A preliminary estimate of the Apennine brown bear population size based on hair-snag sampling and multiple data source mark–recapture Huggins models

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Abstract: Although the brown bear (*Ursus arctos*) population in Abruzzo (central Apennines, Italy) suffered high mortality during the past 30 years and is potentially at high risk of extinction, no formal estimate of its abundance has been attempted. In 2004, the Italian Forest Service and Abruzzo National Park applied DNA-based techniques to hair-snag samples from the Apennine bear population. Even though sampling and theoretical limitations prevented estimating population size from being the objective of these first applications, we extracted the most we could out of the 2004 data to produce the first estimate of population size. To overcome the limitations of the sampling strategies (systematic grid, opportunistic sampling at buckthorn [*Rhamnus alpina*] patches, incidental sampling during other field activities), we used a multiple data-source approach and Huggins closed models implemented in program MARK. To account for model uncertainty, we averaged plausible models using Akaike weights and estimated an unconditional population size of 43 bears (95% CI = 35–67). We urge caution in interpreting these results because other expected but undefined sources of heterogeneity (i.e., gender) may have biased this estimate. The low capture probability obtained through the systematic grid prevented the use of this sampling technique as a stand-alone tool to estimate the Apennine bear population size. Therefore, further applications in this direction will require a substantial improvement of field procedures, the use of a multiple data-source approach, or both. In this perspective, we used Monte Carlo simulations to compare the relative performance of the 3 sampling approaches and discuss their feasibility to overcome the problem of small and sparse DNA data that often prevent reliable capture–mark–recapture applications in small bear populations.

Key words: Abruzzo, brown bear, DNA, hair sampling, Huggins model, mark–recapture, population size, Program MARK, sampling strategies, *Ursus arctos marsicanus*

The small population of the Apennine brown bear (*Ursus arctos marsicanus*) is geographically isolated in the Apennines of central Italy, and its unique conservation value is currently jeopardized by the lack of reliable data on its status, trends, and basic ecology (Ciucci and Boitani 2008). Since the beginning of the last century, its range has been gradually shrinking to the present range, the last remnant of a once larger distribution along the Apennines (Carpaneto and Boitani 2003). Because the Apennine brown bear is considered a distinct conservation unit, based both on genetic (Randi et al. 1994, Lorenzini et al. 2004) and morphological (Vigna Taglianti 2003, Loy et al. 2008) traits, this population represents the only source for the natural

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recovery of the species. Despite its relevant conservation value, however, no reliable estimates of population size are available for the Apennine brown bear (Ciucci and Boitani 2008). During the past 30 years, rough estimates based on miscellaneous and informal approaches depicted 25–100 bears in the core of the range (Posillico et al. 2002). These attempts were mostly based on expert opinion and lacked any statistical inference and quantification of precision. Therefore, especially in the light of the high human-caused mortality during the 1980s (Wilson and Castellucci 2006), formal estimation of population size remains of paramount importance for the conservation of the Apennine brown bear.

Although estimating size and trends of small brown bear populations is considered an essential step for designing effective conservation strategies (<100 bears; Servheen et al. 2000), technical problems arise when dealing with particularly small populations. Brown bears living in mountainous and heavily forested areas are difficult to observe, and their low density, large home ranges, and elusive behavior make estimation of their number technically and logistically difficult (Mills et al. 2000). In addition, small populations (i.e., <100 bears) may yield sample sizes too small and sparse to reliably apply capture–mark–recapture (CMR) models unless capture probabilities are high (Boulanger et al. 2002).

In recent years, hair-snagging has become widely used to obtain individual DNA profiles and produce mark–recapture estimates of population size for brown bears. This technique has been extensively applied in Canada (Woods et al. 1999, Mowat and Strobek 2000, Poole et al. 2001, Boulanger et al. 2004b) and has several potential advantages when compared with traditional methods (McLellan 1989, Mace et al. 1994, Miller et al. 1997). Individual marks can be obtained without physically capturing and handling animals, which not only reduces behavioral responses and the risks of trap-related injuries, but also enables data collection in a single season over a large scale. Potential problems in applying CMR methods to brown bears (e.g., geographic closure, heterogeneity in capture probability) can be more effectively addressed through carefully designed field protocols and recently developed analytical procedures (Boulanger et al. 2006). However, the individual heterogeneity in capture probability expected in hair-snagging bear studies (Woods et al. 1999, Boulanger and McLellan

2001) may still represent a major problem for CMR applications to small populations. CMR models that account for individual variation in capture probability (Chao 1989, Huggins 1991, Norris and Pollock 1996, Pledger 2000) often require large sample sizes, which are difficult to obtain in small bear populations. For these reasons, field protocols with high capture probabilities and a strong reduction of individual heterogeneity are commonly considered fundamental requirements for the application of CMR models to small populations (White et al. 1982, Boulanger et al. 2004c).

Since 2001, the Italian Forest Service (hereafter, CFS) has applied the hair-snag technique to the Apennine bear population (Posillico et al. 2002). Rather than a formal estimation of population size, the main objective of these attempts was the assessment of the minimum number of bears in the population (Lorenzini et al. 2004, Potena et al. 2004, Randi et al. 2006). Sampling design and sampling frames varied from year to year, and both opportunistic collection at buckthorn (*Rhamnus alpina*) aggregation sites and a systematic grid of baited traps were used to collect hair samples. In addition, hair samples were also incidentally collected by Abruzzo Lazio and Molise National Park (hereafter, PNALM) and CFS personnel during normal management and patrolling activities.

In 2004, several institutions participated in sampling bear hair using snags in an attempt to systematically and thoroughly cover most of the current bear range, using standardized field protocols and a robust sampling strategy (Woods et al. 1999). The 2004 systematic sampling had 3 main objectives (Posillico et al. 2004): (a) to determine the minimum number of bears within current range, (b) to refine field and lab remote genetic sampling techniques for Apennine brown bears, and (c) to evaluate hair-snagging rate and efficiency according to a systematic grid design. In the same year, the CFS also collected hair samples at some intensively used buckthorn aggregation sites. Thus, data in 2004 were a composite of hair snagged at baited traps and at known aggregation sites, and incidentally collected throughout the study area.

Due to theoretical limitations of CMR estimators as applied to the (expected) small number of bears as well as their low and variable capture probabilities, we did not originally consider estimation of population size a feasible objective (Posillico et al. 2004). However, given the lack of any previous formal

estimate of the Apennine brown bear population size, coupled with the recent availability of modelling techniques able to handle capture heterogeneity according to individual covariates (Boulanger and McLellan 2001), we used the 2004 composite, hair-snag dataset to produce a statistically based estimate of the population.

We detail the results of the 2004 hair-snag sampling effort and report a first estimate of the Apennine brown bear population using Huggins (1991) closed population model. We also compare systematic with opportunistic sampling designs in estimating the size of small brown bear populations. We discuss implications of sampling requirements for the application of CMR models to small bear populations, and define possible scenarios for further hair-snagging applications to the Apennine brown bear population.

Study area

The 1,462 km^2 study area crosses the Apennines mountain chain in southern-central Italy (41°50′N; 13°54′E) and is centred on the PNALM. It is characterized by high elevations, with many peaks exceeding 2,000 m, and is mainly covered with deciduous forests (56%) of beech (*Fagus sylvatica*) and oak (*Quercus* sp.). Timberline is usually located from 1800 to 1900 m, and high elevation open habitats (grasslands, bare rocks) cover 30% of the area. High elevation grasslands provide an important food source for bears during spring and summer, whereas buckthorn patches are intensively used from late summer to fall. Mean temperature in the coldest month (Jan) is around 0°C and around 16–18°C in the warmest month (Jul). Snowfall usually occurs from late November to March. Rainfall (usually >1000 mm/year) is typically concentrated in spring and autumn. A few small rivers and streams occur in the area, which is mainly limestone and rich in karst. Most roads are located through valley bottoms and mid-elevation plateaus (14% of total area), which are characterized by a mixture of agricultural landscape, settlements, fragmented woodland, and pastures. Livestock breeding is common but not intensively practiced, and some thousands of sheep and cattle are raised in high elevation pastures during summer, also by pastoralists.

Wolf (*Canis lupus*), wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and Abruzzo chamois (*Rupicapra pyrenaica ornata*) also occur throughout the study area. Hunting is banned inside the borders of PNALM, infrastructure development is minimal, and traditional economic activities (livestock raising and forest harvesting) are strictly managed by PNALM regulations. In outer buffer area of the PNALM, bears are common, year-round hunting, mainly of wild boar, is allowed (Zunino and Herrero 1972, Ciucci and Boitani 2008), and development and natural resources exploitation are less rigorously regulated and monitored.
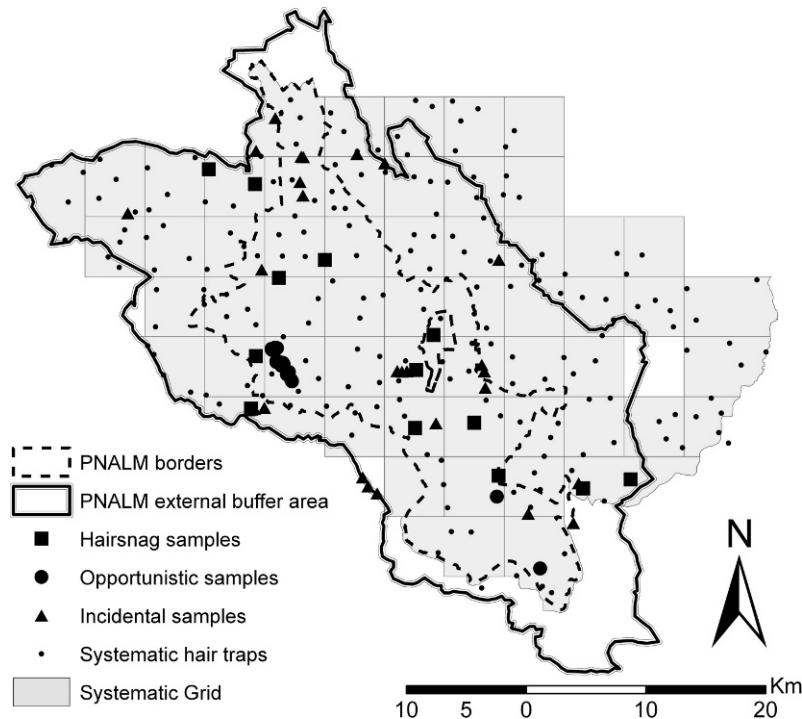
Methods
Study design and field methods

In 2004, field work was carried out by a joint CFS and PNALM team (Potena et al. 2004). Hair-snag sampling was based on a systematic grid. Additional hair samples were collected opportunistically by CFS at feeding aggregation sites and incidentally throughout the study area. Based on mileage, labor costs, and materials used, we computed costs of each sampling strategy; only lab costs are reported for incidental sampling.

Systematic grid sampling. Systematic sampling was carried out between 4 October and 25 November 2004. We activated 219 grid traps during 4 sessions using a sampling grid divided into 56 5 x 5 km cells and covering the whole PNALM, its external buffer area, and other portion of the bear range to the east and northwest, for a total of 1,462 km^2 (Fig. 1). The sampling area coincided with the core of the Apennine brown bear range (Posillico et al. 2002). According to Roth et al. (1994), Eusepi and Latini (2003), and L. Gentile and R. Latini (PNALM Scientific Service, personal communication, 2004), this cell size approximated the home range of 3 Apennine bear radiocollared females. It was also the smallest gridcell size reported in previous hair-snagging applications in Canada (Woods et al. 1999, Mowat and Strobek 2000, Poole et al. 2001, Boulanger et al. 2004b).

Trap sites were moved within the cells at 10-day intervals to ensure that all bears encountered the trap sites. The contours of cells that were placed at the border of the study area were drawn along definite landscape features (i.e., ridges, valley bottoms, rivers) to increase geographical closure of the sampling grid (Mowat and Strobek 2000). Within each cell, field teams identified trap locations through their knowledge of the terrain and using 1:10,000 aerial and 1:25,000 topographic maps;

Fig. 1. Grid borders, trap location, and brown bear hair collection sites of the hair-snag sampling in the Abruzzo Lazio and Molise National Park (fall 2004).

logistical constraints and road accessibility were also considered. Sites with sign of domestic ungulates were rejected because previous experience had shown that livestock easily pull apart barbed wire fences. Suitability of trees at the site was also evaluated to ensure proper trap dimension and bait positioning. For each cell, 1 trap location was selected, baited, and activated for a 10-day session after which it was moved 3 times to a total of 4 different trap sites within the cell during 4 sequential sampling sessions. At the end of each session the team checked the trap, collected hair samples if present, deactivated the trap, and activated a new one ≥1.7 km from any previous trap site.

Traps consisted of a single strand of barbed wire nailed to at least 4 trees approximately 50 cm from the ground, with a bait bucket hung to a tree at about 3 m height in the middle of the trap (Woods et al. 1999). As bait, the team used 15 L of rotten fish and water in equal proportions. During trap inspection, experienced field personnel macroscopically identified and categorized sampled hair into "bear," "other species," and "unidentified species." Samples clearly not belonging to a bear were discarded, whereas all bear and unidentified samples

were collected using disposable gloves and temporarily stored in a paper envelope. Each hair cluster found on a different barb, even if adjacent, was considered a distinct sample. Samples with ≥5 hairs were stored in 20–25 ml 90% ethanol and sent to the laboratory for genetic analysis.

Opportunistic sampling. Between 1 September and 31 October 2004, the CFS collected bear hair samples opportunistically at 2 feeding (buckthorn) aggregation sites in the western and southern part of the study area. Buckthorn hair traps consisted of a single barbed wire about 50 cm from the ground and completely surrounding buckthorn shrubs. About 2–4 buckthorn shrubs were wired in each aggregation site. Buckthorn traps were simultaneously activated at the beginning of the sampling period (when bears were expected to begin using *Rhamnus* following ripening) and were checked but not moved every 10–15 days. Categorization and storage of samples followed procedures described for systematic sampling.

Incidental sampling. Between 11 September and 9 November 2004, experienced CFS and PNALM personnel collected bear hair samples incidentally to other patrolling activities, along fixed trails or during

verification of depredations or damage by bears. Incidental hair-snag samples were collected in 6 fenced cultivations (total area = 2.1 ha) maintained by the CFS as a source of supplemental feeding, 2 of which were inside the PNALM.

Genetic analyses. Samples were analyzed at the conservation genetics lab of the Italian Wildlife Institute (INFS). DNA was extracted using a guanidine thiocyanate/silica protocol (after Gerloff et al. 1995) and genotyped by PCR-amplification of 9 microsatellites: Mu05, Mu11, Mu15, Mu50, Mu51, Mu59, G10B, G10C, G10L (Taberlet et al. 1997, Paetkau et al. 1998; Bellemain and Taberlet 2004), and the Amelogenin locus (AMG) to assess gender, using a multiple tube procedure.

Individual genotypes were determined using an ABI 3130 automated sequencer and software GENE-MAPPER V.3.0 (Applied Biosystems, Foster City, California, USA). Each locus in each sample was amplified twice, and all samples which produced $\leq 50\%$ positive PCR were discarded (a positive PCR should produce a detectable amplification of at least 1 allele at the typed locus). Remaining samples were further processed to obtain a total of 4 PCR replicates per locus. All multilocus genotypes were analyzed with software RELIOTYPE (Miller et al. 2002), which estimates the maximum-likelihood of each genotype in the sampled population (reliability score R) and eventually indicates a number of additional replications at loci that are not reliable enough. Samples were accepted as reliable if $R \geq 0.95$. Consensus genotypes were determined using GIM-LET V.133 (Valière 2002), with the threshold method in which alleles were retained as consensus genotypes if they scored at least two times.

The consensus genotypes were used as true reference genotypes to compute the frequency of allelic drop-out and false alleles. The multiple-tube procedure was repeated in samples showing only 1 or 2 mismatches. The screening step led us to discard 27% of the hair samples; 81% of the selected samples obtained reliability scores $R \geq 0.95$. In these samples allelic drop-out was 0.070, and false alleles were 0.012, on average across loci and samples. The microsatellite panel produced cumulative values of probability of identity $PID_{unb} = 0.0002$, and $PID_{sib} = 0.0125$. Hair samples from uncertain species were diagnosed by sequencing a fragment of about 250 nucleotides of the mitochondrial DNA control-region (Randi et al. 1994). DNA extractions and amplifications were done in separate rooms. PCRs were done in a room dedicated to non-invasive DNA, working under a sterile air-flow hood, which was regularly cleaned with ultraviolet light.

Population estimation

To account for heterogeneity in capture probabilities, we separately treated the 3 data sources corresponding to the different sampling designs. Whereas incidental (sessions 1–5) and opportunistic (sessions 6–8) samples were sorted according to 5 15-day sessions encompassing the whole 10-week sampling period, bear captures from the systematic grid sampling (sessions 9–12) were tallied according to the 4 10-day original sessions. For each sampling design we assigned each bear capture to a specific session according to the date of hair collection. In total, we obtained 12 sessions because opportunistic samples were collected for only 3 sessions.

We used the Huggins closed population model (Huggins 1991) incorporated in Program MARK (White and Burnham 1999) to estimate the size of the Apennine brown bear population. The Huggins estimator allows capture probability to be a function of individual covariates, producing more robust estimates than other closed population models when heterogeneity of capture probability can be linked to some individual continuous variable (Boulanger and McLellan 2001). In our case, sampling effort was particularly intensive and localized where opportunistic sampling was allowed by the highly clustered buckthorn feeding aggregations, but was less intense across the sampling grid (systematic sampling). As a consequence, we expected a strong decrease in capture probability with increasing distance from the buckthorn sampling areas, therefore creating heterogeneity variation with effects stronger than those of factors commonly revealed in brown bear hair-snagging projects (sex, age, previous capture events, etc; Woods et al. 1999, Boulanger et al. 2004b, Boulanger et al. 2006, 2008). Therefore, we considered distance from buckthorn patches as an individual covariate and measured it for each genotyped bear as the linear distance between the mean location of its hair-snag sites and the closest buckthorn patch. We then used linear and quadratic functions in program MARK to model spatial heterogeneity in capture probability using distance as a covariate, standardized by the mean and standard deviation of the observed distances (White et al. 2001). In addition, to account for differences in capture probability between the 3 sampling ap-

proaches, we built models with unique capture probabilities for each of the different data sources. An assumption of using multiple data sources (in our case sampling sessions and designs) is that they are independent, as this will translate in minimal bias with the Huggins estimator (Boulanger et al. 2007). In our case, independence among data sets could be an issue for sessions conducted simultaneously, and estimating covariance between data types is problematic with our sparse data set. However, we believe correlation in capture probabilities of bears among data types to be minimal, if any, due to the different sampling method employed.

We evaluated model fit using the Akaike Information Criterion adjusted for low sample sizes (AIC_c). The model with the lowest AIC_c was considered to be the most supported by the data (Burnham and Anderson 1998). Changes in AIC_c (ΔAIC_c) were also used to assess the fit of different models, and all models with ΔAIC_c <2 were considered for population estimation. To account for model uncertainty, we also estimated population size and the associated variance using the model averaging procedure in MARK. Confidence intervals in MARK do not account for the minimum number of bears on the sampling area, and therefore we computed log-based corrected CIs using the unconditional SE from model averaged estimates (White et al. 2001).

We considered the assumption of demographic closure to be reasonable because we collected hair-samples within reasonably short time (i.e., about 3 months), and because bear mortality rates are generally low (McLellan et al. 1999). In addition, we considered geographic closure to be a minor source of bias in our estimation because our study area appears very clearly demarcated by topography and habitat types, and bear presence outside the area is rarely reported. Unfortunately, no radiocollars were deployed on bears during the 2004 hair-sampling period so we could not quantitatively account for closure violation using telemetry data (White and Shenk 2001, Boulanger et al. 2004b). However, geographic closure seems to be supported by GPS (global positioning system) telemetry data collected from up to 15 adult Apennine bears from 2005 to 2007 (Ciucci et al., unpublished data).

Simulated relative performance of different sampling designs

To compare sampling designs for collecting hair samples to estimate population size, we used Monte Carlo simulations under conditions expected for our bear population. In particular, we aimed to assess if the composite data source provided better accuracy and precision than a 'buckthorn only' or a 'hair-snag only' design, and evaluated how the performance of a sampling design varied according to a range of expected values of capture probability. In the simulations, we did not consider incidental sampling due to the unpredictability in sampling effort.

Rather than assessing the absolute bias of population estimates, we aimed to support the hypothesis that when remote genetic datasets are too small or sparse to be a stand-alone tool for population estimation, joint use of DNA data collected under different sampling strategies may improve the performance of CMR models. Simulated encounter histories were generated (SAS Institute Inc. 1989) with a population size of 43 bears, average capture probability of 0.1–0.3, and a uniform distribution of bears across the study area.

To simulate heterogeneity in capture probability due to gender, which has been previously reported in other hair-snagging projects on brown bears (Woods et al. 1999, Boulanger and McLellan 2001), we used a distribution of 2 finite mixtures with different capture probability. Capture probability values for each mixture were defined using a $CV(\theta) = 0.2$, where θ is the average capture probability of each finite mixture (Carothers 1973, Boulanger et al. 2002). A random additive factor, ranging from -0.1 to 0.1, was added to simulate individual heterogeneity. With the exception of the gender effect, which we could not model in the original data due to limited sample size, the above simulation parameters were derived from the results of our Huggins model population estimation because these represented the most plausible approximation of population size and variation of capture probability currently available for the Apennine brown bear population. Capture probabilities were simulated on the logit-scale using parameter estimates from the Huggins analysis. Based on these parameters, 3 designs were implemented using the simulation model: an opportunistic sampling, in which capture probability was a function of the individual distance from buckthorn patches; a systematic hair-snag sampling, based on an intercept-only model with constant capture probability; and a multiple data-source design, which combined the first two. We ran 1,000 iterations for each trial and used percent relative bias, CI coverage, and coefficient of variation of

Table 1. Hair-snag sampling of the Apennine brown bear population according to the 3 sampling designs (Abruzzo Lazio and Molise National Park, 1 Sep–25 Nov 2004). Cells labelled ''n/e'' were not estimated or not pertinent. Cost figures used a rate of 1.5 €/$.

	Systematic grid	*Rhamnus* patches	Incidental
Samples collected	61	228	54
Samples analyzed	52	198	54
Bear samples (mtDNA)	24	198	44
Samples from other species	22	2	3
Unidentified samples	6	28	7
Identified bear samples[a]	19	150	40
Positive cells (%)	23%	n/e	n/e
Positive traps (%)	6%	52%	n/e
Individual bear genotypes	11	16	9
Female:male	10:1	12:4	7:2
Mean genotypes per session	2.5[b]	11.3[c]	2.8[c]
Bear samples used in CMR analysis[d]	12	35	9
Field costs[e] ($)	34,500	52,350	n/e
Lab costs (at $150/sample)	7,800	29,700	8,100
Total cost/sample ($)	813	414	150
Total cost ($)/CMR used genotype	3,525	2,345	900

[a]9 microsatellite loci
[b]According to the original 10-day sessions
[c]According to the 15-day sessions used for the analyses
[d]Recaptures of the same genotype within the same session were discarded in CMR analyses
[e]Converted from euros at 1.5 €/$; includes salary, materials, and mileage

population estimates to evaluate the relative performance of each design.

Results
Hair collection and field procedures

Of 61 collected samples, 52 were macroscopically categorized as bear or undetermined hair sample. Of these, 24 were mtDNA confirmed as bear samples, and 28 were either attributed to other species or DNA extraction failed (Table 1). In total, 23% of cells and 6% of traps were positive for bear hair samples (Table 1), with an overall hair-snagging success rate of 0.11 bear sample/trap (Table 2). From the 24 bear samples, 19 provided reliable genotypes belonging to 11 individuals (10F, 1M; Table 3). Individual genotypes were recaptured up to 4 times during the sampling period, and 37% of recaptures could not be used in our CMR analysis because they occurred within the same sampling session (Table 1).

We obtained 198 bear hair samples from 42 traps in buckthorn patches for opportunistic sampling. In total, 52% of these traps were positive for bear hair samples (Table 1), with an overall hair-snagging success rate of 3.57 bear sample/trap. DNA extraction failed for 48 of these samples. The remaining 150 were successfully genotyped, identifying 16

different bears (12F, 4M). Individual genotypes were recaptured up to 39 times during the sampling period, but 70% of recaptures could not be used in CMR analysis because they occurred within the same sampling session (Table 1).

Hair collected incidentally provided 54 bear samples, 40 of which were successfully analyzed and attributed to 9 different genotypes (7F, 2M). Individual genotypes were recaptured up to 11 times during the sampling period, and 23% of recaptures could not be used in our CMR analysis because they occurred within the same sampling session (Table 1).

Overall, we identified 30 individual bears during the sampling period (Table 3). No 9-locus genotypes matched at all loci; but 1, 20, and 44 similar pairs of genotypes matched at all but 1, 2, or 3 markers, respectively. Overall, we estimated a sex ratio of 3.2:1 (F:M), and, although it varied by sampling approach, was always female biased (Table 1). The majority (40%) of these genotypes were sampled only at buckthorn patches, whereas 20% were sampled exclusively through systematic sampling, 20% through incidental sampling, and 20% from combining 2 sampling approaches (Table 2). Excluding genotypes recaptured within the same session (Table 1), from 1 to 2 genotypes were recaptured once using systematic or incidental sampling, whereas 5–7 genotypes were recaptured

Table 2. Statistics for DNA-based mark–recapture projects in Canada and for grid based hair-snagging in the Abruzzo Lazio and Molise National Park, Italy, fall 2004.

Study area (Reference)	Grid area (km²)	Cell size (km²)	Cells	Sessions	Traps	Bear samples/trap[a]	Bear genotypes	Genotypes/trap[a]	$\hat{N}$	$\hat{P}$
Abruzzo Lazio and Molise National Park (this work)	1,462	25	56	4	219	0.11	11	0.05	44	0.03
Jumbo Pass (Strom et al. 1999)	1,650	25	66	4[b]	264	–	33	–	45	0.26
British Columbia (UCR[c] 1996) (Woods et al. 1999, Boulanger et al. 2004b)	4,096	64	64	4	256	1.58	55	0.21	108	0.16
British Columbia (Mowat and Strobeck 2000)	9,866	64	76	5	381	1.60	109	0.29	262	0.10
Alberta (Mowat and Strobeck 2000)	5,030	64	73	4	321	0.52	37	0.12	74	0.13
British Columbia (Poole et al. 2001)	8,527	81	103	5	515	1.06	98	0.19	138	0.17
British Columbia (UCR[c] 1997) (Boulanger et al. 2004b)	1,900	25	76	5[b]	380	–	40	0.11	55	0.20
British Columbia (UCR[c] 1998) (Boulanger et al. 2004b)	2,350	25	94	5[b]	470	–	40	–	92	0.12
Alberta (Unit 4) (Boulanger et al. 2007)	8,477	49	173	4	692	–	41	–	47	0.52
Alberta (Unit 3) (Boulanger et al. 2007)	8,820	49	180	4	720	–	39	–	53	0.33
Alberta (Unit 5) (Boulanger et al. 2006)	7,369	49	172	4	688	–	85	0.06	133	0.25

[a] For North American studies, using bear samples/trap to compare projects is compromised by presence of American black bear (*Ursus americanus*) samples and if they were subsampled for genetic censoring. Capture probability should be used in these cases to compare projects.

[b] Trap sites were not moved between sampling sessions

[c] Upper Columbia River

1–2 times using opportunistic sampling (Fig. 2). Merging hair samples from the sampling approaches, 43.3% of the 30 genotypes were trapped only once, 26.7% twice, 23.3% 3 times and 6.7% 4 times during the sampling period (Table 3).

Considering both field and laboratory costs, we expended €88,300 on the 2004 hair-snagging application to the Apennine bear population, with subcomponents varying from € 5,400 for incidental to €54,700 for opportunistic sampling approaches (Table 1). Weighted on a single, CMR usable-bear genotype-basis, systematic sampling was the most expensive (€2,350/genotype), and incidental sampling the least (€600/genotype) expensive (Table 1).
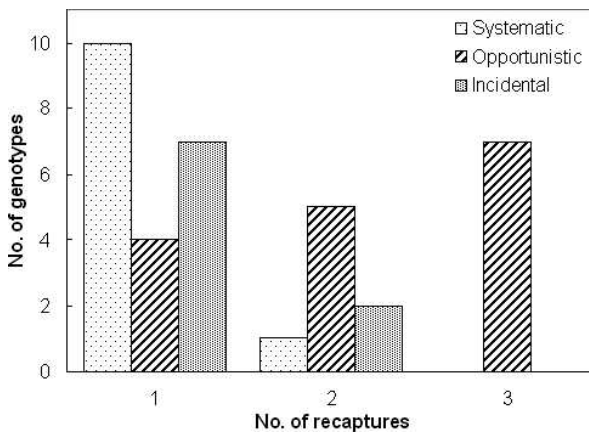
Population estimation

We first investigated capture probability variation according to sampling design and found a noticeable increase in capture probability for sampling in buckthorn areas (opportunistic sessions 6, 7, and 8). Therefore, to contrast opportunistic and other sampling designs, we used session as a covariate to model capture probability variation by assigning a unique estimate of capture probability to buckthorn sessions and another estimate to all remaining sessions. In addition, to account for differences in capture probability between the incidental and systematic sampling designs, we built models where each sampling design was assigned a single estimate of capture probability. The 2 most supported models both contained an effect of the linear distance of mean bear capture location from the closest buckthorn trapping area for the opportunistic sampling design only, and an additive effect of sampling design (models 1 and 2, Table 4). Population estimates from these models were 44 (95% CI = 33–66) and 42 bears (95% CI = 34–69) respectively. Less supported models (models 3–8, Table 4) implied a less plausible effect of the distance from the buckthorn areas on capture probability for all sampling designs. We obtained a model averaged population estimate of 43 bears (95% CI = 35–67 bears; Table 4).

Estimates of capture probability from the most supported model showed a rapid decrease as the distance from the closest buckthorn patch increased (Fig. 3). Under this model, capture probability at buckthorn aggregations was ≥0.5 for bears sampled at ≤1 km from the buckthorn patch, but it approached zero for distances >6 km. From the same model, capture probabilities for the systematic and incidental sampling were not influenced by the

Table 3. Bear genotypes identified through hair-snagging based on the 3 sampling designs and number of recaptures useful for mark–recapture population estimation. Recaptures (in parentheses) within the same session were not used for mark–recapture analysis. Genotypes are ranked according to the overall recapture rate useful for mark–recapture population estimation (Abruzzo Lazio and Molise National Park, fall 2004).

Genotype	Sex	Systematic		Opportunistic		Incidental		Overall
		Number of captures (recaptures)						
19	F	1	(2)	3	(21)	–	–	4
44	F	1	(2)	3	(7)	–	–	4
10	M	–	–	3	(5)	–	–	3
12	F	1	(1)	2	(13)	–	–	3
18	F	1	(1)	–	–	2	(7)	3
31	F	–	–	3	(14)	–	–	3
38	F	–	–	3	(39)	–	–	3
50	F	–	–	3	(7)	–	–	3
51	M	–	–	3	(12)	–	–	3
25	F	–	–	2	(5)	–	–	2
32	F	–	–	2	(6)	–	–	2
34	F	2	(4)	–	–	–	–	2
36	F	–	–	–	–	2	(7)	2
41	F	–	–	2	(15)	–	–	2
43	F	1	(1)	–	–	1	(4)	2
49	M	–	–	2	(2)	–	–	2
55	F	–	–	1	(1)	1	(1)	2
2	F	1	(2)	–	–	–	–	1
7	F	–	–	–	–	1	(11)	1
11	M	–	–	–	–	1	(3)	1
23	F	1	(1)	–	–	–	–	1
33	F	1	(2)	–	–	–	–	1
37	F	1	(1)	–	–	–	–	1
45	M	1	(2)	–	–	–	–	1
46	M	–	–	–	–	1	(5)	1
47	F	–	–	1	(1)	–	–	1
48	F	–	–	–	–	1	(1)	1
52	M	–	–	1	(1)	–	–	1
53	F	–	–	1	(1)	–	–	1
54	F	–	–	–	–	1	(1)	1

Fig. 2. Recaptures of the 30 hair-snagged bear genotypes according to 3 sampling approaches (Abruzzo Lazio and Molise National Park, fall 2004). Individual recaptures not used for mark–recapture estimates (within the same session) were not included.

distance of hair-collection from the buckthorn trapping areas (Fig. 3), with estimates of 0.03 (95% CI = 0.006–0.149) and 0.11 (95% CI = 0.083–0.158), respectively.

Simulated relative performance of different sampling designs

Choice of sampling design had a large effect on accuracy and precision of population estimates. At low average capture probabilities ($\hat{P} = 0.1$, such as in our conditions), opportunistic sampling at buckthorn patches performed poorly if used alone, with 29.6% relative bias and CV = 67.6% (Table 5). Model failure rate was also high, with 23.5% of estimates >2 times higher than the actual population size, with extremely high SE values. These estimates strongly affected the average statistics of the simulations, and were therefore removed from the analyses. Under this minimal capture probability

Table 4. Results of the Huggins model selection to estimate size of the Apennine brown bear population using hair-snagging and DNA-based data (fall 2004).

Model	Capture probability	AIC_c	Parameters	ΔAIC_c	w_i	$\hat{N}$	95% CI
1	Design[a]+dist$_{opp}$[b]	298.96	4	0	0.5660	44	36–66
2	Design$_{opp}$[c]+dist$_{opp}$+dist$_{inc+sys}$	299.49	4	0.53	0.4336	42	34–69
3	Design$_{opp}$+dist$_{all}$	314.82	3	15.86	0.0002	33	30–43
4	Design$_{opp}$+dist$_{all}$+dist$_{all}^2$	316.72	4	17.76	0.0001	32	30–44
5	Design[a]	316.77	3	17.81	0.0001	31	30–40
6	dist$_{all}$	321.69	2	22.73	0.0000	38	32–57
7	constant	324.88	1	25.92	0.0000	35	32–45
8	dist$_{all}^2$	326.15	2	27.19	0.0000	36	32–47

[a]Capture probability variation was modeled as a function of the 3 sampling designs. A unique value of $\hat{P}$ was assigned to sessions 1–5 (incidental sampling), 6–8 (opportunistic sampling), and 9–12 (systematic sampling).
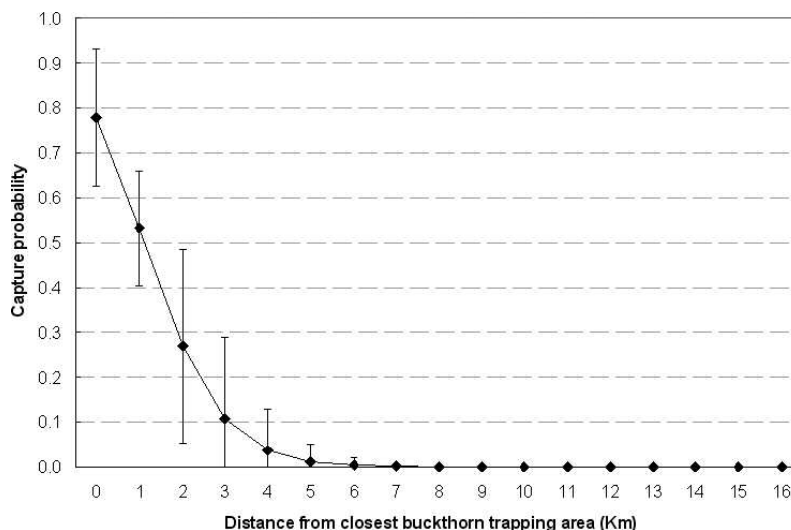
[b]The individual covariate was calculated as the distance (dist) of mean bear capture location from the buckthorn trapping area. It was modeled as a linear or quadratic function (*opp*: opportunistic sampling only; *inc*: incidental sampling only; *sys*: systematic sampling only; *all*: systematic, opportunistic, and incidental sampling).

[c]Capture probability variation was modeled as a function of opportunistic versus other sampling designs. A unique value of $\hat{P}$ was assigned to sessions 6, 7, and 8, during which samples were opportunistically collected at buckthorn sites, whereas another value was assigned to other sampling sessions (incidental and systematic sampling).

scenario, the systematic hair-snagging design performed better, with reduced bias, increased precision, and no simulation failures (Table 5). A further improvement was obtained by combining the 2 designs (as used in our study), with lower bias and higher precision than the other 2 designs (Table 5). At higher values of capture probability (i.e., $0.25 \leq \hat{P} \leq 0.3$), the systematic grid and the combined sampling design performed similarly, whereas the buckthorn-only design still suffered from low precision and high failure rate (Table 5).

Discussion

The application of CMR models to hair-snag based DNA data is a promising and powerful tool to estimate bear population size. However, reliable application of this method requires sampling strategies that reflect statistical assumptions and model requirements (Boulanger et al. 2002). The systematic collection of hair samples through a grid of hair traps across the entire study area appears optimal for applications aimed at estimating population size, because it spreads capture effort throughout the

Fig. 3. Estimated DNA-based capture probabilities (±SE) of Apennine brown bears as a function of the distance of each genotype mean sampling location (hair snag) from the closest buckthorn trapping area. Estimates refer to the most supported model (Model 1, Table 4), according to which capture probability for the incidental and systematic sampling (not shown) are not affected by the distance from buckthorn patches.

Table 5. Results of Monte Carlo simulations using a logistic regression model and according to 3 sampling designs for DNA-based mark–recapture population estimation of the Apennine bear population. Simulations (1,000 iterations) were based on sex and individual heterogeneity in capture probability using 2 finite mixtures with different capture probabilities and a true population of 43 bears.

P	Sampling design	N	SE	Bias (%)	CV (%)	CI coverage	Model failure[a] (%)
0.10	Hair-snag only	43.49	24.63	−1.15	56.63	79.5	16.7
0.15		45.54	16.91	3.50	37.12	91.7	5.5
0.20		44.20	10.43	0.46	23.59	94.5	1.0
0.25		44.18	7.57	0.40	17.13	94.6	0.0
0.30		43.63	5.52	0.84	12.65	93.9	0.0
0.10	Buckthorn only	30.94	20.92	−29.69	67.62	49.4	22.5
0.15		32.39	21.29	−26.39	65.75	51.2	21.6
0.20		33.16	20.20	−24.63	60.91	52.6	20.2
0.25		34.03	20.51	−22.66	60.26	53.6	19.7
0.30		33.95	19.10	−22.85	56.58	54.7	19.1
0.10	Joint data-source (hair-snag +	43.09	8.79	−2.07	20.40	92.6	0.6
0.15	buckthorn)	43.78	6.38	−0.50	14.57	93.4	0.1
0.20		43.13	4.53	−1.98	10.50	93.4	0.0
0.25		43.20	3.51	−1.82	8.13	93.9	0.0
0.30		43.31	2.83	−1.57	6.53	93.8	0.0

[a]Model failure was defined as a population estimate of >2 times the actual population size.

whole population, reduces capture heterogeneity, and accounts for closure violation better than alternative sampling approaches.

In small bear populations, however, systematic hair-snag sampling may result in small and inconclusive datasets, especially if field procedures are not efficient. In our case, the systematic sampling effort was particularly intensive: we used a relatively small cell size and 4 sampling sessions with trap sites moved between sessions. Nevertheless, trapping success was low (0.11 bear samples/trap) compared with other hair-snagging projects conducted on North American brown bear populations at densities similar to the Apennine bear population (range: 0.51–1.60 bear samples/trap; Mowat and Strobek 2000, Boulanger et al. 2006).

One potential reason for the lower systematic hair-snagging efficiency we obtained in our study could be that our sampling occurred in the fall rather than spring when sampling was conducted in North American projects and when bears shed hair more easily and are more likely to respond to scent lures (M. Proctor, Department of Biological Sciences, University of Alberta, Edmonton, Canada, personal communication, 2007). Lower efficiency of the systematic grid, in turn, resulted in lower capture probabilities ($\hat{P} = 0.03$) than those reported in analogous applications to similarly sized brown bear populations (Table 2; see Boulanger et al. 2002 for a review). The mean capture probability we obtained by systematic hair-snagging was well below the $\hat{P} >$

0.3 criterion suggested by CAPTURE as the minimum needed to properly detect heterogeneity and estimate size of populations <100 individuals (White et al. 1982), and below the $\hat{P} > 0.2$ suggested under more relaxed sample size constraints using program MARK (M. Proctor, University of Alberta, Edmonton, Alberta, Canada, personal communication, 2007). Whereas our outcomes may result from the pioneering nature of the 2004 systematic hair-snagging effort, they also underline how relevant fine-scale improvements in field procedures are for application of DNA-based CMR models to small bear populations. Bait type, trap site selection, trapping effort and season, trap structure as well as the expertise of field personnel should all be optimized to maximize collection of hair samples (Mowat and Strobek 2000). In this perspective, it might prove particularly important to evaluate the feasibility of the technique through an ad hoc pilot study and simulation work.

Although more problematic on theoretical grounds, opportunistic hair-snagging may prove more efficient than systematic sampling in small bear populations. In this study, sampling at buckthorn patches provided most of the hair samples collected and bear genotypes identified. Similar to studies which adopted analogous sampling approaches (e.g., grizzly bear aggregations at salmon [*Oncorhynchus*] escapement sites in North America: Boulanger et al. 2004a, LaVern et al. 2005), buckthorn areas represented a favorable sampling

opportunity for the Apennine brown bear population. Higher capture rates also corresponded to a higher redundancy of information and proportionally higher lab costs, as 77% of the genotypes were recaptured within the same sampling session and thus could not be used in our CMR analyses. However, if based on the number of genotypes used for CMR analysis, total costs of opportunistic sampling were lower than those of systematic sampling because the former provided a higher number of usable captures. In such a situation, an alternative analytical option could be represented by single session models (Miller et al. 2005, Petit and Valiere 2006, Puechmaille and Petit 2007) that allow pooling the entire dataset in a single session and eliminate the need to remove duplicate genotypes observed during a session. We did not consider this approach because single session models do not allow explicit modeling of heterogeneity arising from 3 sampling designs, which was also the main source of variation in capture probability.

On the other hand, opportunistic sampling is flawed by theoretical limitations which prevent it being applied as a stand-alone sampling strategy for population estimation. Among others, a basic CMR assumption is that all individuals in the population should have a non-zero probability of being captured (Pollock and Otto 1983), but our modeling showed that this was not the case for buckthorn sampled bears in the peripheral part of the study area, or as distance from buckthorn patches increased. The relationship between capture probability and distance from buckthorn patches revealed the high heterogeneity in capture probability associated with this sampling approach; its negative effects on CMR performance were further highlighted by contrasting the sampling designs through simulations. According to the most plausible population size and mean capture probability for the Apennine brown bear, our simulations suggest that estimates of population size were negatively biased and more imprecise when based on opportunistic samples alone. In addition to distance from buckthorn areas, configuration of bear home ranges, individual habitat use patterns, and social interactions may affect the degree to which *Rhamnus* patches are frequented by individual bears in late summer, therefore representing additional and undetected sources of heterogeneity if opportunistic samples were used for CMR models.

Finally, another potential problem of hair samples collected at buckthorn aggregation sites is the risk of obtaining mixed samples (the chance that 2 or more individuals leave hair tufts on the same barbed wire). Mixed samples are usually identified and removed by the amplification of more than 2 alleles at one or more loci (Alpers et al. 2003), but a non-trivial percent of them might resemble unique, legitimate genotypes, especially for populations with high levels of allele sharing (Roon et al. 2005) or when allelic dropout is undetected (Taberlet et al. 1996). In such cases, collection of mixed samples could be minimized by using ad hoc field techniques (single-catch snares; LaVern et al. 2005) or by frequently checking trap sites. Potential effects of mixed samples on the performance of CMR models can be assessed through simulations (Roon et al. 2005).

Hair samples collected incidentally by CFS and PNALM personnel during other field activities also contributed to the 2004 DNA dataset. They provided a comparable amount of information (unique bear genotypes) and relatively more samples than the systematic grid sampling without requiring additional field effort. However, as with opportunistic samples, redundancy of information (total number of recaptures per genotype per session) was higher for incidental than for systematic sampling, and corresponded to higher lab costs. In addition, although sources of heterogeneity as extreme as those associated with opportunistic sampling should not be expected, collection of incidental samples may be biased by area, habitat, or bear behavior. Patrolling surveys, damage verification, and other field activities by Forestry and PNALM personnel occurred most likely and most often in areas closer or more accessible to humans, and the incidental collection of hair samples cannot be considered random.

Notwithstanding the many problems associated with opportunistic and incidental sampling of DNA genotypes for population estimation, heterogeneity in capture probability can be modeled in a way that may allow careful use of such data in combination with other data sources (Boulanger et al. 2007, this study), especially with small bear populations, whose systematic hair-snag sampling might not reliably support CMR models. Most notably, with multiple data sources bear capture probability can be zero for one of the data types as long as it is above zero for at least one of the other data types, allowing the use of all data types in one analysis. Considering each data

source in a separate session improves the power of detecting heterogeneity in capture probability among different sampling strategies, as well as overall robustness of population estimates (Boulanger et al. 2007). However, the temporal sequence of individual captures is not a requisite of CMR closed models that do not model behavioral response. Behavioral response is possible if trapping sites are not moved between sampling sessions; however, multiple studies have shown that if sites are moved then there will be minimal behavioral response (e.g., Boulanger et al. 2002, 2006).

Simulations tailored to the Apennine bear population confirmed the important contribution of multiple data sources, especially when sampling conditions and encounter rates cause particularly low capture probabilities. In this case, the joint use of opportunistic and sampling datasets substantially reduced the relative bias of population size estimates while improving their precision. However, the advantage of joint data sources became less evident with increasing capture probability, and for capture probabilities >0.2, accuracy and precision of population estimates stabilized at values similar to those obtained using the systematic data type alone. On the other hand, the opportunistic design alone displayed a lower performance even at medium to high capture probabilities, supporting the argument that, regardless of sample size, it cannot be considered a stand-alone sampling strategy for population estimation when non-zero capture probabilities cannot be assumed for all bears in the population.

One restriction of the joint data source approach is that data obtained through different sampling designs should not be correlated, as this would reduce the performance of Lincoln-Petersen, Huggins, and Pledger estimators (Boulanger et al. 2007). In this study, due to their limited sample size, we could not check for correlation among opportunistic, incidental, and systematic data sources. However, we believe that correlation among the datasets, if any, was minimal due to neutral behavioral responses expected by bears sampled at buckthorn patches and hair-snagged in baited traps, or vice versa; accordingly, observed distribution of genotypes and their number of recaptures did not match among sampling strategies (Table 3).

The main advantage of the Huggins model over other estimators (Otis et al. 1978, Norris and Pollock 1996, Pledger 2000) is that population size is estimated as a derived parameter, therefore allowing the use of individual covariates to model variation in capture probability (Huggins 1991). These covariates may range from the distance of capture location from the edge of a sampling grid (Boulanger and McLellan 2001) to sampling methods and operators (Collier et al. 2007), sex and accessibility of trapping sites (Bowden et al. 2003), and previous capture events (Boulanger et al. 2008). In our case, using the distance from the closest buckthorn trapping area as an individual covariate in a multiple data source context allowed us to reduce the positive bias caused by undetected heterogeneity on the estimates of capture probability for the 3 designs, thus also removing a relevant source of negative bias in the estimation of population size. This is evident when noting that population estimates resulting from the 2 most supported models (models 1 and 2 in Table 4), are around 30% higher than those of all other models (models 3–8 in Table 4). Nevertheless, not every source of variation in capture probability can be modeled when sampling wildlife, and heterogeneity remains a problematic aspect of mark–recapture studies, especially for population estimation using closed models (Link 2003). Although it could be possible to account for undefined heterogeneity with the Huggins model using mixture models (Pledger 2000), these models require at least 4 sampling sessions and high capture probabilities. Thus, rigorous and coherent data collection procedures still remain a fundamental requirement for applying CMR models to estimate population size. In the present study, we used individual covariates with the Huggins model and treated each data source separately according to its sampling design, and this allowed us to model sampling and spatial heterogeneity in capture probabilities.

However, other undetected sources of individual variation might have affected the encounter rates we observed, possibly affecting the accuracy of population size estimates. For example, the unbalanced sex ratio of our samples suggest differences in capture probability (Boulanger et al. 2004c). However, pooling data across sampling designs, only 7 male bears were captured, and the small sample size negatively affected the power to detect a sex effect. Similarly, we could not take into account time variation in capture probability because sample size was too small to support time dependent models. Neglecting these additional sources of heterogeneity may have affected the accuracy of our population

estimates and contributed to their relatively large magnitude of uncertainty.

The value of our Apennine brown bear population size estimate lays primarily in representing a first, formal assessment of population abundance. Although it may suffer to unknown extent by undefined sources of heterogeneity, our estimate depicts an order of abundance needed to put this reduced population into perspective and to delineate feasible conservation scenarios. The fact that important sources of heterogeneity (such as gender) could not be modeled may imply that capture probability is overestimated and therefore the population underestimated (albeit to an unknown extent). Higher accuracy and improved precision could be obtained in future remote genetic applications to this bear population by adopting improved hair-snag applications to increase sample size and mean capture probability. In this perspective, we showed that the use of DNA joint datasets and Huggins estimator may provide useful tools to overcome the limitations of systematic hair-snagging for the Apennine bear populations.

Management implications

Although our estimate does confirm a very limited population size that corresponds to high risks of demographic extinction, different factors suggest that accuracy of this estimate may be limited by some negative bias: (1) the low level of genetic variability which characterizes this population (Randi et al. 1994, 2006; Lorenzini et al. 2004) may reduce the possibility to genetically discriminate individuals; (2) additional forms of heterogeneity in capture probability, such as gender, which we could not model due to limited sample size; (3) the very low capture probability and the important effect played by buckthorn patches both suggest that part of the population was not catchable during the study. The assessment of the latter represents the critical issue for future CMR applications. However, our results support the notion that there is a definite utility in collecting DNA using multiple sampling methods, as recently suggested by Boulanger et al. (2007). Accordingly, indications from our simulations (minimum threshold values of mean capture probability) are currently being used to evaluate, by means of a pilot study, the improvements expected in field techniques for future and more conclusive DNA-based CMR applications to the Apennine bear population.

This study is also a clear example of how a proper survey design is essential for conservation programs, especially for small populations. In addition, it should warn against hair-snag applications aimed solely to produce a population index (i.e., minimal number alive, [Otis et al. 1978]), as these can be costly but of little conservation value (Anderson 2001, Conn et al. 2004). Authorities responsible for bear conservation in Abruzzo (Ministry of Environment, Regional Governments, and PNALM) should commit to developing a long-term monitoring program using standardized methodology and sound sampling designs.

Finally, we stress the importance of monitoring the Apennine bear population using as many radio-collared bears as feasible. While this is of uttermost importance to monitor survival of individual bears and to assess their mortality causes and rates, it would also allow evaluation of geographic closure and range, which should be surveyed in future CMR applications.

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