Weathered antlers: a valuable source of DNA useful for Cervidae conservation purposes

Claudia Greco[1], Anna Maria De Marinis[1], Francesco Riga[1], Nadia Mucci[1]

[1]Italian Institute for Environmental Protection and Research ISPRA

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Short title
Cervidae conservation through DNA from weathered antlers

Corresponding author
Claudia Greco
Italian Institute for Environmental Protection and Research ISPRA; email: claudia.greco@isprambiente.it

Short Note

Abstract: Conservation genetics pinpoint the use of biological materials collected without stressing wildlife and antlers seem to fit with this need. In the State Nature Reserve “Bosco della Mesola”, Northern Italy, occurs the only native red deer population of peninsular Italy, recently recognized as a distinct subspecies (*Cervus elaphus italicus*) and characterized by peculiar phenotype. We tested the applicability of a simple and inexpensive biomolecular protocol to obtain information for the conservation of endangered deer with no stress for animals. DNA obtained by drilling both well preserved and highly degraded antlers was amplified with three types of molecular markers to get comprehensive information at individual, population and species level. Our results pointed out that only the seal yielded amplifiable DNA. The study attested that weathered antlers can be a suitable source of DNA not only in dry but also in Mediterranean climates, characterized by strong seasonal fluctuations. Antlers are an efficient and cost-effective non-invasive sampling for molecular genetic

studies on rare or threatened species of cervids. We tested and provided easy to use and cheap laboratory protocols implementing all the previous knowledge.

Genetic studies have assumed an increasingly important role in the management and conservation of wildlife (Arif, et al., 2011; Holderegger, et al., 2019), also in cervids (Kumar, et al., 2018; Schwartz, et al., 2007). Different biological materials, like tissue, blood, hairs, faeces and bones, are a suitable source of DNA, but sampling of tissues and blood is invasive and may cause stress and trauma to animals, even death (Blumstein, et al., 2015; DeNicola, et al., 1997; Powell, et al., 2003). Moreover, they need appropriate preservation methods (Kumar, et al., 2019; Seutin, et al 1991; Wang, et al., 2001) to avoid DNA degradation. On the other hand, in ungulates, common non-invasive samples, such as faeces from several individuals, can be found in foraging areas; they often provide low quality and quantity DNA (Forgacs, et al. 2019; Maudet, et al. 2004) but can be also prone to genotyping errors, because of the proximity and overlapping of pellets. Bones like mandibles and skulls are a good source of DNA, but they are only from dead individuals. It follows that antlers represent a promising alternative approach in genetic studies of deer populations because they can be collected in the field without hunting or stressing animals (Hoffmann, et al., 2015; Venegas, et al. 2020). Collectors can access cast antlers in the wild, but also in private or public collections and in museums as trophies or historical artefacts. Here, they are stored under controlled conditions, allowing their use in population genetics studies over time (Gizejewska, et al. 2016; Hoffmann, et al., 2015; Wandeler, et al., 2007) and among extant and historical or fossil specimens (Kang, et al., 2007; Meiri, et al., 2018; Schnitzler, et al., 2018).

Antlers are composed of bone, cartilage, fibrous tissue, skin, nerves, and blood vessels, and develop from permanent frontal outgrowths, the pedicles. Different types of stem cells determine the pedicle and first antler formation, the annual regeneration and the rapid antler growth (Zhang et al. 2021); these cells, located in the pedicle and antler tip tissues, are self-renewing and can differentiate into multiple lineages (Seo et al. 2014). Antlers are cast once a year and are weathered on the ground

by abiotic (as temperature, solar irradiation, rains, etc.), and biotic (as rodent gnawing activity) agents that cause warping, cracking, and breakage (Jin and Shipman 2010). Physical stresses of repeated heating and cooling, and wetting and drying, increased the bone weathering process (Behrensmeyer, 1978) and broke antlers, transforming them into small whitish remains.

Antler drilling allows DNA extraction from a small amount of powder (Hoffmann, et al., 2013; Lopez, et al., 2012; O'Connell, et al., 1999; Røed, et al. 2014), but any differences in PCR success depending on drilling position have never been investigated (Hoffmann, et al., 2013; Lopez, et al., 2012). In some studies authors drilled the base (Lopez, et al., 2012), other researchers did not specify the drilling place (O'Connell, et al., 1999; Røed, et al. 2014), or choose the position only to make less evident the borehole (Hoffmann, et al., 2013).

Previous studies regarded antlers collected in dry climates (Lopez, et al., 2012) or housed in museums (Wandeler, et al., 2007) but DNA extraction from antlers collected in habitats characterized by strong seasonality and humid climates was never tested. Humidity and warm temperatures, as those characterizing the Mediterranean area, favour DNA degradation more than dried or cold habitats, thus leading to different amplification success rates when the same biological material has been exposed to different conditions (Alaeddini, et al. 2010).

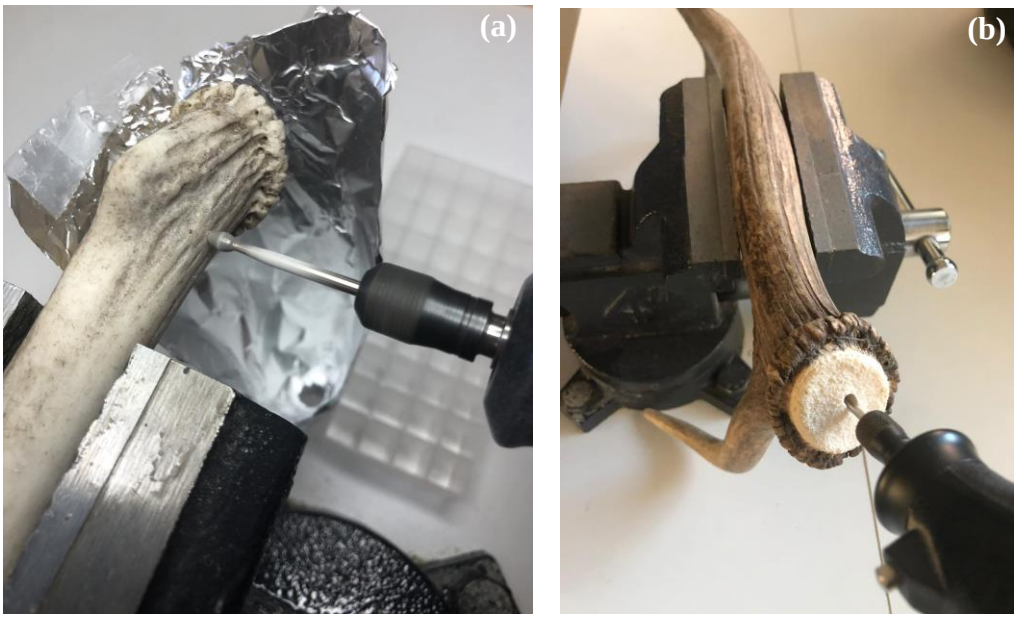
The Mesola red deer (*Cervus elaphus italicus*), is the only native red deer of the Italian peninsula, recently recognized as a distinct subspecies (Zachos, et al., 2014). It became nearly extinct in the 20th century while nowadays has slightly recovered (Lovari, et al., 2010) despite it has never been subject to restocking. The geographical isolation in the Po delta area has produced a rustic ecotype, adapted to a scarcely productive environment, with morphological characteristics and antlers structure partially different from other deer (Lovari, et al., 2010; Mattioli, et al., 2014; Mattioli, et al., 2003). The unique relict nucleus lives in the fenced area of the State Natural Reserve “Bosco della Mesola” (Northern Italy) where some cast antlers remain in the field for years although most of them are stored in a sheltered warehouse.

In our study, we utilized antlers conserved under different conditions to address the following aims: i) determine if DNA extraction is influenced by environmental conditions and/or antler drilling position (seal *vs* beam); ii) verify PCR amplification success of different markers from antlers DNA; iii) test methods to provide detailed, easy to use and cheap laboratory protocols implementing all the previous knowledge.

The Reserve (1058 ha) is an enclosed area composed of woodland (93%), wetland (4%) and grassland (3%) and is characterized by high seasonal fluctuations in temperature varying from - 3 in January to 33°C in August, in monthly precipitation from 30 mm in January to 59 mm in October and in relative monthly humidity from 55 in July to 85% in December.

In 2014, four antlers were gathered by rangers as soon as they were cast and housed at room temperature in a warehouse until we carried out the genetic analysis in 2018. In 2018 we also collected in the fields additional four cast antlers that have been remained on the ground and exposed to different environmental conditions for years (Table 1).

After securing antlers in a clamp, we pierced the beam and the seal (respectively, Fig. 1a and 1b) of the four weathered samples with a Dremel precision drill with a 3 mm diamond engraving drill bit. The single hole of around 8 mm depth produced a small amount of powder that was collected through a clean tinfoil funnel in a 2 ml empty safe-lock sterile tube. We took all precautions to prevent the dust from spreading in the air and avoid cross-contamination using for the drill bit a transparent side shield and for each sample a tinfoil collection funnel wrapping the diamond tip during drilling procedure. The drill bit was cleaned and UV-sterilized after each drilling and also the decontamination of the instrumentation including the work bench was performed between each sample.



DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) on the robotic workstation QIAcube extractor (Qiagen) and was quantified by electrophoresis through a 2% E-Gel™ Agarose Gels with SYBR™ Safe (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) using Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). We carried out a screening of the DNA extracted by sequencing an intronic region 600 bp long of the Growth Hormone (GH). As only DNA from the seal yielded reliable results for all the weathered samples for this molecular marker, we drilled exclusively the seal of the four well-preserved antlers (Figure 1b) and excluded all the beams from the successive analysis. Three different types of molecular markers (nuclear and mitochondrial genes, microsatellite loci) that are routinely used in Conservation Genetics of wildlife were amplified to test the efficiency of each DNA extraction: an intronic region (intron 1) of the Growth Hormone (GH) (Blumstein, et al., 2015; Lioupis, et al., 1997; Wallis, et al., 2006), as functional genes can explain phenotypical or functional characters; the starting region of the control region of mitochondrial DNA (Douzery, et al., 1997; Peakall, et al., 2012) to investigate phylogeny; and twelve microsatellite loci (Al-Atiyat, et al., 2018; Barendse, et al., 1994; Bishop, et al., 1994; Ede, et al., 1995; Kemp, et al., 1995; Penty, et al., 1993; Røed, et al., 1998; Talbot, et al., 1996) to describe genetic variability and allow individual identification.

We used a unique PCR protocol for all the markers in 8 µl of total volume containing 0.8 µl Reaction buffer 10X, 0.8 µl BSA (0.2%), 0.48 µl $MgCl_2$ (25 mM), 0.4 µl dNTPs (2.5 mM), 0.04 µl Qiagen Hotstart Taq, 0.20 µl Primer forward and reverse (10 mM), 3.28 µl Double distilled water and 2 µl DNA. Termocycler protocol consisted of initial denaturation at 94°C for 15" followed by 35 cycles (94°C x 40", 55°C x 40", 72°C x 40") then final extension at 72°C for 10 min. Primer utilized and combined in the same amplification reaction in duplex, and bibliographic references are listed in Table 2. Four replicates were performed for STRs to attest to the reliability of the results.

Amplicons of the control region and GH gene were purified with Exonuclease I and Phosphatase and sequenced with Big Dye terminator chemistry (Thermo Fisher Scientific) following the manufacturer's protocol in a 10 µl final volume. Sequences and STRs fragments were separated on an ABI prism 3500 Genetic Analyzer (Thermo Fisher Scientific). We verified, validated and checked data respectively sequences with the software Seqscape 3.0 and microsatellite with GeneMapper 5.0 (Thermo Fisher Scientific).

DNAsp (Librado, et al., 2009) was used to compute haplotype diversity for sequence analysis while GeneAlEx 6.05 (Peakall, et al., 2012) was utilized to calculate variability indices (observed and expected heterozygosity, allele number, percentage of polymorphic loci) in STRs.

In Gimlet we computed the success rate and the genotyping errors (Allelic Drop Out and False Alleles) between STRs replicates. In GenAlEx, we also compared the individual multilocus genotypes obtained from microsatellite loci to verify whether they could belong to different individuals. We computed the number of mismatches differing each genotype from the others and calculated the Probability of Identity that represents the probability that two individuals share by chance the same genotype among unrelated (P_{ID}) or sibling (P_{ID_SIB}) individuals to verify the power of the STRs panel in identifying single individuals.

The concentration of the DNA extracted from the weathered antlers ranged from 0 to 1 ng/ul for the beams and from 2 to 7 ng/ul for the seals. The concentration of the DNA extracted from the seals

of the antlers stored in the warehouse ranged from 2 to 11 ng/ul. The screening by sequencing the Growth Hormone (GH) yielded reliable results only DNA from the seal.

A sequence around 400-600 bp long was obtained for the GH gene for all the antler seals. The specimens shared the same unique haplotype, corresponding to the variant B haplotype found in cervids (Genebank Acc. N. AM049993). The amplification of the short mtDNA region produced an alignment 200 bp long in 7 out of the 8 samples. Antlers shared an identical haplotype, corresponding to AF291887 of Genebank (*Cervus elaphus italicus*).

The analysis of STRs replicates led to eight unique multilocus genotypes. All the genotypes showed a different allelic composition with no less than three mismatches between genotypes, thus supporting the assignment of antlers to eight different males. The amplification success value computed on the four replicates was 95 %, with low mean values of genotyping errors recorded between replicates (ADO =0,03; FA=0). All the STRs analysed resulted polymorphic.

Despite the fence has hampered the gene flow with other deer populations, we recorded discrete levels of genetic variability. Mean number of alleles was equal to 3.007 $\pm$ 0.265, and observed and expected heterozygosity values were respectively 0.351 $\pm$ 0.053 and 0.433 $\pm$ 0.052. Departure from Hardy Weinberg Equilibrium (HWE) was significant ($p < 0.05$), probably due to the low number of samples collected. Low P_{ID} values were recorded ($P_{ID} = 1{,}0 \times 10^{-6}$; P_{ID} SIB = 1.9×10^{-9}).

Our results confirmed that antlers can be considered a valuable source of DNA even if they are weathered for years and deteriorated from biotic and abiotic agents. An easy sampling procedure combined with a simple DNA extraction protocol makes this methodology easy to use and cost-effective. In the case of weathered antlers, we suggest drilling in the middle of the seal at the junction with the pedicle. Quantifying extracted DNA, we obtained similar concentration for seal in well-preserved and weathered antlers, higher than beam that was close to zero ng/ul. High genotyping and sequencing success rates suggest that this bone material is less affected by biotic and abiotic factors than organic traces left from animals, like faeces and hairs, on the ground in which DNA degradation is very fast (Venegas, et al., 2020). Despite we did not record high levels of Allelic Drop Out and

False Allele, we suggest the application of multitube approach in the monitoring projects that will use antlers as a source of DNA. We have obtained useful preliminary genetic data for Mesola red deer conservation that is worth presenting. Although the small number of samples and the possibility of inbreeding in a low consistence and isolated population occupying a restricted and fenced area, all the 12 STRs were polymorphic. Our results confirmed previous studies and the good chance to obtain information from old antlers (Lopez, et al., 2012, Hoffmann, et al., 2013). As weathering does not seem to preclude the use of DNA extracted from old antlers, also ancient trophies or artefacts can be a good DNA source for genetic studies. Medieval crafts people's artefacts and manufacture's wasting products obtained from reindeer antlers provided good quality DNA (Røed, et al., 1998). Moreover, antler artefacts allow knowing past human societies, for example, by reconstructing trade routes (Stanton, et al., 2016).

Exposure to sunlight and rain determines usually weathering rate of bones (Behrensmeyer, 1978). The marked Mediterranean seasonality of the study area with fluctuation in temperature, monthly precipitation and relative monthly humidity does not affect the amplifiability of weathered antlers DNA samples. Additional analysis involving more individuals should be carried out to confirm these results.

Despite these genetic data can be available only for males, the easy extraction method combined with the variety of different markers make antlers a valuable tool for species identification, intrapopulation and interpopulation variability, phylogenesis, demography and functional genes, individual genotyping and kinship studies. Considering antler anatomy and in particular bone marrow location, the protocols presented in this study can be extended to all cervids.

Antlers are a source of DNA that, until now, has been rarely used for genetic analysis (Venegas, et al., 2020) but indeed offers a good chance to get information avoiding stress during capture and allows a wide non-invasive genetic sampling for comparative studies. For continuous monitoring of Mesola red deer, this approach would be recommended to avoid any disturbance to this relict autochthonous population. Antlers revealed to be a useful tool for the evaluation of genetic variability

of the endangered Mesola red deer in management and future reintroduction projects outlined in the action plan (Lovari, et al., 2010; Mattioli, et al., 2003).

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Table 1 Samples list. Years: number of years between the antlers casting and collection. Type: samples collected in the field and soon after stored in a warehouse (W) or weathered samples collected in the field after several years from casting (F). Collection: year of the gathering of cast antlers. Extraction: month and year of samples DNA processing. Drill. position: position of drilling on each antler respectively in the middle of the seal or in the basal portion of the beam. For all the samples we tested all the molecular markers listed in Table 2.

ID	Years	Type	Collection	Extraction	Drill. position
Cel518	<1	W	2014	Sept 2018	seal
Cel519	<1	W	2014	Sept 2018	seal
Cel520	<1	W	2014	Sept 2018	seal
Cel521	<1	W	2014	Sept 2018	seal
Cel522	>3	F	2018	Sept 2018	seal, beam
Cel523	>3	F	2018	Sept 2018	seal, beam
Cel524	>3	F	2018	Sept 2018	seal, beam
Cel525	>3	F	2018	Sept 2018	seal, beam

Table 2 Complete primer list and references. Three categories (Type) of molecular markers utilized: loci microsatellite – single tandem repeats (STR), Growth hormone (GH) and Sequence (SEQ) of mitochondrial DNA. We reported the number of alleles (N°A), range (R) and multiplex (M) for loci microsatellite. In the last two rows of the table we listed sequence length (L) of the mtDNA control region (LCAPPRO) and Growth Hormone (GH-GHR) intron 1. We obtained shorter sequences (*) for weathered antlers, respectively mtDNA around 150 base and GH around 400 nucleotides.

Marker	Type	N°A	R/L	Multiplex	Reference
BM1258	STR	4	100-120	M1	Bishop et al., 1994
BM203	STR	3	213-229	M2	Talbot et al., 1996
BM4107	STR	3	145-168	M1	Talbot et al., 1996
BM5004	STR	4	120-140	M3	Talbot et al., 1996
ILSTS008	STR	1	165-171	M4	Kemp et al., 1995
NVHRT16	STR	2	155-157	M3	Roed and Midthjell, 1998
NVHRT48	STR	4	79-103	M2	Roed and Midthjell, 1998
NVHRT73	STR	2	208-230	M5	Roed and Midthjell, 1998
OARAE129	STR	4	146-158	M5	Penty et al., 1993
OARFCP26	STR	4	134-160	M4	Ede et al., 1995
OARFCB304	STR	3	123-139	M6	Talbot et al., 1996
TGLA53	STR	3	162-170	M6	Al-Atiyat et al., 2018
LCAPPRO	SEQ	/	350*		Douzery and Randi, 1997
GH-GHR	SEQ	/	600*		Lioupis et al. 1997, Wallis et al. 2006