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Loxodonta Localizer: A Software Tool for Inferring the Provenance of African Elephants and Their Ivory Using Mitochondrial DNA

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Abstract

Illegal hunting is a major threat to the elephants of Africa, with more elephants killed by poachers than die from natural causes. DNA from tusks has been used to infer the source populations for confiscated ivory, relying on nuclear genetic markers. However, mitochondrial DNA (mtDNA) sequences can also provide information on the geographic origins of elephants due to female elephant philopatry. Here, we introduce the *Loxodonta* Localizer (LL; www.loxodontalocalizer.org), an interactive software tool that uses a database of mtDNA sequences compiled from previously published studies to provide information on the potential provenance of confiscated ivory. A 316 bp control region sequence, which can be readily generated from DNA extracted from ivory, is used as a query. The software generates a listing of haplotypes reported among 1917 African elephants in 24 range countries, sorted in order of similarity to the query sequence. The African locations from which haplotype sequences have been previously reported are shown on a map. We demonstrate examples of haplotypes reported from only a single locality or country, examine the utility of the program in identifying elephants from countries with varying degrees of sampling, and analyze batches of confiscated ivory. The LL allows for the source of confiscated ivory to be assessed within days, using widely available molecular methods that do not depend on a particular

platform or laboratory. The program enables identification of potential regions or localities from which elephants are being poached, with capacity for rapid identification of populations newly or consistently targeted by poachers.

Keywords: forensics, forest elephant, poaching, savanna elephant, wildlife trafficking

The poaching (illegal hunting) of elephants for their ivory is a major threat to populations across much of Africa. An upward trend in the amount of ivory illegally traded has persisted since 2008, with the overall weight of ivory in illegal trade estimated to be 3 times greater in 2016 than in 2007 (CITES 2017). There were 22 large-scale seizures of batches of ivory in 2016–each weighing more than 500 kg (CITES 2017). As a result of the illegal trade in ivory, currently more elephants in Africa die from poaching than die from natural causes, and the number of elephants in Africa is estimated to have fallen by ca. 110 000 in the decade prior to 2016 (CITES 2017). The recent decline in population size has been documented as greater than 60% for forest elephants (*Loxodonta cyclotis*) (Maisels et al. 2013) and 30% for savanna elephants (*Loxodonta africana*) (Chase et al. 2016). Recent analyses of population datasets from 73 locations believed to carry half of Africa's elephants concluded that the number of elephants across these locations was less than 25% of what would be expected if poaching were not a factor (Robson et al. 2017). Among these 73 locations, 30% were estimated to have a population size of less than 5% of the expected numbers (Robson et al. 2017). Recently, efforts to reduce demand for ivory and to protect source populations appear to have reduced poaching intensity (Hauenstein et al. 2019).

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has mandated that large-scale seizures of elephant ivory be subject to forensic analysis, which could help to establish the geographic source of the ivory (CITES 2016; CITES 2017). DNA can be extracted from ivory, even from older samples kept at room temperature, in sufficient concentration to permit genetic analysis (Mailand and Wasser 2007; Winters et al. 2018). Progress in establishing the provenance of confiscated elephant ivory has largely relied on the use of microsatellite markers present in nuclear DNA (nDNA). With statistical methods such as spatial smoothing and tessellation, location-specific allele frequencies have been derived for both forest (*Loxodonta cyclotis*) and savanna (*L. africana*) elephants. These have been used to estimate the geographic origin of confiscated ivory (Wasser et al. 2004; Wasser et al. 2007), identify poaching hotspots (Wasser et al. 2015), and infer the tactics of smuggling operations (Wasser et al. 2008; Wasser et al. 2018).

The use of mitochondrial (mt) DNA sequences for establishing the provenance of ivory has also been proposed (Ishida et al. 2013). Among elephants, social groups consist of closely related females and calves of both sexes (Moss and Poole 1983). Upon maturity, female elephants remain with their core social groups, but male elephants disperse (Moss and Poole 1983). But dispersing males cannot propagate mtDNA because offspring of both sexes inherit mtDNA only from their mothers. Therefore, mutations in mtDNA are likely to persistently remain geographically localized (Roca et al. 2015; Ishida et al. 2018). The genetic diversity of mtDNA may be slower to erode among populations than nuclear DNA diversity, because the latter would be affected by a high degree of male–male competition among elephants (Petit and Excoffier 2009; Roca et al. 2015). Importantly, the phylogeographic patterns displayed by elephant mtDNA may starkly differ from patterns exhibited by nuclear DNA (Debruyne 2005; Roca et al. 2005; de Flamingh et al. 2018). Thus mtDNA can provide information on the geographic origins of elephants or their ivory that may not be apparent if only nuclear markers are relied upon (Ishida et al. 2013). Another advantage of mtDNA is that it has a higher copy number per cell than nuclear DNA, a consideration for samples with less abundant or lower quality DNA.

The potential utility of mtDNA sequences for establishing the provenance of elephants has been suggested by previous analyses of the geographic distributions of mtDNA haplotypes. Ishida and colleagues sequenced 4258 bp of mtDNA in 653 savanna and forest elephants from 22 localities in 13 countries (Ishida et al. 2013). Among 108 distinct haplotypes identified, 72% were detected at only one of the 22 localities. This sequence included a shorter segment of mtDNA that forms part of the control region. A 316 bp sequence of hypervariable region 1 of the control region (Johnson et al. 2007) from these elephants was then combined with the same control region sequence reported by previous studies of African elephants to yield a combined dataset representing elephants from 81 localities across 22 countries. In this dataset, 101 distinct haplotypes were detected, with 62% of the distinct haplotypes detected in only a single country (Ishida et al. 2013). The limited geographic distribution of distinct haplotypes suggests that mtDNA sequences would provide a useful tool for examining the provenance of seized ivory (Ishida et al. 2013).

Here, we report the development of software, designated the *Loxodonta* Localizer (LL; www.loxodontalocalizer.org), that uses as input a 316 bp sequence of hypervariable region 1 of the control region of mtDNA generated from African elephant samples to assess the provenance of ivory. The input sequences are queried against previously generated sequences for the genus *Loxodonta* (Barriel et al. 1999; Eggert et al. 2002; Nyakaana et al. 2002; Debruyne et al. 2003; Charif et al. 2005; Debruyne 2005; Archie et al. 2006; Johnson et al. 2007; Ishida et al. 2013; Brandt et al. 2014; Finch et al. 2014; Mondol et al. 2015). The studies that generated these sequences examined a total of 1917 African elephant individuals across 24 range states (Supplementary Table S1). The range states are divided by the IUCN into 4 broad geographic regions, representing western, central, eastern, and southern Africa. For each of these regions, elephant sequences from 6 countries are currently included in the LL, so that each of the regions is well covered by the LL. As output, the LL shows on a map of Africa the geographic locations from which identical or similar sequences have been reported. The map also shows each geographic locality from which any African elephants have been sequenced for the same mtDNA segment. The program provides an assessment of where in Africa elephants with identical or similar haplotypes to the query sequence have previously been sampled. If the query sequence haplotype has a broad geographic distribution, it will not be very informative for assessing the geographic origins of confiscated ivory. But a haplotype with limited geographic distribution would be informative about place of origin. The required DNA methods are widely accessible, provide quick turnaround times, are independent of the platform

used, and allow for ready comparisons across laboratories and research studies. The software is intended to indicate the regions or localities where elephants are being poached, and may especially be useful for quickly identifying elephant populations being newly or most targeted by poachers.

Methods

Programming of the LL

The LL was developed in the Django web application framework (Django Software Foundation 2018) and is hosted at www.loxodontalocalizer.org using the AWS Lambda serverless computer platform. The source code is available at https://github.com/kaizhao86/loxodontalocalizer. The application uses a Django Object-Relational-Model (ORM) managed SQLite database, which manages the architecture of the database through Django interfaces in the Python programming language. The database containing published elephant haplotypes, their respective GenBank accession numbers and publication information, along with the locations of sample collection and write/modify/delete access is restricted to administrators for the web service. The entity-relationship diagram for the LL is shown as Supplementary Figure S1, which lists the information included in the database tables.

User-submitted queries are compared against all haplotypes in the database using the BioPython global pairwise aligner (Cock et al. 2009). Matches are shown in an interactive web viewer. Haplotypes are ordered by number of mismatches to the queried sequence. Localities, where elephants are found with each respective haplotype, are shown utilizing Google Maps. To ensure that errors and omissions were not present in the LL databases, systematic testing was conducted, including 3 major sets of tests, which are described in the Supplementary Text.

DNA Extraction, Amplification, and Sequencing of Seized Ivory

Ivory pieces were decalcified and DNA recovered following the protocol of Mailand and Wasser (Mailand and Wasser 2007). A 522 bp of the elephant control region of mtDNA was targeted using the primers CR-F1/CR-R2 (Brandt et al. 2014) in a 20 µL reaction (14 µL mastermix, 2 µL of each primer and 2 µL of DNA) using a Veriti Thermal Cycler (Applied Biosystems) with the following program: 95°C for 5 min; followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Appropriate negative and positive controls were included throughout. Following treatment of the PCR products with Exonuclease I and shrimp alkaline phosphatase (Hanke and Wink 1994), Sanger sequencing was undertaken on an in-house 3500 sequencing platform (Applied Biosystems) or outsourced, using both forward and reverse primers to produce a consensus sequence which was subsequently edited to 316 bp using Geneious 9.0 (https://www.geneious.com). DNA sequence data were queried against the LL for analysis.

Software Description and Results

Home Page and Output Page for the LL

The LL home page may be viewed at www.loxodontalocalizer.org. The home page introduces the LL; links to frequently asked questions (FAQ); and links to protocols describing DNA extraction from ivory, amplification and Sanger sequencing, and the workflow for trimming of sequences to the appropriate size. Additional details are given in the Supplementary Text. A 316 bp control region sequence from an African elephant is used as a query, and a pull-down menu is available to show existing sequences in the database. The query may be entered in FASTA format, so that the first line(s) of the entry box (preceded by a header line that begins with ">") can include a description, sample number(s), or other identifying information for the sequence. This information will also be shown by the program on the output page.

The LL generates output showing the geographic distribution of identical or similar sequences that have previously been published for African elephants (Figure 1). The output consists of a map, a timestamp and copy of the input sequence including the information entered as the header line of the FASTA format, a listing of results by number of mismatches to the sequence, and a number of options provided to the user. The default output map shows all of sub-Saharan Africa, and thus includes all of the African elephant range states (Thouless et al. 2016) as a Google map (https://www.google.com/maps). It shows an icon at every location for which a sequence is included in the LL database. At locations at which the haplotype(s) most closely matching the input sequence have been reported, the rectangular icons are replaced with pins. Clicking on any of the pins or rectangular icons on the map will produce a box providing information for the closest-matching haplotype to the query at that location, indicating the name of the location, the type of location, the name of the African country, the haplotype LL number, the number of matches and mismatches when compared to the query sequence, and the author(s) reporting the most closely matching sequence.

A listing to the right of the map reiterates the query sequence in FASTA format entered on the input page, and also generates a timestamp that lists the date and time that the output was generated (in Greenwich Mean Time). Results of the search are then listed, showing all of the haplotypes in the LL databases in the order in which they match the query, and including links to corresponding published papers and GenBank accession number(s) (https://www.ncbi.nlm.nih.gov/genbank/). The users can print the output page to save a record of the input sequence, timestamp, and output map and listing. Additional information on the output page is included in the legend of Figure 1 and in the Supplementary Text.

Results From the LL: Haplotypes With Limited Distribution

A haplotype reported from only one locality or country would provide useful information about the likely origin of a batch of confiscated ivory, especially if supported by the sequences of multiple tusks in the batch. Some African elephant mtDNA sequences are carried by elephants from many different countries in a region of Africa, and thus would only be useful for suggesting that regions of Africa from which they have not been detected as unlikely to be the potential source of confiscated ivory. More useful for establishing provenance are the haplotype sequences that have only been reported from a small number of adjacent countries, or from a single country, or even from only a single locality within a country. Those haplotypes that have the most limited geographic distribution would be the most useful for assessing the geographic provenance of confiscated ivory. One such haplotype, LL005, is highlighted in Figure 1. It has been reported only once in the literature, from Ngwasha National Park in Botswana (Eggert et al. 2002). When DNA from a batch of confiscated ivory is sequenced, haplotypes with limited

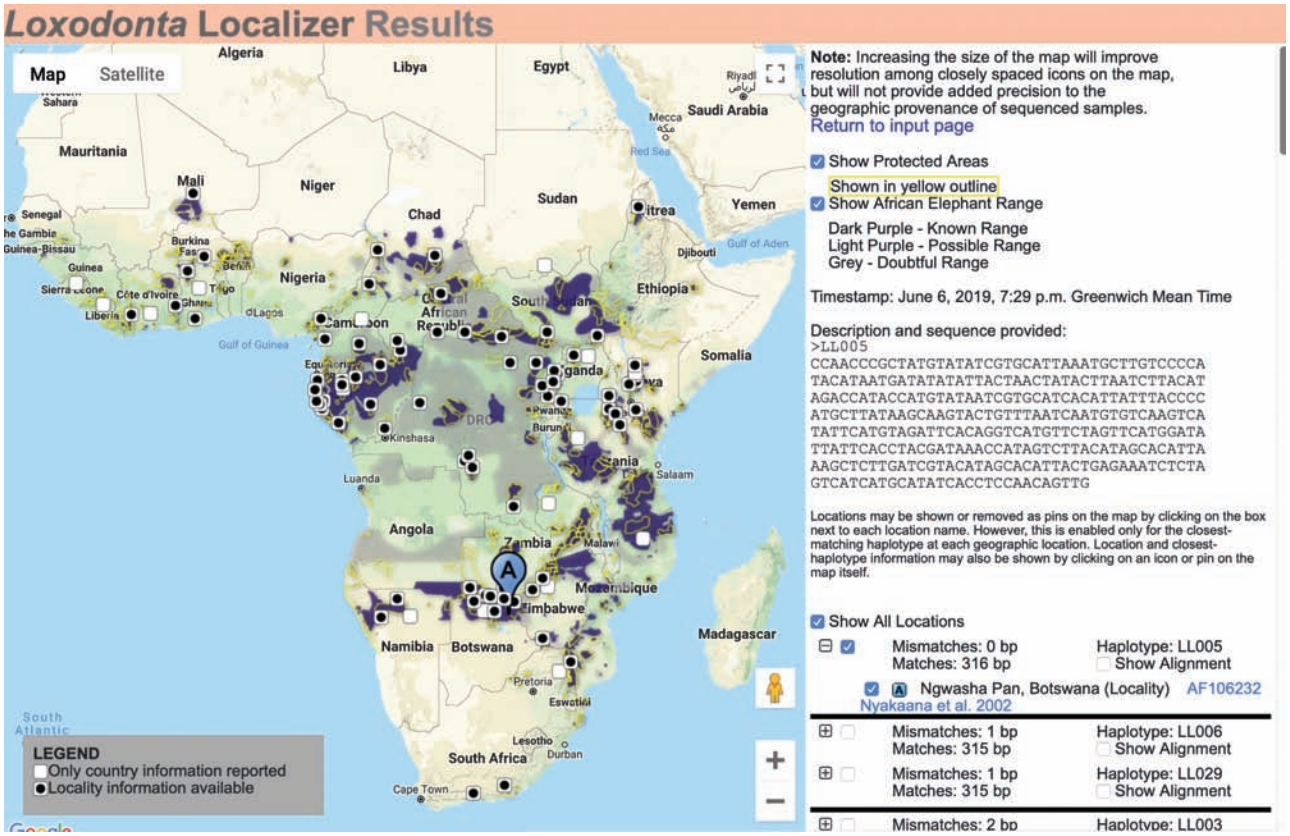

Figure 1. Results page for the LL. The map on the left shows an icon at each location where a reference sequence from an African elephant is available. As indicated by the key, when a specific geographic locality or region is available for the sequence, a white square with a black dot is used; when only country information has been reported for the sequence, the white square does not have a black dot. The user can shift the regions covered by the map or zoom in or out of the map. In a listing to the right, the user is given the option to also show protected areas or the current African elephant range on the map (both are shown) (Thouless et al. 2016). To the right of the map, a timestamp is also shown that lists the date and time that the LL output was generated (in Greenwich Mean Time), and the description and sequence query that were entered in the input page. This is followed by a listing of the search results, with haplotypes ordered by the number of mismatches to the queried sequence. Each reference haplotype result returned includes: the LL haplotype designation, the number of mismatches and matches, the option to show an alignment of the entry with the LL haplotype (not shown), the location and country from which the reference sequence was reported, the corresponding GenBank accession number (with link to GenBank) and the paper reporting the sequences (with a link to the published paper). Reference sequences with exact matches are automatically shown as blue icons with letter codes. It is also possible for the user to select on the list of reference sequences that have mismatches, in which case red pins would appear on the map (not shown). On the map, one may click on a pin (shown) or location icon (not shown), which then displays a box above the pin or icon, with information on the closest-matching reference haplotype at that location (not shown). The example shown has one match at one locality; other haplotypes may provide less precise geographic information.

geographic distributions such as this one would be especially helpful for the assessment of provenance. The utility of such haplotypes is more compelling when the haplotypes are present in regions that have been heavily sampled both extensively (many geographic locations within a region) and intensively (large numbers of elephants, from different social groups). Depending on the sequence, it may be helpful to have the LL also display on a map the geographic distribution of haplotypes with only 1 or 2 mismatches to the identified sequence, to examine the degree to which these may cluster within a precise geographic region.

Results From the LL: Geographically Isolated Populations

Elephant range across Africa is becoming increasingly fragmented, with many populations becoming isolated. An example of an isolated elephant population is that of the Gash-Barka region of Eritrea (Brandt et al. 2014). The elephants of Eritrea are not contiguous with other populations, and population size has been estimated as ca. 80–120 (Shoshani et al. 2004; CITES 2017). mtDNA sequences have been reported for 15 elephant individuals from Eritrea, which carried 3 distinct haplotypes (Brandt et al. 2014). We used each of the 3 haplotypes known to be carried by Eritrean elephants (Brandt et al. 2014) as queries to the LL. One of the Eritrean haplotypes, carried by 3 of 15 Eritrean elephants (Figure 2), has been reported for elephants across the eastern and southern regions of the continent (Brandt et al. 2014). This haplotype is so geographically widespread that it would not be helpful for establishing provenance. By contrast, each of the other 2 haplotypes has only been reported from elephants in Eritrea, and thus would be quite useful for inferring the provenance of an elephant or of ivory as originating from Eritrea or a nearby location (Figure 2). Thus 2 of 3 haplotypes found in Eritrea, carried by 12 of 15 sequenced Eritrean elephants, would provide information useful in assessing the provenance of a sample of unknown origin. While Eritrea's elephant population is not known to be subject to poaching (Yacob et al. 2004), this example highlights the potential of the LL to identify novel locations being targeted by poachers, when rare haplotypes unique to such regions are evident among confiscated ivory.

Figure 2. LL results for Eritrean elephants. The maps show the geographic distribution of each haplotype reported from the population of elephants in the Gash-Barka region of Eritrea (Brandt et al. 2014; CITES 2017). This may be taken as representative of a query sequence to the LL from a set of elephant samples from an isolated population in the increasingly fragmented range of elephants in Africa. Mitochondrial DNA sequences of 15 elephant individuals from Eritrea (out of a total population estimated as ca. 80–120 individuals) comprised 3 distinct haplotypes (Shoshani et al. 2004; Brandt et al. 2014; CITES 2017); the LL output is shown for each haplotype. (**A**) Haplotype LL116 was carried by 11 of the 15 elephants sequenced for Eritrea. (**B**) Haplotype LL117 was carried by 1 of the 15 elephants (Brandt et al. 2014). (**C**) Haplotype LL062 was carried by 3 of 15 Eritrean elephants (Brandt et al. 2014), but has also been reported in elephants across eastern and southern African localities and would not provide information on geographic provenance. Overall, 12 of 15 Eritrean elephants carried haplotypes reported only from Eritrea. This example highlights the potential of the *Loxodonta* Localizer to identify novel locations targeted by poachers, should haplotypes unique to such regions become evident among confiscated ivory.

Results From the LL: Previously Un-Sampled Countries

The addition of more mtDNA sequences, especially for elephants from countries and regions that are not well-sampled, would add to the accuracy and precision of the LL, for example, by identifying novel locally restricted haplotypes. As an example, sequences recently generated for 6 Zambian elephants (de Flamingh et al. 2018) were examined. The 4 distinct haplotypes in these Zambian elephants were queried in the LL (not shown) before these sequences were added to the LL database. Two of the haplotypes from Zambia had exact matches to haplotypes detected in nearby countries. The other 2 haplotypes from Zambia were novel, and there was a high degree of divergence (4 and 6 mismatches) compared to the closest sequence on the LL database. The more mismatches there are between the query sequence and the closest LL haplotype, the less reliable the geographic information provided by the mismatching haplotypes, so these 2 haplotypes would have been geographically uninformative if their provenance had been unknown. Thus novel and divergent mtDNA haplotypes in a batch of ivory may be an indication that it originates in geographic locations not heavily sampled.

Forensic Use of the LL

In 2012, the largest elephant ivory seizure in over a decade (6 tons, approximately 2300 whole or cut tusks) was confiscated in Malaysia, arriving in a shipment from Togo. A subsample of 266 small ivory sections was later transported to the United States for nuclear DNA analysis (Wasser et al. 2004; Wasser et al. 2007). Estimates of geographic provenance were made available almost a year later (Wasser et al. 2015) for 79% of the samples where sufficient nuclear DNA was recovered. During the initial subsampling of the ivory, the Malaysian Department of Wildlife and National Parks (PERHILITAN) collected, where possible, duplicate ivory samples for every sample being transferred to the United States. While individual results for each tested sample were not published alongside the estimate of geographic provenance, which highlighted, in particular, the countries of Tanzania and Gabon (Wasser et al. 2015), they were included for a subset of 80 samples in the supplementary data of a later publication (Cerling et al. 2016). Of these 80 ivory samples, 74 duplicate samples of ivory remained with PERHILITAN and were tested for DNA geographic provenance using the LL.

The mtDNA assessment of provenance was consistent with the geographic provenance previously estimated using nuclear markers, as were the conclusions about the mixed origin of the ivory in this seizure (Wasser et al. 2015). The mtDNA identified countries that overlapped with those identified by nuclear DNA, or identified geographically adjacent countries. This demonstrated that the LL can provide information on the geographic origins of a batch of confiscated ivory with accuracy. The tusks with haplotypes previously reported only from elephants within a single locality or country, or from a few adjacent countries, would be especially helpful for establishing provenance, and this outcome was illustrated by showing output data as 2 sets, one for more narrowly distributed and the other for more widespread haplotypes (Supplementary Table S2).

Following the promising agreement between both nuclear and mtDNA testing of the Malaysia ivory samples, ivory samples were then DNA tested from 2 additional ivory seizures:

(1) From a 7.2 ton ivory seizure in Hong Kong seized by Customs in July 2017. Ninety samples were selected by Hong Kong Customs for subsampling before being transported to Malaysia, with appropriate CITES permits, to undergo mtDNA analysis using the LL as described below. The 90 samples likely represented 78 individuals, as 12 samples were likely from the second tusk of a previously sampled individual (based on morphology; the mtDNA sequences also matched for the 2 tusks for each of these 12 pairs). The samples were all assigned to the Tridom region of central Africa, and therefore represented a single area of origin, assuming that the initial ivory subsampling, which was not entirely consistent with best practice for subsampling (UNODC 2014), was representative of the entire seizure.

(2) From a 3 ton ivory seizure in Malaysia, confiscated by Customs in 2017. One hundred ninety ivory samples were selected for subsampling following appropriate guidelines (UNODC 2014) and DNA sequences analyzed using LL as described below. Again almost all samples were assigned to the Tridom region of Africa as a source for the seized ivory.

For these sets of ivory, the time from the start of analysis to obtaining the sequence results was 6 days and a report showing the provenance of ivory was ready for quick dissemination to source countries within the following days. This highlights the speed at which results can be obtained by using the LL for a preliminary assessment of the provenance of ivory, and the ability of facilities within countries where ivory is confiscated to generate results quickly and effectively in local forensic laboratories. Rapid assessment of the geographic provenance of seized ivory is key to enabling law enforcement agencies to work collaboratively with colleagues from other countries of interest in relation to a particular seizure, and therefore help tackle the transnational nature of this illegal trade.

The simplicity and low cost of the LL approach also make it suitable for ivory DNA testing from seizures below the suggested >500 kg weight proposed by CITES, and even for cases of single tusk seizures, as well as from worked ivory pieces, a sample type that is often less suitable for nuclear DNA tests. On seized ivory pieces, a high success rate (>95%) for DNA extraction, amplification, and sequencing indicates that the LL will be useful for examining these (unpublished data).

The LL could be used to examine and compare haplotypes within or between seizures for further insights into the origins of the ivory. Within a seizure, one may examine whether the geographic distributions of haplotypes are consistent with a common provenance for the tusks. Elephant mtDNA haplotypes fall into 8 subclades (Ishida et al. 2013), many of which do not overlap geographically. Thus, in many cases, 2 different seizures of ivory would not overlap in mtDNA haplotypes, establishing that the 2 sets of ivory have different geographic origins. Previous research has established that many localities even within the same region of Africa do not overlap in haplotypes carried by elephants. For example, in southern Africa, mtDNA haplotypes present among elephants in northern Namibia (n = 60 elephants; 9 unique haplotypes) did not overlap with the haplotypes present among elephants in Kruger (n = 50 elephants; 9 unique haplotypes) (Ishida et al. 2013). To determine whether 2 tusks belong to the same individual, a single locus such as mtDNA is never sufficient, but if 2 seizures of ivory show no overlap in mtDNA haplotypes, this would also preclude the necessity for further analyzing the 2 seizures to determine whether 2 tusks in the different shipments were from the same individual elephant (Wasser et al. 2018). By contrast, if tusks in 2 different seizures both carried a rare mitochondrial haplotype, the 2 tusks could be specifically targeted for nuclear DNA analysis for individual identification. Even if haplotypes are shared between ivory seizures, one could split the haplotypes in each seizure into those with a geographically widespread, and those with a geographically limited distribution. Comparing only the geographically limited haplotypes between the 2 seizures would give an indication as to whether the ivory in each seizure may have originated in the same geographic region.

Discussion

The LL does not specifically address the taxonomy of African elephants because CITES lists all African elephants as a single protected taxon regardless of species or subspecies. The only exceptions to the placement of elephants in Africa in CITES Appendix I have been granted to individual countries, and not by taxonomic unit, so that species or subspecies within Africa are not a legal consideration. However, in the FAQ we include information on our published method for identifying species of origin using nuclear SNPs that have fixed differences between the species (Ishida et al. 2011). The mtDNA phylogeography of elephants is consistent with the pattern detected among many pairs of hybridizing species, for which mtDNA is a poor marker of species boundaries when females are the non-dispersing sex (Petit and Excoffier 2009), as is the case for elephants (Supplementary Text). This mtDNA pattern does not affect the ability of unique haplotypes to provide geographically useful information (Ishida et al. 2013), indeed it is the matrilocality of females that allows many mtDNA haplotypes to enable assessment of geographic provenance. Discrepant mtDNA and nuclear DNA phylogeographic patterns suggest that analyses including both nuclear and mitochondrial genetic markers would provide improvements in the accuracy and precision of geographic assignment of provenance, and that mtDNA data can reduce uncertainty of assignment for some samples examined using nuclear DNA (Ishida et al. 2013). The LL could also be used to examine in greater detail the evolutionary history of African elephants, which were heavily impacted by climate and habitat changes during the Pleistocene (Roca et al. 2015; Ishida et al. 2018). These events likely shaped phylogeographic patterns in mtDNA, which continue to persist because females do not disperse (Roca et al. 2015; Ishida et al. 2018).

In principle, mtDNA would also be a useful marker for establishing the provenance of confiscated wildlife products from other species, notably those for which dispersal is low among females. Similar software has already been developed for various taxa, notably to distinguish among species within several vertebrate groups (http://www.dna-surveillance.auckland.ac.nz/) (Ross et al. 2003). It would be possible to develop similar software for other species, although separate databases would have to be established for the other species, which would also have to be examined for the geographic distributions of distinct haplotypes. To facilitate the development of such software, the entity-relationship diagram for the LL is included as Supplementary Figure S1, along with the information included in the database tables.

The LL can be used to estimate the place of origin of elephants (or their ivory) using geographically restricted mtDNA haplotypes (Ishida et al. 2013). Analysis of tusks from a seizure of ivory in Malaysia established that mtDNA could be effectively used to examine the provenance within Africa of confiscated ivory of unknown origin, as results were consistent between nuclear and mtDNA. The LL has a number of advantages; for example, DNA extraction, amplification, sequencing, and sequence assembly are straightforward and well established in molecular laboratories, and mtDNA sequences can be obtained quickly, typically in less than a few weeks. There are also a number of potential limitations, though some of the limitations could be overcome with additional enhancements to the LL. While many mtDNA sequences have been reported from only a single country or even a single locality, some of the 316 bp control region haplotypes in the LL show geographically quite widespread distributions (Figure 2) (Ishida et al. 2013). Such haplotypes could only establish the broad region of Africa from which an elephant's ivory originated. One possible future improvement to the LL would be to use longer mtDNA sequences from elephants across Africa. While the 316 bp control region sequences

used by the LL are highly variable, there are many mutations outside of the control region that would provide greater geographic precision through the use of longer mtDNA sequences. Currently, the query sequence must be 316 bp long as this corresponds to the region of the mtDNA control region that was sequenced by all of the major previous surveys of African elephants, and allows inclusion in the LL of shortest sequences reported by transnational studies of elephant mtDNA (Johnson et al. 2007). In the future, it would be possible to provide more precise results by using longer sequences in the LL, ultimately including the full mitogenome sequences of elephants. Given this potential, although the 316 bp segment is currently being used by the LL, sequencing of longer regions of the mitogenome is encouraged for its potential future utility.

A more intensive sampling in terms of numbers of elephants sequenced would also serve to improve the LL. As greater numbers of elephants are sequenced at a geographic location, it becomes more likely that rare recent mutations limited to elephants in the local region would be detected. The detection of these rare and locally distributed sequences in shipments of ivory would then help to more precisely assess the locations being targeted by poachers, thereby also permitting a faster response by law enforcement and conservation agencies to local poaching pressures. There is also a need for extensive sequencing of elephants from novel geographic regions of Africa. The addition of more geographic regions may demonstrate that some haplotypes are more widespread than is currently established, which would serve to improve the reliability of the LL. Importantly, more widespread geographic coverage is also likely to identify rare haplotypes with recent mutations unique to the newly sampled localities or countries, improving the ability of the LL software to identify the provenance of ivory for a greater number of locations.

One major advantage of the LL is that the results of mtDNA sequencing can be readily compared across laboratories, allowing for results to be combined across studies. Because sequences can be accumulated from many different sources, researchers within each African elephant range state could potentially collect samples locally, then extract, amplify and sequence mtDNA from elephants within their country. This would pave the way for capacity building in range countries and in transit or destination countries where trafficked ivory is confiscated. Sampling can be non-invasive and straightforward, because sequences can be obtained from dung. Sequences can also be obtained from museum samples, to provide information from geographic areas where sampling may not be currently possible or where populations are now extirpated. These factors together allow for greater numbers of laboratories to generate more intensive and extensive surveys of African elephant mtDNA. Surveys can be independently published by range state researchers before the sequences are incorporated into the LL. As noted above, improvements to the LL are likely to involve the use of longer regions of mtDNA, and eventually the use of complete mitogenomes for the assignment of geographic provenance, providing greater accuracy and precision to the LL. Thus the widespread generation by range state laboratories of elephant sequences much longer than the 316 bp currently used by the LL would be strongly encouraged.

Supplementary Material

Supplementary material can be found at *Journal of Heredity* online.

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References

Archie EA, Moss CJ, Alberts SC. 2006. The ties that bind: genetic relatedness predicts the fission and fusion of social groups in wild African elephants. *Proc Biol Sci*. 273:513–522.

Barriel V, Thuet E, Tassy P. 1999. Molecular phylogeny of Elephantidae. Extreme divergence of the extant forest African elephant. *C R Acad Sci III*. 322:447–454.

Brandt AL, Hagos Y, Yacob Y, David VA, Georgiadis NJ, Shoshani J, Roca AL. 2014. The elephants of Gash-Barka, Eritrea: nuclear and mitochondrial genetic patterns. *J Hered*. 105:82–90.

Cerling TE, Barnette JE, Chesson LA, Douglas-Hamilton I, Gobush KS, Uno KT, Wasser SK, Xu X. 2016. Radiocarbon dating of seized ivory confirms rapid decline in African elephant populations and provides insight into illegal trade. *Proc Natl Acad Sci U S A*. 113:13330–13335.

Charif RA, Ramey RR, Langbauer WR, Payne KB, Martin RB, Brown LM. 2005. Spatial relationships and matrilineal kinship in African savanna elephant (*Loxodonta africana*) clans. *Behav Ecol Sociobiol*. 57: 327–338.

Chase MJ, Schlossberg S, Griffin CR, Bouché PJ, Djene SW, Elkan PW, Ferreira S, Grossman F, Kohi EM, Landen K, *et al.* 2016. Continent-wide survey reveals massive decline in African savannah elephants. *PeerJ*. 4:e2354.

CITES. 2016. *Resolution Conf. 10.10 (Rev. CoP17): trade in elephant specimens*. Geneva (Switzerland): Convention on International Trade in Endangered Species of Wild Fauna and Flora.

CITES. 2017. *SC69 Doc. 51.1: elephant conservation, illegal killing and ivory trade*. Geneva (Switzerland): Convention on International Trade in Endangered Species of Wild Fauna and Flora.

Cock PJ, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, *et al.* 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics*. 25:1422–1423.

de Flamingh A, Roca AL, van Aarde RJ. 2018. Origin and phylogeography of African savannah elephants (*Loxodonta africana*) in Kruger and nearby parks in southern Africa. *Conservation Genetics*. 19:155–167.

Debruyne R. 2005. A case study of apparent conflict between molecular phylogenies: the interrelationships of African elephants. *Cladistics*. 21: 31–50.

Debruyne R, Van Holt A, Barriel V, Tassy P. 2003. Status of the so-called African pygmy elephant (*Loxodonta pumilio* (NOACK 1906)): phylogeny of cytochrome b and mitochondrial control region sequences. *C R Biol*. 326:687–697.

Django Software Foundation. 2018. Django (Version 2.1.3). Lawrence (KS): Django Software Foundation. Available from: https://djangoproject.com

Eggert LS, Rasner CA, Woodruff DS. 2002. The evolution and phylogeography of the African elephant inferred from mitochondrial DNA sequence and nuclear microsatellite markers. *Proc Biol Sci*. 269:1993–2006.

Finch TM, Zhao N, Korkin D, Frederick KH, Eggert LS. 2014. Evidence of positive selection in mitochondrial complexes I and V of the African elephant. *PLoS One*. 9:e92587.

Hanke M, Wink M. 1994. Direct DNA sequencing of PCR-amplified vector inserts following enzymatic degradation of primer and dNTPs. *Biotechniques*. 17:858–860.

Hauenstein S, Kshatriya M, Blanc J, Dormann CF, Beale CM. 2019. African elephant poaching rates correlate with local poverty, national corruption and global ivory price. *Nat Commun*. 10:2242.

Ishida Y, Demeke Y, van Coeverden de Groot PJ, Georgiadis NJ, Leggett KE, Fox VE, Roca AL. 2011. Distinguishing forest and savanna African elephants using short nuclear DNA sequences. *J Hered*. 102:610–616.

Ishida Y, Georgiadis NJ, Hondo T, Roca AL. 2013. Triangulating the provenance of African elephants using mitochondrial DNA. *Evol Appl*. 6: 253–265.

Ishida Y, Gugala NA, Georgiadis NJ, Roca AL. 2018. Evolutionary and demographic processes shaping geographic patterns of genetic diversity in a keystone species, the African forest elephant (*Loxodonta cyclotis*). *Ecol Evol*. 8:4919–4931.

Johnson MB, Clifford SL, Goossens B, Nyakaana S, Curran B, White LJ, Wickings EJ, Bruford MW. 2007. Complex phylogeographic history of central African forest elephants and its implications for taxonomy. *BMC Evol Biol*. 7:244.

Mailand C, Wasser SK. 2007. Isolation of DNA from small amounts of elephant ivory. *Nat Protoc*. 2:2228–2232.

Maisels F, Strindberg S, Blake S, Wittemyer G, Hart J, Williamson EA, Aba'a R, Abitsi G, Ambahe RD, Amsini F, *et al.* 2013. Devastating decline of forest elephants in central Africa. *PLoS One*. 8:e59469.

Mondol S, Moltke I, Hart J, Keigwin M, Brown L, Stephens M, Wasser SK. 2015. New evidence for hybrid zones of forest and savanna elephants in Central and West Africa. *Mol Ecol*. 24:6134–6147.

Moss CJ, Poole JH. 1983 Relationships and social structure of African elephants. In: Hinde RA, editor. *Primate social relationships*. Sunderland (MA): Sinauer. pp 315–325.

Nyakaana S, Arctander P, Siegismund HR. 2002. Population structure of the African savannah elephant inferred from mitochondrial control region sequences and nuclear microsatellite loci. *Heredity (Edinb)*. 89:90–98.

Petit RJ, Excoffier L. 2009. Gene flow and species delimitation. *Trends Ecol Evol*. 24:386–393.

Robson AS, Trimble MJ, Purdon A, Young-Overton KD, Pimm SL, van Aarde RJ. 2017. Savanna elephant numbers are only a quarter of their expected values. *PLoS One*. 12:e0175942.

Roca AL, Georgiadis N, O'Brien SJ. 2005. Cytonuclear genomic dissociation in African elephant species. *Nat Genet*. 37:96–100.

Roca AL, Ishida Y, Brandt AL, Benjamin NR, Zhao K, Georgiadis NJ. 2015. Elephant natural history: a genomic perspective. *Annu Rev Anim Biosci*. 3:139–167.

Ross HA, Lento GM, Dalebout ML, Goode M, Ewing G, McLaren P, Rodrigo AG, Lavery S, Baker CS. 2003. DNA surveillance: web-based molecular identification of whales, dolphins, and porpoises. *J Hered*. 94: 111–114.

Shoshani J, Hagos Y, Yacob Y, Ghebrehiwet M, Kebrom E. 2004. Elephants (*Loxodonta africana*) of Zoba Gash Barka, Eritrea: Part 2. Numbers and distribution, ecology and behaviour, and fauna and flora in their ecosystem. *Pachyderm*. 36:52–68.

Thouless CR, Dublin HT, Blanc JJ, Skinner DP, Daniel TE, Taylor RD, Maisels F, Frederick HL, Bouche P. 2016. *African Elephant Status Report 2016: an update from the African Elephant Database. Occasional Paper Series of the IUCN Species Survival Commission, No. 60*. Gland (Switzerland): IUCN.

UNODC. 2014. *Guidelines on Methods and Procedures for Ivory Sampling and Laboratory Analysis*. Vienna: United Nations Office on Drugs and Crime. Available from: http://www.unodc.org/documents/Wildlife/Guidelines_Ivory.pdf

Wasser SK, Brown L, Mailand C, Mondol S, Clark W, Laurie C, Weir BS. 2015. Genetic assignment of large seizures of elephant ivory reveals Africa's major poaching hotspots. *Science*. 349:84–87.

Wasser SK, Joseph Clark W, Drori O, Stephen Kisamo E, Mailand C, Mutayoba B, Stephens M. 2008. Combating the illegal trade in African elephant ivory with DNA forensics. *Conserv Biol*. 22:1065–1071.

Wasser SK, Mailand C, Booth R, Mutayoba B, Kisamo E, Clark B, Stephens M. 2007. Using DNA to track the origin of the largest ivory seizure since the 1989 trade ban. *Proc Natl Acad Sci U S A*. 104:4228–4233.

Wasser SK, Shedlock AM, Comstock K, Ostrander EA, Mutayoba B, Stephens M. 2004. Assigning African elephant DNA to geographic region of origin: applications to the ivory trade. *Proc Natl Acad Sci U S A*. 101:14847–14852.

Wasser SK, Torkelson A, Winters M, Horeaux Y, Tucker S, Otiende MY, Sitam FAT, Buckleton J, Weir BS. 2018. Combating transnational organized crime by linking multiple large ivory seizures to the same dealer. *Sci Adv*. 4:eaat0625.

Winters M, Torkelson A, Booth R, Mailand C, Hoareau Y, Tucker S, Wasser SK. 2018. Isolation of DNA from small amounts of elephant ivory: sampling the cementum with total demineralization extraction. *Forensic Sci Int*. 288:131–139.

Yacob Y, Shoshani J, Hagos Y, Kebrom E. 2004. The elephants (*Loxodonta africana*) of Zoba Gash Barka, Eritrea: part 3. Ecological and other data from tusks, teeth and carcasses. *Pachyderm*. 37:45–58.