An accurate molecular method to sex elephants using PCR amplification of Amelogenin gene

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Abstract

The use of molecular methods to identify the sex of elephants from non-invasive samples is essential for studies of population dynamics and population genetics. We designed a new technique for sex identification in elephants using Amelogenin (AMEL) genes. The X-Y homologs of AMEL genes are suitable for sex determination in pigs and some species in the family Bovidae. The use of AMEL genes was more successful than previous methods that relied on genes found exclusively on Y-chromosomes, such as SRY, to distinguish males from females. We designed a common forward primer and two reverse primers for X- and Y-specific AMEL genes to obtain 262bp and 196bp PCR amplicons from Y and X genes, respectively. We tested the primers for the identification of the sex of 132 elephants. Using our approach, the sex of 126 individuals (95.45%) matched the reference samples, while 6 (4.54%) did not match. This discrepancy observed was due to high grass content in which reduces the ability to accurately sex young individuals in the field. Through our stool samples results, we have shown that the use of only three primers for AMELX/Y provides a highly accurate PCR-based method for sex identification in elephants. The method is fast and shows more success than the SRY system by avoiding the inherent ambiguities of the previous PCR-based methods that made it difficult to distinguish between female samples and failed amplification reactions. Our sex identification method is non-invasive, and can be applied in population genetic studies and forensics tests with elephant species.

Introduction

Molecular genetics is a powerful tool with many potential applications for conservation biology and wildlife management (Ortega et al. 2004). One of these applications includes the development of simple and accurate methods to determine the sex of wildlife from non-invasive samples. African savannah elephants face population decline as a result of illegal poaching for their ivory. An accurate method to sex elephants non-invasively becomes crucial to determine sex ratio for ecological studies and for the identification of the forensic samples of elephant origin. Several methods have been developed that use PCR amplification of sex determination regions on sex chromosomes (Fernando and Melnick 2001; Gupta, Thangaraj, and Singh 2006; Vidya et al. 2007; Ahlering et al. 2011).

A method of sex identification for elephants, that has been widely used on non-invasive sample types, is based on three pairs of PCR primers for amplification of the genes AMELY2, SRY1, and PLP1 (Ahlering et al. 2011). The method requires conducting multiplex PCR such that females exhibit a single band (PLP1) and males exhibit all three bands owing to the presence of Y-chromosome genes in males only. Although this method was reliable and more efficient than the previous methods (Ahlering et al. 2011), there were some incidences where amplification was not successful. Tests based on SRY amplification can be problematic as non-amplification of the Y-marker represents either female or a failed PCR (Peppin et al. 2010). Although this method (Ahlering et al. 2011) used two Y-markers (AMELY2 and SRY1) for males, there were occasions when one of the markers would fail to amplify, resulting in ambiguous results.

A more accurate method was designed based on the existing differences in sequence length between X- and Y-specific Amelogenin (AMEL) genes in a variety of species (Weikard, Pitra, and Kuhn 2006). The X-Y homologs of AMEL genes (AMELX and AMELY) have been shown to be suitable for sex determination at the molecular level and have been used to identify sex in the family Bovidae (Weikard, Pitra, and Kuhn 2006) and in pigs (Sembon et al. 2008). A single primer pair can be designed to amplify both chromosome-specific forms of the genes. The aim of this paper was to design primers from AMEL genes specific for elephants to accurately determine the sex of elephants from non-invasive samples.

Materials and Methods

Fresh elephant fecal samples were collected from external parts of dungs and stored in Queen's College buffer solution buffer (20% DMSO, 0.25 M EDTA, 100mM Tris, pH7.5, saturated with NaCl). We collected 132 fecal samples from the Serengeti and Tarangire-Manyara ecosystems in northern Tanzania (Table 1). Four tissue samples (2 males and 2 females) from elephants who died naturally were also included as positive controls for known sex. While in the field, an individual's sex was determined through observations of genitalia, their overall body size, and the shape of the head and the thickness of the tusks, especially for adults. Photographs were taken for each individual to confirm their sexes. Through this close field observations, the sex of all 132 individuals were identified while collecting their fecal samples and these were used to test the new method. DNA was isolated using company protocols with DNA extraction kits (QIAamp DNA Stool Mini Kit) with minor modifications (Eggert, Maldonado, and Fleischer 2005). Three PCR primers were designed using SnapGene® software (GSL Biotech LLC), based upon the AMELX (Reference Sequence: NW_003573459) and AMELY (Genebank accession AY823322) in *Loxodonta Africana* by a common forward primer "AMELXY" 5'-TTCTGGAATCTGGTTTGAGGCT-3', X-specific "AMELX-R"5'-ATC TTT ACAACA AAA CAA TTG TTA ACC ATG CTC-3' and Y-specific "AMELY-R" 5'-TCAGATTCA GAGTTTCCT TCATGC AGTAG-3' reverse primers.

Three primers would amplify both X and Y if present (i.e. males) yielding two DNA bands of different size, whereas females would generate only one band. The PCR reaction was performed the initial polymerase activation step at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing temperature at 56°C for 45 seconds, extension at 72°C for 30 seconds for 35 cycles. Each PCR mixture contained 3µl of 5X Green GoTaq reaction buffer (promega) ,a final concentration of 0.67µM of forward primers and 0.33 µM for each of the reverse primers, 0.13µM of dNTP (Quanta bio), Bovine serum albumin (BSA) 0.1µg/µl, and 3µl of DNA template of unknown concentration in a 15µl volume. For each PCR reaction with test samples, we also ran a negative control (no DNA) and a positive control using DNA from known tissue samples. 6µl of the PCR product was used for electrophoresis in Tris-Acetate EDTA running buffer at 120V for 45 min on a 2% agarose gel stained with GelRed (Biotium). Experiments were repeated twice to verify the results. Samples that gave ambiguous results were tested three times under the standard conditions described above.

To confirm the origins of the PCR products, amplicons from at least three samples for each primer pair were sequenced and performed a BLASTn alignment search on NCBI database to confirm the sequences.

Results and Discussion

A total of 126 dung samples were successfully sexed using our AMELX/Y sexing method (Table 1). By this PCR-based method, 6 of the 132 individuals identified in the field as being of known sex, were shown to be misidentified (4.45% false-positive rate). Four of these were young elephants with age less than ten years. In the field, it is more difficult to accurately identify the sex of young elephants compared to older elephants due to limited visibility in long grass or savanna woodlands. A similar challenge was previously reported by (Jones et al. 2017). Therefore, all mismatches observed were likely due to observational errors in the field and not the failure of PCR-based sexing method.

Table 1. The number of females and males identified using AMELX/Y primers. For each location, the number of males and females are shown for reference sex and AMELX/Y sexing method.

Gender	Reference sex	AMELY/X sexing method	Misidentification
Females	67	64	3
Males	65	62	3
Total	132	126	6

As expected, known male samples produced two DNA amplification bands whereas only one DNA band was amplified from female samples (Figure 1). A similar method was used to identify the sex of species in family Bovidae (Weikard, Pitra, and Kuhn 2006) and pigs (Sembon et al. 2008) using tissue samples. Because we used DNA derived from scats, which were significantly degraded, we chose PCR priming sites that produced short fragment sizes of 262bp and 196bp for AMELY and AMELX respectively.

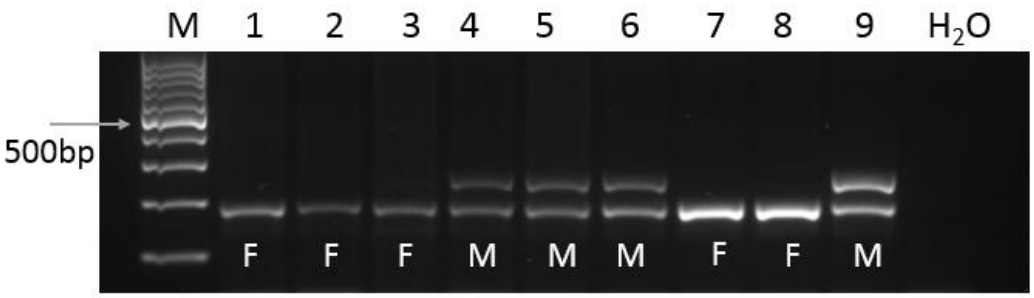

Figure 1. Agarose gel electrophoresis (2% agarose) of PCR product using AMELX and AMELY primer sets for Loxodonta africana.

Males (M) show two PCR bands (AMELX 196 bp and AMELY 262bp) whereas females (F) show only one PCR band (AMELX 196 bp). Lane M is 100bp DNA ladder (GBiosciences) and water was used as a negative control. An arrow indicates a 500bp size of the ladder. Lanes 1, 2, 3, 7 and 8 represent females and lanes 4, 5, 6, and 9 represents males

Our AMELX/Y sexing method is particularly useful for partially degraded DNA samples, such as those derived from fecal samples, because the amplicons produced are relatively short. The method also allows for the direct positive scoring of females. When the DNA quality is low, there is a chance of high PCR failure rates. Using the previous multiplexing method of Ahlering et al. (2011), we unexpectedly found a high number of mismatches from the reference samples, which made it difficult to distinguish males from females. A similar challenge was experienced in other studies. For example, a recent study could not determine the sex of some individuals (16%) due to repeated failure to discriminate DNA bands as either males or females (Lobora et al. 2017). Such a high failure rate results in a large amount of missing data needed to understand important aspects of population structure, such as effective population size. Our technique for identifying the sex of elephants can be used in a wide variety of studies aimed at the conservation of elephants, including those that address human-elephant conflicts, population dynamics, and anti-poaching efforts. Future improvements possible for this method, such as an automated assay, will further facilitate genetic analysis of free-ranging elephant populations and could make significant contributions to the management and conservation of other threatened species.

In this article, we described an improved technique to sex elephants using a non-invasive method. To our knowledge, this is the first work to report the use of this AMELX/Y method for sexing elephants based on DNA collected from fecal samples in the field. The use of this method will

help researchers save time and resources and can be applied to other mammalian species. Using our method, we could easily distinguish males from females, thus the method is more reliable than the previous approached.

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