Satellitentelemetrische Studien an Rothirschen (*Cervus elaphus*) und retrospektive Betrachtung von Änderungen in der genetischen Konstitution freilebender Rothirsch-Populationen mit Hilfe alter DNA aus Geweihen

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Inhalt

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Kapitel 6 befindet sich in Bearbeitung und soll im Journal „Molecular Ecology“ eingereicht werden:

Hoffmann, G.S., Johannesen, J., Griebeler E.M.: A quite complete genotypic- and phenotypic turnover in an evolutionary short timespan due to translocation events in a natural red deer population.

Kapitel 11 befindet sich in Bearbeitung und soll auf Vorschlag des Journals beim “European Journal of Wildlife Research“ als „Short Communication“ wieder eingereicht werden:

Hoffmann, G.S., Griebeler, E.M.: Home range and habitat use of red deer in the Alps detected from satellite telemetry.

Zusammenfassung

Während der Rothirsch (*Cervus elaphus*) von der Späteiszeit bis in die Neuzeit hinein über ganz Mitteleuropa verbreitet war, unterlag seine Verbreitung in der jüngeren Zeit extremen Schwankungen, insbesondere aufgrund anthropogener Einflüsse (Bützler 2001; Raesfeld & Reulecke 1988). Ihren Tiefststand erreichten die Populationen nach der Revolution im Jahre 1848, der den Rothirsch an den Rand der Ausrottung drängte (Herzog 1995). Obwohl sich die Bestände in der Folgezeit steigerten, wurde die Rückkehr in viele Gebiete aufgrund des hohen Schadenspotenzials in der Land- und Forstwirtschaft verhindert. Als störungsanfällige Art gilt sie als typische Leitart für große, störungsarme, zusammenhängende Waldgebiete (Grau 2005; Herzog et al. 2010).

In der hier vorliegenden Arbeit wird der Rothirsch mit verschiedenen methodischen Ansätzen untersucht. Die Individuen - Ebene und das Raum – Zeitverhalten werden mit Hilfe satellitentelemetrischer Technik beleuchtet. Zur Untersuchung auf Populationsebene werden populationsgenetische Methoden, wie Mikrosatelliten und mitochondriale Sequenzen genutzt.

Bei der Satellitentelemetrie werden Tiere mit einem Empfänger ausgestattet, der die aktuelle Position des Tieres mit Hilfe des von GPS- Satelliten abgestrahlten Signals ermittelt. Insgesamt wurden im Rahmen dieser Arbeit 20 Individuen mit Halsbandsendern ausgerüstet, die in einem frei wählbaren Turnus die jeweilige Position bestimmen. Die Untersuchungsgebiete liegen in zwei unterschiedlichen Naturräumen, zum einen im Hochgebirge im Lechtal in den Alpen, zum anderen in einem kleinräumigeren, mittelgebirgsartigen Gebiet im „Naturpark Saar Hunsrück“. Durch die Nutzung moderner Satelliten- Aufnahmen und Vermessungstechniken konnten zum ersten Mal abiotische Faktoren auf den durch die Tiere genutzten Flächen untersucht werden, wie Sonneneinstrahlung oder Hangneigung. Zusätzlich wurden geschlechtsspezifische Unterschiede insbesondere in Streifgebietsgrößen und auch in der Flächennutzung dargestellt. Neben den GPS Koordinaten werden von den Halsbandsendern auch permanent Beschleunigungsdaten der Bewegungen der Tiere in horizontaler wie vertikaler Richtung detektiert und gespeichert. Durch die

korrekte Interpretation dieser Beschleunigungsdaten, steht nicht mehr nur eine GPS-Koordinate zur Verfügung, sondern es werden Aussagen über die Tätigkeiten und über das Verhalten der Tiere möglich (Moreau et al. 2009; Nathan et al. 2012; Brown et al. 2013). Problem dieser Daten ist, dass es ohne eine Eichung auf die jeweilige Tierart nicht möglich ist, spezielle Beschleunigungswerte spezifischen Tätigkeiten zuzuordnen. Aus diesem Grund haben wir zwei Tiere im Tierpark Hanau mit Halsbandsendern ausgerüstet und ihr Verhalten dokumentiert. So konnten einfache Tätigkeiten (ruhen, gehen, laufen, fressen) aufgezeichneten Beschleunigungsdaten zugeordnet werden.

Durch die hohe jagdliche Nutzung des Rothirschs existieren Trophäen (von Jägern präparierte Geweihe) aus spezifischen geographischen Gebieten, die teilweise bis zu mehrere hundert Jahre alt sind. Diese große Menge an Probenmaterial bietet einen riesigen Fundus zur retrospektiven Betrachtung der Veränderung der genetischen Konstitution in Populationen über die Zeit. Zur Nutzung der Trophäen als Probenmaterial wurde im Rahmen dieser Arbeit ein Verfahren entwickelt, welches die Extraktion hochwertiger mitochondrialer und genomischer DNA ermöglicht. Damit konnten bis zu über 200 Jahre alte Trophäen erfolgreich beprobt werden. Während die Extraktion mitochondrialer DNA aus altem Gewebe seit längerer Zeit erfolgreich praktiziert wird, ist die hier durchgeführte Gewinnung von qualitativ hochwertiger genomischer DNA eine Neuerung. Die zur populationsgenetischen Untersuchung verwendeten Proben stellen die derzeit ältesten Proben zur Mikrosatelliten – Analyse weltweit dar. Durch den Vergleich der genetischen Konstitution innerhalb Populationen in verschiedenen Zeitspannen, konnte der Einfluss anthropogenen Handelns auf Populationen durch übermäßige Bejagung und auch durch das Aussetzen gebietsfremder Individuen nachgewiesen werden.

1. Allgemeine Einleitung

Der Rothirsch (*Cervus elaphus*) ist eines der größten freilebenden Säugetiere Mitteleuropas. Während diese Art von der Späteiszeit bis in die Neuzeit hinein über ganz Mitteleuropa verbreitet war, unterlag das Verbreitungsgebiet in der jüngeren Zeit extremen Schwankungen in der Größe, insbesondere aufgrund anthropogener Einflüsse (Raesfeld & Reulecke 1988; Bützler 2001). Besonders die übermäßige Bejagung nach der Deutschen Revolution in den Jahren 1848 und 1849 führte zu starken Verringerungen der Populationsgrößen und zur gebietsweisen Ausrottung des Rothirschs (Herzog 1995). Obwohl sich die Bestände in der Folgezeit erholten und örtlich sogar stark steigern konnten, wird vom Menschen bis in die heutige Zeit ein sehr ambivalenter Umgang mit dieser Art gepflegt. Während Jäger aufgrund der jagdlichen Bedeutung eine Erhöhung der Populationsgröße durch intensive Hegemaßnahmen und Wiederansiedlungen forcierten, wurde die Rückkehr in vielen Gebieten aufgrund des hohen Schadenspotenzials für die Land- und Forstwirtschaft nicht geduldet. Als rein pflanzenfressende, wiederkäuende Art können sowohl Pflanzen mit hochresorbierbaren Zellinhaltsstoffen (Gebert & Verheyden-Tixier 2001), als auch zellulosehaltige Pflanzen von ihr aufgenommen werden (äsen) (Hofmann 1989). Wegen des hohen Nahrungsbedarfs von bis zu 20 Kilogramm pro Individuum pro Tag (Raesfeld & Wagner 1911) kann es bei selektiver Nutzung bestimmter Pflanzenarten zu enormen Änderungen in der Artzusammensetzung und Häufigkeitsverteilung der im Biotop vorkommenden Flora führen (Borowski & Kossak 1975). Im Falle des Äsens von land- und forstwirtschaftlich genutzten Pflanzen kann es zu erheblichen ökonomischen Schäden kommen.

Resultierend aus der intensiven Nutzung des Rothirschs durch verschiedenste Interessensgruppen kam es wie auch bei den meisten anderen Großsäugerarten in Europa zu ständigen Schwankungen in seinen Populationsgrößen und dem Verbreitungsgebiet. Daraus resultierende genetische Unterschiede in durch anthropogene Einflüsse isolierten Rothirschpopulationen (Hartl et al. 1990; Schreiber et al. 1994) bis hin zu Inzuchterscheinungen in einzelnen Populationen (Zachos et al. 2007) wurden bereits festgestellt.

Neben diesem modellhaften Charakter des Rothirschs zur Detektion anthropogener Einflüsse auf die genetische Konstitution von Populationen für Arten, die stark durch den Menschen genutzt sind, bietet der Rothirsch auch die Möglichkeit der Bearbeitung und Beantwortung naturschutzfachlicher Aspekte und Fragestellungen. Sein hoher und vor allem permanenter Nahrungsbedarf (mehrere Äsungsperioden pro Tag) macht ihn zu einer der störungsanfälligsten Arten in der Kulturlandschaft. Eine Hinderung oder auch nur Verschiebung dieses festen Äsungsrhythmus durch anthropogene Störungen führt schnell und zwangsläufig zu einer Meidung des Gebietes und zu einer Verschiebung des durch die Tiere genutzten Areals. Dies qualifiziert ihn seit längerer Zeit zu einer Leitart für große störungsarme und unfragmentierte Lebensräume (Grau 2005; Herzog et al. 2010).

Aufgrund dieses Modellcharakters habe ich mich in der hier vorliegenden Doktorarbeit mit dem Rothirsch beschäftigt. Um sowohl individuenbasierte, naturschutzfachliche Fragestellungen nach dem Verhalten und der Lebensraumnutzung, als auch Einflüsse auf Populationsebene detektieren und quantifizieren zu können, wurden von mir zwei methodisch verschiedene Ansätze gewählt. Um individuenbasierte Fragestellungen, wie die Wirkung anthropogener Störungen auf das Raum- und Zeitverhalten von Individuen aber auch zur Beantwortung ökologischer Grundlagenfragen wurden von mir satellitentelemetrische Studien durchgeführt. Hierzu habe ich Tiere mit Halsbandsendern ausgestattet, die mittels GPS Empfänger permanent die Position des besenderten Tieres aufzeichnen.

Um die Art auf Populationsebene untersuchen zu können habe ich genetische Studien durchgeführt. Mit verschiedenen genetischen Markern sollte hier die Kausalität zwischen menschlichem Handeln und der parallel verlaufenden Änderung in der genetischen Konstitution der Populationen hergestellt werden und so der Effekt und die Auswirkungen der jahrhundertelangen Nutzung für ausgewählte Populationen quantifiziert und beschrieben werden.

Diese beiden methodisch verschiedenen Ansätze der genetischen Untersuchung und der Satellitentelemetrie von Populationen werden im folgenden Teil der Arbeit separat voneinander dargestellt.

2. Einleitung Genetik

Populationen freilebender Tiere und Pflanzen unterliegen einem ständigen Wandel ihrer demographischen Organisation und genetischen Struktur. Ursachen dafür sind ökologische Faktoren und evolutionäre Prozesse. Das komplexe Wirkungsgefüge ist zum bedeutenden Teil das Resultat aus inter- und intraspezifischer Konkurrenz, abiotischen Faktoren, sowie anthropogenen Einflüssen. Die Anteile der einzelnen Faktoren an den beobachteten Veränderungen, sowohl im Hinblick auf globale, als auch lokale Ursachen, sind noch weitgehend unbekannt.

Aufgrund der überwiegenden Verwendung von rezentem Material als DNA Quelle (z.B. Muskelgewebe) können die meisten populationsgenetischen Studien keine direkten Informationen über die Veränderung der genetischen Konstitution von Populationen über die Zeit liefern, da genetische Referenzdaten aus der Vergangenheit fehlen. Dies begrenzt generell die Nutzbarkeit genetischer Daten, da anthropogen verursachte und auch natürliche Veränderungen der Lebensbedingungen nicht direkt mit Veränderungen des Genpools von Populationen in Verbindung gebracht werden können (Wandeler et al. 2007).

Evolution allerdings ist die Veränderung von Arten und deren Genpools über die Zeit. Während langandauernde und lange zurückliegende evolutionäre Prozesse anhand fossiler Funde dokumentiert und analysiert werden können (Wandeler et al. 2007), ist die Erforschung und das Verständnis mikroevolutionärer Prozesse oft limitiert auf Organismen mit kurzen Generationszeiten (Balanya et al. 2006). Durch die Entwicklung neuer molekulargenetischer Verfahren bieten Museen und andere alte Sammlungen eine neue wichtige DNA-Quelle von Populationen der Vergangenheit in Form von alten Bälgen, Knochen, Präparaten oder sonstigen dort eingelagerten Geweben. Dieser riesige Fundus an Probenmaterial eröffnet uns die Möglichkeit populationsgenetische Studien durchzuführen und zum Beispiel rezente mit vergangenen Zuständen von Populationen zu vergleichen, erstmalig auch für Organismen mit langen Generationszeiten (Wandeler et al. 2007). Obwohl diese alten Proben vielfältige Möglichkeiten für retrospektive Analysen bieten, sind ihre

Einsatzmöglichkeiten insbesondere hinsichtlich genetischer Analysen dennoch limitiert. Neben niedriger DNA Qualität ist oft auch die Anzahl an Proben aus einer spezifischen geographischen Region begrenzt, was aber eine essenzielle Voraussetzung für die Betrachtung genetischer und demographischer Einflüsse auf Populationen darstellt.

In den letzten Jahrzehnten wurden mehrere genetische Untersuchungen europäischer Rothirschpopulationen unter Verwendung von rezentem Probenmaterial (Muskelgewebe) durchgeführt (Martinez et al. 2002; Kuehn et al. 2003; Kuehn et al. 2004; Frantz et al. 2006; Zachos et al. 2007). Diese konnten Isolation und auch Inzuchterscheinungen in Populationen als Folge einer starken anthropogenen Nutzung des Rothirschs in Mitteleuropa nachweisen (Hartl et al. 1990; Schreiber et al. 1994; Zachos et al. 2007). Durch die jahrhundertelange hohe jagdliche Bedeutung und Nutzung des Rothirschs unterlagen seine Populationsgrößen enormen Schwankungen, die zudem auch stark abhängig von der jeweiligen Gesetzeslage, der politischen Situation und durch Neugründungen von Populationen durch Aussetzungen von Tieren bedingt waren (Fernandez-Garcia et al. 2014). In den letzten Jahrzehnten hat sich sicher auch der starke Selektionsdruck durch die gezielte Bevorzugung von männlichen Tieren mit großen und endenreichen Geweihen (Hartl et al. 2003) in Rothirschpopulationen genetisch ausgewirkt. Aufgrund der hohen jagdlichen Relevanz dieser Art existieren aber auch große und teilweise jahrhundertealte Sammlungen von Trophäen und „Abwurfstangen“ (Geweihe, die jahresperiodisch abgeworfen wurden), die einen Zugriff auf Probenmaterial von historischen Populationen bieten.

Vor diesem Hintergrund habe ich mich in meiner Doktorarbeit unter anderem mit der Extraktion von qualitativ hochwertiger DNA aus alten Geweihknochen beschäftigt. Ein von mir publiziertes Verfahren (Hoffmann & Griebeler 2013) liefert hierbei beste Ergebnisse und ermöglichte mir die Nutzung von über zweihundert Jahren alten Geweihen als DNA Quelle für genetische Analysen verschiedener Populationen. Aufgrund der guten Lagerungsbedingungen von Trophäen (Rankin et al. 1996; MacHugh et al. 2000;), sind sowohl Untersuchungen mit mitochondrialer, als auch

nukleärer DNA möglich. Während die Extraktion mitochondrialer DNA aus alten Proben seit längerer Zeit erfolgreich Anwendung findet, stellt die Verwendung von nukleärer DNA und hier insbesondere die Analyse von Mikrosatelliten-Markern eine Besonderheit dar. Wegen den hohen Mutationsraten von Mikrosatelliten und ihrer Selektionsneutralität stellen diese genetischen Marker das perfekte Werkzeug für vergleichende populationsgenetische Studien rezenter und historischer Rothirschpopulationen dar.

2.1 Geleistete Vorarbeiten, DNA Extraktion

Knochengewebe wird bereits seit den neunziger Jahren als Quelle zur Gewinnung alter DNA verwendet (Höss & Pääbo 1993; Krings et al. 1997). Allerdings sind der Nutzbarkeit alter DNA aufgrund ihrer zunehmenden Degradierung über die Zeit Grenzen gesetzt. Während mitochondriale DNA wegen ihrer hohen Anzahl an Kopien pro Zelle und der doppelten Zellmembran der Organellen oft in ausreichender Menge und Länge extrahiert werden kann, ist die Extraktion von genomischer DNA oft nicht möglich. Wegen ihrer biparentalen Vererbung und ihrer hochvariablen Abschnitte ist aber gerade die Kern-DNA in idealer Weise geeignet um populationsgenetische Studien durchzuführen und auch kurzzeitige mikroevolutionäre Prozesse zu untersuchen.

Für eine erfolgreiche Extraktion von alter genomischer DNA müssen aus diesen Gründen hochspezifische und zuverlässig zu amplifizierende Marker gefunden werden, insbesondere da die Ziel-DNA in stark degradierten Proben nur noch in sehr geringer Anzahl vorhanden sein kann.

Deshalb habe ich den Beginn meiner Arbeit der Etablierung einer möglichst effizienten Methode hinsichtlich DNA-Qualität und Kosten zur Amplifizierung von geringen Mengen an Ziel-DNA aus alten Geweihen gewidmet. Beste Ergebnisse lieferte hierbei die Amplifizierung der verwendeten Mikrosatelliten-Loci mit dem Quiagen Multiplex PCR Kit. Gegenüber den vorher von mir ausgetesteten herkömmlichen PCR Methoden mit Fluoreszenz-Markierung in der PCR-Reaktion (M13-Tail) (Schuelke 2000) oder mit Beads, konnte ich so deutlich bessere Ergebnisse und um ein Vielfaches höhere

Fluoreszenzwerte erreichen. Durch die sukzessive Verringerung des Reaktionsvolumens von 50 µl auf schließlich 10 µl Endvolumen pro PCR-Reaktion konnte ich auch eine erhebliche Kostenreduktion für die einzelne Probe erreichen, da nun die fünffache Anzahl an Proben mit identischer Menge an Reagenzien bearbeitet werden kann.

Die so mit qualitativ hochwertiger DNA aus Muskelgewebe gefundenen beiden Multiplexsysteme mit jeweils fünf Primern erzeugen sehr hohe, eindeutige und somit gut auswertbare Peaks für die Mikrosatellitenmarker, was nach meiner Meinung die Grundvoraussetzung für ein erfolgreiches Arbeiten mit qualitativ minderwertiger und degradierter DNA darstellt. Das so in Zusammenarbeit mit der Bachelorkandidatin Silke Gabel-Scheurich optimierte und etablierte Multiplex-System vereint sehr gute Bedingungen für eine effektive PCR-Reaktion in Bezug auf Reaktionsvolumen, Annealing-Temperaturen sowie eingesetzter Primermenge.

Eigene Versuche haben gezeigt, dass die Menge des eingesetzten Primers die Menge an PCR-Produkt maßgeblich beeinflusst, während die Menge an Ziel-DNA eher als zweitrangig anzusehen ist. Auch deshalb ist ein perfekt auf den jeweiligen Organismus abgestimmtes System gerade bei der Arbeit mit DNA von minderwertiger Qualität oder in sehr geringen Konzentrationen unabdingbar. Die Verwendung universeller Primer und von Standard-Protokollen ist hier höchstwahrscheinlich selten zielführend.

Um an geeignetes älteres Probenmaterial zu gelangen wurden von mir Adelshäuser in ganz Deutschland angeschrieben, die möglichst große zusammenhängende Waldgebiete mit dauerhaftem Rothirschbestand besitzen. Nur so konnte ich sicher sein, dass alte Trophäen aus einem definierten geografischen Gebiet in ausreichender Anzahl für populationsgenetische Studien vorhanden sind.

Die Extraktion von DNA aus diesen Geweihen wurde mit den derzeit gängigen Verfahren erprobt (Magnetic Beads, Adhäsion an Silica, Phenol-Chloroform-Fällung), die Ergebnisse untereinander verglichen und das erfolgreichste Verfahren (Phenol-Chloroform) sukzessive verbessert und optimiert (Kapitel 3, Hoffmann & Griebeler 2013).

Nach der in Kapitel 3 beschriebenen und publizierten Methode zur erfolgreichen Extraktion qualitativ hochwertiger genomischer DNA aus Rothirschgeweihen, war für mich der nächste logische Schritt die Erprobung, ob nur Rothirschgeweihe eine hochwertige DNA-Quelle darstellen oder ob dies auch Geweihe anderer Cerviden-Arten sind. Hierzu habe ich Verbindung zum Tierpark Berlin aufgenommen, der derzeit den artenreichsten Cerviden Bestand Deutschlands beherbergt. Dort konnte ich Geweih-Proben von 17 Arten und fünf Unterarten zur genetischen Untersuchung nehmen. Da in dem Artikel „An improved high yield method to obtain microsatellite genotypes from red deer antlers up to 200 years old“ (Kapitel 3, Hoffmann & Griebeler, 2013) ausschließlich Mikrosatelliten, also genomische DNA verwendet wurde, wurde hier (Hoffmann et al. 2014) auch die erfolgreiche Extraktion und Verwendbarkeit mitochondrialer DNA aus Geweihen nachgewiesen. Dadurch wird die Bandbreite an Einsatzmöglichkeiten der in Kapitel 3 vorgestellten Methode (Hoffmann & Griebeler 2013) nochmals erhöht. Während die erfolgreiche Extraktion und Amplifizierung hochmutierender Kern-DNA Abschnitte populationsgenetische Studien und das Studium mikroevolutionärer Prozesse ermöglicht, können mit Hilfe mitochondrialer DNA auch phylogenetische und auch biogeografische Fragestellungen beantwortet werden.

2.2 Populationsgenetik

Nach diesen eher methodischen Publikationen mit artenschutzfachlichen und phylogenetischen Teilaspekten, gingen meine Bemühungen in Richtung populationsgenetischer Studien und Grundlagenforschung. Das sich als erfolgreich herausgestellte Verfahren der DNA-Extraktion sollte nun zur retrospektiven Betrachtung der genetischen Konstitution freilebender Rothirsch Populationen eingesetzt werden. Wie bereits erwähnt, ist die Nutzbarkeit von historischen Sammlungen als DNA-Quelle stark limitiert durch die begrenzte Anzahl an verfügbarem Probenmaterial und auch durch den jeweiligen Erhaltungszustand der Exponate. Für die Extraktion genomischer DNA ist beste DNA-Qualität und für die Durchführung populationsgenetischer Studien außerdem eine möglichst hohe Stichprobengröße aus definierten geographischen Gebieten erforderlich. Beim

Rothirsch sind diese Faktoren vereint in den Sammlungen von Adeligen, bei denen sich große Landflächen teilweise seit Jahrhunderten in Privatbesitz befinden. Zur Beschaffung von Probenmaterial habe ich diese Adelshäuser kontaktiert und um die Möglichkeit der Probenentnahme an Trophäen gebeten. So konnte ich aus unterschiedlichsten Gebieten Deutschlands Geweihproben zur anschließenden genetischen Analyse gewinnen. Bei der Auswahl potenzieller Untersuchungsgebiete habe ich darauf geachtet, dass neben ausreichender Individuenzahl, die Populationshistorie bekannt ist und die Population möglichst zur Beantwortung populationsgenetischer oder mikroevolutionärer Grundlagenfragen geeignet ist.

2.3 Population Neuwied

Die Population Neuwied ist eine bis heute existierende und stabile Population auf dem Grundbesitz der Fürsten zu Wied. Die ältesten dort im Schloss vorhandenen Trophäen sind über 200 Jahre alt. Aus dieser ersten Zeitperiode (1813-1861) sind insgesamt sechs Individuen erhalten, die ich auch erfolgreich beprobt habe. Aus einer zweiten Zeitperiode zwischen den Jahren 1923 und 1940 wurden weitere acht Trophäen von mir beprobt. Rezentes Probenmaterial (Muskelgewebe) konnte von 17 Individuen von mir entnommen werden.

Wie in den meisten Rothirsch-Gebieten wurden die Populationen bis zum Jahre 1848 von den zumeist adligen Besitzern bewirtschaftet und bejagt. Während und nach der Revolution im Jahre 1848 wurden die Rothirschbestände durch das Außerkrafttreten von Gesetzen unbeschränkt gewildert und so deutschlandweit stark dezimiert und gebietsweise sogar völlig ausgerottet. Auch die Population in Neuwied wurde in dieser Zeit extrem reduziert, konnte sich aber nach Beruhigung der politischen Lage, in den folgenden Jahrzehnten wieder vergrößern und räumlich ausbreiten. Als Besonderheit im Gegensatz zu vielen anderen großen Populationen Deutschlands sind hier keine Aussetzungen von Individuen aus geographisch weit entfernten Regionen bekannt.

Heute ist die Population stabil und wird von den Fürsten bejagt und bewirtschaftet. Da die Population durch den Rhein, die Verstädterung der Region und auch durch

Autobahnen geographisch isoliert ist, könnte der gegenwärtige genetische Austausch mit anderen Populationen erschwert sein.

2.4 Population Bad Berleburg

Die zweite von mir untersuchte Population ist die Population in Bad Berleburg. Auch die Historie dieser Population ist bekannt, da es sich hier um eines der berühmtesten Rothirsch-Gebiete der Welt handelt. Seit nunmehr fast drei Jahrhunderten werden Rothirsche hier gezielt bewirtschaftet, erhalten und bejagt. Die Größe und Stärke der derzeit dort vorkommenden Tiere ist im deutschlandweiten Vergleich überdurchschnittlich.

Auch der Rothirsch folgt der Bergmann´schen Regel und nimmt an Körpermasse und damit verbunden meist auch an Geweihgewicht innerhalb seines Verbreitungsgebietes nach Norden/ Osten hin zu. Diese Tatsache hat man sich in vielen Rothirsch-Gebieten Europas zu Nutze gemacht und Tiere aus nord-östlich gelegenen Gebieten nach Mitteleuropa transportiert. Ziel dieser Translokationen war die Stärkung der einheimischen Bestände und das Erreichen eines höheren Trophäengewichtes, was aus jagdlicher und auch wirtschaftlicher Sicht erstrebenswert war und immer noch ist. Auch in die Rothirschpopulation in Bad Berleburg wurden seit den 1960er Jahren gezielt stark fremde Tiere eingesetzt.

Über den Erfolg und insbesondere den Reproduktionserfolg dieser eingebrachten Tiere ist allerdings wenig bekannt. Zwar haben die Hirsche in dieser Region sichtbar an Stärke zugenommen, ob dies allerdings einen genetischen Ursprung hat (Introgression), oder aber durch verbesserte Nahrungs- und Fütterungsbedingungen erreicht wurde ist bisher rein spekulativ. Das Aussetzen fremder Individuen, nicht nur wie hier zur Erhöhung der Trophäenstärke, sondern auch zur genetischen Auffrischung (genetische Verarmung, Vermeidung von Inzucht), sowie zur Erhöhung der Populationsgröße ist insbesondere bei seltenen Arten ein gängiges und wichtiges Werkzeug des Artenschutzes. Die Sinnhaftigkeit dieses Wiederansiedelns und Aussetzens von Individuen steht und fällt mit der Fähigkeit der ausgesetzten Tiere sich erfolgreich in den dort ansässigen Populationen zu reproduzieren. Zahlreiche

Mechanismen, die zu einer reproduktiven Isolation dieser neu hinzugekommenen Individuen führen könnten sind denkbar, wie zum Beispiel eine zeitlich verschobene Paarungsbereitschaft, Unterschiede in Signalen (Ruf, Duft) und eine selektive Partnerwahl (sortengleiche Paarung). Auch aus artenschutzfachlichen Gründen ist damit die retrospektive Betrachtung des Paarungserfolges der eingesetzten Tiere relevant.

In dem nachfolgenden Manuskript bleiben meine Bemühungen unerwähnt einen geeigneten molekularen Marker für den paternalen Vererbungsweg zu finden. Die Detektion fremder Tiere und auch die Quantifizierung ihres Fortpflanzungserfolgs alleine über Mikrosatelliten erwiesen sich nämlich als schwierig. Aufgrund der hohen Mutationsrate und der Rekombination werden fremde Allele, besonders wenn sie in hoher Frequenz in der Population vorhanden sind, schnell übernommen und sind so als Anzeiger einer Introgression ungeeignet. Die im nachfolgenden Manuskript (Kapitel 5) verwendete D-Loop Sequenz ist ein geeigneter Marker, um den maternalen Vererbungsweg von Allelen zu untersuchen. Der Fortpflanzungserfolg der eingesetzten weiblichen Tiere ist so direkt erkennbar. Da allerdings fünfzig Prozent der eingesetzten Tiere männlichen Geschlechts waren, wäre ein polymorpher, rein paternal vererbter Marker nötig, um auch den Erfolg dieser Tiere direkt beobachten zu können. Um dies zu erreichen, haben wir etliche y-chromosomale Mikrosatelliten-Loci und auch codierende Sequenzen auf Polymorphie und damit Eignung zur Beantwortung meiner Fragestellung untersucht. Leider konnten wir im Rahmen der Arbeiten bezüglich der Population Bad Berleburg keinen geeigneten Marker finden. Das hier präsentierte Kapitel zur Population Bad Berleburg basiert auf der Masterarbeit von Frau Rebecca Nagel.

3. An improved high yield method to obtain microsatellite genotypes from red deer antlers up to 200 years old

3.1 Abstract

Analysis of DNA from older samples, such as museum specimen, is a promising approach to studying genetics of populations and ecological processes across several generations. Here, we present a method for extracting high quality nuclear DNA for microsatellite analysis from antlers of red deer (*Cervus elaphus*). The genotyping of individuals was based on nine microsatellite loci. Because the amount of DNA found in antlers was high, we could reduce the amount of sample and chemicals used and shorten the decalcification time in comparison to other methods. Using these methods, we obtained genotypes from antlers up to 200 years old.

3.2 Introduction

Utilizing temporal sampling to genotype populations at different time periods allows for a comparison of and distinction between historical and extant processes that might shape the genetic constitution of populations (Pertoldi et al. 2001; Flagstad et al. 2003). The patterns of temporal variation in the genetic constitution of populations is therefore important in several ecological and population genetic contexts, as well as in species conservation (Hedmark & Ellegren 2005). Knowledge of foreign introductions, stocking practices including breeding regimes, and control of studbooks are of important management concerns for many game and/ or domesticated animals.

Despite the significance of temporal information, it is often difficult to acquire adequate sample sizes of old specimens needed to study the temporal genetic structure within populations. Old tissue samples also often come from different sampling sites or may be of unknown geographical origin (Nielsen et al. 1999; Heath et al. 2002). A further problem encountered when studying historical genetic processes is the high degradation of DNA obtained from old samples. Dry, cold and constant environmental conditions in combination with above ground storage are the most important factors for conserving DNA at a high quality (Rankin et al. 1996; MacHugh et al. 2000).

Red deer (*Cervus elaphus*) are important and traditional game animals in central Europe with a known history of stocking and artificial population founding. There is an abundance of ecological information about population sizes and spatial distributions of this species in the wild. Population genetic analyses have detected significant differentiation among artificially separated populations (Hartl et al. 1990; Schreiber et al. 1994) or inbreeding depression in isolated populations (Zachos et al. 2007). All population genetic studies conducted so far for this species have examined extant populations using muscle tissue for genotyping; to the best of our knowledge, no studies have analysed old DNA from antlers. Because antlers from males have been collected as trophies for many centuries, extensive sample sizes (trophy collections) from different regions and different ages exists, often with the exact age and origin of the antlers known.

Due to the profound history of stocking, translocations, population founding, and bottlenecks in many red deer populations, an analysis of the temporal genetic constitution has the potential to provide novel insights not only into the demographics of natural populations, but also to test hypotheses on population development. Non-invasive sampling and genotyping of antlers is also useful in conservation efforts to answer, among other things, whether past habitat fragmentation and changes and/or hunting has influenced the genetic diversity of populations.

Red deer shed their antlers every year and regrow them during the following year. Deer heads can weigh up to 15 kg, meaning that antler material is plentiful in comparison to other tissue samples from rare and precious museum samples usually available for genetic studies. Large antlers collected and displayed in trophy collections are commonly longer than one meter (Figure 3.1 A). The thickness of the compact, hard outer bone from which the samples for genotyping antlers are taken can be up to one centimetre (Figure 3.1 B).

In this study, we report a method for extracting high quality DNA from red deer antlers up to 200 years old. This method may also be applicable in other cervids. The extracted old DNA was successfully used to amplify nine microsatellite loci commonly applied in population genetic studies utilizing DNA from muscle tissue of red deer (Kuehn et al. 2003). To assess DNA quality in terms of DNA concentration, we used a photometer

(NanoDrop 1000) for all samples and a fluorometer for a smaller sample set (Qubit® Fluorometer). To rate genotyping reproducibility, we compared microsatellite amplifications from DNA extracted from old antlers and fresh muscle tissue in addition to genotyping seven antlers shed by the same individual.

3.3 Materials and methods

3.3.1 Sampled animals

Forty-two red deer antlers between 10 and 199 years old were genotyped at nine microsatellite loci commonly applied in red deer (Kuehn et al. 2004). Twenty-two antlers were from a German population (Neuwied, origin of samples was the trophy collection at castle Neuwied) with ages ranging from 27 to 198 years. The remaining twenty antlers were from an Austrian population (Steeg) between 11 and 51 years old. In total, six antlers were older than 150 years, eight were between 70 and 150 years old, and 20 antlers were aged between 10 and 50 years.

To assess DNA quality and rate genotyping reproducibility of antler tissue, we also extracted DNA from the fresh muscle tissue of 18 individuals from the German population and of 72 individuals from the Austrian population. These samples were genotyped at the same nine microsatellite loci as the antler DNA. Samples were taken during the years 2009 and 2010. They were stored in 99% alcohol and frozen at -20 °C. Amplified fragments of microsatellite loci obtained from muscle and antler samples were compared in terms of shape, quantity, height and length.

To rate the reproducibility of the genotype, we genotyped seven antlers shed by the same individual; the antlers ranged from 9 to 15 years old.

3.3.2 DNA Extraction from antlers and muscle tissue and DNA amount

DNA was extracted from 0.2 g of antler material, attained with an 8 mm drill. Each drilling gains ca. 0.6 g of antler tissue, which provides enough material for at least two DNA extractions.

For antler sampling, we used an autoclaved drill bit normally used for metal due to the hard and compact material of antlers. To avoid cross contamination between two different samplings, we always sterilized the bit between each use with tissue and applied the nucleic acid decontamination reagent DNA-ExitusPlus™ (AppliChem). We then heated the bit with a Bunsen burner, put it in 99% alcohol and flamed it again. To minimize the risk of DNA contamination from any cells on the surface of the antler, we rejected the antler chips obtained from the outermost millimetre of the drilled core where the antler is brown coloured (Figure 3.1 B). Bone marrow from the inside of the antlers was also avoided because it has high fat content, which complicates DNA extraction and reduces the amount and quality of DNA. Bone marrow is a much softer material than the compact outer bone and is easier to drill into, which makes avoiding this inner bone material relatively easy. We drilled into the base of the antlers on its backside so the trophies were not damaged and the borehole was on the far side (Figure 3.1 A). We had previously tested material from different areas of the antler, all of which revealed a sufficient amount of white bone chips from the outer bone for DNA extraction. The best compromise found for acquiring compact white bone material for DNA extraction while limiting the size and visibility of the borehole was to drill in the area at the base of the antlers (for variability in DNA amounts at different areas of the antler see Table S 3.1). The collected antler chips were stored at -20 °C until DNA extraction.

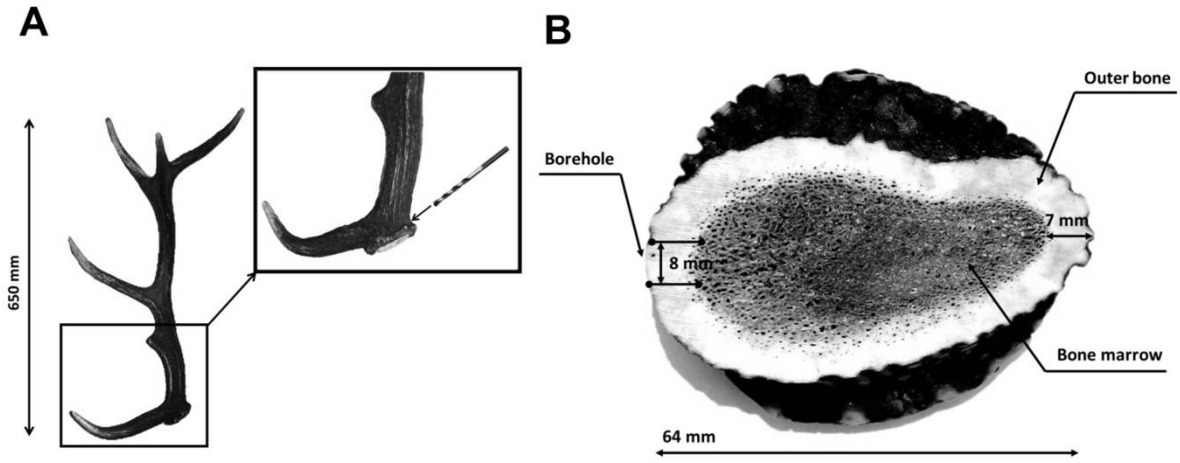


Figure 3.1 A: Best point for drilling into an antler to gain antler material. The hole is located at the backside of the base of the antler and on the far side of a wall mounted trophy. **B**: Cross section through the base of an antler of average size. The antler sample shown here is from a middle-aged red deer.

3.3.3 Extraction method

The antler chips (0.2 g) were crushed in a Retsch-mill at a frequency of 30 Hertz for 120 seconds. The powdered antler material was decalcified in an extraction buffer (consisting of 750 µl 0.5M EDTA, pH 8.5; 75µl 0.5% N-lauryl sarcosine; 20 µl proteinase K). The total volume of the extraction buffer and the antler chips was ca. 900 µl. Tubes containing the antler material and extraction buffer where placed on a rotary shaker for 24 hours at 37 °C. For DNA extraction, we used a phenol/chloroform/isoamyl-alcohol solution (25:24:1) twice before adding chloroform. After the addition of each solution, the solid matter and lipophilic components were separated by centrifugation; the aqueous solution (ca. 800 µl) was used while remaining components were dropped. After these three steps, the nucleic acids remaining in the aqueous solution were precipitated with cold ethanol. The remaining pellet was diluted with 50 µl elution buffer (included in the “High pure PCR template preparation Kit” (Roche Diagnostics)). To avoid the inhibition of amplification in the following PCR, we purified the DNA solution with the “High pure PCR templpreparation

Kit" (Roche Diagnostics) using the standard protocol recommended by the manufacturer. Finally, the DNA was stored at -20 °C.
The microsatellite amplification of old antler DNA was compared to amplifications from fresh muscle tissue isolated from a total of 90 individuals. The extraction of DNA from muscle tissue was again performed with the "High pure PCR template preparation Kit" (Roche Diagnostics) using the standard protocol. The amount of muscle tissue used was ca. 0.04 g for each extraction.
The DNA content of every sample (antlers and muscle tissue) was measured with a NanoDrop 1000 (manufacturer). We also determined the 260/280 nm ratio to evaluate potential contamination of DNA samples with proteins. Before PCR, every sample was diluted to a final DNA concentration of 50-100 µg/ml (measured with NanoDrop 1000). The amount of DNA in 12 additional samples originating from muscle tissue (4) and antlers of different ages (8) was determined using a Qubit®1.0 fluorometer using the Qubit®dsDNA BR Assay Kit. Contrary to the NanoDrop device, the fluorescence-based Qubit device is highly selective for double-stranded DNA through the use of a DNA specific dye.

3.3.4 PCR reaction and genotyping of microsatellites

The DNA extracted from antler and muscle tissue was treated identically. For PCR, we used the Qiagen Multiplex Kit (Qiagen, Basel, Switzerland) with fluorescently labelled primers. The reaction volume for each sample consisted of 5 µl Quiagen Multiplex PCR Mastermix (contains dNTPs, Qiagen HotStar Taq DNA Polymerase, and 6 mM $MgCl_2$), 1 µl Primer Mix (2 µM each Primer), 2.5 µl RNase-free water and 1.5 µl DNA; the final reaction volume was 10 µl for each DNA sample. PCRs were performed on a Perkin Elmer GeneAmp 9600 thermocycler using recommended multiplex-PCR protocol (Qiagen) with the following cycling conditions: 15 min at 95°C for initial denaturation; then 40 cycles of 40 s at 94°C, 90 s at 57°C and 90 s at 72°C, followed by 30 min at 60°C and a final hold at 4°C.
We used the microsatellite primers Ilsts06, Cer14, CSSM16, Haut14, Inra35, CSSM22, CSSM19, CSSM14, BM1818 (Kuehn et al. 2003; Kuehn et al. 2004). They were

developed for cattle (*Bos primigenius tauris*) but were identified by Kühn *et al*. (2004) as being applicable to red deer. The repeat motif of all loci is a dinucleotide.
PCR fragments were run on a 3130xl capillary sequencer (Applied Biosystems) using GS500 ROX size standard. The genotypes were analysed with GENEMAPPER, version 4.0 (Applied Biosystems). To avoid false allele scoring, we only scored alleles with markedly high numbers fluorescent values (more than 3000) and a distinctive, characteristic form of each locus.

3.3.5 Quality and applicability of antler DNA

Human DNA and cells are ubiquitous in all laboratory settings (Willerslev & Cooper 2005). To exclude the possibility of DNA contamination by humans during sampling and lab work, we extracted the DNA from the blood of the originators and genotyped each sample using the original nine microsatellite primers. Additionally, at least ten percent of all samples genotyped were controls. Control samples were treated identically to DNA samples except that RNAse-free water was used instead of DNA (Figure S 3.1).

We analysed DNA quality and the genotyping reproducibility of antlers based on four approaches. First, DNA from all antlers was amplified and genotyped twice to assess the genotyping error rate. Second, all microsatellite loci were analysed with the software Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) to ensure that there were no scoring errors due to stuttering, no allelic dropouts and no null alleles. Third, we genotyped seven antlers that were shed by the same individual because reproducibility of data by using different samples of the same individual is an important criterion for proving DNA quality (Poinar & Cooper 2000). Finally, we compared the genotypes and similarity of loci from the two ancient populations with the genotypes from the same extant populations characterised from muscle tissue.

3.4 Results

DNA was successfully extracted from all antlers and muscle tissue samples in moderate to high concentrations (NanoDrop values for muscle tissue: 44 – 370 µg ml^{-1}). The concentrations in antler samples were between 65.7 - 774.3 µg ml^{-1} (mean: 252.7 µg ml^{-1}, standard deviation: 156.3). The purity of DNA, assessed by the ratio of absorbance at 260/280 nm, was high (in the range of ca. 1.8) in all antler samples, which is generally accepted as pure DNA (DNA extracted from muscle tissue had similar values) (Table 3.1). As expected, the DNA concentrations of the 12 samples selected for an additional analysis with the Qubit device were lower (NanoDrop: 50-100 µg ml^{-1}; Qubit: 24.3-81.6 µg ml^{-1}), but concentrations were still sufficient for microsatellite analysis (Table 3.2).

Table 3.1 Origin, number of used microsatellite (MS) loci, ranges of age of samples, amplification success, ranges of amount of DNA extracted, and 260/280 nm ratio of antler samples.

Origin	N	MS	Age [years]	Amplification success	Amount of DNA [µg ml^{-1}]	260/280 nm ratio
Germany	16	9	71 - 199	100 %	67.10 - 400.50	1.75 - 1.82
Austria	22	9	11 - 51	100 %	170.20 - 774.30	1.78 - 1.84

Table 3.2 Comparison of DNA concentration of samples measured with a NanoDrop 1000 photometer and a Qubit®1.0 fluorometer. Samples of different ages (young=DNA derived from muscle tissue, middle-aged=DNA from antlers with an age between 72 and 89 years, old= DNA derived from antlers with an age between 151 and 199 yrs) and of different tissues (antlers and muscle tissue) are shown.

Sample material	**Age in years [yrs]**	**NanoDrop [µg ml^{-1}]**	**Qubit [µg ml^{-1}]**
Antler	Old (199 yrs)	72,6 µg ml^{-1}	47,5 µg ml^{-1}
Antler	Old (176 yrs)	72,0 µg ml^{-1}	68,8 µg ml^{-1}
Antler	Old (157 yrs)	81,5 µg ml^{-1}	62,6 µg ml^{-1}
Antler	Old (151 yrs)	82,9 µg ml^{-1}	81,6 µg ml^{-1}
Antler	Middle-aged (80 yrs)	72,6 µg ml^{-1}	43,3 µg ml^{-1}
Antler	Middle-aged (79 yrs)	72,0 µg ml^{-1}	51,5 µg ml^{-1}
Antler	Middle-aged (78 yrs)	81,5 µg ml^{-1}	38,8 µg ml^{-1}
Antler	Middle-aged (72 yrs)	82,9 µg ml^{-1}	57,0 µg ml^{-1}
Muscle tissue	Young (<10 yrs)	67,1 µg ml^{-1}	54,1 µg ml^{-1}
Muscle tissue	Young (<10 yrs)	87,0 µg ml^{-1}	24,3 µg ml^{-1}
Muscle tissue	Young (<10 yrs)	83,8 µg ml^{-1}	32,9 µg ml^{-1}
Muscle tissue	Young (<10 yrs)	55,7 µg ml^{-1}	37,3 µg ml^{-1}

The amplification rate was 100% for antler and muscle samples (Table 3.1) and zero for the control samples (Figure S 3.1). The test for amplification success of the used primers with DNA from the originators was negative. While a few fragments were amplified, they had unique lengths compared to those amplified in the red deer and the signal strength was much weaker than in the red deer samples (Figure S 3.2). These observations corroborate with the findings from Kühn et al. (2004) that the microsatellite primers used in our study work best on cattle and red deer. Since the primers do not bind on human DNA, contamination of samples by humans is unproblematic when genotyping red deer individuals from antlers. Additionally, the genotyping error rate while using these primers was zero (below detection), indicated by an identical multilocus genotype of each individual genotyped twice. The Micro-Checker program also gave no evidence of a scoring error due to stuttering, allelic dropouts or null alleles for any of the loci.

Microsatellite amplification from DNA extracted from antler samples and extant muscle tissue produced identical fragment lengths per locus (between 80bp and 300bp), similar allele numbers (Table 3.3) and comparable signal strength and allele scoring.

The amplification success of microsatellites was independent from fragment length and age of the samples used. Allele scoring was not more difficult in antler samples than in muscle tissue samples. A comparison of the oldest sample and the fresh muscle tissue samples indicated no visible differences in genotyping (Figure S 3.3, S 3.4).

The seven antlers shed by the same individual always revealed the same multilocus genotype (Figure S 3.5).

Table 3.3 Loci, populations and numbers of alleles found in each population. For each population a random subsample of five individuals is presented. Locus: identifier of the microsatellite locus with fragment length range [bp] and number of alleles in brackets; population: young=DNA derived of muscle tissue, middle-aged=DNA from antlers with an age between 72 and 89 years, old= DNA derived from antlers with an age between 151 and 199 yrs, n=number of individuals genotyped; alleles found: fragment lengths and frequency in brackets.

Locus	Population	Alleles found								
CSSM 16 (150-160, 4)	young (n=5)	------	152 (1)	154 (7)	160 (2)					
	middle-aged (n=5)	150 (3)	152 (1)	154 (2)	160 (4)					
	old (n=5)	------	152 (1)	154 (4)	160 (5)					
Inra35 (99-117, 9)	young (n=5)	99 (1)	101 (1)	103 (1)	------	107 (2)	------	111 (3)	------	117 (2)
	middle-aged (n=5)	99 (1)	101 (4)	------	------	------	------	111 (2)	115 (1)	117 (2)
	old (n=5)	------	101 (3)	103 (1)	105 (1)	107 (3)	109 (1)	111 (1)	------	------
CSSM22 (208-214, 3)	young (n=5)	208 (2)	212 (7)	214 (1)						
	middle-aged (n=5)	------	212 (9)	214 (1)						

	old (n=5)	208 (1)	212 (6)	214 (3)					
	young (n=5)	234 (3)	236 (2)	242 (2)	244 (1)	246 (2)			
BM1818 (234-246, 5)	middle-aged (n=5)	234 (1)	236 (2)	242 (7)	------	------			
	old (n=5)	234 (2)	236 (1)	242 (4)	244 (3)	------			
	young (n=5)	------	134 (10)	------					
CSSM14 (132-136, 3)	middle-aged (n=5)	------	134 (8)	136 (2)					
	old (n=5)	132 (2)	134 (7)	136 (1)					
	young (n=5)	------	------	112 (2)	118 (2)	------	130 (3)	142 (3)	
Haut14 (106-142, 7)	middle-aged (n=5)	------	108 (3)	------	------	128 (4)	130 (3)	------	
	old (n=5)	106 (4)	108 (4)	------	118 (1)	------	130 (1)	------	
	young (n=5)	142 (2)	144 (1)	146 (2)	------	150 (2)	------	158 (1)	160 (2)
CSSM19 (142-160, 8)	middle-aged (n=5)	------	------	146 (1)	148 (2)	150 (6)	152 (1)	------	------
	old (n=5)	------	144 (2)	146 (2)	------	150 (4)	------	------	160 (2)

	young (n=5)	------	------	217 (1)	219 (4)	------	225 (2)	227 (2)	229 (1)
Cer14 (211-229, 8)	middle-aged (n=5)	------	------	217 (1)	219 (5)	223 (1)	------	227 (3)	------
	old (n=5)	211 (4)	215 (1)	------	------	------	225 (2)	227 (1)	229 (2)
	young (n=5)	------	------	------	284 (2)	------	292 (3)	294 (3)	300 (2)
ILSTS06 (276-300, 8)	middle-aged (n=5)	276 (1)	------	------	------	------	292 (2)	294 (1)	300 (6)
	old (n=5)	276 (2)	280 (1)	282 (1)	------	290 (1)	292 (2)	294 (3)	------

3.5 Discussion

The oldest antlers used in this study have an age of approximately 200 years, making them, to the best of our knowledge, the oldest samples obtained from a museum or a natural history collection for use in microsatellite analyses (Wandeler et al. 2007). DNA content and amplification success of the DNA from the antlers was equal to that of fresh tissue samples. DNA concentrations derived using our unique extraction methods were sufficient for microsatellite analysis (Table 3.1 and 3.2). There was no evidence for a scoring error due to stuttering, allelic dropouts or null alleles for any of the microsatellite loci, including the old antler samples. Overall, this indicates a high quality of extracted DNA (Pompanon et al. 2005) and applicability of microsatellite primers to antlers.

It is well known that DNA extracted from bone is better preserved than DNA from other tissues (Greenwood 1980; Cooper et al. 1992; Greenwood et al. 1999; Schaerlaekens et al. 2011); the PCR method employed in this study made the retrieval of DNA from specimens collected and stored under controlled conditions for the past 200 years straightforward (Hofreiter et al. 2001).

The finding that the DNA isolated from antlers has amplification success equal to that of fresh tissue is astounding and suggests that even older antler samples could be successfully used in genetic analyses. This is likely explained by the careful storage and treatment of trophies. When a deer is shot, the head is removed immediately and cooked to remove all meat from the cranium (only the parts with meat are cooked, not the antlers). The cranium is then blanched and put on a wall as a trophy. The dry, cold and constant environmental conditions for the above ground storage of these wall mounted trophies by hunters/ gatherers is ideal for the preservation of high quality DNA over time.

The astounding quality of the antler DNA samples could be further explained by the fact that antler tissue is dead and desiccated. After the annually renewed antlers have reached their full size, all processes which normally take place in living cells are arrested. A main component leading to the fast degradation of DNA in tissues after death is the breakdown of cellular compartments that normally sequester catabolic

enzymes. As a consequence, the DNA is rapidly degraded by these enzymes, e.g. by lysosomal nucleases (Pääbo et al. 2004).

Not only do our results indicate that the DNA extracted from antlers has a high quality (Figure S 3.3, S 3.4) but also that genotyping of individuals from antlers is reproducible. The genotype error rate was zero (below detection) and from the seven antlers that were shed by the same individual, the same genotype was always obtained (Figure S 3.5).

Our findings suggest that population genetic studies can be easily conducted with antler and muscle tissue. This includes the genetic identification of individuals and paternity analyses of animals with antler tissue, offering a new, easy, fast and cheap approach for the non-invasive sampling of cervid populations. The fact that males shed antlers and form new ones every year allows for the acquisition of large samples without stunning or killing the animals.

Museums and other natural history collections house worldwide millions of antlers from red deer and other cervid specimens (Wandeler et al. 2007). Specimens for the present study were taken from two trophy collections where red deer antlers were stored under conditions typical for such collections (dry, cold, constant, above ground storing (see above)). Hence, the typical storage of trophies provides acceptable conditions to prevent serious DNA degradation in samples up to two hundred years old. Since many samples spanning several generations of a population exists in museums and antler trophy collections, these aggregations enable the genetic constitution of populations to be analysed over time (Taylor et al. 1994).

The optimised method for DNA extraction presented in this paper is a general method for a cheap and quick extraction of high quality nuclear DNA from old museum samples of red deer and of other cervid species (Hoffmann et al. 2014), and may be applicable to other bone material stored under comparable conditions. While most DNA extractions from bones require several days (Mailand & Wasser 2007), our extraction method is completed within 28 hours. The time is saved thanks to the high amount of DNA in antlers, meaning a shortened decalcification time of 24 hours is sufficient. The large quantity of DNA found in antlers meant we could also minimize the amounts of antler material and chemicals used for extraction. While other methods require at least

0.4 – 1.0 g (Burger et al. 2004; Rohland & Hofreiter 2007) of bone material, we only used 0.2 g. Since a high volume of bone material and reaction solutions requires larger tubes and specific laboratory equipment (such as larger centrifuges, vibraxer, heater, speed vacs, etc.), the extraction of DNA in this manner was previous limited. The reduced antler volume required and subsequent use of 2 ml Eppendorf tubes in this study versus 12 ml Sarstedt tubes presented in Burger *et al.* (2004) or 15 ml tubes in Rohland & Hofreither (2007) makes our methods for DNA extraction from antler fragments feasible in any lab equipped for standard population genetic analyses.

4. Species cross-amplification, identification and genetic variation of 17 species of deer (Cervidae) with microsatellite and mitochondrial DNA from antlers

4.1 Abstract

Strong anthropogenic impact has caused 28 of the currently recognized 55 species of deer (Cervidae) to be listed on the IUCN Red List. Particular threats to vulnerable species include habitat deterioration and hybridization with alien, introduced species. The scarcity of many species has severely hampered genetic analyses of their populations, including the detection of loci for cross-species amplification. Because deer antlers are shed and re-grown annually, antlers offer the possibility for non-invasive genetic sampling of large individual numbers and may provide material for reference genotyping from historical samples stored in zoos, museums and trophy collections of rare and extinct species/populations. In this paper, we report cross-species amplification of 19 nuclear microsatellite loci and the amplification of 16S mtDNA for barcoding from nearly a third of all deer species worldwide based on high quality DNA extracted from antler bone up to 40 years old. Phylogenetic analysis based on mtDNA of seventeen species and five subspecies corroborate previously published phylogenetic data, thus confirming the specific resolution of the DNA extraction methodology.

4.2 Introduction

The deer family (*Cervidae*) is distributed throughout the northern hemisphere as well as in South America and southeast Asia (Gilbert et al. 2006). With 55 species recognized by the International Union for the Conservation of Nature (IUCN), the family constitutes the second most species-rich family of artiodactyls after the *Bovidae* (Grubb 1993). Despite their wide distribution, half of all deer species are today red listed by the IUCN as vulnerable, endangered, critically endangered or extinct in the wild. Overexploitation of natural populations (illegal hunting, etc.) and habitat loss have led to the severe population reductions. Furthermore, deer products, especially antlers and antler powder, are widely employed in animal-based traditional Chinese

medicines. Deer antlers are used to treat ailments ranging from anemia and lumbago to impotence (Mainka & Mills 1995). Consequently, antlers in fresh velvet can cost up to $1,000 per kg.

The antlers of the majority of deer species are trophies, and trophy hunting with paying hunting guests is consequently of economic importance. The economic significance of deer also includes their use as managed game animals and for commercial meat and antler production. These practices have led to numerous translocations of deer species worldwide (Galindoleal & Weber 1994). Translocations may have severe consequences for the conservation of indigenous species (Galindoleal & Weber 1994) due to interspecific competition and/ or the introduction of new diseases and parasites (Geist 1988; Geist 1992), both of which may lead to a reduction in the density of native species. Translocated individuals might also hybridize with native populations of the same or closely related species, consequently impacting the native gene pool of each species. For example, introduced Sika deer (*Cervus nippon*) readily interbreed with native red deer (*Cervus elaphus*) in parts of Great Britain to such an extent that no native red deer without some degree of Sika deer ancestry are currently found in these areas (Lower & Gardiner 1975; Senn & Pemberton 2009).

Cervidae are united by a series of synapomorphies, including the possession of antlers in males (Janis & Scott 1987). Antlers are made of a deciduous bony core covered by velvet skin. Antlers are shed annually and regenerate in the following five months from the permanent pedicles (Li & Suttie 2001). The annual shedding and renewing makes antler an ideal material for non-invasive sampling of deer species. Non-invasive sampling allows for investigations into the biology of elusive, rare and/ or endangered species (Piggott & Taylor 2003) without harming and disturbing or even having to observe individuals. It is therefore of special importance in conservation biology (Broquet et al. 2007). In a previous paper, Hoffmann and Griebeler (2013) established an improved method to isolate and analyze high quality DNA from antlers of red deer and they successfully used the DNA for microsatellite genotyping. In the current paper, we show the applicability of this method for the extraction of high quality DNA for

microsatellite and mtDNA genotyping of antlers from about a third of the world's deer species, including species extinct in the wild.

Microsatellites are versatile tools to investigate paternity, kinship and population structure in both captive breeding programs and hybridizations in the wild (Bruford & Wayne 1993; Bruford et al. 1996). When testing intraspecific populations, knowledge of species-specific loci is adequate. However, for between-species studies such as hybridization tests or for phylogenetic inference, cross-species amplifying loci are required (Goodman et al. 1999). Among the many studies using microsatellites to study various species of deer, there are few studies that use the same sets of primers (Bonnet et al. 2002). We therefore used the DNA from the studied deer species to establish a consistent microsatellite marker system with cross-species amplification tests with 19 microsatellite loci previously used in cattle and red deer (Goodman et al. 2001; Poetsch et al. 2001; Gaur et al. 2003; Kuehn et al. 2003). In total, 17 deer species and five subspecies were genotyped. For some of these species (*Cervus albirostris*, *Rusa unicolor*, *Dama mesopotamica*) and subspecies only a small number of polymorphic loci have been described, until now (Bonnet et al. 2002).

We further amplified 550 bp of the mitochondrial 16S rDNA gene to verify the DNA-extraction method's ability to produce high quality DNA for barcoding (species determination) and phylogeny (Cronin et al. 1991). Barcoding entails the screening of one mitochondrial reference gene to assign unknown individuals to a species (Hebert et al. 2003). DNA barcoding, with its unique reproducibility, sequence versatility and comparability among different species, provides a powerful approach for species authentication (Yan et al. 2013) and enables species determination of unknown samples from various sources such as meat, antlers and trophies, and from the powder of antlers. Our objectives in this study were to characterize the mtDNA 16S rDNA and microsatellite DNA variation from non-invasive tissue sampling (antler) in 17 deer taxa for use in species identification, population genetics and phylogenetic analyses.

4.3 Materials and methods

4.3.1 Sampled animals

We conducted cross-species amplification tests with 19 microsatellite loci and the mitochondrial 16SrDNA for 42 individuals, comprising 17 species and five subspecies with DNA extracted from antlers. Because the subjective nature of subspecies designations (Cronin 2006) we defined subspecies as geographic populations with a largely independent phylogeny (Ball & Avise 1992). Most of the antlers originate from the Tierpark Berlin (TB). The origin of all samples and the species' status on the IUCN Red List are given in Table 1. Although there is no Red List status available for subspecies, all subspecies (except for *Cervus canadensis sibiricus*) in this study are endangered; for example, *C. nippon pseudaxis* is extinct in the wild. The threatened status of some of the researched species limited the number of individuals available for our analysis. The number of analysed individuals per species/ subspecies was between one and three (Table 4.1).

Table 4.1 Investigated species and subspecies, IUCN Red List status, number of successfully amplified loci in the present study, numbers in brackets are number of polymorphic loci identified. N = Number of individuals studied per species. Please note: the IUCN Red List does not include information on subspecies. TB = Tierpark Berlin.

Species	**IUCN Status**	**Number of loci**	**origin**	**Distribution area**
Moose (*Alces alces*) (N = 2)	Least concern	14 (9)	Hunting trophy, Sweden	Northern Europe, Northern Asia, North America
Hog Deer (*Axis porcinus*) (N = 1)	Endangered	13 (9)	TB	South- and Southeast Asia
Roe Deer (*Capreolus capreolus*) (N = 2)	Least concern	13 (5)	Hunting Trophy, Germany	Europe, Asia Minor

White-lipped Deer (*Cervus albirostris*) (N = 2)	Vulnerable	16 (12)	TB	Tibet
Wapiti (*Cervus canadensis*) (N = 1)	Least concern	15 (3)	Hunting Trophy Germany	North America, Siberia, Central Asia
Barasingha (*Cervus duvaucelii*) (N = 3)	Vulnerable	16 (10)	TB	Northern India
Red Deer (*Cervus elaphus*) (N = 3)	Least concern	14 (12)	Shed antlers, Austria	Europe, Northern Africa, Southeastern Asia
Fallow Deer (*Dama dama*) (N = 3)	Least concern	14 (2)	Hunting Trophy, Germany	East Asia, Asia Minor
Persian Fallow Deer (*Dama mesopotamica*) (N = 2)	Endangered	16 (2)	TB	Iran, Israel
Pere David´s Deer (*Elaphurus davidianus*) (N = 3)	Extinct in the wild	16 (6)	TB	Extinct in the wild
Mule Deer (*Odocoileus hemionus*) (N = 1)	Least concern	13 (7)	TB	Western Norhamerica
White-tailed Deer (*Odocoileus virginianus*) (N = 2)	Least concern	15 (8)	TB	North America, Middle America, Northern South America
Reindeer (*Rangifer tarandus*) (N = 3)	Least concern	16 (11)	TB	Northern Eurasia, North America

Eld´s Deer (*Rucervus eldii*) (N = 3)	Endangered	15 (8)	TB	Southeast Asia
Javan Rusa (*Rusa timorensis*) (N = 2)	Vulnerable	15 (2)	TB	Java, Bali
Sambar (*Rusa unicolor*) (N = 1)	Vulnerable	15 (6)	TB	Southern Asia
Subspecies				
Cervus canadensis nannodes (N = 2)	No data available	15 (2)	TB	California
Cervus canadensis sibiricus (N = 1)	No data available	13 (6)	TB	Altai-Mountains, Northwest Mongolia
Cervus elaphus bactrianus (N = 2)	No data available	15 (10)	TB	Central Asia
Cervus nippon mantschuricus (N = 1)	No data available	9 (7)	TB	China, Siberia, Japan, Taiwan
Cervus nippon pseudaxis (N = 1)	No data available	16 (7)	TB	Vietnam

4.3.2 DNA Extraction from antlers

DNA was extracted from a small borehole drilled on the backside of antlers following the method presented in Hoffmann and Griebeler (2013). The antler chips were pulverized in a Retsch-mill at a frequency of 30 Hertz for 120 s. The DNA was therefore extracted from powdered antler samples, similar to that used in traditional Chinese medicine. The DNA content of every sample was measured with a NanoDrop1000 (Peqlab, Erlangen, Germany). Before each PCR, every sample was diluted to a final DNA concentration of 50-100 µg/ml (measured with NanoDrop 1000).

4.3.3 Microsatellite Primers, PCR reactions and microsatellite genotyping

For the amplification of the 19 microsatellites we applied two different PCR-based methods. For eleven fluorescently labelled primers, we used the Qiagen Multiplex Kit (Qiagen, Basel, Switzerland) (Haut 14, ILSTS06, BM1818, CSSM14, CSPS115, CSSM16, MM12, Inra35, Cer14, CSSM22 and CSSM19) (all used primers and primer-sequences are listed in Table S 4.3). The reaction volume for each sample consisted of 5 µl Qiagen Multiplex PCR Mastermix, containing dNTPs, QiagenHotStarTaq DNA Polymerase and 6 mM $MgCl_2$, 1 µl Primer Mix (2 µM each Primer), 2.5 µl RNase-free water and 1.5 µl DNA (final DNA concentration of 50-100 µg/ml) resulting in a final reaction volume of 10 µl for each sample. PCRs were performed on a Perkin Elmer GeneAmp 9600 thermocycler using the recommended multiplex-PCR protocol (Qiagen) with the following cycling conditions: 15 min at 95°C for initial denaturation; then 40 cycles of 40 s at 94°C, 90 s at 57°C and 90 s at 72°C, followed by 30 min at 60°C and a final hold at 4°C.

The remaining eight microsatellite primers (NVHRT48, RT1, RT6, IOBT965, BM4107, NVHRT16, Ca38, Ca60) were labelled during the PCR reaction following the method in (Schuelke 2000). Each reaction consisted of 7.7 µl H_2O with Ready to go PCR Beads (GE Healthcare), ~0,035 µl Forward Primer (100 pM), ~0.14 µl Reward Primer (100 pM), ~0.14 µl M13 Tail (HEX, FAM, NED) and 1 µl DNA resulting in a final reaction volume of 9 µl for each sample. To detect the best annealing temperature, we ran gradient PCRs for each primer on a TGradient Biometra Thermocycler (Biometra). Optimal annealing temperatures were: NVHRT48 (53°C), RT1 (51°C), RT6 (51°C), IOBT965 (54°C), BM4107 (55°C), NVHRT16 (53°C), Ca38 and Ca60 (no amplification, see Results). Subsequent PCRs were performed on a Perkin Elmer GeneAmp 9600 thermocycler using a PCR protocol with the following cycling conditions: 5 min at 94°C for initial denaturation; then 30 cycles of 30 s at 94°C, 45 s at 51-55°C and 45 s at 72°C; then 8 cycles of 30 s at 94°C, 45 s at 53°C and 45 s at 72°C followed by 10 min at 72°C and a final hold at 4°C.

Purified microsatellite PCR fragments were run on a 3130xl capillary sequencer (Applied Biosystems) using a GS500 ROX size standard. The genotypes were analysed with GENEMAPPER, version 4.0 (Applied Biosystems). To avoid false allele scoring, we only scored alleles with markedly high fluorescent values and a distinctive, characteristic form at each locus. Samples with ambiguous peaks were assessed as no amplification success. To assess quality and reproducibility of genotypes, we re-extracted DNA and genotyped more than a fourth (n=11) of all individuals twice (from 7 different species). These eleven individuals were amplified across ten loci to calculate the amplification success rate, false allele rate and allelic dropout rate between replications. We did not test for null allele frequencies due to the low number of individuals per species (Table 4.1).

4.3.4 mtDNA Primers, PCR reactions and 16S rRNA genotyping

We amplified the mitochondrial gene 16SrRNA (550bp, positions 2306-2856 in *Bos taurus*) using universal primer pairs (fwd 5 -CGC CTG TTT ATC AAA AAC AT-3 and rev 5 -CTC CGG TTT GAA CTC AGA TC-3) (Guha et al. 2006). Each 25 µl PCR reaction consisted of 21 µL of H_2O, 1 µL of each respective primer (100 pM) and 2 µL of DNA extract in a 0.5 mL PCR tube with illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare). The final volume of 25 µL contained 200 µM of each dNTP, 10mM Tris-HCL (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, approximately 2.5 units of puReTaq DNA polymerase, reaction buffer and the stabilizers BSA, dATP, dCTP, dGTP and dTTP. Thermocycling included 94°C for 1 minute, 30 cycles of 94°C for 30 seconds, 49°C for 30 seconds and extension at 72°C for 45 seconds. The reaction was stored at 4°C after a final extension at 72°C for 10 minutes. Reactions were performed on a TGradient Biometra Thermocycler (Biometra). PCR products were separated using gel electrophoresis (1% Agarose); fragments were controlled for expected size and band quality. The amplified PCR fragments were purified using the Roche High Pure PCR Product Purification Kit (Roche Diagnostics).

The primary aim of the 16S sequence analysis was to demonstrate the versatility of the DNA extraction method. We show that the extracted DNA can be used to produce high quality DNA sequences for barcoding and phylogeny. We amplified a 550bp fragment of the mitochondrial 16S rDNA for all species and subspecies (primer and primer-sequences listed in Table S 4.3; accession numbers in Table S 4.5). For effective DNA barcoding, sequence variation must be high enough among species to be discriminatory, but low enough within a species so that a clear threshold between intra- and inter-specific genetic variations can be defined (Yan et al. 2013). Due to the low number of individuals per species analyzed in this study (Table 4.1), we blasted the 16SmtDNA sequence for each species in GenBank to evaluate intraspecific sequence variation. We found between two and eleven additional sequences per species (Table S 4.1; accession numbers in Table S 4.5). 16S sequence is frequently used for various species for phylogenetic studies and therefore applicable for barcoding because of the high numbers of comparable sequences in molecular databases.

We established a phylogenetic tree based on haplotype sequences and compared the phylogeny to previously published ones (Gilbert et al. 2006). Phylogenetic inference based on the 550bp 16S rDNA mtDNA fragment was done with Neighbor Joining and Maximum Parsimony using the Tamura 3-Parameter ($\alpha = 0.15$) evolution model, which was the best model detected by MEGA version 5 (Tamura et al. 2011). *Bos taurus* (Bovidae) was used as an outgroup (Accession number: HQ184044.1). Each phylogenetic inference was evaluated with 1000 bootstraps. Only bootstrap scores higher than 70% were considered as significant support of branching.

4.4 Results

4.4.1 DNA extraction

DNA was successfully extracted from antlers in moderate to high concentrations from all individuals. DNA concentrations ranged between 31.06 and 1046.23 µg ml^{-1} (mean: 227.92 µg ml^{-1}, standard deviation: 275.93 (Table S 4.2)).

4.4.2 Cross-species amplification of microsatellite loci

Cross-species amplification of microsatellite loci was tested with 19 loci (primer pairs) for all species and subspecies. Of particular interest were species listed in the IUCN Red List and subspecies in danger of extinction, but also species for which only a few microsatellites have been described (*Cervus albirostris*, *Rusa unicolor*, *Dama mesopotamica*). Table 4.2 summarizes amplification success, and the range and number of alleles found for these specific species. A complete list of amplification success, range and number of alleles for all other species/ subspecies is presented in Table S 4.4. The tested primers, which were developed for cattle (Goodman et al. 2001; Poetsch et al. 2001; Gaur et al. 2003; Kuehn et al. 2003;), showed a high amplification success in all species and subspecies. For each of the investigated species/ subspecies, 9 to 16 primer pairs amplified fragments (Table S 4.4). The loci RT1 and RT6 failed to amplify in all species. The number of polymorphic loci ranged from two (*C. nippon mantschuricus*) to 12 (*C. albirostris, C. elaphus*). In total, nine primer-pairs amplified fragments in all examined species (CER14, NVHRT48, RT1, MM12, INRA35, CSSM22, CSSM19, BM1818, CSPS115).

The test for reproducibility of microsatellite genotyping with 11 individuals at ten loci resulted in a false allele rate between replicates of 2.2% and in the dropout or no amplification of one allele in 1.4% of all replicates. Genotypes were analyzable and amplification success in the first replication was at 96.4% across all of the samples.

Table 4.2 Amplification success for threatened species and subspecies. Number of alleles, allele size range and total number of successfully amplified loci; in brackets: number of polymorphic loci; [1](Kuehn et al. 2003); [2](Poetsch et al. 2001); [3](Goodman et al. 2001). Complete list of amplification success for all species in appendix (Table S 4.6).

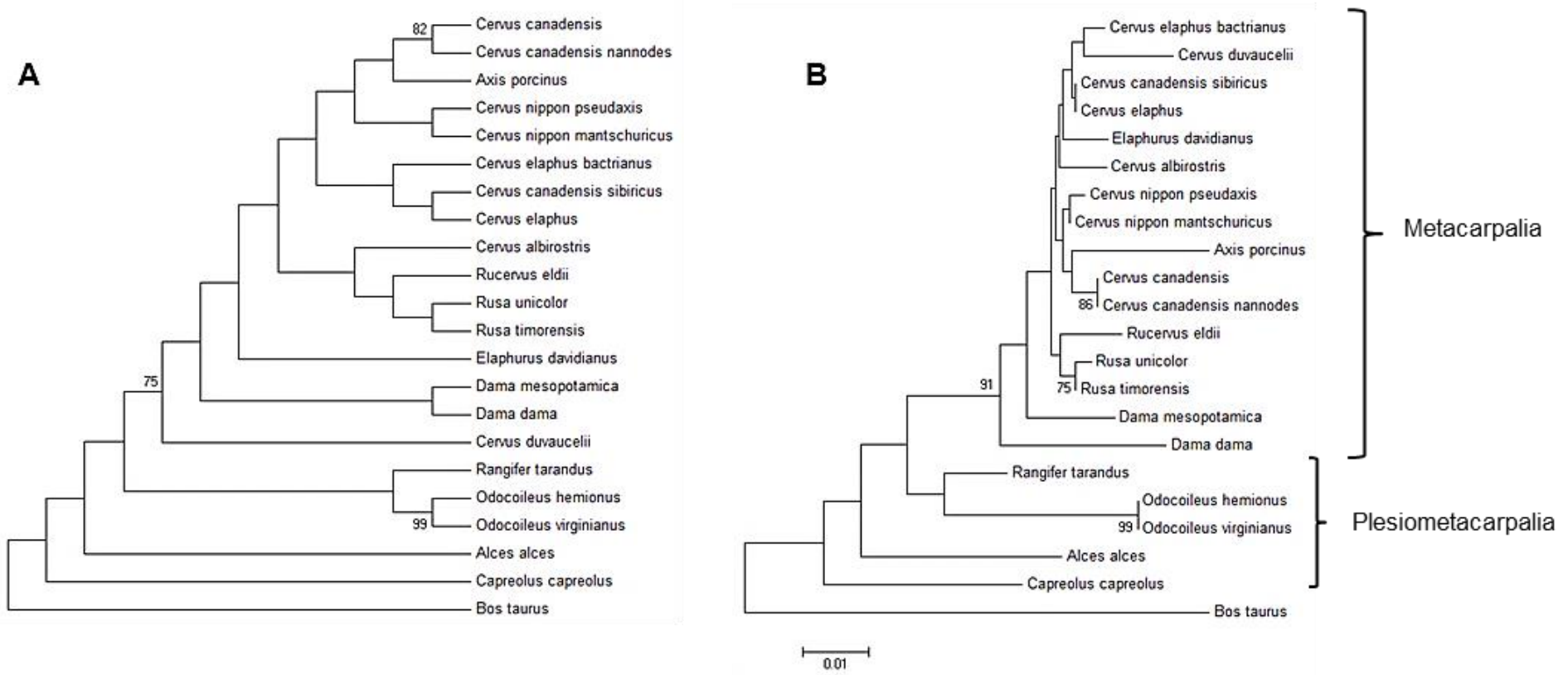
Locusname	***Axis porcinus (N = 2)***	***Cervus albirostris (N = 2)***	***Cervus duvaucelii (N = 3)***	***Dama mesopotamica (N = 2)***	***Elaphurus davidianus (N = 3)***	***Rucervus eldii (N = 3)***	***Cervus elaphus bactrianus (N = 2)***	***Cervus nippon pseudaxis (N = 2)***
	Number of alleles, allele size range	Number of alleles, allele size range	Number of alleles, allele size range	Number of alleles, allele size range	Number of alleles, allele size range	Number of alleles, allele size range	Number of alleles, allele size range	Number of alleles, allele size range
BM1818[1]	2, 243-245	3, 245-251	3, 245-259	1, 231	2, 247-249	2, 247-157	1, 243	1, 237
CSSM14[1]	2, 148-154	2, 122-132	2, 144-146	1, 152	1, 140	No amplification	1, 134	1, 136
CSSM16[1]	No amplification	3, 161-167	3, 155-163	1, 145	2, 161-163	2, 161-169	2, 155-161	2, 159-161
Haut14[1]	2, 118-142	2, 120-126	2, 128-132	1, 106	1, 118	1, 126	2, 120-132	1, 112
ILSTS06[1]	No amplification	2, 283-291	1, 291	1, 283	1, 283	2, 289-291	3, 285-291	2, 287-293
CSPS115[1]	2, 237-239	1, 239	1, 237	1, 245	1, 239	1, 245	1, 237	2, 239-241

MM12[1]	1, 80	1, 84	1, 80	1, 80	1, 82	1, 80	3, 84-88	1, 88
INRA35[1]	2, 116-120	2, 100-104	2, 96-100	1, 92	2, 96-100	2, 96-98	3, 96-114	2, 110-112
CSSM22[1]	1, 213	1, 215	1, 211	1, 213	1, 213	1, 213	1, 211	2, 207-213
CSSM19[1]	1, 155	2, 135-137	4, 155-179	1, 165	1, 147	1, 159	3, 139-155	2, 147-151
CER14[1]	2, 217-223	2, 233-235	3, 201-211	2, 221-229	3, 213-229	2, 210-212	3, 215-231	1, 223
NVHRT48[2]	2, 107-123	2, 128-140	1, 96	1, 98	1, 107	1, 96	No amplification	1, 109
RT1[2]	2, 224-230	1, 221	1, 221	2, 227-233	1, 235	1, 224	2, 233-237	1, 231
RT6[2]	2, 110-115	3, 113-123	5, 115-133	1, 113	2, 108-114	2, 122-134	3, 113-121	2, 111-115
IOBT965[1]	1, 100	4, 120-130	2, 133-135	1, 132	2, 136-140	2, 122-132	2, 129-131	1, 120
BM4107[3]	No amplification	2, 168-176	2, 164-166	1, 172	2, 172-178	3, 170-186	1, 178	No amplification
NVHRT16[2]	No amplification	No amplification	No amplification	No amplification	No amplification	No amplification	No amplification	1, 177
Number of loci	13 (9)	16 (12)	16 (10)	16 (2)	16 (7)	15 (8)	15 (10)	16 (7)

4.4.3 16SrDNA

The mtDNA 16SrRNA fragment (550bp) was successfully amplified in all individuals from DNA extracted from antlers (Table S 4.5). To compare specific/ subspecific sequence diversity for barcoding, we searched GenBank (search date 29.05.2014) and compared the nucleotide diversity for each species with our samples (number of samples per species and intraspecific variation in Supplementary Table 1). Sequence diversities here mean the % of the nucleotides that differ between two sequences. The mean intraspecific haplotype variation across all species, including blasted sequences from GenBank, was 0.6% across the 550 nucleotides. Between species, the genetic variation ranged from 0% (*Odocoileus hemionus*, *O.virginianus*) to 6.8%. Except for *O. hemionus* and *O. virginianus*, no species shared the same haplotype sequence. To further evaluate the reproducibility and phylogenetic accuracy of the 16S sequence data, we calculated phylogenetic trees applying the Neighbor Joining and Maximum Parsimony method (Figure 4.1). The phylogenetic trees are concordant with known phylogenies of *Cervidae*, though the strict monophyly of Plesiometacarpalia and Metacarpalia is not shown.

Figure 4.1 Neighbor Joining (A) and Maximum Parsimony (B) phylogenetic trees of cervid species based on 16SrDNA. Only bootstrap values higher than 70% are shown.



4.5 Discussion

We successfully extracted nuclear and mitochondrial DNA of high quality from antlers of nearly half of the world's cervid species and we used the DNA to establish a microsatellite marker system and 16SmtDNA barcoding inventory. While the extraction method was previously limited to the successful amplification of red deer nuclear DNA (Hoffmann & Griebeler 2013), here we provide proof of the method's applicability to study a wide range of deer species worldwide for conservation and other scientific interests. The astoundingly high concentration and quality of the DNA of all deer species studied can be explained by the almost perfect storage of the antlers as trophies. The fact that antler tissue is desiccated before antlers are shed (Hoffmann & Griebeler 2013) protects the DNA from breakdown into cellular compartments and degradation by catabolic enzymes, e.g. lysosomal nucleases (Pääbo et al. 2004). Easy sampling and the small amounts of bone tissue (0.2 g) needed for analyses makes our method readily applicable for fast and efficient sampling and genotyping of numerous individuals, deer products, antlers, trophies and powder, as well as hunted or poached individuals. No expensive or special equipment is needed for sampling. In addition, the small borehole drilled on the backside of trophies is not visible, which is important when sampling from rare or valuable trophies.

Antlers in this study were between two and 40 years old. For this reason, we cannot attest to DNA preservation >4 decades in each species, as evidenced in red deer antlers up to 200 years old (Hoffmann & Griebeler 2013). Nevertheless, the high structural similarly of cervid antlers indicates that DNA preservation will be equally good in all other species. The high quality of DNA in antlers, preserved for at least 200 years, offers the opportunity for non-invasive genotyping of rare species over time, reconstructing histories of extinct populations and translocations of deer worldwide, without observing individuals (Piggott & Taylor 2003; Broquet et al. 2007). Non-invasive techniques are of great conservation concern and are now routinely used; for instance, feces is used to monitor brown bears in North America (Woods et al. 1999; Paetkau 2003). For cervid populations in captive breeding programmes with or without

complete breeding books, the determination of kinship from old shed antlers or trophies is possible.

In our effort to validate the quality and applicability of DNA extracted from antlers as a reliable tool for non-invasive genotyping, we established a microsatellite marker system for different cervid species/ subspecies. The 19 microsatellite loci were originally developed for cattle and some have already been used individually in cervids. For *Cervus albirostris*, *Rusa unicolor* and *Dama mesopotamica* only a few polymorphic microsatellites were described until now (Bonnet et al. 2002). We found between 9 and 16 primer-pairs that amplified between 2 and 12 polymorphic loci for each of these species. While the number of polymorphic loci was not formerly sufficient for population genetic studies, we could increase the number of applicable polymorphic primers for these threatened species. For the remaining species, for which microsatellite loci are already known, our new marker system identified loci/ primers for inter- and intraspecific studies, including conservation and forensic studies (Felmer et al. 2008; Teletchea et al. 2008). We are well aware that testing 1-3 individuals per species does not capture the genetic diversity of a species or the diagnostic value of specific alleles among closely related species. The low sample size underestimates the number of polymorphic loci, which might also be due to inbreeding in captivity. We therefore can expect that the number of polymorphic loci per species is a lower bound. With heterologous microsatellites, issues with amplification and consistency of scoring might not be apparent until many individuals are scored. Due to low sample size we were also unable to address potential null alleles. There was indeed a high ratio of homozygotes (and monomorphic loci) in the sample. However, based on low sample sizes it is impossible to rule out whether there are technical reasons for this phenomenon (false alleles, bovine origin of primers) or biological/ genetic reasons like null alleles and inbreeding, or if some of the loci are less polymorphic in general. We further assessed the non-invasive genotyping potential of the sampling method based on its ability to produce high quality sequence data for, e.g., barcoding and phylogeny. DNA barcoding must have a low threshold and high efficiency to make it a sensitive method for the identification of different species (Meyer & Paulay 2005) and animal

derived compounds. MtDNA provides this opportunity and was therefore used for cervid species identification (Cronin et al. 1991). Accurate species identification requires a comprehensive comparative molecular database for the assignment of an unknown individual (Meyer & Paulay 2005). In our study, amplification of the 16S gene produced unambiguous sequences with low mean intra-specific sequence diversities (<0.1%; under 0.8% of the nucleotides differ between two sequences when including sequences found in GenBank), whereas inter-specific diversity was much higher (mean 3.3%). In accordance with another study (Cronin et al. 1991), only the two common species *O. hemionus* and *O. virginianus* could not be differentiated because they had identical haplotypes.

The phylogenetic tree calculated with 16S (Figure 4.1) confirmed that 1) individuals of the same species cluster together, while individuals of different species are discriminated and 2) that cervid species in this study are mostly divided in two major clades, Plesiometacarpalia and Metacarpalia, as found in previous studies (Pitra et al. 2004; Gilbert et al. 2006). The fact that the strict monophyletic origin of these two subfamilies is not shown (Odocoileus and Rangifer occur in the large clade with Metacarpalia in the Maximum Parsimony Tree), as also seen in the paraphyletic mtDNA phylogeny within *Odocoileus* (Cronin et al. 1988), reflects the limitations of single gene trees for phylogeny inference. Nevertheless, for our limited analysis of only 550 bp, results are good and we thus conclude that the 16S fragment is efficient in identifying (barcoding) specific species of deer because of high inter-specific and low intra-specific variance; the latter is also evidenced in a larger sample of red deer individuals from different geographical regions in central Europe (Germany and Austria) (GS Hoffmann, unpublished results).

In summary, the extraction method in combination with the different markers used offers a versatile tool for not only species determination, forensic analyses and assessing trafficking of rare species but also is for non-invasive population genetic and conservation studies. A further advantage of the method is that is possible to discriminate species from antler powders, such which are normally used in traditional

Chinese medicine. All DNA samples used in this study were extracted from powdered antler samples (see Materials and Methods).

5. Population dynamics of a natural red deer population over 200 years detected via substantial changes of genetic variation

5.1 Abstract

Most large mammals have constantly been exposed to anthropogenic influence over decades or even centuries. Because of their long generation times and lack of sampling material, inferences of past population genetic dynamics, including anthropogenic impacts, have only relied on the analysis of the structure of extant populations. Here we investigate for the first time the change in the genetic constitution of a natural red deer population over two centuries, using up to 200 years old antlers (30 generations) stored in trophy collections. To the best of our knowledge, this is the oldest DNA source ever used for microsatellite population genetic analyses. We demonstrate that government policy and hunting laws may have strong impacts on populations that can lead to unexpectedly rapid changes in the genetic constitution of a large mammal population. A high ancestral individual polymorphism seen in an out-breeding population (1813-1861) was strongly reduced in descendants (1923-1940) during the mid-19th and early 20th century by genetic bottlenecks. Today (2011), individual polymorphism and variance among individuals is increasing in a constant sized (managed) population. Differentiation was high among periods (FST > ***); consequently, assignment tests assigned individuals to their own period with > 85% probability. In contrast to the high variance observed at nuclear microsatellite loci, mtDNA (D-loop) was monomorphic through time suggesting that male immigration dominates the genetic evolution in this population.

5.2 Introduction

Evolution is change in genepools over time. While long-term evolutionary processes have been studied by analyzing temporal patterns in fossil records (Wandeler et al. 2007) and footprints of selection (Gautier & Naves 2011; Hansen et al. 2010), the study

of micro-evolutionary processes has often been limited to organisms with short generation times of at most a few weeks (Balanya et al. 2006). With the advent of molecular genetic approaches, natural history collections (NHC) have become a new important source of tissue material for population genetic studies, opening up for studies comparing historical and contemporary natural populations of species with long generation times of several years (Wandeler et al. 2007). Using historical samples, anthropogenic influence on the genepool of populations has been documented for migration (gene flow) in the African wild dog (Roy et al. 1994), on population sizes in Greater prairie chicken (Bouzat et al. 1998) and introgression in fish populations due to stocking (Ciborowski et al. 2007). Although NHC offers various possibilities for retrospective analyses of evolution, there are still limitations. Beside DNA degradation through time, the availability of museum samples from a specific geographical region is often scarce, but essential when assessing genetic and demographic impacts on specific populations.

Like most other large mammal species, European red deer (*Cervus elaphus* L.) has for decades or even centuries been exposed to anthropogenic pressures, affecting its genetic structure (Hartl et al. 2003). As an important game animal and meat supplier, population sizes have fluctuated highly depending on government policies, hunting laws and translocations (Fernandez-Garcia et al. 2014). In recent decades, rigorous hunting schedules have favored large, branched antlers by protection of these individuals from harvesting until they could reproduce several times (Hartl et al. 2003), likely causing strong selection pressure on this trait (Hartl et al. 1991; Hartl et al. 1995) . Additionally, significant damages to forestry and agricultural plants have initiated large-scale eradication of red deer populations. Several population genetic studies have shown significant differentiation among artificially separated red deer populations (Hartl et al. 1990; Schreiber et al. 1994), inbreeding depression in isolated populations (Zachos et al. 2007), and have found evidence for translocations (Frantz et al. 2006). All studies conducted so far on red deer have examined extant populations using muscle tissue (Coulson et al. 1998; Zachos & Hartl 2011), thus making retrospective inferences on evolutionary processes.

In the present study, we use DNA from up to 200 years old antlers from a trophy collection to analyze the temporal evolution of a confined red deer (*Cervus elaphus* L.) population. All samples derive from a small geographic area (<10 km^2), which is easily daily covered by red deer, thus representing a locality-based population. We analyzed the change in the genetic constitution of this population over time by sampling antlers from three periods (1813-1861, 1923-1940, 2011), and evaluating ten microsatellite loci. To detect possible translocations of foreign female individuals into the population, we additionally sequenced an 800 bp fragment of the highly variable mitochondrial control region. Microsatellite loci commonly used in genetic studies utilize DNA extracted from muscle tissue (Kuehn et al. 2004), and have successfully been applied to old antler tissue (Hoffmann & Griebeler 2013). Our study reveals dramatic changes in the population's genepool over time for microsatellite loci, but none for the control region. To the best of our knowledge, our study is based on the oldest microsatellites ever used for a population study.

5.3 Materials and methods

5.3.1 Study population

A total of 31 individuals were genotyped through time in a population at Neuwied, Germany (Table S 5.1). The individuals originated from three periods: 1813-1861 (N=6), 1923-1940 (N=8), and 2011 (N=17). In the following text, we refer to these three subsamples as the Old, Middle-aged and Young population, respectively. As samples in population Old and Middle-aged originate from a time period, these two populations represent the average of genetic diversity during these periods rather than instant populations.

5.3.2 Population history

In the period 1813 to 1861 the Neuwied population was large. Although samples derived from the private hunting area of the princes of Neuwied from an area of only 10 km^2 there were other red deer populations around with few barriers to migration and a low degree of fragmentation in an overall continuous habitat. During the German

revolution years 1848 to 1849 no hunting laws were in action. This short period led to massive reductions in red deer population sizes and large-scale extinctions of red deer populations due to poaching and unregulated harvesting. During the second half of the 19th century, hunting laws were reenacted which allowed populations to re-establish in many areas (Kuehn et al. 2003). After re-establishment, population sizes were reduced again due to increasing damages to agriculture, but also poaching during and after World War 1. Our second population samples come from a period representing the end-phase of overexploitation (1923-1940). Following the 1950's, red deer populations expanded into many forested areas. For the first time rigorous hunting schedules favoring large, branched antlers were introduced (Hartl et al. 2003). This schedule mostly spares males with large antlers from being hunted during their reproductive years. Although migration into the population from other areas is possible, the contemporary Neuwied population is more fragmented than in former times. It is confined in the south by the river Rhine, and in the west and east it is fenced in by motorways and highways. Migration and gene flow is presumably highly restricted in this population (further information about population history and study area in Chapter S 5.1 in supplemental material).

5.3.3 DNA preparation

The DNA of individuals from the Old (N=6) and Middle-aged populations (N=8) was extracted from a small borehole drilled on the backside of antlers, following the method of Hoffmann and Griebeler (2013). As only males produce antlers in red deer, all sampled individuals were males. The samples of population Young were randomly taken from hunted individuals (N=17). DNA was extracted from muscle tissue from both sexes (11 females, 6 males) with the 'High pure PCR template preparation Kit' (Roche Diagnostics) using the standard protocol recommended by the manufacturer. All DNA samples were stored at -20 °C. The DNA content of every sample was measured with NanoDrop1000 (Peqlab, Erlangen, Germany). The DNA for PCR was diluted in elution buffer to a final concentration of 50-100 µg/ml.

5.3.4 Microsatellite laboratory procedure

We analyzed 10 microsatellite loci with dinucleotide repeat motifs: Ilsts06, CSPS115, MM12, CSSM16, Haut14, Inra35, CSSM22, CSSM19, CSSM14, BM1818 (Kuehn et al. 2004). All primers were developed for cattle (*Bos primigenius tauris*) but were identified by Kuehn et al. (2004) as applicable to red deer. DNA extracted from antler and muscle tissue was treated identically. The loci were amplified in two multiplex reactions (Table S2) using the Qiagen Multiplex Kit (Qiagen, Basel, Switzerland). The reaction volume for each sample consisted of 5 µl Quiagen Multiplex PCR Mastermix (contains dNTPs, Qiagen HotStar Taq DNA Polymerase and 6 mM MgCl2), 1 µl Primer Mix (2 µM of each Primer), 2.5 µl RNase-free water and 1.5 µl DNA; the final reaction volume was 10 µl for each DNA sample. PCRs were performed on a PerkinElmer GeneAmp 9600 thermocycler using the recommended multiplex-PCR protocol (Qiagen) with the following cycling conditions: 15 min at 95 °C for initial denaturation; then 40 cycles of 40 s at 94 °C, 90 s at 57 °C and 90 s at 72 °C, followed by 30 min at 60 °C and a final hold at 4 °C.

Purified microsatellite PCR fragments were run on a 3130xl capillary sequencer (Applied Biosystems) using a GS500 ROX size standard. The genotypes were analysed with GENEMAPPER, version 4.0 (Applied Biosystems). To avoid false allele scoring, we only scored alleles with fluorescent values above 3000 and a distinctive, characteristic form at each locus. Samples with ambiguous peaks were assessed as no amplification success. In total 60% of all individuals were genotyped twice, after independent PCRs, to ensure reproducibility of genotypes. Null alleles, large allele dropouts or scoring errors due to stuttering were checked with the MicroChecker software (Version 2.2.3) (Van Oosterhout et al. 2004).

5.3.5 MtDNA laboratory procedure

To examine female-based genetic structure, we sequenced the highly variable mtDNA control region (D-loop). The control region has previously been used in a biogeographical study of red deer in Europe to assign foreign haplotypes (Niedzialkowska et al. 2011). The control region was first amplified with self-made

primers (Table S 5.3), which amplify the sequence between Pro-tRNA and Phe-tRNA (800 base pairs). In case of partial amplification success, we re-sequenced the control region in step-wise amplifications with newly designed specific forward and reverse primers (Table S 5.3).

Each 25 µl PCR reaction consisted of 21 µL of H_2O, 1 µL of each respective primer and 2 µL of DNA extract contained in a 0.5mL PCR tube with illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare). The final volume of 25 µL contained 200 µM of each dNTP, 10mM Tris-HCL (pH 9.0, at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, approximately 2.5 units of puReTaq DNA polymerase, reaction buffer and BSA, dATP, dCTP, dGTP and dTTP. Thermocycling started at 95°C for 5 minutes, followed by 40 cycles of 94°C for 75 seconds, then by 60°C for 90 seconds and then by an extension at 75°C for 75 seconds. After a final extension at 72°C for 7 minutes, the reaction was stored at 4°C. Reactions were performed on a TGradient Biometra Thermocycler (Biometra). PCR products were separated using gel electrophoresis (1% Agarose) and fragments controlled for expected size and band quality. The amplified PCR fragments were purified using the Roche High Pure PCR Product Purification Kit (Roche Diagnostics).

MtDNA amplification products were sequenced in both directions with BigDye chemistries on a 3130xl capillary sequencer (Applied Biosystems). Sequence data was aligned and edited with BioEdit (Version 7.1.3).

5.3.6 Population genetics analyses of microsatellites

The genetic analyses were based on three genetic indices providing assessments of within-population dynamics: 1) genetic diversity—including the assessment of the magnitude of genetic drift, 2) inbreeding estimates, and 3) variance of heterozygosity among individuals within periods. While a reduction in genetic diversity is indicative of genetic drift and bottlenecks, estimates of inbreeding are more difficult to assess. Assuming no selection on heterozygotes, negative Fis values may be caused by mating success of foreign individuals or when the individuals who make up the population sample are siblings (Rasmussen 1979; Johannesen & Lubin 1999), while positive Fis values implicate any sort of Wahlund effect (Johnson & Black 1984), which

is a deficiency of heterozygotes compared to Hardy-Weinberg expectations. Variance differences in individual heterozygosity give implications for the stability of the population/breeding system. Low variance is evidence for even breeding success and ancestry, whereas high variance indicates perturbations from equilibrium. The magnitude of change should be manifested in the differentiation among time samples. We first tested for linkage disequilibrium in time samples to assure independency among microsatellite loci (GenePop on the web, Version 4.2, (Rousset 2008). Because of different sample sizes in the three periods, we do not calculate absolute number of alleles per locus, but effective number of alleles in relation to sample size (allelic richness). The effective number of alleles per locus and effective number of private alleles per sample were calculated with HpRare (Version 1.1) (Kalinowski 2005) applying the Rarefaction method. Expected and observed heterozygosities, as well as inbreeding estimates F_{is} and F_{st}, as a measure of variation of allele frequencies, were calculated with Arlequin (Version 3.5.1.2) (Excoffier et al. 2005). To assess the likelihood that an individual belongs to a particular time sample, we used the Bayesian assignment method as implemented in GeneClass (Version 2.0) (Piry et al. 2004). In this test, we excluded each individual to be assigned from the reference population. The magnitude of genetic drift was estimated with BottleSim (Kuo & Janzen 2003) (assumed parameters: random mating, longevity of organism 14yrs, sexual maturity at age of 4, population size pre-bottleneck: 100, post-bottleneck: 40, sex ratio: 1:1, number of iterations: 50) and compared to the observed data. In this analysis, we assessed the level of genetic drift expected in samples Middle-aged and Young based on the observed heterozygosity of the respective previous sample. We estimated two drift scenarios: 1) drift based on heterozygosity in the original population (1813-1861), and 2) drift based on observed heterozygosity in the second sampling period (1923-1940). The model parameter generation time was set as the midpoint of the respective periods. Finally, t-tests (Excel 2010) for samples with homogeneity and also for inhomogeneity of variance as implemented in Excel 2010 were applied to test for differences between heterozygote loci/individual among periods. F-tests of inhomogeneity of variance between samples were also conducted with Excel 2010.

5.3.7 MtDNA analysis

The mtDNA control region sequences were aligned with Mega (Version 5.1) (Tamura et al. 2011). Because only a single haplotype was found (see results) no time-scale analysis was done. Nevertheless, we compared this haplotype to those found in other geographical regions of Europe in a GenBank search.

5.4 Results

5.4.1 DNA extraction and quality

High quality DNA was extracted from all antlers in moderate to high concentrations (44 - 370µg ml^{-1}). Both microsatellites and mtDNA amplified unambiguous peaks with high fluorescent values. Microsatellite amplification success rate was 97.9 %. DNA quality was verified by genotyping 60% of all individuals twice, which yielded 100% concordance (for a complete table of microsatellite genotypes see table S 5.4). The test for null alleles revealed no evidence for null alleles, large dropouts or false alleles due to stuttering (MicroChecker 2.2.3).

5.4.2 Linkage disequilibrium

The tests for pairwise linkage disequilibrium between microsatellite loci revealed three significant values ($p<0.05$) out of 135 comparisons. This frequency is lower than expected by chance (5% level). Thus, these results indicate independent inheritance of the studied loci.

5.4.3 Temporal population genetic indices

When correcting for differences in sample sizes, population Old had the highest effective number of alleles (3.99), followed by population Young (3.49), whereas population Middle-aged had the lowest number (2.71). Consistent with the differences in effective allele numbers, the expected heterozygosity was similar in population Old (H_e = 0.68) and young (H_e = 0.60), but lower in population Middle-aged (H_e = 0.51). Inbreeding estimates F_{is} were significantly positive in the population Middle-aged

(0.148), whereas they were significantly negative in population Young (-0.039) and Old (-0.158) (Figure 5.3).

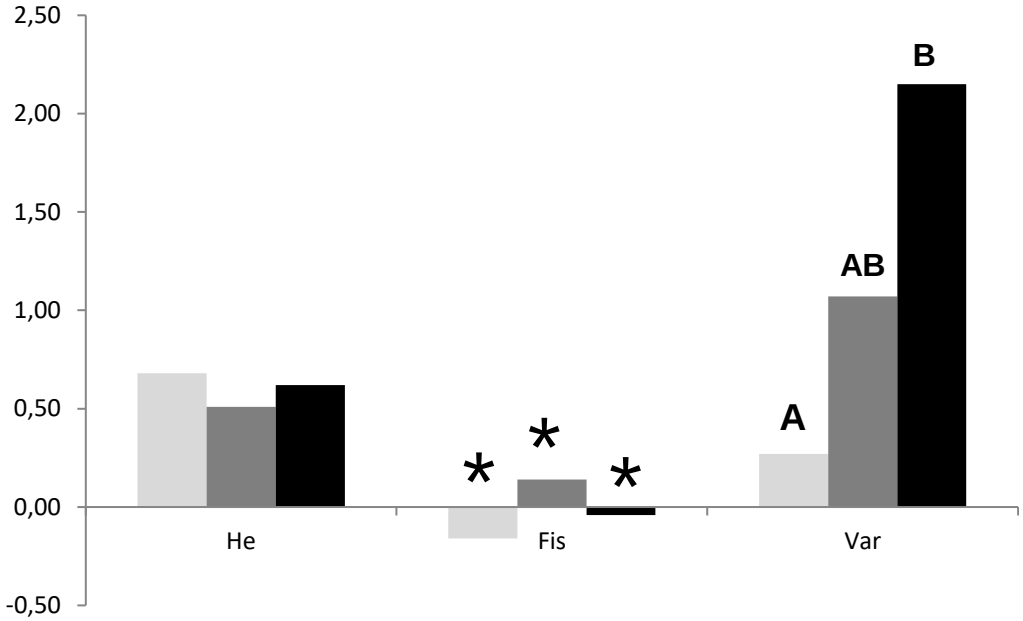


Figure 5.3 Summary of relative estimates of diversity (expected heterozygosity of the time sample, He), inbreeding coefficient of the time sample (F_{is}) and variance among individuals within samples in number of heterozygote loci). Stars (*) indicate estimates significantly different from zero. Different letters show significant differences in the variance in number of heterozygote loci/individual based on 10 microsatellite loci. Different shades of gray mean year of sampling, light gray = 1837, gray = 1930, black = 2011.

The number of heterozygote loci per individual differed significantly between all pairwise comparisons of population samples (periods) (Old vs. Middle-aged: df = 12, t value = -6.295, $p < 10^{-4}$; Middle-aged vs. Young: df = 23, t value = 2.463, p = 0.011; Old vs. Young: df = 21, t value = -3.602, p < 0.001). The average number of heterozygote loci per individual was 7.67 ± 0.52 SD in population Old, 4.75 ± 1.04 SD in population Middle-aged and 6.18 ± 1.47 in population Young (Table 5.1). Variance in individual (i.e. observed) heterozygosity per period increased significantly through time. Variance inhomogeneity was only significant between population Old and Young (df = 16, F = 8.08, p = 0.015; Old vs. Middle-aged: df = 7, F = 4.018, p = 0.072; Middle-aged vs. Young: df = 16, F = 2.011, p = 0.177).

Table 5.1 Genetic diversity estimates for three time-period populations. Number of sampled animals (N), absolute number of alleles per locus (n_a), effective number of alleles per locus (n_e), effective number of private alleles per population (p_e), expected (H_e) and observed (H_o) heterozygosities in studied populations over all loci, mean number of heterozygote loci/individual (N het) and variance of heterozygote loci/ individual (Var).

Population	**N**	**n_a**	**n_e**	**p_e**	**H_e**	**H_o**	**N het**	**Var**
Young	17	4.6	3.49	0.67	0.60	0.62	6.18	2.15
Middle-aged	8	3.0	2.71	0.27	0.51	0.42	4.75	1.07
Old	6	4.2	3.99	1.11	0.68	0.78	7.67	0.27

5.4.4 Genetic drift simulation

In the bottlenecked Middle-aged population, we detected shifts in allele frequencies at most loci (Table S 5.5). As seen in Figure 5.1, the overall proportion of heterozygote loci increased in population Young, but not in each of the individuals.

Genetic diversity in Middle-aged is reduced and lower than expected through drift based on population Old. In contrast, diversity is higher in population Young than predicted by drift based on population Middle-aged (Figure 5.2).

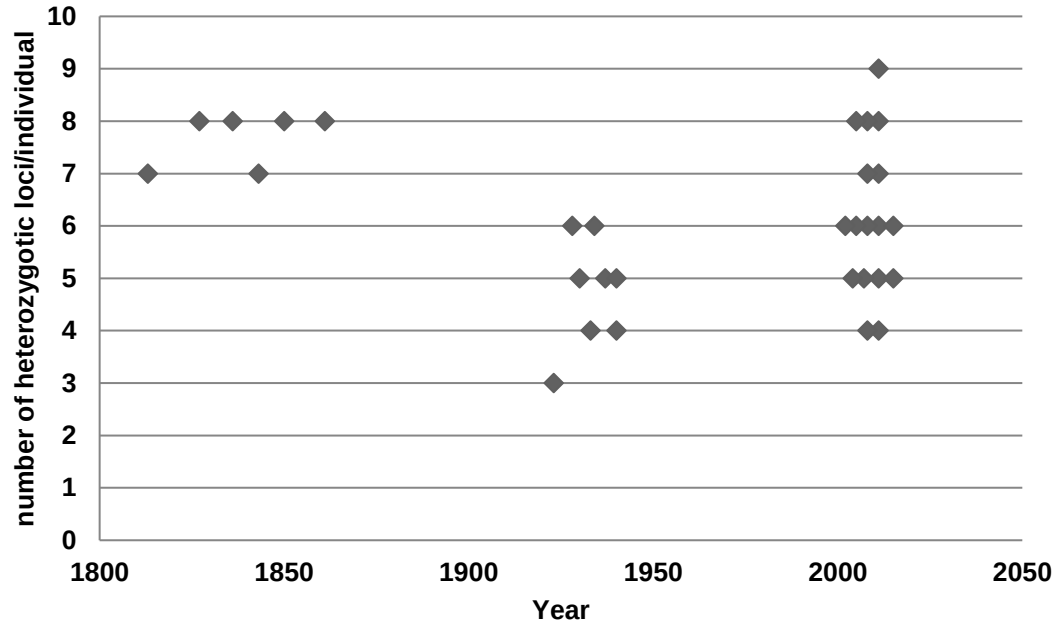


Figure 5.1 Number of heterozygotic loci per individual in the different time periods/years. Although all individuals were sampled in 2011, the multi-year presentation of the young sample is chosen for convenience to demonstrate variability between individuals.

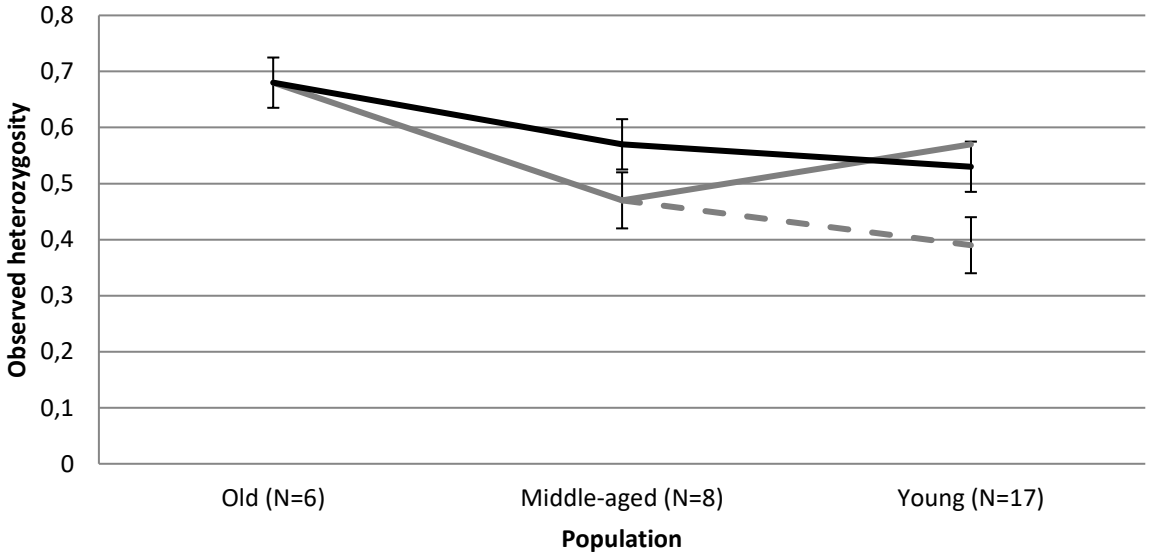


Figure 5.2 Observed heterozygosity of the time samples and genetic drift simulated in BottleSim (Kuo & Janzen 2003) (gray full line = observed heterozygosity, black full line = simulated heterozygosity pre-bottleneck, dotted line = simulated heterozygosity post-bottleneck, error bars = standard error).

5.4.5 Assignment and genetic differentiation among periods

Genetic differentiation among populations from the three periods was significant with moderate to high F_{ST} values (population Young vs. Old: F_{ST} = 0.06; Middle-aged vs. Young: F_{ST} =0.11; Middle-aged vs. Old: F_{ST} = 0.09). The assignment test conducted with multilocus microsatellite genotypes assigned each individual from a time sample with a probability > 0.84 to this sample.

5.4.6 MtDNA analysis

All 31 individuals sampled shared an identical mtDNA haplotype (656 bp, Genbank accession number: KU577263), making a detection of introgression of foreign haplotypes into the population impossible. The haplotype is new, i.e. it is not recorded in Genbank (assessed 05.01.2016). The most similar haplotype found in Genbank was J729E with 99 % similarity (655 out of 656 bp).

5.5 Discussion

Inferences of anthropogenic impacts on natural populations of mammals with long generation times have so far relied on retrospective analyses of contemporary populations. In this study, we analysed the genetic evolution of a single red deer population sampled at three periods over two centuries. Assuming a generation time (mean distance between two generations) of 7 years (Coulson et al. 1998), successive population samples were separated by 11 up to 17 generations. During this time, the population experienced substantial genetic changes at the ten nuclear microsatellite loci analysed, while sequence variation in the normally, highly variable mitochondrial D-loop was absent. The historically high nuclear genetic diversity was followed by a diversity decrease and a subsequent diversity increase. This pattern is consistent with our understanding of hunting pressures during the two centuries (see Population history in Material and methods).

Inferences of population dynamics were obtained by time-based analyses, which provide three basic indices for assessing within-population dynamics: 1) genetic

diversity (H_e), 2) inbreeding estimates (F_{IS}), and 3) variance in individual heterozygosity within periods. The relative levels of these indices among the three samples provide evidence for different structuring processes through time that ultimately led to a substantial change in the genetic constitution of the population over two centuries. We are aware that for the historical samples these indices are only rough estimates. They are based on small population samples and temporal sampling within periods. Nevertheless, individuals within samples were genetically strongly associated (high assignment probabilities) and scored consistently for diversity and variance estimates. For population Old, the relative indices high diversity (H_e = 0.68), negative inbreeding (F_{IS} = -0.158), and low variance in heterozygote loci (0.52) indicate a relative large and stable population. The negative inbreeding estimate was not caused by sampling siblings, but rather evidences immigration into the population or results from differences in mating success of individuals throughout the sampled period. By contrast, in population Middle-aged the relative indices were low diversity (H_e = 0.51), high inbreeding (F_{IS} = 0.148), and intermediate variance in heterozygote loci (1.04). These H_e and F_{IS} values clearly indicate a bottleneck in the population, which is also supported by our genetic drift simulations and the low estimated effective population size. Additionally, the variance in individual heterozygosity increases in the Middle-aged population relative to the Old sample. Interestingly, this drift effect is directly seen at several loci (Table S 5.5), where high frequency alleles increase and low frequency alleles become rarer or even disappear (Nei et al. 1975). The indices for the Young population are an intermediate diversity (H_e= 0.62), no inbreeding (F_{IS} = 0.04), and a high variance in heterozygote loci (1.47). This sample differs from the two historical ones by being sampled at one point in time. Nevertheless, the rapid increase in diversity compared to the Middle-aged population together with the by far highest variance in number of heterozygote loci suggests re-immigration and an admixture of individuals with different pedigree ancestries. Because the studied loci are not linked and there is no evidence for selection on these loci, it is very unlikely that individuals of “different genetic quality” are responsible for the diversity differences among periods. Moreover, all sample material from population Old and Middle-aged originated from trophies and thus represent high quality males despite a significant decrease in

genetic diversity. Likewise, the increase in diversity between Middle-aged samples (trophies) and Young (random samples) is contradictive to quality-linked differences.

Because the mtDNA D-Loop was monomorphic through time, immigration and mating success is most likely dominated by males. For population Old, the identification of a single haplotype was unexpected due to its large estimated effective population size. Compared to other geographical regions, where the D-loop of red deer is highly variable (Ludt et al. 2004; Niedzialkowska et al. 2011), the haplotype in our study is new, and indicates that the female population has been confined through time. Limited immigration by females as a consequence of strong philopatry, as seen in females (Fickel et al. 2012), could have restricted gene flow in the red deer of Neuwied. Limited immigration is common among many polygynous deer species (Nelson 1993; Pitra et al. 2004), as proven by both genetic and telemetry studies (Nussey et al. 2005; Skog et al. 2009). Lack of mtDNA sequence variation further provides evidence against translocations of females in the past decades. In other red deer populations with a history of translocation events, foreign haplotypes have been identified (Skog et al. 2009).

Gender biased gene flow is common in mammals (Kalz et al. 2006; Handley & Perrin 2007). Male-biased gene flow is predicted by inbreeding avoidance theory as well as by local mate competition theory to occur in species with polygynous mating systems, such as in red deer (Cluttonbrock 1989; Perrin & Mazalov 1999, Perrin & Mazalov 2000). In highly mobile red deer, population reductions allow individuals from foreign populations to immigrate in order to search for mating opportunities and thereby introduce foreign alleles. That male red deer contribute more to the genetic diversity of offspring than females (Perez-Gonzalez et al. 2009) further demonstrates the immense impact hunting laws can have on red deer population structure. Males are coveted hunting prey and the culling of dominant males may lead to immigration and reproduction success of individuals from other populations. Skewed mating success very quickly leads to a reduction in genetic variability, especially in areas without hunting laws, when few males remain after several rutting seasons. Also, the rigorous hunting schedules in the last decades which protect individuals with large, branched

antlers from harvesting (Hartl et al. 2003), could have imposed selection on particular males with small antlers prior to sexual maturity. This in combination with the polygynous mating system could also alter the frequencies of alleles within a few generations. The rapid changes observed in allele frequencies over a few generations in our study population highlight the endangerment of small populations exposed to overhunting and habitat fragmentation, but also that red deer populations are able to regenerate and increase population size and genetic diversity quickly within a few generations.

6. A quite complete genotypic and phenotypic turnover in an evolutionary short timespan due to translocation events in a natural red deer population

6.1 Introduction

Large mammals are exposed to strong anthropogenic pressure in the agro-cultivated environment. Red deer (*Cervus elaphus* L.) have for centuries been subjected to a variety of measures that change population characteristics, including exhaustive, illegal hunting, translocations (intentional release of animals to the wild, (Griffith et al. 1989), and stocking (Hartl et al. 2003; Frantz et al. 2006).

All population genetic studies conducted so far on red deer have examined extant populations by using muscle tissue for genotyping of individuals. They detected significant differentiation among artificially separated populations (Hartl et al. 1990; Schreiber et al. 1994) or inbreeding depression in isolated populations (Zachos et al. 2007). So far it was only possible to map differences between extant populations but not to reconstruct and understand changes in the genetic constitution of populations due to anthropogenic impacts. While there is often genetic evidence for translocation events into continuous populations (Frantz et al. 2006), we are mostly unable to figure out whether these translocations indeed altered allele diversity and frequencies of populations in the past or whether introduced individuals had reproductive success. With the advent of new molecular genetic approaches, we are now able to extract high quality DNA from a wide range of tissues enabling us to use old samples from museums or collections as DNA source (Wandeler et al. 2007; Hoffmann & Griebeler 2013; Hoffmann et al. 2016).

Utilizing temporal sampling to genotype populations at different time periods in the past allows a comparison of and distinction between historical and extant processes that might shaped the genetic constitution of populations (Pertoldi et al. 2001; Flagstad et al. 2003). The patterns of temporal variation in the genetic constitution of populations is therefore important in several ecological and population genetic contexts, as well as in species conservation (Hedmark & Ellegren 2005).

In this study, we investigated a red deer population by comparing today's genetic constitution of this population with that around 250 years ago and we related the change in genetic constitution to altered morphometric traits of antlers. While old tissue samples often come from different sampling sites or are of unknown geographical origin (Nielsen et al. 1999; Heath et al. 2002) we had temporal population samples from the same geographical origin. With oldest samples being up to 270 years old we here present the oldest microsatellite genotyping ever conducted from natural history collections (Wandeler et al. 2007). Because of the importance of red deer as game animals, there is a known history of stocking and translocation over the last six decades done in order to improve the trophy quality in our studied population. Using different genetic markers (mtDNA and microsatellites), we addressed questions about changes in the genetic constitution of this red deer population over time and also addressed the question if translocated individuals had really reproductive success and thus caused the expected high turnover and change in allele frequencies due to stocking and translocation. To figure out whether there was indeed an improvement in trophy quality (larger, heavier antlers, more branches) during the last centuries, we recorded morphometric data on antlers. If so, did phenotypic changes in antlers coincide with changes in genotypic composition of the population?

6.2 Materials and methods

Thirty-three individuals were analyzed genetically from the red deer population Bad Berleburg Castle, Germany. DNA from twelve individuals dating from 1750 to 1769 was extracted from trophies. These individuals represent the native red deer population. DNA from the remaining 21 individuals representing the present-day population was extracted from shed antlers of young stags being between two and three years old. As the last known translocation event occurred in the early eighties, which is more than one generation in red deer, the contemporary animals are not translocated individuals. Antler morphology was compared between 12 ancient and 12 contemporary Individuals.

6.2.1 Population history

In the period 1750 to 1769 the Bad Berleburg population was large. During the years 1848 to 1849 new hunting laws led to reductions in red deer population sizes. During the second half of the 19th century, hunting laws changed again and allowed the population to re-grow (Kuehn et al. 2003). Following the 1960's, single male and female individuals were translocated into the continuous population. Today's population density of red deer in the area is around 300 individuals.

6.2.2 Antler Measurements

We studied phenotypic differences between twelve ancient and twelve randomly selected contemporary red deer used for DNA analysis. We therefore used six morphometric antler traits being potentially related to the individual's fitness (Kodric-Brown & Brown 1984; Kruuk et al. 2002): 1) the total length of the main beam, 2) the total number of spikes (any tine longer than 2 cm), 3) the length and 4) width of the brow tine, and the 5) length and 6) width of the trey tine (Figure S 6.1). The width of the brow tine and trey tine was measured with a digital caliper from the front, narrow side of the antler.

To avoid an age-based bias caused by increasing antler sizes, the age of individuals in the ancient population was estimated and compared to similar-aged individuals from the contemporary Bad Berleburg Castle population. As the ancient individuals (trophies) were at least seven years and older, all measurements of the recent population were taken from fifth or sixth cast set of antlers of the 12 contemporary samples. Analysis of the collected data was carried out using IBM SPSS Statistics (Version 22). All values, except the length of the brow tine and number of spikes were normally distributed in a Kolmogorov-Smirnov test of normality (p-value > 0.05). Normally distributed antler traits were tested for significance of equality of means of the ancient and contemporary population using a t-test for independent samples. Non-normally distributed traits were analyzed using the Mann-Whitney U-Test for independent samples.

6.2.3 DNA Extraction from antlers

DNA was extracted from a small borehole drilled on the backside of antlers, following the method of (Hoffmann & Griebeler 2013) and further purified with the 'High pure PCR template preparation Kit' (Roche Diagnostics) using the standard protocol recommended by the manufacturer. All DNA samples were finally stored at -20 °C.

6.2.4 Microsatellite laboratory procedure

We analyzed 10 microsatellite loci with dinucleotide repeat motifs: Ilsts06, CSPS115, MM12, CSSM16, Haut14, Inra35, CSSM22, CSSM19, CSSM14, BM1818 (Kuehn et al. 2004). All primers were developed for cattle (*Bos primigenius tauris*) but were identified by Kuehn et al. (2004) as applicable to red deer. DNA extracted from antler and muscle tissue was treated identically. The loci were amplified in two multiplex reactions (Table S2) using the Qiagen Multiplex Kit (Qiagen, Basel, Switzerland). The reaction volume for each sample consisted of 5 µl Quiagen Multiplex PCR Mastermix (contains dNTPs, Qiagen HotStar Taq DNA Polymerase and 6 mM MgCl2), 1 µl Primer Mix (2 µM of each Primer), 2.5 µl RNase-free water and 1.5 µl DNA; the final reaction volume was 10 µl for each DNA sample. PCRs were performed on a PerkinElmer GeneAmp 9600 thermocycler using the recommended multiplex-PCR protocol (Qiagen) with the following cycling conditions: 15 min at 95 °C for initial denaturation; then 40 cycles of 40 s at 94 °C, 90 s at 57 °C and 90 s at 72 °C, followed by 30 min at 60 °C and a final hold at 4 °C.

Purified microsatellite PCR fragments were run on a 3130xl capillary sequencer (Applied Biosystems) using a GS500 ROX size standard. The genotypes were analysed with GENEMAPPER, version 4.0 (Applied Biosystems). To avoid false allele scoring, we only scored alleles with fluorescent values above 3000 and a distinctive, characteristic form at each locus. Samples with ambiguous peaks were assessed as no amplification success. In total, 60% of all individuals were genotyped twice, after independent PCRs, to ensure reproducibility of genotypes. Null alleles, large allele dropouts or scoring errors due to stuttering were checked with the MicroChecker software (Version 2.2.3) (Van Oosterhout et al. 2004).

6.2.5 mtDNA: MtDNA laboratory procedure

To examine female-based genetic structure, we sequenced the highly variable mtDNA control region (D-loop). The control region has previously been used in a biogeographical study of red deer in Europe to assign foreign haplotypes (Niedzialkowska et al. 2011; Skog et al. 2009). The control region was first amplified with self-made primers (Table S 6.1), which amplify the sequence between Pro-tRNA and Phe-tRNA (800 base pairs). In case of partial amplification success, we re-sequenced the control region in step-wise amplifications with newly designed specific forward and reverse primers (Table S 6.1).

Each 25 µl PCR reaction consisted of 21 µL of H_2O, 1 µL of each respective primer and 2 µL of DNA extract contained in a 0.5mL PCR tube with illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare). The final volume of 25 µL contained 200 µM of each dNTP, 10mM Tris-HCL (pH 9.0, at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, approximately 2.5 units of puReTaq DNA polymerase, reaction buffer and BSA, dATP, dCTP, dGTP and dTTP. Thermocycling started at 95°C for 5 minutes, followed by 40 cycles of 94°C for 75 seconds, then by 60°C for 90 seconds and then by an extension at 75°C for 75 seconds. After a final extension at 72°C for 7 minutes, the reaction was stored at 4°C. Reactions were performed on a TGradient Biometra Thermocycler (Biometra). PCR products were separated using gel electrophoresis (1% Agarose) and fragments controlled for expected size and band quality. The amplified PCR fragments were purified using the Roche High Pure PCR Product Purification Kit (Roche Diagnostics).

MtDNA amplification products were sequenced in both directions with BigDye chemistries on a 3130xl capillary sequencer (Applied Biosystems). Sequence data was aligned and edited with Geneious (Version 9.0.2).

6.2.6 Population genetics analyses

Microsatellites.

We first tested for linkage disequilibrium in pooled temporal population samples to assure independency among microsatellite loci (GenePop on the web, Version 4.2, Rousset 2008). The effective number of alleles per locus and effective number of private alleles per sample were calculated with HpRare (Version 1.1) (Kalinowski 2005) applying the Rarefaction method. Expected and observed heterozygosities, as well as pairwise F_{st} were calculated with Arlequin (Version 3.5.1.2) (Excoffier et al. 2005).

mtDNA.

The finalized mtDNA sequence data obtained from individuals was imported into Mega (version 5.2) (Tamura et al. 2011) and aligned using the ClustalW function. Relationships among haplotypes were visualized in a Minimum evolution tree based on 1000 Bootstrap replications using the Kimura 2-Parameter model. All DNA samples dating from 1750 to 1769 are hereafter labeled as ‘ancient’ and represent the native haplotype(s) in the population. To determine the origin of potentially translocated red deer, contemporary (hereafter present-day) sequences that did not cluster with the ancient haplotype(s) were compared against the NCBI database of assembled reference sequences using a nucleotide Blast search.

As mentioned above, both females and males were released in this area during the last decades. Because of maternal inheritance of mitochondrial DNA, D-Loop sequence data allows us to directly detect descendants and therefore reproductive success of introduced females. In the absence of an adequate marker to detect paternal inheritance, we tried to resolve reproductive success of introduced males using assignment tests for microsatellite data. We assessed the likelihood that an individual belongs to a particular population, using the Bayesian assignment method as implemented in the software GeneClass (Version 2.0) (Piry et al. 2004). Our assumed populations for the assignment tests were the two main groups found in the phylogenetic tree (Figure 6.1), our main indicator for individuals being translocated in

the recent population. For this test, we excluded each individual to be assigned from the respective reference population.

6.3 Results

6.3.1 Antler Measurements

Of the six fitness relevant traits, three differed significantly between ancient and present-day individuals: the mean width of the trey, and of brow tines, and the overall length of the antlers (Table 6.1). The present-day antlers were on average 15.40 cm longer than antlers from ancient individuals of comparable age. This overall length increase significantly correlated with 14.06 cm increase in the mean width of the brow tine (N = 24, r = 0.0800, P < 0.05), where the antler connects to the skull of the red deer and with an increase in mean width of trey tine (N = 24, r = 3,754, P< 0.05).

Table 6.1 The mean value and standard deviation of the six fitness characteristics measured on 12 ancient and 12 present-day Bad Berleburg antlers. * indicates significant p-values < 0.05.

Bad Berleburg	**Mean number of spikes**	**Mean overall length [cm]**	**Mean length of brow tine [cm]**	**Mean length of trey tine [cm]**	**Mean width of brow tine [cm]**	**Mean width of trey tine [cm]**
Ancient *N* = 12	14 ± 2	81.933 ± 8.169	35.658 ± 7.786	32.625 ± 5.935	46.016 ± 5.680	37.925 ± 3.861
Present-day *N* = 12	16 ± 2	97.333 ± 8.544	37.441 ± 3.468	35.458 ± 4.974	60.075 ± 6.619	42.650 ± 3.413
Significance (2-tailed)	0.179 (U-Test)	<0.001* (t-Test)	0.476 (U-Test)	0.218 (t-Test)	0.003* (t-Test)	<0.001* (t-Test)

In addition to the six phenotypic fitness characteristics of antlers measured, the standardized heterozygosity of each individual was calculated from microsatellite data in order to check for a correlation between standardized heterozygosity and each antler trait (Table 6.1). However, mean and variance in heterozygosity did not significantly

differ between present-day and ancient individuals. The Pearson correlation analysis also rejected a relationship between standardized heterozygosity and any of the antler traits measured. This observation further supports the neutrality and linkage equilibrium of the ten microsatellite loci used in this study.

6.3.2 Microsatellites.

Linkage and null alleles

In a global test for linkage disequilibrium across all individuals studied that used probability tests with Markov chain parameters (1000 dememorizations, 100 batches and 1000 iterations per batch), twelve significant values ($p < 0.05$; Fisher's method) were identified. In a local test (ancient, present-day group), all significant linkage values could be attributed to present-day Bad Berleburg individuals. To test for null alleles, the expected and observed heterozygosity was calculated with Levene's correction. Within the Bad Berleburg population, we identified two loci with potential null alleles: CSSM16 in the present-day group and INRA35 in the ancient group. In a global test for heterozygote deficiency across the ancient and present-day population, only CSSM16 showed a significant deficit ($p < 0.05$). To control for these results, five loci were re-sequenced in 16 individuals. Additional ten samples (approximately 30% of the data set) were randomly chosen and blindly re-genotyped to ensure the accuracy of our results.

The mean number of alleles per locus and allelic richness varied only slightly between the present-day and ancient population. Mean number of alleles per locus was 6.000 in the individuals of the present-day population, and 6.300 in the ancient population. Allelic richness was 4.040 in present-day individuals and 4.380 in ancient individuals. Private allelic richness was 1.380 and 1.720, respectively. Neither the present-day nor ancient group deviated from HWE ($P = 0.217$ and $P = 0.399$).

Although the Bad Berleburg population did not deviate from HWE, the significant $F_{ST} = 0.071$ (p-value > 0.050) indicates moderate genetic differentiation between present-

day and ancient individuals. This value is supported by the population specific F_{IS} indices of >0.001 for present-day and 0.019 for ancient individuals.

6.3.3 mtDNA

The D-Loop of the mtDNA was successfully sequenced in all present-day DNA samples. A ClustalW alignment of the 32 mtDNA sequences revealed six haplotypes, with a total of 27 single nucleotide polymorphisms (SNP). The majority of these SNPs are located within a 200 bp range in the middle of the sequence alignment.

The relationships among single trimmed sequences to each other were visualized in a Minimum Evolution Tree (Figure 6.1). Only two present-day individuals clustered with an ancient red deer haplotype (individual new 9 and 17). Five more present-day individuals are in the same clade, but form a own subgroup. Remaining 13 present-day individuals and also the remaining ancient individuals form four separated clades. When BLASTed against the NCBI database of assembled reference sequences, haplotypes of eight individuals (01, 02, 10, 12, 13, 14, 15, 19) from “Bad Berleburg new” (Figure 1) matched with 99% identities (725/726, 1 gap) the Danish H4 haplotype from Jægersborg Dyrehave (GenBank accession number: EU544182; Nielsen *et al.* 2008). “BBnew” individuals 7, 8, 11, 16, 18 (Figure 6.1) matched with 100% identity the Danish H3 haplotype (GenBank accession number: EU544181; Nielsen et al. 2008). The paper attributes this haplotypes to a modern mixture of Danish and introduced red deer from south-eastern Europe. Individuals BBnew 3, 4, 5, 6 matched with two gaps to hippe 9 haplotype (GenBank accession number: KP859321; Lorenzini et al. 2015). All other individuals in “Bad Berleburg New“ and all samples of “Bad Berleburg old” have no identical sequences in the GenBank or show at least two mutations compared to all other sequences deposited in this database.

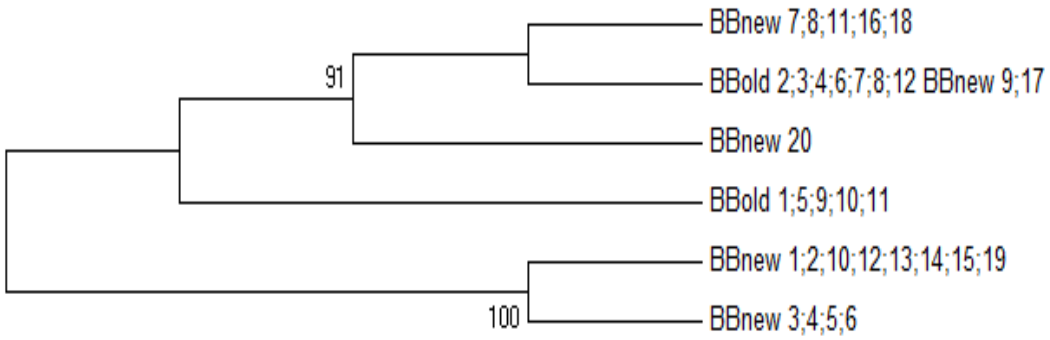


Figure 6.1 Minimum Evolution Tree on the Bad Berleburg population. Cluster based on D-Loop sequences (mtDNA) trimmed to a length of 658 bp. Only bootstrap values larger than 70 of 100 replicates are displayed. BBnew are present-day samples, BBold are ancient samples.

6.3.4 Assignment

To identify potential ancestors of present-day individuals we inspected their microsatellite genotypes and mtDNA haplotypes. We assumed that a high assignment probability to population “new” (>75%) and the presence of a Danish mtDNA haplotype highly indicates that the individual is a descendant of an introduced male and female. A Danish haplotype and a high assignment probability of the individual to the population “old” points to that it descends from a introduced female and a native male. Finally, a haplotype from the population “old” and a high assignment probability to this population indicates that it is a descendant of two native individuals. Based on these criteria we identified one individual being a descendant of the native population, whereas all others were descendants of introduced individuals (Figure 6.2).

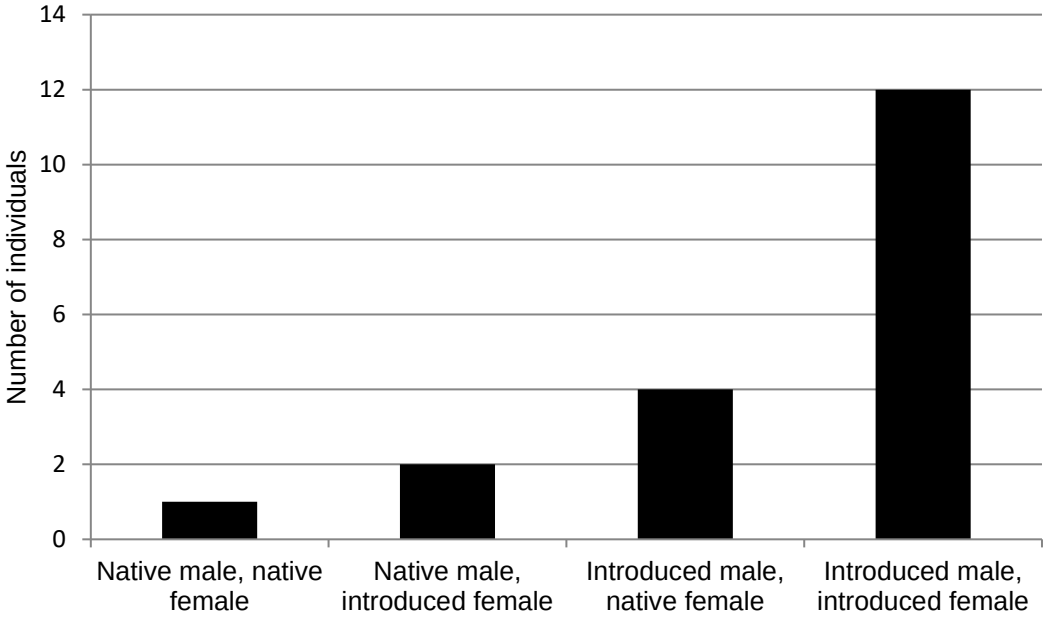


Figure 6.2 Presumed ancestry of tested individuals from the present-day Bad Berleburg population derived from mtDNA and microsatellite data.

6.4 Discussion

6.4.1 Antler size

Antler size was significantly higher in the present-day population than in the ancient population from Bad Berleburg. Especially, in game animals human harvesting can impose strong selection pressures on particular traits and cause changes over shorter time than expected from natural selection (Coltman et al. 2003; Garel et al. 2007; Rivrud et al. 2013). It is often discussed whether harvesting the largest trophies in a population causes a decrease in trophy size as an evolutionary response (Coltman et al. 2003; Garel et al. 2007). Contrary, harvesting of animals with the largest antlers could cause a counteracting selection pressure by removing young males of apparent low quality and leading to increased reproductive success of males with large antler size (Mysterud & Bischof 2010). As antler size is heritable, although with a narrow-sense heritability of 0.327±0.12 (Kruuk et al. 2002), our morphometric antler data would strongly support the second hypothesis.

In fact, we have a relatively poor understanding of the heritability of secondary sexual traits in free living populations, particularly in mammals (Kruuk et al. 2002). Though there is selection and heritability of antler size, there is no evidence for evolutionary response in antler size over decades (Kruuk et al. 2002). If directional selection would act consistently on any heritable trait, we should see a continuous change in the distribution of this trait over time (Falconer et al. 1996; Kruuk et al. 2002) as seen under artificial selection in agricultural breeding schemes or laboratory studies (Kruuk et al. 2002). Despite this, numerous studies on wild animals have reported apparent directional selection on heritable traits without any corresponding response to selection (Larsson et al. 1998; Milner et al. 1999; Przybylo et al. 2000; Kruuk et al. 2001). The problem here is that traits can be highly determined by environmental covariances, for example the individual's healthiness or nutritional state.

In our study antler size increased significantly between sampling periods. Microsatellite and mtDNA sequence data indicate a high genetic turnover between sampling periods in the Bad Berleburg population that is caused by translocation of animals into the native population. Field observations on this population point to increasing antler sizes after these translocation events. Other studies have in fact recorded an abrupt increase in antler size between the years 1958 and 1974 (Rivrud et al. 2013), the main period in which translocations of red deer took place in central Europe. However, these authors did not notice that translocations could drive the increase in antler size, and only tested for changes in management and environmental fluctuations. A further reason for the abrupt increase in antler size observed by these authors could also be a kind of outbreeding enhancement due to outcrossing (translocated and native individuals), i.e. a heterosis effect. There could be also a bias in the morphometric data recorded by these authors. In today's well managed hunting areas translocation events were common in the past and therefore it is very likely that authors measured antlers of translocated individuals.

6.4.2 Translocation

Genetic data indicates that 18 out of 19 individuals investigated of the present-day population are descendants of at least an introduced male or female (Figure 6.2). Twelve out of 19 are descendants of both introduced male and female. As the number of individuals introduced to the ancient population was low (exact number unknown) in comparison to its total size, this indicates a high reproductive success of introduced individuals, and especially of males (16 out of 19). Thus, reproductive isolation of translocated individuals and mate choice of native individuals against introduced individuals can be excluded. If it would be an intended re – introduction, from a nature conservation point of view, translocation was successful.

Astoundingly, past translocations, some generations ago, with high reproductive success of introduced individuals, were quite undetectable when only using microsatellite data. Neither a test for Hardy Weinberg equilibrium (Wahlund effect), nor assignment tests or private alleles indicated translocated individuals. The fast genetic turnover after translocations took place must have altered allele frequencies in a few generations, leading to a significant F_{st} of 0.071 between the ancient and present-day population.

Time evolution takes is relative as it depends on fitness differences. Captive breeding under constant conditions and a systematic selection towards particular traits is known to lead to a fast-evolutionary change in trait values. Quite ideal living conditions for red deer in well managed hunting areas, allow hunters to exert selection pressures comparable to those seen in captive breeding programs that decrease phenotypic plasticity of selected traits. In former times refuges and geneflow may have buffered the effect of selective hunting (Tenhumberg et al. 2004). With increasing exploitation of landscapes and improvement in technology and mobility of hunters, we are now perhaps enabled to select on particular animal traits in larger areas and on quite all individuals in a population.

7. Retrospective analyses of an isolated red deer (Cervus elaphus) population using old antler samples

Ich habe in meiner Arbeit zwei weitere Rothirsch-Populationen deren zeitliche Entwicklung bekannt ist untersucht. Allerdings konnte ich aus Zeitmangel kein fertiges Manuskript für meine Dissertation erstellen, möchte aber dennoch diese beiden Studien hier kurz vorstellen. Die Publikation der Ergebnisse zu den beiden Populationen ist in einer wissenschaftlichen Zeitschrift als „short communication“ geplant.

7.1 Die Population in Hasselbusch

Populationen mit geringer effektiver Populationsgröße sind besonders gefährdet durch eine Kombination aus Drift und Inzucht ihre genetische Diversität zu verlieren. Die durch diesen zufälligen Verlust oder aber durch die Paarung von Individuen mit Allelen desselben Vorfahrens auftretende erhöhte Homozygosität in den Populationen kann zu einer verringerten Fitness von Individuen führen (Hartl & Pucek 1994; Frankham et al. 2002). Starke Reduktionen in der Populationsgröße können neben natürlichen Ursachen, wie z.B. Katastrophen, auch anthropogenen Ursprungs sein, beispielsweise durch Übernutzung von Populationen oder auch durch Habitatfragmentierung, die bis zur vollständigen Isolation von Populationen führen kann. Bei freilebenden Populationen großer und mobiler Arten wurden solche negativen Konsequenzen starker Populationsschwankungen bisher allerdings selten nachgewiesen. Eine Ausnahme bildet die Rothirsch-Population Hasselbusch in Deutschland (Schleswig-Holstein). Bei dieser Population wurden sowohl eine verringerte genetische Diversität als auch morphologische Deformationen festgestellt (Brachyognathie), die auf Inzucht zurückgeführt werden (Zachos et al. 2007). Diese Population wurde um 1870 von 6-8 Individuen gegründet. Da die 2007 von Zachos et al. durchgeführte Studie Muskelgewebe als Probenmaterial verwendete, konnte sie nur die gegenwärtige Zusammensetzung ihres Genpools beschreiben und keinerlei Aussagen über die genetische Veränderung in dieser Population über die Zeit treffen. Um eine retrospektive Betrachtung der genetischen Konstitution und ihres zeitlichen Verlaufs

zu ermöglichen, habe ich 54 Geweihproben von Tieren, die zwischen 1903 und 2011 in diesem Gebiet erlegt oder gefunden wurden, genommen. Neben der Mikrosatellitenanalyse von zehn polymorphen Loci habe ich zusätzlich mitochondriale DNA sequenziert (D-Loop), um gegebenenfalls Genfluss und auch Translokationen in die Population detektieren zu können.

7.2 Vorläufige Ergebnisse und Diskussion

Sowohl die Extraktion als auch die Auswertung mitochondrialer und genomischer DNA der Geweihproben verlief erfolgreich.

Die untersuchte D-Loop Sequenz konnte erfolgreich bei 24 von den 55 Individuen amplifiziert und analysiert werden. Insgesamt habe ich nur zwei verschiedene Haplotypen gefunden, die zufällig verteilt über den gesamten Beobachtungszeitraum auftauchten. Die geringe Anzahl der Haplotypen bestätigt, dass die hier untersuchte Population nicht durch Translokationen in der Vergangenheit beeinflusst wurde und decken sich somit mit den Aussagen der ortsansässigen Jäger.

Um zu überprüfen, ob es durch Drift und Inzuchtpaarungen tatsächlich zu einem Verlust von genetischer Diversität über die Zeit gekommen ist, wurden 50 der beprobten Individuen in 4 verschiedene Zeitperioden eingeteilt (Tabelle 7.1). Aufgrund der niedrigeren Stichprobenanzahl von nur 4 Individuen in der am weitesten zurückliegenden Periode (1895-1908) wurde sie beim Vergleich nicht berücksichtigt. Meine ersten Ergebnisse zeigen eine Abnahme der beobachteten und erwarteten Heterozygosität über die Zeit. Die dargestellten F_{st}-Werte beziehen sich auf den Vergleich jeder hypothetischen Population zur darauffolgenden im nächsten Zeitraum (1 zu 2, 2 zu 3 usw.).

Tabelle 7.1 Gezeigt sind die Anzahl der Individuen pro Population, Lebenszeitraum der beprobten Individuen, die beobachtete (H_o) und erwartete Heterozygosität (H_e), F_{st}-Werte und Abweichungen vom Hardy-Weinberg-Gleichgewicht (p-Werte).

Population	Zeitraum	Ho	He	Fst-Wert	Hardy-Weinberg
1 (N=13)	1922-1935	0,754	0,738	0,0357	0,0017
2 (N=13)	1938-1964	0,628	0,598	0,0322	0,0306
3 (N=12)	1972-1984	0,471	0,547	0,0556	0,0158
4 (N=12)	1988-2008	0,480	0,541	0,0733	0,2651

8. Die Population in Oettingen

Die Rothirsch Population Öttingen (Bayern) ist wie die Population in Haselbusch und viele andere Populationen in ganz Deutschland in der Revolution 1848-1849 ausgerottet worden. Bis heute konnten sich, aufgrund intensiver landwirtschaftlicher Nutzung, keine Populationen mehr in diesem Gebiet etablieren. Von Trophäen, die im Schloss der Fürsten zu Oettingen-Spielberg ausgestellt sind, habe ich Proben von 25 Individuen dieser ausgestorbenen Population entnommen. Interessant ist die Tatsache, dass diese Population, obwohl sie sich kurz vor der Ausrottung befand, keinerlei Anzeichen einer verringerten Diversität durch Drift oder Inzucht zeigt. Im Vergleich mit anderen großen, stabilen Rothirsch-Populationen zeigt sie im Gegenteil hohe Werte für alle Diversitäts-abhängigen Indizes und befindet sich auch im Hardy-Weinberg-Gleichgewicht, vermutlich da genetische Marker nicht in der Geschwindigkeit reagieren, in der der Mensch in diesem Fall die Populationsgröße verringert hat. Die Untersuchung dieser Population beweist eindrucksvoll, dass nicht nur der genetische Zustand über das Überleben von Populationen entscheidet,

sondern dass vielmehr die Gesetzeslage und die politische Situation, völlig unabhängig von Populationsgröße und der Habitatqualität über ihren Fortbestand mitbestimmen.

9. Analyse 1000 Jahre alter Geweihproben aus Haithabu

9.1 Einleitung

Haithabu war eine bedeutende Siedlung dänischer Wikinger. Die frühmittelalterliche Stadt lag auf der kimbrischen Halbinsel am Ende der Schlei, ein Meeresarm der Ostsee (heute ein Teil der Gemeinde Busdorf in Schleswig-Holstein). Von ihrer Gründung im Jahre 770 bis zu ihrer Zerstörung im 11ten Jahrhundert war Haithabu einer der bedeutendsten Handelsplätze für den westlichen Ostseeraum. Ein wichtiges Handelsgut waren Abwurfstangen und Geweihe von Rothirschen, die aufgrund ihrer Härte und guten Bearbeitbarkeit zur Herstellung von Werkzeug, Klingen und Spitzen und auch als Kämme genutzt wurden. Während die Stangen der Geweihe in der Regel vollständig zur Fertigung von Gegenständen genutzt wurden, blieben die Rosen, die wulstartigen Verdickungen an der Stangenbasis als Abfallprodukt erhalten. Diese Rosen wurden in großer Anzahl von den Handwerkern ins Wasser geworfen und schließlich bei archäologischen Grabungen wiedergefunden. Der Erhaltungszustand dieser Geweihteile ist enorm gut, was die Frage aufgeworfen hat, ob diese Rosen nicht eine ideale DNA-Quelle darstellen, um populationsgenetische Studien durchzuführen.

Zur Erprobung der Machbarkeit wurden mir vom Museum Schloss Gottorf fünf Exemplare solcher Rosen zur Verfügung gestellt. Mit einem Alter von ca. 1000 Jahren wären sie mit Abstand die ältesten Gewebeteile bisher, aus denen erfolgreich Kern-DNA zur Mikrosatellitenanalyse extrahiert werden konnte (Wandeler 2007).

9.2 Material und Methoden

Da DNA relativ schnell über die Zeit degradiert, stehen bei alten Proben meist sehr geringe Mengen Ziel-DNA zur Verfügung und die Chance der Kontamination und auch der Verfälschung durch rezentes Material steigt stark an. Aus diesem Grund habe ich

für die Extraktion von DNA aus dem Probenmaterial von Haithabu einen separaten Laborraum benutzt, in dem niemals zuvor mit rezentem Rothirsch-Material gearbeitet wurde. Die Probennahme mit dem Bohrer wurde unter eigens für diesen Zweck bestimmten UV-Kammern durchgeführt. Nach jedem Arbeitsschritt wurden die Kammern mit DNA-Exitus behandelt und anschließend mit UV-Licht bestrahlt. Zur zusätzlichen Sicherheit und um Verunreinigungen auszuschließen, wurden sämtliche Schritte der prä- beziehungsweise post-PCR ebenfalls in einem anderen Labor (bei Frau Prof. Susanne Foitzik) durchgeführt.

Zur DNA-Extraktion wurden von mir die gängigsten Verfahren, wie Ädhasion an Silica, Magnetic Beads und auch Extraktion mit einem Komplett Kit für alle fünf Proben durchgeführt. Die besten Ergebnisse lieferte allerdings die von uns bereits im Vorfeld verwendete und verfeinerte Extraktionsmethode mittels Phenol-Chloroform (Hoffmann & Griebeler 2013). Im Unterschied zu der vorher angewandten Methode wurde hier allerdings 0,5 Gramm Knochenmaterial pro Probe eingesetzt, um die Chance zu erhöhen möglichst wenig degradierte Template-DNA zu finden.

9.3 Ergebnisse und Diskussion

Aus allen fünf untersuchten Geweih-Rosen konnte erfolgreich DNA extrahiert werden. Aber bereits bei der Messung des DNA-Gehaltes (Qubit1) wurde offensichtlich, dass es starke Schwankungen zwischen den einzelnen Proben gab. Während vier der fünf Proben geringe DNA-Gehalte zeigten, befand sich in einer Probe, selbst nach völlig unabhängiger Replikation eine auffällig hohe DNA-Konzentration. Selbst wenn die Extraktion hochwertiger genomischer DNA nur in einem von fünf Fällen erfolgreich verlaufen ist, zeigt dies doch, dass selbst die Amplifizierung von Mikrosatelliten bei entsprechendem Erhaltungsgrad aus sehr altem Probenmaterial möglich ist. Während die bisher älteste Mikrosatellitenprobe 270 Jahre alt war (Population Berleburg), wurde dieses Alter hier um ein Vielfaches überschritten.

Angesichts der riesigen Menge an vorhandenen Geweih-Rosen im Schloss-Gottorf sollte eine erfolgreiche Extraktion mehrerer Individuen und somit populationsgenetische Studien mit diesem Probenmaterial kein Problem darstellen.

Dies ermöglicht zum einen die Bearbeitung ökologischer Fragestellungen über den Rothirsch, zum anderen aber sicherlich auch die Beleuchtung interessanter soziologischer Aspekte der dort Handel treibenden Wikinger. Durch Mikrosatelliten-Analyse und auch die Verwendung mitochondrialer Marker kann zum Beispiel die Herkunft der Hirsche Auskunft über potenzielle Handelspartner, aber auch über ihr historisches Verbreitungsgebiet liefern.

10. Ausblick Genetik

Bisher habe ich mich mit der Auswertung von zeitlichen genetischen Daten auf der Ebene einzelner Populationen beschäftigt. So konnten Auswirkungen anthropogenen Handelns auf die genetische Konstitution der untersuchten Populationen aufgedeckt und miteinander in Bezug gesetzt werden. Änderungen in der Populationsgröße, das Aussetzen gebietsfremder Tiere und eventuell auch die gezielte Selektion einzelner Merkmale haben zu messbaren genetischen Veränderungen in den Populationen geführt.

Als nächster Schritt soll die Populationsebene verlassen werden und die Daten aller Populationen in ihrer Gesamtheit betrachtet werden. Die Suche nach populationsübergreifenden Mustern soll helfen Grundlagenfragen zu beantworten und die Daten auf größerer Ebene übertragbar machen, beispielsweise auf andere Großsäuger-Populationen.

Meine Ergebnisse zeigen klar, dass die genetische Diversität innerhalb der Populationen, in evolutiv gesehen, sehr kurzen Zeiträumen enorm schwankt. Nicht nur in der Population Neuwied (Kapitel 5), sondern auch in der Population Hasselbusch (Kapitel 7). Diese Schwankungen konnte ich sehr gut mit Änderungen in der Gesetzeslage zur Jagd in Verbindung zu bringen. Bei großen jagdbaren Arten entscheidet daher wohl in erster Linie die Gesetzgebung über die genetische Diversität und damit Anpassungsfähigkeit von Populationen an zukünftige Umweltbedingungen. Da diese Arten durch den Menschen schnell in ihrer Populationsgröße reguliert werden

können, ist die gesetzlich geregelte Jagdausübung entscheidender als Habitatverfügbarkeit und dessen Qualität für die genetische Diversität.

Da Untersuchungen über die Änderung der genetischen Diversität über die Zeit bisher für große soziallebende Säugetiere noch nie durchgeführt wurden, könnte es sich bei den von mir beobachteten Schwankungen in der individuellen Heterozygosität aber ebenso um einen natürlichen Prozess handeln. Auf eine Phase hoher individueller Schwankungen in der Anzahl heterozygoter Loci (mit hoher Varianz), folgt eine Phase mittlerer Diversität (Anzahl heterozygoter Loci) mit geringer Varianz zwischen den Individuen.

Inwieweit das Paarungssystem hier Einfluss nehmen könnte ist nicht bekannt. Beim polygamen Rothirsch paart sich in der Regel ein starkes männliches Tier mit mehreren weiblichen Tieren. Gerade in kleinen Populationen und stark bewirtschafteten Gebieten könnte es durch hohen Reproduktionserfolg einzelner starker Hirsche durch Inzucht und Drift zu Verlust von genetischer Diversität in den Folgegenerationen kommen.

Vielleicht folgt auf eine solche Phase eine Periode des gezielten Auswählens genetisch verschiedener Individuen durch die weiblichen Tiere, wie es von anderen Arten bekannt ist oder zumindest vermutet wird (Tregenza & Wedell 2000).

Ein wichtiger Punkt und eine praktische Labortätigkeit die weitergeführt werden sollte ist die Suche nach einem geeigneten paternal vererbten polymorphen Marker. Zwar wurden von mir schon etliche Anstrengungen unternommen und sowohl Sequenzen als auch Mikrosatelliten der SRY-Region (sex determining region) sequenziert, jedoch konnte ich bisher für keinen Abschnitt Polymorphie beobachten.

Ein solcher polymorpher Marker wäre geeignet auch den paternalen Vererbungsweg zu entschlüsseln. So könnten präzisere Aussagen über den Fortpflanzungserfolg eingesetzter gebietsfremder männlicher Tiere getroffen werden und auch die Frage nach dem Fortpflanzungserfolg des sogenannten Platzhirsches durch Untersuchung der Folgegeneration quantifiziert werden. Durch die von mir verwendete

mitochondriale D-Loop Sequenz lässt sich leider nur der maternale Vererbungsweg hervorragend verfolgen, beim paternalen Weg mussten bisher eine Kombination aus mtDNA und auch aus Assignment-Tests mit Mikrosatelliten-Daten zur Hilfe genommen werden (Abschnitt 6).

Hinsichtlich paternal vererbter Mikrosatelliten-Loci habe ich eine Vielzahl potenzieller Kandidaten bereits identifiziert, die auf Polymorphie beim Rothirsch untersucht werden könnten. Da die gesamte Sequenz des Y-Chromosoms vom nah verwandten Rind (*Bos tauris*) bekannt ist, habe ich diese nach Mikrosatelliten durchsucht und so fast einhundert mögliche Loci gefunden. Da auch die meisten derzeit für den Rothirsch verwendeten nicht geschlechts-chromosomal gebundenen Mikrosatelliten vom Rind übernommen wurden (Kuehn et al. 2003), ist es wahrscheinlich, dass auch hier polymorphe Loci gefunden werden könnten.

11. Einleitung Telemetrie

Bei der Satellitentelemetrie werden Tiere mit einem Empfänger ausgestattet, der die aktuelle Position des Tieres mit Hilfe des von GPS- (global positioning system) Satelliten abgestrahlten Signals ermittelt. Die so berechneten Positionen können zusätzlich über eine SIM-Karte per Handynetz an eine Basisstation übermittelt werden. Dies ermöglicht, im Gegensatz zur früher eingesetzten Radiotelemetrie, ein völlig störungsfreies Verfolgen von Tierbewegungen. Besonders für mobile und scheue Arten hat dies zu einer neuen Qualität und auch zu einer neuen Datendichte in Telemetriestudien geführt. Neben diesen reinen Positionsdaten liefert die Satellitentechnik auch eine Vielzahl an Informationen über die von den Tieren genutzten Flächen. Sowohl abiotische Faktoren, wie zum Beispiel die Beschaffenheit des Geländes, als auch die Habitatnutzung der Tiere können aus Satellitenbildern bestimmt und zur Auswertung der Positionsdaten genutzt werden.

Die zur Satellitentelemetrie benötigte Technik ist in Halsbandsendern untergebracht, die den Tieren angelegt werden. Diese Halsbandsender liefern über einen maximal zweijährigen Zeitraum (Leistungsfähigkeit des Akkus), in einem frei wählbaren Turnus, Positionen der besenderten Individuen.

Ich habe in zwei stark unterschiedlichen Naturräumen Rothirsche betäubt und mit Sendern versehen. Das erste Untersuchungsgebiet lag im Hochgebirge in den Alpen (Lechtal), das zweite Gebiet zeigt einen typischen Mittelgebirgscharakter und lag im Naturpark-Saar-Hunsrück (Rheinland-Pfalz, Saarland).

Aufgrund der in der Einleitung bereits beschriebenen Relevanz des Rothirsches, in finanzieller, kultureller und auch naturschutzfachlicher Hinsicht, sollten mit Hilfe dieser beiden Telemetrie-Studien wichtige Erkenntnisse über das Raum-Zeitverhalten dieser Art gewonnen werden. Durch die großen topographischen Unterschiede zwischen den beiden Untersuchungsgebieten werden sie im folgenden Kapitel getrennt voneinander betrachtet.

12. Home range and habitat use of red deer in the Alps detected from satellite telemetry

12.1 Abstract

As there are quite no satellite telemetry studies on spatiotemporal behavior of red deer, particularly for those populations living in the high mountains, we investigated 7 female red deer in the Alps. Over a period of 26 months we got 11,328 validated GPS points. Home range analyses, conducted with the Minimum Convex Polygon (MCP) and the Kernel Density Estimation (KDE) method, were found to differ considerably between individuals. The MCP ranged from 494 to 1568 ha, while the “individual home ranges” (95% KDE) and “core areas” (50% KDE) varied from 129 to 897 and from 11 to 114ha, respectively. Regarding habitat use, in over 50% of all fixed GPS locations, red deer were located in fir forests mixed with other tree species. Of all habitat types in the study area distinguished by us deer preferred undisturbed, covered areas and avoided anthropogenically used areas. Focussing on abiotic habitat factors, deer preferred areas with a high sunshine duration and generally avoided areas higher than 2200m and showing an inclination with more than 58 degrees.

12.2 Introduction

Understanding habitat preferences and home ranges of animals provides manifold insights into the relationship between a species and its environment (Cody 1981; Probst & Crow 1991). Over the last two decades satellite tracking enabled monitoring of individual movements for a large variety of terrestrial, aquatic and aerial vertebrates (Prince et al. 1992; Gudmundsson et al. 1995; Morreale et al. 1996; Polovina et al. 2000; Gillespie 2001; Pamperin et al. 2008; Tarroux et al. 2010). The combination of GPS positions with satellite imagery uncovers areas used by animals and has led to high quality data on movement biology and habitat use for ungulates (Bowyer et al. 1999; Leimgruber et al. 2001; Johnson et al. 2002).

Red deer (*Cervus elaphus*) is the largest, most common ungulate species in central Europe, inhabiting various habitat types from lowland steppes to forested mountains.

Although extensive migrations between summer mountain areas and wintering areas are known for red deer (Atzler 1984; Albon & Langvatn 1992) detailed information on movement and habitat use in mountainous regions is lacking (Georgii, 1980). In the past, it was extremely difficult to get reliable data on shy and mobile red deer in such an impassable landscape via radio tracking. Field observations indicate that characteristic migrations of red deer have changed in the Alps within the last decades due to intensive land use by humans and the feeding of red deer during winter.

These changing migration routes and movements can affect home ranges of red deer, as demonstrated for various deer populations in North America (Miller 1970; Craighead et al. 1973; Drolet 1976; Anderson et al. 2005), in Slovenia (Debeljak et al. 2001), in the Czech Republic (Koubek & Hrabe 1996), in Denmark (Jeppesen 1987), in Germany (Georgii 1980), in Hungary (Szemethy et al. 1994), in Scotland (Clutton-Brock & Guinness 1982) and in Spain (Carranza et al. 1991).

As satellite telemetry studies on home ranges and habitat use of red deer in high mountains are quite completely absent in the literature, we studied seven female red deer in the European Alps. Red deer are important game animals and also important for tourism in this area, but browsing damage caused to forestry by deer is high. So knowledge on the home range and habitat use of animals is of high relevance with respect to economy and lifestyle of people living in this area.

Because all individuals collared by us belonged to different herds, we could effectively monitor the movement of seven independent red deer herds. In particular, we addressed three issues in our study: (1) deer's home range sizes, (2) its habitat use and preference, and (3) abiotic factors of areas used by animals, i.e. altitude, inclination and duration of sunshine.

12.3 Material and Methods

12.3.1 Study area

Our study area was in the upper Lech Valley in Tirol (Austria). The area covers about 7000 ha, and its altitude ranges from 1100 to 2800m. The average total annual precipitation in this area is 1376 mm and the average annual temperature is 4.1°C.

There are urban areas in the valleys; the cliffy hillsides are used for animal husbandry and for forestry. Three artificial winter-feeding stations have been installed in valleys of the study area (more detailed information about study area and history in section S 5.1).

12.3.2 Satellite tracking

During May 2008 and May 2009, we immobilized a total of seven female red deer (Table 12.1) all belonging to different herds. We anaesthetized deer in the feeding areas using a carbon dioxide gun (Dan Inject) and darts filled with “Hellabrunner Mischung” (Xylazin/Ketamin). We fitted deer with GPS-collars (Vectronic Aerospace) programmed to record a GPS-point every 3 hour over a 24-hour day.

To minimize any potential bias on movement, we excluded GPS locations collected during the first two days after immobilization from our dataset. Only GPS points based on at least four GPS satellites were used for analyses.

Table 12.1 Number of animals (n=number of validated positions per individual), time of data collection, sizes of the Minimum Convex Polygons (MCP) and sizes of the 50% and 95% Kernel Density Estimations (KDE) of animals in hectare [ha].

Animal	Time of data collection	MCP [ha]	50% KDE [ha]	95% KDE [ha]
1 (n=1849)	29.04.2008-06.11.2009	873	29	208
2 (n=2623)	13.04.2008-22.06.2010	570	12	95
3 (n=1467)	29.04.2008-13.05.2009	1022	16	232
4 (n=1607)	08.05.2008-25.12.2009	1661	96	802
5 (n=1896)	30.04.2009-18.06.2010	520	9	112
6 (n = 515)	02.05.2009-19.10.2009	479	44	363
7 (n=1302)	08.05.2009-07.09.2010	451	21	312

12.3.3 Home range

We plotted all GPS locations in ArcView 3.2 and analyzed home ranges using the ”Animal Movement Extension“ (Hooge & Eichenlaub 1997) for this software. We used the Minimum Convex Polygon Method (MCP; (Mohr 1947)) and the Kernel Density Estimation (KDE; (Worton 1989)). Individual home ranges were estimated as adaptive kernels, with the smoothing factor h estimated from the data for each individual and optimised via Least Square Cross Validation (LSCV; (Worton 1989)). We defined the 95%-KDEs as “individual home ranges” and the 50%-KDEs as “core areas” (Samuel et al. 1985; Hillen et al. 2009).

12.3.4 Habitat preference, abiotic factors

To estimate habitat use and preference we used a digital map, which provided 17 different land-use types (Tiris maps, Tirol). To assign the GPS points to land-use types, we used ArcGIS10. Habitat preference was calculated with the formula from Lille (Lille 1996): Habitat preference = $\log(r/\sqrt{p})$, where r represents the percentage of time spent in a certain habitat (number of GPS points) and p represents the percentage of this habitat type available within the total area (random use). To avoid any bias caused by winter feeding, we calculated habitat preference based on pooled GPS points of all seven animals for the feeding period (December - April) and for months within the non-feeding period (May - November). The duration of sunshine, altitude, and inclination in the areas corresponding to GPS points were derived using ArcGIS10 (tiris Tirol).

All statistical analyses were conducted in SPSS 20. To test for normal distributions of data, we used the Kolmogorov-Smirnov-test. As all data (altitude, inclination, duration of sunshine) were not normally distributed we used the nonparametric Mann-Whitney U-Test to assess differences in median values between two samples.

12.4 Results and Discussion

We collected reliable data from seven female deer over 26 months in the period from May 2008 to June 2010. The numbers of validated positions varied from 515 to 2623 (Table 12.1) points per individual. The total number of validated positions across all seven individuals was 11,328.

12.4.1 Home ranges

The home range size of mammals depends on different factors, including metabolism requirements, eating habits, mating system (Cameron & Spencer 1985; Sandell & Liberg 1992), population density (Vincent et al. 1995), and predation (Jeppesen 1987). Our study indicates that home ranges of red deer can strongly differ between individuals, independent of the method applied to estimate ranges (MCP or KDE) (Table 12.1).

Animal number 4, covering the longest distance between summer and winter-feeding areas, also had the largest home range size with 1661 ha (MCP-value); it was almost four times higher than the 479 ha covered by the smallest animal (individual 6). When using the KDE method home range sizes of these two individuals differed even stronger, ranging from a factor of 7 (95% KDE) to more than 10 (between 9 and 96 ha) for the core areas (Table 12.1).

While one animal (individual 1), which spent the summer near a feeding station, had only one small core area (11 ha), the long distance migrating animal number 4 had three core areas. The large distance between the area frequented in summer and the area used during feeding in winter demonstrates how large the catchment area during winter feeding can be. Our results indicate that animals can be attracted within many thousands of hectares in the areas with feeding stations, so that the number of individuals at the winter-feeding stations does not reflect the number of animals in this area during the rest of the year. Knowing that there is a massive concentration of individuals at the feeding stations from a large area is not only important for estimating population size but also for avoiding and understanding disease transmission between animals. Home range sizes (MCP values) estimated for red deer in other European countries like Slovenia (Debeljak et al. 2001), Czech Republic (Koubek & Hrabe 1996), Denmark (Jeppesen 1987), Germany (Georgii 1980), Hungary (Szemethy et al. 1994), Scotland (Clutton-Brock & Guinness 1982) and Spain (Carranza et al. 1991) range between 400 and 1500ha and are consistent with the range found in our study in a high mountainous area, although the topography of studied areas considerably differs between all these countries. Normally, we expected larger home range sizes in the high mountains than in lowlands due to migrations between summer and winter-feeding areas, but in our study area the concentration of the animals at winter feeding places seems to diminish home range sizes. Additionally, old natural mixed forests that exist in the study area provide better living conditions for red deer than monocultures and young forests, which naturally increase home ranges (Vanhorne et al. 1988; Happe et al. 1990).

All collared animals in this study belonged to different herds. While there was sometimes a spatial overlap in individual home ranges (95% KDE), all individuals never shared their 50% KDE. There seems to be a moderate territoriality, not at the individual but at a herd level. The small size of all core areas proves that, under good conditions, the area needed by an individual can be quite small, even for a large and mobile species like red deer.

12.4.2 Habitat use and preference

Although monthly habitat use was diverse during the non-feeding period (Figure S 12.1); red deer in this study frequented spruce/ fir and spruce/ pine forests covered by quite 50% of all GPS points, although they cover less than 7% of the whole area (Table S 12.1).

Five of the six most frequented land cover types were different forest types (Figure S 12.2), proving that forests are important retreat areas for deer (Wolff & Van Horn 2003). As found in other studies (Vanhorne et al. 1988), only natural finished mixed forests were preferred, in contrast to monocultures (fir forests) (Figure S 12.2). Fallow land was the only non-forest habitat type preferred by deer. These areas, in different states of succession, offer good coverage and browsing opportunities for deer. All areas strongly avoided by deer (roads, rivers, buildings and mowing areas) were areas under strong human impact (Figure S 12.2).

12.4.3 Duration of sunshine, altitude and inclination

During the non-feeding period, especially in spring, deer significantly concentrated in areas highly exposed to the sun, as these provide better food in terms of quality and quantity (Figure S 12.3). The altitude of areas used by animals was significantly higher in the summer than in the colder and snowier months (Figure 12.1A), with the highest altitude being reached by animals in August (2200m). This annual movement is initiated by early snowfall (Albon & Langvatn 1992), reducing the availability of food and increasing the costs of movement (Parker et al. 1984). In the summer months,

improved food supply at higher altitudes and also high anthropogenic pressure in the lower regions, force deer to frequent higher altitudes.

Besides altitude, inclination is an abiotic factor especially relevant in the high mountains. To the best of our knowledge, the influence of inclination on habitat use has never been investigated for red deer. Inclination of areas frequented by deer shows a similar pattern as altitude of areas (Figure 12.1B). Presumably, identical factors might force deer to migrate to steep hillsides; the attractive new feeding grounds after snowmelt and the increasing presence of tourists and grazing cattle in the lower areas most probably trigger this behaviour. Knowledge about the ability of red deer to move on steep hillsides is for example important for calculating the carrying capacity of a population living in a certain area. Independent of the size and habitat use, tracked deer never used areas with an inclination exceeding 58 degrees. This avoidance either results from a physical limitation and/or deer are displaced in such steep regions by other more competitive and better adapted ungulate species, like chamois and ibex.

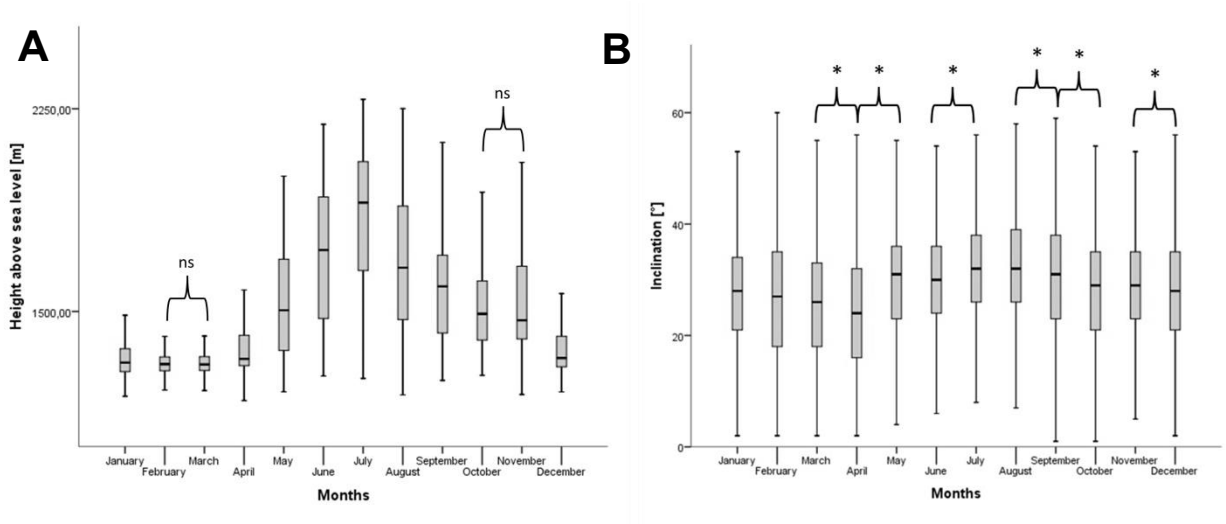


Figure 12.1 Boxplot with average height above sea level (A) and inclination (B) of areas used by deer. Outliers as identified by the software SPSS are not displayed; in A non-significant ($p > 0.05$) monthly differences are characterized with ns. In B significant monthly differences are characterized with * ($p < 0.05$).

13. Einleitung Telemetrie im Naturpark Saar Hunsrück

Der Naturpark Saar Hunsrück hat eine Gesamtfläche von 2055 km^2 und liegt in den Bundesländern Rheinland-Pfalz und Saarland. Aufgrund seiner Größe sind verschiedenste Flächennutzungstypen vertreten; sie reichen von stark bewaldeten Höhen des Hunsrücks über landwirtschaftlich genutzte Flächen, bis hin zu den Weinbergen an Mosel, Saar, Ruwer und Nahe. Die höchste Erhebung im Naturpark ist 816 m ü. NN (Erbeskopf), sein Waldanteil liegt insgesamt bei 50 %.

Im Naturpark wurden zwischen dem 20.03.2010 und dem 21.01.2012 insgesamt 14 Individuen von mir betäubt und mit Halsbandsendern ausgestattet. Im Gegensatz zu meinem Untersuchungsgebiet in Österreich wurden hier sowohl männliche als auch weibliche Tiere besendert (8 weiblich, 6 männlich). Im Untersuchungszeitraum wurden 23.000 validierte Positionen der Tiere aufgenommen (16.000 weiblich und 7.000 männlich).

Da hier Daten beider Geschlechter gesammelt wurden, lag ein Hauptfokus der Studie auch in der Ausarbeitung der geschlechtsspezifischen Unterschiede in der Raumnutzung des Rothirschs. Da eine Publikation zu diesem Thema geplant ist (Sex specific differences in Home Range and habitat use in Red deer, detected by satellite telemetry), wird hier nur eine übersichtsartige Darstellung einiger wichtiger Ergebnisse erfolgen.

13.1 Material und Methoden

Siehe Kapitel 11

13.2 Vorläufige Ergebnisse und Diskussion

Der Begriff „Homerange“ beschreibt den Raum in dem ein Tier sich aufhält. Homerange-Größen von Säugetieren hängen von verschiedensten Faktoren ab, von den Bedürfnissen des Stoffwechsels, von den Fressgewohnheiten, dem Paarungssystem (Cameron & Spencer 1985; Sandell & Liberg 1992) und von einer Kombination weiterer Faktoren wie Alter (Cederlund & Sand 1992), der

Populationsdichte (Vincent et al. 1995) und Prädation (Jeppesen 1987). Nicht zuletzt entscheiden auch die verschiedenen Sozialsysteme der Wildwiederkäuer über ihr Raum-Zeit-Verhalten.

Die im Naturpark Saar-Hunsrück gemessenen durchschnittlichen Homerange-Größen (50% und 95% Kernel) sind insbesondere bei den weiblichen Tieren mit 11 beziehungsweise 148 Hektar im Vergleich zum Untersuchungsgebiet in Österreich (Tabelle 11.1) mit 32 und 303 Hektar sehr klein (Abbildung 13.1). Auch im internationalen Vergleich zu anderen Studien, die meist Werte zwischen 400 und 1500 Hektar (95% Kernel) angeben (Jeppesen 1987; Carranza et al. 1991; Schmidt 1993; Szemethy et al. 1994; Koubek & Hrabe 1996; Debeljak et al. 2001; Szemethy et al. 2003), sind die im Naturpark beobachteten Streifgebietsgrößen gering. Die von den männlichen Tieren genutzten Flächen waren mit 38 (50 % Kernel) und 519 ha (95 % Kernel) im Durchschnitt um ein Vielfaches größer als die der weiblichen Tiere (Abbildung 13.1) und damit auch größer als die Streifgebiete der von mir in Österreich untersuchten weiblichen Tiere. Sicherlich ein Hauptgrund für die große Differenz zwischen den Größen der Homeranges von Weibchen und Männchen ist die Tatsache, dass beim Rothirsch, wie bei vielen Hirschartigen, Genfluss überwiegend durch Ausbreitung und Verbreitung männlicher Individuen stattfindet (Goodman et al. 2001; Zachos & Hartl 2011; Hoffmann et al. 2016). Große und weite Wanderungen, gerade zur Paarungszeit (Brunft) wurden bereits in der Literatur beschrieben und können auch von mir in diesem Untersuchungsgebiet bestätigt werden. Einzelne Hirsche legten in nur 24 Stunden teilweise über 10 Kilometer Luftlinie zurück. Solche großen Wanderungsdistanzen waren bei keinem von mir untersuchten weiblichen Tier zu beobachten. Die Weibchen zeigen in der Regel starke Philopatrie und verbleiben häufig im elterlichen Rudel (Nussey et al. 2005; Nussey et al. 2006; Frantz et al. 2008; Jarnemo 2008).

Die im Vergleich zu den Weibchen durchschnittlich größeren Streifgebiete der männlichen Tiere könnten auch dem erhöhten Nahrungs- und Nährstoffbedarf aufgrund ihrer größeren Körpergröße (Bützler 2001) und auch dem jährlichen Geweihwachstum geschuldet sein. Insgesamt stellen aber die geringen hier festgestellten Streifgebietsgrößen beider Geschlechter unter Beweis, dass der

Flächenbedarf des Rothirschs bei ausreichend verfügbarer Nahrung und Störungsarmut sehr gering sein kann.

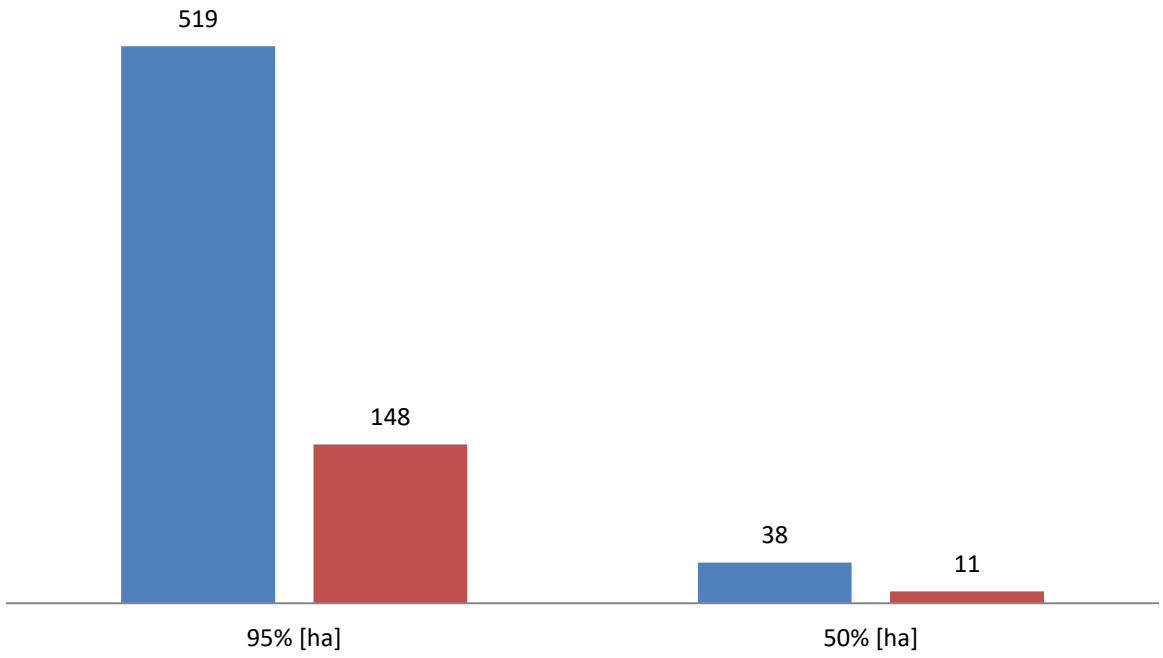


Abbildung 13.1 Durchschnittliche Größe der gemessenen 50% und 95% Kernel Räume bei männlichen Tieren (blau) und weiblichen Tieren (rot) im Vergleich (in Hektar [ha]).

Wie in den Homerange-Größen zeigen sich auch in der Flächennutzung große geschlechtsspezifische Unterschiede (Abbildung 13.2). Im Vergleich zu den männlichen Tieren wird Offenland von den weiblichen Tieren deutlich stärker gemieden. Deckungsreiche, junge und damit dichte Wälder werden dagegen deutlich stärker präferiert. Sicherlich spielt hierbei das Gebären und Vorhandensein von Jungtieren und das damit einhergehende erhöhte Sicherheitsbedürfnis der Weibchen im Vergleich zu den Männchen eine bedeutende Rolle. Aber auch höherer Jagddruck

und Erinnerungsvermögen der älteren weiblichen Rudelmitglieder könnten hier ursächlich sein. Im Gegensatz zu den männlichen Tieren unterliegen die weiblichen Tiere jährlichem Jagddruck, da primär Jungtiere von Jägern erlegt werden müssen.

Die sogenannten Kahlwildrudel bestehen aus weiblichen Tieren jeden Alters und aus männlichen Tieren, die die Geschlechtsreife noch nicht erreicht haben. Geschlechtsreif gewordene Hirsche müssen das elterliche Rudel verlassen und es kommt in der Regel zu einem Zusammenschluss mehrerer Hirsche aus verschiedenen Rudeln zu eigenen Hirschrudeln. Da mittelalte Hirsche in der Regel nicht bejagt werden und alte Hirsche oft alleine während der Paarungszeit (Brunft) erlegt werden, unterliegen die Hirschrudel oft über Jahre keinerlei Jagddruck. Die Kahlwildrudel aus weiblichen Tieren und Jungtieren, die von einem alten erfahrenen weiblichen Tier angeführt werden (Leittier), werden dagegen jährlich bejagt. Zur Reduktion des Gesamtbestandes werden aus diesen Rudeln überwiegend junge Tiere (Kälber), aber auch ältere weibliche Tiere geschossen. So kann das Kahlwildrudel und damit auch das jeweilige Leittier über Jahre Erfahrungen sammeln und sich Feindvermeidungstaktiken aneignen, die an die Rudelmitglieder weitergegeben werden. Daraus könnte die von mir beobachtete Präferenz weiblicher Tiere für dichte, schwer zu bejagende Waldgebiete entstanden sein.

Ein weiterer Punkt, der die Hirsche an der Nutzung dichter und junger Waldkomplexe hindern könnte, ist ihr Geweih. Im fertig ausgebildeten Zustand könnte es die Hirsche in ihrer Bewegung in zu dichten Beständen stark einschränken und somit an schneller koordinierter Flucht hindern. Schwerwiegender ist aber wahrscheinlich die Verletzungsgefahr des sich im Aufbau befindenden Geweihs. Bei allen Cerviden wird das Geweih jährlich abgeworfen und neu gebildet. Die Bildung vollzieht sich unter einem stark durchbluteten Gewebe, der sogenannten Basthaut. Größere Verletzungen an dieser Basthaut würden zu einer unvollständigen oder gestörten Geweihbildung führen. Da ein großes und fertig ausgebildetes Geweih Grundvoraussetzung für eine aktive Teilnahme an der Paarung (Brunft) und somit für Fortpflanzungserfolg ist, müssen die Hirsche jedes Risiko minimieren, welches zu Schädigungen der Basthaut führen könnte.

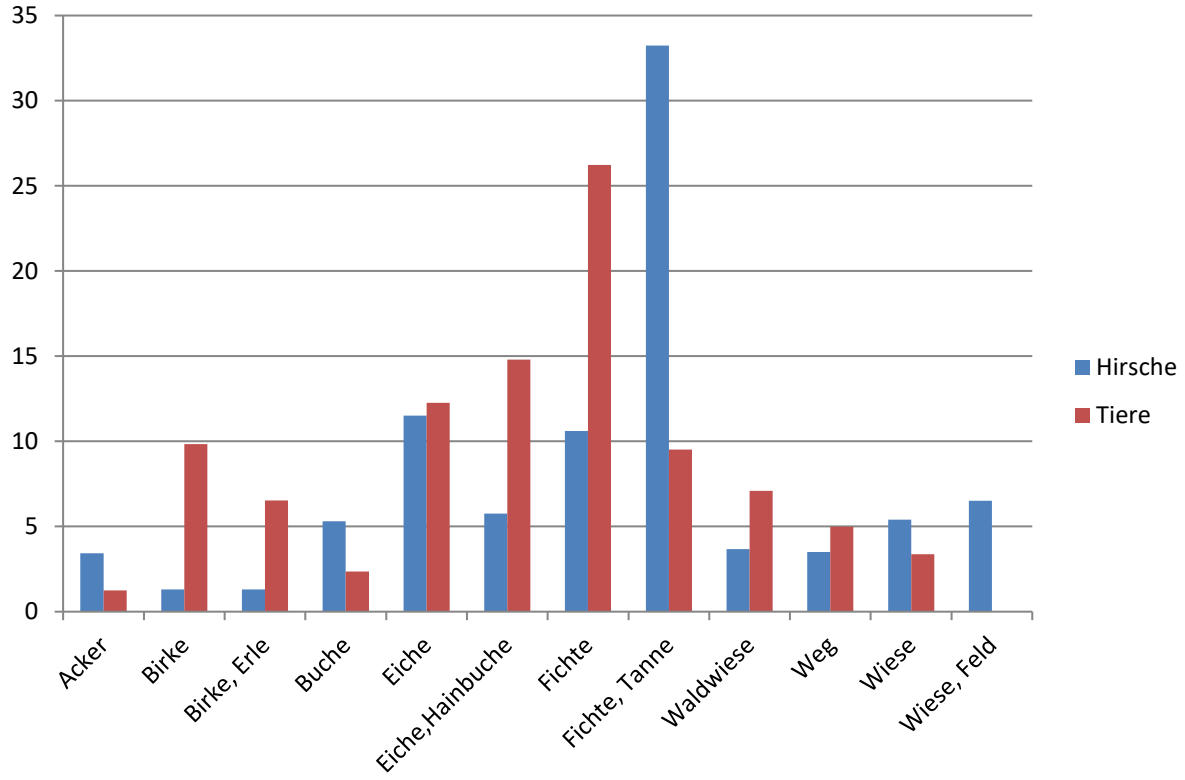


Abbildung 13.2 Flächennutzung der im Naturpark Saar-Hunsrück besenderten Weibchen (rot) und Hirsche (blau).

Vom ursprünglichen Steppen- und Offenlandbewohner (Bützler 2001) ist der Rothirsch, auch durch die steigende Präsenz des Menschen, zu einer typischen Art störungsarmer großer Waldgebiete geworden (Georgii 1981; Boyle & Samson 1985; Jeppesen 1987; Petrak 1996). Dennoch bieten offene Flächen, wie Wiesen und auch Feldfruchtanbau, gute Nahrungsgrundlagen für die Art. Ein Effekt der Meidung anthropogen genutzter Flächen könnte eine erhöhte Nutzung dieser Flächen mit niedriger Vegetation in der Nacht sein, in der es kaum Parallel-Nutzung durch den Menschen gibt. Um dies differenzierter zu betrachten habe ich die Flächennutzung aller Individuen in Tag- und Nachtdaten eingeteilt. Weiterhin wurden alle Flächennutzungstypen nach ihrem Deckungsgrad in „offen“, für wenig Deckung und „geschlossen“ für hohe Deckung eingeteilt.

Unabhängig vom Geschlecht der Tiere zeigte sich eine deutlich höhere Nutzung offener Flächen in der Dunkelheit (Nacht), die jedoch deutlich stärker bei den weiblichen als bei den männlichen Tieren ausgeprägt war. Wie bereits in der Flächennutzung erkennbar, wird Offenland von weiblichen Tieren, unabhängig der Tageszeit, stärker gemieden als von männlichen Tieren. Auch hier ist offensichtlich das Sicherheitsbedürfnis der weiblichen Tiere höher als das der männlichen (Abbildung 13.3).

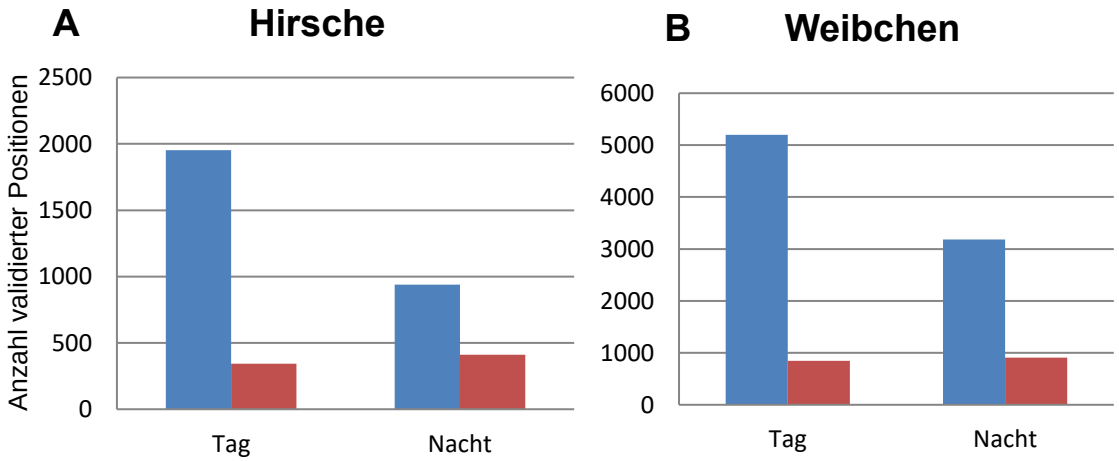


Abbildung 13.3 Anzahl an validierten Ortungen in deckungsreicher, geschlossener Landschaft (blau) und in offenem Gelände (rot) im Tag- und Nacht-Vergleich für Hirsche (A) und Weibchen (B).

14. Ausblick Telemetrie

Neben den GPS Koordinaten werden von den von mir verwendeten Halsbandsendern auch permanent Beschleunigungsdaten der Bewegungen der Tiere aufgezeichnet. Über im Halsband integrierte Beschleunigungssensoren werden Beschleunigungen in horizontaler wie vertikaler Richtung detektiert und gespeichert. Durch die korrekte Interpretation dieser Beschleunigungsdaten, steht nicht mehr nur eine GPS-Koordinate zur Verfügung, sondern es werden Aussagen über die Tätigkeiten und über das Verhalten der Tiere möglich (Moreau et al. 2009; Nathan et al. 2012; Brown et al. 2013). Problem dieser Daten ist, dass es ohne eine Eichung auf die jeweilige Tierart nicht möglich ist, spezielle Beschleunigungswerte spezifischen Tätigkeiten zuzuordnen. Aus diesem Grund haben wir im Rahmen einer Bachelorarbeit von Herrn Sebastian Siebel zwei Tiere im Tierpark Hanau mit Halsbandsendern ausgerüstet und ihr Verhalten über einen vierwöchigen Zeitraum dokumentiert. Es wurden einfache Tätigkeiten (ruhen, gehen, laufen, fressen) mit der jeweils genauen Uhrzeit vom Studenten festgehalten, um sie später den aufgezeichneten Beschleunigungsdaten zuordnen zu können. Seine Studie hat gezeigt, dass Beschleunigungsdaten mit einem hohen Prozentsatz dem richtigen Verhalten zugeordnet werden konnten, was zu einer neuen Dimension in der Satellitentelemetrie des Rothirschs führt. Konnten vorher über aufgezeichnete GPS-Koordinaten lediglich die Aufenthaltsorte der Tiere bestimmt werden, so können mit dieser neuen Technik Aussagen über das Verhalten der Tiere an den jeweiligen Positionen getroffen werden. Eine störungfreie „Beobachtung“ der Tiere kann so, ohne nicht finanzierbaren Personalaufwand, erreicht werden.

15. Literatur

Albon, S. D., & Langvatn, R. (1992). Plant Phenology and the benefits of Migration in a temperate Ungulate. *Oikos, 65*(3), 502-513.

Anderson, D. P., Forester, J. D., Turner, M. G., Frair, J. L., Merrill, E. H., Fortin, D., Boyce, M. S. (2005). Factors influencing female home range sizes in elk (*Cervus elaphus*) in North American landscapes. *Landscape Ecology, 20*(3), 257-271.

Atzler, R. (1984). Feeding supply and pattern of movement of Red Deer in the Ammergauer Mountains. *Zeitschrift Fur Jagdwissenschaft, 30*(2), 73-81.

Balanya, J., Oller, J. M., Huey, R. B., Gilchrist, G. W., & Serra, L. (2006). Global genetic change tracks global climate warming in Drosophila subobscura. *Science, 313*(5794), 1773-1775.

Ball, R. M., & Avise, J. C. (1992). Mitochondrial-DNA phylogeographic differentiation among avian populations and the evolutionary significance of subspecies. *Auk, 109*(3), 626-636.

Bonnet, A., Thevenon, S., Maudet, F., & Maillard, J. C. (2002). Efficiency of semi-automated fluorescent multiplex PCRs with 11 microsatellite markers for genetic studies of deer populations. *Animal Genetics, 33*(5), 343-350.

Borowski, S., & Kossak, S. (1975). The food habits of deer in the Białowieża Primeval Forest. *Acta Theriologica, 20*(32), 463-506.

Bouzat, J. L., Lewin, H. A., & Paige, K. N. (1998). The ghost of genetic diversity past: Historical DNA analysis of the greater prairie chicken. *American Naturalist, 152*(1), 1-6.

Bowyer, R. T., Van Ballenberghe, V., Kie, J. G., & Maier, J. A. K. (1999). Birth-site selection by Alaskan moose: Maternal strategies for coping with a risky environment. *Journal of Mammalogy, 80*(4), 1070-1083.

Boyle, S. A., & Samson, F. B. (1985). Effects of nonconsumptive recreation on wildlife: a review. *Wildlife Society Bulletin (1973-2006), 13*(2), 110-116.

Broquet, T., Ménard, N., & Petit, E. (2007). Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. *Conservation Genetics, 8*(1), 249-260.

Brown, D. D., Kays, R., Wikelski, M., Wilson, R., & Klimley, A. P. (2013). Observing the unwatchable through acceleration logging of animal behavior. *Animal Biotelemetry, 1*(1), 20.

Bruford, M. W., Cheesman, D. J., Coote, T., Green, H. A., Haines, S. A., O'Ryan, C., & Williams, T. R. (1996). Microsatellites and their application to conservation genetics. *Molecular genetic approaches in conservation*, 278-297.

Bruford, M. W., & Wayne, R. K. (1993). Microsatellites and their application to population genetic studies. *Current opinion in genetics & development, 3*(6), 939-943.

Burger, J., Rosendahl, W., Loreille, O., Hemmer, H., Eriksson, T., Götherström, A., Alt, K. W. (2004). Molecular phylogeny of the extinct cave lion Panthera leo spelaea. *Molecular phylogenetics and evolution, 30*(3), 841-849.

Bützler, W. (2001). Rotwild: Biologie, Verhalten. *Umwelt, Hege. BLV München*.

Cameron, G. N., & Spencer, S. R. (1985). Assessment of space-use patterns in the Hispid cotton rat (*Sigmodon-hispidus*). *Oecologia, 68*(1), 133-139.

Carranza, J., Detrucios, S. J. H., Medina, R., Valencia, J., & Delgado, J. (1991). Space use by Red deer in a mediterranean ecosystem as determined by radio-tracking. *Applied Animal Behaviour Science, 30*(3-4), 363-371.j

Cederlund, G. N., & Sand, H. K. (1992). Dispersal of subadult moose (*Alces alces*) in a nonmigratory population. *Canadian Journal of Zoology, 70*(7), 1309-1314.

Ciborowski, K. L., Consuegra, S., de Leaniz, C. G., Wang, J., Beaumont, M. A., & Jordan, W. C. (2007). Stocking may increase mitochondrial DNA diversity but fails to halt the decline of endangered Atlantic salmon populations. *Conservation Genetics, 8*(6), 1355-1367.

Clutton-Brock, T. H., & Guinness, F. E. (1982). *Red deer: behavior and ecology of two sexes*: University of Chicago Press.

Clutton-Brock, T. H. (1989). Mammalian mating systems. *Proceedings of the Royal Society Series B-Biological Sciences, 236*(1285), 339-372.

Cody, M. L. (1981). Habitat selection in birds - the roles of vegetation structure, competitors, and productivity. *Bioscience, 31*(2), 107-113.

Coltman, D. W., O'Donoghue, P., Jorgenson, J. T., Hogg, J. T., Strobeck, C., & Festa-Bianchet, M. (2003). Undesirable evolutionary consequences of trophy hunting. *Nature, 426*(6967), 655-658.

Cooper, A., Mourer-Chauviré, C., Chambers, G. K., von Haeseler, A., Wilson, A. C., & Pääbo, S. (1992). Independent origins of New Zealand moas and kiwis. *Proceedings of the National Academy of Sciences, 89*(18), 8741-8744.

Coulson, T. N., Pemberton, J. M., Albon, S. D., Beaumont, M., Marshall, T. C., Slate, J., . . . Clutton-Brock, T. H. (1998). Microsatellites reveal heterosis in red deer. *Proceedings of the Royal Society B-Biological Sciences, 265*(1395), 489-495.

Craighead, J. J., Craighead, F. C., Jr., Ruff, R. L., & O'Gara, B. W. (1973). Home Ranges and Activity Patterns of Nonmigratory Elk of the Madison Drainage Herd as Determined by Biotelemetry. *Wildlife Monographs*(33), 3-50.

Cronin, M. A. (2006). A proposal to eliminate redundant terminology for intra-species groups. *Wildlife Society Bulletin, 34*(1), 237-241.

Cronin, M. A., Palmisciano, D. A., Vyse, E. R., & Cameron, D. G. (1991). Mitochondrial-DNA in wildlife forensic-science - species identification of tissues. *Wildlife Society Bulletin, 19*(1), 94-105.

Cronin, M. A., Vyse, E. R., & Cameron, D. G. (1988). Genetic-relationships between Mule deer and White-tailed deer in montana. *Journal of Wildlife Management, 52*(2), 320-328.

Debeljak, M., Dzeroski, S., Jerina, K., Kobler, A., & Adamic, M. (2001). Habitat suitability modelling for red deer (*Cervus elaphus L.*) in South-central Slovenia with classification trees. *Ecological Modelling, 138*(1-3), 321-330.

Drolet, C. A. (1976). Distribution and movements of White-tailed deer in southern New Brunswick in relation to environmental factors. *Canadian Field-Naturalist, 90*(2), 123-136.

Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics, 1*, 47-50.

Falconer, D. S., Mackay, T. F., & Frankham, R. (1996). Introduction to quantitative genetics (4th edn). *Trends in Genetics, 12*(7), 280.

Felmer, D. R., Sagredo, D. B., Chavez, R. R., Iraira, H. S., Folch, M. C., Parra, G. L., Ortiz, L. M. (2008). Implementation of a molecular system for traceability of beef based on microsatellite markers. *Chilean Journal of Agricultural Research, 68*(4), 342-351.

Fernandez-Garcia, J. L., Carranza, J., Martinez, J. G., & Randi, E. (2014). Mitochondrial D-loop phylogeny signals two native Iberian red deer (*Cervus elaphus*) Lineages genetically different to Western and Eastern European red deer and infers human-mediated translocations. *Biodiversity and Conservation, 23*(3), 537-554.

Fickel, J., Bubliy, O. A., Stache, A., Noventa, T., Jirsa, A., & Heurich, M. (2012). Crossing the border? Structure of the red deer (*Cervus elaphus*) population from the Bavarian-Bohemian forest ecosystem. *Mammalian Biology, 77*(3), 211-220.

Flagstad, O., Walker, C. W., Vila, C., Sundqvist, A. K., Fernholm, B., Hufthammer, A. K., Ellegren, H. (2003). Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline. *Molecular Ecology, 12*(4), 869-880.

Frankham, R., Briscoe, D. A., & Ballou, J. D. (2002). *Introduction to conservation genetics*: Cambridge University Press.

Frantz, A. C., Hamann, J.-L., & Klein, F. (2008). Fine-scale genetic structure of red deer (Cervus elaphus) in a French temperate forest. *European Journal of Wildlife Research, 54*(1), 44-52.

Frantz, A. C., Pourtois, J. T., Heuertz, M., Schley, L., Flamand, M. C., Krier, A., Burke, T. (2006). Genetic structure and assignment tests demonstrate illegal translocation of red deer (Cervus elaphus) into a continuous population. *Molecular Ecology, 15*(11), 3191-3203.

Galindoleal, C., & Weber, M. (1994). Translocation of deer subspecies - reproductive implications. *Wildlife Society Bulletin, 22*(1), 117-120.

Garel, M., Cugnasse, J.-M., Maillard, D., Gaillard, J.-M., Hewison, A. M., & Dubray, D. (2007). Selective harvesting and habitat loss produce long-term life history changes in a mouflon population. *Ecological applications, 17*(6), 1607-1618.

Gaur, A., Singh, A., Arunabala, V., Umapathy, G., Shailaja, K., & Singh, L. (2003). Development and characterization of 10 novel microsatellite markers from Chital deer (*Cervus axis*) and their cross-amplification in other related species. *Molecular Ecology Notes, 3*(4), 607-609.

Gautier, M., & Naves, M. (2011). Footprints of selection in the ancestral admixture of a New World Creole cattle breed. *Molecular Ecology, 20*(15), 3128-3143.

Gebert, C., & Verheyden-Tixier, H. (2001). Variations of diet composition of red deer (*Cervus elaphus L.*) in Europe. *Mammal Review, 31*(3-4), 189-201.

Geist, V. (1988). How markets in wildlife meat and parts and the sale of hunting privileges jeopardize wildlife conservation. *Conservation Biology, 2*(1), 15-26.

Geist, V. (1992). Deer ranching for products and paid hunting: threat to conservation and biodiversity by luxury markets. *The biology of deer.*, 554-561.

Georgii, B. (1980). Home range patterns of female Red deer (*Cervus-elaphus L*) in the alps. *Oecologia, 47*(2), 278-285.

Georgii, B. (1981). Activity patterns of female red deer (*Cervus elaphus L*.) in the Alps. *Oecologia, 49*(1), 127-136.

Gilbert, C., Ropiquet, A., & Hassanin, A. (2006). Mitochondrial and nuclear phylogenies of Cervidae (Mammalia, Ruminantia): Systematics, morphology, and biogeography. *Molecular Phylogenetics and Evolution, 40*(1), 101-117.

Gillespie, T. W. (2001). Remote sensing of animals. *Progress in Physical Geography, 25*(3), 355-362.

Goodman, S. J., Barton, N. H., Swanson, G., Abernethy, K., & Pemberton, J. M. (1999). Introgression through rare hybridization: A genetic study of a hybrid zone between red and sika deer (genus Cervus) in Argyll, Scotland. *Genetics, 152*(1), 355-371.

Goodman, S. J., Tamate, H. B., Wilson, R., Nagata, J., Tatsuzawa, S., Swanson, G. M., McCullough, D. R. (2001). Bottlenecks, drift and differentiation: the population structure and demographic history of sika deer (*Cervus nippon*) in the Japanese archipelago. *Molecular Ecology, 10*(6), 1357-1370.

Grau, S. (2005). Großflächige Planungen zur Landschaftsentschneidung in Deutschland. *GAIA-Ecological Perspectives for Science and Society, 14*(2), 153-162.

Greenwood, A. D., Capelli, C., Possnert, G., & Pääbo, S. (1999). Nuclear DNA sequences from late Pleistocene megafauna. *Molecular biology and evolution, 16*(11), 1466-1473.

Greenwood, P. J. (1980). Mating systems, philopatry and dispersal in birds and mammals. *Animal Behaviour, 28*(NOV), 1140-1162.

Griffith, B., Scott, J. M., Carpenter, J. W., & Reed, C. (1989). Translocation as a species conservation tool: status and strategy. *Science(Washington), 245*(4917), 477-480.

Grubb, P. (1993). Order Artiodactyla. *Mammal species of the world: a taxonomic and geographic reference. 2nd revised edition.*, 377-414.

Gudmundsson, G. A., Benvenuti, S., Alerstam, T., Papi, F., Lilliendahl, K., & Akesson, S. (1995). Examining the limits of flight and orientation performance - satellite tracking of Brent geese migrating across the greenland ice-cap. *Proceedings of the Royal Society B-Biological Sciences, 261*(1360), 73-79.

Guha, S., Goyal, S. P., & Kashyap, V. K. (2006). Genomic variation in the mitochondrially encoded cytochrome b (MT-CYB) and 16S rRNA (MT-RNR2) genes: characterization of eight endangered Pecoran species. *Animal Genetics, 37*(3), 262-265.

Handley, L. J. L., & Perrin, N. (2007). Advances in our understanding of mammalian sex-biased dispersal. *Molecular Ecology, 16*(8), 1559-1578.

Hansen, M. M., Meier, K., & Mensberg, K.-L. D. (2010). Identifying footprints of selection in stocked brown trout populations: a spatio-temporal approach. *Molecular Ecology, 19*(9), 1787-1800.

Happe, P. J., Jenkins, K. J., Starkey, E. E., & Sharrow, S. H. (1990). Nutritional quality and tannin astringency of browse in clear-cuts and old-growth forests. *Journal of Wildlife Management, 54*(4), 557-566.

Hartl, G. B., Klein, F., Willing, R., Apollonio, M., & Lang, G. (1995). Allozymes and the genetics of antler development in Red deer (*Cervus-elaphus*). *Journal of Zoology, 237*, 83-100.

Hartl, G. B., Lang, G., Klein, F., & Willing, R. (1991). Relationships between allozymes, heterozygosity and morphological characters in Red deer (*Cervus elaphus*), and the influence of selective hunting on allele frequency-distributions. *Heredity, 66*, 343-350.

Hartl, G. B., & Pucek, Z. (1994). Genetic depletion in the European bison (*Bison bonasus*) and the significance of electrophoretic heterozygosity for conservation. *Conservation biology, 8*(1), 167-174.

Hartl, G. B., Willing, R., Lang, G., Klein, F., & Koller, J. (1990). Genetic-variability and differentiation in Red deer (*Cervus elaphus L*) of central-europe. *Genetics Selection Evolution, 22*(3), 289-306.

Hartl, G. B., Zachos, F., & Nadlinger, K. (2003). Genetic diversity in European red deer (*Cervus elaphus L.*): anthropogenic influences on natural populations. *Comptes Rendus Biologies, 326*, S37-S42.

Heath, D. D., Busch, C., Kelly, J., & Atagi, D. Y. (2002). Temporal change in genetic structure and effective population size in steelhead trout (*Oncorhynchus mykiss*). *Molecular Ecology, 11*(2), 197-214.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B-Biological Sciences, 270*(1512), 313-321.

Hedmark, E., & Ellegren, H. (2005). Microsatellite genotyping of DNA isolated from claws left on tanned carnivore hides. *International journal of legal medicine, 119*(6), 370-373.

Herzog, A. (1995). Zur genetischen Struktur isolierter Rotwildpopulationen. *Gemeinsame Lösungsansätze zum Rotwildmanagement in Bayern, Hessen und Thüringen. Schriftenreihe des Landesjagdverbandes Bayern eV, 1*, 31-38.

Herzog, S., Hunger, M., & Krüger, T. (2010). Optimierung der Situation des Rot-wildes (*Cervus elaphus*) durch einen landesweiten partizipativen Prozess: Eckpunkte für ein Rotwildkonzept im Freistaat Sachsen. *Aktuelle Beiträge zur Wildökologie und Jagdwirtschaft in Brandenburg*, 107.

Hillen, J., Kiefer, A., & Veith, M. (2009). Foraging site fidelity shapes the spatial organisation of a population of female western barbastelle bats. *Biological Conservation, 142*(4), 817-823.

Hoffmann, G. S., & Griebeler, E. M. (2013). An improved high yield method to obtain microsatellite genotypes from red deer antlers up to 200years old. *Molecular Ecology Resources, 13*(3), 440-446.

Hoffmann, G. S., Johannesen, J., & Griebeler, E. M. (2014). Species cross-amplification, identification and genetic variation of 17 species of deer (Cervidae) with microsatellite and mitochondrial DNA from antlers. *Molecular biology reports*, 1-9.

Hoffmann, G. S., Johannesen, J., & Griebeler, E. M. (2016). Population dynamics of a natural red deer population over 200 years detected via substantial changes of genetic variation. *Ecology and evolution, 6*(10), 3146-3153.

Hofmann, R. (1989). Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. *Oecologia, 78*(4), 443-457.

Hofreiter, M., Serre, D., Poinar, H. N., Kuch, M., & Pääbo, S. (2001). Ancient DNA. *Nature Reviews Genetics, 2*(5), 353-359.

Hooge, P., & Eichenlaub, B. (1997). Animal movement extension to ArcView (version 1.1). *Alaska Biological Science Center, United States Geological Survey, Anchorage*.

Höss, M., & Pääbo, S. (1993). DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic acids research, 21*(16), 3913-3914.

Janis, C. M., & Scott, K. M. (1987). The interrelationships of higher ruminant families with special emphasis on the members of the cervoidea. *American Museum Novitates*(2893), 1-86.

Jarnemo, A. (2008). Seasonal migration of male red deer (*Cervus elaphus*) in southern Sweden and consequences for management. *European Journal of Wildlife Research, 54*(2), 327-333.

Jeppesen, J. L. (1987). Impact of human disturbance on home range, movements and activity of red deer (*Cervus elaphus*) in a Danish environment. *Danish Review of Game Biology, 13*.

Johannesen, J., & Lubin, Y. (1999). Group founding and breeding structure in the subsocial spider Stegodyphus lineatus (Eresidae). *Heredity, 82*(6), 677-686.

Johnson, C. J., Parker, K. L., Heard, D. C., & Gillingham, M. P. (2002). A multiscale behavioral approach to understanding the movements of woodland caribou. *Ecological Applications, 12*(6), 1840-1860.

Johnson, M. S., & Black, R. (1984). The Wahlund effect and the geographical scale of variation in the intertidal limpet siphonaria sp. *Marine Biology, 79*(3), 295-302.

Kalinowski, S. T. (2005). HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes, 5*(1), 187-189.

Kalz, B., Jewgenow, K., & Fickel, J. (2006). Structure of an otter (*Lutra lutra*) population in Germany - results of DNA and hormone analyses from faecal samples. *Mammalian Biology, 71*(6), 321-335.

Kodric-Brown, A., & Brown, J. H. (1984). Truth in advertising: the kinds of traits favored by sexual selection. *The American Naturalist, 124*(3), 309-323.

Koubek, P., & Hrabe, V. (1996). Home range dynamics in the red deer (*Cervus elaphus*) in a mountain forest in central Europe. *Folia Zoologica, 45*(3), 219-222.

Krings, M., Stone, A., Schmitz, R. W., Krainitzki, H., Stoneking, M., & Pääbo, S. (1997). Neandertal DNA sequences and the origin of modern humans. *cell, 90*(1), 19-30.

Kruuk, L. E., Merilä, J., & Sheldon, B. C. (2001). Phenotypic selection on a heritable size trait revisited. *The American Naturalist, 158*(6), 557-571.

Kruuk, L. E., Slate, J., Pemberton, J. M., Brotherstone, S., Guinness, F., Clutton-Brock, T., & Houle, D. (2002). Antler size in red deer: heritability and selection but no evolution. *Evolution, 56*(8), 1683-1695.

Kuehn, R., Haller, H., Schroeder, W., & Rottmann, O. (2004). Genetic roots of the red deer (*Cervus elaphus*) population in eastern Switzerland. *Journal of Heredity, 95*(2), 136-143.

Kuehn, R., Schroeder, W., Pirchner, F., & Rottmann, O. (2003). Genetic diversity, gene flow and drift in Bavarian red deer populations (*Cervus elaphus*). *Conservation Genetics, 4*(2), 157-166.

Kuo, C. H., & Janzen, F. J. (2003). Bottlesim: A bottleneck simulation program for long-lived species with overlapping generations. *Molecular Ecology Notes, 3*(4), 669-673.

Larsson, K., van der Jeugd, H. P., van der Veen, I. T., & Forslund, P. (1998). Body size declines despite positive directional selection on heritable size traits in a barnacle goose population. *Evolution*, 1169-1184.

Leimgruber, P., McShea, W. J., Brookes, C. J., Bolor-Erdene, L., Wemmer, C., & Larson, C. (2001). Spatial patterns in relative primary productivity and gazelle migration in the Eastern Steppes of Mongolia. *Biological Conservation, 102*(2), 205-212.

Li, C. Y., & Suttie, J. M. (2001). Deer antlerogenic periosteum: a piece of postnatally retained embryonic tissue? *Anatomy and Embryology, 204*(5), 375-388.

Lille, R. (1996). Zur Bedeutung von Bracheflächen für die Avifauna der Agrarlandschaft: eine nahrungsökologische Studie an der Goldammer (*Emberiza citrinella*). *XA-DE*: Haupt.

Lower, V. P. W., & Gardiner, A. S. (1975). Hybridization between red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) with particular reference to stocks in nw england. *Journal of Zoology, 177*(DEC), 553-566.

Ludt, C. J., Schroeder, W., Rottmann, O., & Kuehn, R. (2004). Mitochondrial DNA phylogeography of red deer (*Cervus elaphus*). *Molecular Phylogenetics and Evolution, 31*(3), 1064-1083.

MacHugh, D., Edwards, C., Bailey, J., Bancroft, D., & Bradley, D. (2000). The extraction and analysis of ancient DNA from bone and teeth: a survey of current methodologies. *Anc. Biomol, 3*, 81-102.

Mailand, C., & Wasser, S. K. (2007). Isolation of DNA from small amounts of elephant ivory. *Nature Protocols, 2*(9), 2228-2232.

Mainka, S. A., & Mills, J. A. (1995). Wildlife and traditional Chinese medicine - Supply and demand for wildlife species. *Journal of Zoo and Wildlife Medicine, 26*(2), 193-200.

Martinez, J. G., Carranza, J., Fernandez-Garcia, J. L., & Sanchez-Prieto, C. B. (2002). Genetic variation of red deer populations under hunting exploitation in southwestern Spain. *Journal of Wildlife Management, 66*(4), 1273-1282.

Meyer, C. P., & Paulay, G. (2005). DNA barcoding: Error rates based on comprehensive sampling. *Plos Biology, 3*(12), 2229-2238.

Miller, F. L. (1970). Distribution patterns of black-tailed deer (*Odocoileus hemionus columbianus*) in relation to environment. *Journal of Mammalogy, 51*(2), 248-&.

Milner, J., Albon, S., Illius, A., Pemberton, J., & Clutton-Brock, T. (1999). Repeated selection of morphometric traits in the Soay sheep on St Kilda. *Journal of Animal Ecology, 68*(3), 472-488.

Mohr, C. O. (1947). Table of equivalent populations of North American small mammals. *American Midland Naturalist, 37*(1), 223-249.

Moreau, M., Siebert, S., Buerkert, A., & Schlecht, E. (2009). Use of a tri-axial accelerometer for automated recording and classification of goats' grazing behaviour. *Applied Animal Behaviour Science, 119*(3), 158-170.

Morreale, S. J., Standora, E. A., Spotila, J. R., & Paladino, F. V. (1996). Migration corridor for sea turtles. *Nature, 384*(6607), 319-320.

Mysterud, A., & Bischof, R. (2010). Can compensatory culling offset undesirable evolutionary consequences of trophy hunting? *Journal of Animal Ecology, 79*(1), 148-160.

Nathan, R., Spiegel, O., Fortmann-Roe, S., Harel, R., Wikelski, M., & Getz, W. M. (2012). Using tri-axial acceleration data to identify behavioral modes of free-ranging animals: general concepts and tools illustrated for griffon vultures. *Journal of Experimental Biology, 215*(6), 986-996.

Nei, M., Maruyama, T., & Chakraborty, R. (1975). Bottleneck effect and genetic-variability in populations. *Evolution, 29*(1), 1-10.

Nelson, M. E. (1993). Natal dispersal and gene flow in white-tailed deer in northeastern minnesota. *Journal of Mammalogy, 74*(2), 316-322.

Niedzialkowska, M., Jedrzejewska, B., Honnen, A.-C., Otto, T., Sidorovich, V. E., Perzanowski, K., Zachos, F. E. (2011). Molecular biogeography of red deer Cervus elaphus from eastern Europe: insights from mitochondrial DNA sequences. *Acta Theriologica, 56*(1), 1-12.

Nielsen, E. E., Hansen, M. M., & Loeschcke, V. (1999). Analysis of DNA from old scale samples: technical aspects, applications and perspectives for conservation. *Hereditas, 130*(3), 265-276.

Nussey, D., Coltman, D., Coulson, T., Kruuk, L., Donald, A., Morris, S., Pemberton, J. (2005). Rapidly declining fine-scale spatial genetic structure in female red deer. *Molecular Ecology, 14*(11), 3395-3405.

Nussey, D., Pemberton, J., Donald, A., & Kruuk, L. (2006). Genetic consequences of human management in an introduced island population of red deer (*Cervus elaphus*). *Heredity, 97*(1), 56-65.

Nussey, D. H., Coltman, D. W., Coulson, T., Kruuk, L. E. B., Donald, A., Morris, S. J., Pemberton, J. (2005). Rapidly declining fine-scale spatial genetic structure in female red deer. *Molecular Ecology, 14*(11), 3395-3405.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Despres, V., Hebler, J., Rohland, N., Hofreiter, M. (2004). Genetic analyses from ancient DNA. *Annual Review of Genetics, 38*, 645-679.

Paetkau, D. (2003). An empirical exploration of data quality in DNA-based population inventories. *Molecular Ecology, 12*(6), 1375-1387.

Pamperin, N. J., Follmann, E. H., & Person, B. T. (2008). Sea-ice use by arctic foxes in northern Alaska. *Polar Biology, 31*(11), 1421-1426.

Parker, K. L., Robbins, C. T., & Hanley, T. A. (1984). Energy expenditures for locomotion by mule deer and elk. *Journal of Wildlife Management, 48*(2), 474-488.

Perez-gonzalez, J., Mateos, C., & Carranza, J. (2009). Polygyny can increase rather than decrease genetic diversity contributed by males relative to females: evidence from red deer. *Molecular Ecology, 18*(8), 1591-1600.

Perrin, N., & Mazalov, V. (1999). Dispersal and inbreeding avoidance. *American Naturalist, 154*(3), 282-292.

Perrin, N., & Mazalov, V. (2000). Local competition, inbreeding, and the evolution of sex-biased dispersal. *American Naturalist, 155*(1), 116-127.

Pertoldi, C., Hansen, M. M., Loeschcke, V., Madsen, A. B., Jacobsen, L., & Baagoe, H. (2001). Genetic consequences of population decline in the European otter (*Lutra lutra*): an assessment of microsatellite DNA variation in Danish otters from 1883 to 1993. *Proceedings of the Royal Society B-Biological Sciences, 268*(1478), 1775-1781.

Petrak, M. (1996). Der Mensch als Störgröße in der Umwelt des Rothirsches (*Cervus elaphus L.*). *Zeitschrift für Jagdwissenschaft, 42*(3), 180-194.

Piggott, M. P., & Taylor, A. C. (2003). Remote collection of animal DNA and its applications in conservation management and understanding the population biology of rare and cryptic species. *Wildlife Research, 30*(1), 1-13.

Piry, S., Alapetite, A., Cornuet, J. M., Paetkau, D., Baudouin, L., & Estoup, A. (2004). GENECLASS2: A software for genetic assignment and first-generation migrant detection. *Journal of Heredity, 95*(6), 536-539.

Pitra, C., Fickel, J., Meijaard, E., & Groves, P. C. (2004). Evolution and phylogeny of old world deer. *Molecular Phylogenetics and Evolution, 33*(3), 880-895.

Poetsch, M., Seefeldt, S., Maschke, M., & Lignitz, E. (2001). Analysis of microsatellite polymorphism in red deer, roe deer, and fallow deer - possible employment in forensic applications. *Forensic Science International, 116*(1), 1-8.

Poinar, H. N., & Cooper, A. (2000). Ancient DNA: do it right or not at all. *Science, 5482*, 1139.

Polovina, J. J., Kobayashi, D. R., Parker, D. M., Seki, M. P., & Balazs, G. H. (2000). Turtles on the edge: movement of loggerhead turtles (*Caretta caretta*) along oceanic fronts, spanning longline fishing grounds in the central North Pacific, 1997-1998. *Fisheries Oceanography, 9*(1), 71-82.

Pompanon, F., Bonin, A., Bellemain, E., & Taberlet, P. (2005). Genotyping errors: causes, consequences and solutions. *Nature Reviews Genetics, 6*(11), 847-846.

Prince, P. A., Wood, A. G., Barton, T., & Croxall, J. P. (1992). Satellite tracking of wandering albatrosses (*Diomedea exulans*) in the south-atlantic. *Antarctic Science, 4*(1), 31-36.

Probst, J. R., & Crow, T. R. (1991). Integrating biological diversity and resource-management. *Journal of Forestry, 89*(2), 12-17.

Przybylo, R., Sheldon, B. C., & Merilä, J. (2000). Climatic effects on breeding and morphology: evidence for phenotypic plasticity. *Journal of animal ecology, 69*(3), 395-403.

Raesfeld, F. v., & Reulecke, K. (1988). Das Rotwild. *Naturgeschichte, Hege, Jagdausübung.[Red deer. Natural history, management and hunting]. Verlag Paul Parey, Berlin*.

Raesfeld, F. v., & Wagner, K. (1911). Das Rotwild: Naturbeschreibung, Hege und Jagd des heimischen Edelwildes in freier Wildbahn.

Rankin, D. R., Narveson, S. D., Birkby, W. H., & Lai, J. (1996). Restriction fragment length polymorphism (RFLP) analysis on DNA from human compact bone. *Journal of Forensic Sciences, 41*(1), 40-46.

Rasmussen, D. I. (1979). Sibling clusters and genotypic frequencies. *American Naturalist*, 948-951.

Rivrud, I. M., Sonkoly, K., Lehoczki, R., Csanyi, S., Storvik, G. O., & Mysterud, A. (2013). Hunter selection and long-term trend (1881-2008) of red deer trophy sizes in Hungary. *Journal of Applied Ecology, 50*(1), 168-180.

Rohland, N., & Hofreiter, M. (2007). Ancient DNA extraction from bones and teeth. *Nature protocols, 2*(7), 1756-1762.

Rousset, F. (2008). GENEPOP ' 007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources, 8*(1), 103-106.

Roy, M. S., Girman, D. J., Taylor, A. C., & Wayne, R. K. (1994). The use of museum specimens to reconstruct the genetic-variability and relationships of extinct populations. *Experientia, 50*(6), 551-557.

Samuel, M. D., Pierce, D. J., & Garton, E. O. (1985). Identifying areas of concentrated use within the Home range. *Journal of Animal Ecology, 54*(3), 711-719.

Sandell, M., & Liberg, O. (1992). Roamers and stayers: a model on male mating tactics and mating systems. *The American Naturalist, 139*(1), 177-189.

Schaerlaekens, D. G., Dekker, W., Wickström, H., Volckaert, F. A., & Maes, G. E. (2011). Extracting a century of preserved molecular and population demographic data from

archived otoliths in the endangered European eel (*Anguilla anguilla L.*). *Journal of Experimental Marine Biology and Ecology, 398*(1), 56-62.

Schmidt, K. (1993). Winter ecology of nonmigratory Alpine red deer. *Oecologia, 95*(2), 226-233.

Schreiber, A., Klein, F., & Lang, G. (1994). Transferrin polymorphism of red deer in france - evidence for spatial genetic microstructure of an autochthonous herd. *Genetics Selection Evolution, 26*(3), 187-203.

Schuelke, M. (2000). An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology, 18*(2), 233-234.

Senn, H. V., & Pemberton, J. M. (2009). Variable extent of hybridization between invasive sika (*Cervus nippon*) and native red deer (*Cervus elaphus*) in a small geographical area. *Molecular Ecology, 18*(5), 862-876.

Skog, A., Zachos, F. E., Rueness, E. K., Feulner, P. G. D., Mysterud, A., Langvatn, R., Jakobsen, K. S. (2009). Phylogeography of red deer (*Cervus elaphus*) in Europe. *Journal of Biogeography, 36*(1), 66-77.

Szemethy, L., Matrai, K., Biro, Z., & Katona, K. (2003). Seasonal home range shift of red deer in a forest-agriculture area in southern Hungary. *Acta Theriologica, 48*(4), 547-556.

Szemethy, L., Ritter, D., & Heltai, M. (1994). Preliminary results of radio-tracking of red deer (*Cervus elaphus*) in Hungary. *Recent Developments in Deer Biology. Macaulay Land Research Institute, Aberdeen, UK*, 281.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Tarroux, A., Berteaux, D., & Bety, J. (2010). Northern nomads: ability for extensive movements in adult arctic foxes. *Polar Biology, 33*(8), 1021-1026.

Taylor, A., Sherwin, W., & Wayne, R. (1994). Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat Lasiorhinus krefftii. *Molecular Ecology, 3*(4), 277-290.

Teletchea, F., Bernillon, J., Duffraisse, M., Laudet, V., & Hanni, C. (2008). Molecular identification of vertebrate species by oligonucleotide microarray in food and forensic samples. *Journal of Applied Ecology, 45*(3), 967-975.

Tenhumberg, B., Tyre, A. J., Pople, A. R., & Possingham, H. P. (2004). Do harvest refuges buffer kangaroos against evolutionary responses to selective harvesting? *Ecology, 85*(7), 2003-2017.

Tregenza, T., & Wedell, N. (2000). Genetic compatibility, mate choice and patterns of parentage: invited review. *Molecular Ecology, 9*(8), 1013-1027.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., & Shipley, P. (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes, 4*(3), 535-538.

Vanhorne, B., Hanley, T. A., Cates, R. G., McKendrick, J. D., & Horner, J. D. (1988). Influence of seral stage and season on leaf chemistry of southeastern alaska deer forage. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere, 18*(1), 90-99.

Vincent, J. P., Bideau, E., Hewison, A. J. M., & Angibault, J. M. (1995). The influence of increasing density on body-weight, kid production, home-range and winter grouping in roe deer (*Capreolus capreolus*). *Journal of Zoology, 236*, 371-382.

Wandeler, P., Hoeck, P. E. A., & Keller, L. F. (2007). Back to the future: museum specimens in population genetics. *Trends in Ecology & Evolution, 22*(12), 634-642.

Willerslev, E., & Cooper, A. (2005). Review paper. ancient dna. *Proceedings of the Royal Society of London B: Biological Sciences, 272*(1558), 3-16.

Wolff, J. O., & Van Horn, T. (2003). Vigilance and foraging patterns of American elk during the rut in habitats with and without predators. *Canadian Journal of Zoology-Revue Canadienne De Zoologie, 81*(2), 266-271.

Woods, J. G., Paetkau, D., Lewis, D., McLellan, B. N., Proctor, M., & Strobeck, C. (1999). Genetic tagging of free-ranging black and brown bears. *Wildlife Society Bulletin, 27*(3), 616-627.

Worton, B. J. (1989). Kernel methods for estimating the utilization distribution in home-range studies. *Ecology, 70*(1), 164-168.

Yan, D., Luo, J. Y., Han, Y. M., Peng, C., Dong, X. P., Chen, S. L., Xiao, X. H. (2013). Forensic DNA Barcoding and Bio-Response Studies of Animal Horn Products Used in Traditional Medicine. *Plos One, 8*(2).

Zachos, F. E., Althoff, C., von Steynitz, Y., Eckert, I., & Hartl, G. B. (2007). Genetic analysis of an isolated red deer (*Cervus elaphus*) population showing signs of inbreeding depression. *European Journal of Wildlife Research, 53*(1), 61-67.

Zachos, F. E., & Hartl, G. B. (2011). Phylogeography, population genetics and conservation of the European red deer (*Cervus elaphus*). *Mammal Review, 41*(2), 138-150.

16. Anhang

Figure S 3.1 Plots of five microsatellite loci obtained from Genemapper (Applied Biosystems, Version 4.0) using RNAse free water instead of DNA (controls).

Figure S 3.2 Plots of five microsatellite loci obtained from Genemapper (ABI, Version 4.0) using human DNA from the originator of the samples.

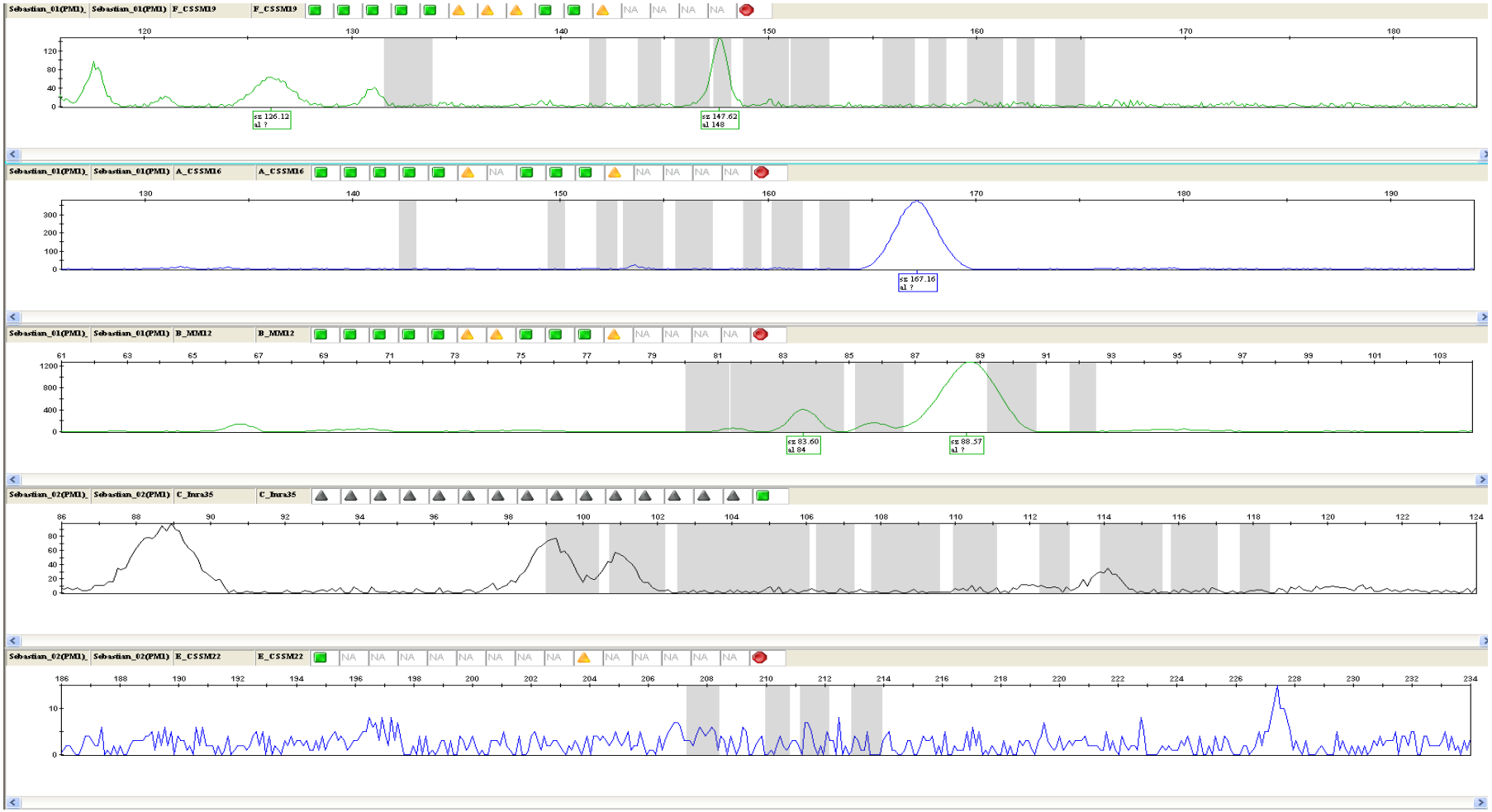


Figure S 3.3 Plots of five microsatellite loci obtained from Genemapper (Applied Biosystems, Version 4.0) using DNA from muscle tissue.

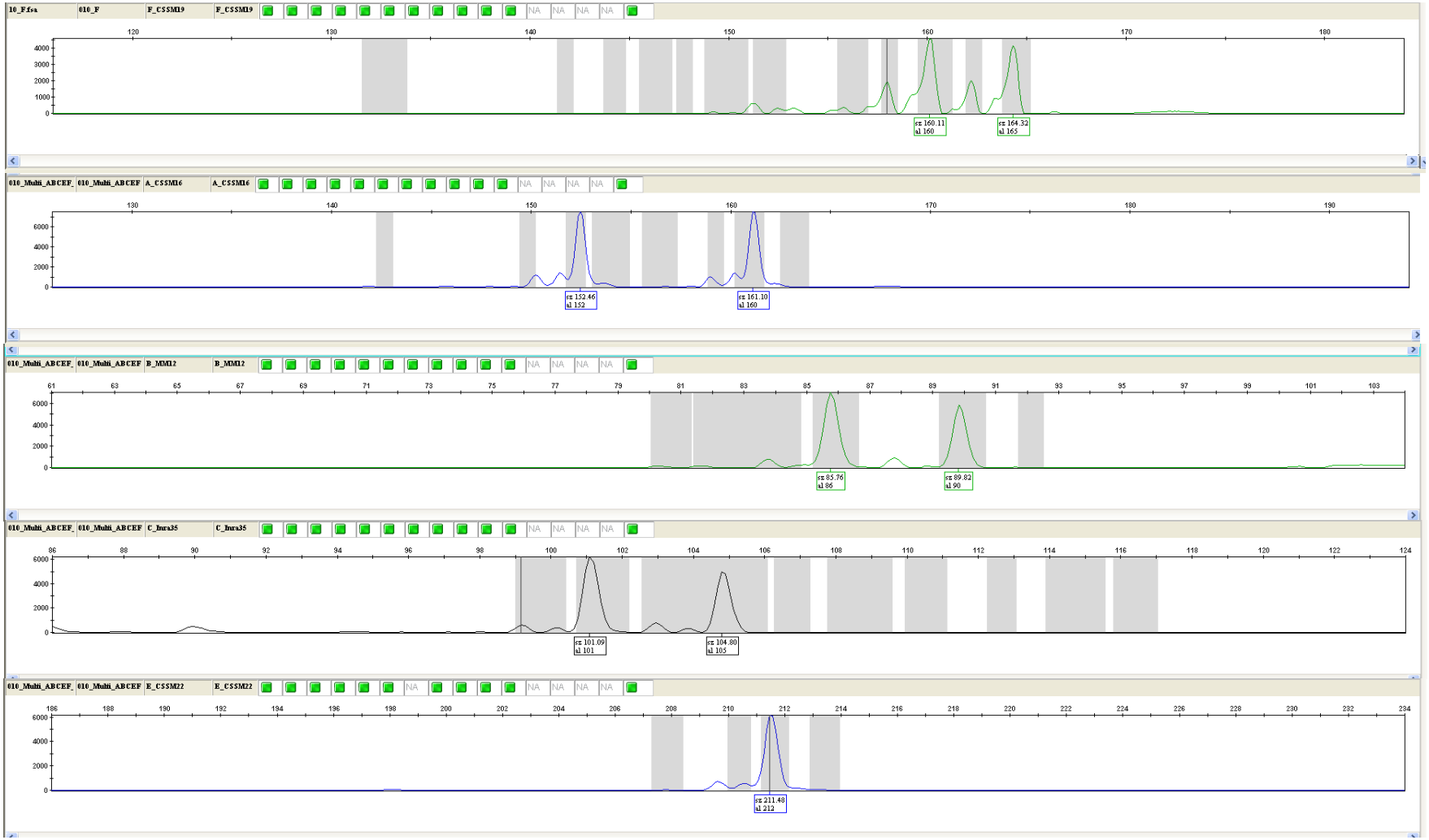


Figure S 3.4 Plots of five microsatellite loci obtained from Genemapper (Applied Biosystems, Version 4.0) using DNA from antler material. This is our oldest sample (199 years).

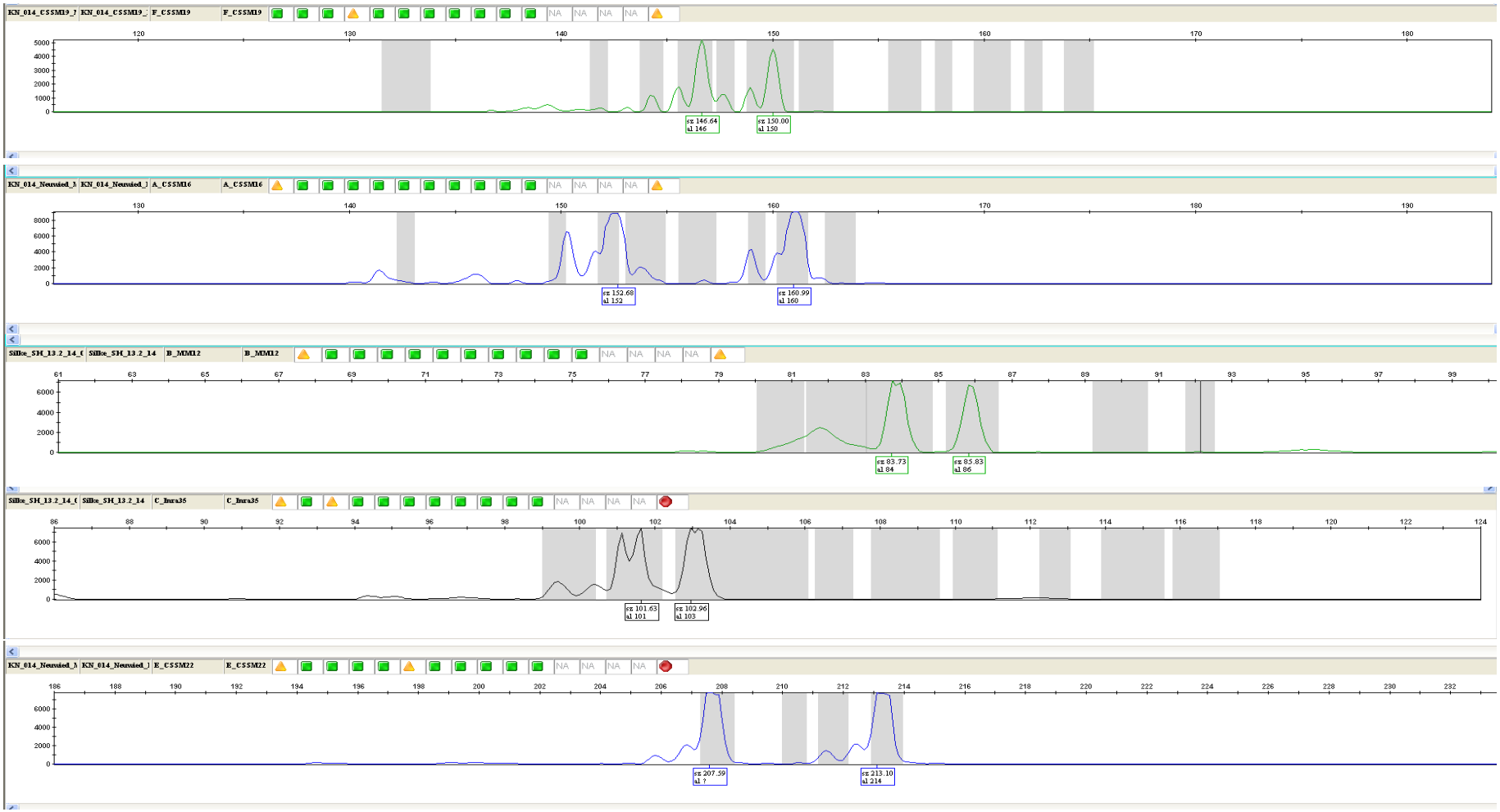
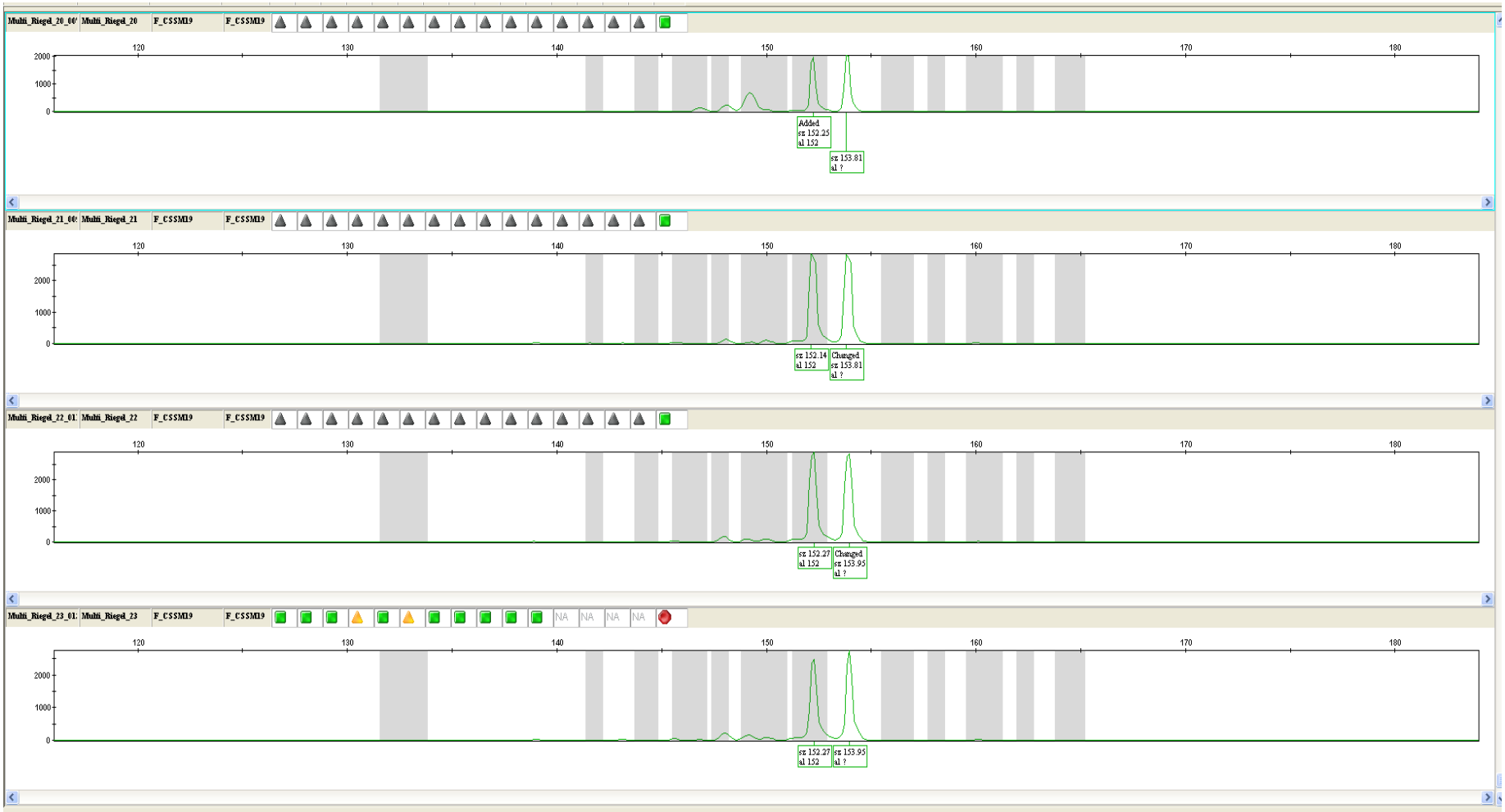


Figure S 3.5 Plots of five microsatellite loci obtained from Genemapper (Applied Biosystems, Version 4.0) using DNA from four shed antlers of one individual. Each plot shows the results of one locus.




Sample File
Sample Name
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Marker
OS
SHP
OBA
SPA
SP
BIN
PHR
LPH
SPU
AN
BD
DP
NB
CC
OVL
GQ
Multi_Riegel_19_00
Multi_Riegel_19
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A_CSSM16
NA
130
140
150
160
170
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4000
2000
0
sz 152.04
al 152
sz 153.73
al 154
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Multi_Riegel_20
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sz 152.14
al 152
sz 153.81
al 154
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Multi_Riegel_21
A_CSSM16
A_CSSM16
sz 152.03
al 152
sz 153.69
al 154
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Multi_Riegel_22
A_CSSM16
A_CSSM16
sz 152.04
al 152
sz 153.73
al 154


Sample File
Sample Name
Panel
Marker
OS
SMP
OBA
SPA
SP
BIN
PHR
LPH
SPU
AN
BD
DP
NB
CC
OVL
GQ
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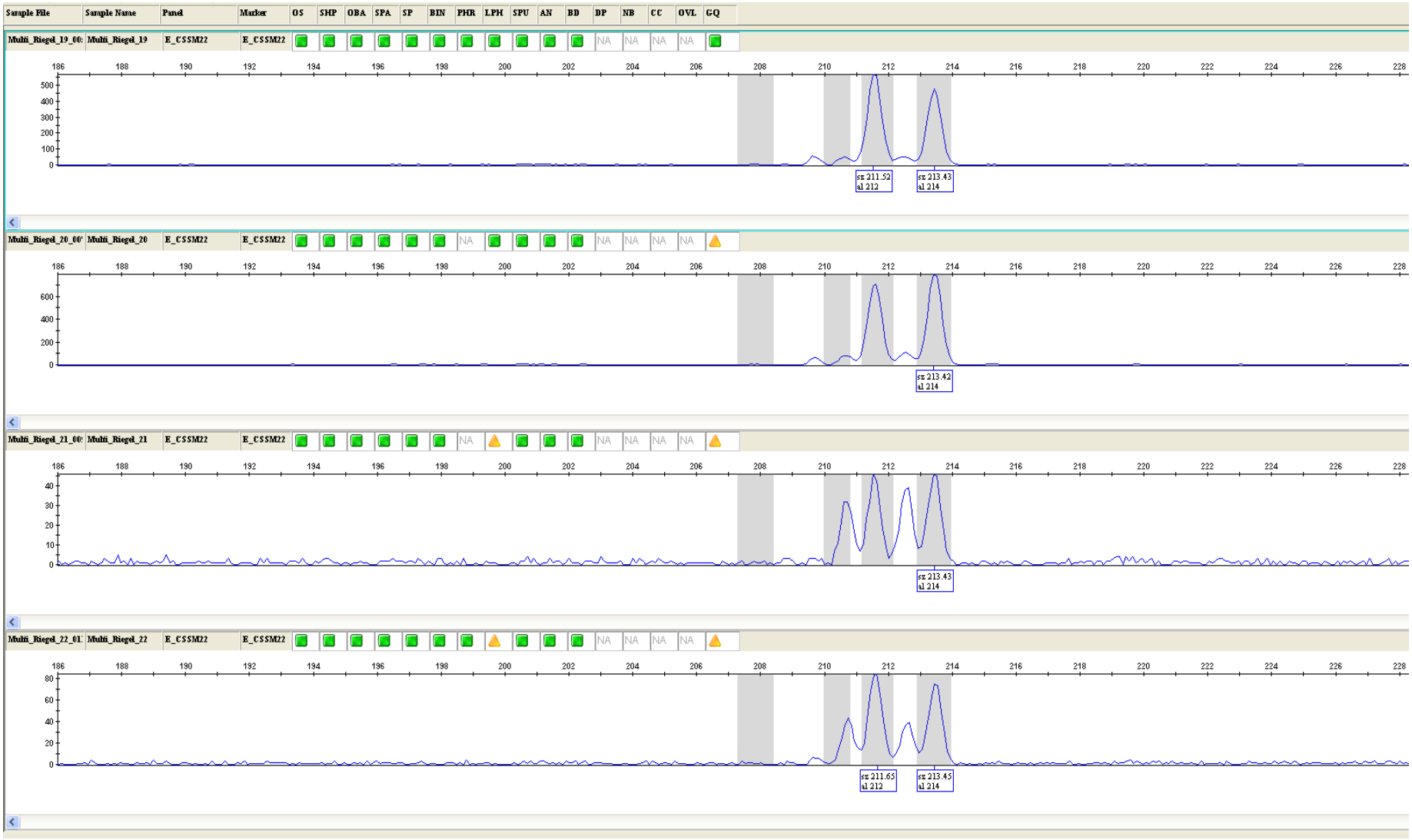
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al 214
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Multi_Riegel_20
sz 213.42
al 214
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Multi_Riegel_21
sz 213.43
al 214
Multi_Riegel_22_01
Multi_Riegel_22
sz 211.65
al 212
sz 213.45
al 214


Sample File
Sample Name
Panel
Marker
OS
SHP
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AN
BD
DP
NB
CC
OVL
GQ
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Multi_Riegel_20
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C_Inra35
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sz 110.41
al 111
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Multi_Riegel_21
sz 106.75
al 107
sz 110.41
al 111
Multi_Riegel_22_01
Multi_Riegel_22
sz 106.76
al 107
sz 110.53
al 111
Multi_Riegel_23_01
Multi_Riegel_23
sz 106.68
al 107
sz 110.46
al 111

Table S 3.1 DNA amounts of samples from different parts of the antler.

Part	**DNA amount [ng µl^{-1}]**
Base	346,52
Middle	84,50
End	159,57
Marrow	196,60

Table S 4.1 Number of 16S sequences found in GenBank (search date 29.05.2013) and percent sequence identity for each of the examined species.

Species	**Number of sequences in GenBank**	**Identity in percent (Number of sequences)**
1. Rangifer tarandus	8	100 % (4); 99 % (4)
2. Elaphurus davidianus	2	100 % (2)
3. Cervus albirostris	2	99 % (1); 98 % (1)
4. Cervus duvaucelii	3	99 % (2); 100 % (1)
5. Rucervus eldii	6	99 % (6)
6. Dama mesopotamica	1	100 % (1)
7. Odocoileus virginianus	11	100 % (6); 99 % (5)
8. Cervus elaphus bactrianus	No data	
9. Axis porcinus	3	99 % (2); 98 % (1)
10. Rusa timorensis	1	99 % (1)
11. Rusa unicolor	11	99 % (11)
12. Cervus nippon pseudaxis	No data	
13. Odocoileus hemionus	7	100 % (1); 99 % (5); 98 % (1)
14. Cervus canadensis sibiricus	No data	
15. Cervus canadensis nannodes	No data	
16. Cervus canadensis	No data	
17. Cervus nippon mantschuricus	No data	
18. Alces alces	3	99 % (3)
19. Dama dama	2	100 % (2)
20. Capreolus capreolus	4	100 % (2); 99 % (1); 97 % (1)

Table S 4.2 DNA concentration of each studied individual measured with a NanoDrop1000 photometer.

Sample ID	Species	DNA concentration [ng/ml]
1	*Rangifer tarandus*	86.64
2	*Rangifer tarandus*	44.26
3	*Rangifer tarandus*	31.06
4	*Elaphurus davidianus*	122.49
5	*Elaphurus davidianus*	175.26
6	*Elaphurus davidianus*	175.47
7	*Cervus albirostris*	288.28
8	*Cervus albirostris*	215.35
9	*Cervus duvaucelii*	82.49
10	*Cervus duvaucelii*	81.65
11	*Cervus duvaucelii*	88.81
12	*Rucervus eldii (Burma)*	509.26
13	*Dama mesopotamica*	1046.23
14	*Dama mesopotamica*	36.7
15	*Odocoileus virginianus*	248.79
16	*Odocoileus virginianus*	61.94
17	*Rucervus eldii*	79.82
18	*Rucervus eldii*	229.79
19	*Cervus elaphus bactrianus*	166.58
20	*Cervus elaphus bactrianus*	38.04
21	*Axis porcinus*	40.63
22	*Axis porcinus*	58.22
23	*Rusa timorensis*	238.6
24	Rusa timorensis	67.03
25	*Rusa unicolor*	88.24
26	*Rusa unicolor*	158.71
27	*Cervus nippon pseudaxis*	86.9

28	*Cervus nippon pseudaxis*	894.95
29	*Odocoileus hemionus*	509.67
30	*Cervus canadensis sibiricus*	48.48
31	*Cervus canadensis nannodes*	71.92
32	*Cervus canadensis nannodes*	493.93
33	*Cervus canadensis*	879.24
34	*Cervus canadensis*	194.16
35	*Cervus nippon mantschuricus*	812.06
36	*Alces alces*	974.05
37	*Dama dama*	42.84
38	*Capreolus capreolus*	113.15
39	Cervus elaphus	67.46
40	Cervus elaphus	85.32
41	Cervus elaphus	67.69
42	Cervus elaphus	91.02

Table S 4.3 Name, sequence and repeat motif of used microsatellite and 16S rDNA primers.

Primername	Primer-sequences 5´-3´	reference	Repeat motif
BM1818	F agtgctttcaaggtccatgc R agctgggaatataaccaaagg	Kuehn et al. (2003)	Dinucleotide
CSSM14	F aaatgacctctcaatggaagcttg R gaattctggcacttaataggattca	Kuehn et al. (2003)	Dinucleotide
CSSM16	F agagccacttgttacaccccaaag R gatgcagtctccacttgattcaaa	Kuehn et al. (2003)	Dinucleotide
Haut14	F ccagggaagatgaagtgacc R tgaccttcactcatgttattaa	Kuehn et al. (2003)	Dinucleotide
ILSTS06	F tgtctgtatttctgctgtgg R acacggaagcgatctaaacg	Kuehn et al. (2003)	Dinucleotide
CSPS115	F aaagtgacacaacagcttctccag R aacgagtgtcctagtttggctgtg	Kuehn et al. (2003)	Dinucleotide
MM12	F caagacaggtgtttcaatct R atcgactctggggatgatgt	Kuehn et al. (2003)	Dinucleotide
INRA35	F ttgtgctttatgacactatccg R atcctttgcagcctccacattc	Kuehn et al. (2003)	Dinucleotide
CSSM22	F tctctctaatggagttggtttttg R atatcccactgaggataagaattc	Kuehn et al. (2003)	Dinucleotide
CSSM19	F ttgtcagcaacttcttgtatcttt R tgttttaagccacccaattatttg	Kuehn et al. (2003)	Dinucleotide

Cer14	F tctcttgcgtctcctgcattgac R aatggcacccactccagtattcttc	Kuehn et al. (2003)	Dinucleotide
NVHRT48	F cgtgaatcttaaccaggtct R ggtcagcttcatttagaaac	Poetsch et al. (2001)	Dinucleotide
RT1	F tgccttctttcatccaacaa R catcttcccatcctctttac	Poetsch et al. (2001)	Dinucleotide
RT6	F: ttcctcttactcattcttgg R: cggattttgacactgttac	Poetsch et al. (2001)	Dinucleotide
IOBT965	F ggggttgtgggtaagcggagtt R gatctagcgccagacagacgtgtcat	Kuehn et al. (2003)	Dinucleotide
Bm4107	F: agcccctgctattgtgtgag R: ataggctttgcattgttcagg	Goodman et al. (2001)	Dinucleotide
NVHRT16	F: attctaagcccaaataatctt R: tctaaggggtctgtgtctt	Poetsch et al. (2001)	Dinucleotide
Ca38	F: caacttgtccaaagttgtgc R: taggtggctttgtctctgct	Gaur et al. (2003)	No information
Ca60	F: gcccttcgtacgtacttgtt R: aaagtcagacagagggaggg	Gaur et al. (2003)	No information
16S rRNA	F: cgcctgtttatcaaaaacat R: ctccggtttgaactcagatc	Guha et al. (2006)	

Table S 4.4 Table of amplification success in different deer species. P = polymorph, M = monomorph; in brackets: size range; number of alleles found; NA = no amplification success; last row = total number of successfully amplified loci; in brackets: number of polymorphic loci ([1]Kühn et al. 2003; [2]Poetsch et al. 2001; [3]Goodman et al. 2001).

Locusname	***Alces alces* (N = 1)**	***Capreolus capreolus* (N = 1)**	***Cervus canadensis* (N = 2)**	***Cervus canadensis nannodes* (N = 2)**	***Cervus canadensis sibiricus* (N = 1)**	***Cervus elaphus* (N = 4)**
BM1818[1]	P (249-251, 2)	M (247, 1)	M (243, 1)	M (243, 1)	M (243, 1)	M (243, 1)
CSSM14[1]	M (132, 1)	NA	M (134, 1)	M (134, 1)	M (134, 1)	M (134, 1)
CSSM16[1]	P (159-161, 2)	P (149-161, 2)	M (161, 1)	M (161, 1)	M (155, 1)	P (155-161, 2)
Haut14[1]	M (104, 1)	NA	M (118, 1)	NA[4]	P (108-134, 2)	P (120-132, 2)
ILSTS06[1]	NA	NA	P (283-285, 2)	M (285, 1)	P (285-301, 2)	P (285-291, 3)
CSPS115[1]	M (239, 1)	M (241, 1)	M (239, 1)	M (237, 1)	M (239, 1)	M (237, 1)
MM12[1]	P (80-84, 2)	M (80, 1)	M (86, 1)	P (86-88, 2)	P (86-92, 2)	P (84-88, 3)

INRA35[1]	P (96-100, 2)	M (94, 1)	M (110, 1)	M (96, 1)	P (96-100, 2)	P (96-114, 3)
CSSM22[1]	P (211-215, 2)	P (211-213, 2)	M (211, 1)	M (211, 1)	M (211, 1)	M (211, 1)
CSSM19[1]	P (149-151, 2)	P (125-127, 2)	M (149, 1)	M (149, 1)	M (151, 1)	P (139-155, 3)
CER14[1]	P (217-227, 2)	P (225-229, 2)	P (221-223, 2)	M (231, 1)	P (225-229, 2)	P (215-231, 3)
NVHRT48[2]	P (90-104, 2)	M (107, 1)	NA	M (120, 1)	NA	NA
RT1[2]	M (235, 1)	P (246-256, 2)	M (233, 1)	M (233, 1)	M (229, 1)	P (233-237, 2)
RT6[2]	P (114-128, 2)	M (98, 1)	M (113, 1)	P (119-121, 2)	P (113-119, 2)	P (113-121, 3)
IOBT965[1]	M (110, 1)	M (94, 1)	M (118, 1)	M (131, 1)	NA	P (129-131, 2)
BM4107[3]	NA	M (172, 1)	P (188-190, 2)	M (178, 1)	NA	M (178, 1)
NVHRT16[2]	NA	NA	NA	NA	NA	NA
Number of loci	14 (9)	13 (5)	15 (3)	15 (2)	13 (6)	15 (10)

Locusname	***Cervus nippon mantschuricus* (N = 1)**	***Dama dama* (N = 1)**	***Odocoileus hemionus* (N = 1)**	***Odocoileus virginianus* (N = 2)**	***Rangifer tarandus* (N = 3)**	***Rusa timorensis* (N = 2)**	***Rusa unicolor* (N = 1)**
BM1818	P (241-243, 2)	M (231, 1)	P (239-249, 2)	P (247-251, 3)	P[1] (247-249[2], 2[3])	M (249, 1)	P (231-235, 2)
CSSM14	P (132-134, 2)	M (150, 1)	M (136, 1)	P (136-138, 2)	M (134, 1)	M (148, 1)	P (144-146, 2)
CSSM16	P (159-161, 2)	M (145, 1)	P (159-161, 2)	M (145, 1)	M (147, 1)	M (155, 1)	P (161-165, 2)
Haut14	P (120-122, 2)	M (108, 1)	P (134-136, 2)	P (104-120, 3)	P (130-158, 2)	M (120, 1)	M (126, 1)
ILSTS06	P (281-291, 2)	NA	M (283, 1)	NA	NA[4]	M (297, 1)	P (279-289, 2)
CSPS115	M (233, 1)	M (245, 1)	M (239, 1)	M (237, 1)	P (231-233, 2)	M (239, 1)	M (245, 1)
MM12	M (84, 1)	M (80, 1)	M (80, 1)	M (80, 1)	M (80, 1)	M (80, 1)	M (80, 1)
INRA35	P (96-100, 2)	M (92, 1)	P (110-112, 2)	P (92-120, 2)	P (110-118, 4)	M (110, 1)	M (96, 1)

CSSM22	P (211-217, 2)	M (213, 1)	P (211-217, 2)	P (209-213, 3)	M (213, 1)	M (211, 1)	M (211, 1)
CSSM19	NA[4]	P (159-167, 2)	P (135-152, 2)	P (149-165, 3)	P (137-165, 6)	M (161, 1)	M (153, 1)
CER14	NA	NA	P (207-213, 2)	M (208, 1)	P (209-223, 3)	M (219, 1)	P (201-235, 2)
NVHRT48	NA	M (98, 1)	NA	P (115-119, 2)	P (123-129, 3)	M (130, 1)	NA
RT1	NA	M (233, 1)	M (237, 1)	M (236, 1)	P (226-246, 3)	P (239-241, 2)	M (222, 1)
RT6	NA	P (113-121, 2)	M (99, 1)	M (100, 1)	P (119-121, 2)	P (109-115, 2)	P (113-129, 2)
IOBT965	NA	M (132, 1)	NA	M (132, 1)	M (132, 1)	M (127, 1)	M (130, 1)
BM4107	NA	M (170, 1)	NA	P (158-162, 2)	M (154,1)	NA	M (166, 1)
NVHRT16	NA	NA	NA	NA	P (191-199, 2)	NA	NA
Number of loci	9 (7)	14 (2)	13 (7)	15 (8)	16 (11)[5]	15 (2)	15 (6)

Table S 4.4 Variable sites of the 16S sequence for all species.

Nucleotide position	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**16**	**18**	**19**	**23**	**40**	**42**	**43**	**47**	**73**	**89**	**96**	**104**	**112**	**123**
Rusa unicolor	-	-	-	A	C	T	A	G	T	A	T	G	A	G	C	G	C	C	A	A	C	T	G	T	A
Rusa timorensis	C	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rucervus eldii	-	-	-	-	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Odocoileus hemionus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	T	.	A	.	.
Elaphurus davidianus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Dama mesopotamica	-	-	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus nippon pseudaxis	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.
Cervus elaphus bactrianus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
Cervus duvaucelii	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
Cervus albirostris	C	C	C	.	.	.	T	T	G	T	A	T	G	A	.	.	.	.	.	.	.	.	.	.	.
Alces alces	A	T	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.
Capreolus capreolus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	A	.	.
Dama dama	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.
Cervus canadensis	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Cervus canadensis nannodes	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Cervus canadensis sibiricus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Axis porcinus	-	T	A	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Odocoileus virginianus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	T	.	A	.	.
Rangifer tarandus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	T	.	A	.	.
Cervus nippon mantschuricus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	A	.	.	.	.	.	.	.	.	.
Cervus elaphus	A	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bos taurus	A	T	T	C	.	C	.	.	.	.	.	.	.	.	T	A	.	T	T	.	T	.	A	.	G

	124	**151**	**152**	**170**	**187**	**188**	**190**	**191**	**192**	**193**	**194**	**197**	**230**	**231**	**232**	**234**	**240**	**241**	**242**	**244**	**245**	**247**	**249**	**250**	**252**
Rusa unicolor	C	C	A	T	A	A	A	T	A	T	T	T	T	T	A	C	-	G	A	A	C	A	T	T	C
Rusa timorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Rucervus eldii	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	C	.	T
Odocoileus hemionus	A	.	.	.	.	.	.	C	C	A	.	.	.	.	.	T	-	.	.	.	T	.	.	.	A
Elaphurus davidianus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Dama mesopotamica	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	-	.	.	.	.	.	C	.	.
Cervus nippon pseudaxis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
Cervus elaphus bactrianus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	T	.	.	.	.
Cervus duvaucelii	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	A	.	.	.	T	.	.	.	.
Cervus albirostris	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Alces alces	A	.	.	.	.	.	G	A	.	C	.	.	.	.	.	.	-	.	.	.	T	.	C	.	A
Capreolus capreolus	A	.	C	C	G	.	.	.	.	A	.	.	.	.	.	.	-	.	.	.	T	G	C	.	A
Dama dama	.	T	.	.	.	.	.	.	G	C	C	.	.	.	.	.	-	.	.	T	.	.	.	.	T

Cervus canadensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.				
Cervus canadensis nannodes	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.				
Cervus canadensis sibiricus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	T	.	.	.	.				
Axis porcinus	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	-	.	.	.	T	.	C	C	.					
Odocoileus virginianus	A	.	.	.	.	.	C	C	A	.	.	.	.	.	.	T	-	.	.	.	T	.	.	.	A					
Rangifer tarandus	A	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	-	.	.	.	T	.	.	.	A					
Cervus nippon mantschuricus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.					
Cervus elaphus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	T	.	.	.	.					
Bos taurus	.	.	.	.	.	.	G	C	.	C	A	.	C	C	.	.	G	A	G	.	T	.	.	.	A					

	253	**254**	**255**	**256**	**258**	**263**	**264**	**266**	**269**	**272**	**274**	**278**	**279**	**280**	**283**	**284**	**286**	**287**	**288**	**289**	**292**	**295**	**297**	**307**	**317**
Rusa unicolor	A	T	T	A	C	A	A	C	C	C	C	T	T	A	G	G	T	A	A	C	C	T	G	C	C
Rusa timorensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rucervus eldii	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Odocoileus hemionus	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Elaphurus davidianus	.	.	.	.	T	.	.	.	.	T	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.
Dama mesopotamica	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
Cervus nippon pseudaxis	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus elaphus bactrianus	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus duvaucelii	.	.	.	G	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus albirostris	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Alces alces	.	C	.	.	T	.	G	.	T	T	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.
Capreolus capreolus	.	C	C	.	.	T	.	T	.	T	A	C	.	.	.	.	.	.	.	.	T	.	.	.	.
Dama dama	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	T
Cervus canadensis	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus canadensis nannodes	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus canadensis sibiricus	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Axis porcinus	.	.	C	G	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
Odocoileus virginianus	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Rangifer tarandus	.	C	A	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus nippon mantschuricus	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus elaphus	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bos taurus	.	C	C	.	T	-	.	T	.	.	A	C	C	.	A	.	.	G	G	T	T	C	.	.	T

	319	**320**	**322**	**325**	**326**	**336**	**336**	**336**	**336**	**336**	**336**	**337**	**337**	**337**	**339**	**344**	**344**	**344**	**345**	**345**	**347**	**349**	**349**	**351**	**356**	**356**	
Rusa unicolor		A	G	A	T	G	A	T	A	C	C	T	T	T	A	A	C	C	A	A	G	T	C	A	C	A	
Rusa timorensis		.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	-	
Rucervus eldii		.	.	.	.	.	.	C	.	.	.	A	.	.	T	.	.	.	.	.	.	.	.	.	A	-	
Odocoileus hemionus		G	A	T	C	A	.	.	.	.	.	A	C	.	.	.	T	.	.	.	C	.	.	A	-		
Elaphurus davidianus		.	.	T	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	A	-		

Dama mesopotamica	.	.	C	.	.	.	.	.	.	.	A	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
Cervus nippon pseudaxis	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus elaphus bactrianus	.	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cervus duvaucelii	.	A	T	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	A	-
Cervus albirostris	.	.	T	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
Alces alces	.	A	T	C	.	.	.	G	T	T	A	C	.	-	.	T	.	.	C	.	.	.	.	.	.	.	.	.
Capreolus capreolus	.	A	T	.	A	C	.	G	.	.	A	.	.	-	.	.	.	.	.	.	.	T	G	-	-
Dama dama	.	.	T	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	-	-
Cervus canadensis	.	.	T	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	-	-	-
Cervus canadensis nannodes	.	.	T	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	-	-
Cervus canadensis sibiricus	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	-
Axis porcinus	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	-
Odocoileus virginianus	G	A	T	C	A	.	.	.	.	.	A	C	.	.	.	.	T	.	.	.	C	.	.	.	-
Rangifer tarandus	G	A	T	.	A	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	-
Cervus nippon mantschuricus	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	-	-	-
Cervus elaphus	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bos taurus	.	A	T	C	A	.	.	.	.	.	.	C	C	C	.	.	.	.	.	.	.	T	.	.	G

Table S 4.5 Genbank accession numbers of all our sequences and numbers of our BLAST results.

Species	**Accession number for 16SrRna**	**Accession numbers of BLAST results**
1. Rangifer tarandus	KJ870169	AB245426.1, AY122047.1, DQ318376.1, DQ318375.1, DQ318373.1, DQ318374.1, DQ318372.1, EU078985.1
2. Elaphurus davidianus	KJ870155	JN399997.1, JN632632.1
3. Cervus albirostris	KJ870160	JN632690.1, HM049636.1
4. Cervus duvaucelii	KJ870159	JN632696.1, EU084668.1, DQ989296.1
5. Rucervus eldii	KJ870153	HM138200.1, AY391772.1, AY391771.1, DQ989293.1, AF108041.1
6. Dama mesopotamica	KJ870156	JN632630.1

7. Odocoileus virginianus	KJ870168	HQ332445.1, JN632672.1, JN632671.1, DQ318370.1, DQ318365.1, DQ318363.1, DQ318362.1, DQ318361.1, JN632673.1, M35874.1, JN315624.1
8. Cervus elaphus bactrianus	KJ870158	No data
9. Axis porcinus	KJ870167	JN632600.1, AY391767.1, AY391768.1
10. Rusa timorensis	KJ870152	JN632699.1
11. Rusa unicolor	KJ870151	DQ989636.3, DQ832273.1, M35875.1, AY391769.1, AY391770.1, GQ411195.1, GQ411196.1, JN714134.1, JN714135.1, EU223368
12. Cervus nippon pseudaxis	KJ870157	No data
13. Odocoileus hemionus	KJ870154	DQ318364.1, DQ318368.1, DQ318367.1, DQ318366.1, DQ318369.1, DQ318371.1
14. Cervus canadensis sibiricus	KJ870166	No data
15. Cervus canadensis nannodes	KJ870165	No data
16. Cervus canadensis	KJ870164	No data
17. Cervus nippon mantschuricus	KJ870170	No data
18. Alces alces	KJ870161	JN632595.1, DQ318382.1, JN315627.1
19. Dama dama	KJ870163	JN632629.1, DQ922639.1
20. Capreolus capreolus	KJ870162	JN632610.1, AY122048.1, AY947553.1, KC984213.1

Table S 5.1 List of individuals. Age refers to tissue age. Generation refers to the estimated past generation assuming a generation time of seven years.

Individual	Population	DNA content	Age	Generation
1-17	Young	nm	2011	0
18	Middle-aged	400.5	1940	-11.1
19	Middle-aged	224.7	1923	-13.6
20	Middle-aged	66.2	1930	-12.6
21	Middle-aged	182.6	1937	-11.6
22	Middle-aged	180.7	1933	-12.1
23	Middle-aged	119.8	1930	-12.6
24	Middle-aged	123.7	1934	-12.0
25	Middle-aged	240.6	1932	-12.3
26	Old	67.1	1836	-26.0
27	Old	178.8	1843	-25.0
28	Old	306.3	1827	-27.3
29	Old	93.0	1861	-22.4
30	Old	123.0	1855	-23.3
31	Old	76.1	1813	-29.3

nm = not measured

Table S 5.2 Name, sequence, multiplex system, dilution, annealing temperatures and dye of used microsatellite primers.

Locus	Primer Sequence (5' - 3')	System	Dilution	Annealing Temperature	Dye
Haut14	**F** CCAGGGAAGATGAAGTGACC **R** TGACCTTCACTCATGTTATTAA	2	/	56.0°C	FAM
ILSTS06	**F** TGTCTGTATTTCTGCTGTGG **R** ACACGGAAGCGATCTAAACG	2	/	56.0°C	NED
BM1818	**F** AGTGCTTTCAAGGTCCATGC **R** AGCTGGGAATATAACCAAAGG	2	1:5	56.0°C	HEX
CSSM14	**F** AAATGACCTCTCAATGGAAGCTTG **R** GAATTCTGGCACTTAATAGGATTCA	2	1:3	56.0°C	NED
CSPS115	**F** AAAGTGACACAACAGCTTCTCCAG **R** AACGAGTGTCCTAGTTTGGCTGTG	2	1:2	56.0°C	FAM
CSSM16	**F** AGAGCCACTTGTTACACCCCAAAG **R** GATGCAGTCTCCACTTGATTCAAA	1	1:5	56.0°C	FAM
MM12	**F** CAAGACAGGTGTTTCAATCT **R** ATCGACTCTGGGGATGATGT	1	1:4	56.0°C	HEX
INRA35	**F** TTGTGCTTTATGACACTATCCG **R** ATCCTTTGCAGCCTCCACATTC	1	1:5	56.0°C	NED
CSSM22	**F** TCTCTCTAATGGAGTTGGTTTTTG **R** ATATCCCACTGAGGATAAGAATTC	1	1:3	56.0°C	FAM
CSSM19	**F** TTGTCAGCAACTTCTTGTATCTTT **R** TGTTTTAAGCCACCCAATTATTTG	1	1:3	56.0°C	HEX

Table S 5.3 Primer sequences and annealing temperatures of specific forward and reverse primers for the amplification of the D-Loop region between Pro-tRNA and Phe-tRNA of the mtDNA.

Primer	Primer Sequence (5' – 3')	Annealing Temperature
Forward	ACCCCCTGGAGTGCTAATTT	59.9°C
F2	TTTCATGAGTCAACCCTAAGATC	57.4°C
F3	GGATGCTTGGACTCAGCAAT	60.2°C
F4	TTTCAGGGCCATCTCACCTA	60.5°C
F5	TATCCCGTCCCCTAGATCAC	58.9°C
Reverse	CAGCTTTCCACTCAACATCCA	61.2°C
R1	ATGGCAGTCAATGGTCACAG	59.6°C
R2	CTAATCAGCCCATGCTCACA	59.8°C
R3	CCATAAATTGTGGGGGTAGC	59.2°C
R4	GATGTTGTTCATCGTACATAG	58.2°C
R5	CATCCACTAACCACACAACAAAA	59.8°C

Table S 5.4 Microsatellite genotypes of all 33 individuals in the three different periods Old (1813-1861), Middle-aged (1923-1940) and Young (2011).

Locus	**CSSM16**		**MM12**		**Inra35**		**CSSM22**		**CSSM19**	
Individual	Allel1	Allel2	Allel1	Allel2	Allel1	Allel2	Allel1	Allel2	Allel1	Allel2
Young1	154	162	84	84	101	107	208	212	142	?
Young2	156	162	84	86	101	117	212	212	150	160
Young3	156	162	84	86	101	117	208	212	150	160
Young4	154	162	84	86	107	111	208	212	142	160
Young5	154	154	84	84	117	117	212	212	144	146
Young6	154	162	84	84	111	117	208	212	146	160
Young7	162	162	84	84	111	111	208	212	142	142
Young8	154	162	84	84	111	117	212	212	146	150
Young9	156	156	84	86	111	117	212	212	142	150
Young10	154	154	84	84	99	115	208	212	150	150
Young11	154	156	84	86	111	111	212	212	160	160
Young12	154	162	84	84	99	109	208	212	150	160
Young13	154	162	84	84	107	111	208	212	146	150
Young14	162	162	84	84	99	115	212	212	142	146
Young15	154	162	84	84	99	99	212	212	146	150
Young16	156	156	84	84	111	117	212	214	142	150
Young17	156	162	84	84	101	111	212	212	150	160
Middle1	162	162	84	86	101	115	212	212	150	150
Middle2	162	162	84	84	101	101	212	212	150	150
Middle3	154	154	84	86	111	117	212	214	150	150
Middle4	154	162	84	84	101	117	212	212	150	152
Middle5	162	162	84	86	101	111	212	212	150	150
Middle6	154	154	86	86	111	117	208	212	150	160
Middle7	154	162	84	84	101	117	212	212	150	152
Middle8	156	162	84	86	111	117	212	212	150	160
Old1	156	162	84	84	101	107	212	212	146	150

Old2	162	162	84	84	107	111	212	212	144	160
Old3	156	162	84	84	107	107	212	214	150	160
Old4	156	156	84	86	101	103	212	214	144	150
Old5	156	164	84	86	101	107	212	212	144	150
Old6	154	162	84	84	101	109	208	214	146	150

Locus	**Haut14**		**CSPS115**		**BM1818**		**ILSTS06**		**CSSM14**	
Individual	Allel1	Allel2	Allel1	Allel2	Allel1	Allel2	Allel1	Allel2	Allel1	Allel2
Young1	118	142	240	244	243	243	292	294	134	134
Young2	130	142	244	244	237	243	296	300	134	134
Young3	118	124	240	244	237	237	282	292	134	134
Young4	130	142	240	244	243	245	292	294	134	134
Young5	?	?	?	?	237	245	292	294	134	134
Young6	116	118	238	244	247	247	294	296	134	136
Young7	128	142	244	244	235	245	292	300	134	134
Young8	116	118	238	238	247	247	292	300	134	134
Young9	118	118	238	240	235	237	294	294	134	136
Young10	118	142	240	244	243	247	280	294	134	134
Young11	118	142	244	244	243	243	284	294	134	134
Young12	104	142	244	244	235	243	280	284	134	134
Young13	118	142	238	244	243	247	284	292	134	134
Young14	122	142	244	244	237	243	288	300	134	134
Young15	118	122	238	244	243	243	288	294	134	134
Young16	118	118	240	244	235	237	284	294	134	134
Young17	118	142	244	244	235	243	280	284	134	134
Middle1	130	130	244	244	237	243	276	300	134	134
Middle2	128	128	236	238	235	243	300	300	134	136
Middle3	106	106	?	?	243	243	292	292	136	136
Middle4	106	106	238	244	243	243	294	300	134	134

Middle5	?	?	?	?	243	243	300	300	134	134
Middle6	130	130	238	238	243	243	294	300	134	134
Middle7	106	130	238	244	243	243	292	294	134	134
Middle8	128	?	244	244	243	243	292	300	134	134
Old1	104	106	236	238	235	243	282	292	132	134
Old2	106	108	244	246	243	245	276	294	132	134
Old3	106	114	236	240	237	243	280	294	134	136
Old4	104	118	238	244	235	245	276	290	134	134
Old5	104	?	236	240	243	247	276	276	134	134
Old6	104	?	236	244	243	245	292	294	134	134

Table S 5.5 Allele frequencies at ten loci of population Old, Middle-aged and Young.

Locus 1

Population	Alleles			
	154	156	162	164
Young	0.353	0.235	0.412	0.000
Middle-aged	0.100	0.100	0.800	0.000
Old	0.100	0.300	0.500	0.100

Locus 2

Population	Alleles	
	84	86
Young	0.853	0.147
Middle-aged	0.625	0.375
Old	0.833	0.167

Locus 3

Population	Alleles							
	99	101	107	109	111	115	117	119
Young	0.147	0.118	0.000	0.088	0.029	0.324	0.059	0.235

Middle-aged	0.000	0.375	0.000	0.000	0.000	0.250	0.062	0.312
Old	0.000	0.333	0.083	0.417	0.083	0.083	0.000	0.000

Locus 4

Population	Alleles		
	208	212	214
Young	0.235	0.735	0.029
Middle-aged	0.062	0.875	0.062
Old	0.083	0.667	0.250

Locus 5

Population	Alleles					
	142	144	146	150	152	160
Young	0.188	0.031	0.188	0.344	0.000	0.250
Middle-aged	0.000	0.000	0.000	0.750	0.125	0.125
Old	0.000	0.250	0.167	0.417	0.000	0.167

Locus 6

Population	Alleles										
	104	106	108	114	116	118	122	124	128	130	142
Young	0.031	0.000	0.000	0.000	0.062	0.406	0.062	0.031	0.031	0.062	0.312
Middle-aged	0.000	0.417	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.417	0.000
Old	0.250	0.375	0.125	0.125	0.000	0.125	0.000	0.000	0.000	0.000	0.000

Locus 7

Population	Alleles				
	236	238	240	244	246
Young	0.000	0.188	0.188	0.625	0.000
Middle-aged	0.083	0.417	0.000	0.500	0.000
Old	0.333	0.167	0.167	0.250	0.083

Locus 8

Population	Alleles

	235	237	243	245	247
Young	0.147	0.206	0.382	0.088	0.176
Middle-aged	0.062	0.062	0.875	0.000	0.000
Old	0.167	0.083	0.417	0.250	0.083

Locus 9

Population	Alleles									
	276	280	282	284	288	290	292	294	296	300
Young	0.000	0.088	0.029	0.147	0.059	0.000	0.206	0.294	0.059	0.118
Middle-aged	0.062	0.000	0.000	0.000	0.000	0.000	0.250	0.188	0.000	0.500
Old	0.333	0.083	0.083	0.000	0.000	0.083	0.167	0.250	0.000	0.000

Locus 10

Population	Alleles		
	132	134	136
Young	0.000	0.941	0.059
Middle-aged	0.000	0.812	0.188
Old	0.167	0.750	0.083

Chapter S 5.1 Study area and history

The area is located at the westernmost edge of a nature preserve ("Rhein Westerwald"). The landscape is typical for the river Rhine; land use alternates between vineyards and steep forested hills. About 45 % of the area is forested while *c*. 34 % is used for agriculture. The 10 km^2 area, from where samples came from, belongs to the private estate of the princes of Neuwied who were the only people hunting and managing red deer there. In Germany, hunting is area based, not license based as in many other parts of the world. The 10 km^2 is part of a larger area that has never been completely fenced during the last centuries, so gene flow with other populations was not restricted.

In the period 1813-1861 the Neuwied population was large. There were few barriers to migration and there was a low degree of fragmentation in an overall continuous habitat. Prior to 1848 only nobles were allowed to hunt. They managed the red deer populations and deer were only harvested in a few hunts per year. The most popular hunting items were older males with large antlers. During the revolution years 1848-1849 there were no hunting laws. This period saw massive reductions and large-scale extinctions in red deer populations nation-wide. During the second half of the 19th century, hunting laws were again put into action which allowed populations to re-establish in many areas (Kuehn et al. 2003). After re-establishment, population sizes were once again reduced as a response to increasing damages to agriculture, but also caused by poaching during and after World War 1. Our second population samples come from the period representing the end-phase of overexploitation (1923-1940). Following the 1950's, red deer populations expanded into many forested areas. For the first time, rigorous hunting schedules favoring large, branched antlers were introduced (Hartl et al. 2003). Young males with small antlers were harvested while males with large antlers were allowed to reach maturity and reproduce. Middle-aged to old males up to 10 years with large antlers were normally not harvested. The contemporary Neuwied population is more fragmented than in former times. It is confined to the south by the river Rhine, and in the west and east it is fenced by motorways and highways. Migration and gene flow is presumably highly restricted in

this population. Translocation events into the continuous population are not known. We do not know the population sizes of red deer during the three time periods except that the population was nearly eradicated during and after the revolution in 1848.

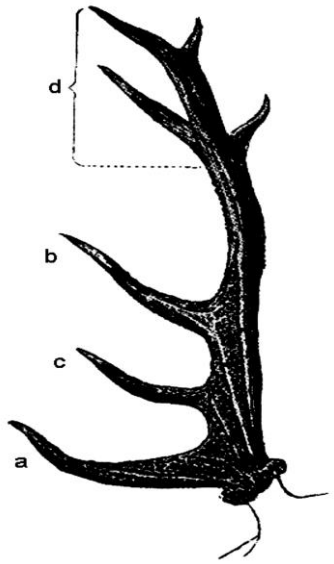

Figure S 6.1 The basic anatomy of a red deer antler, side view. The spikes include (a) brow tine (b) trey tine (c) bey tine (d) crown. The base of the antler is connected to the skull at the burr. Figure taken from the *Nordisk familjebok* (1911), vol. 15, page 19.

Table S 6.1 Designed forward and reverse primers for the amplification of the D-Loop region on the mtDNA.

PheHB/ProL D-Loop Sequence Primer	**Primer-Sequence (5' – 3')**	**Annealing Temperature**
Forward	ACCCCCTGGAGTGCTAATTT	59.9°C
F2	TTTCATGAGTCAACCCTAAGATC	57.4°C
F3	GGATGCTTGGACTCAGCAAT	60.2°C
F4	TTTCAGGGCCATCTCACCTA	60.5°C
F5	TATCCCGTCCCCTAGATCAC	58.9°C
Reverse	CAGCTTTCCACTCAACATCCA	61.2°C
R1	ATGGCAGTCAATGGTCACAG	59.6°C

R2	CTAATCAGCCCATGCTCACA	59.8°C
R3	CCATAAATTGTGGGGGTAGC	59.2°C
R4	GATGTTGTTCATCGTACATAG	58.2°C
R5	CATCCACTAACCACACAACAAAA	59.8°C

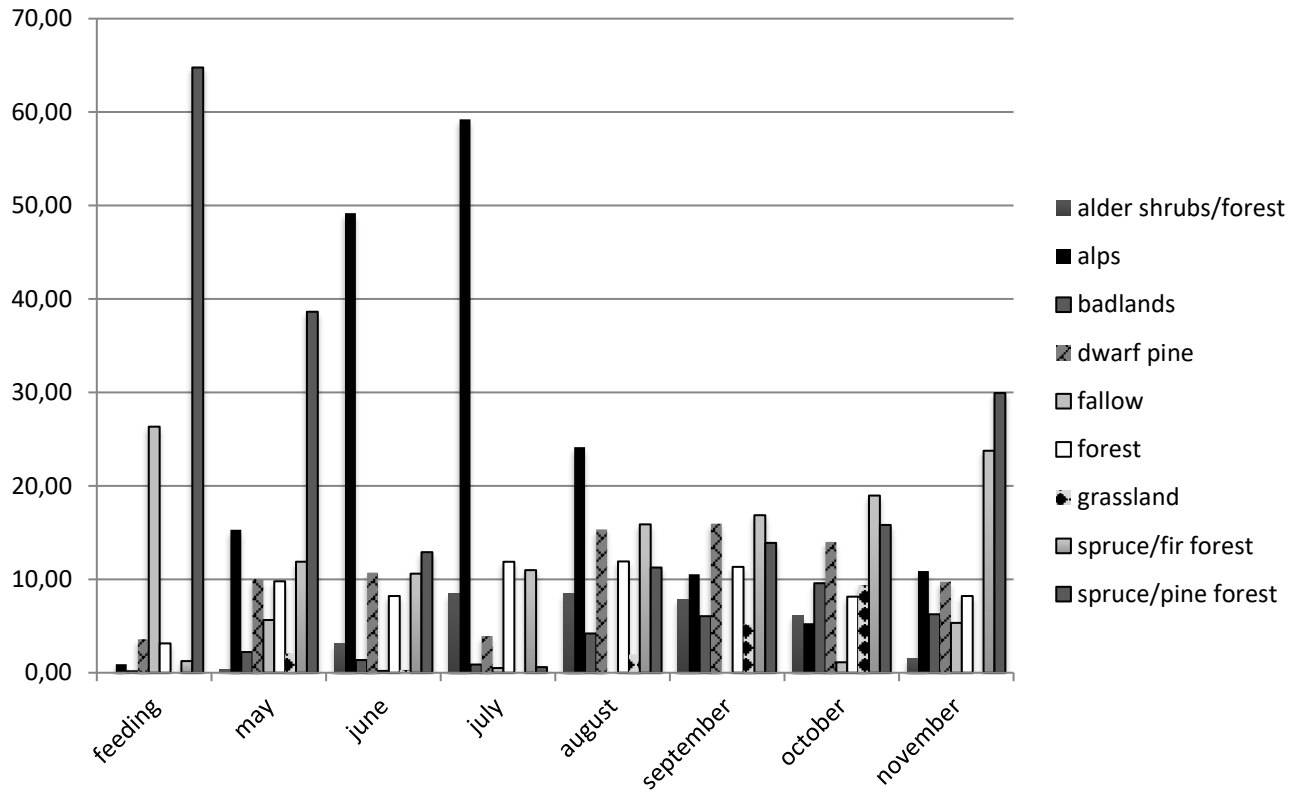

Figure S 12.1 Monthly habitat use of deer. Only habitat types with a percentage greater than 5% of monthly fixes were included in this graph.

Table S 12.1 Different types of habitat used in the investigated area, size and percentage of the total area. Percentages of animal positions (GPS fixes) in the particular habitat types are in the far-right column.

Habitat use	size [ha]	Percentage of total area	Percentage used by the animals
alps	3066.31	45%	9%
buildings	18.26	0%	0%
mowing	48.87	1%	0%
fallow	25.91	0%	5%
spruce/pine forest	262.45	4%	37%
spruce/fir forest	114.64	2%	10%
fir forest	563.90	8%	2%
water	45.14	1%	0%
alder shrubs/forests	64.60	1%	6%
floodplain	12.89	0%	1%
rangeland	116.34	2%	1%
dwarf pine	455.17	7%	12%
badlands	1304.49	19%	4%
road	39.44	1%	0%
forest	383.37	6%	10%
grassland	260.15	4%	3%

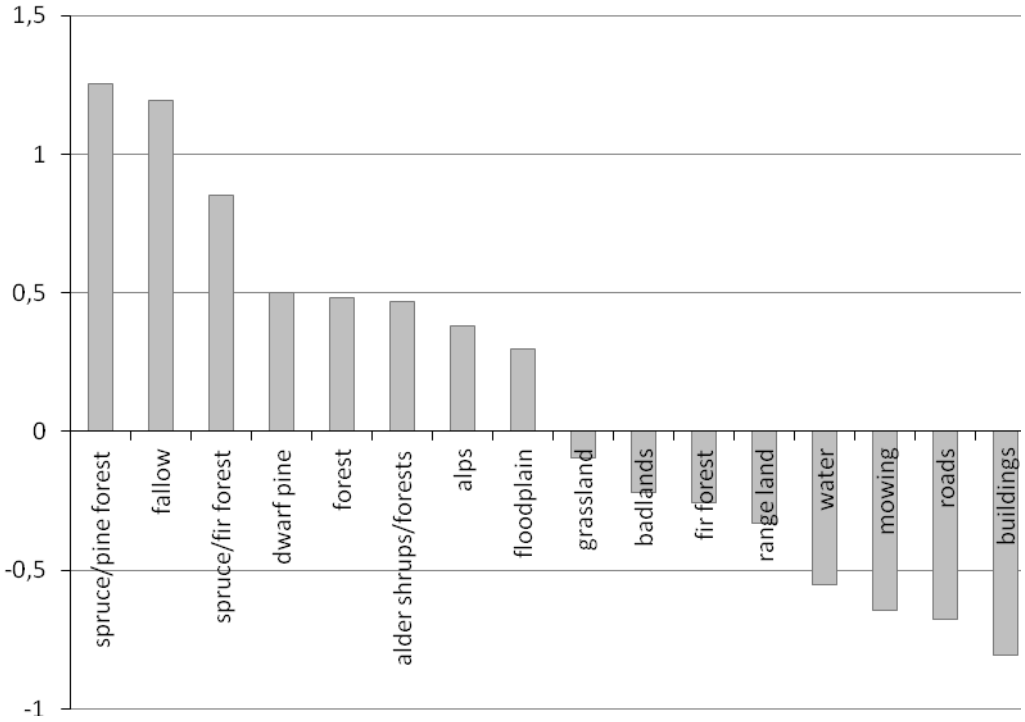

Figure S 12.2 Habitat preference of the animals for the different habitat types. The higher the value, the higher is the preference of the animal for this habitat type. Negative values indicate avoidance.

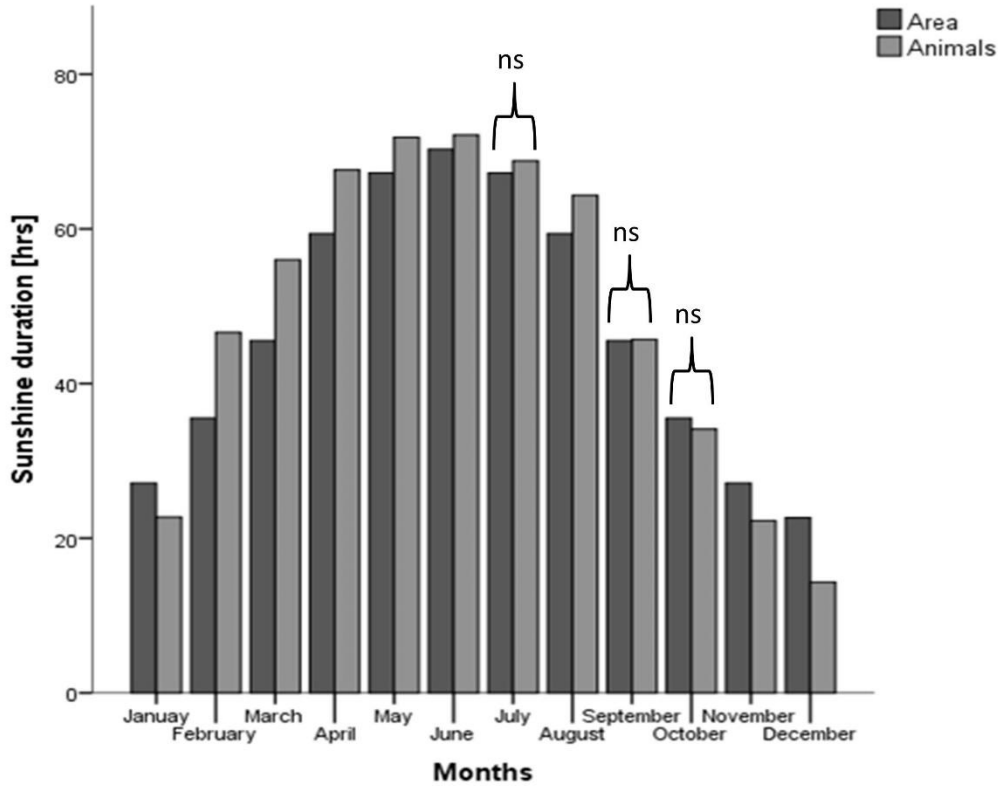

Figure S 12.3 Usage of sun exposed areas. Light grey bars represent the total number of hours of sunshine per month in a particular area used by deer and dark grey barks the average number of hours of sunshine in our study area. Non-significant ($p > 0.05$) monthly differences are marked with ns. All other monthly differences are significant ($p \leq 0.05$)

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Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig und nur mit Hilfe der angegebenen Personen und Mittel (Literatur, Apparaturen, Material) angefertigt zu haben. Bei den von mir durchgeführten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der Satzung der Johannes-Gutenberg-Universität Mainz niedergelegt sind, eingehalten.

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1. Betreuung von Praktika in der Ökologie (Bachelor of science, Bachelor of education, Master of science, Master of education).

2. Teil „Populationsgenetik“ (inkl. Vorlesung und praktischer Teil) im Mastermodul.

3. Veranstaltung von Exkursionen für Master und Bachelor „Fischbestandsaufnahmen mittels Elektrofischerei“

4. Betreuung von F2 Praktika im Diplom- und Masterstudiengang mit Themenschwerpunkt Populationsgenetik und Auswertung von Telemetriedaten.

Betreute Abschlussarbeiten

1. Würth D. (2014): Populationsgenetische Untersuchungen an Bachforellen *(Salmo trutta forma fario).* Diplomarbeit

2. Nagel R. (2013): A population genetics study of Red Deer (*Cervus elaphus*). Master of science.

3. Siebel S. (2014) Satelitentelemetrische Untersuchungen an Rothirschen (*Cervus elaphus*). Bachelor of science.

4. Scheurich S. (2012) Test zweier Multiplex-Systeme für *Cervus elaphus* und Cross-Amplifikations-Test bei anderen Hirscharten (Cervidae). Bachelor of science.

Publikationen

Peer-reviewed

Hoffmann, G. S., Johannesen, J., & Griebeler, E. M. „Population dynamics of a natural red deer population over 200 years detected via substantial changes of

genetic variation.“ *Ecology and evolution*, 6(10), (2016): 3146-3153.

Hoffmann G. S., Johannesen J., Griebeler E. M. "Species cross-amplification, identification and genetic variation of 17 species of deer (Cervidae) with microsatellite and mitochondrial DNA from antlers." *Molecular biology reports* (2014): 1-9.

Hoffmann G. S., and Griebeler E. M. "An improved high yield method to obtain microsatellite genotypes from red deer antlers up to 200 years old." *Molecular ecology resources* 13, no. 3 (2013): 440-446.