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Historical and non-invasive samples: a study case of genotyping errors in newly isolated microsatellites for the lesser anteater (*Tamandua tetradactyla* L., Pilosa)

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Abstract

Tamandua tetradactyla **(Pilosa), the lesser anteater, is a medium-size mammal from South America. Its wide distribution through different landscapes, solitary and nocturnal habits, and the difficulty to capture and contain specimens limit the amount of individuals and populations sampled during fieldworks. These features along with the lack of specific molecular markers for the lesser anteater might be the causes for paucity in population genetic studies for the species. Historical samples from museum specimens, such as skins, and non-invasive samples, such as plucked hair, can be supplementary sources of DNA samples. However, the DNA quantity and quality of these samples may be limiting factors in molecular studies. In this study, we describe nine microsatellite loci for *T. tetradactyla* and test the amplification success, data reliability and estimate errors on both historical and non-invasive sample sets. We tested nine polymorphic microsatellites and applied the quality index approach to evaluate the relative performance in genotype analysis of 138 historical samples (study skin) and 19 non-invasive samples (plucked hair). The observed results show a much superior DNA quality of non-invasive over historical samples and support the quality index analysis as a practical tool to exclude samples with doubtful performance in genetic studies. We also found a relationship between the age of non-invasive samples and DNA quality, but lack of evidence of this pattern for historical samples.**

Keywords: genotyping errors, historical samples, microsatellite loci, non-invasive samples, quality index, *Tamandua tetradactyla*

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Introduction

The lesser anteater (*Tamandua tetradactyla*) is a medium-sized mammal of the Pilosa order. The species occurs east of Andes, from Colombia, Venezuela, Trinidad to the Guyanas, and south to northern Argentina and northern Uruguay, from the sea level up to 1600 m (Wetzel 1975; Gardner 2005; revised in Hayssen 2011). It is able to occupy different types of habitats, from savannas and wetlands to rain forests. Mostly nocturnal and solitary, it uses the ground and trees for moving and foraging and eventually may use armadillo's burrows and hollow trees to hide (Montgomery & Lubin 1977). Despite the wide distribution, the characteristics and habits of the species hinder the obtainment of biological samples for molecular studies, especially when populations are addressed. Searching for a lesser anteater in the field is arduous and time-consuming, and the use of a trap is not feasible for capture. Once detected, the animal must be properly immobilized with chemicals by a veterinarian to collect blood, a delicate procedure that includes risks to the animal and that is not always possible due to fieldwork logistic issues.

Therefore, the use of specimens from natural history collections, hereafter referred to as historical samples, as sources of DNA for population genetic studies in the species is a good strategy to achieve an adequate sample size with reasonable geographical coverage, besides adding a time dimension to the analysis, and the possibility to sample from currently extinct populations (Wandeler *et al*. 2007).

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Another strategy for sampling difficult free-ranging species is to collect non-invasive samples, such as plucked hair or faecal samples, as there is no need for animal immobilization, and in some cases, no need even for animal visualization, allowing researchers to address questions in natural populations that would not be possible with traditional methods (Taberlet *et al.* 1999; Waits & Paetkau 2005; Broquet *et al.* 2007; Beja-Pereira *et al.* 2009).

Despite the advantages of both strategies, there are limitations concerning the quantity and quality of DNA samples. In the case of historical samples, the preservation treatments, age and storage conditions seem to be an important issues (Wandeler *et al.* 2007; Casas-Marce *et al.* 2010). However, for both types of sample, the presence of inhibitors during the amplification, possibility of contamination, intersample quality variance and low quantity of DNA are also limitations for their use (Taberlet *et al.* 1996; Hall *et al.* 1997; Wandeler *et al.* 2007).

The most common challenges in genotyping degraded samples are the high rate of amplification failure, the presence of false alleles (FA), which are essentially PCR artefacts, and allele dropout (ADO), the nonamplification of an allele from a heterozygote resulting in false homozygotes (Taberlet *et al.* 1999). Moreover, there is also the occurrence of null alleles, which probably happens due to mutations within the primer annealing regions, resulting in nonamplification of some alleles (Shaw *et al.* 1999), also augmenting the amount of homozygotes. In fact, empirical estimates showed that about two-thirds of all genotyping errors can originate from the presence of null alleles and imperfect repetitions (Kelly *et al.* 2011).

To account for genotyping errors in microsatellite analysis in both sample types, we calculated the quality index (QI) described by Miquel *et al.* (2006) upon a set of newly polymorphic microsatellite loci developed for the lesser anteater. Besides being a powerful tool for the evaluation of genetic diversity and population structure in future studies involving the species, these molecular markers are appropriated to address degraded samples because amplified loci are usually small DNA fragments, with <200 base pairs (bp), easier to be amplified (Wandeler *et al.* 2007).

Even though several authors have addressed the relevance of estimating genotyping errors not only in problematic samples but in all microsatellite studies (Bonin *et al.* 2004; Broquet & Petit 2004; Pompanon *et al.* 2005; Morin *et al.* 2009), once these errors are always present regardless of the method used, few of them have indeed proposed a method capable of a broader use and possibility of comparison between data. Miquel *et al.* (2006) have proposed a standardized method to test the reliability of the genotyping process that allows comparison between samples, loci, protocols and different studies.

In this study, we used study skins as representatives of historical samples and plucked hair as representatives of non-invasive samples for comparing the genotyping errors generated in each data set. By relating the QI of historical and non-invasive samples, we aim to determine whether their use is reliable for further population genetics studies, considering beforehand the potential misinterpretations to which these sample sets may lead, and test whether there are differences regarding their quality during the genotyping procedure.

Materials and methods

A total of 138 historical samples were used for this study, collected from four different natural history collections (Museu Nacional – MN, Universidade Federal de Santa Catarina – UFSC, Museu de História Natural do Capão da Imbuia – MNHCI, and Museu de Biologia Professor Mello Leitão – MBML) all located in Brazil. The samples were originated from several localities in different Brazilian biomes, collected from 1908 to 2008. All samples consisted of a piece of hide (dried skin) obtained from study skin specimens ranging about 1 cm^2. Even though bones and claws are reported to be better sources of DNA than hides (Casas-Marce *et al.* 2010), we did not have access to bone tissue in the collections, and we could not damage any piece of the claws, because those are considered taxonomic markers for *T. tetradactyla*.

A total of 19 non-invasive samples of plucked hair from individualized specimens were used, collected during fieldwork in Mato Grosso do Sul and Ceará states, or donated by the CETAS/IBAMA from Bahia state (a screening centre for wild animals), also in Brazil. The samples were collected directly from the animal in the field, transported in sterile tubes at room temperature and stored at 4 °C until processed for DNA extraction. They were collected from 2006 to 2012. All biological samples were collected under SISBIO-IBAMA permission number for scientific activities 24001-2/53695225.

Besides historical and non-invasive sample sets, we also used a total of 23 fresh tissue samples of *T. tetradactyla* collected during fieldwork or donated from institutions and researchers, representing populations of Minas Gerais and São Paulo state. These samples were used for the construction of genomic libraries, selections of microsatellites and as a source of comparison for the occurrence of genotyping errors in the previous data sets. All information regarding the samples is encompassed in Table S1 (Supporting Information).

Extraction of genomic DNA was performed with proteinase K digestion enhanced with DDT 1M, followed

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by salt precipitation (Sambrook *et al.* 1989). For historical samples, the hide was first cleaned with ultrapure water and ethanol 70% and then hydrated in TE solution for at least 24 h, which has been reported to improve digestion (de Moraes-Barros & Morgante 2007). The entire procedure was performed in a sterile environment dedicated to low-quantity DNA samples, exposed to UV light prior to use and segregated physically from laboratories handling PCR products. A blank control was included in each step of all extractions to identify any contamination. Extracts were stored at −20 °C before used.

The isolation of microsatellite polymorphic loci followed the method of enriched genomic libraries (Billotte *et al.* 1999), with two biotinylated microsatellite probes of motifs $(CT)_8$, $(GT)_8$, $(GATA)_4$ and $(GACA)_4$. The selected fragments were amplified by PCR, cloned into a pGEM-T vector (Promega, Fitchburg, WI, USA), transformed into chimiocompetent *Escherichia coli* XL1-Blue cells and cultivated in agar plates containing 100 mg/mL of X-galactosidase and ampicillin for the blue/white selection of positive clones. A total of 272 clones containing inserts were sequenced with the vector's primers (M13F: 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ and M13R: 5′-TCACACAGGAAACAGCTATGAC-3′) using ABI Prism Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and run in ABI 3100. Forty sequenced clones containing microsatellite motifs were found using the Gramene Project SSR Tool (Ware *et al.* 2002). From those sequenced clones, 34 primer pairs could be designed. PCR conditions were optimized using fresh tissue samples. These were also used to screen for polymorphism and to have a reference for the general composition of alleles and their range. Software PEAKSCANNER v. 1.0 (Applied Biosystems) was used to inspect the peaks. PCR was performed in 25 μL reactions containing 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 pmol of fluorescent labelled primers, 0.1 unit/μL of GO-Taq (Promega) and 1–2 μL of template DNA. Cycling conditions consisted of an initial denaturation at 95 °C for 11′, 35 cycles at 94 °C for 1′, 54–60 °C for 1′, 70 °C for 1′ and a final extension at 60 °C for 60′. DMSO (up to 5%) was occasionally used as adjuvant. For historical and non-invasive samples, bovine serum albumin (BSA, 1.6%) was routinely used as adjuvant. Finally, nine polymorphic microsatellite loci were identified, namely H5, E12, G3, E3, F1R, B2, C10, A9 and A8 (Table 1). The sequences corresponding to each microsatellite locus were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the Accession nos KF746177–KF746185. These loci were tested for linkage disequilibrium (LD) using software ARLEQUIN v. 3.5 (Excoffier & Lischer 2010) with the fresh tissue samples, and no significant and/or consistent LD was found, so they were used for genotyping historical and non-invasive samples. All samples were amplified with 3–5 independent PCR replicates (multiple tube approach). Alleles were considered as 'real alleles' if they appeared at least twice in independent PCR replicates (e.g. outliers in microsatellite range were identified taking the fresh tissue samples as references). After assigning a consensus genotype based on simultaneous observation of peaks for all replicates, scores were given for each replicate. When a

Table 1 Characteristics of the nine microsatellite loci isolated for *Tamandua tetradactyla*

Locus	Repeat structure	Primer sequence (5′–3′)	Annealing temperature (°C)	Size range (bp)	No. of alleles	No. of effective alleles	Dye label
H5	$(TC)_6$	F-CCCGCAGTATAGAAGCAG R-CCACGTCACAATCACCT	54–60	214–216	2	1.019	FAM
E12	$(GT)_{10}$	F-GGGTTTCCTGTCCCCATTAT R-GTTCTCCTTGGGAAGCTG	54–60	225–237	7	4.477	VIC
G3	$(GT)_{18}$	F-TGGACCCGCCATATAAACAT R-TGGACTAACTGGGCTTCTGC	54–60	174–222	19	9.039	NED
E3	$(GT)_{23}$	F-CACCACGACACCACACTACC R-TGCTTACGCGTGGACAAAT	54–60	104–134	13	1.762	VIC
F1R	$(CA)_8$	F-TCCCTAGGGCATCATCGTTA R-AGCAGCCACGTTCTCAGACT	54–60	208–216	5	1.299	VIC
B2	$(TG)_9$	F-CCTTTGGGTCCTGATTGAGA R-AATGGTGGGGCACTAAGATG	54–60	191–233	23	11.184	NED
C10	$(CA)_6$	FCTGGCCCTTAGCAGGGTTAT R-TCTGGTTTCAGGAAGGGTT	58	166–176	6	1.700	PET
A9	$(AC)_8$	F-TCCAAGTCTCAGGTCCCAT R-TGTGAGCCACTGATCGTGTT	54–60	158–194	9	1.787	VIC
A8	$(TGTC)_8$	F-ACAGGCTGTTTGAGTGCCA R-CCACTGGCACGTTATCGTTT	56	179–199	7	2.622	FAM

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genotype in one repeat was identical to the inferred true genotype, a score of '1' was assigned. Otherwise, if this genotype presented allelic dropout (ADO), false allele (FA) or even failure of amplification, a score of '0' was assigned. This procedure was carried out for all PCR replicates. The amount of score '1' amplifications was divided by the total amount of replicates. Then, QIs were calculated for each sample, each locus, and globally, based on the average of obtained values, according to descriptions in Miquel *et al.* 2006, for the historical and non-invasive sample sets separately. The QI value can vary from zero to one. ADO was considered when homozygote amplification was found in a sample that consistently showed heterozygote genotypes for other different independent amplifications. FA was considered when any allele other than those considered 'real alleles' was found.

The package *stats* implemented in R Project for Statistical Computing program (R Development Core Team 2008) was used to conduct statistical analysis of data. To compare QI values for historical and non-invasive data sets at loci and samples level, a *t*-test was performed (95% confidence interval). Pearson's *r* correlation index was used to test the strength of association between the age of study skin or plucked hair samples (time since it was collected, prepared and stored) and QI values, representing DNA quality. The significance was tested at a 95% confidence interval. Different procedures of study skin treatment and storage can affect their performance for molecular studies. To test whether the QI values for historical samples (study skins) had more influence from the age of collection or from the museum, they were stored, an ANCOVA test was performed (the four different museums as treatments, age and QI as quantitative variables).

To verify the occurrence of null alleles in microsatellite genotyping of all sample sets (historical, non-invasive and fresh tissue samples), the software MICRO-CHECKER v. 2.2.3 (Van Oosterhout *et al.* 2004) was used (set to 95% confidence interval with 1000 randomizations). According to Van Oosterhout *et al.* 2004, estimations of null alleles are more accurate if the sample set used represents a natural population, as it relies on Hardy–Weinberg equilibrium (HWE) estimates of heterozygotes and homozygotes. Thus, each sample set representing a natural population was used (from historical samples, a subset of Paraná state, $n = 29$; for non-invasive samples, a subset of Mato Grosso do Sul state, $n = 8$; and for fresh tissue samples, a subset of Minas Gerais state, $n = 13$) (Table S1, Supporting Information). These sample sets representing natural populations were also used for estimating observed and expected heterozygosity (H_O and H_E), using Arlequin v. 3.5 (Excoffier & Lischer 2010).

Results

Most of the fresh tissue samples amplified successfully for all nine loci (18 of 23, 78.3%), with a mean amplification success of 97.1%. Due to the high percentage of amplification success and the absence of observed ADO and FA in fresh tissue samples, data regarding genotyping errors and QI values will be presented solely to historical and non-invasive sample set. A total of 138 historical samples were analysed at nine microsatellite loci. Of these, we used only 101 samples that amplified for five or more loci for further analyses. All specimens used in this study, as well as natural history collections of origin, individual ages (years since collection) and QI values were assembled in the Table S1 (Supporting Information). The genotyping dataset is available in the file Data S1, Supporting Information.

In total, 24 (23.8%) historical samples amplified successfully for all loci. The mean amplification success for all historical samples per locus was 77.4%. All 19 non-invasive samples were used, of which ten (52.6%) amplified successfully for all nine loci. The mean amplification success per locus was 89.5%. In general, historical samples had a reduced amplification success across microsatellite loci and fewer complete individual genotypes than non-invasive samples (Fig. 1).

During the calculation of QI for historical samples, some individuals could not be assigned to any consensus genotype due to the presence of ADO, FA or nonamplification of individual repeats (from 0% in A8 to 16.8% in E3). ADO ranged from 1.3% in A8 and A9 to 4.3% in G3 and C10. FA ranged from 1.7% in H5 and E12 to 7.6% in E3. The error rates were also observed for non-invasive samples, but in a lower rate: individuals with no consensus genotype ranged from 0% to 5.3% in E12 and C10, FA ranged from 0% to 7% in C10 and ADO ranged from 0% to 5.3% in E12 and A9 (Table 2).

The same pattern emerged from QI values. For each locus, the lowest QI values were observed for historical samples (Table 2, Fig. 1). The QI value for each sample ranged from 0.26 to 0.92 in historical and from 0.50 to 0.93 in non-invasive samples. The QI values for samples and loci were both significantly higher for non-invasive samples than for historical samples (samples, *P*-value = 0.000289; loci, *P*-value = 0.001217) (Fig. 2). The global QI across all samples and loci was 0.58 for historical samples and 0.78 for non-invasive samples, reflecting the overall tendency.

There was no significant correlation between the age of historical samples (time since the study skins were prepared and deposited in the collection) and their QI values ($r = 0.0420774$, *P*-value = 0.6954), while the correlation was negative and significant when analysing non-invasive samples age (time since the

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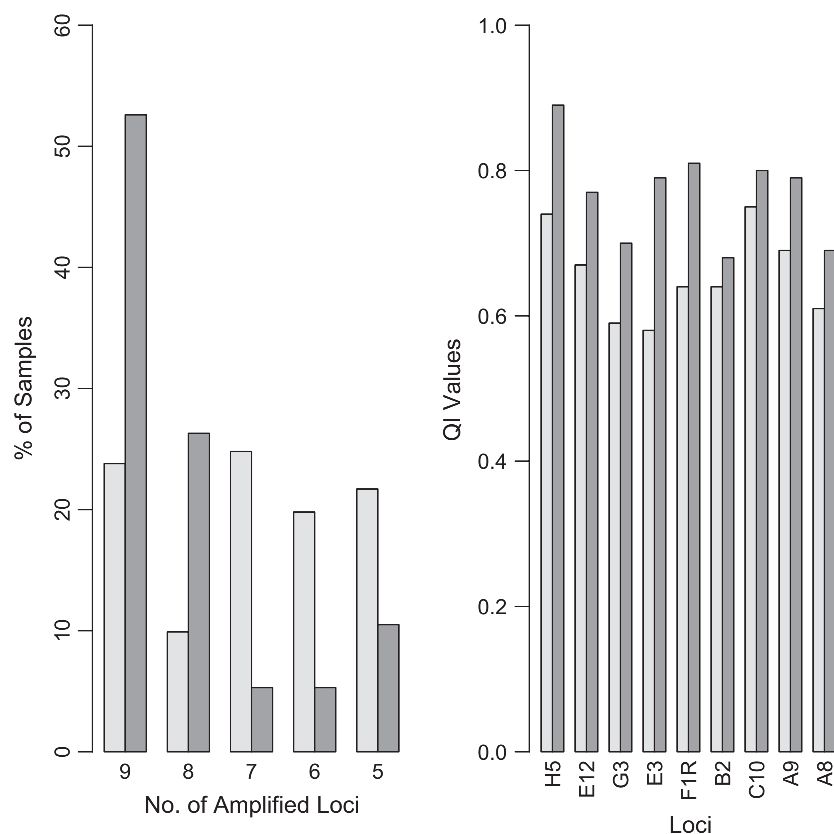

Fig. 1 Number of loci amplified, from a maximum of nine to a minimum of five, for historical, n = 101, and non-invasive samples, n = 19 (left); locus quality index values (QI) for historical and non-invasive sample sets (right).

Table 2 Amplification success, quality index (QI), error rate (Allele Dropout, ADO; False Allele, FA) and samples without a consensus genotype for each locus per sample type (historical/non-invasive). All values are percentages, except for QI

Locus	H5	E12	G3	E3	F1R	B2	C10	A9	A8
Amplification success	98.0/100	71.3/84.2	74.3/89.5	85.2/94.7	72.3/94.7	66.3/73.7	96.0/100	75.2/94.7	57.4/73.7
Locus QI	0.75/0.89	0.67/0.77	0.59/0.7	0.58/0.79	0.64/0.81	0.64/0.68	0.75/0.8	0.69/0.79	0.61/0.69
ADO	2.6/0	3.9/0	3.9/0	1 1/18	1 5/18	2.3/0	43/70	1.3/0	13/18
FA	1.0/0	10/53	1 5/6	1 2/5	13/18	3.6/0	2.6/0	2.3/0	0/0
No consensus	4.9/0	39/53	12.9/0	16.8/0	5.6/0	10.5/0	1 16/53	4.9/0	0/0

plucked hair was collected and stored, $r = -0.5057731$, P-value = 0.02716) (Fig. 3). The non-invasive data set (plucked hair) had an outlier in terms of age, while most of the samples were a few months to 2 years old, one sample was 6 years old (TTPR01, Table S1, Supporting Information). To check whether this particular individual was biasing the correlation, we also performed the correlation test excluding this sample. The results remained the same, showing even a stronger negative correlation ($r = -0.7193593$, P-value = 0.0007655, Fig. S1, Supporting Information). The ANCOVA test for the influence of the natural history collections in the QI of samples showed that there was no response of the quality index values to the institution from which it was collected (F = 1.0086, P-value=0.3931). When plotting QI values against age (years), taking into account the institution of origin of the samples, no linear relationship is found, and samples from the same origin and age achieve a wide range of QI values (Fig. S2, Supporting Information).

The software MICRO-CHECKER showed the presence of null alleles in E3 locus within the fresh tissue sample set from Minas Gerais. For historical samples, four of the nine microsatellite loci showed evidence of null alleles in Paraná population: E12, E3, B2 and A9. For the latter, it also suggested stuttering effect, which we judged not to be true, once the visualization of peaks for this locus was

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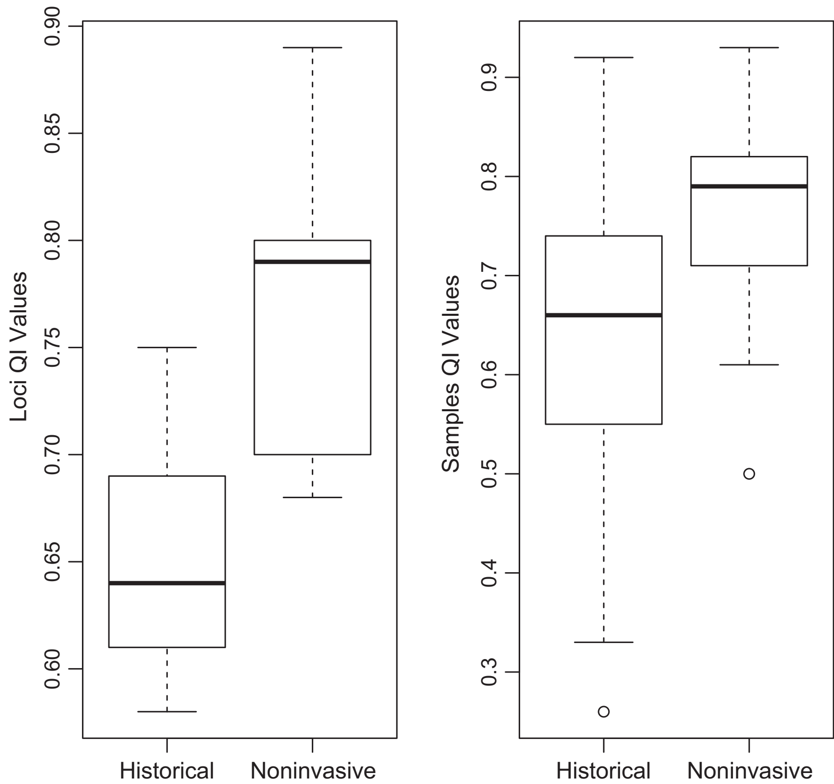

Fig. 2 Differences in quality index values for loci (P-value = 0.001217) and samples (P-value = 0.000289), between historical and non-invasive data sets.

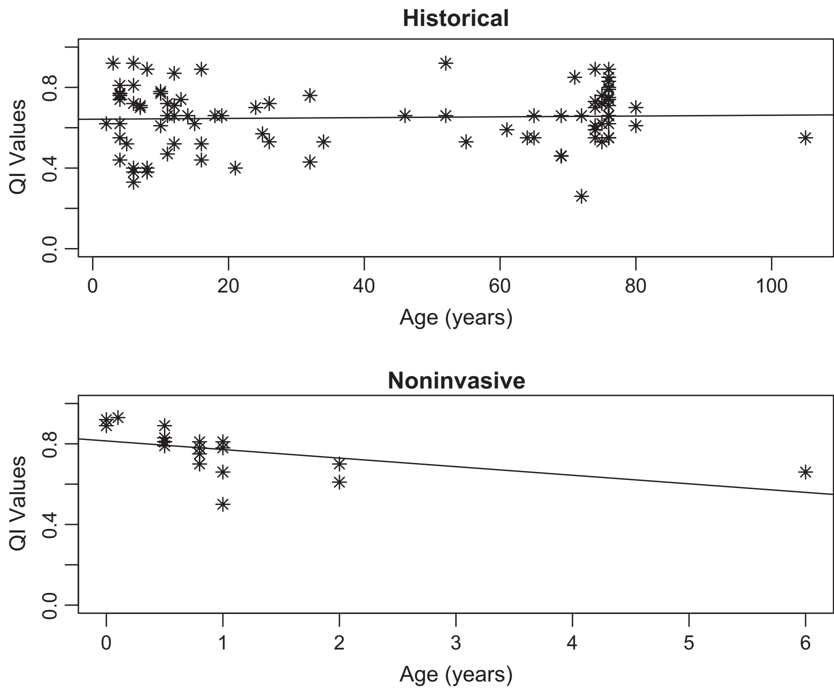

Fig. 3 Relationship between the age (years) of samples and their quality index evaluated through Pearson's correlation coefficient r, for historical samples (r = 0.0420774, P-value = 0.6954) and non-invasive samples (r = −0.5057731, P-value = 0.02716).

very clear and easy to assign. In non-invasive samples, the subset from Mato Grosso do Sul presented null alleles only in locus E3 (Table 3). Estimates of H_E and H_O in each of the populations showed significant deviations from HWE expectations for several loci in all sample sets (Table 3).

Discussion

We have analysed the genotyping success and errors in 101 historical samples and 19 non-invasive samples using nine newly described polymorphic microsatellite loci for the lesser anteater. This is the first study to

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Table 3 Summary information of genotyped loci for population subsets of fresh tissue (n = 13), historical (n = 29) and non-invasive samples (n = 8)

Locus	No. of alleles	Null alleles	H_O	H_E	P-value
Fresh tissue samples					
H5	2	No	0.08696	0.08502	1.00000
E12	5	No	0.52174	0.56522	0.23394
G3	9	No	0.50000	0.83827	0.00005*
E3	4	Yes	0.27273	0.49049	0.00028*
F1R	2	No	0.26087	0.23188	1.00000
B2	10	No	0.50000	0.82664	0.00120*
C10	2	No	0.08696	0.16232	0.13266
A9	5	No	0.30435	0.31111	0.36815
A8	4	No	0.20000	0.59231	0.00010*
Historical samples					
H5	2	No	0.03448	0.03448	1.00000
E12	7	Yes	0.53846	0.78054	0.05794
G3	9	No	0.43478	0.86957	0.00002*
E3	4	Yes	0.27778	0.57302	0.00376*
F1R	3	No	0.16667	0.23050	0.14900
B2	15	Yes	0.65217	0.91594	0.00000*
C10	5	No	0.40741	0.52760	0.00673*
A9	4	Yes	0.15385	0.46078	0.00000*
A8	3	No	0.36842	0.39118	0.71062
Non-invasive samples					
H5†	1	—	—	—	—
E12	4	No	0.33333	0.75758	0.04698*
G3	5	No	0.40000	0.84444	0.03370*
E3	5	Yes	0.25000	0.53333	0.01448*
F1R	2	No	0.28571	0.43956	0.44130
B2	6	No	0.60000	0.88889	0.14675
C10	3	No	0.25000	0.24167	1.00000
A9	4	No	0.50000	0.59167	0.19607
A8	2	No	0.25000	0.25000	1.00000

*Significant P-values on a 0.05 confidence level.
†Locus H5 was monomorphic within the population sampled for non-invasive data set.

describe species-specific genetic markers for *T. tetradactyla*.

Our results show that the quality of plucked hair is legitimately superior to that of study skin samples, as demonstrated by the higher QI global value for loci and samples (Figs 1 and 2), higher mean amplification success (89.5%), lower error rates (Table 2) and statistical support. Even though we compared two different sample sizes, we feel confident with the results, as QI calculation is entirely based on weighted averages, and we had significant differences showed by statistical results. Given the clearly distinct tendency found in non-invasive samples, we would expect to observe the same pattern even if this sample size increased.

The mean amplification success observed for historical samples (77.4%) was concordant with what have been reported in literature (40–88% in Rohland *et al.* 2004; 64% in de Moraes-Barros & Morgante 2007; 74% in Arandjelovic *et al.* 2009; 43–74% in Polanc *et al.* 2012). However, genotyping errors were high, and frequently, the consensus genotypes could not be inferred.

The QI values of historical samples varied greatly, and many of them were low. Miquel *et al.* (2006) argued that a threshold above which samples should be considered cannot be defined a priori and depends on the objectives of each study. This value may be different depending on the type of study performed (e.g. QI > 0.625 in Miquel *et al.* 2006; QI > 0.4 in Polanc *et al.* 2012). For a threshold of QI > 0.5, 13 historical samples would be excluded, and 88 (87.1% of total) would pass the criteria for further use. As expected, increasing this threshold would decrease the number of samples for further use, for example, with QI > 0.7, 77 samples would remain, and with QI > 0.8, only 64 (63.4% of total) samples would remain. Using the latter QI threshold, the selected samples show more complete individual genotypes (less missing data), and therefore, they would more suitable to further population genetics analysis. That shows, as pointed by Miquel *et al.* 2006, that QI is a valuable measure to design experiments from a pilot study and predict how many samples must be collected as a function of the average obtained QI. It is important to notice, however, that the low QI values found in historical samples, as well as the low amplification success can be related to the type of material used, that is, study skins. These are known to perform worse than other types of material, such as bones and claws (Casas-Marce *et al.* 2010). When there is an alternative to use of dried skins, it should be preferred.

The amplification success for non-invasive samples (89.5%) was also concordant with literature (95.2% in Luikart *et al.* 2008; 90% in Arandjelovic *et al.* 2009; 71–100% in Mondol *et al.* 2009; 72–88 % in Boston *et al.* 2012). Concerning the QI threshold, all samples were above QI > 0.5, 18 (94.7% of the total) had a QI > 0.7 and 15 (78.9%) had a QI > 0.8. Over half of the samples had the complete genotype for all microsatellite loci (Fig. 1), confirming that a non-invasive sampling approach such as the use of plucked hair is valid for molecular biology studies.

The quality of historical samples depicted by QI values showed not to be correlated with the time since the skins were dried (r = 0.0420774, P-value = 0.6954). Even though it seems logical to think that more recent specimens should yield better results than older ones, one major factor affecting the DNA conservation through the years is actually the type of preservation treatment of the hides (Wandeler *et al.* 2007), as well as the conditions in which they are maintained (presence of humidity, control of temperature, regular care, *etc.*). Depending on that, a higher level of contamination and PCR inhibition

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may be encountered (Hall *et al.* 1997). Therefore, great variance of DNA quality is frequently found among historical samples, not essentially correlated with their age. In fact, among the samples excluded, for example, on a QI > 0.5 threshold, there were old samples (from 1908 to 1950) and relatively new samples (from 2002 to 2008). However, we could not infer a relationship between the effect of the each natural history collection (different preparation and storage) and the quality of the samples. We had no data on how the skins were prepared in each institution (e.g. protocols for skin preparation, if the procedure changed over time, or if conditions of storage were altered), and, therefore, we cannot make statements on this problem. Nevertheless, this issue should be further investigated so that studies using such sample type can account for this effect.

Conversely, the quality of DNA from plucked hair (non-invasive samples) seems to be dependent on time since it was collected. The negative and significant correlation found ($r = -0.5057731$, P-value = 0.02716, and after exclusion of outlier: −0.7193593, P-value = 0.0007655) indicates that the older the samples get (increasing number of years since collection), the worst the DNA quality gets. This could be explained by the lack of conservation methods applied to this type of material, as plucked hair samples are usually kept in closed recipients without any further protective reagent, such as ethanol, buffers or others. Also, it is important to notice that the exposure to environmental conditions after collection, such as high temperatures and humidity, can affect the performance on molecular studies (Kelly *et al.* 2012). As our samples were all collected the same way, placed in closed recipients since collection and kept at constant temperatures (except for transportation time), we believe our results were not significantly influenced by this factor.

In spite of showing better overall quality over historical samples, non-invasive samples should be used right away after collected to guarantee a reasonable performance on molecular studies. However, we would like to emphasize that these results apply to plucked hair samples only, which were the aim of this study. Other sources of non-invasive samples (for example, scats) may behave in a different way and should be handled in an appropriate manner.

Historical samples showed the presence of null alleles in four microsatellite loci. It is known that null alleles can generate a bias in F_{ST} and genetic distances overestimating these measures as they create false homozygotes (Chapuis & Estoup 2007). Furthermore, studies show that loci affected by null alleles probably do not alter the overall outcome of assignment testing, although it lowers the power of assignment tests and accuracy of F_{ST} (Carlsson 2008). These problems can be addressed by accounting for the downward bias resulting from the null alleles (e.g. software MICRO-CHECKER). Adjusting the allele and genotype frequencies of the amplified alleles to account for the excess of homozygotes can allow the use of these loci in analysis that require frequency estimates of natural populations (not individual-based analysis, once genotypes are not modified). Nevertheless, caution should be taken with this approach because it assumes, necessarily, that the population is in Hardy–Weinberg equilibrium (Van Oosterhout *et al.* 2004). Ideally, microsatellite loci less susceptible to null alleles should be preferred. Non-invasive samples showed null alleles only in one locus, E3, which was also indicated for historical samples and for fresh tissue samples, the latter being the most appropriated source of comparison, for showing the best genotyping results. Moreover, E3 locus also showed the highest occurrence of FA and individuals without an inferred consensus genotype (Table 2). Thus, considering its low QI as well, we judge it would be best to discard this locus for further population genetic analysis, as it could add bias to results. Historical samples showed both the highest amount of null alleles and lowest QI values. It is also possible that the higher occurrence of null alleles in historical samples is an effect of the different samples sizes of data sets and different characteristics of the populations used for calculation (discussed below) and may be considered an artefact.

Several loci showed significant deviations from HWE in all sample sets, as displayed in Table 3. These results are difficult to evaluate, once the event causing such deviation may come from different sources. Besides technical issues (e.g. genotyping errors, addressed in this study), there may be also violations from HWE assumptions in the populations used for testing, such as cryptic genetic substructure, unstable population size and small sample sizes, which may hinder P-value estimates (Hartl & Clark 2006).

Conversely, it has been shown that even small levels of laboratory errors, such as very few homozygote individuals for a rare allele, can result in an overestimation of HWE deviations (Morin *et al.* 2009). We have no reason to believe that few individuals in the historical sample set could alter the general results, once the HWE test was also performed excluding the samples with QI below 0.5, 0.7 and 0.8, successively, and the results remained unaltered (data not shown).

Deviations of HWE found in loci among different data sets can also have influenced our estimates of null alleles. The excess of homozygotes observed (Table 3) may reflect violations of assumptions as described above, and/or the presence of null alleles, as its detection also takes into account HWE calculation. The populations of each data set used for HWE estimations may have violated one or more assumptions. For example, the population of historical data set contained individuals

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from various ages, some overlapping generations in the species (Table S1, Supporting Information). When performing the test again with a group of only 18 samples, all under 10 years old, the results remained equal (the same loci showed HWE deviation, data not shown). This problem was not encountered in non-invasive and fresh tissue data sets. On the other hand, we cannot guarantee the individuals are completely unrelated, once anteaters present lonely behaviour, and unless long-term observations are carried out, simply field collection in different localities is not enough to exclude kinship. Therefore, due to these restrictions, the estimation of null alleles may not be comparable between data sets, as these estimates may be overestimated reflecting the data set characteristics. In this case, QI, ADO and FA estimates may be better sources of comparison between data sets.

In this study, we presented a comprehensive comparison of the performance of historical (study skins) and non-invasive (plucked hair) samples through the genotyping of newly described polymorphic microsatellite loci for the lesser anteater. We have reported the superior quality of plucked hair over study skins and depicted the main error sources in each sample set. We have confirmed that QI measures are valuable to identify problematic samples and loci, and select the best ones for further population genetic analysis. Even though we detected different error types and the presence of null alleles in historical samples (as well as in non-invasive and fresh tissue, but in a smaller scale), we do not discourage their use in molecular studies, once they represent a valuable source of populations that no longer exist or that have undergone changes through time. We recommend a careful inspection of data, excluding nonsatisfactory samples, and applying appropriate methods to judge the results when performing analysis to address population genetic questions.

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All authors contributed with the research design. C.L.C. assembled sample sets, performed wet laboratory work, analysed the data and wrote the manuscript. N.M.B., F.R.S. and J.S.M. assisted with data interpretation and with preparation and revision of the manuscript.

Data Accessibility

For detailed information regarding genotyped microsatellite loci (primers and PCR conditions), please see Table 1.

Microsatellite sequences of loci were deposited in GenBank under the Accession nos KF746177-KF746185.

Specific information about samples used in this study can be found in Table S1 of Supporting Information.

Genotyping data set can be found in Data S1 (Supporting Information).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Relationship between the age (years) of non-invasive samples and their QI values, evaluated through Pearson´s correlation coefficient *r* ($r = -0.7193593$, *P*-value = 0.0007655), for the data set without the outlier TTPR01 (Table S1, Supporting Information), which was an 6 years old sample.

Fig. S2 Plot of QI values of samples against age (years) depicted by the natural history collections of origin (symbols in legend).

Table S1 List of samples used in this study.

Data S1 Genotyping data set.

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