Master thesis

Development of a standardised SNP panel for genetic assessment of population structure in European bison (*Bos bonasus*)

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Declaration

I hereby confirm that I wrote this submitted master's thesis myself without using any help besides the referenced sources and material as well as noted in the acknowledgements. This applies also to all graphics, maps and images included in the thesis. This work has not been submitted or published as examination material for either this or any other degree.

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date Gerrit Wehrenberg

Dedication

To all the people who took and take part of protecting the European bison from the edge of extinction since the first meeting in Berlin initiating wisent conservation almost 100 years ago. Considering the political landscape back then, those people achieved an extraordinary international cooperation especially between Poland and Germany which overcame even World War II. With this following study, utilising modern methods, I want to contribute a small part to this legacy of so many people to carry on wisent conservation into a future those participants of the meeting at the 25th August 1923 could only dream of: an united Europe with free-roaming populations of European bison.

"Alle Arbeit für den großen und herrlichen Gedanken des Naturschutzes muß Stückwerk bleiben, wenn sie nicht auf dem Boden der Internationalität gedeiht. Naturschutz ist heute nicht nur eine unabweisbare Forderung unserer Zeit, sondern er ist auch eine Wissenschaft geworden, die sich zur allgemeinen Anerkennung durchgerungen hat."

– Dr. KURT PRIEMEL, former director of the Frankfurt Zoo and first president of the *International Society for the Protection of the European Bison* (1923)

Abstract

The European bison or wisent (*Bos bonasus* LINNAEUS 1758) was saved from the brink of extinction due to considerable conservation efforts since the early 20th century. The current global population descends from a total of 12 captive individuals, representing a severe bottleneck event. Although the population size has since increased to more than 7 500 individuals worldwide via successful *ex situ*-breeding and reintroductions into the wild, the species is still threatened by an extremely low level of genetic variability and high inbreeding. Today, the molecular analysis of genetic diversity is a crucial tool for conservation management of endangered species. Due to their low allelic diversity, traditional molecular tools, such as microsatellites, fail to provide sufficient resolution for accurate assessments of genetic diversity, individualisation and relatedness in European bison. This has so far hampered genetic assessments of *ex situ* breeding management as well as non-invasive monitoring of the reintroduced and isolated populations. Here, I present a reduced SNP panel for microfluidic genotyping of low-quality and degraded samples from European bison. The panel accommodates 96 informative markers allowing for (i) sex determination, (ii) individualisation, (iii) parental assignment, (iv) breeding line discrimination, (v) assessment of genetic diversity and (vi) cross-species detection. I successfully genotyped various non-invasively collected sample types, such as faeces, hair and saliva from 137 captive and wild living wisent individuals. With approx. 300 sampled individuals, I have collected the most extensive non-invasive collection of extant European bison in the frame of this thesis, providing a comprehensive sample set for marker testing, and optimisation as well as for genetically assessing the global population. In addition to marker panel development, I provide a 'best practice method' for sampling, preservation and DNA extraction of wisent dung. Due to the low costs, high resolution and suitability for various sample types, the new SNP panel will allow to tackle crucial tasks in wisent conservation management, including the accurate genetic monitoring of reintroduced wild populations, as well as the molecular assessment of pedigree data documented in the world's oldest studbook of a threatened species, reaching back more than one hundred years. Thus, this showcase provides a unique possibility for an informative evaluation of the added value when applying novel genetic tools in conservation. The new SNP panel will be implemented for the accurate monitoring of reintroduced European bison herds as well as for optimisation of captive breeding.

Zusammenfassung

Molekulare Analysen der genetischen Diversität sind ein erfolgsversprechendes Werkzeug für das Management zum Schutz bedrohter Arten. Der Wisent oder auch Europäischer Bison (*Bos bonasus* LINNAEUS 1758) wurde durch erhebliche Artenschutzbemühungen Anfang des 20. Jahrhunderts vor dem Aussterben bewahrt. Die heutige globale Population stammt von insgesamt nur zwölf Gründertieren ab, wodurch die Art einen starken genetischen Flaschenhals durchlaufen hat. Zwar ist die Population durch eine erfolgreiche Erhaltungszucht und Wiederansiedelungen in angestammten Regionen weltweit wieder auf über 7 500 Individuen herangewachsen, ist aber weiterhin bedroht durch eine sehr niedrige genetische Variabilität und Inzucht. Verursacht durch die niedrige Allelvariablität, versagen traditionelle molekulare Methoden wie Mikrosatelliten, die für die Bewertung von genetischer Diversität oder für Verwandtschaftsanalysen erforderliche Auflösung für diese Art zu leisten. Dies hat genetische Untersuchungen, für das *ex situ*-Management oder das nicht-invasive Monitoring von ausgewilderten und isolierten Populationen stark erschwert. Hiermit präsentiere ich ein reduziertes SNP-Panel für mikrofluides Genotypisieren von Proben vom Wisent mit niedriger Qualität. Dieses Panel enthält 96 informative Marker für (i) Geschlechtsbestimmung, (ii) Individualisierung, (iii) Zuordnung von Elterntieren, (iv) Zuchtliniendiskriminierung, (v) Evaluierung der genetischen Diversität und (vi) die Erkennung anderer Arten. Ich konnte verschiedenste im Vorhinein gesammelte nicht-invasive Probentypen, wie Kot, Haare, und Speichel von 137 Wisenten in menschlicher Obhut als auch aus der Wildnis erfolgreich genotypisieren. Mit in etwa 300 beprobten Individuen habe ich die wohl umfangreichste Sammlung einer lebenden Population von Wisenten zusammengetragen, die sowohl für das Testen und Optimieren der Marker, als auch für die genetische Untersuchung der Gesamtpopulation ein repräsentatives Probenset bietet. Zusätzlich zur Entwicklung des Markerpanels, stelle ich eine *Best Practice*-Methode für das Sammeln, Lagern und die DNS-Extraktion von und aus Wisentdung bereit. Durch die niedrigen Kosten, die hohe molekulare Auflösungskraft, als auch die Anwendbarkeit für verschiedenste Probentypen, kann das neue SNP-Panel wichtige Aufgaben in den aktuellen Artenschutzbemühungen zum Wisent bewältigen. Dazu gehört ein präzises genetisches Monitoring von wiederausgewilderten Herden, als auch der molekulare Vergleich mit den ältesten Zuchtbuchdaten einer bedrohten Art überhaupt, die mehr als 100 Jahre zurückreichen. Letzteres ermöglicht eine unvergleichbare Gelegenheit dieses neuentwickelte genetische Werkzeug mit bereits vorhandenen Daten abzugleichen. Dieses neue SNP Panel kann und wird zum erstmaligen Monitoring wilder Populationen und zur Verbesserung der Erhaltungszucht eingesetzt.

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Abbreviations

µl	microliter
ADO	allelic dropout
AE buffer	animal elution buffer (Qiagen)
AICc	Akaike information criterion
AL buffer	animal lysis buffer (Qiagen)
ANOVA	analysis of variance
approx.	approximately
ASL buffer	animal stool lysis buffer (Qiagen)
ASP	Allele-Specific Primers
ATL buffer	animal tissue lysis buffer (Qiagen)
AW1 buffer	animal wash buffer 1 (Qiagen)
AW2 buffer	animal wash buffer 2 (Qiagen)
B. b. bonasus	*Bos bonasus bonasus*
B. b. caucasicus	*Bos bonasus caucasicus*
BIC	Bayesian information criterion
bp	base pair(s)
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
cf.	confer/conferatur; english: compare
EAZA	European Association of Zoos and Aquaria
EBCC	European Bison Conservation Center
EBCC-RZ	European Bison Conservation Center-Regionalzentrum (-regional center)
EBPB	European Bison Pedigree Book
EDTA	Ethylenediaminetetraacetic acid
EEP	European Endangered Species Programme
ESU	evolutionarily significant unit
et al.	et alia (English: and others)
EtOH	ethanol
F	fixation index/inbreeding coefficient ($F = \frac{H_E - H_O}{H_E}$)
F_1 hybrid	filial 1 hybrid
FA	false alleles

F_{IS}	fixation index/inbreeding coefficient (from the **i**ndividual to the **s**ubpopulation) ($F_{IS} = \frac{H_S - H_I}{H_S}$)
F_{IT}	fixation index/inbreeding coefficient (from the **i**ndividual to the **t**otal population) ($F_{IT} = \frac{H_T - H_I}{H_T}$)
F_{ST}	fixation index/inbreeding coefficient (from the **s**ubpopulation to the **t**otal population) ($F_{IT} = \frac{H_T - H_S}{H_T}$)
Fwd	forward sequence
g	gram
gDNA	genomic DNA
GD	gene diversity (in this study always referring to pedigree-based data; ($GD = 1 - \overline{mk} = 1 - \overline{mF}$))
GE	genotyping error
GLMM	generalized linear mixed model
ha	hectare
H_E	expected heterozygosity
HL line	highland line
H_O	observed heterozygosity
HWE	Hardy-Weinberg equilibrium
IFC	integrated fluidic circuit
in prep.	in preparation
ISB	International studbook
IUCN	International Union for Conservation of Nature
K	number of populations (assumption)
kg	kilogram
Lab#	laboratory number/ID
LC line	lowland-Caucasian line
LL line	lowland line
LSP	Locus-specific primer (Rev)
M	molar mass
MCMC	Markov Chain Monte Carlo estimation
$\overline{mF}$	mean inbreeding coefficient
mg	milligram
$\overline{mk}$	mean kinship (in this study always referring to pedigree-based data; ($\overline{mk} = 1 - GD$))

ml	millilitre
mtDNA	mitochondrial DNA
MVP	minimum viable population
n	sample size
NA	not applicable
N_e	effective population size
ns	not significant
NTC	no template control
PBS	phosphate buffered saline
PCoA	Principal Coordinates Analysis
PCR	polymerase chain reaction
PIC	polymorphic information content
PID	probability of identity
PIDsib	probability of identity among siblings
PO	parent-offspring
Rev	revers sequence
RFU	Relative Fluorescent Unit
RH	relative humidity
rpm	revolutions per minute
RT	room temperature (~23 °C)
s	seconds
SE	standard error
sex code	e.g. 1.3.2 (1 male, 3 females and 2 individuals with unknown sex)
SNP	single-nucleotide polymorphism
sp.	species
spp.	species (plural)
SSC	Species Survival Commission
SSP	American Species Survival Plan
STA	Specific target amplification primer
TAG	Taxon Advisory Group
uH_E	unbiased expected heterozygosity (correction for sample size)
v	version

1 Introduction

The European bison or wisent (*Bos bonasus* LINNAEUS 1758) represents a textbook example of successful *ex situ*-population management and reintroductions after severe bottlenecks and extinction in the wild in 1927. Indeed, it was the world's first threatened species for which a studbook (European Bison Pedigree Book; EBPB) for conservation purposes was established (KRASIŃSKA and KRASIŃSKI 2013). With 1 745 European bison living in captivity, 5 036 in wild and 399 in semi-wild conditions (RACZYŃSKI 2018) a global population size of 7 180 is the result of this successful population management during the last almost 100 years (KRASIŃSKA and KRASIŃSKI 2013). Despite this success, the species is still threatened by genetic erosion due to a small gene pool and uneven founder representations (SLATIS 1960; PUCEK et al. 2004; TOKARSKA et al. 2011). Indications of inbreeding were found in the species' demography, fertility (SLATIS 1960; OLECH 1987; BELOUSOVA 1993; but see OLECH 1998) and morphology (KOBRYŃCZUK 1985; RAUTIAN et al. 1998; WOŁK and KRASIŃSKA 2004; KRASIŃSKA et al. 2008; CZYKIER et al. 2016; but see KRASIŃSKA and KRASIŃSKI 2002). Lower genetic diversity decreases the species' ability to adapt to environmental changes and also makes it more prone to diseases threatening the population like in the case of posthitis or balanoposthitis, an inflammation of male reproductive organs, respectively (KRASIŃSKA and KRASIŃSKI 2013).

1.1 The European bison: an overview

Classified as an even-toed ungulate (order: Artiodactyla OWEN 1848), ruminant (suborder: Ruminantia SCOPOLI 1777) and bovid (family: Bovidae GRAY 1821) the wisent is the last surviving wild member of the subfamily Bovinae (GRAY 1821) and the tribe Bovini (GRAY 1821) in Europe (GROVES et al. 2011). Despite a long research and conservation history, the systematic status of the bison species on and below the genus level is still not fully agreed on. Although American bison (*Bos bison* LINNAEUS 1758) and European bison are cross-fertile (KLÖS and WÜNSCHMANN 1993) but commonly recognised as different sister species (GRUBB 2005; GROVES et al. 2011; GROVES and GRUBB 2011; but see GROVES 1981). Three natural subspecies of the European bison have been described (KRASIŃSKA et al. 2014), which should generally not be confused with the different anthropogenic breeding lines:

The so called 'lowland line' (LL line; often 'lowland-Białowieża line') represents the solely pure breeding line of a natural subspecies in *B. bonasus*. All individuals of the LL line are assigned as European lowland bison (*Bos bonasus bonasus* LINNAEUS 1758). Historically distributed in the North European Plain located in Central and Eastern Europe, the last wild individual died in 1919 in the Białowieża forest in Eastern Poland (KRASIŃSKA and KRASIŃSKI 2013; KRASIŃSKA et al. 2014). Just five years earlier, 727 individuals had lived there, but were heavily reduced in population size during World War I (KRASIŃSKA and KRASIŃSKI 2013). Prior to this extinction event, the European bison had experienced declining population trends during the past centuries. Only the regional royal game

protection enabled the continual natural maintenance of the Białowieża forest and thus the survival of this large bovid. Thereafter, the LL line, *inter alia*, the majority of founders of the current global population, survived only in European private and zoo collections, which in turn entirely descended from caught individuals from Białowieża forest (KRASIŃSKA and KRASIŃSKI 2013; KRASIŃSKA et al. 2014). The often mentioned and former distinguished 'Pszczyna/Pleß line' within the LL line contains individuals derived from a private collection in Pszczyna (former Pleß in German), originally received as a gift from Białowieża from the Russian Tsar in 1865 by the Duke of Pleß Hans Heinrich XI Hochberg. Founded by only 1.4.0 animals (throughout this work individual count denoted separated by sex as followed: ♂♂.♀♀.??), the Pleß line (n = 6) played a major role in establishing the LL line later on (SLATIS 1960; PUCEK et al. 2004; KRASIŃSKA and KRASIŃSKI 2013). Formerly, the 'Bialowies line', already extinct at the time of the establishment of the managed breeding program, and the 'Tiergarten line' (mixed breeding line between the Bialowies and Pleß line) were additionally distinguished within the LL line (KLÖS and WÜNSCHMANN 1993) but are not recognised anymore.

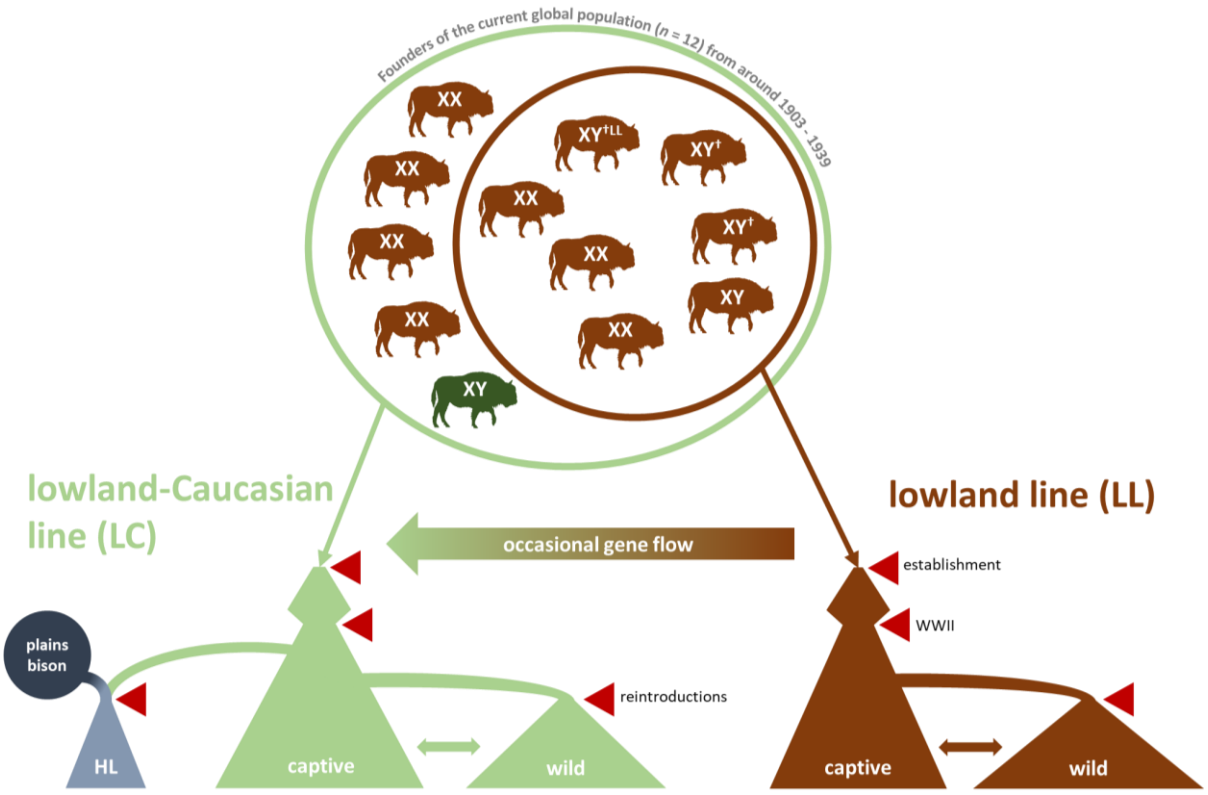

Figure 1: Simplified visualisation of the population history of the European bison since the establishment of the managed breeding. The 12 founders of the current global population are visualised with wisent symbols: Brown wisent: European lowland bison, dark green wisent: Caucasian bison; XX: female, XY: male, †: Y-chromosome of this founder has gone extinct in the current population, †LL: Y-chromosome of this founder has gone extinct in the current LL line (TOKARSKA et al. 2011). Brown colours symbolise the LL line, light green colours symbolise the LC line, dark blue colours plains bison, light blue colours the highland line (HL)/mountain bison. Horizontal arrows show direction of gene flow. Red arrows: bottleneck events. Demographic structures show the population size development over the decades until today from the top to the bottom (only symbolic, not exact).

In contrast to the LL line, individuals of the 'lowland-Caucasian line' (LC line), also recorded in the EBPB, carry additional genetic material of a single wild-caught and respectively last captive Caucasian bison (*Bos bonasus caucasicus* (TURKIN & SATUNIN 1904)). This male named 'Kaukasus' (studbook number 100 (hereafter called 'EBPB#100')) was brought to the *Tierpark Hagenbeck* in Stellingen (Hamburg, Germany) in 1908. All further founders of the LC line (4.7) are assigned to *B. b. bonasus* (SLATIS 1960; KRASIŃSKA et al. 2014), including all seven founders of the LL line plus four additional lowland bison individuals (TOKARSKA et al. 2011; KRASIŃSKA and KRASIŃSKI 2013; Figure 1). Genetically pure Caucasian bison went extinct in the Caucasian mountains in the mid to late 1920s after rapid population declines (KRASIŃSKA and KRASIŃSKI 2013; KRASIŃSKA et al. 2014).

The Carpathian or Transylvanian bison (*Bos bonasus hungarorum* (KRETZOI 1946)) probably became extinct around the second half of the 18th century in the Carpathian Mountains and is not represented in any breeding line today (KRASIŃSKA et al. 2014). It was scientifically described only based on few subfossils after its extinction (GROVES and GRUBB 2011). The validity of its status as a separate subspecies is questioned but cannot be solved due to the holotype destruction during the Hungarian Uprising in 1956 (GROVES and GRUBB 2011; KRASIŃSKA and KRASIŃSKI 2013).

A third separate breeding line called 'highland line' (HL line) or sometimes 'mountain bison' represents hybrids (*Bos bonasus bonasus* × *Bos bonasus caucasicus* × *Bos bison bison*) with American plains bison (*Bos bison bison* LINNAEUS 1758; ZABLOTSKAYA et al. 2004; KRASIŃSKA and KRASIŃSKI 2013) and was even described as a new subspecies (*Bos bonasus montanus* (RAUTIAN et al. 2000)). However, this 'subspecies' status is considered controversial (GROVES and GRUBB 2011; KRASIŃSKA et al. 2014). These animals originate from five bison-wisent hybrids from the Askania Nova steppe reserve (southern Ukraine) and the successful introgression from eight males of the LC line. Some were brought to the Caucasus Biosphere Reserve in the central part of the Caucasus main massif in 1940 (PUCEK et al. 2004; KRASIŃSKA and KRASIŃSKI 2013). Further herds of these hybrids were later established also in other locations of Russia (PUCEK et al. 2004). These approx. 1 180 individuals (SIPKO et al. 2018) assigned to the HL line are neither registered in the EBPB (RACZYŃSKI 2018) nor part of the European Endangered Species Programme (EEP) (Species360 2019).

1.2 Conservation in European bison

Why, however, should conservation put further effort in this particular species today? Certainly, the emblematic European bison deserves specific conservation efforts out of cultural values and simply, the intrinsic value every species has. Though, critics towards conservation only concentrating on single species versus biodiversity-focused conservation regarding generally available resources and current rapid biodiversity decreases are reasonable (MYERS et al. 2000). Not contradictory, however, the implementation of the ecological concept of keystone species could help to increase the positive

effects of conservation efforts towards biodiversity (MILLS and DOAK 1993). The wisent enhances local biodiversity by heterogenisation of its native habitat as it keeps landscapes open and prevents scrub encroachment (CROMSIGT et al. 2017; VALDÉS-CORRECHER et al. 2018). In addition, the wisent also plays an important role regarding zoochory for many plant species (JAROSZEWICZ 2013; SCHULZE et al. 2014). Thus, the wisent clearly fulfils to the keystone category 'modifier' (MILLS and DOAK 1993). Consequently, the reintroduction of the keystone species *B. bonasus* re-establishes an ecological niche not provided by other megafaunal species (CROMSIGT et al. 2017; VALDÉS-CORRECHER et al. 2018) and ultimately induces increasing biodiversity evidently (JAROSZEWICZ 2013; EVSTIGNEEV and SOLONINA 2016).

With moderate morphological (KOBRYŃCZUK 1985) and genetical differences (ŁOPIEŃSKA et al. 2011; KAMIŃSKI et al. 2012; TOKARSKA et al. 2015; WOJCIECHOWSKA et al. 2017), wisent conservation authorities managed and reintroduced the LL and LC lines separately since its establishment with occasionally gene flow from the LL line into the LC line in captivity (PUCEK et al. 2004; RACZYŃSKI 2018). This latter introgression is tolerated because all seven founders of the LL line represent founders of the LC line, subsequently managed as an open population. An introgression of genetic material originated from Caucasian bison into the LL line is not desired and its prevention is still a priority for the conservation strategy in the wisent (PUCEK et al. 2004; Figure 1). This overarching purity requirement for conservation actions is only assessed with pedigree data from the EBPB (OLECH and PERZANOWSKI 2002; PUCEK et al. 2004). However, some problems have occasionally been noted regarding the reporting of pedigree data (OLECH 1999; 2000; 2003; 2006; 2007; RACZYŃSKI 2018) as well as uncertainties concerning the purity of the breeding lines due to neglection of some holders to keep both lines separately (OLECH 2006; 2007). Today, the EBPB provides the only documentation of a genealogy of an entire species after its restitution in the early 20th century. Thus, since the establishment of wisent conservation, the EBPB is a comprehensive, but also the only guidance for conservation-oriented breeding management and reintroduction facing the small gene pool (OLECH and PERZANOWSKI 2002; PUCEK et al. 2004). Additionally, the EBPB does not provide the genealogical relationships of wild-born individuals (RACZYŃSKI 2018), which currently makes a monitoring of free-roaming herds impossible. A further weakness with pedigree data lies in the statistical default assumption of kinship calculations not allowing for the representation of the actual relatedness of the founders, resulting in most likely incorrect initial kinship estimations (BALLOU and LACY 1995; GOUDET et al. 2018). In the case of the wisent already three founder individuals were known descendants of three further founders of in total 12 designated founders of the current global population (KOBRYŃCZUK 1985). Thus, the current pedigree data, though being still very valuable, cannot constitute the only source of information concerning the genetic value of individuals regarding genetic variability and breeding line purity (WOJCIECHOWSKA et al. 2017). However, in

accompaniment with this long-term genealogical documentation, pedigree data enables unique comparative molecular studies and makes this large ungulate an exemplary subject for conservation genetics.

After the establishment of a pure-bred population the European bison experienced its last population bottleneck during World War II. Only 67 bison survived and gave rise to the current global population (BELOUSOVA and KUDRIAVTSEV 1997; Figure 1). Additionally two less admixed subpopulations of the LC line developed in Eastern and Western Europe caused by political separation during the Cold War (BELOUSOVA and KUDRIAVTSEV 1997). Since the late 1950s European bison were sent to zoos in Africa, Asia and the Americas to establish decentralised reserve populations. However, most of those zoo populations expired, leaving only 1.5.0 individuals in Canada and 3.1.0 individuals in Indonesia (RACZYŃSKI 2018). Hence, Europe as the native continent of the wisent, holds with the entire captive population also the only source for reintroductions. Previous genomic investigations suggest that beside those more recent bottleneck events during both World Wars, currently low heterozygosity is also caused by severe declines of the effective population in European bison during the last 20 000 years. These (pre)historic events causing decreased population sizes in the wisent are associated with human activities such as hunting intensification and land use, especially after the development of agriculture (KUEMMERLE et al. 2012; GAUTIER et al. 2016). Therefore, the wisent has on the one hand always been in conflict with humans as potential prey or, as large herbivore with high spatial requirements (OLECH and PERZANOWSKI 2015), competitor for forestry or livestock, but on the other hand as an emblematic protected animal stimulating exceptional efforts in conservation. In light of the above, the European bison experienced strong influences on its genetic diversity from humans for a long time to the present day.

After the establishment of a reserve in Białowieża National Park in 1929 and subsequent captive breeding, the first reintroduction took place in 1952 *ibidem* (KRASIŃSKA and KRASIŃSKI 2013). Today, free-roaming herds of pure-bred European bison are present in Poland, Belarus, Russia, Ukraine, Slovakia, Lithuania, Latvia, Romania and Germany (VAN DE VLASAKKER 2014; RACZYŃSKI 2018). After habitat suitability mappings (KUEMMERLE et al. 2011; BLEYHL et al. 2015) introductions started in the Azerbaijanian Caucasus mountains very recently as well (WWF 2019). To date, the IUCN (*International Union for Conservation of Nature*) listed the European bison as vulnerable (VU) on species-level. The same status was assigned for its subspecies *B. b. bonasus*, synonymous with the LL line, whereas due to recent population declines, the LC line is ranked as endangered (EN) (OLECH 2008). Worth to mention here is that there are genetic indications that the Belarusian population of the supposed pure LL line at the Białowieża National Park at least partly carries genetic material of the Caucasian bison, which in turn could further halve the supposed global population size of *B. b. bonasus* by definition (TOKARSKA et al. 2015).

1.3 Conservation genetics

The implementation of evidence-based conservation is often demanded (PULLIN et al. 2004; SUTHERLAND et al. 2004; COOK et al. 2010). For the European bison, KLOSE (2018) recommended a further professionalisation of the wildlife management including comprehensive monitoring. Other authors and contributors urged more explicitly for a genetic assessment of the species (PUCEK et al. 2004; VAN DE VLASAKKER 2014; HOMES 2018; OLECH 2018; SIPKO et al. 2018; ZENTNER 2018). Therefore, a comprehensive genetic assessment implemented with a reliable molecular method within the existing structures is needed to be able to further preserve the already low intraspecific genetic diversity. The ongoing development of genetic markers in the last decades provides a crucial molecular toolkit for many research fields. Driven by improvements in accuracy, costs and speed of next generation sequencing (NGS), single nucleotide polymorphisms (SNPs) are nowadays well established due to their broad applicability (MORIN et al. 2004; GROVER and SHARMA 2016). By now, population genetics, ecology and conservation studies have applied this genetic marker type successfully on non-model organisms (MORIN et al. 2004; WAITS and PAETKAU 2005; BROQUET et al. 2006; BEJA-PEREIRA et al. 2009; HELYAR et al. 2011; OGDEN 2011) both in wild (WILLING et al. 2010; KRAUS et al. 2015; DE GROOT et al. 2016; HINDRIKSON et al. 2017; HOLDEREGGER et al. 2019) and in *ex situ*-populations (IVY and LACY 2010; WITZENBERGER and HOCHKIRCH 2011; OGDEN et al. 2012; LABUSCHAGNE et al. 2015).

When compared with formally preferred mitochondrial DNA (mtDNA) and microsatellites utilised in (non-invasive) genotyping, SNPs are discussed to be a valuable alternative, providing specific advantages. Firstly, beside the genome-wide character of SNPs (MORIN et al. 2004), nuclear DNA seems to be more resistant during storage than mtDNA (SOTO-CALDERÓN et al. 2009). Secondly, standardisation of SNP analyses between laboratories is easier due to their biallelic nature and straight-forward genotype calling processes (VON THADEN et al. 2017). However, such a single biallelic locus has an approx. two to four times lower informative power regarding individualisation than a multiallelic locus. Though, a high number of SNPs, possible to combine in a single multiplex, could potentially generate higher quality genotypes. Thus, the success of individualisation depends mainly on the number of unlinked markers, the evenness of their heterozygosity and less on the number of alleles per locus (MORIN et al. 2004). Lower explanatory power per locus in biallelic markers is more pronounced for relatedness measurements, which require higher levels of genotype precision compared with individualisation. Here, considerably higher numbers of biallelic loci are needed to achieve a similar explanatory power compared with multiallelic loci (MORIN et al. 2004). This especially applies for relationships beyond single-generation and parent-offspring (PO) pairs (GLAUBITZ et al. 2003). Loci selection for high heterozygosity (approaching 0.5) increases power for parental assignment (MORIN et al. 2004). Generally low genetic variability, uneven founder

distribution and Y-chromosomal loss are long known in the European bison (TOKARSKA et al. 2011; KRASIŃSKA and KRASIŃSKI 2013). This low genetic variability in European bison was proven molecularly with several marker systems: most studies used microsatellites (GRALAK et al. 2004; LUENSER et al. 2005; ROTH et al. 2006; FLISIKOWSKI et al. 2007; NOWAK and OLECH 2008; TOKARSKA et al. 2009a; 2009b; 2015; MIKHAILOVA and VOITSUKHOVSKAYA 2017; DOTSEV et al. 2018) and SNPs (FLISIKOWSKI et al. 2007; PERTOLDI et al. 2009; TOKARSKA et al. 2009a; PERTOLDI et al. 2010a; KAMIŃSKI et al. 2012; TOKARSKA et al. 2015; WOJCIECHOWSKA et al. 2017; OLEŃSKI et al. 2018; 2020). In general, microsatellites proved to be appropriate genetic markers to evaluate population structures and individual kinships (SELKOE and TOONEN 2006). Due to the small gene pool, however, it has been shown that a microsatellite panel of 17 loci was not sufficient to assess identity and paternity in European bison whereas SNPs were argued to be the more promising approach (TOKARSKA et al. 2009a). Additionally, HARTL and PUCEK (1994) admonished the use of too few markers for genetic diversity estimations especially of populations which experienced bottleneck events.

Until now, no marker system that was specifically developed for bison is published. SNPs utilised in wisent originate from the BovineSNP50 Genotyping BeadChip and BovineHD Genotyping BeadChip (Illumina) developed from domestic cattle (*Bos primigenius taurus* (LINNAEUS, 1758) and *Bos primigenius indicus* (LINNAEUS, 1758); FLISIKOWSKI et al. 2007; PERTOLDI et al. 2009; TOKARSKA et al. 2009a; PERTOLDI et al. 2010a; 2010b; KAMIŃSKI et al. 2012; OLEŃSKI et al. 2015; TOKARSKA et al. 2015; WOJCIECHOWSKA et al. 2017; OLEŃSKI et al. 2018; DRUET et al. 2020; OLEŃSKI et al. 2020; for further information see Appendix 6.3). IVY et al. (2016) showed that utilising selected SNPs from those chips for related species provide similar sufficient data for accurately estimating relationships compared with loci obtained by species-specific SNP discovery, even within an extremely bottlenecked population. So far, those before mentioned marker panels tested for the European bison are not comprehensively applicable for conservation management. In contrast to often impractical and undesired invasive sampling, the ability to use non-invasive samples to assess viable genetic population data from appropriate numbers of individuals could be a valuable tool for monitoring wild, particularly rare and elusive species or for the use in behavioural studies (TABERLET et al. 1997; 1999; MILLS et al. 2000; PALOMARES et al. 2002; EGGERT et al. 2003; PIGGOTT and TAYLOR 2003; BELLEMAIN et al. 2005; WAITS and PAETKAU 2005).

Until now, no non-invasive genetics were done in the European bison. Since the introduction of polymerase chain reaction (PCR; MULLIS et al. 1986) the range of viable sample sources extended to those associated with low-quantity and quality DNA, such as ancient (HOFREITER et al. 2001) or non-invasively collected samples. Molecular marker systems, such as microsatellites or SNPs utilise short DNA fragments and allow to obtain viable information even from samples with potentially degraded DNA (TABERLET et al. 1999). Compared with microsatellites, SNPs, especially implemented in

microfluidic array systems, provide a technology to counter cost- and time-efficient assessments with such non-invasive samples shown for several species (KRAUS et al. 2015; VON THADEN et al. 2017). Subsequently, research on non-invasive genetic assessments in ecological, population and conservation biology especially for vertebrates has increased (BEJA-PEREIRA et al. 2009). Due to its reliability, non-invasive genetics has led to conservation recommendations and is an integral part of conservation itself (TABERLET et al. 1997; RANDI 2011; DE GROOT et al. 2016; HINDRIKSON et al. 2017; HOLDEREGGER et al. 2019). A variety of non-invasive sources for genetics from environmental residuals of animals like hair has been shown (e.g. GAGNEUX et al. 1997; TABERLET et al. 1997). Post-lethal samples like tissue from found carcasses (RANDI et al. 2001; VERARDI et al. 2006; JONES et al. 2008; HERTWIG et al. 2009; NUSSBERGER et al. 2014), museum specimens (PAYNE and SORENSON 2003; ROWE et al. 2011) or teeth (SASTRE et al. 2009) can also be used as valuable non-invasive sources for DNA-based studies. Further possible residuals like keratinous parts in the form of nails (HOGERVORST et al. 2014; TRUONG et al. 2015) or horn (HARPER et al. 2013; YAN et al. 2013) were also investigated. In most cases, however, a comprehensive and continuous population monitoring relies on frequently found residuals in form of secretions or excretion that allow to obtain considerably high and more representative sample sizes of populations (WASSER et al. 1997). Sampling and subsequent genotyping from residual saliva (BLEJWAS et al. 2006; INOUE et al. 2007; SUNDQVIST et al. 2008; SASTRE et al. 2009; WHEAT et al. 2016; ISHIZUKA et al. 2019) plays an important role especially in wildlife management of larger mammals (NICHOLS et al. 2012; HARMS et al. 2015; DE GROOT et al. 2016). Nasal secretion and urine are less recognised but a similar promising source for genotyping (TAHIR et al. 1995; HAYAKAWA and TAKENAKA 1999; VALIERE and TABERLET 2003; HAUSKNECHT et al. 2007). Despite the undeniable complications the utility of sampling faeces is well proven (e.g. WAITS and PAETKAU 2005). Besides individual and population data faecal samples could also provide trophic information (FARRELL et al. 2000; CASPER et al. 2007; DEAGLE et al. 2007; BERGMANN et al. 2015; KOWALCZYK et al. 2019).

While the opportunities given by non-invasive sampling are immense for genetic population assessments, they certainly have their challenges and limitations (TABERLET et al. 1999; PIGGOTT and TAYLOR 2003; BROQUET and PETIT 2004). With higher risk of genetic contamination as well as decreased qualities and quantities of DNA found in many non-invasive samples genotypes are often accompanied by genotyping errors (GEs; TABERLET et al. 1999; SMITH and WANG 2014). Two types of GEs are recognised: not detectable alleles, called 'allelic dropouts' (ADOs) causing false homozygosity (NAVIDI et al. 1992; GAGNEUX et al. 1997) and contamination or amplification artefacts could lead to 'false alleles' (FAs; TABERLET et al. 1999) both resulting in incorrect genotypes. Co-extracted DNA damaging substances or PCR inhibitors further complicate the generation of reliable genotypes (VON THADEN et al. 2017). In particular, the large molecular diversity of secondary metabolites in plants as the diet of European bison (ROSENTHAL and BERENBAUM 1992) holds a significantly higher potential for

substances with a negative effect on DNA processing (DEHESTANI and KAZEMI TABAR 2007). The large amounts of codominant alien DNA in faeces from the diet and the intestinal microbiome are avoided by specificity of the utilised primers (BROQUET et al. 2006). However, the risk of amplification of non-target DNA with herbivore faeces is expectedly lower compared with carnivore faeces due to food range (MURPHY et al. 2003). To prevent those weaknesses, a multiple-tubes approach is recommended (NAVIDI et al. 1992; TABERLET et al. 1999). However, it was pointed out that additional PCRs from the same extract do not necessarily enhance an initially failed product used for genotyping. Therefore, it is also recommended to take up more than one sample per individual for separated extractions and further genotype validation (FRANTZEN et al. 1998). In general, it is recommended to evaluate the species-specific sample methodology to account for those mentioned weaknesses (TABERLET et al. 1999). Hence, I conducted a pilot study to test for a best practice in faecal sampling and sample storage for European bison, before starting the collection of samples for the main study.

Consequently, a comprehensive genetic assessment with a reliable molecular method accompanying the existing conservation management in the wisent is needed to enable further preservation of the already low intraspecific diversity in the long-term. In this study a reduced 96 SNP panel for (i) sex determination, (ii) individualisation, (iii) parental assignment, (iv) assessment of genetic diversity within the population, (v) breeding line discrimination and (vi) cross-species detection based on non-invasive samples from European bison was developed. Within this framework, the modularly composed SNP markers were separately evaluated for basic genotyping performance and their respective analysis power in the relevant species-specific questions. In particular, resolution of parental assignment and genetic diversity measures were compared with data available in the world's oldest pedigree book of a threatened species. As providing a viable molecular tool, a review on the scientific justification of the current conservation management practice separating the European bison into two breeding lines is provided. Finally, direct applications and recommendations for conservation regarding the SNP panel as well as promising perspectives are discussed.

2 Material and Methods

All statistical evaluation and several of the graphical visualisations were conducted using *R* v3.6.0 (R Core Team 2019) within *RStudio* v1.0.43 (RStudio Team 2016).

2.1 Pedigree data

All EBPB editions from 1947 to 2018 were reviewed to assess genealogical data and create a total pedigree data set of all European bison sampled in this study ($n = 337$) up to the founders. The software *mPed* (JANSSON et al. 2013) was used to convert the pedigree data into a readable format for *PMx* v1.5.20180429 (BALLOU et al. 2018).

2.2 Sampling and sample storage

Several different non-invasive sample types commonly used for wildlife population genetic studies (see Introduction) were collected and tested for the first time in *B. bonasus*. A concomitant collection of additional eleven Bovini species in 18 subspecies or rather major lineages (the latter from hereof are referred as evolutionarily significant units (ESUs: LL and LC breeding lines in wisent, taurine, indicine (zebu), African humpless shorthorn and sanga cattle (KLÖS and WÜNSCHMANN 1993; MWAI et al. 2015) as well as the river- and swamp-types of the water buffalo (KUMAR et al. 2007; YINDEE et al. 2010)) was done, while focussing on a representative sample collection from European bison (Figure 4, Figure A 5). This sample collection includes all species, subspecies and ESUs currently kept in Europe. In the case of mountain anoa (*Bubalus quarlesi* (OUWENS 1910)) the last specimen at least outside of Asia was sampled as well.

Captive sampling was done in 37 institutions from eight European countries. The majority of samples originates from Germany, which has the highest concentration of captive breeding centres and consequently the highest number of captive European bison worldwide (RACZYŃSKI 2018). The representative sampling aimed to obtain a collection containing a sufficient number of members from both breeding lines and descendants from all founders. Samples from free-roaming LL line individuals originate from the Białowieża and Knyszyńska forests in Poland and a single male shot near Lebus in Germany in 2017. Samples from free-roaming LC line individuals were collected in Russia and the Rothaar mountains in Germany (Figure 2). The collection includes samples from captive individuals which were recently reintroduced in Romania and Azerbaijan. Not all samples or individuals could be genotyped within the framework of this master thesis. Samples from other non-Bovini species were provided by the conservation genetics section of the Senckenberg Research Institute and Natural History Museum in Gelnhausen, where all samples of this study are stored.

This study focused mainly on the collection of faecal samples, however, hair, saliva and nasal secretion as valuable non-invasive sample types for future applications were also collected. Other

sample types like tissue, blood as well as further environmental residuals like urine, bone, teeth and horn are not representative, but were also sampled and partly tested as potential DNA sources (Table 1).

All samples were taken with nitrile gloves (StarGuard® Comfort), which were changed if a sample item or other objects with the potential for contamination were touched. All samples were stored and extracted at room temperature (RT; 20 – 21 °C) in a laboratory dedicated to processing of non-invasively collected sample material (TABERLET et al. 1999). Invasively collected samples were stored at RT and extracted in a separate laboratory dedicated to process invasive samples. Blood samples and a single lower jawbone with teeth were stored at -20 °C.

For faecal sampling and for sampling of body liquids like urine, saliva, nasal secretion or blood sterile cotton swabs were used (small (tip-⌀ 4 – 5.5 mm) Rotilabo®-cotton buds, wood, sterile (ROTH) or STX®705W CleanTips® Swabs (ITW Texwipe)). Beside faecal swab samples in buffer all swab, horn and hair samples were stored in a filter paper and pressure lock bags including a silica gel sachet (in the following *dry bags*).

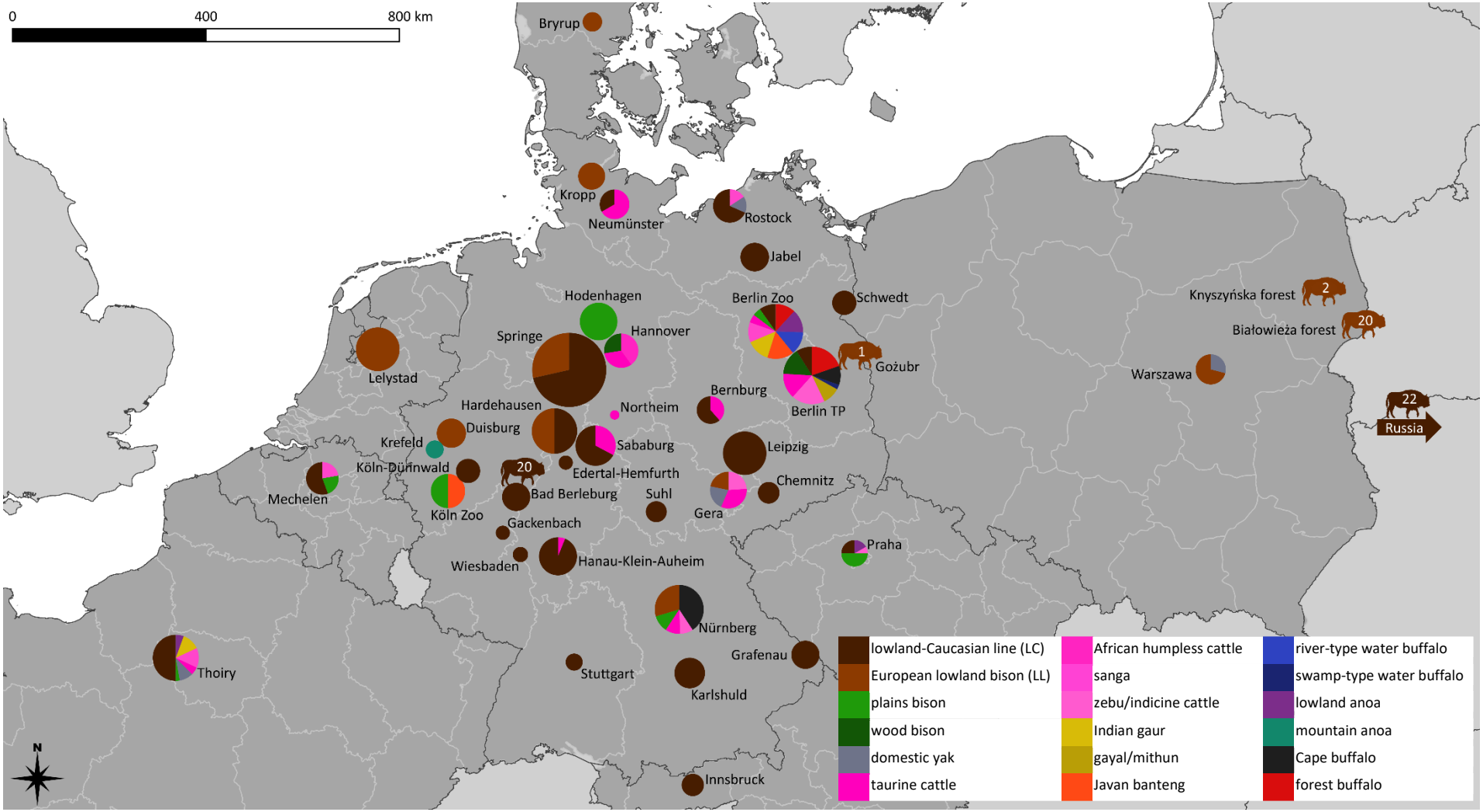

Figure 2: Map of sample distribution collected in this study throughout Europe. Sizes of the pie charts represent the sample sizes per location (total: *n* = 1 613). Most samples originate from the Wisentgehege in Springe (*n* = 186), whereas three samples from the Bauernhof Lutze in Northeim represent the lowest sample size. The pie chart segments represent the proportion of the sampled species/subspecies/ESUs (Figure A 5) at a location. Wisent symbols represent samples from wild European bison, where the numbers show the sample sizes. The location of samples collected in Russia are not depicted and are rather denoted by an arrow. Subspecies and ESUs of a species share the basic colour: brown (wisent/European bison), green (American bison), pink (domestic cattle), yellow (gaur) and blue ((domestic) water buffalo). Countries in which samples were collected are highlighted in a darker grey.

Table 1: Collected samples ordered by sample type, their sample sizes and associated taxa as well as ESUs within Bovini (Figure A 5). If all taxa and ESUs under higher taxa were sampled, the associated taxon is noted. Sample types marked with an asterisk were at least partly sampled invasively (see 2.2.2). Bold upper categories summarise all subordinated methods regarding the sample types (e.g. all faecal methods). Underlined sample type/preservation methods are mainly used in this study. There are several intermixed samples containing saliva and nasal secretion due to the oral-nasal secretion pattern of bovids. All those were assigned as saliva samples. For detailed descriptions of every sample type and preservation method see 2.2. Total sample list can be found in the supplementary file 'Sample list_all Bovini_wisent project.xlsx'.

sample type and preservation method	***n***	**Bovini taxa/ESUs**
total	**1 613**	**Bovini**
Faeces (dung)*	**1 135**	**Bovini**
Full faecal sample in EtOH*	405	Bovini
Faecal swab in EtOH	4	LC line
Faecal swab in inhibitex buffer*	705	Bovini
Faecal swab in ASL buffer	12	LC line
Faecal swab in dry bag	4	LC line
Faecal flocked swab	5	LC line
Saliva*	**172**	**wisent, American bison, cattle, Javan banteng, gaur, anoas, river-type water buffalo, *Syncerus***
Saliva in dry bag*	167	wisent, American bison, cattle, Javan banteng, gaur, anoas, river-type water buffalo, *Syncerus*
Saliva in InhibitEX buffer	5	LC line
Nasal secretion*	**61**	**wisent, plains bison, taurine cattle, sanga, zebu, Cape buffalo**
Nasal secretion in dry bag*	58	wisent, plains bison, taurine cattle, sanga, zebu, Cape buffalo
Nasal secretion flocked swabs	3	LC line
Hair*	**126**	**wisent, plains bison, yak, taurine cattle, zebu, gayal, anoas, Cape buffalo**
Hair in dry bag*	93	wisent, plains bison, yak, taurine cattle, zebu, gayal, anoas, Cape buffalo
Hair in EtOH	2	LC line
Urine*	**30**	**wisent, wood bison, yak, taurine cattle, zebu, sanga, mountain anoa, *Syncerus***
Urine swab in dry bag*	27	wisent, yak, cattle, mountain anoa, *Syncerus*
Urine flocked swab	3	LC line
Earwax	**1**	**swamp-type water buffalo**
Horn	**2**	**LC line**
Tissue in EtOH*	**49**	**wisent, taurine cattle**
Blood*	**33**	**wisent, American bison, taurine cattle, zebu, Cape buffalo**
Blood in EDTA*	25	wisent, American bison, taurine cattle, zebu, Cape buffalo
Blood plasma in EDTA*	5	LC line
Lyophilized blood	2	LL line
Blood swab*	1	taurine cattle
Bone/teeth	**2**	**LC line**
Bone in EDTA	1	LC line
Bone/teeth at -20 °C	1	LC line

2.2.1 Non-invasive sampling

2.2.1.1 Faeces

In order to obtain optimal and useful faecal samples for genetic analysis, several sampling and preservation methods were validated in a pilot study (see Appendix 6.6): One-way forceps were used to isolate a piece of up to 10 – 15 g faecal matrix for conservation in 33 ml 96 % EtOH (70 ml cup, SARSTEDT; from hereof *full faecal sample*; Figure A 10). For the methodological experiment in the pilot study four different types of preservation methods were tested: swab sample directly in DNA lysis buffer, swab samples in dry bags, swab samples in 33 ml 96 % EtOH and in dry flocked swab tubes (4N6FLOQSwabs genetics™ regular size tips in 109 mm long tube with Active Drying System (COPAN flock technologies)). Furthermore, faecal swab samples were taken separately from the faecal surface and faecal interior. All faecal samples were stored at RT.

Based on the results obtained in the pilot study, two types of faecal sampling were selected for further collection: full faecal samples in 96 % EtOH and faecal swab samples in InhibitEX.

2.2.1.2 Hair, urine, saliva and nasal secretion

Non-invasive hair samples were taken from rubbing sites such as brushes (Figure A 6) or stable walls. Non-invasive saliva and nasal secretion samples were taken up with swabs from surfaces of e.g. offered treats or feeding troughs. Non-invasive urine samples were taken up with swabs from the ground, if possible, or from not soiled objects like plants. Most pure urine samples were collected from urine-soaked snow in winter (VALIERE and TABERLET 2003). All hair, saliva, nasal secretion, and urine samples were stored in dry bags at RT.

2.2.1.3 Bone and horn

Bone and horn samples originated from individuals that died by natural causes and were opportunistically provided by cooperation partners. Horn samples were stored dried in a plastic pressure lock bag at RT. A vertebra from a found carcass was stored in 96 % EtOH at RT, whereas a lower jawbone with teeth from a second found carcass was stored dry in a plastic bag at -20 °C. Those samples were not used for further analyses in this study.

2.2.2 Invasive sampling

2.2.2.1 Tissue and blood

In the context of this study no invasive tissue samples were actively collected. Invasive tissue (n = 40), lyophilised blood (n = 2) and lyophilised DNA samples (n = 1) from dead free-roaming wisent from Poland (LL line) and Russia (LC line) were provided by the *Mammal Research Institute of the Polish Academy of Sciences* in Białowieża, Poland. These samples were collected between 1990 and 2016. Two tissue samples from one individual of the free-roaming wisent herd in the German Rothaar

mountains were sampled in 2017. A sole tissue sample originated from the first self-immigrated free-roaming wisent bull (publicly known as 'Gożubr') into Germany, shot around 2 km from Lebus in Brandenburg at the 13th September 2017. Further samples were provided by holders from mostly culled bovids sampled in 2018 and 2019. Two of those tissue samples were collected after punched out by the earmark from individuals still alive. As by-product, such occasionally biopsy punches are predestined sources for high quality samples without additional actions or harms towards the animals. All tissue samples were stored in 96 % EtOH at RT.

Further blood samples were stored with Ethylenediaminetetraacetic acid (EDTA) at -20 °C. Beside from dead individuals some fresh blood samples independently originate from veterinarian procedures occurring alongside this study. Some beforehand stored blood samples were also provided by some holders (collected between 2014 – 2019).

2.2.2.2 Faeces, hair, urine, saliva and nasal secretion

Some faecal, but also hair, saliva and nasal secretion samples were directly taken from animals and therefore are also invasive, which is rarely possible with captive individuals (Figure A 7). Few invasive faecal samples were taken by the holders during anaesthetisation for transportation or veterinarian treatment. Invasive hair samples were also collected utilising a corral system (Figure A 8). A similar case to gain invasive hair samples would be while the individual is penned in an animal trailer. Rectal obtained faecal samples are predestined for individual genotype assignment. As well as the non-invasive samples innocuous invasive saliva, nasal secretion and urine samples were collected with swabs. These swabs and hair samples were also stored in dry bags at RT. Very few saliva swab samples were collected in InhibitEX buffer. Together with blood samples taken from living animals those innocuous samples are also called non-destructive samples (TABERLET et al. 1999). Additionally, a sole urine sample from an individual Hungarian grey cattle was taken directly from the bladder after culling.

2.3 DNA extraction

The QIAamp DNA Investigator Kit (Qiagen) and the QIAamp Fast DNA Stool Mini Kit (Qiagen), respectively, were used to extract DNA on the QIAcube system (Qiagen) generally following manufacturer's instructions with some adjustments (see 6.4 Detailed protocols for DNA extraction).

The nucleic acid concentrations of DNA extracts from invasive samples were determined with a NanoDrop ND-1000 (ThermoFisher Scientific). All invasive DNA extracts were normalised to 5 ng/µl for the following PCR (see 6.5). Isolated DNA was stored at 4°C until use.

2.4 Pilot study: faecal sampling, preservation and sample storage methodology

To account for the aforementioned methodological challenges, this study tested for best practice in faecal sampling, sample preservation and DNA extraction from wisent dung. This complies with the recommended method development procedure of TABERLET et al. (1999). Pilot studies to molecularly evaluate never before analysed non-invasive samples of one species are recommended because analysis methods used in other species or populations are not reliably transferable, due to potentially different sample qualities and zygosities (TABERLET et al. 1999).

With the focus on faeces, both invasive and non-invasive samples of the European bison were analysed with a set of 21 microsatellite markers from non-coding regions originally developed for different even-toed ungulate species and a sex determination marker (WESTEKEMPER et al., *in prep.*) to evaluate the applicability of the enumerated sampling methods. In the present study, 16 of these markers were applied for the first time to European bison. Using GLMMs, I statistically evaluated sampling, sample preservation and DNA extraction of wisent dung and used these results to extrapolate the best practice (see 6.6).

2.5 Selection of SNP loci and SNPtype assay design

A set of 232 informative SNP loci for the European bison was selected from available publications for initial testing (see supplementary file 'SNP_marker_list_details.xlsx'): 14 SNPs with the strongest association to posthitis (OLEŃSKI et al. 2015), 43 most polymorphic SNPs from KAMIŃSKI et al. (2012), respective 43 loci from OLEŃSKI et al. (2018) filtered by probability of identity (PID), additionally 44 SNP loci from M. TOKARSKA (*pers. comm.*; unpublished data) by high polymorphic information content (PIC) and 82 SNPS for breeding line discrimination using loci with highest contrary allele frequencies between the LL and LC line. It is noted that other promising SNP loci from the study WOJCIECHOWSKA et al. (2017) for more accurate breeding line discrimination were not available due to missing indication of used loci. All aforementioned SNP loci were derived from the BovineSNP50 Genotyping BeadChip (Illumina) and BovineHD Genotyping BeadChip (Illumina). For sex determination, a SNP (in the following ZFXY) found in the homologous zinc finger gene distinguishing between the gonosomal ZFX and ZFY with a C/T transition (AASEN and MEDRANO 1990) was included. Five gonosomal SNPs were identified in the amelogenin gene of European bison, plains bison (*Bos bison bison* (LINNAEUS 1758)), taurine cattle (*Bos primigenius taurus* (LINNAEUS 1758)) and zebu (*Bos primigenius indicus* (LINNAEUS 1758)), yak (*Bos mutus grunniens* (LINNAEUS 1766)), banteng (*Bos javanicus* D'ALTON 1823) and gayal (*Bos gaurus frontalis* (LAMBERT 1804)) using sequence information from GenBank® (www.ncbi.nlm.nih.gov/genbank; see supplementary file 'SNP_marker_list_details.xlsx'). Subsequently, SNPtype assays were designed based on sequence information of approx. 300 bp for each SNP locus using the web-based D3 assay design tool (Fluidigm corp.). SNPs were rejected from

the initial selection if not traceable at the European Bioinformatics Institute (EMBL-EBI; http://www.ebi.ac.uk) to avoid SNP duplicates or if primer design by Fluidigm corp. was not possible.

2.6 96 SNP panel development and SNP genotyping

This study followed the development guidelines for genotyping degraded samples with reduced SNP recommended in VON THADEN et al. (2020) to obtain a final 96 SNP panel for implementation into a microfluidic chip system. For this stepwise approach the following sample sets were used during the complete testing phase: 46 invasive samples (LL line: $n = 17$; LC line: $n = 21$; taurine cattle: $n = 6$; plains bison: $n = 2$) as the reference sample set and 90 non-invasive samples. Only if an individualised DNA extraction was depleted, was it replaced by a sample from the same breeding line and with comparable quality. For initial wet laboratory testing of a first batch of SNP markers, I used 150 *in silico* SNPtype assays in two partitioned genotyping runs to filter for (i) markers with proper amplification and (ii) their informative value regarding the projected analyses. Assays with no amplification or indistinct clustering were excluded after this test. All reference samples with nucleic acid concentrations under 60 ng/µl or above approx. 120 ng/µl were normalised (see 6.5) before genotyping towards the recommended concentration of 60 ng/µl (Fluidigm). Those samples did not undergo a STA (specific target amplification primer) pre-amplification step to multiply the target regions for SNP genotyping.

In the next step, serial dilutions of the reference sample set were prepared to concentrations of 5 ng/µl, 1 ng/µl and 0.2 ng/µl and genotyped with the remaining pool of SNPs after filtering to test the applicability of single SNP makers on low nucleic acid concentrations and subsequent pre-amplification. Thereafter, I included a second batch of 82 putative breeding line discrimination SNP markers in the testing due to later availability of required sequence information for assay design. Next, 90 selected markers from the initial 150 SNP panel and 30 selected markers of the 82 SNP panel of putative breeding line markers were fused after the first test runs with the reference sample set to a preliminary panel of 120 SNP markers. This was tested further with the dilution series of the reference sample set and with 90 non-invasive samples to screen for suitable markers for low and degraded DNA samples.

2.7 Specific target amplification and SNP genotyping

The SNP genotyping procedure using 96.96 Dynamic Arrays™ with integrated fluidic circuits (IFCs; WANG et al. 2009) was conducted according to the manufacturer's protocol for SNPtype™ Assays for SNP Genotyping (Advanced Development Protocol 34, Fluidigm corp.). Low DNA samples were pre-amplified in a modified STA (Specific target amplification, Fluidigm corp.) for enrichment of the target loci before the SNP genotyping PCR. Primer assay volumes were adjusted to process > 96 marker during the testing phase in each one single SNP mix. The original protocols are designed for 96

marker (+24 for ease of pipetting). An overview of those standard STA and SNP genotyping protocols as well as modified protocols used in this study are described as followed or can be found in the supplementary file 'SNP genotyping protocol adjustments.xlsx'.

The pre-amplification of the target regions was conducted using the recommended 14 cycles with diluted (5/1 ng/µl) extracts from invasive samples and 28 cycles with and diluted extracts from invasive samples (0.2 ng/µl) and extracts from non-invasive samples (not diluted) in a T1 thermocyclers (Biometra, Analytik Jena) with the following program: Initial denaturation at 95 °C (15 min); followed by 40 cycles denaturation at 95 °C (15 s), annealing and extension at 60 °C (4 min) followed by cooling at 6 °C. A 1:10 dilution with 2 µl of every STA product and 18 µl DNA suspension buffer (Tris 10 mM, EDTA 0.1 mM; pH 8.0, Dnase/Rnase tested; TEKNOVA) was done before loading the SNP chip. STA products were stored at -20 °C.

For more resource-efficiency, adjusted smaller amounts than recommended (40 µl) of the SNPtype Assay Mix were prepared and still resulted in the required concentrations (see supplementary file 'SNP genotyping protocol adjustments.xlsx'). The SNPtype Assay Mix was stored at -20 °C and protected from light.

The protocols for preparing the final assay and sample mixes were not changed: the Assay Pre-Mix was prepared with 300 µl (2.5 µl per microwell) 2× Assay Loading Reagent (Fluidigm, PN85000736) and 180 µl (1.5 µl per inlet) PCR-certified water. 4 µl Assay Pre-Mix with 1 µl SNPtype Assay Mix per microwell resulting in 5 µl 10× Assay for chip loading. A total of 420 µl (3.5 µl per microwell) Sample Pre-Mix was prepared with 360 µl (3 µl per microwell) Biotium 2× Master Mix (Biotium, PN 31005) including the polymerase, 36 µl (0.3 µl per microwell) SNPtype 20× Sample Loading Reagent (Fluidigm, 100-3425), 12 µl (0.1 µl per microwell) SNPtype 60× Reagent (Fluigdim, 100-3402), 4.3 µl (0.036 per microwell) of the fluorescent dye 50X ROX (Invitrogen, PN 12223-012) and 7.7 µl (0.064 µl per microwell) PCR-certified water. 2.5 µl gDNA extractions were added to obtain 6 µl of a Sample Mix per microwell.

The reagent mixes were performed in 96-well plates (semi-skirted, 4titude Ltd., Surry, UK). Priming and loading the Dynamic Array IFC was done with an IFC Controller HX (Fluidigm). The genotyping PCR was done in a FC1™ Cycler (Fluigdim). The allele-specific fluorescence signals were measured after 34×/38×/42× amplification runs. All sample setups included STA NTCs (no template controls) and NTCS with not pre-amplified RNA-free water. In all experiments NTCs and samples were replicated.

2.7.1 Validation of SNP markers and scoring procedure

Raw data analyses of all runs were conducted after 38× thermal cycles (see 2.7) with *Fluidigm SNP Genotyping Analysis* v4.1.2 software (Fluidigm). Automated clustering and allele scoring of every SNP

marker was manually checked and corrected if needed according to VON THADEN et al. (2017). During the development phase every SNP cluster was compared to its profile in former chip runs to keep uniformity in allele scoring. This was especially important in loci for the sex determination or cross-species detection because here often only two zygosities were shown. If the clustering pattern of SNP markers diverged to the pattern in former runs the complete marker was disregarded and scored as 'No Call' (= missing data) for all samples. Alleles appearing too far from the centre of a cluster were ranked as FAs and were also scored as 'No Call'.

2.7.2 Validation of genotyping errors

Genotyping errors of each single replicate were calculated based on a consensus multilocus genotype (subsequently called reference genotype) which was built using all replicates of a sample (for consensus genotypes see supplementary file 'Genotype_list.xlsx'). Accordingly, the following rules were applied: generally, the major scored genotype over all replicates was assigned. If 50 % of the overall replicates for one locus were scored heterozygous and 50 % homozygous, the reference genotype was assigned as heterozygous for this locus. If one homozygosity was not scored and > 50 % of all replicates were scored as the opposite homozygosity the latter was assigned. For all autosomal loci: if a locus was scored partly to be heterozygous and both opposite homozygous genotypes were found at least twice in other replicates, the genotype was defined as heterozygous. If every possible zygosity was shown in triplicates, the locus was considered to be heterozygous as well. If both homozygous genotypes were scored the more frequent zygosity was assigned. If both homozygosities were scored with 50 %, no zygosity was assigned in the consensus. If the information of the individual sex were available in the metadata of a sample, the given sex was used as reference for calculation of the sex markers` GE.

To evaluate the GE rates and some of the interference factors for the 96 SNP panel applied on non-invasive samples, I set up seven test runs with sample replicates. Six plates with the same setup of 88 samples and eight NTCs for interspecific and one plate with six replicates of 15 samples and six NTCs for intraspecific interference factors. Half of the NTCs of each plate contained 2.5 µl NTCs from the STA pre-amplification whereas the other half contained 2.5 µl RNA-free water (double distilled, ROTH). With 39 samples from the LC line and 41 samples from the LL line both breeding lines were almost equally represented. Six samples from three individuals were included to evaluate the genotypes in between individualised samples. GLMMs (Equation 1) were applied for the assessment of interference factors such as plate run, sample (n = 88), sample type (dung in EtOH: n = 174; faecal swab in InhibitEX: n = 114; hair: n = 132; saliva: n = 168; nasal secretion: n = 24; urine: n = 6) and species (European bison: n = 80; American bison: n = 8).

Equation 1: GLMM with the GE, ADO and FA rates as response variable and the NC rate as predictor over 95 autosomal loci. *Random effect factors: Lab# represents the replicated sample, SNPrun the microfluidic SNP chip on which the genotype was processed and the sample type dung in EtOH or hair. For more information on the GLMMs see 2.9.*

$$glmer(cbind(95 - GE/ADO/FA, GE/ADO/FA)$$
$$\sim cbind(95 - NC, NC) + (1|Lab\#) + (1|SNPrun) + (1|sample\ type), family\ =\ binomial)$$

2.8 96 SNP panel

The final reduced SNP panel of 96 informative loci was compiled from in total 232 markers (see supplementary file 'SNP_marker_list_details.xlsx'). Considering the genomic approach, the 96 SNPs of the final panel were distributed throughout all *B. primigenius* chromosomes except autosome 25, whichh was not represented in the initially tested 282 SNPs (see supplementary file 'SNP_marker_list_details.xlsx'). With $2n = 60$, European bison carry the same number of chromosomes (NGUYEN et al. 2008) which suggest a similar distribution of the SNPs found in both species.

Several applications of *GenAlEx* v6.5 (PEAKALL and SMOUSE 2012) implemented in *Microsoft® Excel®* for *Office 355 MSO* v16.0.12527.20260 was used for evaluation and assessment of the molecular data as explicitly noted below. A test for linkage disequilibrium (LD) of the 90 autosomal markers polymorphic in the European bison was conducted using the likelihood-ratio test (number of permutations = 10,000; number of initial conditions for expectation-maximization (EM) algorithm = 2; unknown gametic phase) in *Arlequin* v3.5.2 (EXCOFFIER et al. 2007) and with squared allelic correlation (R^2) utilising the *R* package *LDheatmap* v (SHIN et al. 2006).

All genotypes used for analysis after the testing phase were generated separately with the final 96 SNP panel, regardless whether they had been genotyped before.

2.8.1 Individualisation

The discriminative power of the polymorphic autosomal SNP set (90 loci) and of the microsatellite panel (11 loci, data from pilot study) was assessed by estimating the probability of identity (PID) and the probabilities of identity among siblings (PIDsib) in *GenAlEx*. The markers were sorted according to the highest expected heterozygosity (H_E) before input.

The minimal number of loci with allele mismatches between genotypes with assigned individuals until individualisation was not possible, were compared: The lowest number of allowed allele mismatches were expected between close relatives and were used as a guidance threshold for individualisation.

2.8.2 Parental assignment

To test for the best resolution power in parental assignment, 137 individual genotypes consisting of both, the aforementioned 90 SNP markers and a subset of 64 SNP (which were in HWE and without

loci in LD (see 3.2)) were used in different statistical approaches for identification of parent-offspring relationships (PO) in comparison to the pedigree data. Therefore, the software *ML-Relate* (KALINOWSKI et al. 2006), using maximum likelihood estimations, and *Colony* v2.0.6.5 (JONES and WANG 2010a), using the Full-likelihood analysis method were utilised (Best (ML) cluster, Best (ML) configuration and pairwise approach outputs were compared). The Full-likelihood method was chosen because it was shown to be the most accurate method of *Colony* (WANG 2012). In *ML-Relate*, the allele frequencies were calculated with a subset of 58 non-first-order relatives out of 137 successful SNP-genotyped European bison. This was done to avoid an estimation bias due to allele frequencies obtained from a sample set with a substantial proportion of close relatives potentially causing underestimations of relatedness between those close related individuals (WANG 2014; WANG 2017). The estimations in *Colony* were computed with default assumptions except the following settings: male and female polygamy and inbreeding were assumed since both cases were present in the data set. High likelihood precision with allele frequency updates in three long runs were executed. All 137 individuals were put in as offspring and assigned to their sex with the probability of a sire or a dam in the data set = 0.5. No parental sibling inclusion or exclusion were added. It was only excluded for every individual to be their own parent. Genotyping error rates were assumed to be 0.0001 per locus because the used consensus genotypes were generated from at least triplicates and assumed to be reliable.

The example of a family network of 23 individuals was chosen because it includes three generations from different parks (different sample types from different collectors), many possible parents in siblinghoods, a case of inbreeding, individually assigned and not assigned samples as well as individuals with undocumented maternities and thus, visualise the applications for parental assignment (Figure 9).

2.8.3 Breeding line discrimination

Based on individual genotypes of 58 not directly related individuals (non-parent-offspring, non-full and non-half siblings) *GenAlEx* was used to identify markers with highest F_{ST} in each of the breeding lines. Therefore, PO, full and half siblings were excluded to minimise an allele frequency bias by relatedness. If both parents were genotyped, the offspring were removed to obtain the highest allele variation possible. Using a F_{ST} threshold of 0.415, 29 markers were selected showing the best descriptive resolution utilised in 137 genotyped individuals. Two methods for genetic clustering were applied to the selected 29 descriptive markers to test the robustness of the breeding line marker subset across different statistical approaches. A minimum breeding line discrimination threshold of 60 % probability was set for both genetic clustering methods. For data exploration the *Population Assignment* function of *GenAlEx* was utilised with the default setup additionally to the two methods described below.

2.8.3.1 Bayesian genetic clustering

To infer the presence of a distinct breeding line structure the systematic Bayesian clustering approach of *STRUCTURE* v2.3.4 (PRITCHARD et al. 2000; FALUSH et al. 2003; PRITCHARD et al. 2010) was used for microsatellite (Figure A 4) and SNP genotypes (Figure 10) with burn-in periods of 250 000 repetitions and 500 000 MCMC (Markov Chain Monte Carlo) repeats. The simulations were set with $K = 1 – 10$ each with 20 iterations. *STRUCTURE HARVESTER* (EARL and VON HOLDT 2012) was used to select the most likely *K* value. *CLUMPP* v1.1.2 was used to combine the iterations of the most likely *K* value with the *FullSearch* algorithm among 10 *K* (JAKOBSSON and ROSENBERG 2007).

2.8.3.2 Maximum-likelihood genetic clustering

The function *snapclust* (BEUGIN et al. 2018) implemented in the *R* package *adagenet* v2.1.1 (JOMBART 2008; JOMBART and AHMED 2011) was used to infer the presence of distinct genetic structures between the two breeding lines. The Bayesian information criterion (BIC) among $K = 1 – 10$ was used to estimate the most likely *K* value.

2.8.4 Assessment of molecular genetic diversity

To select a marker subset for the assessment of genetic diversity in the European bison all markers not being in Hardy-Weinberg equilibrium (HWE) or monomorphic within 58 not directly related individuals were discarded utilising χ^2 test in *GenAlEx* and *Arlequin* visualised in ternary plots performed with the *R* package *HardyWeinberg* v1.6.3 (GRAFFELMAN and CAMARENA 2008; GRAFFELMAN 2015). Allelic richness, expected (H_E), unbiased expected (uH_E) and observed heterozygosity (H_O) as well as the *F*-statistics were used to measure total, partial genetic diversities (between breeding lines). Molecular based heterozygosities and *F*-statistics (F_{IT}, F_{ST}, F_{IS}) were calculated in *GenAlex* and *FSTAT* v2.9.4 (GOUDET 2003).

PMx, a commonly used software in population management (LACY et al. 2012), was used to generate genetic values from pedigree data. *PMx* provides two methods to calculate pedigree-based gene diversity (GD): from kinship matrix as well as gene drop method (TRAYLOR-HOLZER 2011). For the latter method genetic default assumptions (1 000 gene drop iterations, autosomal mendelian inheritance mode) were used. GD is equivalent to H_E (NEI 1973; TRAYLOR-HOLZER 2011) and was therefore used for pedigree-molecular data comparisons. For clarification, GD will always refer to the pedigree-based values within this study, whereas H_E is referring to molecular-based values. Additionally, pedigree-based F_{ST}, F_{IS} and F_{IT} were generated in *ENDOG* v4.8 (GUTIÉRREZ and GOYACHE 2005), whereby the latter two values are not provided by *PMx*.

The pedigree-based and SNP-based F_{IS}-, F_{IT}- and F_{ST}-values were also compared. In order to do this, two pedigree data sets are used for *PMx*: for a direct comparison the pedigree-based genetic values were computed including only the successfully SNP-genotyped individuals with known genealogy

(*n* = 99) and their assigned ancestors (*n* = 982) up to the founders. To evaluate the representativeness of those pedigree-based genetic values, the same calculations were conducted with all sampled individuals with known genealogy in this study (*n* = 227) and their assigned ancestors up to the founders (in total *n* = 1 296). At the time of the analysis 338 individuals of those 1 296 European bison were alive and represent the basis of the pedigree-based genetic values. Ten sampled individuals with known pedigree were dead at the time of the analysis. Two of these were successfully genotyped (EBPB#13293, EBPB#13826) but were included (assumed to be alive) to increase data power for the genetic evaluation.

2.8.5 Cross-species detection

In total, 24 taxa or rather ESUs for the cross-species test were selected on the basis of the following criteria: taurine cattle, zebu, river-type water buffalo (*Bubalus arnee bubalis* (LINNAEUS 1758)), horse (*Equus ferus caballus* (LINNAEUS 1758)), Eurasian elk (*Alces alces alces* (LINNAEUS 1758)), common red deer (*Cervus elaphus elaphus* LINNAEUS 1758), Central European wild boar (*Sus scrofa scrofa* LINNAEUS 1758), European brown bear (*Ursus arctos* arctos LINNAEUS 1758), European grey wolf (*Canis lupus lupus* LINNAEUS 1758), European red fox (*Vulpes vulpes crucigera* (BECHSTEIN 1789)) are potentially sympatric with the European bison (WILSON and REEDER 2005; WILSON and MITTERMEIER 2009) and represent candidates for potential confusion in environmental traces such as faeces and stripping damage or sample contamination due to faecal wallowing. All further Bovini (13 ESUs), representing the closest living relatives up to the tribe level collectable in Europe (Figure 2), were also included for cross-species detection. Human (*Homo sapiens* LINNAEUS 1758) was included to test for methodological contamination. Besides taurine cattle (*n* = 2; two breeds) and American bison (*n* = 10; both subspecies), one individual per species or ESUs was genotyped. All samples with a SNP call rate over 80 % were analysed with a Principal Coordinates Analysis (PCoA) using all 84 autosomal loci not in LD executed in *GenAlEx*.

2.9 Generalised linear mixed models

Binomial generalised linear mixed models (GLMMs) fit by maximum likelihood (Laplace Approximation) and ANOVA (Wald χ^2 test) were used via the *R* packages *lme4* v1.1-21 (BATES et al. 2015) and *car* v3.0-3 (FOX and WEISBERG 2019), to statistically test the influence of the preselected parameters of sampling after following testing assumption: normality of residuals was evaluated with the Shapiro-Wilk test (KÖHLER et al. 2012) and diagnostic plots of the *R* package *DHARMa* v0.2.4 (HARTIG 2019) using scaled residuals, which were not always fulfilled. All predictor variables of all models in this study were categorical, thus the data was nested (ZUUR et al. 2009) and the linear models showed overdispersion. Furthermore, random effects were added (ZUUR et al. 2009) to account for influential factors of the experimental design. The selected models were compared with the corrected Akaike information criterion (AICc; BURNHAM and ANDERSON 2010) to the null model,

where the predictor = 1 and in the case of the pilot study to the model with interaction terms as well as without the random effects. Δ AICc ≤ -2 shows a better model by definition. If Δ AICc = [-2, 2], the less complex model was chosen. If the null model shows Δ AICc = [-2, 2], the explanatory model was chosen, instead. The AICc was chosen as it corrects for small sample sizes (BURNHAM and ANDERSON 2010). The marginal ($R^2_{GLMM(m)}$) and the conditional pseudo-R^2 ($R^2_{GLMM(c)}$) for the GLMMs were calculated to describe the amount of explained variance. The $R^2_{GLMM(m)}$ describes the proportion of variance explained by the fixed factor alone whereas the $R^2_{GLMM(c)}$ describes the proportion of variance explained by both the fixed and random factors (NAKAGAWA and SCHIELZETH 2013). This study uses the pseudo-R^2 based on the distribution-specific (theoretical) variance (NAKAGAWA et al. 2017). AICc and pseudo-R^2 are calculated with the *R* package *MuMIn* (BARTOŃ 2019). In the present study a significance level of 5 % (α = 0.05) was used.

2.10 Visualisation and data set conversion

Boxplots and scatterplots were generated with the *R* packages *ggplot2* v3.2.0 (WICKHAM 2016) and *gridExtra* v2.3 (AUGUIE and ANTONOV 2017). *QGIS* v3.4.12 '*Madeira*' was utilised for mapping the geographical allocation of samples used in this study. The mapped administrative country boundaries were downloaded from http://www.diva-gis.org/Data (assessed: 22nd January 2020). The cladogram of the Bovini and other non-target species was conducted in *Mesquite* v3.61 (build 927) (MADDISON and MADDISON 2019). For pedigree visualisation the *R* package *kinship2* v1.8.4 (SINNWELL et al. 2014) was used. *CONVERT* v1.31 (GLAUBITZ 2004) was used to adjust data sets for implementation in several analysis programs. The *R* package *genetics* v1.3.8.1.2 (WARNES 2012) was used to transform data sets into partly required genotype data sets.

3 Results

3.1 Pedigree data

In literature only seven individuals for the LL line and twelve individuals for the LC line are assigned as traceable founders (TOKARSKA et al. 2011; KRASIŃSKA and KRASIŃSKI 2013). The individuals 'Begründer' (EBPB#15), 'Bismarck' (EBPB#147) and 'Plewna' (EBPB#35) are assigned as founders but have known pedigrees. These founder statuses were assigned to the most recent common ancestors of the current population. However, since inbreeding within the Bismarck-lineage, the ancestors 'Biber' (EBPB# 123), 'Birke' (EBPB# 122) and 'Biene' (EBPB# 124) have different founder representations (cf. Figure 3 and Figure 4). Additionally, with the available pedigree data it is possible to exclude siblinghoods or even more distant kinships between 'Begründer', 'Bismarck' and 'Plewna'. Thus, genetic calculation with pedigree data leads to unnecessary, imprecise assumptions particularly if the founder population was as small as in the case of the wisent. Therefore, all available genealogical data was included in the present study, resulting in 16 instead of twelve founder individuals for the LC line and ten founders for the LL line, excluding the traditionally designated founders 'Begründer', 'Bismarck' and 'Plewna' from this definition. This extension of the pedigree gives the most possible pedigree depth, which was then compared to molecular-based values.

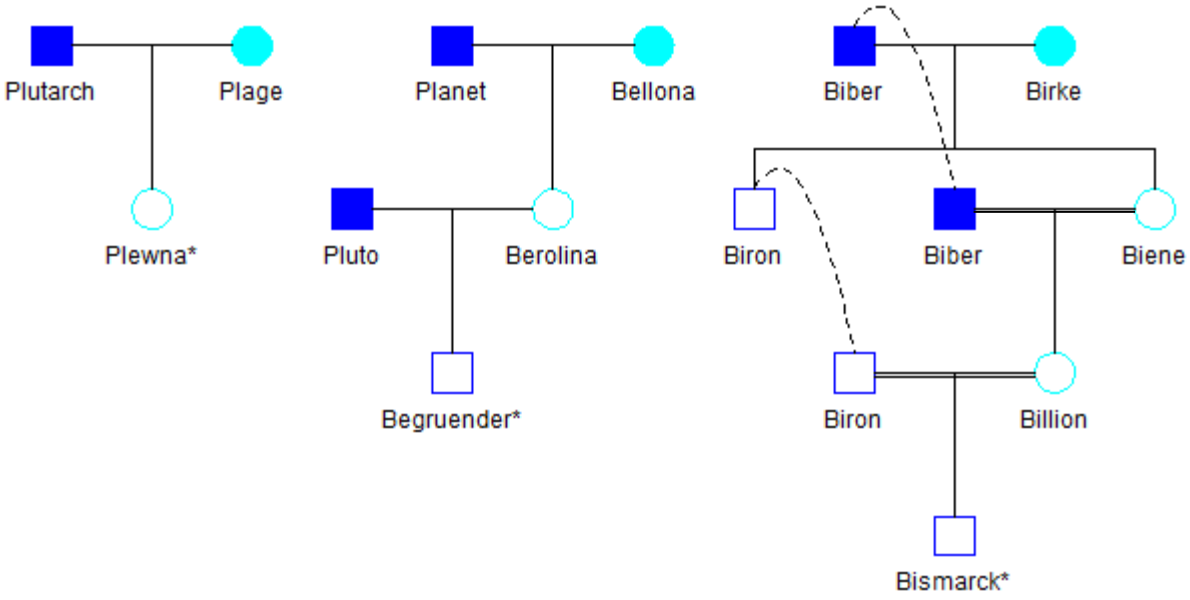

Figure 3: Known pedigrees of three of the traditionally designated founders (marked with asterisk; all other plotted individuals are traditionally not considered as founders). The animals included here were born between 1881 – 1925. Blue squares: male individuals; cyan circles: female individuals; filled individual symbols: 'real founders' without further genealogical information; dashed line: same individual; double line: known inbreeding.

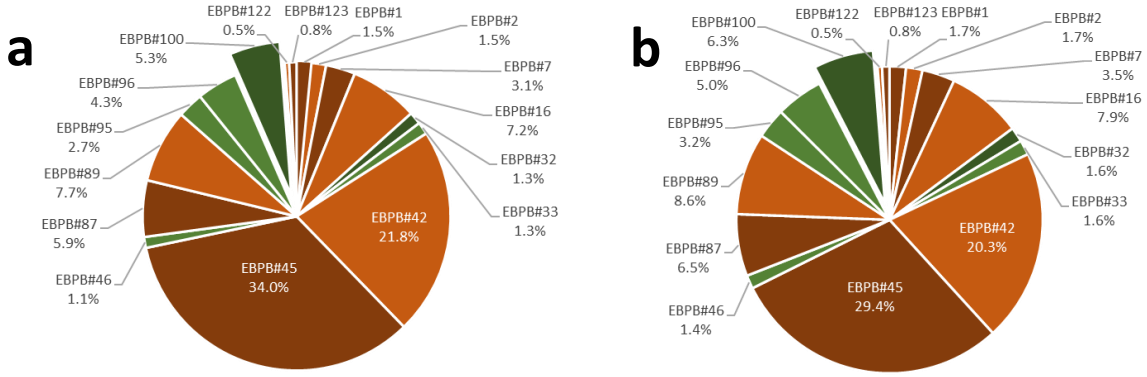

Figure 4: Pie charts of Founder representations in (a) all 337 sampled and (b) all genotyped individuals with pedigree information based on genealogy documented in the EBPB. Brown: Founders of both breeding lines; green: founders exclusive for the LC line. Darker colours: males; lighter colours: females. Detached pie piece resembles the Caucasian bison founder 'Kaukasus' (EBPB#100).

In total ≤ 337 European bison were sampled (99.223.15; LC: *n* = 229, LL: *n* = 108; 224 individuals were directly assigned to samples; Figure A 5). Thereof, the pedigree of 277 individuals (84.193.0; LC: *n* = 191; LL: *n* = 86; 178 individuals are directly assigned to samples) is known up to the founders and overall includes 1 296 European bison (489.807.0; LC: *n* = 885, LL: *n* = 410, Caucasian bison: *n* = 1 (EBPB#100)) and was used as the total pedigree for this study (Table 2). Dependent on the individual age and genealogy this data set reflects eight to 22 generations since the founders. From 38 successfully genotyped individuals (13.25.0; LC: *n* = 17, LL: *n* = 21) virtually no genealogical information exists (wild wisent) or genotypes could not be explicitly assigned to documented individuals. The pedigree of the 99 successfully assigned and SNP-genotyped wisent with known genealogy (35.64.0; LC: *n* = 59, LL: *n* = 40) includes 981 individuals overall (389.592.0; LC: *n* = 640, LL: *n* = 340, Caucasian bison: *n* = 1 (EBPB#100); Table 2). 77.9 % of the pedigree of these 277 sampled individuals and 72.6 % of the pedigree of these 99 successfully SNP-genotyped individuals with documented genealogy are known up to the founders.

Three out of the 137 successfully SNP-genotyped European bison are documented F_1 breeding line hybrids not being full or half siblings (Figure 10). 95 of 1 356 individuals (7 %; 95 of 923 individuals of the LC line (10.3 %)) are F_1 breeding line hybrids in the total pedigree in this study. The living 281 of 337 sampled and 106 of 137 successfully SNP-genotyped individuals represent approx. 4 % and 1.5 % of the current living global population, respectively.

3.2 96 SNP panel

Occasional fluorescence of NTCs are known in SNP genotyping and is considered to be no concern due to marker-specificity and inconsistency in genotype yields from NTCs (KRAUS et al. 2015). With the marker GTA0242130 all NTC showed fluorescence and solely clustered with the homozygous YY cluster. Nevertheless, this marker was kept because of the overall good clustering.

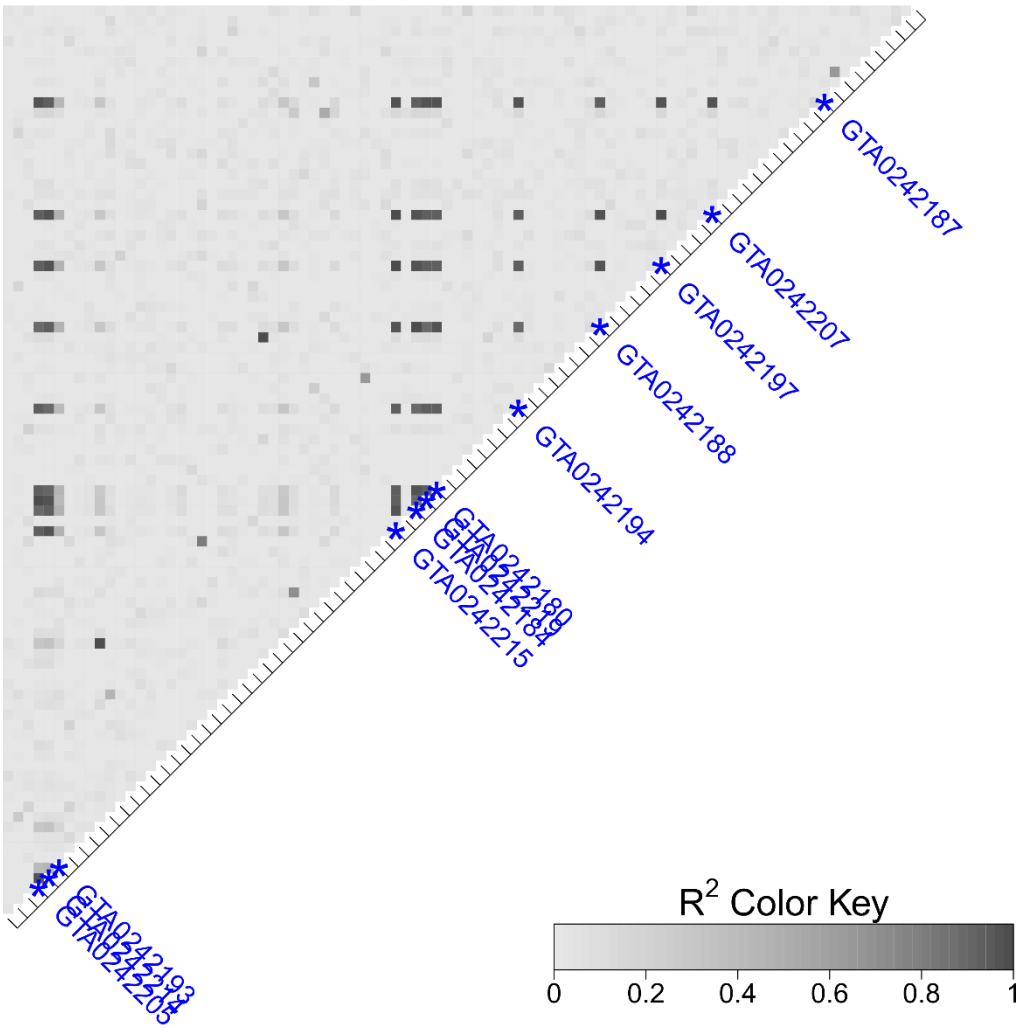

Figure 5: Pairwise linkage disequilibrium heatmap. Pairwise linkage disequilibrium (R^2) calculated for 90 autosomal SNPs polymorphic in the European bison in 58 non-first-order relatives. Regardless of their LD, all 12 markers with an association to posthitis (OLEŃSKI et al. 2015) are labelled in blue.

Significant linkage disequilibria were found in all 90 autosomal markers with the likelihood-ratio test ranging from at least two up to 33 linked loci per marker (see supplementary file 'LD_90SNPs_Arlequin.xlsx').

Utilising *Arlequin* and *GenAlEx*, 74 loci were consistently in HWE across 58 genotyped non-first-order relatives and were consequently used to calculate all genetic diversity values of all 137 European bison in this study.

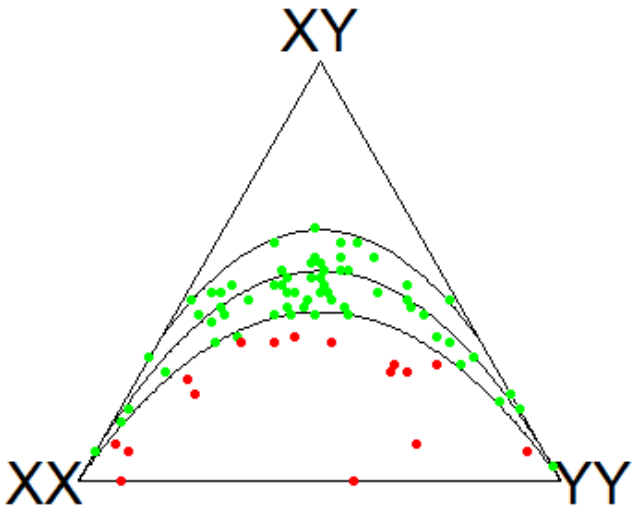

Figure 6: Ternary Hardy-Weinberg-Equilibrium (HWE) plots showing heterozygote deficiencies or excesses of 90 autosomal markers of 58 European bison. Green dots represent the 74 loci in HWE whereas 16 loci (red dots) are deviating from HWE. The HWE parabola (intermediate curve) and acceptance region (between lower and upper curves) for the χ^2 test ($\alpha = 0.05$) are shown. XX and XY symbolise both the monomorphic and XY the polymorphic states of the markers marking the genotype count vectors. Table with *p*-values per locus for HWE can be found in the supplementary file 'SNP_marker_list_details.xlsx'.

3.2.1 Sex determination

The final SNP panel includes a single sex marker in the Y-chromosomal amelogenin gene after selecting markers for non-invasive genotyping. Five out of six markers (AmelY1, AmelY2, AmelY3, AmelX1, ZFXY) were functional with invasive samples of which four were excluded from the panel due to failing with non-invasive samples. AmelY1 was the only marker applicable for non-invasive samples. Though, NTCs were amplified within the X-chromosomal cluster, the locus was still found to be informative due to the distinct Y-chromosomal-associated allele cluster.

AmelY1 showed a GE rate of 0.066 with accurate sex determination failing for six European bison cows out of a total of 137 individuals. Three of those individual samples showed three FAs in six replicates whereas three individual samples show four FAs out of six replicates. Four of those six failed sex determinations were showcased in in the exemplary family network (Figure 9). One bull out of a total ten American bison had three ADOs in the sex marker over six replicates. Within the Bovini samples, only the genotyped river-type water buffalo and gayal females failed sex determination.

3.2.2 Individualisation

The microsatellite panel with eleven loci used in the pilot study did not reach enough resolution for PID and PIDsib ≤ 0.0001 (Figure 7, Figure A 11), which is considered to be a sufficiently low threshold for natural populations (WAITS et al. 2001). In contrast, the SNP subset of 90 polymorphic markers reached a PID ≤ 0.0001 with ≤ 10 markers and PIDsib ≤ 0.0001 with ≤ 18 markers (Figure 7).

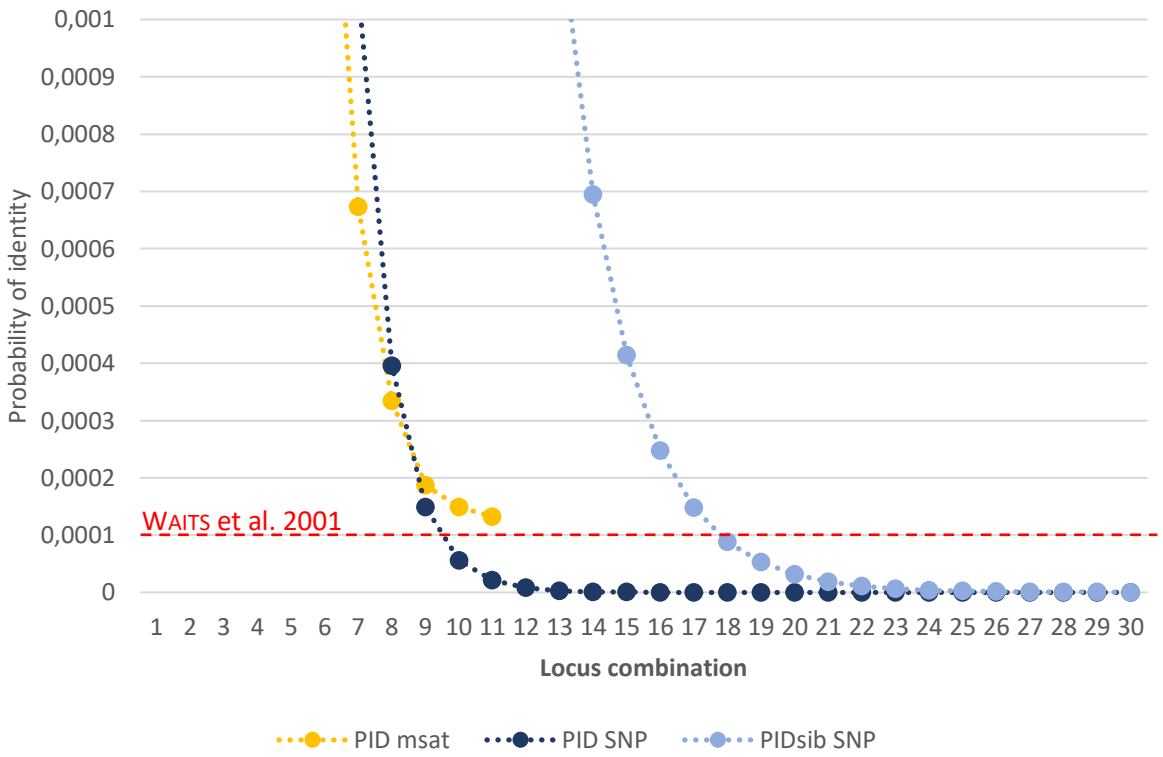

Figure 7: Probability of identity for two marker panels (microsatellites (msat) vs SNPs) for European bison. PID and PIDsibs are depicted for both marker panels (microsatellite panel: *n* = 11; SNP panel: *n* = 90). Dashed red line: PID threshold for natural populations by WAITS et al. (2001) is not overcome by the microsatellite panel. SNP-based PID reaches threshold at approx. 10, PIDsib at approx. 18 loci. Approximations of PID and PIDsib close to zero are reached approx. with 13 and 23 loci, respectively. The x-axis was cut at locus combination of 30 loci for more conciseness whereby the approximation of the SNP-based PIDs does not change after 30 loci. PIDsibs estimations of the microsatellite panel are outside of the scale (x-axis; cf. Figure A 11*).*

The minimal mean allele mismatches found between genotypes for distinct individualisation within the total wisent population were 28.12 loci (LC line: 29.53 loci; LL line: 26.38 loci), for American bison 13 loci and highest for domestic cattle with > 40 loci. Lowest minimal allele mismatches for individualisation were 16 loci between first-degree relatives: full siblings (EBPB#13517 and EBPB#14062) and PO (EBPB#12017 and EBPB#14173)). The lowest number of minimal allele mismatches in the American bison was 10 loci between half siblings (wood bison 'Catori' and 'Yuka'; Figure 8).

23 genotyped samples originated from eleven European bison. Twelve samples which were not assignable in the field were assigned to nine individuals based on the genotypes.

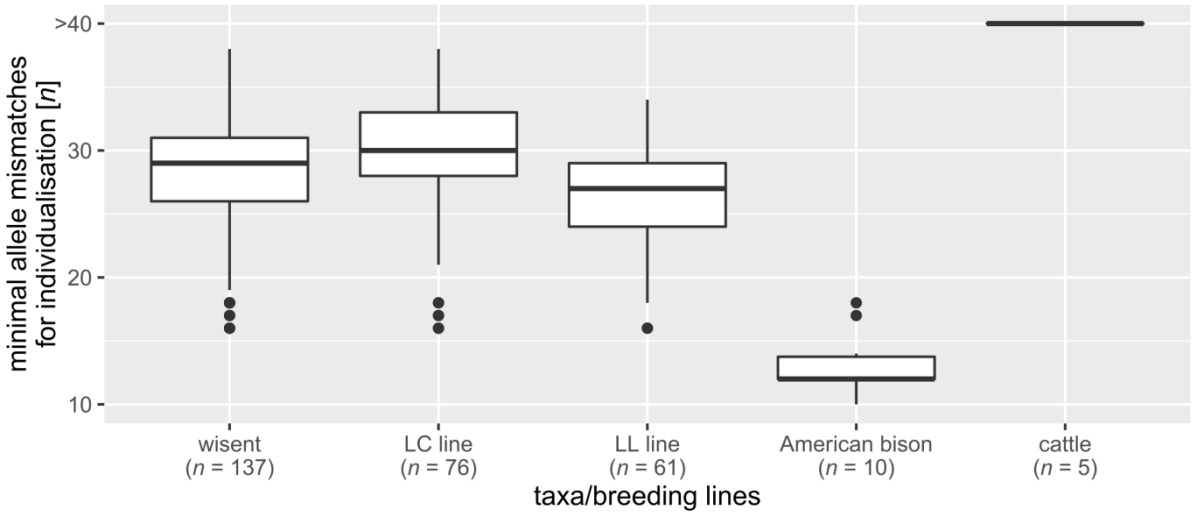

Figure 8: Detected number of mean minimal allele mismatches (loci) between individual genotypes (96 SNP panel) of European bison and within the two breeding lines, as well as for American bison and cattle. Individual sample size per group is noted (*n*). Minimal allele mismatches for cattle are > 40 loci for all five genotyped unrelated individuals.

3.2.3 Parental assignment

After comparing all outputs from *ML-Relate* and *Colony*, the Full-likelihood method with 64 loci in HWE and without SNPs in LD was chosen as it showed the most correct assignments and least false-positive parental assignments based on the available metadata (see supplementary files 'Parental assignment_ml-relate_64SNPs_137IDS+msat.xlsx' and 'Parental assignment_COLONY_90_64SNPs_137IDs.xlsx'). Parental assignment of 137 individual genotypes was conducted for comparison with the pedigree book data. According to the pedigree book, 45 parental assignments were expected to be detected between the available genotypes. From those, 33 maternal and paternal relationships were correctly identified. In five cases, the PO relationship was detected but the offspring was assumed to be the parent or vice versa, due to a missing genotype from the second parent. In seven cases the expected PO relationship was not identified. In eight cases, PO relationships were estimated false-positively compared to pedigree data. Six of these false positives were assigned to second-degree relatives and one third-degree relative mostly with recent inbreeding involved. Another false-positively parental assignment between a wild ('Gożubr' and EBPB#13293) and a captive individual could not be further investigated due to the lack of pedigree data for the wild individual. All false-positive parental assignments between individuals were obtained if no actual parental genotypes were available in the molecular sample set. No false-positive parental assignments between individuals of the two breeding lines were estimated.

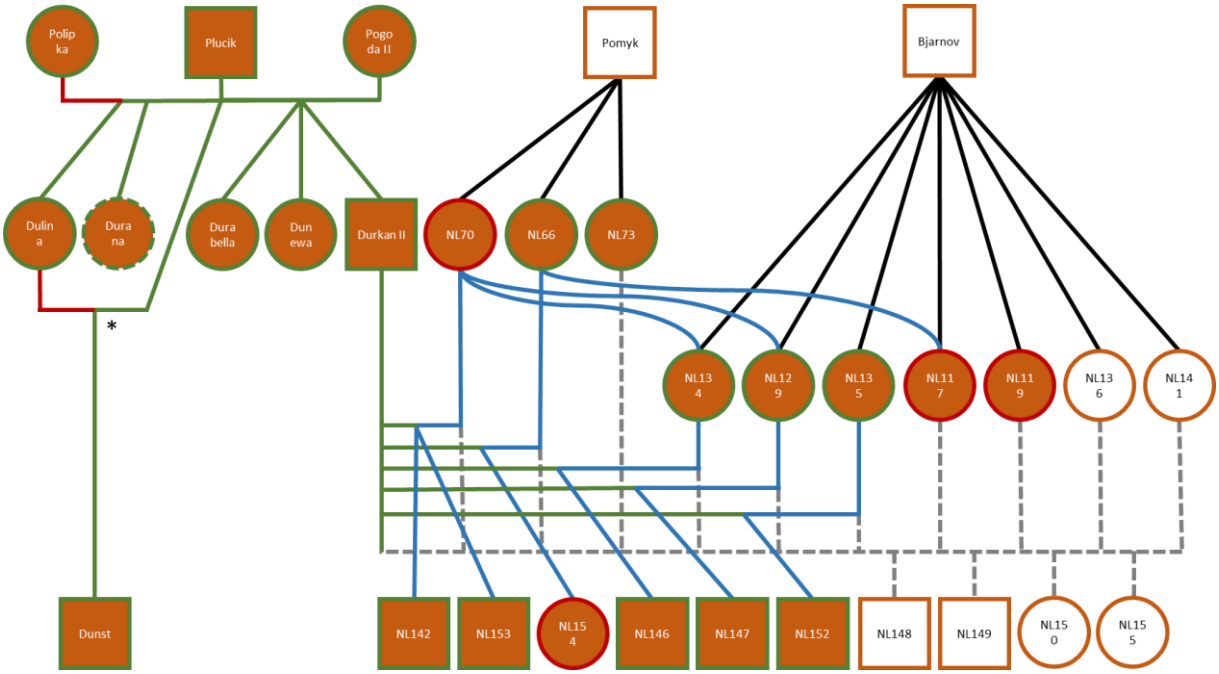

Figure 9: An exemplary family network to document the integration of molecular-genetic kinship analysis into the present pedigree data from the EBPB. Three generations of 23 individuals assigned to the LL line were sampled and genotyped from three locations (Duisburg (Zoo), Lelystad (Natuurpark) and Springe (Wisentgehege)). Circles represent female individuals and squares male individuals (filled symbols: genotyped). Green edges around the individuals represent successful molecular sex verification, whereas red edges represent unsuccessful molecular sex verification. All individuals were genotyped with a single sample. Dashed edges: sample was not individually assignable in the field but was assigned with the genotype. Different colours of the genealogical lineages represent different verification states: green: genetically verified kinships from the EBPB; blue: genetically assigned kinships with lacking data in the EBPB; red: kinship from the EBPB not genetically verified; black: kinships genetically not verifiable due to missing genotypes in the set. 10 parental assignments (from 'Pomyk' and 'Bjarnov') without unknown maternities from the EBPB were included to visualise the at least HS relationships of the females/potential mothers in Lelystad (Naturpark)); grey dashed: presumed kinships not verifiable due to missing genotypes and missing data in the EBPB. Asterisk: Case of inbreeding. All breeding line assignments of the displayed individuals were genetically verified (not noted here).

Looking at the exemplary family network in (Figure 9) two maternal relationships were not estimated with the highest probabilities (red lineages). However, both relationships were not excluded and estimated with lower probabilities (Polipka – Dulina (0.2457); Dulina – Dunst (0.2352)) as the second suggestion of the Full-likelihood method in *Colony*.

It was possible to assign two (LabID#X190221; LabID#X190815) out of twelve in the field individually unassignable but genotyped samples (= nine individualisations) to known individuals documented in the EBPB through their genotyped parents: 'Durana' (EBPB#11813) was at the collection date along other cows the only offspring of 'Plucik' (EBPB#9763) and 'Polipka' (EBPB#10380) in the herd in Springe (Wisentgehege) (Figure 9). 'Odila' (EBPB#13951) was assigned to her sire 'Benno' (EBPB#12102) excluding the also possible cow 'Tilowina' (EBPB#11783) with a different genealogy.

All 64 non-linked markers in HWE (see 3.2) were used for the genetic assessment of the European bison in comparison to pedigree-based genetic values. Only a sole posthitis-associated marker (GTA0242214) was considered, due to exclusion of the others based on LD.

3.2.4 Assessment of genetic diversity

Table 2: Genetic diversity measures based on SNP genotypes and pedigree data for different sample sets of European bison individuals. The molecular values are based on 64 SNP loci in HWE. For all 277 sampled individuals with known genealogy (total population) it was possible to generate pedigree-based genetic values (based on 338 individuals). Genealogical information was not available for all successfully genotyped individuals, whereby a complete pedigree-based assessment is not possible. Thus, molecular and pedigree-based genetic diversity values were calculated for an overlapping set of 99 successfully SNP-genotyped individuals with available genealogical data. Sample sizes (*n*) in brackets show the number of individuals included in the associated pedigree. Values in brackets below the genetic values represent the associated standard error (SE). SE from values computed in *FSTAT* were calculated manually based on output per locus. *F*-statistics in *GenAlEx* were partly calculated in two different ways: [1] arithmetic averages; [2] calculated based on the average H_S and H_T over loci. Mean H_E and H_S calculated in *GenAlEx* are homologous. $F'_{ST}{}^{FSTAT}$ (Nei 1987) is corrected for sample size. Pedigree-based genetic diversity values in *PMx* were calculated utilising two methods: [3] based on kinship matrix; [4] based on gene drop.

		SNP genotypes							**pedigree**			
(sub)population/ sample set	*n*	Allelic richness	mean $H_O{}^{GenAlEx}$ mean $H_O{}^{FSTAT}$	$H_S{}^{GenAlEx}$ mean u$H_E{}^{GenAlEx}$ $H_S{}^{FSTAT(Nei)}$	$H_T{}^{GenAlEx}$ $H_T{}^{FSTAT(Nei)}$	$F_{IT}{}^{GenAlEx}$	$F_{IS}{}^{GenAlEx1}$ $F_{IS}{}^{GenAlEx2}$ $F_{IS}{}^{FSTAT(Nei)}$	$F_{ST}{}^{GenAlEx1}$ $F_{ST}{}^{GenAlEx2}$ $F_{ST}{}^{FSTAT(Nei)}$ $F'_{ST}{}^{FSTAT(Nei)}$	GD^{PMx1} GD^{PMx2}	$F_{IT}{}^{ENDOG}$	$F_{IS}{}^{ENDOG}$	$F_{ST}{}^{PMx}$ $F_{ST}{}^{ENDOG}$
Wisent (total)						F_{IT}						
all sampled with pedigree (total)	338 (1296)	-	-	-	-	-	-	-	0.8252 0.8248	0.0587	0.0219	0.0243 0.0376
all genotyped	137	128	0.396 (0.012) 0.396 (0.015)	0.402 (0.011) 0.405 (0.012) 0.405 (0.015)	0.415 (0.015) 0.417 (0.015)	0.050 (0.012)	0.017 (0.011) 0.015 (0.011) 0.023 (0.011)	0.034 (0.005) 0.033 (0.006) 0.029 (0.005) 0.056 (0.009)	-	-	-	-
all genotyped with pedigree	99 (982)	128	0.395 (0.013) 0.395 (0.016)	0.392 (0.011) 0.397 (0.011) 0.397 (0.014)	0.410 (0.015) 0.412 (0.015)	0.037 (0.015)	-0.005 (0.013) -0.007 (0.014) 0.003 (0.013)	0.043 (0.006) 0.043 (0.007) 0.038 (0.006) 0.073 (0.011)	0.8034 0.8037	0.0574	0.0105	0.0546 0.0474
LC line												
all sampled with pedigree (total)	243 (1032)	-	-	-	-	-	-	-	0.8248 0.8209	-	-	-
all genotyped	76	128	0.400 (0.014) 0.400 (0.014)	0.415 (0.013) 0.417 (0.013) 0.417 (0.013)	0.415 (0.013) 0.417 (0.013)	-	-	-	-	-	-	-
all genotyped with pedigree	59 (785)	128	0.398 (0.014) 0.398 (0.014)	0.410 (0.013) 0.413 (0.013) 0.413 (0.013)	0.410 (0.013) 0.413 (0.013)	-	-	-	0.8119 0.8074	-	-	-
LL line												
all sampled with pedigree (total)	95 (410)	-	-	-		-	-	-	0.6110 0.6041		-	-
all genotyped	61	123	0.392 (0.020) 0.392 (0.020)	0.389 (0.019) 0.392 (0.019) 0.392 (0.019)	0.389 (0.019) 0.392 (0.019)	-	-	-	-	-	-	-
all genotyped with pedigree	40 (340)	123	0.393 (0.021) 0.393 (0.021)	0.375 (0.019) 0.380 (0.019) 0.380 (0.019)	0.375 (0.019) 0.380 (0.019)	-	-	-	0.5673 0.5625	-	-	-

Generally, GD and uH_E were not consistent between molecular and pedigree data, whereas the *F*-statistics showed similar values between both data sets. The LC line showed a consistently higher genetic diversity than the LL line.

3.2.5 Breeding line discrimination

To identify candidate markers with the highest resolution for breeding line discrimination, different subsets of markers were tested. The subset with the highest resolution was identified, when the F_{ST} threshold per locus was set to a minimum of 0.0415. The resulting marker subset contained 29 markers and provided the lowest false-positive rate in breeding line assignments compared with metadata while including the highest number of markers possible. The latter was considered to provide a high genetic resolution even if markers are failing particularly in non-invasive samples.

The final selected 29 SNP subset for breeding line discrimination excludes two out of six loci with private alleles (Table 3) found in the LC line among 137 individuals. In GTA0250956 a low scoring success was obtained (see 3.2.7) but gives an added value for breeding line discrimination due to a fixation above average in the LC line (F_{ST} = 0.088).

Table 3: Allele frequencies (Freq.) of the private alleles in six SNP markers within the LC line.

Locus	GTA0250939	GTA0078270	GTA0250944	GTA0250904	GTA0250943	GTA0250892
Freq.	0.066	0.033	0.112	0.191	0.092	0.138

The most likely *K* from the Bayesian clustering was 2. Considering the BIC for the maximum-likelihood clustering *K* = 3 to 6 was assumed to be more optimal (Figure A 12). However, the strong decrease of the BIC in assuming *K* = 2 reflects the actual separation of the global wisent population into two breeding lines for which the markers were selected in the first place. Thus, the subsequent analysis was conducted assuming *K* = 2. With the subset of 29 selected breeding line markers the mean F_{ST} per locus between the LC and LL line was 0.094 (SE = 0.012) among 137 individuals (LC: *n* = 76; LL: *n* = 61).

Twelve individuals with the Bayesian genetic clustering (*STRUCTURE*) and six individuals with the maximum likelihood genetic clustering (*adagenet*) were false-positively assigned to a breeding line (Bayesian: total: *n* = 5, LC: *n* = 5, LL: *n* = 0; Maximum Likelihood: total: *n* = 6, LC: *n* = 5, LL: *n* = 1) or were not assignable (probability of > 40 % to < 60 % for a population; Bayesian: total: *n* = 7, LC: *n* = 3, LL: *n* = 4; Maximum Likelihood: total: *n* = 0; Figure 10). Besides four 'Russian' samples, false-positively assigned to the LL line, all individuals were correctly assigned with the *Population Assignment* function in *GenAlEx*.

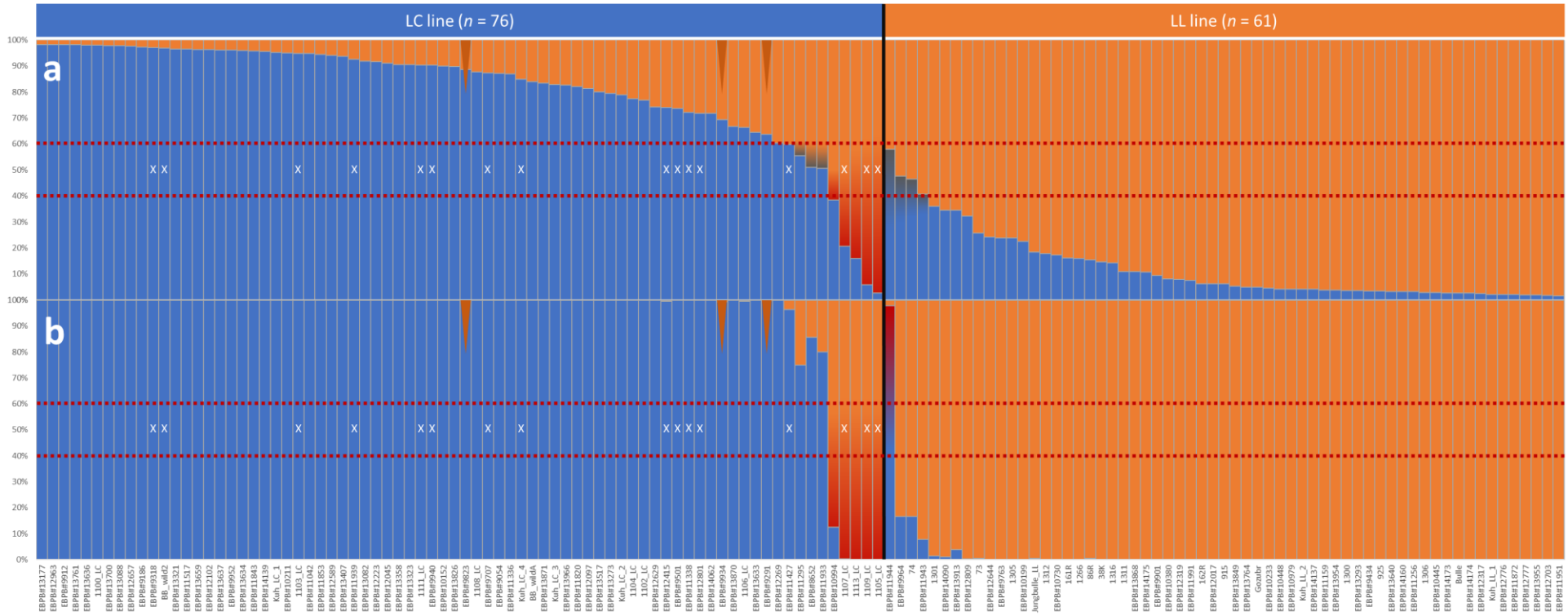

Figure 10: Barplots of assignment probabilities [%] from 29 loci selected for breeding line discrimination between the LC (*n* = 76) and LL line (*n* = 61) in the European bison. Two methods are compared: (a) Bayesian genetic clustering computed with *STRUCTURE*; (b) Maximum-likelihood genetic clustering computed with *adegenet*. A black line separates the two breeding lines (LC line: blue; LL line: orange) based on metadata. Dashed red lines mark the assignment thresholds: bars tarnished red mark individuals with false-positive assignments to a breeding line based on genotypic data compared to metadata; bars tarnished grey mark individuals not assignable with genotypic data according to the assignment threshold. Brown arrows: F_1 breeding line hybrids. White crosses: LC individuals without any of the six private alleles found in the LC line. Individuals (EBPB# and study internal names can be found at the bottom) are ordered within their breeding line (according to the metadata) after assignment probabilities computed with the Bayesian clustering.

3.2.6 Cross-species detection

Five cross-species markers (GTA0250958, GTA0250953, GTA0250963, GTA0250909, GTA0250962) were selected to be monomorphic in the European bison and polymorphic in the most common sympatric bovine species (domestic cattle) or sister species (American bison), respectively. Those five markers were utilised for cross-species detection only.

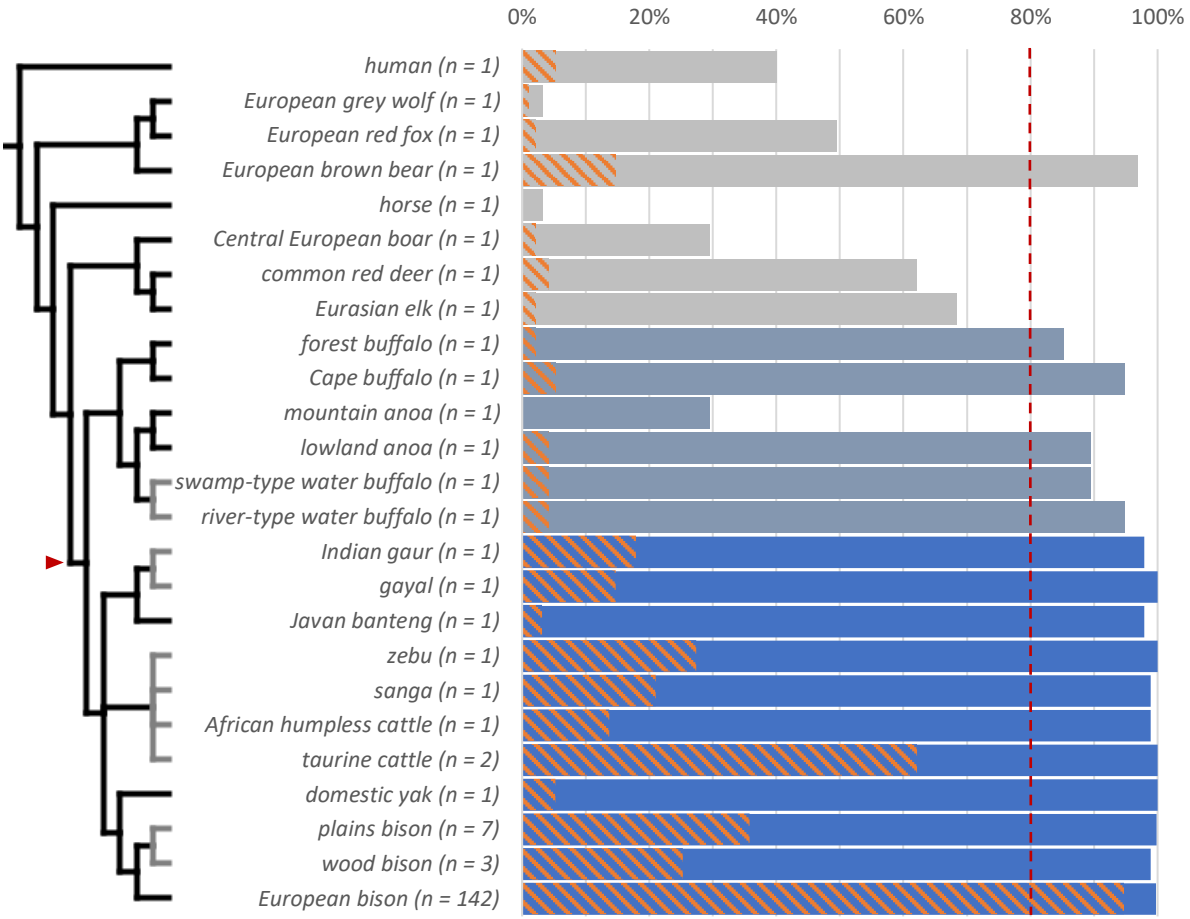

Figure 11: SNP call rate [%] for 95 autosomal markers in 18 non-target species and the European bison with associated sample sizes. If sample size for a species > 1 the mean call rate of all samples is shown. Blue bars reflect all groups classified to the genus *Bos*, blue-grey bars groups classified to the subtribe Bubalina and grey bars species outside of Bovini. A SNP call rate of at least 80 % call rate (red dashed line) is the threshold for inclusion into further analysis. The orange-hatched bars show the percentage of found polymorphism over 95 loci within the groups. The cladogram reflects the known relationships (GARRICK and RUVINSKY 2015) of all species, subspecies and other ESUs genotyped. Grey lineages in the cladogram represent subspecies or other ESUs within species. The red arrow points out the tribe of Bovini.

Samples from not closely related taxa showed tendencies of lower call rates and SNP polymorphism. Except of human, all samples originate from Laurasiatheria species, whereas the majority represents Artiodactyla. All non-wisent taxa or groups with SNP call rates over 80 % (16 ESUs in 10 species) can be distinguished from *B. bonasus* in a PCoA based on a 84 loci genotypes (Figure 12) including all close relatives within the Bovini. This also applies for the brown bear, which showed a conspicuously high call rate and was consequently included in the PCoA analysis.

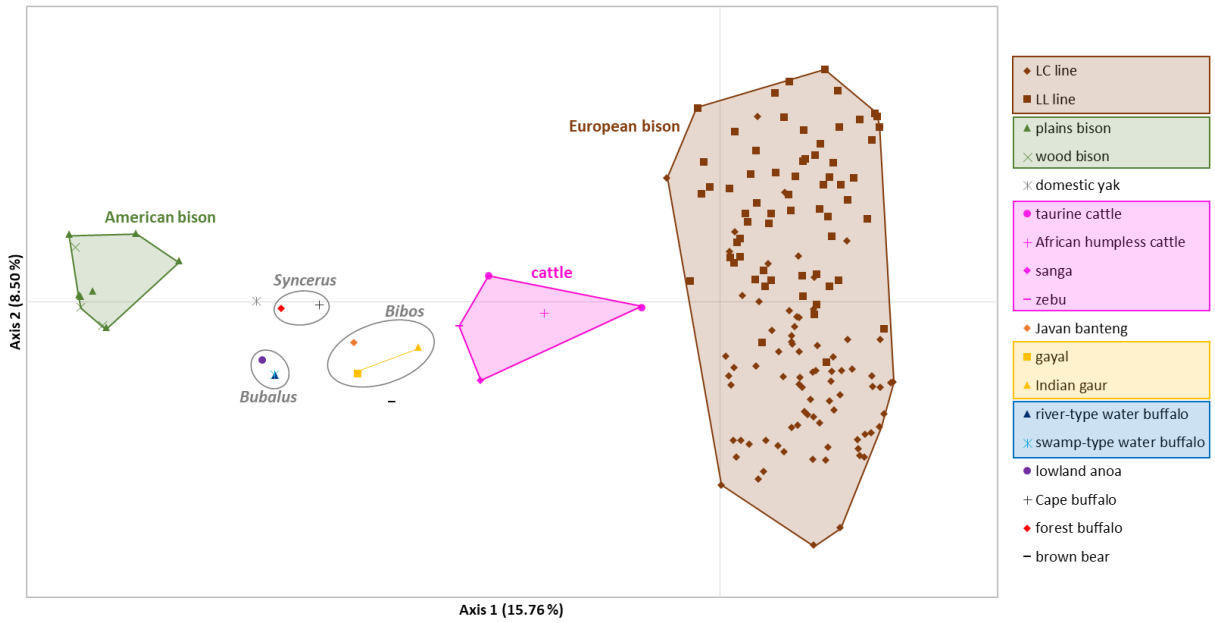

Figure 12: PCoA of 142 European bison and 25 individuals of 10 non-target species (16 ESUs) with a SNP call rate over 80 % utilising 84 SNP loci. Minimal extension of clusters is edged for species with samples sizes > 1. Clusters above species-level are marked for the genera *Bubalus* and *Syncerus* as well as for the subgenus *Bibos* (HODGSON 1837). Eigenvalues: axis 1: 90.075; axis 2: 48.567.

3.2.7 Genotyping error rates

Sample ID, sample type and array run were integrated in the GLMMs as random effects after improving the model. Species as a factor (European or American bison) had no impact on GE/ADO/FA and showed a marginal deterioration (ΔAICc = 2), if added as a random effect. Thus, this factor was removed from the model in favour of model simplicity.

35.4 % (mean NC rate per replicate = 2.6) of all replicate genotypes showed no missing data (= no calls) and 30.1 % no GEs (mean GE per replicate = 1.8), 66.0 % no ADOs (mean ADO per replicate = 1.5) and 79.5 % no FAs (mean FA per replicate = 0.3). NC rates as predictor showed a significant positive relationship on ADO and GE rates as response variables but not for FA rates (Table 4). The call rates from invasive samples were approx. 100 %, while the GE rates were close to 0 (see supplementary file 'SNP_marker_list_details.xlsx').

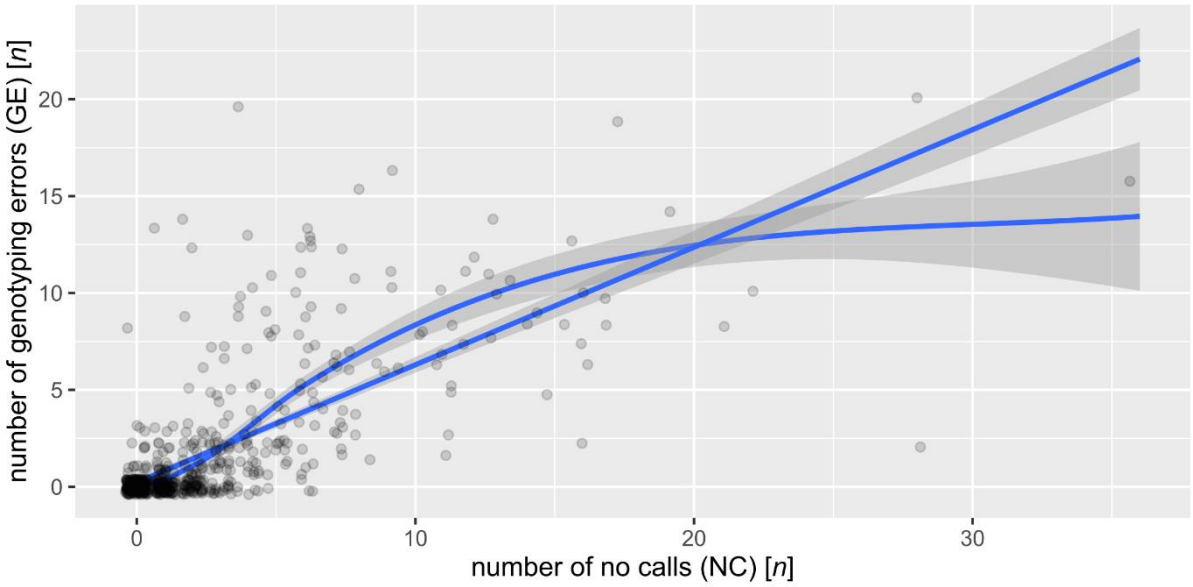

Figure 13: Scatter plot of the number of no calls (NC) and genotyping errors (GE) from 618 genotypes of 88 samples. A high density of low rates of GE and NC is visualised with overlapping data points. Two curves show the relationship of NC and GE: linear relationship and the smoothed conditional mean each with 95 % confidence intervals. It is not an exact visualisation of the GLMM tested here.

Table 4: Results from the GLMMs (cf. Equation 1) on the relationship of no call (NC) rates on genotyping error (GE) rates. Additionally, the same models were applied for allelic dropout (ADO) rates and false allele (FA) rates. The slope estimate for the predictor NC represents the difference from the intercept. For every model a null model was executed. The significance of the ANOVA (Wald χ^2 test) is coded as followed: not significant 'ns', < 0.1 '.', < 0.05 '*', < 0.01 '**', < 0.001 '***'.

Model		estimate	SE	ANOVA	$R^2_{GLMM(m)}$	$R^2_{GLMM(c)}$	AICc
GE	intercept	9.095185	1.271819	***	4.693301e-04	0.7124737	1304.284
	NC	-0.018214	0.007768	*			
GE null		7.3150	0.9853	***	0	0.6970351	1307.788
ADO	intercept	10.810156	1.451256	***	0.0009300123	0.7581565	1144.213
	NC	-0.027957	0.008621	**			
ADO null		8.029	1.110	***	0	0.7359774	1152.775
FA	intercept	6.18746	2.05862	**	8.228590e-04	0.56613912	564.4249
	NC	0.01963	0.02083	ns			
FA null		8.0820	0.4791	***	0	0.58648719	563.2563

4 Discussion

4.1 Dung as source for genetic analyses in European bison

Faeces are a frequently used environmental sample type that has been proven to be a viable source for DNA in numerous genetic assessments (WASSER et al. 1997; TABERLET et al. 1999; MURPHY et al. 2003; WAITS and PAETKAU 2005; GARDIPEE 2007). Many bovids (Bovidae GRAY 1821) utilise specific localised defaecation sites or latrines for urination and/or defaecation often resulting in dung piles known as 'dung heaps' or 'dung middens' (HASSANIN et al. 2012; BIBI 2013; ZURANO et al. 2019). While this behaviour might hamper genetic assessments due to intraspecific cross-contamination among individuals, it has not yet been observed in European bison. In contrast, TAYLOR (1954) noted that taurine cattle do not even show any sign of field division regarding foraging and defaecation. In this regard, Bovini is the only tribus within the Bovinae which does not contain species which are reported to utilise such latrines (cf. WALTHER 1964; JARMAN and JARMAN 1974; HENDRICHS 1975; ESSGHAIER and JOHNSON 1981; SCHÜTZE 2002; WRONSKI et al. 2006; LESLIE 2008; SHARMA et al. 2009; LUNT 2011; WRONSKI et al. 2013). Additionally, taurine cattle showed avoidance towards their own faeces to a certain extent (TAYLOR 1954). Therefore, pristine Bovini dung provides a viable sample type for genetic monitoring with a relatively low risk of intraspecific cross-contamination but might hold complications in other bovids. Furthermore, the wisent, with a daily intake of as much as 30 kg of vegetable biomass with low digestibility defaecates between 5 – 7 kg of dung per day (OLECH and PERZANOWSKI 2015) providing an exceptional frequent and therefore pivotal source for non-invasive sampling. Even though, dung probably represents the optimal sample source in non-invasive genetic monitoring in the European bison, it is not completely free from potential contamination risks which should always be considered: licking each other exhibited in grooming behaviour especially between mother and calf or in sexual behaviour between male and female (KRASIŃSKA and KRASIŃSKI 2013; KRASIŃSKA et al. 2014) are potential intraspecific sources of cross-contaminants in e.g. faecal samples (WASSER et al. 1997).

Even if often proven as a viable DNA source for genetic studies, faeces still represent low-quality samples and need to be evaluated regarding the optimal sampling and sample storage strategy as measured by their PCR amplification success (TABERLET et al. 1999). During quantification of nucleic acids in the samples, different initial concentrations and longevity of both DNA types during storage could lead to, to a certain extent, false conclusions to use amplification values of mtDNA to evaluate nuclear DNA in faeces (MORIN et al. 2001). Consequently, (nuclear) microsatellites were used to evaluate the reliability of different faecal sampling, DNA extraction and storage methods, considering that the amplification success of microsatellites was identified as good indicator for genotype

qualities of SNPs (VON THADEN et al. 2017) beyond their actual meaningfulness for population genetics in the European bison.

This pilot study provides a faecal sampling methodology evaluation on which (i) DNA extraction kit, (ii) sampled faecal part, (iii) sample storage duration, (iv) sampling and storage type is the most promising for genotyping and applicability in a comprehensive genetic population monitoring of European bison. Additional comments on other (non-)invasive sample types are included (further detailed material and methods, results and discussion see Appendix 6.6). In conclusion, dung samples of European bison were identified as a suitable source for genetic analyses. The generated high-quality SNP genotypes allowed for investigating several population genetic questions in European bison. While collecting faecal swab samples directly in InhibitEX buffer had shown to entail the best success rates with relatively low error rates, the collection of full samples in EtOH clearly has the advantage of providing back-up material for further genetic analysis as well as additional investigations, such as diet studies if needed. Thus, the collection of decent faecal swab samples in InhibitEX buffer and full faecal samples in 96 % EtOH with a contemporary DNA extraction are recommended and were used for further comprehensive sampling in the main study, consistent with others (VELLI et al. 2019). In general, minor evidence of cross-contamination was found in dung samples genotyped with microsatellites. Beside the fact that Bovini do not use latrines, the pilot study represents the molecular proof of the viability of dung as a DNA source for genetic assessment in this group.

4.2 Comparison of marker systems

Overall, SNPs are discussed to have advantages over mtDNA and microsatellites being nuclear, abundant and widespread in many species' genomes while also inhabiting simple mutation patterns (MORIN et al. 2004). Previously, it was shown that microsatellites and microfluidic SNP panels are able to assign the same individuals but genotype recovery for non-invasive samples was higher using SNPs (VON THADEN et al. 2017). This might be connected with the shorter amplicon lengths needed for the analysis of SNPs complying the recommended short-length approach for effective genotyping (FRANTZEN et al. 1998). The microsatellites used in the pilot study, showed a low allelic richness relative to what is commonly found in other species (MORIN et al. 2004) but consistent with other studies on European bison: for instance, analysis with a set of 17 microsatellites shows limited resolution regarding basic questions like paternity and breeding line discrimination (TOKARSKA et al. 2009a). In contrast, parental assignment with 15 microsatellites was shown to be possible in American bison and domestic cattle (SCHNABEL et al. 2000). This discrepancy is most likely caused by the general low genetic diversity present in the recent population of *B. bonasus* (TOKARSKA et al. 2009a; 2011). Since the microsatellite set in the current study was neither able to resolve individuals

(Figure 7) nor breeding line discrimination (Figure A 4), no further investigations were conducted with the microsatellites in this study.

Despite those advantages, the proper usage of SNPs for non-model organisms has been discussed (e.g. MORIN et al. 2004; HELYAR et al. 2011). In general, individual and population selection during the SNP discovery process can lead to an ascertainment bias in population genetic inferences and should be taken into account during the SNP selection process (ALBRECHTSEN et al. 2010; MALOMANE et al. 2018). SNP panels normally developed for humans or economically important livestock are commonly utilised in ecological and conservation genetics for related non-model organisms (LAUNHARDT et al. 1998; SMITH et al. 2000; OGDEN et al. 2012) such as in wisent genetics, where the SNPs were originally detected in domestic cattle (PERTOLDI et al. 2009; TOKARSKA et al. 2009a; 2009b; PERTOLDI et al. 2010a; KAMIŃSKI et al. 2012; WOJCIECHOWSKA et al. 2017; OLEŃSKI et al. 2018; 2020). All SNPs utilised in this study, whether self-designed or taken from those commercially available bovine chips, were also originally detected in related species but identified to be polymorphic in the European bison. Thus, these SNPs are most likely located in orthologous regions derived from the last common ancestor. This cross-species amplification approach is currently less time-consuming than *de novo*-SNP discovery (LAUNHARDT et al. 1998), and can potentially be used for species within a clade (SMITH et al. 2005) but is also prone for such an ascertainment bias due to different allele frequencies. In general, it could upwardly bias the studied diversity estimations of a population with lower diversity if the SNPs were discovered in a more diverse population or species (SCHLÖTTERER and HARR 2002; MORIN et al. 2004) conceivable in the case of the European bison. Thus, several aspects were considered to reduce an ascertainment bias in the current 96 SNP panel. The utilised SNPs were already tested and filtered for the European bison before tested and filtered with own samples. As it is recommended for SNP selection via genotyping, the sample set representative for the global population of the European bison (see 3.1) reduces an ascertainment bias as well. While the captive population of European bison is highly admixed (DRUET et al. 2020), reintroductions represent bottleneck events with a potential negative effect on genetic diversity in the wild herds. It was shown that if an ancestral population was used to develop an informative SNP panel no evidence for bias was observed, if used for derived populations (SCHLÖTTERER and HARR 2002). Until today, reintroductions of the wisent always are sourced from the captive population and therefore resembles an ancestral population from which the majority of individuals for the SNP selection process originated. Nevertheless, most of the SNP panel applications like individualisation, parental assignment and breeding line discrimination are less sensitive towards ascertainment bias anyway (MORIN et al. 2004).

Regardless, SNPs were selected from previous studies on European bison, not all loci could be verified for their intended applications. For instance, SNP alleles to be private to a breeding line

published by KAMIŃSKI et al. (2012) could not be confirmed in the current study as discriminative. This can be explained by the considerably small and therefore not representative sample size of only ten individuals genotyped in the aforementioned study (LL: n = 5; LC: n = 5).

4.3 96 SNP panel

The high rate of detected LD with the likelihood ratio test using the EM algorithm might be overestimated in the 90 autosomal loci and might represent an inherent issue of this statistical method caused by the species' overall low genetic diversity (MACK et al. 2014). Thus, only the results of the R^2-based LD calculations were considered in this study, which do not show LD for every locus but also provide independently detected and comprehensible values for the posthitis-associated loci.

4.4 Genotyping error and SNP call rates

The mean GE rates found in this study (see supplementary file 'SNP_marker_list_details.xlsx') tend to be slightly higher than those found in comparable literature (TABERLET et al. 1999; KRAUS et al. 2015). In contrast to most published GE rates, however, no pre-selection for good-quality samples was conducted. Furthermore, additional 15 low-quality samples (with higher GE and NC rates) were replicated twelve times instead of six times to be able to detect GEs and investigate inter- and intraspecific causes of errors. Together, this increased GE rates for the SNP panel in this analysis. However, on average, 2.6 NCs per locus across the 618 genotypes generated from 88 samples caused no significant issues for the different downstream analyses. In individual cases noticeably higher NC rates were accepted: a single autosomal marker (GTA0250956) showed a drastically higher NC rate of 8 % even in consensus genotypes (average NC rate over all marker = 0.13). Since this marker is highly informative for breeding line discrimination (F_{ST} = 0.088 in a set of 58 individuals not in a first-degree relationship) with a low GE rate, it was kept. Further evaluations for GE rates without failed genotypes including estimations on how many replicates are needed in very low-quality samples utilising this SNP panel are recommended. The already generated genotypes, especially the extensively replicated low-quality genotypes, together with the extensive sample collection from this study yields the potential for such continuing investigations. Invasive samples generally showed nearly complete call rates and minor GE rates and thus no need to be replicated with the current SNP panel.

The risk of false genotypes caused by GEs is a well-known problem in non-invasive genetics but can be reduced with adaptations in the experimental setup: foregone studies induced a multiple-tubes approach (NAVIDI et al. 1992; TABERLET et al. 1999) and a stochastically reasonable sample sizes of three to six faeces per individual to provide reliable genotypic data (FRANTZEN et al. 1998). Three (SOTO-CALDERÓN et al. 2009) or four (MORIN et al. 2001) replicates were recommended, whereas in this study triplicates were used for non-invasive genotyping.

Generally, low SNP call rates are good predictors for non-reliable genotypes (VON THADEN et al. 2017). In this study, 28 cycles were performed for the pre-amplification step to improve successful amplification of loci in samples with a low quantity of DNA. By doing this, amplification artefacts might be elevated as well and consequently hamper the recognition of erroneous genotypes. Here, I introduced GLMMs to test for a predictory relationship of NC to GEs. Subsequently, this could be used to set a NC threshold as a guidance during genotyping as an additional criterion for genotype quality evaluation. The GLMMs showed that NC rates could function as such a predictor for GEs and ADOs. However, it was not possible to find a significant predictory relationship between NC rates and FA rates. This itself might be due to the known overall low occurrence of FAs (TABERLET et al. 1999; VON THADEN et al. 2020; own data). Approximately 80 % of all replicates showed no FAs. Since the FA rate was included in the GE rate there is no special need to predict FA specifically. Though, the $R^2_{GLMM(m)}$ in all models was relatively low, the $R^2_{GLMM(c)}$ showed that with the additional random effects the models could explain up to 76 % variance. Here, it was not important to find the most impactful predictor but to show a predictory relationship between NC rates and GE rates to be able to evaluate the quality of obtained genotypes. On average, around 25 NCs were needed to exceed minimal allele mismatches of 16 loci measured between first-degree relatives in the current wisent sample set for individualisation (cf. Figure 13 and 3.2.2). More sensitive measurements like parental assignment or breeding line discrimination with reduced genotypes would require lower GE rates to be feasible. This has to be tested separately but was not further investigated here. The predictory relationship of NCs on GEs allows a data-based evaluation of genotypes after SNP scoring, as shown with similar evaluations (VON THADEN et al. 2020). However, for such genotype validation the variation of this relationship has to be considered, especially if more GEs per NC than average are observed. Such an evaluation criterion is particularly useful if the costly multiple-tubes approach is not utilised.

4.5 Sex determination

For population characterisation, the determination of the individual sex is a crucial information to exclude e.g. considerable individuals for genealogical analysis in a genetic monitoring. Beyond this, the possibility to determine the sex might be very useful in future non-invasive studies about population structure or sex-related behaviour. Suppositions of an inbreeding effect by uneven sex ratios in the European bison were mentioned before (OLECH 2006). Thus, the single sex marker introduced here has a much higher informative value compared with all the single autosomal loci in the panel. The GE rate of 0.06 for this marker led to six failed sex determinations of in total 137 European bison with viable autosomal genotypes and represents an effective sex marker for non-invasive samples. If this single sex marker fails and there is no further sample material, but the autosomal genotype is viable, a secondary sex determination can be achieved via individualisation and further parental assignment if listed in the EBPB. This is exemplarily demonstrated with the cow

'Nl-70' (EBPB#10445) which provided a sample in which the sex marker failed but could be successfully assigned within the pedigree based on the remaining autosomal genotype (Figure 9). Perceptively, corroborative sex markers could be included at the cost of other markers to strengthen the reliability of sex determination of this SNP panel.

The sex marker was functional in all Bovini with the only exception of three genotyped individuals (American bison, gayal and river-type water buffalo), which is reasonable since the SNP was detected in species of the genus *Bos*. In all three non-wisent cases of failed sex determination within the Bovini, conspecific individuals (American bison, gaur, water buffalo) were successfully genotyped for their sex. Thus, the aforementioned failed sex determinations represent individual cases but not overall results for those species and should be investigated further. The most distant relatives with validated successful molecular sex determination belong to both the Cervidae (GOLDFUSS 1820) species and goes along with the phylogeny (WILSON and MITTERMEIER 2009). This finding reduces the chance of cross-contamination by many sympatric species or by humans during sampling or laboratory work.

4.6 Individualisation

With a sample set of European bison individuals selected for a pedigree-based representative genetic diversity including both breeding lines, family networks, captive and wild individuals, all genotyped animals could be individualised with the SNP panel presented here. Additionally, individualisation within other Bovini species with > 1 genotyped individuals was possible for American bison, domestic cattle, gaur and water buffalo. The lowest number of hypothetical allele mismatches found, still allowing a secure individualisation, was 16 loci between first-degree relatives in the wisent. This is two to three times higher than allele mismatch thresholds allowing individualisation known from similar SNP panels (NUSSBERGER et al. 2014; VON THADEN et al. 2020). Thus, a sixth of all markers could fail until the sample would be falsely assigned to a closely related individual. Over the averaged population, approx. 28 loci would be allowed to fail (Figure 8), making the SNP panel presented here less prone to GEs and NCs found especially in genotypes from non-invasive samples. Nevertheless, if approx. 75 loci are identical between genotyped cases of first-degree relatives, the approx. 23 loci needed to individualise (predicted with PIDsib with the same set of genotypes) might be underestimated. If the probability threshold for natural populations by WAITS et al. (2001) is considered, even approx. 18 SNPs would be sufficient (Figure 7). Consequently, it is still necessary to carefully analyse the genotypic data and not only rely on PID estimations to conceptualise the marker panel especially in a species with low genetic variation.

23 samples which were not assigned to an individual in the field were clearly individualised as eleven European bison based on their genotypes. In one exemplary case, three samples (one urine and two

dung samples from two different wisent pats), belonging to one individual, were narrowed down to three juvenile bulls (EBPB#13746, EBPB#14023, EBPB#14039). Those bulls were part of a sampled herd in Springe (Wisentgehege und Falkenhof) together with eight cows at the day of sample collection. The assignment to those three individuals was possible due to successful molecular sex determination and additional metadata documented during the collection. Further samples from related individuals would be needed to assign the sample to one of those bulls with SNP-based parental assignment. As described below (see 4.7) two dung samples not individually assignable in the field, could be assigned to two individuals because parental genotypes were available. This showcases the versatile diagnostic power of the different SNP panel subsets to assess populations.

The twelve putatively posthitis-associated markers with high LD lack discriminative power for individualisation compared with loci in linkage equilibrium. However, since not every allele is linked to all the other eleven loci, those markers still hold information on occasion. With the non-invasive approach, those markers still provide information in the case of amplification failure of another of those loci. If not used for a posthitis-related application in wisent conservation, those markers could be substituted by unlinked markers in the long-term.

4.7 Parental assignment

Marker-based pedigree reconstructions for conservation management already had been successfully utilised in other threatened species (GUERIER et al. 2012; IVY et al. 2016; MCLENNAN et al. 2018). Nevertheless, parentage analysis requires high quality genotypes (MORIN et al. 2004). Previous studies conclude that 50 – 60 SNPs selected for high heterozygosity would be enough to resolve paternity in the European bison (TOKARSKA et al. 2009a; WOJCIECHOWSKA et al. 2017). The number of required loci depends on the breeding line and known parents (TOKARSKA et al. 2009a; WOJCIECHOWSKA et al. 2017; OLEŃSKI et al. 2018). A 100 SNP panel was published for parental assignment for the LL line exclusively (OLEŃSKI et al. 2018) of which a portion of markers were included in the current panel. With the new reduced 64 SNP subset, parental assignment was successfully expanded to the LC line and to the range of samples that can be utilised due to its non-invasive approach. Although sufficient but not perfect (see 3.2.3), the results from genotypic data must be validated by existing metadata from e.g. pedigrees of known relationships from former assessments and vice versa. However, this is state of the art in other comprehensive genetic population monitoring studies (MUELLER et al. 2020) and in line with the conclusion of other authors that if one parent is known, parental assignment is possible with less loci (TOKARSKA et al. 2009a; WOJCIECHOWSKA et al. 2017; OLEŃSKI et al. 2018). This is confirmed when considering the exemplary family network (Figure 9) where the two red marked lineages were not estimated with the highest probabilities but as the second suggestion with lower probabilities (no other individuals were assigned for alternative maternity). In addition to the

involved inbreeding in this particular part of the shown family network, those individuals are part of the LL line, which is expected to require more loci to resolve PO relationships (WOJCIECHOWSKA et al. 2017). Here, the combination of the known and assessed relationships and other metadata could help to put results into a proper context. The software *Colony* enables the inclusion of such additional metadata with excluding PO relationships and parental siblings as well as including known parental siblings between individuals in the genotyped sample set. It is not only recommended to add known relationship information with the inclusion/exclusion-options provided by *Colony* but to also include assumed first-degree relatives e.g. already known from the herd in question. If genotypes from the actual dam and sire are included into the analysis, the parental assignment results are satisfying with lower chances to false-positively assign second-degree relatives especially if recent inbreeding is involved. Such expectable reliability of the accuracy of marker-based pedigree-reconstructions on a certain completeness of population sampling, additional to the number and polymorphism of loci available in a marker panel, has been discussed before (JONES and WANG 2010b; TAYLOR et al. 2015).

4.8 Assessment of genetic diversity

In conservation, the assessment of genetic diversity can help to manage population optimally in order to prevent its loss (WITZENBERGER and HOCHKIRCH 2011). In this chapter, I discuss whether it is possible to assess genetic diversity in populations of European bison measurable from non-invasive samples utilising a reduced SNP panel. From a total of 90 autosomal polymorphic loci, 64 markers in HWE and not in LD were selected and therefore useful for the direct non-invasive assessment of genetic diversity in the wisent. The ternary plot (Figure 6) visualises the discarded 16 loci for HWE caused by tendentially low heterozygosities. In general, this could indicate inbreeding within the genotyped population (GRAFFELMAN 2015). However, since eleven of those 16 loci not in HWE represent markers for breeding line discrimination, it has to be seen as an ascertainment bias, which consequently is removed from the subsequent analysis.

Studies molecularly investigating genetic diversity often face the problem of incompleteness of their population sampling (WITZENBERGER and HOCHKIRCH 2011). Here, the founder representation of the genotype set was compared with larger data sets to evaluate its representativity before assessing the genetic diversity of the wisent population. Based on pedigree data, the 99 genotyped and 338 sampled individuals showed comparable founder representations (Figure 4). Overall, they represent 1.5 % and 4 % of the current global population, respectively.

From all applications provided by the SNP panel presented here, the assessment of the genetic diversity is most prone to an ascertainment bias as already mentioned above. SNPs selected for high polymorphism derived from a more diverse population or species would upwardly bias diversity

estimations (MORIN et al. 2004). In this case, however, GD computed from pedigree data was estimated approx. twice as high as the mean H_E from SNP genotype data, which argues against a conclusion of a strong ascertainment bias among the markers. Moreover, the SNP-based values for H_E still range within independent molecular-based estimations based on presumably neutral markers (0.28 to 0.50), also showing low genetic diversity in the European bison (GRALAK et al. 2004; LUENSER et al. 2005; TOKARSKA et al. 2009a; 2009b). Regarding the mentioned weaknesses of pedigree-based values, it could represent overestimations of genetic diversity if using the EBPB.

Thus, both GD and H_E were not suitable for a direct comparison. *F*-statistics are more suitable for a comparison due to the more relative approach. Though, GD as a measure of genetic diversity might be overestimated mainly by falsely assuming low inbreeding in the founders, the relationship of expected and observed GD, expressed in the *F*-statistics is comparable to the SNP-based results. With a certain confusion in literature (BHATIA et al. 2013) using estimators for population structure such as F_{ST}, G_{ST} or ϑ and its associated corrections, the F_{ST}, as part of the classical *F*-statistics (WRIGHT 1943), is recommended for SNPs especially. The F_{ST} was developed for biallelic markers, whereas the other estimators assume multiallelic markers and therefore higher amounts of unique alleles in every subpopulation (MEIRMANS and HEDRICK 2011). Not only this biallelic nature but also the allelic variation of the utilised SNPs within the LL line included in that of the LC line contradicts with this assumption. Accompanying with this, comparing the SNP based F_{ST} and F'_{ST} to pedigree-based F_{ST} values of this study showed that the F'_{ST} is noticeably higher than both more comparable F_{ST} values from different data sources (Table 2).

General deviations in the genetic diversity values, whether computed from SNP genotypes or pedigree data, occur according to the program and the implemented methods. Although not suitable for a direct comparison, heterozygosities showed the same tendencies as the pedigree-based GD, where the diversity is relatively stable in the total population as well as in the LC line but declined noticeably in the LL line. The same effect was consistently observable if the sample size is reduced in all sample sets (Table 2). The accuracy of pedigree-based genetic values, computed at least in *ENDOG*, suffers from increased kinship (equal to expected homozygosity under random mating) caused by small sample sizes, inbred populations or sample sets with a big portion of relatives, which is the case in the LL line in particular (GUTIÉRREZ et al. 2010). A similar effect of inbreeding and small sample sizes can be seen with the SNP genotype-based data: the tendency of $H_O > H_E$ in small sample sets containing LL individuals (Table 2) caused negative values in the *F*-statistics. It is well known that if biallelic SNPs are utilised for a reliable assessment of genetic diversity, substantially higher numbers of markers are required (WANG 2016). However, especially since these 64 loci were selected after HWE and LD, it is not necessarily an issue caused by the markers itself. It is known that small sample sizes, but also sample sets containing high numbers of relatives or inbred populations, causes

underestimations of H_E (HARRIS and DEGIORGIO 2017). In most cases, the underestimated H_E could be successfully corrected for small sample sizes (uH_E) which is recommended to use for real data in general (PEAKALL and SMOUSE 2012). Like in the sample set of 40 LL individuals, however, H_O was still higher than the associated uH_E. There is an unbiased estimator H_{BLUE} which not only corrects for small sample sizes, but also for samples sets containing related and inbred individuals of any ploidy, subsequently improving estimates of population differentiation statistics as well (HARRIS and DEGIORGIO 2017). In the framework of this study, it was not possible to implement H_{BLUE} to correct for such a relatedness bias due to extensive adjustments to the data sets and the provided *R* script required. Additionally, the *R* script cannot handle missing data in the genotypes properly. However, besides the extension of the genotype data set itself, an utilisation of this estimator is recommended since first results have been promising. The required kinship matrix can be generated from modified relatedness matrices from e.g. *ML-Relate*, where the coefficients of relatedness are divided by two. This would also allow to use this estimator in a genetic monitoring of wild European bison without pedigree data because the current SNP panel also provides kinship estimation. In summary, despite all data corrections, the molecular resolution for assessing the genetic diversity of the European bison suffers in the same way as the pedigree-based calculations from too small sample sizes in relation to its small gene pool. Thus, estimation accuracy will be increased by larger sample sizes and decreasing sampling variance of reference genotypes (WANG 2016), particularly within the breeding lines. As long as the breeding lines are managed separately, estimates of intra-lineage diversities are of interest. If separate genetic assessments are utilised, it is required to test the markers for HWE and LD on each breeding line separately. However, this reduces the number of loci for the LL line. Accompanying with building up a more representative genotype reference database, a continuous check for HWE and LD is recommended due to possible shifts in allele frequencies with additional genotypes and/or over time. The number of SNP markers is a known important factor for the marker resolution (roughly 300 SNPs per morgan are recommended to yield estimates equal to true genomic values). Not surprisingly, pedigree-based estimations of relatedness or inbreeding with sufficient pedigree extent are generally more accurate than marker-based estimates (WANG 2016). However, often no pedigree data is available for conservation-related population studies. Even for the otherwise well documented wisent, this is true for reintroduced free-roaming herds. Uncertainties towards the correctness of all parental assignments in the pedigree over the last nearly 100 years based on observation have been mentioned (OLECH 2006; OLECH 2007) which cannot be quantified for the entire pedigree such as GEs for the SNP panel. The case-study with parental assignment (Figure 9) additionally shows the difficulty to obtain the pedigree information in the first place, even in captive herds (see 4.11.1). Considering the low number of suitable loci used of this reduced SNP panel, it provides a surprisingly viable tool to not only validate, revise and construct pedigrees from

which e.g. inbreeding can be evaluated but serves as a sufficient and direct measurement for population structures where no pedigree data is available. It is also worth to mention here, that the approach of non-invasive genotyping limits the number of informative markers substantially. Thus, the new SNP panel represents an optimised compromise between the needed non-invasive sampling method, cost-efficiency needed for the application in conservation and the resulting informative accuracy, which is demonstrably and reasonably sufficient for the purpose it was developed for.

With $F_{ST} \approx 0.03$ the population differentiation between the breeding lines was, as expected, small but comparable with values between populations of different mammal species where interspecific admixture is involved (VON HOLDT et al. 2016). Here, it must be considered that 18 of those 64 markers presented here, were selected for breeding line discrimination by their F_{ST} with a subsequent statistical differentiation power and may not reflect such a biological meaningful distinctiveness (HEDRICK 1999). Nevertheless, the molecular values agree with the pedigree-based values (Table 2). When comparing the F_{IT}, F_{IS} and F_{ST} values, a reduction of heterozygosity in the total population to the average of both the breeding lines can be observed. The relatively low F_{IS} is caused by high intermixture within the breeding lines, whereas rare gene flow between the LC and LL line, manifested in the second highest fixation estimated in the F_{ST}. The highest fixation seen in the F_{IT} is caused by different allele frequencies within the breeding lines compared to the total population (without considering breeding lines). This is known as the Wahlund effect (WAHLUND 1928). Though, if the European bison is such an inbred species originating from only 12 individuals – why do we get such low *F*-values? A high coefficient of inbreeding (*F*) of an individual is usually a sign of inbreeding due to higher homozygosity rates than expected, or in other words, high fixation of allele frequencies. Among populations, changes in fixation indices can be caused by other population dynamics such as genetic drift, gene flow, migration or bottleneck events (FRANKHAM et al. 2015). Though, the European bison holds a small gene pool, we observe a high degree of admixture and subsequent conserved heterozygosity over the population. This is consistent with a recent study utilising 22 602 SNPs and is a consequence of the successful population management of the last decades (DRUET et al. 2020). It becomes even clearer if the F_{ST} of the 29 markers selected for breeding line discrimination is looked at: it showed still a minor fixation ($F_{ST} = 0.088$) within the breeding lines. Since one of the biggest threats for the European bison with its small gene pool is genetic erosion, damaging effects like gene drift could now be observed with this SNP panel for establishing stable populations in the wild.

4.9 Breeding line discrimination

Previously, breeding line discrimination was achieved with a set of 1 536 selected SNPs (WOJCIECHOWSKA et al. 2017). The 29 SNP subset presented here provides a comparable resolution with only 1.9 % of the number of markers. Additionally, those SNPs are applicable on low-quality

samples, not relying on blood or tissue. The mentioned study genotyped slightly less individuals (LL: *n* = 57; LC: *n* = 72) from as well captive and free-roaming individuals and thus comparable regarding sample size and population representativity.

In total (137 individual genotypes), 4.4 % with the maximum-likelihood clustering and 8.8 % with the Bayesian clustering were genetically false-positive assigned to a breeding line or not assignable according to the individual metadata and the assignment threshold. Four individuals (1105_LC, 1107_LC, 1109_LC, 1113_LC) from 'Russia' documented as LC individuals were the only ones clearly clustering in LL regardless of the utilised method (see 3.2.5). Wild herds founded only by LL individuals in Russia are known (SIPKO 2009). Though, due to the lack of more detailed information regarding those four samples, no final explanation for the genetic signal can be deduced here. One further supposed LC individual (EBPB#10994) clustered within the LL line with both genetic cluster methods (Figure 10). Together with closely related individuals which showed also higher assignment probabilities towards the LL line and partly were not assignable in the maximum-likelihood clustering, reflect the documented genealogy. A single LL female (EBPB#11944 'Pociemna') clustered clearly within the LC line (Figure 10). No gene flow should occur from the LC line into the LL line and the documented pedigree data gives no explanatory indication for this molecular signal. Across all triplicates, the assigned dung sample (Lab#X191495) showed a GE rate of 0 but one breeding line discrimination marker (GTA0250956) failed completely (see supplementary file 'Genotype_list.xlsx') and could have a crucial impact on the result. To exclude laboratory issues such as genetic contamination or sample confusion another sample assigned to 'Pociemna' (EBPB#11944) should be genotyped for comparison. Overall, the subset of 29 SNPs gives insight into anthropogenically induced lineage differentiations with a constant one-directional gene flow from the LL line into the LC line since the establishment of the breeding program. This gene flow from the LL line, whose genetic diversity is comprised within the genetic diversity of the LC line (Figure 1), dilutes the genetic distinctiveness of the LC line. The estimated optimal $K > 2$ (see 3.2.5 and Figure A 12) for the maximum-likelihood genetic clustering methods might also reflect the population substructures due to closely related individuals within two anyway mixed and therefore similar lineages.

The finding of six private alleles within the LC line is not surprising since this breeding line carries genetic material of five additional founders including one bull from a separate subspecies which is expected to be genetically more distinct. The F_{ST} for two of the six loci carrying private alleles in the LC line were below the threshold of 0.0415 and were consequently not included into the discriminative SNP panel subset. With the mentioned discrimination error rate of 4 – 9 % (depending on the genetic clustering method), the panel subset of 29 SNPs holds uncertainties for assignments to a breeding line within the European bison. If we assume the same fixation of those private alleles found in the genotyped sample collection, these six loci could be used as supporting characters for

assigning individuals to one breeding line: if one or more of the private alleles are found in a genotype, it could be assigned to the LC line. This would not only validate most results but would enhance the decision making regarding an assignment to a breeding line. Due to the lack of private alleles found in the LL line, there is no affirmative equivalent vice versa (Figure 10). The absence of any of six private alleles in 16 individuals of the LC line (Figure 10) shows the low information content just relying on those markers and the need for a more discriminative marker panel when aiming at accurate breeding line separation as presented here. However, if considered to be private to LC individuals among the entire population, the presence of such an allele still holds an informative value and could be an additional considerable factor for breeding line discrimination.

In comparison to the Bayesian clustering, the maximum-likelihood clustering estimated one more false-positive breeding line assignment and increased the number from five to six of in total 137 individuals. In contrast, however, it also increased the number of correctly assignable individuals by six for both breeding lines. Considering these results, in this specific case it is recommended to use the maximum-likelihood genetic clustering together with the presented SNP panel to achieve breeding line discrimination in the European bison. Further investigations of the false-positives and more genotypes are needed. The high discrimination success shows the viability of this molecular tool even with non-invasive samples but cannot serve as an absolute definite instrument. *STRUCTURE* provides a Boolean flag option to incorporate predefined population assignments into the Bayesian clustering (PRITCHARD et al. 2010). This could lead to consideration of a certain genotypic variance of the genetically very similar breeding lines and enhance the discriminative success.

It was not possible to explicitly assign the F_1 breeding line hybrid status to individuals with the current SNP panel. Following the official management definition those F_1 hybrids between the breeding lines are formally assigned to the LC line. Thus, all three documented F_1 breeding line hybrids in this study were correctly assigned to the LC line based on SNP genotyping data in any analysis (Figure 10). Here, the presented SNP panel resolves an important overarching issue within wisent conservation regarding the current purity requirement for conservation actions.

Since the here selected SNPs were originally detected in *B. primigenius*, the loci are considered to be evolutionary more conserved and therefore chances might be lower to find discriminative markers between breeding lines within the European bison. This study considered all previous works and, if accessible, most SNPs. Restriction site associated DNA sequencing (RAD-seq) or whole-genome sequencing are alternative opportunities for further SNP-detection directly within *B. bonasus*. For such applications a potential reference genome of a male wisent has already been published (WANG et al. 2017).

Considering the observed impact of family networks in the breeding line discrimination analysis, the breeding lines must be seen as just slightly different lineages on the level of interbreeding communities defined by artificial separation with one-directional gene flow and random gene drift as part of the founder effect. Neither the private alleles nor the other discriminative markers have assignable genetic origins from *B. b. bonasus* or *B. b. caucasicus*. Those genetic patterns could also be random due to e.g. past gene drift and subsequently cannot be assigned without a comprehensive genetic comparison with ancient or historical specimens itself assignable to certain subspecies. In summary, the marker subset for breeding line discrimination presented here, is not suitable for a validation of both the breeding lines as ESUs themselves. This panel is only able to assign the majority of individuals to the currently predefined anthropogenic breeding lines. Though, this SNP panel provides a viable tool for wisent conservation to evaluate breeding line assignments in demand, but it is inherently no argument for a separation of the two breeding lines within the European bison. The following review deals with the breeding line discrimination within the wisent practiced in current conservation from a scientific point of view.

4.9.1 Review on breeding line discrimination in European bison conservation

Due to managed individual exchanges in captive breeding and reintroductions of free-roaming herds, both the breeding lines form more or less separated reproductive entities despite their geographical locations. Since its establishment in the 1930s, this separation of the LL and LC line is still an important part of conservation management in the European bison (PUCEK et al. 2004). From the viewpoint of population biology, this breeding line separation resembles the concept of assortative mating. Assortative mating describes the mating preference by sexual selection caused by similar phenotypes and can lead to decreasing genetic diversity. In the case of the European bison, the breeding lines would represent the phenotypes and the sexual selection is replaced by anthropogenic selection. If just considering the total genetic diversity of the European bison, both breeding lines should interbreed to maintain as much genetic variability as possible in the long-term. This study's genotypic data confirms this and is in line with the current knowledge of genetic diversity in the European bison (see 4.8) and support a viewpoint in conservation of admixing ESUs even up to the subspecies level (cf. discussions on tigers (WILTING et al. 2015) or Sumatran rhinoceroses (AMATO et al. 1995)) to rescue a species with the highest genetic diversity possible. Accordingly, it would be beneficial for the total genetic diversity of *B. bonasus* to manage both breeding lines as one reproductive population.

Thus, is this current conservation policy of separating these breeding lines in the European bison scientifically tenable? The population separation in the wisent has two major motivations: on the one hand, to conserve the last remaining genetic material of *B. b. caucasicus in vivo* without outbreeding Caucasian genotypes and on the other hand to conserve pure *B. b. bonasus* in the LL line. With a

recent increase in genetic knowledge, similar discussions about handling natural purity and *ex situ*-hybrids are not restricted to the European bison and arise in other managed species as well. In the *ex situ*-breeding program of Asian elephants, for instance, further hybridisation between different subspecies while having a small reproductive population is discussed (SCHMIDT and KAPPELHOF 2019). The significance of genetic purity of intraspecific genetic lineages such as subspecies or subpopulations in conservation is a longstanding controversial topic (ROJAS 1992). Despite subspecific taxonomical dissension and subspecies hybridisation in e.g. the sister species of the wisent, *B. bison*, huge efforts were undertaken to conserve pure populations on the subspecies level (GATES et al. 2010). In some very rare taxa, however, species survival in general is prioritised above subspecific purity in current *ex-situ* conservation due to very low self-sustaining population sizes as mentioned above. Therefore, individuals of different subspecies are brought together for breeding, which was already practiced with the sole Caucasian bison (EBPB#100) in the last century. An important argument of supporters of the conservation of subspecies/population purity is the concept of 'evolutionarily significant units' (ESUs) or 'distinct population segments' (CONNER and HARTL 2004). These naturally evolved units are potentially adapted to their habitat and therefore possess traits that may not be present in other subspecies or populations. These adaptations would affect the ecological fitness and therefore have a significance for the long-term survival and hence conservation success. Admixture of putative ESUs not recognised in managed breeding could lead to problems in conservation actions later on: in the case of the Socorro dove (*Zenaida graysoni* (LAWRENCE 1871)), which is extinct in the wild, the later recognition of the status as a distinct species of the mourning dove (*Zenaida macroura* (LINNAEUS 1758)) lead to interspecific captive cross-breeding (SOORAE 2010). Consequently, a still existing not purely bred captive population in North America is excluded from further *ex-situ* conservation breeding and reintroduction plans in the future, while probably containing unique genetic variability of *Zenaida graysoni*. The *IUCN Species Survival Commission Guidelines on the Use of Ex situ Management for Species Conservation* applies to 'species, subspecies or different groupings of these' (IUCN SSC Conservation Breeding Specialist Group 2014), which would include the subspecies and breeding lines of the wisent as often referred management units (e.g. WITZENBERGER and HOCHKIRCH 2011). Accordingly, as in the American bison, the Status Survey and Conservation Guidelines of the European bison recommend to keep ESUs as separate non-interbreeding units even if their inner and outer taxonomical validity is controversial discussed (PUCEK et al. 2004; BOYD et al. 2010).

For obvious reasons, the systematic evaluation of an extinct taxon like *B. b. caucasicus* is difficult. Relatively recently, the Caucasian bison was lifted into species rank (*Bos caucasicus*) only based on consistently distinguishable morphological characteristics (GROVES and GRUBB 2011). In fact, GROVES and GRUBB (2011) state that all cited authors distinguished the Caucasian bison as a subspecies. There

is in fact a whole scientific debate about 'taxonomic inflation' versus 'taxonomic conservatism' in the Bovidae (HELLER et al. 2013; COTTERILL et al. 2014). Contrarily, other authors classify the European bison as a monotypic species and do not recognise valid species or even subspecies of certain populations in European bison at all (GROVES et al. 2011). In this study, the Caucasian bison is conservatively assigned as a subspecies of *B. bonasus* because of the contradicting literature and the fact that the species rank was given after its extinction. Recently, genomic data let suggest that *B. b. caucasicus* is not only a monophyletic sister taxon to *B. b. bonasus* but also had population substructures with different contributions in modern wisent. However, the authors themselves mention the limited informative power of their genomic approach towards the inferred phylogeny. Those putative Caucasian admixtures were found in founders and derived individuals of the LL line on the genomic level. Based on their results, varying admixture among the founders were proposed while, despite of the ancestry of 'Kaukasus' (EBPB#100), recent admixture between the LL and LC line is unlikely. This could be explained by admixture between European lowland and Caucasian bison prior to the extinction in the wild and the establishment of the recent managed population in the 1930s. The authors consider it as reasonable that admixture between *B. b. bonasus* and *B. b. caucasicus* was part of the natural population dynamics but found the first genetic evidence for the monophyly of the Caucasian bison (WĘCEK et al. 2016). Even if the LC line does not represent a natural subspecies founded by eleven individuals of *B. b. bonasus* and only a sole male of *B. b. caucasicus* (SLATIS 1960; TOKARSKA et al. 2011; KRASIŃSKA and KRASIŃSKI 2013), it is the only possibility to conserve unique genetic traits of the Caucasian bison *in vivo* and therefore fulfils the aim of conservation to protect biodiversity measured also in genetic diversity (MACE et al. 2005). Furthermore, the LC line exhibits morphological traits consistent with *B. b. caucasicus* (KOBRYŃCZUK 1985) although the genetic contribution is low. Possible maladaptations to its mountainous habitat in the HL line with minor genetic contribution from the plains bison generations ago is mentioned below (see 4.12.1). This latter case might be a close example for minor genetic contribution of a separate taxon but with phenotypic and subsequently ecological relevance.

Beside arguments to conserve pure ESUs, there might be also other reasons to manage the LL and LC line separately further in the future. Surprisingly, the LL line with only seven founders shows less signs of inbreeding depression in contrast to the LC line (with five additional founders) possibly because of successful purging of deleterious alleles. A slightly stronger relationship between mortality rate and inbreeding was shown in descendants of 'Kaukasus' (EBPB#100) compared to descendants of the founders of pure *B. b. bonasus* (SLATIS 1960). While this can certainly be due to a higher genetic load in this animal, another alternative explanation for this observation might be outbreeding depression (FRANKHAM et al. 2015). At least in recent history, however, the Caucasian bison was not affected by constantly low population sizes as in the European lowland bison but went

extinct in a short series of rapid declines, respectively. With estimations of 2 000 individuals in the 19th century the Caucasian bison was more prevalent than the nominate subspecies at that time (KRASIŃSKA and KRASIŃSKI 2013). Miscellaneous anthropogenic influences in the Caucasus mountains forced *B. b. caucasicus* back into the area between the Belaya and Luba rivers. Only 442 Caucasian bison were counted by the 1890s and subsequently the population was reduced to 50 animals in the beginning of the 20th century by an epizooty originated from domestic cattle. Even the establishment of the Caucasus Reserve in 1924 could not prevent the Caucasian bison from extinction around two years later. In contrast, the population of European lowland bison founders were always restricted and isolated to Białowieża for much longer (KRASIŃSKA and KRASIŃSKI 2013). Additionally, the Pszczyna/Pleß line, making up the majority of the *B. b. bonasus* contribution, experienced high inbreeding for years and thus a large portion of lethal alleles might have been eliminated (SLATIS 1960). On the other hand, posthitis, a necrotic inflammation of the prepuce, until now occurred mainly in individuals of the LL line. This could be an example of the loss of lethal alleles by high inbreeding but also less adaptive capacity towards environmental pressures like diseases. The perspective to utilise the current 96 SNP panel to genetically assess posthitis will be discussed below (see 4.12.2).

With the recent population size of 7 180 individuals in 2018 (LL: n = 2 011; LC: n = 3 339; RACZYŃSKI 2018) in conjunction with the knowledge of stabilised population admixture (DRUET et al. 2020), the capacities for a self-sustaining separate breeding approach in the European bison are in place. Therefore, it is reasonable to proceed with the current breeding management policy of separate breeding lines and their reintroductions according to the distribution of the former subspecies. The newly developed SNP panel can provide a genetic tool needed by conservationists to execute this conservation maxim.

4.10 Cross-species detection

The appearance of Bovini pats are well distinguishable from other faeces due to their size, shape and consistency. Even with their phenological differences in appearance it is easy to recognise a wisent pat in the field (JĘDRZEJEWSKI et al. 2010). The European bison is the only recent wild cattle in its current distribution (GROVES et al. 2011). However, within all native regions of the European bison, taurine cattle occur as livestock (FELIUS 1995) and could potentially cause confusion during sampling in the field. The traditional westernmost occurrence of pure-bred zebu (FELIUS 1995) potentially overlaps as well with the wisent distribution since the most-recent reintroductions in the Shahdag National Park in the Southern Caucasian mountains of Azerbaijan (WWF 2019). This zebu breed, the Caucasian dwarf zebu, has also been genotyped in this study. The Mediterranean water buffalo, included here as well, resembles the river-type breed kept in Europe. Regarding the current wisent

distribution, especially in Romania (BORGHESE and MAZZI 2005), this water buffalo breed holds potential for confusion during sampling. Together with all the Bovini forms genotyped here, all of those domestic breeds that are potentially important for wisent monitoring can be distinguished from the European bison with the SNP panel presented here (Figure 12). The tendency of the species and ESUs to cluster according to their taxonomy can be seen as an independent confirmation of the cross-species detection resolution provided here. The American bison cluster appears to be most distinctive along axis 1 (Figure 12) because of selectively chosen SNP loci for wisent-American bison discrimination of the panel. The proximity of the cattle cluster to the European bison cluster can be attributed to the fact that all autosomal SNPs in this study were originally detected in *B. primigenius*. This also causes the strikingly high SNP polymorphism in this species (Figure 11). Hence, five SNP markers monomorphic in the European bison but polymorphic in cattle were included into the total SNP panel. A representative reference set of genotypes from species of interest is needed for cross-species detection with the PCoA (Figure 12), which is provided here but must be extended for accurate discrimination. Axis 1 explains only 15.76 % of the variation (Figure 12) but represents a SNP panel only selected for LD to avoid cluster artefacts and not for the highest discrimination power. 84 loci from a SNP panel not designed to discriminate between species, were chosen to be utilised for cross-species detection due to simplicity, while providing enough resolution for this task. Exemplary, seven non-Bovini species sympatric with the wisent were also tested and could be detached with the SNP panel presented here. Regarding methodological reasons, the human genotype from the author was tested and could be excluded as well, while executing all lab work and part of the sample collection. Nevertheless, the samples of the grey wolf, horse, and within the Bovini, the mountain anoa most likely represent failed genotypes. Higher call rates and SNP polymorphism can be expected from those taxa. The high call rate found in the brown bear sample is caused most likely by genetic contamination. Since the latter genotype could clearly be excluded from European bison samples in the PCoA analysis, this case shows the robustness of the SNP panel for cross-species detection even in exceptional samples. The representiveness of the exact call rates but primarily of the SNP polymorphism rates in all non-target species are limited due to their very small sample sizes. However, they show clear tendencies of reflecting the known phylogeny as well. Since the SNPs were originally detected in *B. primigenius*, but still polymorphic in *B. bonasus*, they might be more conserved and subsequently more likely to be found in all descendants originated from the last common ancestor. This is supported by the generally higher call and polymorphism rates in the genus *Bos* while the more distant clade of Bubalina showed lower rates (Figure 11). This makes it likely to use this SNP panel developed for the European bison for species at least within *Bos* for applications like molecular individualisation from non-invasive samples (see 4.12.3). However, to confirm this, further tests are needed.

4.11 Implementation in population management

PERTOLDI et al. (2009) mention that genetic assessments, in addition to the traditional practice based on the EBPB, could enhance conservation breeding strategies in the European bison. The SNP panel presented here was specifically developed for current questions and needs in *ex* and *in situ*-conservation for the European bison after communication with stakeholders.

4.11.1 *Ex situ*-management

The majority of the samples in this study originate from captive animals in zoos, wildlife parks and holders within the European Union (EU). Like museums with their collections, zoos are an important source for conservation biology and genetic research (KITCHENER 1997; BUERKI and BAKER 2016). Furthermore, participation of zoos within the EU in scientific research is a measurement of the Council Directive 1999/22/EC of 29 March 1999 relating to the keeping of wild animals in zoos, which defines the role of zoos in society and conservation. Zoos could provide vital resources not only with the foundations for reintroduced populations but also for genetic material for research and beyond that for the development of such molecular tools with the potential of supporting wildlife conservation shown here.

Even more than 50 years since the first reintroductions, the captive wisent population is still the source for current rewildings. Therefore, an assessment of the *ex situ*-population must go hand in hand with re-establishing Europe's last species of wild bovines. If sufficient pedigree data is available for a species, breeding strategies based on mean kinship (*mk*) are tested to be efficient (RUDNICK and LACY 2008; GIGLIO et al. 2018). Until today, this pedigree data is utilised for breeding, culling and reintroduction recommendations (OLECH and PERZANOWSKI 2002; PUCEK et al. 2004). However, a weakness of pedigree-based estimations on genetic diversity is the default assumption that the founders were unrelated (RUDNICK and LACY 2008). *PMx* provides a function to assign kinship values to the founders based on molecular data (BALLOU et al. 2018). Since the last founder died in 1939 (KRASIŃSKA and KRASIŃSKI 2013), such inclusion of values based on founder genotypes might not be possible but is still interesting for managing reintroduced populations. Solely regarding the raised doubts towards the complete correctness of the pedigree reaching back to individuals born in late 19th century, an independent assessment is needed. Unintended documentation errors in the EBPB are possible due to certain husbandry conditions or natural behaviours in wisent: if several mature bulls (in an enclosure) could have had prior contact to impregnated females, several paternities may be assumed. Even in captivity, with spacious enclosures, like those of the Dutch *Natuurpark* in Lelystad (45 ha), a certain maternal assignment is often not possible by pure observation alone (DE VRIES and DE BEER, *pers. com.*). Generally, allomaternal behaviour and calf adoption are known in American bison (RIEDMAN 1982; HERMAN et al. 2014). Such alloparental care, in form of kindergartens

mostly guarded by younger females within the herd, is regularly observed even in captivity (HENNIG, *pers. com.*). Occasional non-maternal suckling is also already known in European bison (CABOŃ-RACZYŃSKA et al. 1983; RIEDL, *pers. com.*) and could equally lead to false maternity assignments. Thus, it makes sense to extend the current practice of relying exclusively on pedigree-based data by supplementing SNP derived molecular evidence. For both recent bison species, SNP-based marker-assisted breeding strategies have been recommended to prevent genetic drift (PERTOLDI et al. 2010b). This might be especially true for populations with high inbreeding, where it is presumably more important to practice population management based on genetic diversity instead of management purely based on heredity. One recommended genetic analysis of the captive population (OLECH 1999) has been made accordingly (OLECH 2006) but has not been published yet and did not result in a comprehensive global management to the knowledge of the author. The inclusion of commonly used programs like *PMx* in this study could make an implementation of the new molecular data easier. The existing EEP is currently factually inactive due to past difficulties (see 6.2) but, with some adaptions, could provide a good framework for a centralised management, particularly, since nearly the entire captive population is located in Europe. Accordingly, there should always be a combination of methods for optimal management of a population. Such additional aspects for individual selection regarding e.g. reintroduction are equally important for a success: for example, a functional social structure is a major criterion for successful implementation of free herds as well (KASPARI 2018).

4.11.2 *In situ*-management

The free-roaming individuals are not listed individually in the EBPB and therefore lack genealogical documentation (RACZYŃSKI 2018). The SNP panel provided here allow the assessment of the relationships of genotyped individuals in wild populations for the first time without the need to catch or harm the animals. Continuous genetic monitoring enabled by such marker panels is recommended and can improve *in situ*-conservation efforts (WILSON et al. 2006). Trained rangers, for instance, could concomitantly collect non-invasive samples on their patrols for SNP genotyping. Nevertheless, supportive pedigree data of the introduced founders from the EBPB could be helpful to assess the studied population structure, especially if founders are still alive like in the recent reintroduction in the Rothaar mountains in Germany (TILLMANN et al. 2013; SCHMITZ et al. 2015) or the Tarcu region in Romania (VAN DE VLASAKKER 2014; see 4.7). To enhance the genetic monitoring of wild populations in the long-term, it is strongly recommended to initially genotype all reintroduced individuals. With genotyping the founders of wild populations, the basis for future investigations regarding populations structures and dynamics is set. This study was able to successfully genotype five founder individuals reintroduced in regions of Romania (1.1.0) and Azerbaijan (0.3.0). A genetic monitoring of reintroduced populations also allows to assess the development of home range and social structure (HAGEMANN et al. 2019).

One potentially meaningful application of the panel could be the estimation of effective population sizes (N_e), from which the minimum viable population size (MVP) can be derived. However, populations which are much smaller than the generalised recommended size of $N_e > 500$ are not automatically doomed to go extinct (FRANKHAM et al. 2015). The European bison with its 12 founders might be a textbook example for that. Regarding this, it is supposed that a low genetic load of lethal alleles by genetic depletion before the last bottleneck event took place and could explain the mild effects of inbreeding depression observed in the wisent until today (SLATIS 1960; TOKARSKA et al. 2011). Nevertheless, due to reduced genetic variability, it is highly likely that the adaptive potential of this species is reduced, making it more vulnerable towards environmental changes (FRANKHAM et al. 2015). In several free-roaming herds supplementary feeding during winter is practiced to reduce environmental pressure and consequently mortality rates (KRASIŃSKA and KRASIŃSKI 2013). In current reintroductions of the LC line in the Carpathians major efforts have been undertaken to establish viable populations by providing a diverse founder representation based on pedigree data (PERZANOWSKI and OLECH 2007). Complementing this approach with a continuous genetic monitoring would allow to rack genetic diversity over time and help disentangle the effects of e.g. gene drift, population isolation or adaptive processes after reintroductions for adequate conservation actions, now possible (see 2.8.4).

The new SNP panel could further provide solutions and services for human-wildlife conflicts and therefore could also be of public interest. There are cases where classical human-wildlife conflicts are still an issue: local extinctions after reintroduction due to low protection efforts and poaching were reported from Russia and Azerbaijan (PUCEK et al. 2004; VAN DE VLASAKKER 2014). Other reasons why the return of this once extinct in the wild megaherbivore can also arise public issues: rewilding of big animals like European bison especially in densely populated regions has considerable conflict potential. First, intended and unintended, potentially dangerous encounters between wisent and humans are simply more likely in those regions. In 2017, after hundreds of years, the first wisent self-immigrated from Poland into the German federal state of Brandenburg (genotyped in this study). Unfortunately, it was shot after a few hours, motivated by the feeling of insecurity in the presence of a free-roaming wisent close to inhabited areas. Thus, a certain lack of knowledge towards this long-gone native species was the major issue here. Furthermore, financial loss due to damages in forestry or agriculture could be the issue of dispute for local stakeholders. In Germany, bark stripping damage in forestry by the local free-roaming individuals of the Rothaar mountains are object of judicial disputes (SCHRÖDER et al. 2019). Beside, scientifically evaluated regions regarding habitat suitability for wisent (KUEMMERLE et al. 2011; 2017) the judgments of those cases will be path-breaking for the further reintroduction in the densely populated Germany. Species identification based on mtDNA obtained from browsed twigs was previously shown in deer species even after weeks (NICHOLS et al.

2012) and could probably be methodologically transferred to browsing and bark stripping marks of the European bison. This study provides protocols for using such non-invasive samples from the environment and could be tested to be applicable in such forensic issues.

In general, genetic population monitoring not only allows scientific research but also the assessment of information important for decision makers and could therefore help raise public awareness. The lack of knowledge and experience towards such species or populations and the associated ambiguous fears in humans might be a major obstacle in the acceptance of returning wildlife. In such cases, science as part of the society has the obligation to not only study the contentious population but also to communicate achieved facts. The case of the Rothaar mountain European bison demonstrates how vital the public perception for a successful reintroduction in highly populated countries like Germany is (DECKER et al. 2010). Thus, the need of transparency towards such species of public interest is high but contradicts with limited monitoring possibilities in the field and wildlife protection aspects. Wildlife genetics analysis services may help to provide the foundation for public awareness due to generating data from individuals up to populations (SUTHERLAND et al. 2004; YOUNG et al. 2005; BROOKS et al. 2006; WHITE and WARD 2010; REDPATH et al. 2013). Due to the non-invasive approach of the SNP panel, a gentle and affordable method for wildlife monitoring is now provided.

4.12 Outlook on further applications and perspectives

The current SNP panel could be directly used to resolve issues with relevance for science and conservation: little is known about the influence of mating behaviour of dominant males on the genetic structure and effective population size of the species. Furthermore, due to its robustness towards low quality samples, the analysis of collection specimens (ROWE et al. 2011) and historical hunting trophies (HOFFMANN and GRIEBELER 2013; HOFFMANN et al. 2016) could provide interesting insights into the development of genetic diversity over time. The *Mammal Research Institute Polish Academy of Sciences* in Białowieża holds a comprehensive collection of wisent skulls going back for decades and has already shown interest in a respective collaboration (KOWALCZYK, *pers. comm.*).

4.12.1 Monitoring of breeding line admixture and hybridisation

The current SNP panel could be interesting to observe migration and admixture of different kinds of free-roaming populations: a recent recognition of a certain genetic contribution of *B. b. caucasicus* in the Belarussian population of Białowieża (Belaweschskaja Puschtscha) but not in the Polish population (TOKARSKA et al. 2015) could imply further conservation actions. Currently, both populations are separated by a fence on the Polish-Belarussian border originally built for political reasons. In fact, this would reduce the global number of pure LL bison dramatically. In that case, the Polish herds of the LL line would again represent the last remaining wild population of presumably pure *B. b. bonasus* globally. The potential of interbreeding with Belarussian individuals exhibiting

genetic material of the Caucasian bison and therefore spreading into pure bred LL populations increases with time and is dependent on the maintenance of the border fence system. This case also entails a shift in the expected distribution of genetic diversity of the lowland subspecies aka LL line. A feasible genetic monitoring tool for breeding line discrimination of free-roaming herds might soon become important.

Another case could be the HL line in the Caucasian mountains: those animals carry genetic material of plains bison and are not part of any conservation efforts, but are rather considered to be a threat by introgression of American bison material into the European bison population. These interspecific hybrids show signs of maladaptation towards their environment. This includes constantly migration out of the reserve originally established to preserve Caucasian bison, proposed negative ecological destruction of the reserve itself and severe mortality rates during winter seasons not observed in other sympatric ungulates, not even in nearby herds of the LC line (ZABLOTSKAYA et al. 2004).

Beside the HL line, other cases of interspecific hybridisations are known in *B. bonasus*. Several documented anthropogenic forced interspecific crossbreds underline the close relatedness of the species in the *Bos*-complex: European and American bison are completely cross-fertile (KLÖS and WÜNSCHMANN 1993). At least European and American bison, yak (MEDUGORAC et al. 2017), gaur (with the gayal) and the banteng (with bali cattle (*Bos javanicus domesticus* GANS 1915); NIJMAN et al. 2003; HARTATI et al. 2015) are cross-fertile with domestic cattle (KLÖS and WÜNSCHMANN 1993). Wisent × cattle hybrids, called *żubrońs*, are well known from captivity but until now have not been observed in the still limited range of wild European bison (KRASIŃSKA and KRASIŃSKI 2013). Additionally, fertile hybridisations or at least interspecific gene flow in Bovini are reported (KLÖS and WÜNSCHMANN 1993; HASSANIN and ROPIQUET 2007b). Hybridisation as the evolutionary origin of the modern *Bos* spp. including the wisent (see 6.1) are scientifically discussed (HASSANIN and ROPIQUET 2004; GALBREATH et al. 2006; HASSANIN et al. 2006; HASSANIN and ROPIQUET 2007a; MEI et al. 2016). Following Haldane's rule, only females are fertile in the F_1 hybrid generation (HALDANE 1922) and make permanent interspecific introgression possible. Primarily cattle but more scarcely also American bison, domestic yak and domestic water buffalo are kept in Europe for economic reasons (SAMBRAUS 2006). Hybridisation in wild European ungulates is common and due to the uncertainty on how those unintended anthropogenic introgressions of non-native taxa or domestic breeds affects native populations is unwelcome in modern conservation (PUCEK et al. 2004; IACOLINA et al. 2018). Though, viable hybrid development to the blastocyst stage were shown *in vitro*, but no reports of cattle-water buffalo hybrids are known despite co-rearing in many countries (KOCHHAR et al. 2002). Thus, an introgression of water buffalo is therefore also unlikely for the wisent. Nevertheless, species hybridisation within *Bos* is a potential issue. Such bovine hybrids should be tested on the current SNP panel to evaluate the possibility to recognise hybridisation in European bison while establishing this

species with high efforts in a highly anthropogenic influenced environment found in Europe and the presence of free-roaming HL line herds.

4.12.2 Functional genetic diversity

Assessing functional genetic diversity, e.g. to evaluate the immunocompetence, is an additional reasonable way to use genetics for conservation decisions. Genetic variation in functional loci may differ considerably from that observed in neutral loci, mostly explained by selective pressure (HARTMANN et al. 2014; KNAFLER et al. 2016). However, it has been warned to only rely on functional genetic diversity to understand the genetic population structure especially in populations that experienced bottleneck events and consequently to execute population management actions based on erroneous conclusions (VRIJENHOEK and LEBERG 1991; HARTMANN et al. 2014). Beside necessary for applications like kinship analyses neutral loci, as utilised here, are able to directly measure neutral genetic diversity in populations of the European bison (see 4.8). To gain a necessary and more complete picture of wisent genetics it may, however, be of interest to extend the genetic assessment to functional genetics (PERTOLDI et al. 2009), partly because of past epizooties from livestock already causing extinction in European bison populations and specific conservation-relevant diseases like posthitis or balanoposthitis, respectively (KRASIŃSKA and KRASIŃSKI 2013). Having such SNP panels with neutral and functional loci would also allow to observe population structure development under selection compared between captive and wild populations utilising for example Tajima's D. Some studies have already looked into the variation of the major histocompatibility complex (MHC) of *B. b. bonasus* (UDINA et al. 1994; UDINA and SHAIKHAEV 1998; RADWAN et al. 2007; 2010; ŁOPIEŃSKA et al. 2011; BABIK et al. 2012; MIKHAILOVA and VOITSUKHOVSKAYA 2017). Recently, the focus on posthitis-associated SNPs (OLEŃSKI et al. 2015; 2020) paves the way for an utilisation in conservation management. In prospect, posthitis-associated markers were included into the current SNP panel. Due to the lack of presence-absence information of posthitis in the genotyped individuals of this study, there was no further investigation possible. However, the genotypic results give interesting insights since individuals from both breeding lines were genotyped: the clinical picture of posthitis or balanoposthitis occurs mainly in the LL line in Białowieża (KRASIŃSKA and KRASIŃSKI 2013). Interestingly, none of the posthitis-associated loci were found to have significant discriminative value between the LC and LL line. These results may provide a hint that posthitis is in fact not associated exclusively to a breeding line based on those loci. An alternative explanation would be that those markers in fact are not linked to posthitis susceptibly.

4.12.3 Applications in other Bovini

The IUCN red list contains 12 Bovini species (*Syncerus* spp. included in this study are recognised as conspecific) of which 10 species are listed as threatened (VU: $n = 3$; EN: $n = 4$; CR: $n = 3$) (OLECH 2008; BOYLES et al. 2016; BURTON et al. 2016a; 2016b; BUZZARD and BERGER 2016; DUCKWORTH et al. 2016;

GARDNER et al. 2016; TIMMINS et al. 2016; AUNE et al. 2018; IUCN SSC Antelope Specialist Group 2019; KAUL et al. 2019; TIMMINS et al. 2020). The EAZA TAG (cattle and camelids) is willing to focus more towards conservation of threatened Bovini species with, for example seven partly new EEPs (EAZA 2019b). A genetic assessment of those wild cattle, similar to the European bison is therefore of considerable interest. The SNP marker panel presented here was strictly developed for *B. bonasus*. However, all autosomal SNPs were originally discovered in *B. primigenius* (the gonosomal sex marker also in other *Bos* spp.) but are still polymorphic in the European bison. This fact arises the probability of evolutionary conserved SNPs at least since the last common ancestor and for the potential utilisation for genotyping in several close-relative species. Therefore, it is worth to test this SNP panel towards relevant applications in other Bovini for at least sex determination and individualisation. The applicability for at least all six recent species within the genus *Bos* is highly likely but would need a validation with more individuals tested. A substantially higher number of individuals of those species was sampled during collection and would be ready for genotyping. Demonstrably, this SNP panel can be utilised for sex determination in any Bovini species as well as for individualisation in domestic cattle (with all four major lineages sampled in this study) and American bison (both subspecies) from non-invasive samples. Therefore, this SNP panel for non-invasive genotyping developed for the European bison has instant potential for basic populations genetics or conservation questions in other threatened bovines.

4.13 Conclusions

The newly developed reduced 96 SNP panel provides a valuable genetic tool to answer a variety of questions relevant for wisent *ex* and *in situ* conservation, such as (i) sex determination, (ii) individualisation, (iii) parental assignment, (iv) assessment of genetic diversity, (v) breeding line discrimination and (vi) cross-species detection. Markers are selected to provide reliable results for non-invasive samples and thus dramatically expand the range of usable DNA sources and applications. Accordingly, protocols for the best practice for faecal sampling, as a known pillar of non-invasive genetic monitoring, and other non-invasive samples types are provided. Through constant comparison with the existing long-term pedigree data from the EBPB, as the basis of conservation management in the European bison today, the implementation with current practice is also already provided here. Moreover, the assessment of genetic diversity with SNPs overcame the inevitable weaknesses of pedigree-based estimations important for population management. The high potential of this modular marker panel for not yet evaluated conservation applications is discussed. Unprecedentedly, the genetic structure of free-roaming populations of European bison can be comprehensively assessed non-invasively, allowing henceforth for a reliable monitoring of reintroductions of Europe's largest herbivore, once extinct in the wild.

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6 Appendix

6.1 Evolutionary history, systematics and taxonomy of the European bison

A hypothesis of the evolutionary origin of *B. bonasus* proposes an interspecific hybridisation based on the same mitochondrial lineage in the aurochs (*Bos primigenius* BOJANUS 1827) (VERKAAR et al. 2004; SOUBRIER et al. 2016) extinct in 1627 (BRADLEY et al. 1996; KRASIŃSKA and KRASIŃSKI 2013), which is also the ancestor of the domesticated cattle namely taurine cattle and zebu or indicine cattle. In contrast, the wisent is clustering with American bison based on nuclear data (PITRA et al. 1997; BUNTJER et al. 2002) and on the genomic level (WANG et al. 2017). It is assumed that female aurochs reproduced with males of the as well extinct steppe bison (*Bos priscus* (BOJANUS 1827)) (VERKAAR et al. 2004; SOUBRIER et al. 2016). Recently, this hypothesis that *B. bonasus* originated from a species hybridisation between *B. priscus* and *B. primigenius* was rejected by others. A more complex situation was concluded, were the separate mitochondrial lineages originated during a long period of incomplete speciation, also called incomplete lineage sorting (GRANGE et al. 2018; WANG et al. 2018), which was driven by sex depended reproduction behaviour (GRANGE et al. 2018) with later minor interspecific gene flows between the bisontine and taurine lineages after divergence between the taurine and indicine cattle. But it could not determined whether the gene flow occurred prior or posterior to the taurine domestication. All taurine introgressions were shown to occurred prior the establishment of the managed population and supposed to be occurred under natural conditions (GAUTIER et al. 2016; WĘCEK et al. 2016).

Traditionally, the genus *Bison*, including *Bos* (*Bison*) *bison* and *Bos* (*Bison*) *bonasus* (GRUBB 2005), was assigned separately but appears to be nested in *Bos,* including, additional to the mentioned recent forms, the yak (*Bos mutus*, (PRZEWALSKI 1883)), gaur (*Bos gaurus*, SMITH 1827), banteng (*Bos javanicus*, D'ALTON 1823) and each their domestic derivates as well as the recently extinct kouprey (*Bos sauveli*, URBAIN 1937) (JANECEK et al. 1996; SCHREIBER et al. 1999; HASSANIN and ROPIQUET 2004; VERKAAR et al. 2004; GUO et al. 2006; MA et al. 2010; HASSANIN et al. 2012; BIBI 2013; MASSILANI et al. 2016; SOUBRIER et al. 2016; PALACIO et al. 2017; GRANGE et al. 2018; WU et al. 2018; ZURANO et al. 2019). Whereby, *Bos* would be paraphyletic, *Bison* was synonymized (GROVES 1981; GROVES and GRUBB 2011) after its type species indeed being originally described as *Bos bison* by LINNAEUS (GRUBB 2005). The synonymisation of *Bos* and *Bison* is supported by others with the suggestion of a new genus name *Boson* (GRANGE et al. 2018), but which in turn would violate the International Code of Zoological Nomenclature (RIDE 1999). Accordingly, *Bos* would be the sole genus within the subtribe Bovina (GRAY 1821). In which between the majority of its species several past interspecific gene flows could be shown (HASSANIN and ROPIQUET 2007a; GAUTIER et al. 2016; WĘCEK et al. 2016; WU et al. 2018). These results reflect the complex and close relationship and therefore supports the synonymisation of *Bos* and *Bison* as well. However, there are concerns that the synonymising with *Bos* could disrupt an established history of

public policy and scientific community identification with the genus *Bison* and therefore complicate especially conservation of *B. bison* and *B. bonasus* with each their associated subspecies (GATES et al. 2010). Though, the synonymising of *Bison* with *Bos* does not affect the validity or inner taxonomy of both species. Thus, to account for the recent knowledge in phylogeny, this study assigns all bison taxa to the genus *Bos* in opposite to the traditional and often used nomenclature of the genus *Bison*. Furthermore, this study recognises all mentioned domesticated forms as conspecific with their wild relatives (BRADLEY et al. 1996; WANG et al. 2010) and nevertheless nomenclaturally follows Opinion 2027 (International Commission on Zoological Nomenclature 2003; GENTRY et al. 2004).

6.2 Conservation history of the European bison

To preserve Europe's largest recent land animal the Society for the Protection of the European Bison was founded at the Berlin Zoological Garden in 1923 under the leadership of Dr. KURT PRIEMEL, then director of the Frankfurt zoo. This resulted in the first establishment of a published pedigree book (EBPB) of a wild threatened species and managed breeding (KRASIŃSKA and KRASIŃSKI 2013). Under the editorship of GERD VON DER GROEBEN, ERNA MOHR and JAN ŻABIŃSKI it was possible to recognise 54 (29.25.0) individuals with known pedigree which could traced back to seven founders in the lowland line and 12 for the lowland-Caucasian line (KRASIŃSKA and KRASIŃSKI 2013). Big efforts were undertaken by ERNA MOHR and JAN ŻABIŃSKI to establish the verified pedigree of the post-war population (KRASIŃSKA and KRASIŃSKI 2013) which in this regard experienced it's last genetic bottleneck (BELOUSOVA and KUDRIAVTSEV 1997).

The EBPB represents the oldest pedigree documentation of a threatened wild species, provides genealogical data for assessing genetic values. The EBPB contains the global captive population of European bison independent of any kind of membership of the holder. Individuals derived from hybridisations with other species like cattle or American bison are not registered (RACZYŃSKI 2018). Today, genealogical records documented in studbooks are commonly used to calculate genetic diversities, kinships and inbreeding coefficients in many breeding programs of managed population of mainly threatened (sub)species (BALLOU and LACY 1995). The most comprehensive of such studbooks are coordinated within the World Association of Zoos and Aquariums (WAZA) namely International Studbooks (ISBs), for Europe the European Endangered Species Programmes (EEPs) as well as European Studbooks (ESBs) within the European Association of Zoos and Aquaria (EAZA) or Species Survival Plans (SSPs) within the Association of Zoos and Aquariums (AZA) in North America and similar breeding programs around the world (WAZA 2019).

In 1996 the EAZA established an EEP for the European bison (PUCEK et al. 2004). In the following years several issues were reported from the EEP coordination: Then, only one-third of the global population was represented in the EEP (OLECH 2007) and the inclusion of non-EEP holders was

recommended from the beginning (OLECH 1999; OLECH 2000). This is due to the high husbandry and management standards within the EAZA (EAZA 2015; EAZA 2019a). A further obstacle was the suboptimal communication with the holders which hindered the management implementation (OLECH 1999; OLECH 2000; OLECH 2003; OLECH 2006; OLECH 2007). This included the neglection of some participants (OLECH 2006; OLECH 2007) to follow the view hold by the EEP coordination of the importance to separate the two breeding lines (OLECH 2007). Lastly, the EEP programme was seen not to be a solution for this species. A new form of programme for the wisent was worked out (OLECH 2006), which resulted in the establishment of the European Bison Conservation Center (EBCC) in 2008 (EBCC 2019). The plan is to gather also non-EAZA breeders under the umbrella organisation of the European Bison Friends Society (OLECH 2007). A important inclusive difference to the EEP is that the participation is free of charge (EBCC 2018), which, especially for smaller holders might be a crucial reason advantage. Currently, in Belarus, Czechia, Hungary, Poland, Romania, Russia, Spain and Sweden national EBCCs were established to coordinate the breeding program more efficiently. The German EBCC is split into four regional EBCCs (EBCC-RZs) due to the by far highest national captive population size worldwide (RACZYŃSKI 2018). In other countries with wisent holdings an EBCC is still vacant (EBCC 2019). In parallel, the EAZA still holds the EEP for the wisent under the current coordination by DOUGLAS RICHARDSON (EAZA 2018). Currently, under 10 % of the global population but nearly 30 % of the captive population were coordinated through the EEP (RACZYŃSKI 2018; Species360 2019). The IUCN/SSC Bison Specialist Group and the IUCN/SSC Conservation Breeding Specialist Group recommended that the standardised EEP should expanded to the global captive population (PUCEK et al. 2004). A closer cooperation between the EBCC and EEP was suggested as well (HOMES 2018). Today, the EBPB provides the only documentation of an entire genealogy of a species after its restitution in the early 20th century. Unfortunately, some holders of European bison still do not correspond regularly or not at all with the EBPB editor (PUCEK et al. 2004) comparable to issues in the EEP. The *Status Survey and Conservation Action Plan of the European bison* recommends a management of the entire captive population of European bison through the EEP (PUCEK et al. 2004) or similar existing programs (PERZANOWSKI and OLECH 2007), which I would fully agree with. With regard to the development of the holder distribution the European bison today has become a species increasingly kept in wildlife parks with more native or Nordic species than in traditional zoological gardens (RACZYŃSKI 2018) unlike other EEP species. But these smaller holders mostly have less funds and resources available. The participation fee and strict regulations of the EAZA towards EEP participants are meaningful but major obstacles for many holders of European bison to participate in the program. Therefore, the majority of the population is not part of the EEP of this species (RACZYŃSKI 2018). Nevertheless, *PMx* and *ZIMS* are purposeful and established programs for population management to handle genetic data of a population important for recommendations.

These software uses the still available pedigree data but also allow to integrate molecular data assessed with tools like I developed in this study (TRAYLOR-HOLZER 2011). Though, *PMx* is a freeware accessible online but needs input files from *SPARKS* or *ZIMS* which are restricted programs to EAZA members or at least EEP participants. Utilising *mPed* for pedigree data conversion still needs additional data set adjustments. As long as the rules and regulations within the EEP are not going to be changed, the program can never acquire enough holders to do a severe and by experts recommended population management of the European bison with such an incomparable ex-situ conservation history. Finally, the EBCCs and the EEP based on the pedigree data of the EBPB are in place and play important roles in conservations breeding and reintroductions but still do not comprehensively cover the global population. Additionally, a *Bison Rewilding Plan* from *Rewilding Europe* (VAN DE VLASAKKER 2014) and a *Best practice manual for protection of European Bison* from *Best for Biodiversity* (OLECH and PERZANOWSKI 2015) were published, both financed by the European Commission through the LIFE programme.

6.3 Genetics in European bison

The appropriate selection of the molecular marker system depends on the study system (GROVER and SHARMA 2016). Microsatellites showed to be appropriate genetic markers to evaluate population structures and individual kinships (SELKOE and TOONEN 2006). Until now no marker system specifically developed for bison were published. But it has been shown that microsatellites originally developed for other species (predominantly cattle) are utilisable in American bison (MOMMENS et al. 1998; WILSON et al. 2002) and European bison as well (GRALAK et al. 2004; LUENSER et al. 2005; ROTH et al. 2006; TOKARSKA et al. 2009a; TOKARSKA et al. 2009b; TOKARSKA et al. 2015). But, SNPs were shown to be favourable over microsatellites regarding assessing individual identification and paternity (TOKARSKA et al. 2009a). SNPs utilised in wisent originate from the BovineSNP50 Genotyping BeadChip and BovineHD Genotyping BeadChip (Illumina) developed from domestic cattle (FLISIKOWSKI et al. 2007; PERTOLDI et al. 2009; TOKARSKA et al. 2009a; PERTOLDI et al. 2010a; PERTOLDI et al. 2010b; KAMIŃSKI et al. 2012; OLEŃSKI et al. 2015; TOKARSKA et al. 2015; WOJCIECHOWSKA et al. 2017; OLEŃSKI et al. 2018; DRUET et al. 2020; OLEŃSKI et al. 2020). As recited above, up to genomic level studies were done in the European bison primarily to investigate the complex evolution of the entire *Bos* clade and the species itself. Additionally, low genetic variability in European bison was proven molecularly with several marker systems: Blood serum proteins (GEBCZYŃSKI and TOMASZEWSKA-GUSZKIEWICZ 1987; SIPKO et al. 1996), allozyms (HARTL and PUCEK 1994), blood-group systems (SIPKO et al. 1995), mtDNA (TIEDEMANN et al. 1998; BURZYŃSKA et al. 1999; WÓJCIK et al. 2009), milk protein genes (SIPKO et al. 1994; UDINA et al. 1994; BURZYŃSKA and TOPCZEWSKI 1995), bovine prion protein (PRNP) genes (CZARNIK et al. 2009), minisatellites and telomeric markers (SEMENOVA et al. 2000), MHC genes (UDINA et al. 1994; UDINA and SHAIKHAEV 1998; RADWAN et al. 2007; ŁOPIEŃSKA et al. 2011; BABIK et al. 2012; MIKHAILOVA and

VOITSUKHOVSKAYA 2017), microsatellites (LUCY et al. 1993; GRALAK et al. 2004; LUENSER et al. 2005; ROTH et al. 2006; FLISIKOWSKI et al. 2007; NOWAK and OLECH 2008; TOKARSKA et al. 2009a; 2009b; TOKARSKA et al. 2015; MIKHAILOVA and VOITSUKHOVSKAYA 2017; DOTSEV et al. 2018) and SNPs (FLISIKOWSKI et al. 2007; PERTOLDI et al. 2009; TOKARSKA et al. 2009a; PERTOLDI et al. 2010a; PERTOLDI et al. 2010b; KAMIŃSKI et al. 2012; TOKARSKA et al. 2015; WOJCIECHOWSKA et al. 2017; OLEŃSKI et al. 2018; OLEŃSKI et al. 2020). With a ~ 10 x coverage genomic data of two individuals of the LL line a genetic variability comparable with taurine cattle breeds was estimated (GAUTIER et al. 2016). This found was interpreted by poorly related founders and effective population management (GAUTIER et al. 2016).

The bottleneck event in the early 20th century resulted in an extreme depletion of Y-chromosomes nowadays: three of four founder Y-chromosomes in the LL line, only leaving the one inherited from 'Plebejer' EBPB#45, and two of originally five founder Y-chromosomes in the LC line were outbred. The total extinction of two Y-chromosomes and partly the current unequal founder representation can be explained by the fact that three of the founders, namely the males 'Begründer' EBPB#15 and 'Bismarck' EBPB#147 as well as the female 'Plavia' EBPB#16, left only one female decedent ('Beste' EBPB#524) (KRASIŃSKA and KRASIŃSKI 2013). This knowledge about the Y-chromosome representation assessed via pedigree data is representative for the general genetic depletion mainly forced by former or current genetically overrepresentations of certain founders (PUCEK et al. 2004; TOKARSKA et al. 2011). Still, WÓJCIK et al. (2009) showed on mtDNA that all maternal founder haplotypes of the LL line survived at least in the free-roaming population of Białowieża. Although, especially in the LL line the maternal founder representation is also very unequal (TOKARSKA et al. 2011).

Until 2015 no genetic connection to *posthitis* were found in European bison (RADWAN et al. 2007; WÓJCIK et al. 2009). But after a genome-wide study few SNPs show a significant association with this disease were found (OLEŃSKI et al. 2015; OLEŃSKI et al. 2020).

6.4 Detailed protocols for DNA extraction

6.4.1 Faeces

All faecal samples were processed in a laboratory determined for non-invasively collected sample material. DNA extraction was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen). To evaluate the applicability of the DNA extraction kit for the non-invasive approach in European bison DNA extraction was additionally carried out using the QIAamp DNA Stool Mini Kit (Qiagen). Pieces (~100 – 200 mg) of the faecal full samples were isolated and handled with forceps, which were decontaminated after every usage per sample. The isolated pieces from the faecal full samples were air-dried on tissue cloths to reduce the EtOH content before adding DNA lysis buffer. Depending on the original consistence of the collected dung the full faecal sample might resolved in EtOH. In this case, it was transferred with cautious draining and subsequently air-drying directly in the reaction

tube. Whether, if it was a faecal full sample piece or a faecal swab sample (dried and 96 % EtOH) were transferred into a 2.0 ml reaction tube (Eppendorf).

Depending on the used DNA extraction kit either 1000 µl InhibitEX buffer (QIAamp Fast DNA Stool Mini Kit) or 1600 µl ASL buffer (QIAamp DNA Stool Mini Kit) were added and samples were suspended into buffer with a lab shaker (Vortex-Genie® (Scientific Inductries)) and shaked at 1000 rpm overnight in a Vibramax 110 (Heidolph) at RT. The addition of lysis buffer is obsolete for the faecal swab sampled stored directly in ASL or InhibitEX buffer. Subsequently, a centrifugation at 13.3 rpm (Micro Star 17 (VWR)) is carried out to separate the suspension from rough faecal matrix. To each sample in ASL buffer an InhibitEX tablet is added. The tablet is suspended by vortexing for 1 min and incubation for at least 1 min at RT. After 3 min of centrifugation at full speed the supernatant is transferred into a new reaction tube. After an additional centrifugation for separating the suspension of further tablet residuals, 600 µl of the supernatant is transferred into 2.0 ml reaction tube with 1 µl carrierRNA (Qiagen). The following steps for DNA isolation were performed automated using the QIAcube system (Qiagen). Therefore, a modified version of the human stool protocol (Qiagen) was used, which differs from the standard protocol mainly by including a two-step DNA elution function: beginning with a centrifuge step at full speed for 3 min followed by adding 25 µl proteinase K and 600 µl AL buffer and vortexing for 15 s. This is followed by an incubation for 10 min at 70 °C. Subsequently, followed by adding 600 µl 96 % EtOH and mixing by vortexing. 600 µl lysate are filtrated with a QIAamp spin column with a 1 min centrifugation at full speed. The latter step is repeated two additional times followed by adding 500 µl AW1 buffer with an 1 min centrifugation at full speed. Then, 500 µl AW2 buffer was added with centrifuge for 3 min at full speed. The eluation differs from the standard protocol: after incubation for 1 minute at RT for each sample DNA was eluted in two steps with 60 µl ATE buffer each, resulting in a 120 µl DNA extract per sample.

6.4.2 Saliva, nasal secretion and urine

Saliva, nasal secretion and urine swab samples were processed in a laboratory determined for non-invasively collected sample material. DNA extraction was performed using the the QIAamp DNA Investigator Kit (Qiagen). Maximally, two swab tips were detached into a spin basket, which is supplied in the Investigator® Lyse&Spin Basket Kit (Qiagen). Following, 400 µl ATL buffer and 25 µl Proteinase K were added and incubates for 3 h in a thermoshaker (BioShake iQ (QUANTIFOIL Instruments)) at 1000 rpm and 56 °C. The suspension is centrifuged into 2.0 ml reaction tube and 1 µl carrierRNA were added. The following steps for DNA isolation were performed automated using the QIAcube system (Qiagen). Therefore, a modified version of the human stool protocol (Qiagen) was used, which differs from the standard protocol mainly by including a two-step DNA elution function:

400 µl AL buffer were added with following mixing by vortexing for 15 s. This is followed by an incubation step for 10 min at 70 °C while shaked at 900 rpm. Subsequently, followed by adding 150 µl 96 % EtOH and mixing by vortexing for 15 s. The lysate was transferred into a QIAamp MinElute column and filtered with a 1-minute centrifugation at 8000 rpm. The latter step is repeated two additional times followed by adding 500 µl AW1 buffer with 1-minute centrifugation at 8000 rpm. Then, 700 µl AW2 buffer was added with centrifuge for 1 min at 8000 rpm. Further, 700 µl of 96 % EtOH were added with centrifugation for 1 minute at 8000 rpm and an additional centrifugation step for 3 min at full speed. Then, the column is incubated at 56 °C for 3 min. The eluation step differs from the standart protocol: after incubation for 1 minute at RT for each sample DNA was eluted in two steps with 40 µl ATE buffer each, resulting in an 80 µl DNA extract per sample at 14 000 rpm.

Five saliva swab samples were collected in InhibitEX buffer and therefore were extracted following manufacturer's instructions of the QIAamp Fast DNA Stool Mini Kit (QIAgen).

6.4.3 Hair

Selected hair with visible follicles ($n \geq 3$) were processed with the QIAamp DNA Investigator Kit (QIAgen). Follicles are well visible at the often darker coloured guard hair and, if possible, were preferred over the tight woolly undercoat hair. Especially, guard hair can be cut above the follicle into the reaction tube with 300 µl ATL buffer. All used forceps and scissors were disinfected beforehand. Following, 20 µl proteinase K and 20 µl dichlorodiphenyltrichloroethane (DDT) were added and rotary incubated in an Enviro-Genie (Scientific Industries Inc.) at for at least 3 h or overnight at 56 °C. Before transferring into a QIAcube (QIAgen) 1 µl carrierRNA were added. The same extraction protocol as with saliva, nasal secretion and urine swab samples (see 6.4.2) were executed by a QIAcube (QIAgen) with two eluation steps of 40 µl with resulting in 80 µl DNA extract per sample.

6.4.4 Tissue

Especially, for the SNP panel development samples with high DNA concentrations and possibly low contaminations tissue samples were used.

Tissue samples were manually processed separately in a laboratory dedicated to process invasive samples using the Dneasy® Blood & Tissue Kit (QIAgen). Therefore, a ≤ 25 mg tissue piece was cut into a reaction tube and processed following the manufacturer's instructions: adding 180 µl ATL buffer and 20 µl proteinase K and completely lysing by incubating and shaking in a BioShake iQ (QUANTIFOIL Instruments) at 56 °C. Subsequently, 200 µl AL buffer and 200 µl 96 % EtOH were added with each a following vortexing step. The mixture was pipetted into a Dneasy Mini spin column (QIAgen) and centrifuged at 8000 rpm (Micro Star 17) for 1 min into a 2.0 ml safe-lock tube (Eppendorf). The Dneasy Mini spin column was transferred into a second 2.0 ml safe-lock tube, filled

with 500 µl AW1 buffer and centrifuged for 1 min at 8000 rpm (Micro Star 17). After a transfer into a third 2.0 ml safe-lock tube 500 µl AW2 buffer were added and centrifuged at 13 300 rpm for 3 min. The centrifuge step was repeated with a fourth 2.0 ml safe-lock tube again at 13 300 rpm for 3 min. The spin column was transferred into 1.5 ml safe-lock tube (Eppendorf) and dried at 56 °C in a BioShake iQ for 10 min. DNA elution was performed in two steps of adding 100 µl AE buffer, incubating each for 5 min at RT and a following centrifuge step at 8 000 rpm resulting in 200 µl DNA extract per sample.

If possible, a second extraction was done for the SNP panel if an eluate was measured with a concentration of nucleic acid > 50 ng/µl. The concentration measurements were done with a spectrophotometer (NanoDrop ND-1000; ThermoFischer). Therefore, the last step was altered with a single eluation step of 30 µl AE buffer per sample to yield a DNA extract with a higher concentration.

To obtain more DNA bound at the spin column a separate eluation with 60 µl AE buffer, incubating for 5 min at RT and a following centrifuge step at 8000 rpm. The minimum volume of 60 µl is recommended by the manufacturer (QIAgen).

6.4.5 Blood

Blood samples were manually processed with the Dneasy® Blood & Tissue Kit (QIAgen). Therefore, a 100 µl blood in EDTA was piped into a reaction tube with adding 100 µl phosphate buffered saline (PBS) and 20 µl proteinase K and completely lysing by incubating and shaking in a at 56 °C.

Subsequently, 200 µl AL buffer was added with a following incubation at 56 °C for 10 min (BioShake iQ (QUANTIFOIL Instruments)) with following vortexing. Additionally, 200 µl 96 % EtOH were added with a following vortexing step. The mixture was pipetted into a Dneasy Mini Spin Column (Dneasy® Blood & Tissue Kit (50) (QIAgen)) and centrifuged at 8000 rpm (Micro Star 17) for 1 min into a 2.0 ml safe-lock tube (Eppendorf). The Dneasy Mini spin column was transferred into a second 2.0 ml safe-lock tube, filled with 500 µl AW1 buffer and centrifuged for 1 min at 8 000 rpm (Micro Star 17). After a transfer into a third 2.0 ml safe-lock tube 500 µl AW2 buffer were added and centrifuged at 13 300 rpm for 3 min. The centrifuge step was repeated with a fourth 2.0 ml safe-lock tube again at 13 300 rpm for 3 min. The spin column was transferred into 1.5 ml safe-lock tube (Eppendorf) and dried at 56 °C in a BioShake iQ (QUANTIFOIL Instruments) for 10 min. Finally, two eluation steps of adding 100 µl AE buffer, incubating each for 5 min in room temperature and following centrifuge at 8000 rpm resulting in 200 µl DNA extract per sample. Blood swab samples were placed in a Dneasy® Mini Spin Column while setting the sample volume at 0 µl. This resulted in adding 250 µl of AL buffer and 250 µl 96 % EtOH before transfer into a Dneasy Mini Spin Column. All following steps are processed as described before. Two eluation steps of 100 µl AE buffer resulting in 200 µl DNA extract per sample.

6.5 Normalisation of DNA extracts

To obtain DNA extracts with the recommended concentration of ≥ 60 ng/µl nucleic acid for the first SNP panel analysis without STAs (fluigdim) DNA extracts from tissue were concentrated with the following protocol: 60 µl DNA extract mixed with 2.5× 96 % EtOH and 0.1× NaCl (5 M) shake 5 – 10 times and invert for 3 – 4 times. The mixture was centrifuged at 4 °C at 152000 rpm (Heraeus™ Megafuge™ 16R (Thermo Scientific™)) for 15 min. The supernatant was carefully discarded with following drying at 35 °C for 10 min. A washing step with 150 µl 70 % EtOH was followed by 10 min centrifuge at 152000 rpm at 4 °C. After discarding the EtOH the pellet was dried for 10 min at 35 °C. Finally, the pellet was solved in 20 µl AE buffer.

For dilution the necessary volume of AE buffer was added.

6.6 Pilot Study: best practice for faecal sampling, preservation and DNA extraction

6.6.1 Detailed material and methods

6.6.1.1 Sampling and preservation procedures

For the main study objective, 38 faecal samples from two wisent pats with several sampling types were collected at the 8th August 2018 in the *Wildpark Alte Fasanerie* in Hanau-Klein-Auheim, Hesse, Germany. Therefore, occasional defaecation of every wisent was observed to secure individual assignment of the sampled wisent pats. Accordingly, those two wisent pats originating from two ('Falka' EBPB#9318 and 'Abia' EBPB#13637) out of four captive individuals were obtained in order to test for an optimal faecal sampling as following: one-way forceps were used to isolate a portion of up to 10 – 15 g faecal matrix for full conservation in 33 ml 96 % EtOH (70 ml cup, SARSTEDT) (in the following *full faecal sample*). For the methodological preservation evaluation three types of swab storing conditions were used: (i) directly in DNA lysis buffer (ASL buffer (QIAamp DNA Stool Mini Kit (Qiagen)) and InhibitEX buffer (QIAampFast DNA Stool Mini Kit (Qiagen)), (ii) in dry bags and (iii) in 33 ml 96 % EtOH. Faecal cotton swab samples were taken separately from the faecal surface and faecal interior. Secondary, pats were sampled with dry flocked nylon swabs (4N6FLOQSwabs genetics™ regular size tips in 109 mm long tube with Active Drying System (COPAN flock technologies)). The environmental temperatures ranged from 29 – 32 °C whereas the humidity was measured between 40 – 55 % relative humidity (RH) during sampling. Environmental temperature and RH were assessed with a WindMate™ 300 (Speedtech Instruments).

Not included in the statistical evaluation of the best practice for faecal sampling are two faecal samples collected from the captive herd in Bad Berleburg (Wisent-Wildnis am Rothaarsteig) as full samples in EtOH. Those samples represent older dung samples not assignable to an individual by observation in the field but specifically sampled to genotype aged wisent pats.

6.6.1.1.1 Reference samples

In the context of this study no invasive tissue samples were actively collected. Invasive tissue (*n* = 40) lyophilised blood (*n* = 2) and lyophilised DNA samples (*n* = 1) from dead free-roaming wisent from Poland (LL line) and Russia (LC line) were provided by the Mammal Research Institute of the Polish Academy of Sciences in Białowieża, Poland. These samples were collected between 1990 and 2016. Two additional tissue samples from one individual of the free-roaming wisent herd in the German Rothaar mountains sampled in 2017 and a sole tissue sample originating from the first German self-immigrated free-roaming wisent bull (publically known as 'Gożubr') shot around 2 km from Lebus in Brandenburg in at the 13th September 2017 were included in the analysis. All tissue samples were stored in 96 % EtOH at RT.

6.6.1.2 Microsatellite genotyping and analysis

The 21 microsatellite primers were allocated in three multiplex mixes (Multiplex A to C) with adjusted concentrations of each marker (Table A 2). Each primer premix (in total 800 µl) was prepared with 640 µl DNA-free water, 80 µl reveres primer (Rev) and 80 µl forward primer (For). The concentration ratios of the fluorescence-labelled forward primers (ForLab) and the non-labelled forward primers (For) were also adjusted for each marker beforehand (Table A 2).

The multiplex PCR premix included 5 µl 2× Hot StarTaq Master Mix (QIAgen), 1.4 µl primer premix, 4 µg Bovine serum albumin (BSA, Molecular Biology Grade, B9000S, New England Biolabs® Inc.), 1.4 µl DNA-free water and 3 µl DNA extract.

The microsatellite sequencing target DNA was amplified with a PCR in T1 thermocyclers (Biometra, Analytik Jena) with the following program: Initial denaturation at 95 °C (15 min); followed by 40 cycles denaturation at 94 °C (30 s), annealing at 56 °C (1 min) and extension at 72 °C (1 min); followed by a final extension at 72 °C (10 min); followed by cooling at 10 °C.

PCR products were separated and detected on the ABI 3730 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Allele sizes were determined based on the GeneScan™ 600 LIZ size standard (ThermoFisher Scientific) using GeneMarker® v2.6.3 (Softgenetics®). All automatic scorings were visually checked and if applicable manually corrected. Threshold for calling was set at a minimal florescence of 100 Relative Fluorescent Units (RFUs) for peaks in markers < 200 bp and a florescence of 80 RFUs for peaks in markers > 200 bp if the background noise of fluorescence is moderate around zero. Scoring errors and null alleles were evaluated with *Micro-Checker* v2.2.3 (VAN OOSTERHOUT et al. 2004) with a confidence interval of 95 %.

Table A 1: Overview of all utilised microsatellite markers. All bold printed markers ($n = 14$) were used for the faecal sampling and storage methodological experiment. All other markers were rejected by disfunction or homozygoty. Marker premix and multiplex protocols from WESTEKEMPER et al., *in prep.*. References (Ref.): (1) BISHOP et al. (1994), (2) TALBOT et al. (1996), (3) MOORE et al. (1992), (4) MOORE et al. (1994), (5) KUEHN et al. (2003), (6) HIRANO et al. (1996), (7) MEZZELANI ET AL. (1995), (8) RØED and MIDTHJELL (1998), (9) WILSON et al. (1997), (10) STEFFEN et al. (1993), (11) DEWOODY et al. (1995), (12) VAIMAN et al. (1994), (13) MOMMENS et al. (1994), (14) BRINKMAN and HUNDERTMARK (2009).

Locus	*Primer sequence* ($\frac{forward}{reverse}$)	*Motif*	*Fluorescent label*	*Multi-plex*	*Ref.*	*Accession#*
BM4208	**TCAGTACACTGGCCACCATG**	**(GT)**	**PET**	**A**	**1, 2**	**G18509.1**
	CACTGCATGCTTTTCCAAAC					
CSSM66	AATTTAATGCACTGAGGAGCTTGG	(GT)	NED	A	3, 4, 5	AF232764.1
	ACACAAATCCTTTCTGCCAGCTTGA					
DIK082	**CCCACTCTGTCTCCAGTTTG**	**(GT)**	**6-FAM**	**A**	**6**	**D83304.1**
	TATCCTGAGAAAAGCTGCTAGA					
IDVGA59	**CAGTCCCTCAACCCTCTTTTC**	**$(AC)_{23}$**	**VIC**	**A**	**7**	**X85074.1**
	AACCCAAATATCCATCAATAG					
NVHRT21	GCAGCGGAGAGGAACAAAAG	$(GT)_{16}$ $(GC)_4GT$	VIC	A	8	AF068207.1
	GGGGAGGAGCAGGGAAATC					
NVHRT48	CGTGAATCTTAACCAGGTCT	(GT)	NED	A	8	AF068214.1
	GGTCAGCTTCATTTAGAAAC					
RT1	TGCCTTCTTTCATCCAACAA	(GT)	PET	A	9	U90737.1
	CATCTTCCCATCCTCTTTAC					
BM1818	**AGTGCTTTCAAGGTCCATGC**	**(GT)**	**PET**	**B**	**1**	**G18391.1**
	AGCTGGGAATATAACCAAAGG					
BM203	**GGGTGTGACATTTTGTTCCC**	**(GT)**	**6-FAM**	**B**	**1**	**G18500**
	CTGCTCGCCACTAGTCCTTC					
BMC1009	**GCACCAGCAGAGAGGACATT**	**$(AC)_{15}$?**	**NED**	**B**	**1**	**?**
	ACCGGCTATTGTCCATCTTG					
CSSM14	**AAATGACCTCTCAATGGAAGCTTG**	**(GT)**	**NED**	**B**	**3, 4, 5**	**AF232759**
	GAATTCTGGCACTTAATAGGATTCA					
CSSM19	**TTGTCAGCAACTTCTTGTATCTTT**	**(GT)**	**VIC**	**B**	**3, 4, 5**	**AF232761**
	TGTTTTAAGCCACCCAATTATTTG					
CSSM22	**TCTCTCTAATGGAGTTGGTTTTTG**	**(GT)**	**NED**	**B**	**3, 4, 5**	**AF232762**
	CTTTCTCTTCAATCAATCCTCATC					
ETH225	ACATGACAGCCAGCTGCTACT	(GT)	6-FAM	B	5, 10	AF232767.1
	GATCACCTTGCCACTATTTCCT					
CER14	TCTCTTGCGTCTCCTGCATTGAC	(GT)	6-FAM	C	5, 11	L35583.1
	AATGGCACCCACTCCAGTATTCTTC					
CSSM16	**AGAGCCACTTGTTACACCCCAAAG**	**(GT)**	**NED**	**C**	**5**	**AF232760**
	GATGCAGTCTCCACTTGATTCAAA					
Haut14	**CCAGGGAAGATGAAGTGACC**	**(GT)**	**VIC**	**C**	**5**	**AF236378**
	TGACCTTCACTCATGTTATTAA					
IDVGA55	**GTGACTGTATTTGTGAACACCTA**	**$(AC)_{12}$**	**NED**	**C**	**7**	**X85071**
	TCTAAAACGGAGGCAGAGATG					
INRA35	TTGTGCTTTATGACACTATCCG	(GT)	PET	C	5, 12	X68049
	ATCCTTTGCAGCCTCCACATTC					
MM12	**CAAGACAGGTGTTTCAATCT**	**(GT)**	**6-FAM**	**C**	**5, 13**	**Z30343**

	ATCGACTCTGGGGATGATGT					
KY1/2	**GCCCAGCAGCCCTTCCAG**	**AmelY/ AmelX**	**PET**	**C**	**14**	**FJ434497.1**
	TGGCCAAGCTTCCAGAGGCA					

Table A 2: Overview of primer concentrations in the multiplex mixes A to C (µl/reaction) and ratios of the fluorescence-labelled forward primers and not labelled forward primers.

Multiplex A			**Multiplex B**			**Multiplex C**		
Primer	µl/rxn	For:ForLab	Primer	µl/rxn	For:ForLab	Primer	µl/rxn	For:ForLab
NVHRT48	0.2	1:5	CSSM19	0.2	1:5	KY1/2	0.2	1:10
DIK082	0.2	1:5	CSSM22	0.2	1:10	MM12	0.2	1:10
NVHRT21	0.2	1:5	ETH225	0.2	1:10	Haut14	0.3	1:4
BM4208	0.3	1:3	BMC1009	0.2	1:5	CSSM16	0.2	1:20
CSSM66	0.4	1:2	BM203	0.2	1:5	CER14	0.3	1:3
RT1	0.4	1:3	BM1818	0.2	1:5	INRA35	0.2	1:5
IDVGA59	0.4	1:5	CSSM14	0.2	1:5	IDVGA55	0.2	1:5

6.6.1.2.1 Faecal sampling validation

All faecal samples of both wisent individuals from the wildlife park ('Falka' EBPB#9318 and 'Abia' EBPB#13637) were used for the validation of faecal sampling and preservation methods as well as two DNA extraction kits. Based on these multiple-time sampled and genotyped non-invasive samples, reference genotypes were built to determine the GE rates for each single triplicate genotype. In total 207 genotypes ('Falka' EBPB#9318: $n = 105$; 'Abia' EBPB#13637: $n = 102$) from 41 samples were generated. Those are comprised of 38 faecal samples and a sole saliva sample collected at the same day in Hanau Klein-Auheim, complemented by two saliva samples from the same two individuals collected in a former sampling to verify the reference individual genotype. The full faecal samples in EtOH were extracted six times (three after one week and three after five weeks). With the exception of the faecal swab samples in lysis buffer all samples were extracted with both the ASL and InhibitEX buffer. All extracts were triplicated for genotyping, while single non-informative triplicates due to missing data or technical error were excluded. The threshold for a valid allele per locus were matching alleles $n > 10$ over all genotypes per individual (≙ 10 % of all genotypes per individual). Three triplicates of a saliva sample (lab#X180120) collected from a feeding trough surface were excluded from analysis due to contamination presumably with DNA of a second individual.

The *amplification success rate*, *genotyping success rate*, *allelic dropout rate* and *false allele rate* as proportional response variables were used to evaluate the applicability of every faecal sampling method, storage and DNA extraction (predictors). The amplification success rate is the number of successful amplified and scored loci per genotype over the total number of loci ($n = 14$). This response variable reflects the applicability measured in successful sequenced PCR products

disregarding the final consensus genotype (BROQUET et al. 2006). The genotyping success rate is the number of successful amplified and scored loci per genotype over the total number of loci matching with the individual consensus genotype. Thus, the latter response variable reflects the applicability measured in the result of providing the true genotype, excluding amplification failure (missing data) and genotyping errors (ADOs and FAs) (BROQUET et al. 2006). The ADO rate is the number of observed ADO over the number of total successfully amplified and scored loci per genotype (= amplification success). It was recommended to determine GE rates only as the observed number of GEs over the number of loci where those error can be detected (CREEL et al. 2003; BROQUET and PETIT 2004). Since all samples in the present study originate from only two individuals and the 14 microsatellite maker were not specifically selected to be heterozygous at every locus, the ADO rate is not calculated from the total number of heterozygous loci based on the consensus genotype ('Falka' EBPB#9318: $n = 5$; 'Abia' EBPB#13637: $n = 2$). ADOs in homozygous loci are a minor problem since they cause no erroneous genotypes like FAs (BROQUET and PETIT 2004). The FA rate is the number of observed FA over the number of total successfully amplified and scored loci per genotype (BROQUET and PETIT 2004). The definition of the ADO rate facilitate a comparison with the FA rate in this context and was used before in similar approaches (BAYES et al. 2000; SMITH et al. 2000; DALLAS et al. 2003).

Three models per response variable were implemented to test all predictors of the sampling methodology due to the fact that not all samples were allocable to every predictor character (Equation A *1* - Equation A *3*). Single reactions showing erroneous raw data files (Mix A: $n = 5$; Mix B: $n = 5$; Mix C: $n = 0$) were rejected entirely (total: $n = 10$; 'Falka' EBPB#9318: $n = 8$; 'Abia' EBPB#13637: $n = 2$) for the GLMMs to not bias the explanatory power of the predictor variables on the success or error rates. Those erroneous raw data files were not possible to be displayed into GeneMarker v2.6.3 (SoftGenetics) and represent digital errors.

Several sampling and storage methods were tested on the dependence of their success and GE rates to find out a convenient best practice for a genetic study based on non-invasive faecal samples in the European bison. First, the full faecal samples in 96 % EtOH and faecal uptake by swabs were compared. Several containment types of the swab samples are included: cotton swab sample directly in (i) DNA lysis buffer, (ii) cotton swab samples in dry filter paper arranged in a dry bag, (iii) cotton swab samples in 96 % EtOH and (iv) flocked nylon swabs with integrated drying agent (Equation A 1;Equation A 3). The impact of two different DNA extraction kits on all sampling methods were tested with the exception of the flocked nylon swabs (only extracted with the InhibitEX buffer) (Equation A 1 – Equation A 3). Furthermore, the possible impact of swabs samples from the faecal surface and faecal interior (Equation A *3*) and storage duration on the full samples in EtOH (Equation A *2*) were also tested. Additional to the amplification triplets for each sample, three extraction triplicates of

two faecal full samples (lab#X180110; lab#X180111) from two individuals are included per DNA extraction kit and storage duration.

Two random effect groups were implemented in every GLMM: genotypes generated from amplification triplets of the identical sample (laboratory number (lab#)) and the sample assemblage for each automated extraction run in the QIAcube (QIAcube run, 12 samples per run).

Equation A 1: GLMM for testing the influence of the categorical predictor variables (sampling method and DNA extraction kit) on the response variable (amplification/genotyping success per locus (S_i) of all microsatellite markers (n = 14) per reaction/genotyping errors per locus (E_i) of successfully amplified and scored microsatellite markers per locus ($AmpS_i$) per reaction). The random effect variable of the lab number (lab#) represents the single triplicated samples. The random effect variable of the DNA extraction run (QIAcube run) represents the sample assemblage of the automated DNA extraction (this variable was additionally tested). The distribution of the response variable is assumed to be binomial.

$$glmer(cbind(S_i, 14 - S_i)$$
$$glmer(cbind(E_i, AmpS_i - E_i)$$
$$\sim sampling\_method + DNA\_extraction\_kit + (1|Lab\#)[+(1|run)], family = binomial)$$

Due to the limited number of samples the impact of the storage duration (one week and five weeks after collection) of faecal samples were tested only on full samples in EtOH. The DNA extraction kit is an additional predictor for the success and GE rate (Equation A *2*).

Equation A 2: GLMM for testing the influence of the categorical variables (storage duration and DNA extraction kit) on the response variable (amplification/genotyping success of the sequenced microsatellite markers (n = 14) per reaction/ genotyping errors per locus (E_i) of successfully amplified and scored microsatellite markers per locus ($AmpS_i$) per reaction). The random effect variable of the lab number (lab#) represents the single triplicated samples. The random effect variable of the DNA extraction run (QIAcube run) represents the sample assemblage of the automated DNA extraction (this variable was additionally tested). The distribution of the response variable is assumed to be binomial.

$$glmer(cbind(S_i, 14 - S_i)$$
$$glmer(cbind(E_i, AmpS_i - E_i)$$
$$\sim storage\_duration + DNA\_extraction\_kit + (1|Lab\#)[+(1|run)], family = binomial)$$

Models with the predictors faecal part, sample method and DNA extraction kit were utilised on the success and GE rates to test the reliance of the sampled part of the wisent pat. Since a differentiation between faecal surface and faecal interior in the full faecal samples in EtOH was not possible, the latter sampling method was excluded from this model (Equation A *3*).

Equation A 3: GLMM for testing the influence of the categorical variables (faecal part, sampling method and DNA extraction kit) on the response variable (amplification/genotyping success of the sequenced microsatellite markers (n = 14) per reaction/ genotyping errors per locus (E_i) of successfully amplified and scored microsatellite markers per locus ($AmpS_i$) per reaction). The random effect variable of the lab number (lab#) represents the single triplicated samples. The random effect variable of the DNA extraction run (QIAcube run) represents the sample assemblage of the automated DNA extraction (this variable was additionally tested). The distribution of the response variable is assumed to be binomial.

$$glmer(cbind(S_i, 14 - S_i)$$
$$glmer(cbind(E_i, AmpS_i - E_i)$$
$$\sim faecal\_part + sampling\_method + DNA\_extraction\_kit + (1|Lab\#)[+(1|run)], family = binomial)$$

6.6.1.2.2 Non-faecal sampling

For the pilot study, additional to the reference samples (tissue and samples with lyophilised blood and DNA), two non-invasive urine, one invasive nasal secretion, one invasive and three non-invasive saliva samples from five captive individuals ((LC line) (Hanau-Klein-Auheim (Wildpark Alte Fasanerie) and Bad Berleburg (Wisent-Wildnis am Rothaarsteig)) were genotyped, but not considered for statistical evaluation due to the small sample size.

6.6.2 Detailed results

6.6.2.1 Microsatellites

Nearly all microsatellites used in wisent genetics so far are in non-coding regions. Only FLISIKOWSKI et al. (2007) used a not named microsatellite located in the growth hormone receptor (GHR) gene given by LUCY et al. (1993). MIKHAILOVA and VOITSUKHOVSKAYA (2017) did not published their microsatellite marker set and therefore cannot compared with other studies.

Seven of 21 microsatellite markers were rejected by homozygosity or non-function: four microsatellites (NVHRT48, NVHRT21, CER14, INRA35) by non-function in *B. bonasus* and three (CSSM66, RT1, ETH225) were not suitable for the non-invasive approach. The gonosomal and twelve autosomal polymorphic microsatellites were viable for the analysis of European bison samples (Table A 3). IDVGA55 was the only monomorphic marker of the microsatellites applicable for the non-invasive approach and only used in evaluation of faecal sampling methodology.

Table A 3: Characterisation of tested microsatellite markers appropriate for non-invasive samples from *Bos bonasus* found in 51 individuals from both breeding lines (LL: *n* = 22; LC: *n* = 29). Allele frequencies are given for all individuals (wisent) and separately for both breeding lines (LC, LL). Private alleles per breeding line in the genotyped individuals are underlined. In two individuals IDVGA59 could not be successfully amplified and scored. In these both cases only non-invasive samples were available.

Locus	*Multiplex*	*Allele*		*wisent*	*LC*	*LL*
DIK082	**A**		**N**	**51**	**22**	**29**
		100		0.363	0.341	0.379
		112		0.039	0.000	0.069
		124		0.598	0.659	0.552
IDVGA59	**A**		**N**	**49**	**20**	**29**
		244		0.265	0.275	0.259
		264		0.735	0.725	0.741
BM4208	**A**		**N**	**51**	**22**	**29**
		158		0.167	0.114	0.207
		160		0.833	0.886	0.793
BM203	**B**		**N**	**51**	**22**	**29**
		218		0.971	0.955	0.983
		222		0.029	0.045	0.017
CSSM19	**B**		**N**	**51**	**22**	**29**
		140		0.118	0.182	0.069
		142		0.569	0.568	0.569
		148		0.314	0.250	0.362
CSSM14	**B**		**N**	**51**	**22**	**29**
		134		0.627	0.614	0.638
		136		0.088	0.023	0.138
		138		0.284	0.364	0.224
CSSM22	**B**		**N**	**51**	**22**	**29**
		214		0.990	0.977	1.000
		216		0.010	0.023	0.000
BMC1009	**B**		**N**	**51**	**22**	**29**
		276		0.941	0.932	0.948
		278		0.010	0.023	0.000
		280		0.049	0.045	0.052
BM1818	**B**		**N**	**51**	**22**	**29**
		260		0.480	0.614	0.379
		264		0.520	0.386	0.621
MM12	**C**		**N**	**51**	**22**	**29**
		108		0.382	0.318	0.431
		110		0.618	0.682	0.569
Haut14	**C**		**N**	**51**	**22**	**29**
		142		0.559	0.523	0.586
		144		0.441	0.477	0.414
CSSM16	**C**		**N**	**51**	**22**	**29**
		159		0.196	0.159	0.224
		171		0.794	0.818	0.776
		173		0.010	0.023	0.000
IDVGA55	**C**		**N**	**51**	**22**	**29**
		199		1.000	1.000	1.000
KY1/2 (sexmarker)	**C**		**N**	**51**	**22**	**29**
		170		0.167	0.205	0.138
		233		0.833	0.795	0.862

No statistical evidence for null alleles (Table A 4) or scoring errors were found (level of significance: > 5 % (α = 0.05)).

Table A 4: Comparison of estimated null allele frequencies of the microsatellites of this study on European bison (*n* = 51) using four algorithms (CHAKRABORTY et al. 1992; BROOKFIELD 1996; VAN OOSTERHOUT et al. 2004).

Locus	*van Oosterhout*	*Chakraborty*	*Brookfield 1*	*Brookfield 2*
DIK082	0.078	0.0856	0.0515	0.0515
IDVGA59	-0.0328	-0.0307	-0.0181	0.1432
BM4208	0.035	0.0389	0.0167	0.0167
BM203	-0.0311	-0.0155	-0.0018	0
CSSM19	0.0175	0.0123	0.0088	0.0088
CSSM14	0.0597	0.0694	0.0428	0.0428
CSSM22	-0.0103	-0.0051	-0.0002	0
BMC1009	-0.0626	-0.0271	-0.0058	0
BM1818	-0.0534	-0.0494	-0.0346	0
MM12	0.0513	0.0557	0.0342	0.0342
Haut14	0.0628	0.0694	0.0428	0.0428
CSSM16	-0.0593	-0.0462	-0.0233	0

6.6.2.2 Faecal sampling and sample storage methodology

Both DNA extraction kits were developed for human faeces. However, the QIAgen DNA stool mini kit was already successfully used for DNA extraction from taurine frozen faeces and faecal swab samples for microbial investigation (INGLIS and KALISCHUK 2003; GIOFFRÉ et al. 2004; INGLIS et al. 2004). Additionally, I can verify the applicability for both extraction kits for all sampled species of Bovini.

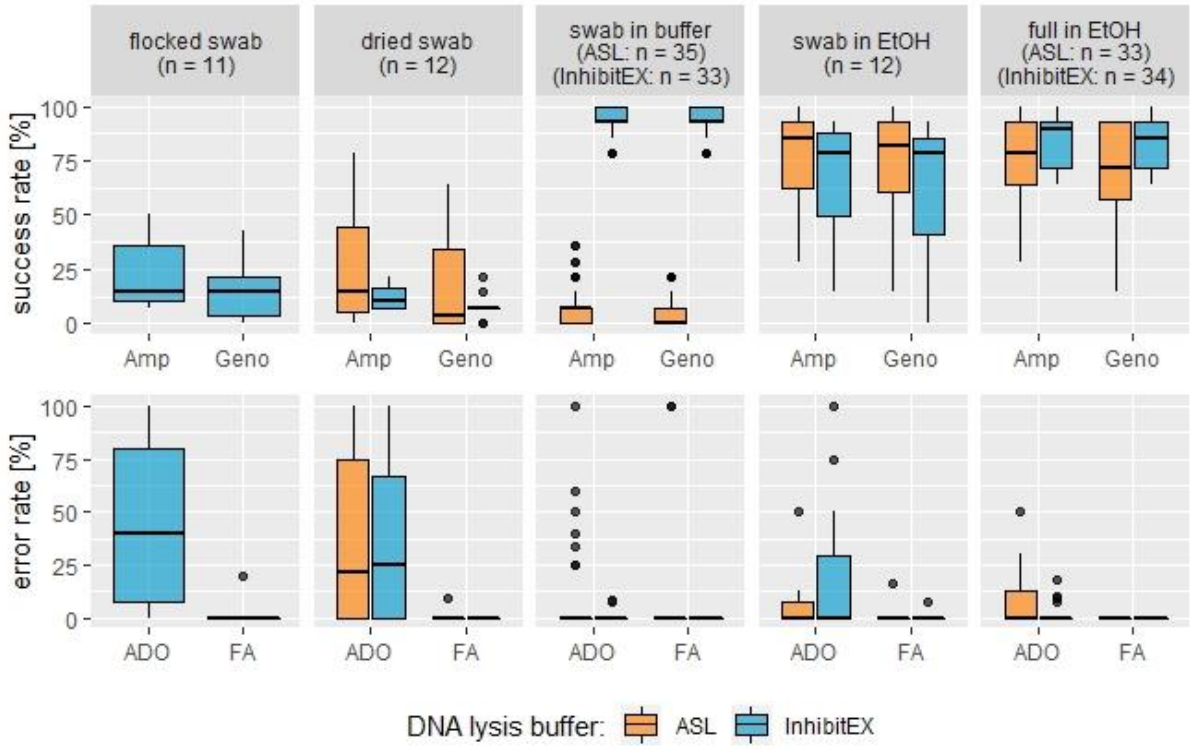


Figure A 1: Success rates and genotyping error rates of triplicated genotypes from faecal samples (*n* = 194) collected with five sampling methods and two DNA lysis buffer. Sample sizes per sampling method and DNA lysis buffer can be found above the boxplots and represent triplets of in total 68 DNA extractions. Those genotypes originate from two female individuals ('Falka' EBPB#9318: *n* = 94; 'Abia' EBPB#13637: *n* = 100). Amplification success rate (Amp): successful scored loci over total number of loci (*n* = 14). Genotyping success (Geno): genotyping errors (allelic dropout and false alleles) deducted from amplification success over the total number of loci. Allelic dropout rate (ADO): loci with not amplified alleles based on the consensus genotype over the number of successful amplified and scored loci (= amplification success). False allele rate (FA): amplified artefacts scored as allele based on the consensus genotype over the number of successful amplified and scored loci.

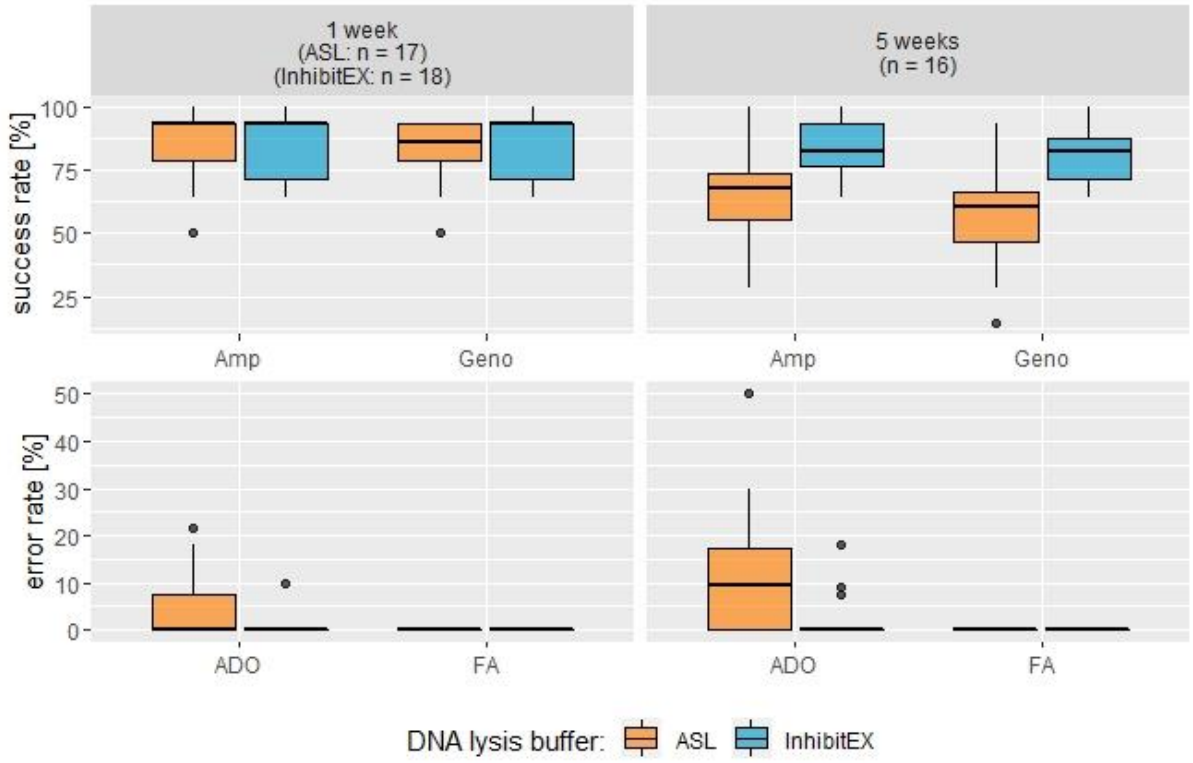


Figure A 2: Success and genotyping error rates of triplicated genotypes from full faecal samples (*n* = 67) extracted after one and five weeks using two DNA lysis buffers corresponding to two DNA extraction kits. Sample sizes per storage duration and DNA lysis buffer can be found above the boxplots and represent triplets of in total 24 DNA extractions. Those genotypes originate from two female individuals ('Falka' EBPB#9318: *n* = 32; 'Abia' EBPB#13637: *n* = 35). Amplification success rate (Amp): successful scored loci over total number of loci (*n* = 14). Genotyping success (Geno): genotyping errors (allelic dropout and false alleles) deducted from amplification success over the total number of loci. Allelic dropout rate (ADO): loci with not amplified alleles based on the consensus genotype over the number of successful amplified and scored loci (= amplification success). False allele rate (FA): amplified artefacts scored as allele based on the consensus genotype over the number of successful amplified and scored loci.

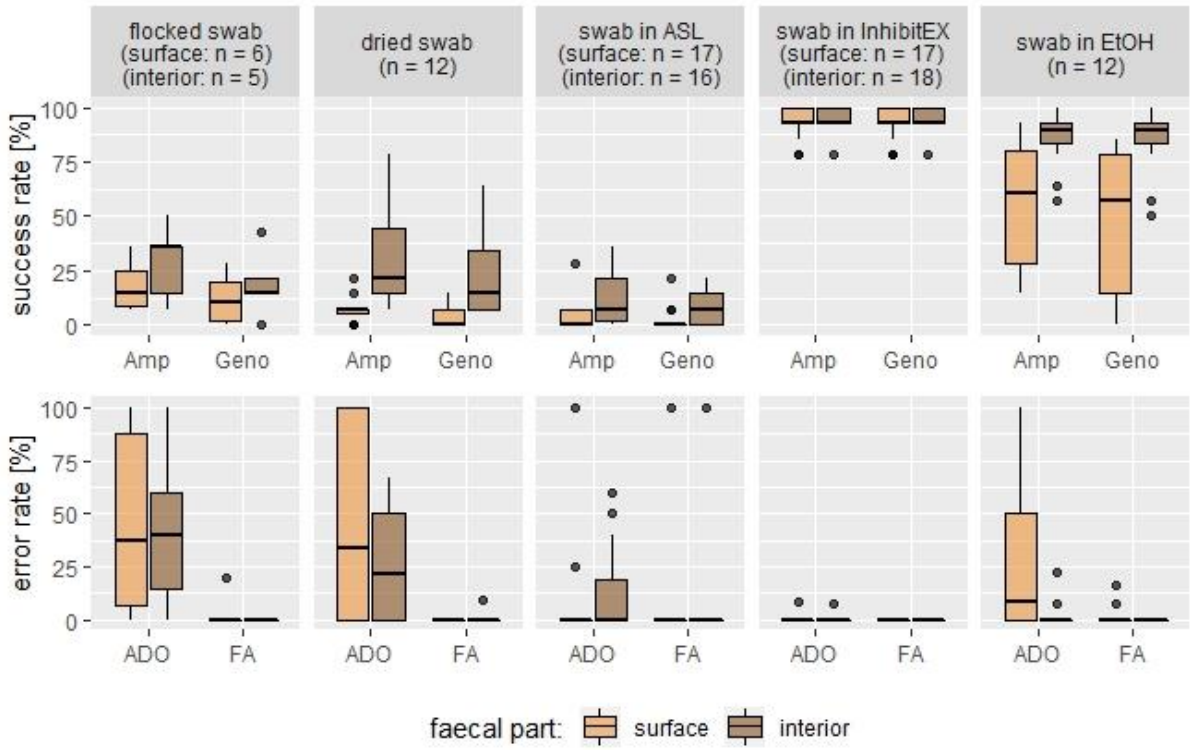


Figure A 3: Success and genotyping error rates of triplicated genotypes from faecal samples (*n* = 127) collected with five sampling methods and two part of the wisent pat to evaluate faecal sampling methodology. Sample sizes per sampling method and DNA lysis buffer can be found above the boxplots and represent triplets of in total 43 DNA extractions. Those genotypes originate from two female individuals ('Falka' EBPB#9318: *n* = 62; 'Abia' EBPB#13637: *n* = 65). Amplification success rate (Amp): successful scored loci over total number of loci (*n* = 14). Genotyping success (Geno): genotyping errors (allelic dropout and false alleles) deducted from amplification success over the total number of loci. Allelic dropout rate (ADO): loci with not amplified alleles based on the consensus genotype over the number of successful amplified and scored loci (= amplification success). False allele rate (FA): amplified artefacts scored as allele based on the consensus genotype over the number of successful amplified and scored loci.

Table A 5: AICcs for the GLMMs of the success rates with/without the additional random effect 'QIAcube run', interaction terms and the null model. Models in bold were selected. For the selected GLMMs the *p*-value on normality of the residuals (executed with the Shapiro-Wilk-test) are shown as well.

Model	AICc	Shapiro-Wild-test (*p*-value)
Amplification success rate		
Sampling method + DNA extraction kit (Equation A 1)	**652.7255**	**0.2055**
+ random effect group (run)	654.9254	
Interaction terms	655.1201	
Null model	740.2407	
Storage duration + DNA extraction kit (Equation A 2)	**238.9371**	**0.005541**
+ random effect group (run)	241.2755	
Interaction terms	240.4805	
Null model	239.967	
Faecal part + sampling method + DNA extraction kit (Equation A 3)	**404.5755**	**0.04195**
+ random effect group (run)	406.8936	
Interaction terms	410.2526	
Null model	474.2571	
Genotyping success rate		
Sampling method + DNA extraction kit (Equation A 1)	**635.1107**	**0.2134**
+ random effect group (run)	637.3107	
Interaction terms	636.4667	
Null model	720.6068	
Storage duration + DNA extraction kit (Equation A 2)	**247.3894**	**0.5164**
+ random effect group (run)	249.7279	
Interaction terms	248.9009	
Null model	251.5641	
Faecal part + sampling method + DNA extraction kit (Equation A 3)	**374.3496**	**0.02461**
+ random effect group (run)	376.6679	
Interaction terms	440.3392	
Null model	1726.25	

Table A 6: AICcs for the GLMMs of the genotyping error rates with/without the additional random effect 'QIAcube run', interaction terms and the null model. Models in bold were selected. The relationship of the storage duration and the DNA extraction kit on false alleles was not possible to determine because no false allele was detected in this experimental setup (grey). For the selected GLMMs the *p*-values on normality of the residuals (executed with the Shapiro-Wilk-test) are shown as well. No *p*-values concerning the false allele rates are given, because no GLMM was executed.

Model	AICc	Shapiro-Wilk-test (*p*-value)
Allelic dropout rate		
Sampling method + DNA extraction kit (Equation A 1)	**289.6554**	**3.789e-08**
+ random effect group (run)	291.8696	
Interaction terms	288.3367	
Null model	315.5074	
Storage duration + DNA extraction kit (Equation A 2)	**106.6175**	**9.049e-05**
+ random effect group (run)	108.956	
Interaction terms	108.947	
Null model	115.7784	
Faecal part + sampling method + DNA extraction kit (Equation A 3)	**177.7566**	**7.098e-07**
+ random effect group (run)	180.0747	
Interaction terms	187.4496	
Null model	199.4994	
False alleles rate		
Sampling method + DNA extraction kit (Equation A 1)	58.31521	
+ random effect group (run)	60.5151	
Interaction terms	62.04845	
Null model	**54.26459**	
Storage duration + DNA extraction kit (Equation A 2)	NA	
+ random effect group (run)	NA	
Interaction terms	NA	
Null model	NA	
Faecal part + sampling method + DNA extraction kit (Equation A 3)	**54.86338**	
+ random effect group (run)	57.18151	
Interaction terms	68.69846	
Null model	58.24566	

Effects of single samples or QIAcube runs were considered as random effects in the GLMMs. No explanatory improvements for the models were shown with the QIAcube runs as random effect groups and were subsequently excluded. Therefore, this variable is neglected in the following evaluation of best practice for the faecal sampling method.

Table A 7: Summary of intercepts, standard errors, z-values and the *p*-value (Pr(>|z|)) for the predictor factors in the GLMM with the response variable 'amplification success rate'. For each model sample sizes (*n*) are attached. The sample size (*n*) for the random effect groups represents the physical samples (divided by the triplicates used in the model). Significance codes: not significant 'ns', < 0.1 '.', < 0.05 '*', < 0.01 '**', < 0.001 '***'.

predictor	intercept	Standard error	z-value	Pr(>|z|)	
Sampling method + DNA extraction kit (Equation A 1)	*n* = 194				
	-1.35625	0.68447	-1.981	0.047538	*
sampling_methodfull_EtOH	2.97495	0.65128	4.568	4.93e-06	***
sampling_methodswab_buffer_ASL	-1.61580	0.78466	-2.059	0.039471	*
sampling_methodswab_buffer_InhibitEX	4.50090	0.70075	6.423	1.34e-10	***
sampling_methodswab_dry	-0.46640	0.73464	-0.635	0.525517	ns
sampling_methodswab_EtOH	2.49529	0.72857	3.425	0.000615	***
DNA_extraction_kitInhibitEX	0.06884	0.37243	0.185	0.853345	ns
Random effect groups (lab#): *n* = 68					
Storage duration + DNA extraction kit (Equation A 2)	*n* = 67				
	1.8247	0.3579	5.098	3.43e-07	***
storage_time5	-0.8722	0.4082	-2.137	0.0326	*
DNA_extraction_kitInhibitEX	0.4922	0.4071	1.209	0.2267	ns
Random effect groups (lab#): *n* = 24					
Faecal part + sampling method + DNA extraction kit (Equation A 3)	*n* = 127				
	-0.1002	0.7113	-0.141	0.888016	ns
faecal_partsurface	-1.2139	0.3281	-3.700	0.000216	***
sampling_methodswab_buffer_ASL	-2.2727	0.7713	-2.947	0.003212	**
sampling_methodswab_buffer_InhibitEX	4.4421	0.5987	7.420	1.17e-13	***
sampling_methodswab_dry	-0.7923	0.6502	-1.219	0.223017	ns
sampling_methodswab_EtOH	2.1832	0.6427	3.397	0.000682	***
DNA_extraction_kitInhibitEX	-0.5841	0.4971	-1.175	0.239983	ns
Random effect groups (lab#): *n* = 44					

Table A 8: Summary of intercepts, standard errors, z-values and the *p*-value (Pr(>|z|)) for the predictor factors in the GLMM with the response variable 'genotyping success rate'. For each model sample sizes (*n*) are attached. The sample size (*n*) for the random effect groups represents the physical samples (divided by the triplicates used in the model). Significance codes: not significant 'ns', < 0.1 '.', < 0.05 '*', < 0.01 '**', < 0.001 '***'.

predictor	intercept	Standard error	z-value	Pr(>|z|)	
Sampling method + DNA extraction kit (Equation A 1)	*n* = 194				
	-2.3221	0.7780	-2.985	0.002838	**
sampling_methodfull_EtOH	3.6359	0.7415	4.904	9.41e-07	***
sampling_methodswab_buffer_ASL	-1.0694	0.8900	-1.202	0.229519	ns
sampling_methodswab_buffer_InhibitEX	5.2927	0.7913	6.688	2.26e-11	***
sampling_methodswab_dry	-0.2897	0.8435	-0.343	0.731253	ns
sampling_methodswab_EtOH	3.0608	0.8248	3.711	0.000206	***
DNA_extraction_kitInhibitEX	0.2426	0.4118	0.589	0.555739	ns
Random effect groups (lab#): *n* = 68					
Storage duration + DNA extraction kit (Equation A 2)	*n* = 67				
	1.75959	0.41008	4.291	1.78e-05	***
storage_time5	-0.24583	0.09885	-2.487	0.0129	*
DNA_extraction_kitInhibitEX	0.77095	0.39431	1.955	0.0506	.
Random effect groups (lab#): *n* = 24					
Faecal part + sampling method + DNA extraction kit (Equation A 3)	*n* = 127				
	-0.7504	0.8464	-0.887	0.375330	
faecal_partsurface	-1.4529	0.3867	-3.757	0.000172	***
sampling_methodswab_buffer_ASL	-1.9827	0.9185	-2.159	0.030876	*
sampling_methodswab_buffer_InhibitEX	5.2674	0.7075	7.445	9.72e-14	***
sampling_methodswab_dry	-0.7389	0.7854	-0.941	0.346871	ns
sampling_methodswab_EtOH	2.6680	0.7661	3.483	0.000497	***
DNA_extraction_kitInhibitEX	-0.6320	0.5875	-1.076	0.282042	ns
Random effect groups (lab#): *n* = 44					

Table A 9: Summary of intercepts, standard errors, z-values and the *p*-value (Pr(>|z|)) for the predictor factors in the GLMM with the response variable 'allelic dropout rate'. For each model sample sizes (*n*) are attached. The sample size (*n*) for the random effect groups represents the physical samples (divided by the triplicates used in the model). Significance codes: not significant 'ns', < 0.1 '.', < 0.05 '*', < 0.01 '**', < 0.001 '***'.

predictor	intercept	Standard error	z-value	Pr(>|z|)	
Sampling method + DNA extraction kit (Equation A 1)	*n* = 194				
	0.1994	1.0522	0.189	0.849711	ns
sampling_methodfull_EtOH	-3.6215	1.0373	-3.491	0.000481	***
sampling_methodswab_buffer_ASL	-2.0819	1.3121	-1.587	0.112589	ns
sampling_methodswab_buffer_InhibitEX	-5.3668	1.2478	-4.301	1.7e-05	***
sampling_methodswab_dry	-0.2634	1.1227	-0.235	0.814489	ns
sampling_methodswab_EtOH	-2.7152	1.1081	-2.450	0.014278	*
DNA_extraction_kitInhibitEX	-1.0372	0.6175	-1.680	0.093020	.
Random effect groups (lab#): *n* = 68					
Storage duration + DNA extraction kit (Equation A 2)	*n* = 67				
	-4.1505	0.7781	-5.334	9.59e-08	***
storage_time5	0.4102	0.1801	2.278	0.02274	*
DNA_extraction_kitInhibitEX	-2.1223	0.7342	-2.891	0.00384	**
Random effect groups (lab#): *n* = 24					
Faecal part + sampling method + DNA extraction kit (Equation A 3)	*n* = 127				
	-1.7393	1.1149	-1.560	0.1187	ns
faecal_partsurface	1.1082	0.6068	1.826	0.0678	.
sampling_methodswab_buffer_ASL	-0.2933	1.3000	-0.226	0.8215	ns
sampling_methodswab_buffer_InhibitEX	-5.2974	1.1475	-4.616	3.9e-06	***
sampling_methodswab_dry	0.5210	1.0205	0.511	0.6097	ns
sampling_methodswab_EtOH	-2.0247	1.0130	-1.999	0.0456	*
DNA_extraction_kitInhibitEX	0.4006	0.8029	0.499	0.6178	ns
Random effect groups (lab#): *n* = 44					

With only 6 observed FAs (in 6 genotypes, 5 samples and 3 markers) in 1564 successful scored PCR reactions no meaningful statistical dependency testing could be implemented. In the experimental setup of the GLMM including the explanatory variable of storage duration (Equation A 2) no FA was detected at all. In two single PCR reactions exclusively, FAs were amplified and scored and therefore are only accounted in the genotyping success but not in the amplification success.

Included in every model the two DNA extraction kits do not show a significant different impact on the success rates (Table A 7 – Table A 9). Only in the ADO rate of the GLMM including the storage duration the used DNA extraction kit showed significant differences (Table A 9). Especially, in the DNA extractions after five weeks compared to DNA extractions after one week (Figure A 2). In contrast, sampling with a swab directly in InhibitEX buffer is significant positive different than sampling with a swab directly in ASL buffer in all models. The latter method was even significantly negative different than every dry sampling method. All dry sampling methods were significantly negative different to sampling in EtOH as either a swab sample, a full sample or InhibitEX buffer. To

collect a faecal sample from a wisent pat surface was highly significant negative different to take samples from the faecal interior in the success rates. However, it showed only a marginal significant effect on the ADO rate. The storage duration of four more weeks showed a significant negative effect on the success and ADO rates (Figure A 2; Table A 7 – Table A 9).

Also genotyped, but not part of the statistical evaluation for the best practice in faecal sampling were two selectively collected aged faecal samples. It was possible to assign one sample (lab#X180174, amplification success: 59 %) to the bull ('Horno' EBPB#11338) due to a reference genotype. The second sample (lab#X180176, amplification success: 83 %) could not assigned to an individual because no matching reference genotype was available. An assignment to the individual 'Quelle' (EBPB#12045) can be ruled out.

6.6.2.2.1 Non-faecal sampling

The triplicated tissue samples showed only one missing data and no GEs at all. No missing data or genotyping errors were observed in the samples from lyophilised blood and DNA.

Overall good quality genotypes were generated from urine, saliva and nasal secretion. Only a sole non-invasive saliva swab sample (lab#X180120) from a feeding trough showed clear genetic contamination due to several loci exhibiting more than two alleles. Two individual urine swab samples with in total six generated replicate genotypes showed two missing data and two ADOs occurred only associated with a sole sample. The single invasive saliva swab sample showed no missing data and two ADOs in all triplicates. Both non-invasive saliva swab samples with no indication of genetic contamination from two females showed no missing data and only one ADO. The single invasive nasal secretion swab sample showed no missing data or any GEs.

6.6.2.3 Probability of identity

The probability of identity (PID) for the 11 heterozygous microsatellite markers (Figure 7; Figure A 11) and 51 European bison individuals is 1.322×10^{-04}. The probability of identity of siblings is 1.141×10^{-02}.

6.6.2.4 Breeding line discrimination

Beside the non-functional microsatellite markers all monomorphic and sex microsatellite markers (Table A 3) were excluded for further evaluation of genetic population structure. This results in a set of 12 heterozygous microsatellites markers.

Figure A 4: Structure barplot based on 12 microsatellite markers (, not including the homozygous and sex markers) genotyping of 51 individuals of European bison including both breeding lines (*K* = 2).

I tested 51 wisent of which 22 individuals were assigned to the LL line and 29 individuals to the LC line. With the selected set of twelve polymorphic autosomal microsatellites no discrimination of subpopulations or breeding lines are achievable (Figure A 4).

6.6.3 Detailed discussion

6.6.3.1 Sampling and sample storage methodology

With the mentioned complications of non-invasive genetics this study, besides few other studies (LAUNHARDT et al. 1998; BAYES et al. 2000; SMITH et al. 2000), provides a comparative approach with a majority of the collection representing individually assigned samples additionally of different types (e.g. faeces, saliva etc.) from animals with documented pedigrees with the example of the wisent.

Beside a broader collection approach for gaining samples from individuals in the first place the comparatively error-prone nature of non-invasive samples regarding correct genotypes could be another reason to collect different sample types for a genetic monitoring: A genotype generated by several sample types per individual reduces the potential of negative effects of single sample type.

Due to the comparable low risk of genetic contamination and high frequency wisent pats are good candidates for non-invasive genetic sampling (see Introduction). An optimised faecal sampling is vital for a successful genetic monitoring and therefore was investigated in this pilot study.

6.6.3.1.1 Faeces

Utility of faecal samples for genetic population assessment brings specific problems but provides a frequent informative DNA source for genetic monitoring and is well-established in population and conservation genetics. Due to very different success rates of DNA amplification from faeces caused by methodology, including the sampling method, the storage method and duration as well as the extraction method, but also dependent towards other factors like environmental conditions (MILES et al. 2015) and also by the biology of the studied organism such studies remain taxa-specific (NSUBUGA et al. 2004). Pilot studies for examine the best practice are recommended (TABERLET et al. 1999; MILES et al. 2015).

To find the best practice for faecal sampling, preservation and DNA extraction in European bison different methods and scenarios were tested with GLMMs. Statistical research on GLMMs is still in

progress and e.g. model selection is less defined as in other modelling techniques (ZUUR et al. 2009). But with a set of altogether categorical predictors, not normal distributed, overdispersed data and random effects, the GLMM provided the most suitable approach. It is recommended to keep every model as simple as possible (ZUUR et al. 2009). Therefore, based on the sample set (see 2.4 three independent simple models were simulated and models with interaction terms were tested but reasonable rejected (Table A 7; Table A 8; Table A 9).

The microsatellite marker set, not specifically selected for the wisent, was only used for the evaluation of the sampling and storage methodology since it has limited explanatory power for this species (e.g. Figure A 4). The inclusion of only two individuals in this methodology evaluation leads to certain explanatory weaknesses. Especially the presented ADO rate lacks informative power due to many homozygous loci in both individuals and the calculation based on the amplification success instead on the number of successful amplified and scored heterozygous loci (CREEL et al. 2003; BROQUET and PETIT 2004). But with the consistent calculation this error rate is still comparable and conclusive within the experiment because its trend shown in the faecal samples is consistent with former studies on other species. Here too, ADO seems to be the most serious GE (GAGNEUX et al. 1997). This goes along with my results of only six FAs along in total 1 564 amplified and scored alleles.

The faecal consistency of wisent dung is highly variable and depends on the seasonal food composition in the wild. During spring and summer while feeding on mostly herbaceous plants wisent dung show a very loose consistency compared to winter months while feeding on shoots of trees, shrubs and hay (JĘDRZEJEWSKI et al. 2010). MAUDET et al. (2004) showed a significant seasonal dependence in GE rates from faecal samples of two caprine species. Samples were collected in spring months showed higher error rates compared with samples collected in winter maybe due to high forage quality in less harsh months. European bison also show such a seasonal diet selection due to their distribution in the European temperate zone (CROMSIGT et al. 2017; ZIELKE et al. 2017). In some holdings such seasonal foraging is present due to big enclosures with naturalistic vegetation and less supplementary feeding. The proportion of supplementary hay and concentrated feed depends on each institution's husbandry. All faecal samples of this study were collected in August 2018 during environmental conditions like high temperatures and humidity not advantageous for non-invasive genetic sampling in general. Still, the present study shows successful genotyping with non-invasive samples. Since the heterogeneous husbandries as well as additional feeding with e.g. hay is a common practice even in free-roaming herds (KRASIŃSKA and KRASIŃSKI 2013) the present data are applicable for wild populations regarding nutrition concerns. Likewise, GARDIPEE (2007) sampled faecal material of wild plains bison (*B. b. bison*) in two summers with an overall high amplification success and low error rates. Furthermore, HÁJKOVÁ et al. (2006) point out that MAUDET et al. (2004)

did not mentioned the lower temperatures during winter and its potential effect on DNA degradation. High temperatures and humidity increase the activity of hydrolytic enzymes in faeces causing more rapid degradation of DNA (NSUBUGA et al. 2004; HÁJKOVÁ et al. 2006). Based on study results (HÁJKOVÁ et al. 2006) in general recommends sampling in colder seasons, if possible even frozen faeces. But this might be unrealistic in continuous monitorings due to temporal, geographical or project-related circumstances. Based on my experience, sampling wisent dung during winter with temperatures > -0 °C could be difficult due to their sheer size. Splitting off collectable samples from frozen wisent pats are potentially sources of genetic contamination, because heavy tools are required and disinfection in the field is often impractical. An important aspect that this study could show concerns the practicability to store the samples at RT. This fact is of interest because it is not always possible to provide a continuous cooling chain. Precipitation is another, probable more severe factor than temperature or humidity, negatively influencing DNA quality in faeces (BRINKMAN et al. 2010; WEDROWICZ et al. 2013; AGETSUMA-YANAGIHARA et al. 2017). Sampling and preserving shortly after defaecation is recommended to prevent such negative impacts and improve the ultimate genetic assessment (TABERLET et al. 1999; SANTINI et al. 2007; BRINKMAN et al. 2010; AGETSUMA-YANAGIHARA et al. 2017; SCHULTZ et al. 2018; VELLI et al. 2019). Previous studies showed success with sampling within 1 h up to 24 h after defaecation in captive conditions and approximately 6 h up to 21 days under field conditions (WASSER et al. 1997; MURPHY et al. 2002; BERRY et al. 2007; MILES et al. 2015; CANU et al. 2017). So far, plains bison dung of free-roaming individuals were collected within 10 – 15 min for genotyping (GARDIPEE 2007). The obvious advantage of faeces detection in the latter case was the weald of the habitat of *B. b. bison* and, not only with the more forest-dwelling *B. bonasus* (KRASIŃSKA et al. 2014), seldom represents the given field conditions. In a genetic nutrition study for European bison fresh faecal samples were collected after GPS-tracking of collared individuals (KOWALCZYK et al. 2019), which might also not be possible for a comprehensive genetic population monitoring. Thus, it might be pivotal for e.g. monitoring programs and studies to utilise faecal samples a few days old. Yet, the genotyping success is not always predictable by the physical faecal appearance (BROWN et al. 2014). In this regard, samples collected months after defaecation showed an expectable increasing uncertainty towards genetic assignment sensitivity estimations (0.786 for a 200-day-old faecal sample) (RAMSEY et al. 2015). Accordingly, this holds still a certain informative power but might not be transferred one to one regarding the faecal consistency and environmental associated with European bison. With the two occasionally sampled aged faeces the present study could show that genotyping is potentially possible. Regarding this, marker sets consisting of more loci than microsatellite panels, such utilised in SNP panels, would be favourable. The single biallelic SNP locus holds less information for the genotypes but in turn would cause a lower GE if failed.

It was presumed that diet has an impact on DNA extraction from faeces (REED et al. 1997; FARRELL et al. 2000; GOOSSENS et al. 2000). But overall, genotyping success from faecal samples seems to be not heavily affect by the diet, but most likely by other parameters (BROQUET et al. 2006). Exemplary, in omnivorous brown bears (*Ursus arctos*, LINNAEUS 1758) general differences in dietary ratios of carnivory, herbivory and dietary fiber itself did not affect DNA yield (MURPHY et al. 2003). But, the digestion system in ruminants is radical different in contrast to a monogastric mammal (MITTERMEIER 2011). In general, wisent dung with a loose consistency was very common while collection. Thus, compared with more compact faeces from e.g. Canoidea (KRETZOI 1943) species wisent pats may do not strip the same amount of mucosa cells from the intestinal wall visible as light grey slough resulting in higher chances to gain higher host DNA yield from the faecal surface (MURPHY et al. 2003; HÁJKOVÁ et al. 2006; MILES et al. 2015; AGETSUMA-YANAGIHARA et al. 2017; VELLI et al. 2019). Such slough was not determinable on wisent pats. Nevertheless, differences in the amplification and genotyping successes of swab samples from the faecal surface versus the faecal interior were found here. The significant negative effect of samples from the faecal surface in comparison to sampling from the wisent pat interior might be connected to its environmental exposure and therefore to outer forces accelerating DNA degradation, such as UV light. Technically, it is more difficult to take up pure surface material than faecal substance from the wisent pat interior. Therefore, samples from the interior provides more faecal material, which in turn could positively influence the genetic analysis from such a sample. Consequently, in the following sampling it was recommended to cover the cotton tip of the swab decently with faecal material, no matter from which part of the wisent pat. Uneven distributions of intestinal cells in the faecal matrix itself, not showing explicitly mucosa slough and therefore randomly chosen for DNA extraction, leads to the additional problem of non-reproducible results of amplifiable DNA yields (KOHN et al. 1995; GOOSSENS et al. 2000). This can be avoided by homogenise the faecal sample during the collection process (WASSER et al. 1997; MURPHY et al. 2002). The advantage of preservation of full faecal samples in 96 % EtOH is that the comparable loose matrix of wisent dung could be homogenised afterwards within the permanent storage container if necessary.

Faecal sampling with swabs is cheaper, more practical and less prone to genetic contamination (VELLI et al. 2019). But the success of gaining genotypes also depends on the type of swab sample storage. Both methods presented here including drying faecal samples (flocked swab and dried swab samples) not only showed significant lower amplification rates but the highest ADO rates resulting in decreased genotyping success (Figure A 1). Drying faeces for subsequent DNA extraction by silica gel has been shown to be an applicable preservation and storage alternative to Drierite, freezing, freeze-drying, the usage of 70 – 100 % EtOH, preservative solutions for nucleic acids (RNA) or directly in DNA lysis buffer partly on different temperature levels (WASSER et al. 1997; FRANTZEN et al. 1998; NSUBUGA

et al. 2004). Especially, for long-term storage other studies found that silica gel together with RNA*later*™ Stabilization Solution (Invitrogen™) are more effective than 95 % EtOH (SOTO-CALDERÓN et al. 2009). A two-step storage procedure combining collecting in EtOH and subsequent drying with silica gel was shown to increase DNA amplification rates significantly (NSUBUGA et al. 2004). In contrast, the less successful results presented here with faecal samples collected and subsequently dried goes along with studies on long-term storage and silica dried faeces, which showed the lowest success in amplification rates and highest GE rates compared with preservation and storage in EtOH, DMSO/EDTA/Tris/salt solution (DETs) and by freezing (MURPHY et al. 2002). The DNA extraction for all faecal swab samples in the present study were done after five weeks after collection. The observation that some faecal samples moulded after a few days and subsequently may not dry fast enough to prevent DNA from further degradation were mentioned before (MURPHY et al. 2002; NSUBUGA et al. 2004). Especially, in large species like the wisent the amount of collected faecal matrix is a considerable factor for the silica drying approach. Even with collecting and drying swabs with relatively small amounts of faeces in this study the latter obstacle could not be eliminated. Thus, higher amounts of non-invasive samples could increase extracted DNA quantities. But concentrations of PCR inhibiting substances would also increase particularly in faeces (TABERLET et al. 1999). The faecal moisture content of taurine cattle varies between less than 80 to 90 % (VALIELA 1969) with a comparable faecal structure to the closely related *B. bonasus* (JANECEK et al. 1996; HASSANIN and ROPIQUET 2004; VERKAAR et al. 2004; KRASIŃSKA and KRASIŃSKI 2013; SOUBRIER et al. 2016; PALACIO et al. 2017). In contrast, the faecal moisture content of e.g. dogs (*Canis lupus familiaris* (LINNAEUS 1758)) dependent on diet, body size and breed, but was measured to be mostly under 80 % (MEYER et al. 1999). Here, this relatively high moisture content in wisent dung might be a further complication regarding the storage of dried faecal samples for genetic assessments compared with other species. This also applies potentially for storing faecal samples in EtOH or other solutions regarding a certain dilution effect by the contained water. But if used in a comprehensive genetic monitoring dried faecal samples like handled in this study are not recommended due to the risk of partial informative loss of the collection. If sampling in silica is required, subsequently desiccation with another method is recommended (MURPHY et al. 2002).

Beside the common method to store faecal samples in EtOH collecting swab samples directly in DNA lysis buffer are already established (HAYAISHI and KAWAMOTO 2006; AGETSUMA-YANAGIHARA et al. 2017; VELLI et al. 2019). In general, preservation in liquid solutions (DNA lysis buffer and 96 % EtOH) was more successful in the present study. Faecal samples (full faecal and faecal swab samples) stored in 96 % EtOH showed a higher variance in success rates compared with faecal samples stored in DNA lysis buffer (Figure A 1). Consequently, storage of faecal samples in EtOH represents an overall less consistently reliable method.

Other DNA extraction kits were used for faecal samples before, for instance the Blood & Tissue kit and DNeasy Tissue Kit (Qiagen Inc. Valencia, CA) for faecal full samples (MAUDET et al. 2004; BRINKMAN et al. 2010). Both DNA extraction kits utilised in the present study did not show a significant different impact on the amplification or genotyping success. Notably, storing faecal swab samples in ASL buffer shows highly significant negative different success and ADO rates to all other storage methods in liquids. The major difference between these two DNA extraction kits is that the ingredients that remove PCR inhibiting substances present in faeces are separately added to the ASL buffer during the extraction process in the form of a tablet, while the InhibitEX buffer already contains such inhibitors removing chemistry (see Qiagen kit instructions). The ingredients of both this InhibitEX tablet (QIAamp DNA Stool Mini Kit) and the InhibitEX buffer (QIAamp Fast DNA Stool Mini Kit), are not fully provided by the manufacturer (Qiagen) but might be very similar and consequently show a similar result for the DNA extraction itself. The fact that the InhibitEX buffer already contains these ingredients, might be the explanation for the significant positive effect of sampling faeces directly in the latter buffer (Figure A 1). DNA of the faecal samples collected directly in the ASL buffer might be less protected by lacking direct inhibitor suppression. The dehydrate quality of 96 % EtOH leads to a similar positive effect in this experiment, by inhibiting enzymatic activity degrading DNA (BEJA-PEREIRA et al. 2009). However, both faecal swab sample types (ASL/InhibitEX) were extracted in separate QIAcube runs. Thus, the possibility of an impact by handling failures during DNA extraction on the effect in the results regarding the strong differentiation cannot be ruled out. Since, searching for not only successful but practical methods the noticeably longer handling time in the extraction procedure for the samples stored in ASL buffer has to be considered. Longer handling durations as an important factor regarding sampling methodology evaluations was mentioned before (VELLI et al. 2019). Collecting faecal swab samples in InhibitEX buffer showed not only the highest success rates but also low variance and is therefore the best practice. Therefore, it is totally reasonable to choose the latter DNA extraction kit without further experiments including storing faecal samples in ASL buffer. Additionally, the QIAamp DNA Stool Mini Kit with the ASL buffer will become not commercially available in the near future (Qiagen pers. comm.).

Expectably, the storage duration had a negative effect on the success rates and positive effect on the ADO rate. Here, the presented data does not represent a continuous rate of DNA degradation but enables to recommend a contemporary DNA extraction based on a significant difference in success and error rates. Notably, the amplification and genotyping success was higher after five weeks after collection from samples stored in InhibiteEX buffer than the amplification success from full samples in EtOH after only one week after collection, which represents the both best practices tested here.

The sample collector field experience showed an effect especially on genotyping success from nuclear DNA of faecal samples. To reduce the negative effect from heterogenic skilled collectors

initial sampling training is recommended (RUIZ-GONZÁLEZ et al. 2013). Since the collection for this pilot study was done by only two collectors the possible error is equal and therefore neglectable. For further sampling throughout Europe detailed instructions were provided for the cooperation partners.

6.6.3.1.2 Non-faecal sampling

All 44 tissue samples (sampled between 1990 – 2018), two samples of lyophilised blood (2006) and a sole sample of lyophilised DNA (2006) from both breeding lines genotyped with the microsatellite panel provided overall error-free genotypes despite their partly high ages.

It was possible to generate a complete consensus genotype from every non-faecal sample presented here including from urine, saliva and nasal secretion.

Though, collection of frozen wisent dung brings potential difficulties during winter months (see 6.6.3.1.1) beside beneficial low temperatures regarding DNA degradation sampling in snow opens the possibility to utilise urine for genetic assessment (VALIERE and TABERLET 2003). Occasional swab samples from urine-soaked snow in the follow-up study represent a further collection opportunity without any additional preparation. Nevertheless, this sampling method relies on snow and might only be complementary in a comprehensive genetic population monitoring. Two urine swab samples directly taken from a meadow after urination in summer were also successful. Nevertheless, it was possible to generate the entire genotype from only this urine sample. But due to difficult visibility and evaporation pure urine samples could only be occasionally found and are not a promising frequent sample source in European bison.

Hair as a well proven and potential non-invasive source for genotyping was collected in the further comprehensive sampling. During moulting wisent intensively rubbing against tree trunks and stumps sometimes called ‘bison combs’ (KRASIŃSKA and KRASIŃSKI 2013). Especially for free-roaming herds those bison combs are potentially sources for non-invasive hair samples but also characterised by become polished, therefore heavily used and prone for genetic contamination. Such bison combs were not sampled within this study. Only in occasional cases hair samples were collected non-invasively utilising comparable objects like brushes and stable walls (Figure A 6).

This non-invasive saliva sample with genetic contamination was not only excluded from further evaluations but generally shows a weakness of non-invasive samples from the environment more prone to genetic contamination in general to be aware of.

6.6.3.2 Marker system

6.6.3.2.1 Microsatellites

Ten autosomal polymorphic microsatellite marker and the applicable sex marker are new for European bison. MM12 was successfully tested in American bison (MOMMENS et al. 1998). BM203

was previously determined as monomorphic in the LL line (LUENSER et al. 2005) but shows private alleles in three individuals of the LC line (allele frequency: 0.0306).

Previously it was shown that 17 microsatellites do not provide enough informative power to assess issues like individual identification and paternity assignment in European bison whereas SNP panel of down to 50 – 60 most heterozygous loci would be sufficient (TOKARSKA et al. 2009a). Expectably, with a new set of autosomal microsatellite markers this study can support these previous results. No sibling (see 6.6.2.3) and breeding line discrimination (see 6.6.2.4) was possible. The PID suggests that seven markers are sufficient to discriminate individuals but is disproven due to the fact that three individuals of the LC line from Russia (reference sample: lab#X161076, lab#X161076, lab#X161091) showed the same genotype. But the microsatellites used here were not preselected to address any issues for the European bison. Instead, they were implemented for the methodology evaluation regarding the optimal faecal sampling.

6.7 Tables

Table A 10: Overview of all digital files including detailed result tables (attached CD).

File name	content
Sample list_all Bovini_wisent project.xlsx	Complete sampling list of all Bovini samples with individual details
Genotype_list.xlsx	Complete SNP genotype list (96 loci) with individual details
SNP_marker_list_details.xlsx	List of all SNP markers tested in this study with individual details and additional data for all 96 loci of the final panel. Details on sex marker design.
SNP genotyping protocol adjustments.xlsx	Adjustments for the SNP genotyping protocols during the development phase
LD_90SNPs_Arlequin.xlsx	Detailed results with figures on LD of 90 loci polymorphic in *Bos bonasus* from Arlequin (not used)
Parental assignment_ml-relate_64SNPs_137IDS+msat.xlsx	Detailed results for parental assignment from *ML-Relate* in several matrices for microsatellites and SNPs (not used)
Parental assignment_COLONY_90_64SNPs_137IDs.xlsx	Detailed results from all SNP-based analyses on parental assignment from *COLONY* including pedigree data and the individualised samples that were assigned to individuals based on metadata.

6.8 Figures

Wild cattle
Tribe Bovini GRAY, 1821 (*n* ≤ 526)
Subtribe Bovina GRAY, 1821
Genus *Bos* LINNAEUS, 1758 (*n* ≤ 486)

European bison or wisent
(*Bos bonasus* LINNAEUS, 1758) (*n* ≤ 337)

lowland bison (recent lowland line (LL))
(*Bos bonasus bonasus* LINNAEUS, 1758) (*n* ≤ 108)

lowland-Caucasian line (LC)
(*B. b. bonasus* × *B. bonas caucasicus*) (*n* ≤ 229)

American bison
(*Bos bison* LINNAEUS, 1758) (*n* ≤ 43)

plains bison
(*Bos bison bison* LINNAEUS, 1758) (*n* ≤ 29)

wood bison
(*Bos bison athabascae* (RHOADS, 1898)) (*n* = 14)

yak
(*Bos mutus* PRZEWALSKI, 1883) (*n* ≤ 7)

domestic yak
(*Bos mutus grunniens* (LINNAEUS, 1766)) (*n* ≤ 10)

cattle
(*Bos primigenius* BOJANUS, 1827) (*n* ≤ 78)

taurine cattle
(*Bos primigenius taurus* (LINNAEUS, 1758)) (*n* = 33)

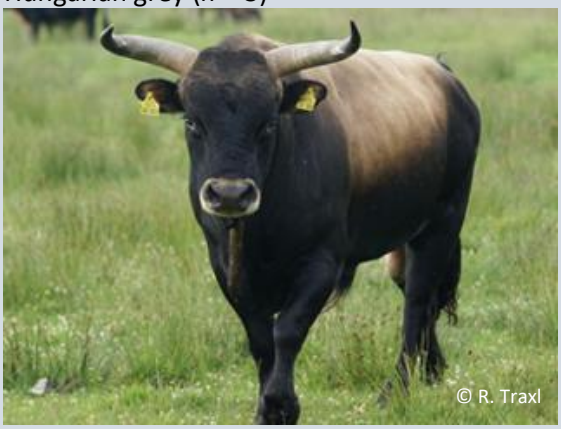
Oldgerman black pied (*n* = 2)

Harz red mountain (*n* = 2)

Hinterwald (*n* = 1)

Hungarian grey (*n* = 3)

Scotish highland (*n* = 12)



Heck (*n* = 6)

Holstein-Friesian (*n* = 3)

Fjäll (*n* = 1)

red mountain (*n* = 3)

African humpless shorthorn cattle (*Bos primigenius taurus*) (*n* = 3)

sanga cattle (*Bos primigenius taurus* × *Bos primigenius indicus*) (*n* ≤ 25)

Lagune or Dahomey (*n* = 3)

Ankole or Watusi (*n* ≤ 25)

indicine cattle or zebu
(*Bos primigenius indicus* (LINNAEUS, 1758)) (*n* = 17)

dwarf zebu (*n* = 11)

Caucasian dwarf zebu or Azerbaijani (*n* = 2)

Nelore zebu (*n* = 4)

Gaur
(*Bos gaurus* C. H. SMITH, 1827) (*n* = 10)

Indian gaur
(*Bos gaurus gaurus* C. H. SMITH, 1827) (*n* = 6)

gayal or mithun
(*Bos gaurus frontalis* (LAMBERT, 1804)) (*n* = 4)

banteng
(*Bos javanicus* D'ALTON, 1823) (n = 8)

Javan banteng
(*Bos javanicus javanicus* D'ALTON, 1823) (*n* = 8)

Buffalos
Subtribe Bubalina RÜTIMEYER, 1865 (*n* = 40)
Asian buffalos
Genus *Bubalus* HAMILTON-SMITH, 1827 (*n* = 13)
water buffalo (*Bubalus arnee* (KERR, 1792)) (*n* = 5)

Mediterranean water buffalo (river-type)
(*Bubalus arnee bubalis* (LINNAEUS, 1758)) (*n* = 4)

carabao (swamp-type)
(*Bubalus arnee bubalis* (LINNAEUS, 1758)) (*n* = 1)

lowland anoa
(*Bubalus depressicornis* (C. H. SMITH, 1827)) (*n* = 7)

mountain anoa
(*Bubalus quarlesi* (OUWENS, 1910)) (*n* = 1)

African buffalos
Genus *Syncerus* HODGSON, 1847 (*n* = 27)

Cape buffalo
(*Syncerus caffer* (SPARRMAN, 1779)) (*n* = 14)

forest buffalo
(*Syncerus nanus* (BODDAERT, 1785)) (*n* = 13)

Figure A 5: Systematic catalogue with exemplary photographs of all sampled Bovini taxa and ESUs in this study with the numbers of sampled individuals (*n*). Samples sizes with n smaller or equal represents the maximal individual sample size, due to not individually assignable samples while collecting in the field. Photos show not necessarily sampled individuals. If not labelled otherwise the pictures were taken by the author.

Figure A 6: Non-invasive sampling of wisent hair from a rubbing brush into a sip-lock bag with silica gel. Photo: VICTORIA REUBER

Figure A 7: Invasive sampling of hair from the forehead of a female wisent (LL line). This exact method is not possible in the majority of collecting. Most invasive hair samples were taken while anaesthetisation or in corral systems. Photo: FELIX RUDZINSKI

Figure A 8: Corral systems like in Lelystad (Natuurpark) are recommendable installations to sample e.g. invasive but innocuous hair samples without anaesthetisation. Photo: RANDY VAN DOMSELAAR

Figure A 9: Collecting faecal swab sample into a 2.0 ml reaction tube with lysis buffer. A decent amount of faecal matrix should be transferred to 100 µl buffer. The wisent pat shown here has a comparable solid consistance. Photo: VICTORIA REUBER

Figure A 10: Collecting full faecal sample into a collection cub with 33 ml 96 % EtOH with an one-way forceps. The wisent pat shown here has a comparable loose consistance, which is more common. Photo: KAJA HEISING

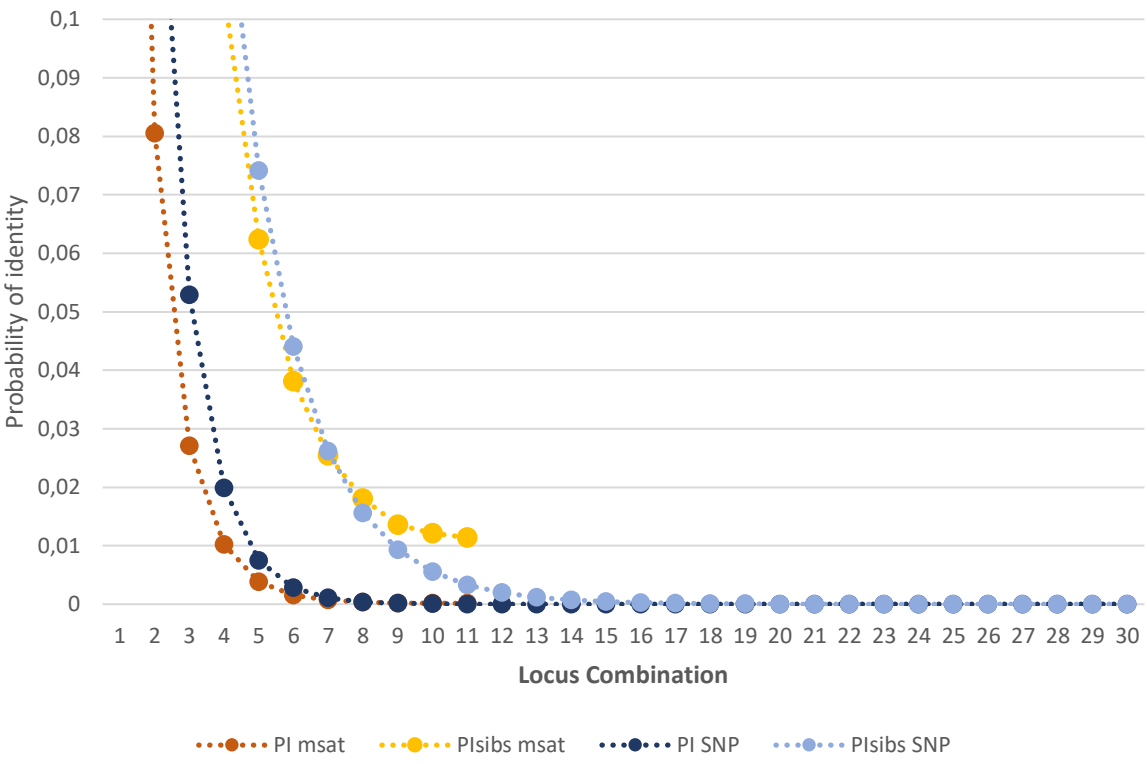


Figure A 11: Probability of identities for two marker panels (microsatellites (msat) vs SNPs) for European bison. PID and PiDsib are depicted for poth marker panels (microsatellite panel: $n = 11$; SNP panel: $n = 90$). X-axis was cut at locus combination of 30 loci for more conciseness. The approximation does not change after 30 loci.

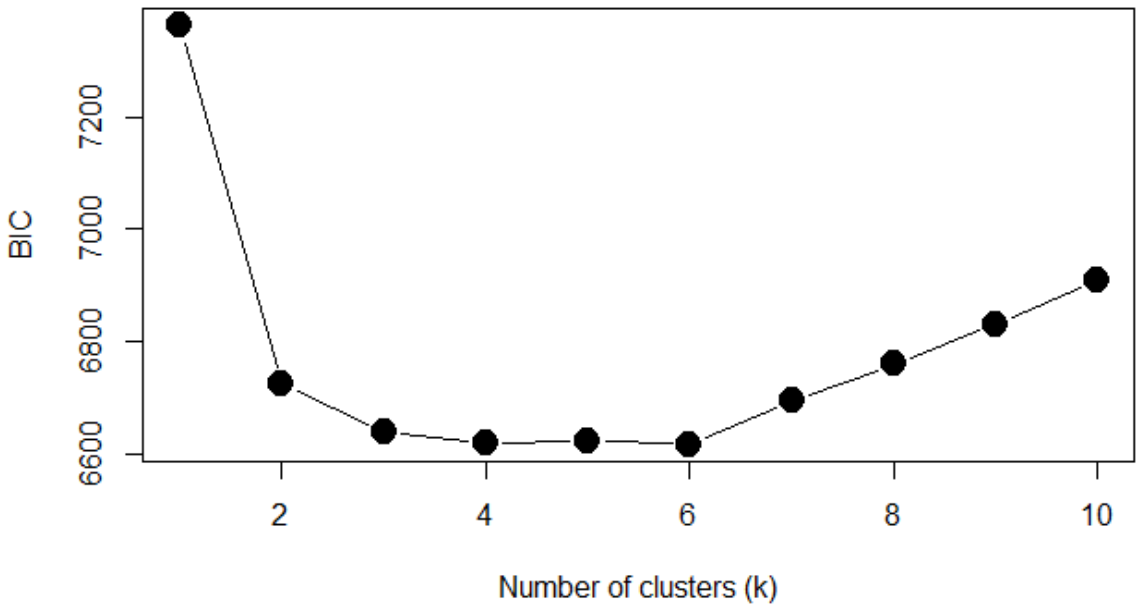


Figure A 12: BIC for one to ten assumed *K* from maximum-likelihood genetic clustering with 29 SNP markers and 137 European bison. The subset of 29 markers was selected to desriminate between two breeding lines in the wisent. The lower BICs for $K = 3$ to 6 might reflect genetic structures of close related individuals.

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