Population structure, reproductive behaviour and population density of the European wild boar, *Sus scrofa*, based on STR marker studies of hunting bags in Lower Saxony, Germany

PhD Thesis

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Population structure, reproductive behaviour and population density of the European wild boar, *Sus scrofa*, based on STR marker studies of hunting bags in Lower Saxony, Germany

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"One of the most significant and disturbing factors affecting the success of wildlife management is the management itself" (*Oliver Keuling on several occasions*)

Summary

Population densities and relative abundance of the European wild boar, *Sus scrofa*, has been steadily increasing throughout Europe for decades. Due to climate changes, wild boar is able to expand into climatic regions that have long been unsuitable habitats. Intensified agricultural use has resulted in the availability of large quantities of crop fields as a habitat and food source. The omnivorous choice of food enables an increasing expansion even into urban areas. Furthermore, wild boar has exceptionally high reproduction rates. This overpopulation leads to a variety of problems e.g. intensive crop damage or increasing numbers of vehicle collisions in both rural and urban areas. In addition, the wild boar is host to numerous zoonoses, of which African swine fever (ASF) in particular is a great endangerment to the pig fattening industry.

Despite a significant intensification of hunting measures, it has not yet been possible to reduce the populations. High reproduction rates, enormous plasticity and inadequate hunting strategies makes harvest rates insufficient. In addition, hunting success has been difficult to quantify in concrete numbers. This makes it difficult to realise the aforementioned population reduction.

Great efforts have also been made by researchers to find out more about the biology and behaviour of wild boar. For 20 years, the use of DNA markers, such as STRs, in ecology has been one of the central instruments for determining e.g. kinship relationships to demonstrate the influence of anthropogenic barriers or habitat fragmentation on gene flow between populations.

One of the most important sources of information for managing wild boar are the annual culling numbers, the hunting bags. However, the hunting bag is not suitable for population estimates based on simple extrapolations, as the actual population size is significantly underestimated. The central task of this work is to link hunting bag as a source of genetic samples and data with the use of STR markers to answer specific questions concerning genetic structuring, extent of alternative reproductive tactics and density of populations using kinship-based capture-mark-recapture (CMR) methods.

Using 14 microsatellites and 910 wild boar samples from different hunting bags, it was possible to identify eight distinct subpopulations in the study area. The reasons for the substructuring were not always the same and the genetic exchange between populations is rather low. In addition, multiple paternity rates, i.e. a litter of a pregnant female is fertilised by more than one male, of 23%-30% were detected in some populations. The assumption that male biased hunting strategies could influence MP rates does not seem to be confirmed. With the development of a genetic CMR estimator, it was possible to establish a population density calculation method that can compute robust population sizes when the necessary sampling requirements are met. The practical application of the g-CMR estimator resulted in an underestimation of the actual population size, mainly due to too small sampling sizes.

This study has shown that the hunting bag is a suitable tool for collecting genetic samples and generating genotype data, which can be used to answer a wide range of questions, but here is still a greater need for optimisation when calculating population densities. Unrestricted use of the hunting bags can therefore not yet be recommended at the present time. It therefore seems reasonable and purposeful to optimise the procedure presented here in further studies in order to use the full potential of genetic studies of the hunting bag.

Contents

Abbreviations

%	Percentage
°C	Degree Celsius
µ	Micro
µl	Microliter
A	Adenine or Number of Alleles per Locus
AFLP	Amplified Fragment Length Polymorphisms
ASF	African swine fever
bp	Base Pair
C	Cytosine
CE	Capillary Electrophoresis
CI	Confidence Interval
CMR	Capture-Mark-Recapture
COI	Cytochrome-c-Oxidase
COX	Cytochrome-c-Oxidase
CR	Capture-Recapture
CsCl	Caesium Chloride
CSF	Classical swine fever
CT	Camera Trap
DS	Distance Sampling
DC	Drive Counts
DJV	Deutscher Jagdschutz-Verband e. V.
DMK	Deutsches Maiskomitee e. V.
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
F(Null)	Frequency of Null Alleles
FAWF	Forschungsanstalt für Waldökologie und Forstwirtschaft Rheinland-Pfalz
FOXP3	Forkhead-Box-Protein P3
g	Gram
G	Guanine
g/cm^3	Gram per Cubic Centimetre
g-CMR	Genetic Capture-Mark-Recapture
HB	Hunting Bag
H_{exp}	Expected Rate of Heterozygosity
H_{obs}	Observed Rate of Heterozygosity
HPLC	High Performance Liquid Chromatography
HWE	Hardy-Weinberg Equilibrium
K	Number of Subpopulations/Cluster
kb	Kilo Base
kg	Kilogram
km	Kilometer
km^2	Square Kilometers
LnP(D)	Log-Likelihood Score
MCMC	Markov Chain Monte Carlo
mL	Milliliters
mM	Millimole
MS	Microsatellite
mtDNA	Mitochondrial DNA

n/km2	Animal per Square Kilometre
NN	No Data Available
NS	Not Significant
ODl	Other Deciduous Trees with a Long Lifespan
ODs	Other Deciduous Trees with a Short Lifespan
PCR	Polymerase Chain Reaction
P_{HWE}	Probability of Deviation from Hardy-Weinberg Equilibrium
PIC	Polymorphism Information Content
PMS2	Mismatch Repair Protein
POPs	Parent-Offspring Pairs
Pr (X│Y)	Posterior Probability for each K
Q	Estimated Membership Coefficient
REM	Random-Encounter-Model
RFLP	Restriction Fragment Length Polymorphisms
satDNA	Satellite DNA
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SP	Summer Population
SSP	Surrounding Saupark
SSR	Single Sequence Repeat
STR	Short Tandem Repeat
Stdev	Standard Deviation
STR	Short Tandem Repeats
T	Thymine
VNTR	Variable Number of Tandem Repeats
WP	Winter Population

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1. Introduction

1.1. Genetic variation at multiple loci and different marker types in ecology: a brief history

Genetic variations describe the differences in deoxyribonucleic acid (DNA) between different individuals of a species, between different populations of a species, but also the differences between species (Mitchell-Olds, 1995; Nevo, 1978; Schielzeth & Husby, 2014). The driving force behind this variation process, or the emergence of such differences, are chemical structural changes in the DNA, so-called mutations. Mutations can change the order of a nucleotide sequence. The sequence of the four nucleotides occurring in DNA, adenine (A), guanine (G), cytosine (C) and thymine (T), can be permanently changed by point mutations such as substitution, deletion or insertion. This process results in polymorphic variants of a specific DNA sequence range, also known as allele. An allele can therefore consist of different variants. Instead of a single point mutation, entire groups of nucleotides, e.g. base triplets, can also be affected. In both cases, there can be a change in the nucleotide sequence as well as a change in the sequence length. These processes can affect protein-coding areas of the DNA as well as non-protein-coding areas of the DNA. Therefore, an allele is not necessarily a gene or part of a proteinogenic gene sequence. However, mutations can also affect the entire genome, e.g. by changing the number of chromosomes or altering the spatial structure of an individual chromosome.

Describing DNA variations at multiple loci and their genetic markers, including allozymes, mitochondrial or nuclear DNA sequence regions, satellite DNA markers such as mini-, midi- or microsatellites, or SNPs, have been essential for answering a wide range of ecological questions for almost six decades. With the discovery of allele-related, enzymatic polymorphisms, which gave the allozymes their name, it was possible for the first time to describe the extent of genetic variation within and between populations. Methodologically, the analysis was identical for both, fresh tissue samples of animal or plant origin, as all tissue types contain enzymes (review Allendorf, 2017; Harris, 1966; Lewontin & Hubby, 1966). Prior information about the sampled species was therefore not necessary, because species specific DNA markers were not necessary. In addition, due to their low mutation rate, allozymes allow conclusions to be drawn about barriers restricting gene flow, level of genetic variation in protein-coding genes or the recolonization of habitats since the last ice age (Dobrynin et al., 2015; Selkoe & Toonen, 2006).

Using restriction enzymatic analyses of mitochondrial DNA (mtDNA), sequence variations within and between populations of rodents were detected for the first time, which represent the origin of phylogeography (Avise et al., 1979a; Avise et al., 1979b). At the same time, the female inheritance lines of mtDNA were used to determine the sex of lizards (Brown & Wright, 1979). mt-DNA analyses, in particular cytochrome-c-oxidase (COX or COI) barcode analyses, have subsequently established themselves as a fundamental tool for understanding species diversity, biodiversity and species conservation (review e.g. Avise et al., 2016; Hebert et al., 2003).

In contrast to most genomic regions containing satellite DNA, genetic analyses with single nucleotide polymorphisms (SNP) have the advantage that they are not limited to the non-coding regions of the genome. This allows analysing an almost unlimited number of SNP loci in the entire genome. SNPs enable much clearer representations of population structures than allozymes and have therefore replaced them in many research areas (see Dann et al., 2013). As SNPs are found in non-coding and coding regions of DNA, so-called non-synonymous SNPs in coding areas can lead to an amino acid change at the codon affected and consequently to an altered protein structure. Such point mutations are the cause of various human genetic diseases that can affect membrane proteins (e.g. serotonin 5-HT2A receptors), the immune system (e.g. FOXP3) or sperm DNA damage and risk of male infertility (e.g. PMS2). SNPs represent the most frequent nucleotide change, accounting for around 90 % of all genetic variations in the human genome.

One of the first approaches in population genomics was the simultaneous study of numerous loci or genomic regions, which provided comprehensive insights into evolutionary processes such as mutations, natural selection, random genetic drift and gene flow, which can influence variation between genomes and populations (Luikart et al., 2003). Following the work of Hohenlohe et al. (2010), Allendorf (2017) analogously describes population genomics as follows: '*In a narrower sense, population genomics involves sampling the mapped genome at sufficient density to detect forces affecting a particular genomic region (e.g. regions of reduced recombination), i.e. rather than using a representative sample of loci to study the average effect of processes acting throughout the genome, population genomics characterises the variation of these processes along regions of the genome.*'

The temporal gap between the establishment of mitochondrial sequence analyses and SNPs is filled by a family of markers that are found specifically in highly repetitive regions of the genome. Differences in the GC content of certain genome fragments form the eponymous basis of satellite DNA and its markers.

1.2. Satellite DNA and its marker types

The observation that gave satellite DNA (satDNA) its name dates back to the 1960s (see i.a. Kit, 1961; Sueoka, 1961). The underlying idea was to centrifuge macromolecules in a caesium chloride solution (CsCl) at very high speed and for a sufficiently long time (up to 48h). The CsCl solution then forms a stable density gradient and the dissolved macromolecules come to a standstill in the so-called isopycnic zone, which corresponds to their buoyant density (g/cm^3). In density gradient centrifugation with DNA molecules, besides the main peak, which contains the main nuclear genomic DNA, other, larger and smaller fractions could be found. These flanking peaks have since been known as satellite DNA (Britten & Kohne, 1968). The fractions below the main peak are GC-rich DNA molecules, the fractions above AT-rich DNA molecules (Kit, 1961; review also Thakur et al., 2021). These satellite peaks were subsequently identified as large centromeric tandem repeats (Britten & Kohne, 1968; review also Ellegren, 2004). Contrary to previous assumptions, the eukaryotic genome is full of repetitive DNA patterns (Ellegren, 2004), including animals, plants and insects (Biscotti et al., 2015). These abundant, tandemly repeated noncoding DNA sequences of classical satellite DNA consist of a core DNA sequence, a so called core motif, of 5-300 base pairs (bp), in every possible bp combination, that is repeated up to a million times, resulting in sequence lengths between 100-5000kb (Butler, 2005; Munk et al., 2017). The total amount of these satDNAs in the genome, also known as 'satellitome', of some plants and animals can account for 30% or more. Although satDNAs do not encode proteins, they play a vital role in chromatin modulation and the establishment of centromeres (Rošić et al., 2014) and other distinct cellular functions (review Thakur et al., 2021).

It was then subsequently shown that even smaller, so-called minisatellites (Jeffreys et al., 1985a) can be found throughout the genome (see e.g. Bell et al., 1982). These sequences, also known as 'variable number of tandem repeats' (VNTR) (Nakamura et al., 1987a), differ from classic satellite DNA by having both shorter core motifs (approx. 10-100 bp) and shorter total lengths (approx. 100 bp to 20 kb) (Chambers & MacAvoy, 2000; Munk et al., 2017; Tautz, 1993). The even shorter repetitive core motifs between 1-6 bp, which are only repeated a few hundred times at most, have become known as short tandem repeats (STR), simple sequence repeats (SSR) or just microsatellites (Litt & Luty, 1989; Weber & May, 1989). However, the nomenclature is not always standardised with regard to motif length and the degree of motif repetition (e.g. Tautz, 1993) and so there are overlaps in the length specifications, especially for mini and microsatellites. In addition, motifs with only 1-2 bp or significantly more than 300 bp are also found in classical satDNA (e.g. Tautz, 1993). For example, sequences were found early on in the human genome that, in strict terms, are too long for a minisatellite but too short for a classic satDNA. Such intermediate sequences are also known as midisatellite' (Nakamura et al., 1987b), although they can also be described as exceptions to the rule - minisatellites (Tautz, 1993).

1.2.1. Fragment length polymorphisms

1.2.1.1. Restriction fragment length polymorphism

The discovery that repetitive DNA patterns occur in the human genome to an extent not previously thought possible was further revolutionised by another discovery. The average heterozygosity of human DNA can be regarded as rather low at ~ 0.001 per bp (Cooper & Schmidtke, 1984; Jeffreys, 1979). Restriction endonucleases can therefore be used specifically to cleave DNA fragments of the same length in single-copy DNA from different individuals (Botstein et al., 1980; Jeffreys, 1979). However, even small changes in the DNA sequence, usually due to base substitutions, but also deletions and insertions, can have a lasting effect on the very specific cleavage sites of restriction endonucleases. Such sequence changes can either make existing cleavage sites unusable for an enzymatic reaction or create new cleavage sites elsewhere in the DNA (Cooper & Schmidtke, 1984; Jeffreys, 1979). In both cases, compared to the original copy and fragment length, one or more new fragments of different lengths are created, the so-called restriction fragment length polymorphism (RFLP) (Botstein et al., 1980; Nathans & Smith, 1975). The disadvantage of these fragments was initially the fact that they are often only dimorphic, i.e. the original cleavage site is either present or absent. Accordingly, the information content of these fragments was only of limited use. In kinship analyses with homozygous individuals, for example, the information content is often zero (Jeffreys et al., 1985a). Nevertheless, it was possible to prove that gene loci or alleles can be polymorphic.

1.2.1.2. Variable number of tandem repeats

Using the human genome, it was shown for several genes, including ß-globulin (Miesfeld et al., 1981), cardiac actin (Hamada et al., 1982) or somatostatin (Shen & Rutter, 1984), that large quantities of simple tandem (TG)n repeats can be found there. Comparisons between different individuals show that the number of these tandem repeats is hypervariable, leading to differences in sequence length and consequently to allelic polymorphisms (Jeffreys et al., 1985a). Genetic sequences that contain such tandem repeats but only represent a single locus were then designated as VNTR (Nakamura et al., 1987a). Based on Botstein's RFLP technique (Botstein et al., 1980), the first successful identity tests were carried out with human DNA and parental genotypes were clearly identified by visualising fragment size differences, creating the basis for DNA fingerprinting (Jeffreys et al., 1985b).

1.2.1.3. Short tandem repeats

The fact that microsatellites also exhibit such fragment length polymorphism was also shown only a shortly time later (Litt & Luty, 1989). Subsequently, the combination of Botstein's definition of the polymorphism information content (PIC) (Botstein et al., 1980) and the development of exponential amplification of DNA fragments using polymerase chain reaction (PCR) (Saiki et al., 1988) made it possible to make both quantitative and qualitative conclusions about hypervariable microsatellites (Litt & Luty, 1989). There are several reasons why microsatellites have been used successfully for almost 30 years in a wide variety of research and diagnostic applications.

As already mentioned, they show the necessary allelic polymorphism to display individual genotypes, but they also offer a number of other advantages. Despite the desired variability regarding the number of core motifs, the flanking DNA sequences of a microsatellite are highly conserved and stable against mutations, an important prerequisite for the immutability of primer binding sites (Litt & Luty, 1989; Selkoe & Toonen, 2006; Tautz, 1989; Weber & May, 1989). Some of these binding sites are conserved to such an extent that some different species share the same microsatellite (Selkoe & Toonen, 2006). Due to rather small core motifs of one to six nucleotides per motif, which are usually repeated 5 to 40 times, the fragments can be easily amplified via PCR and their fragment length can be determined with base accuracy using capillary electrophoresis (CE). Nuclear motifs with 2-4 nucleotides per motif, so-called di-, tri- and tetranucleotides, are usually favoured (Ellegren, 2004). In addition, microsatellites are inherited co-dominantly and are located in non-protein coding and selection-neutral regions of DNA (Ellegren, 2004; Fan & Chu, 2007).

Nevertheless, there are a number of exceptions. In humans, microsatellites play a central role in some cases of neurodegenerative diseases and in some types of cancer (Tóth et al., 2000).

In total, more than 40, often trinucleotide STR-associated diseases are known in humans that can be found in coding regions (Orr & Zoghbi, 2007). These include, for example, Huntington's disease (Dayalu & Albin, 2015; Ha & Jankovic, 2011) and various forms of ataxia (Matsuura et al., 2000).

With mutation rates between 10^{-2} and 10^{-6} per locus per generation, microsatellites mutate significantly faster than non-repetitive DNA, which mutates at a rate of 10^{-9} (Ellegren, 1995, 2000, 2004; Fan & Chu, 2007; Selkoe & Toonen, 2006). However, the mutation rates of the different alleles vary and depend on the number of motif repeats, motif type, sex, age and other factors (Ellegren, 2000; Schlötterer, 1998; Selkoe & Toonen, 2006; Wierdl et al., 1997)

Apart from these exceptions, all requirements are fulfilled for microsatellites to serve as a general source to display the relationships of individuals within a population, as well as between populations (Tautz, 1989). This enables the determination of family relationships, population genetic studies, demographic patterns, genome mapping and forensics (Ellegren, 1995, 2004). The reliable identification of individuals by microsatellites enables accurate estimates of parent-offspring relationships as well as sibling and half-sibling relationships.(Christie, 2010; Costa et al., 2012; Putnova et al., 2003).

1.3. The European wild boar, *Sus scrofa*

1.3.1. Origin and present distribution in Europe

The European wild boar, *Sus scrofa* (Linnaeus, 1758) has become one of the most prominent ungulate wild animal species in almost all Europe (Acevedo et al., 2014; ENETWILD consortium et al., 2022a; Massei et al., 2015; Neil et al., 2016; Velickovic et al., 2016). The originally geographical distribution in longitude direction reached about 165 latitudinal lines over three continents, Europe, Africa and Asia, from ca. 15° western longitude to 150° eastern longitude. In latitude direction probably from ca. 60° northern latitude to 15° southern latitude (Briedermann, 2009; Heptner et al., 1966; Markov et al., 2022; Markov et al., 2019). From the 17th century to the 1930s of the past century in many European regions mostly hunted into extinction, today there are few countries and territories without continually growing wild boar stocks. Even Norway, supposed to be wild boar-free until the early 2000s, is settled since nearly 10 years. At present, Ireland is the last European country without free-living wild boar stocks. These pan-European territory recoveries initially happened slowly from the eastern parts of the former USSR, but in recent times, they reached almost exponential levels.

Concurrently with this, the original distribution boundaries extended in all directions. Especially the northern distribution reached 66° northern latitude nowadays (Markov et al., 2019). Even urban areas were settled more and more (Podgorski et al., 2013). Cities like Barcelona (Cahill et al., 2012; Cahill et al., 2003; Hagemann et al., 2022) or Berlin (Kotulski & König, 2008; Stillfried et al., 2017) register a persistent wild boar increase since two decades. Free-living wild boar populations in southern parts of the USA, Argentina, Brasilia, Australia and New Zealand are the direct result of anthropogenic influences (Briedermann, 2009; Cowled et al., 2008; Delgado-Acevedo et al., 2011; Gongora et al., 2004; Sparklin et al., 2009). The European wild boar can be therefore considered as a ubiquitous species today. For further and more detailed information see also de Jong et al. (2023).

1.3.2. Sounder structure and organisation

Female wild boars are philopatric and highly social mammals, organised in matriarchic dominated family groups, the sounders (Briedermann, 1986; Keuling et al., 2010). In most cases a wild boar sounder is usually composed of one or two older, but not necessarily adult females who leads the family group (Briedermann, 2009). Groups with a higher number of older females are rather rare. There is often a genetic relationship between these females in parent-offspring terms, or a sibling or aunt relationship (Kaminski et al., 2005). However, the female members of the sounder are subject to strict social ranking structures and only a single sow represents the alpha female, which accordingly determines all social activities of the group (Keuling et al., 2014). These activities include territory selection, home ranges size and synchronisation of the oestrous cycle (Keuling et al., 2008a). The numerical majority of a sounder is often represented by the offspring of the adult and subadult members, the piglets of either sex. However, the occurrence of sounders with only one adult mother animal and its piglets is not uncommon, especially in areas with high adult female densities or low quality territories (Happ, 2017). Older males are never member of such a sounder. When the male members of the group have reached about their first year of life and reach sexual maturity, they are chased out of the family group (Andrzejewski & Jezierski, 1978; Happ, 2017). Female subadults did not necessarily remain in their natal sounder as well. Subadult animals of both sexes can form yearlings-sounders of limited duration. These communities of purpose can consist of mixed sex members or are male-only. The females of such mixed groups will usually try to establish their own sounder, but often stay close to the natal home range (Briedermann, 1990). In such cases it can also happen that unrelated subadult females form new sounders in which the genetic relationship described above does not exist (Iacolina et al., 2009). The males, in turn, become permanent solitary, at the latest when their first reproductive season starts. Accordingly, the degree of distribution of wild boar living in a particular habitat is male-biased (Keuling et al., 2010).

1.3.3. Reproduction parameters, mortality and alternative reproductive tactics

As mentioned above, wild boar family groups are controlled by older and experienced females and sexually mature males are only tolerated in the sounders periphery during the reproductive season. The beginning of the sexual maturity in males usually takes place between 15th to 18th month of life (Happ, 2017; Meynhardt, 1989), under favourable environmental and nutrition conditions even sooner, between the 9th to 14th month of life (Keuling et al., 2010), tendency to fall further (Keuling et al., 2014). Even though the amount of sperm produced can fluctuate by around 25% in either direction over the course of the year, the males are capable of reproducing all year round (Bonet et al., 2013), but do not provide male parental care. Females can attain sexual maturity even earlier. If the environmental and nutrition conditions are beneficial as well, female piglets can reach sexual maturity between the 5th and 8th month of life (Gethöffer et al., 2007) and the average gestation period is 114 days. Due to the seasonality of mating and farrowing times, females are described as seasonally polyoestrous, i.e. they can ovulate several times during a mating season (Bonet et al., 2013; Gethöffer et al., 2007).

The average litter size of wild boar is higher than that of comparable sized ungulates (Carranza, 1996; Rosell et al., 2012), even in comparison with other mammals (Millar & Zammuto, 1983). There can be significant regional fluctuations in litter size due to the influence of food or weather. Therefore, Europe-wide fluctuations of 2-7 (Lombardini et al., 2014), 2-8 (Fonseca et al., 2010), 2-10 (Müller et al., 2018), 2-9 or 3-10 (Gethöffer et al., 2007), and 1-14 foetuses per litter (Servanty et al., 2007) have been documented. Accordingly, there are large differences in the average litter size, ranging from 3 in Andalusia, Spain (Fernandez-Llario & Carranza, 2000) to 6.7 in Hungary (Nàhlik & Sándor, 2003). Statistically significant differences in litter size between adult and subadult animals are hardly quantifiable in some regions and reach between 5-7.6 embryos per litter for both age classes (Gethöffer et al., 2007). At the same time, the embryonic mortality is comparatively low, with only 8%-25% (Ahrens, 1984; Gethöffer et al., 2007; Nàhlik & Sándor, 2003) with a declining tendency. Embryonic and perinatal mortality rates about 25% often originate from study during the mid- and late-eighties until late-nineties of the past century (Ahrens, 1984; Martys, 1982). Studies in the recent 15-20 years reach barley more than 15% (Gethöffer et al., 2007; Nàhlik & Sándor, 2003).

If the main causes of postnatal mortality, hunting and, to a certain extent, traffic collisions, are excluded, mortality rates are low (Keuling et al., 2013; Toïgo et al., 2008). Natural-postnatal mortality in wild boar is essentially made up of starvation due to extreme weather conditions, diseases and, in some cases, wolf predation (Massei et al., 2015). A central reason for low natural mortality rates is the intensive and months-long care of the piglets by the female parents (Kaminski 2005). On the one hand, this results in a mother-daughter bond that sometimes lasts for years and, on the other hand, it forms the basis for the often high genetic relatedness between adult and subadult females of a sounder, as mentioned above (Kaminski et al., 2005; Keuling et al., 2010).

A traditional and widely accepted view is that a single strong adult male monopolises all fertile female members of a sounder (Briedermann, 1986). Accordingly, males are polygamous and females should be monogamous. The ability of females to ovulate several times during a mating season basically allows them to copulate with different sires. This behaviour is well documented in various domestic pig breeds (Sumption & Adams, 1961; Sumption et al., 1959). In wild boar, evidence from the recent past suggests that this behaviour is also practised (Delgado et al., 2008; Poteaux et al., 2009) In addition to the postulated monogamous behaviour of females, sperm competition occurs in these cases, which is synonymous with alternative reproductive tactics.

Sperm production can be costly for males and therefore is a limiting factor and can restrict the number of offspring (Dewsbury, 1982). However, multiple mating can be advantageous for males because it than increases the chance to sire more offspring. In contrast to this, females cannot necessarily increase their direct number of offspring by mating with several males due to a limited number of mature oocytes. A potential benefit for females actively practicing multiple mating is sperm competition (Parker, 1970). In mating systems where dominant males try to monopolize several females, as assumed in European wild boar, sperm competition is unavoidable if these monopolization attempts fail. This pre- and postcopulatory competition enables females to select for the most suitable or viable sperm for their offspring. In addition, multiple mating offers the females several advantages, such as enhanced genetic diversity (Bergeron et al., 2011) and genetic quality (Jennions & Petrie, 2000) of their offspring, a reduced homozygosity (Charlesworth & Charlesworth, 1987), or optimised resistance to parasites (Coltman et al., 1999). However, multiple mating are not necessarily made by choice, but can also be the result of sexual harassment (Cappozzo et al., 2008; Carranza & Valencia, 1999; Fitze et al., 2005; Fox, 2002). In wild boar populations with no or very few dominant adult males, such behaviour could occur due to a surplus of subadult and juvenile males (Gayet et al., 2016; Gayet et al., 2021).

1.3.4. Population trends and reasons for the continuous population growth

Over the past four decades, there has been a steady increase in wild boar populations across Europe (Augustsson et al., 2024; Boitani et al., 1995; Keuling et al., 2013; Markov et al., 2022; Massei et al., 2015). While the total number of wild boar hunted in Germany in 1982/1983 hunting year was 123,165, it rose to 882,231 in the all-time record hunting year 2018/2019 and most recently reached 462,220 (see Figure 1.1) (Source: Deutscher Jagdschutz-Verband e. V. (DJV): https://www.jagdverband.de/jagd-und-wildunfallstatistik, 2024.01.15).

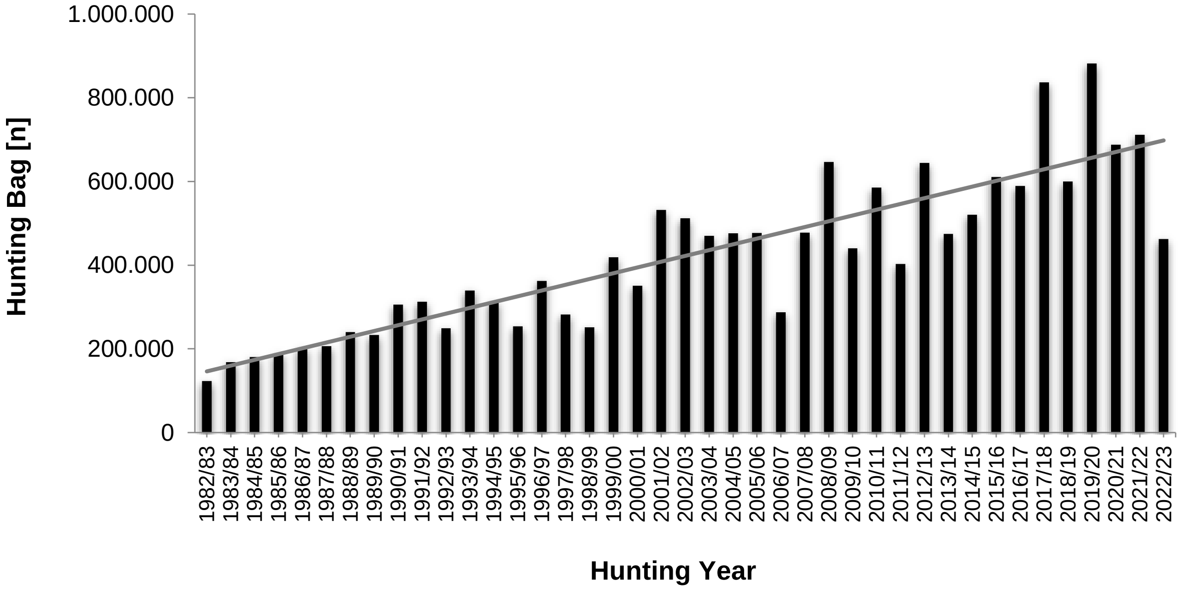

Figure 1.1: Historical development of annual hunting distances in Germany since 1982/83. The grey trend line shows the increase over time. (Source: Deutscher Jagdschutz-Verband e. V. (DJV): https://www.jagdverband.de/jagd-und-wildunfallstatistik, 2024.01.15)

Similar trends can be observed in many other European countries (Baubet et al., 2004; Cahill et al., 2003; Croft et al., 2020; Ferreira et al., 2009; Tsachalidis & Hadjisterkotis, 2009). The reasons for this significant increase are numerous. The climate changes of recent years have made the wild boar one of the major winners (Geisser & Reyer, 2005; Vetter et al., 2020). In this context, the years with full mast, years in which the amount of acorns, chestnuts and beechnuts is particularly high, have increased, resulting in an enormous improvement in food availability (Övergaard et al., 2007). Full masts are one of the main components of the wild boar's diet (Briedermann, 1986; Meynhardt, 1989), and can have a positive effect on population growth (Bieber & Ruf, 2005; Stier & Keuling, 2009). This optimised food availability has a direct influence on the reproduction rates of female wild boar. It is not the age of the females that is relevant for the number of offspring, as originally assumed, but weight and body mass (Frauendorf et al., 2016; Gamelon et al., 2013; Gethöffer et al., 2007; Müller et al., 2018). Due to the favourable physiological conditions, sexual maturity can also be reached even earlier and juvenile females participate in reproduction at an increasingly earlier age (Frauendorf et al., 2016; Gethöffer et al., 2007). The intensification of agriculture also leads to an improved food supply (Geisser & Reyer, 2005; Herrero et al., 2006; Schley & Roper, 2003), but also facilitates spatial dispersal, as maize and rapeseed fields provide a substitute for undergrowth and thickets in summer and autumn (Herrero et al., 2006; Keuling et al., 2008a, 2009; Rosell et al., 2012). The omnivorous and unpretentious selection of diet also contributes to the fact that wild boars can colonise a wide variety of habitats (Baubet et al., 2004; Gamelon et al., 2013; Keuling et al., 2008a; Schley & Roper, 2003). The ability to colonise new habitats (Geisser & Reyer, 2005; Markov et al., 2019), including the recent trend of urbanisation (Cahill et al., 2012; Hagemann et al., 2022; Stillfried et al., 2017), continues to contribute to their steady population increase. In addition, natural mortality decreases in warmer winters with less snow, especially among young and subadult animals (Geisser & Reyer, 2005; Stier & Keuling, 2009). If supplemental food is then provided by humans despite mild winters (Keuling et al., 2008a), food availability is practically guaranteed for the entire year with all the consequences described above.

1.3.5. Causes of conflicts

With the steadily increasing population sizes and densities and the continuing geographical expansion, there are a variety of problems and conflicts. This population expansion primarily affects agricultural landscapes, where wild boar can cause enormous economic problems and damage (Augustsson et al., 2024; Cecchini et al., 2023; Goedbloed et al., 2014; Herrero et al., 2006; Rosell et al., 2012; Schley et al., 2008). Their foraging behaviour can change entire habitat characteristics and ecosystem structures (Genov et al., 2017; Massei et al., 2015; Valente et al., 2020). The aforementioned expansion into urban areas is also leading to ever-increasing human-wildlife conflicts (Cahill et al., 2012; Hagemann et al., 2022; Podgorski et al., 2013; Stillfried et al., 2017). The number of vehicle collisions is therefore increasing not only in rural areas but also in urban areas due to rising populations (Häggmark et al., 2014; Miraglia & Di Brita, 2023; Thurfjell et al., 2015; Zuberogoitia et al., 2014). Although it has been shown many times that the reproductive potential of females in particular has increased significantly in the recent past (Frauendorf et al., 2016; Gethöffer et al., 2007), this continues to be underestimated (Keuling et al., 2016). The same applies to population densities, which are too often incorrectly rated and underestimated (Keuling et al., 2013; Massei et al., 2015; Massei et al., 2011).

One of the most serious problems associated with high population densities is the apparently unstoppable spread of African swine fever (ASF) (Bergmann et al., 2021; Podgorski & Smietanka, 2018; Sauter-Louis et al., 2021a). It arrived in the EU in 2014 and has been spreading ever since (Jori et al., 2021; Linden et al., 2019; Mazur-Panasiuk et al., 2020). The high mortality caused by the virus can have a greater impact on a population than hunting mortality (Morelle et al., 2020). However, the death of the animals does not mean that the virus has disappeared from the remaining population, but remains infectious even after the death of an infected host due to its high environmental resistance (Rogoll et al., 2024). As vaccination is not yet possible, the only way to contain the virus is to regulate the wild boar populations or prevent them from spreading further (Podgorski & Smietanka, 2018).

1.3.6. The hunting bag as genetic information supplier

Throughout Germany and Europe, hunting bags are at a very high level and numbers continue to increase, with occasional periods of stability (Keuling et al., 2013; Massei et al., 2015). As a source of information on relative abundance or population structures and dynamics in the form of sex ratios or age class distributions, the hunting bag is a very suitable and reliable resource (Briedermann, 2009; ENETWILD consortium et al., 2018). Reproductive parameters such as the average litter size can also be reliably calculated from the hunting bag (Fonseca et al., 2010; Gethöffer et al., 2007; Lombardini et al., 2014). However, hunting bags cannot be compared regionally and cross-regionally per se. Due to a wide variety of influences, hunting bags can give a very biased impression of the actual population. Some of the most common influencing biases are legal restrictions in protected areas or legal requirements in the form of shooting quotas, but also different hunting methods and traditions, and different or changing hunting efforts (ENETWILD consortium et al., 2018; Keuling et al., 2021). Therefore, the hunting bag is not always related to the actual development of the population, but reflects the varying hunting effort. In principle, comparisons between different hunting regions and also between European countries are possible, but the factors described must be taken into account when making them.

Although population trends can be identified from the hunting bags as a long-term source, they are no suitable indicator of the current and accurate population size or density, e.g. due to the varying hunting efforts described above. Although summer and winter populations can be approximately calculated using simple statistical extrapolations, underestimates by a factor of three are not uncommon (Keuling et al., 2014). In this context, the incorrect assumption is often made that the size of the hunting bag reflects actual population sizes, which leads to the inaccurate conclusion that a quantitative decline in a hunting bag compared to previous years is equivalent to a fundamental population reduction (Acevedo et al., 2007; Baber & Coblentz, 1986; ENETWILD consortium et al., 2018).

In general, the hunting bag is a central source of information for studies focussing on genetic issues. However, it is surprising that there are comparatively few studies on the causes of e.g. genetic diversity or structuring etc., particularly in the area of larger mammals such as ungulates, and thus also wild boar (Frantz et al., 2012; Griciuvienė et al., 2021). The potential of hunting bags as a genetic information source is widespread and enables a variety of different questions to be addressed, such as litter size (Frauendorf et al., 2016), phenotypic variation in highly variable environments (Gamelon et al., 2013), population bottlenecks (Ferreira et al., 2009), genetic diversity and differentiation (Choi et al., 2014; Mihalik et al., 2020), alternative reproductive tactics (Poteaux et al., 2009), effects of habitat fragmentation (Griciuvienė et al., 2021), or urban island populations (Hagemann et al., 2022). For estimating population sizes and densities, the hunting bag has not yet been used as an underlying source of genetic information. In this context, conventional methods such as camera traps (Random-Encounter-Model approach), drive counts, or distance sampling with thermography and others are used (ENETWILD consortium et al., 2018). As hunting in Germany is often based on the 'Lüneburger model' (Hennig, 1998; Teuwsen, 1980), there is comparability between the respective hunting grounds from which the hunting bags originate. None of the sexes should be excessively focussed on hunting and, in accordance with the model's specifications, the proportions of culled age classes should also be similar (Keuling et al., 2014). Under such conditions, the hunting bag should be a representative reflection of the actual population.

1.3.7. Aims and structure of the present thesis

The central aim of this study is to determine whether, in view of the ever-increasing wild boar populations throughout Europe and the associated permanent increase in culling numbers, these hunting bags can be used as a source of genetic data to answer a wide range of questions. The use of STR markers in ecology has been one of the central tools for determining specific relationships in populations for 20 years. The individual display of genotypes allows (i) genetic population structuring to be characterised, (ii) the extent of alternative reproductive tactics, and (iii) the abundance and density of populations to be determined using kinship-based capture-mark-recapture (CMR) methods, in particular using parent-offspring pairs (POPs). In general, to answer these and similar questions, separate efforts are made to obtain appropriate genetic sample material. Accordingly, a lot of time and resources should be saved if the same data source contains all the necessary information to answer all questions. All the following chapters therefore have the same protagonist: the hunting bag.

In Chapter 2, the genetic relationships of all wild boar sampled by the hunting bag and their corresponding population membership will be presented. A Bayesian clustering approach using multilocus genotype data will be used to determine the most likely number of possible subpopulations. By quantifying the population clusters, it should be possible to determine the extent of genetic exchange between the respective subpopulations, and whether and which barriers, natural, anthropogenic or behavioural, cause or favour this structuring. In this context, potential indicators that could bias the cluster calculations will also be identified. In addition, this chapter presents the topographical and forestry characteristics of all forestry districts that took part in the study. In addition, a descriptive evaluation of the hunting bags is provided in terms of age and sex classes as well as the most important microsatellite indices of all analysed genotypes.

Chapter 3 focusses on a very specific part of the sampled hunting bag, i.e. exclusively female parent animals for which the extent of multiple paternities is determined on the basis of uterine examinations. The male sires are not known in the present case, but their genotypes can be reconstructed manually from the sampled foetuses and by computer-aided parentage analyses. Whether multiple paternity populations occur at all in German wild boar and to what extent age and weight could play a role is not yet known and should be quantified accordingly. Based on current knowledge, the extent and intensity of multiple paternity is possibly caused by different hunting strategies on the male members of a population, which can lead to changes in the usual mating behaviour. As the present study assumes moderate hunting, the rates of multiple paternity are expected to be lower than in studies with correspondingly more intensive hunting strategies. In addition, possibilities of cryptic female choice and associated sperm competition are also presented. In this context, a causality between varying numbers of foetuses per uterus in the presence of multiple sires would also be possible.

Chapters 4 & 5 evaluate the possibilities of using the hunting bags of two sub-areas of the present study as a data basis for determining population density using kinship-based or genetic capture-mark-recapture (g-CMR) methods in the form of parent-offspring pairs (POPs). Since no physical recapture of an already culled animal is possible, the use of genetic information in a hunting bag, in this case the detection and number of parent-offspring pairs, must be used as genetic recapture. The underlying assumption is that the larger a population is, the lower the probability that parents and offspring will occur together in the hunting bag subsample. Chapter 4 therefore aims to clarify whether a classic capture-mark-recapture method (CMR), such as the Lincoln-Petersen or Chapman estimator, can be adapted and extended to a genetic-based capture-mark-recapture method (g-CMR) by adding the genetic element of kinship. In addition, the type and extent of possible biases are to be identified and quantified. It can be assumed that the size of the subsample or the non-random hunting of parents and offspring can have a massive influence on the validity of the calculated population size. Accordingly, the practical feasibility of the g-CMR estimator will be examined in Chapter 5. Adequate sampling of the hunting bags should provide sufficient numbers of parent-offspring pairs. In order to have comparative values, simple extrapolations were made from the hunting bag, but experience has shown that these can be assumed to significantly underestimate the actual population size. Accordingly, the values calculated with the g-CMR should be higher. Parallel to the present study, other methods were also used to determine population densities in the same study areas (but review Keuling et al., 2014). This should allow sufficient validation of the g-CMR results and potential biases to be identified.

In chapter 6, all the previous results will be reviewed and summarised and, where appropriate, contextualised in the latest state of knowledge. Finally, the quality, advantages and disadvantages of the hunting bag as an exclusive data source will be assessed and evaluated. The thesis concludes with an outlook on the current technical possibilities for improving existing laboratory procedures in order to achieve the current state of opportunities and modern research.

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2. Descriptive and demographic analysis of genotype data and hunting bag metadata

2.1. Introduction

The European wild boar (*Sus scrofa*) is one of the biggest winner in the European agricultural landscape and of global climate change (Vetter et al., 2020). While distribution and abundance of many species are declining, other species are adapting better to increasingly longer summer and shorter and milder winter periods together with an increasingly changing agricultural landscape, and their population numbers and abundance are growing (Ozgul et al., 2010; Parmesan, 2006). Barley any other ungulate species has reproduced more successfully and colonised new habitats in the past four and a half decades than wild boar (Markov et al., 2022; Markov et al., 2019). They are no longer found exclusively in forests. They have long since extended their living and roaming areas into the parks and front yards of the suburbs of cities (Cahill et al., 2012; Hagemann et al., 2022; Stillfried et al., 2017) and have relocated their summer residences to cropland like maize or rapeseed (Herrero et al., 2006; Keuling et al., 2008a; Rosell et al., 2012). The ecological plasticity and adaptability of this species seems to know no bounds and all climatic or agricultural changes seem to favour further population growth. The expansion is currently only limited by extreme polar winters and extremely dry deserts or desert-like areas (Markov et al., 2022; Wehr, 2021). Conditions that hardly ever occur in Europe, with the exception of localised periods of aridity. The Central European mild winters of recent years have significantly reduced natural mortality, especially in the juvenile classes, piglets and subadults (Arnold, 2005; Briedermann, 2009; Keuling et al., 2013; Massei et al., 2015; Stier & Keuling, 2009; Toïgo et al., 2008). At the same time, despite these mild conditions, annual supplemental food with up to 1,000 kg per square kilometre is still provided by hunters and year-round decoy feeding is used to make it easier to kill game and increase shooting numbers (Cellina et al., 2006; Hespeler, 2000; Hohmann & Huckschlag, 2010; Keuling et al., 2008a, 2009; Massei et al., 2015). However, in view of the increasing frequency of mast and full mast years in oak, beech and chestnut, supplementary winter feeding in particular appears to be completely superfluous (Bieber & Ruf, 2005; Cellina, 2008; Hennig, 1998; Meynhardt, 1989). As a direct consequence, a feed deficit for the majority of wild boar populations can no longer be assumed at any time of the year. Parallel to the steady increase in the wild boar population, the area of farmed maize has also increased significantly. In the 1960s, the German area cultivated for grain maize was 60 km^2, in the 1980s it was already 1200 km^2 and in 2023 it was 4700 km^2 (Source: © Statista 2024, https://de.statista.com/statistik/daten/studie/28893/umfrage/anbauflaeche-von-koernermais-in-deutschland-seit-1960/, 2024.05.09). The area under silage maize, mainly grown as forage maize and partly for the production of biogas, is around 20,000 km^2 (as of 2022, Deutsches Maiskomitee e. V. (DMK), 2024.05.09). Due to the adaptability of the wild boar, an area of 25,000 km^2 can be used as a thicket replacement and food source in the summer and autumn months before harvest (Keuling, 2001; Keuling et al., 2014; Keuling et al., 2008a, 2009). Accordingly, it is not surprising that for the wild boar a reproductive capacity of 300% can be assumed under optimal conditions (Briedermann, 2009; Cellina, 2008; Gethöffer et al., 2007; Keuling et al., 2014).

The combination of smaller forest areas in landscapes with increasing agricultural land use, appears to favour an expansion of wild boar populations in areas that were previously unsuitable as permanent habitat; wild boar populations were therefore rare and only found in small numbers (Herrero et al., 2006; Keuling et al., 2014; Schley et al., 2008). The seasonal habitat selection of wild boar, with smaller foraging areas in the winter months and more extensive foraging areas in summer, e.g. when crops have reached a sufficient height to serve both as undergrowth, thicket replacement and as a food source, also favours further expansion (Herrero et al., 2006; Keuling et al., 2008a, 2009; Meynhardt, 1986). The fact that populations with previously rather small and manageable numbers can increase rapidly and significantly due to the short-term increase in food availability and new summer habitats therefore seems plausible. The extent to which wild boar migrate from their original areas to these newly available habitats is almost completely unknown, as there is hardly any data available on such migration movements. Accordingly, it is not known from which geographical direction or location animals migrate into new habitats. Wild boars are considered to be very territorial or philopatric, with rather small sized home ranges between 0.5 km^2 - 9.0 km^2. At the same time, foraging trips through their own home range, especially at night, tend to be rather short at 4 km (Keuling et al., 2014; Podgorski et al., 2013). In contrast, however, nocturnal migration movements within the home range up to16 km are also known, but were found in urban environments (Podgorski et al., 2013). Larger migrations of entire groups or individual animals are therefore typically not expected and are not part of such studies. However, if groups or individual animals do leave their original territory, they usually do so over a longer period of time. In this context, migration movements of 30-40 km in a linear distance over a period of up to 6 months have been documented (Keuling et al., 2014). Especially animals of the juvenile classes, between 1-2 years old, roam around in this way and can double their weight during this time. Such data is mostly derived from radio telemetry studies that reflect the movement patterns of individuals or small family groups. In particular, differences in spatiotemporal behaviour between different habitats or different sexes and age classes, home range sizes diurnal movements and activity patterns can be documented excellently in this way (Johann et al., 2020; Keuling et al., 2008a, 2009; Podgorski et al., 2013). However, by using telemetry it is not possible to visualise migration movements of entire (sub)populations or between populations.

From a wildlife management perspective, information on gene flow in particular would be of great benefit if 1) one wanted to determine the extent of population structuring and which populations in an area are in exchange with each other and 2) to what genetic extent. 3) Migration directions of populations into previously not or rarely colonised areas could also be depicted in the same way. The present study was part of a 3-year survey in central northern Lower Saxony, that was focused on radio-telemetric space and time utilisation studies and camera trapping to monitor activity patterns and population numbers (Keuling et al., 2014). In addition, tissue samples from the annual hunting bags, including single-, hide- and drive hunts, were collected in the study area over the entire study period. The study area is rather unusual in many respects with regard to a classic wild boar area. On the one hand, the proportion of forest is rather low compared to the rest of Lower Saxony and on the other hand, the contiguous forest areas of 5-6 km^2 are rather small for larger ungulate wild life populations. In addition, the proportion of the area used for agriculture is very high in some cases at 75%. The proportion of wet marshes and moors and the resulting high proportion of grassland is also considerably higher than in many other parts of Lower Saxony. Although such a habitat composition cannot be regarded as optimal for larger wild boar populations, the annual harvest numbers have been rising steadily for years.

In addition, classical swine fever (CSF) was already detected in wild boar populations in neighbouring areas of the study region between 1992 and 2002 (Sodeikat et al., 2010). Since 2020, African swine fever (ASF) has also continued to spread in Germany (Richter et al., 2023; Sauter-Louis et al., 2021b). However, in order to be able to take preventive measures to control diseases in wild boar, for example, it is urgently necessary to determine the genetic spread and distribution corridors that can serve as dispersal routes for a large number of wild life diseases such as ASF, CSF or Aujeszky's disease (pseudorabies).

This study focuses on analysing and evaluating information concerning genetic structuring, age and sex distribution from several forestry districts in northern Lower Saxony and their hunting bags of European wild boar. The annual hunting bags are the most frequently used source of information for wild boar management activities and strategies or abundance and density calculations (Briedermann, 2009; ENETWILD consortium et al., 2018). As a long-term data source, the hunting bags provide a reasonably reliable overview of the general population developments and trends as well as the age distribution and sex ratio in recent years. However, particularly with regard to population trends, this is exclusively a look into the past. The extent of natural fluctuations in population size and culling numbers between individual hunting years can therefore only be determined with a time delay of several years in some cases. Simple statistical projections and extrapolations of current annual population densities from a hunting bag have so far been difficult to determine with great inaccuracies (for a detailed review see ENETWILD consortium et al., 2020). Genetic analysis of samples from a hunting bag is rarely carried out (but see Chapter 5 and Müller et al., 2018; Müller et al., 2020). Accordingly, there is hardly any information available as to whether and to what extent genetic exchange takes place between the populations of different hunting grounds or forestry districts, if there are several (sub)populations at all. Therefore, no statements can be made as to whether the anthropogenic forestry district boundaries are home to one or more populations of wild boar. The central question of the present study is therefore whether sampling of the respective forestry district hunting bags is a suitable means of detecting population (sub)structuring. Although hunting bags are generally balanced overall, at least in Central Europe (Keuling et al., 2013), regional and seasonal differences and biases in the age and sex of hunted animals in particular can occur (Keuling et al., 2010; Toigo et al., 2008). As a result, the kinship proportions in the corresponding hunting bags could be over- or underrepresented, which could represent a further biasing factor. If biases of this kind can be detected, they must be taken into account in the population structuring calculations. In principal, hunting bags should contain all the necessary genetic information to establish a link between a spatial area or habitat, the location of the cull and a genetic population membership. The individual genetic identification of each sample from the hunting bag by means of multilocus genotyping using 14 microsatellite loci is expected to reveal a population (sub)structure. If the aforementioned forest and habitat fragmentation in the study area results in a loss of habitat connectivity, or if there is a more complex infrastructure network consisting of roadways or comparable barriers, the free exchange of genes should at least be affected and should be able to be identified as distinct subpopulations. A Bayesian clustering approach using multilocus genotype data (Hubisz et al., 2009; Pritchard et al., 2000) should enable the number of possible subpopulations to be determined, and consequently the extent of genetic exchange between the different forestry districts and how far possible genetic clusters extend spatially. In newly or recently colonised habitats, possible founder populations could be identified if they have been sampled. The possibility to assign all sampled individuals of a hunting bag to a specific population represents a source of information that is still rarely used in practice in order to expand, optimise and sustainably improve the possibilities of wildlife management. If, for example, genetic dispersal and distribution corridors could be identified on the basis of hunting bags, measures such as the establishment of ASF protection corridors and high-risk corridors could be taken to reduce genetic and thus disease-transmitting exchange.

2.2. Materials and methods

2.2.1. Study area

2.2.1.1. Topographical and forest conditions in Lower Saxony

With an area of around 47,614 km², Lower Saxony is the second largest federal state in the Federal Republic of Germany and the fourth largest federal state in terms of human population with around 8.14 million inhabitants (as at 31 December 2022). By far the largest part of Lower Saxony, around 60% (approx. 28,500 km^2), is agricultural land. Around 22% (approx. 10,500 km^2) of the state is woodland. Here, coniferous forests account for around 50%, while mixed and deciduous forests each represent around 25%. Only just over 12% (approx. 6000 km^2) is used for buildings (including open spaces) or transport areas. The remaining utilisation type is made up of recreational areas, water areas, etc. (Source: Lower Saxony State Office for Statistics). Although Lower Saxony belongs to the temperate climate zone of Central Europe, it is located in the transition zone between the maritime climate of Western Europe and the continental climate of Eastern Europe. Accordingly, the north-west is characterised by an Atlantic (North Sea coast) to sub-Atlantic climate, with comparatively low temperature amplitudes over the course of the year and a water balance surplus. The climate of the south-east is increasingly influenced by the continent, characterised by greater temperature differences between the summer and winter seasons as well as lower and unevenly distributed precipitation throughout the year. The average annual temperature is 8 °C (Source: Lower Saxony, represented by the Lower Saxony State Chancellery: https://www.niedersachsen.de/startseite/land_leute/das_land/zahlen_fakten/niedersachsen-in-zahlen-20094.html, 2024.05.09).

The federal state of Lower Saxony can be divided into three different topographical regions, some of which differ greatly in terms of their respective forested areas and forest composition. The proportion of woodland in the 1) western lowlands of Lower Saxony is 15%, in the 2) eastern Lowlands it is 41% and in the 3) Lower Saxony highlands it is 33%. The study area of the present study and its respective districts were located in the western lowlands of Lower Saxony. In terms of forest composition, there is an east-west gradient in Lower Saxony on the one hand and a north-south gradient on the other. In the densely forested eastern lowlands, conifer species such as pine (*Pinus sylvestris*) and spruce (*Picea abies*), but also larch (*Larix sp.*), dominate the tree population of the forests with an average of 70%. In the sparsely wooded western lowlands, the proportion of pine in particular is reduced by almost 25%, whereas the proportion of oak (*Quercus sp.*) and other deciduous tree species such as birch (*Betula sp.*), alder (*Alnus sp.*), poplar (*Populus*) and willow (*Salix sp.*) is increasing. In the south-facing highlands of Lower Saxony, spruce is the dominant conifer species, with pine hardly being found. The deciduous tree species there are mainly characterised by beech (*Fagus sylvatica*), followed by oak (see Figure 2.1) (Source: Lower Saxony Ministry for Nutrition, Agriculture and Consumer Protection: https://www.ml.niedersachsen.de/startseite/themen/wald_holz_jagd/walder_fur_niedersachsen/die-bundeswaldinventur-3-127660.html, 2024.05.09).

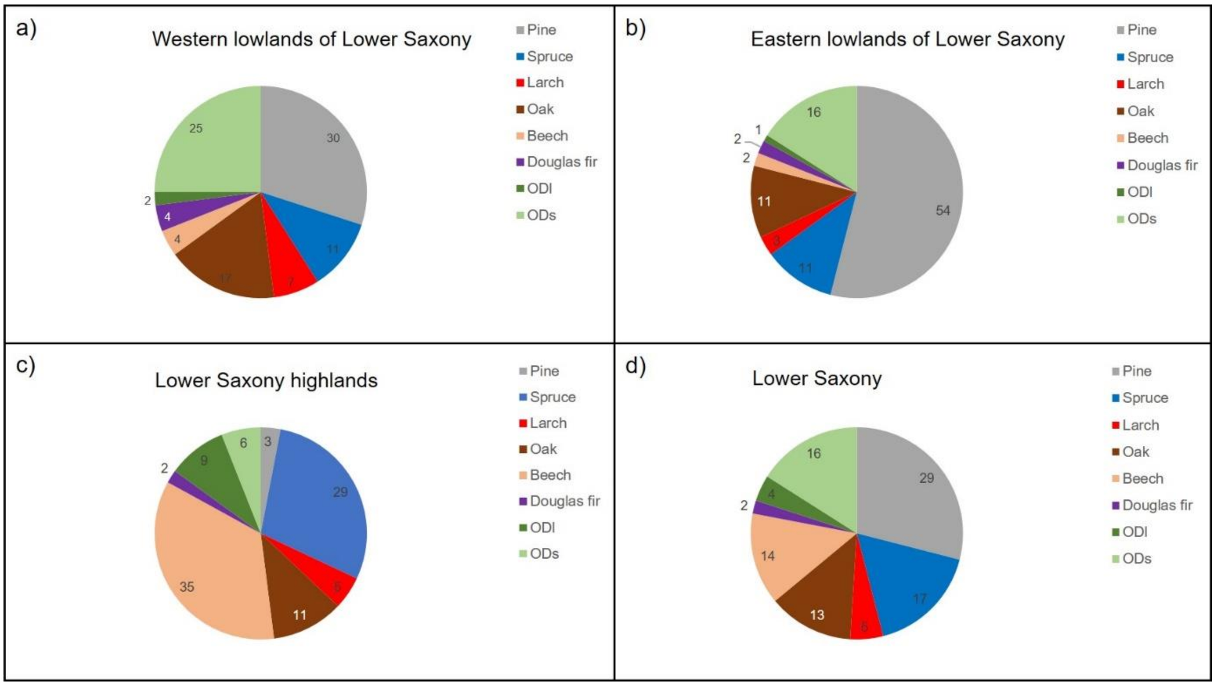

Figure 2.1: Various species gradients between conifers and deciduous trees in different areas of Lower Saxony (a-c) and in the state as a whole (d). The study took place in the western lowlands of Lower Saxony (a). Abbreviations in the legend: ODl = other deciduous trees with a long lifespan; ODs = other deciduous trees with a short lifespan (Source: Lower Saxony Ministry for Nutrition, Agriculture and Consumer Protection: https://www.ml.niedersachsen.de/startseite/themen/wald_holz_jagd/walder_fur_niedersachsen/diebundeswaldinventur-3-127660.html, 2024.05.09).

2.2.1.2. Sampling sites

The present study was part of the "Wild boar management in Lower Saxony" survey, carried out from 2011 to 2013 (Keuling et al., 2014) in the Harsefeld forestry commission office in northern Lower Saxony. In addition to this project, samples were taken from other forestry commission offices in order to obtain a more comprehensive impression of wild boar population structures in Lower Saxony. Like all other German federal states, Lower Saxony is divided into forestry commission offices, which in turn are subdivided into further, so-called forestry districts. In total, samples were collected in 12 different forestry districts over the course of the project. The first samples were already taken in 2010 in three forestry districts: Betzhorn (Unterlüß forestry commission office), Danndorf (Wolfenbüttel forestry commission office) and Hämelerwald (Fuhrberg forestry commission office). The aforementioned forestry commission offices were already the subject of intensive wild boar investigations in previous years (for a detailed review see Keuling et al., 2011; Sodeikat et al., 2010). In 2011, the above-mentioned project was launched, which was heavily focussed on the Bevern and Hallah forestry districts. The district Bevern covers an area of about 480 km^2 and the district Hallah about 520 km^2. Both district consist of 60% agricultural area, 36% woodland and other habitat structures, such as marsh or hedgerow and 4% housing area (Keuling et al., 2014). However, the contiguous forest areas are a maximum of 5-6 km^2 in size and accordingly, this region of Lower Saxony tends to be characterised by rather small forest areas that are often completely surrounded by agricultural land, mainly maize (*Zea mays*) but also rapeseed (*Brassica napus*). The forest composition in both districts consists of ca. 70% conifers like spruce and pine and 30% deciduous tree species such as beech and oak. The maize cultivation area includes 50% in Bevern, but only 37% in Hallah. Concerning the proportion of grassland, the situation is almost reverse. The study areas in Bevern and Hallah were supplemented by more northerly forestry districts in Holzurburg and Rüstje, which are characterised by similar topographical conditions. Beginning in 2012, data collection and sampling was expanded to include another wild boar population in “Süsing” (Oerrel forestry office) in the east of Lower Saxony. The "Süsing" forest area, which covers around 6.5 km^2, is part of a contiguous and cross-district woodland with a total size of around 40 km^2. The dominant tree species is pine, followed by oak and spruce. Douglas fir (*Pseudotsuga menziesii*), larch , beech and other deciduous trees occur to a lesser extent. To the north and south, the forest area is bordered by agricultural land that is predominantly characterised by maize cultivation. Also in 2012, sampling of the southernmost area of this study began. The focus there was on a largely fenced-in (basically walled-in) wild boar population of the former imperial hunting lodge in Springe, Jagdschloss Springe (Saupark forestry commission office). Neighbouring areas in the forestry districts of Brünninghausen, Jägerhaus and Coppenbrügge complete the present study and the sampling sites. In the area under the management of the Saupark, beech forms the dominant forest community in almost all locations. The forest is supplemented by ash (*Fraxinus excelsior*), sycamore maple (*Acer pseudoplatanus*), cherry (*Prunus sp.*) or lime (*Tilia sp.*) on nutrient-rich sites or spruce and Douglas fir on nutrient-poor sites. For a geographical distribution of the study areas described, see Figure 2.2.

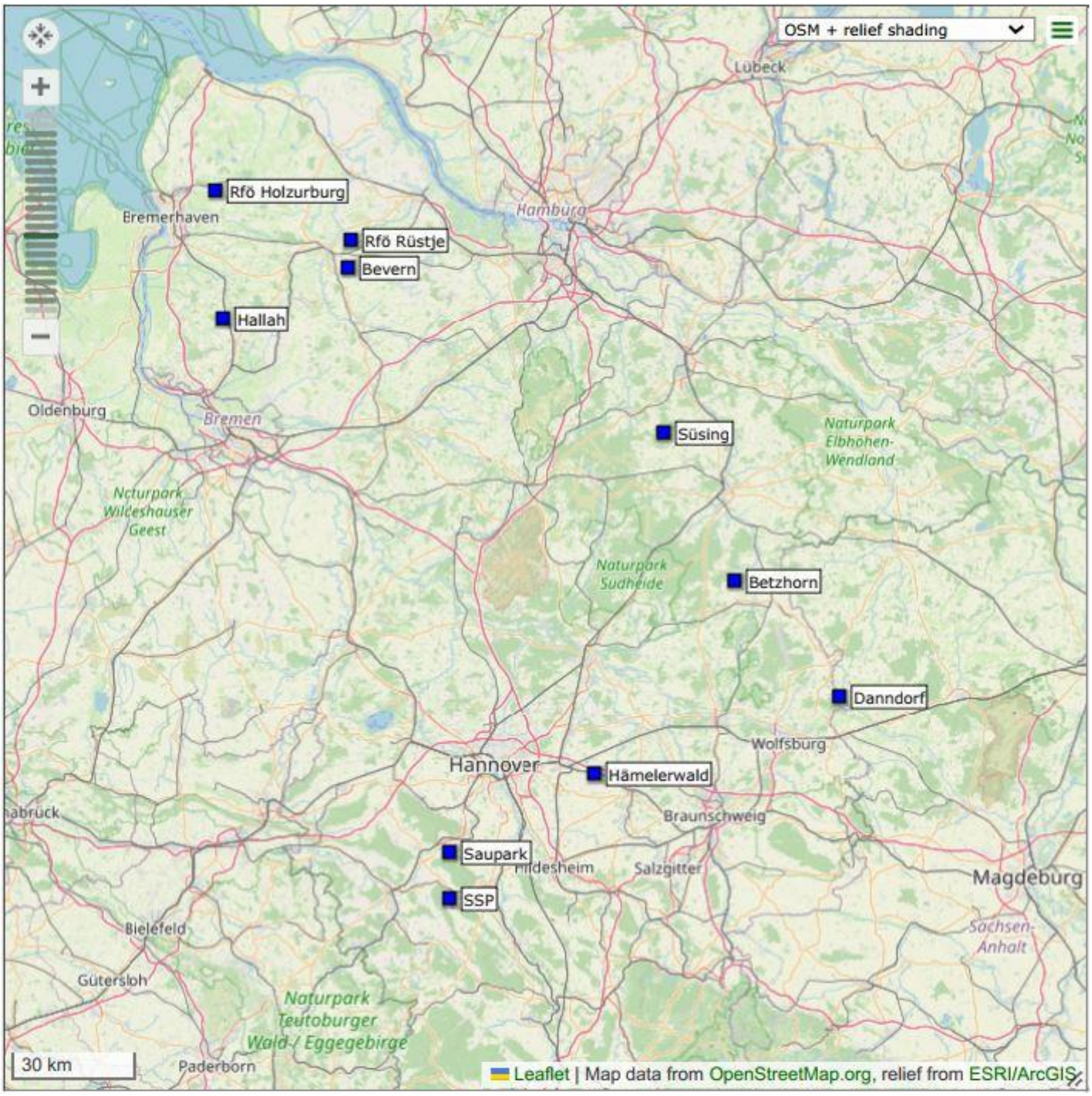

Figure 2.2: Overview on the location of the different study sites in central-northern Lower Saxony (Source: GPS Visualizer © 2003-2019; red = motorways; yellow = federal highways; black = railway lines; blue = rivers

2.2.2. Metadata sampling

The start and end of a German hunting year differ from those of a normal and regular year. As the reproduction and breeding seasons of many native wild animal species, especially ungulates, extend from the last quarter of one year to the first quarter of the following year, e.g. the hunting year of 2011 began on the 1st of April 2011 and ended on 31st March 2012. Accordingly, the 2012 hunting year begins on the 1st of April 2012 and not on the 1st of January 2012. This must be taken into account when referring to a hunting year in the following.

For all tissue samples taken during the study period 2010-2013, a sample accompanying document was prepared. All data that was collected for the corresponding tissue sample of an animal was entered on this form. This data included age, weight, sex, and date and location of the cull. In the present study, an approximate age categorisation into adult, subadult and piglet was estimated visually based on the body size and body mass. To some extent, a precise age categorisation of wild boar is generally possible based on dental abrasion, at least in a monthly range. However, this method is rather unusual in practice due to its time-consuming nature and was not used in the present study. However, when using body size and mass as indicators of age, a greater measurement error must be expected than in the dental assessment. The weight of the wild boar is taken as the so-called dressed weight (without digestive system, heart, lungs, liver, reproductive tract and blood). This means that all entrails and organs are removed before the weight is taken. A reliable sex determination is possible in every age group and was done visually.

2.2.3. Muscle tissue sampling

In the forestry districts of Bevern, Hallah, Holzurburg, Rüstje and Süsing the sampling was carried out as follows: muscle tissue samples of both sexes and all age classes were collected on drive hunts from October 2011 until February 2012, October 2012 until February 2013 and from October 2013 until December 2013. Additionally, sampling from single and hide hunts were collected consistently during the entire study. The first samples were taken there in April 2011 and the last in December 2013. Tissue samples (5–15 g) that were taken from all individuals, were stored in 5 mL tubes containing 4 mL 99.8% ethanol at 4°C for subsequent kinship analyses. The age classes were defined as follows: piglets aged up to 12 months, subadults aged 13–24 months, and adults aged 25 months and older.

An exception to the above-described sampling procedure is the Saupark. The hunting conditions there are different from any of the other districts. In the Saupark, only two short successive winter drive hunts are organised each year. Single or hide hunts are not practised during the rest of the year.

2.2.4. Uterine tissue sampling

The samples from the Betzhorn, Danndorf and Hämelerwald forestry offices from 2010 represent a special case within the present study. Here, only female wild boars that were pregnant were sampled on drive and single hunts between October 2010 and February 2011 (Müller et al., 2018; Sodeikat et al., 2010). This part of the data set is therefore made up of the female parents and their direct offspring. All uteri samples, pregnant females and their full litters, were dissected, examined, and measured in the lab (University of Veterinary Medicine Hannover, Foundation). The amount of tissue removed from both, mothers and embryos, the alcohol volume and concentration, and storage temperature were identical to the conditions described above.

2.2.5. DNA extraction, amplification of microsatellite primers and PCR-Conditions

DNA extraction was performed following the Chelex^{TM} 100 (Bio-Rad) protocol (Walsh et al., 1991). All 14 microsatellites used in this study were amplified by PCR with Peqlabs "Taq all inclusive" kit (formerly PEQLAB Biotechnologie GmbH, now VWR International, LLC). A detailed overview of all microsatellites used, the PCR conditions and all other necessary steps concerning the DNA laboratory can be found in Chapter 5.

2.2.6. Genotyping and allele binning

Genotyping was performed on an automated CEQ 8000 or CEQ 8800 series Genetic Analysis Systems (both Beckman Coulter). PCR products were diluted with 20 µl HPLC water and 2 µl were mixed with 30 µl CEQ Sample Loading Solution (Beckman Coulter) and 0.15 µl CEQ DNA Size Standard Kit 400 bp (Beckman Coulter). Fragment sizing was performed according to the Beckman Coulter standard protocol for the above mentioned CEQ series and GenLab software version 9.0.25 (Beckman Coulter). Allele binning was performed using R 3.3.2 (R Development Core Team (2016)) and the MsatAllele 1.02 package (Alberto, 2009).

2.2.7. *Descriptive statistic of the loci*

The *CERVUS 3.0.7* software (Kalinowski et al., 2007; Marshall et al., 1998; Slate et al., 2000) was used for the descriptive statistics of the 14 microsatellite loci used. The software calculates the number of different alleles found per locus, the rate of expected heterozygosity (H_{exp}) and observed heterozygosity (H_{obs}) Further, the polymorphic information content (PIC) (Botstein et al., 1980) of the individual loci can be determined. The PIC allows a direct qualitative categorisation of the microsatellites. Generally, loci with values between 1 and > 0.5 are considered highly informative. A PIC between 0.5 and 0.25 is still considered reasonably informative, while values < 0.25 are classified as less informative.

The probability of deviations from Hardy-Weinberg equilibrium (HWE) is another key parameter that can be determined with *CERVUS*. The HWE describes an ideal, unchanging population. Assuming this, mating in such a population would be completely random, the mating probability and success would be the same for all members of the population and there would be no changes in the frequencies of alleles or the frequencies of genotypes. If there is a significant deviation from the HWE, this indicates a considerable homozygote excess. Although *CERVUS* evaluates deviations from the HWE using a chi-square goodness-of-fit test (Nei, 1987), this was partly replaced by a more powerful and accurate test. This probability test is based on the Markov chain method using *Genepop 4.7.5* (Raymond & Rousset, 1995; Rousset, 2008; Weir, 1996) using the following settings: Dememorization number = 5,000; Number of batches = 500; Number of iterations per batch = 5,000 (http://genepop.curtin.edu.au; Option 1).

Another value to be calculated by *CERVUS*, provides information about the presence or absence of null alleles. These are alleles that can not be visualised by genotyping (Dakin & Avise, 2004). Accordingly, an actually heterozygous locus could be misinterpreted as a homozygous locus. If such null alleles occur too frequently, this would result in an artificial deviation from the HWE. *CERVUS* calculates the frequency using an iterative algorithm based on the observed and expected frequencies of the different genotypes (Summers & Amos, 1997). An estimate of the null allele frequency close to zero indicates that there are no null alleles, while negative and large positive estimates indicate an excess of homozygotes.

2.2.8. *Population sub structure*

The three districts of Brünninghausen, Osterwald and Coppenbrügge were grouped together as "Surrounding Saupark" (SSP) due to their very close proximity, as none of the three districts is more than 6 kilometres apart and all samples were taken on the same day. Accordingly, in all subsequent calculations, the “Surrounding Saupark” was treated as a single district. Accordingly, the number of districts in which samples were taken was reduced from 12 to 10.

If no information on the hunting location was available for some samples, they were assigned to a separate, theoretical district. If these samples actually originate from the study area, they should be able to be assigned to their original clusters based on their genetic profile.

The main questions was whether the number of the 10 districts in which samples were taken also represents the number of actual genetically distinct (sub)populations, so-called clusters (K). Although the number K could correspond to the number of forestry districts, even if only approximately, without further knowledge, e.g. about geography or topography, K could also be larger or smaller. One way to determine the most probable number K is a Bayesian modelling approach using a Markov Chain Monte Carlo algorithm (MCMC) with *Structure 2.3.4* (Falush et al., 2003, 2007; Hubisz et al., 2009; Pritchard et al., 2000). *Structure* is a software package for the use of multilocus genotype data, e.g. microsatellites, RFLPs and AFLPs and SNPs to analyse population structure. It is essentially used to infer the presence of distinct populations and to assign individuals to populations. This allows hybrid zones to be analysed, migrants and admixed individuals to be identified and the allele frequencies of populations to be estimated if many individuals are migrants or admixed.

Although K was not known in the present case, preliminary studies had already revealed a tendency for K to be equal to or smaller than the number of 11 districts (Jarausch, 2014; unpublished data from Master Thesis). Accordingly, it can be assumed that the most likely number of K is between 1 and 15. If the calculated results came to the conclusion that $K \geq 15$, the calculations would be repeated with a correspondingly higher K (16-20). With this defined range for K = 1-15, *Structure* calculates the allele frequencies for each cluster and assigns the corresponding individuals to it. The following settings for burnin and number of MCMC repetitions were used: burnin was set at 200,000 and MCMC at 500,000. Each K (1-15) was repeated 10 times independently with these settings, resulting in a total of 150 runs.

Assuming that each individual shares some fraction of the genome with each of the K populations, runs were conducted using the correlated allele frequency model as well as the admixture model. These estimated membership fractions of the individuals to the several clusters are represented by the Q-value (Pritchard et al., 2007). *Structure* then calculates the posterior probability for each K; $\Pr(X \mid Y)$ and scores an estimated log-likelihood [LnP(D)].

The most probable number of clusters (K) was inferred based on the Delta K statistic in Evanno et al. (2005). The ΔK statistic is based on the second order rate of change of the likelihood, ΔK = mean [L''K]/Stdev [LnP(D)], using the freely available web based software *StructureSelector* (http://lmme.qdio.ac.cn/StructureSelector/) (Li & Liu, 2018). However, the performance and accuracy of *Structure* is compromised, for example, when to many close relatives are included in the dataset (Anderson & Dunham, 2008; Pritchard et al., 2010; Rodríguez-Ramilo et al., 2014; Rodríguez-Ramilo & Wang, 2012), the number of samples in the respective study areas are very unevenly distributed (Puechmaille et al., 2011) or when isolation by distance is present (Frantz et al., 2010; Jombart et al., 2010; Pritchard et al., 2010). In such cases, the actual number of clusters (K) may be significantly miscalculated (Janes et al., 2017; Puechmaille, 2016). Therefore, the actual number of clusters should be calculated and determined using alternative methods. In this case, in addition to Evanno's ΔK method, the Puechmailles method, also integrated in *StructureSelector*, was used to determine K (Puechmaille, 2016), as it takes into account the potential bias factors listed above. Despite correction, misjudgements of K can still occur if the proportion of close relatives is too high in some putative subpopulations or the available number of samples between certain putative subpopulations is too uneven (Puechmaille et al., 2011).

As no statements could be made in advance as to whether and to what extent possible bias effects could be found in the data matrix, two different calculation models were created. Model I included all animals, divided into the corresponding 10 districts, plus the theoretical district including all unknown samples. In Model II, possible biases were to be taken into account to a greater extent by removing some of the closely related animals and by adjusting the quantitatively unevenly distributed sample numbers in the forestry districts. In the present case, both options could be combined by excluding closely related animals from the quantitatively strongest study areas for the cluster calculation. To decrease this disproportion without losing too much genotypic informative value, reducing the number of piglets is the most reliable option. As wild boar, with the exception of solitary adult males and partly also subadult males, live in family groups and piglets in particular follow their mothers on their forays, hunting inevitably leads to the culling of closely related animals, especially full siblings. The proportion of full siblings also increases, as in this case, due to the use of uterine samples. Due to the dependence of the piglets on the subadult and adult female members of the sounder, the genotypic population membership of the piglets can be defined by the parent animals, provided that these are themselves part of the genotype data matrix to a sufficient extent. By removing some or all piglets, the bias caused by too many close relatives should be minimized.

On the other hand, removing especially the piglets from the disproportionately overrepresented forestry districts reduces the numerical imbalance between them, which can also lead to population structure bias.

To calculate the optimal assignment of all individuals to the different clusters, the clusters output by the independent runs (K = 1-15, repeated 10 times each) was permuted by *CLUMPAK* - Clustering Markov Packager Across K (Kopelman et al., 2015). Individuals were assigned to the corresponding subpopulations based on their highest Q-value (estimated proportion of the individual belonging to the individual clusters) (Pritchard et al., 2007).The following settings were used: Search method = LargeKGreedy with 2000 repetitions, and the default settings for MCL: threshold for similarity scores and DISTRUCT: threshold for minimal cluster size. *CLUMPAK* is also integrated in *StructureSelector* and can be used as an additional option.

2.3. Results

2.3.1. Metadata sampling: sampling performance, age and sex structure

During the entire study period from 2010-2013 a total number of 910 samples was collected. There were clear differences in the number of animals sampled in the individual hunting years. With 396 wild boars, the 2012 hunting year was by far the year with the highest number of samples, while 2013 was the lowest with 189. In the 2011 hunting year, 220 animals were sampled. Reference is made to the special nature of the 2010 hunting year, which did not involved 104 individual samples, but 18 pregnant females and their full litters (n = 86 embryos). In the case of one sample, the accompanying document was either not completed or was lost. The corresponding data was therefore not recorded (see Figure 2.3).

With 60% of all samples taken, the age group distribution was piglet and foetuses-sided, i.e. animals under one year of age. Around 25% of all wild boar came from the sub-adult age group. Around 13% of all samples were adult animals. No information on the age class was available for around 1%. In summary, 86% of all samples came from animals that had not yet reached adulthood (see Figure 2.4).

The summarised sex distribution in the study period 2010 - 2013 shows a female surplus in all age groups (see Figure 2.5). Although more males were sampled in the subadult and piglet age groups in 2012 hunting year, this is superimposed by the other years. No age information was available for 3 females and 7 males.

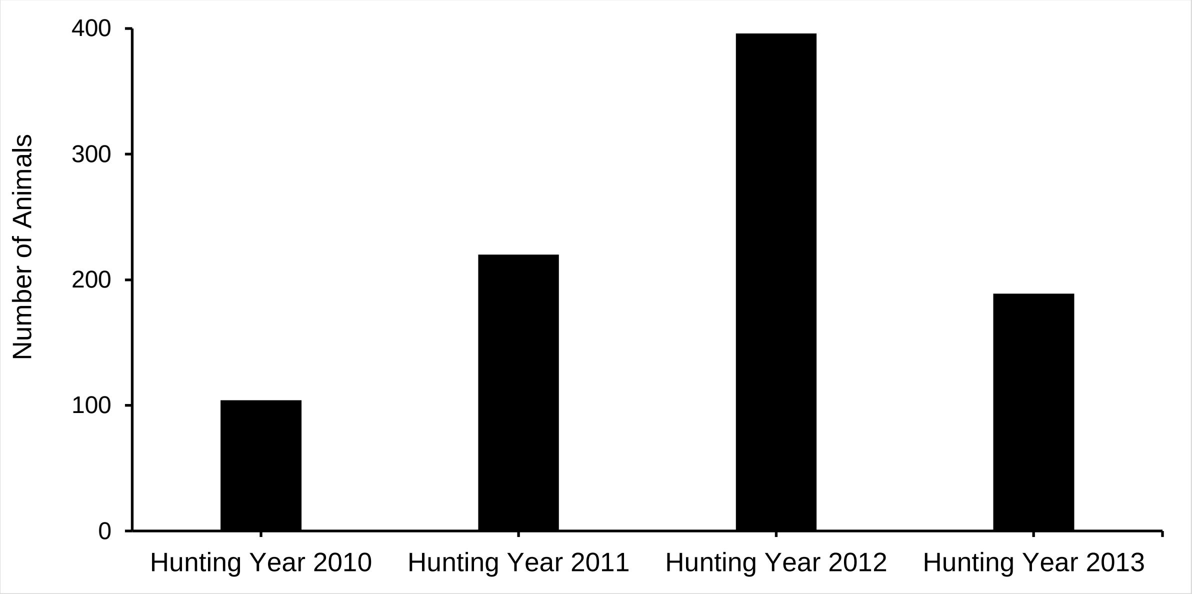

Figure 2.3: Sampling progress over the duration of the study from 2010-2013. One sample could not be assigned to a year (data not shown).

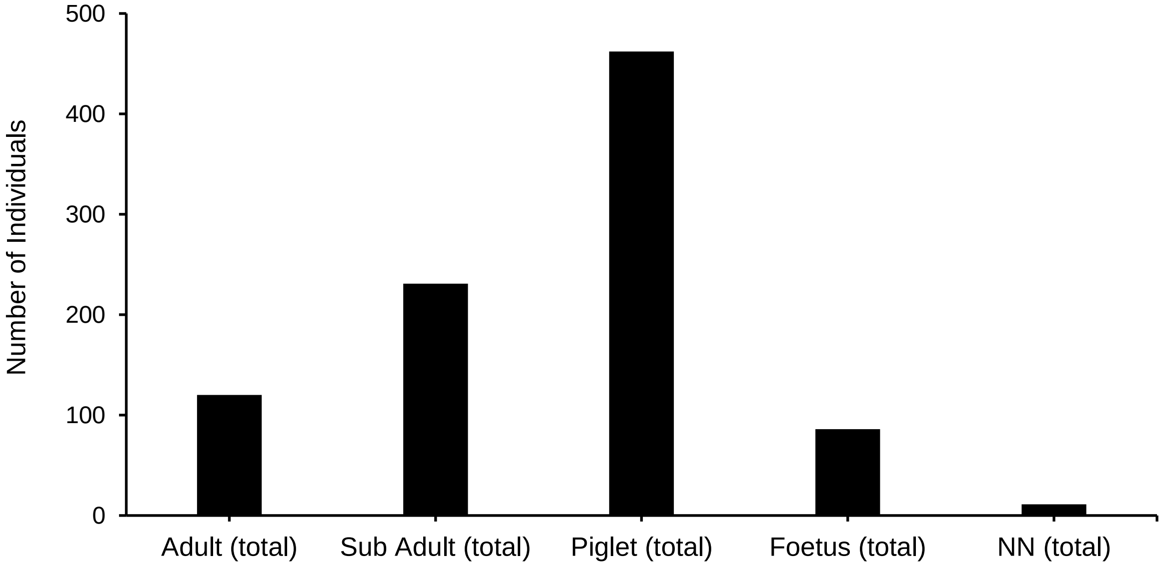

Figure 2.4: Shown is the age class distribution of all 910 wild boar during the entire study period. No age class information was available for 11 samples (NN).

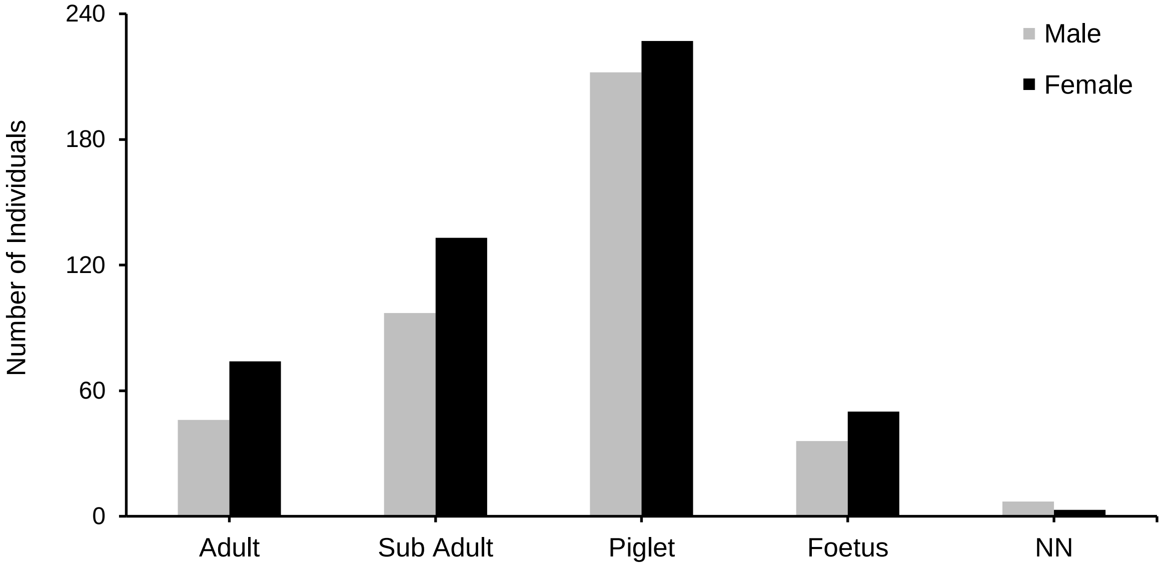

Figure 2.5: Shown is the sex distribution for the entire study period. The grey bars show the number of males for each age group, adult, subadult, piglet and foetus. The black bars show the female proportion. No age information was available for 10 Individuals (NN). The sex was unknown for 25 individuals (Data not shown).

2.3.2. Muscle and uterine sampling data

The collection of the 910 tissue samples in the respective forestry districts was distributed as follows: In forestry district Bevern 367 wild boars were collected and 170 in Hallah. 65 samples were taken in the nearby forestry district Holzurburg and 22 in district Rüstje. A total of 44 samples were collected in Süsing. In Betzhorn forestry district 17 wild boars were harvested, in Danndorf 63 and in Hämelerwald 24 (as the sum of the uterine sampling of 18 pregnant females and their full litters (n = 86 embryos)). 106 samples were taken in Saupark and 30 samples in the "Surrounding Saupark" (SSP) (16 in districts of Brünninghausen and seven each in Osterwald and Coppenbrügge). No information was available on the hunting location of 2 samples. For one of these samples, the year of cull was also unknown. However, it should be possible to subsequently assign these samples to a population on the basis of their genotypic profile. Accordingly, all collected samples remained part of the data matrix for Model I. An overview of the sampling and study area, including a categorisation by collection year, is shown in Table 2.1.

Table 2.1: Overview of the study areas and all forestry districts and the associated samples from the corresponding study years 2010 - 2013. The last column summarises the total sample volume over the entire duration of the project. In the case of two samples (NN), no data was available on the location or year of the harvest. The Brünninghausen, Osterwald and Coppenbrügge forestry districts are still listed separately here, but are combined into the "Surrounding Saupark" (SSP) district, especially for the number of subpopulations to be calculated, as the sampling was carried out on one day and none of the districts is more than 6 km apart. At the same time, the "total" column also represents the number of samples used for Model I (n = 910).

Forestry Districts	2010	2011	2012	2013	total
Bevern	0	164	137	66	367
Hallah	0	40	93	37	170
Holzurburg	0	11	49	5	65
Rüstje	0	5	12	5	22
Süsing	0	0	44	0	44
Betzhorn	17	0	0	0	17
Danndorf	63	0	0	0	63
Hämelerwald	24	0	0	0	24
Saupark	0	0	60	46	106
Brünninghausen	0	0	0	16	16
Osterwald	0	0	0	7	7
Coppenbrügge	0	0	0	7	7
NN	0	0	1	0	2

2.3.3. *Descriptive statistic of the loci*

The average overall success rate for genotyping was 96.75%. Microsatellite SW742 had the lowest rate at 93.736% and MS SW936 the highest at 99.89%. The average number of different alleles (A) per locus was 11.5, with the lowest allelic diversity in MS Sw72 with 4 alleles and the highest in MS S0005 with 25 alleles. The expected rate of heterozygosity (H_{exp}) was always higher than the observed rate of heterozygosity (H_{obs}). The only exception was locus TNFB. The mean polymorphic information content (PIC) was lowest in MS 387A12F at 0.42 and highest in MS KVL 9495 at 0.89. Across all MS, the mean PIC was 0.713. According to Botstein (Botstein et al., 1980), all MS are at least reasonably informative and the majority, 12 out of 14, are even highly informative. With the exception of the loci KVL 9807, TNFB and S0226, all other MS showed significant deviations from Weinberg equilibrium (HWE). A possible indication of the presence of null alleles (data not shown here) only occurred in loci TNFB. A complete overview of all microsatellite diversity indices can be found in Table 2.2.

Table 2.2: Diversity indices of the 14 microsatellites for all 910 samples tested. Shown are the microsatellite systems (MS-system), the number of alleles per locus (A), the number of genotyped individuals (N), the observed and expected rate of heterozygosity (H_{obs}, H_{exp}), the polymorphism information content (PIC), deviations from Hardy-Weinberg equilibrium (HWE) and the mean genotyping success rate per locus in percent (%). In the case of the HWE column, 1-3 asterisks indicate a significant deviation. NS = not significant.

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	Mean genotyping success (%)
SW742	11	853	0.637	0.668	0.643	***	93.736
KVL 9495	21	894	0.846	0.899	0.890	*	98.242
KVL 9807	12	883	0.811	0.869	0.854	NS	97.033
TNFB	9	885	0.759	0.749	0.707	NS	97.253
CGA	23	860	0.753	0.861	0.845	***	94.505
S0090	7	882	0.736	0.824	0.799	***	96.923
SW936	11	909	0.633	0.781	0.745	***	99.890
Sw632	11	904	0.716	0.789	0.757	***	99.341
Sw24	5	858	0.662	0.735	0.691	***	94.286
Sw72	4	905	0.602	0.694	0.641	***	99.451
S0226	6	860	0.505	0.534	0.429	NS	94.505
S0655	6	863	0.660	0.756	0.716	***	94.835
387A12F	10	889	0.406	0.450	0.420	***	97.692
S0005	25	881	0.767	0.855	0.842	***	96.813

2.3.4. Comparison of the sampling locations

Since the observation of the descriptive statistics of the loci for the entire data set did not necessarily reflect the distribution of the alleles and the associated indices in the different sample locations, these were analysed separately in order to be able to visualise possible differences between them. Accordingly, for better comparability, the average was calculated for all MS indices. In addition, any deviations from the HWE and the frequency of null alleles (F) were calculated for each study area. The average number of different alleles (A) was highest in Bevern (8.143) and lowest in Betzhorn (3.786). The expected rate of heterozygosity (H_{exp}) was always higher than the observed rate of heterozygosity (H_{obs}). The only exception was the district Hämelerwald. In Hallah both values were almost the same. The highest average polymorphism information content (PIC) was 0.669 in Saupark and the lowest 0.538 in Hämelerwald. The average values for deviations from the Hardy-Weinberg equilibrium (HWE) deviated significantly from it in all districts except for Hallah, Holzurburg, Danndorf and Saupark. However, with the exception of the district in Rüstje, no indications of a significant occurrence of null alleles could be found (see Table 2.3).

Table 2.3: Diversity indices of the 14 microsatellites separately for all 10 study areas. Shown are the forestry districts (Sample location), the mean number of alleles per locus (A), the number of genotyped individuals (N), the mean observed and expected rate of heterozygosity (H_{obs}, H_{exp}), the mean polymorphism information content (PIC), the results of the Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HWE}) and the calculated frequency of null alleles (F(Null)). All indices but HWE were calculated by Structure. Deviations from HWE were calculated by Genepop.

Sample location	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
Bevern	8.143	367	0.687	0.705	0.664	6.33E-21	0,0198
Hallah	7.286	170	0.680	0.678	0.635	0.000	0,0306
Holzurburg	6.429	65	0.669	0.680	0.631	0.000	0,0337
Rüstje	5.714	22	0.690	0.712	0.651	0.0000051	-0,0072
Süsing	6.857	44	0.655	0.709	0.663	6.32E-38	0,0670
Betzhorn	3.786	17	0.659	0.676	0.596	2.47E-15	0,0560
Danndorf	6.714	63	0.677	0.707	0.653	0.000	0,1029
Hämelerwald	4.571	24	0.655	0.598	0.538	2.16E-40	0,0460
Saupark	7.714	106	0.678	0.720	0.669	0.000	0,0497
Surrounding Saupark	5.714	30	0.633	0.670	0.612	<0.001	0,0732

2.3.5. *Estimating K*

2.3.5.1. *Model I*

The estimation of K using the settings for Model I (11 different forestry districts and 910 animals) at a burnin 200k and MCMC 500K, using *Structure 2.3.4*, resulted in 2 different clusters. The values of Mean LnP(D) did not show a clear plateau that would allow an estimation of K (see Figure 2.6). On the contrary, the delta K values calculated by *StructureSelector* according to Evanno et al (2005), on the contrary, showed a clear maximum at K = 2 (see Figure 2.7).

According to K=2, there is a clear gradient between the northern areas of Bevern, Hallah, Holzurburg and Rüstje, forming cluster 1, and the south-eastern areas of Süsing, Betzhorn, Danndorf, Hämelerwald, Saupark and Surrounding Saupark (SSP), which form cluster 2. Despite the clear genotypic demarcation between the two population clusters, the data showed also mutual exchanges and individual animals migrate to the other population. A more intensive genetic exchange movement from cluster 1 towards cluster 2 can be observed. However, within the 2nd cluster, only the Süsing and Danndorf areas are affected. Betzhorn, Hämelerwald and SSP showed hardly any genetic profile of cluster 1. Saupark was almost completely without genetic exchange with cluster 1. Based on the parameters of Model I at K=2, the two individuals from the theoretical cluster NN would belong to the northern cluster 1 (see Figure 2.8).

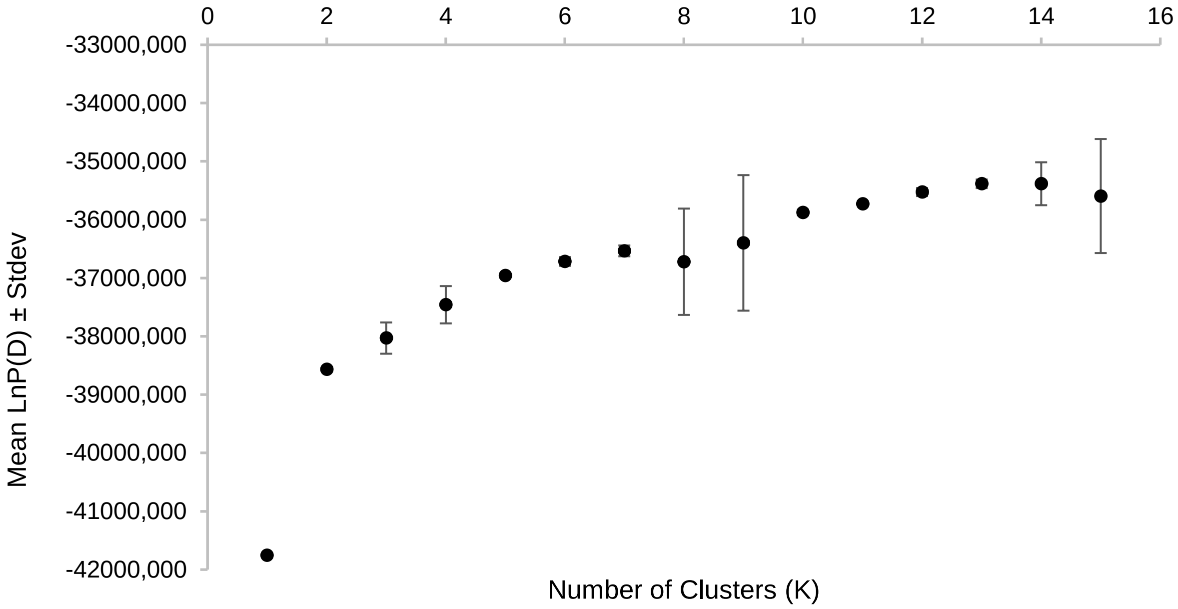

Figure 2.6: Bayesian clustering results of Structure for determining the true number of subpopulations (K) using the log-likelihood values. The number of clusters (K) on the x-axis is plotted against the mean values of the estimated log-likelihood [LnP(D)] (with standard deviation (Stdev)) on the y-axis.

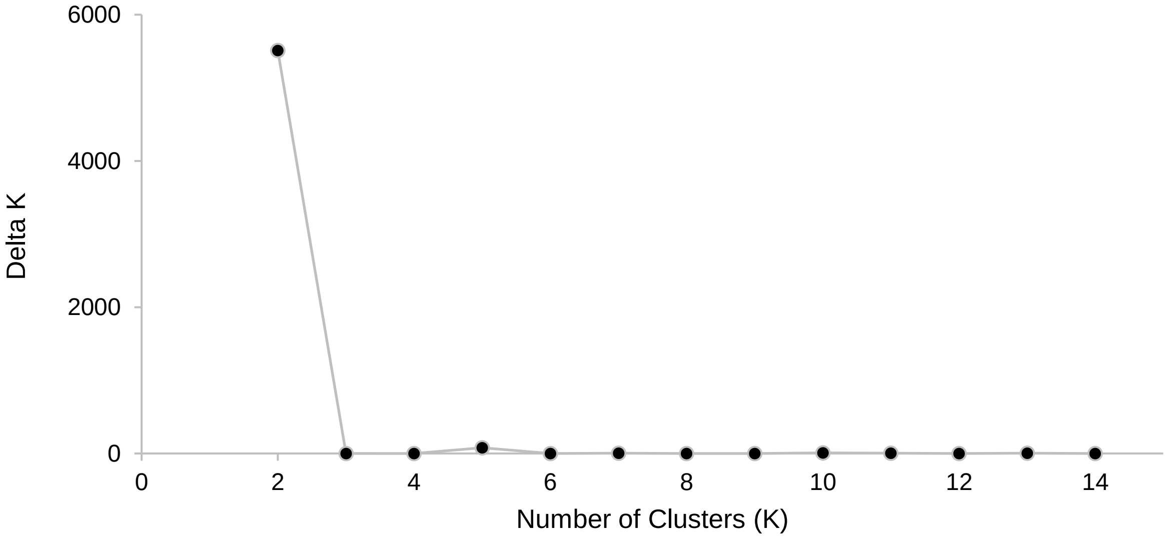

Figure 2.7: The DeltaK method used according to Evanno et al (2005) shows a clear peak at K=2. The x-axis shows the possible clusters and the y-axis the ΔK values calculated with StructureSelector.

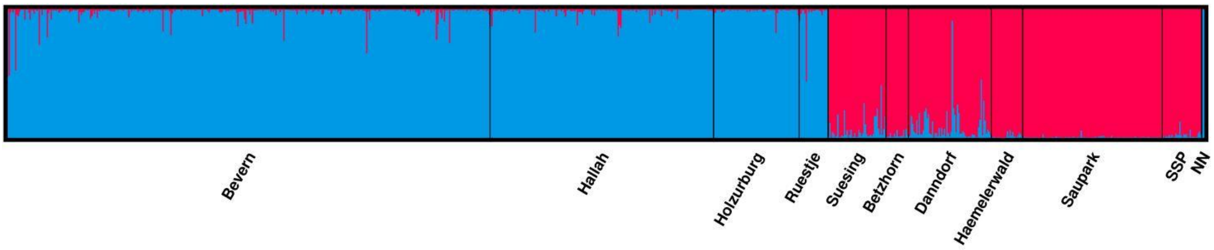

Figure 2.8: Estimated population structure K=2 according to Model I, calculated by Structure according to Evanno et al., 2005. Each individual is represented by a thin vertical line/bar, divided into two coloured portions (blue and red) representing the estimated membership proportions (Q) of the individual in each cluster. The different districts are separated from each other by thin black lines. The respective sample locations are annotated below the figure.

As already mentioned, the determination of the most probable number of clusters K by *Structure* can lead to significant misjudgements in the presence of e.g. closely related animals or an unequal number of samples in the subpopulations. Due to the close family structures in the wild boar sounders and an average mortality probability of approx. 40-50% for each individual caused by hunting (Keuling et al 2014), the sampling of closely related animals (parents and their offspring, several full siblings from one litter, etc.) from the hunting bag is basically unavoidable. In the case of uterine samples, the high degree of kinship is a given from the outset. In addition, there is an uneven number of samples in the study areas and sample locations. The maximum of 367 samples was found in Bevern and the minimum of 17 samples in Betzhorn (see also Table 1). Accordingly, an underestimation of K by *Structure* seems possible in this case. An independent consideration of the most probable number of clusters, which takes into account the possible bias factors mentioned above and corrects them accordingly, is therefore essential. The 10 repetitions of the number of clusters K=1-15 serve as the data basis. Using a cluster algorithm, the most probable K is determined by the median of medians, the median of means, the maximum median value and the maximum mean value. For a detailed review, see Puechmaille (2016).The determination of the most probable number of different clusters according to the Puechmaille method resulted in a number of clusters of K=5 for all four approaches (see Figure 9a-d).

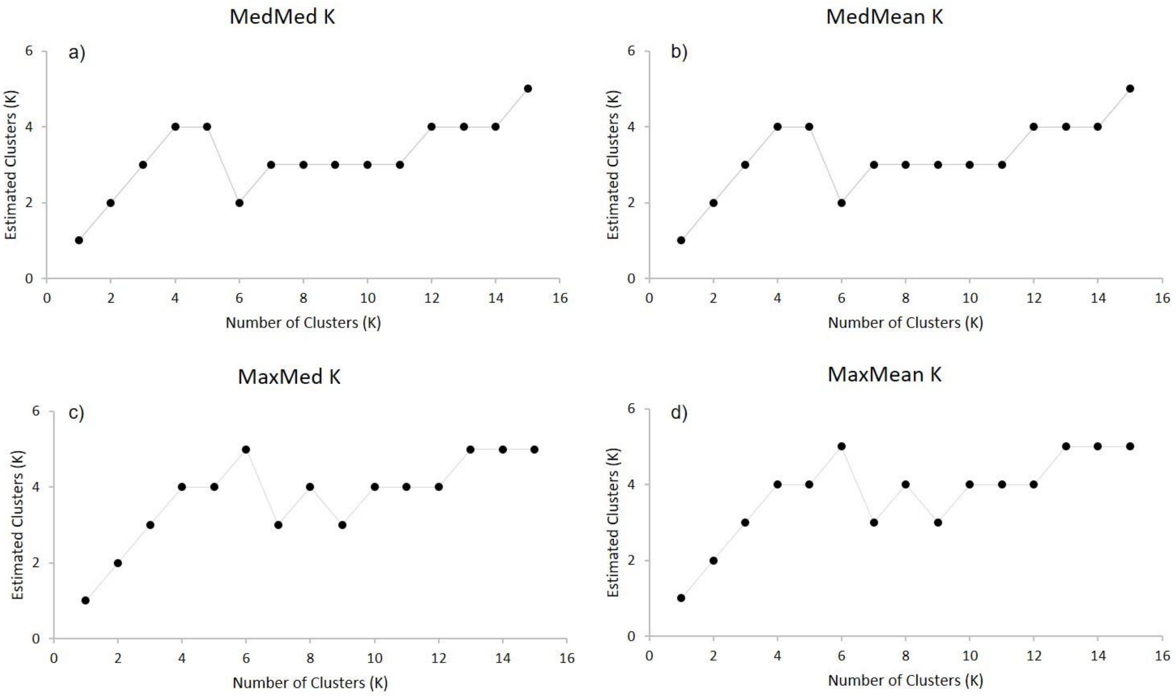

Figure 2.9: Figure (a) shows the with StructureSelector calculated median of medians (MedMed K), figure (b) the median of means (MedMean K), figure (c) the maximum median value (MaxMed K) and figure (d) the maximum mean value (MaxMean K) of all estimated Cluster (K), according to Puechmaille (2016).

Using the maximum number of clusters K=5 calculated by *StructureSelector* and the method according to Puechmaille 2016, the gradient between the northern (3 clusters) and south-eastern (2 clusters) areas could still be recognised. By increasing the membership fraction (Q) of each individual from K=2 to K=5, a more dispersed pattern of population structuring emerged. A clear population delimitation could only be found in Saupark, which represented one cluster, and in the south-eastern areas of Süsing, Betzhorn, Danndorf, Hämelerwald and SSP, which together formed one further cluster. However, movements of individuals between these two clusters could also be observed. The 3 remaining northern clusters covered the other areas of Bevern, Hallah, Holzurburg and Rüstje. However, none of these four districts represented a cluster of its own, but three mixed and overlapping clusters. There was a recognisable east-west gradient between Bevern/Rüstje and Hallah/Holzurburg. The parallel presence of the three different clusters was most evident in the districts of Bevern and Hallah. However, one of these three clusters was found to a much lesser extent in the Hallah/Hozurburg area. With K=5, genetic exchange patterns between the 3 northern clusters (cluster 1 at K=2) and the two south-eastern clusters (cluster 2 at K=2) were thus significantly reduced. Although a large number of animals moved from the Saupark in a north-easterly direction towards Betzhorn and Süsing, little or no genetic tracks could be found in Hämelerwald or Danndorf. Even in the nearby SSP there was comparatively little genetic profile of the Saupark.

Conversely, the walled Saupark was not a completely demarcated area, as significant genetic traces of SSP were found. Based on the parameters of Model I at K=5, the two individuals from the theoretical cluster NN would again belong to the northern cluster. However, one individual tended to belong more to the Hallah/Holzurburg cluster and the second to the Bevern/Rüstje cluster. (see Figure 2.10).

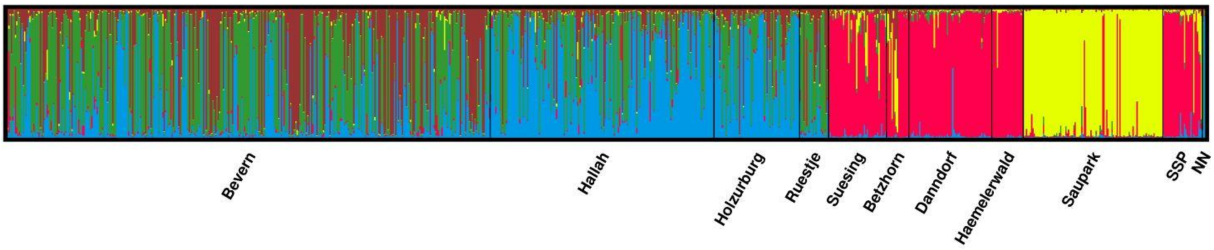

Figure 2.10: Estimated population structure K=5, calculated by StructureSelector according to Puechmaille 2016. Each individual is represented by a thin vertical line, which in this case is divided into 5 coloured segments representing the estimated membership proportions (Q) of the individual in each cluster. The different districts are separated from each other by thin black lines. The respective sample locations are annotated below the figure. The two individuals from the NN cluster belong to the northern cluster. However, one individual tends to belong to the Hallah/Holzurburg cluster (blue), while the second probably belongs to the Bevern/Rüstje cluster (green/brown).

In a final step, in order to visualise the respective proportions and distributions of the membership fraction (Q) in the calculated clusters and the respective districts most clearly, all individuals within their cluster were sorted according to the highest Q value (see Figure 11).

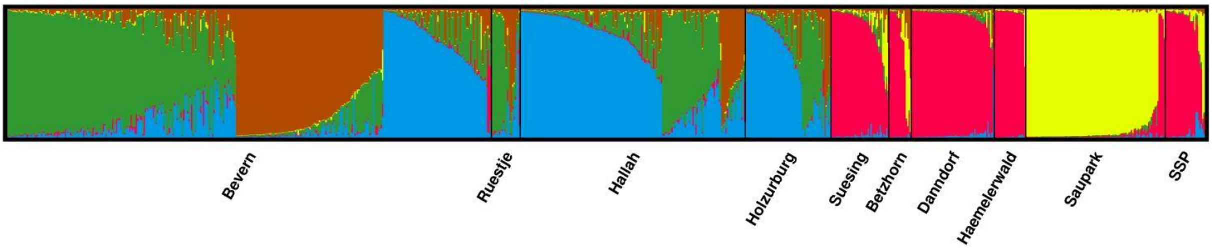

Figure 2.11: Estimated population structure K=5 with Model I settings, calculated by StructureSelector All individuals are sorted according to their proportion of membership (Q), which is segmented into five coloured components, within their determined cluster. The different districts are separated from each other by thin black lines. The locations of the samples are indicated below the figure. Because of the recognisable east-west gradient between Bevern/Rüstje and Hallah/Holzurburg, these labels were rearranged to the previous figure 8 and 9.

2.3.5.2. *Model II*

Based on the previous results (see *2.3.2 Muscle and uterine Sampling data*), Bevern (n = 367) and Hallah (n = 170) were the quantitatively strongest districts. In order to take greater account of possible biases, some of the closely related animals were removed and the unevenly distributed sample numbers in the forestry districts were adjusted. In the present case, both options could be combined by excluding closely related animals from Bevern and Hallah for further cluster calculation. Accordingly, 216 piglets were removed in Bevern and 110 in

Hallah, leaving a total of n = 584 animals in Model II (see Table 2.4). The sample sizes in the other forestry districts and their total number 11 remained unchanged.

Table 2.4: Adjusted sample size for calculation Model II (n = 584). The piglets from the Bevern and Hallah districts were removed in order to take into account possible biases due to an excessive proportion of closely related animals or unevenly distributed sample sizes in the forestry districts. All other districts remain unchanged (see Table 1).

Forestry Districts	**2010**	**2011**	**2012**	**2013**	**total**
Bevern	0	72	44	35	151
Hallah	0	13	39	8	60

Using the adapted Model II settings for 11 different populations and 584 animals, using the same above mentioned *Structure* parameters, the estimation of K resulted in 2 different clusters. The values of Mean LnP(D) again did not show a clear plateau that would allow an estimation of K (see Figure 2.12), but delta K values calculated by *StructureSelector* again showed a clear maximum at K=2 (see Figure 2.13).

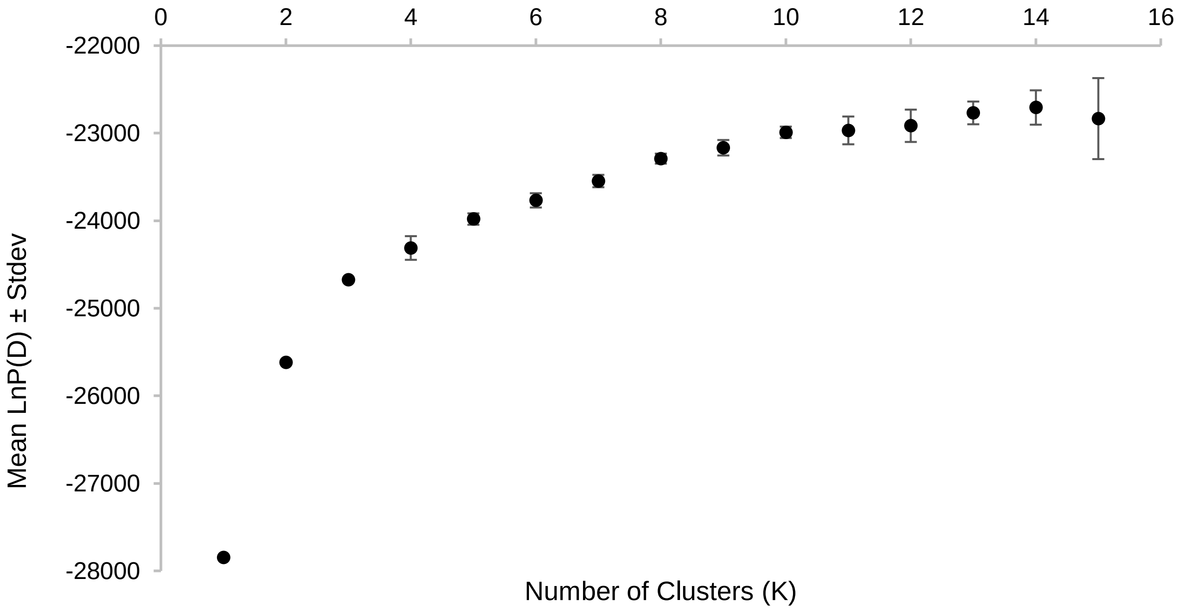

Figure 2.12: Bayesian clustering results of Structure for determining the true number of subpopulations (K) using the log-likelihood values. The number of clusters (K) on the x-axis is plotted against the mean values of the estimated log-likelihood [LnP(D)] (with standard deviation (Stdev)) on the y-axis.

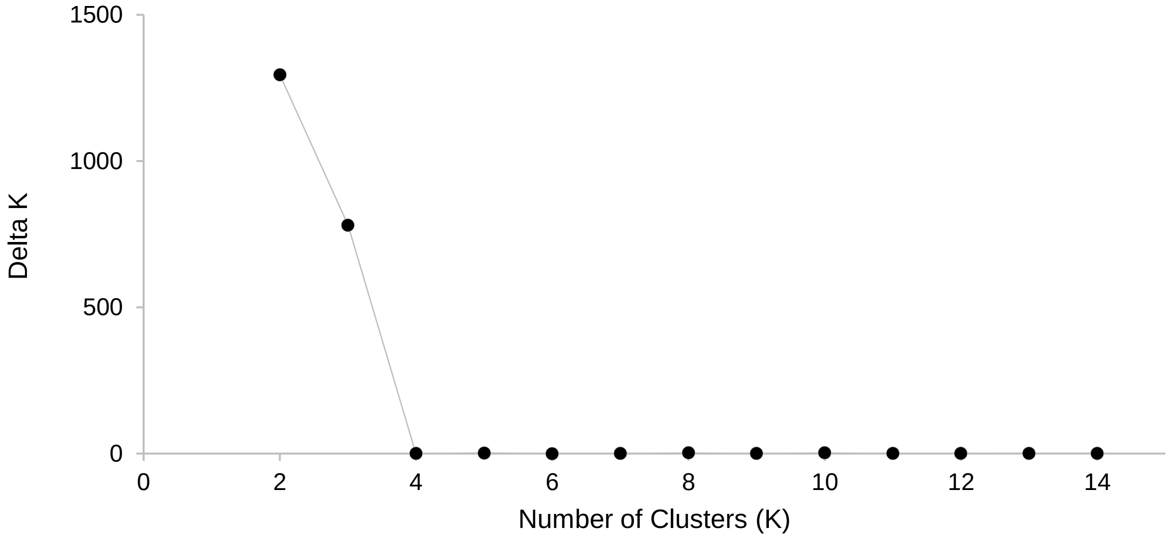

Figure 2.13: The DeltaK method used according to Evanno et al (2005) shows a clear peak at K=2. The x-axis shows the possible clusters and the y-axis the ΔK values calculated with StructureSelector.

According to K=2, using Model II, there was still a clear gradient between the northern districts of Bevern, Hallah, Holzurburg and Rüstje and the south-eastern districts of Süsing, Betzhorn, Danndorf, Hämelerwald, Saupark and Surrounding Saupark (SSP). Even under the conditions of Model II, there was mutual genetic exchange between the two populations. There do not appear to be any major movements of individual animals or entire groups between the populations, with the exception of 2 animals. Given the data available here, the two animals from the hypothetical District 11 (NN) would belong to the northern cluster (see Figure 2.14).

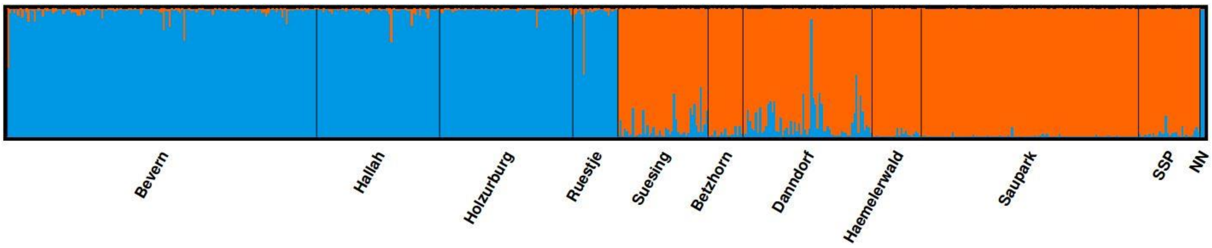

Figure 2.14: Estimated population structure K=2 according to Model II, calculated by Structure according to Evanno et al., 2005. Each individual is represented by a thin vertical line, divided into two coloured segments (blue and orange) representing the estimated membership proportions (Q) of the individual in each cluster. The different districts are separated from each other by thin black lines. The respective sample locations are annotated below the figure

The determination of the number of different clusters according to the Puechmaille method resulted in a number of clusters of K=7 for the two Median approaches and K=8 for the two Maximum approaches (see Figure 2.15).

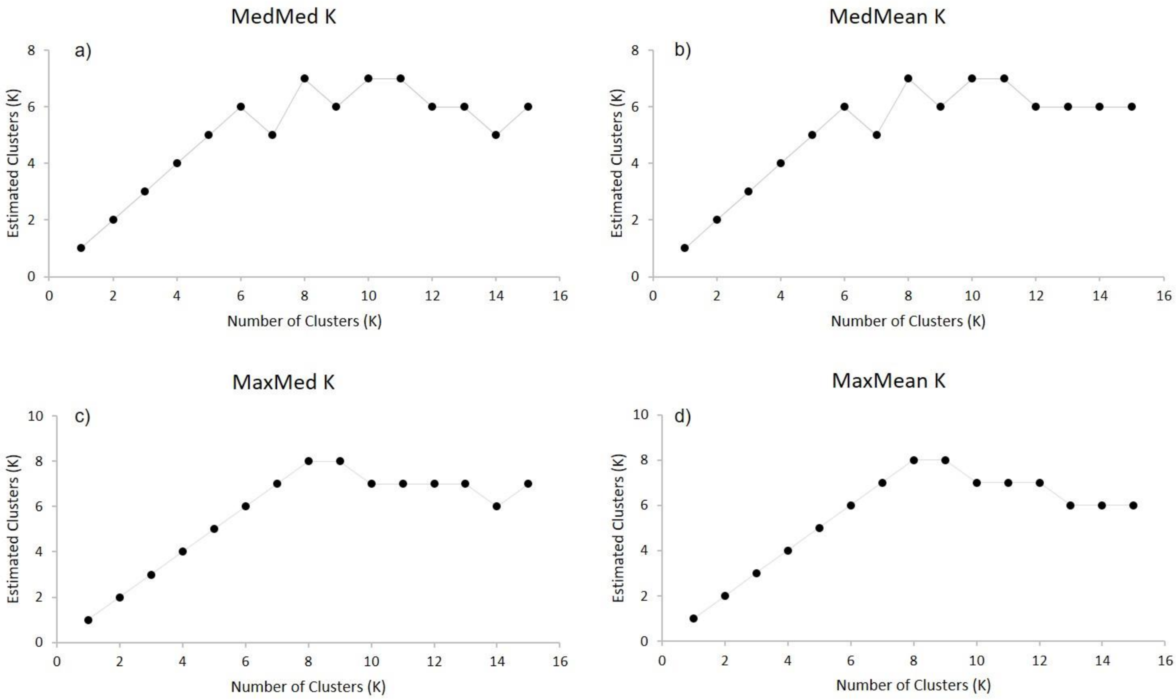

Figure 2.15: Figure a shows the with StructureSelector calculated median of medians (MedMed K), figure b the median of means (MedMean K), figure c the maximum median value (MaxMed K) and figure d the maximum mean value (MaxMean K) of all estimated Cluster (K), according to Puechmaille (2016).

Comparing the results of the cluster calculations for Model I and the offspring reduced Model II for K=5, a clearly different distribution of the clusters could be observed. The basic gradient between the northern areas (Bevern, Rüstje, Hallah and Holzurburg) and the south-eastern areas (Süsing, Betzhorn, Danndorf, Hämelerwald, Saupark and SSP) was still present. The hinted east-west gradient from Hallah and Holzurburg to Bevern and Rüstje rather disappeared by the reduction from 3 (Model I) to 2 (Model II) clusters. The two calculated subpopulations extended freely across all 4 study areas. The Saupark remained a clearly defined cluster of its own, but the Surrounding Saupark (SSP) also represented one combined cluster with the Hämelerwald district. The Süsing area remained one cluster together with Betzhorn and Danndorf (see Figure 2.16).

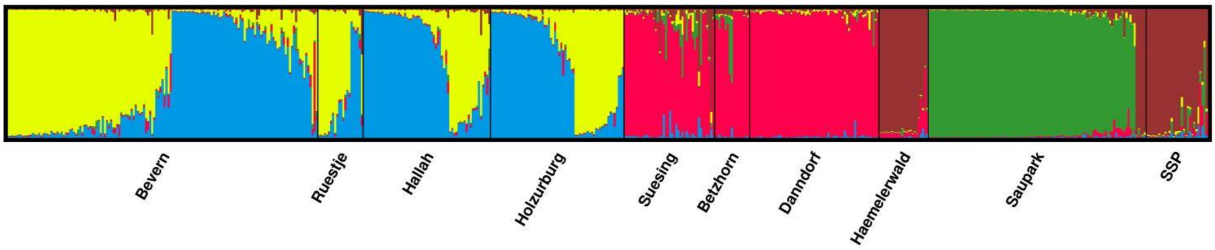

Figure 2.16: Estimated population structure K=5 with Model II settings, calculated by StructureSelector. All individuals are sorted according to their proportion of membership (Q), which is segmented into five coloured components, within their determined cluster. The different districts are separated from each other by thin black lines. The locations of the samples are labelled below the figure. For reasons of comparability with Model I and K=5, the labels were sorted in the same way.

If K=8, as supported by the MaxMed and MaxMean results by *StructureSelector*, it could be seen that there is still a visible gradient between the northern areas of Hallah, Holzurburg, Bevern and Rüstje and the south-eastern areas of Süsing, Betzhorn, Danndorf, Hämelerwald, Saupark and Surrounding Saupark (SSP), as could also be seen with K=2 in both models. However, Model 2 K=8 revealed even more boundaries within this gradient. Both Saupark and Sourrounding Saupark each formed their own cluster. The same applied to Hämelerwald and Süsing. Betzhorn also formed its own cluster, although this also extended into the Danndorf district. In addition to Betzhorn's share of the cluster, Danndorf was home to another independent cluster. Therefore, 2 different clusters occured in parallel in the Danndorf area (see Figure 2.17).

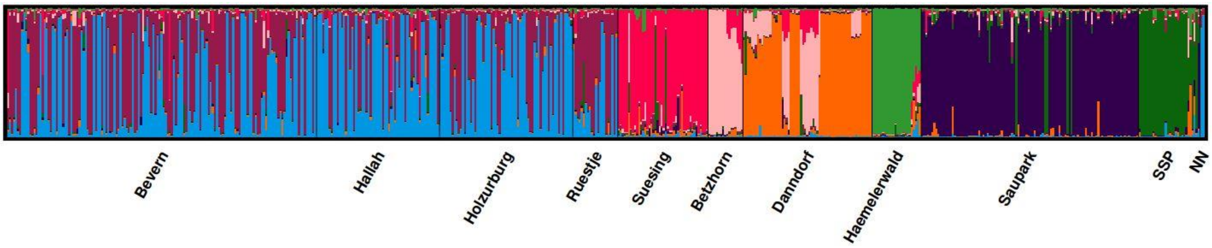

Figure 2.17: Estimated population structure K=8 with Model II settings, calculated by StructureSelector. Each individual is represented by a thin vertical line, which in this case is divided into 8 coloured segments representing the estimated membership proportions (Q) of the individual in each cluster. The thin black lines separate the different districts from each other. The respective districts are annotated below the figure.

In order to visualise the respective proportions and distributions of the membership fraction (Q) in the calculated 8 clusters and the respective districts most clearly, all individuals within their cluster were sorted according to the highest Q value and the two formerly unknown samples were assigned to their respective clusters (see Figure 2.18).

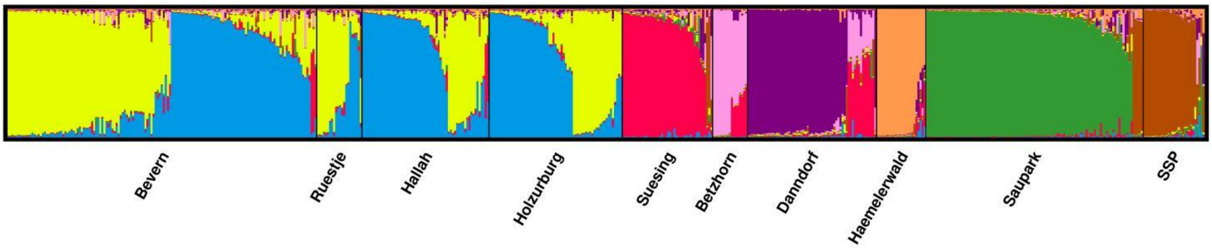

Figure 2.18: Estimated population structure K=8 with Model II settings, calculated by StructureSelector. All individuals are sorted according to their proportion of membership (Q), which is segmented into eight coloured components, within their determined cluster. The different districts are separated from each other by thin black lines. Sample locations are labelled below the figure. For better comparability with Model I and II with K=5, forestry district labels were sorted in the same way.

2.3.6. Most probable number of different population clusters K

Although the used models I and II and the different methods to calculate the most probable number of populations (Evanno’s ΔK and Puechmailles method) gave different results regarding the number of distinct clusters, a clear trend towards a genetic gradient between the northern (Bevern, Rüstje, Hallah and Holzurburg) and south-eastern districts (Süsing, Betzhorn, Danndorf, Hämelerwald, Saupark and SSP) was observed in all cases. This genetic gradient was clearest with Evanno’s ΔK at K=2, regardless of the model chosen. Under these conditions, the direction of genetic dispersal from the northern districts to the south-eastern districts was stronger than in the opposite direction. Taking into account possible quantitative biases in the number of samples between districts and disproportionately high kinship densities, especially in Bevern and Hallah, the most likely number of different clusters using Puechmaille's method was K=5 for Model I. The described gradient from north to south-east remained visible and genetic exchange across this gradient was low. Genetic flow in Model I K=5 occurred mainly in and between the 3 northern clusters. In the two southern clusters, genetic exchange took place to a much lesser extent. In addition, the results suggested that there was slightly more genetic flow out of the Saupark cluster than in.

With the settings and parameters of Model II, there was a cluster shift at K=5. The number of northern clusters decreased from 3 to 2, while the south-eastern clusters increased from 2 to 3. In addition, the genetic proportion that migrated out of the Saupark into the surrounding clusters decreased. However, the calculated most probable number of clusters in Model II according to Puechmaille's method was K=8. Compared to K=5 in Model II, the two northern clusters remained largely unchanged, but there were significant changes in the south-eastern districts due to the increase in calculated clusters from 3 to 6. Based on these results, all 6 forestry districts formed independent genetic clusters with varying degrees of genetic influence from the other clusters. The north-south-east gradient was also maintained in Model II and K=8. There were individual genetic profiles from the Süsing district in both Bevern and Hallah. Conversely, genetic traces from Bevern and Hallah could be found in the south-eastern districts. In summary, the present results suggest that there was no evidence of genetic flow on a broader level during the 2010-2013 study period.

2.4. Discussion

2.4.1. *Sex and age structure*

The sex ratio in the hunting year of 2012/2013 for the state of Lower Saxony was 53.6% males and 46.4% females. The long-term mean from previous years showed a sex ratio of 53:47 in favour of males (Keuling 2014). In the study areas, the average sex ratio over the entire period 2010-2013 was 44.97% males and 55.03% females. Since no male parent animals were sampled in 2010, there is a minimal bias in the calculated sex ratio, which was 45.91% males to 54.09% females without the dams from that year. This shift in the sex ratio in favour of females across all age groups is therefore rather unusual. Adult and subadult females are often deliberately spared by hunters to avoid the unintentional shooting of pregnant or lactating animals or females in principle (Keuling et al., 2013; Keuling et al., 2014; Toïgo et al., 2008). From a hunting perspective, this shift in the sex ratio in the hunting bag like in this study is generally to be favoured, as it is the females that are significantly involved in the population increase (Keuling et al., 2014; Vetter et al., 2020). From a statistical point of view, such a change could be problematic, especially if the culling of females is intentional. Under these circumstances, independent sampling would no longer be ensured and derived results would be biased. However, such intentional intervention in very specific age groups is inevitable (Keuling et al., 2014; Vetter et al., 2020) if the existing high population densities are to be sustainably reduced. It is irrelevant whether the hunting scenario favours the shooting of piglets or sub-adults or an equal reduction of all age groups. In all cases, independent and random sampling is only possible to a limited extent. The present study also appears to be affected by the resulting bias. This was particularly evident in the different determinations of the optimal number of clusters (K) between Models I and II and the different results from Evanno's ΔK method and Puechmailles method. However, the influence of such biases also affects further analyses of hunting bags. Statistical extrapolations of population density based on the annual hunting bag would also show biased results if sex and kinship ratios of the hunting bag are no longer randomly distributed to a sufficient extent (for a detailed review see Chapter 5 and Müller et al., 2020).

Following the so-called 'Lüneburger model' (Hennig, 1998; Teuwsen, 1980), the hunting guidelines of Lower Saxony recommend a piglet proportion of 70% of the annual hunting bag, a sub-adult proportion of 20% and an adult proportion of 10%. This proportional age group distributions should be approximately similar in the vital population as in the hunting bag but with some significant variation from year to year (Keuling et al., 2014). According to this model, hunting should skim off the annual growth and prevent a further population increase. The annual hunting bags in Lower Saxony in the three survey years fell well short of these targets with an average 52.433% piglet proportion, 38.967% sub-adult proportion and 8.267% adult proportion in all age classes (Keuling et al., 2014). The distribution of age classes in the data set of the present study differs even further. The average proportion of piglets and foetuses is 60.219%, the sub-adult 25.385% and the adult 13.187% (see also Figure 4). It should be noted that the number of foetuses is neither taken into account nor recorded in the regular hunting bag statistics. However, only foetal samples and the corresponding dams are available from the study areas of Betzhorn, Danndorf and Hämelerwald. This can result in a ratio between potential parents (adults and sub-adults) and potential offspring (piglets and foetuses) in parts of the existing data set that, on the one hand, does not optimally reflect the actual population ratios between parents and offspring in quantitative terms. On the other hand, the guaranteed genetic relationship between the female parent and the foetuses may result in artificially high relationship ratios, which could bias the display of population structures. Accordingly, the proportion of offspring in the present data set is at least 10% too low and the proportion of sub-adult and in particular the adult parents is correspondingly at least 10% too high. Such an imbalance must be considered critical. The total reproductive proportion of piglet dams in the population is around 34%, despite a significantly lower average reproductive capacity than sub-adults and adult females (Frauendorf et al., 2016; Gethöffer et al., 2007; Müller et al., 2018). However, their age group accounts for nearly two thirds of the population, which is why piglets are the main factor responsible for population growth. Accordingly, each per cent difference between the required number of piglets harvest and the actual number of piglets harvest leads to a further increase in the population in subsequent years (Keuling et al., 2014). The sample disproportion between the age groups and the unavoidable relationship between dam and foetus therefore leads to a certain conflict between independent and representative sampling, which is desirable from a statistical point of view. The resulting biases in the present case must therefore either be taken into account when analysing and interpreting the calculated results or the parts of the data set causing the bias must be removed, provided this is permissible from a statistical perspective. A quantitative reduction of the data set, in particular by closely related piglets, is to be considered permissible in this context and was carried out (Jarausch et al., 2023).

2.4.2. Microsatellite marker analysis

The conclusive identification of individuals using genetic markers such as microsatellites is the most fundamental step in many studies, from capture-recapture methods (Ebert et al., 2012; Ebert et al., 2021; Müller et al., 2020), cryptic mate choice (Gayet et al., 2016; Müller et al., 2018; Vanpe et al., 2009) to population or landscape genetics (Frantz et al., 2012; Jarausch et al., 2023). Depending on the research issue and the desired genetic resolution, six to sixteen different microsatellites are considered a sufficient number to visualise the individual genotype and the resulting relationships (Iacolina et al., 2009; Müller et al., 2018; Suárez-Menéndez et al., 2024; Vernesi et al., 2003). The 14 microsatellites used for the present study thus represented a sufficient number to reveal the genetic structure of a wild life population. Moreover, with an average polymorphic information content (PIC) of 0.713, minimum 0.42 (locus 387A12F), maximum 0.89 (locus KVL 9495), for all samples (n = 910), 12 of the microsatellites used were highly informative and 2 others were reasonably informative (Botstein et al., 1980). With an average of 11.5 alleles per locus, minimum 4 alleles (Sw72), maximum 25 alleles (locus S0005), all microsatellites were sufficiently polymorphic. Other studies on population structure, in which up to 14 microsatellites were used, also include loci with only 3 or fewer alleles (Frantz et al., 2012; Frantz et al., 2006; Vernesi et al., 2003). Furthermore, with an average genotyping success rate of 96.75% for all microsatellites, no relationships should remain undetected or the average allele frequencies should be biased by gaps in the data set.

Looking at the different forestry districts, the respective average PIC of all microsatellites together is above 0.5 in all study areas (minimum 0.538 in Hämelerwald and maximum 0.669 in Saupark) and is therefore always highly informative.

Analysing all loci of the entire data set (n = 910), significant deviations from the HWE are found in all MS except KVL 9807, TNFB and S0226. Isolated deviations from HWE at one or two loci could be interpreted as an indicator of a genotyping error. However, deviations from the HWE at 11 of 14 loci indicate a structuring of the population, which would be consistent with the results of the Bayesian clustering analysis. In addition, these deviations indicate a significant homozygote excess, which may be caused by non-random mating or gene flow, for example (Andrews, 2010). The spatial distances between some of the forestry districts on the one hand and habitat fragmentation, especially in Bevern and Hallah, on the other hand, are possible factors explaining limited random mating. In addition, a very constant reduction in observed heterozygosity compared to expected heterozygosity can be observed. Such continuous deviations are indicators for consanguineous mating in local populations. Here too, spatial distances and habitat fragmentation are an obvious explanation. Since no null alleles that could cause an artificial increase in homozygous alleles can be detected in the data set, with the exception of the TNFB locus, the assumption of spatial distances and habitat fragmentation as causative factors is further confirmed and accordingly no influencing genotyping error is present.

At a forestry district level, only one third of the districts - Hallah, Holzurburg, Danndorf and Saupark - are not affected by deviations from the HWE. In this case, however, it must be taken into account that the microsatellite indices were calculated separately for each district. In the remaining districts, with the exception of Bevern, it is noticeable that the number of samples in these districts is significantly lower than in the districts mentioned above, e.g. Süsing n = 44 or Betzhorn n = 22 (cf. Table 1, Chapter 2.3.2). In addition, in Süsing, Betzhorn, Hämelerwald and SSP districts, samples were only collected in one hunting year, sometimes only on a single date. The deviations from the HWE observed there could therefore be caused by an insufficient and unrepresentative sample size. As a consequence, genotyping errors cannot be assumed to be the causative factor in this case either, but rather a data structural imbalance. That the Bevern district also deviates from the HWE, despite a sufficiently high sample number and constant sampling over 3 years, can be explained on the one hand by the too high proportion of closely related individuals. Among the piglets in particular, the number of full and half siblings is too high (see Müller et al., 2018 and results in Chapter *5.3.4 Parent-offspring detection and assignment rates*), which means that the actual allele distribution in the population does not correspond to the allele distribution in the sampled hunting bag. On the other hand, the forest and habitat fragmentation in Bevern, Hallah, Holzurburg and Rüstje in particular can lead to limited mating between unrelated individuals. The resulting increase in the proportion of homozygous individuals due to mating between relatives would therefore result in a deviation from the HWE.

2.4.3. Determining the number of subpopulations

The characterisation of individual genotypes by microsatellites or SNPs enables bioinformatic assignment methods to allocate individuals or groups of individuals to one or more genetically distinct populations (Manel et al., 2005; Raj et al., 2014). By determining the population membership of these individuals or entire groups, migration movements, the degree of genetic mixing between two or more populations or overlapping and hybrid zones can be detected (Pritchard et al., 2000). This enables, for example, distribution patterns and directions of species to be visualised, which would not be possible without knowledge of the genetic population membership, or only on a much smaller scale, e.g. by radio telemetry of individual animals or smaller groups.

In the present study, the *Structure* software was used as an assignment approach to define and visualise population structures and to identify subpopulations of wild boar populations in northern Lower Saxony, Germany. Since *Structure* can be inaccurate under certain circumstances and conditions, such as too many close relatives or unevenly distributed sample numbers across populations, it can fail to calculate the correct number of clusters (Gilbert, 2016; Puechmaille, 2016). To consider this, Puechmaille's method has been used in addition to Evanno's ΔK method to determine the most probable number of different clusters. The results of the present study show that it can be assumed in the data set for Model I that both types of bias described occur and the number of clusters (K) must be determined accordingly. On the one hand, a quantitative sample mismatch can be observed between the forestry districts, in particular Bevern and Hallah (see Chapter 2.3.2, Table 1). On the other hand, the high proportion of piglets in the two districts mentioned suggests that the proportion of closely related animals, piglets in particular, is too high for Model I (see Chapter 2.3.5.2, Table 4). A detailed description of the relationships, especially for Bevern and Hallah, is provided in Chapter 5.

2.4.3.1. Number of different populations for Model I

The rate of change of the likelihood distribution [LnP(D)] by continuously increasing log likelihood values showed no clear plateau which therefore enables no determination of the true number of subpopulations. Using Evanno's ΔK method to identify the number of sub populations, resulted K=2.

The resulting population split into a northern cluster, consisting of the four areas Bevern, Rüstje, Hallah and Holzurburg, and a south-eastern population cluster, consisting of the remaining areas Süsing, Betzhorn, Danndorf, Hämelerwald, Saupark and SSP, does not appear to be entirely unrealistic due to the spatial distance between the clusters (minimum distance ≈ 55 km, maximum distance ≈ 180 km). In addition, clear genetic migration movements can be seen, particularly from the north to the areas of Süsing, Betzhorn and Danndorf in the southern cluster. However, sufficient evidence has already been shown that could lead to a misinterpretation of the correct number of clusters according to Evanno's DeltaK method and could affect the present study.

Accordingly, the number of clusters K=5 calculated by the alternative determination method according to Puechmailles appears more reliable and would also more plausibly reflect the corresponding spatial distances and structural barriers, particularly in the south-eastern districts. Especially if K≥3, the walled-in Saupark becomes always an independent genetic cluster. Even if the present study did not aim to localise the precise location of gene flow barriers, it can be assumed that the wall around the Saupark, the urbanised area of Hanover, but also Wolfsburg and Bremen, several motorways, the artificial waterway ‘Mittellandkanal’ and rivers such as the Aller and Leine together restrict the movements of wild boar in the study area or dictate certain migration directions. The fragmentation of the northern areas of Bevern, Rüstje, Hallah and Holzurburg into 3 different genetic clusters must therefore be regarded as difficult or questionable. The areas, or respectively the individual sampling sites, are at a maximum distance of barely 35-40 km from each other, but often much closer to each other, and in this very rural part of Lower Saxony there are no major structural barriers that would explain such a fragmentation. At the same time, in this Model I, (n = 910), K=5, Süsing and SSP, among others, are summarised in a common cluster. Not only is the spatial distance here over 100 km, but the Hanover city area, the Mittellandkanal and various other natural and anthropogenic barriers are also located exactly on the route. It therefore seems less plausible than initially assumed that the number of clusters to be distinguished can actually be determined as 5. In addition, there is the shown significant imbalance between the northern areas of Bevern and Hallah and most of the south-eastern districts in terms of the number of available samples. The high number of closely related animals in the Bevern and Hallah area also seems to confirm the assumption of a biased and inaccurate distribution of the clusters under the present conditions. Overall, the existing biases are too significant even for Puechmaille's method. Therefore, Model I cannot conclusively clarify whether K=5 could possibly represent the correct number of clusters or whether the individuals themselves were clustered incorrectly or whether the number of clusters is not equal to 5 after all.

Regardless of whether the number of clusters is 2 or 5, in both cases the two samples (NN) could be assigned without any doubt, at least to the northern districts, without a known sampling site.

2.4.3.2. *Number of different populations for Model II*

The estimation of K using the adapted Model II (n = 584) settings with a reduced number of close related individuals from Bevern and Hallha does not initially lead to any changes compared to Model I. Again, the rate of change of the likelihood distribution [LnP(D)] by continuously increasing log likelihood values showed no clear plateau which therefore enables no determination of the true number of subpopulations. Using Evanno's ΔK method to identify the number of sub populations, again resulted K=2. Therefore, the resulting population split into one northern population cluster and one south-eastern cluster still remains. Accordingly, it was not possible to calculate a reliable number of clusters (K) with the available data using either *Structure* itself or Evanno's ΔK method.

Despite a clear adjustment in the sample size between the forestry districts and a clear reduction in closely related animals, there still seems to be a high degree of bias. In Model I, on the other hand, it was shown that Puechmaille's method resulted in a higher number of clusters, but it was not possible to clarify whether the number of clusters was incorrect or whether the assignment of individuals to clusters was erroneous. The latter case applies to data sets in which there is a clear surplus of closely related animals (Gilbert, 2016). Accordingly, after the reduction of these animals and the additional adjustment of the sample size between the forestry districts, a changed and more precise pattern of population substructuring should result using Puechmaille's method. As expected, in Model II and assuming K=5, there is a shift in the clusters. Accordingly, there are 2 clusters in the northern districts, while the south-eastern districts form 3 clusters. Despite the shift of only one cluster from north to south-east, the distribution of the clusters is the most plausible result to be found to this point, especially geographically. The clear genetic differentiation between north and south-east remains. Due to the spatial distance, the formation of a Süsing-Betzhorn-Danndorf cluster, which is separate from a Hämelerwald-SSP cluster, appears more realistic than the calculated cluster grouping from Model I. Even if distances of ≈ 30 km between the Süsing, Betzhorn and Danndorf districts and ≈ 50 km between Hämelerwald-SSP leave reasonable potential for hypotheses that there is a more extensive sub structuring than K=5.

This hypothesis is confirmed by the calculations of the settings for Model II. The clear reduction in closely related animals from the Bevern and Hallah districts, which were sampled more frequently than proportionally, appears to reduce the biasing causes to such an extent that the number of different clusters can be calculated correctly enough using Puechmaille's method. Removing the piglets from the data set does not represent an artificial bias in the data set. Firstly, the survival of the piglets is highly dependent on the dams and secondly, their home ranges correspond to those of their female parents. Piglets for which the dam is genetically known are therefore represented in the population structure via this genetic profile. Piglets for which the maternal genotype is missing can at least be assigned to the correct population with a high probability via the place of harvest. According to MaxMed and MaxMean, the number of clusters calculated in this way is K=8. The number of northern clusters remains unchanged at 2, and the Saupark also remains a separate cluster. The north-south-east population caesura observed from the beginning also remains unchanged. The number of independent clusters in the remaining territories increases from 2 to 5. The distances of 30-50 km between these territories Süsing, Betzhorn, Danndorf, Hämelerwald and SSP are therefore sufficient to define each of these territories as a separate genetic cluster.

The individuals from the Süsing district appear to have the greatest range of movement. Their genetic profile is found both in the northern forestry districts of Bevern and Hallah, but especially in the Betzhorn and Danndorf clusters to the south of Süsing. The Danndorf cluster shows the most notable genetic migration of the entire data set. The Danndorf district forms its own genetic cluster, which can also be clearly distinguished from all the other 7 clusters. In addition to this genetic cluster, however, there is an additional mixed cluster from Süsingen-Betzhorn in the spatial area of the Danndorf forestry district. The available results suggest that there must have been a very recent migration of closely related animals from Süsing-Betzhorn to Danndorf. In this case, 14 individuals were involved, 3 older piglet dams and their 11 sampled foetuses. Two of these dams originate genetically from Süsingen. The genotypes of the foetuses suggest that the sires originate from Betzhorn. The third dam belongs to the Danndorf cluster, but the genetic profiles of the foetuses indicate a mixed paternal ancestry from Betzhorn-Süsingen. Accordingly, a genetic exchange across both sexes appears to be present.

The Saupark, which is surrounded by a wall, is the object of immigration, mainly of animals from the immediate neighbourhood (SSP), rather than emigration. Although an individual from the Saupark was also found in the SSP. The fact that the Saupark cannot be more clearly distinguished genetically from its surroundings is mainly due to several gates that are not continuously closed. In addition, the wall, which was completed in 1839, has now been partially replaced by a fence, which is occasionally damaged, making it passable for wild boar in both directions.

The spatial distribution of the 2 clusters in the north differs very clearly from all other clusters. While in all south-eastern clusters a comparatively clear demarcation to the other clusters and especially to the other forestry districts can be detected, this is not possible in the 4 northern forestry districts. No clear genetic boundary can be drawn between any of the 4 forestry districts or the 2 genetic clusters. In all 4 forestry districts, both clusters occur in parallel. While in the south-eastern clusters distances of 30 km were sufficient to make genetically clear classifications, this is not possible in the north, despite comparable distances between the Hallah-Bevern/Rüstje, Hallah-Holzurburg and Bevern/Rüstje-Holzurburg districts. Generally speaking, the two highways running through the area and the river Oste could represent spatial and corresponding genetic barriers (Bennett, 2017; Epps et al., 2005; Graves et al., 2012; Jackson & Fahrig, 2011). However, wild boar in particular are often not affected by such barriers in their movement patterns (Frantz et al., 2012; Griciuvienė et al., 2021). The genetic structuring can therefore be explained more in terms of behavioural biology and topography. As already explained, this northern part of the study area is characterised by extensive maize and rapeseed fields, particularly in the summer, which serve as forest and thicket substitutes for wild boar during this period. Even though the home ranges of wild boar in agriculturally dominated landscapes are often smaller than in large woodland areas, extensive roaming areas are still possible, or individual groups/sounders migrate relatively far from their winter woodland habitats to more distant crop fields and spend the summer months there. In contrast, the forest areas in the study area are rather small at 5-6 km^2. When the maize and rapeseed harvests in late summer and early autumn deprive the wild boar of their summer alternative habitats, the family groups are forced to return to their small forest habitats to spend the winter months there. Accordingly, in all habitats still available for the winter, either both clusters are found in parallel in one forest area or one cluster randomly dominates a forest area and the other cluster a neighbouring forest area. Wild boar is philopatric, but they do not typically defend their home ranges from other sounders, and individuals from separate sounders generally have non-exclusive overlapping home ranges, which has the potential to facilitate gene flow (Graves, 1984; Hampton et al., 2004; Sparklin et al., 2009), especially as winter is also the main breeding season. Accordingly, the mosaic-like distribution of small forest patches throughout the northern study area leads to an equally parallel genetic cluster distribution. At the same time, the genetic exchange between the clusters is not permanently blocked.

In view of the constantly spreading ASF, basic information on migratory movements and directions of spread is of significant advantage when it comes to successfully controlling or at least limiting the spread of the disease. In this context, it could be seen in this study that individual animals are found some 60 km away from their population of origin. While genetic exchange between populations is desirable and necessary, e.g. to counteract inbreeding depression, larger migratory movements also involve risks if the migrating individuals are carriers of zoonoses and thus accelerate the spread of ASF, for example.

2.4.4. Conclusions and outlook

The present study was able to show that the use of 14 microsatellites is sufficient to reveal the genetic structure of wild boar populations, even if there are biasing elements in the data set due to hunting. The removal of closely related animals, especially from those parts of the data set with a disproportionately high number of samples, can compensate for this bias. Furthermore, the results of this study show that the wild boar population in eastern Lower Saxony has a genetic structure that is caused by both habitat fragmentation and species-specific behaviour, in particular breeding behaviour and pronounced site fidelity with simultaneous territory overlap tolerance. This enables the parallel presence of two different genetic clusters in the same topographical area. In addition, the wall of the Saupark Springe could be identified as a barrier to gene flow, although the results also indicate that a certain gene flow into and out of the Saupark probably exists.

The available data at least suggest that the spread of the wild boar into the northernmost areas of Lower Saxony did not take place, or only to a small extent, through migration from the south-eastern populations studied here. No major genetic migrations can be found across the described north-south-east caesura. A migration from a western direction, Oldenburg, the Wildeshauser Geest behind Bremen up to Nienburg/Weser, north-west of Hannover, would also be conceivable. However, samples from these areas were not available, which is why no statements can be made about migration from this direction.

In this context, it would be advisable to repeat such studies on a regular basis, even with this sample size, and also to sample the areas that have not yet been considered. Due to the enormous adaptability of wild boar, it must always be expected that migration movements and directions will change over the years. Therefore, an observation on how an overlapping population structure such as that in Bevern, Rüstje, Hallah and Holzurburg develops over the years would be extremely important. Based on the available results, sampling the hunting bags throughout the year is most suited to such long-term genetic monitoring. It is important, however, to ensure that the different hunting bags are sampled in sufficient numbers in order to avoid biasing effects due to excessive differences in sample numbers. Furthermore, if there are too many closely related individuals in parts of the data set, these should be identified in advance using suitable computer-aided kinship analyses and reduced accordingly. In addition, alternative DNA marker systems, e.g. mtDNA markers, could be added in order to map phylogeographic patterns, genetic structuring or population boundaries in the European wild boar even more accurately (Niedziałkowska et al., 2021).

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3. Mother's baby, father's maybe: Occurrence and frequency of multiple paternities in the European wild boar

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Summary

Multiple paternity (i.e. when the litter of a pregnant female is fertilized by more than one male) is common in a variety of animal taxa, including several ungulate species. In addition to that, multiple paternity has been observed in both, socially polygamous and socially monogamous species. The occurrence of multiple paternities can have two major advantages for females. Firstly, the number of offspring can be increased and secondly, genetic diversity. However, multiple paternity is not always a result of female interests, but can also be enforced by sexual harassment on the male's initiative. It is generally believed that dominant males of European wild boar (Sus scrofa) monopolize several females, suggesting that multiple paternity is a rare phenomenon in this species. However, recent studies from different populations across Europe suggest that multiple paternity occurs more often in wild boar than had previously been assumed. However, previous studies were either strongly theory-based or based on rather small sample sizes or had very strong hunting pressure on males. Germany, which has one of the largest wild boar populations in Europe, has so far lacked any quantitative and qualitative studies on this topic. Therefore, the aim of this study is to clarify the number and frequency of multiple paternities in European wild boar under moderate and balanced hunting conditions, in Germany.

For this purpose, the uteri of 35 pregnant female wild boars of all age classes were collected on drive and single hunts between October 2010 and February 2011, and again between October 2011 and February 2012. Colleting took place in different but nearby hunting areas in Lower Saxony (Germany). The genetic analysis of the uterine and embryo tissue was carried out using eight highly polymorphic microsatellite markers. Subsequently, the putative paternal genotypes were visually reconstructed based on the available genotype data of the offspring and the corresponding female parent. The frequency of occurrence of multiple paternity was calculated by using different software packages.

Almost 23% (8 of 35 the uteri) of the embryonic genotypes suggested at least two different sires. A minimum of 45 different fathers had to have been involved. In fact, due to hidden incidences where both parents carried the same alleles, maximum likelihood calculations suggested an even higher rate of multiple paternity of >31% (11 of 35 the uteri).

The present study provides conclusive evidence for the regular occurrence of multiple paternity in wild boars in Germany in a range of approximately 20-30% of the uteri analysed. In contrast to some previous studies, multiple paternity appears not to be a rare phenomenon

but a regular trait. The high proportion of females mating several times in this study is in line with research in other European countries, which showed rates of multiple paternity of ca. 30%-60%. All these results suggest that this behaviour is much more common than previously assumed and clearly not a local phenomenon. While the occurrence of this behaviour can be considered valid according to all available results, the causes remain unknown or are not clear. It might be beneficial for females to engage in multiple mating as a protection against functional infertility of their mates. Cryptic female choice is also a possibility, in which case females would release competent spermatozoa from a sperm reservoir accumulated through mating with competing sires. Social changes caused by male selective hunting pressure, resulting in a sounder of adult females losing the protection of their dominant male during the oestrous cycle, could also lead to an increase of multiple paternities. In such a case, multiple paternities could be caused by a new dominant male. Alternatively, in the absence of a dominant male, an excess of sexually mature but non-adult males can lead to an increase of multiple paternities. It is known that populations with a strong overall male-selective harvest may switch to a more promiscuous and polyandrous mating system owing to the lack of dominant and monopolizing males.

In conclusion, it was shown that multiple paternity in wild boar litters is a relatively common phenomenon in Germany. Among the questions to be clarified in future research, is whether this behaviour is related to age, for example, i.e. whether adult females may be less affected than subadult animals and piglets.

4. Population estimates based on the frequency of parent-offspring relationship within a subsample

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Summary

Estimating population density as precise as possible is a basic requirement for managing many wild animal species. This can be a difficult task if the species in question is elusive or difficult to count due to its high numbers. In addition, not every method for estimating population densities is equally applicable for every species. Low-density tending species, which concerns many carnivores, can be counted rather easily with hair traps. This often does not apply to many high-density ungulate species. Other methods that are particularly suitable (all with typical advantages and disadvantages.) for ungulate species are drive counts or camera traps. Collecting faecal samples is another widely used method for species monitoring and estimating population trends. In addition to the classical approach of this method by determining a defecation rate in a defined area and timescale to estimate the density, dung counts offer an advantage that no other method, except perhaps hair samples, can provide. DNA analyses of the collected faeces can be used to determine the number of different individuals and then the density can be determined using classical capture-mark-recapture (CMR) methods. However, there are several significant disadvantage of this method such as the choice of appropriate transects, high staff expenses and weather-dependent faeces quality and therefore also DNA quality. All that can make faeces counts and genetic analyse of the faecal pellets a time-consuming process with an uncertain outcome. These disadvantages could be minimised by sampling the hunting bags of relevant species, especially ungulates like wild boar, roe deer, fallow deer or red deer. Hunting bags of drive hunts provide a whole range of additional genetic information, which usually remains unexploited, in particular the genetic relationships between the animals, such as parent–offspring pairs and kinship structures within and between populations. In particular, the detection and number of parent–offspring pairs may act as a genetic recapture. Most of all, such a genetic-based capture–mark–recapture (g-CMR) model would provide a unified, reliable, and comparable method for estimating abundance and density of many ungulate species.

Present here is a new, mathematically derived estimator (of classical CMR models as the Lincoln–Petersen estimator or the Chapman estimator) for population size, where the estimation is based solely on the frequency of genetically assigned parent–offspring pairs within a subsample of an ungulate population. By use of molecular markers like microsatellites, the number of these parent–offspring pairs can be determined. The study's aim was to clarify whether a classical capture–mark–recapture (CMR) method can be adapted or extended by this genetic element to a genetic-based capture–mark–recapture (g-CMR). The presented estimator

(and the corresponding variance estimates) was numerically validated and the R code for the computation of population size estimates including confidence intervals was provided. The presented method provides a new framework to precisely estimate population size based on the genetic analysis of a one-time subsample.

An estimator for the population size based on the number of parent-offspring pairs in a subsample was successfully derived and validated. Monte Carlo simulations were used to show that the estimator is only slightly biased when relative subsample sizes are small and asymptotically unbiased when the relative subsample size increases. Calculated confidence limits showed a reasonable coverage probability. The estimation procedure is robust to certain deviations from random sampling and smaller sample sizes. A deviation of less than 10% of the real population size appears to be tolerable. In that case, the estimator rather tends to overestimate the real population size, but as the magnitude of the error can be estimated, it can be considered for future management actions. However, too small sample sizes or subsamples appears rather unrealistic for many European ungulates. But when investigating, for example, small carnivore populations, the results of the here developed estimator must be treat with caution. For species with a hunting regime that is focused on specific or purposefully harvest on parent–offspring pairs, the estimator seems to become inaccurate and inappropriate and tends to underestimate the real population size. It is therefore recommended not to rely on one method alone, but to always use at least two different methods for mutual validation. The combination of the estimator presented here with e.g. a non-invasive sampling and density estimation method could provide more than promising results that enable both long-term and sustainable wildlife management.

5. Wild boar population size estimated by hunting bag genotyping

5.1. Introduction

For 40 years now, the European-wide densities, abundance and areal distribution of wild boar (*Sus scrofa*) have almost exclusively been pointing in one direction: upwards (Augustsson et al., 2024; Baubet et al., 2004; Boitani et al., 1995; Cahill et al., 2003; Croft et al., 2020; Ferreira et al., 2009; Keuling et al., 2013; Markov et al., 2022; Massei et al., 2015; Sodeikat, 2004; Tsachalidis & Hadjisterkotis, 2009). With higher numbers of individuals, the general risk of crop and forest damage increases, causing widespread economic and ecological damage (e.g. Bueno et al., 2009; Gren et al., 2019; Welander, 1995). Furthermore, as a vector for various diseases and zoonoses (e.g. classical and African swine fever), the wild boar also poses a threat to industrial pig farming (Costard et al., 2009; Jori et al., 2021; Keuling et al., 2013; Keuling et al., 2018; Richter et al., 2023; Rossi et al., 2005a; Rossi et al., 2005b; Sauter-Louis et al., 2021a). Moreover, the growing population densities generate certain human-wildlife conflicts like animal-vehicle collisions (Häggmark et al., 2014; Putman et al., 2011; Stillfried et al., 2017; Thurfjell et al., 2015). This poses a problem that will be further intensified by the increasing urbanisation of wild boar (Cahill et al., 2012; Kotulski & König, 2008; Stillfried et al., 2017).

Germany has one of the highest wild boar stocks all over Europe (Keuling et al., 2013; Keuling et al., 2021; Massei et al., 2015). For instance, the German hunting season from April 2019 until March 2020 had the highest hunting bag of all times with approximately 882,000 shot wild boars. For comparison, the mean hunting bag ± SD for the previous ten years was 570,410 ± 116,758 wild boars. This means a 47% growth compared to the previous hunting season from April 2018 until March 2019, and a mean increase of almost 55% compared with the period from 2010-2020. This hunting bag increase in 2019/2020 was at least partly owed to the occurrence of the African swine fever (ASF) in Poland (Nov. 2017 and Nov 2019 (Mazur-Panasiuk et al., 2020)) and Belgium (Sept. 2018 (Linden et al., 2019)) and the associated attempt to significantly increase the hunting pressure. Two of these efforts were a nationwide, temporary suspension of the closed season for adult wild boar and government bonus payments for additional culls for intensified hunting pressure in some federal states (e.g. Bavaria and Brandenburg). However, these actions have not been able to stop the spread of ASF into

German wild boar populations. Accordingly, the first ASF detection on German territory in the federal state of Brandenburg in September 2020 did not come as a complete surprise (Sauter-Louis et al., 2021b). The fact that this was not an isolated case is equally unsurprising, as the ASF virus was also detected in the federal state of Saxony in October 2020 and has continued to spread since then (Richter et al., 2023). Since mid-June 2024, the ASF virus has also been detected in the Hessian district of Groß-Gerau, which borders directly on Rhineland-Palatinate and is only around 40 kilometres away from Baden-Wuerttemberg and Bavaria.

There were reasons enough to be sceptical that the enhanced hunting bag in 2019/2020 constitute a sustainable and consistent reduction of wild boar populations in Germany. With ~688,000 and ~711,000 wild boar shot in the 2 following years, the German hunting bag remained at a consistently high level. Although the current hunting bag from the 2022/2023 hunting season has fallen below 500,000 for the first time in almost 10 years, no sustained population decline can be inferred from this. Strong population fluctuations from one year to the next, which have also occurred in the past, do not mark a permanent population change, but are rather to be understood as natural fluctuations. A conclusive population trend based on hunting bag numbers can only be determined over many years or decades. The exceptional reproductive capacity of wild boar (Boitani et al., 1995; Frauendorf et al., 2016; Geisser & Reyer, 2005; Gethöffer et al., 2007; Servanty et al., 2011; Taylor et al., 1998), additionally favoured by constant and high food availability (Frauendorf et al., 2016), combined with alternative reproductive tactics (Gayet et al., 2016; Müller et al., 2018), will most likely quickly compensate the hunting bags from 2020-2022. It is therefore highly questionable whether the applied hunting strategies (Briedermann, 2009; Happ, 2017; Keuling et al., 2010; Keuling et al., 2008b; Keuling et al., 2021) are sufficient to reach a permanent population reduction (Boitani et al., 1995; Geisser & Reyer, 2005; Keuling et al., 2013; Keuling et al., 2016; Keuling et al., 2021; Massei et al., 2015).

For a sustainable wild boar management, which has the claim to regulate population sizes in any intended direction, reliable numbers of living animals in their habitat are essential because hunting bag statistics alone are not reliable enough (ENETWILD consortium et al., 2018). This means that either the remaining vital animals of a population must be reliably counted or powerful extrapolations or back-calculations of the living population must be made on the basis of the annual hunting bag. In recent years, various methods have been developed to determine the density and abundance of ungulates or wild animals in general (e.g. Borkowski et al., 2011; Buckland et al., 2001; Rowcliffe et al., 2008; Valente et al., 2016) and, in this case, for wild boar in particular (e.g. Acevedo et al., 2007; Hebeisen et al., 2008; Jiménez et al., 2017). In practical application, consistent and standardised methods are needed to enable comparability between different hunting areas, hunting success rates, habitat properties and populations characteristics in order to predict the required hunting effort and hunting strategy for the forthcoming hunting seasons. At the same time, it is important to measure hunting efficiency at a local scale in order to be able to make adjustments very quickly and guide hunting in the desired direction. Comparing population dynamics over several years or between different populations is difficult and is done too rarely (but see e.g. Melis et al., 2006) due to a lack of standardised practical application and a mismatch between available methods and research aims. The ENETWILD project has for the first time summarised and standardised a variety of commonly used methods for recording wild boar density and abundance, including their advantages and disadvantages, as well as their possibilities and limitations (ENETWILD consortium et al., 2018).

One of the most widely used source of information for wild boar management activities and strategies or abundance and density calculations is the annual hunting bag (Briedermann, 2009; ENETWILD consortium et al., 2018). Using this numbers as long-term data, they provide a quite reliable overview of general population developments and trends as well as the age distribution and sex ratio within the hunting bag. Simple statistical projections based on the number of annual culled animals are comparatively easy to accomplish but the validity of the results is limited and comparisons between different hunting grounds are difficult (for a detailed review see ENETWILD consortium et al., 2020). Moreover, a common but incorrect assumption is that hunting bags reflect actual population size, leading to the conclusion that a quantitative decline of a hunting bag compared to previous years is equivalent to a fundamental stock reduction (Acevedo et al., 2007; Baber & Coblentz, 1986; ENETWILD consortium et al., 2018). However, in order to verify that game populations have been reduced, other counting and estimation methods like camera traps (CT), drive counts (DC) or distance sampling (DS) must be used (Acevedo et al., 2007; ENETWILD consortium et al., 2018). In this context, however, it must be noted that each of these methods has its limitations. They must always be adapted to the conditions of the study area and the corresponding life history traits of the species under investigation. Furthermore, unforeseeable events can bias results at any time.

Despite their high accuracy, genetic methods for estimating wild boar populations (Ebert et al., 2012) are often avoided because they are considered expensive and time-consuming (ENETWILD consortium et al., 2018). In general, faecal pellets are used as a DNA source for genetic estimation of population densities, e.g. in red deer (*Cervus elaphus*) (Ebert et al., 2021), and in wild boar (Kolodziej et al., 2012; Kolodziej et al., 2011). However, faecal samples have to be collected and genotyped in a comparatively time-consuming process. In addition, the faecal samples collected during a defined period also only reflect the genetic diversity of a population and all the findings concluded from it for this period. It would therefore be beneficial to access other DNA sources that provide similarly valid population density estimates with less collection time. In principle, non-invasive sampling, as with faecal samples, would also be preferable. While hair samples are a good alternative for other animal species, e.g. European badger (*Meles meles*) (Judge et al., 2017), they are rather unsuitable for wild boar (Ebert et al., 2009), even if recent results indicate that at least some evidence of abundance is possible (Evans et al., 2024). Although hunting bags are not non-invasive, they are the most effective alternative for collecting genetic samples. In particular, drive hunts that take place in the winter months allow fast and easy access to the hunting bag and allows rapid muscle tissue sampling in large numbers. The high DNA quality of a fresh muscle sample is another benefit of using the hunting bag as a sample source. In addition, the hunting bag can be used to obtain additional genetic information that would normally remain unused, such as the genetic relationships between the sampled animals or the rate of multiple paternities (Müller et al., 2018; Müller et al., 2020). A muscular tissue sample can also be taken during single and hide hunts. Sampling is ideally carried out by the hunters themselves, as is the temporary storage of the samples, which means that any population can be sampled throughout the year (Keuling et al., 2014).

In the present study, the genetic relationship structures within the hunting bags are of particular interest. While classical capture-mark-recapture (CMR) methods like the Lincoln-Petersen estimator (Lincoln, 1930; Petersen, 1896) allow an estimation of the population size by the proportion of capture and recapture events, genetic capture-mark-recapture (g-CMR) enables modelling of the actual population size based on the genetically determined number of parents and offspring in the hunting bag (Müller et al., 2020). The aim of the present study is to determine the extent to which a classic capture-mark-recapture can be transferred to a hunting bag in which recapture in the true sense is no longer possible because the animals are dead. By taking tissue samples from these animals, it should be possible to identify the genetic relationships between parents and their offspring if the sample size is large enough. In this case, the genetically determined genotypes of the parent animals should serve as capture and mark and a positive assignment of an offspring, also via its genotype, would correspond to its recapture. The sampling intensity or the size of the subsample should be approximately 10% of the total population in order to avoid any bias in the density extrapolations. (Larroque & Balkenhol, 2023; Müller et al., 2020). Therefore, if the sample size of the hunting bag is sufficiently large, it can be expected that with a high number of genetic recaptures in a hunting bag, the remaining population should be comparatively smaller than in hunting bags with low recapture rates. Furthermore, it is investigated in the present study whether sampling the annual hunting bag of wild boar allows for reliable and robust population estimate. In order to validate the calculated population sizes by g-CMR, they are compared with other methods like extrapolation of the annual hunting bag, camera traps and distance sampling, which took place at the same time in the same study area. Finally, a population in Rhineland-Palatinate, Germany, which was already investigated in 2008, was used for comparison (for a detailed review see Ebert et al., 2012). As already mentioned, the population density in this study was determined by capture-recapture modelling with genotyping of faecal samples. As samples from the hunting bag from the same period and study area are also available here, the densities can be calculated using the method presented here and allow a direct comparison.

5.2. Materials and methods

5.2.1. Study site and sampling

The study area is part of the Forestry commission office Harsefeld, in the northern part of Lower Saxony, Germany. The Forestry commission is segmented in seven forestry districts. The present study was focused on the two districts of Bevern and Hallah (Keuling et al., 2014). The area under hunting in district Bevern includes about 480 km^2 and in district Hallah about 520 km^2. Both districts are not directly connected, but separated by three other hunting districts (https://www.landesforsten.de/forstaemter/harsefeld/forstamt-harsefeld-wir/). Both district consist of 60% agricultural area, 36% woodland and other habitat structures, such as marsh or hedgerow and 4% housing area (Keuling et al., 2014). The forest composition in both districts consists of about 70% conifers like spruce (*Picea abies*) and pine (*Pinus sylvestris*) and 30% deciduous tree species such as beech (*Fagus sylvatica*) and oak (*Quercus* spp.). The maize cultivation area includes 50% in Bevern, but only 37% in Hallah. Concerning the proportion of grassland, the situation is almost reverse.

Samples of all age classes were collected on drive hunts from October 2011 until February 2012, October 2012 until February 2013 and from October 2013 until December 2013. Additionally, sampling from single hunts were collected consistently during the entire study.

The first animals were sampled in April 2011 and the last in December 2013. All sampled animals were grouped in three age classes in the following way: piglets up to 12 months, sub adults from 13 to 24 months and adults older than 24 months. 5 – 15 g muscle tissue samples of all individuals were stored in 5 ml tubes containing 4 ml 99.8 % ethanol at 4°C for subsequent kinship analyses.

Although regional and seasonal differences regarding age and sex of the hunted animals may occur in the hunting bag (Keuling et al., 2010; Toïgo et al., 2008), it is usually balanced overall, at least in Central Europe (Keuling et al., 2013). A balanced distribution of age and sex across sample regions and seasons is an important prerequisite for independent and random sampling thereby leading to reliable genetically-based population density estimates.

5.2.2. DNA extraction, amplification of microsatellite primers and PCR-Conditions

DNA extraction was conducted following the Chelex™ 100 (Bio-Rad) protocol (Walsh et al., 1991) with 25 mg tissue for each sample. With a NanoDrop™ 1000 spectrophotometer (formerly PEQLAB Biotechnologie GmbH, now VWR International, LLC) the DNA concentration of each sample was measured and uniformed to 70 ng/µl by ultrapure water dilution (LiChrosolv®, Merck KGaA). This procedure avoids DNA-amplification interferences during PCR caused by excessive amounts of DNA. A set of 14 microsatellites was used as follows: four tetranucleotides (387A12F, KVL9495, KVL9807 and S0655) (Karlskov-Mortensen et al., 2008; Robino et al., 2008), one trinucleotide (TNFB) (Rohrer et al., 1994) and nine dinucleotides (CGA, S0005, S0090, S0226, Sw24, Sw72, Sw632, SW742 and SW963) (Ernst et al., 1998; Kato et al., 1991; Rohrer et al., 1996; Rohrer et al., 1994). For genotyping the forward sequence of each microsatellite it was labelled with a fluorescent marker (biomers.net GmbH) (see Table 5.1).

PCR was done with Peqlabs “Taq all inclusive” Kit (formerly PEQLAB Biotechnologie GmbH, now VWR International, LLC): for amplifications we used a 10 µl reaction volume containing 4.15 µl ultrapure water, 1 µl buffer solution Y, 0.2 µl dNTP (10 mM), 2 µl enhancer solution P, 2 µl DNA (70 ng/µl), 0.3 µl forward (10 pmol/µl), 0.3 µl reverse (10 pmol/µl) primer and 0.05 µl peqGold Taq DNA Polymerase. In cases where microsatellite systems shared identical PCR condition (e.g. KVL9495 and KVL9807), all primer pairs where added in one 10 µl reaction volume, but the amount of ultrapure water was reduced at the same volume. The used primer systems and PCR conditions are listed in Table 5.2 and 5.3.

Table 5.1: Primer sequences of the 14 microsatellites. For capillary electrophoresis, a specific fluorescent label (Cy5 = blue, BMN6 = green, Dy-751 = black) was attached to the 5´end of every forward primer of each primer pair (primer designed after [1] Rohrer et al. (1994), [2] Ernst et al. (1998), [3] Robino et al. (2008)). Repeat length of the loci varies from 2 – 4 base pairs. Where possible the microsatellite’s location within the genome is specified (SSC = porcine chromosome). The same applies for the accession numbers.

Microsatellite	Label	Repeat length	Forward Primer	Reverse Primer	SSC	Accession No.
387A12F [3]	BMN-6	4	5’ GCT TTT ACA GAT GCC CAA GG 3’	5’ ATC GCT TTG CTG TAC ACC TG 3’	12	-
KVL9495	BMN6	4	5‘ CAC AGC TGG.GCG AAG TTA AC 3‘	5‘ CTC CTT TAA AAG CTC CTT GTG AGA G 3‘	NA	EU010900.1
KVL9807	Cy5	4	5‘ AAG TAT TAA GCA GAA CCC.AGC GTG 3‘	5‘ CCA GTT CTT TTC AGA CCC AGA CTC 3‘	NA	EU011210.1
S0655	Dy-751	4	5’ CCA ATT GGA CCC CTA GTC TG 3’	5’ AGC CCC CTA GTC CCA AAT TA 3’	7	AJ251829.1
TNFB [1]	Dy-751	3	5‘ CTG GTC AGC CAC CAA GAT TT 3‘	5‘ GGA AAT GAG AAG TGT GGA GAC C 3‘	7	X54859.1
CGA	BMN-6	2	5‘ ATA GAC ATT ATG TAA GTT GCT GAT 3‘	5‘ GAA CTT TCA CAT CCC TAA GGT CGT 3‘	1	D00768.2
S0005	Cy5	2	5‘ TCT TCC CTC CTG GTA ACT A 3’	5’ GCA CTT CCT GAT TCT GGG TA 3’	5	M97232.1
S0090	Dy-751	2	5’ CCA AGA CTG CCT TGT AGG TGA ATA 3’	5’ GCT ATC AAG TAT TGT ACC ATT AGG 3’	12	M95002.1
S0226 [2]	Cy5	2	5’ GCA CTT TTA ACT TTC ATG ATA CTC C 3’	5’ GGT TAA ACT TTT NCC CCA ATA CA 3’	2	-
Sw24 [1]	Dy-751	2	5’ CTT TGG GTG GAG TGT GTG C 3’	5’ ATC CAA ATG CTG CAA GCG 3’	17	-
Sw72 [1]	BMN-6	2	5’ ATC AGA ACA GTG CGC CGT 3’	5’ TTT GAA AAT GGG GTG TTT CC 3’	3	-
SW632 [1]	Cy5	2	5’ TGG GTT GAA AGA TTT CCC AA 3’	5’ GGA GTC AGT ACT TTG GCT TGA 3’	7	-
SW742 [1]	Dy-751	2	5‘ AAT TCT ACT TCT GGG GAG AGG G 3‘	5‘ CTT TTG GGA ACA TTT CTG CC 3‘	16	EF130727.1
SW936 [1]	BMN-6	2	5’ TCT GGA GCT AGC ATA AGT GCC 3’	5’ GTG CAA GTA CAC ATG CAG GG 3’	15	-

Table 5.2: Used PCR conditions for all primer systems without S0005

Microsatellite	**Denaturation** (Temperature [°C]/ Duration [s])	**Cyclic denaturation, annealing and elongation** (Temperature [°C]/ Duration [s])	**Number of cycles**	**Final elongation and cooling** (Temperature [°C]/ Duration [s])
SW742	*94°C/300s*	**94°C/30s** **60°C/30s** 68°C/30 s	*35*	**68°C/900s** *4°C/∞*
KVL 9495 **KVL 9807**	**95°C/300s**	**95°C/60s** **58°C/60s** **72°C/60 s**	**35**	**72°C/900s** **4°C/∞**
TNFB **CGA**	**94°C/300s**	**94°C/30s** **55°C/30s** **68°C/30s**	**35**	**68°C/1200s** **4°C/∞**
S0090 **SW632**	**94°C/300s**	**94°C/30s** **55°C/30s** **72°C/30s**	**32**	**72°C/900s** **4°C/∞**
SW936	**94°C/300s**	**94°C/30s** **60°C/30s** **72°C/3*0 s***	**32**	**72°C/900s** **4°C/∞**
Sw24	**94°C/300s**	**94°C/30s** **55°C/30s** **72°C/30s**	**35**	**72°C/900s** **4°C/∞**
Sw72 **S0226**	**94°C/300s**	**94°C/30s** **52°C/30s** **72°C/30s**	**32**	**72°C/900s** **4°C/∞**
S0655 **387A12F**	**94°C/240s**	**94°C/20s** **61.5°C/40s** **72°C/30s**	**35**	**70°C/1200s** **4°C/∞**

Table 5.3: Used touchdown-PCR (TD-PCR) conditions for primer system S0005 only.

Micro-satellite	Denaturation (Temperature [°C]/ Duration [s])	1st cyclic denaturation, annealing and elongation (Temperature [°C]/ Duration [s])	Number of cycles	2nd cyclic denaturation, annealing and elongation (Temperature [°C]/ Duration [s])	Number of cycles	Final elongation and cooling (Temperature [°C]/ Duration [s])
S0005	*94°C/300s*	**94°C/ 30s** **55°C (-1°C per cycle)/30s** 68°C/30s	5	**94°C/30s** **56°C/30s** *72°C/30s*	*30*	**72°C/900s** 4°C/∞

5.2.3. Genotyping and allele binning

Genotyping was carried out on an automated CEQ 8800 series Genetic Analysis Systems (Beckman Coulter). All PCR products were diluted with 20 µl HPLC water and 2 µl of this was mixed with 30 µl of CEQ Sample Loading Solution (Beckman Coulter) and 0.15 µl of CEQ DNA Size Standard Kit 400 bp (Beckman Coulter). Sizing of the fragments followed the Beckman Coulter standard protocol for the CEQ 8800 series and the GenLab software version 9.0.25 (Beckman Coulter). Allele binning was performed with R 3.3.2 (R Development Core Team (2016)) and the package MsatAllele 1.02 (Alberto, 2009).

5.2.4. Descriptive statistics of the loci

Calculations of allele frequency, observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}), polymorphism information content (PIC) (Botstein et al., 1980), deviations from Hardy-Weinberg Equilibrium (HWE) and presence of null alleles were performed with *CERVUS 3.0.7* (Kalinowski et al., 2007; Marshall et al., 1998; Slate et al., 2000). Values close to zero indicate that no null alleles are present. Negative and strongly positive values are indicators of an excess of null alleles and thus indicate an excess of homozygotes. However, the values do not represent conclusive proof of the presence of a null allele.

5.2.5. Repeatability and error rates

For quality assurance, each PCR contained a positive control (sample with known fragment length) and a negative control (HPLC water instead of DNA) (Budowle et al., 2005; Selkoe & Toonen, 2006). To calculate the overall genotyping error rate (Bonin et al., 2004), about 10% of both sample sets (38 samples from Bevern and 18 samples from Hallah) (DeWoody et al., 2006; Selkoe & Toonen, 2006) were randomly repeated (http://www.random.org/lists/) by independent DNA Extraction, PCR and genotyping. Runtime deviations of more than one half of the repetitive motive length, resulting in different scoring alleles, were evaluated as errors. Experience has shown that error rates of up to 15% can occur (Pompanon 2005). In the present case, on the basis of preliminary studies (data not shown here), error rates of less than 5% are to be expected. Nevertheless, to compensate all potentially undetected errors, we assumed a 5 % error rate in all used software packages that require one. If the calculated error rate is higher than 5%, the correspondingly higher value was used.

5.2.6. Calculation of population parameters

5.2.6.1. Parent-offspring detection

The kinship relations were conducted independently for both, hunting district and hunting year. Consequently, all sampled animals were grouped in three years and two districts. At the same time, animals were grouped either as potential offspring or as potential female or male parent. All animals equal or younger than 12 months are considered as offspring. Accordingly, all animals equal or older than 13 month were considered as potential parents (Keuling et al., 2014; Müller et al., 2018). The paternity analysis was calculated with *COLONY 2.0.7.0* (Jones & Wang, 2010b; Wang, 2004; Wang, 2013; Wang & Santure, 2009). *COLONY* utilises a full maximum likelihood method for joint parentage and sibship assignment using individual multilocus genotypes at a range of codominant or dominant marker loci. It can be used to determine parentage but also to estimate full and half sibling relationships and to infer polygamous or monogamous mating systems. In particular, those animals from the pool of potential and genotyped offspring that are not genetically assigned to a potential and genotyped parent by *COLONY*, e.g. because this parent was not sampled, allow conclusions to be drawn about the overall population. If no genotyped parent can be assigned to a genotyped offspring, a hypothetical genetic parent is generated instead. In this way, the minimum number of parents that must be present in the population can be estimated, even if no actual genetic samples are available for them. Only then can the presence of all genotyped offspring be genetically explained. The following settings were used: 'Mating System – I' = polygamy for both, male and female parents; 'Mating System – II' = without inbreeding and without clones; 'Sibship Prior' = No Prior; 'Analysis Method' = Full-Likelihood (FL); 'Likelihood Precision' = High;

‘Length of Run’ = Long; default settings were used for ‘Species’, ‘Run Specifications’ and Paternal Sibship Size.

5.2.6.2. Reproductive capacity and missing parents

By calculating the relationships in the hunting bag, important population parameters can be calculated. One parameter is the average reproductive capacity of the potential parents in the sub-sample and the second parameter is the minimum number parents, which not have been sampled but their offspring, and vice versa. The genetic assignment rate between potential offspring and putative parents calculated by *COLONY*, allow for an approximate estimate of the number of offspring per parent. If, for example, in a data matrix with 100 potential offspring and 20 putative female parents, 40 of the potential offspring could be assigned to 10 of these female parents, this would initially result in a reproductive capacity of 4 offspring/female parent. *COLONY* can also be used to determine the minimum number of missing female parents that would be necessary to genetically explain the 60 remaining unassigned potential offspring. Assuming that this value would be 12, this would result in a corrected reproductive capacity of about 4.5 offspring/female parent. In addition, there are 10 female parents in the data matrix to which no offspring could be assigned. Assuming that these putative parents also have offspring to a similar extent as described above, this results in an additional 45 offspring. The minimum population size would therefore be 145 offspring and 32 parents. In this way, the reproductive capacity and the minimum number of offspring and parents can be calculated. The male putative parent proportion of the population is determined in the same way.

5.2.7. Calculation of the population size and density by g-CMR

The population density calculations for Bevern and Hallah were done using the R-script ‘ESTIMATE_function’ (Müller et al., 2020). Based on the calculated numbers of parent-offspring pairs with *COLONY* we prepared input files for the estimator. Every potential offspring used, is a single element in the input file. For the case that both parents could be assigned to an offspring, this was treated as one recapture event. This is to prevent underestimation of the population due to an artificially high number of genetic parentage assignments. The ‘ESTIMATE_function’ calculates the number n of adult females and males as well as juveniles in the population based on the parent-offspring-relationships in the sub-sample, including upper and lower confidence intervals.

5.2.8. Validation by simulation

The results of the three hunting years for both populations were validated by simulating 20 virtual populations each, based on the calculated number of parent-offspring pairs. To create a virtual population, four variables are necessary: 1) the number *n* of the calculated adult females and 2) adult males from the ESTIMATE_function; 3) the mean number of piglets per sow, which results from the quotient of the total number of offspring and females; 4) the quantitative estimate of the subsample, i.e. the number of animals removed from the simulated population. For this estimation, the percentage assignment rate of the piglets, calculated by *COLONY*, is taken, because the number of parent-offspring pairs in the hunting bag should reflect the efficiency of hunting. If the assignment rates of the above example of reproductive capacity were used, this would result in a percentage assignment rate of 40%. The number of bootstrap iterations is freely selectable. In the present case, 2000 repeats were performed. Virtual populations were than simulated with the R-script (Test_Beispiel) described in Müller et al. (2020). A total of 20 independent simulations were carried out for each district and each hunting year. The mean values of the simulated population sizes and the corresponding upper and lower confidence intervals allow a direct comparison with the population calculation of the subsamples.

5.2.9. Validation by comparing different methods

Population estimates from other methods in the same period and the same study areas were used to compare with the g-CMR results calculated here.

One of the most commonly used methods for estimating population density is direct extrapolation from the annual hunting bag. However, such extrapolating or back-calculating of a population density from a hunting bag is considered rather problematic, since one never knows how effectively hunting was carried out and how high the actual reproduction was. But in principle the following applies: summer population = winter population + increase/growth (assuming that hunting skims off the increase/growth). Accordingly, the increase/growth is equal to the hunting bag. The summer population then corresponds to the winter population + hunting bag. With an estimated postnatal growth of about 220% (Frauendorf et al., 2016; Keuling et al., 2013) the winter population corresponds to the hunting bag divided by 320%, WP = HB/320%. According to this approach, the population densities of 2011 and 2012 in Bevern and Hallah were calculated on the basis of the total annual hunting bag.

Camera traps (CT) were also used to estimate population density, using the random encounter model (REM) that needs no individual recognition for density estimations (Rowcliffe et al., 2008; Rowcliffe et al., 2016). The study period in Bervern covered spring 2012, while in Hallah population densities were carried out in winter 2012/2013 and summer 2013 (Keuling et al., 2014).

Distance sampling (DS) as “random” point counts (e.g. from hide-outs) (Keuling et al., 2014; Keuling et al., 2018) were used as a third method of comparison. The surveys in Bevern and Hallah took place in March and April 2012.

As part of the research project "Reproductive success, relationships and kin selection in wild boar, *Sus scrofa*" in Rhineland-Palatinate, a local hunting bag of 2007/2008 was sampled and analysed in the same way as described for Bevern and Hallah. At the same time, a capture-recapture study was carried out in the same study area using wild boar faecal samples, collected by transect sampling (Ebert et al., 2012). This allows the results of both CMR methods to be directly compared and mutually validated. The project was supervised by the ‘Rhineland-Palatinate Research Centre for Forest Ecology and Forestry’ (Forschungsanstalt für Waldökologie und Forstwirtschaft Rheinland-Pfalz, FAWF).

5.3. Results

5.3.1. Sampling

During the sampling period from 2011 to 2013, 366 wild boars were collected in district Bevern, of which 191 individuals were female and 159 male. No information on sex was available for 16 individuals. There were 216 individuals classified as piglets and the remaining 147 were sub-adults or adults. No age information was available for 3 individuals. In Hallah, 170 samples were taken during the same period. There, 89 females and 75 males were sampled. No information on sex was available for 6 animals. A total of 110 animals were classified as piglets and the remaining 60 were sub adults or adults. A detailed overview of the age and sex distribution per hunting year can be found in Figure 5.1.

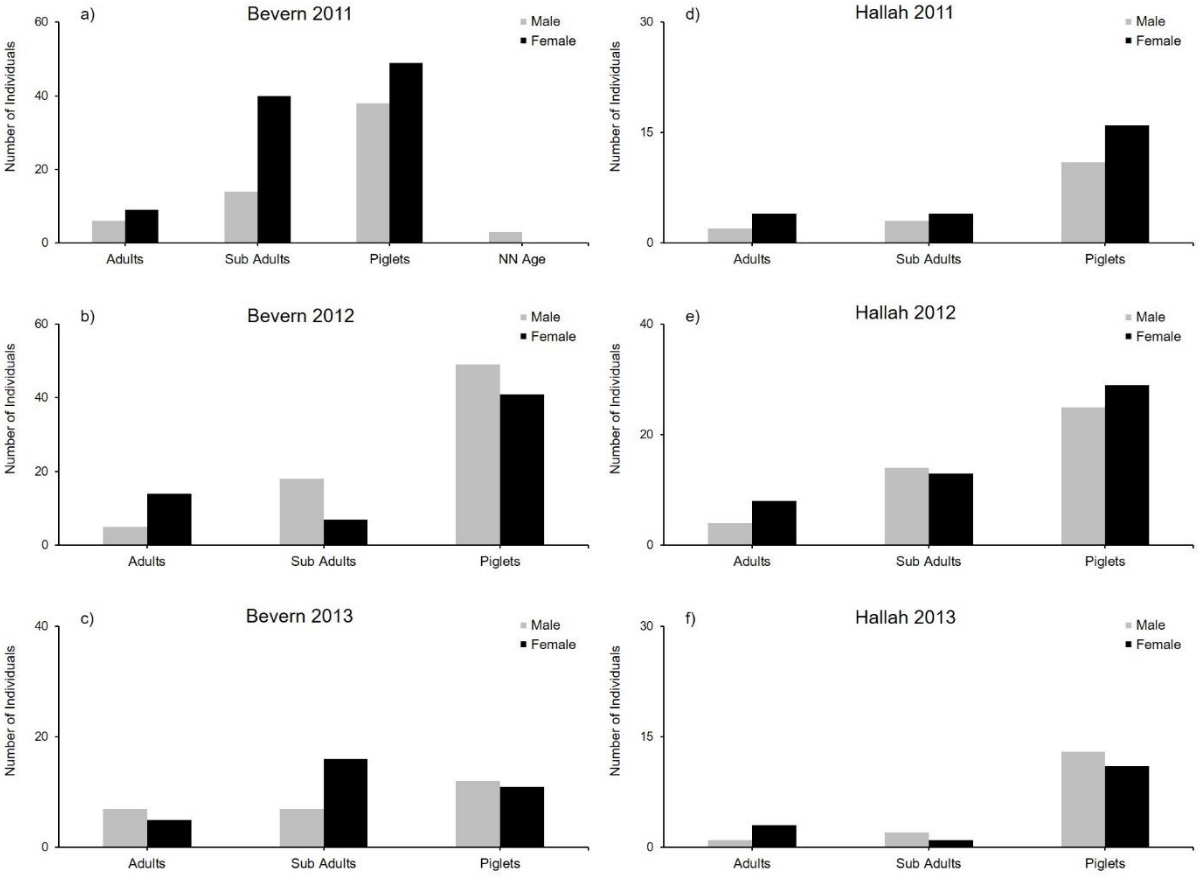

Figure 5.1: Sex and age distribution for the hunting years 2011-2013 in the Bevern study area (a-c) and in the Hallah study area (d-f). Males and females are displayed as grey and black bars, respectively.

In 2011, the highest number of animals was collected in Bevern with 164 sampled animals, whereas the lowest number was collected in Hallah in 2013 with 37 samples. In the latter case, it must be assumed that the subsample of the total population may not be sufficient to be able to carry out a successful density extrapolation. The same applies to Hallah in 2011, with 40 samples, and Bevern in 2013, with 66 samples. However, without a sufficient number of genetic parentages to be verified, extrapolations can become unreliable and may even be impossible. In the latter case, a simulation series as described above could at least provide an approximation of the failed population calculations.

5.3.2. Descriptive statistics of the loci

In district Bevern all microsatellite loci were highly polymorphic and suitable for individual discrimination purposes, with the exception of S0226 and 387A12F, which are only reasonably or slightly informative (see Table 5.4 a-c) (Botstein et al., 1980; Waits et al., 2001). The polymorphism information content (PIC) ranged from a minimum of 0.264 for locus 387A12F to a maximum of 0.848 for KVL9807 with a mean of 0.66 ± 0.152 ($\overline{x}$ ± SD). There was one significant deviation from HWE in 2011 (Sw632), but none in 2012 and 2013. In contrast, there are some indications of null alleles in all three years of the study. In 2013, this applies to 10 of the 14 loci.

Table 5.4: Diversity indices of the 14 microsatellites for the Bevern study area for the successive hunting years from 2011 to 2013 (a-c). Shown are the microsatellite system (MS-system), the number of alleles per locus (A), the number of genotyped individuals (N), the observed and expected rate of heterozygosity (H_{obs}, H_{exp}), the polymorphism information content (PIC), the results of the Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HWE}) and the calculated frequency of null alleles (F(Null)). P_{HWE} legend: NS = Not Significant; ND = Not Done; * = significant deviation.

a: Bevern 2011

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
387A12F	4	157	0.268	0.294	0.264	ND	0.0519
KVL 9495	14	163	0.804	0.840	0.819	NS	0.0227
KVL 9807	9	160	0.713	0.840	0.819	NS	0.0839
S0655	5	148	0.662	0.706	0.649	NS	0.0309
TNFB	7	161	0.851	0.733	0.689	NS	-0.0828
CGA	11	153	0.745	0.801	0.768	NS	0.0372
S0005	16	153	0.817	0.847	0.829	NS	0.0100
S0090	5	162	0.673	0.748	0.706	NS	0.0522
S0226	4	156	0.513	0.517	0.404	NS	-0.0003
Sw24	5	157	0.650	0.685	0.631	NS	0.0279
Sw72	4	164	0.585	0.586	0.543	NS	-0.0009
Sw632	8	162	0.765	0.743	0.703	*	-0.0306
SW742	6	154	0.584	0.618	0.577	NS	0.0329
SW936	5	164	0.768	0.768	0.727	NS	-0.0015

b: Bevern 2012

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
387A12F	4	129	0.341	0.334	0.295	ND	-0.0084
KVL 9495	14	134	0.813	0.847	0.826	NS	0.0203
KVL 9807	10	133	0.872	0.867	0.848	ND	-0.0061
S0655	5	124	0.685	0.705	0.650	NS	0.0121
TNFB	5	128	0.664	0.699	0.642	NS	0.0298
CGA	7	123	0.780	0.803	0.770	NS	0.0109
S0005	15	129	0.729	0.803	0.779	NS	0.0470
S0090	6	128	0.727	0.740	0.698	NS	0.0108
S0226	3	126	0.413	0.511	0.391	NS	0.1059
Sw24	5	115	0.774	0.757	0.712	NS	-0.0135
Sw72	4	135	0.719	0.646	0.596	NS	-0.0592
Sw632	8	136	0.757	0.769	0.732	NS	0.0030
SW742	5	119	0.622	0.593	0.550	NS	-0.0154
SW936	5	136	0.779	0.755	0.709	NS	-0.0173

c: Bevern 2013

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
387A12F	4	64	0.375	0.382	0.339	ND	-0.0100
KVL 9495	12	60	0.783	0.857	0.835	ND	0.0438
KVL 9807	8	61	0.885	0.844	0.818	ND	-0.0318
S0655	5	63	0.794	0.748	0.695	ND	-0.0355
TNFB	5	66	0.742	0.725	0.669	NS	-0.0174
CGA	6	60	0.667	0.794	0.754	ND	0.0846
S0005	12	63	0.889	0.833	0.805	ND	-0.0415
S0090	7	62	0.790	0.791	0.751	ND	-0.0058
S0226	3	59	0.508	0.537	0.422	NS	0.0238
Sw24	5	65	0.677	0.749	0.699	NS	0.0485
Sw72	4	64	0.563	0.568	0.520	NS	-0.0046
Sw632	7	66	0.833	0.756	0.712	NS	-0.0567
SW742	5	63	0.619	0.582	0.539	NS	-0.0401
SW936	5	66	0.788	0.770	0.724	ND	-0.0141

Also in district Hallah, all microsatellite loci were highly polymorphic and suitable, again with the exception of S0226 and 387A12F, which are only reasonably or slightly informative. (see Table 5.5 a-c). The PIC ranged from a minimum of 0.186 for locus 387A12F to a maximum of 0.86 for KVL9495 with a mean of 0.63 ± 0.166 ($\overline{x}$ ± SD). However, particularly in 2011 and 2013, the deviation at most loci can no longer be calculated. Evidence for the presence of null alleles can be found in all three years.

Table 5.5: Diversity indices of the 14 microsatellites for the Hallah study area for the successive hunting years from 2011 to 2013 (a-c). Shown are the microsatellite system (MS-system), the number of alleles per locus (A), the number of genotyped individuals (N), the observed and expected rate of heterozygosity (H_{obs}, H_{exp}), the polymorphism information content (PIC), the results of the Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HWE}) and the calculated frequency of null alleles (F(Null)). P_{HWE} legend: NS = Not Significant; ND = Not Done.

a: Hallah 2011

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
387A12F	2	38	0.237	0.212	0.187	ND	-0.0578
KVL 9495	10	40	0.950	0.853	0.824	ND	-0.0659
KVL 9807	8	39	0.667	0.826	0.791	ND	0.1003
S0655	5	35	0.657	0.721	0.666	ND	0.0434
TNFB	6	40	0.875	0.739	0.683	ND	-0.0927
CGA	7	40	0.775	0.728	0.686	NS	-0.0286
S0005	8	38	0.658	0.722	0.681	NS	0.0464
S0090	5	39	0.692	0.768	0.719	ND	0.0513
S0226	2	37	0.432	0.462	0.352	ND	0.0263
Sw24	5	40	0.675	0.704	0.637	ND	0.0177
Sw72	4	40	0.600	0.528	0.461	ND	-0.0686
Sw632	8	38	0.789	0.782	0.737	ND	-0.0087
SW742	5	38	0.737	0.626	0.575	NS	-0.1258
SW936	6	40	0.800	0.773	0.727	ND	-0.0207

b: Hallah 2012

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
387A12F	4	93	0.172	0.204	0.186	ND	0.0983
KVL 9495	15	89	0.944	0.877	0.860	ND	-0.0402
KVL 9807	11	86	0.767	0.849	0.827	ND	0.0480
S0655	5	90	0.700	0.717	0.669	NS	0.0038
TNFB	5	88	0.739	0.767	0.721	ND	0.0164
CGA	7	87	0.759	0.731	0.690	NS	-0.0201
S0005	12	90	0.700	0.777	0.746	NS	0.0486
S0090	7	88	0.761	0.778	0.738	NS	0.0055
S0226	3	87	0.529	0.494	0.400	NS	-0.0409
Sw24	5	84	0.631	0.685	0.616	NS	0.0401
Sw72	4	92	0.620	0.573	0.510	NS	-0.0416
Sw632	7	93	0.731	0.761	0.719	NS	0.0016
SW742	5	87	0.552	0.515	0.467	NS	-0.0420
SW936	5	93	0.699	0.726	0.671	NS	0.0163

c: Hallah 2013

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
387A12F	3	36	0.361	0.335	0.304	ND	-0.0354
KVL 9495	10	37	0.838	0.848	0.819	ND	0.0001
KVL 9807	10	37	0.865	0.822	0.787	ND	-0.0373
S0655	5	36	0.806	0.695	0.628	ND	-0.0828
TNFB	5	37	0.865	0.775	0.725	ND	-0.0673
CGA	6	32	0.750	0.631	0.590	ND	-0.1156
S0005	9	37	0.865	0.782	0.740	ND	-0.0588
S0090	6	36	0.861	0.802	0.760	ND	-0.0427
S0226	3	29	0.379	0.399	0.327	ND	0.0111
Sw24	5	37	0.703	0.670	0.596	NS	-0.0290
Sw72	4	37	0.595	0.591	0.527	ND	-0.0177
Sw632	7	37	0.730	0.765	0.714	ND	0.0179
SW742	5	31	0.677	0.639	0.584	ND	-0.0500
SW936	5	37	0.703	0.722	0.659	ND	0.0094

5.3.3. Repeatability and error rates

The repetition of randomly chosen 10% of the whole dataset with all primer systems showed one deviation from the first results in the data proportion for Bevern but none for Hallah. Accordingly, the detectable genotyping error in the present study is 2.632% in Bevern and 1.786% overall. As explained above, *CERVUS*, for example, requires a pre-estimation of the genotyping error. The default settings recommend an error rate of 5%, which is significantly lower in this case. In order to compensate for any undetected errors, the default settings were retained.

5.3.4. *Parent-offspring detection and assignment rates*

For the parentage calculations with *COLONY*, all females were separated by hunting year, 2011, 2012 and 2013, and the age classes sub adult and adult were defined as putative female parents. The putative male parents were defined in the same way. The potential offspring contains both sexes and was accordingly only classified by hunting year. The full maximum likelihood described above is used to assign the offspring to one or both potential parents, if they are present. In 2011, the assignment rate in Bevern was 77.174% (n=71) and in Hallah 40.741% (n=11) of all offspring were assigned to at least one parent. In the 2012 hunting year, the assignment rate for Bevern was 56.522% (n=52) and in Hallah 51.852% (n=28). In the 2013 hunting year, it was 58.065% (n=18) in Bevern and 41.379% (n=12) in Hallah. The absolute numbers can be seen in Figure 5.2.

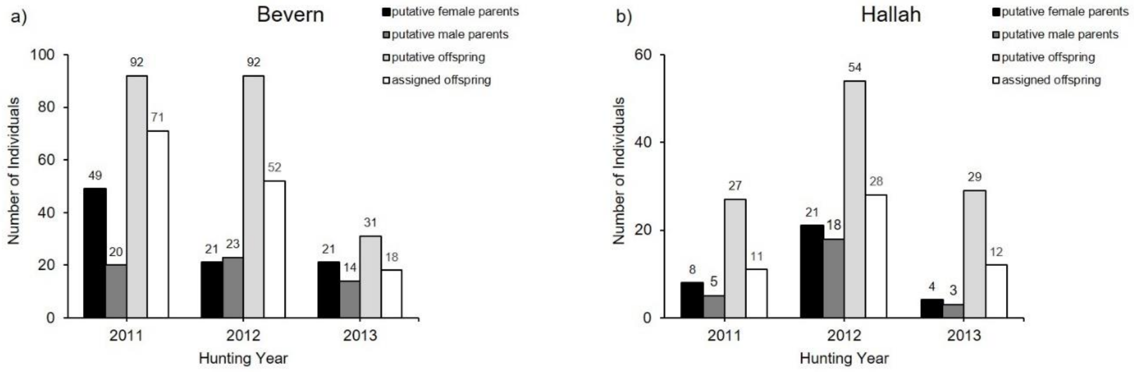

Figure 5.2: Number of genotyped putative female parents (black bars), putative male parents (dark grey filled bars) and putative offspring (light grey filled bars) as well as the number of genetically assigned offspring to at least one putative parent (white bars) based on COLONY. Results are shown for the respective hunting years (2011-2013) in the study areas (a) Bevern and (b) Hallah.

By calculating the assignment rates for the offspring, the number of animals to which no parent could be assigned was also determined. The genetic pool of these animals requires additional parent animals, which could be quantified by *COLONY* (see Figure 5.3a-f).

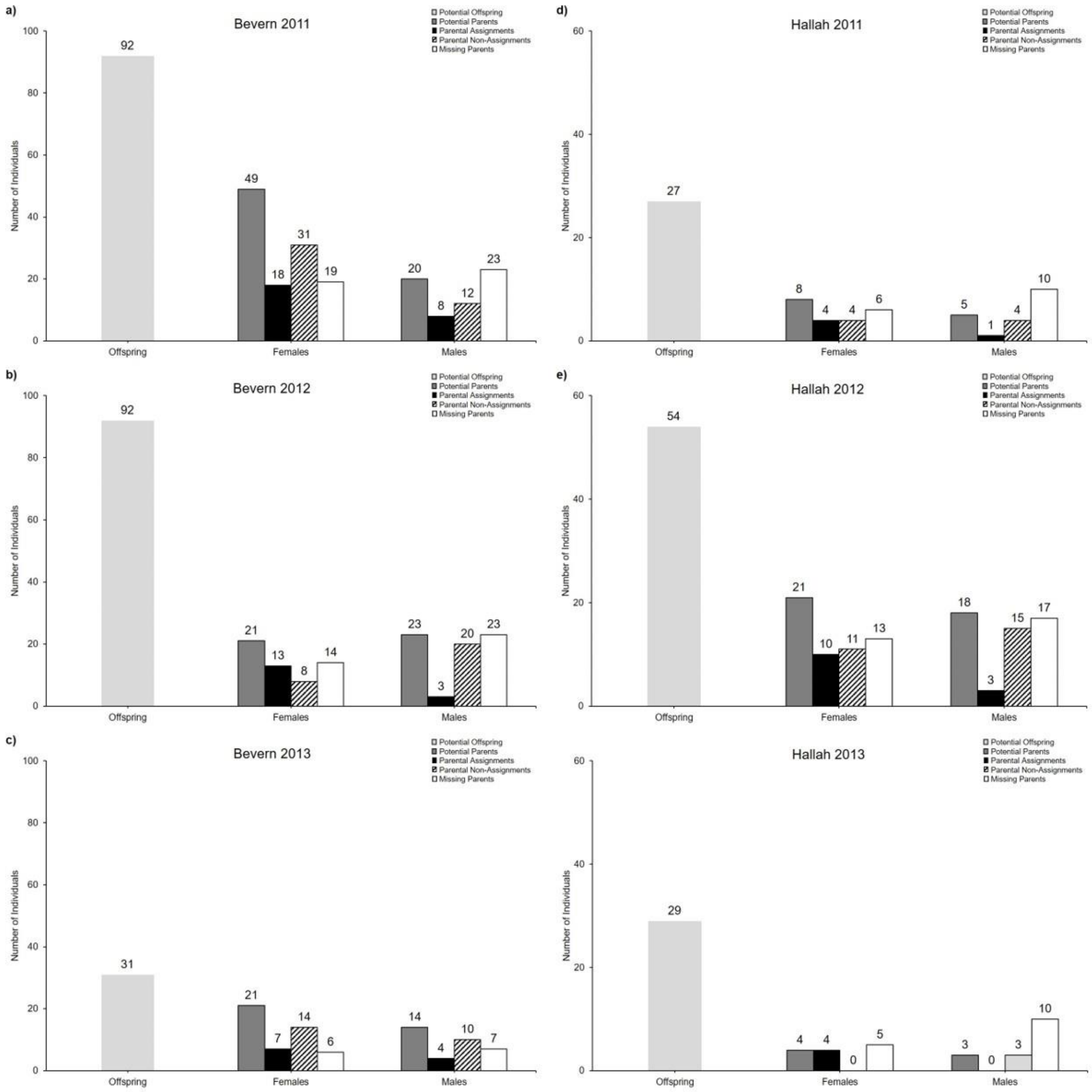

Figure 5.3: Number of genotyped putative offspring (light grey filled bars), all potential parents from the respective hunting year and study area (dark grey filled bars), genetically assigned parents (black bars) from the 'all potential parents' stock, genetically not assigned parents (black striped bars) from the 'all potential parents' stock as well as the number of missing parents (white bars). The number of these additional parents is calculated by COLONY to account for the offspring that remained without a parental assignment. Results are shown for the respective hunting years (2011-2013) in the study areas (a-c) Bevern and (d-f) Hallah.

5.3.5. *Calculation of the population size and density by g-CMR*

Based on the determined parent-offspring pairs, the population size, including 95% confidence intervals, was calculated using the described ESTIMATE_function (Müller et al., 2020). The population densities (n/km^2) were also calculated based on the population sizes and the associated CIs. A hunting area of 480 km^2 was used for Bevern and 520 km^2 for Hallah. In 2013, as already mentioned, it was not possible to calculate the density in Hallah due to the lack of molecularly detectable parent-offspring pairs in the data set. The population sizes and densities for all other hunting years and districts are shown in Table 5.6.

Table 5.6: Determined population sizes (left) and densities (n/km^2) (right) in Bevern and Hallah for the hunting years 2011, 2012 and 2013, also including the calculated confidence intervals. Due to missing relationships in Hallah in 2013, a calculation of the population size was not possible (NA).

	Population Size	Lower 95% CI	Upper 95% CI	Population Density (n/km^2)	Lower 95% CI	Upper 95% CI
Bevern 2011	393	274	907	0.8	0.6	1.9
Bevern 2012	675	342	3263	1.4	0.7	6.8
Bevern 2013	176	107	540	0.4	0.2	1.1
Hallah 2011	222	100	582	0.4	0.2	1.1
Hallah 2012	576	288	2250	1.1	0.6	4.3
Hallah 2013	NA	NA	NA	NA	NA	NA

5.3.6. Validation by simulation

As already explained above, population sizes can also be calculated using simulations if the necessary parameters such as reproductive capacity (see Chapter 5.2.6.2) are known. Accordingly, for example, a reproductive capacity of 2.5 could be determined for Bevern in 2011 (92 offspring, 49 putative female parents, 18 assigned putative female parents, 31 unassigned putative female parents and 19 missing female parents; see Figure 3a). Even if assuming an average of only 2 offspring per female parent, this means that there must be at least a further 62 piglets in the population. If the calculated 2.5 were used as a basis, there would have to be 78 more piglets. The average reproductive capacity of wild boar is significantly higher at around 5-7 offspring per sow (Frauendorf et al., 2016; Gethöffer et al., 2007; Müller et al., 2018). Accordingly, an overestimation of the offspring contingent seems rather unlikely and the simulated population densities should therefore be regarded as the minimum existing population. The male putative parent proportion of the population is determined in the same way. Based on these frame values, 20 independently simulated populations carried out for all calculated subsample-based population sizes in each district and each hunting year. The respective mean values were determined from the population densities calculated by the simulations, including the upper and lower confidence intervals, and compared with the population densities of the sub-sample calculated by the g-CMR. This shows that in all cases the two results from the sub-sample and the average simulation are very close to each other.

The simulated population densities tend to be slightly higher than those of the g-CMR's Estimate_function (see Figure 5.4 a-f). Since no population size could be determined due to the lack of genetic relationships in the subsample of Hallah's hunting bag in 2013, the mean value of the simulations can be used to determine at least a population dimension that can be assumed to be the minimum population density. On average, a population of 168 wild boars and a density of about 0.3 individuals/km^2 (lower 95 % CI: 0.1 individuals/km^2; upper 95% CI: 0.8 individuals/km^2) was calculated (see. Figure 5.4 f).

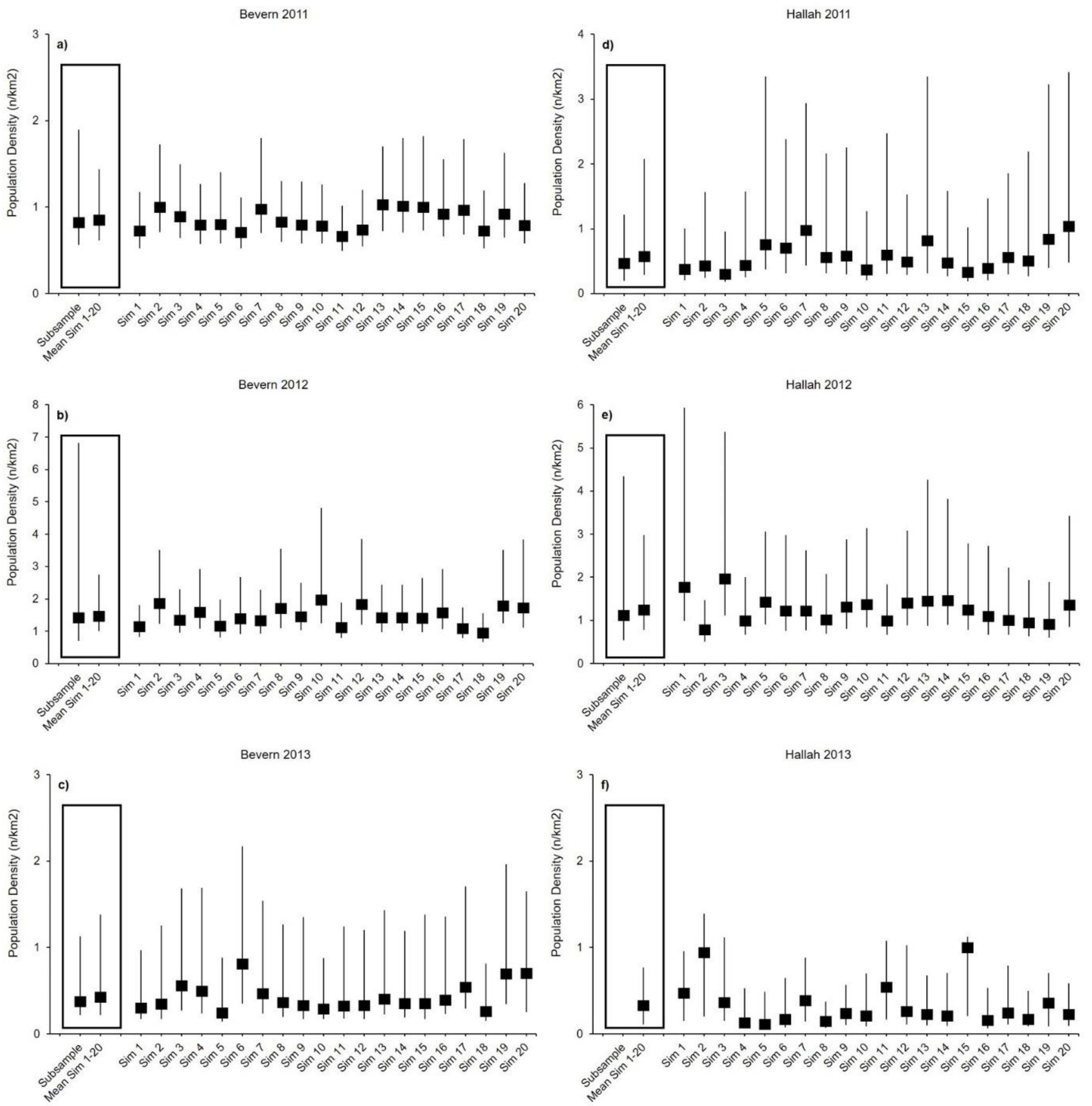

Figure 5.4: Comparison between determined and simulated population densities (number of individuals/km2) for the three hunting years 2011, 2012 and 2013 in Bevern (a, b, c) and Hallah (d, e, f). Vertical lines at each data point show the upper and lower 95% confidence intervals. For comparison with the by g-CMR determined densities, the mean density out of the 20 individual simulations is also shown for each hunting year and district, respectively (data within black framed boxes). No population density could be determined for Hallah in 2013 as no kinship relationships were found.

5.3.7. Validation by comparing different methods

As explained above, summer and winter populations can be extrapolated directly from the total annual hunting bag, assuming that hunting skims off the growth. The total hunting bag in Bevern was 418 wild boars in 2011 and 391 wild boars in 2012. 327 animals were culled in Hallah in 2011 and 334 in 2012. Based on the above-mentioned hunting ground sizes, the summer and winter population sizes and densities can be estimated from the hunting bags and compared directly with the g-CMR and the simulation results of the respective territories and hunting years. In this context, a clear discrepancy between the hunting years becomes apparent. In the 2011 hunting year in Bevern, but especially in Hallah, the average population sizes and densities of the g-CMR are in some cases significantly lower than those of the annual hunting bag and especially below the extrapolated summer populations. Accordingly, in both cases, Bevern and Hallah, the vital population would be smaller than the annual hunting bag. Overall, the simulated population sizes and densities are closer to the annual hunting bag, at a similar level in the case of Bevern and only slightly lower in Hallah. As the simulated results also represent an annual average, the hunt would have accordingly skimmed off the entire population. In the 2011 hunting year, it should therefore be assumed for both hunting grounds and both estimation methods that the actual population sizes were underestimated. A completely different pattern emerges in the 2012 hunting year. While the annual hunting bags remain at a comparable level to the previous year, as do the summer and winter populations, the calculated population sizes and densities of both the g-CMR and the simulations are considerably higher than the hunting bag. While an underestimation of the actual population size should again be assumed for 2012 with regard to the hunting bag extrapolation, it can be assumed for the population sizes of the g-CMR and the simulations that they reliably represent at least the minimum population size of the annual average of the vital populations (see Figure 5.5).

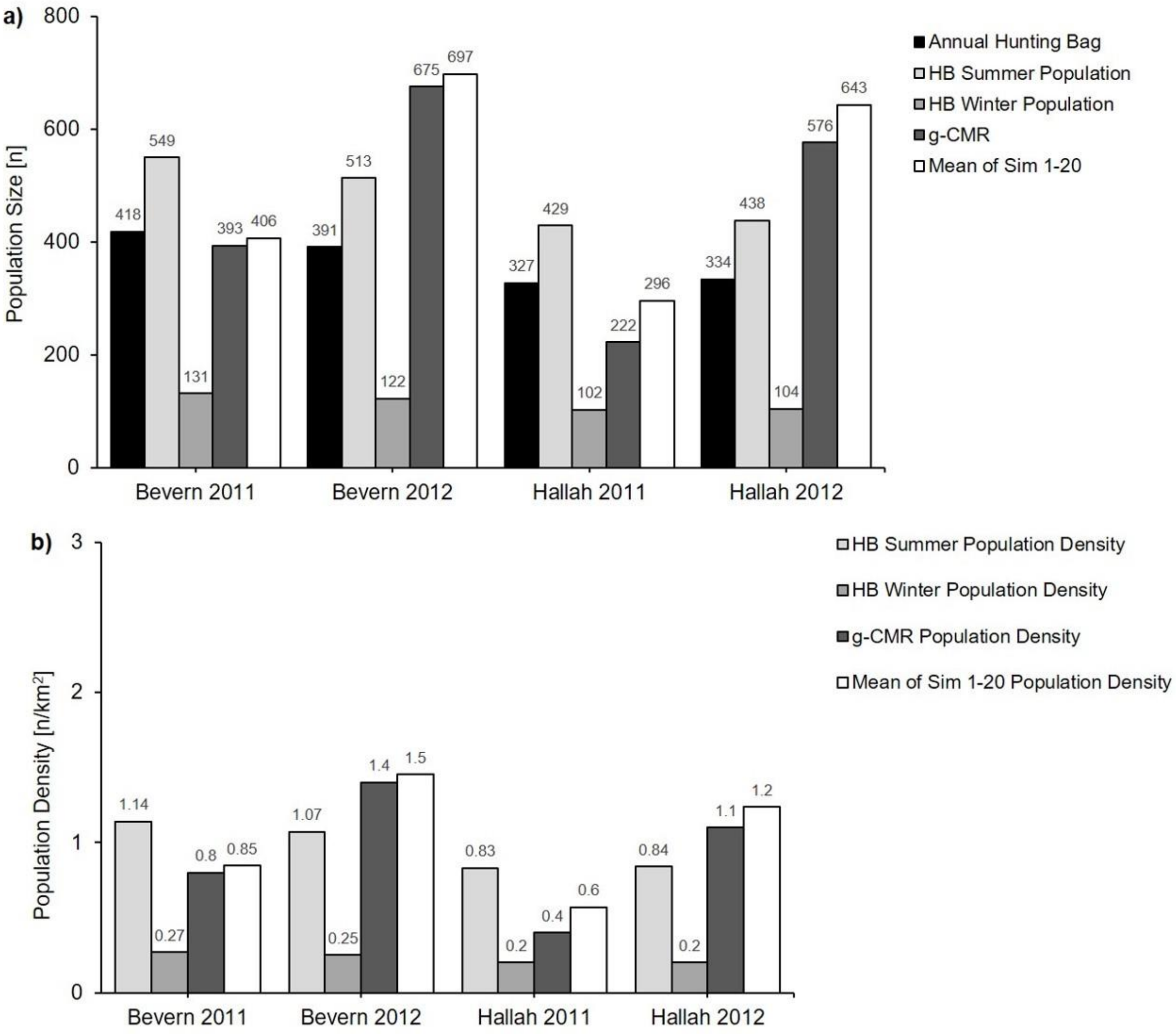

Figure 5.5: The upper part (a) shows the annual hunting bags (black bars) in the corresponding districts and the predicted population sizes for the hunting bag (HB) summer population (light grey bars) and hunting bag (HB) winter population (middle grey bars) by hunting bag extrapolation. The population sizes calculated by the g-CMR (dark grey bars) and the mean values of the simulations (white bars) enable a direct comparison. The lower part (b) shows the population densities (same colour code), excluding the annual hunting bags.

The population densities calculated with camera traps (CT) using the random encounter model (REM) came to the following result: the population densities determined in Bevern in spring 2012 came to a result of 1.05 ± 0.56 ($\bar{x} \pm$ SD) wild boars/km^2. In Hallah in winter 2012/2013, 3.5 ± 2.5 ($\bar{x} \pm$ SD) wild boars/km^2 were recorded and in summer 2013 0.7 wild boars/km^2. Accordingly, only a single spring measurement from 2012 is available for the Bevern district, which largely corresponds to the summer population extrapolation of the hunting bag from 2012. Based on the previous estimates for the hunting bag extrapolation for 2011 and 2012, it can be assumed that the population estimate of the camera traps also underestimates the actual population. The population density of 3.5 wild boars/km^2 in Hallah calculated for the winter of 2012/2013 is over 4 times higher than the hunting bag extrapolation of the winter population 2012 in Hallah. The annual average of 1.1 wild boars/km^2 calculated with the g-CMR is also considerably lower than the REM results. The population density of 0.7 wild boars/km^2 calculated for summer 2013 can only be evaluated and compared to a limited extent due to the lack of results from the g-CMR model. However, summer populations should be around 3 times larger than the winter population (Keuling et al., 2014). Accordingly, it must also be assumed in these two cases that the population densities were not accurately measured.

The population densities calculated with distance sampling (DS) in March and April 2012 were 0.3 (CI 95%: 0.16) wild boars/km^2 in Bevern. Due to missing sightings no density could be determined in Hallah. The single calculated spring population density of 0.3 wild boars/km^2 in 2012 is only slightly higher than the winter population extrapolation of the hunting bag from the same year, at 0.25 wild boars/km^2. Therefore, the DS was insufficient to produce results that could be compared across time with the results of the other methods for almost the entire study period.

As no capture-recapture study based on faecal samples was carried out during the project period, such results from a Rhineland-Palatinate study in 2007 were used for the method comparison. The calculated population densities ranged from 4.5 (2.9-7.8) to 5 (4.0-7.0) wild boars per km^2 (Ebert et al., 2012). During the same time, the hunting bag was also sampled and analysed as described above. The density measurements in the Rhineland-Palatinate population were based on 66 putative female parents, 98 putative male parents and 121 piglets as potential offspring. (Microsatellite diversity indices are not shown, but see Müller 2011). The calculated population size with the 'ESTIMATE_function' was 880 (lower CI 637, upper CI 1706) animals. For the FAWF study area of 160 km^2, this corresponds to a population density of 5.5 wild boars/km^2 (lower CI 4.0 wild boars/km^2, upper CI 10.67 wild boars/km^2). Also for this population, 20 simulations were carried out with a mean (Sim 1-20) population density of 6.5 wild boars/km^2. As the faecal samples were collected in January 2008, the results represent the winter population. The population average calculated with the g-CMR for the entire year is correspondingly 0.5-1 wild boars per km^2 higher. If the average of the 20 simulations were used as reference value, the population density would be 1.5-2 wild boars per km^2 higher (see Figure 5.6).

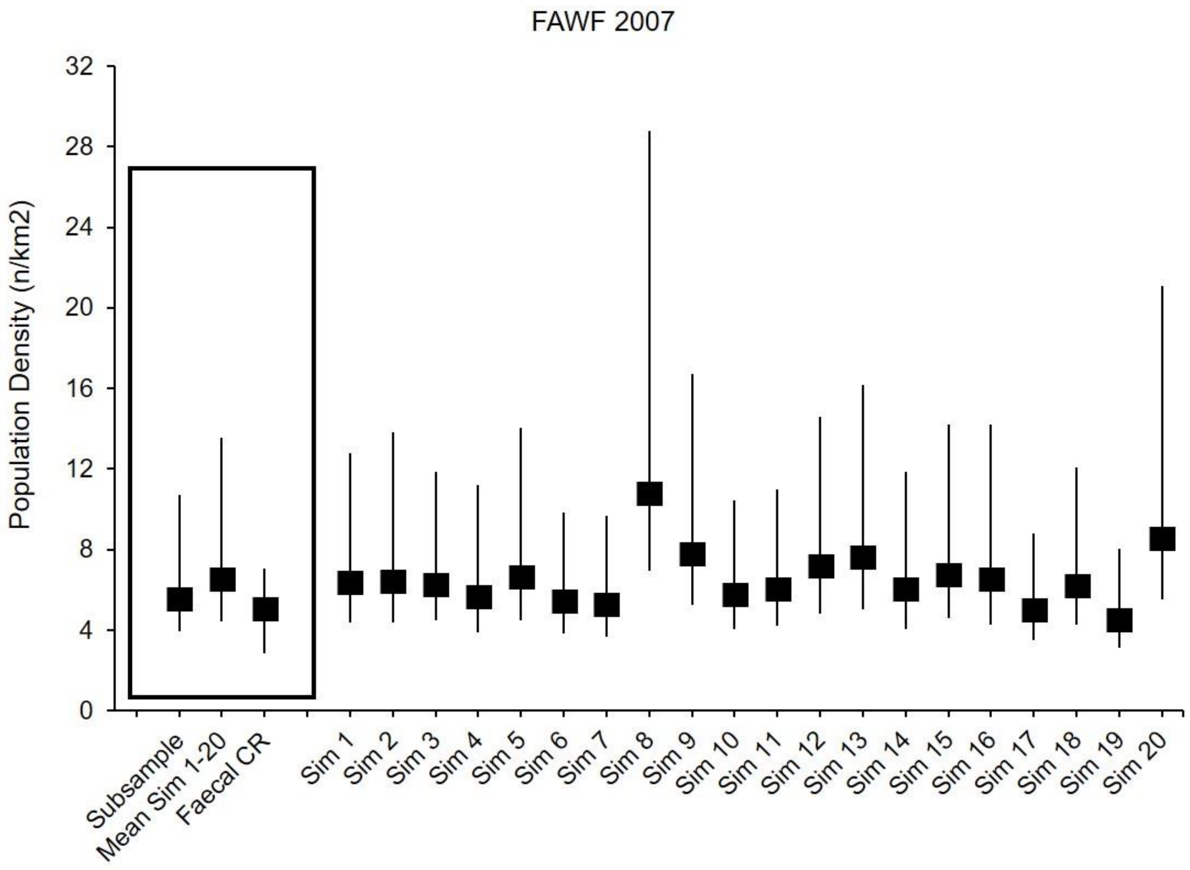

Figure 5.6: Comparison between g-CMR, simulated and faecal Capture-Recapture (CR) determined population densities (number of individuals/km2) for the hunting year 2007 (Rhineland-Palatinate Research Centre for Forest Ecology and Forestry (FAWF). Vertical lines at each data point show the upper and lower 95% confidence intervals. For comparison with g-CMR calculated densities (Subsample), the mean density out of the 20 individual simulations is also shown (Mean Sim 1-20) as well as the Faecal CR densities (data from left to right in the black framed box) followed by the 20 independent simulations in ascending order.

5.4. Discussion

5.4.1. Microsatellite marker analysis

The number of microsatellites used and the calculated indices are of sufficient quality for an accurate determination of the parent-offspring relationships. A mean PIC of > 0.6 shows the sufficiently high quality of the allelic diversity of the data. With one exception (loci Sw632 in Bevern 2011), there are no deviations from Hardy-Weinberg equilibrium (HWE). However, CERVUS often fails to calculate the HWE and accordingly no results are obtained. This applies in particular to Hallah in all three survey years, but also in Bevern in 2013. In Hallah, this is mainly due to the small sample sizes. The most sample-intensive year 2012 shows 4 of 14 loci not calculated, in the sample-weak years it is 12 in 2011 and 13 in 2013. For Bevern, this also only applies to the sample-weak year 2013, where HWE could not be calculated for 8 of 14 loci. As deviations from the HWE do not occur in the presence of sufficiently high sample numbers, a population-wide deviation from the HWE is unlikely. The comparatively frequent indications of null alleles are therefore also more likely to be a side effect of insufficient sample sizes. Since significant deviations from HWE are caused in particular by the presence of null alleles (Pemberton et al., 1995), there should have been significantly more deviations in the years in which HWE could be calculated. However, with only a single significant deviation at loci Sw632, this does not appear to be the case. If a single or only a few microsatellites deviate from HWE, genotyping errors are usually the cause. However, a genotyping error rate of less than 3% in Bevern, or less than 2% overall, show high levels of reliability and reproducibility of the data (Pompanon et al., 2005).

5.4.2. g-CMR calculated Population size and density in Bevern

As a result of the not-independent sampling in Bevern in 2011, the population size and density calculated with the g-CMR are likely to be underestimated (Müller et al., 2020). Accordingly, the total annual hunting bag in Bevern in 2011 was 418 animals but the average annual population size calculated using g-CMR for this period was only 393 (406 by Mean Sim 1-20), which would be equivalent to a complete removal of the population, which seems to be a rather unrealistic scenario. The situation for Hallah appears to be similar to that in Bevern during the same study period. Here again, the average population density of 222 (296 by Mean Sim 1-20) animals calculated using g-CMR is lower than the annual hunting bag of 327 animals. Looking in particular at the average values of the 20 simulations carried out, a clear trend towards higher populations can be seen across all years and districts studied. For the period 2011, this confirms the assumption for these two study areas that both g-CMR and the classic hunting bag extrapolation underestimated the real population densities.

The data situation for the following year 2012 is substantially different, particularly with regard to the g-CMR. With about 57%, the offspring assignment rate for Bevern was 20% lower than in the previous year, despite otherwise similar conditions in terms of the number of available putative parents and offspring. The importance of independent and random sampling through hunting, which was emphasised several times in advance, appears to be guaranteed in the 2012 survey year. In addition, other counting methods, REM and DS, carried out in the same period provide more possibilities for comparison. Using the g-CMR, an average annual population density of around 1.4 wild boars/km^2 was calculated in Bevern. Based on the extrapolation of the hunting bag, the summer and winter populations vary between 1.07 and 0.25 wild boars/km^2. Using camera traps and REM, a population density of 1.05 wild boars/km^2 was determined for spring 2012. The distance sampling carried out in March/April 2012 resulted in a density of 0.3 wild boars/km^2. Initially, it appears that the results of the REM and the hunting bag extrapolation seem to be in a similar and comparable range. The DS in spring, on the other hand, is close to the extrapolated winter population and thus appears to be too low. However, if the calculated summer and winter populations are considered at least approximately as the corresponding largest and smallest extent of the population, the annual average in 2012 in Bevern was then substantially less than 1 wild boar per km^2. In contrast, the annual average calculated by the g-CMR is 1.4 wild boars per km^2 and the simulated annual average is 1.5 wild boars per km^2. As both, the calculations with the ESTIMATE function and the simulations were carried out conservatively, i.e. reproduction rates of 2.5 or 3 offspring per sow were assumed and not 4-7 as repeatedly shown in previous studies (Frauendorf et al., 2016; Gethöffer et al., 2007; Müller et al., 2018). Moreover, the results of the g-CMR modelling tend to represent the minimum population that must have been present on average in 2012. It can therefore be assumed that the calculated and extrapolated population sizes and densities of both, the hunting bag and DS represent a rather significant underestimation of the actual population size. The REM-based spring population density of 1.05 therefore fits more closely with the population pattern calculated by g-CRM and confirms the assumption that the extrapolation of the hunting bag and DS was too low. However, a single REM result from just one season of year is not a suitable means of determining an annual average and should therefore be seen more as a snapshot of the population density. Although DS is a cost-effective method for determining population densities of wild boar, it has fundamental methodological difficulties.

On the one hand, the positions of the high seats in the study area were not randomly distributed, and on the other hand, the counts were often carried out before and during twilight. However, the main activity of wild boar usually begins at night (Keuling et al., 2008b, 2009). A randomly distributed observation position can only be guaranteed if existing high seats are not used, as these are oriented towards well-frequented game crossings or open shooting fields. The existing high seats must therefore be replaced by randomly selected observation locations. Adjusting the observation period to the activity rhythms of the wild boar, on the other hand, is a problem that can certainly be solved. Additionally, it would be advisable to carry out a DS for a longer period, e.g. March to May, and to repeat it later in the year. For instance, other methods cover significantly longer periods of the year, or almost the entire year. In particular, the data from the g-CMR model, which is based on all-year sampling, provides a much more solid database in this case and therefore more robust results.

5.4.3. g-CMR calculated population size and density in Hallah

In the same period for Hallah (2012), densities of 1.11 wild boars/km^2 were determined by g-CMR and a simulated annual average of 1.2 wild boars/km^2. The extrapolated numbers of the hunting bag determine summer and winter densities between 0.84 and 0.2 wild boars/km^2. Using the REM in winter 2012/2013 density values of approximately 3.5 wild boars/km^2 were recorded. In comparison, the DS did not provide any results due to a lack of sightings. Again assuming that the extrapolation of the hunting bag for Hallah also represent the approximate maximum expansion of the population upwards and downwards over the year, an annual average population density of significantly less than one wild boar per km^2 must also be presumed here. Comparable to Bevern in 2012, Hallah also has an annual density average calculated by the g-CMR, which is at least around 24% higher at around 1.1 wild boars/km^2. Due to the increase in the simulated annual average from 1.1 to 1.2 alone, the difference between hunting bag extrapolation and g-CMR would already be 32%. However, the conservative approach described above should also be regarded as the minimum of the existing average population density in Hallah.

The population density of 3.5 wild boars/km^2 determined by the REM in winter 2012/2013 can only be brought into the density dimensioning calculated by the g-CMR to a limited extent. If one were to assume that the winter population is actually 3.5 wild boars/km^2, one would have to assume subsequently that the summer population would be approximately 7-11 wild boars/km^2, if conservatively considered only 2-3 times higher. However, the REM calculations for the summer of 2013 result in a density of 0.7 wild boars/km^2 and thus only one-tenth or less of the population density that would have to be present if the calculated winter densities were correct. However, these seasonal density ratios should tend to be reversed. With regard to the summer population, telemetry data has shown that large parts of the population moved from their forest habitats to mainly maize but also to rapeseed (*Brassica napus*) fields in summer. As soon as these crops have reached a sufficient height and provide adequate visual cover for all members of the sounder, fields are used as temporary forest replacement (for a detailed review see Keuling et al., 2014). The sounders remain in the fields until the harvest before returning to the forest. Accordingly, the number of camera observations in the forest decreases and the REM generates incorrect data. At the same time, median and bootstrap re-calculations of the REM in winter 2012/2013 show a winter population that assumes only about 2.5 wild boars/km^2 (Keuling 2014). In addition, the REM does not take into account the total area of the study area, but only the sub-area equipped with camera traps.

In 2013, the final year of the project, it was no longer possible to carry out valid population calculations using the g-CRM. The number of available samples was too small, which means that very few or no relationships can be found in the analysed sample and no reliable calculations can be made.

5.4.4. g-CMR and faecal based CR in Rhineland-Palatinate

The faecal sample based capture-recapture study carried out in Rhineland-Palatinate in 2008, estimated population densities of 4.5 to 5.0 wild boars/km^2 (Ebert et al., 2012). The analysis of the 2008 hunting bag using g-CMR estimated population densities of 5.5 wild boars/km^2 and 6.5 wild boars/km^2 by simulating. The main difference between the two studies is that the results of the faecal samples refer the winter population, sampled during 14th-31st January 2008, while the hunting bag reflects the annual average of the population. However, Bayesian-based estimates of population densities can also be used to extrapolate the calculated reproductive output for the faecal sample based CMR. This resulted in average spring population densities of 11.3 (95% CI: 7.2-19.3) wild boars per km^2 (Ebert et al., 2012). Conversely, the g-CMR can also be used to calculate the winter population only. In this case, the calculations and simulations would only use those animals from the corresponding hunting years that originate from the single-, hide- and drive hunts that take place in the winter months. An exclusive calculation of a spring or summer population is also possible in principle, but requires a sufficient sample size of the subsample. With their respective estimates, both approaches show the potential that these methods can provide. With a winter population of up to 5.0 wild boars/km^2, the expected summer population densities are likely to be 2-3 times higher. With average annual densities of 5.5 boars/km^2, correspondingly high summer populations can likewise be expected. In a direct comparison of the calculated densities using the two CMR methods, the faecal sample based CMR results in slightly higher densities. However, the calculated values were corrected downwards by around 0.5-1 wild boars/km^2 after the data has been recalculated (Ebert et al., 2012, unpublished data). In both cases, g-CMR and faecal based CR, important assessments and recommendations can be made for wildlife management and the coming hunting season. By quantifying the hunting effort, in this case for the year 2008, plans and measures can also be prepared to determine the extent to which hunting should impact on the population in the following year

In many aspects, the use of muscle samples from the hunting bag seems to be a more suitable DNA source compared to other DNA sources such as faeces or hair. Muscle samples can be stored easily and long-term and the DNA samples are also of high quality, as they are not subject to any weather influences, for example. The presence of foreign DNA, such as in faecal samples, is also not to be expected. Furthermore, non-invasively collected samples often contain less DNA and is usually already degraded to varying degrees. Accordingly, a DNA screenings may have to be carried out prior to genetic analyses in order to be able to sort out samples with no or too little DNA content at an early stage. This can reduce the number of repeat PCR runs and thus increase cost efficiency.

5.4.5. Conclusions and outlook

The results of this study show very clearly how important a sufficiently large and representative sample size is for the successful calculation of a reliable population size. As the hunting bag itself is already a subsample of the total population, it is all the more important that it is sampled adequately and representatively. Given that the project started in October 2011, individuals from single and hide hunts from spring and summer 2011 are missing in both study areas. This data gap could not be filled in Hallah by the drive hunts that took place in winter. The lack of kinship data from this time interval then inevitably leads to a misjudgement of the population size. In Bevern, although the availability of data was better, there was a high proportion of related animals. This high degree of relatedness (77% offspring assignment rate) of animals from the winter drive hunts leads to a population density bias. Similar to the start of the project, a much lower sample volume was again recorded in the final phase of 2013. Here, too, only partially valid or no population densities can be calculated. As expected, the sample size was largest in the middle of the project in 2012 and the calculated results can be regarded as the most representative and valid of the entire project duration. For future projects, it would therefore be advisable if both the start and end of the project could be selected in such a way that the annual hunting bags and all other samples from e.g. individual nocturnal or hide hunts are included in the data collection to the maximum extent. If the required sample size or an independent sampling for valid calculations is only achieved in one of three project years, a consistent and well-founded presentation of the population trends cannot be achieved in a sufficient way. This also means that no clear recommendations can be made regarding future adjustments for hunting and management strategies. Considering reliable and valid population size estimates with adequate sample sizes, as provided in 2012 in Bevern and Hallah and in the comparative study in Rhineland-Palatinate, the relationship between proper population sampling and the calculated population sizes is evident. At the same time, it should be noted that the g-CMR approach as presented here reaches its limits in areas with low wild boar populations densities and small hunting bags. In this case, it must be ensured that the entire available hunting bag is sampled almost exhaustively or the sample size is either expanded by additional collection methods or another survey method is carried out in parallel. An inadequate data basis was also the cause of significant misjudgements of summer and winter population densities in 2012 using REM and DS calculations. This shows that even small events such as the migration of a population from the forest to the directly neighbouring maize and rapeseed field can have considerable consequences for the calculated results. In particular, the DS must be methodically adapted to the conditions of the study area, especially randomly distributed observation points, and to the corresponding day or night activity patterns of the species under investigation. The extrapolations of the annual hunting bags in this study also represent only an extremely rough method of representing the actual population size. In all cases, it can be assumed that the population sizes have been significantly underestimated. Only the g-CMR method was able to provide an accurate representation of the actual population size and density for the entire year, especially in 2012. Despite the poor data situation, approximate estimates of the existing population sizes and densities were nevertheless possible for the years 2011 and 2013.

In any case, it is recommended to never rely on only one method to determine a population density, but use at least two methods that are mutually supportive and consistent instead. The combination of the estimator presented here with e.g., a non-invasive sampling and density estimation method (Ebert et al., 2012; Kery et al., 2011; Mollet et al., 2015) or camera traps (ENETWILD consortium et al., 2022b; ENETWILD consortium et al., 2022c), could provide mutually validating results that allow both long-term and sustainable wildlife management. At the same time, incorrect wildlife management recommendations that can be caused by an inadequate data basis as described above can be minimised. The available data also show that density estimations by simple extrapolations of the annual hunting bag or DS are not methods that provide valid results. An area-wide DS, e.g. in Bevern and Hallah, with a total area of 1000 km^2, seems hardly practicable and extremely unrealistic for several reasons. For reasons of comparability of the data to be collected, all observation sites would have to be randomly and reasonably evenly distributed in the overall study area. All counts would have to be carried out under very similar conditions such as comparable times (day- and clock times), similar weather and visibility conditions and on the same date if possible. In addition, such counts must be repeated regularly in order to be more than just a brief snapshot of a subpopulation. If these conditions are not fulfilled, DS can at best be implemented in small and localised areas, whereby the comparability with DS results from other areas must be questioned and viewed critically. Extrapolations from the annual hunting bag must be viewed in a similarly critical light. Since at no point in time is it known, based solely on the number of culled animals, how efficiently they were actually hunted (cf. also Chapter *2.4.1 Sex and age structure*, hunting according to the 'Lüneburger model'). Such extrapolations can therefore only be regarded as extremely rough population estimates.

The results of the present study, with particular focus on the hunting bag as a central DNA sample source, have clearly shown that, on the one hand, it can provide a reliable and accurate method for estimating population size. On the other hand, genotyping data used for kinship relations can also provide valuable information on other relationships within and between populations that can provide answers to even more complex questions such as population sub structuring (Griciuvienė et al., 2021; Hagemann et al., 2022), mating preferences and strategies (Müller et al., 2018; Say et al., 2012), genetic variability (Gamelon et al., 2018) as well as sounder structure and (sex dependent) migration rates (Broquet et al., 2009). This complementary data could help to improve management decisions in order to achieve long-term stable population development (for a detailed review see Chapter 2 - Demographic analysis of hunting and metadata).

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6. General Discussion

6.1. Synthesis of the study

After almost four decades of continuous population growth and expansion of the European wild boar, *Sus scrofa*, into new rural, agricultural and urban areas in almost all of Europe (Acevedo et al., 2011; Acevedo et al., 2014; Augustsson et al., 2024; Cahill et al., 2012; ENETWILD consortium et al., 2022a; Hagemann et al., 2022; Kotulski & König, 2008; Schley & Roper, 2003), a sustainable regulation and reduction of the populations is more urgent than ever before. The extent of crop damage (Augustsson et al., 2024; Cecchini et al., 2023; Herrero et al., 2006; Schley et al., 2008), increasing traffic accidents (Häggmark et al., 2014; Thurfjell et al., 2015), growing human-wildlife conflict (Coz & Mathevet, 2024; Podgorski et al., 2013), negative impact on other species (Barrios-Garcia & Ballari, 2012; Graitson et al., 2019) and host animal of numerous wildlife zoonosis such as African and classical swine fever (Artois et al., 2002; Bergmann et al., 2021; Costard et al., 2009; Richter et al., 2023; Sauter-Louis et al., 2021a) is constantly increasing. As a consequence of these known subsequent problems caused by the population increase and spread, long overdue measures must therefore be taken to manage wild boar populations efficiently. The basis and requirement for all planning of regulatory measures is a fundamental knowledge of the characteristics in which wild boar populations are structured and interact with each other: which sex and age classes make the major contribution to population growth and what reproductive strategy they pursue, and the resulting population trends regarding size and abundance.

The use of microsatellite markers (STR's) in ecology has been one of the most widely used genetic tools for the past 20 years for the visualisation of general relationships in a population, for specific parent-offspring detection, full- and half-sibling analyses or the identification of population structuring and boundaries and hybrid zones (review e.g. Carling et al., 2003; Costa et al., 2012; Flanagan & Jones, 2018; Hodel et al., 2016; Jarausch et al., 2023; Müller et al., 2018; Pizzigalli et al., 2024; Selkoe & Toonen, 2006; Suárez-Menéndez et al., 2024). The central question of the present study was to find out if the annual hunting bags of several forestry districts in Lower Saxony, Germany, can provide the data basis to develop standardised procedures for (i) characterising genetic population structuring, (ii) the extent of alternative reproductive tactics, especially in females, and (iii) the determination of abundance and density of populations using STR markers.

6.2. Population structure

In a first step (Chapter 2), the membership of the individuals to a potential population was determined by genotyping all available samples from several hunting bags. In the present case, despite some biases in the data, a clear structuring of eight genetically distinct subpopulations was identified. Based on the available data and results, it was shown that there are various reasons for such population structuring. In the southern forestry districts of Saupark and the surrounding Saupark areas (SSP), a physical barrier in the form of a stone wall is the causal factor of sub-structuring. Nevertheless, despite this anthropogenic barrier, genetic interactions between Saupark and other subpopulations were detected, as a certain permeability is provided by several gateways. In this context, it is remarkable that genetic profiles of several individuals that can be clearly assigned to the SSP cluster were culled in the Saupark. It is not clear whether these individuals came for mating reasons or simply to feed on one of the many winter feeding stations. Although both options seem possible, due to the low genetic intermixing between Saupark and SSP, feeding is considered more likely.

In the four south-east located forestry districts Süsing, Betzhorn, Danndorf and Hämelerwald, no comparable barriers as in the Saupark were found, but all four forestry districts can be genetically distinguished clearly from each other and form own subpopulations. These substructures result as a direct consequence of the sufficient size of the available habitats and breeding-site fidelity or philopatric behaviour in combination with rather small home ranges of the wild boar (Keuling et al., 2014; Keuling et al., 2008a; Miettinen et al., 2023). However, there are also cross-subpopulation genetic migrations, especially from the Süsing district towards Betzhorn and Danndorf, and Betzhorn towards Danndorf. Accordingly, the direction of movement is clearly orientated towards the south. The structuring of these four subpopulations is therefore characterised by behaviour and habitat.

The most surprising structuring of subpopulations in the present study is found in the northern forestry districts of Bevern, Rüstje, Hallah and Holzurburg. Here, the number of forestry districts does not correspond to the number of specific subpopulations, as in the other study site districts. Instead, two large genetic population clusters can be identified. However, these two clusters cannot be spatially separated by any visible natural or anthropogenic barriers. It is known for ungulate species like red deer (*Cervus elaphus*) or desert bighorn sheep (*Ovis canadensis nelson*), that federal roads, motorways and fences represent massive restrictions of movement and can hinder gene flow and greatly reduce genetic diversity, which results in a sub structuring of the population (Dellicour et al., 2011; Epps et al., 2005; Frantz et al., 2012). Such barriers and the mentioned consequences usually do not affect wild boar (Frantz et al., 2012; Griciuvienė et al., 2021). This also applies to the present study. If, for example, a federal road or a river were to restrict the genetic exchange between the two existing subpopulations, the two clusters would have to split up along such a barrier. However, this is not the case, as the present results show. Rather, both clusters occur in largely equal proportions in all four forestry districts in parallel. It can therefore be assumed that there is another reason for the diffuse distribution of the clusters across the four forest districts. Due to the intensive agricultural use in these districts, different numbers and different types of habitats are available to wild boar depending on the seasons. During winter and spring, the main available habitats consist of small, non-contiguous patches of forest, while in summer and autumn, additional habitats become available, consisting of maize and rapeseed fields (Keuling et al., 2014). Similar to road and river barriers, a fragmentation of the available habitats can negatively affect the genetic diversity and connectivity of populations (Dellicour et al., 2011; Dixo et al., 2009). However, this is again not the case with wild boar (Griciuvienė et al., 2021). Nevertheless, it can be assumed that the parallel presence and diffuse distribution of the two clusters, due to the existing habitat fragmentation and in particular their seasonally varying availability and presence, favours population structuring, as in the present case. In winter, when maize and rapeseed fields are harvested and mating and reproduction take place, wild boars are forced to move their territories to small and non-connected forest areas. Despite wild boars local loyalty and territoriality, the female-dominated family groups in particular have overlapping territories and no clear territorial boundaries, which are only rarely defended by aggressive behaviour (Keuling et al., 2014). In most cases, the group with the younger and less experienced alpha sow leaves the field to the group with the older and more experienced alpha sow (Keuling et al., 2014). This conflict-avoidance behaviour allows two family groups to use the same small area of forest in winter. Such family groups in a single territory do not necessarily have to belong to the same genetic cluster. At the same time, however, this also leads to genetic exchange between the clusters. This genetic exchange can also be demonstrated in all four districts of Bevern, Rüstje, Hallah and Holzurburg. In the summer and autumn months, when the offspring make the family groups grow, the food-rich maize and rape fields can once again serve as a replacement for the forest. As a result of these constant migratory movements, there would rarely be major conflict situations over food and habitat availability. In winter and spring, when the family groups are comparatively small, the groups can avoid each other in the shared forest territory. In the food-rich summer and autumn months, there is no significant competition for food or habitat in the fields. As long as year-round food availability is ensured, the habitat size, type of habitat (forest or crop fields), even urban areas, appears to be of secondary importance (Keuling et al., 2009; Podgorski et al., 2013; Stillfried et al., 2017).

Based on the available data, no founding populations originating from the south-eastern districts can be detected for the two northern clusters. The original founder individuals therefore either originate from other districts not sampled in this study or the two existing clusters have been isolated for long enough to form their own clearly defined genotypic differentiations and subpopulations over time (Ferreira et al., 2009; Hagemann et al., 2022; Wandeler et al., 2003). Overall, the genetic exchange can be categorised as rather low. The different clusters can be clearly distinguished from each other despite the sometimes very small spatial distances between them (~ 35-40 km, in some cases significantly less in the northern forestry districts or between Saupark and SSP). Accordingly, it is hardly possible to identify migration corridors along which the ASF virus or other zoonoses could spread. However, the migration of single individuals is already sufficient for the transmission of such zoonoses. Individual movement patterns of this kind have been demonstrated several times. In particular, genetic profiles from the Süsing are not only found in Betzhorn and Danndorf, but also in a northerly direction, in the Bevern and Hallah districts. Overall, it can be assumed in this context that the spatial dispersal of the animals tends to occur through individual animals or small family groups (Miettinen et al., 2023), which successfully establish themselves in a new territory under favourable conditions. After successful dispersal, the number of individuals can than grow rapidly due to the high reproductive capacity (Gethöffer et al., 2007; Keuling et al., 2014; Lombardini et al., 2014; Sprem et al., 2016). On the one side, the present results confirm the multiple descriptions of the local fidelity of wild boar through clearly delimited populations. On the other side, there is sufficient evidence for genetic migration between populations that are further apart. This shows another facet of the enormous behavioural plasticity of the wild boar.

6.3. Multiple Paternities

As early as the late 1970s, it was observed visually that female wild boars copulate multiple times during their mating season and different mating partners could not be excluded (Briedermann, 1986; Meynhardt, 1978). In breeding lines of various domestic pig breeds such as Duroc, Hampshire and Yorkshire, it had already been shown ten years earlier that sows successfully mate with different sires, resulting in multiple paternity (Sumption & Adams, 1961; Sumption et al., 1959). Therefore, all necessary mechanisms seem to be available for cryptic female choice (Bonet et al., 2013; Sumption & Adams, 1961). However, the conclusion from these observations was that only in a few exceptional cases these multiple copulations would lead to multiple paternities in wild boars. This is because the basic opinion and assumption was that only a few, mainly adult males in a population monopolise the majority of reproductive females for themselves (Briedermann, 1986). Accordingly, the opinion of many hunters and scientists was and is that there is a clear behavioural sexual dimorphism in wild boar: male members of a population behave in a polygamous manner, while the female members are monogamous (Poteaux et al., 2009). In Germany in particular, this opinion was further supported by the assumption that the adult alpha female of a sounder synchronise the fertility cycle of all other female members of the sounder (Meynhardt, 1979), but also other females of the population, and suppresses reproduction of subordinate sows and fertile piglets (Briedermann, 1990). The last two points in particular are considered highly controversial and inapplicable (Hohmann, 2005; Keuling et al., 2014). However, such synchronisation of fertility would make it easy for a single dominant male wild boar to inseminate all female fertile members of a sounder within a very short time and thus make multiple paternity significantly more difficult. About 30 years later, the idea that a single litter of a pregnant female could be fertilised by more than one male was revisited (Delgado et al., 2008). In this study, statistical modelling showed that such multiple paternities could theoretically occur, but were classified as very unlikely. The idea that male mate guarding plays the decisive role in the mating system of wild boar was therefore confirmed. Only a short time later, a genetically focussed study was able to disprove that multiple paternity is a rather theoretical construct and a difficult phenomenon to prove in practice (Poteaux et al., 2009). However, the idea of a rather rare event that is not based on changes in the social structures of a population or female reproductive tactics was still maintained.

In the following years, this assumption was largely revised. Genetic studies in wild boar populations in Portugal, Spain and Hungary (Costa et al., 2012; Perez-Gonzalez et al., 2014), France (Gayet et al., 2016; Say et al., 2012) and with the present study in Germany (Müller et al., 2018) were able to show that, with multiple sire rates between 20%-60%, it is neither a localised phenomenon nor that only a negligible small proportion of the female population is affected. For the time being, it remains unclear whether this is a female reproductive strategy or what other reasons cause this phenomenon. If it is not a reproductive strategy, the cause of the high rates of multiple paternity observed might be human-induced changes in social population structures. For example, a particularly high harvesting pressure towards dominant males in particular or selective hunting of all male members of a population in general can lead to a change from the original polygynous mating system to a promiscuous/polyandrous mating system and causes high rates of multiple paternity compared to other populations (Gayet et al., 2016). If the dominant males were missing, monopolisation of the sounders would hardly be possible. Accordingly, younger males would move up in the hierarchy, but they would not be able to monopolise a sounder to the same extent. As a consequence, more young males would participate in reproduction. Even with male-dominated hunting across all age classes, the result would be an increasing number of reproducing young boars. The rates of multiple paternity determined under these hunting conditions were between 30%-60%, depending on the calculation model (Gayet et al., 2016).

For a German population under rather moderate hunting pressure, concrete numbers on the general occurrence and extent of multiple paternity were lacking up to this point. The aim of the present study was to determine these dimensions by analysing the uteri samples of various hunting bags, including both drive and single hunts (Müller et al., 2018; Chapter 3). Since hunting in Germany often follows the Lüneburg model (Hennig, 1998; Teuwsen, 1980), the focus is therefore on the culling of subadults and piglets, whilst the sex ratio is balanced, the number of multiple paternities should be lower than described above, if hunting pressure is the causal factor. The results of the present study shows a proportion of multiple paternities between 20%-30%, also depending on the calculation model. This is comparable to Costa et al., 2012, where 33% is calculated, also without specifically increased hunting pressure. With an average of approximately 25% multiple paternity even under moderate hunting conditions, it cannot be assumed that hunting or increased hunting pressure is the cause of this polyandrous-promiscuous behaviour. Consequently, intensified hunting, as shown by Gayet et al., 2016, can strongly increase the already existing rates of multiple paternity but do not causes them. Comparing the hunting methodology and the results between the present study (Müller et al., 2018) and that of Gayet 2016, it becomes evident that hunting pressure cannot be linked in a generalised way to increasing rates of multiple paternity. The extent and impact of hunting pressure or management regulations on the proportion of females that mate multiple times cannot be clearly clarified with the available data.

Accordingly, these variables, hunting pressure and management regulations, have to become comparable, quantifiable and measurable (Gayet et al., 2021). The (i) hunting pressure or hunting rate, *hr*, can be defined as the number of animals culled during a specified time period divided by the size of the hunting area. For (ii) management regulations, this is difficult to do, as these can change constantly within an area and are not always identical between different areas and therefore hard to compare (Gayet et al., 2021). Therefore, a proxy measure needs to be created, that allows for comparability between different areas. The hypothesis in this context is that the male’s weight or the proportion, *Prop,* of heavy males in a population is a proxy measure of the ability to monopolise females in higher numbers (Gayet et al., 2021). The following applies to this proxy: yearly proportions of heavy males in the hunting bag were estimated as the proportion of males with a weight over 80% of the weight of the heaviest male culled that year (Gayet et al., 2021). As a result, populations with high *prop* should have lower rates of multiple paternity. By determining the *hr*, these two measures can be directly related to each other. This allows the rate of multiple paternity to be compared in different areas, even if hunting pressure varies. Contrary to their expectations, Gayet et al. (2021) have shown that in five different populations in France the rates of multiple paternity do not differ significantly from each other and are all at a very similar high level of around 50%, although hunting pressure (*hr*) varies significantly in the different populations. These consistently high rates of multiple paternity must therefore have causes other than hunting. Gayet et al. (2021) therefore argue that since all populations studied originate from very similar and ecologically beneficial habitats, these greatly favour the population development of wild boar. Therefore, the high rates of multiple paternity in the present case are more likely to be explained by favourable ecological parameters such as temporal and spatial distribution of partners and resources as well as fluctuations in population density. However, the exact causes of multiple paternity are still unclear. Up to now, it has only been clearly demonstrated that increasing rates of multiple paternity also lead to an increase in population densities, as the litter size increases significantly with the presence of multiple sires (Gayet et al., 2016; Müller et al., 2018).

6.4. Population density estimations

As mentioned before, profound knowledge of population densities forms the basis of regulating and sustainable wildlife management. A central point in the context of constantly increasing wild boar populations (Croft et al., 2020; Markov et al., 2022) and the associated and frequently described problems (Gren et al., 2019; Richter et al., 2023; Thurfjell et al., 2015) is the identification and optimisation of suitable solutions for a reliable estimation of current densities (Guerrasio et al., 2022). These approaches range from simple extrapolations of annual hunting bags, counting snow tracks or faecal pellets, distance sampling, camera traps to classic or genetic capture-recapture methods (Ebert et al., 2021; ENETWILD consortium et al., 2018; Müller et al., 2020). The data bases collected are as diverse as the number of approaches. All of these methods have advantages and disadvantages (ENETWILD consortium et al., 2018). A common disadvantage of many of these methods is the intensive amount of time required to collect samples and data. The costs of the methods, whether personnel or equipment or both together, can also be a major challenge (ENETWILD consortium et al., 2018; Luikart et al., 2010). Accordingly, the main objective of the genetic capture-mark-recapture model (g-CMR) developed here (Müller et al., 2020; Chapter 4) was to use existing structures and procedures in hunting and wildlife management, but at the same time to be able to use every available DNA source, muscle, faeces, hair, etc., as far as possible. The estimator should therefore orientate itself mostly to existing data sources and not adapt the data sources to the estimator. This is particularly necessary if a hunting bag is to serve as a data source, because on the one hand it is not possible to physically recapture an individual there and on the other hand this is not desirable or meaningful. Especially in high-density populations such as wild boar, where the need for reduction is a key management objective rather than release. Recapture in this case is achieved through genetic relatedness between parents and their offspring. The use of DNA data as a component of recaptures instead of physical recaptures to estimate population densities in a CMR environment provides, as in the present case (Müller et al., 2020), results with high quality and accuracy (Ebert et al., 2012; Ebert et al., 2021; Kery et al., 2011). Multiple simulations of virtually generated populations show that the present estimation method is robust to deviations in relative subsample size and random harvesting of parent-offspring pairs. A calculated deviation from the actual population size of less than 10% can be considered tolerable. However, if the subsample size is too small, the estimator tends to overestimate the actual population size, which must be taken into account when interpreting the data. For most European ungulate species, however, small sample sizes are probably the exception rather than the rule (Bassi et al., 2020; Burbaitė & Csányi, 2009, 2010; Toïgo et al., 2008). This will not always be the case for carnivorous species with inherently low population densities (Kery et al., 2011; Meuret et al., 2021). Accordingly, the results must also be interpreted with caution here. If an excess of parent-offspring pairs in the subsample is due to random hunting, the g-CMR estimator is also robust and only shows slight population overestimates. The estimator becomes imprecise and inaccurate if the presence of such parent-offspring pairs in the subsample is not due to chance but for example because of hunting interests (Milner-Gulland et al., 2004; Milner et al., 2007). In this case, the calculated population size will be significantly underestimated. This would particularly affect species in which the hunting regime is partly based on the combined shooting of offspring and parent animals, such as red deer, where in the vast majority of cases the hind has only one calf (Clutton-Brock et al., 1984). Such data pairs should either not be included in the population density calculation or an alternative, independent estimation method should be used.

Together with three other kinship- or parent-offspring pairs (POPs) based CMR methods, *Creel-Rosenblatt Estimator* (Creel & Rosenblatt, 2013), *Close-Kin Mark-Recapture* (Bravington et al., 2016) and the *Moment estimator* (Hettiarachchige & Huggins, 2018), the estimator *g-CMR* (Müller et al., 2020) presented here was independently analysed for sensitivity to sample size, fecundity values, sampling strategy, i.e. hunting regulations, hunting preferences, hunting methods (e. g., single or drive hunts), and animal behaviour like social or solitary species (Larroque & Balkenhol, 2023). The demographically contrasting strategies of the two terrestrial game species wild boar and red deer were compared. In principle, all POP approaches mentioned show the required accuracy with regard to population density estimation if the corresponding preconditions are met. However, here too, biases occur due to non-optimal sample sizes or certain hunting practices.

The fertility values, i.e. the number of offspring per female, have a greater influence on the precision of the density results than previously assumed for the g-CMR estimator. The optimal values for g-CMR are between four and six offspring/female (Larroque & Balkenhol, 2023). Ungulate species with high reproductive numbers such as wild boar (Frauendorf et al., 2016; Gethöffer et al., 2007) but also carnivore species with corresponding litter sizes such as fox and wolf (Ferreras-Colino et al., 2021; Ruette & Albaret, 2011) are therefore more suitable for the g-CMR estimator. At the same time, deer species such as roe deer, fallow deer or red deer with litter sizes between one and four (Clutton-Brock et al., 1984; Flajšman et al., 2017) tend to be less suitable and the calculated population sizes must be considered with corresponding caution.

In addition to the fertility values, the magnitude of the sample size has the expected significant influence on the reliability of the calculated population density with g-CMR. Depending on the optimal fecundity value, ideally 30%-40% of the total population must be sampled in order to keep the confidence levels of the calculated population densities within a range of ≤ 20%. As already mentioned, it can be assumed that this value appears realistically achievable with exhaustive sampling of the annual hunting bags for most ungulates such as wild boar, red deer and roe deer (Burbaitė & Csányi, 2009, 2010; Massei et al., 2015). Where this is not possible, the number of samples could be supplemented by alternative DNA sources such as faeces and hair samples (Ebert et al., 2012; Evans et al., 2024; Ferretti et al., 2016; Zhang et al., 2016). However, this would again lead to a significant increase in field work. If the level of sampling required is not achievable, a rough estimate of the population size can still be made by accepting higher confidence intervals, but in extreme cases up to 280% (Larroque & Balkenhol, 2023). On the one hand, a roughly approximated result is more helpful than no result and, on the other hand, population fluctuations over time can also be represented reasonably adequately with such values, as long as the bias remains constant over the years within a certain range. In general, however, the calculated results should be handled with care in such cases.

The argument that non-random sampling strategy can have a strong influence on the results is already illustrated by the example of combined offspring-parent hunting mentioned above (Müller et al., 2020). However, selective trophy hunting for certain age and sex classes can also lead to social disintegrated population structures and, as a result, a hunting bag not reflecting the actual conditions in the vital population (Milner-Gulland et al., 2004; Milner et al., 2007). Hunting regulations aimed exclusively at harvesting certain age and sex classes also have similar consequences with regard to the actual population proportions (Massei et al., 2015; Massei et al., 2011). In both cases, trophy hunting and hunting regulations, there would be a miscalculation of the actual population size. For a detailed review of the methods and results of the three other POP estimators mentioned above, see Larroque and Balkenhol (2023).

In summary, it is concluded that the g-CMR estimator presented here is most suitable for species with large populations that can be adequately sampled, and high female reproductive rates. In principle, species with distinct social and familial structures are more suitable. In this case, the aforementioned hunting regimes focussing on the combined shooting of offspring and parents would be unsuitable for such populations and the bias caused by such hunting methods would be avoided. If the necessary preconditions like sample size, fecundity values, etc., are met, the calculated population densities including confidence levels can be considered very robust (Larroque & Balkenhol, 2023; Müller et al., 2020).

When using the real genotype data of the existing hunting bags from Bevern and Hallah for the years 2011 to 2013, there is a tendency towards a generalised underestimation of the calculated population densities. The main reasons for this are non-compliance with the defined and necessary requirements such as a sufficient sample size, an excess of non-random sampling of parent-offspring pairs and deviations from the sex ratio. In the study year 2011, 38.517% of the annual hunting bag was sampled in Bevern. It can therefore be assumed that the ideally required 30-40% of the total population as the necessary basis for calculation is not given, as the hunting bag is already a subsample. In combination with an assignment rate of 77.174% between offspring and parents, it can be further assumed that the proportion of non-random sampling of parent-offspring pairs in 2011 was too high and therefore also does not fulfil the ideal calculation requirements. The third biasing factor is a very unbalanced sex ratio among the parents. With 1.42/0.58, this was clearly in favour of female parents. As a result, a population size is determined that is smaller than the hunting bag, which corresponds to a very clear but also expected underestimation of the population. In the study year 2012, 34.782% of the annual hunting bag was sampled in Bevern. Here, too, the sampling size of the total population is not optimal. The assignment rate between offspring and parents was 56.522% in 2012. The sex ratio of the parents was almost balanced at 1.05/0.95 and should therefore have no distorting influence on the calculations. The calculated population density for 2012 was 675 wild boar, which would mean a 58% reduction in the vital population when measured against the hunting bag of 391 wild boar. In principle, such culling numbers are possible, but they represent exceptionally successful years and are rather the exception. Accordingly, the calculated population size for 2012 is still within a realistic range, but must also be regarded as an underestimate of the actual population size. Calculations for 2013 are hardly possible. With less than 20% samples from the entire hunting bag, the sample size is very small. In addition, the proportion of sampled piglets has fallen by two thirds compared to the two previous years and still the assignment rate is 58.065%. Accordingly, the fecundity rate is too low on the one hand and the proportion of non-random sampling of parent-offspring pairs is too high in this context on the other hand. The calculated population size of 176 individuals is therefore probably a very clear underestimate of the actual population size.

For Hallah, only small sample sizes of the respective hunting bags are available in all three survey years. In 2010, 12.232% of the hunting bag was sampled, in 2012 it was 27.844% and in 2013 barely 10%. Accordingly, it was only possible to determine the parent-offspring pairs required for the calculation to a limited extent in 2011 and not at all in 2013. The calculated population density of 222 individuals for 2011 is therefore considerably lower than the hunting bag of 327. Due to the lack of parent-offspring pairs in 2013, it was no longer possible to calculate the population density. Only in the 2012 hunting year a population density of 576 individuals could be calculated, which was higher than the hunting bag. Similar to Bevern 2012, in this case approximately 60% of the entire population would have been harvested. However, the sample ratio of parents to offspring and thus the fecundity rate is lower than optimal. Accordingly, an underestimation can also be assumed here.

Summarising, it must be stated that, particularly in the first and last year of the study, the sampling from the hunting bags was in many respects insufficient to generate adequate and robust population densities from them. Although the fecundity rates, which are important for the extrapolations, essentially fulfil the requirements, it was not possible to sample the hunting bags to a sufficient extent, contrary to the expectations of this study. As the annual hunting bag of wild boar accounts for around 30%-60% of the total population (Bassi et al., 2020; Merli et al., 2017; Toïgo et al., 2008), at least 70% of the hunting bag should always be sampled. Ideally, it should be sampled in an exhaustive manner. Supplementing the tissue samples from a hunting bag with faeces or hair samples would be possible in principle, but cannot be carried out retrospectively. Another negative influence on the results of the population density is that the assignment rates tend to be too high in combination with insufficient sample numbers. In the present case, it can therefore be assumed that the proportion of co-harvested parent-offspring pairs is disproportionately overrepresented in the data sets, resulting in a consistent underestimation of population sizes.

6.5. Synopsis, conclusion and outlook

Using STR markers, the present study has clearly shown that extensive genetic analyses of the European wild boar hunting bags can be a fundamental tool to answer a variety of questions concerning geographical distribution, population structures even in the smallest areas (Chapter 2), reproductive behaviour (Müller et al., 2018; Chapter 3) and population size and density (Müller et al., 2020; Chapter 4 and 5). With 500,000-800,000 wild boar harvested annually over the past 10 years, this data source has so far been used far too little from a genetic perspective for data collection and the resulting findings and insights to finally solve the multitude of problems caused by the high populations and to guide management strategies such as permanent population reduction in the desired directions. It has been shown that populations can be structured and separated from each other for a variety of reasons, such as food availability, physical or behavioural barriers. On the one hand, the individuals of a population show the well-known territorial and breeding site fidelity, on the other hand they migrate many kilometres to other populations and thus contribute to gene exchange between the populations. By displaying over 900 individual genotypes over a period of 3 years, it is possible to illustrate the use of space and habitat to an extent that is hardly possible with radio telemetry studies, for example. It can be shown across how many human-made forestry district boundaries a single or several populations extend. These findings are of great benefit for cross-territory hunting in the winter months. Only when the extent and spatial boundaries of a population are known can the entire population be hunted effectively.

The extent of alternative female reproductive potential in the form of multiple paternities can also be determined by consistently sampling the uteri from the hunting bag. Even if the exact reasons cannot yet be explained, it can be shown that neither moderate nor intensive hunting has a significant influence on the number of multiple paternities and does not cause them. However, there is clear proof of a significant correlation between an increasing litter size in the presence of multiple sires. With increasing rates of multiple paternity, further increases in population densities must be expected.

The hunting bag can also form the underlying data source for the realisation of reliable projections of population size and density using kinship-based CMR methods. By modelling and simulating virtual populations, the necessary framework conditions and potential pitfalls could be identified. The theoretical models show the potential of these methods, but also highlight that non-compliance with the framework conditions can cause significant underestimates of population sizes.

The hunting bag, as sampled in this study, contains too many limitations to be considered a gold standard data source. Even if the nationwide hunting bag in Germany can be considered balanced in terms of the sex ratio, this is not necessarily the case, especially at hunting or forestry district level, and affects the present study. The same applies to the distribution of age classes. If the potential parents are not part of the hunting bag or sampling, or only to a small extent, no robust extrapolations can be made. If the proportion of offspring, i.e. the fecundity values, is too low, the population size will also be underestimated. One of the main aims of this study was to identify such difficulties, which is considered successful, even if the population densities in particular still too often do not fulfil the intended objectives. However, the greatest and unexpected weakness of the hunting bag is the lack of sampling, especially in the core areas of the project, Bevern and Hallah. In no survey year has it been possible to collect more than 40% of the available samples from the hunting bag. Despite enormous and excellent support from the forestry district managers and hunters involved, too many samples of hunting bags were either not taken at all or not handed in at the communicated collection sites. In the last year of the study in particular, there was also a certain lack of knowledge. It was often the case that samples were not collected because it was assumed that the project period had ended. Accordingly, some of the problems mentioned above, fecundity values etc., were also caused by the lack of too many samples. For future projects of this kind, it is therefore urgently necessary that all participants are aware and repeatedly made aware that every single sample in the entire study area and period is indispensable. The enormous potential of a kinship-based CMR using the hunting bag should be emphasised once again, because the limitations and weaknesses identified here could be addressed more clearly in future projects and reduced accordingly. In particular, the practising hunters must be more closely involved with regard to exhaustive sampling. Hunters in Germany are usually involved in recording data (age, sex, weight, etc.) of the game species hunted. In some countries, other tasks such as the collection of jawbones or ovaries are added (Cretois et al., 2020). Such practices could also be applied to the sampling of all harvested wild boar, as they require very moderate additional time investment during fieldwork. For a better determination of population size, structure, etc., such practices integrated into the normal workflow could make an enormous contribution. The collection of uteri could also be done in this way. It seems unlikely that this is practicable for every sow harvested, in every district and every hunting bag. However, for a limited period of time and at a local scale, such measures would be feasible and at the same time very helpful in clarifying unanswered questions in the context of multiple paternity. In addition, they would significantly improve the general monitoring of the population in terms of the average number of offspring per sow, ideally pre- and postnatal, as well as the most accurate estimate possible of the piglets' current expected birth rate.

With regard to genetic studies in game science, enormous costs are repeatedly postulated (ENETWILD consortium et al., 2018). These are then often cited as a reason for not realising such studies or not long enough as would actually be necessary to generate comparable data over a suitably long period of time. These concerns must be addressed by optimising and reducing laboratory costs, particularly with regard to DNA extraction, PCR and fragment length analysis. Efforts must also be made to utilise recent and most cost-effective methods. The costs of DNA extraction depend largely on the quality and type of the source tissue. Fresh muscle tissue, taken directly from the hunting bag, is probably one of the most suitable starting materials in this context. Faecal samples are susceptible to foreign DNA contamination and degenerated DNA (Ebert et al., 2021), which means that PCR and fragment length analysis may need to be repeated several times. Hair and feather samples often lack important accompanying information about the animals, as hair samples in particular are collected anonymously from sticky traps or similar methods. Blood and saliva samples are more complex to obtain, store and extract. On the PCR side, the focus should be much more on multiplex PCR approaches, i.e. all STR markers used should be amplified in one PCR and under the same conditions. The use of a uniform and standardised STR marker set would also be beneficial in order to improve comparability between different studies and working groups. The increased use of less error-prone tetra nucleotide STRs, as has long been standard in forensic biology (Linacre et al., 2011), should also be focussed on more intensively. This would save laboratory consumables and work would not have to be carried out several times. However, there could be great potential to reduce costs, particularly in fragment length analysis. The use of STR chips (Cornelis et al., 2019) could be one solution here. New sequencing methods, e.g. via nanopore sequencing (Rang et al., 2018) are also promising technologies that can not only reduce costs but also provide additional information.

In individual cases, damage caused by game in grassland or crops can amount to 5,000-15,000€ per damage event (Keuling et al., 2014; Linderoth & Elliger, 2002; Pegel, 2011). In this context, it should be considered whether investment in research and thus in the prevention of damage caused by wildlife should be increased in order to promote methods such as those presented here. On the one hand, this would lead to a better basic understanding of the biology of wild boar and, on the other hand, contribute to sustainable and long-term population regulation.

6.6. References

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Appendix

Chapter 3

Publication: Mother’s baby, father’s maybe: Occurrence and frequency of multiple paternities in the European wild boar

Chapter 4

Publication: Population estimates based on the frequency of parent-offspring relationship within a subsample

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Mother's baby, father's maybe: the occurrence and frequency of multiple paternity in the European wild boar

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ABSTRACT

Background: Multiple paternity (i.e. when the litter of a pregnant female is fertilized by more than one male) is common in a variety of animal taxa, including several ungulate species. It is generally believed that dominant males of European wild boar (*Sus scrofa*) monopolize several females, suggesting that multiple paternity is a rare phenomenon in this species. However, recent studies from different populations across Europe suggest that multiple paternity occurs more often in wild boar than had previously been assumed. However, previous studies were based on small sample sizes or a background of very strong hunting pressure on males.

Aim: Clarify the number and frequency of multiple paternities in European wild boar under moderate and balanced hunting conditions.

Method: We analysed eight highly polymorphic microsatellite markers in the embryonic and uterine tissues of 35 gestating female wild boars from different but nearby hunting grounds in Lower Saxony (Germany). Then, we visually reconstructed the putative paternal genotypes. We calculated the frequency of occurrence of multiple paternity using a variety of software packages.

Results: Almost 23% (8 of 35 the uteri) of the embryonic genotypes suggested at least two different sires. A minimum of 45 different fathers had to have been involved. In fact, due to hidden incidences where both parents carried the same alleles, maximum likelihood calculations suggested an even higher rate of multiple paternity.

Keywords: alternative reproductive tactics, European wild boar, microsatellites, multiple paternity, sexual conflict, *Sus scrofa*.

INTRODUCTION

Multiple paternity (i.e. when a pregnant female carries a litter sired by more than one male) is a common phenomenon that has been observed in insects (Song *et al.*, 2007), fish (Girndt *et al.*, 2012), amphibians (Knopp and Merila, 2009), birds (Birkhead and Møller, 1992), reptiles (Meister *et al.*, 2012),

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and mammals (Dugdale *et al.*, 2007; Glen *et al.*, 2009; Vanpe *et al.*, 2009; Falcon *et al.*, 2011), and is very well documented in several ungulate species, including white-tailed deer [*Odocoileus virginianus* (DeYoung *et al.*, 2002; Sorin, 2004)], wild pronghorn antelopes [*Antilocapra americana* (Carling *et al.*, 2003)], and roe deer [*Capreolus capreolus* (Vanpe *et al.*, 2009)]. Multiple paternity has been observed not only in socially polygamous (Firman, 2014) but also in socially monogamous species (Jennions and Petrie, 2000; Arnqvist and Kirkpatrick, 2005).

Multiple mating is advantageous for males because it increases the chance to sire more offspring. However, the production of sperm can be costly for males and therefore is a limiting factor and will finally restrict the number of offspring (Dewsbury, 1982). By contrast, females cannot necessarily increase their direct fitness by mating with several males owing to a limited number of mature eggs. But a potential benefit for females actively practising multiple mating is sperm competition (Parker, 1970). Especially in mating systems where dominant males try to monopolize several females, as assumed in European wild boar, sperm competition is unavoidable if these monopolization attempts fail. This pre- and post-copulatory competition allows females to select for the most viable or suitable sperm for their offspring. Furthermore, multiple mating represents a possibility for females to enhance the genetic diversity (Bergeron *et al.*, 2011) and genetic quality (Jennions and Petrie, 2000) of their offspring, to reduce homozygosity (Charlesworth and Charlesworth, 1987), or to reduce resistance to parasites (Coltman *et al.*, 1999). However, in some cases multiple mating does not happen by choice but is the consequence of sexual harassment (Carranza and Valencia, 1999; Fox, 2002; Fitze *et al.*, 2005; Cappozzo *et al.*, 2008).

Traditionally, it was assumed that multiple paternity in European wild boar litters was unlikely due to the monopolization of females by the strongest boars (Briedermann, 1986). It was first suggested that female wild boars copulate with two or more males during a reproductive cycle, resulting in multiple paternity, some years ago (Delgado *et al.*, 2008). But the authors of that study assumed this behaviour to be rare and only statistically provable. Shortly afterwards, the first physical evidence was provided in France (Poteaux *et al.*, 2009). However, it was still considered an infrequent occurrence because only two cases of multiple paternity were identified in the 21 uteri investigated. Further support for the existence of multiple paternity emerged from a transnational study conducted in Portugal, Spain, and Hungary (Costa *et al.*, 2012), in which multiple paternity was identified in 5 of 15 uteri collected, and from a multi-year study in a French population but with very strong selective hunting pressure on males (Gayet *et al.*, 2016). Over six hunting seasons, 165 pregnant females and their full litters were collected. Depending on the analytical model used, multiple paternity rates of between 33.8% and 60.6% were observed (Gayet *et al.*, 2016). However, populations with a strong overall male-selective harvest may switch to a more promiscuous and polyandrous mating system (Gayet *et al.*, 2016). As a sequel to these events, litter sizes can increase and the number of multiple paternities will be biased (Gayet *et al.*, 2016). While these examples show that multiple paternity in wild boar is more common than assumed, a study is required of its occurrence in a population where there is only a small or no impact of hunting based on selection by sex or age.

Germany has one of the highest wild boar stocks in Europe (Massei *et al.*, 2015). Only moderate and balanced hunting of wild boar is allowed under German hunting law. Most of the hunters in our sampling area follow the ‘Lüneburger Model’ (Teuwsen, 1980; Hennig, 1998), which mandates a significant reduction of young boars (piglets and subadults) regardless of sex, but only a sustainable cull of adults (Keuling *et al.*, 2014). Even if the numbers of harvested piglets and female subadults are below those required, the total sex ratio of the animals killed is balanced (Keuling *et al.*, 2013). A shift to a more promiscuous and polyandrous mating system with all its consequences owing to sex-specific hunting would, therefore, be unlikely.

The first aim of this study was to confirm the occurrence of multiple paternity in a German wild boar population because to date there is no physical evidence of this. To detect multiple paternity, we used a set of eight microsatellites (Kato *et al.*, 1991; Rohrer *et al.*, 1994, 1996; Karlskov-Mortensen *et al.*, 2008) to analyse *in utero* kinship relations. If this analysis was successful, our second aim was to estimate the mean frequency of multiple paternity in the study area and to determine which females favour this behaviour. In particular, we investigated differences among maternal age classes, such as differences in body mass or mean number of embryos, and their possible influences on multiple paternity.

MATERIALS AND METHODS

Study site, sampling, and age classes

Although the European wild boar is mainly monoestrous (Briedermann, 2009) and shows a distinct seasonality of reproductive performance from March to May, pregnant piglets and subadult sows have been observed up until late summer (Gethöffer *et al.*, 2007). Gravid sows, therefore, can be observed most of the year and sampling is thus feasible throughout the year.

Sampling took place in Lower Saxony, northern Germany. Samples were taken from hunting grounds up to 100 km to the north and east of the state capital Hannover (Gethöffer *et al.*, 2007; Keuling *et al.*, 2014; Frauendorf *et al.*, 2016). The uteri of females of all age classes were collected on drive and single hunts between October 2010 and February 2011, and again between October 2011 and February 2012. The age classes were defined as follows: piglets aged up to 12 months, subadults aged 13–24 months, and adults aged 25 months and older. The body mass of each individual was weighed after dressing, i.e. after its entrails were removed. The uteri were dissected, examined, and measured in the lab (University of Veterinary Medicine Hannover, Foundation). We wished to determine whether the mean number of embryos per litter was influenced by the body mass or age class of the pregnant females. The uteri of 35 visibly pregnant females and their full litters ($n = 213$ embryos) were weighed. The tissue samples (5–15 g) that were taken from all individuals, both mothers and embryos, were stored in 5 mL tubes containing 4 mL 99.8% ethanol at 4°C for subsequent paternity analyses.

DNA extraction, amplification of microsatellite primers, and PCR conditions

DNA extraction was conducted in line with the Chelex™ 100 (Bio-Rad) protocol (Walsh *et al.*, 1991) using 25 mg tissue for each sample. The DNA concentration of each sample was measured with a NanoDrop™ 1000 spectrophotometer (Peqlab Biotechnologie GmbH) and made uniform to 70 $\text{ng} \cdot \mu\text{L}^{-1}$ by ultrapure water dilution (LiChrosolv®, Merck KGaA) to avoid DNA-amplification interference during polymerase chain reaction (PCR) caused by too much DNA. PCR was done with Peqlab's 'Taq all inclusive' kit (Peqlab Biotechnologie GmbH): amplifications used a 10 μL reaction volume containing 4.15 μL ultrapure water, 1 μL buffer solution Y, 0.2 μL dNTP (10 mM), 2 μL enhancer solution P, 2 μL DNA (70 $\text{ng} \cdot \mu\text{L}^{-1}$), 0.3 μL forward primer (10 $\text{pmol} \cdot \mu\text{L}^{-1}$), 0.3 μL reverse primer

Table 1. Primer sequences of the eight microsatellites

Microsatellite	Label	Repeat length	Forward primer	Reverse primer
KVL9495	BMN6	4	5′ CAC AGC TGG GCG AAG TTA AAC 3′	5′ CTC CTT TAA AAG CTC CTT GTG AGA G 3′
KVL9807	Cy5	4	5′ AAG TAT TAA GCA GAA CCC AGC GTG 3′	5′ CCA GTT CTT TTC AGA CCC AGA CTC 3′
TNFB	Dy-751	3	5′ CTG GTC AGC CAC CAA GAT TT 3′	5′ GGA AAT GAG AAG TGT GGA GAC C 3′
CGA	BMN6	2	5′ ATA GAC ATT ATG TAA GTT GCT GAT 3′	5′ GAA CTT TCA CAT CCC TAA GGT CGT 3′
Sw24	BMN6	2	5′ CTT TGG GTG GAG TGT GTG C 3′	5′ ATC CAA ATG CTG CAA GCG 3′
Sw632	Cy5	2	5′ TGG GTT GAA AGA TTT CCC AA 3′	5′ GGA GTC AGT ACT TTG GCT TGA 3′
SW742	Dy-751	2	5′ AAT TCT ACT TCT GGG GAG AGG G 3′	5′ CTT TTG GGA ACA TTT CTG CC 3′
Swr1941	Dy-751	2	5′ AGA AAG CAA TTT GAT TTG CAT AAT C 3′	5′ ACA AGG ACC TAC TGT ATA GCA CAG G 3′

Note: For capillary electrophoresis, a specific fluorescent label (Cy5 = blue, BMN6 = green, Dy-751 = black) was attached to the 5′ end of every forward primer of each primer pair. The repeat length of the loci varies from 2 to 4 base pairs.

Table 2. PCR conditions for primer systems

Microsatellite	Denaturation (temp./duration)	Cyclic denaturation, annealing and elongation (temp./duration)	No. of cycles	Final elongation and cooling (temp./duration)
KVL9495 KVL9807	95°C/5 minutes	95°C/60 seconds 58°C/60 seconds 72°C/60 seconds	35	72°C/15 minutes 4°C/∞
TNFB CGA	94°C/5 minutes	94°C/30 seconds 55°C/30 seconds 68°C/30 seconds	35	68°C/20 minutes 4°C/∞
Sw24 Swr1941	94°C/5 minutes	94°C/30 seconds 55°C/30 seconds 72°C/30 seconds	35	72°C/15 minutes 4°C/∞
Sw632	94°C/5 minutes	94°C/30 seconds 55°C/30 seconds 72°C/30 seconds	32	72°C/15 minutes 4°C/∞
SW742	94°C/5 minutes	94°C/30 seconds 60°C/30 seconds 68°C/30 seconds	35	68°C/20 minutes 4°C/∞

(10 pmol·µL^{-1}), and 0.05 µL peqGold Taq DNA polymerase. We used a set of eight highly polymorphic microsatellites: two tetranucleotide [KVL9495 and KVL9807 (Karlskov-Mortensen *et al.*, 2008)], one trinucleotide [TNFB (Rohrer *et al.*, 1994)], and five dinucleotide [CGA, Sw24, Sw632, SW742, and Swr1941 (Kato *et al.*, 1991; Rohrer *et al.*, 1994, 1996)] primer pairs. For genotyping, the forward sequence of each microsatellite was labelled with a fluorescent marker (biomers.net GmbH) (see Table 1). Amplification was done using the PCR conditions listed in Table 2.

Genotyping

Genotyping was carried out using an automated CEQ 8000 series Genetic Analysis System (Beckman Coulter). PCR products were diluted with 20 µL HPLC water, 2 µL of which was mixed with 0.15 µL of CEQ DNA Size Standard Kit 400 bp (Beckman Coulter) and 30 µL of CEQ Sample Loading Solution (Beckman Coulter). Sizing of the fragments was done following the Beckman Coulter standard protocol for the CEQ 8000 series and using GenLab software v.10.2.3 (Beckman Coulter). Allele binning was performed with R v.2.15.3 (R Development Core Team, 2013) and the package MsatAllele v.1.02 (Alberto, 2009). Calculations of allele frequency, observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}), deviations from Hardy-Weinberg equilibrium (HWE), polymorphism information content (PIC) (Botstein *et al.*, 1980), probability of identity ($P_{ID/SIB}$), and presence of null alleles were performed with ARLEQUIN v.3.5.1.3 (Excoffier and Lischer, 2010), CERVUS v.3.0.3 (Marshall *et al.*, 1998; Slate *et al.*, 2000; Kalinowski *et al.*, 2007), and GIMLET v.1.3.3 (Valiere, 2002).

Repeatability, error rates, and descriptive statistics

For quality assurance, each PCR run contained an additional positive control (sample with known fragment length) and a negative control (HPLC water instead of DNA) as recommended in the literature (Budowle *et al.*, 2005; Selkoe and Toonen, 2006). Finally, to calculate the overall genotyping error rate, 10% (21 samples) of the total sample set (DeWoody *et al.*, 2006; Selkoe and Toonen, 2006) was randomly repeated (http://www.random.org/lists/) by independent DNA extraction, PCR, and genotyping steps for all eight microsatellites. Deviations in run time of more than one-half of the repetitive motif length, resulting in different numbers of alleles, were evaluated as errors. The calculated error rate is defined as the ratio of the number of deviations resulting in errors to the total number of alleles compared (Bonin *et al.*, 2004). None of the repeated samples resulted in different fragment length compared with the first run. Nevertheless, for all software packages used that require a genotyping error rate, a 5% error rate was assumed. This procedure will compensate all potentially undetected errors. Descriptive statistics and non-parametric tests were conducted with R v.2.15.3 (R Development Core Team, 2013).

Multiple paternity detection

In addition to the computational parentage analysis using the programs described in the following paragraph, microsatellite data of each sow and her piglets allowed a visual reconstruction by hand of the minimum number of paternal genotypes based on Mendelian rules of inheritance. To avoid overestimation of the number of sires, only the minimum number of paternal alleles required was used here to explain the offspring genotypes; for example, in cases where only one unambiguous paternal allele could be found, the father was assumed to be homozygous. These fathers could also be heterozygous but owing to the absence of a second allele, the proposed assumption seems to be the most conservative way to avoid overestimating the number of alleles. In cases where two unambiguous paternal alleles were observed, only one heterozygous sire was assumed although two different homozygous fathers could also be a possible explanation. Only if the number of detected alleles allowed no other explanation was a case of multiple paternity assumed. To exclude mutations as a reason for altered allele sizes, multiple paternity was assumed only for embryos with alleles that could not be explained by a sole sire on at least two different microsatellite loci. If a case of multiple paternity was detected, the DNA of the whole family was again extracted and PCR and genotyping repeated to ensure that no technical, chemical or human factor biased the results. This was performed in addition to the above mentioned error rate calculation.

From the various available statistical programs for paternity analysis (Jones and Ardren, 2003; Jones and Wang, 2010a), we chose COLONY v.2.0 (Wang, 2004; Wang and Santure, 2009; Jones and Wang, 2010b), CERVUS v.3.0.3 (Marshall *et al.*, 1998; Slate *et al.*, 2000; Kalinowski *et al.*, 2007), and PEDIGREE v.2.2 (Smith *et al.*, 2001; Wilson *et al.*, 2003; Butler *et al.*, 2004) to identify cases of multiple paternity in combination with the visual reconstruction of paternal genotypes. While CERVUS assigns parentage based solely on a pair-wise likelihood comparison approach, COLONY infers sibship and parentage simultaneously using a full-pedigree likelihood method. PEDIGREE served as an independent means of assessing the number of sires solely based on the occurrence of half-sibs in the litters.

To estimate critical values for CERVUS log-likelihood statistics with a 95% level of confidence, we applied a simulation with the following parameters: 100,000 cycles, 266 candidate parents (all maternal and all possible combinations of paternal genotypes), 0.8 for the proportion of candidate parents sampled, 0.99 for the proportion of loci typed, 0.05 for the proportion of loci mistyped, and a 0.05 error rate in likelihood calculations. COLONY was either provided with the same dataset (all parents known) or with the option 'all males unknown' in a polygynous-polyandric mating system with inbreeding and a full-likelihood model with no sibship prior. PEDIGREE was provided with the same dataset and the following parameters: 10 runs, one million iterations, full-sib constraint, temperature of 5 to 50°C in steps of 5°C, weight 1, and random seed.

RESULTS

The 35 analysed gestating sows represented all age classes: piglets ($n = 11$), subadults ($n = 17$), and adults ($n = 7$). Litter sizes ranged from 2 to 10 with a mean of 6.057 ± 2.222 ($\pm$SD) embryos per litter (median = 6; see Fig. 1).

We found significant differences between maternal age groups in the number of embryos (Kruskal-Wallis rank sum test: $\chi^2 = 11.215$, $df = 2$, $P < 0.004$; see Fig. 1). Subadult females had significantly more embryos than piglet females (Mann-Whitney *U*-test: $W = 21.5$, $P < 0.001$) but did not differ from adult females ($W = 48.5$, $P = 0.499$), while adults tended to have more embryos than piglets ($W = 57$, $P = 0.098$; see Fig. 1). The number of embryos increased with female body mass regardless of their age (Spearman rank correlation: $r = 0.556$, $P = 0.0005$). However, this held true only because of the highly significant correlation between body mass and number of embryos for females with a body mass below 60 kg ($r = 0.796$, $P < 0.001$); no such correlation was observed for females with a body mass of 60 kg and above ($r = 0.019$, $P = 0.942$) (see Fig. 2).

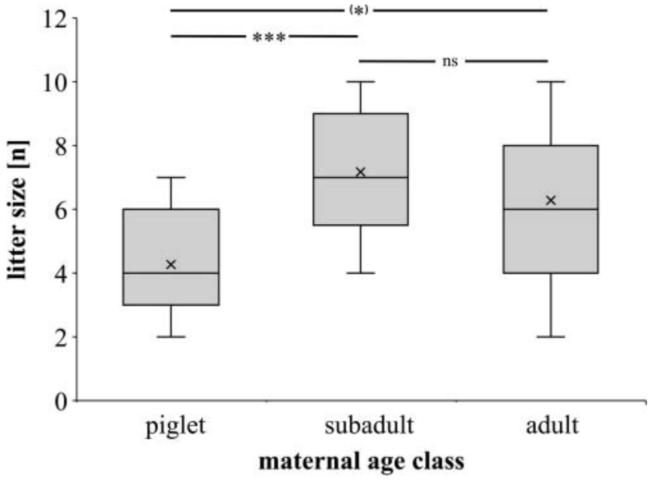

Fig. 1. Mean litter size of piglets, subadults, and adults. Shown are mean (×), median, first and third quartiles, and range span. $***P < 0.001$, (*) = non-significant but a tendency $0.1 > P < 0.05$, ns = $P > 0.05$.

All microsatellite loci were highly polymorphic and suitable for individual discrimination purposes (see Table 3) (Botstein *et al.*, 1980; Waits *et al.*, 2001). The mean gene diversity over all loci was estimated at 0.765 ± 0.403 (±SD) and the polymorphism information content (PIC) ranged from a minimum of 0.572 for locus Swr1941 to a maximum of 0.929 for locus KVL9495 with a mean of 0.769 ± 0.129. The combined non-exclusion probability of identity (P_{ID}), the probability that the genotypes do not differ between two randomly chosen individuals, was estimated at 3.78×10^{-11}, and between full-sibs (P_{SIB}) at 3.16×10^{-4}. There was no significant deviation from HWE except for locus TNFB and no evidence for null alleles F(Null).

As mentioned previously, by visually reconstructing by hand the minimum number of paternal genotypes based on Mendelian rules of inheritance, at least 45 different sires would be required to explain the genotypes of all 213 embryos. In 6 of the 35 investigated uteri, one or more embryos could only be explained by two or more different sires. For another two uteri, the allelic distribution of the embryos could only be explained by three or more different sires. In summary, 8 of 35 females were fertilized by multiple males.

With these 45 reconstructed putative paternal genotypes set as priors in the genetic analyses, all eight cases of multiple paternity could be validated by adjacent CERVUS and COLONY runs. Without prior male genotype information, the CERVUS, COLONY, and PEDIGREE analyses provided slightly different results. In addition to the 8 cases of multiple paternity confirmed by visual reconstruction (8 of 35), CERVUS suggested an additional two cases of cryptic multiple paternity (10 of 35) where the combination of alleles was likely due to multiple sires. The analysis by COLONY suggested an additional three cases of cryptic multiple paternity (11 of 35). Using PEDIGREE for clustering the offspring in full- and half-sib groups resulted in 10 cases of multiple paternity. All confirmed and suggested cases of multiple paternity were for the same individuals, except for the eleventh case proposed by COLONY (see Fig. 2).

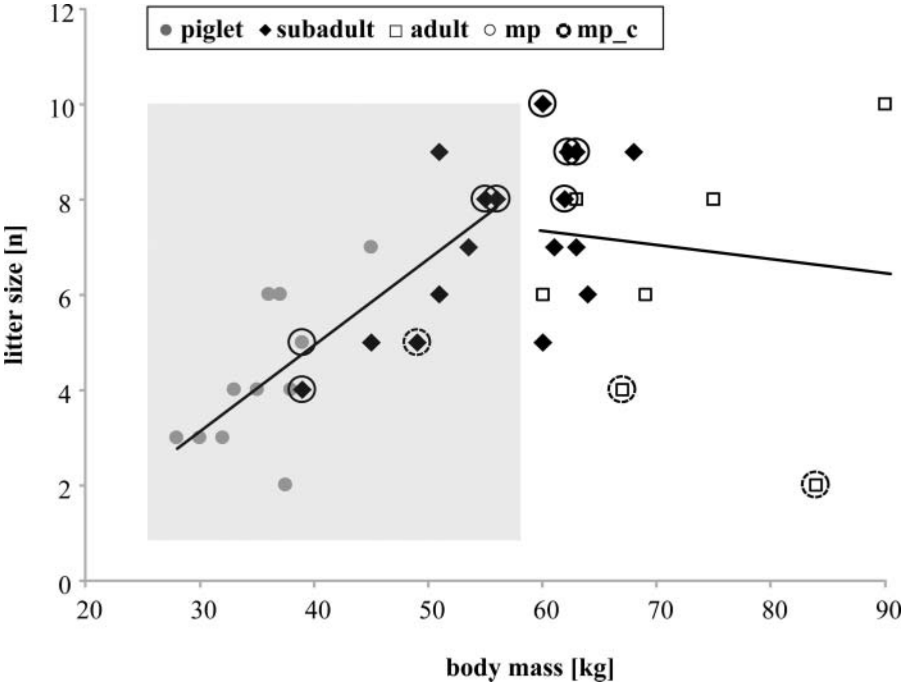

Fig. 2. Correlation between female body mass and litter size. The grey dots (●) show piglets, the solid rhombi (◆) subadults, and the open squares (□) adults. There was a highly significant correlation between body mass and number of fetuses (highlighted by the grey background) for females below 60 kg, but not for females of 60 kg or above. The eight unbroken black circles indicate females with multi-paternal offspring (mp) from a visual reconstruction by hand of the minimum number of paternal genotypes based on Mendelian rules of inheritance. The three broken circles indicate females with multi-paternal offspring (mp_c) using the program COLONY.

Table 3. Diversity indices of the eight microsatellites for mothers and fetuses

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
TNFB	7	239	0.619	0.727	0.687	<0.001	0.0913
CGA	20	242	0.831	0.902	0.891	0.0144	0.0409
KVL 9495	24	246	0.886	0.934	0.928	0.0241	0.0251
KVL 9807	13	245	0.869	0.883	0.869	0.0914	0.0054
SW 742	16	242	0.736	0.843	0.829	0.0185	0.0697
Swr1941	5	248	0.605	0.619	0.572	0.1699	0.0037
Sw24	5	247	0.680	0.701	0.646	0.0444	0.0080
Sw632	8	248	0.617	0.685	0.633	0.1444	0.0515

Note: Microsatellite system (MS-system), number of alleles per locus (A), number of genotyped individuals (N), observed (H_{obs}) and expected rate of heterozygosity (H_{exp}), polymorphism information content (PIC), results of Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HWE}), and calculated frequency of null alleles F(Null).

Table 4. Different computations revealed different numbers of sires and multiple paternities

	Visual	CERVUS	COLONY	PEDIGREE
Sires	45	45	39	45
Multiple paternities	8	10	11	10

While all 45 visually reconstructed putative paternal genotypes were confirmed with and without prior male genotype information by CERVUS, only 39 sires were suggested by COLONY without prior male genotype information, assuming some of the females to be inseminated by the same males (see Table 4).

Although none of the litters of adult females resulted in an unambiguous case of multiple paternity, the effect of female age on the occurrence of multiple paternity was not significant (Pearson's chi-squared test: $\chi^2 = 4.2805$, $df = 2$, $P = 0.1176$). However, litters that are the result of multiple paternity tend to be larger than those that are not (Mann-Whitney *U*-test: $W = 163$, $P < 0.031$).

DISCUSSION

The present study provides conclusive evidence for the regular occurrence of multiple paternity in wild boars in Germany. In 22.8% of the investigated uteri, clearly recognizable cases of multiple paternity were highlighted by reconstruction of paternal genotypes. According to this, two or more different sires would need to be involved to explain all fetal genotypes. When including those cases where the allelic distribution makes a single sire statistically less likely than a case of multiple paternity, this should be considered as the minimum frequency of occurrence in the study area. The software tools used to calculate the occurrence of these cryptic events – CERVUS, COLONY, and PEDIGREE – proposed two to three additional cases of multiple paternity. This results in a total of 11 of 35 cases, which means about 30% of all examined gestating females successfully engaged in multiple copulations with different sires. Repetitive DNA extraction, amplification, and genotyping of all potential cases of multiple paternity should have minimized possible errors to a rate below that expected to influence the study results significantly.

In contrast to previous studies (Delgado *et al.*, 2008; Poteaux *et al.*, 2009), multiple paternity appears not to be a rare phenomenon but a regular trait. The high proportion of females mating several times in this study is in line with research in other European countries, which showed rates of multiple paternity of 33% (Costa *et al.*, 2012), 50% (Say *et al.*, 2012), and even 60% (Gayet *et al.*, 2016). With half-sibs in every second to fourth litter, this behaviour is far more common than originally thought (Briedermann, 2009) and clearly not a local phenomenon – it has been shown from Portugal and Spain to Hungary (Costa *et al.*, 2012), France (Say *et al.*, 2012; Gayet *et al.*, 2016), and now Germany. While the occurrence of this behaviour is clear, its causes remain unknown. It might be beneficial for females to engage in multiple mating as a protection against functional infertility of their mates (Sheldon, 1994). Cryptic female choice is also a possibility (Eberhard and Cordero, 1995), in which case females would release competent spermatozoa from a sperm reservoir accumulated through mating with competing sires (Parker, 1970; Birkhead and Møller, 1998; Simmons, 2001). To date, no proof has been provided for cryptic female choice in wild boar. All necessary mechanisms seem to be available though: females are able to store and release male sperm for up to 30 hours (Gualtieri and Talevi, 2003; Talevi and Gualtieri, 2004; Satake *et al.*, 2006; Bonet *et al.*, 2013), they usually engage in multiple copulations during their fertile period (Meynhardt, 1978; Briedermann, 1986), and boar semen is potentially highly variable (Kozdrowski and Dubiel, 2004; Smital, 2009; Klimas *et al.*, 2012). However, alterations in the population structure might be an additional factor in female selection behaviour. Such a social change might be created by selective hunting pressure, resulting in a sounder of adult females losing the protection of their dominant male during the oestrous cycle. It is known that populations with a strong overall male-selective harvest may switch to a more promiscuous and polyandrous mating system owing to the lack of dominant and monopolizing males (Say *et al.*, 2012; Gayet *et al.*, 2016). Thus, frequencies of multiple paternities of up to 60% have been documented (Gayet *et al.*, 2016). Also, a surplus of sexually mature young males (i.e. sexually mature male piglets and subadults), owing to the selective hunting of older males, influences the population structure and hence the reproductive behaviour of piglets and subadults (Meynhardt, 1978; Briedermann, 1986). Both may induce a shift from pre- to more post-copulatory selection mechanisms to counteract the increasing risk of genetic incompatibility (Zeh and Zeh, 1997) in reduced and oversupplied male contingents.

Interestingly in the present study, although litters that had multiple paternity tended to be larger than those that did not, which is supported by other findings (Gayet *et al.*, 2016), all cases of multiple paternity were observed in the two younger age classes. We could not detect any unambiguous case of multiple paternity in adult female litters. Even though all cases of multiple paternity were assigned to younger females (piglets and subadults), no age-dependent influence on the occurrence of multiple paternity was observed. The findings of the present study rather suggest that the body mass of females is a better predictor of reproductive performance than their age. Accordingly, recent studies have revealed that nutritional status is an important factor in the onset of sexual maturation and consequently litter size (Gethöffer *et al.*, 2007; Frauendorf *et al.*, 2016). Female age as well as female body mass and number of embryos showed the expected associations (Briedermann, 2009). Older and heavier females had more embryos than younger and lighter ones but with one proviso: a highly significant correlation was observed between body mass and number of embryos for females with a body mass below 60 kg, whereas no such correlation was observed for females with a body mass of 60 kg and above. This result is predicted by the ‘coin-flipping’ hypothesis proposed for wild boar (Gamelon *et al.*, 2013). Coin-flipping is a form of phenotypic plasticity. Fertile heavy female wild boars seem to be able to diversify their offspring’s phenotype by differential investment in the embryos within a litter. In contrast, lighter females tend to produce similar-sized embryos. In this context, it would be reasonable to assume that lighter females therefore increase the genetic diversity of their offspring instead of phenotypic diversity as a result of multiple mating.

It is conceivable that female boars with a lower body mass might be more vulnerable to sexual harassment by stronger males. Sexual harassment could also be induced by a surplus of sexually mature non-adult males, as mentioned above. Especially in the case of polygynous species, sexual coercion and sexual harassment arise owing to intense male–male competition for mates (Smuts and Smuts, 1993). Also, the females of such species may voluntarily engage in multiple copulations to dilute the probability of inbreeding when close relatives or male littermates are involved (Meynhardt, 1978).

In conclusion, we have shown that multiple paternity is a relatively common phenomenon among wild boar litters in Germany. Questions to be addressed in future research include whether this behaviour is related to age (i.e. are adult females less affected by this than subadults and piglets) and whether there is support for the ‘coin-flipping’ hypothesis. The underlying mechanisms why females engage in multiple mating with different sires is a more complicated question to answer and will require more research on larger data sets.

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ORIGINAL RESEARCH

Population size estimates based on the frequency of genetically assigned parent–offspring pairs within a subsample

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Abstract

Estimating population density as precise as possible is a key premise for managing wild animal species. This can be a challenging task if the species in question is elusive or, due to high quantities, hard to count. We present a new, mathematically derived estimator for population size, where the estimation is based solely on the frequency of genetically assigned parent–offspring pairs within a subsample of an ungulate population. By use of molecular markers like microsatellites, the number of these parent–offspring pairs can be determined. The study's aim was to clarify whether a classical capture–mark–recapture (CMR) method can be adapted or extended by this genetic element to a genetic-based capture–mark–recapture (*g*-CMR). We numerically validate the presented estimator (and corresponding variance estimates) and provide the R-code for the computation of estimates of population size including confidence intervals. The presented method provides a new framework to precisely estimate population size based on the genetic analysis of a one-time subsample. This is especially of value where traditional CMR methods or other DNA-based (fecal or hair) capture–recapture methods fail or are too difficult to apply. The DNA source used is basically irrelevant, but in the present case the sampling of an annual hunting bag is to serve as data basis. In addition to the high quality of muscle tissue samples, hunting bags provide additional and essential information for wildlife management practices, such as age, weight, or sex. In cases where a *g*-CMR method is ecologically and hunting-wise appropriate, it enables a wide applicability, also through its species-independent use.

KEYWORDS
density estimations in ungulates, genetic-based capture–mark–recapture, microsatellites, *Sus scrofa*, wildlife management

1 | INTRODUCTION

Reliable information of population dynamics is a major pillar of estimating the impact of wildlife management actions. A key factor for population dynamics is an accurate estimation of numbers of animals living in a certain area. Counting wild animals, whether it is carnivores or herbivores, is often a demanding task, especially in nocturnal or elusive species (Bellemain, Swenson, Tallmon, Brunberg, & Taberlet, 2005; de Oliveira, do Couto, & Duarte, 2019; Eggert, Eggert, & Woodruff, 2003; Kery, Gardner, Stoeckle, Weber,

& Royle, 2011). Not every method for estimating population densities is equally applicable for every species. Low-density tending species, which concerns many carnivores (Kery et al., 2011), must be counted differently. For instance, hair traps seem to be suitable for low-density populations (Balestrieri et al., 2010; Steyer, Simon, Kraus, Haase, & Nowak, 2013), but rather inappropriate for some high-density ungulate species (Ebert, Huckschlag, Schulz, & Hohmann, 2009).

Collecting fecal samples is a widely used method for species monitoring and estimating population trends. They can be used in two different ways. Standard dung counts use a defecation rate in a defined area and timescale for density estimation (Eggert et al., 2003; Pfeffer et al., 2018). Alternatively, DNA analyses of the collected feces can reveal the number of different individuals, and with classical capture–mark–recapture methods (CMR), the density can be evaluated (Bellemain et al., 2005; Ebert, Knauer, Spielberger, Thiele, & Hohmann, 2012; Kery et al., 2011; Petit & Valiere, 2006). Counting fecal pellets can provide reliable results (Ferretti, Fattorini, Sforzi, & Pisani, 2016; Plhal, Kamler, & Homolka, 2014) but in some cases can lead to over- or underestimated densities compared to other methods (Barnes, 2001; Pfeffer et al., 2018). The choice of appropriate transects, often unavoidably high staff expenses, aggravated long-term storage for later DNA analysis and DNA amplification, and weather-dependent feces quality can make feces counts and genetic analyze of the fecal pellets a protracted and time-consuming process with an uncertain outcome (Barnes, 2001; Bellemain et al., 2005; Kolodziej, Nikolov, Schulz, Theissinger, & Schulz, 2013; Soto-Calderon et al., 2009).

Drive counts are another widely used method for estimating densities, particularly in ungulate species (Noss, Salidas, & Crespo, 2006). However, the accuracy of the results is known to be insufficient, when a high level of reliability of the estimated density is needed (Borkowski, Palmer, & Borowski, 2011). Over the last decade, camera traps became a commonly used method to estimate population density using the random encounter model (REM) by Rowcliffe, Field, Turvey, and Carbone (2008) that needs no individual recognition for density estimations (Pfeffer et al., 2018; Rowcliffe et al., 2008).

The European wild boar (*Sus scrofa*) is a species that is hard to count accurately, which can partly be explained by its complex social behavior and nocturnal activity (Briedermann, 2009; Cahil, Llimona, & Gràcia, 2003) and an exceptionally high reproductive potential compared to other ungulates of similar body size (Carranza, 1996). For more than 30 years, a Europe-wide continuous increase of the size of wild boar populations can be observed (Baubet, Bonenfant, & Brandt, 2004; Boitani, Trapanese, Mattei, & Nonis, 1995; Cahill et al., 2003; Ferreira, Souto, Soares, & Fonseca, 2009; Keuling et al., 2013; Tsachalidis & Hadjisterkotis, 2009). This steady and partly immense population growth is basically recognizable and measurable by two factors/conditions: (a) by the annual increase in the number of shot animals in hunting bags and (b) by increased crop damages (Keuling et al., 2013). Germany has one of the highest wild boar stocks all over Europe (Keuling et al., 2013; Massei et al., 2015), and particularly, the hunting season 2017/2018 had the highest hunting bag of all times with almost 837,000 shot animals. Nevertheless, there is no clear perception of how many living wild boars there are in absolute numbers (Briedermann, 2009). But especially, the progress of African swine fever (ASF) (More et al., 2018) necessitates a reliable and accurate method to estimate their density and to adjust a proper wildlife management.

Even in Germany, the size of the remaining living populations remains mostly ambiguous due to the lack of a unified applied and exact procedure to calculate wild boar population density. Comparisons concerning the wild boar population development extending over several years or between different populations are therefore barely achievable.

The currently most common method for wild boar stocktaking is hunting bag-based abundance calculation (Briedermann, 2009; Keuling et al., 2018). However, the annual rate of increment (between 100%–300%) or the hunting ground-specific ecological conditions can only be guessed. Different hunting grounds are therefore difficult to compare, and the impact of current management actions is hardly assessable. Even with further attempts of improvement based on camera trap detections, drive counts, and distance sampling (Keuling et al., 2018), the comparability of estimated population sizes remains difficult without unified, standardized methods to estimate population size.

Hunting wild animals like carnivores or ungulates is not always without bias. Sport and trophy hunting, as well as sex- and age-specific hunting, can affect reproduction, offspring sex ratio, body weight etc. (Milner, Nilsen, & Andreassen, 2007). Accordingly, selective harvesting can influence the age and sex composition of a hunting bag and, with that, kinship structures. Since many ungulates practice parental care, parent(s) and offspring often appear close together in their home range. This could lead to an increased proportion of parent–offspring pairs in a hunting bag, when these animals are shot together. Both circumstances could bias the results of an estimator that is based on this proportion. Regular wild boar hunting can be assumed as mostly unbiased with respect to the sex of the animals (Keuling et al., 2013; Keuling, Lauterbach, Stier, & Roth, 2010; Toigo, Servanty, Gaillard, Brandt, & Baubet, 2008), as well as unbiased toward hunting close relatives intentionally. Hunters try to avoid shooting adult females (Toigo et al., 2008) or females with piglets in general (Keuling et al., 2013), coincident with increased hunting efforts toward male yearlings and adults. A sex-biased hunting bag toward males is not found in central Europe per se. There are both regional and annual variations, but the trend is rather toward balanced sex ratios (Keuling et al., 2013). Therefore, there seems to be no significant influence on the parent–offspring pairs in the annual hunting bag. Because of that, we expect a random and representative sample size of close relatives (like parents and offspring, full-sibs, half-sibs) within a sampled hunting bag.

The common data acquisition of hunting bags mainly consists of information concerning, that is, long-term tendencies of population size, sex ratio, age composition, and sometimes state of pregnancy and litter size. But hunting bags additionally provide a whole range of genetic information, which usually remains unexploited, in particular the genetic relationships between the animals.

Genotypic analyses of fecal samples were used to develop a CMR estimator of the actual population density (Ebert et al., 2012; Ebert, Knauer, Storch, & Hohmann, 2010; Kolodziej, Schulz, et al., 2012; Kolodziej, Theissinger, Brün, Schulz, & Schulz, 2012) but require a considerable extra amount of money, work, and workforce. Some of the difficulties with fecal samples can be low DNA concentrations and low genotyping rates (e.g., Kolodziej et al., 2013). Taking fresh and high-quality muscle tissue samples from the hunting bag could solve these problems.

With these data, kinship relations like parent–offspring pairs and kinship structures within and between populations can be revealed. Based on adaptions of classical CMR models as the Lincoln–Petersen estimator (Lincoln, 1930; Petersen, 1896; Seber, 1973; Southwood & Henderson, 2000) or the Chapman estimator (Chapman, 1951, 1954; Southwood & Henderson, 2000), these genetic relationships can be used to estimate population densities. Although no physical recapture for an already shot animal is feasible, the use of genetic information in a hunting bag, and in particular the detection and number of parent–offspring pairs, may act as a genetic recapture. Most of all, such a genetic-based capture–mark–recapture (*g*-CMR) model would provide a unified, reliable, and comparable method for estimating abundance and density of wild boar and other (ungulate) species.

Despite the possibility of using newer molecular methods (Genotyping by Sequencing, GBS) (Hodel et al., 2016; Sonah et al., 2013), microsatellites (Simple Sequence Repeats, SSR) still belong to the most frequently used genetic markers for reliable individual identification (reviewed in Hodel et al., 2016; Selkoe & Toonen, 2006). Microsatellites allow for accurate estimations of parent–offspring as well as full-sib and half-sib relationships (Christie, 2010; Costa et al., 2012; Putnova, Knoll, Dvorak, & Dvorak, 2003).

In principle, the number of parent–offspring pairs can be determined from all available DNA sources, for example, muscle tissue, hair samples, or feces. Furthermore, it should be possible to estimate the size of a population based on the number of these parent–offspring pairs within a subsample: The larger the population is (while keeping the size of a subsample constant), the lower is the chance that parents and offspring appear together within the subsample. Thus, the estimated population size should increase with a decreasing number of parent–offspring pairs within the subsample. We thus aimed to create a protocol and R-script that allows for estimating and simulating population densities together with the opportunity to validate the results. In order to estimate a possible influence of the above-mentioned hunting bias on a hunting bag, different scenarios should be considered. Below, we mathematically derive and numerically validate corresponding population estimators.

2 | DERIVATION OF THE ESTIMATORS

Let N be the true size of a closed population N, where N_F is the number of adult females, N_M is the number of adult males, and N_J is the number of juveniles, thus $N = N_F + N_M + N_J$. Let further n be the size of a subsample of N, where n_F is the number of adult females in the subsample, n_M is the number of adult males in the subsample, and n_J is the number of juveniles, thus $n = n_M + n_F + n_J$. Finally, m_{FC} is the number of father–offspring pairs in the subsample, and m_{MC} is the number of mother–offspring pairs.

The average chance of any juvenile being in the same subsample as their parent depends on the relative portion of all N_M adult males (N_F females) in this subsample: If for example 50% of the adult males are within the subsample, for each juvenile in the subsample the average chance that its father is also within the subsample is 0.5. It follows for the expected values that

$$\mathbb{E}(m_{FC}/n_J) = \mathbb{E}(n_M/N_M) = \mathbb{E}(n_M)/N_M$$

respectively

$$\mathbb{E}(m_{MC}/n_J) = \mathbb{E}(n_F/N_F) = \mathbb{E}(n_F)/N_F$$

Assuming that there is always at least one mother–offspring respectively one father–offspring pair within the subsample (i.e., $m_{FC} > 0$ and $m_{MC} > 0$, otherwise the population size cannot be estimated), simple rewriting of the equation leads to

$$N_M = \mathbb{E}\left(\frac{n_M * n_J}{m_{FC}}\right)$$

respectively

$$N_F = \mathbb{E}\left(\frac{n_F * n_J}{m_{MC}}\right)$$

Leading to the corresponding estimators

$$\hat{N}_M = \frac{n_M * n_J}{m_{FC}}$$

respectively

$$\hat{N}_F = \frac{n_F * n_J}{m_{MC}}$$

Thus, an estimator for all adult animals $N_A = N_M + N_F$ is given by

$$\hat{N}_A = \frac{n_F * n_J}{m_{MC}} + \frac{n_M * n_J}{m_{FC}} = n_J * \left(\frac{n_F}{m_{MC}} + \frac{n_M}{m_{FC}}\right).$$

which is strongly related to the Lincoln–Petersen estimator (Lincoln, 1930; Petersen, 1896) that is frequently used in the capture–mark–recapture analysis. In our derived estimator, we require randomly chosen males (females, juveniles) from the entire population of males (females, juveniles), but the proportion of males versus females versus juveniles in the subsample does not have to be representative. This is an important issue, especially when the catching probability of animals depends on sex or age.

However, if the true number of juveniles N_J has to be estimated as well, an estimator for the average number of juveniles per adult is required, in the following denoted by $\hat{J}$. If such an estimator is given, N can be estimated via

$$\hat{N} = \hat{N}_A + \hat{J} * \hat{N}_A$$

If the proportion of males versus females versus juveniles in the subsample can be assumed to be representative for the entire population (i.e., the catching probability does not depend on sex or age), a straight forward definition of $\hat{J}$ is given by

$$\hat{J} = n_J/(n_M + n_F)$$

thus

$$\hat{N} = \hat{N}_A + \frac{n_J}{n_M + n_F} * \hat{N}_A$$

$$= n_J * \left(\frac{n_F}{m_{MC}} + \frac{n_M}{m_{FC}} \right) * \frac{(n_J)^2}{n_M + n_F} * \left(\frac{n_F}{m_{MC}} + \frac{n_M}{m_{FC}} \right)$$

$$= n_J * \left(\frac{n_F}{m_{MC}} + \frac{n_M}{m_{FC}} \right) * \left(1 + \frac{n_J}{(n_M + n_F)} \right)$$

3 | VARIANCE ESTIMATION

In the following, we estimate the variance of the estimators by using bootstrap methods, which have been shown to yield reliable variance estimations if the analytical calculations are complex (Canty, Davison, Hinkley, & Ventura, 2006; Davison & Hinkley, 1997; Efron & Tibshirani, 1994). Especially, for each subsample of size n, we create $j = 1, \ldots, n_{\text{boot}}$ random resamples with replacement (of size n) and calculate for each resample j the corresponding estimator value; the final variance is then calculated based on the quantiles of the n_{boot} estimator values.

4 | VALIDATION OF THE ESTIMATORS

In order to validate the estimators, it is worth mentioning that for a given population N, the estimators $\hat{N}_M$, $\hat{N}_F$, $\hat{N}_A$, and $\hat{N}$ differ on average only by one (not necessarily even) number, so that the relative bias (i.e., the population estimate normalized by the true population size, $\hat{N}/N$) should show an identical behavior for all estimators. It is thus sufficient to numerically validate one of them, for example, $\hat{N}$.

The validation of the bias and coverage probability has been done based on Monte Carlo methods. Specifically, we first created a virtual population of 100 males, 100 females, and 200 offspring (thus $N = 400$), where the number of offspring per mother has been randomly generated based on a Poisson distribution with expected value $\lambda = 2$, and the father has been randomly assigned for each offspring. In a second step, we randomly selected a subsample of size n, and based on this subsample, we finally estimated the population size as well as 95% confidence intervals, and the latter based on 200 bootstrap resamples. The second step has been repeated for $n = 20, 21, 22, \ldots, 360$; thus, the relative subsample size n/N ranged between 0.05 and 0.9. The corresponding results are shown in Figure 1a. It appears that the estimator is only slightly positively biased for small values n/N and appears to be unbiased with increasing n/N. However, for $n/N = 0.05$ the relative bias is still <10%. Additionally, the calculated confidence intervals show a reasonable experimental coverage, even for small values of n/N.

To estimate a possible hunting distortion on a hunting bag, we simulated the following two scenarios: (a) the influence of random harvest efforts on parent–offspring pairs staying together in their home range and (b) the influence of harvest efforts on parent–offspring pairs when not random, but purposefully caused by hunt. In Figure 1b, the bias is plotted against the number of parent–offspring pairs in the hunting bag, demonstrating that the estimator works well (respectively is only slightly positively biased) if only a few pairs are available. Finally, Figure 1c demonstrates that when parent–offspring pairs are harvested together (e.g., by hunters' purpose), the estimator shows a strong negative bias, revealing the importance of considering only populations (respectively, hunting techniques and conventions) where parent–offspring pairs do not per se have a higher chance to occur together in the same hunting bag.

5 | DISCUSSION

We derived and validated an estimator for the population size based on the number of parent–offspring pairs in a subsample. Based on Monte Carlo simulations, we demonstrated that the estimator is only slightly biased when relative subsample sizes are small and asymptotically unbiased when the relative subsample size increases. Calculated confidence limits showed a reasonable coverage probability. Finally, we provided the R-code for the calculations as well as some examples in order to make this method easily available for any researcher (see Appendix S1–S3). The presented approach allows an accurate estimate of population size based on the genetic analysis of a subsample and thus offers a simple and attractive alternative to frequently used capture–mark–recapture methods, especially if the latter are difficult or impossible to apply.

An appropriate wildlife management for wild boar or any other wild animal species can only be implemented when it is based on reliable and current data. For more than 30 years, people recognized the importance of having information concerning the annual growth rate and the relationship between the living stock and the hunting bag to calculate population trends as precise as possible (Briedermann, 1986, 2009). However, calculating reliable trends needs long-term data (Acevedo et al., 2007) but such data are only of limited benefit for calculating current population density (Keuling et al., 2018). Since the annual population density and hunting bag can

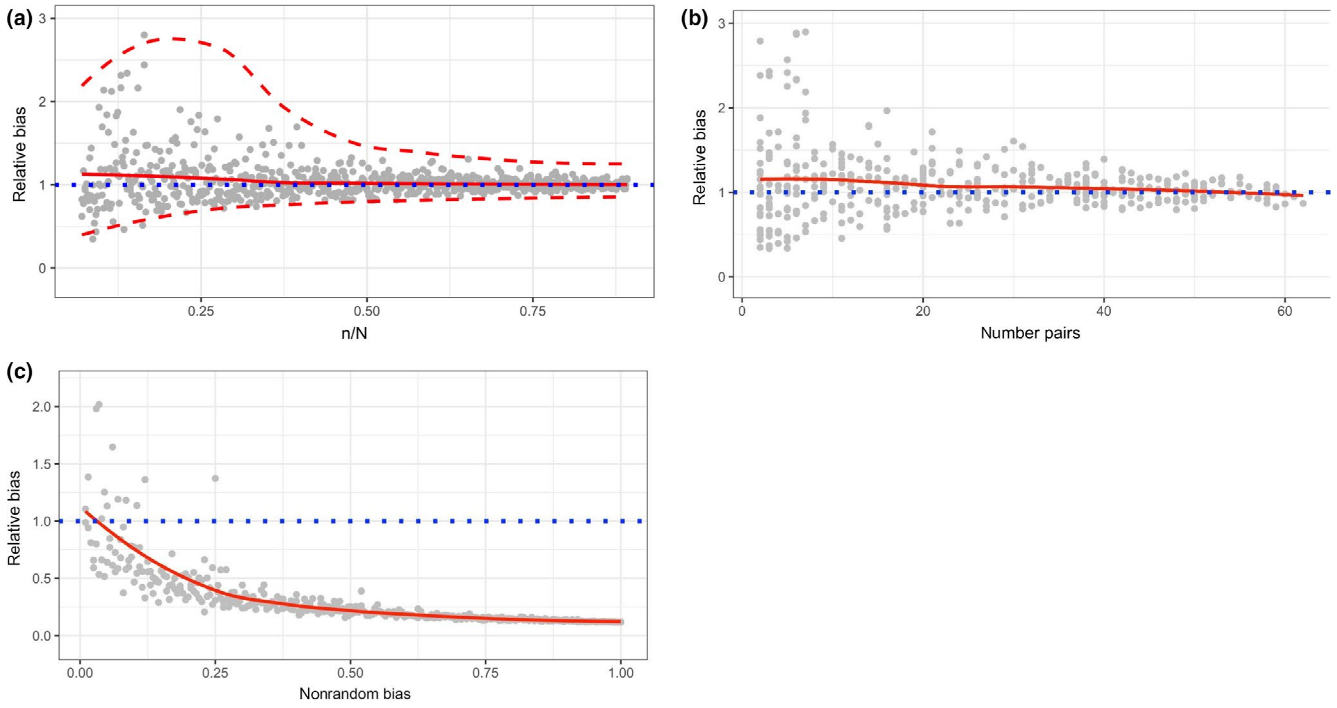

FIGURE 1 (a) Numerical (Monte Carlo) validation of the relative bias and the coverage probability of the estimator. Especially, the influence of different relative subsample sizes *n*/*N* is investigated. Gray dots: Relative bias estimated for a single subsample; red continuous line: average smooth of the single estimates; red dashed lines: average smooth of the upper and lower 95% confidence limits as calculated based on 200 bootstrap resamples; blue dotted line: unbiased value at 1. Smooths are based on LOESS smoothing, the total size of the virtual population is *N* = 400. (b) The same simulation framework and legend as in (a), but estimator bias is plotted against the total number of parent–offspring pairs per sample. (c) The same simulation framework and legend as in (a, b) (except a fixed subsample size of *n* = 50) but "nonrandom bias" in the hunting bag has been additionally introduced and plotted against estimator bias. Especially, a nonrandom bias = 0 means that there is no per se increased chance that parents and offspring occur together in the hunting bag; a nonrandom bias = 0.5 means a 50% probability for an offspring being shoot together with either its mother or father; a nonrandom bias = 1 means a 100% probability for an offspring being shoot together with its mother and father

strongly fluctuate, it is essential to update the actual density every year (Briedermann, 2009). The technical advances, particularly in molecular genetic protocols combined with the here presented *g*-CMR, shall help to take an important step forward to accomplish this objective.

The usage of classical capture–mark–recapture (CMR) provides reliable and high-grade results concerning animal densities. However, there are some disadvantages making this method outdated. Capturing, for example, ungulates is time-consuming and, in some countries, demands extensive administration. In high-density populations, where the need for reduction is a main management purpose, releasing captured animal seems paradox (Keuling et al., 2018). Simultaneously, modern spatial capture–recapture models (SCR) are replacing traditional CMR in wildlife monitoring (Jimenez, Higuero, Charre-Medellin, & Acevedo, 2017; Kery et al., 2011). Using DNA data as a component of recapture instead of physical recapture for estimating population densities in a CMR or SCR environment also provides results with high quality and accuracy (Ebert et al., 2012; Kery et al., 2011). Compared to DNA-based sampling methods like feces sampling (Bellemain et al., 2005; Ebert et al., 2012; Kolodziej et al., 2013), genotyping of the hunting bag requires less time and sampling effort and provides a steady and faster genotyping success due to superior DNA quality of muscle tissue. Additionally, this method gives a surplus of information regarding the population. For any sampled individual, it is possible to collect information concerning the weight, approximately age, the general body condition etc. For females, period of gestation and number of embryos can be calculated. Basically, the sex can be determined by molecular methods (e.g., Fontanesi, Scotti, & Russo, 2008), but is normally not required when sampling a hunting bag. Combining all available information results in an improved insight in the investigated population. Further statistical analyses could reveal different preferences of individuals and age classes (piglets, subadult and adult) in terms of mate choice preferences or the individual reproductive success. All that appears helpful for further improvements in managing populations. Knowing the density of a population as precisely as possible is one part of wildlife management. To know the impact, and its reasons, of the different population members in the population growth is another part.

The estimation procedure is robust to certain deviations from random sampling and smaller sample sizes. A deviation of less than 10% of the real population size appears to be tolerable. In that case, the estimator rather tends to overestimate the real population size, but as the magnitude of the error can be estimated, it can be considered for future management actions. However, too small sample sizes or subsamples appears rather unrealistic for many European ungulates. But when investigating, for example, small carnivore populations (Kery et al., 2011), the results of the here developed estimator must be treat with caution.

For species with a hunting regime that is focused on specific or purposefully harvest on parent–offspring pairs, the estimator seems to become inaccurate and inappropriate and tends to underestimate the real population size. It is therefore recommended not to rely on one method alone, but to always use at least two different methods for mutual validation. Combining the here presented estimator with, for example, a noninvasive sampling and density-estimating method (e.g., Ebert et al., 2012; Kery et al., 2011; Mollet, Kery, Gardner, Pasinelli, & Royle, 2015), could provide more than promising results, allowing both long-term and sustainable wildlife management.

Both CMR models, Lincoln–Petersen and Chapman estimator, assume that the investigated population in question is closed and without any migration. This also applies to our model. Although real populations are always affected by both immigration and emigration, as a first approach it makes sense to treat populations as closed, especially as reliable data on migration are hard to come by. In case of wild boar, this error is rather small since especially females are faithful to their habitats and have comparatively small home ranges (Keuling, Stier, & Roth, 2008, 2009). However, this may not be true for other species, both ungulates and carnivores. Further studies may aim to extend the present model with data on migration.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Björn Müller: Conceptualization (equal); Formal analysis (supporting); Methodology (equal); Project administration (lead); Writing-original draft (lead); Writing-review & editing (lead). **Moritz Mercker:** Conceptualization (equal); Formal analysis (lead); Methodology (equal); Software (lead); Writing-original draft (supporting); Writing-review & editing (supporting). **Jörg Brün:** Conceptualization (equal); Methodology (equal); Supervision (lead); Writing-original draft (supporting); Writing-review & editing (supporting).

DATA AVAILABILITY STATEMENT

All generated R-scripts will be available as online electronic appendices or will be downloadable on our institute website.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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