

Short Communication

Non-invasive genetic identification confirms the presence of the Endangered okapi *Okapia johnstoni* south-west of the Congo River

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Abstract The okapi *Okapia johnstoni*, a rainforest giraffid endemic to the Democratic Republic of Congo, was recategorized as Endangered on the IUCN Red List in 2013. Historical records and anecdotal reports suggest that a disjunct population of okapi may have occurred south-west of the Congo River but the current distribution and status of the okapi in this region are not well known. Here we describe the use of non-invasive genetic identification for this species and assess the success of species identification from dung in the wild, which varied throughout the range. This variation is probably attributable to varying okapi population densities and/or different sample collection strategies across the okapi's distribution. Okapi were confirmed to occur south-west of the Congo River, in scattered localities west of the Lomami River. We demonstrated that non-invasive genetic methods can provide information on the distribution of cryptic, uncommon species that is difficult to obtain by other methods. Further investigation is required to genetically characterize the okapi across its range and to investigate the biogeographical processes that have led to the observed distribution of okapi and other fauna in the region.

Keywords Democratic Republic of Congo, dung sample, Endangered, endemic species, Giraffidae, okapi, protected area

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The okapi *Okapia johnstoni* is a monotypic species of the family Giraffidae, endemic to the Democratic Republic of Congo. In 2013 the species was recategorized as Endangered on the IUCN Red List but there is still a lack of information regarding its range and population sizes (Mallon et al., 2013). Most of the okapi's range lies to the north and east of the Congo River (Kingdon, 1997; Stuart & Stuart, 1997; IUCN, 2008; Hart, 2013). However, there are anecdotal historical (dating back at least to 1926; Royal Museum for Central Africa, Tervuren) and present-day reports of okapi also occurring south-west of the river (Fig. 1), although these are unconfirmed. It is important to determine the validity of these reports because rediscovery of a southern okapi population would imply a geographically and potentially evolutionarily distinct population and reinforce efforts to gazette protected areas within this region.

Okapi are elusive (the first camera-trap image of an okapi was obtained in 2008; Nixon & Lusenge, 2008) and are consequently difficult to monitor in the wild. Previously, dung counts have been used to determine okapi presence/absence (Beyers, 2008; Vosper et al., 2012). However, studies of other species have shown that accurate visual identification of animal dung can be difficult (Busby et al., 2009; Faria et al., 2011). According to field researchers okapi dung is likely to be confused with bongo *Tragelaphus eurycerus* dung in the wild (J. Hart & S. Nixon, pers. comm.). Non-invasive genetic methods provide a useful way of testing this possibility (Taberlet & Luikart, 1999; Busby et al., 2009; Faria et al., 2011).

Here we present an update on the distribution of the okapi south and west of the Congo River, based on genetic identification of putative okapi dung. Our results show the potential utility of this non-invasive, cost-effective method as a means to confirm species' identities where numbers are low and confusion with other species is possible. We used two mitochondrial DNA (mtDNA) primers, OJ1 and OJ2 (OJ1-F (15162–15180, based on Genbank okapi mt genome, Genbank accession number: NC_020730.1): ATGA ATCGGAGGACAACCA, OJ1-R (15359–15380): GGCCTC TTCTTTGAGTCTTAGG, 217 bp; OJ2-F (15359–15380): CCTAAGACTCAAAGAAGAGGCC, OJ2-R (15525–15542): TGCTGCGTTAAGGCTGTG, 184 bp) from Stanton et al. (2014). Primer OJ1 is sited in the okapi cytochrome b

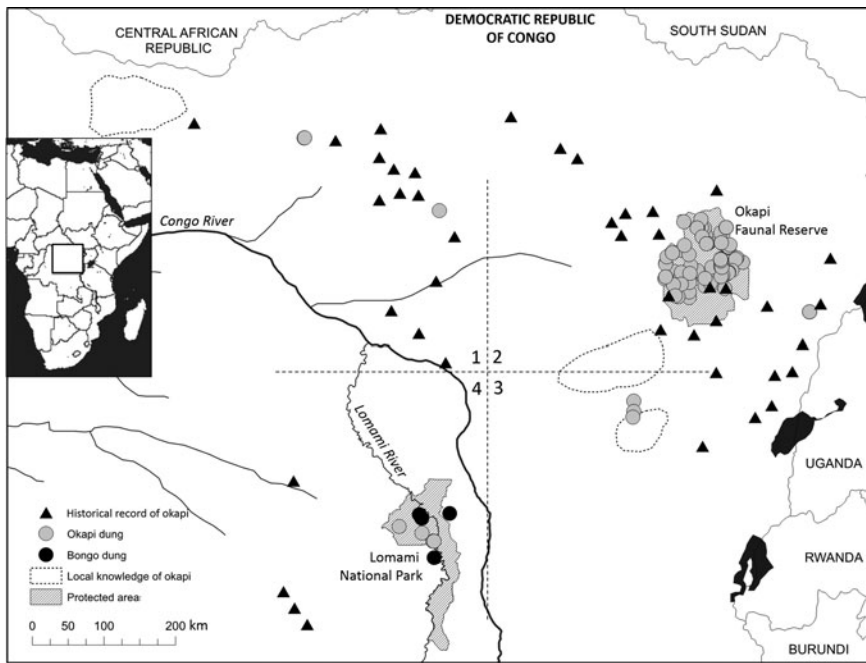


FIG. 1 Map of the study area in the Democratic Republic of Congo, showing the locations where dung samples with viable DNA were found, and of historical records of okapi *Okapia johnstoni* presence (Royal Museum for Central Africa, Tervuren, and the Centre de Recherche en Science Naturelles, Lwiro, DRC). The study area is divided into four sampling regions. Areas with local knowledge of okapi presence and/or evidence of okapi observed in a local village are delineated by dashed lines, based on information collected from the 2013 Okapi Conservation Workshop.

(Cyt b) and tRNA genes, whereas the reverse primer for OJ2 is sited in the control region. Cyt b is usually more conserved between taxa than the control region, and therefore whereas primer OJ1 may amplify DNA from other taxa as well as okapi, primer OJ2 should only amplify DNA from okapi. This combination of primers may therefore be useful for species identification, based on their relative amplification successes, without the need for DNA sequencing. We analysed 247 putative okapi dung samples collected during 2010–2013 throughout the okapi's known range. Seven samples were from sampling region 1, 209 from region 2, seven from region 3 and 24 from region 4 (Fig. 1). Faecal samples were collected either by (1) walking along randomly placed transects through forest sites and collecting any faeces observed, or (2) identifying putative okapi sign (dung or prints, for which the reliability of correctly identifying okapi in the wild is unknown) and searching the surrounding area for faeces. Sampling methodology (1) was used in areas of high okapi density (the Okapi Faunal Reserve; Fig. 1) and (2) was used in areas of low okapi density (everywhere else in the range where faecal samples were found). Sample collection in the Reserve was carried out as part of a separate large-mammal survey (Vosper et al., 2012). Elsewhere in the range, areas for sample collection were chosen based on the historical distribution of okapi (Fig. 1; Kingdon, 1997; Stuart & Stuart, 1997; IUCN, 2008; Hart, 2013), attempting to maximize spatial coverage within logistical limits. In sampling locations where it was not clear whether dung piles were from one or more individuals, samples were genotyped with microsatellites (Stanton et al., 2010) to distinguish between individuals.

Details of mtDNA PCR (polymerase chain reaction) reagents and conditions are in Supplementary Tables S1 & S2,

respectively. Products were visualized on a 3% agarose gel and sequenced by Macrogen Europe. For successfully sequenced PCR products, species were identified using the GenBank (BLAST) database. The formulae from Faria et al. (2011) were used to quantify and describe the accuracy of species identification based on the observed pattern of PCR bands on agarose gel before DNA sequencing had been carried out. These formulae estimate the error of omission (where dung from a given species is overlooked by this method), error of commission (where dung is incorrectly attributed to a particular species) and identification accuracy (rate of identification accuracy, both within species and overall). Here, the estimation of identification accuracy reflects only the accuracy of this method for identifying okapi and is therefore equivalent to one minus the error of omission.

Of the 247 dung samples tested 122 did not amplify successfully using either of the mtDNA primers (four of seven (57.1%), sampling region 1; 101 of 209 (48.3%), sampling region 2; four of seven (57.1%), sampling region 3; 13 of 24 (54.2%), sampling region 4), probably because of degraded DNA. Details of OJ1 and OJ2 primer amplification and species identification are in Supplementary Material 1. All samples in which primer OJ2 could be amplified were okapi samples, based on a Genbank (BLAST) of sequenced PCR products. Primer OJ2 failed to amplify a fragment in all bongo samples. Successful PCR amplification of primer OJ2 can therefore be used to differentiate between okapi and bongo without the need for DNA sequencing, with primer OJ1 acting as a positive control. Using the species identification formulae from Faria et al. (2011) we estimate an error of omission of 5%, an error of commission of 0%

and an identification accuracy of 95% for correctly identifying an okapi individual by the presence/absence of a band for primer OJ2. This is because the primer pair OJ1 primes in the Cyt b and tRNA genes, whereas the reverse primer of the OJ2 primer pair primes in the control region, which is much less conserved between species (Supplementary Table S3). Assuming an error of commission of 0% using the pre-sequencing method described above, 119 of the 125 samples containing usable DNA were from okapi and six from bongo. All six bongo samples, misidentified as okapi, were from sampling region 4, south-west of the Congo River. The six okapi samples found in region 4 were from four individuals and were all found on the western side of the Lomami River (Fig. 1).

Our findings show that okapi still occur south-west of the Congo River, although they appear to be localized and at low density, with an encounter rate in the Lomami area of < 0.02 dung samples per km, even in the zones where okapi are known to occur. This compares with encounter rates of 0.11–0.51 dung samples per km in the Okapi Faunal Reserve (Hart et al., 2008; Vosper et al., 2012). This study demonstrates the utility of a molecular approach for differentiating between dung samples from okapi and bongo, the species with which okapi are most likely to be confused in the field. In particular, we demonstrate the possibility of differentiating between okapi and bongo dung without the need for DNA sequencing. These primers provide a simple diagnostic test to identify okapi from non-invasively collected samples, such as dung, using only PCR amplification and gel electrophoresis. However, this methodology is only being tested for positive identification of okapi samples. The 5% error of omission implies that an investigator using this method may class some okapi as bongo (this is essentially allelic dropout; Taberlet & Luikart, 1999); however, this is easily accounted for by sequencing the putative bongo samples. The 0% error of commission implies a high accuracy for samples identified as okapi. It is unlikely that the dung of any species other than bongo, which has an overlapping range with okapi, would be confused with that of okapi (J. Hart & S. Nixon, pers. comm.). Duiker dung has previously been confused with bongo (Faria et al., 2011) but we found no evidence of this in our dataset.

Accurately determining distribution and abundance is a priority for species conservation (Jenkins et al., 2013) and is especially challenging in species that are cryptic, uncommon and locally distributed, as the okapi is in many parts of its range. Fig. 1 shows regions within the putative okapi range where there is local knowledge, based on anecdotal information such as unconfirmed community reports at the Okapi Conservation Workshop (ZSL, 2013). These should be priority areas for implementing the methods we describe here, to improve our knowledge of the current distribution of okapi.

Although we confirmed the occurrence of okapi in Congo's central basin, the species is uncommon and

apparently no longer exists in some areas where it was reported historically, whereas bongo appear to be more common in the areas surveyed south-west of the Congo River than in the Okapi Faunal Reserve (J. Hart, pers. comm.). These differences in density may account for the higher occurrence of bongo dung samples in sampling region 4. In addition, given the low abundance of putative okapi dung outside the Reserve, field teams were asked to collect anything that could be okapi dung, whereas for the surveys within the Reserve they were asked to differentiate between species. Thus, variation in species identification success between sample collectors does not necessarily reflect the identification ability of the field workers. Taken together these results imply that okapi and bongo dung can be identified correctly in the field by eye. However, correct identification should not be assumed, and molecular methods should be used to confirm species identity.

Of the samples found in the Lomami National Park, only the ones west of the Lomami River could be confirmed as okapi. This does not imply that okapi are not present elsewhere south-west of the Congo River but we did not have enough samples to investigate this. The presence of okapi south-west of the Congo River raises the possibility that there are genetically differentiated populations either side of the Congo (and Lomami) River if, as seems likely, the river acts as a dispersal barrier for the species. Okapi, *Cercopithecus lomamiensis* and Thollon's red colobus *Procolobus tholloni* are all restricted to the west side of the Lomami River and absent from the Lomami–Lualaba inter-fluve (Hart et al., 2012). Further studies are needed to investigate the biogeographical processes that have influenced the fauna of this region, and to genetically characterize okapi throughout their range.

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Biographical sketches

DAVID STANTON is a molecular ecologist, whose research interests include inferring the demographic history of populations or species and investigating domestication and human-mediated wildlife translocations. JOHN HART has studied and monitored okapi throughout their range since 1985. He is involved in the establishment of the Lomami National Park in the Democratic Republic of Congo, which will provide legal protection for okapi and other endemic large mammals of the Congo River's left-bank forests. ASHLEY VOSPER is a field scientist and has coordinated a large number of conservation surveys, predominantly in Central and West Africa. NOELLE KÜMPEL's work focuses on conservation, research and policy, specializing in tropical forest conservation, in particular wildlife hunting and trade, monitoring, and sustainable livelihoods. She has managed field projects in the Democratic Republic of Congo since 2007, including a multi-partner project on the range-wide conservation of the okapi. JINLIANG WANG is interested in developing population genetics models and methods of analysis of empirical data to address issues in evolutionary and conservation biology. JOHN EWEN's work focuses on the biology and management of small populations, often involving reintroduction. MICHAEL BRUFORD is a molecular ecologist who works on threatened species, especially those in fragmented and disturbed habitats. He is also interested in the development of conservation policy and management tools.