# Ex-situ conservation of the pancake tortoise (*Malacochersus tornieri*) – novel DNA markers for the genetic characterisation of captive breeding populations

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## Introduction

The pancake tortoise (*Malacochersus tornieri*) is an East African species that inhabits specific microhabitats in rocky outcrops or kopjes in the arid and semi-arid *Acacia-Commiphora* bushland. These outcrops have suitable crevices that provide access to close foraging material, an altitude of less than 1000m, rock that has been exposed to weathering and erosion and rock that is sheltered by vegetation which may prevent overheating and desiccation of the tortoises during the dry season (Kyalo 2008). Due to the habitat requirements of the species, populations are discontinuously scattered and are often separated by large areas of unsuitable habitats or physical and climatic barriers. The species has experienced major population declines due to heavy exploitation for the exotic pet trade (Bombi *et al.* 2013). Three distinct populations have been identified in Kenya, Tanzania and Zambia, with the latter suspected to be an introduced population in recent decades (Chansa & Wagner 2006). The pancake tortoise is classified as vulnerable on the IUCN Red List (Tortoise & Freshwater Turtle Specialist Group 1996) and listed on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Despite a classification of vulnerable in the wild and being CITES Appendix II listed, the tortoises are often illegally collected and transported for the pet trade. International interest and trade in the species is significantly increasing and along with habitat loss and degradation it is posing a significant threat to the species. When individuals are poached the rocks are often broken and

  

Fig. 1. Young pancake tortoises (*Malacochersus tornieri*) hatching at Bristol Zoo. Photo by Adam Davis.

vegetation cleared to access the tortoises, destroying the crevices they prefer to inhabit and making the habitat unsuitable for the tortoise (Malonza 2003). Agricultural practices and livestock grazing also threaten these populations. The species is now the subject of a coordinated breeding programme within the UK and other European zoos, and several young pancake tortoises have been hatched successfully in Bristol Zoo's incubators (Fig. 1).

Examining the genetics of the captive population is necessary to enable better management of the breeding programme. At present, many pancake tortoises in captivity are animals that have been confiscated at customs; thus, information regarding their geographical origin as well as the level of genetic diversity or relatedness between individuals is missing. Studies by Darlington and Davis (1990) indicate that the breeding success of *M. tornieri* in captivity was highly variable and sporadic, with low fertility and hatch rates being recorded in captive animals. The high frequency of infertile eggs observed suggested that different populations may have had difficulty in interbreeding. A possible explanation is that the discontinuous distribution of the tortoise results in isolated populations with variability high enough that there is genetic incompatibility and so reduced offspring viability. The ability to identify differing population origins in captive animals could therefore better inform breeding programmes, helping to select which individuals to breed in order to maintain genetic diversity, avoid health issues and reduce breeding failures (Hamon 2000).

The main aim of this project was to develop a platform that allows the genetic analysis of the pancake tortoise. This includes the identification of molecular markers specifically designed for the genetic characterisation of pancake tortoise breeding populations, and the development of a simple, reliable method to collect DNA samples from individuals.

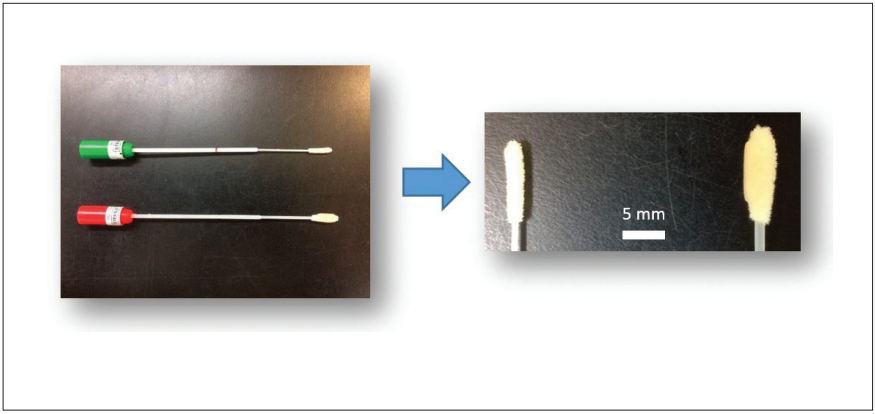


Fig. 2. Green and red buccal swabs (FLOQSwabs™, COPAN).

**Sample collection and DNA extraction for the study**

Pancake tortoise blood and buccal swabs collection from zoos affiliated to the British and Irish Association of Zoos and Aquariums (BIAZA) and the European Association of Zoos and Aquaria (EAZA) was coordinated by T. Skelton. Blood and buccal swab samples were obtained from three tortoises (turtle ids 6, 11 and 12) at Bristol Zoo. These samples were used as references to assess the quality of the DNA obtained from different swab collection approaches, and were also analysed to generate next-generation sequencing Illumina data used to identify genetic markers. All remaining tortoise DNA samples in the study were collected using FLOQSwabs™ buccal swabs (COPAN). All DNA samples were extracted using GeneJET Whole Blood Genomic DNA Purification Kit (ThermoFisher) following the manufacturer's protocol.

**DNA quality using buccal swabs**

DNA collection was assessed from buccal swab samples sent from all participant zoos to Bristol via post. This method of sample collection was deemed to be more practical than blood sampling, having a limited impact on the tortoises and not requiring a home office licence for UK importation. Two synthetic swabs (FLOQSwabs™), wide red ones and narrower green ones, were used to collect samples from 76 individuals (Fig. 2). DNA extracted from buccal swabs was tested with five SSR markers (see Nuclear Simple Sequence Repeat section).

Three to five SSR loci were amplified from 83% of all red swabs tested (49/59) while no amplification was observed from the green swabs (0/17). This confirmed that buccal swabs can be used for collection of pancake tortoise DNA, but smaller swabs do not yield sufficient DNA for molecular analysis to be performed.

**Developing molecular markers specific to *Malacochersus tornieri***

Molecular markers have become a valuable tool for the conservation of threatened species. The wide range of marker types available have a multitude of applications, from the analysis of genetic variation as mentioned previously, to the prevention of poaching and illegal trade of endangered species. No single molecular marker can be regarded as optimal for all of these approaches and a combination of different markers may be most useful to employ in any one conservation programme (Wan *et al.* 2004). Recent developments in Next-Generation Sequencing (NGS) technology have enabled researchers to rapidly sequence uncharacterised species genomes, and identify large numbers of molecular markers for conservation studies (Castoe *et al.* 2012). In the present study, Illumina sequencing analysis was conducted by A. Croxford from the University of Adelaide, Australia, on the DNA extracted from the blood and buccal swabs of the three adult pancake tortoises from the Bristol Zoo (6, 11 and 12). Over 15 million sequences of 600 base pairs (bp) were generated across all six samples. These were analysed for mitochondrial and nuclear specific markers.

***Mitochondrial markers***

Markers designed from the mitochondrial genome are typically less variable genetically and inherited maternally. They have been shown to be extremely useful as geographical specific markers (Zink & Barrowclough 2008). Hundreds of thousands of copies of mitochondrial genome may exist in a single cell, making it easier to purify and sequence than a single copy of nuclear DNA per cell (Butler & Levin 1998). To characterise the pancake tortoise mitochondrial genome, Illumina sequences were demultiplexed according to each of the three DNA samples (6, 11 and 12), with both buccal and blood samples combined for each tortoise. Using the only published pancake tortoise mitochondrial genomic sequence (GenBank accession number: NC_007700) as a reference, scaffolds were built and three new complete sequences were generated on the NGS analysis web platform GALAXY (Goecks *et al.* 2010; Blankenberg *et al.* 2010; Giardine *et al.* 2010) (Fig. 3).

A sufficient number of sequences were available to generate a complete sequence of the mitochondrial genome for each tortoise (6, 11 and 12). Illumina sequences which were generated from the buccal swabs are also likely to include DNA contaminants present in the mouth of the tortoise (such as microbes and food), but preliminary analysis has revealed a higher level of mitochondrial sequences in buccal swabs Illumina data set, compared to those of blood samples. This is likely to be due to a higher mitochondrial copy number in buccal cells and has potential for direct large-scale screening of multiple individuals for complete mitochondrial genomes. When comparing sequences 6, 11 and 12 to the reference published sequence, a total of 23

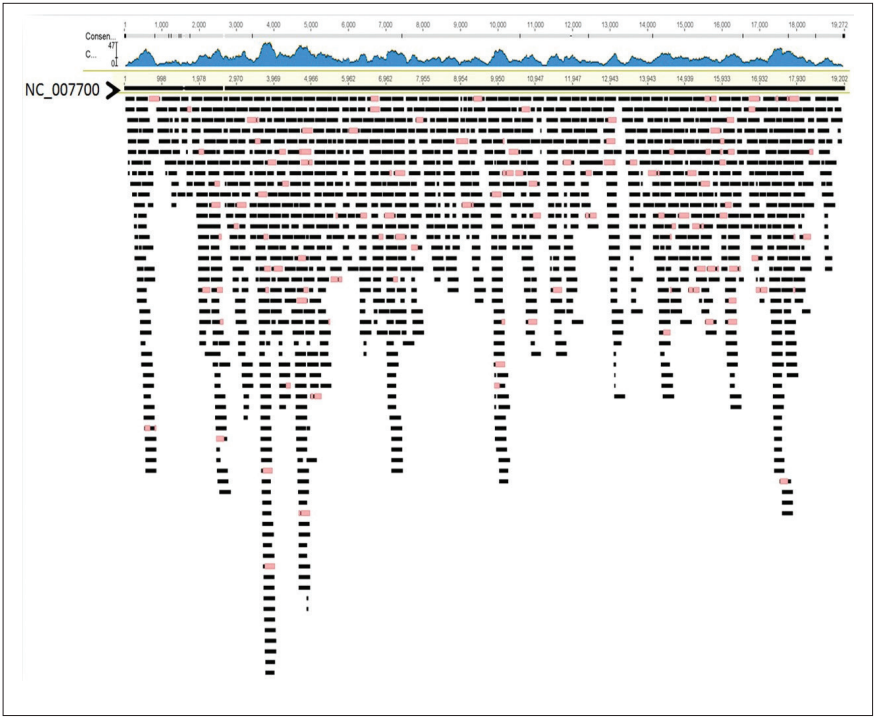


Fig. 3. Assemble of 1324 Illumina sequences from the mitochondrial DNA of pancake tortoise id 11 according to the reference mitochondrial genome NC_007700 (GenBank accession number). Each black line corresponds to a single sequence that has been mapped to NC_007700 using a sequence alignment tool on the Web Server Galaxy (Goecks *et al.* 2010; Blankenberg et al. 2010; Giardine et al. 2010). Enough sequences were obtained to cover the whole mitochondrial genome sequence.

Single Nucleotide Polymorphisms (SNP) were detected across the 19,000 bp of the mitochondrial genome (example of two of these SNPs highlighted in Fig. 4).

### *Nuclear Simple Sequence Repeat (SSR)*

SSRs, or microsatellites, are short sequences of DNA consisting of tandem repeating nucleotide units. Motif lengths of SSRs range from one to six bases, with tri- and tetra nucleotide units being the most popular for population studies, due to their combination of polymorphism and ease of analysis. SSRs were the first Polymerase Chain Reaction (PCR) based markers to become widely used in genetic analysis, and have remained a popular choice for many applications. The popularity of these markers is due to a combination of their relatively high polymorphism, abundance in the genome, their ease of amplification using PCR and their codominant nature (Ryberg *et al.* 2002). SSR

analysis is based on differences in the length of the SSR between homologous chromosomes within an individual, and in different individuals. Primers are designed to amplify DNA fragments containing SSRs and these fragments are then analysed using capillary electrophoresis to determine differences in their lengths (Arif *et al.* 2010). So far, due to high mutation rates in the flanking sequences of SSRs, the design of primers with broad universal applications to multiple species has been limited. In part this may be due to the laborious process historically required to identify microsatellites, as this factor has also contributed to the limited number of species that have had microsatellites identified. Despite the methodology involved in identifying SSRs, they remain some of the most versatile and useful markers currently utilised for conservation projects.

The data obtained from the three blood samples corresponded only to a portion of the species nuclear genome, but provided a vast number of candidate sequences containing diagnostic nuclear markers. The Illumina sequence dataset was preliminarily screened for sequences containing SSR motifs using the software PHOBOS (Mayer 2006-2010) with over 10,000 sequences identified. Not all of these sequences were appropriate to be used as nuclear markers and to reduce the number of potential hits the screen was restricted to trinucleotide repeats which are more stable and are the simplest marker to score. Around 2000 sequences were selected and screened using Primer3 (Untergasser *et al.* 2012), to identify those that could be converted to PCR assays (Fig. 5). PCR primer pairs for around 400 potential trinucleotide SSR markers were identified and 10 were randomly selected to be screened on DNA samples extracted from buccal swabs.

**Marker assessment**

All markers defined from bioinformatics data needed to be assessed to verify their usefulness for conservation genetic purposes. Each molecular marker was therefore assessed by PCR.

***Mitochondrial markers***

Indirect sequencing approaches such as High Resolution Melt (HRM) can be used to identify the presence of SNPs (Croxford *et al.* 2008). HRM analysis is a technique that measures the dissociation of double stranded DNA at high temperature resolution, using double stranded DNA binding dyes (Lochlainn 2011). HRM PCR assays from four of the SNPs identified in the mitochondrial genome were designed and screened on eight DNA samples extracted from buccal swabs. All four assays enable the rapid identification of a SNP category at a specific sequence position of the mitochondrial genome (Fig. 6).

These markers will permit preliminary rapid identification of the geographical origin of individuals and will inform on which individual

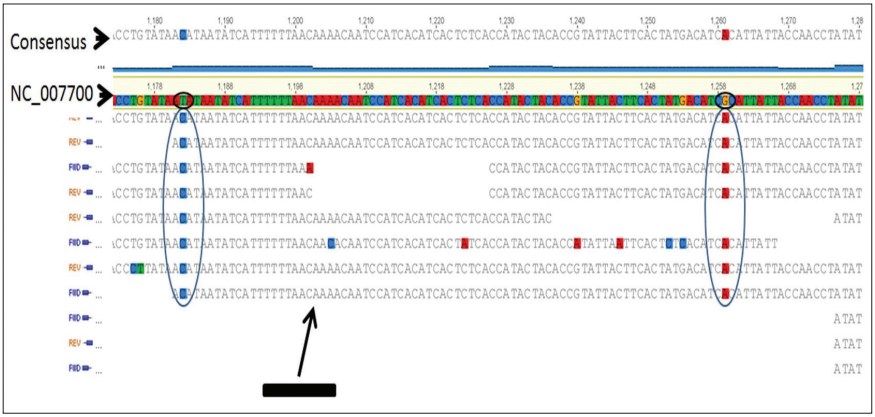


Fig. 4. Detail of the mapping of Illumina sequences from the mitochondrial DNA of pancake tortoise id 11, according to the reference mitochondrial genome NC_007700 (GenBank accession number). Two single nucleotide polymorphisms (SNPs, circled in blue) have been identified (C to T in position1184, and A to G in position 1261). The consensus new sequence is indicated at the top of the alignment.

```
OLIGO             start  len      tm      gc%  any th  3' th  hairpin  seq
LEFT PRIMER          24   20   58.34    50.00    0.00   0.00     0.00  ACAAATCAGCCAGAGGAGGA
RIGHT PRIMER        176   20   58.74    55.00    0.00   0.00     0.00  CAGTGTAGTGCTCCCTCAGT
SEQUENCE SIZE: 180
INCLUDED REGION SIZE: 180

PRODUCT SIZE: 153, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00
TARGETS (start len

     1 GTTAGGTGCCTTCCTCATTTCACACAAATCAGCCAGAGGAGGAGATAGAAGTGGTGGTGG
                               >>>>>>>>>>>>>>>>>>>>          **********

    61 TGGTGGTGGTGGTGGTGGTGCACCCCTTTTATAACTTCTAGTCCAATGGTGGTTATTGAA
       ********************

   121 TTATTTATCCACTGTGGAGCAGAGTCAATAGGAGAGACTGAGGGAGCACTACACTGGAAT
                                             <<<<<<<<<<<<<<<<<<<<
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Fig. 5. Example of primer design using Primer3 software (Untergasser *et al*. 2012). Rapid screening of the sequences that could be used to design SSR PCR assays was performed on bulk sequences. For the sequences not rejected during that screen, primer pairs were automatically designed and all information regarding these and the expected PCR product described. The primer position is indicated with arrows along the sequence above.

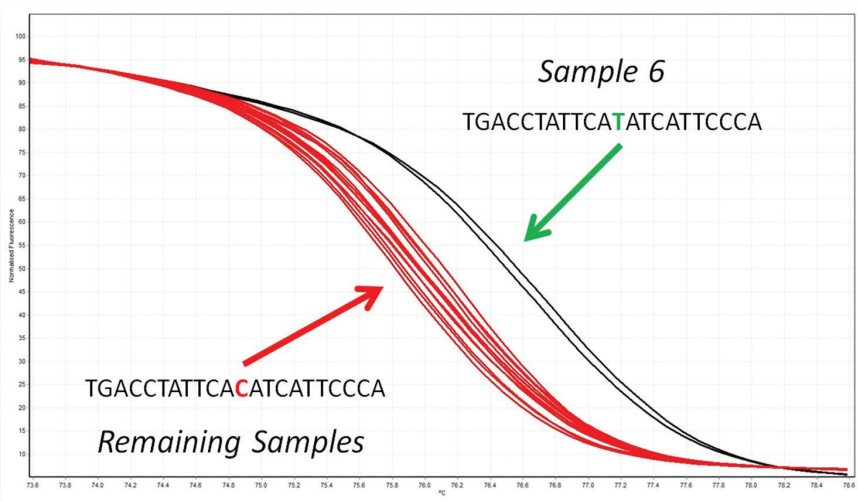


Fig. 6. Example of High Resolution Melt (HRM) profile of pancake tortoise mitochondrial DNA targeting a single nucleotide polymorphism (SNP) found on turtle id 6 (T to C change). The assays were run using duplicate reactions for all eight samples of pancake tortoises screened. This SNP is only found in sample id 6, as revealed by the difference in melting curve observed between id 6 (black curve) and the other samples (red curves).

should be selected to represent specific geographical regions (i.e. Kenya, Tanzania or Zambia), thus allowing the preservation of the genetic integrity of specific ecotypes.

***Nuclear Simple Sequence Repeat (SSR)***

Preliminary screening of 10 randomly selected trinucleotide SSR assays was performed on eight DNA samples from a range of tortoises. PCR products were screened via fluorescent capillary electrophoresis to assess product size variation related to sequence repeat. Four SSR loci did not yield any PCR products and one amplified three alleles and therefore targeted multiple sites on the tortoise genome. These five loci were rejected. Three SSRs were polymorphic and two monomorphic within the eight samples tested and deemed suitable for population studies (only one to two alleles per individuals; Fig. 7).

These five markers were further screened across 42 individuals. Good allelic diversity was observed for all SSRs with a specific high level of heterozygosity in three loci (TB6-11: seven alleles, TB12-4: 28 alleles and M175: 12 alleles). This preliminary screen revealed complete differentiation of all individuals tested, and indicated that these markers are suitable to characterise the relatedness of populations, both in the wild and in captivity. Pedigree analysis typically requires up to 20 SSR markers and this

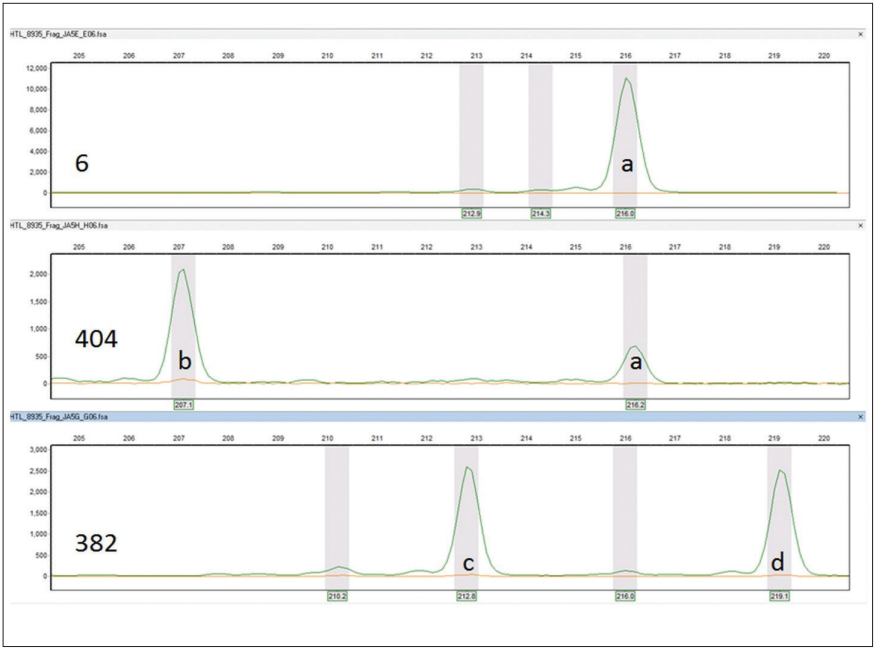


Fig. 7. Example of SSR Fluorescent capillary analysis applied to three pancake tortoises (id 6, id 404 and id 382), using GeneMarker software. Four alleles can be observed; a: 216bp, b: 207bp, c: 213bp and d: 219bp, with sizes varying by three bases, as expected for trinucleotide repeats.

can easily be achieved with a conservative selective success rate of 10% of the remaining 400 trinucleotide microsatellites identified.

## Conclusions

We have confirmed that good quality DNA samples can be collected from pancake tortoises using buccal swabs, thus facilitating a wider collection from many individuals in captivity. We have identified the mitochondrial and nuclear DNA markers required to select the best individuals for the breeding of pancake tortoises, with more samples to be assessed as they are obtained from European zoos collaborating in this project. This invaluable resource can also be now used to characterise the genetic diversity of wild populations and support application for funding to visit East Africa to collect reference DNA samples from individuals across the geographical range of the species. This will further inform ex-situ breeding strategies. Finally, markers developed in this study can be directly applied to the forensic study of the illegal trade of *M. tornieri.*

**Acknowledgements**

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