RESEARCH ARTICLE

Infanticide in brown bear: a case-study in the Italian Alps – Genetic identification of perpetrator and implications in small populations

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Abstract

Sexually Selected Infanticide (SSI) is thought of as a male reproductive strategy in social mammalian species, because females who lose cubs may quickly re-enter oestrus. SSI has rarely been documented in non-social mammals and, in brown bears, SSI has been studied mainly in an eco-ethological perspective. The authors examined the first genetically documented infanticide case which occurred in May 2015 in brown bears in Italy (Trentino, Central-Eastern Alps). The infanticide killed two cubs and their mother. Hair samples were collected from the corpses as well as saliva, through swabs on mother's wounds, with the aim of identifying the genotype of the perpetrator. The samples were genotyped by PCR amplification of 15 autosomal microsatellite loci, following the protocol routinely used for individual bear identifications within the Interregional Action Plan for Brown Bear Conservation in the Central-Eastern Alps (PACOBACE). Reliable genotypes were obtained from the mother, cubs and putative perpetrator. The genotypes were matched with those populating the PACOBACE database and genealogies were reconstructed. Both mother and perpetrator genotypes were already present in the database. Kinship analyses confirmed mother-cubs relationships and identified the father of the cubs. In this study, for the first time, the authors used the open-source LRmix STUDIO software, designed to analyse human forensic genetic profiles, to solve a case in wildlife. Through LRmix STUDIO, those alleles that do not belong to the

victims were isolated and, finally, the perpetrator was identified. This study presents a method that allows, through the application of different models, the genetic identification of the conspecific perpetrator with the highest probability. The identification of the infanticidal male is relevant for the better management and conservation of wild populations with small effective population size (Ne) and low population growth rate, especially in the case of recently established populations in human-dominated landscapes. This procedure will have predictably wide applications, supplying important data in the monitoring of small and isolated populations.

Introduction

Infanticide, the killing of dependent offspring by conspecifics, has been thought of as a component of intersexual conflicts in social mammals (Hrdy 1979, Hrdy and Hausfater 1984). Most reports describe adult and sub-adult males killing young-of-the-year or yearling cubs (Taylor et al. 1985, Dean et al. 1986, Olson 1993, Craighead et al. 1995, Derocher and Wiig 1999). Others document the killing of cubs by unmated females (Dean et al. 1986, Hessing and Aumiller 1994). Although the victim was consumed in most instances, cannibalism could not be invoked as a general cause of aggression (Dean et al. 1986, Hessing and Aumiller 1994, Karamanlidis et al. 2015). Similar to other species, such as African lions *Panthera leo* (Packer and Pusey 1984) and red deer *Cervus elaphus* (Bartoš and Madlafousek 1994), infanticide in bears is most likely related to male reproductive success (Swenson et al. 1997). SSI can be a male reproductive strategy where the killing of unrelated offspring induces premature oestrus in mothers and increases the opportunity to breed with them (Hrdy 1979). SSI is common in size-dimorphic species with a polygamous mating system (van Schaik 2000) and occurs during the mating season in seasonal breeders that have extended maternal care and lactational anoestrous (Bellemain et al. 2006a, Zedrosser et al. 2009). SSI has been described in some North American and Scandinavian brown bear populations (Wielgus and Bunnell 1995, Swenson et al. 1997, Bellemain et al. 2006a, Libal et al. 2011). Resistance to infanticide may be costly: a female may sustain serious injuries in defending her offspring and sometimes the mother dies in an attempt to defend her cubs. In isolated populations with a small number of reproductive adults, SSI can negatively impact the long-term conservation of the species, especially in the case where the female is killed while protecting her cubs. Taking this into account, the genetic identification of the perpetrators could give concrete indications for the management of small populations (e.g. placing radio-collars on infanticidal males to track them). Nevertheless, genetic studies for identifying infanticidal males have received little attention.

In this paper, the authors report the first observation of infanticide in brown bears documented through genetic analysis in Italy. On 10 May 2015, as part of the field and genetic long-term monitoring of the brown bear population re-introduced in Trentino,

central-eastern Italian Alps (De Barba et al. 2010a), in Costa Lugiangia (Tuenno), the corpses of three brown bears were found: an adult female and two cubs-of-the-year. Death was not caused by anthropogenic causes. Their injuries, in fact, led to the hypothesis that the aggressor was an adult male bear. The cubs were killed and partially consumed and the female was consumed as well and covered with plant material. The mother (the only one with substantial parts still to be consumed) was found covered with earth, branches and foliage, which is typical of the behaviour observed in bears in an attempt to defend carcasses from scavengers. Another typical ursine behaviour involves dragging carcasses to places inaccessible to scavengers: the corpses were found, indeed, in a very dense forest area. Moreover, the day before, the person who found the first cub's corpse had heard very loud noises coming from the woods and these could have been derived from an intense struggle. This is just one of the indications that the mother had defended her cubs fiercely. It was observed that trees had been broken and that there were claw marks on the trunks. The suspects are, therefore, all the adult male bears considered present in the population in 2015.

Since an infanticidal bear can affect the growth rate of a small and isolated population (Gosselin et al. 2015), the results of this study are particularly relevant for the management of other wild small populations located in structurally fragmented and human-dominated landscapes.

Methods

Study area, population and sampling

In 2015, the brown bear population, re-introduced in central-eastern Italian Alps, extends mainly in the western part of the Trento Autonomous Province (PAT), across an area of ca. 20794 km² (including movements of young dispersal males). The females permanently occupy a smaller area (1303 km²) entirely located within the PAT. The Extent of Occurrence is estimated as 100% minimum convex polygon, delimited by all the validated indices of presence (Groff et al. 2016). In 2015, the bear density in the territory occupied by females was approximately 3.4 bears/100 km² (44 individuals in total, including the cubs-of-the-year).

This population has been continuously monitored during the last 17 years by both genetic and direct observation procedures (AA VV 2010, De Barba et al. 2010a). The main monitoring targets are: the identification of most of the individuals; the assessment of all reproductive events; the reconstruction of the population pedigree and demographic structure. Moreover, the reference genetic database allows identifying problematic bears that have a high level of interface with humans with humans and allows implementing effective actions for conservation, prevention and mitigation of conflicts. Although such bears represent only a small part of the bear population, they usually cause the majority of all human-bear conflicts, while most bears come into conflict with humans only rarely or never.

In the period between 2003 and 2015, the authors collected and analysed ~7800 biological samples, of which ~7700 (hair samples, scat, saliva samples, urine and blood samples on snow) were collected through non-invasive techniques during the monitoring programmes and ~100 were of an invasive origin (tissue, hair samples, teeth and bone sample). Tissue, teeth and bone samples were taken during necropsies of animals which had died of natural causes or had been killed by traffic or poaching, while hair samples came from animals live-captured for radio-tracking studies.

During the genetic monitoring carried out in 2015, 45 different bears (24 females and 21 males) were identified of which 25 were adults: 13 females (>3 years old, reconstructed on the basis of field data and genetic pedigrees) and eight males (>4 years old). In addition to these bears, the authors considered in the population even those bears that had been sampled during 2012–2015 (the last sample collected not before than 2012) and not known as dead in 2015. Thus, the total number of individuals present in 2015 was 57 (of which 14 were adult females and 12 were adult males).

Following the infanticide case, in 2015 two types of biological samples were collected: three samples of hairs from the corpses and four saliva swabs, by swabbing the mother's injuries, hoping to isolate the DNA of the perpetrator. The samples were preserved dry until the DNA extraction.

DNA isolation and amplification

DNA from hairs was extracted using the ZYMO Research ZR-96 Genomic DNA™ – Tissue MiniPrep Kit (CA, U.S.A.) and DNA from swabs was extracted using the QIAGEN QIAamp® DNA Investigator Kit (Hilden, Germany), following the manufacturer's protocol. The amplification and analysis of microsatellites were carried out by updating the protocol described by De Barba et al. (2010b). For the parentage analysis, the authors amplified 15 loci (Ostrander et al. 1993, Paetkau and Strobeck 1995, Taberlet et al. 1997, Paetkau et al. 1998, Bellemain and Taberlet 2004) in three multiplex-PCR: M1 (cxx20, G10M, G10P, Mu11, Mu15), M2 (G1D, G10X, Mu23, Mu50, Mu59) and M3 (G10C, G10H, G10L, Mu09, Mu10); for the individual identification, the authors used routinely only 10 loci (M1 and M2). Sex was identified using the amelogenin gene (AMG, Ennis and Gallagher, 1994) and confirmed using the SRY gene (Taberlet et al. 1993). A multitube approach was used (Taberlet et al. 1996, Adams and Waits 2007) with positive and negative controls in each step (Pompanon et al. 2005). One primer of each pair was 5'-labelled with 6-FAM, HEX, NED or PET dyes. STR fragments were detected and sized on an ABI Prism 3130XL Genetic Analyzer DNA sequencer (Thermo Fisher Scientific, Waltham, MA USA). The electropherograms were collected by the DATA COLLECTION Software v.3.0 and analysed by the GeneMapper Software v.4.0 (Applied Biosystems by Thermo Fisher Scientific).

Statistical analysis

Low genetic variability in the sampled population and small numbers of markers used in genotyping, might lead different individuals to show the same multilocus genotype. This shadow effect (Mills et al. 2000) can be minimised by increasing the number of loci genotyped. Two probability-of-identity formulations were used (Waits et al. 2001): PID_{unb} (unbiased for small sample size) and PID_{sibs} (the expected PID between sibs), which defined respectively the lower and upper bounds assuming that the sampled population included only unrelated individuals or sibs. GenAlEx v.6.502 (http://biology-assets.anu.edu.au/GenAlEx) was used to evaluate the suitability of the marker set chosen for individual (and victims') identification. The matches were calculated amongst the genotypes with the option MATCHES in the MULTILOCUS menu, which automates the detection of repeated genotypes within the dataset, by comparing the profiles of the victims directly with those present in the reference database, to find out from which one they originate.

To ensure as much as possible the proper genetic reconstruction of the cubs' pedigree, the paternity probabilities were calculated by comparing the results of two software taking into account the allelic frequencies of the population and the error rate per locus: COLONY v.2.0.5.0 (http://www.zsl.org/science/software/colony) and FRANz v.2.0.0 (http://www.bioinf.uni-leipzig.de/Software/FRANz/). COLONY implements a maximum likelihood method to assign sibship and parentage jointly, using individual multilocus genotypes at a number of co-dominant or dominant marker loci. FRANz reconstructs pedigrees (family trees) using polymorphic, co-dominant markers. [See Suppl. material 1 (Parameters used for parentage analysis) for the settings used].

Two different statistical models were used aiming to obtain reliable results from the analysis of the Low Template-DNA (LT-DNA) from the swab samples: the "classical" biological model (Caragine et al. 2009, Benschop et al. 2011, Pfeifer et al. 2012) and the statistical or probabilistic model (Curran et al. 2005, Gill et al. 2008, Gill and Buckleton 2009, Gill and Haned 2013, Benschop et al. 2015).

Following the biological model, the genetic profiles from the four swabs were interpreted, not individually, but in an integrated manner, by comparing the results obtained from the four independent replicates of each swab. Scientific literature describes two main approaches, both of which were applied to the evaluation of the genetic profiles of the single trace:

- The consensus method (Gill et al. 2000, Benschop et al. 2011, Benschop et al. 2013) which provides that, for each locus, an allele, that meets pre-specified acceptability criteria, can be considered reliable and it can contribute to a virtual consensus profile, only if it is confirmed in the replicates. The authors considered reliable only the alleles that appeared in at least half of the replicated genetic profiles. This approach allowed the authors to partially reduce the drop-in phenomenon because only the alleles consolidated in the replicates were considered (Benschop et al. 2011).

- The composite method (Bright et al. 2012, Pfeifer et al. 2012) which provides that, for each locus, each allele considered responsive to predetermined acceptability criteria and observed in the replicates may contribute to a virtual composite profile, which consists of the sum of all observed alleles. This approach allowed the authors to partially reduce the drop-out phenomenon because all the alleles presented in the replicates were considered (Pfeifer et al. 2012).

The results from the biological model analyses were compared with the results obtained by the statistical model. There are three groups of statistical methods to evaluate the Weight-Of-Evidence from the traces through the calculation of the likelihood value (Likelihood Ratio – LR), based on different algorithms and classified as: binary models (traditional methods of calculation), semi-continuous models and continuous models (Gill et al. 2015). All the statistical methods cited are based on the LR calculation, an adimensional value that, on the basis of the genetic profiles obtained and the assumptions formulated, objectively identifies which one of two possible and mutually exclusive hypotheses is the more likely in a given scenario. The use of the binary statistical methods is not recommended in cases of LT-DNA traces, in which stochastic events may occur. In addition, to date, the continuous statistical methods that use all the quantitative information (intensity of allelic signals) have found a restricted application in the LT-DNA analysis, because they can provide misleading information when the signals are of low intensity, there are stochastic phenomena and the analytical noise background is relevant (Gill et al. 2007, Benschop et al. 2011, Taylor et al. 2013). In this study, an algorithm has been applied of a semi-continuous calculation that evaluates, in terms of LR, the possibility that one or more comparison subjects contributed with their own genetic material to the genetic profiles detected in the traces, including consideration of possible stochastic events of drop-out (Pr(D) – drop-out probability) and drop-in (Pr(C) drop-in, or contaminations, probability).

Specifically in the case of conspecifics, the low amount of DNA mixtures can be treated as the DNA traces usually found in a crime scene. For the interpretation of the low-level complex DNA mixtures with the statistical model, an open-source software was used: LRmix STUDIO (version 2.1.3-CommunityEdition, 2013–2016 Netherlands Forensic Institute, freely available at http://lrmixstudio.org). This software is dedicated to the semi-continuous approach and explicitly accommodates for uncertainty in the DNA profile from the allelic drop-out (ADO) and drop-in (contaminations) phenomena. LRmix STUDIO estimates these quantities from the available data and uses those estimates to generate LR. LRmix STUDIO was used to compute the LR for each suspected male (reference DNA profile) and to compare the global consensus profile and the global composite profile, obtained from the comparison of each trace, with all the reference profiles. The authors performed:

- A LR calculation defining the prosecution (Hp) and the defence (Hd) hypotheses. Under each hypothesis, the authors defined the contributors (Hp: victims and 1

suspect, Hd: victims and 1 unknown), the drop-out probabilities (victims: 0.01, suspect and unknown: 0.6, afterwards replaced with the average value of drop-out, calculated over all suspects, by the drop-out estimation of the sensitivity analysis), the Pr(C) and the rare alleles frequency (the default values: 0.05 and 0.001 respectively), the allelic frequencies of the population (calculated by GenAlEx on the reference database) and the Theta correction (0.03 for small and isolated populations);

- A Sensitivity Analysis (SA) that plots the log10 LRs along with the likelihoods of the Hp and Hd. The SA, showing the variation of the LR value when $0 \leq Pr(D) \leq 0.99$, allows the verification of the range of the most likely values of Pr(D) (from the 5th to 95th percentile) using a Monte-Carlo simulation method (Haned et al. 2015). The corresponding most likely range of LR values is compared with the LR value initially obtained. The drop-out estimation is a qualitative estimator of the Pr(D) of the whole profile, based on the average number of alleles observed in the profile. The ADO value obtained is an interval of the plausible range of drop-out, plotted on the SA, but also displayed as a highlighted area in the plot (see Suppl. material 2 and Suppl. material 3 for detailed LRmix STUDIO results on Global Consensus and Global Composite respectively);

- A Non-contributor test for better understanding the case specific LR (Gill and Haned 2013). This test consists of the evaluation of the LR value when the subject of interest (the suspected male) is replaced by N subjects taken at random (N = No. of iterations), in which the genetic profile is simulated based on the allelic frequencies. The distribution of LR for N iterations is shown in a barplot where the case-specific log10 LR is displayed in red (LR(POI)) and the minimum, the maximum, the 1-50-99 percentiles (LRs) are displayed in grey (see Suppl. material 2 and Suppl. material 3 for detailed LRmix STUDIO results on Global Consensus and Global Composite respectively). If the LR calculation model worked efficiently and if Hp is true, then the LR(POI) will be >1 and LRs will be <1.

Results

Reliable genotypes were obtained from all the hair samples collected from the three corpses. The DNA profiles obtained from each victim were compared with the profiles in the reference database: 93 genotypes (9 founders and 84 offspring in 15 years) of which 45 are females and 48 are males. This comparison allowed the identification of the mother (a female called BJ1), while the cubs (one male and one female) were unknown and these were added them to the reference database, with the names of M33 and F22 (Table 1). The low PID values at 15 loci ($PID_{unb}=3.7\times10^{-13}$ and $PID_{sibs}=6.6\times10^{-06}$) indicated that a shadow effect is unlikely, given the size of the studied population.

BJ1 was genetically identified as the mother of M33 and F22. Moreover, both software used for parentage analysis agree in identifying a known male, MJ4, as the father of the cubs (Table 2a, COLONY results; Table 2b, FRANz results).

Table 1. List of samples with matching multilocus genotypes at all loci.

Sample	cxx20		G10M		G10P		G10X		G1D		Mu11		Mu15		Mu23		Mu50		Mu59		G10C		G10H		G10L		Mu09		Mu10	
M22	134	134	123	123	165	171	133	139	106	108	80	86	136	146	118	120	102	106	123	123	94	106	253	253	151	151	193	195	118	130
Positive Control	134	134	123	123	165	171	133	139	106	108	80	86	136	146	118	120	102	106	123	123	94	106	253	253	151	151	193	195	118	130
BJ1	118	118	117	119	151	171	139	143	102	116	88	88	136	142	120	122	98	104	103	123	94	106	253	253	151	151	177	185	128	130
Victim 1 (mother)	118	118	117	119	151	171	139	143	102	116	88	88	136	142	120	122	98	104	103	123	94	106	253	253	151	151	177	185	128	130
Victim 2 (male cub – M33)	118	118	117	123	171	171	133	139	102	106	78	88	136	146	120	122	98	98	111	123	94	94	253	253	151	151	185	185	118	128
Victim 3 (female cub – F22)	118	118	119	123	171	171	133	143	102	116	88	88	136	146	120	122	102	104	101	103	94	94	253	253	151	153	185	193	118	130

Table 2. Parentage analysis. (**a**) Colony results; (**b**) FRANz results.

(a)

Offspring ID	Inferred Mum	Posterior	Mismatches	Inferred Dad	Prob. Mum	Prob. Dad
M33	BJ1	1.000	0	MJ4	1.000	1.000
F22	BJ1	1.000	0	MJ4	1.000	1.000

(b)

Offspring	Parent 1	Parent 2	LOD	Posterior	Mismatches	n_f	n_m	Pair LOD Parent 1	Pair LOD Parent 2	Posterior Parent 1	Posterior Parent 2
M33	BJ1	MJ4	2.104E+01	1.000	0	14	12	9.377E+00	9.835E+00	1.000	1.000
F22	BJ1	MJ4	2.298E+01	1.000	0	14	12	1.126E+01	7.903E+00	1.000	1.000

Due to the small quantities of DNA extracted from the swabs, only 10 loci (PID$_{unb}$=3.0×10^{-10} and PID$_{sibs}$=1.3×10^{-04}) were typed. Although drop-in phenomena cannot be excluded, the presence in most of the STR loci of more than two alleles suggests a genetic mixture, produced from organic material from at least two individuals. The presence of the allelic signal Y (male-specific), appreciable both in Amelogenin and in SRY, in three out of four swabs, suggests that the contribution to the formation of the traces is derived from at least one male, in addition to the dominant genetic component originating from the blood of the mother. As a whole, for the extrapolation of genetic profiles, all the replicates of the traces were considered useful and these were all used for comparison (Table 3).

Comparison of the results obtained using the biological model provides quantitative information regarding the degree of concordance or discordance between each genetic profile of comparison (potential infanticidal males) and the genetic profiles from the traces, based on the consensus and the composite methods (Table 3, see Suppl. material 4 for detailed results of the biological model). For each comparison, the authors calculated, excluding molecular sexing: (1) the ratio of the number of alleles present in each suspect's genetic profile to the number of alleles found in the genetic profile from the single traces (No (%) of matching alleles); (2) the ratio of the number of alleles from the single traces to the number of alleles not found in the genetic profile of each suspect (No (%) of divergent alleles). The results obtained were presented in terms of percentage of concordances and discordances with a histogram to allow a more intuitive graphical display (Figure 1). The bear that most likely contributed to the analysed traces is the one that receives the lowest values of percentage of discordances. Apparently, the culprit seems to be MJ4, but this is the father of the cubs and the high value of allelic concordance and the low value of allelic discordance can be explained by the fact that he shares half of his genetic heritage with the killed cubs. Previous studies have demonstrated, through DNA analysis, that infanticidal males are unrelated to the infants they attack (Bellemain et al. 2006a). The putative perpetrator is, therefore, the second one: M7. Applying the consensus method, there is present only one discrepancy between the alleles of M7 and those from the traces: at the locus cxx20, alleles of M7 have not been detected in the genetic profiles of the traces.

About the results obtained using the statistical model, the Hp was tested by comparing the values of Log10(Pr(E|Hp)) and Log10(Pr(E|Hd)) for every suspect (Figure 2): if Log10(Pr(E|Hp))>Log10(Pr(E|Hd)) and the difference (Δ) between these two values is greater than zero, it is more likely that the DNA, belonging to the individual tested, is present in the analysed traces. The mean value of ADO was corrected for the data presented in Figure 2, based on the results of the Drop-out Estimation calculated during the SA (0.65 for Global Consensus and 0.55 for Global Composite). The bear that most likely contributed to the analysed traces is the one that achieves the highest values: the biostatistical analysis allowed the authors to observe some compatibility between the genetic profile of one male (M7) and the genetic profiles from the traces and a high degree of incompatibility of the genetic profiles of the remaining suspects

Table 3. Results of typing of the four replicates for each swab sampled on the female's injuries.

|		Genetic profiles										Sexing	
		cxx20	G10M	G10P	G10X	G1D	Mu11	Mu15	Mu23	Mu50	Mu59	AMG	SRY
I SWAB	I	118/118	117/119	151/171	139/143	102/116	88/88	136/142	120/122	98/104	103/123	158/212	75/75
	II	118/118	117/119	151/171	139/143	102/116	88/88	136/142	120/122	84/98/104	103/123	158/212	75/75
	III	118/118	117/119	151/171	139/143	102/116	88/88	136/142	120/122	84/98/104	103/123	158/212	75/75
	IV	118/118	117/119	151/171	139/143	102/102	88/88	136/142	120/122	84/98/104	103/123	158/212	75/75
Biological model results	I Consensus	118/118	117/119	151/171	139/143	102/116	88/88	136/142	120/122	84/98/104	103/123	158/212	75/75
	I Composite	118/118	117/119	151/171	139/143	102/116	88/88	136/142	120/122	84/98/104	103/123	158/212	75/75
II SWAB	I	118/118	119/123	171/171	133/143	102/102	88/88	136/146	120/122	102/104	101/103	212/212	0
	II	118/118	119/123	171/171	133/143	102/116	88/88	136/146	120/122	102/104	101/103	212/212	0
	III	118/118	119/123	171/171	133/143	102/116	88/88	136/146	120/122	102/104	101/103	212/212	0
	IV	118/118	119/123	171/171	133/143	102/116	88/88	136/146	120/122	102/104	101/103	212/212	0
Biological model results	II Consensus	118/118	119/123	171/171	133/143	102/116	88/88	136/146	120/122	102/104	101/103	212/212	0
	II Composite	118/118	119/123	171/171	133/143	102/116	88/88	136/146	120/122	102/104	101/103	212/212	0
III SWAB	I	118/118	111/117/119	151/171	139/143	102/102	88/88	0	120/122	98/104	0	158/212	0
	II	118/134	111/117/119	151/171	139/143	102/102	78/88	0	120/122	84/98/104	103/103	158/212	75/75
	III	118/118	111/117/119	171/171	139/143	102/102	78/88	0	122/122	84/98/104	103/103	212/212	75/75
	IV	0	111/117/119	151/171	139/143	102/102	78/88	0	120/122	84/98/104	103/103	158/212	0
Biological model results	III Consensus	118/118	111/117/119	151/171	139/143	102/102	78/88	0	120/122	84/98/104	103/103	158/212	75/75
	III Composite	118/134	111/117/119	151/171	139/143	102/102	78/88	0	120/122	84/98/104	103/103	158/212	75/75
IV SWAB	I	118/118	117/123	171/171	133/139	102/106	78/88	136/146	120/122	98/98	111/123	158/212	75/75
	II	118/118	117/123	171/171	133/139	102/106	78/88	136/146	120/122	98/98	111/123	158/212	75/75
	III	118/118	117/123	171/171	133/139	102/106	78/88	136/146	120/122	98/98	111/123	158/212	75/75
	IV	118/118	117/123	171/171	133/139	102/106	78/88	136/146	120/122	98/98	111/123	158/212	75/75
Biological model results	IV Consensus	118/118	117/123	171/171	133/139	102/106	78/88	136/146	120/122	98/98	111/123	158/212	75/75
	IV Composite	118/118	117/123	171/171	133/139	102/106	78/88	136/146	120/122	98/98	111/123	158/212	75/75
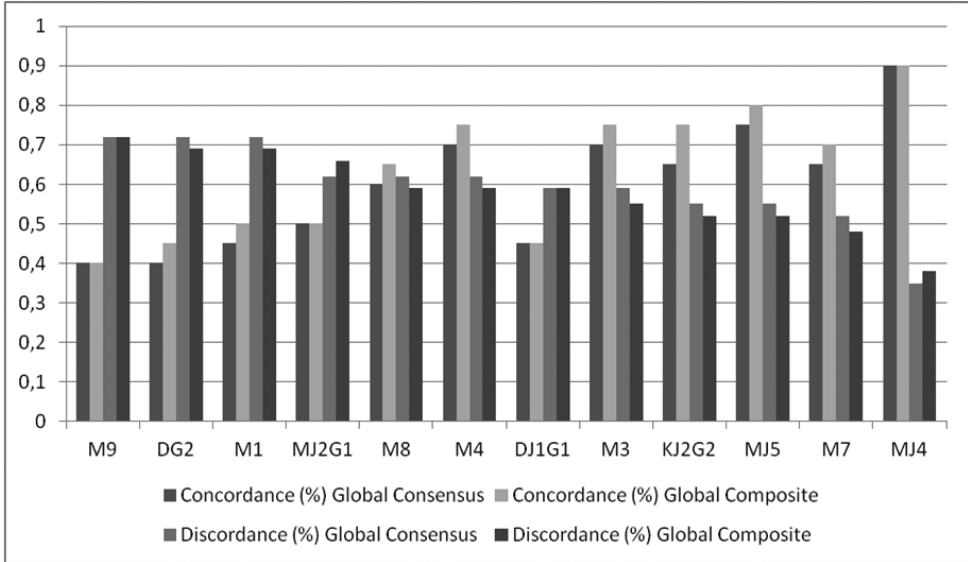
Figure 1. Classical biological model. Biological model results obtained in terms of percentage of concordances and discordances for each suspected male.
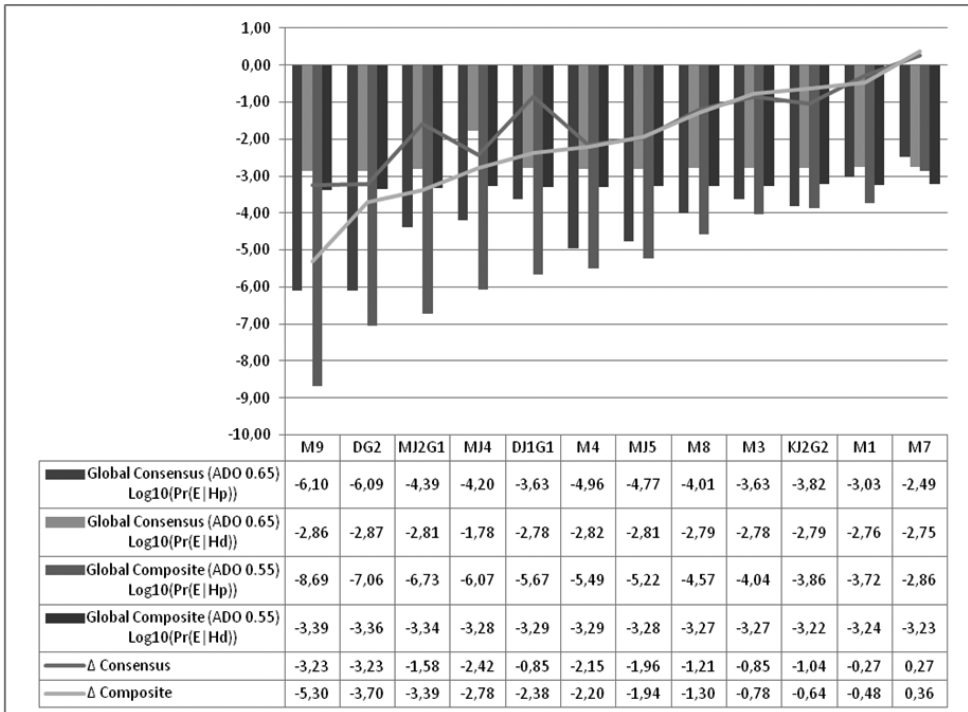

Figure 2. Test for the veracity of the prosecution hypothesis. Likelihood ratio of the prosecution hypothesis (Log10(Pr(E|Hp))) versus the defense hypothesis (Log10(Pr(E|Hd))) for each suspected male. The Δ value gives an idea of the veracity of the hypothesis tested.
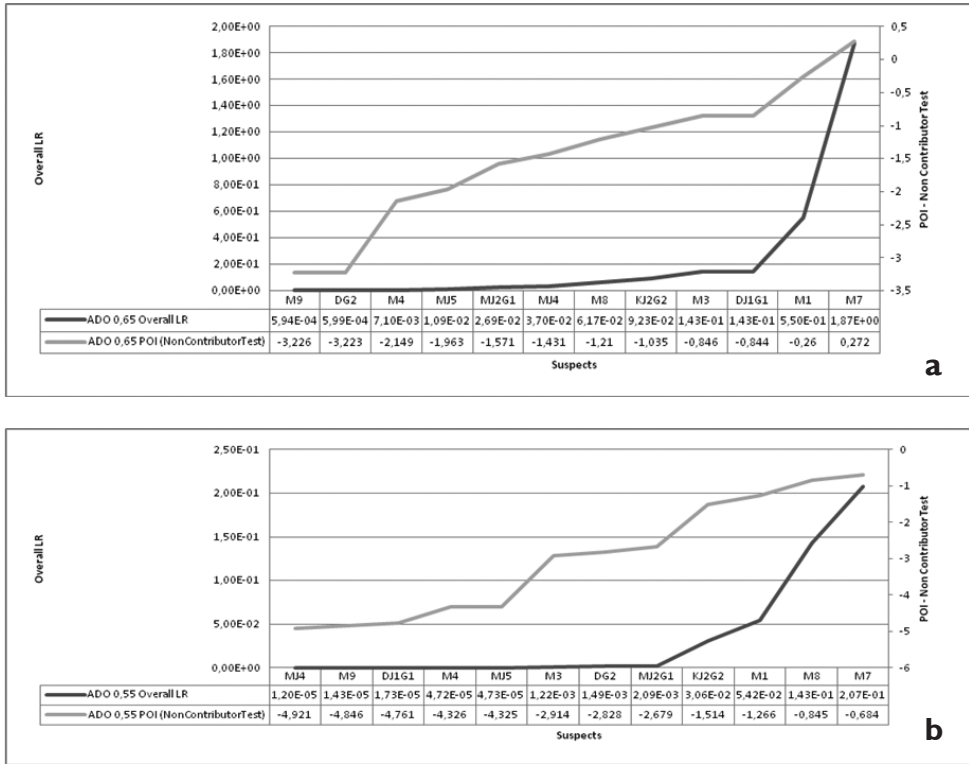
Figure 3. Non-contributor test results in comparison to the Overall LR value for each tested subject. **a** Global Consensus with ADO 0.65 **b** Global Composite with ADO 0.55.

(Figure 3). Allelic components attributable, consistently and coherently, to neither M7 nor any of the victims are absent in the traces. Suppl. materials 2 and 3 describe the detailed results of LRmix STUDIO (SA, Non-contributor test and LR values obtained for each suspect, for single STR locus and for the entire genetic profile – Overall LR).

Discussion

A comparative analysis of the multilocus genotypes of the 12 adult males considered present in the PAT during 2015 and the genetic profiles found in the analysed traces (biological model) has highlighted a variable percentage of discrepancies depending on the suspect, both in the consensus and in the composite profile (from 0.35 to 0.72). In particular, for some subjects (DG2, MJ2G1, M1, M4, M8 and M9), there is clear evidence about the absence of a genotype consistent with the alleles of the suspects on almost all of the STR loci from the traces (percentage of divergent alleles >0.60). These findings are an important support to the hypothesis of exclusion of these subjects as

contributors to the genetic traces found on the swabs. As expected, alleles from MJ4, the father of the killed cubs, have been detected in the biological traces, not because the infanticidal male is the father (which is highly unlikely, Bellemain et al. 2006a-b), but because the swabs were probably contaminated with the cubs' blood, which shares half of their alleles with the father. It is difficult to determine with certainty the dynamics of what happened, but it is clear that the struggle lasted for several minutes, during which time the perpetrator repeatedly attacked both the cubs and the mother. The male, therefore, was contaminated with the blood of both. Samples were collected from the mother's injuries, which were caused by the male during the fight with the cubs, so it is highly probable that the male wounded the female and cubs alternately, leaving traces of the cubs' blood also on the mother. Moreover, comparison between the genetic profile of the suspects and the genetic profiles of the analysed traces has highlighted numerous concordances with the male M7. Actually, with the exception of only six alleles not observed on the swabs (allele 120 on locus cxx20, allele 165 on locus G10P, allele 108 on locus G1D, alleles 118/124 on locus Mu23 and allele 113 on locus Mu59), there is a full correlation between the alleles of the genetic profile of M7 and the alleles of the consensus and the composite genetic profile from the traces. Given that: (i) the traces appear in conditions of LT-DNA and are likely affected by stochastic phenomena (primarily drop-in and drop-out) and therefore are to be evaluated with extreme caution, in agreement with international guidelines (Gill et al. 2007), (ii) the discrepancies found on locus cxx20, that emerge applying the consensus method, can reasonably be due to ADO and (iii) the discrepancies found are anyway subjected to biostatistical evaluation with the probabilistic method of interpretation described above, according to the latest guidelines ISFG (Gill et al. 2012), the results presented indicate that M7 contributed with its biological fluids to the composition of the swabs. The remaining allelic components not attributable to M7 in more than one STR locus, suggest a genetic mixture condition and all of them derive from the blood of the victims (mother and cubs).

LRmix STUDIO (statistical model) estimates the likelihood and the Weight-Of-Evidence by comparing two hypotheses: the accusatory hypothesis (Hp) and the defensive hypothesis (Hd). Each one of the 12 adult males considered in the population during 2015 was tested individually. LRmix STUDIO tests what is the probability that DNA of the suspect contributing to the formation of the traces (DNA mixture extracted from swabs). The alternative hypothesis (Hd), is that the subject did not contribute to the formation of the traces. The calculation of the LR value is the result of the statistical analysis and it gives an estimate of the weight of the two hypotheses that were explored: the subject is present in the traces or the subject is absent. A high LR value indicates that Hp is much more reasonable than Hd; on the contrary, a low LR value indicates that the Hd is preferred. The LR values, obtained from the statistical analysis, range between 5.94×10^{-4} and 1.87 in the case of Global Consensus (Pr(D) = 0.65) and between 1.2×10^{-5} and 2.07×10^{-1} in the case of the Global Composite (Pr(D) = 0.55). The only suspect that obtains values of LR greater than 1 is M7. These values, considered with the results of the Sensitivity Analysis and Non-contributor test (see Suppl. material 2, and Suppl. material 3 for detailed LRmix STUDIO results on

Global Consensus and Global Composite respectively), exclude the hypothesis that the genetic material of MJ4, MJ5, DG2, KJ2G2, MJ2G1, DJ1G1, M1, M3, M4, M8 and M9 is present in the genetic traces, confirming the assessment expressed by applying the biological model and raising the inference that M7 has contributed to the traces with its own biological material.

The use of the statistical model is certainly preferable to the use of the classical model, as it eliminates the bias due to the presence of the father of the killed cubs between the suspected bears. Numerically, the LR value can range between 0 (absolute non-involvement) and +∞ (certain identification) and can express three consequences: (i) LR>1 means "strong support for the hypothesis of identification"; (ii) LR~1 means "neutrality" (the result of the genetic analysis does not allow support for either Hp or Hd, since it has not yielded useful results, i.e. it was inconclusive); (iii) LR<1 means "strong support for the hypothesis of exclusion", in a manner much more accentuated as LR tends to 0; if LR=0, the non-identity between the suspect and the perpetrator can be assured.

Finally, in light of the evaluations expressed, the question can be answered: Who is the perpetrator of the killing of BJ1 and her cubs? The genetic analysis conducted on the four swabs showed the presence of a very small amount of genetic material, resulting from the contribution of more subjects, which led to considering the samples in complex analytical conditions. The genetic typing, carried out with multitube protocol procedure on the traces, allowed the authors to obtain four genetic profiles largely overlapping amongst them and, on the whole, suitable for comparisons. The comparison was carried out for each adult male considered in the bears' population during 2015 and the genetic results were obtained from the traces, interpreting the results on the basis of both the biological model and the statistical model, in accordance with the strictest and updated protocols of interpretation, drawn from international scientific literature. The outcome of this comparison excludes the hypothesis that the genetic material of MJ4, MJ5, DG2, KJ2G2, MJ2G1, DJ1G1, M1, M3, M4, M8 and M9 is present in the analysed traces and that, therefore, these subjects may have contributed to the genetic traces; on the contrary, the overall assessment of the interpretative analysis carried out supports the hypothesis that the genetic material of M7 is present in the traces. Therefore, M7 is probably the killer of M33, F22 and BJ1.

Conclusions

Infanticide occurs in brown bear populations and it is an important cause of mortality, which can affect even the demographic evolution of the population (Gosselin et al. 2015). Similarly to that which was reported by the authors, in Sweden it was found that none of the four infanticidal males that have been genetically identified, was the father of the killed cubs (Bellemain et al. 2006a), likely due to the fact that infanticidal males can distinguish their own cubs from those of others, possibly because they recognise the mother (Bellemain et al. 2006b). Moreover, in Sweden, it has been seen

that, in two of the eight cases of infanticide studied, the male also killed the mother (Bellemain et al. 2006a). Furthermore, in support of SSI theory, in Cantabria (Spain), it has been found that all the confirmed cases of infanticide occurred during the mating season, between April and June (Palomero et al. 2011). In the evolutionary scenario, females of brown bear have developed strategies to limit the consequences of infanticidal behaviour. One of these is sexual promiscuity, as a mechanism to confuse males and generate uncertainty about future paternity, in the brown bears as in other animals (Wolff and Macdonald 2004, Bellemain et al. 2006b). In the Italian alpine population of brown bear, the authors have genetically determined only one case of multipaternity: in 2006, the female MJ2 gave birth to two cubs, one of these being a cub of the then dominant male (Joze) and the other being a cub of the only other adult male, which started to reproduce exactly in 2006 (Gasper). Following Joze's disappearance, Gasper became the dominant male until 2015, the year of his death.

Analogously to what happens in bear populations subject to hunting pressure (LeCount 1987, Wielgus 2002, Wielgus and Bunnell 1994, 2000), in Trentino, the case of a reported infanticide occurred when the dominant male disappeared. Death or disappearance of older adult males frequently coincided with an influx of younger immigrant males, which apparently contributed with SSI to low reproductive rate and population decline (Wielgus and Bunnell 1994). M7, indeed, is a male born in 2009 by female DJ3 and male Gasper. During the juvenile dispersal, M7 moved outside the brown bear core area in Trentino: it was sampled in Lombardia (Bergamo and Sondrio provinces) from 2011 to 2013. In late 2013, it returned to Trentino, where it was sampled in 2014 and 2015, the year of the death of his father, the dominant male Gasper. Around 10 May 2015, the date of discovery of the corpses of BJ1 and her cubs, M7 was sampled near the site of infanticide. M7 probably returned to the core area after years of absence and, to increase its reproductive opportunities, has eliminated the cubs of an adult female. The mother's reaction, however, brought M7 to killing her and failing in his attempt at copulation. There is no evidence to suggest that M7 has reproduced until now.

Future monitoring actions should allow the supervision of the behaviour of infanticidal males (e.g. using radiotelemetry) and, in the case of risk of repeated infanticide, should facilitate suitable conservation actions (e.g. deterrence plans that can include some level of active and passive dissuasion activities). In small and isolated populations, in fact, behaviour that leads to the killing of cubs and adult females could lead to a further decrease in the Ne and a potential reduction in the population growth rate. Wildlife managers should be cautious when dealing with small populations of vulnerable and threatened species. The small populations, in fact, must be studied to understand their dynamics. The monitoring of litters is a fundamental tool for the management of bear populations: it has allowed the authors to genetically confirm the existence of cases of infanticide and in the future may facilitate the retrieval of information necessary to assess the impact of SSI on demographic trends. In the Italian Alps, although infanticide does not seem to be a serious problem and the population seems to be in progressive and continuous growth, it is imperative to continue to gather further information.

Acknowledgements

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Supplementary material 1

Text S1. Parameters used for parentage analysis

Authors: Francesca Davoli, Mario Cozzo, Fabio Angeli, Claudio Groff, Ettore Randi

Data type: statistical analysis

Link. https://doi.org/10.3897/natureconservation.25.23776.suppl1

Supplementary material 2

Text S2. Detailed results of LRmix STUDIO for each suspected male: Global Consensus (ADO 0.65)

Authors: Francesca Davoli, Mario Cozzo, Fabio Angeli, Claudio Groff, Ettore Randi

Data type: statistical analysis

Link. https://doi.org/10.3897/natureconservation.25.23776.suppl2

Supplementary material 3

Text S3. Detailed results of LRmix STUDIO for each suspected male: Global Composite (ADO 0.55)

Authors: Francesca Davoli, Mario Cozzo, Fabio Angeli, Claudio Groff, Ettore Randi

Data type: statistical analysis

Link. https://doi.org/10.3897/natureconservation.25.23776.suppl3

Supplementary material 4

Table S1. Detailed results of the biological model (consensus and composite)

Authors: Francesca Davoli, Mario Cozzo, Fabio Angeli, Claudio Groff, Ettore Randi

Data type:statistical analysis

Link. https://doi.org/10.3897/natureconservation.25.23776.suppl4