

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

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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.

Keywords Non-invasive sampling · Barcoding · Artiodactyla · Phylogeny · Population genetics · Museum samples

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Introduction

The deer family (*Cervidae*) is distributed throughout the northern hemisphere as well as in South America and southeast Asia [1]. 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* [2]. 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 [3]. Consequently, antlers in fresh velvet can cost up to \$1,000 per kg.

The economic significance of deer as managed game animals, for trophy hunting and commercial meat and antler production have led to numerous translocations of deer species worldwide [4]. Translocations may have severe consequences for the conservation of indigenous species [4] due to interspecific competition and/or the introduction of new diseases and parasites [5, 6], 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 [7, 8].

Cervidae are united by a series of synapomorphies, including the possession of antlers in males [9]. Antlers are made of a deciduous bony core covered by velvet skin. Antlers are shed annually and regenerate in the following 5 months from the permanent pedicles [10]. 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 [11] without harming and disturbing or even having to observe individuals. It is therefore of special importance in conservation biology [12]. In a previous paper, Hoffmann and Griebeler [13] 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 [14, 15]. 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 [16]. Among the many studies using microsatellites to study various species of deer, there are few studies that use the same sets of primers [17]. 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 [18–21]. 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 [17].

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 [22]. Barcoding entails the screening of one mitochondrial reference gene to assign unknown individuals to a species [23]. DNA barcoding, with its unique reproducibility, sequence versatility and comparability among different species, provides a powerful approach for species authentication [24] 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.

Materials and methods

Sampled animals

We conducted cross-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 [25] we defined subspecies as geographic populations with a largely independent phylogeny [26]. 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 1).

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 [13]. The antler chips were pulverized in a Retsch-mill at a frequency of 30 Hz 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).

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 Supplementary Table S3). The reaction volume for each sample consisted of 5 µl Qiagen Multiplex PCR Mastermix, containing dNTPs, Qiagen-HotStarTaq DNA Polymerase and 6 mM MgCl₂, 1 µl

Table 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

Species	IUCN status	Number of loci	origin	Distribution area
Moose (<i>Alces alces</i>) (N = 2)	Least concern	14 (9)	Hunting trophy, Sweden	Northern Europe, Northern Asia, North America
Hog deer (<i>Axis porcinus</i>) (N = 1)	Endangered	13 (9)	TB	South- and Southeast Asia
Roe deer (<i>Capreolus capreolus</i>) (N = 2)	Least concern	13 (5)	Hunting trophy, Germany	Europe, Asia Minor
White-lipped deer (<i>Cervus albirostris</i>) (N = 2)	Vulnerable	16 (12)	TB	Tibet
Wapiti (<i>Cervus canadensis</i>) (N = 1)	Least concern	15 (3)	Hunting trophy Germany	North America, Siberia, Central Asia
Barasingha (<i>Cervus duvaucelii</i>) (N = 3)	Vulnerable	16 (10)	TB	Northern India
Red deer (<i>Cervus elaphus</i>) (N = 3)	Least concern	14 (12)	Shed antlers, Austria	Europe, Northern Africa, Southeastern Asia
Fallow deer (<i>Dama dama</i>) (N = 3)	Least concern	14 (2)	Hunting Trophy, Germany	East Asia, Asia Minor
Persian fallow deer (<i>Dama mesopotamica</i>) (N = 2)	Endangered	16 (2)	TB	Iran, Israel
Pere David's deer (<i>Elaphurus davidianus</i>) (N = 3)	Extinct in the wild	16 (6)	TB	Extinct in the wild
Mule deer (<i>Odocoileus hemionus</i>) (N = 1)	Least concern	13 (7)	TB	Western North America
White-tailed deer (<i>Odocoileus virginianus</i>) (N = 2)	Least concern	15 (8)	TB	North America, Middle America, Northern South America
Reindeer (<i>Rangifer tarandus</i>) (N = 3)	Least concern	16 (11)	TB	Northern Eurasia, North America
Elás deer (<i>Rucervus eldii</i>) (N = 3)	Endangered	15 (8)	TB	Southeast Asia
Javan Rusa (<i>Rusa timorensis</i>) (N = 2)	Vulnerable	15 (2)	TB	Java, Bali
Sambar (<i>Rusa unicornis</i>) (N = 1)	Vulnerable	15 (6)	TB	Southern Asia
Subspecies				
<i>Cervus canadensis nannodes</i> (N = 2)	No data available	15 (2)	TB	California
<i>Cervus canadensis sibiricus</i> (N = 1)	No data available	13 (6)	TB	Altai-Mountains, Northwest Mongolia
<i>Cervus elaphus bactrianus</i> (N = 2)	No data available	15 (10)	TB	Central Asia
<i>Cervus nippon manschuricus</i> (N = 1)	No data available	9 (7)	TB	China, Siberia, Japan, Taiwan
<i>Cervus nippon pseudaxis</i> (N = 1)	No data available	16 (7)	TB	Vietnam

The IUCN Red List does not include information on subspecies

N number of individuals studied per species, TB Tierpark Berlin

Primer Mix (2 μ M each Primer), 2.5 μ l RNase-free water and 1.5 μ l DNA (final DNA concentration of 50–100 μ g/ml) resulting in a final reaction volume of 10 μ l 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 $^{\circ}$ C for initial denaturation; then 40 cycles of 40 s at 94 $^{\circ}$ C, 90 s at 57 $^{\circ}$ C and 90 s at 72 $^{\circ}$ C, followed by 30 min at 60 $^{\circ}$ C and a final hold at 4 $^{\circ}$ C.

The remaining eight microsatellite primers (NVHRT48, RT1, RT6, IOBT965, BM4107, NVHRT16, Ca38, Ca60)

were labelled during the PCR reaction following the method in [27]). Each reaction consisted of 7.7 μ l H₂O with Ready to go PCR Beads (GE Healthcare), \sim 0.035 μ l Forward Primer (100 pM), \sim 0.14 μ l Reward Primer (100 pM), \sim 0.14 μ l M13 Tail (HEX, FAM, NED) and 1 μ l DNA resulting in a final reaction volume of 9 μ l 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 $^{\circ}$ C), RT1 (51 $^{\circ}$ C), RT6 (51 $^{\circ}$ C), IOBT965 (54 $^{\circ}$ C), BM4107 (55 $^{\circ}$ 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 seven 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 1).

mtDNA primers, PCR reactions and 16S rRNA genotyping

We amplified the mitochondrial gene 16S rRNA (550 bp, 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) [28]. Each 25 μ L PCR reaction consisted of 21 μ L of H₂O, 1 μ L of each respective primer (100 pM) and 2 μ L of DNA extract in a 0.5 mL PCR tube with illustraTM puReTaq Ready-To-Go PCR Beads (GE Healthcare). The final volume of 25 μ L contained 200 μ M of each dNTP, 10 mM Tris–HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM MgCl₂, 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 min, 30 cycles of 94 °C for 30 s, 49 °C for 30 s and extension at 72 °C for 45 s. The reaction was stored at 4 °C after a final extension at 72 °C for 10 min. 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 550 bp fragment of the mitochondrial 16S rDNA for all species and subspecies (primer and primer-sequences listed in Supplementary Table S3; accession numbers in Supplementary Table S6). 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 [24]. Due to the low number of individuals per species analyzed in this study (Table 1), we blasted the 16S mtDNA sequence for each species in GenBank to evaluate intraspecific sequence variation. We found between two and eleven additional sequences per species (Supplementary Table S1; accession numbers in Supplementary Table S6). 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 [1]. Phylogenetic inference based on the 550 bp 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 [29]. *Bos taurus* (Bovidae) was used as an outgroup (Accession number: HQ184044.1). Each phylogenetic inference was evaluated with 1,000 bootstraps. Only bootstrap scores higher than 70 % were considered as significant support of branching.

Results

DNA extraction

DNA was successfully extracted from antlers in moderate to high concentrations from all individuals. DNA concentrations ranged between 31.06 and 1,046.23 μ g ml⁻¹ (mean: 227.92 μ g ml⁻¹, standard deviation: 275.93 (Supplementary Table S2)).

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 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 Supplementary Table S4, S7. The tested primers, which were developed for cattle [18–21], showed a high amplification success in all species and subspecies. For each of the investigated species/subspecies, 9–16 primer pairs amplified fragments (Supplementary Table S4, S7). 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, CSPA115).

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.

16S rDNA

The mtDNA 16S rRNA fragment (550 bp) was successfully amplified in all individuals from DNA extracted from antlers (Supplementary Table S5). 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 S1). 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 (Fig. 1). The phylogenetic trees are concordant with known phylogenies of *Cervidae*, though the strict monophyly of Plesiometacarpalia and Metacarpalia is not shown.

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 16S mtDNA barcoding inventory. While the extraction method was previously limited to the successful amplification of red deer nuclear

DNA [13], 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 [13] protects the DNA from breakdown into cellular compartments and degradation by catabolic enzymes, e.g. lysosomal nucleases [30]. 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.

Although we cannot make general statements about the extraction method's applicability for non-antler bone, we have successfully extracted DNA from jaw (80 year old) and femur bone stored in museums (GSH, unpublished data).

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 [13]. Nevertheless, the high structural similarity 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 [11, 12]. 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 [31, 32]. 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 [17]. 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

Table 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

Locus name	<i>Axis porcinus</i> (N = 2)	<i>Cervus albirostris</i> (N = 2)	<i>Cervus davaucei</i> (N = 3)	<i>Dama mesopotamica</i> (N = 2)	<i>Elaphurus davidianus</i> (N = 3)	<i>Rucervus eldii</i> (N = 3)	<i>Cervus elaphus bactrianus</i> (N = 2)	<i>Cervus nippon pseudaxis</i> (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)

Complete list of amplification success for all species in appendix (Table S4, S7)

¹ Ref. [18]² Ref. [19]³ Ref. [20]

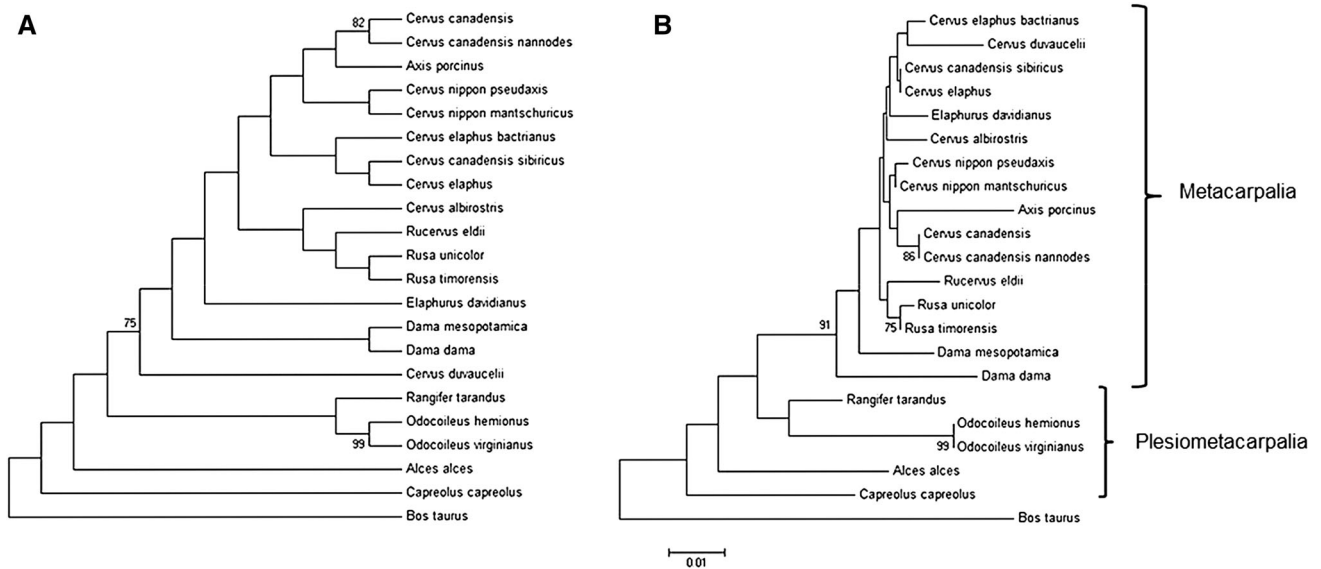


Fig. 1 Neighbor joining (a) and maximum parsimony (b) phylogenetic trees of cervid species based on 16S rDNA. Only bootstrap values higher than 70 % are shown

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 [33, 34]. 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 [35] and animal derived compounds. mtDNA provides this opportunity and was therefore used for cervid species identification [22]. Accurate species identification requires a comprehensive comparative molecular database for the assignment of an unknown individual [35]. 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 [22], 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 (Fig. 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 [22, 30]. The fact that the strict monophyletic origin of these two sub-families 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* [36], 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).

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