Evaluation of Microsatellite Markers for Populations Studies and Forensic Identification of African Lions (*Panthera leo*)

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Running title: Microsatellite marker evaluation for African lion

Abstract

The South African lion (*Panthera leo*) population is highly fragmented. One third of its wild lions occur in small (<1000 km^2) reserves. These lions were reintroduced from other areas of the species' historical range. Management practices on these reserves have not prioritised genetic provenance or heterozygosity. These trends potentially constrain the conservation value of these lions. To ensure the best management and long-term survival of these subpopulations as a viable collective population, the provenance and current genetic diversity must be described. Concurrently, poaching of lions to supply a growing market for lion bones in Asia may become a serious conservation challenge in the future. Having a standardised, validated method for matching confiscated lion parts with carcasses will be a key tool in investigating these crimes.

We evaluated 28 microsatellites in the African lion using samples from 18 small reserves and one captive facility in South Africa, two conservancies in Zimbabwe, and Kruger National and Kgalagadi Transfrontier Parks to determine the loci most suited for population management and forensic genetic applications. Twelve microsatellite loci with a match probability of 1.1×10^{-5} between siblings were identified for forensics. A further ten could be added for population genetics studies.

Keywords: conservation genetic management, population genetics, wildlife forensics

Introduction

Across Africa lions (*Panthea leo*) are increasingly under threat due to habitat loss (Riggio et al. 2013), human conflict (Woodroffe & Frank 2005), bushmeat snaring (Becker et al. 2013) and poorly regulated trophy hunting (Loveridge et al. 2007; Lindsey et al. 2013). In South Africa fencing has reduced these threats (Funston 2008), but has resulted in fragmentation and isolation with subsequent inbreeding (Trinkel et al. 2008; 2010) and overpopulation (Kettles & Slotow 2009, Miller et al. 2013), which undermine the conservation value of these lions (Slotow & Hunter 2009). As a result, there is an urgent need to apply a national metapopulation approach to lion management in South Africa (Funston 2008; Hayward & Kerley 2009; Slotow & Hunter 2009; Trinkel et al. 2010; Ferreira & Hofmeyr 2014). This approach should also be applied to other isolated populations of lions across Africa.

Steps towards this have been proposed by the Lion Management Forum (LiMF; Miller et al. 2013), who have recognised the importance of using genetic tools for the successful

implementation of the plan. Concurrently, a Biodiversity Management Plan is being developed for lions in South Africa, which could be better informed by pertinent genetic data.

Another issue of concern is that lion bones are replacing tiger (*Panthera tigris*) bones in traditional Asian medicine (Gratwicke et al. 2008). Export of lion bones from South Africa to Asia has increased in recent years (Lindsey et al. 2012). Poaching of lions, presumably in part to supply this Asian market, is also occurring in South Africa (G. Vermeulen, Senior Superintendent, South African Police Services Forensic Laboratory, pers. comm. 2012). Having a standardised, validated method for matching confiscated lion parts with recovered carcasses or DNA samples already in a database will be a key tool to combat poaching. Guidelines for standardisation of wildlife forensic analysis using microsatellites, the currently preferred genetic markers in forensic testing, have been established by SWGWILD (2012). Forensic standards for several species have been developed including domestic dog (*Canis lupus familiaris*; Wictum et al. 2013), Eurasian badger (*Meles meles;* Ogden et al. 2008), domestic cat (*Felis catus;* Menotti-Raymond et al. 1997; 2012) and black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros (Harper et al. 2013).

Menotti-Raymond (1995) first described the use of microsatellites developed for the domestic cat in lions. Since then many microsatellite markers isolated from the domestic cat genome have been used for lion genetics studies including: determining genetic variation in Asiatic lions (Shankaranarayanan et al. 1997); exploring genetic structure (Spong et al. 2002), dispersal distances (Spong & Creel 2001) and kinship (Spong & Creel 2004) in lions of the Selous Game

Reserve (GR) in Tanzania; examining timescales of demographic events in wild felids (Driscoll et al. 2002); in combination with other techniques to define historical lion distribution (Antunes et al. 2008) and lion conservation units (Dubach et al. 2013); subspecies definition of Ethiopian lions (Bruche et al. 2012); and confirming mating structure and paternity in lions of Etosha National Park (NP; Lyke et al. 2013). Only one limited study has been conducted on South African lions in small reserves (Grubich 2001), and a second is currently underway for the Kruger NP (J. Hofmeyr, pers. comm. 2012). No extensive genetic studies have been done on the captive lion populations in South Africa. A wide range of samples from southern African lions, mostly from free-ranging lions in South Africa, was collected for the present study. In this manuscript we evaluate microsatellite markers originally developed for the domestic cat for applicability in forensics and genetic management of lions in South Africa.

Materials and Methods

Samples

Blood, skin or hair samples from archived collections and private reserves, totalling 401 individual lions, were used. Blood samples were either collected in EDTA tubes or on FTA® filter paper (Whatman, GE Healthcare, Florham Park, NJ). Skin samples were collected from tranquilised animals or using biopsy darts. Samples were from free-roaming lions on small reserves (<200 individuals) in South Africa and Zimbabwe, Kruger National Park (NP), Kgalagadi Transfronteir Park (TP), Greater Mapungubwe Transfrontier Conservation Area (TFCA) and a captive white lion breeding centre in South Africa (Table 1, Figure 1). Some of the populations were of mixed genetic provenance (Table 1). DNA was extracted from tissue and EDTA blood

Table 1 : Sources of tissue samples indicating geographic location of collection and known or suspected provenance

Reserve (source of collection)	Total	Known/ suspected provenance(s)	
	Single provenance		
1	Madjuma GR	3	Etosha
2	Pilanesberg NP	31	Etosha
3	Shambala GR	3	Etosha
4	Tembe Elephant Park	15	Etosha
5	Addo Elephant NP	8	Kgalagadi
6	Karoo NP	1	Kgalagadi
7	Kgalagadi TP	11	Kgalagadi
8	Karongwe GR	4	Kruger
9	Kruger NP	53	Kruger
10	Thornybush GR	4	Kruger
11	Greater Mapungubwe TFCA	2	Mapungubwe
12	Venetia Limpopo NR	16	Mapungubwe
	Multiple provenance		
13	Hluhluwe-iMfolozi Park	63	Etosha, Kruger
14	Ka'Ingo GR	3	Etosha, Kruger
15	Madikwe GR	6	Etosha, Kruger
16	Thanda GR	1	Etosha, Kruger
17	Greater Makalali GR	14	Etosha, Kruger, Kgalagadi
18	Mun-ya-wana GR	43	Etosha, Kruger, Kgalagadi
19	Welgevonden GR	33	Etosha, Kruger, Kgalagadi
20	Marakele NP	7	Kgalagadi, UNK
21	Bubye Conservancy, Zimbabwe	23	Zimbabwe, Etosha
22	Save Valley Conservancy, Zimbabwe	10	Zimbabwe, Etosha
23	Gonarezhou NP, Zimbabwe	1	Zimbabwe, UNK
24	Ukutula GR[a]	44	Kruger, white lions, plus unknown
	Unknown provenance		
	Forensic case, South Africa	2	UNK
	Total	401	

UNK, Unknown; WR, Wildlife Reserve.

[a]Captive population.

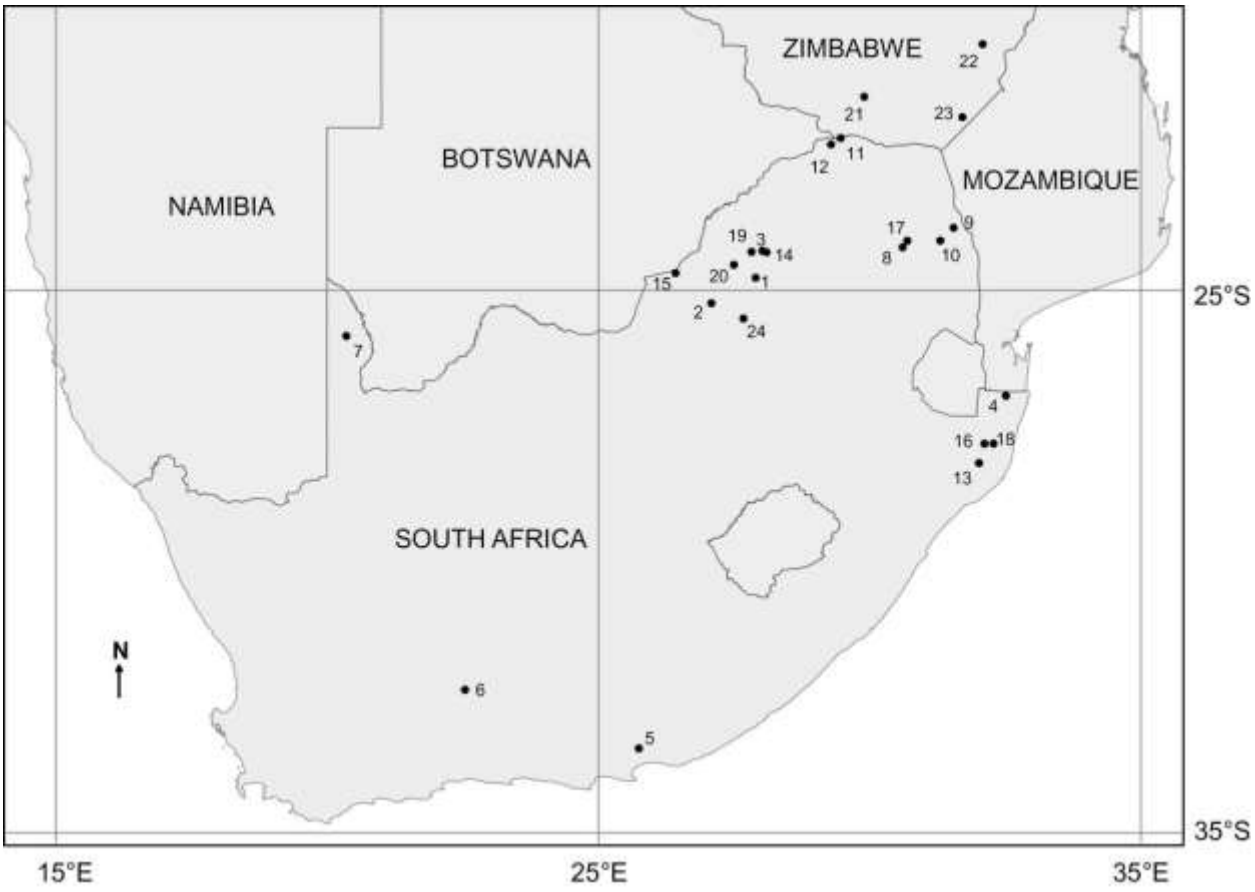

Figure 1. : Locations of DNA sampling as outlined in Table 1.

samples using a phenol:chloroform protocol modified from Sambrook & Russel (2006a,b); from FTA® filter paper using "protocol 4" in Smith & Burgoyne (2004); and from hair samples using a protocol modified from Bastos *et al*. (2000).

Amplification of Microsatellite Loci

Initially 32 microsatellite loci listed in Table 2 were combined in PCR multiplexes. These included 24 dinucleotide, seven tetranucleotide repeat loci (Menotti-Raymond et al. 1999) and a zinc finger for sex determination (Pilgrim et al. 2005). These were chosen based on success in other wild felids and communications with other labs using microsatellites for lions (Table 2

Table 2 : Multiplexing details for microsatellite loci for lions

Locus	Forward primer[a]	Reverse primer[a]	Nucleotide repeats	Dye label	Concentration	Multiplex[b]	STRUCTURE Analysis
FCA506S	AATGACACCAAGCTGTTGTCC	AGAATGTTCTCTCCGCGTGT	Di	NED	0.07	3	Y
FCA230A	AAGAATGGACTTGGGAAATGG	AAACCACAACAGGCAAAAGG	Di	6-FAM	0.11	4	Y
FCA391SA	GCCTTCTAACTTCCTTGCAGA	TTTAGGTAGCCCATTTTCATCA	Tetra	NED	0.08	1	Y
FCA075D	ATGCTAATCAGTGGCATTTGG	GAACAAAAATTCCAGACGTGC	Di	NED	0.08	2	Y
FCA096D	CACGCCAAACTCTATGCTGA	CAATGTGCCGTCCAAGAAC	Di	6-FAM	0.08	2	Y
FCA628S	CCCCTGCTCATTCTCTCTCA	GTGCCTGGCACAGCATAAG	Di	VIC	0.08	3	Y
FCA001SD	TGCTTGTCCTCTCCCTCG	TGACTGCGCCATAGCTTTC	Di	NED	0.085	4	Y
FCA224DA	CTGGGTGCTGACAGCATAGA	TGCCAGAGTTGTATGAAAGGG	Di	6-FAM	0.10	1	Y
FCA097D	TAATGTTCAACTTGAATTGCTTCC	GAACAGTAGTTTGCCCATACAGG	Di	VIC	0.11	4	Y
FCA126DA	AAGGCAACATGGCCCCTGAT	ACTCTAGCACAGAGCCAAGAGC	Di	PET	0.08	3	Y
FCA453	AATTCTGAGAACAAGCTGAGGG	ATCCTCTATGGCAGGACTTTG	Tetra	VIC	0.035	1	Y
FCA240	TCTTTAAGATGGCCGGACTG	TCCCCTCAAATATGCAAAGG	Di	6-FAM	0.11	3	Y
F41	GTCTGCATCTTCAAATAGGA	GTACCTGAGTTGGCTGTTGA	Tetra	PET	0.085	2	
FCA057D	AAGTGTGGGATTGGGTGAAA	CCATAAGAGGCTCTTAAAAACTGA	Di	VIC	0.06	3	Y
FCA193D	TATCTGGTAAGCCCCTGGG	CCCTGTTTTTATGCCTCTGC	Di	VIC	0.08	1	Y
FCA085A	CTGTACATTTCTCTTCCCATTGC	CCCCTACTGGGTGCACTG	Di	PET	0.07	4	Y
FCA113D	TGGGACGTCATTGCTCCT	CCCACATTGGGCATAGAAAT	Di	VIC	0.07	1	Y
FCA42	CCCACGTGGACTAATCAAAT	CACTGCACAAATTAAGAGGC	Tetra	PET	0.08	1	Y
FCA026D	GGAGCCCTTAGAGTCATGCA	TGTACACGCACCAAAAACAA	Di	6-FAM	0.1	2	Y
FCA275D	TTGGCTGCCCAGTTTTAGTT	ACGAAGGGGCAGGACTATCT	Di	NED	0.05	3	Y

Locus	Forward primer[a]	Reverse primer[a]	Nucleotide repeats	Dye label	Concentration	Multiplex[b]	STRUCTURE Analysis
FCA272	ACCTTTACCTCCTTCCAAAAAG	CACCTTTCCATCCAATAAATTC	Di	6-FAM	0.11	1	Y
FCA031	GCCAGGGACCTTTAGTTAGATT	GCCCTTGGAACTATTAAAACCA	Di	6-FAM	0.06	1	Y
FCA008[SA]	ACTGTAAATTTCTGAGCTGGCC	TGACAGACTGTTCTGGGTATGG	Di	VIC	0.05	2	Y
FCA069[DA]	AATCACTCATGCACGAATGC	AATTTAACGTTAGGCTTTTTGCC	Di	6-FAM	0.065	3	
FCA441[A]	ATCGGTAGGTAGGTAGATATAG	GCTTGCTTCAAAATTTTCAC	Tetra	PET	0.11	1	
FCA310[D]	TTAATTGTATCCCAAGTGGTCA	TAATGCTGCAATGTAGGGCA	Di	PET	0.06	3	
FCA105[DA]	TTGACCCTCATACCTTCTTTGG	TGGGAGAATAAATTTGCAAAGC	Di	NED	0.085	2	
F37	CGCCTTTCTCACATTACCAT	CACTGACAGATCTGATCCTG	Tetra	PET	0.08	2	
ZnF	AAGTTTACACAACCACCTGG	CACAGAATTTACACTTGTGCA	X and Y	PET	0.025	4	
F115	CTCACACAAGTAACTCTTTG	CCTTCCAGATTAAGATGAGA	Tetra			X	
FCA023	GACGCTTCACTGACTGAGCCAC	GCAACTCTTAATCAAGATTCCATT	Di			X	
FCA651	CAGGGGCCCTGATTTCTAG	GGCCTACAAATTGGCAAAGA	Di			X	

[S, D, A]Indicate use in previous studies on lions—S: Spong et al. (2002); D: Driscoll et al. (2002); A: Antunes et al. (2008).

[a]As found in Driscoll et al. (2002).

[2]X indicates discarded.

indicates those used in published lion literature). Three of the loci were subsequently excluded (Table 2). The forward primers were labelled with 6-FAM™, NED™, VIC™ or PET™ dyes and the reverse primers were tailed to facilitate accurate genotyping (Brownstein et al. 1996; Applied Biosystems, Warrington, UK). Polymerase Chain Reactions (PCR) and electrophoresis were performed in four multiplexes of 10 µl reactions using the KAPA2G™Fast Multiplex PCR Kit

(Kapa Biosystems, Cape Town, South Africa, Table 2). The concentration of some primers was adjusted to optimise the peak signals (Table 2). The amplification PCR was performed on a GeneAmp® PCR System 9700 (Perkin Elmer, Midrand, Gauteng) as follows: denaturation at 95°C for three minutes; thirty cycles of 95°C for 15 seconds, 59°C for 30 seconds and 72°C for 30 seconds; a final amplification at 72°C for 10 minutes. Electrophoresis was performed on a 3130x Genetic Analyzer (Applied Biosystems). Allele sizes and peak heights were determined using GENEMARKER®V2.4.0 (SoftGenetics, State College, PA) using GeneScan™ 500 LIZ® size standard (Applied Biosystems). Dinucleotide loci "bins" were set to 0.5 on either side of an average size for each allele. Tetranucleotide loci "bins" were set to 1.0 on either side of an average size for each allele. Allelic heights were required to be a minimum of 100.

Deviations from Mendelian inheritance of alleles were determined where possible and unusual alleles from F115 and FCA113 were sequenced. For sequencing, the same primer sequences were used, but they were unlabelled and unmodified (Whitehead Scientific, Cape Town, South Africa). Reactions were carried out as follows: Twenty microlitre reactions containing 0.2 µM forward and reverse primers, 0.0125U Super-Therm Gold DNA Polymerase with 1x Buffer and 1.5 mM $MgCl_2$ (Separation Scientific, Johannesburg, South Africa), 250 µM dNTPs (Life Technologies, Johannesburg, South Africa) and 2.5 to 25 ng DNA were set up for each primer pair. The PCRs were performed on a GeneAmp® PCR system 9700: denatured at 95°C for 5 min; 35 cycles of 95°C for 1 min, 58°C for 30 sec and 72°C for 30 sec; and a final extension of 72°C for 30 min. The PCR product was cleaned up with the MSB® Spin PCRapace kit (Invitek, Berlin, Germany) following Protocol 1, with a final elution volume of 20 µl. The ABI Prism® BigDye®

Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used for the sequencing reactions following manufacturer's instructions, however a quarter of the recommended concentration of BigDye® Terminator v3.1 was used. The sequencing reaction was performed on a GeneAmp® PCR system 9700 following the recommended temperature and time requirements for the kit. Sequencing products were purified with an ethanol precipitation and analysed on a 3130xl Genetic Analyzer. Geneious Version 6.1.0 created by Biomatters (Available from http://www.geneious.com) was used to analyse the sequences.

Optimisation of DNA Concentration for PCR and Genotyping Repeatability

Relative DNA quality and concentration were measured using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Dilutions of three samples were made to approximately 160, 80, 40, 20, 10, 5 and 1 ng/µl. Two of the samples were from the same animal: one EDTA blood and the other a skin sample. The dilutions were tested on the microsatellite panels to determine the ideal concentration range for the PCR. One of these samples was repeated three times at all concentrations. A further sample was repeated ten times at an ideal concentration. Average allele sizes and standard deviations were calculated in Excel. Based on these results, all samples were diluted to a concentration of between 20 and 40 ng/µl.

Statistical Analyses

Overall summary statistics were calculated for each locus: number and size range of alleles, amplification success and polymorphic information content (PIC) were calculated using Cervus

v. 3.03 (Kalinowski et al. 2007), allelic richness was calculated using FSTAT v. 2.9.3.2 (Goudet 1995) and linkage disequilibrium was calculated in GENEPOP v. 4.1.4 (Rousset 2008). Match probabilities were calculated for siblings for individual loci and cumulatively using the GenAlEx macro v. 6.501 for Microsoft Excel (Peakall & Smouse 2006). GenAlEx was used to identify rare alleles for the sample set as a whole, and within samples of known or suspected provenance.

Loci were ranked using PIC values and we determined the minimum number of loci needed to discriminate between all the samples in this study by eliminating one locus at a time, starting with the lowest PIC score. Known parentages (based on field observations) from Mun-ya-wana Private Nature Reserve (NR), Karongwe Game Reserve (GR), De Beers Venetia-Limpopo NR, Addo Elephant Park, Greater Makalali Private GR, Thornybush GR, Bubye Conservancy and Ukutula Lion Park were used to confirm inheritance patterns of alleles where possible. Parentages were confirmed manually.

For analyses where mixed origins may influence the outcome, such as testing for Hardy-Weinberg (HW) equilibrium, only unrelated animals with single, known or suspected provenance (based on genealogical data) were used. They were then grouped as follows: Greater Kruger NP (from small reserves), Kruger NP (from recent sampling in Kruger NP), Etosha NP, Kgalagadi TP and Greater Mapungubwe TFCA. MICRO-CHECKER v. 2.2.3 was used to test for scoring error, large allele dropout and null alleles within each group (van Oosterhout et al. 2004). Observed and expected heterozygosity and Hardy-Weinberg equilibrium of each marker

in each group were calculated using GenAlEx and the Bonferroni correction was applied to account for multiple comparisons (Rice 1989).

Structure Analysis

STRUCTURE (Pritchard et al 2000) software was used to analyse the data using 22 of the microsatellites (Table 2). K-values from two to six were tested with a "burn-in" of 100 000 and data collection of 100 000 with 50 replicates per K-value. The "Admixture" model was used as we suspected mixing between genetic provenances. STRUCTURE HARVESTER web version 0.6.93 (Earl and vonHoldt 2012) was used to perform the Evanno method (Evanno et al. 2005) for determining the best value of K and to prepare the data for input into CLUMPP (Jakobsson and Rosenberg 2007). CLUMMP was run for all K-values with the "Greedy" algorithm and 1000 repeats to average the results from the STRUCTURE analyses. CLUMPP output files were converted to PS files with Distruct (Rosenberg 2004).

Case Studies

We evaluated the applicability of the optimised forensics marker panel in one poaching case study and in one parentage assessment case study. For the poaching case the aim was to confirm the species and to determine the number of animals involved. Four full skins and three partial bone/tissue samples that were morphologically consistent as lions were tested. In the second case study, parentage based on field observations for nine litters of "reserve A" were compared to parentage analysis, based on the multi-locus genotypes, in Cervus. The twelve loci recommended for forensics were used. The simulation parameters were: 100 offspring, 10

candidate mothers, with a 0.50 proportion of the sampled mothers, 10 candidate fathers, with a 0.50 proportion of the sampled fathers, and a minimum of eight loci typed. All mismatches between expected and actual parentage were confirmed manually. In all cases on reserve A the mother had been assigned based on observational data. As male lions often form coalitions and any member of the coalition can father the cubs, no observation data could help determine which member of a coalition had fathered the cubs. This is where the DNA analysis was expected to be most helpful.

In fulfilment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad.

Results

Microsatellite Amplification Success and Consistency

Of the 401 samples, 361 amplified and could be used in further analyses. The samples that failed were of questionable quality and we were unable to extract DNA despite multiple attempts. The majority of the loci had regular 2 or 4 base pair repeats, however, locus FCA441, thought to represent a tetranucleotide repeat, manifested as a dinucleotide repeat. Several loci had some unusual alleles that were segregating according to Mendelian expectations. For example, dinucleotide locus FCA113 had five alleles (149, 151, 154, 156 and 159); sequencing confirmed the sizes of the alleles with single base insertions in the flanking regions of alleles 154 and 156 causing the switching between odd and even allele sizes in the range (Figure S1). A complete list of the allele sizes and frequencies can be found in the Supplementary Data (Table

S1). Allele sizes did not vary by more than 0.1 either side of the average among replicates of individual samples and between samples by not more than 0.5 for dinucleotide repeats and 1.0 for tetranucleotide repeats. There was one exception: the FCA441 (dinucleotide) 163 allele varied by more than 0.5 on either side of the average.

Peak height ratios for heterozygous individuals varied between 0.3 and 0.9 HR in Table 3. The peak of the first allele was sometimes smaller than the second in four of the loci (FCA069, FCA272, FCA031 and F41). MICRO-CHECKER found no evidence of scoring error, stutter or large allele dropout. There was some evidence of null alleles (see deviations from HW equilibrium indicated in Table 3), however, there was no evidence of any null alleles in parentage matching.

Three of the original 31 loci tested were excluded from subsequent analyses; F115 had inconsistent allele sizes and sequencing revealed that it was not a regular tetranucleotide repeat - including two alleles only one base pair apart and homoplasy of one of these alleles (Figure S2, S3); FCA651 was monomorphic in initial testing; FCA023 had only two alleles and was very difficult to amplify in multiplexes.

Samples with DNA concentrations above 80 ng/µl and below 10 ng/µl did not amplify consistently (Table S2). Concentrations between 10 and 80 ng/µl yielded variable results between samples; concentrations from 20 to 40 ng/µl were the most reliable (Table S2). The sample that was repeated 10 times at this concentration gave a consistent result (Table S3). Although we found locus FCA441 to be polymorphic and informative it could not be reliably

Table 3 : Microsatellite descriptive statistics and recommendations for forensic and population genetic studies of lions in South Africa

	Overall *n* = 361							ENP provenance, *n* = 23		GMTFCA provenance, *n* = 6		KNP provenance[a], *n* = 11		KTP provenance, *n* = 18		KNP origin[a], *n* = 53		
Locus	***N***	**Amp (%)**	**Size range**	**HR**	**PIC**	**LD**	**P_{IDsibs} cumulative**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**Comments**
Loci recommended for forensic matching[b]																		
FCA506[c]	13	97	199–233	0.46	0.81		3.5E-01	0.39***	3.32	0.17	2.00	0.46	2.45	0.65	3.39	0.62	4.76	
FCA230[c]	10	96	98–116	0.69	0.80		1.2E-01	0.70	2.84	0.50	2.82	0.73	4.35	0.72	4.47	0.72	4.85	
FCA391[c]	7	94	205–233	0.87	0.78		4.4E-02	0.70	3.12	0.00	1.00	0.91	4.25	0.82	4.53	0.73	3.98	
FCA075[c]	9	99	209–217	0.67	0.75	FCA069	1.7E-02	0.78	3.55	0.40	2.99	0.91	4.58	0.61	3.49	0.79	4.35	
FCA096[c]	8	95	205–218	0.68	0.74		6.5E-03	0.64	3.39	0.67	3.97	0.91	4.14	0.50	2.98	0.76	4.19	
FCA628[c]	7	95	108–119	0.75	0.72		2.6E-03	0.48	2.98	0.17	2.00	0.70	3.47	0.18**	2.31	0.73	4.15	
FCA001[c]	9	93	133–169	0.41	0.66		1.1E-03	0.74	3.57	0.20	2.00	0.55	2.98	0.29	2.07	0.65	3.76	
FCA224	10	100	158–175	0.76	0.78		4.0E-04	0.65	3.11	0.17	1.83	0.82	4.92	0.67	4.01	0.82	4.72	
FCA097	10	96	144–164	0.78	0.73		1.6E-04	0.48	2.52	0.40	2.82	0.82	5.15	0.89	5.41	0.65	3.50	
FCA126	6	99	186–222	0.68	0.70		6.4E-05	0.61	2.21	0.50	2.99	0.82	4.68	0.56	2.00	0.72	4.60	
FCA453	5	100	192–204	0.94	0.70	FCA085	2.6E-05	0.52	2.86	0.67	2.00	0.73	3.71	0.56	2.25	0.78	3.45	
FCA240	9	96	189–211	0.78	0.67		1.1E-05	—	2.00	—	2.82	—	3.36	—	3.65	—	4.20	X chromosome
F41[d]	2	94	101–110	0.77	0.03	20[e]	1.1E-05	0.04	1.22	0.00	2.00	0.00	1.00	0.06	1.29	0.00	1.00	Lion-specific
ZnF	2	93	165–	0.33	—	—	—	—	—	—	—	—	—	—	—	—	—	Sex marker

	Overall *n* = 361							ENP provenance, *n* = 23		GMTFCA provenance, *n* = 6		KNP provenance[a], *n* = 11		KTP provenance, *n* = 18		KNP origin[a], *n* = 53		
Locus	***N***	**Amp (%)**	**Size range**	**HR**	**PIC**	**LD**	**P_{IDsibs} cumulative**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**Comments**
			168															
Additional loci recommended for population studies																		
FCA057	8	92	166–180	0.77	0.66			0.59	2.63	0.33	1.83	0.64	3.82	0.38	3.14	0.72**	4.86	
FCA193	5	99	111–119	0.77	0.66			0.52	2.63	0.17	2.99	0.55	2.45	0.61	3.11	0.59	3.18	
FCA085	7	93	122–142	0.81	0.61	FCA453		0.65	3.48	0.33	3.82	0.55	4.02	0.41	1.98	0.60	3.74	
FCA113	5	99	149–159	0.71	0.60	FCA105		0.65	3.65	0.67	2.99	0.27	2.68	0.78	2.94	0.59	2.77	
F42	5	97	234–249	0.91	0.59	FCA069, FCA008		0.83	2.93	0.17	3.50	0.55	2.90	0.88	3.61	0.69	3.61	
FCA026	7	99	135–152	0.82	0.59			0.57	2.63	1.00	4.80	0.91	3.42	0.44	2.46	0.70	4.24	
FCA275	5	99	128–136	0.77	0.56			0.48	2.00	0.33	2.82	0.46	2.39	0.83	3.57	0.53	3.36	
FCA272	4	96	105–111	0.72	0.56			0.59	2.65	0.17	3.50	0.73	3.62	0.47	2.93	0.57	3.08	
FCA031	7	99	239–251	0.85	0.55			0.13	1.53	0.50	2.99	0.64	2.94	0.56	2.97	0.65	3.54	
FCA008	3	92	125–141	0.61	0.54	FCA105		0.44	2.56	0.60	3.82	0.73	2.70	0.64	2.70	0.62	2.56	
Loci containing rare population-specific alleles																		
FCA069	6	98	105–113	0.76	0.60	4[e]		0.41	2.40	0.17	1.99	0.36	2.00	0.67	3.42	0.42	2.73	
FCA441	4	98	151–163	0.90	0.52			0.39	1.96	0.33	4.50	0.46	2.39	0.28*	2.63	0.47	2.70	
FCA310	4	97	125–	0.81	0.35	5[e]		0.23*	2.00	0.50	2.99	0.46	2.00	0.72	2.00	0.43	2.17	

	Overall *n* = 361							ENP provenance, *n* = 23		GMTFCA provenance, *n* = 6		KNP provenance[a], *n* = 11		KTP provenance, *n* = 18		KNP origin[a], *n* = 53		
Locus	***N***	**Amp (%)**	**Size range**	**HR**	**PIC**	**LD**	**$P_{IDsibs\ cumulative}$**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**H_O**	**AR**	**Comments**
FCA105	7	96	128 209–217	0.73	0.30	FCA113, FCA008		0.00	1.00	0.33	2.99	0.36	2.39	0.60	3.26	0.34	2.90	
F37	3	98	221–225	0.79	0.05	10[e]		0.00	1.00	0.00	1.00	0.27	1.86	0.00	1.00	0.23	1.84	

Amp, amplification success; AR, allelic richness; ENP, Etosha National Park; GMFTCA, Greater Mapungubwe Transfrontier Conservation Area; H_O, observed heterozygosity; HR, height ratio; KNP, Kruger National Park; KTP, Kgalagadi Transfrontier Park; LD, linkage disequilibrium $P > 0.05$; *n*, number of samples; *N*, number of alleles; $P_{Idsibscumulative}$, average nonexclusion probability for related individuals; size range, of alleles.

[a]"KNP origin" refers to samples from small reserves with known KNP origin; "KNP provenance"refers to recent samples directly from KNP.

[b]Based on lion population estimates in Africa of not more than 40000 lions, these loci will discriminate, in theory, between 90000 lions.

[c]Loci successfully discriminating between 381 samples in this study.

[d]Lion-specific allele.

[e]The number of other loci that this locus is linked to. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ significant Hardy–Weinberg deviation after Bonferroni correction. Shading indicates possible null alleles based on Microchecker analysis.

genotyped within the multiplex (Table S2). An example of the GeneMarker output for a sample at an ideal concentration is presented in Figure S4.

Tissue type did not affect the allele sizes, peak height ratio or optimal DNA concentration for blood and skin tissue samples. Hair samples could not be tested at the same concentrations as blood and skin. Determining DNA concentrations of extracts from these hair samples did not yield accurate results on the NanoDrop®; the hair samples used did however yield good results without the need for dilution of the original DNA preparations. Duplicate extractions from one individual (hair and EDTA blood) yielded identical genotypes at all loci, except for FCA230 which did not sufficiently amplify from the hair sample.

Microsatellite Summary Statistics

The match probability for related individuals when all 28 loci were included was 4.8×10^{-10}. Reducing the number of loci to 12 reduced the match probability between related individuals to 1.1×10^{-5}. All successfully genotyped samples (N=381) could be differentiated using a minimal set of seven microsatellite markers (Table 3).

Polymorphic information content values ranged from 0.81 to 0.03 (Table 3). Based on Hardy-Weinberg expectations, four loci had significantly lower heterozygosity than expected in only one population group, the rest were in equilibrium. FCA230 is on the X-chromosome and so HWE could not be determined (Table 3). Allelic richness varied from 5.41 to 1.00 (monomorphic

within the population group). Some loci may be linked, but unlikely physically linked, as the loci were chosen based on the domestic cat linkage map (Table 3).

Seventy seven alleles from 26 microsatellite loci occurred in 5% or less of the samples (Table 4). Several alleles were specific to samples from known or suspected provenance, including four alleles that were only found in one of two samples in a captive population (Table 4). Locus F41 was not very variable with only two alleles, however, the 101 allele appeared in all samples and may be useful as a lion-specific allele. When compared to cheetah (*Acinonyx jubatus*), leopard (*Panthera pardus*) and tiger results in our database the allelic size range at this locus was more variable, with larger allele sizes than in the lion (cheetah range: 196 – 232; leopard range: 114 – 154; tiger range: 120 – 156; unpublished data; leopard 123-139 (Mondol et al. 2009)), suggesting that the 101 allele was unique to lion samples.

Structure analysis using 22 microsatellites found clear divisions amongst the samples with the Evanno ΔK statistic indicating the strongest population division at K = 4 (Figure 2, Table S5). There were many admixed individuals (Figure 2).

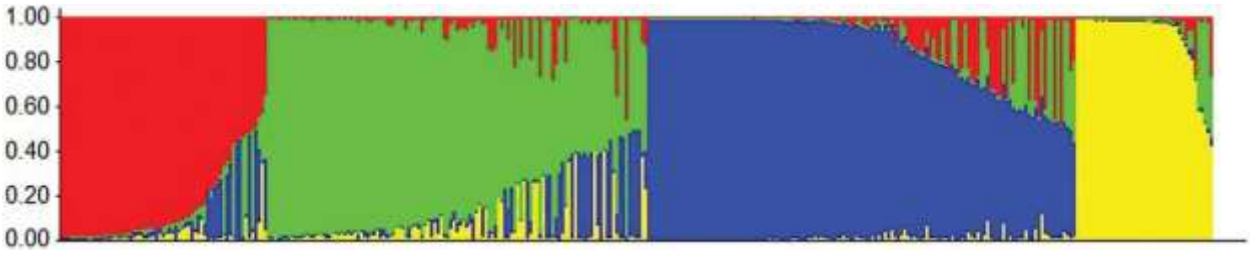

Figure 2. STRUCTURE analysis of 361 lion samples at K = 4

Table 4 : Rare alleles specific to genetic provenance in southern African lions

Number of rare alleles in overall population (%)					Alleles specific to a genetic provenance/population								
Locus	***N***	**<1**	**2**	**<5**	**KNP origin[a]**	**KNP[a]**	**HiP**	**ENP**	**KTP**	**GMTFCA**	**BC**	**SVC**	**ULP**
FCA506	13	4	5	7	229	186	231		215				186, 209, 217
FCA230	10	3	3	4							100		108
FCA391	7	1	1	2					233				201
FCA075	9	—	4	4									115
FCA096	8	2	3	3		220		201					
FCA628	7	1	1	1									110
FCA001	9	4	4	4		129, 165, 174					133		
FCA224	10	2	4	4		159, 177		167					177
FCA097	10	1	3	7					156		162		156
FCA126	6	—	1	2									
FCA453	5	1	1	1		208							
FCA240[b]	9	2	4	5		207					189, 207		
F41	2	—	1	1									
FCA057	8	2	3	3		172, 178	178						
FCA193	5	1	1	1			113	113					128
FCA085	7	1	2	3		116					134	134	
FCA113	5	—	—	1									
F42	5	—	1	2									
FCA026	7	1	2	4	145								
FCA275	5	1	1	2									
FCA272	4	—	—	—									
FCA031	7	1	2	4		241, 247				241			
FCA008	3	—	—	—									
FCA069	6	2	2	2		115			111			111	
FCA441	4	1	1	1						163			
FCA310	4	2	2	2		121, 136							132
FCA105	7	3	4	5			197	203					207
F37	3	1	2	2	225	223, 225							
Total	185	37	58	77									

BC, Bubye Conservancy; ENP, Etosha National Park; GMTFCA, Greater Mapungubwe Transfrontier Conservation Area; HiP, Hluhluwe-iMfolozi Park; KNP, Kruger National Park; KTP, Kgalagadi Transfrontier Park; *N*, number of alleles; SVC, Savé Valley Conservancy; ULP, Ukutula Lion Park.

[a]KNP origin refers to samples from small reserves with known KNP origin; KNP refers to recent samples directly from KNP.

[b]On X chromosome.

Table 3 summarises the recommendations for forensic matching of samples and population genetics studies. Twelve loci are recommended for forensics plus a zinc finger molecular sexing marker and F41 for species confirmation. An additional ten of the remaining 15 loci would be useful for population genetics studies of South African lions.

Case studies

All samples tested in the poaching case had the 101 allele at locus F41, suggesting that all samples originated from lions. Four of the samples were suspected as being from multiple lions based on the number and size of the skins. Based on the microsatellite results, we determined that they originated from separate individuals. Two of the three bone/skin samples provided partial profiles that indicated that the samples were from different animals, while the remaining one matched the genotype of one of the skin samples. All six individuals could be discriminated from each other using the twelve loci recommended for forensic applications.

Many cubs on “reserve A” had an unknown father, as there were often two males in a coalition that held tenure at the time of conception. Fathers were assigned (from the expected coalitions) and mothers confirmed for 24 cubs in eight litters. Two cubs did not match their expected parents: In one litter of four cubs, one cub did not match the mother (four exclusions in the 12 recommended loci) and the father was different to the rest of the litter, but matched the other member of the coalition. In another litter of four cubs, all four matched the mother, but one did not match the father of the rest (seven exclusions in the 12 recommended loci).

Discussion

Twenty eight microsatellite loci and a zinc finger molecular sexing marker were successfully evaluated for lion genotyping. Waits *et al.* (2001) recommend that the more conservative probability of identity for related animals be used rather than the probability for unrelated animals. Following this suggestion a subset of 12 of the evaluated markers (all with PIC values above 0.6) and the sexing marker are recommended for use in forensic matching of samples. The resulting match probability between related individuals should discriminate between 90 000 individual lions. As there are fewer than 40 000 free-roaming lions remaining in Africa (Riggio et al. 2013), this should be an appropriate cut-off to allow for any inbreeding that may exist in some isolated populations (Waits et al. 2001). Furthermore it should minimise the risk of genotyping error associated with larger numbers of loci (Waits et al. 2001).

The inclusion of locus F41 is further recommended when species confirmation is required. The available genotype database suggests allele 101 to be "lion-specific". While the amplification of this allele is not conclusive proof that a sample comes from a lion, it could be used in combination with other evidence to help substantiate or refute such a claim. The absence of the 101 allele must be treated with caution, especially if there is no amplification of this locus, as it could be caused by allelic drop-out. Larger sets of genotypes will be available in the near future to confirm our results and determine the rate of allelic drop-out. In the short-term a species-specific single nucleotide polymorphism (Rosenhart 2012) or short DNA barcode (such as from the mitochondrial DNA genome; Ivanova et al. 2012) could be used to confirm source identity in cases where molecular confirmation is the only option.

Some of the allele sizes at a few loci indicated abnormalities in repeat patterns. Sequencing of alleles where homoplasy or other abnormalities are suspected was recommended by Selkoe & Toonen (2006). The value of sequencing when choosing loci has been demonstrated here with one locus being eliminated based on sequencing results and another validated. Ideally all twelve recommended loci would be sequenced to confirm their suitability as forensic loci. However, due to financial constraints this was not possible. The data for the twelve recommended loci suggest that even though there were some transitions from odd to even allele sizes, these were stable and thus should be suitable.

The recommended forensic panel was successfully applied to a poaching case where skins and other tissue samples were submitted for testing. These loci were used to determine the number of animals involved in the case and all sample genotypes included the locus F41 “lion-specific” allele 101, confirming that they all originated from lions.

The clarification of field observation-based parentage using genotypic data of lions on one reserve was presented as a second case study. All but two of the offspring matched as expected based on field observations and the fathers that were in doubt (where there were two males in a coalition) were determined. Of the two cubs with unexpected results one did not match the expected mother and the other the expected father. The mismatches were over a large number of loci, thus it is most likely that they were not due to a scoring error, null alleles or any other artefact. As samples from all the animals on the reserve were not available, we were not able to

determine the actual parents in these two cases. Both of these mismatches were from litters of four cubs suggesting that the reserve records are not completely accurate. DNA analysis can therefore be used to clarify ambiguous data for reserve managers and ultimately aid in future management decisions.

For population genetics studies it may be useful to include more loci than for forensic matching, depending on the goals of the project (Hoban et al. 2013). Väli et al. 2008 warn against using too few microsatellite loci, even if they are polymorphic, when determining population origin. Therefore, if genetic provenance is of interest, some or all of the additional ten recommended loci should be considered, as some alleles have been identified that are specific to lions of different genetic provenance. Some of these additional markers showed evidence of genetic linkage with other loci. These loci were not physically close on domestic cat chromosomes (Menotti-Raymond et al. 1999). Care should be taken when deciding which additional markers to use to avoid possible linkage. SPOTG is a new online application that has been designed to aid researchers in planning genetics studies and may be a useful starting point for determining the most useful loci for various applications (Hoban et al. 2013). We have briefly demonstrated the power of using 22 microsatellite loci to discriminate between samples of four different provenances and to identify individuals where admixture has likely occurred. Further analysis of a sub-set of the results presented here (using the 22 recommended microsatellite loci) to assess the current genetic provenance and heterozygosity within small reserves in South Africa is in preparation (Miller et al. in preparation). The remaining five of the 28 tested loci appear to be of limited value for lion genetics studies based on linkage, low PIC values and/or unreliability.

Locus FCA506 may not be appropriate for population, relatedness and parentage studies as it deviated from Hardy-Weinberg equilibrium and had evidence of null alleles in the Etosha NP samples. While null alleles are not problematic for individual identity matching, they can be problematic for parentage and relatedness analyses, as well as in population studies (Dakin & Avise 2004). There are ways of accounting for null alleles for parentage analysis (Dakin & Avise 2004), relatedness analysis (Wagner et al. 2006) and population differentiation (Chapuis & Estoup 2007). This would be preferable to elimination of this locus as it has such high allelic richness. Primers can also be redesigned for the focal species, as the use of primers designed from distantly related species, such as the domestic cat loci used here, may be prone to amplification failure for some alleles due to mutations in the priming regions (Selkoe & Toonen 2006).

Most of the loci recommended here have been found to be variable in other genetic studies of African lion (Spong, Stone, Creel, & Björklund, 2002; Driscoll et al., 2002; Antunes et al., 2008). This suggests that the forensic and population loci recommendations should be applicable to other lions across the continent. Confirmation is still necessary, especially for the forensic panel given the need for accurate traceability. Ideally a minimum set of loci would be used in all lion studies allowing for more rigorous comparisons between studies as has been described by Skrbinšek et al. (2012).

Some captive lion samples were included in this study and a number of unique alleles were found in this population. This suggests that there may be some genetic diversity in the captive

populations that has been lost from the wild populations. This warrants further research into captive populations in South Africa to determine if unique diversity has been preserved in these captive populations and could be restored to the wild populations at a later date.

Conclusions

A set of 12 microsatellite loci have been defined for confirmation of individual identity of lion samples within South Africa. A likely lion-specific allele has also been identified that can be used to aid in species confirmation. Standardization of microsatellite panels across Africa would be invaluable in wildlife forensics. A further 15 microsatellite loci have been evaluated and their usefulness for population genetics studies has been highlighted. These microsatellite loci will be useful for conservation planning for lions in South Africa and, if evaluated across more lion populations, the rest of the continent.

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Supplemental Data

Figure S1. Sequencing of four alleles of the FCA113 locus in African lions

Figure S2. Sequencing of seven alleles of the F115 locus in African lion.

Figure S3. Homoplasy at the 208 allele in locus F115 in African lion.

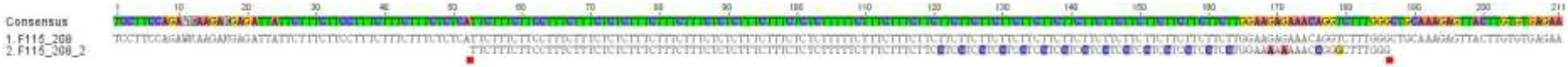

Table S2. Average allele sizes for 10 replicates of one sample at 29 microsatellite loci

	Allele 1		Allele 2	
Locus	Average	SD	Average	SD
		Multiplex 1		
F42	238.0	0.12		
FCA031	241.3	0.12	249.0	0.13
FCA113	154.1	0.07		
FCA193	116.9	0.08		
FCA224	165.1	0.05		
FCA272	107.4	0.06		
FCA391	221.2	0.10		
FCA441	154.9	0.00	159.0	0.00
FCA453	196.2	0.08		
		Multiplex 2		
F37	220.8	0.05		
F41	101.2	0.05		
FCA008	127.4	0.05	141.4	0.05
FCA026	139.8	0.05	147.0	0.05
FCA075	121.2	0.04	130.9	0.05
FCA096	210.9	0.05	216.1	0.07
FCA105	212.8	0.07		
		Multiplex 3		
FCA057	175.9	0.05		
FCA069	105.3	0.07	109.0	0.07
FCA126	185.3	0.05		
FCA240	194.5	0.06		
FCA275	131.7	0.07	133.8	0.07
FCA310	125.3	0.07	127.4	0.07
FCA506	219.0	0.03		
FCA628	111.7	0.06	119.3	0.07
		Multiplex 4		
FCA001	136.9	0.06	163.2	0.05
FCA085	128.3	0.06		
FCA097	153.3	0.04		
FCA230	97.6	0.09		
ZnF	165.2	1.02	168.1	0.05

Figure S4. Example of multiplex results.

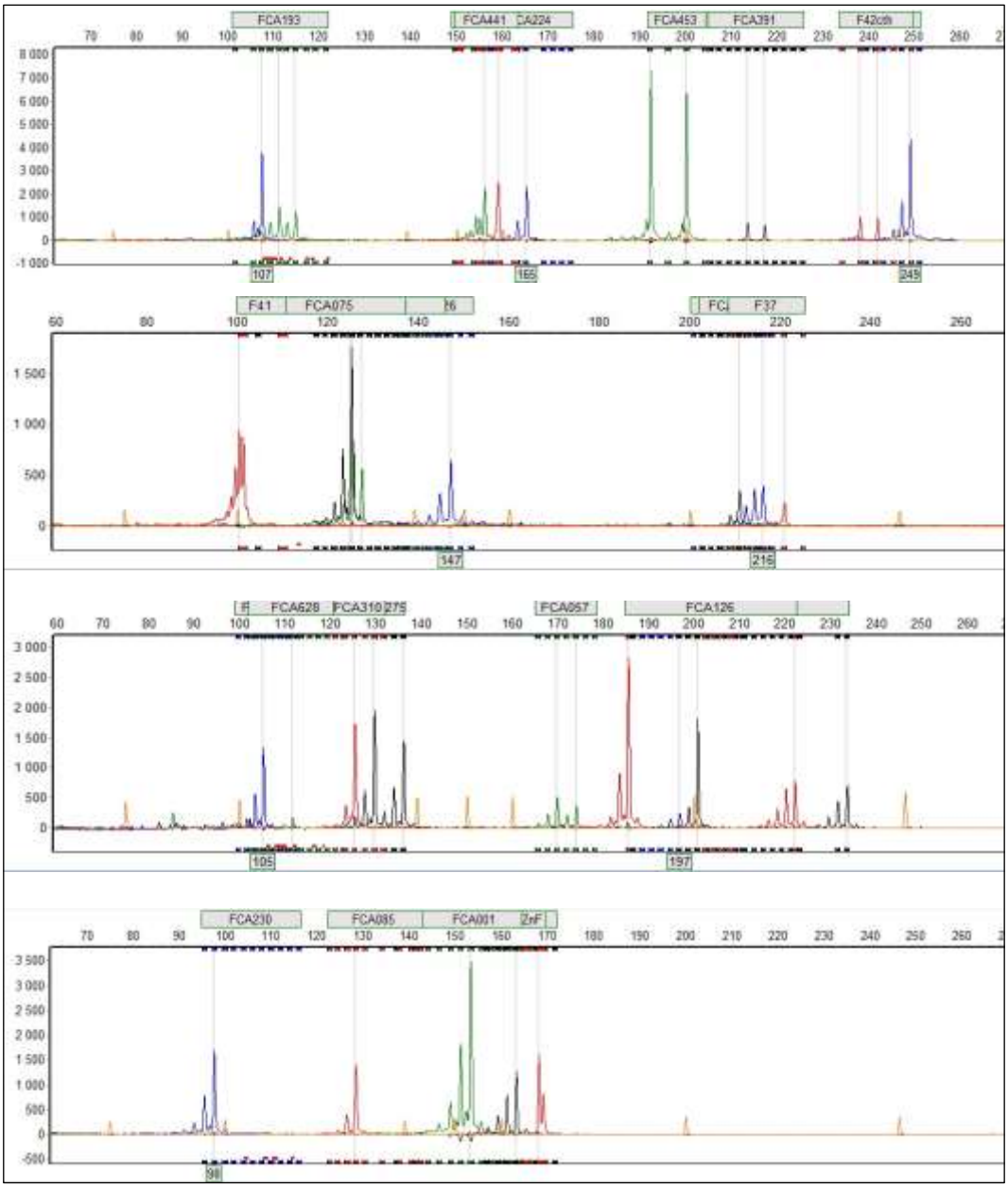

Table S4. Evanno results for STRUCTURE analysis

# K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2	50	-23021.3	1.80	NA	NA	NA
3	50	-22230.9	1109.98	790.38	48.68	0.04
4	50	-21391.9	87.05	839.06	348.37	4.00
5	50	-20901.2	91.63	490.69	88.52	0.97
6	20	-20499	75.37	402.17	NA	NA