Development and characterization of 20 polymorphic microsatellite markers for the white-bellied pangolin *Phataginus tricuspis* (Mammalia, Pholidota)

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Abstract

Pangolins, or scaly anteaters, have recently been flagshiped as one of the most illegally traded mammals, and as a corollary, as potential intermediate hosts at the origin of the COVID-19 pandemic. In order to improve the traceability of their trade, we developed 20 polymorphic microsatellite loci for the white-bellied pangolin (*Phataginus tricuspis*), the species most frequently found on African bushmeat markets. We genotyped 24 white-bellied pangolins from the Douala market, Cameroon, originating from the Ebo forest c. 75 km north-east of Douala. The number of alleles per locus ranged from 4 to 12 (mean=6.95), and mean observed and expected heterozygosities were 0.592 (0.208-0.875) and 0.671 (0.469-0.836), respectively. Genetic diversity was higher than that cross-estimated from microsatellite loci developed for other species of pangolins. Two loci deviated from Hardy-Weinberg equilibrium and two loci showed linkage disequilibrium. Genetic variance (PCoA) was increased with the addition of 13 pangolins of unknown origin, possibly suggesting that the Douala market is fed from differentiated source populations of white-bellied pangolins. Each of the 37 individuals had a unique multilocus genotype. The unbiased probability of identity (uPI) and the probability of identity among siblings (PIsibs) were both very low (uPI=8.443 e-21; PIsibs=1.011 e-07). Only five microsatellite loci were needed to reach the conservative value of PIsibs < 0.01, overall indicating a powerful discriminating power of our combined loci. These 20 newly developed microsatellite loci might prove useful in tracing the local-to-global trade of the white-bellied pangolin, and will hopefully contribute to the DNA-assisted implementation of future conservation strategies at reasonable costs.

Introduction

The illegal wildlife trade is a flourishing, parallel economy that threatens the worldwide biodiversity [1]. Pangolins, or scaly anteaters, have recently been flagshiped as one of the most illegally traded mammals, being literally eaten to extinction [2]. Based on reports from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), a minimum number of c. 895,000 African and Asian pangolins were trafficked during the last decade (2010-2019). They were mainly destined to Asian markets including China and Vietnam [3], where they feed the growing demand from the Traditional Chinese Medicine market [4]. As a corollary, the pangolin trade has very recently been pinpointed as posing a serious threat to human health, pangolins being potentially involved in the COVID-19 pandemic [5,6]. Pangolins are also part of the traditional wild meat intake and pharmacopeia in most of their range countries [7,8], and as such suffer from the ‘bushmeat crisis’ that threaten the survival of many vertebrates in the tropics [9].

The traceability of the pangolin trade is rendered difficult by the elusive, international criminal networks that fuel the market, but also by the various forms under which pangolins are traded. Indeed, a large spectrum of pangolin-related ‘items’ are involved in the trade, from scales alone to smoked skins, boiled and ‘pealed’ carcasses, chopped meat, scale powder and embryo soups [10-12]. The societal demand for mitigating the pangolin trade [13] implies to develop tools capable of accurately identifying the trafficked

species of pangolins, tracing their geographic origins (sources) and estimating the number of pangolins when body parts and scales are seized.

The genetic 'tool box' may constitute an efficient support to reach these objectives. To date, a certain number of mitochondrial, nuclear and Y-borne genes have been sequenced in pangolins [14-17], providing useful resources for the accurate 'tagging' of the species involved in the trade [18]. On the other hand, our ability to trace the geographic origins of seized pangolins has remained limited because of a lack of exhaustive DNA registers and the reduced geographic resolution of traditional gene sequencing [19-23]. Although microsatellite loci were developed in an Asian (*Manis javanica*) and an African species (*Smutsia temminckii*) [24,25], their application to the issue of the traceability of the trade remains limited or pending [26,27]. More recently, single nucleotide polymorphic markers (SNPs) have successfully been used to assign seizures of *M. javanica* to potential geographic sources in South-East Asia [28]. So far, no genotyping approach has been applied to assess the number of individuals involved in large (scale) seizures, despite the potential advantage this would have for seizure processing [29].

Pangolins are highly sensitive to habitat degradation and have slow reproduction rates [30]. They are one of the mammalian lineages most prone to extinction (http://www.edgeofexistence.org/). In the last years, there have been pressing initiatives for increasing awareness on the unsustainability of the pangolin trade and for promoting law enforcement at the international level. All eight species were moved to the Appendix I of CITES in 2016 [31] and have recently been upgraded to "Vulnerable", "Endangered" or "Critically Endangered" on the IUCN Red List of Threatened Species [32].

Recent seizures indicate that, as Asian pangolins are becoming rarer, traffickers are now sourcing from Africa possibly via the same criminal networks used for the trade of ivory [33,34]. The white-bellied pangolin (*Phataginus tricuspis*) is the most abundant species of pangolin in Africa, with a widespread sub-Saharan distribution covering the African lowland rainforests [30]. It is one of the most frequent mammalian species observed on the stalls of the range countries' bushmeat markets [35-38]. The species is also likely undergoing heavy international trade pressure as it has been the constituent of large seizures both in Africa and Asia since 2010 [39].

Recently, an unexpected level of cryptic diversity was revealed across the species range through the existence of six geographically traceable genetic lineages [17]. Although those results might constitute valuable support to delimitate the sources of traded pangolins at the regional scale, deeper resolution is needed to improve our ability to trace the trade at the local scale. We therefore developed and characterized 20 microsatellite loci from the genome of *P. tricuspis*, with the objective of providing a tool applicable at reasonable costs to range countries and international agencies that would be keen to implement the genetic tracing of the pangolin trade.

Materials and Methods

A total of 37 samples of white-bellied pangolins were collected from the Douala market network. A first sample set included 24 samples from the Ebo forest (population 'Ebo'), c. 75 km north-east of Douala, as ascertained by circumscribing the supply area that receives bushmeat from Ebo together with interviews with bushmeat sellers (data not shown). A second sample set included 13 samples likely originating from diverse geographic sources, possibly including Ebo ('altEbo'), according to the same interviews. Samples consisted of fresh and smoked tissues (tongue or muscle) collected from dead animals sold on the market. Sampling was done, after explaining the aim of our study, with the initial agreement of the seller and following the guidelines of the Comité d'Ethique Institutionnel de la recherche pour la Santé humaine de l'Université de Douala (CEI-UDo). All the samples were preserved in 90% ethanol.

Genomic DNA was extracted with the NucleoSpin® Tissue Kit (MACHEREY-NAGEL, Hoerdt, France), following the manufacturer's recommendations. The elution step was repeated twice in 50 µL BE to maximize DNA yield and concentration. DNA concentration was quantified using the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific). All the DNA extracts were stored at -20 °C.

Microsatellite markers were developed at Ecogenics, Balgach, Switzerland (https://www.ecogenics.ch/home.html). An Illumina TruSeq nano library was built from a single DNA extract, which was enriched for simple sequence repeat content using magnetic streptavidin beads and biotin-labeled CT and GT repeat oligonucleotides. The library was sequenced on an Illumina MiSeq sequencing platform using a nano 2 (500 cycles) sequencing chip. The resulting paired-end reads which passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals. Subsequently, the quality of the surviving reads was checked with the software FastQC 0.117 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). In a next step, the paired-end reads were merged with the software USEARCH 10.0.240 [38] to reform *in silico* the sequenced molecule. The resulting reads were screened with the software Tandem Repeats Finder 4.09 [41]. After this process, 8,488 merged reads contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Primer design was performed with Primer 3 4.1.0 [42]. Suitable primer design was possible in 6,025 microsatellite candidates. Individual tests for amplification and polymorphism of the 48 best primer-designed loci were performed on 14 individuals (from the western central African and Gabon lineages; see [17]), after which 20 polymorphic markers were retained (Table 1). Overall, eight loci were di-nucleotide, three tri-nucleotide and nine tetra-nucleotide repeats.

We used MULTIPLEX MANAGER 1.2 [43] to optimize a design into four PCR multiplexes (4-6 loci) using four different ABI fluorescent dyes (Table 1). PCR amplifications for each multiplex were carried out in 20 µL reaction mixture containing approximately 20 ng of genomic DNA, 1x Multiplex PCR Master Mix (QIAGEN Multiplex PCR Plus Kit; Qiagen, Courtaboeuf, France) and 0.2 µM of each primer pair. PCR thermoprofiles included an initial denaturation step (95 °C for 5 min), followed by 32 cycles of denaturation (95 °C for 30 sec) – annealing (60 °C for 90 sec) – elongation (72 °C for 30 sec), and a final elongation step (60 °C for 30 min).

The PCR products were run on an ABI 3730 DNA Analyser (Thermo Fisher Scientific) at GeT-PlaGe (Génotoul, Institut National de Recherche Agronomique, Castanet-Tolosan, France; https://get.genotoul.fr/). Allele scoring and final extraction of genotypes were performed in Geneious 9.0.5 [44] with the Microsatellites plugin (https://www.geneious.com/features/microsatellite-genotyping/).

As a geographically coherent population, Ebo (N=24) was used for the validation of our 20 microsatellite loci. We ran the detection of potential scoring errors and null alleles in MICROCHECKER 2.2.3 [45] Deviations from the Hardy-Weinberg equilibrium were tested for each locus with GenAlEx 6.503 [46]. We used a permutation test under GENETIX 4.05.2 [47] to estimate linkage disequilibrium (LD) between each pair of loci (1000 permutations). The Bonferroni correction was applied to each of those statistical procedures.

The number of alleles per loci (Na) and the observed (Ho) and expected heterozygosities (He) were estimated in GenAlEx. Allelic richness (A_R) was calculated in FSTAT 2.9.3.2 [48].

We used the whole sample set (Ebo+altEbo; N=37) to conduct a Principal Coordinates Analysis (PCoA) in GenAlEx with pairwise population matrix unbiased genetic distances [49] in order to explore genetic variance among individuals sold in the Douala markets.

Values of unbiased probability of identity and probability of identity among siblings (uPI and PIsibs) were calculated in Gimlet 1.3.3 [50]. We used the Multilocus tagging option in GenAlex to detect identical genotypes in our dataset (sub-option 'Matches').

Results and Discussion

The 20 loci were successfully amplified (with 100 % PCR success rate), with only 0.84% missing data in our final multilocus dataset (Table 1). Null alleles were detected in five loci (PT_338821, PT_796077, PT_839522, PT_1453906, PT_1594892). Two of those loci significantly deviated from the Hardy–Weinberg equilibrium (Table 1). Two loci were subjected to linkage disequilibrium (PT_353755, PT_1225378). Deviation from Hardy-Weinberg equilibrium and linkage disequilibrium among loci can be due to genetic differentiation [51,52], as was also suspected from loci developed for other pangolin species [24,25]. Our results may suggest that we genotyped several populations from the Ebo forest, the latter covering > 1,500 km², although we cannot totally discard the possibility that the Ebo sample set was not as accurately circumscribed as expected from our interviews.

The mean number of alleles per locus was 6.95 (from 4 to 12 alleles) and mean A_R was 6.74. Mean He was 0.671 (0.469-0.836) and mean Ho was 0.592 (0.208-0.875). The number of alleles and levels of genetic diversity were higher than in previous cross-amplification studies (mean number of alleles = 2.61-4; [24,25]). Those results were expected given the reduced sample set (N=2-10) used in the

previous studies and the bias related to cross-amplification among phylogenetically divergent species of pangolins [18].

The 20 newly developed microsatellite loci represent the first markers isolated from the genome of *P. tricuspis* and may prove useful in tracing the local-to-global trade of the species. Each of the 37 individuals had a unique multilocus genotype. The unbiased probability of identity (uPI) and the probability of identity among siblings (PIsibs), that is, the probability that two individuals drawn at random from a population, including or not including siblings, will have the same genotype, were both very low (uPI=8.443 e-21; PIsibs=1.011 e-07; Fig. S1). Only five microsatellite loci were needed to reach the conservative value of PIsibs < 0.01 [53], overall indicating a powerful discriminating power of our combined loci. This could have important conservation implications, as notably regarding the individual-based management of *in situ* and *ex situ* populations [54,55] and the accurate assessment of the number of individuals contained in large seizures of scales [29]. Although China has temporarily banned the wildlife trade, the durable effect and efficiency of law enforcement on this illegal market remains questionable [56] and accurate tools will be needed to trace the consumption of pangolins.

Genetic variance was increased through the addition of pangolins with unknown origin (altEbo), involving the potential presence of ‘outliers’ along the axes 1 and 2 of the PCoA (14.88 % cumulated variation; Fig. 1), in agreement with the interviewees’ answers regarding the origin of pangolins. Although our results are preliminary, this may suggest that the Douala market network is fed from differentiated source populations of white-bellied pangolins, but this working hypothesis will have to be tested further with an accurate geographic representation of pangolin populations. The set of microsatellite loci that we have developed significantly adds to the molecular toolbox available for the species, and will hopefully contribute to the fine-scale implementation of future conservation strategies at reasonable costs. Because the previously published, cross-amplified loci were not validated on a sufficient number of geographically traceable samples, we recommend the use of our 20 newly developed microsatellite loci for population genetic studies on the white-bellied pangolin and related conservation and forensic applications.

Declarations

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Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

Research involving Human Participants and/or Animals

No animal testing was performed during this study. Sampling was done following the guidelines of the Comité d'Ethique Institutionnel de la recherche pour la Santé humaine de l'Université de Douala.

Informed consent

Not applicable.

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Tables

Table 1 Characterization of 20 polymorphic microsatellite loci in the white-bellied pangolin (*Phataginus tricuspis*) from Cameroon.

Locus	Primer sequences (5'à3')	Repeat Motif	Na	Size range – bp	Ho	He	A_R	P_{HW}	Genbank Acc. No.	Dye	Multiplex
PT_34432	F: AGATATGGCACCCCAATGTTTC R: AAGTGTATGCACCCCTTTGC	$(AC)_{21}$	9	107-141	0.667	0.608	8.498	0.950	MT109130	VIC	II
PT_276641	F: TGAGTCCTCCAGATCAGCAC R: GAACTCAGGGAAATGGTGGC	$(TGAG)_9$	5	182-198	0.542	0.549	4.75	0.397	MT109129	VIC	IV
PT_308752	F: CTGCAACGGGATTTCCCTAC R: TGTTTCCCCAGGGCTTAGAG	$(TTCC)_{16}$	8	144-208	0.625	0.784	7.749	0.391	MT109128	NED	II
PT_338821	F: AGTCAACGATGCAATAGGAAAC R: TGGCATCTTTCTAGGCGTGG	$(TG)_{22}$	9	232-254	0.571	0.836	9.000	0.000*	MT109127	PET	III
PT_353755	F: GGTCACATGCTGAAGTGCTC R: TGCAATGAGGTAGGGACACG	$(TTA)_9$	8	206-242	0.75	0.76	7.847	0.021	MT109126	6-FAM	IV
PT_378852	F: CCGCTGTCTTTCCAGAATGC R: GTCCCCACGGAGCTTACTAC	$(CCT)_7$	3	163-175	0.5	0.546	2.999	0.389	MT109125	6-FAM	IV
PT_464918	F: TGGCAATACTGGTGTTTAGGAAC R: CTGTCATGGGCTAGTCACAC	$(AAT)_{10}$	8	110-131	0.667	0.755	7.847	0.428	MT109124	6-FAM	II
PT_619913	F: AGCGGAAGGGTCACTAATGG R: TCTGTGCTGCTTCATAAAAGAGG	$(TGAA)_8$	7	202-226	0.5	0.637	6.737	0.728	MT109123	NED	III
PT_739516	F: ACATGTGGCATAGTCTGTTGG R: AGAGGCCTATGGGACAGTAAG	$(TTTG)_8$	5	226-246	0.417	0.545	4.750	0.691	MT109122	VIC	III
PT_796077	F: GGGACAATGCCAAAGGGTAG R: CAAACATTTGGGGCGAGTTG	$(TTTA)_{13}$	9	157-201	0.542	0.829	8.871	0.105	MT109121	VIC	I
PT_839522	F: CAGAGCTTGTACGCCATCAG R: CAGTGACAGTGTAATCCTGCG	$(AC)_{20}$	6	203-217	0.435	0.783	5.988	0.000*	MT109120	PET	I
PT_1162028	F: CCTTTGTTATCCACCGACCC R: AGAACAGTGCCTTATGGAAGC	$(CATC)_9$	7	142-166	0.708	0.619	6.624	0.842	MT109119	6-FAM	I
PT_1225378	F: GGGACTTGATAATAGGGGGTC R: AGCTGGGAATCTCAAACTGG	$(AATT)_8$	5	227-247	0.542	0.469	4.873	0.978	MT109118	6-FAM	III
PT_1453906	F: GACCCCCATCTTTATGGAAGC R: AGTATGCCACTTGATGGATGG	$(ATCT)_9$	4	177-193	0.208	0.489	3.874	0.003	MT109117	6-FAM	II
PT_1594892	F: AAAACGGAAGTGAAATTAAAGCC R: TGTTGTTCAAGGGCCAACTG	$(TG)_{12}$	6	202-218	0.458	0.624	5.999	0.012	MT109116	VIC	II
PT_1669238	F: ACCCACAGGATGTGCTACTC R: AGATTTCCCATCTACCCCGC	$(GT)_{14}$	8	178-204	0.667	0.715	7.623	0.780	MT109115	PET	II
PT_1753627	F: GTCCATCTACGCAACCCAAG R: GTTTAACCAGCTCCCACAATTTC	$(TG)_{16}$	7	234-252	0.75	0.782	6.848	0.797	MT109114	6-FAM	I
PT_1849728	F: TCAAGGAACTTAGCTTCAGTTTG R: AGACTTTCGGTGTCATCCCC	$(TG)_{11}$	5	112-120	0.583	0.526	4.998	0.560	MT109113	6-FAM	IV
PT_1973508	F: GGCTAATGTACAGTCTGATGTTCTC R: GGGCCCACGTGATTCTATGG	$(CA)_{23}$	12	221-249	0.833	0.827	11.36	0.962	MT109111	NED	I
PT_2019332	F: CAGTGTAACCAAAAAGAGTCTGC	$(ATCT)_{10}$	8	190-	0.875	0.731	7.625	0.967	MT109112	PET	IV

	R: ACAGTTTTGGCACCTTCAATTTATC			222							

Na number of alleles, Ho observed heterozygosity, He expected heterozygosity, A_R allelic richness, and P_{HW} P-values for tests of Hardy–Weinberg equilibrium after Bonferroni correction.

*Loci with significant departure from Hardy-Weinberg equilibrium (adjusted P-value=0.0025)

The number of individuals was 24 for all loci

Figures

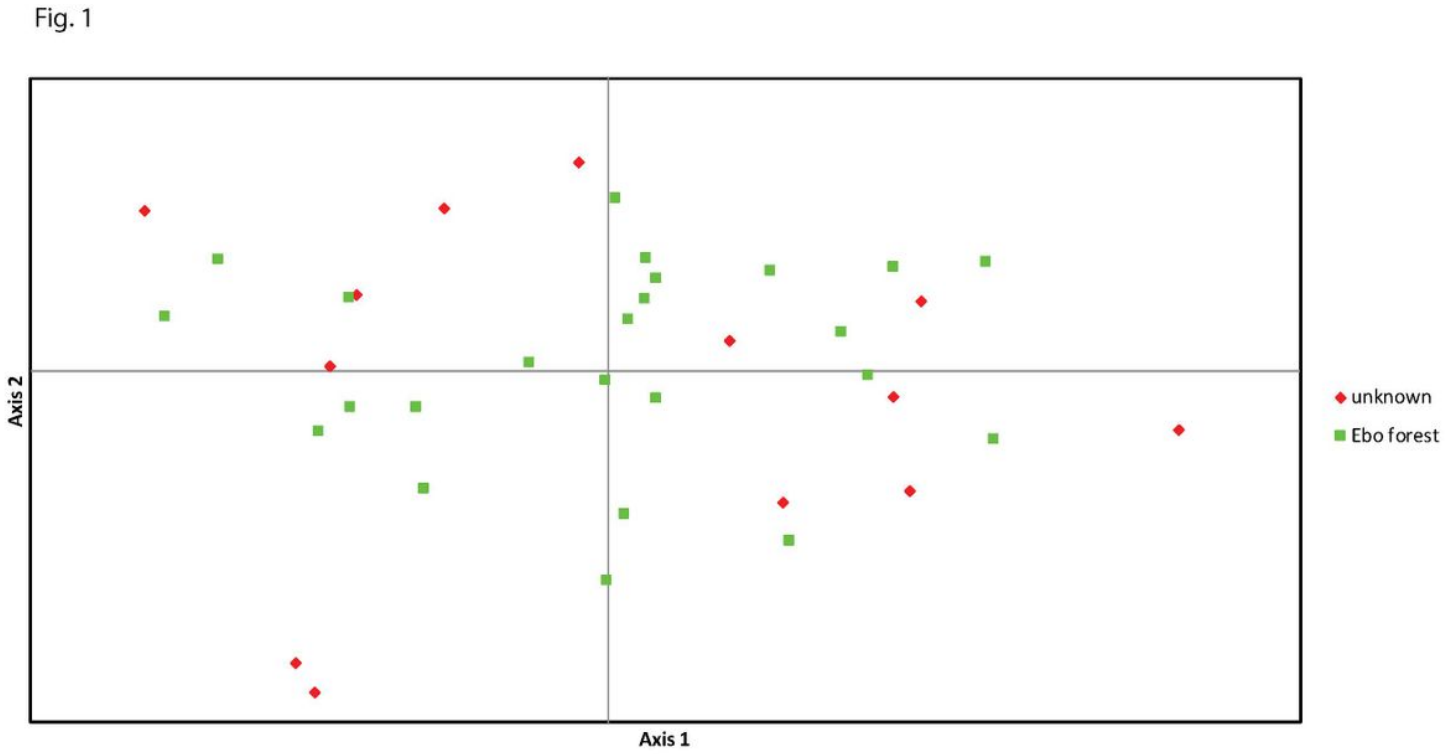

Figure 1

Principal Coordinates Analysis (PCoA) based on microsatellite data in white-bellied pangolins from the Douala market originating from Ebo forest and unknown sources. Percentages of variance explained: axis 1 = 7.79 %; axis 2 = 7.09 %.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.jpg