DIPLOMAMUNKA

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Universal genetic method for fetal sex determination from maternal plasma in unipara mammalian species

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LIST OF ABBREVIATIONS

A, C, G, T	adenine, cytosine, guanine, thymine (bases)
AmelX	amelogenin X
AmelY	amelogenin Y
bp	basepair
BSA	bovine serum albumin
cfDNA	cell free DNA
cffDNA	cell free fetal DNA
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EAZA	European Association of Zoos and Aquaria
g	acceleration of gravity
HMG	high mobility group
ng	nanogram
NIPD	non-invasive prenatal diagnostics
PCR	polymerase chain reaction
rpm	revolutions per minute
SOX	SRY-related HMG-box
SRY	sex determining region Y
TSPY	testis-specific protein Y-encoded
UV	ultraviolet
µl	microlitre

1. INTRODUCTION

Fetal sex-determination has long been a widely applied, routine service in human prenatal diagnostics. Regarding other mammalian species, sex determination of the yet unborn fetus is way less advanced. Its feasibility is still limited to a very few species, mainly farm animals. The applied methods are various, one working in certain species but not in others. To promote the breeding activities of both farm animals, but even more importantly, a wide range of captive species, the existence of a single, universal method applicable to nearly all mammalian species would be extremely remarkable.

Literature review

1.1. The importance of fetal sex-determination in domestic animal breeding and wildlife conservation

Fetal sex-determination has significance mainly in the case of unipara mammalian species, species that normally give birth to only one calf at a time. Major unipara species include primates, elephants, cetaceans, seals, several even and odd toed ungulates. Among these, the breeding of farm animals (such as cattle, sheep, horse) and wild species found in captivity (such as elephants, giraffes, zebras, rhinos, dolphins, seals and several primates) is of great importance. Since the gestation period of these species is rather long, some lasting even up to 21 months, prenatal diagnostics has substantial benefits. In the light of the gender of the offspring to be born, several important breeding-related questions and activities can be clarified and arranged in advance. The related issues are fundamentally different though for domestic farm animals and captive bred wild species.

In case of intensive animal husbandry, economic benefit is the primary consideration behind acquiring the knowledge of gender. Gender affects phenotypic traits, that contribute to the economic value of the individual in each breeding sector. In the dairy cattle industry, a heifer calf is more valuable; in the beef cattle sector however, a bull calf has higher value due to better growth rate and feed conversion (Silversides et al, 2001). Therefore, knowing the gender of the offspring in advance facilitates revenue and production planning for the owner and has the advantage of asking for a higher price when marketing heifers pregnant with offspring of a certain sex. In sheep-breeding, knowledge of fetal sex can also increase the value of ewes, provide the opportunity to a better planned, more tailored feeding program of the ewes (Santos et al, 2007), and facilitate making appropriate management and commercial decisions, such as the culling of animals and the selection of sex in breeding programs (Kadivar et al, 2013). As regards of horse-breeding, the desirable sex and the value

of an animal is more of a matter of individual preference (Panarace et al, 2014). However, while stallion thoroughbreds are suitable for both racing and hobby purposes, a mare quarter horse is considered specifically suitable for racing and thus its value exceeds that of the stallions (Chezum & Wimmer, 1997; Lansford et al, 1998). Again, knowing the gender of the offspring in advance can be beneficial for the breeder when making sales planning, for both the offspring and the pregnant mares.

Fundamentally different considerations are applied to captive wild animal species present in zoos. Many of them being threatened, the relevant aspect in this case is the protection and maintenance of the species. Zoological societies, such as the European Association of Zoos and Aquaria (EAZA), link together hundreds of organizations and lead coordinated breeding programs of wild animals in captivity in order to support successful *ex situ* conservation of the species (?). The carefully designed breeding and exchange programs, with cooperation of zoos around the world, allow for the maintenance of a healthy, genetically diverse captive population of the species. Individuals are regularly exchanged between zoos, and the breeding of each individual is designed and recorded. In certain species, due to aggression of the males, sex ratio of individuals held in each zoo is also regulated by international rules (Papp 2015; Andréka & Gunics, 2015; Abelló et al, 2017). Being aware of the gender of an offspring well before birth provide zoo management the opportunity to plan and arrange solutions and procedures related to exchange and breeding programs in advance. Finding and arranging placement, arranging transportation, preparing the necessary documents are only some of the essential works related to the relocation of an animal, and the sooner a decision is made, the sooner institutions can begin the tasks that need to be fulfilled (Olson, 2002; Abelló et al, 2017). Species with harem-structured populations, such as elephants, rhinos or zebras, are of particular interest, since in these cases only one male can be housed with several females; therefore, while the demand for females is usually high, the placement of males can pose great challenges. Beyond the exchange programs, subsequent breeding programs also benefit from early sex-determination, which allows for taking into account the individuals to be born during a planning process. In addition, the risk and probability of stillbirth at some species, such as rhinoceros, can be significantly higher in case of a male fetus (Kennedy, 2016), therefore fetal sex determination can allow for a more careful pregnancy monitoring and a better prepared veterinary care during calving (Stoops et al, 2018). Especially since captive breeding of some species is extremely challenging and pregnancies are rare events (Snyder et al, 1996), prior knowledge can be crucial to prosperous breeding programs.

1.2. The variance and limitations of currently applied methods in fetal sex-determination of animals

Despite the potential and the wide range of benefits, fetal sex determination is not such a widely applied process in the animal kingdom. The primary reason behind is the limitations of available methods regarding feasibility. Farm animals are in a relatively good position, however, prenatal sex determination of captive wild species is still in need of development.

1.2.1. Ultrasound

Ultrasound has been the first and still the most basic form of fetal-sex determination, in both the human, and later in the animal field. The method is based on the examination of the fetal gonad with the help of an ultrasound device, providing a black-and white visualization of the fetus in the uterus. Among farm animals, ultrasound has been quite prevalently applied in pregnant mares, heifers and ewes, with an accuracy even up to 100%, but is suitable only at a specified, short time period of the pregnancy (Curran, 1992; Coubrough & Castell, 1998), limiting its applicability. In captive wild species, as opposed to farm animals, ultrasonography is not at all as easy to implement, can only be performed during anesthesia, that puts significant stress on both the animal and its fetus, and potentially increase the risk of abortion (Taylor, 1997). As such invasive procedure, it is rarely applied by zoos, only in last resort. At certain species, such as elephants, due to the large internal organs of the female, the fetus cannot even be reached by ultrasound (Pushpakumara et al, 2019), therefore this method is completely outside possibility.

1.2.2. Hormon level measurement

During pregnancy, significant changes in hormone levels of the maternal blood occur. Measurement of maternal testosterone level can be relatively reliable in predicting fetal sex of certain species. In Asian elephants, testosterone is proved to increase in the maternal blood to a significantly higher level in case of a male fetus, than in case of a female fetus, after the first year of pregnancy – when organogenesis of the fetus is completed. After this time, measurement of peripheral maternal testosterone concentration produces a nearly 100% level of accuracy in Asian elephants (its accuracy is disputed in African elephants) (Duer et al, 2002). Since most captive individuals are relatively accustomed to blood sampling due to routine veterinary inspections (Ilycsin 2016; Koroknai et al, 2016; Sápi, 2016), hormone level measurement is a preferred alternative to ultrasound, frequently applied by zoos. Nevertheless, the method is proven to be exact in few species only, and since repeated measurements are needed, requiring blood sample collection over a longer period of time, it can be exhausting for both staff and animal.

1.2.3. Genetic fetal sex-determination

A relatively new method with a different approach, the genetic sex-determination of the fetus based on cell free fetal DNA (cffDNA) present in the maternal blood is currently available (apart from humans) for a few animal species only: cattle, horse and sheep as regards of farm animals, elephants and rhinos as regards of zoo animals, and macaque and rhesus monkey as model animals in respect of biomedical research. The principle of a cffDNA-based assay is the detection of the male-specific Y chromosome in the maternal blood by molecular methods. A positive result indicates a male fetus, while in case of a negative one, if the pregnancy is already proven, a female fetus is likely. This method has the advantage of being non-invasive to the fetus, applicable since an early stage (commonly after the first trimester) of pregnancy, easy to implement, even with a single blood test, with no significant stress effect on either the mother or the fetus. Though the sensitivity of the DNA-based fetal sex determination varies among species, the accuracy of the developed tests is up to 90-100% that along with the additional benefits make the cffDNA-based assay great both as additional and alternative method to the previous ones. However, the tests developed mostly being species-specific, the scope of the cffDNA-based method is currently narrow, slowly expanding.

1.3. Cell free fetal DNA (cffDNA) and its role in prenatal diagnostics

CffDNA refers to extracellular DNA with fetal origin, circulating freely in the maternal blood. Since its discovery in 1997 (Lo et al, 1997) it has revolutionized the field of non-invasive prenatal diagnostics (NIPD), first and primarily in the human field, but increasingly in the animal field as well. The detection and analysis of cffDNA present in the maternal blood has replaced chorion villi biopsy and amniocentesis (known to carry a relatively high risk of abortion) (Mujezinovic & Alfirevic, 2007) in many areas of human prenatal diagnostics, such as detecting aneuploidy (Chiu et al, 2011; Bianchi et al, 2014; Jeon et al, 2014), diagnosing monogenic diseases (Tsui et al, 2011), determining Rh status (Finning et al, 2008), gender and chromosome-associated diseases (Liao et al, 2011). In small quantities cffDNA derives from the apoptosis of fetal hematopoietic cells (Lo et al, 1997; Sekizawa et al, 2000), and DNA segments already present in a free form crossing the placenta (Sekizawa et al, 2003), however, its primary source is the constant turnover of trophoblastic cells of the placenta, being replaced and destructed, shedding into the maternal circulation (Jackson, 2003; Alberry et al, 2007) (Figure 1). In the process of apoptotic body formation fetal DNA becomes heavily fragmented, and subsequently released in such fragmented form (Bischoff et al, 2005). The vast majority of cffDNA segments in the maternal blood are under 300 base pairs (bp) in length, with an even greater portion of them being sub-100 bp (Li et al, 2004). CffDNA can be detected in the maternal blood from the 6th week of pregnancy in humans (Lo et al, 1997), which allows for early examination and diagnosis, well before ultrasound. As the pregnancy progresses, and the contact surface of mother and fetus gradually extends, concentration of cffDNA increases in the maternal blood. Though it varies significantly between individuals (Wang et al, 2013), in average cffDNA accounts for 3-6% of the total cell free DNA present in the maternal blood (Lo et al, 1998), in some cases reaching even up to 10-20% to the final stage of pregnancy (Lun et al, 2008) (the remaining proportion being the typically longer segments of cell free DNA with maternal origin, normally present in the blood). The sensitivity of DNA-based sex determination varies according to gestational age, the volume of maternal blood obtained or copy number of target sequences (Primacio et al, 2017). Within hours to days after delivery cffDNA is rapidly cleared and finally completely eliminated from the maternal circulation (Lo et al, 1999; Hui et al, 2008) therefore does not affect diagnostics during a subsequent pregnancy.

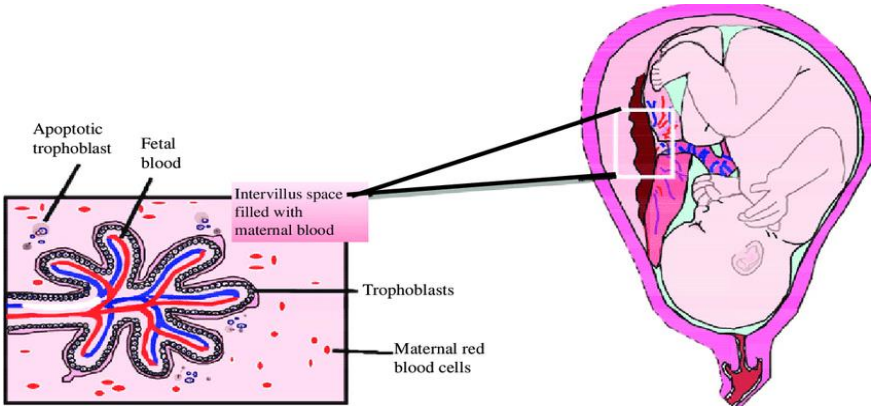

Figure 1. Main source of cell free fetal DNA in the maternal blood.

Source: Kotsopoulou et al, 2015.

In the animal field cffDNA analysis has become adopted primarily in terms of prenatal sex determination. Rhesus monkeys were the first species after humans to be examined for cffDNA in the maternal blood, primarily with the aim of promoting biomedical research (Jimenez & Tarantal, 2003; Mitsunaga et al, 2010). Later, for similar reasons, a test for Javan macaca was also developed (Yasmin et al, 2015). The utilization of cffDNA in farm animal husbandry soon followed (Davoodian & Kadivar, 2016); the development of several molecularly diverse tests in cattle (Wang et al, 2010; Da Cruz et al, 2012; Davoudi et al, 2012), horse (Leon et al, 2012, Kadivar et al, 2016) and sheep (Kadivar et al, 2013; Kadivar et al, 2015; Asadpour et al, 2015; Saberivand & Ahsan, 2016) have been described over the past decade to promote successful breeding-related activities. Just recently, a test for the Arabian camel has been developed (Abdulla et al, 2020). Captive wild species have come into focus only in the last two years; a test applicable to all four existing rhinoceros species were developed (Stoops et al, 2018), followed by another for African and Asian elephants (Vincze et al, 2019).

By overcoming the limitations of the currently applied method of ultrasound and hormone level measurement, the cffDNA-based assay has the potential to conquer the process of prenatal sex determination throughout unipara mammalian species and broaden the range of species possible to be examined. In theory a cffDNA-based assay could be feasible in all relevant species (Figure 2), however, anatomical structure of the placenta (such as the type of fetal implantation, the number of tissue layers separating maternal and fetal blood circulation, the location of chorion villi and connecting points on the surface of the chorion) shows significant difference between groups of mammalian species (Chavatte-Palmer & Tarrade, 2016) and may strongly affect the extent to which cffDNA enters the maternal blood. Unlike in the case of humans, little is clearly known about the presence and

detectability of cffDNA in the maternal circulation throughout the different mammalian species. However, the results achieved so far are promising, and suggest that cffDNA could potentially be detected and applied to fetal-sex determination in other, not yet examined mammalian species as well.

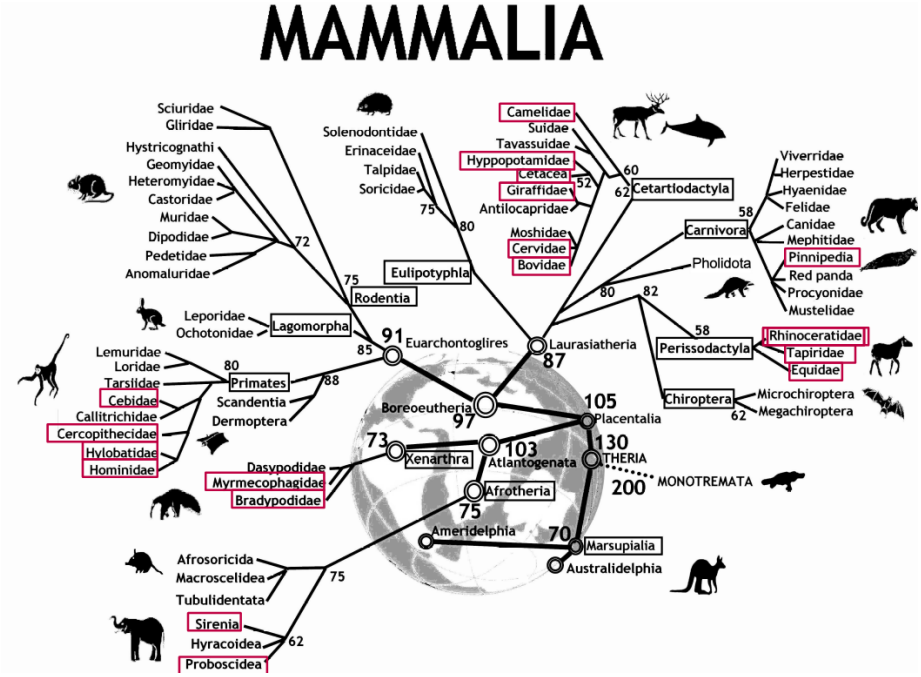

Figure 2. Main groups of unipara mammals relevant for fetal sex determination, indicated by red frame
Source: Graphodatsky et al, 2011

1.3.1. Molecular process of cffDNA-based genetic fetal sex-determination

In the process of the cffDNA-based assay cffDNA (together with maternal cfDNA) is first extracted from the maternal plasma sample. Then, one or more selected marker segments of the male specific Y chromosome are amplified by polymerase chain reaction (PCR) using specific primer pairs and detected by electrophoresis. The detection of an autosomal, mitochondrial or X-chromosomal marker segment found in both sexes (and could potentially have maternal as well as fetal origin) is normally also applied to indicate the proper functioning of the reaction even in the absence of the Y specific markers. Therefore, the presence of the Y specific marker along with the reaction control marker indicates the mother is carrying a male fetus, while the absence of the Y specific markers and detection of the reaction control designates a female fetus (other cases indicate a faulty reaction that needs to be repeated). However, before evaluating the ability of the test to determine fetal sex based on cffDNA of the maternal blood, preliminary testing and optimization process are necessary on known DNA samples of male and female individuals, potentially deriving from hair, feces, muscle or blood.

1.3.2. Cff-DNA-based tests for fetal sex determination developed in animal species to date

Cff-DNA based fetal sex-determination has been developed in few animal species so far. Though the principle of the method is the same, the tests developed are various, regarding the molecular details, the genetic markers selected, and the molecular techniques applied in amplification and detection (Table 1).

Table 1. Details of cffDNA-based tests developed in animal species to date.

Species	**Molecular techniques** ***(Accuracy of detection)***	**Y-marker** ***Reaction Control***	**Length (bp)**	**Reference**
Rhesus macaque *Macaca mulatta*	Real-time PCR (TaqMan) *(NA)*	SRY *ε-globin*	65 63	Jimenez & Tarantal, 2003
	Nested PCR (conventional/real-time) Polyacrylamide gel electrophoresis *(♂, ♀:100%)*	SRY *GAPDH*	239/75 194	Mitsunaga et al, 2010
Javan macaque *Macaca fascicularis*	Multiplex real-time PCR *(♂, ♀:100%)*	SRY DYS14 *no control*	122 95 -	Yasmin et al, 2015
Cattle *Bos taurus*	Nested PCR Gel electrophoresis *(♂:100%; ♀:85,7-92,5%)*	SRY *no control*	182/124 -	Wang et al, 2010
	Conventional multiplex PCR Agarose gel electrophoresis *(88,6%)*	ChrY_1 ChrY_2 *Chr1 (autos.)*	210 196 280	Da Cruz et al, 2012
	Conventional multiplex PCR Agarose gel electrophoresis *(♂, ♀:100%)*	Y_BC1.2 AmelY TSPY *AmelX*	190 341 260 467	Davoudi et al, 2012
Equine *Equus caballus*	Real-time PCR Two-round PCR Agarose gel electrophoresis *(♂:72,7-90,9%; ♀:100%)*	SRY *GAPDH*	182 150	Leon et al, 2012
	Nested real-time PCR *(♂:85,7%; ♀:90,9%)*	SRY *GAPDH*	155/91 193	Kadivar et al, 2016
Sheep *Ovis aries aries*	Real-time (PCR) Conventional PCR Gel electrophoresis *(♂, ♀:100%)*	SRY *GAPDH*	286 467	Kadivar et al, 2013
	Real-time PCR Agarose gel electrophoresis *(♂:96,5%; ♀:87,5%)*	AmelY_ins *GAPDH*	111 467	Kadivar et al, 2015
	Conventional PCR Agarose gel electrophoresis *(♂:80%; ♀:76%)*	AmelY *AmelX*	217 280	Asadpour et al, 2015
	Conventional PCR Agarose gel electrophoresis *(♂, ♀:100%)*	SRY AmelY *AmelX*	171 182 242	Saberivand & Ahsan, 2016
Rhinoceroses *Diceros bicornis,* *Certaotherium simum.,* *Rhinoceros unicornis,* *Dicerorhinus sumatrensis*	Two-round PCR Agarose gel electrophoresis *(♂:71-100%; ♀:100%)*	SRY *Amel*	177 232	Stoops et al, 2018
African/Asian elephant *Loxodonta africana,* *Elephas maximus*	Multiplex PCR Capillary electrophoresis *(♂, ♀:100%)*	SRY AmelY *PlpX*	85 75 147	Vincze et al, 2019
Arabian camel *Camelus dromedaries*	Conventional multiplex PCR Agarose gel electrophoresis *(♂, ♀:100%)*	SRY *GAPDH*	251 152	Abdulla et al, 2020

In most studies segments of the sex-determining region Y (SRY) gene were applied as an Y marker. The testis-specific protein Y (TSPY), and the amelogenin gene, which can be found on both the X (AmelX) and Y chromosome (AmelY) of mammals was also applied in some cases. For reaction control, segments of the autosomal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were the most frequently used. All the tests described have an accuracy in the range of 70-100%, however, these results also depend on the number of individuals tested and the stage of pregnancy examined, that also vary throughout the studies.

Since developments to date were focused on particular species, in most studies the primers applied were designed to be complementary to the DNA sequence of the one (or few closely related) species examined. This approach resulted mostly in species-specific tests applicable for one or a few related species, potentially reducing the risk of false-positive results deriving from contamination from another species. However, the process of developing additional tests for new species one by one is extremely time, cost and energy consuming, and results are in slow expansion of usability. Primers earlier applied for equine were later successfully used for rhino species though, suggesting that primers used previously might be successfully applied in the development of a test for certain other, closely related species. This opportunity could slightly fasten the progress of genetic testing of additional species, but preliminary testings on male and female control samples would still be necessary in each case.

2. AIM OF STUDY

Based on the importance of prenatal sex-determination throughout unipara mammalian species and on the variance and limitations of currently applied methods our intention is to provide a solution for fetal sex-determination in a wide range of unipara species.

Since a cffDNA-based assay could potentially be feasible in all relevant species, thinking further its application we aim to develop a single, generalized, universally applicable assay by identifying highly conservative Y chromosome-specific segments across relevant species.

By developing a universal test instead of species-specific ones, the process of pre-testing and optimization related to the development of individual tests could be saved, therefore this approach would be highly time- and cost-efficient and could make tremendous progress in the genetic prenatal sex determination of animal species within the foreseeable future.

With due diligence during the procedure avoiding contamination a universal cffDNA-based test could be successfully applied in the case of all relevant mammalian species, both economically important ones, but even more importantly wild species found in a captive environment, for many of which there is practically no possibility to determine fetal sex.

Management of both zoos and farms would have the opportunity to have the gender of a fetus determined throughout various species with a single, easy to implement method, both eliminating the inconvenience of applying different methods for each species (which are financially unfavorable) and promoting the exchange and breeding programs of several species at the same time.

3. MATERIALS AND METHODS

3.1. In silico analysis

Initially, *in silico* analysis was carried out to identify potential species to be examined and DNA sequences might be suitable for development of a universal cffDNA-based test.

3.1.1. Relevant species

We compiled a list of major relevant unipara species with a relatively long gestation period. Both aquatic and terrestrial animal species found across zoos and animal farms were recorded (Table 2).

Table 2. List of major unipara species relevant for fetal sex-determination, indicating length of pregnancy and conservation status (CR: Critically Endangered, EN: Endangered, VU: Vulnerable, NT: Near Threatened, LC: Least Concerned, DD: Data Deficient).

MAJOR RELEVANT UNIPARA SPECIES	LENGTH OF PREGNANCY (MONTHS)	CONSERVATION STATUS
PRIMATES		
Western/ Eastern gorilla *(Gorilla gorilla/ beringei)*	8.5	CR
Bornean/ Sumatran orangutan *(Pongo pygmaeus/ abelii)*	8-9	CR
Bonobo *(Pan paniscus)*	8	EN
Silvery gibbon *(Hylobates moloch)*	8	EN
Golden snub-nosed monkey *(Rhinopithecus roxellana)*	7	EN
Mandrill *(Mandrillus sphinx)*	6	VU
PROBOSCIDS		
African elephant *(Loxodonta africana)*	22	VU
Asian elephant *(Elephas maximus)*	18-22	EN
PILOSA		
Anteaters		
Giant anteater *(Myrmecophaga tridactyla)*	6.5	VU
Sloths		
Pygmy three-toed sloth (Bradypus pygmaeus)	4-7	CR
ODD-TOED UNGULATES		
Tapirs		
Malayan/ Baird's/ Mountain tapir *(Tapirus indicus/ bairdii/ pinchaque)*	13	EN
Rhinoceroses		
Black rhinoceros *(Diceros bicornis)*	16	CR
White rhinoceros *(Certaotherium simum)*	16	CR
Sumatran rhinoceros *(Dicerorhinus sumatrensis)*	16	CR
Javan/ Indian rhinoceros *(Rhinoceros sondaicus/ unicornis)*	15-16	CR/VU

Equids		
Grévy's/ Plains/ Mountain zebra *(Equus grevyi/ quagga/ zebra)*	12-13	EN/NT/VU
Wild horse (*Equus ferus)*	11-12	CR
Horse (*Equus caballus*)	11-12	(Domesticated)
Donkey *(Equus asinus)*	11-14	(Domesticated)
CARNIVORES		
Seals		
Galápagos/ California sea lion *(Zalophus wollebaeki/ californianus)*	11-12	EN/LC
Hawaiian monk seal *(Neomonachus schauinslandi)*	9	EN
Walruses		
Walrus *(Odobenus rosmarus)*	15	VU
SIRENIANS		
West Indian manatee *(Trichechus manatus)*	11	VU
EVEN-TOED UNGULATES		
Giraffids		
Giraffe *(Giraffa camelopardalis)*	13- 16	VU
Hippos		
Nile hippopotamus *(Hippopotamus amphibius)*	7-8	VU
Pygmy hippopotamus *(Choeropsis liberiensis)*	6-7	EN
Cetaceans		
Common bottlenose dolphin *(Tursiops truncatus)*	12	LC
Orca (*Orcinus orca)*	15-18	DD
Beluga whale (*Delphinapterus leucas)*	14-15	NT
Bovids		
Water buffalo *(Bubalus bubalis)*	9-11	(Domesticated)
European bison *(Bison bonasus)*	8-9	VU
Cattle *(Bos taurus)*	9-10	(Domesticated)
Sheep *(Ovis aries)*	5	(Domesticated)
Takin *(Budorcas taxicolor)*	8	VU
Camelids		
Wild Bactrian camel *(Camelus ferus)*	13	CR
Domestic Bactrian camel/ Dromedary *(Camelus bactrianus/ dromedarius)*	13-15	(Domesticated)
Lama *(Lama glama)*	11	(Domesticated)
Alpaca *(Vicugna pacos)*	11-12	(Domesticated)
Cervids		
Thorold's deer (*Cervus albirostris)*	8.5	VU
Sika deer *(Cervus nippon)*	7.5	LC
Reindeer *(Rangifer tarandus)*	7.5	LC

3.1.2. Search of DNA sequences in NCBI GenBank database

DNA sequences of the Y-specific TSPY, AmelY and SRY gene and of the autosomal GAPDH gene of relevant species were obtained from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Since not all relevant species had available homologous sequence in the database, available sequences of related species, not held in a captive environment and not relevant for breeding were also selected and included in further *in silico* analysis, to ensure finding a region that would presumably be conservative indeed across all relevant species.

3.1.3. Sequence alignment and search of conservative regions in MEGA program

The selected homologous sequences, separately for each gene, were aligned in MEGA (MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar, Stecher, Li, Knyaz, and Tamura 2018)) to identify regions of similarity. Regarding the Y-specific markers, TSPY and AmelY were neglected due to the scarcity and/or high variability of the available homologous sequences. Therefore, in further analysis, only the SRY and the GAPDH gene was included, having sequences available from a great variety of species (Figure 3 and 4.).

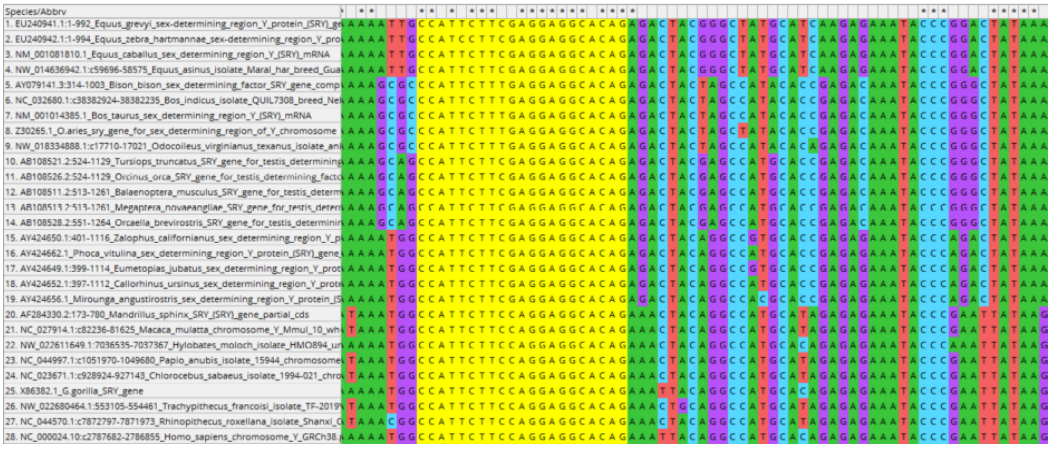

Figure 3. A highly conservative region of the SRY gene identified by MEGA.

Figure 4. A highly conservative region of the GAPDH gene.

3.1.5. *Primer design*

Considering the variable positions in the highly conservative homologous sequences, degenerate bases were occasionally used in some positions of the primer sequences to generate universal primers. Primers were designed using the Primer Designer 4 software (http://www.scied.com) and the Multiple Primer Analyzer of Thermo Fisher Scientific, shifting, extending, or shortening the original sequences by a few base pairs to reach optimal conditions for the primer pairs.

For the Y-marker SRY gene, initially two primer pairs were designed: one non-degenerate primer pair (*SRY non-deg*), and a degenerate primer pair (*SRY deg*), both amplifying the same 81-bp-long segment, the only difference being three bases in the sequence of the forward primer. Later on, several additional degenerate primer pairs, amplifying segments of varying length (80, 82, 119, 137 bp) were designed. For the reaction control GAPDH gene, two degenerate primer pairs were designed, with the same reverse, but different forward primers, one of them amplifying a longer, 404-bp-long segment (*GAPDH deg1*), the other one amplifying a shorter, 292-bp-long segment (*GAPDH deg2*).

Sequences of the designed primers are shown in Table 3.

Table 3. Primer pairs for SRY and GAPDH. Degenerate nucleotides are marked according to IUPAC codes (Y=C, T; S=C, G, D=A, G, T; R=A, G; K=G, T; H=A, C, T; V=A, C, G; B=C, G, T) and highlighted.

Primer pair	**Position**	**Sequence 5'-3'**	**Size**
SRY deg	Forward	CCATYCTTYSAGGAGGCACAG	81 bp
	Reverse	CTTCCGACGAGGTCGATA	
SRY non-deg	Forward	CCATTCTTCGAGGAGGCACAG	81 bp
	Reverse	CTTCCGACGAGGTCGATA	
GAPDH deg1	Forward	GCCATCACYGCCACCCAGAAGAC	404 bp
	Reverse	TACCAGGAAATGAGCTTSAC	
GAPDH deg2	Forward	TGGGCAAGGTCATCCCDGAG	292 bp
	Reverse	TACCAGGAAATGAGCTTSAC	
SRY 1	Forward	CATTCTTCGAGGAGGCAC	82 bp
	Reverse	GCCTTCCGACGAGGTCGA	
SRY 2	Forward	CTGGGATRYSAGTGGAAAAKGC	80bp
	Reverse	TGCATGGCYHGTAGTYTCTG	
SRY 3	Forward	AGGCGVAAGRTSGCTCT	137 bp
	Reverse	TGCATGGCYHGTAGTYTCTG	
SRY 4	Forward	GRTSGCBCTAGAGAAT	119 bp
	Reverse	GTAGTYTCTGYGCCTCC	

Specificity of the primers was checked in the NCBI Nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Initially all the primers were tested without fluorescent labelling. Following pre-testing, the most appropriate ones were ordered with fluorescent labelling.

3.2. *Sample collection*

Sample collection began in parallel with the *in silico* analysis, and continued throughout the research, aiming to collect control and maternal plasma samples from all relevant species possible. Up to this point hair, blood, muscle and feces control samples (Figure 5.) from males and females of several relevant species (Grévy's and Chapman's zebra, rhinoceros, hippopotamus, orangutan, gorilla, sloth, takin, California sea lion, water buffalo, European bison, mouflon, sheep, cattle, equine, fallow deer, red deer), and maternal plasma of Grévy's zebra, equine and cattle (Figure 6.) were collected. The samples were provided by the Budapest Zoo and Botanical Garden, Sóstó Zoo, Pilisvölgye Hunting Company, the Oasis Wildlife Fuerteventura, dairy farm of South-Pest County Agricultural Ltd., and the Department of Animal Breeding and Genetics, University of Veterinary Medicine Budapest.

Hair samples with intact roots were collected into plastic bags or envelopes and stored at room temperature. Fresh feces samples collected into plastic tubes were stored frozen until use. Peripheral blood samples, dropped on sterile glauze, were stored on room temperature. Maternal blood samples were collected into EDTA tubes, and centrifugated for 15 minutes with 1000g to obtain the plasma containing cell free DNA. The plasma samples were stored in Eppendorf-tubes on -20°C until the DNA extraction process. All procedures were performed in accordance with relevant guidelines and regulations in force.

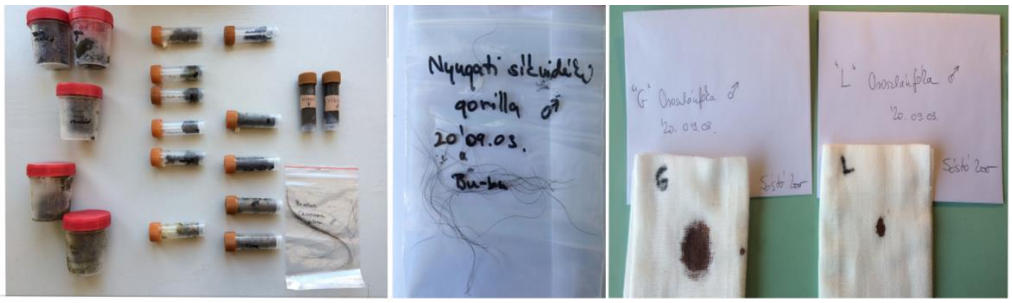

Figure 5. Collected feces, hair and blood samples from a variety of captive species.

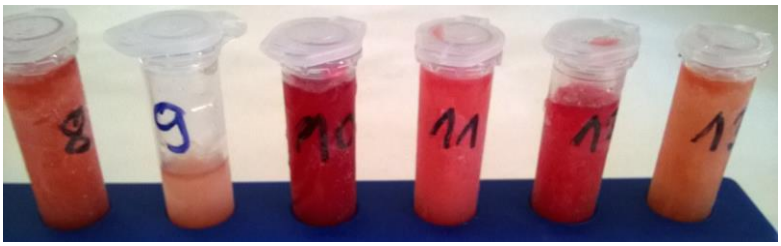

Figure 6. Maternal plasma samples of cattle.

3.3. In vitro study

The *in vitro* study was carried out in the laboratory rooms at the Department of Animal Breeding and Genetics, University of Veterinary Medicine Budapest.

3.3.1. Preliminary testing on control samples

Initially, control samples were used to test the designed primers, the detectability of the targeted DNA segments, and to optimize the reaction.

3.3.1.1. DNA extraction from various samples

All samples were prepared on a sterile surface using sterile tools to avoid contamination. Genomic DNA of control samples was extracted from feces (QIAamp Fast DNA Stool Mini Kit, Qiagen), hair and blood samples (QIAamp Mini Kit, Qiagen) according to the protocol provided by the manufacturer. Extracted DNA samples were stored at 4°C until subsequent analysis.

3.3.1.2. Primer testing with simplex PCR

The designed primers were initially tested in simplex PCR reactions (containing one primer pair only). Apart from the forward and reverse primers specific for the marker to be amplified, each PCR reaction requires the following essential reagents: extracted DNA sample, ultrapure water (UP), bovine serum albumin (BSA), and the Master Mix. The Master Mix contains the DNA polymerase, the dNTPs, magnesium chloride and PCR buffer. The BSA promotes the efficiency of the amplification. The PCR tubes were placed on ice while pipetting the reagents into each tube. Exact amount of each component in a given PCR reaction mixture is described in Table 4.

Table 4. Composition of the PCR mixture.

Reagent	**Amount**
Master Mix	4 µl
Primer mix (forward and reverse primer)	1 µl (5 µM)
DNA	1-10 µl (1-10 ng/µl, depending on the DNA concentration)
BSA	1 µl (2 ng/µl)
+ UP	up to 20 µl
Total	20 µl

The amount of DNA added depended on the concentration of extracted DNA samples that was examined on agarose gel prior to the PCR process. In the case of higher concentration a lower ratio, while in the case of lower concentration/lower quality DNA a higher ratio was

added to the reaction mixture (between 1-10 ng). Primer mixes were previously created from the forward and reverse primers, which therefore contained a primer pair specific to each targeted segment. A negative control reaction mixture containing no DNA sample was also included to indicate potential contamination.

The PCR process for each primer pair was optimized on male and female genomic DNA samples of horse, cattle, mouflon, sheep and zebra. Based on the calculated melting temperatures, the following annealing temperatures were tested using conventional PCR programs (Table 5).

Table 5. Tested PCR conditions for SRY and GAPDH primer pairs in simplex reaction.

Marker	Initial denaturation	*Denaturation*	*Anellation*	*Extension*	Final extension
	5 min	30 s	30 s	30 s	10 min
SRY	94˚C	94˚*C*	45/46/48/55/58˚C	72˚C	72˚C
GAPDH	94˚C	94˚C	50/52/54˚C	72˚C	72˚C
			32/34 cycle		

Based on the strength and specificity of the PCR products, certain primer pairs were selected, ordered with fluorescent labelling and tested on control samples of several additional species.

3.3.1.3. Detection of PCR products

The amplified DNA fragments were initially detected by agarose gel electrophoresis. A 2% agarose gel containing DNA-intercalating dye (GR Safe) was used. 5 µl from each PCR sample was loaded into each well as well as a 100 bp molecular ladder to check the size of the products. A running time of 40 minutes at 230V voltage was applied. By UV scanning the finished gel digital recordings were made for examination and evaluation of the results of the PCR. Amplicons were expected to be seen in the form of DNA bands at positions designated by the molecular ladder.

For more precise detection and determination of length, PCR products amplified with fluorescently labelled primers were subsequently detected by capillary electrophoresis as well (after purification of the PCR products).

3.3.1.4. Purification and sequencing of PCR products

For subsequent DNA sequencing, the amplicons were purified from the PCR reaction mix using the GenElute^{TM} PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's protocol. Sequencing of the amplified and purified PCR products was carried out by the Seqomics Biotechnology Ltd. in Mórahalom. The received sequences

were evaluated using Sequencher™ 4.1.2 (Gene Codes Corp), and aligned with the SRY and GAPDH sequences available in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) to confirm the identity of the detected region to the target region.

3.3.1.5. Duplex PCR

Based on the results of the simplex PCR programs, a duplex (two marker) PCR reaction was later optimized for co-amplification and detection of the SRY and GAPDH gene sequences. In this case, primer pairs of both markers were added to the PCR reaction mixture. *Touch-down* PCR was applied to ensure optimal conditions for both markers. The annealing temperature was reduced from 52°C to 46°C over 12 cycles, by 0.5°C per cycle. The other parameters were the same as those used in the simplex programs.

3.3.2. Examination of maternal plasma samples

Following preliminary testing on control samples, the effectiveness of the selected primers and the PCR procedure in fetal sex determination was tested on maternal plasma samples.

3.3.2.1. Extraction of cell-free fetal DNA from maternal plasma

For extracting cffDNA (together with maternal cfDNA) from maternal plasma, the Quick-cfDNA™ Serum & Plasma Kit (Zymo Research) was applied according to the recommended protocol. The amount of purified DNA was checked with a Qubit system.

3.3.2.2. PCR and detection

The PCR procedure and the detection of PCR products were the same as described in 3.3.1.2. and 3.3.1.3., respectively. Up to this point however, maternal plasma samples were tested with simplex PCR programs only.

3.3.3. Evaluation of reliability

Specificity, sensitivity and accuracy of the test will be assessed using standard descriptive statistics and binomial tests after the final optimization of the procedure and when sufficient number of samples are available.

4. RESULTS

4.1. Collection of reference samples

Control samples of known sexes were successfully collected from a wide range of targeted, unipara species (17 species in total), representing almost all major groups of unipara mammals.

4.2. DNA extraction

Genomic DNA (of varying quality and quantity) was successfully extracted and visible on agarose gel in case of almost all control samples (Figure 7.).

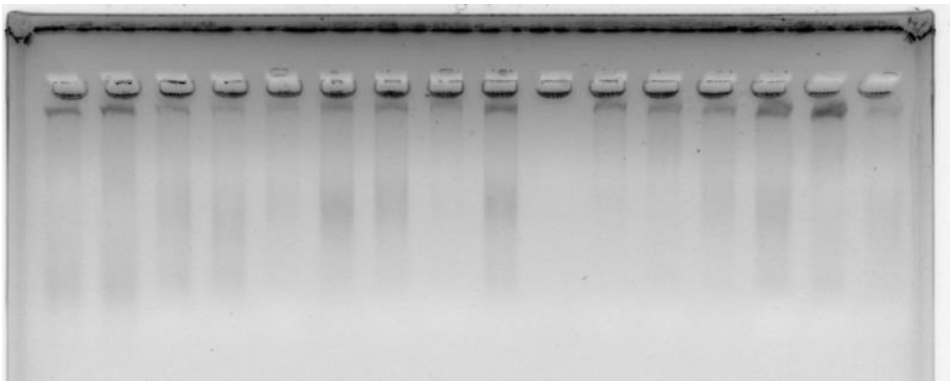

Figure 7. Examination of the quantity and quality of extracted DNA on agarose gel.

4.3. Primer design and optimization process

Based on the conservative segments identified within the SRY and GAPDH genes, we were able to design several primer pairs that could be successfully used for universal amplification in the targeted species (see Table 3).

In the optimization process both primer pairs designed for the GAPDH gene proved to be equally effective at an annealing temperature of 52°C (Figure 8). Amplified segments of the expected length were detectable in both male and female samples, as expected. Out of the two primer pairs, the *GAPDH deg2* was selected, based on its shorter sequence length, and used as a reaction control in further assays.

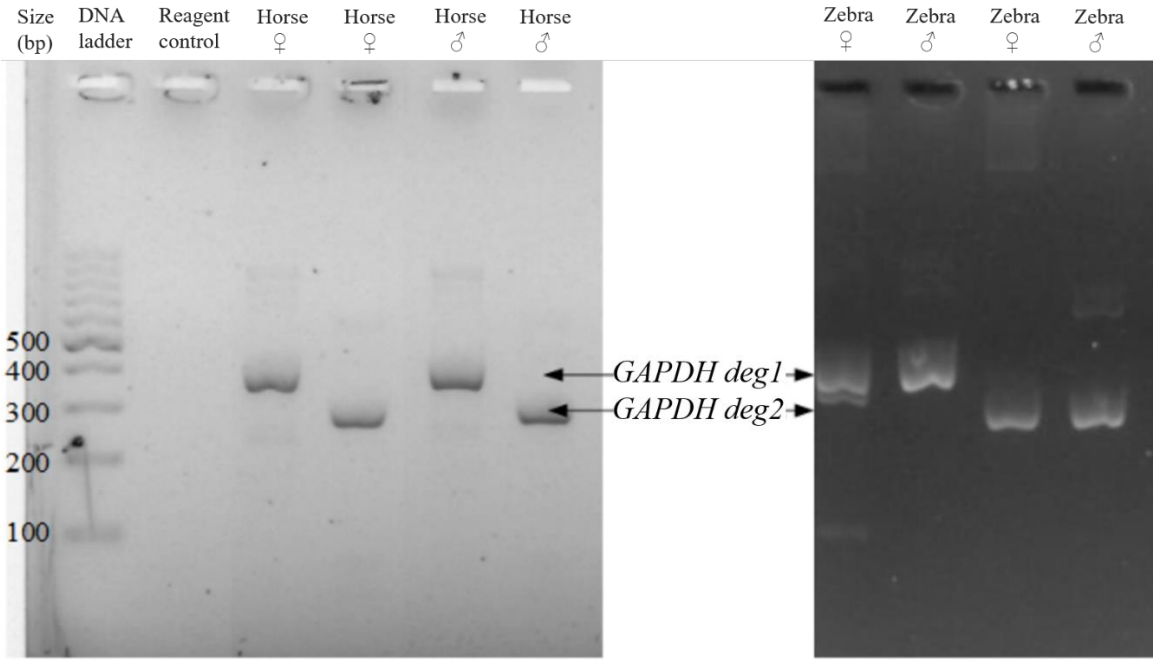

Figure 8. Preliminary testing of GAPDH primer pairs by simplex PCR programs.

Regarding the SRY marker, the initially designed and tested *SRY deg* and *SRY non-deg* primer pairs successfully amplified the targeted segment exclusively in male samples; however, in some cases they resulted in very low intensity DNA bands (Figure 9). Out of these two primer pairs, the non-degenerate primer pair (*SRY non-deg*) seemed to be more effective, and was therefore applied in further testing, with an annealing temperature of 46°C.

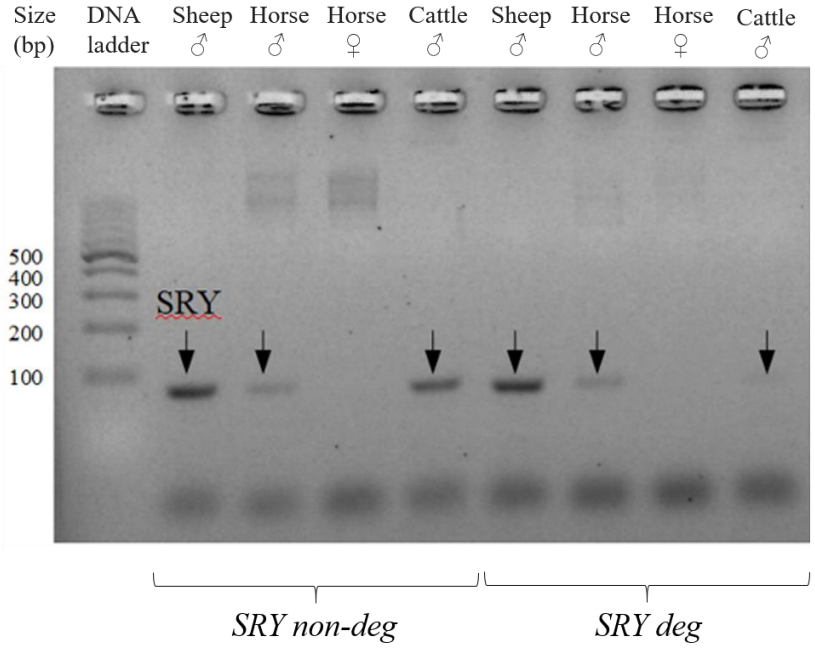

Figure 9. Preliminary testing of the initially designed SRY primer pairs (SRY non-deg, SRY deg) by simplex PCR programs.

The additional SRY primer pairs designed in the later phase of the study have so far been tested in the optimization process, but not on additional control and plasma samples. While the *SRY 2* and *SRY 4* primer pairs provided weak results in case of all tested conditions, the *SRY 1* and *SRY 3* primer pairs seems to be highly effective, with 36 cycles, at an annealing temperature of 60°C (Figure 10).

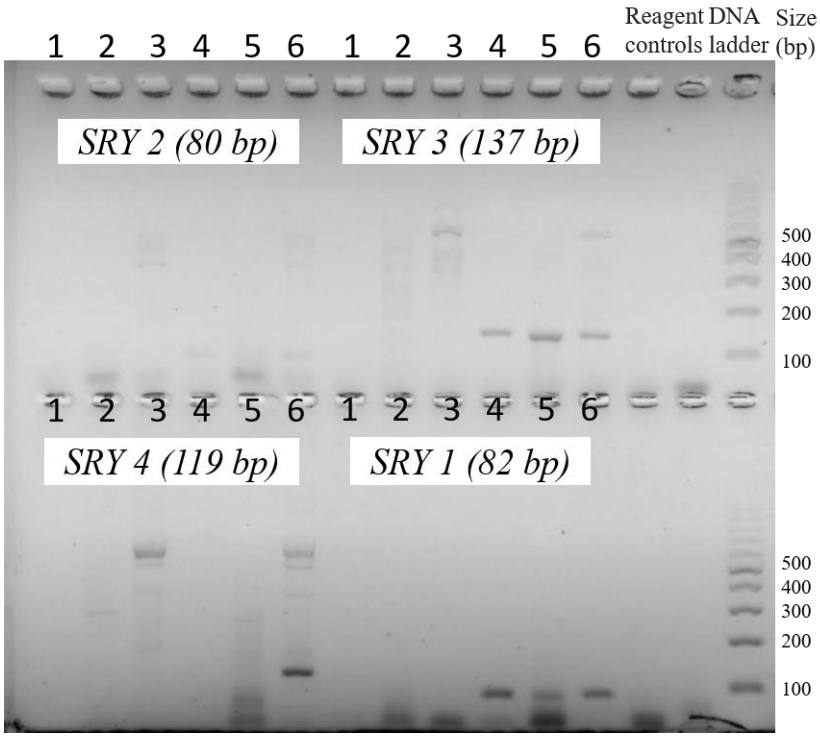

Figure 10. Preliminary testing of the additional SRY primer pairs (SRY 1, SRY 2, SRY 3, SRY 4) by simplex PCR programs.

1: Cattle ♀, 2: Horse ♀, 3: Sheep ♀, 4: Cattle ♂, 5: Horse ♂, 6: Sheep ♂

4.4. *Examination of species and sex specificity on control samples*

After the optimization process, the GAPDH marker was successfully detected in nearly all male and female control samples of additional, more exotic species as well (Figure 11, 12). In some cases of DNA samples extracted from feces the PCR reaction was unsuccessful (Figure 11, sample 4, 6, 10, 13, 14, 15).

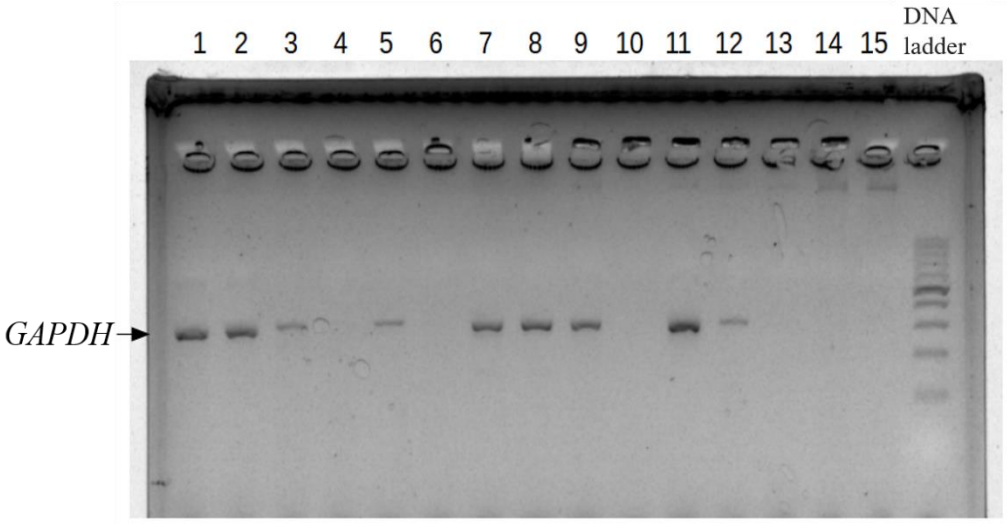

Figure 11. Detection of GAPDH in faecal samples of zoo species.

1: Takin ♀, 2: Takin ♂, 3: European bison ♀, 4: Water buffalo ♀, 5: Orangutan ♂, 6: Orangutan ♂, 7: Orangutan ♀, 8: Orangutan ♀, 9: Orangutan ♀, 10: Arctic wolf ♀, 11: Sloth ♀, 12: White rhinoceros ♀, 13: White rhinoceros ♂, 14: Chapman's zebra ♂, 15: Chapman's zebra ♀

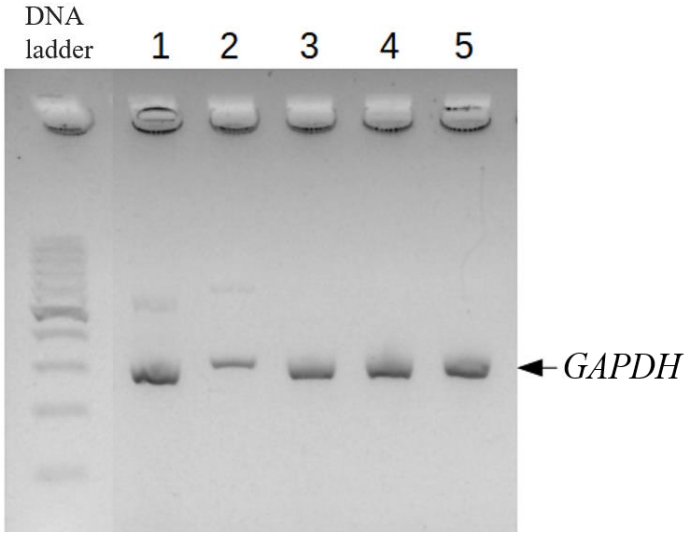

Figure 12. Detection of GAPDH in blood samples.

1: Fallow deer ♂, 2: Grévy's zebra ♂, 3: Gorilla ♂, 4: White rhinoceros ♂, 5: California sea lion ♂

Applying the *SRY non-deg* primer pair, the SRY marker was successfully detected in good quality, but not in lower quality male samples. As expected, it was not detected in any of the female samples (Figure 13).

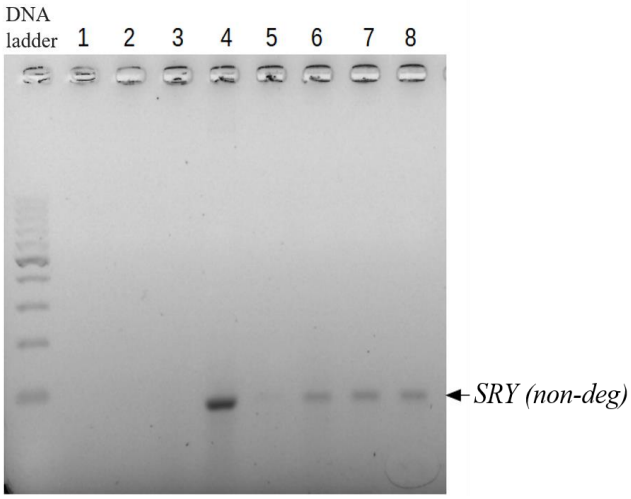

Figure 13. Detection of SRY in faecal and blood samples.

1: Takin ♀, 2: Takin ♂, 3: Orangutan ♂, 4: Fallow deer ♂, 5: Grévy's zebra ♂, 6: Gorilla ♂, 7: White rhinoceros ♂, 8: California sea lion ♂

4.5. Duplex PCR

Applying the touch-down PCR technique, the Y specific SRY marker and the reaction control GAPDH were successfully co-amplified and co-detected in a number of male control samples (although, larger non-specific products were also produced) (Figure 14).

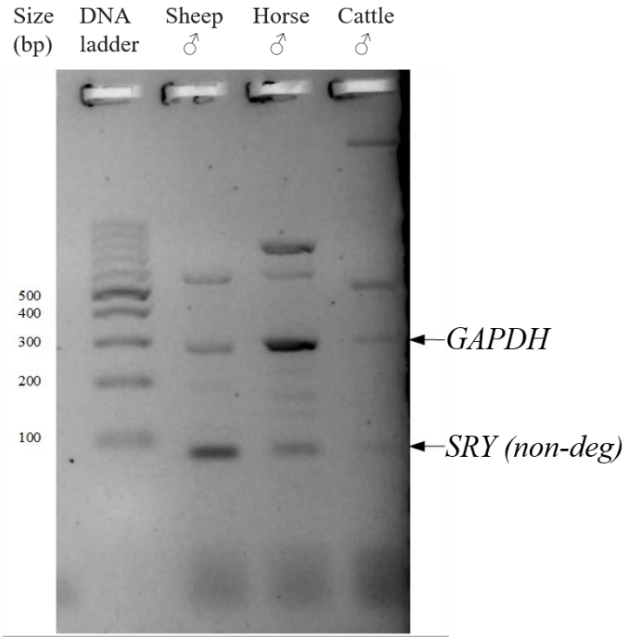

Figure 14. Agarose gel electrophoresis of duplex reaction (SRY and GAPDH).

Capillary electrophoresis of the segments amplified with fluorescently labelled primers confirmed the exact length of the SRY and GAPDH markers, being 81 and 292 bp, respectively (Figure 15).

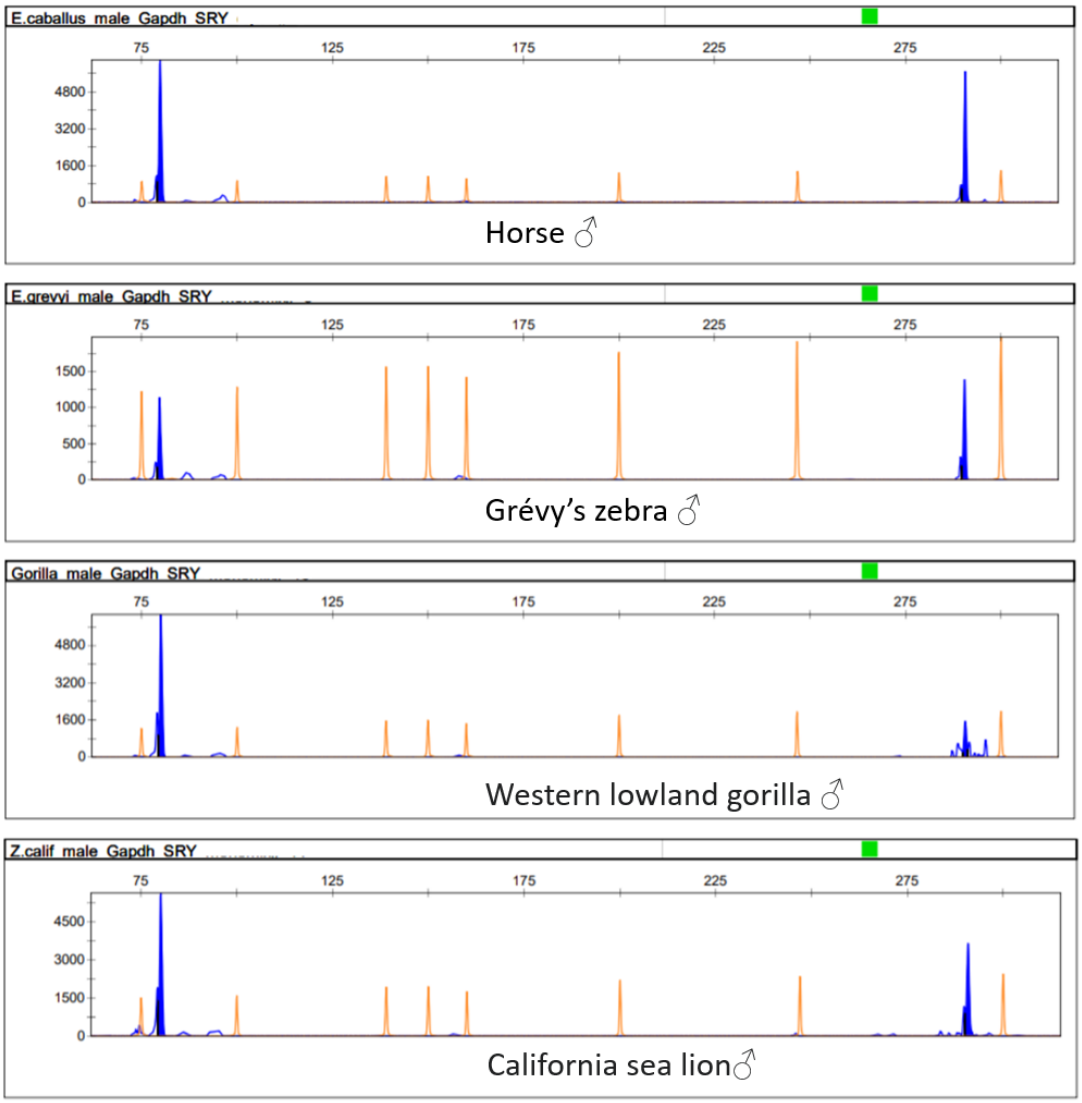

Figure 15. Capillary electrophoresis of duplex reaction (SRY and GAPDH).

Base sequences of the segments submitted for sequencing was identical to the reference sequences of the given species available in the NCBI GenBank database (Figure 16).

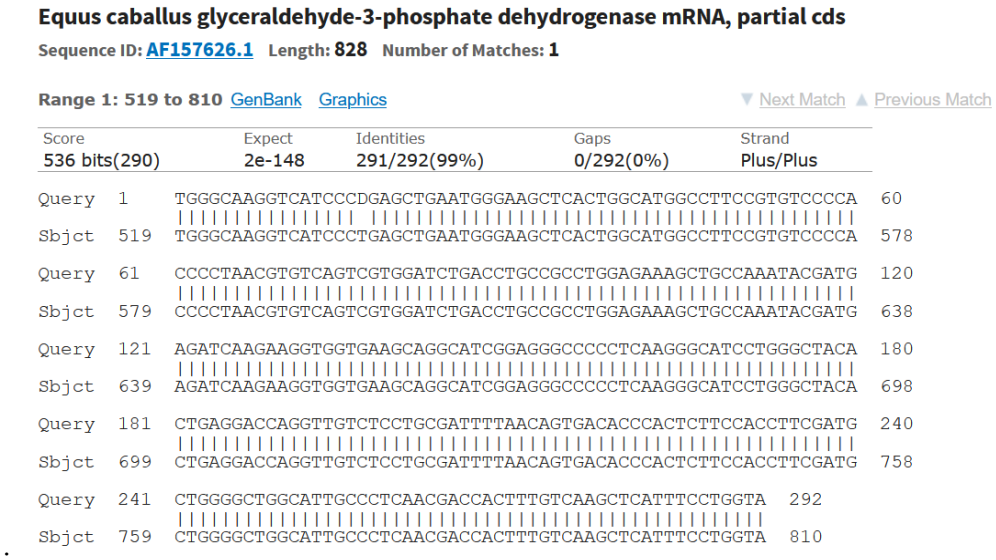

Figure 16. Alignment of received horse GAPDH sequence data to reference genome.

Overall, both the SRY and the GAPDH markers were successfully detected in a sex-specific manner in the majority of species studied; however, the assay did not work effectively in low-quality samples (Table 6).

Table 6. Success of detection of the SRY és GAPDH genes in the species examined ("+": successful, "-": unsuccessful, "NA": no sample was available)

	♂		♀
	SRY	*GAPDH*	*GAPDH*
Cattle	+	+	+
Horse	+	+	+
Sheep	+	+	+
Mouflon	+	+	+
Chapman's zebra	-	-	-
Grévy's zebra	+	+	+
White rhinoceros	+	+	+
Orangutan	+	+	+
Western lowland gorilla	+	+	+
Sloth	NA	NA	+
Takin	-	+	+
Water buffalo	NA	NA	-
European bison	NA	NA	+
Fallow deer	+	+	+
Red deer	+	+	+
California sea lion	+	+	NA
Hippopotamus	-	-	-

4.6. Maternal plasma samples

Maternal plasma samples of six pregnant cattle (4 male, 2 female offspring), two pregnant horses (1 male, 1 female offspring), and a pregnant Grévy's zebra (male offspring) were tested up to this point of the study. Amplification and detection of GAPDH was successful in each of the samples, however, the SRY marker was not detectable by either agarose gel electrophoresis (Figure 17) or capillary electrophoresis in any of the relevant samples.

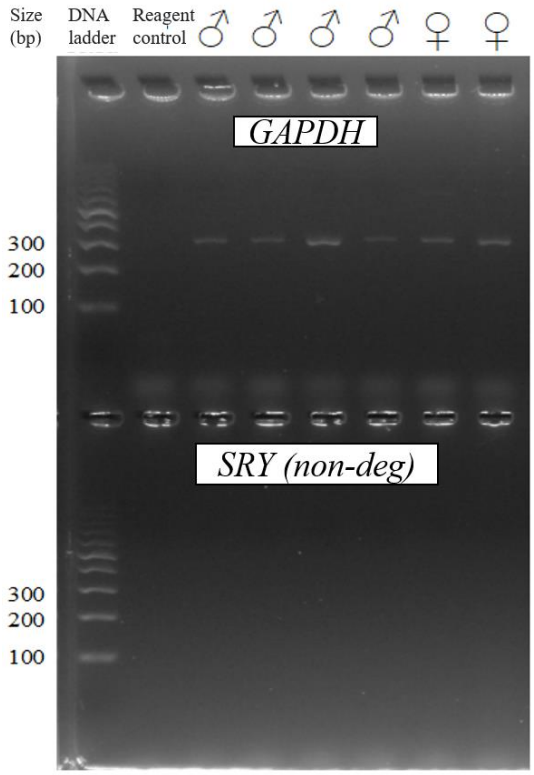

Figure 17. Maternal plasma samples of six pregnant cattle with offspring of known sex (4 males, 2 females) examined on agarose gel after PCR.

5. CONCLUSION AND DISCUSSION

Based on our results obtained so far, it seems clear that the development of a widely applicable DNA-based sex determination assay can be indeed possible. The markers identified using gene bank data and sequence alignment software were successfully detected in a wide range of unipara mammalian species.

Universal primers for the AmelY and TSPY genes could not be designed due to the small number and/or high variability of available sequences in the database.

Primers designed for the reaction control GAPDH gene were successfully used in all species tested, both in male and female control samples and maternal plasma samples. In the few cases where GAPDH was not detectable, the amount of subanalytic DNA and the presence of inhibitors could be assumed in the preparations of the faecal samples.

Primers initially designed for the SRY gene (*SRY deg, SRY non-deg*), although ideal based on *in silico* studies (meeting all criteria important for primer design), have been shown to be less effective in *in vitro* tests. The marker was detectable in good quality male DNA samples extracted from blood, hair follicles, and muscle, however, testing of control samples of lower quality and maternal plasma samples was unsuccessful. Although unsuccessful reactions in control samples may be associated with poor quality DNA samples that may contain inhibitors, the low sensitivity of the primers appears to be clear from the results of testing the plasma samples. Therefore, in the second phase of the research several new SRY primers were designed. Although these primer pairs have only been tested on few samples so far, two of them seems particularly encouraging based on the optimization process, regarding both their specificity and sensitivity. Further testing is already in progress.

Since the concentration of cffDNA in maternal blood, and thus the copy number of target sequences, is usually extremely low, especially in the earlier stages of pregnancy (Lo et al., 1998), a high degree of sensitivity of primers and detection techniques is essential for successful reaction. Since the GAPDH is present in the maternal circulation not only in the form of fetal but also in maternal cfDNA (and also it is present in two copy in each cell as opposed to the single SRY gene copy), its amount is obviously significantly higher in the maternal blood than that of the exclusively fetal Y chromosomal marker. This could explain successful detection of the GAPDH, and the more challenging detection of the SRY. Nevertheless, sex and species specificity of the designed primers proved to be adequate in all cases.

Also, since most of the cffDNA present in maternal blood is present in a short, highly fragmented form (Li et al., 2004), the success of the test depends largely on the length of the target sequence within the marker genes. The SRY segments between 80 and 137 bp we targeted should be optimal for both successful PCR and subsequent detection of the product.

The selected target regions within the SRY gene are part of the HMG (high-mobility group box) region of the SRY gene. While much of the gene is considered to be highly variable inter-species, the HMG box is the most conserved region of SRY (Whitfield et al., 1993), which plays a role in DNA binding. An HMG box with a sequence similar to the SRY HMG box occurs in the SOX (SRY-related HMG-box) genes (Bowles et al., 2000), which are not Y-specific. However, the sequences of the primer annealing sites we selected were sufficiently different that they did not cause a false positive result in either the *in silico* or *in vitro* assay. The sequence of GAPDH, a gene encoding a vital enzyme for glycolysis, is more conserved among species (Kisters-Woike et al., 2000), therefore in this case the design of universally functioning primers was significantly easier.

We continue our research by further testing with the newly designed primers on our existing samples as well as samples of better quality and from additional species. Eventually, even a three-marker test (two Y chromosome markers, plus reaction control) may be generated, allowing for greater reliability.

If the developed method already works reliably on all tested control samples of the relevant species and on maternal plasma samples of the species already studied in previous research, its universal applicability in fetal sex determination will depend mainly on the presence of cffDNA in maternal circulation of the given species. Since the species studied in previous research represent all four basic types of fetal placentation (epitheliochorial placenta in horses and rhinos; syndesmochorial placenta in cattle and sheep; hemochoric placenta in macaque and monkey; endotheliochorial placenta in elephants) (Wooding & Flint), presumably, cffDNA enters the maternal bloodstream in all mammalian species. However, there might be other influencing factors, and therefore the test may not necessarily be applicable in practice in all species to determine the sex of the fetus.

Overall, we strongly believe that after careful design, comprehensive laboratory testing and validation a reliable DNA-based assay will be ready for use in a wide range of mammalian species. Even apart from fetal sex determination, the assay could be just as well suitable for sex determination of individuals in many other areas of research (e.g. forensic animal research, population genetics).

Regarding universal fetal sex determination, zoos and livestock farms would benefit significantly from the advantages of the method, both in terms of usability, simplicity, time- and cost-efficiency; the method could both replace the various, currently available methods that are often difficult to implement, and provide a solution in case of several species not currently possible to examine.

At the same time, results deriving from maternal plasma samples may for the first time provide information on the presence and amount of cffDNA in the maternal circulation of several species not examined before. Thereby, our research can also significantly contribute to the further development of prenatal diagnostics (e.g. early detection of genetic disorders) in the animal kingdom.

6. SUMMARY

Universal genetic method for fetal sex-determination from maternal plasma in unipara mammalian species

Fetal-sex determination of unipara mammals with long gestation can benefit the management of economically important as well as captive wild populations, but feasibility of currently available methods is limited to very few species. Based on the hypothesized presence of cell free fetal DNA (cffDNA) in maternal circulation of all unipara species, the primary objective of this study was to develop a single, universal cffDNA-based assay for fetal-sex determination.

Highly conservative regions of Y chromosome-specific SRY gene and the autosomal GAPDH gene (used as a reaction control) were identified by *in silico* analysis, and universal primers were designed for the most appropriate gene segments. Selected primer pairs were tested on control samples (hair, feces, muscle, blood) from individuals of known sex collected from a wide range of relevant species, representing primates (e.g. gorilla), even and odd toed ungulates (e.g. cattle, takin, mouflon, equine, zebra, rhinoceros), proboscideans (e.g. African elephant) and carnivores (e.g. sea lion).

After primer testing with simplex (single marker) PCR programs, the base sequence of specific products was confirmed by sequencing. Subsequently, we optimized a duplex (two-marker) reaction, using touch-down PCR technique. The amplified fragments were detected by agarose gel and capillary electrophoresis.

After the optimization process, as expected, both Y-chromosomal and autosomal segments were detected in high-quality male samples, and only the GAPDH gene segment was detected in female samples.

Following preliminary testing, to evaluate the ability of the assay to determine fetal sex from maternal blood plasma samples of pregnant zebra, equine and cattle with a known gender of the offspring were tested up to the current stage of the study. Testing of samples from additional species is necessary and in progress to accurately determine reliability, specificity, and sensitivity of the test.

By successful development of such a universally applicable method our research could rapidly promote both economic-, but most notably conservation-related breeding activities of a wide range of species, and beyond it could be the first to provide information about the presence of cffDNA in maternal blood of various, not yet studied mammalian species.

Univerzális, genetikai alapú magzati ivarmeghatározás anyai vérplazmából unipara emlősökben

A magzati ivarmeghatározás a hosszú vemhességi idejű unipara emlős fajok − mind a gazdaságilag jelentős, mind pedig a fogságban élő egzotikus állatok − tenyésztésében nagy jelentőséggel bír, azonban a jelenleg elérhető módszerek a releváns fajoknak csak szűk körében kivitelezhetők. A sejtmentes magzati DNS (cffDNS) anyai vérkeringésben való előfordulását az emlős fajok széles körében feltételezve, kutatásunk elsődleges célja volt egy cffDNS-en alapuló univerzális genetikai ivarmeghatározó teszt kidolgozása.

Az Y-kromoszómán lokalizált, hím specifikus SRY gén, és az autoszómás, reakció kontrollként használandó GAPDH gén erősen konzervatív régióit *in silico* vizsgálattal azonosítottuk, majd a legtöbb szempontnak megfelelő génszakaszokra univerzális primereket terveztünk. A kiválasztott primerpárokat a releváns fajok széles köréből gyűjtött, ismert ivarú egyedektől származó kontroll mintákon (szőr, ürülék, izom, vér) teszteltük. A vizsgált állatok köre kiterjedt a főemlősökre (pl. gorilla), páros és páratlanujjú patásokra (pl. szarvasmarha, takin, muflon, ló, zebra, rinocérosz), ormányosokra (pl. afrikai elefánt) és ragadozókra (pl. oroszlánfóka).

A simplex (egy markeres) PCR programokkal történő primer-tesztelés után a specifikus termékek bázissorrendjét szekvenálással igazoltuk. Ezt követően optimalizáltuk a diplex (két markeres) reakciót is, ún. touch-down technika segítségével. A sokszorosított fragmenseket agarózgél- és kapilláris elektroforézis technikával mutattuk ki.

A kidolgozott protokollal megfelelő minőségű hím eredetű minták esetén a vártnak megfelelően mind az Y-kromoszómás és autoszómás, míg nőstény minták esetén kizárólag a GAPDH génszakasz volt kimutatható.

Az érzékenységi és előtesztelési vizsgálatok után az anyai vérből történő magzati ivarmeghatározáshoz ismert ivarú vehemmel rendelkező zebra, ló és szarvasmarha plazmamintáit teszteltük a kutatás jelen fázisáig. További fajok mintáinak tesztelése folyamatban van, mellyel a teszt megbízhatóságát, specificitását és szenzitivitását tervezzük pontosan meghatározni.

Az univerzális teszt létrehozásával a kutatás jelentősen elősegítheti az állatok tenyésztésével kapcsolatos tevékenységeket, különös tekintettel a fogságban élő vadállat fajok konzervációs programjára, s emellett elsőként nyújthat információt a cffDNS anyai vérkeringésben való jelenlétéről számos, korábban nem vizsgált emlős fajban.

7. REFERENCES

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