives. Since mtDNA is maternally inherited, we decided to construct the genealogical trees showing mares only, to track particular dam lines more easily. We later marked the pedigree mtDNA haplotypes of the mares tested in our previous study (Cieslak *et al.* 2017) and decided which individuals should be targeted for molecular testing to cover, if possible, all the major branches of the maternal line phylogenetic tree. When a putative pedigree error (the presence of more than one mtDNA haplotype in the same maternal line) was detected, we tested as many archival DNA samples as possible and consulted the documentation kept in national and private PPH breeding centres. Such an approach allowed us to illuminate the history and establish the period and potential location in which the horses were substituted. The genomic DNA samples (n = 396) representing all of the 16 PPH maternal lines were derived from the collections of the Horse Genetic Marker Laboratory (Poznań University of Life Sciences, Poznań, Poland) and the Laboratory of Molecular Genetics (National Research Institute for Animal Production, Balice, Poland). An ethical review and approval were waived for this study, since we used samples already stored in our laboratories as remains from the commercial parentage verification analyses commissioned by the horses' owners (no new samples have been collected for this study). All of the samples tested in this study are listed in the supplementary Table S1 (SM.01).

Molecular methods

The DNA was isolated from frozen blood samples using a Sherlock AX extraction kit (A&A Biotechnology, Gdańsk, Poland), following the manufacturer's instructions. The quality and quantity of the extracted DNA were assessed using a NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA). The 510 bp fragment of the equine mtDNA D-loop sequence containing the hypervariable region 1 (HVR1) was amplified using primers as described by Cothran *et al.* (2005). The primers spanned positions 15343-15852 in the GenBank X79547 mtDNA sequence (as a reference NC_091244.1). The PCR was carried out in a T100 Thermocycler (Bio-Rad, Hercules, CA, USA) using the following programme: initial denaturation (95°C/10 min); 25 cycles of denaturation (95°C/30 s), primer annealing (56°C/45 s), elongation (72°C/45 s) and final synthesis (72°C/10 min). For each genomic DNA sample (concentration 25 ng/µl), we used 1U of onTaq DNA Polymerase (EURx, Gdańsk, Poland). The amplification efficiency and PCR product integrity were determined using electrophoresis in 1.5% agarose gel stained with SimplySafe (EURx, Gdańsk, Poland). After an enzymatic cleaning of the unused primers and by digestion with FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Fisher Scientific, Waltham, MA, USA) (37°C/30 min; 80°C/15 min), the sequencing reaction was carried out in a T100 thermocycler as follows: initial denaturation: 95°C/5 min; 25 cycles of denaturation: 95°C/30 s, primer annealing (50°C/10 s) and elongation (60°C/4 min). The samples were then filtered through a 96-well plate containing Sephadex (Sigma-Aldrich, St Louis, MO, USA) by centrifugation at 3,180 × g for 3 min, and separated in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In silico analysis of the obtained sequences

The sequencing results were analysed using Lasergene SeqMan Pro software (DNASTAR, Madison, WI, USA). The nucleotide sequences were aligned to the mitochondrial genome (GenBank X79547 as

a reference NC_091244.1) and to sequences representing particular previously described PPH mtDNA haplotypes (Cieslak *et al.* 2017), and were aligned with the MegAlign Pro tool (DNASTAR, Madison, WI, USA). To easily compare this study with the previous one, we used identical mtDNA haplotype numbering, where PPH1 is the haplotype representing the equine mtDNA reference sequence and PPH2 to PPH20 are the haplotypes found within the examined PPH dam lines.

Results

MtDNA haplotypes detection and their distribution within the studied population

An analysis of the 510 bp fragment of the mtDNA D-loop sequence revealed the presence of nineteen haplotypes within the PPH population, which is concordant with the results of our previous study (Cieslak *et al*. 2017). Combining both the molecular and pedigree data indicated that the frequency of particular haplotypes is highly uneven: the PPH3 (27%), PPH10 (16.3%), PPH4 (14.2%) and PPH13 (12.4%) haplotypes are common and cover about 70% of the tested population; while the remaining haplotypes occur with frequencies ranging between 0.2 (PPH21) and 5.7% (PPH12) (Fig. 2).

Comparison of molecular and pedigree data

Tracking the presence of particular haplotypes in all sixteen official maternal lines revealed that the segregation of one specific haplotype occurs in only four lines: Dzina I (PPH7), Popielica (PPH12), Geneza (PPH6) and Bona (PPH17). Supplementary Fig S1 (SM.02) shows an example of a pedigree chart for the Geneza line which turned out 'genetically pure' (all tested individuals had the same PPH6 mtDNA haplotype). The remaining lines showed a segregation of more (2-6) mtDNA haplotypes. However, on the basis of their frequencies and with the use of a pedigree analysis, we were able to pinpoint the putative unique founder haplotype for most of the dam lines (Table 1). For the Karolka and Tunguska lines, we observed the same predominant haplotype (PPH4), which may suggest that they have a common female ancestor and represent the same strain in the genetic sense. The prevalence of PPH4 and other haplotypes in both maternal lines is presented in Fig. S2 (Karolka) and Fig. S3 (Tunguska). A similar situation (shared predominant PPH3 haplotype) was also noted for the Tarpanka I and Zaza maternal lines. Since the number of officially recognised PPH pedigree lines (16) is smaller than the number of detected mtDNA haplotypes (19), we also checked the possible origin of haplotypes which cannot be easily assigned as characteristic of any of the known maternal lines. In one case (PPH2 and PPH16), the sequence difference is clearly the effect of a single mutation that occurred at some point in the breeding history. Since the PPH16 haplotype sequence (GenBank: MF120542) is

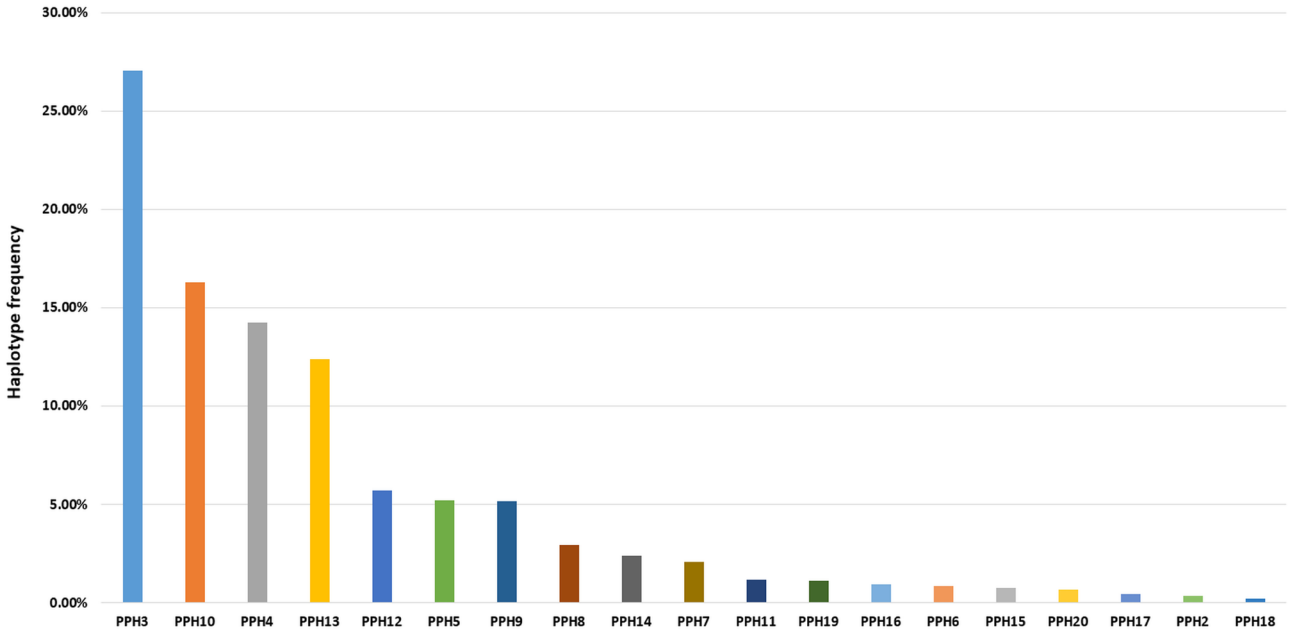

Fig. 2. The mtDNA haplotype frequency within the tested PPH population (based on molecular and pedigree data).

Table 1

Mitochondrial DNA haplotype distribution within particular maternal lines (based on molecular and pedigree data)

Maternal line	N_{ped} (%)	N_{mol}	HapN	Main haplotype (%) #	Other haplotypes (%)
Tarpanka I	1668 (17.9%)	41	6	PPH3 (69%)	PPH12 (19%), PPH14 (8%), PPH13 (3%), PPH10 (0.8%), PPH4 (0.2%)
Traszka	1523 (16.4%)	37	2	PPH10 (99%)	PPH3 (1%)
Zaza	1275 (13.7%)	21	2	PPH3 (94%)	PPH19 (6%)
Karolka	1139 (12.2%)	34	4	PPH4 (65%)	PPH13 (25%), PPH14 (8%), PPH19 (2%)
Liliputka I	1084 (11.6%)	53	6	PPH5 (45%)	PPH9 (26%), PPH4 (23%), PPH20 (3%), PPH13 (2%), PPH12 (1%)
Urszulka	839 (9.0%)	25	4	PPH13 (94%)	PPH20 (2%), PPH4 (2%), PPH12 (2%)
Tunguska	360 (3.9%)	37	2	PPH4 (76%)	PPH3 (24%)
Wola	315 (3.4%)	30	3	PPH8 (87%)	PPH4 (11%), PPH20 (2%)
Tygryska	259 (2.8%)	13	2	PPH9 (77%)	PPH3 (23%)
Dzina I	192 (2.1%)	16	1	PPH7 (100%)	–
Popielica	184 (2.0%)	19	1	PPH12 (100%)	–
Misia II	123 (1.3%)	15	2	PPH11 (85%)	PPH12 (15%)
Białka	122 (1.3%)	11	3	PPH16 (71%)	PPH2 (27%), PPH3 (2%)
Ponętna	89 (1.0%)	20	5	PPH15 (79%)	PPH13 (8%), PPH11 (7%), PPH19 (4%), PPH17 (2%)
Geneza	79 (0.8%)	16	1	PPH6 (100%)	–
Bona	39 (0.4%)	5	1	PPH17 (100%)	–
Baśka*	20 (0.2%)	3	2	PPH18 (95%)	PPH12 (5%)

N_{ped} – total number of mares in the reconstructed pedigree, N_{mol} – number of molecularly tested mares, HapN – number of haplotypes, # calculated in relation to the total number of mares in the given maternal line pedigree (N_{ped}), *not an officially recognized maternal line.

identical to many other sequences previously deposited in the NCBI database, while PPH2 (GenBank: MF120550) seems to be exclusive to our studies, we can suppose that PPH16 was the original haplotype of the Białka maternal line whereas the PPH2 is a result of its single A>G substitution (position 15810, according to the GenBank X79547 reference mtDNA sequence), which took place before 1990 (the birth year of the oldest mare in our experiment with the PPH2 haplotype). This slight nucleotide sequence difference should not constitute a reason for classifying the mares carrying PPH2 and PPH16 haplotypes as belonging to different genetic lines.

Interestingly, combining the molecular and pedigree data on the PPH18 haplotype revealed that it segregates within a pedigree branch that does not belong to the pedigree of any officially recognised maternal lines. The oldest female ancestor present in this strain and recorded in the Polish Horse Breeders Association database is a mare named ‘Baśka’

(probably born in the 1980s), but unfortunately, her detailed origin is unknown. In tracking the history of the other rare haplotypes (PPH14, PPH19 and PPH20), we cannot exclude the possibility that they are remnants of PPH dam lines that have been considered extinct, but whose presence was obscured by the numerous mistakes in the registry – mistakes made before the era of accessible genetic testing. For example, an analysis of the pedigree and breeding documentation of horses carrying the PPH19 haplotype reveals that some of the ancestors were kept in the same places as mares derived from the Hajnówka maternal line, which seemed to die out in the 1970s. It should also be noted that, in several cases, our study might have pinpointed the putative place and time where horses from two different maternal lines were mistakenly substituted. Such information can be important for correcting the PPH pedigrees and better managing the maternal genetic diversity – one of the main goals of the breed conservation programme. This is particularly crucial for those maternal lines that prove to have been genetically subdivided into two or more strains (carrying various mtDNA haplotypes) long ago. For example, our studies on archival DNA samples have revealed that, for the Tunguska maternal line, the pedigree error must have occurred more than thirty years ago, since some mares born in the years 1994-1995 had two different mtDNA haplotypes: PPH3 and PPH4. This led to the subdivision of this particular dam line pedigree into two genetically separate branches (Fig. S3). Interestingly, the PPH12 haplotype, which was assigned to the Popielica maternal line (as all tested Popielica horses carried PPH12), was also found in a large branch of the Tarpanka I line pedigree. This is important information from the conservation point of view, since until now the Popielica line has been considered as one of the rarest lines, needing extensive development. Our study has shown that the situation regarding this particular line is probably better than was previously thought, as pedigree errors in (most likely) the 1980s mean that some horses of the Popielica line origin were wrongly assigned to the Tarpanka I maternal line (Fig. S4). Signs of the presence of individuals of the Popielica line (such as carrying of the PPH12 haplotype) were also observed in Misia II, Urszulka and Liliputka I animals, as well as within the officially unrecognised Baśka line (Table 1).

Practical utility of the presented findings: an example of the Polish Society for Bird Protection herd

A very good example of the potential application of our results to the PPH conservation programme is the analysis of changes in maternal genetic diversity within the herd kept by the Polish Society for Bird Protection (PTOP) over the five-year timespan from 2019 to 2024. In 2019, there were 46 broodmares in this herd belonging to twelve of the sixteen officially

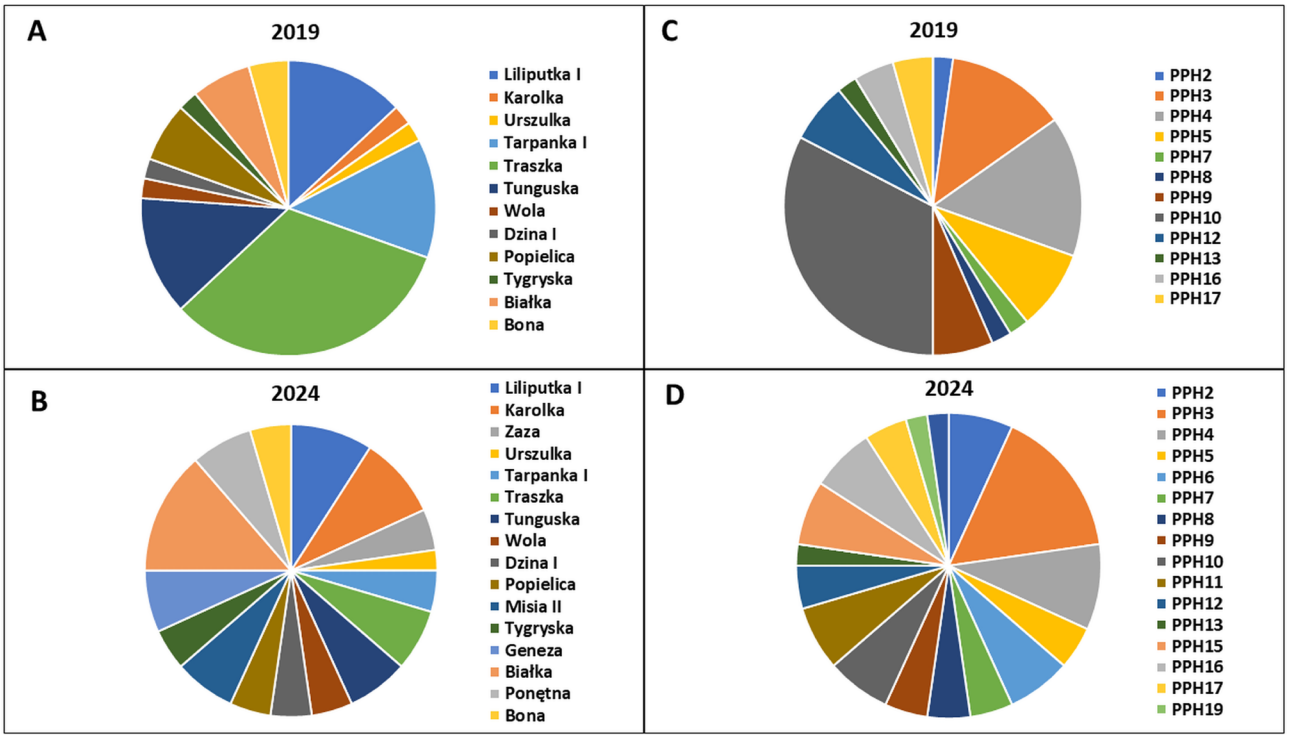

Fig. 3. Changes in genetic diversity of PPH mares in the PTOP herd between 2019 (n = 46) and 2024 (n = 44). A, B: officially recognized maternal lines; C, D: mtDNA haplotypes.

recognised maternal lines (Fig. 3A) and carrying twelve of the nineteen known mtDNA haplotypes (Fig. 3C). PTOP's collaboration with Poznań University of Life Sciences on both molecular and pedigree studies helped to significantly increase the maternal genetic diversity of the herd, which currently contains 44 broodmares representing all sixteen of the officially recognised maternal lines (Fig. 3B) and spanning seventeen of the nineteen recognised mtDNA haplotypes (Fig. 3D). To our knowledge, this is currently one of the most diverse PPH herds in Poland, in the context of maternal lines. This example clearly demonstrates that the genetic testing of horses and a comparison of the obtained results to each PPH maternal line pedigree can assist in making informed breeding decisions, in order to significantly increase the maternal genetic diversity of the herd.

Discussion

The mtDNA sequence's maternal inheritance and high level of variability (especially within the D-loop regulatory region) make it a valuable tool for genetic diversity studies and for tracking the history of equine dam lines (Cieslak *et al.* 2010). Investigations of mtDNA sequence variations have significantly enhanced our knowledge of the evolution, domestication and phylogenetic relationship between different equid species and horse breeds (Oakenfull *et al.* 2000; Jansen *et al.* 2002; Achilli *et al.* 2012). Such analyses can also outline the paths by which the domestic horse has expanded to different regions of the world. Recently, an analysis of the earliest complete equine mtDNA showed that the specimen belonged to a Caribbean horse from the sixteenth century, which putatively came to Haiti from the Iberian Peninsula in the Colonial Era (Delsol *et al.* 2022). Moreover, the study by Engel *et al.* (2022) showed the potential usefulness of mtDNA studies for predicting equine sports performance. The authors described the significant associations between 20 mtDNA sequence variants and the estimated breeding values (EBVs) for dressage and show jumping disciplines in the Holstein breed. Finally, studies employing mitochondrial DNA sequence variations are common for the equine breeds covered by conservation programmes, and for those in which the tradition of distinguishing and breeding particular maternal lines is still strongly cultivated. The best-known example of this is the Arabian horse population, in which the breeders often refer to eight historical maternal strains (RASANs): Kahlila, Saklawia, Abiah, Shweemat, Muanakii, Hamadania, Dahmaa and Hadbaa. Traditional breeders in the Middle East desert have focused on breeding pure Arabians (avoiding potential admixtures of other horse breeds) and keeping rigorously separated RASANs. However, the mtDNA-based study by Khanshour & Cothran (2013) revealed that this separation is not clearly visible on the molecular level, and that some mtDNA haplotypes are shared by many traditionally distinguished maternal strains. On the other hand, a recent study on the endangered Cleveland Bay horse has shown that the majority of the mtDNA-tested individuals were clustered into separate clades of the neighbour-joining tree, and this division clearly reflected the previously described maternal ancestry lines (Dell *et al.* 2020).

Although the historical data indicates that as many as 35 PPH dam lines survived the Second World War, nineteen of these are now considered to be extinct, leaving only sixteen lines active in the breeding population (Jaworski, 1997). One of the main goals of the PPH conservation programme is to ensure the balanced development of the remaining lines to prevent them from potential extinction. Tracking the results of previous studies using microsatellite markers (Mackowski *et al.* 2015; Szwaczkowski *et al.* 2016; Fornal *et al.* 2021) or mtDNA sequence variants (Cieslak *et al.* 2017; Musiał *et al.* 2024), we realised that reaching this goal is likely to be challenging without an extensive revision of the breeding strategy.

Even though several studies on the genetic diversity of PPHs have been published so far, this is the first investigation to show the possibility of using both mtDNA and pedigree data for PPH breed conservation purposes. Since the beginning of the conservation programme, the number of PPH herds in Poland has increased significantly, and currently over 1600 mares are registered, which is an undoubted success. However, to our knowledge, the percentage distribution of particular maternal lines has not changed noticeably, and some lines can still be considered as endangered. Since the total number of PPH horses registered in the conservation programme is now generally high, we suggest focusing more on protecting the genetic diversity, such as by emphasising the development of the rarest maternal lines and reducing the number of horses belonging to the lines that are overrepresented. We have shown that this goal can only be reached by using pedigree data that has been corrected on the basis of a molecular analysis, since the number of inconsistencies found between the breeding documentation and the molecular data indicates that the official registry

does not accurately reflect the real genetic diversity of the breed.

Pedigree errors are a well-documented issue in equine genetics and have been reported across multiple horse breeds. The pedigree discrepancies are not rare and emphasise the importance of genetic verification in breed management. The study by Seyedabadi and Sofla (2017) highlighted the occurrence of pedigree errors in the Turkmen horse population, showing discrepancies between the recorded pedigrees and the STR genetic data. This underscores the need for routine parentage verification using molecular markers to ensure accuracy. Moreover, the study by Sereno *et al.* (2008) used 12 microsatellite markers for a genetic analysis of 101 Pantaneiro horses to verify the parentage and control the pedigree records. The results demonstrated a high polymorphism of markers and the effectiveness of a DNA analysis in identifying misassignments in the parentage records. This underscores the importance of genetic markers in conservation programmes and breeding management for the Pantaneiro horse. The utility of using mtDNA markers in pedigree verification and a reconstruction of the 'maternal history' has been carried out successfully before, such as in the case of Arabian, Lipizzan and Maremmano horses (Bowling *et al.* 2000; Čačić *et al.* 2011; Giontella *et al.* 2020). Mitochondrial sequence data has also been successfully used to monitor the genetic diversity of many local horse breeds, such as the Szekler (Gáspárdy *et al.* 2023), Italian Salernitano horse (Criscione *et al.* 2015) and the Finnhorse (Kvist *et al.* 2019). The above findings confirm that pedigree inconsistencies are prevalent across various horse breeds, reinforcing the importance of genetic validation in breeding programmes. Integrating an mtDNA analysis with microsatellites or SNP-based genotyping can significantly enhance the pedigree accuracy and improve breed management practices.

Since, as mentioned, the current number of PPH individuals harboured by the conservation programme is generally high, it would seem that now is a good moment to revise the main goals of the programme and to make changes to the breeding strategy, where necessary. One of the most important objectives of the PPH programme, as specified by its authors, was to preserve the unique phenotype of the breed, including the primitive body conformation. At the same time, the programme also suggests that PPH individuals should be selected to improve their usefulness for riding, driving and other purposes. This seems impossible to do without losing part of the breed's genetic diversity (as the genomic regions under selection pressure will become more homozygous) and without changing the conformation of the horse. A clear ROH (run of homozygosity) has been already detected in the PPH breed within the region of the TBX3 gene (Gurgul *et al.* 2019), which is responsible for the dun coat colour pigmentation – the only colour permitted by the PPH phenotypic pattern (Cieslak *et al.* 2021). Although selecting for the most desired coat colours has sometimes been suggested for other primitive horse breeds covered by conservation programmes (Yoshihara *et al.* 2025), prioritising horses of a high utility value (e.g. for riding use) is uncommon, because as aforementioned, this is impossible without altering the characteristic body conformation. However, the study by Pasicka *et al.* (2017), which was based upon biometric data covering a period of nearly one hundred years, has indicated that the PPH breed is under putative selection pressure for its riding utility, since the number of conformation traits has significantly changed during the breeding history. These examples raise serious questions regarding the compatibility of the goal of preserving unique genetic and phenotypic features with that of making the breed more rider-friendly. Similar doubts have also been formulated in the context of other horse breeds, in which the need to protect genetic diversity coexists with the market demand for animals of a high utility value (Ablondi *et al.* 2020; Ivanković *et al.* 2021). Taking into account the significant growth of the PPH population since the beginning of the conservation programme, it is perhaps worth considering reducing the programme to include only the most genetically and phenotypically valuable individuals (that is, to those with the most pronounced primitive characteristics and who represent the most differentiated genetic lines). In contrast, the rest of the population could be selected for different types of utility, without the significant restrictions aimed at maintaining genetic diversity.

We can speculate that, perhaps paradoxically, the numerous errors committed in the PPH pedigree are responsible for the representatives of some maternal lines that are officially considered extinct still being present in the breeding population. It is thus fundamental to consider the molecular data in the breeding strategy, to protect those individuals with uncommon mtDNA haplotypes. However, the example of the officially unrecognised Baśka line with the unique PPH18 haplotype shows that our results should be considered carefully, because the incompleteness of the pedigree data means that we cannot verify whether this haplotype is a relic of one of the old maternal lines or is instead a sign of the admixture of the PPH breed. On the other hand, this case

is a good example of how the management of PPH conservation can be improved, as the programme clearly states that only mares representing one of the sixteen officially recognised maternal lines can be included. Our study has shown that there are some exceptions to this, such as mares that do not belong to the sixteen known maternal lines being nonetheless active in the breeding population.

Although the results of our investigations will undoubtfully contribute to a better management of the PPH breeding, studies focusing on the breed's genetic diversity should be continued. The conservation programme could benefit, e.g. by expanding mtDNA analyses to include the entire mitochondrial genome sequence. As was indicated in a recent study by Musiał *et al.* (2024), examining longer sequences results in the detection of a higher number of variants and haplotypes, which was also shown in other breeds, i.e. the Holstein Horse (Engel *et al.* 2021). This could be useful for a more detailed analysis of the maternal lines sharing identical mitochondrial haplotypes in the present study. Furthermore, the authors demonstrated the application of Y chromosome haplotypes studies in reflecting the male counterpart of PPH genetic diversity. Such analyses, along with comprehensive pedigree studies, would benefit the PPH conservation programme that is aimed not only at protecting the sixteen maternal lines, but also the six officially recognised male lines of which some (Glejt I and Liliput) are considered less frequent and need development (Pasicka 2013).

Conclusions

Our study has confirmed the great usefulness of mtDNA analyses in outlining the history of equine dam lines and in assessing the maternal genetic diversity. The example of the PPH herd of the Polish Society for Bird Protection (PTOP) shows that the consideration of mtDNA sequence data in the breeding programme may result in a significant increase in genetic diversity within a relatively short time. Such changes, however, would not be possible without improving the pedigrees using molecular data.

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Author Contributions

Research concept and design: K.R.-M., J.C.; Collection and/or assembly of data: M.M., A.B., R.K., A.B.; Data analysis and interpretation: W.S., R.K., A.M., J.C.; Writing the article: W.S., J.C.; Critical revision of the article: M.M., A.B., R.K., A.M., A.B., K.R.-M.; Final approval of article: W.S., M.M., A.B., R.K., A.M., A.B., K.R.-M., J.C.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary Materials to this article can be found online at:
http://www.isez.pan.krakow.pl/en/folia-biologica.html
Supplementary files:
SM.01. Table S1. The list of tested samples.
SM.02. Figures S1-S4: Geneza, Karolka, Tunguska and Tarpanka lines pedigree charts.

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