Biological Conservation 197 (2016) 154–163

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/bioc

Towards understanding isotope variability in elephant ivory to establish isotopic profiling and source-area determination

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ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form 29 February 2016
Accepted 7 March 2016
Available online xxxx

Keywords:
Elephant ivory
Stable isotopes
Wildlife forensics
Assignment simulation

ABSTRACT

We present here new isotopic data ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{2}H$, and $\delta^{34}S$) from pulverised ivory powder, measured by continuous flow isotope ratio mass spectrometry from an unprecedented large dataset of 507 ivory samples, derived from twenty-eight African and six Asian elephant range states. The aim of this study is to assess the accuracy of isotopic fingerprinting and to evaluate its forensic potential and limitations to predict the provenance of ivory of unknown origin. We constructed a nominal assignment framework for the African reference samples, consisting of 208 different sites and applied the weighted *k*-Nearest Neighbor Classifier with reference site as classifier and inferred the accuracy of the assignments of samples from the African elephant species to their correct provenance. Our results show that isotopic profiling of African elephant ivory works on regional scales and we were able to assign 50% of all samples within 381 km, and the majority of the remaining samples within 1154 km. Source area determination is hampered by the fact that within-site and within-individual variation in ivory is immense because elephants as ecological generalists use a wide diversity of plant resources. We propose that forest elephant diets differ more between individuals (i.e. dietary niche partitioning is more significant) than in savanna elephants where individual diets overlap more. Increasing sampling effort in order to decrease median distance of the nominal assignment framework and to better understand within-site variance of the studied isotopic systems are imperative to establish isotopic profiling in the context of law enforcement and wildlife forensics.

1. Introduction

Illegal killing of elephants for the international ivory trade is a serious threat in many range states and may be leading to a dramatic decline in some populations, particularly in central Africa (CITES, 2012). Many large-scale ivory seizures are linked to Asian destinations, and long-term preservation of the elephant populations of central Africa will only be possible with concerted actions to reduce illegal killing of elephants and to curb the illegal trade in elephant specimens (Wasser et al., 2010, 2015). Court-proof results will need internationally recognized analytical methods and reference materials to facilitate legal prosecution (UNODC, 2014).

Forensic analytical methods can play an important role in conserving and managing wild populations as well as in the investigation of wildlife crime through identification and profiling of tissue samples (Voigt et al., 2012, Moncada et al., 2012, CITES, 2012). Molecular approaches to track the provenance of elephant ivory include analyses of nuclear microsatellite DNA markers (Wasser et al., 2004, 2008) and mitochondrial DNA (Ishida et al., 2013). Based on elephant relatedness rather than habitat signatures, these methods are thought to provide cost-efficient markers to support wildlife conservation (Wasser et al., 2004). Stable isotope analysis, by contrast, investigates intrinsic chemical tissue signatures that provide information on local foodwebs, climate and other environmental parameters (Peterson and Fry, 1987; Tieszen and Boutton, 1988; Tieszen, 1991; Michener and Schell, 1994; Hobson, 1999). Isotopic analysis has the advantage of allowing insights into the origin of elephant ivory even when the sample does not contain DNA, or if DNA material is degraded (Ehleringer and Matheson, 2007; UNODC, 2014).

Quantitative measurements of stable isotopes have been used extensively in the fields of biology, ecology, geology as well as in forensic investigation and identification (Hobson, 1999; Ehleringer and Matheson, 2007). The use of natural abundance of isotope variation as geographic tracers to determine the provenance of food commodities has been established for materials that are naturally less variable

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http://dx.doi.org/10.1016/j.biocon.2016.03.008
0006-3207/

isotopically, such as beef, dairy products and beverages but also for products and derivates that derive from species with distinct distribution areas, such as honey (Kelly et al., 2005 and references therein). On the contrary, species with vast distribution ranges, such as elephants often show considerable variation in isotope ratios (Ziegler et al., 2012). Stable isotopes can play an important and efficient role in the investigation of wildlife crime (Bowen et al., 2005). In the past 20 years, carbon, oxygen, nitrogen, strontium and lead isotopes in elephant tusks have been evaluated and used to determine the geographic source of ivory (Van der Merwe et al., 1988; Vogel et al., 1990; Cerling et al., 1999, 2003, 2004, 2007). Van der Merwe et al. (1988), for example, firstly documented that carbon isotopic ratios in elephant ivory, expressed as $\delta^{13}C$, had a linear relationship with tree density, which can be used to distinguish forest elephants from savannah populations. Later, van der Merwe et al. (1990) analysed more than 100 ivory samples from ten African countries and were able to distinguish most elephant populations by combining $\delta^{13}C$, $\delta^{15}N$ and $\delta^{87}Sr$ isotopic ratios. Cerling et al. (1999, 2003, 2004, 2007) combined $\delta^{13}C$- and $\delta^{18}O$-ratios of elephant ivory and distinguished different regions based on variable diet preferences and different sources of water. Lastly, Ishibashi et al. (1999) compared 163 ivory samples from 11 range states of *Loxodonta africana* and concluded that $\delta^{13}C$- and $\delta^{15}N$-ratios were useful for ivory sourcing.

Several authors referred to general expectations of biome characteristics associated with foodweb ^{13}C and ^{15}N values (Van der Merwe et al., 1990; Ishibashi et al., 1999), but one shortcoming of the current framework is that when applied across an entire community, the metrics do not fully incorporate the natural variability within the system into the subsequent summary statistics (Jackson et al., 2011). Furthermore, none of the authors conducted assignment tests to validate the potential of the isotopes more quantitatively to provide predictable and complementary markers for determining the provenance of ivory despite the fact that any source-area determination must have a secure scientific basis that can withstand legal scrutiny (Koch and Behrensmeyer, 1992).

In this paper, we assess and compare new measurements of single and multiple isotope signatures of carbon, nitrogen, sulfur, oxygen and hydrogen from a newly collected, unprecedentedly large sample set (507 samples) of elephant ivory (*Loxodonta africana*, *Elephas maximus*) throughout the species' range of distribution in Africa and Asia. We use these new data to construct a nominal assignment framework with a focus on Africa and to describe spatial variability of elephant isotope ecology. Our aim is to test this reference framework for conservation managers and decision makers by assessing the accuracy of isotopic fingerprinting and to evaluate its forensic potential and limitations for law enforcement to predict the provenance of ivory of unknown origin.

2. Material and methods

2.1. Sampling

507 ivory samples were collected between 2009 and 2012 from 28 African and six Asian elephant range states from European museums and collections, trophy hunters and via protected areas and CITES management authorities in African elephant range states (Table 1). The oldest sample in the dataset is from India and dates back to 1795, while the youngest sample was collected in 2012 in Botswana. Exact provenance of sampled material was not always known, but geographic locations, such as proximity to a village or a river or coordinates were curated for 487 tusks from 208 reference sites in Africa with 1–28 samples (Fig. 1; Table A1).

In cases where sampling was conducted by one of the authors (SZ), ivory fragments of at least 30 mg and less than 2 mm thickness were taken from the most proximal end of the tusk by using a small handsaw or a pincer. This section is largely composed of cementum, is less than six months old and thus minimizes within-individual variation. As this is the most recently-formed and youngest part of the tusk, the isotopic signal reflects the most recent living environment. In cases where samples were provided by others the material often was thicker, and was presumably composed of a mixture of cementum and dentine. The fragments were stored in polyethylene bags until analysis. Ten samples each were available from ten African range states and India (Table 1). The collections from Botswana, Burkina Faso, Democratic Republic of Congo, Malawi, Mozambique and South Africa consist of 362 samples altogether. All data from this study are accessible at http://www.ivoryid.org which is a searchable online database.

Ivory, i.e. the dentine of the elephant's tusk is secreted in increments at the margin of the inner pulp cavity (Raubenheimer et al., 1990); therefore the youngest ivory is formed along this margin with progressively older increments towards the tip of the tusk. Transverse growth rates are ca. 5 mm per year (UNODC, 2014), while the longitudinal growth of a tusk probably continues until late in life or until death (Perry, 1954). In order to evaluate the effect of multi-year isotope signatures upon the variation within individuals, we measured isotope ratios in two individual tusks by drilling powders from the tusks every three centimetres, starting at the proximal end and covering a distance of approximately 42 cm. The tusks were of unknown African origin and were obtained from the German CITES Management Authority. Isotopic measurements of carbon, nitrogen, oxygen, hydrogen and sulfur were carried out on the powders (Table A2). We detected pronounced individual within-tooth isotopic variability, which culminated in high regression coefficients and significant linear relationships between isotopic ratios and distance from the proximal end for all isotopes with the exception of nitrogen (Table 2). However, interpretation of correlations must not be generalised since the direction of isotope change is not consistent in all individual tusks, but depends on elephant migration patterns and nutritional changes during the lifetime of the species (Codron et al., 2012). Thus, attempts to assign an individual elephant to the environment where it died should restrict sampling to the most recently-formed part of the tusks. However, limited sampling size bears the risk of cutting natural variation in the respective reference sites so that samples may be traced to a completely different area.

2.2. Isotopic analyses

Samples were analysed at the Agroisolab Facility for Stable Isotope Research in Jülich, Germany between January 2011 and September 2012. After pulverization in a steel ball mill (Retsch MM200) with the grinding jar continually cooled with liquid nitrogen at −196 °C, samples were cleaned with dichloromethane for six hours to extract apolar substances, such as tissue fat, and then air-dried at 60 °C for 36 h. Samples were then stored in a desiccator to avoid humidification. Subsamples of 1–4.5 mg were subjected to analysis by loading them into 4 × 6 mm tin capsules for carbon, nitrogen and sulfur isotopic measurements. Silver capsules (3.3 × 5 mm) were used for oxygen and hydrogen analysis of another split of the powdered samples. We used continuous flow isotope ratio mass spectrometers and measured five different stable isotope ratios (carbon and nitrogen: Nu Horizon; oxygen: Isoprime JB332; hydrogen: Isoprime JB102; sulfur: Optima A27). Results are reported relative to the Vienna PeeDee Bemennite ($\delta^{13}C$), atmospheric N_2 ($\delta^{15}N$), Standard Mean Ocean Water ($\delta^{2}H$, $\delta^{18}O$), and Canyon Diablo Troilite ($\delta^{34}S$) respectively and measured isotopic ratios (R) are expressed in δ units in the conventional permil notation where $\delta = [(R_{sample}/R_{standard}) - 1] \times 1000$. Samples were also measured against a set of secondary standards (carbon: IAEA-CH-6, IAEA-CH-7; nitrogen: IAEA-N-1, IAEA-N-2; oxygen: IAEA-601; hydrogen: IAEA-CH-7; sulfur: IAEA-S-1; IAEA-S-2, IAEA-S-3) and laboratory standards (carbon and nitrogen: Leucin; oxygen and hydrogen: 1,4-Dihydroxyanthrachinon; sulfur: Cystein). In order to assess precision of the analyses, we performed at least two replicate measurements for each sample. Analytical uncertainties, based on these replicate analyses were typically between 0.1‰ ($\delta^{13}C$, $\delta^{15}N$), 0.2‰ ($\delta^{34}S$), 0.4‰ ($\delta^{18}O$),