Project LifeNAT/IT/000160 "ARCTOS" – Action E3

Non-invasive, integrated datasource survey of the core Apennine bear population (*Ursus arctos marsicanus*) in 2011

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Executive summary

From 1 June to 30 September 2011 we conducted a non-invasive survey of the Apennine bear population in the core of its range, represented by the National Park of Abruzzo Lazio and Molise (PNALM), its external buffer zone and adjacent areas in the central Apennines. Following an *ex-ante* and *ex-post* approach, our aim was to produce a reliable and precise estimate of population size in the first year of the UE Life Project 'Arctos' (Action E3) to be compared with the same estimate at the end of the project (2014) as to assess the efficacy of the intervened conservation actions. We sampled bears by 4 sampling methods (systematic hair snagging, rub tree sampling, opportunistic sampling at buckthorn patches, and sampling incidentally to other management activities), and we also recorded visual and/or camera-trap detections of previously marked bears (GPS-collars and/or eartags; n=23) in the population. Using these data within an integrated data source approach for capture-recapture, closed population modelling (Huggins models in program MARK), our aim was to ensure a high capture probability and an adequate sample size to allow for a reasonably accurate and precise final estimate of population size. Overall, we collected 599 hair samples, ranging from 65 by incidental sampling to 253 by rub tree sampling, 529 of which were analysed. Of these, 426 proved positive to DNA extraction and multilocus genotyping, with a success rate of 80.5%. In total, we non-invasively sampled 45 different bears, with a sex ratio of 1.25:1 females to males, including cubs and management bears. Fifteen of these genotypes matched those of previously live-captured bears, 14 matched those of bears non-invasively detected in previous surveys (2000 – 2008), and 16 were bears never detected before (either already present in the population during previous surveys but gone undetected, or newly added bears to the population after the 2008 survey). The most supported AIC_c models included sampling methods and their interactions with sex, time, and rub trees sampling effort among the major descriptors of capture heterogeneity. Accordingly, our final estimate of the population size was 49 (47 – 61 95%CIs) bears (closure-corrected density: 38 bears/1000 km^2), with a precision (CV=7%) adequate enough to allow for a meaningful comparison with the population size that will be estimated in 2014. The estimate of population size includes all age cohorts (i.e., cubs, yearlings, subadults and adults), and also management bears, of which at least 3 are known to recurrently cause damages to crops and bee-hives. Conservation-wise, these bears should be subtracted from the number of effective bears composing the overall population.

Compared to the point estimate of the population size in 2008, there has been a slight increase in the population, although this conclusion can be partly confounded by the inherent uncertainty associated to these estimates, the expected sampling variability, as well as methodological and modelling differences. In addition, population increase in 2011 mostly account for an increase in the number of males, and much less so in the number of females, probably accounting for the higher reported mortality of females in the 2008-2011 period (of 7 bears reported dead during this period, 5 were females of which 3 of reproductive age). In any event, we did not detect any negative trends in the population compared to its 2008 estimated size, and this confirms that the relict Apennine bear population, notwithstanding substantial levels of human mortality, is still reproductively active and potentially capable of positive growth, at least within the core distribution, potentially providing bear dispersers across a larger geographical scale. Although this tentative interpretation needs to be confirmed in the light of the 2014 estimate that will be produced within the Life Arctos project, it currently provides hope and support for renewed conservation efforts.

From a methodological point of view, this survey provided the opportunity to evaluate complementary sampling methods, among which rub tree sampling that was applied for the first time to this bear population and proved very successful in terms of collected samples, and hence recapture rates, although it was not particularly efficient in terms of uniquely detected genotypes. Empirical data obtained from this survey will be used to further evaluate relative efficiency of the various sampling method aimed to their optimal integration within an ideal sampling strategy for future surveys of this bear population.

Samples collected in the 2011 survey have been analysed by WGI (Wildlife Genetics International, B.C., Canada), differently from previous surveys (2004-2008), whose samples have been analysed by ISPRA. Provided that comparability of scored genotypes between labs was a fundamental prerequisite for ensuring the continuity of a comprehensive genetic database for this bear population over larger temporal and spatial scales, WGI made available calibration factors computed using blood samples of 25 bears whose genotypes were known from the previous analyses at ISPRA. However, given the low level of genetic variability observed in this bear population, WGI reassessed a larger set of markers (n=30), including those previously used by ISPRA, in order to formally select those most appropriate for individual identification. To allow comparison between labs, final selection included a total of 14 markers (plus sex), 10 of which in common with ISPRA. However, 2 of the 11 markers (plus sex) originally used by ISPRA (*MU15* and *G10P*) were considered little informative for the Apennine bear population, as they revealed $0.04 \leq H_E \leq 0.22$. Conversion factors to translate WGI-scored into ISPRA-scored bear genotypes are included in this report. Based on them, the 45 bears detected in the 2011 survey (whose genotypes are individually listed here both in WGI and ISPRA scores) have been compared to those detected in previous surveys of the same population (2000 – 2008); this matching process highlighted a few equivocal cases (i.e., 1 and 2 MM-pairs) that need further consideration prior to their definitive inclusion in a comprehensive dataset, especially in the light of the application of open population models. Finally, the marker selection evaluation suggested that all 10 markers common to both labs be retained for future analyses, and that 2 of the additional loci used by WGI (*G10X* and *MSUT-2*) can be dropped without loss of information, thus defining an ideal marker system of 11 markers (plus gender) for future individual identification in the Apennine bear population.

Riassunto

Con il fine ultimo di valutare l'efficacia delle azioni messe in atto nell'ambito del progetto Life Arctos per le conservazione della popolazione di orso bruno marsicano, abbiamo adottato un approccio *ex-ante* e *ex-post* che prevede una stima accurata della dimensione della popolazione nell'areale centrale di presenza (PNALM, inclusa Zona di Protezione Esterna e aree adiacenti) sia ad inizio (2011) che a fine (2014) progetto. Per quanto riguarda la fase *ex-ante*, dal giugno al settembre del 2011 abbiamo quindi condotto un campionamento genetico non invasivo della popolazione di orso bruno marsicano nell'intero areale centrale di presenza utilizzando quattro tecniche di campionamento complementari: trappole per peli, posizionate sistematicamente sull'intero territorio (hair-snagging); campionamento presso i grattatoi (rub trees); campionamento opportunistico nei pressi di alcuni ramneti di quota; e campionamento accidentale realizzato durante altre attività di campo e/o pattugliamento. Nello stesso periodo abbiamo anche registrato gli avvistamenti di orsi marcati durante una precedente fase di ricerca, o il loro riscontro di presenza tramite trappole fotografiche; 23 orsi precedentemente marcati erano presenti nella popolazione al momento del campionamento. La necessità di ricorrere ad una strategia integrata di raccolta dati ai fini della stima di popolazione nasce dalle difficoltà statistiche intrinseche nel produrre stime di precisione per una popolazione di così ridotte dimensioni; in questi casi è infatti imperativo assicurare un'elevata probabilità di cattura individuale ed una dimensione campionaria sufficiente da permettere un'adeguata precisione della stima finale; questa, a sua volta, deve essere tale da rendere plausibile, alla luce dell'attesa variabilità di campionamento, un confronto numerico tra stime prodotte in anni successivi. Dal punto di vista analitico, ciò ha previsto la composizione di una matrice integrata di catture individuali, dove ciascuna strategia di campionamento corrisponde a più sessioni di cattura; tale matrice è stata a sua volta trattata con modelli di cattura, marcatura e ricattura per popolazioni chiuse tramite il modello di Huggins (programma MARK).

Cumulativamente, sono stati raccolti 599 campioni di pelo, variando tra i 65 raccolti tramite campionamento accidentale ai 253 raccolti presso i grattatoi; di questi, 529 sono stati inviati al laboratorio di analisi genetiche per il riconoscimento genotipico individuale: 426 hanno prodotto DNA in quantità e qualità sufficiente da rendere genotipi affidabili, con un successo di estrazione dell'80.5%. In totale, i 426 campioni sono rivelati appartenere a 45 orsi differenti, con un rapporto sessi di 1,25:1 (FF:MM), ad inclusione dei cuccioli dell'anno e di individui problematici. Quindici dei 45 genotipi combaciano con orsi precedentemente catturati a scopo di ricerca, mentre altri 14 combaciano con orsi campionati tramite tecniche non invasive negli anni passati (2002 – 2008); i rimanenti 16 corrispondono quindi a genotipi mai rilevati in precedenza e che, a loro volta, includono sia orsi già presenti nella popolazione prima del 2008 (anno dell'ultimo survey) ma mai campionati, sia orsi comparsi nella popolazione dal 2008 in poi. I modelli di stima che sono risultati più adeguati, tramite procedura di selezione AIC_c, contemplano come principali fattori esplicativi sia il metodo di campionamento che varie interazioni tra sesso, fattori temporali e sforzo di campionamento; questi, nella loro globalità, rispondono maggiormente all'elevata eterogeneità di cattura individuale da noi osservata. In base alla media dei parametri dei modelli più verosimili, la nostra stima finale è di 49 orsi (IF95%: 47 – 61 orsi) con una densità, corretta per l'eventuale violazione dell'assunto di chiusura della popolazione, di 38 orsi/1000 km^2 e una precisione (CV) del 7%, considerata sufficientemente adeguata per effettuare un confronto con la stima che verrà realizzata nel 2014. La stima da noi effettuata include tutte le classi d'età (cuccioli, piccoli di un anno, giovani e adulti), così come individui considerati confidenti e/o problematici, di cui almeno 3 sono da tempo noti per i danni ripetutamente causati alle coltivazioni in alcuni settori del parco e della zona di protezione esterna. Da un punto di vista di conservazione, gli individui problematici non dovrebbero essere conteggiati in termini del loro effettivo contributo demografico nel lungo periodo alla popolazione.

Da un confronto delle stime puntuali del 2008 e del 2011 si rileva un leggero incremento della popolazione nell'area di studio ($\lambda = 1.075$); tuttavia, ciò deve essere necessariamente e attentamente valutato alla luce della reale possibilità che tale incremento sia anche in solo parte dovuto alla variabilità intrinseca di tali stime e ad alcune differenze nei modelli statistici utilizzati nelle stime del 2008 e del 2011. È inoltre da sottolineare come l'incremento rilevato interessi essenzialmente il segmento maschile della popolazione e non quello femminile, probabilmente in risposta ai più elevati livelli di mortalità sostenuti dalle femmine negli anni tra il 2008 ed il 2011; in questo periodo, di 7 orsi rinvenuti morti 5 erano femmine, di cui 3 in età riproduttiva. In definitiva, in base alla stima del 2011, non sono state comunque rilevate tendenze negative nella popolazione rispetto al 2008. Questo conferma che la popolazione di orso bruno marsicano, nonostante gli elevati livelli di mortalità per cause antropica, è ancora riproduttivamente attiva e potenzialmente in grado di mantenersi demograficamente, perlomeno per quanto concerne la porzione centrale del suo areale, continuando ad esercitare una potenziale pressione di dispersione su più vasta scala geografica. Sebbene questa interpretazione necessiti di essere ulteriormente verificata in base alla stima che verrà prodotta nell'ambito del progetto Life 'Arctos' nel 2014, la situazione descritta rappresenta al momento un'importante fonte di speranza a supporto di una rinnovata strategia di conservazione.

Da un punto di vista metodologico, le attività di campionamento condotte nel 2011 ci hanno inoltre permesso di sperimentare alcune tecniche innovative di raccolta dei campioni, e tra loro complementari in un'ottica di monitoraggio a lungo termine. Tra queste, abbiamo applicato per la prima volta alla popolazione di orso bruno marsicano la tecnica del campionamento non invasivo presso i grattatoi (rub tree); tale tecnica, sfruttando la naturale tendenza degli orsi a strofinarsi sui tronchi degli alberi, è logisticamente più semplice e non comporta l'uso di esche olfattive; nell'ambito del survey del 2011 questa tecnica ha avuto un margine di successo superiore alle aspettative, portando alla raccolta di un elevato numero di campioni, incluse le ricatture degli stessi individui valide ai fini della stima. Il campionamento presso rub

tree, tuttavia, non è risultato particolarmente efficiente rispetto alle altre tecniche, né il numero di genotipi unici, ovvero rilevati esclusivamente tramite una singola tecnica, è paragonabile rispetto a quelli rilevati dalle altre tecniche di campionamento. I dati ottenuti dal campionamento del 2011 saranno quindi utilizzati, tramite simulazioni, per disegnare una strategia di campionamento funzionale ed efficiente per i survey futuri, ma soprattutto sostenibile per il monitoraggio a lungo termine di questa popolazione.

Differentemente rispetto ai survey pregressi (2004 – 2008), quando i campioni raccolti sono stati analizzati dai laboratori di genetica dell'ISPRA, i campioni raccolti nel 2011 sono stati analizzati nei laboratori della WGI (Wildlife Genetics International, B.C., Canada). Premesso che la comparabilità dei genotipi tra i due laboratori è stata una condizione fondamentale nello svolgimento delle analisi, al fine di assicurare l'integrità e la continuità della banca dati genetica per la popolazione di orso bruno marsicano, WGI ha calcolato fattori di calibrazione a partire dai genotipi di 25 orsi (campioni di sangue) precedentemente tipizzati dal laboratorio dell'ISPRA. Inoltre, data la scarsa variabilità genetica che caratterizza questa popolazione, WGI ha reputato utile riesaminare un più ampio numero di loci nucleari microsatellite (n=30), ad inclusione di quelli precedentemente utilizzati da ISPRA, al fine di determinare quelli più appropriati per l'identificazione individuale. In quest'ottica, e per permettere comunque la confrontabilità dei genotipi analizzati dai due laboratori, WGI ha selezionato 14 loci in totale (oltre al sesso), 10 dei quali in comune con ISPRA. Tuttavia, 2 (*MU15* e *G10P*) degli 11 loci (oltre al sesso) originariamente utilizzati da ISPRA sono stati considerati di scarso valore informativo, corrispondendo a valori di $0{,}04 \leq H_E \leq 0{,}22$. I fattori di conversione per tradurre i genotipi WGI in genotipi ISPRA (e viceversa) vengono inclusi nella presente relazione. Sulla base di questi, i 45 orsi rilevati tramite campionamento non invasivo nel 2011 (individualmente elencati nella presente relazione sia con punteggi WGI che ISPRA) sono stati confrontati con quelli rilevati nei precedenti campionamenti realizzati sulla stessa popolazione (2000 – 2008). Da questo confronto sono emersi alcuni casi dubbi (coppie 1 e 2 MM) che meritano un'ulteriore valutazione congiunta prima di una loro definitiva conferma all'interno della banca dati genetica, specialmente in previsione dell'applicazione di modelli per popolazioni aperte. Infine, dalla procedura di valutazione del numero (e tipo) di loci emerge la necessità di mantenere per le analisi future tutti i 10 loci in comune ai due laboratori, per continuare a garantire la confrontabilità dei genotipi, e che 2 (*G10X* e *MSUT-2*) dei loci integrati da WGI possono essere tralasciati senza conseguenze apprezzabili, per un totale effettivo di 11 loci (oltre al sesso) ai fini di un riconoscimento affidabile dei genotipi individuali in questa popolazione di orso bruno marsicano.

1. INTRODUCTION

The EU Life Natura project (LIFE+ NAT/IT/000160 "ARCTOS"), co-funded by the European Union and conducted by several national and regional conservation agencies, started in September 2010 and aims to promote several practical conservation measures in order to enhance bear conservation by improving long-term coexistence with humans. Within this project, the Department of Biology and Biotechnologies of the University of Rome "La Sapienza" is responsible for the assessment of the bear population size in the PNALM at the first (2011) and last (2014) year of the project (Action E3). Comparing population size at the beginning and at the end of the 5-year project, not only would reflect the efficacy of conservation interventions implemented during the project, but would also provide an opportunity to assess for the first time population trends over a biologically meaningful stretch of time (i.e., 2004 – 2014).

The Apennine brown bear, endemic to the central Apennines and believed by some to represent a subspecies (*Ursus arctos marsicanus*; Loy et al. 2008, Colangelo et al. 2012), is mostly relegated to a limited area centered around the Abruzzo Lazio and Molise National Park (PNALM), comprising a single population whose size and trends have been scarcely investigated prior to 2008 (Ciucci and Boitani 2008). Whereas a formal survey was conducted for the first time in 2004 (Gervasi et al. 2008), a more reliable assessment was carried out in 2008 by means of non-invasive genetic sampling coupled with an integrated data sources CR, closed population modelling approach (Gervasi et al. 2012). Based on a composite sampling strategy, this approach has been shown to provide reasonable accuracy for the PNALM bear population, notwithstanding the small population size and an expectedly small data set for CR applications. The same approach can therefore be used to assess population trends over a longer time frame, while allowing the accumulation of genetic data (i.e., multilocus genotypes) for the future application of open population models.

Based on the above, and through a collaboration with the Park Authority and the National Forestry Service, we carried out a non-invasive genetic survey to estimate population size of the Apennine bear in spring-summer 2011. In particular, our aims were:

- to estimate, with adequate precision, the size of the Apennine brown bear population in the core of its distribution (cf. Ciucci and Boitani 2008);
- to assess hair collection at rub-trees as an additional sampling method for our bear population;
- to provide empirical data (sampling, population size, distribution and trends) to run realistic simulations to design an optimal sampling scheme to allow a cost-effective, long-term monitoring of this population for the future;
- to contribute additional genetic data (i.e., multilocus genotypes) through intensive sampling to monitor the population over a larger geographical and temporal scale (e.g., dispersal, survivorship, trends).

Both in summer 2011 and 2012, we also conducted unduplicated counts of females with cubs (Knight et al. 1995, Keating et al. 2002) on a yearly basis to aid interpretation of estimates of population size and trends. Results of FWC counts in 2011 and 2012 have been reported elsewhere (Ciucci et al. 2011a, 2012).

In addition, as the samples we collected in the 2011 survey were analyzed by a different lab (WGI: Wildlife Genetics International, BC, Canada) with respect to the previous surveys, this provided also an additional opportunity to assess the overall quality of the genetic approach, similarly to other non-invasive applications (Kendall et al. 2008). This also allowed us to re-evaluate the set of markers best suited for the demographic assessment of the Apennine bear population, while ensuring comparability with previously detected multilocus genotypes. This was ensured by WGI by providing

conversion factors and by scoring, in addition to newly added markers, the same set of markers used by the previous lab.

The aim of this report is therefore twofold. First, we detail technical aspects of the genetic analyses, including the selection of markers and their power for individual discrimination in our bear population. By doing so, we also report WGI conversion factors for translating WGI scores to those of the previous lab, ensuring comparability of individual multilocus genotypes and consolidation of a comprehensive dataset for the Apennine bear population. To this end, we make reference to information and results previously provided by a WGI technical report to BBCD (Paetkau 2012, *in litteris*). Practical implications of these results for the consolidation of a comprehensive dataset for the overall 2000 – 2011 period are briefly summarized in this report, but they have been illustrated in greater detail elsewhere (Ciucci et al. 2012a). Second, we report the results of the 2011 survey both in terms of sampling design, sampling effort and achieved sample size, as well as in terms of demographic assessment (i.e., population size estimate).

Although intermediate, progress reports have already been made available (Ciucci and Gervasi 2010, Ciucci et al. 2011b, 2012a, Paetkau 2012 *in litteris*), this is the first comprehensive report of the 2011 survey, dealing with its design, sampling strategies, genetic aspects, and population assessment. We also briefly discuss some practical implications of this non-invasive application for the design of similar surveys for the Apennine bear population in the future.

2. METHODS

Due to an expectedly sparse dataset and low capture probability, hair-snagging alone has been deemed inappropriate, if used alone, to produce accurate estimates of the Apennine bear population size (Gervasi et al. 2010). This problem was anticipated in the 2008 survey adopting an integrated data source approach (Boulanger et al. 2008) by composing individual encounter histories through a combination of sampling methods, namely hair-snagging, live captures (i.e., number of live-captured and collared bears known to be alive in the study area), and direct observations of collared females with cubs (Gervasi et al. 2012). The latter method, in particular, was instrumental to include the cubs in the estimate, as in the Apennine bear population this cohort seems to escape sampling with hair traps set at 50 cm (Gervasi et al. 2008, 2012).

In 2011, the survey was conducted using the same DNA-based, noninvasive CR modeling techniques adopted in 2008, although with some sampling modifications due to changes in field conditions and a decreased availability of collared bears for individual recognition. In 2011 no bears with active VHF- or GPS-collars were available, and just three females with cubs were wearing (exhausted) collars, raising two potential problems: (a) the integration of live-captures and/or direct observations into the individual encounter histories, and (b) how to account for cubs in the final population estimate. The rationale behind the 2011 survey strategic planning (Ciucci et al. 2011b), detailing field, logistic and analytical options, was therefore aimed to account for one or both of the above problems. Likewise, we drafted a field protocol providing field work and implementation details for each sampling method to be officially adopted and circulated among field operators (Ciucci and Gervasi 2010). Sampling strategies and corresponding field methods are detailed below with reference to differences with respect to the 2008 survey.

2.1 Preliminary activities and communication

Preliminary activities and communication within the survey team have been carefully planned as they were deemed critical to enhance success of the 2011 survey. In particular, with reference to sampling methods and strategies we:

- field visited, during April-May 2011, each of the 2008 survey hair-trap locations in order to mark them, for subsequent recognition by the field crew. According to indications collected in 2008, some problematic trap locations (e.g., rocky or uneven substrate, steepness, lack of trees) were moved within the same general locality and sampling grid as to further enhance capture probability. Two additional grid were added for the 2011 survey, and a total of 215 potential hair traps were marked;
- conducted a rub-tree sampling workshop with all park wardens in October 2010 in order to solicit extensive searching for such trees in the PNALM which were never tallied before. In order to maximize searching efforts we provided for each area of the PNALM a detailed map of travelling paths by GPS-instrumented bears during 2006-2010;
- computed an index of use of buckthorn patches by bears in the PNALM based on the 2004 survey (Gervasi et al. 2008), GPS data and direct observation (2005-2010). We then ranked buckthorn patches according to a composite index accounting for the highest number of different bears visiting/captured by each patch, and used simulations to design an optimal sampling strategy for the 2011 survey (see § 2.2.3);
- stored, by December 2010, 1000 kg of ground fish and 1000 lt of beef blood to be successively used for the hair trap lure. These compounds were stored for the following 6 months within barrels inside a greenhouse built in an open area to allow high temperatures. The greenhouse was located outside the study area as to avoid any contact or habituation to the lure by resident bears prior to the survey;
- drafted a hair-snag sampling plan and calendar by assigning cluster of hair-snag sampling cells to each field crew, and establishing for cluster date of activation and deactivation as to ensure the correct timing of the hair-snag survey (5 sessions of 12 days each, see below). Most sampling crews comprised 2 operators from the three institutions participating in the survey. They were assigned a given set of sampling cells on the basis of both their personal knowledge of the sampling locations and to minimize travel costs;
- conducted training and motivational workshops with all field personnel, and all field and sampling material was distributed to each sampling crew in occasion of the last training workshop; during the survey, regular meetings among all field personnel further ensured proper coordination and standardization of sampling techniques;
- conducted three preliminary coordination meetings with the three different institutions involved in the survey to ensure proper coordination of the survey; coordination was further ensured by circulating progress reports at the end of each hair-snag sampling session.

2.2 Sampling strategies and field methods

In order to take advantage of the integrated data source approach (Boulanger et al. 2008), we adopted multiple sampling strategies: systematic hair-snagging, rub-tree sampling, opportunistic sampling at buckthorn patches, and incidental sampling (Gervasi et al. 2008). Hair-snagging ranged June – July 2011, but the other sampling methods extended through September.

Regardless of the sampling method, we considered a hair sample as a tuft of hairs entangled in one set of barbs (Woods et al. 1999). We collected each sample possibly containing guard hairs with bulbs with sterilized surgical forceps, and placed each sample in a paper envelope labeled with a uniquely numbered barcode. We then passed a flame under the barbs to remove any trace of hair to avoid contamination between sessions (Kendall et al. 2009). Paper envelopes containing samples where then stored in a dark place within a box with silica gel to avoid DNA degradation.

During sampling, but particularly for rub tree sampling, we also collected ≥1 sample believed to be left by the same bear in a single sample occasion (i.e., 'replicated' samples) based on proximity of the samples on the barbed wire. Some of these replicates were sent to the lab for genetic analyses to provide additional source of DNA in case the primary sample did not yield a reliable genotype. Upon sample collection we discarded on the field all hair samples of other species, and used microscopic

characteristics (Teerink 1991) to distinguish less obvious cases. Therefore, only macro- and microscopically pre-selected bear samples have been considered for genetic analysis.

Obtaining noninvasive samples from scats was not considered feasible due to the low extraction rate of DNA (R. Prive, pers. com.). However, in addition to the noninvasive sampling methods above, we also recorded direct sightings of bears live-captured and marked (i.e., VHF or GPS-collars and/or eartags) during a previous (2005-2010) study, of which 23 were expected to be still alive and within the study area during the 2011 survey (Appendix 1). We also obtained photos of marked bears by opportunistically placing camera-traps throughout the study area. Similarly to the 2008 survey, observations and photo-traps were thought to potentially represent additional capture sessions to be included in the integrated data sources approach for CR modeling (but see § 2.4.5). However, no VHF- or GPS collars of previously live-trapped bears were still active at the time of the survey so, differently from similar applications (Kendall et al. 2008, Gervasi et al. 2012), we could not include this as an additional data source.

2.2.1 Hair-snagging

We adopted systematic hair-snagging using 5x5 km grid cells covering the entire core area and 5 sampling sessions of 12 days each, and moved traps within each cell to increase trapping efficiency and to reduce the risk of behavioral responses. Number of cells and criteria for trap locations were slightly revised with respect to the 2008 survey in order to slightly increase the sampling area and capture probability. Overall, 43 cells were surveyed in 2011, for a total of 215 traps in an area of 1221 km^2 (Fig. 1).

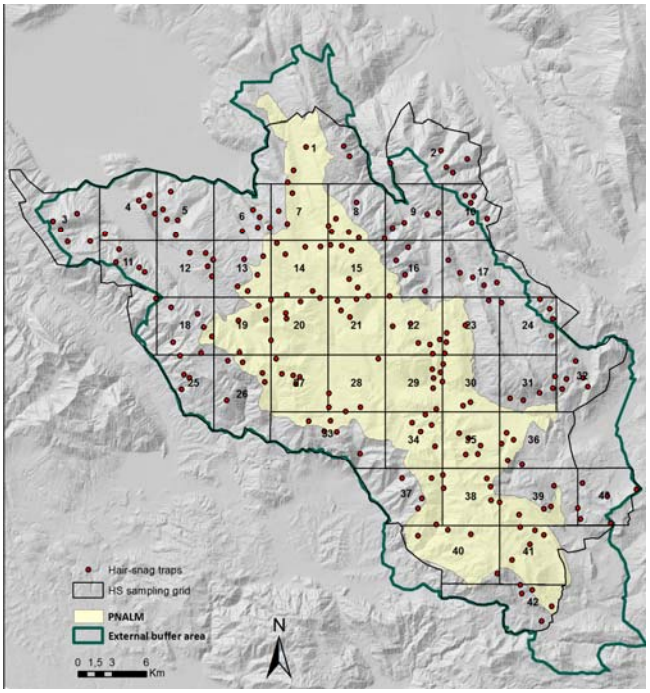

Figure 1. – Hair-snag (HS) sampling grid adopted for the survey of the Apennine bear population in the PNALM area (June – July 2011). In total, 43 sampling grids (5x5 km each) have been used, with some peripheral grids ≥25 km^2. Five sampling sessions of 12 days each were used, and hair-trap locations were moved between successive sessions for a total of 5 traps/cell and 215 traps (red dots) for the entire survey.

Hair-snagging extended for 8 weeks, from the beginning of June to the end of July, and the starting date was about 2 weeks later than that of the 2008 survey. Six field teams, of 2-4 operators each, worked simultaneously during the hair-snag survey, and they included personnel from the University of Rome, the PNALM authority, and the Forest Service (UTB and CTA). Each field team was pre-assigned a given set of sampling grids, whose

location and number (2-12 traps per session) were based on logistics, availability of personnel, and knowledge of the area by the operators (Fig. 2). Lures were composed of about 3 L of a 50:50 mixture of cattle blood and rancid fish poured over wood debris piled in the center of the hair traps. All hair traps were dismantled at the end of the session and moved to the new trap location.

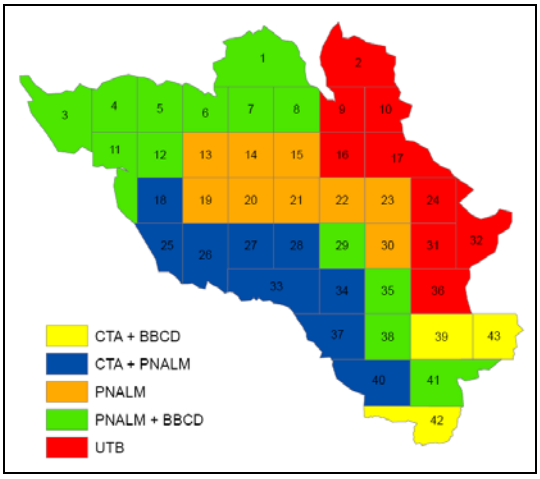

Figure 2. – Spatial partitioning of the 43 hair-snagging grids used for the survey of the Apennine brown bear population (PNALM, June – July 2011). Six field teams were simultaneously active (1-12 cells per session), with each team of mixed affiliation (CTA: Forest Service, Coordinamento Territoriale per l'Ambiente; BBCD: Dept. Biology and Biotechnoligies University of Rome; PNALM: National Park of Abruzzo Lazio and Molise; UTB: Forest Service, Ufficio Territoriale per la Biodiversità of Castel di Sangro).

In contrast to other hair-snagging studies (e.g., Kendall et al. 2009), cubs in our population cubs are apparently inaccessible to hair-snagging during spring and summer using traditional 50m-high barbed wire traps (Gervasi et al. 2012). Along with the overall lower capture probability of cubs (Tab. 6, Kendall et al. 2009), this is possibly due to their smaller size compared to other brown bear populations, their particularly restricted movements, and the elusive behavior of their mothers, including their lower attraction to lured traps. As a result, no cubs were hair sampled during the 2008 survey, although a minimum of 10 cubs in 6 family units were visually detected in the same year. Because using a lower than 50 cm height for the barbed wire strand in hair traps might have reduced the efficiency of hair snagging adult bears, and double-stranded hair traps would have been logistically unfeasible, we addressed the inaccessibility of cubs to hair-snagging adopting a complementary sampling strategy at patches (see below).

2.2.2 Rub-tree sampling

Sampling at rub-trees (RTs) has been proven to be an efficient way to obtain non invasive samples from brown bear populations (Kendall et al. 2008, 2009, Stetz et al. 2010). Although rubbing behavior in the Apennine bear was never described before, during a 3-month period prior to the survey (October – December 2010) we identified a minimum of 40 RTs in the core of the PNALM, at a minimum density (8 RTs/100 km^2) which was comparable to those revealed in other study areas (e.g., 8-10 RTs/100 km^2 and 20 RTs/100 km^2, in Glacier National Park and in the North Continental Divide Ecosystem, respectively; Kendall et al. 2008, 2009). As we expected to identify more rub trees by the time of the survey, RT sampling had the potential to provide a practical source of samples in our bear population. We therefore tentatively included this sampling method in the 2011 survey based on the rationale that it would have at least provided some additional samples, similarly to incidental sampling (Ciucci et al. 2011b).

Out of 147 rub trees inventoried by May 2011, we installed and visited hair traps in 97 of them, attaching 4-6 short (30-40 cm each) strands of barbed wire to the rubbing surface of the tree in a zig-zag pattern at about 30 – 170 cm from the tree base (Kendall et al. 2008). Similarly to the other hair traps, we collected hair samples only from the barbs and passed a flame to avoid contamination between successive sessions. At each sampling occasion, we often found more than one hair sample on the same RT, possibly left by the same bear in a

rubbing event; in these cases we selected the samples with more guard hairs and bulbs, but often collected up to 4 samples farthest apart from each other, both because we were not sure if they were actually left by the same or more bears, and to provide a backup sample in case the others would yield a reliable genotype. Similarly to other noninvasive genetic surveys that adopted RT sampling (i.e., Kendall et al. 2009), we started rub-tree sampling in June and extended sampling through September. Once activated through the installation of barbed wire, we visited rub trees every 1-13 days and a subsample of these were monitored with IR camera-traps to better assess RT use by bears and sampling success, as well as to obtain information as to enhance sampling success. We de-installed hair traps at rub trees by the end of the survey.

2.2.3 Opportunistic sampling at buckthorn patches

Although not very efficient, an effective means to sample our bear population is to opportunistically locate hair-traps at buckthorn patches, where bear congregate in late summer to feed on ripening *Ramnus* fruit (Gervasi et al. 2008). In addition, by using hair traps at buckthorn sites we intended to include cubs in our 2011 population estimate, as hair traps used at buckthorn patches were built as to increase the capture probability of this cohort, otherwise difficult to sample with systematic hair traps (see also §2.2).

Because the park authority generally restricts public access to these areas during summer in order to avoid potential disturbance to bears, we had to reduce potential sampling effort at a minimal rate in order to avoid controversies and public concerns. We did so by selecting only the buckthorn areas most used by bears (see §2.1) and restricting sampling to the most *Ramnus* productive 4-5 weeks in August-September, based to the ripening periods of the selected buckthorn areas at the different latitudes of the park. In order to design optimal sampling at buckthorn patches under current conditions, we ran simulations to evaluate what would constitute a minimal sampling effort (i.e., number of buckthorn areas to sample) based on the data (number of samples and bear genotypes) collected during the 2004 and 2005 surveys. In particular, we evaluated the distribution of all genotypes sampled through opportunistic traps at buckthorn patches over the years, to assess which patches could have provided the highest sampling efficiency while, at the same time, reducing the potential impact on bear feeding behavior. The aim has been to eliminate redundant traps and to limit sampling only to those areas in which an effective contribution (in terms of new genotypes) was expected based on the data collected in previous surveys. In the simulations, we simultaneously considered the sampling efficacy (number of genotypes detected per patch), redundancy of sampling (number of genotypes re-sampled at different buckthorn areas), and visibility of the buckthorn area to the public (high or low), and defined a minimum sampling scenario comprising 7 buckthorn areas from the total of 36 for which we detected use by bears during 2004-2008.

In each selected buckhorn area we constructed from 1 to 4 long peripheral hair-traps, each encircling cohesive aggregations of buckthorn patches, for a total of 19 hair traps for all sampled buckthorn areas. We used a double strand of barbed wire at 1.5 m from the nearest buckthorn shrub, and placed it at 2 different heights (30 and 50 cm) to increase capture probability of cubs (see § 2.2.1). As most of these patches occur above timberline, we anchored the barbed wire to steel pegs dug into the ground. The perimeter of individual hair traps within buckthorn sites averaged 32 (±25) m, ranging from 15 – 130 m, and it took about 1.5 (±1) hours for a field crew of 3-5 to build traps in each buckthorn site. Due to the remote location and inaccessibility of some of the buckthorn hair traps, the Forest Service made a helicopter available to carry the material. All hair traps were removed by the time of the last sampling occasion.

2.2.4 Incidental sampling

Hair samples have been collected by experienced park wardens during their patrolling and management activities (e.g., damage assessment), as this sampling technique has been previously shown to increase sample size considerably (Gervasi et al. 2008). Whereas in the 2004 survey incidental sampling was limited to 2 months, in the 2011 survey we collected incidental samples from June to September.

2.3 Genetic analyses

Genetic analyses were conducted at Wildlife Genetic International (WGI) using quality assurance protocols (Paetkau 2003) that have been shown to ensure accurate individual identification (Kendall et al. 2009). Blood and tissue samples (n=25) from live-trapped bears were used to provide marker

selection data, as well as to allow calibration between WGI and the previous Italian lab (ISPRA; Gervasi et al., 2008, 2012). Non-invasive samples during the 2011 survey (n=599 hair samples) were analyzed for individual multilocus genotype identification and subsequent CR modelling.

2.3.1 DNA extraction

Twenty-five blood samples drawn from live-trapped bears were provided to WGI by means of soaked dried cotton swabs. DNA was extracted from the blood swabs by clipping a small (~3mm x 3mm) piece of cotton swab, and processing the clippings with QIAGEN DNeasy Blood and Tissue Kits according to the instructions for tissue (for details http://www.qiagen.com/). The same procedure was used for hair samples, which were washed in warm water before being placed in the extraction solution.

Hair samples were excluded from analysis if they contained no guard hair roots, and <5 underfur. For the samples which were analysed, we aimed to use 10 guard hair roots where available. When underfurs were used, the number recorded was an estimate because entire clumps of whole underfur were used rather than clipping individual roots.

Analysis of the hair samples started with a first pass during which all extracted samples were analyzed at 7 of the 14 markers (13 microsatellites plus gender), and we culled samples that had high-confidence scores (Paetkau 2003) for ≤ 3 of 7 markers. The first pass was followed by a cleanup phase in which we reanalyzed data points that were weak or difficult to read the first time (i.e. scored with low- confidence), using 5 µl of DNA per reaction instead of the 3 µl used during the first pass. Multiple rounds of reanalysis were used to confirm persistently weak data points. The first pass and cleanup process was then repeated at the other 7 markers with the non-culled hair samples.

2.3.2 Marker selection and calibration with ISPRA genotypes

We used the 25 blood samples to generate marker selection data, as genotypes for these bears had been generated in the ISPRA lab for 11 microsatellite markers (Gervasi et al. 2008). We analysed these samples at 27 microsatellite markers available at WGI from previous projects. Adding data for the same bears from 3 other ISPRA markers (MU05, MU11 and MU15), we used the program GENEPOP to summarize the variability of 30 markers in the Apennine bear population (Table 1). Nine of 30 markers were 'fixed' for a single allele in the blood sample data, and were thus of no interest. Another 7 markers showed only 2 alleles, with 1 allele being so common that H_E was ≤ 0.35. These 7 included 2 of the 11 markers in the existing ISPRA dataset (MU15 and G10P): such markers do so little to lower the match probability in an analysis of individual identity that the cost of their analysis could only be justified in the absence of other alternatives. Fortunately, alternatives were available in the form of 14 microsatellite markers with $H_E \geq 0.44$ (Table 1). While many of the these 14 markers were less variable than the ideal, we had little choice but to use 4 of the least variable of these markers (MU11, MU05, MU50 and G10L) because they were used by ISPRA and were therefore needed for comparisons between labs.

Marker selection was originally done using the blood samples from 25 bears, although Table 1 summarizes variability data using all individual bears identified (i.e., including all analysed blood and hair samples collected in the 2011 survey). With the original sample size, G10H appeared to be the least variable of the 14 microsatellites that were candidates for individual identification. This marker is also comparatively long (alleles over 250 bp), so one might anticipate that it would have a lower success rate with marginal samples, as the efficiency of PCR drops off with the length of the sequence being amplified. We were quite certain that individual identification could be done well with 13 microsatellites, plus a ZFX/ZFY gender marker, so we decided at this point not to use G10H for individual identification. The inclusion of gender data at an early stage of the analysis — rather than limiting the analysis to 1 sample per individual after the microsatellite analysis is finalized — roughly halves the match probability, even for close relatives, and streamlines the process while also reducing opportunities for sample handling errors. We expected that a satisfactory match probability could be achieved with < 13 microsatellites, but given the potential for a high degree of consanguinity in the study population, we felt that any decision to further reduce the number of markers should be postponed until the end of the project, when data were available from more individuals (see § 3.3.2). In addition to the 13 selected markers, we also used G10P at a later stage of the analysis to better discriminate between

controversial cases (e.g., 1 MM- and 2 MM-pairs) when comparing genotypes detected in 2011 with the previous ones scored by ISPRA (see § 2.3.3).

Locus	n	H_E	H_O	A
CXX20	55	0.62	0.67	3
REN144A06	55	0.61	0.65	3
G1D	55	0.58	0.64	3
MU51	55	0.57	0.47	3
G10B	55	0.52	0.47	3
G10C	55	0.50	0.56	3
MU59	55	0.49	0.53	2
MSUT-2	55	0.49	0.36	3
G10X	55	0.47	0.35	2
MU05	55	0.47	0.47	2
G10L	55	0.44	0.51	2
MU50	55	0.44	0.47	2
MU11	55	0.44	0.38	2
G10H	25	0.46	0.52	2
MSUT-6	23	0.35	0.35	2
G1A	23	0.26	0.30	2
D123	23	0.26	0.30	2
G10P	24	0.22	0.25	2
CXX110	24	0.13	0.13	2
G10U	23	0.04	0.04	2
MU15	26	0.04	0.04	2
CXX173	18	0.00	0.00	1
MU26	22	0.00	0.00	1
G10O	23	0.00	0.00	1
G10J	25	0.00	0.00	1
REN145P07	23	0.00	0.00	1
CPH9	22	0.00	0.00	1
MU23	23	0.00	0.00	1
G10M	23	0.00	0.00	1
D1A	22	0.00	0.00	1
13-Locus mean		*0.51*	*0.50*	*2.5*
30-Locus mean		*0.28*	*0.28*	*1.9*

Table 1. – Measures of variability including the observed number of alleles (A), and expected (H_E) and observed (H_O) heterozygosity. The 13 markers used at WGI for individual multilocus genotyping are those above the dashed line, plus G10P (see text) and sex.

After completing the analysis of the blood samples, we compared our genotype scores for the 10 markers (all except MU15) that had at this point been analyzed in both labs. This comparison identified a conversion factor for each marker that can be added to the ISPRA lab's allele scores to convert them to WGI's scoring (Table 2). WGI uses a scoring convention wherein the database treats 2-digit allele scores as missing data when assigning individual identity. To accommodate this convention, WGI added 100 bp to the allele scores for any marker that has alleles shorter than 100 bp (explaining why 3 markers have scores that differ by roughly 100 bp between labs). For marker MU05, not used before by WGI, scoring was calibrated to match ISPRA existing data. MU11 was treated similarly, but 100 was added to ISPRA allele scores to avoid 2-digit scores for shorter alleles.

The calibration process provided an excellent opportunity to check for genotyping errors, since the data from different labs were generated strictly independently. WGI encountered 2 genotyping errors at marker G10B, with reasonably certainty that these errors were not in WGI genotypes, because these were replicated in a large number of hair samples that were subsequently matched to the same individuals (M09 and M10). Both errors appeared to be caused by allelic dropout, the most common type of error to affect data from sparse DNA samples like hair. With 2 errors in data from 500 data points (2 alleles * 10 markers * 25 individuals), the

results from ISPRA appear to be at the high end of the spectrum of data quality, making it possible to reliably compare data between labs.

Marker type		Conversion[a]	Notes
WGI	ISPRA		
G10B	G10B	+28	
G10C	G10C	+102[b]	
G1D	G1D	+20/+22	- ISPRA ≤150 bp: +22 - ISPRA >150 bp: +20
G10L	G10L	+9	less variable
MSUT-2	-	-	
MU59	MU59	+128	
REN114A06	-	-	
CXX20	-	-	
MU50	MU50	+32	less variable
MU51	MU51	+92[b]	
G10X	-	-	
MU05	MU05	0[c]	less variable
MU11	MU11	+100[b]	less variable
G10P	G10P	-7	much less variable
-	MU15	-	n.a.[d] (effectively invariable)
Sex	Sex		

[a]: to obtain WGI score from ISPRA score
[b]: markers with alleles <100 bp actual length are scored 100 bp higher at WGI to accommodate use of 2-digit allele scores for low-confidence (≈failed) results (see text)
[c]: WGI did not use this marker before, and calibrated its scoring to match the ISPRA score
[d]: this marker was not analysed at WGI; data from ISPRA

Table 2. – Markers used by WGI (data from 2011 survey) and ISPRA (data from 2004-2008 surveys) for multilocus genotyping of individual bears for demographic assessments of the Apennine bear population. The conversion factor is the amount to add or subtract to the ISPRA allele scores to convert them to WGI scoring. ISPRA used 11 markers, plus one for sex, and WGI used 13 loci, plus one for sex. Of these markers, 10 were in common between labs. WGI added 4 new markers among those available with more expected variability, but deleted MU15 as considered little informative (cf. Table 1). G10P was also considered little informative but it was retained to allow calibration and comparison between labs for controversial samples (e.g., 1- and 2-MM pairs).

2.3.3 Microsatellite genotyping

Analysis of the hair samples started with a first pass during which all extracted samples were analyzed at 7 of the 14 markers (13 microsatellites plus gender). After first pass we culled samples that had high-confidence scores for ≤ 3 of 7 markers, by using a combination of objective (i.e. peak height) and subjective (i.e. appearance) criteria to classify genotype scores (Paetkau 2003). In WGI experience, no amount of effort will produce complete, accurate genotypes from such samples. The first pass was followed by a cleanup phase in which WGI reanalyzed data points that were weak or difficult to read the first time (i.e. scored with low-confidence, 2-digit alleles), using 5 µl of DNA per reaction instead of the 3 µl used during the first pass. In some cases multiple rounds of reanalysis were used to confirm persistently weak data points. This process (first pass and cleanup) was then repeated at the other 7 markers with the non-culled hair samples, and further samples were eliminated after the cleanup phase of this second round of 7-locus genotyping. Samples left after this final cull had high-confidence scores for all 14 markers.

Multilocus analysis finally addressed error-checking, where we searched for and reanalyzed any pair of genotypes that was similar enough to have conceivably been created by genotyping error (Paetkau 2003). Intensive testing with blind control samples has shown that this protocol effectively prevents the recognition of false individuals through genotyping error (Kendall et al. 2009), although it does not claim to eliminate genotyping errors in cases where only one sample has been analyzed from a given individual. During error-

checking, 6 errors were found and corrected, of the sort expected when working with sparse DNA sources like hair follicles. After correcting these errors, the most similar pair of genotypes in the dataset mismatched at 2 markers (i.e., 2MM-pairs), and those mismatching data points had been solidly replicated to rule out genotyping error.

The last quality control phase involved interaction between the genetic lab (WGI) and field personnel (BBCD), as already done in previous genetic sampling projects on the Apennine brown bear (Gervasi et al. 2012). We performed a series of cross-controls between the information provided by the genotyping process and that included in field data (dates and locations of sample collection, GPS data from radio-collared bears, position of samples on the trap, etc.). The aim was to further investigate some cases from the 2011 survey (i.e., unique samples, some mismatching) by: (a) comparing locations of samples attributed to previously radio-collared bears with GPS locations from the same bears; (b) plotting the distance and time between samples attributed to the same genotype, to check if any samples had been collected at unexpectedly large distances; (c) checking the consistency of the results at each trap, evaluating dates of collection, the position of samples on the trap, and the number of mismatching loci among genotypes sampled at the same trap.

We also cross-checked any 1 MM-, 2MM- and 3 MM-pairs, or other potentially equivocal results, which emerged during comparison of multilocus genotypes detected in 2011 (scored by WGI) with those detected in previous surveys (2000 – 2008) and scored by ISPRA. To this aim, we added in this step of the analysis the *G10P* marker to better discriminate between samples that had suspicious MM-pairs between labs (51 out of 452 analyzed samples). Evaluation of these cases has been based on the number and type of samples (i.e., hairs vs. scats) and markers involved, sampling dates, re-sampling rates, and geographic appraisal of their distribution. Remedy actions, needed to ensure the quality of the aggregated dataset, that emerged from this control phase were summarized for current and future reference (Ciucci et al. 2012). Based on this final step, we preliminarily merged previous (ISPRA) with current (WGI) bear genotypes in a final database, although its definitive consolidation needs further interactions between both labs to clarify a few equivocal cases.

2.4 CR modelling and model selection

We used Huggins closed population models (Huggins 1991) in Program MARK (White and Burnham 1999) to estimate the size of the Apennine brown bear population. As a first step, we combined data from the 4 non invasive sampling methods described above (i.e., excluding re-sight and camera-trap sessions on marked bears) to construct individual encounter histories. For each sampled bear, we recorded hair-snag captures in sessions 1-5, captures at buckthorn aggregations in sessions 6-8, rub tree samples in sessions 9-12, and incidental genetic samples in session 13. This approach is allowed in a context of closed population capture-recapture models, as the relative order of sessions is irrelevant to parameters estimation, unless any behavioural response is expected in the data (Boulanger et al. 2008). In our case, we assumed our sampling design to be minimally affected by any behavioural response. The issue did not involve incidental samples, as these data source was summarized into a single session, thus a-priori preventing any possible response. As to hair-snag, data traps were moved between successive sessions, providing no reward to sampled bears, whereas both buckthorn and rub tree sampling took advantage of a natural behaviour by bears, without enhancing or stimulating it in any way (Gervasi et al. 2012). After building the encounter histories, we constructed candidate models for each data source, and combined them into a most parameterized starting model. The variables included in the initial most parameterized model were selected based on a-priori knowledge of bear biology and spatial behaviour, on previous non invasive applications in North America (Boulanger et al. 2008, Kendall et al. 2008), and on our own previous experience sampling this population (Gervasi et al. 2010, 2012). These variables are summarized in the following sections for each data source.

2.4.1 Hair-snag data

For the hair-snag sampling, we first tested for a temporal variation in capture probability, both through a simple *time effect* (one parameter for each session) and through a *trend effect*, aimed at detecting an increasing or decreasing capture probability during the whole survey. We tested if a previous live-trapping

event was associated with a decreased capture probability, as previously found in other brown bear hair-snag studies (Kendall et al. 2008, 2009). We also tested if capture probability varied as a function of *gender*, and if a hair snagging event during previous years produced a decreased capture probability, as an effect of the expected awareness by previously-hair snagged bears (i.e., no food reward). As many of the bears born before 2008 had been sampled at least once through hair snag (4 efforts have been conducted between 2003 and 2008 in the same area), whereas no new genotype had been detected between 2007 and 2010 (Gervasi et al. 2012), this variable was also expected to be correlated with a bear's age. Bears with a previous hair-snag history were older than 4 years, whereas most of the bears with no previous hair-snag history were expected to be younger than 4 years. Based on the hair-snag capture probability estimates of our 2007 and 2008 samplings (see Gervasi et al. 2010, 2012 for details), the probability for a 4 years old male bear not to be detected until 2011 was less than 5%, whereas the same probability for a female of the same age was about 28%. These probabilities further decrease for bears older than 4 years.

2.4.2 Buckthorn data

Similarly to hair-snag data, we tested a simple *time effect* (one parameter for each session) and a *trend effect* also for the buckthorn sampling. As the buckthorn sampling was performed during a period of two months, we expected that the progression of the *season*, affecting the ripening of berries and the extent of use of buckthorn aggregations by bears, could generate different capture probabilities among sessions. As the length of each session was slightly different among sessions and sampling sites, we also assessed if the temporal variation in sampling effort during the 3 buckthorn sessions affected the variation in capture probability. As an estimate of effort, we used the cumulative number of trap nights in each session multiplied by the cumulative length of the barbed wire of all traps by each buckthorn site. Also, as the study area lies on a broad NW-SE gradient, we expected the ripening of buckthorn berries to occur later in the season in the Northern part; we therefore tested an interaction between the time effect and the *latitude* of the central sampling point of each bear. Finally, we included a *gender effect* and an effect of a previous hair-snag sampling (see above), under the same hypotheses described for the hair-snag sampling.

2.4.3 Rub tree data

Rub tree sampling was modeled according to 4 monthly session, from June to September, because rub trees were not all simultaneously installed or de-installed. The heterogeneity in sampling effort per session was modeled according to the cumulative number of rub tree sampling nights for each session, an index of sampling effort. We also modeled additional temporal variation in capture probability with 2 alternative variables: *i)* a simple *time effec*t (one parameter per session); *ii)* a *trend effect*. In addition, because of the uneven distribution of installed rub trees, we expected the spatial variation in capture probability to be markedly affected by variation in sampling effort (i.e., number of installed rub trees in different portions of the study area); to model it, we first calculated the center of all sampling locations for each bear; we then created a buffer equivalent to the average seasonal home range, for males and females separately (115 and 50 km^2, respectively; Tosoni 2010), and finally calculated the number of RTs in each individual "home range" weighted (i.e., multiplied) by the actual sampling nights (RT-nights within individual home ranges). We also included a *gender effect* and an effect of a previous hair-snag sampling (see hair snag data), and tested for a possible interaction between these two variables. As described above, the binary variable separating bears with previous hair-snag events from the ones never detected before through this method was expected to be highly correlated to a bear's age class. As the use of rub trees is known to be especially frequent in adult male bears (Green and Mattson 2003), we expected the interaction effect to be supported by the data.

2.4.4 Incidental samples data

For the incidental samples we did not have many modelling opportunities, as it was not feasible to estimate sampling effort; we therefore summarized the data in a single session. As a consequence, we only tested for a *gender effect* and for the effect of previous hair-snag events (see above).

2.4.5 Re-sights and photo-traps data

Similarly to incidental samples, all visual and/or camera-trap detections of marked bears during the sampling period can be combined into a single session, and eventually modeled according to a gender effect and recognizing the binary nature of this variable in the population (i.e., marked vs. unmarked bears). However, as additional data sources (i.e., rub tree sampling and opportunistic sampling at buckthorn patches) are available for the 2011 with respect to the 2008 survey, inclusion of this data source, due to some inherent limitations, will be considered only if it contributes significantly to the overall precision of the final estimate.

Besides including the effect of the variables potentially affecting variation in capture probability for each data source, we also tested for two additional forms of spatial variation in capture probability, likely acting in an additive way on all sampling methods: *i)* a violation of the geographic closure assumption, generating decreasing capture probability in the peripheral part of the study area; *ii)* a spatial heterogeneity in sampling efficiency, due to a differential knowledge of bear space and habitat use patterns in different portions of the study area. Regarding the first effect, we pooled all captures for each individual and used the distance of the center of these locations from the grid edge (DFE) to assess if some closure violation was supported by the data. We also tested a Log(DFE) and DFE^2 functions to assess if different shapes of the relationship were more supported by the data. Different from the issue of closure violation was that of a possible difference in sampling efficiency in the different parts of the study area. This hypothesis emerged from the fact that for some data sources (especially hair-snag and rub trees), the sampling performance was likely affected by our ability to identify good trap sites, proportionately more toward the central part of the study area than toward its external portions. To spatially describe this potential effect, we applied two approaches: *i)* as the study area broadly lies on a NW-SE gradient (represented by the main Apennine chain), and because most field and patrolling activities were concentrated in the proximity of the divide, we estimated for each bear the distance of its mean sampling location from the NW-SE backbone of the study area (DFC). This is as a measure of the distance of each sampled bear from the core of our field activity, and we tested for this effect both additively on all data sources, and separately for each of them; *ii)* we modeled the interaction between the latitude and the longitude of each bear sampling center. Although this provided a less mechanistic modeling of the process, this interaction potentially allowed to reveal if some portions of the sampling grid were consistently associated with higher (or lower) capture probability. Similarity to DFC, we tested for this effect both cumulatively for all data sources, and individually for each of them.

After generating the most parameterized general model, we fitted reduced models and assessed their relative support using the sample size adjusted Akaike's Information Criterion (AIC_c) of model fit. The model with the lowest value of the AIC_c was considered to be the most parsimonious (Burnham and Anderson 2002). We started from the most parameterized model, including all the above described effects and interactions for each data type. Then, we fitted less parameterized models for the hair-snag part only, while keeping the same structure for the rest of the design, and we identified the most parsimonious parameterization for this data type. Once the most supported variables were identified for the hair-snag part, we kept them constant for the rest of the model selection procedure, and repeated the same model selection approach with each of the remaining parts of the analytical design, thus finally identifying the most parsimonious general model. To account for the degree of uncertainty in model selection, we model averaged parameter estimates from all the fitted models, using the Akaike weights as an index of their relative support (Burnham and Anderson 2002). We calculated 95% log-based confidence intervals of model averaged population size estimates (White et al. 2002), accounting for the minimum number of bears known to be alive and in the study area, through all the available sampling tools (i.e., non invasive genetic sampling, observations, photo-traps).

Finally we estimated population size using a reduced sampling design that excluded hair-snagging (the most expensive and time consuming data source). By contrasting the two sampling scenarios in terms of the point estimate and its precision, we evaluated to what extent the population estimate depended on the hair-snag data, and thus whether this sampling method was essential to the upcoming estimate in summer 2014.

3. RESULTS

3.1 Preliminary activities

For a timely realization of the composite sampling strategy, we: searched and inventoried RTs with signs of bear use throughout the study area (October 2010 – July 2011); field checked and marked each individual hair-trap for its subsequent activation (April-May 2011); ranked buckthorn areas according to previous indications of bear use, and field investigated those most appropriate for the 2011 survey. Both for buckthorn and hair-snag sampling, the material for hair-trap construction was carried by foot at the time of trap activation, with the exception of a few remote locations for which we used a helicopter. In addition, preliminary activities (Table 3) included the preparation of the sampling material of each hair-trap, and the mixture of the final hair-snag lure and its partitioning into single jars of 5-10 lt each. To ensure the correct application of field protocols, we also conducted workshops with field personnel prior and during the actual survey. During the survey, we drafted and circulated progress reports after each sampling session (hair-snag sampling).

Activity	**Date** (from – to)
Organizational meetings (3)	October 2010 – April 2011
Search and inventory of RTs	October 2010 – July 2011
Selection and field investigation of buckthorn areas	April – July 2011
Preliminary field investigation and marking of individual hair-snagging traps	April 8 – May 23 2011
Training workshops with field personnel	April – August 2011
Preparation of barbed wire and tools for hair-snagging	May 15 – June 10 2011
Lure partitioning for single-trap use	May 25 – May 27 2011

Table 3. – Preliminary activities of the 2011 non-invasive sampling survey of the Apennine brown bear population.

3.2 Collected samples

Overall, from June to September 2011 and based on all non invasive genetic sampling techniques, we collected 679 bear hair samples, 599 of which (88.2%) were sent to the genetic lab for multilocus genotyping (Table 8); this proportion varied from 87.8% by opportunistic sampling at buckthorn sites to 100% by hair snagging.

3.2.1 Systematic hair-snagging

We hair-snagged bear samples in 25 (58.1%) out of 43 sampling cells, reflecting bear distribution across the entire study area (Fig. 3). Most (76%) successful grid cells provided bear samples in only 1 session, whereas 12% provided samples in 2 sessions, and an additional 12% in 3 sessions. From 5 to 10 traps provided samples in each sampling session, for a total of 34 successful hair traps out of 215 during all 5 sessions (15.8%). We cumulatively collected 159 bear samples during the 5 sampling sessions, ranging 12 – 76 samples per session, with an average (±SD) of 0.74 (±0.6) samples per trap (Table 4). All 159 collected bear samples were delivered for genetic analyses.

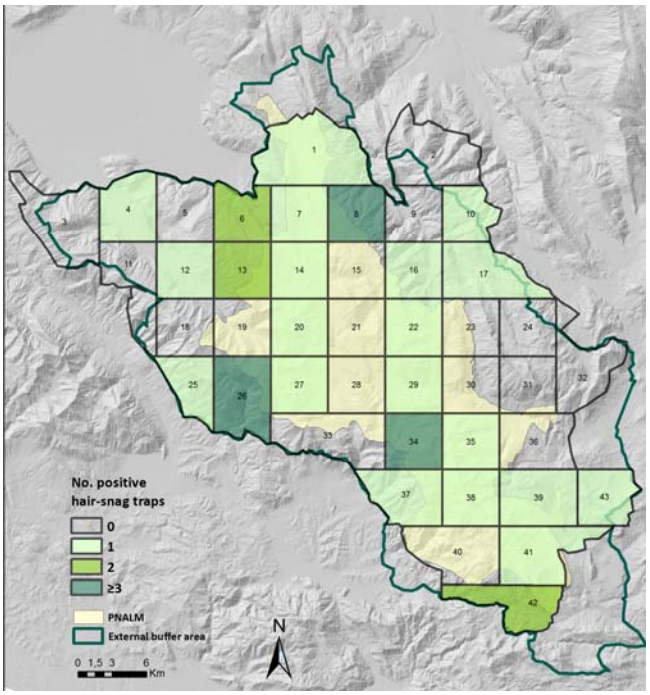

Figure 3. – Hair-snagging sampling grid adopted for the survey of the Apennine bear population (June – July 2011), and grid cell distribution based on the number of successful traps per grid. Sampled area encompass the PNALM and portions of its external buffer area and has been designed along topographic, habitat and anthropogenic features to ensure population closure.

Sampling session	Date[a]	Successful traps[b]	Bear samples	Bear samples/trap	
				mean	*range*
1	1 – 12 June	10 (23%)	76	1.8	2 – 27
2	12 – 23 June	5 (12%)	32	0.7	1 – 16
3	23 June – 4 July	7 (16%)	24	0.6	1 – 7
4	4 – 15 July	6 (14%)	15	0.4	1 – 4
5	15 – 26 July	6 (14%)	12	0.3	1 – 4
Total		*34 (15.8%)*	*159*	*0.74 (±0.6)*	*1 – 27*

[a]: because the date of deactivation of the trap could have been anticipated or postponed by 1-2 days, the average length of each sampling session (12 days) might have varied ±2 days

[b]: in parenthesis percentage of successful traps per sampling session

Table 4. – Results of hair-snag sampling by sampling session (Apennine bear population survey in the PNALM, June – July 2011).

3.2.2 Rub-tree sampling

From October 2010 to June 2011 we inventoried 147 RTs within the core area of the PNALM (about 28 RTs/100 km^2), 97 of which were installed with barbed wire for sampling (19 RTs/100 km^2). Surveyed rub trees, however, were unevenly distributed across the PNALM and all but one were exclusively within the PNALM (Fig. 4). Date of rub trees installation ranged from 2 June – 8 August 2011, even though 92% of surveyed rub trees were activated by the end of June. Sampling period ranged from 53 to 120 days per RT ($\overline{X}$ ±SD=107±14 days/rub tree), during which we visited installed rub trees at a frequency of 1-13 days, for an overall average of 9 (±4) visits per RT. Thirteen activated rub trees were also discontinuously monitored by means of camera-trapping (April – October 2011), 8 of which provided a total of 16 clips of bears rubbing or investigating rub trees (see §§3.2.5 and 3.3.4).

Overall, we collected 278 bear samples by rub tree sampling. Fifty-six of the armed RTs (57.1%) provided bear samples on at least one sampling session (Fig. 4), for an average of 5 (±3.6) bear samples/rub tree; in each session, from 23 to 41% installed rub trees proved successful, providing from 2 to 3.5 bear samples/rub tree

(Table 5). As the 278 collected bear samples included 25 inadequate samples (few hairs and/or no bulb), so that only 253 were sent for genetic analysis, comprising 33 back-up samples (i.e., samples believed to belong to the same bear already sampled in the same sampling occasion).

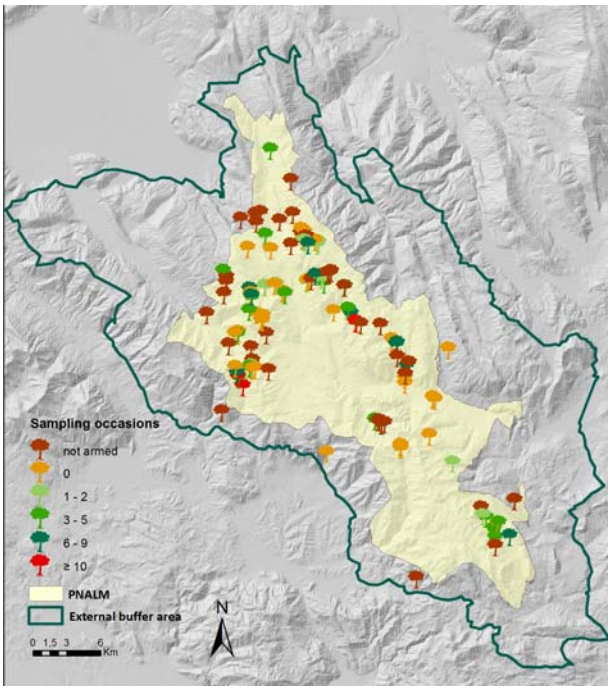

Figure 4. – Distribution of rub trees inventoried (n=147) within the PNALM (October 2010 – August 2011), 97 of which have been installed with barbed wire to collect samples for the assessment of the Apennine bear population (June – September 2011). Installed rub trees are ranked according to their overall sampling success (bear samples/rub tree throughout the whole sampling period).

Sampling session	Date[a]	Installed rub trees (no.)	Rub tree effort[b]	Rub trees with bear hair[c]	Bear samples	No. bear samples/rub tree[d]
1	3 – 30 Jun	72	1123	26 (36.1%)	61	2.4 (±2.5)
2	1 – 31 Jul	85	2511	35 (41.2%)	123	3.5 (±2.0)
3	1 – 31 Aug	86	2263	20 (23.2%)	43	2.2 (±1.2)
4	1 – 30 Sept	89	3096	25 (28.1%)	51	2.0 (±1.1)
Total		*97*	*8993*	*56 (57.1%)*	*278*	*5 (±3.6)*

[a]: for modeling purposes, sessions were aribtrarily defined according to monthly intervals
[b]: cumulative number of installed rub trees multiplied by the number of days each has been surveyed within the session
[c]: in parenthesis percentage of successful rub trees per sampling session
[d]: mean ± SD

Table 5. – Results of rub tree sampling by sampling session (Apennine bear population survey in the PNALM, June – July 2011).

3.2.3 Opportunistic sampling at buckthorn patches

We cumulatively installed 19 hair traps for non-invasive, opportunistic sampling in 7 buckthorn sites (Fig. 5), visiting traps every 7 –12 days since installation, for an average session length of 8 (±1) days from August 17 through September 2011. Due to differences in the actual installation date, total sampling period by buckthorn site ranged from 32 to 40 days, for an average of 36 (±3) days per site (Table 6).

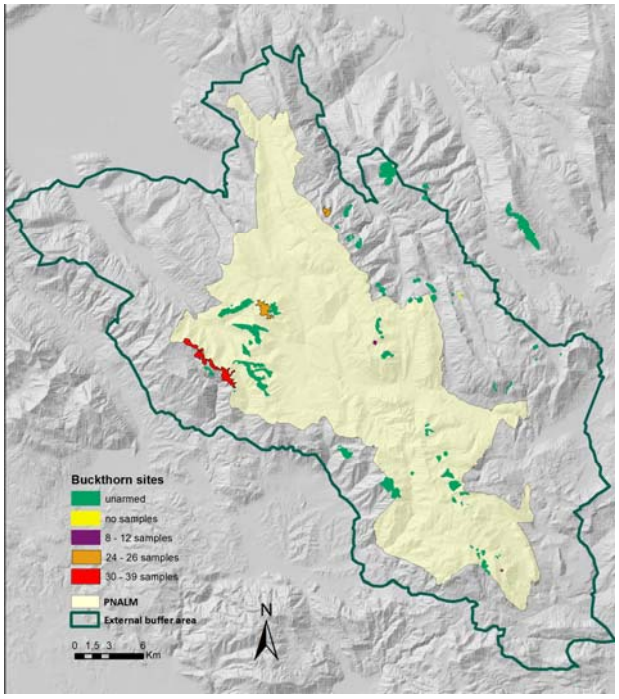

Figure 5. –Distribution of the inventoried buckthorn sites (n=36), 7 of which were armed with hair traps (17 Aug – 29 Sept 2011) to provide opportunistic samples for the assessment of the Apennine bear population. Armed buckthorn sites are ranked according to their sampling success (bear samples collected across the whole sampling period).

Overall, 85.7% (n=6) of the installed buckthorn sites proved successful, each providing on average of 19.9 (±14) bear samples, ranging from none to 39 (Table 6), for a total of 139 collected bear samples. During each session, successful buckthorn sites provided 1 – 30 bear samples per site at a mean rate of 4 (±4.2) samples/site, although with high variability from session to session (Table 7). Out of the 139 collected samples, 122 were considered feasible for genetic analyses. As we found no hair samples in all buckthorn sites during the last 2 sessions (Table 7), we excluded these two sessions from the individual encounter history for CR modelling.

Buckthorn site	**No. traps**	**Total trap length (m)**	**Sampling period**		**Bear samples**
			from – to[a]	days	
1	1	130	17 Aug – 24 Sept	38	12
2	3	68	18 Aug – 23 Sept	36	0
3	3	80	18 Aug – 26 Sept	39	8
4	4	80	19 Aug – 28 Sept	40	39
5	2	74	22 Aug – 27 Sept	36	26
6	3	65	23 Aug – 26 Sept	34	30
7	3	104	27 Aug – 28 Sept	32	24

[a]: date refer to installation date and date of last visit (de-installation) of hair trap in each buckthorn sites

Table 6. - Results of sampling at the 7 buckthorn sites. Each site was activated with two strands of barbed wire encircling most productive buckthorn patches, and 1-4 traps of different perimeter length were activated per site (Apennine bear population survey in the PNALM, August – September 2011).

Sampling session[a]	Successful sites[b]	Sampling effort[c]	Bear samples	Bear samples/site	
				mean	*range*
1	4 (57.1%)	5065	44	6.3 (±11)	1 – 30
2	6 (85.7%)	4829	69	9.9 (±7)	5 – 19
3	5 (71.4%)	4314	26	3.7 (±5)	1 – 13
4	0	5318	0	-	-
5	0	2384	0	-	-
Total	*6 (85.7%)*		*139*	*4.0 (±4.2)*	*1 – 30*

[a]: actual session dates depend on installation date and vary by buckthorn site (cf. Table 5)

[b]: in parenthesis percentage of successful buckthorn sites per sampling session

[c]: total number of trap-nights per session multiplied by length of barbed wire installed

Table 7. – Results of opportunistic sampling at buckthorn sites by sampling session (Apennine bear population survey in the PNALM, June – July 2011).

3.2.4 Incidental sampling

From June through September 2011, 67 bear samples were collected incidentally to field and patrolling activities, during verification of alleged damages by bears, or provided by other researchers (ARP Lazio) from a localized hair-trap in a peripheral site of the PNALM (Fig. 6). Sixty-five (64 plus 1 backup) of the incidentally collected samples were delivered for genetic analyses.

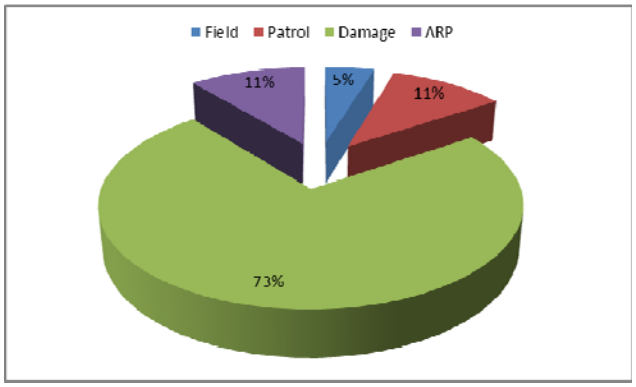

Figure 6. – Distribution of hair samples (n=67) collected incidentally to field (Field) or patrolling (Patrol) activities, during verification of alleged damages by bears (Damage), or provided by other researchers (ARP) (PNALM, June – September 2011).

3.2.5 Direct observations and photo-traps

We cumulatively detected 12 bears (9 females and 3 males) through direct sightings and camera-trapping of previously marked bears during the non-invasive survey period (June – September 2011), 11 by direct sightings and 3 by camera-trapping. Whereas bears detected through camera-trapping had all been sampled through the other sampling techniques, 3 of the bears detected through direct observations had not been genetically sampled (Appendix 1). Assuming all 23 marked bears (13 females and 10 males) were still potentially available to sampling during the 2011 survey, the maximum theoretically achievable probability of being detected was 0.43 (0.69 for females and 0.30 for males) and 0.13 by direct observations and camera-traps, respectively. Several camera-trap detections of other marked bears occurred after the end of the genetic sampling project, especially in October and November, as a result of an enhanced effort; however, including these data would have required extension of the sampling period to 6 months, perhaps stretching the demographic closure assumption. Based on the marked bears which have been sampled and/or observed during the 2011 survey (n=18; Appendix 1), there are 9 remaining bears among those previously (2005-2010) marked (n=27), 4 of which have been reported dead and the other 5 have been not detected since 2007 – 2010 (Appendix 2).

In any event, as sample size and probability of capture determined through the other genetic techniques in the 2011 survey was satisfactory, we deemed preferable not to include this data source into the final modeling as to avoid unnecessary complexity and uncertainty (due to untested assumptions; see Discussion), and also

because it would have contributed with only 2 additional (unique) bears with a negligible effect on the final estimate's precision.

From 22 April through 1 September 2011, we obtained 16 clips of bears interacting (rubbing and/or investigating) at 8 different rub trees among the 13 rub trees which we monitored by means of camera-traps (see also § 3.3.4). Other clips at the same rub trees portrayed other wildlife (wolves, foxes, stoat, badger, red deer, wild boar, porcupine, red squirrel) and domestic (dogs, cattle, mule, horse) species using bear rub trees as scent communication posts.

3.3 Genetic analyses

3.3.1 Success rate and culled samples

In total, 599 alleged bear hair-samples collected by all four sampling techniques have been considered for genetic analyses, including 34 replicated samples (33 from RT sampling and 1 from incidental sampling). Most (42.2%) of the collected hair samples (n=599) were obtained by RT sampling, followed by HS (26.5%), buckthorn (20.4%) and incidental (10.9%) sampling (Table 8). Out of these, 28 samples have not been used since they were replicates of samples that were analyzed successfully, and 42 were not analyzed as they contained no guard hair roots and <5 underfur; many of these were broken guard hair shafts lacking roots, the majority of which from hair-snag and buckthorn samples (n = 34).

	Total	**Sampling method**			
		HS	RT	OPP	INC
Collected	679	159	278[a]	139	67
to lab	599	159	253[b]	122	65[c]
replicates not analyzed	28	-	27	-	1
inadequate	42	17	4	17	4
Analyzed	529	142	222	105	60
culled	103	40	40	6	17
successful	426	102	182	99	43
Genotypes	45	26	21	22	10
Unknown genotypes[d]	16	8	4	8	2
Unknown/all genotypes	0.36	0.31	0.19	0.36	0.20
Uniquely detected genotypes[e]	-	8	2	8	2
Genotypes sampled only once	4	4	1	5	6
Genotypes/analyzed sample	0.09	0.18	0.09	0.21	0.17
Uniquely detected genotypes/ analyzed sample	-	0.06	0.01	0.08	0.03
Euro/genotype[f]		325	600	284	298

[a]: including 43 alleged back-up samples (i.e. replicated samples from the same rub tree sampling event)
[b]: including 33 alleged replicated samples
[c]: including 1 alleged replicated sample
[d]: number of genotypes unknown from previous non-invasive surveys (2000 – 2008) and live-trapping projects (2006 – 2010)
[e]: number of genotypes uniquely sampled by a given sampling method
[f]: based on analyzed samples (n=529)

Table 8. – Descriptive statistics of 599 bear hair samples collected in the National Park of Abruzzo Lazio and Molise (June – September 2011) by four sampling methods (HS: systematic hair snagging; RT: rub-tree sampling; OPP: opportunistic sampling at buckthorn patches; INC: incidental sampling).

Of the remaining samples which were analyzed (n=529), 103 were culled whereas 426 samples, including 6 replicates of previously failed samples, had high-confidence scores for all 14 markers (Fig. 7). Overall success

rate was 80.5%, ranging from 71.8% by HS to 94.3% by buckthorn sampling, with rub tree and incidental sampling at intermediate values (Table 8).

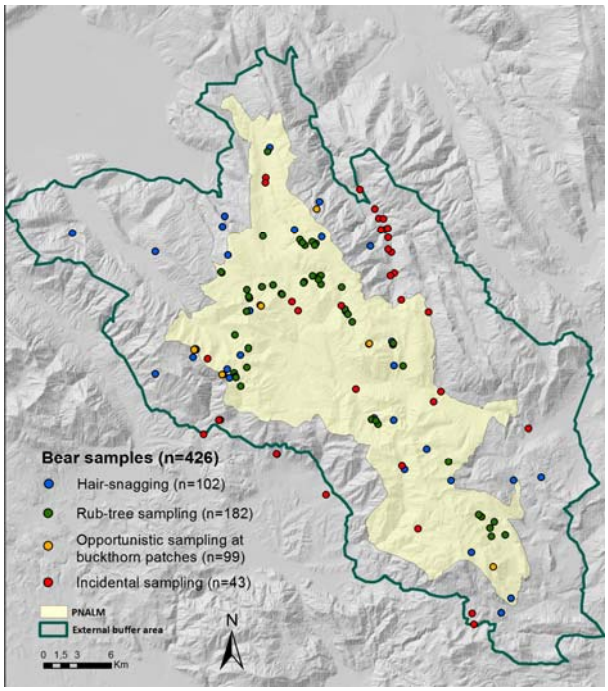

Figure 7. – Distribution of the 426 successufully genotyped bear samples, based on the four sampling strategies (PNALM, June – September 2011).

Proportions of culled samples by sampling method differed by both proportions of collected and analyzed samples ($18.1 \leq G_{adj} \leq 19.8$, d.f.=3, $p < 0.0005$), and proportionally more samples were culled for HS and incidental sampling than for rub tree and buckthorn sampling (Fig. 8). Overall, HS identified the largest number of bears, but had the greatest share of failed and inadequate samples, which cumulatively accounted for 35.8% of HS collected samples, whereas rub tree and buckthorn sampling had the lowest (18.9% and 19.5%, respectively; Table 8).

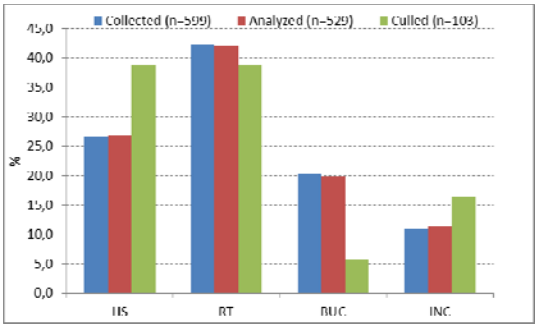

Figure 8. – Distribution, by sampling technique, of bear hair samples which have been collected and successively used (successful) or culled for genetic analysis (PNALM, June – September 2011).

Success rates also varied according to the amount of hair available for extraction. Unsurprisingly, the best group was extracts based on > 2 guard hair roots, which enjoyed an 85% success rate. By contrast, the success rate from 1 or 2 guard hair roots was 72%, and extracts from underfur yielded 69% success. The lowest mean number of guard hair roots per extract (treating underfur as equivalent to 0.2 guard hairs) was for HS samples, at 5.7, while the incidental samples had the highest value, at 7.9, indicating that differences in success rate between collection methods were not caused entirely by variation in the amount of hair collected.

In terms of post-quality control, all the samples attributed to previously collared bears were consistent with their estimated home-ranges during the previous years, and all non invasive samples attributed to the same individual were spatially distributed within expected distances and at reasonable patterns, revealing no suspicious cases. However, due to some potentially equivocal results (e.g., single samples, samples collected from the same hair trap in adjacent barbs and assigned to different individuals) some samples have been regarded as candidates for further genetic control and evaluation (Appendix 3). From this process, 1 out of 5 equivocal samples was culled as possibly being a mixture, and 4 were confirmed for the 2011 population assessment.

3.3.2 Marker power for individual identification

Having used data replication to rule out genotyping inaccuracies as a meaningful source of error, we turn to the task of estimating the probability that we sampled one or more pairs of individuals with identical multilocus genotypes ('0MM- pairs'). This would lead to underestimating the true number of individuals from which these samples were obtained, a non-trivial subject for a study population with such a low variability.

Calculated match probabilities vary by orders of magnitude depending on what assumptions one makes about the degree of relatedness among the sampled animals. For example, siblings have vastly higher match probabilities than unrelated animals, but the proportion of siblings in the dataset is unknown. As a result, calculated match probabilities provide little practical insight into the risk of a false match within a given dataset, especially in a study area where consanguinity might be expected to be high. By contrast, extrapolation from an observed distribution of genotype similarity is robust across a range of marker variability and degrees of consanguinity (Paetkau 2003 and subsequent experience), and thus provides a reasonable estimate of how many 0MM-pairs we might have sampled with a given set of markers.

We observed no 1MM-pairs and just a single 2MM-pair among the 55 individuals sampled in 2011 (25 from blood and hair samples, and 45 from hair samples) and identified using 14-locus data. Extrapolation from this curve suggests no realistic chance of having sampled any 0MM-pairs (Fig. 9). In other words, we used more markers than strictly necessary to achieve a low match probability.

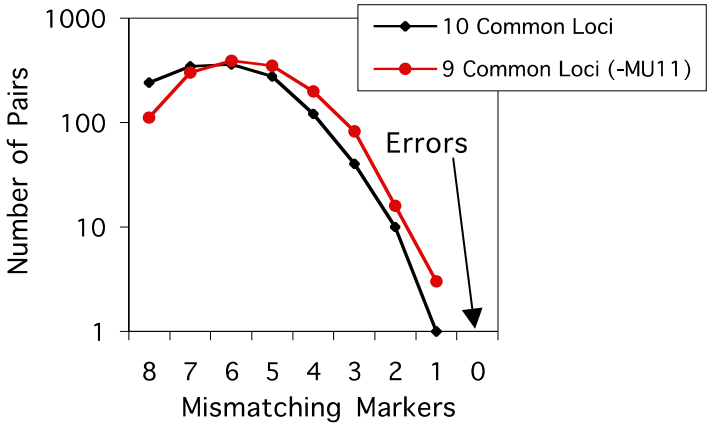

Figure 9. – Mismatch distributions for the 55 individuals in the current results based only on markers common to both WGI and ISPRA labs. The increased number of 1MM- and 2MM-pairs when MU11 is removed from the dataset probably indicates an unacceptable increase in match probability. We therefore recommend that all 10 common markers continue to be used in future analyses (cfr. Tables 1 and 2).

This conclusion leads to the subtler question of how many markers would be ideal for this study population. In fact, it is not desirable to analyze more markers than necessary, because each additional marker adds to cost, creates new opportunities for genotyping error, and takes away from success rate, as the same amount of DNA has to be spread across more markers (Waits and Leberg 2000, Paetkau 2004). As previously discussed (see § 2.3.2), WGI faced a constraint as enough markers of those previously used by ISPRA had to be retained in order to ensure a low match probability in comparisons between labs. With this constraint in mind, we produced a mismatch distribution: (a) for the 10 ISPRA markers (including gender) that were included in the WGI set of 14, and (b) for a dataset without MU11, the least variable of the 9 microsatellites common to both labs (Fig. 9). In

other words, we evaluated if it was possible to drop 1 of the 9 microsatellites common to both labs but still ensure reliable comparison of genotypes between labs. However, this did not seem to be advisable, as the 9-locus mismatch distribution depicted 3 1MM-pairs, illustrating the real possibility of encountering false matches in any comparison should they be limited to these 9 markers. Even the 10-locus mismatch distribution, which included 10 2MM-pairs and 1 1MM-pair, suggested some risk of encountering 0MM-pairs if the number of individuals in the dataset grows significantly. This provided the first information required to identify an ideal marker system, as this must include all 10 of the markers currently common to both labs (cf. Table 2).

If any of the 10 common markers (9 microsatellites) cannot be dropped, then the best candidates for elimination are MSUT-2 and G10X, which have only been used at WGI, and which have HE < 0.5 in the current results (Table 1). WGI therefore created mismatch distributions with successively fewer markers, dropping first G10X (a problematic marker to start with, requiring excessive rounds of cleanup), and then MSUT-2. WGI also considered the removal of MU11, since this would be the logical candidate to remove if we were to drop from 12 to 11 markers (Fig. 10).

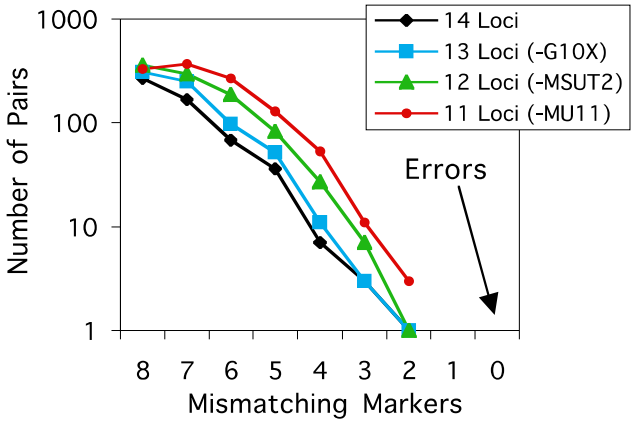

Figure 10. – Mismatch distributions for increasingly smaller subsets of the current group of 14 markers. The removal of G10X and MSUT-2 has little meaningful impact on the implied match probability, which remains very low with 12-locus data. The further removal of MU11, a marker common to both labs, causes a jump in the number of 2MM- pairs, and is thus less attractive (even less so for comparisons between labs; Fig. 9).

This exploration of marker number continued to find just a single 2MM-pair, and no 1MM-pairs, with 12 markers, after removing both G10X and MSUT-2. Given the cost reduction (about € 4.6 per sample) and efficiency gain that this removal would effect, we recommend that these 2 markers not be used for individual identification in future non-invasive genetic surveys. It has been already concluded that further reductions in the number of markers common to both labs are unwise (Fig. 9), but the 11-locus mismatch curve without MU11, where we see 3 2MM-pairs (Fig. 10), confirmed that this marker needs to be retained in future analyses. In summary, we recommend that future analyses of individual identity involving the Apennine bear population use 12 markers, including gender and the 11 microsatellites listed above the middle line in Table 1 (i.e., all 13 microsatellites except G10X and MSUT-2). In addition, as it was done for the analyses hereby illustrated, marker *G10P* can also be used to better assess suspicious mismatch cases when comparing sampled between labs.

3.3.3 Bear genotypes detected in 2011

The 426 hair samples which provided successful genotypes were assigned to 45 bears, 15 of which were among the 25 previously live trapped bears whose multilocus genotype was typed from blood smears (of which at least 4 died before the 2011 survey; Appendix 2). The remaining 30 non-invasively sampled genotypes comprised 14 bears non-invasively sampled in previous surveys (2002 - 2008) and recaptured in 2011, and 16 bears which had never been sampled before. The latter include, in unknown proportions, bears already in the population in the years before 2008 (i.e. the year of the last survey) but that were never sampled during previous genetic surveys, and new bears added to the population after 2008.

We hypothesized that the newly detected genotypes might also include the 3 cubs of marked female F10, the only litter we estimated to be born in the population in 2011 (Ciucci et al. 2011a). Therefore, to assess if the new genotypes comprised one or more of the F10 cubs, we compared all genotypes simultaneously sampled along with that of F10, by assuming that her cubs would have been sampled at least once at the same location and sampling occasion along with their mother. Although F10 was unfortunately sampled only once, 3 genotypes are nevertheless strong candidates for F10s cubs based on the following:

(a) they have never been sampled in previous surveys (2000 – 2008);
(b) they share at least one allele with F10 at all 13 loci (excluding gender; Table 9);
(c) no other genotypes but one[1], among the 16 newly detected in the 2011 survey, have such a close similarity to F10's genotype, displaying from 1 – 6 loci with different alleles than F10;
(d) they have been sampled all together at the same buckthorn site and sampling occasion along with F10, and at the same trap number, side and portion of the trap (barbs 5-31; Tables 9, 10);
(e) the three candidate cubs were sampled always together and at the same buckthorn site and trap in 2 different sampling occasions (3 and 11 September) (Table 10).

Based on the above, these putative 3 cubs have been detected from 3-5 samples each, and comprise one female and two males (Table 10). In addition, when the putative maternal contribution of F10 is subtracted from the genotypes of the 3 offspring, the remaining (paternal) alleles can all be matched to the same male (M13, an adult male sampled by different sampling methods in the same general area; Table 10). In fact, it is far less likely to see a random male and female account for all of the alleles in a set of offspring's genotypes than for a random female alone to share 1 allele per locus, particularly in a population where many markers have only 2 circulating alleles.

Genotype	*G10B*	*G10C*	*G1D*	*G10L*	*MSUT-2*	*MU59*	*REN144A06*	*CXX20*	*MU50*	*MU51*	*G10X*	*MU05*	*MU11*	*Sex*
F10	140.156	203.207	172.172	157.163	203.203	229.235	109.127	137.137	136.136	206.206	129.129	135.135	188.188	F
RAM'042	140.156	203.203	172.172	157.163	203.203	229.235	109.127	137.139	132.136	206.206	129.129	135.137	188.188	M
RAM'048	140.156	207.207	172.186	157.163	203.203	229.229	109.127	137.137	132.136	206.212	129.129	135.137	188.192	F
RAM'045	140.140	203.203	172.186	157.163	203.203	229.235	127.127	137.137	132.136	206.212	129.129	135.137	188.188	M
HS'058	140.156	203.203	172.172	163.163	203.203	229.235	127.127	137.139	132.132	206.206	129.129	137.137	192.192	M
M13	140.156	203.207	172.186	157.157	195.203	229.235	109.127	137.139	132.132	206.212	129.135	137.137	188.192	M

Table 9. – Multilocus genotypes detected by sampling at buckthorn site no. 7 in the same sampling occasion (3 Sept 2011) when F10 was sampled (see Table 8). From direct observations (Ciucci et al. 2012b), F10 was known to be part of a family unit with 3 cubs. Bold letters in the first column indicate candidate genotypes for F10's cubs among the other 5 genotyped detected in the same sampling occasion. Excluding M13, which is a known adult male (and putative father of F10's cubs; see text), the three bold genotypes share with F10 one (highlighted in green) or both (no background color) alleles at all loci, whereas the other sample collected in the same occasion (HS058) has unshared alleles with F10 at three loci (highlighted in red). Only genotypes collected on Sept 3 are shown, but the same check has been done with all other genotypes detected in the 2011 survey (see text).

Recapture rates by rub-tree sampling were strongly male-biased (1 female every 4 males), whereas they were female-biased by opportunistic sampling at buckthorn patches (1 male every about 3 females), with somewhat intermediate values for the other two sampling methods (Table 11). However, based on the total number of genotypes (n=45) detected by all sampling methods, we empirically revealed an overall female-biased sex-ratio of 1.25:1 in the population, although it varied slightly based on sampling method (Table 11). For reference, the sex-ratio based on 26 live-trapped bears from 2005 to 2010 was 1.0 FF:MM.

[1] sample RT187, never sampled before the 2011 survey, was detected twice in 2011 (9 and 16 July) at the same rub tree located within the annual home range of F10. Analogously to RAM042, RAM045 and RAM048, also RT187 share at least one allele with F10 at all 13 loci; in addition, similarly to M13 for the above 3 putative cubs of F10 in 2011, alleles of M12 match the paternal contribution to RT187 genotype.

Genotype	Sample ID	Date	Trap no.	level	Trap side	Barb no.
RAM042	RAM042	03/09/2011	71	lower	1	5
	RAM046	03/09/2011	71	lower	1	30
	RAM074	11/09/2011	71	lower	5	59
	RAM076	11/09/2011	71	upper	1	37
	RAM083	11/09/2011	73	upper	2	50
RAM045	RAM045	03/09/2011	71	lower	1	27
	RAM049	03/09/2011	71	upper	2	36
	RAM050	03/09/2011	71	upper	2	37
	RAM079	11/09/2011	73	upper	2	9
	RAM082	11/09/2011	73	lower	2	36
RAM048	RAM048	03/09/2011	71	lower	1	31
	RAM073	11/09/2011	71	lower	1	44
	RAM078	11/09/2011	73	lower	1	60
F10	RAM043	03/09/2011	71	lower	1	11

Table 10. – Sampling chronology of the three genotypes (in bold) candidate to be the 3 offspring of F10, known from direct observations to be part of a family unit with 3 cubs. Samples were all collected at the same buckthorn site (no. 7) in 2 sampling occasions, the first of which (3 Sept) along with F10, their putative mother (see Table 7).

Sampling method	Samples			Genotypes		
	Females	Males	Sex-ratio	Females	Males	Sex-ratio
HS	53	49	1.08	14	12	1.17
RT	35	142	0.25	11	11	1.00
OPP	72	27	2.67	14	8	1.75
INC	19	24	0.79	5	7	0.71
total	*179*	*242*	*0.74*	*25*	*20*	*1.25*

Table 11. – Recapture rate by sex (samples) and sex-ratio (genotypes) based on hair samples collected in the bear population in the PNALM (June – September 2011) and sampling method (HS: systematic hair-snagging; RT: rub-tree sampling; OPP: opportunistic sampling at buckthorn patches; INC: incidental sampling).

3.3.4 Genotypes detected by rub-tree and incidental sampling

Detection of genotypes allowed us to investigate if sampling methods such as rub tree and incidental sampling reflected patterns due to gender and individual behaviour, and whose implications could be relevant for the survey itself or from a management point of view. For instance, rubbing behaviour by bears is expected to be primarily displayed by males during the mating period (Green and Mattson 2003), although no information has ever been reported for the Apennine bear. Similarly, as incidental sampling mostly accounted for alleged damages by bears to crops, beehives and livestock, we were interested in assessing how many of these events were actually caused by bears and if some of these bears were proportionally more often involved than others in causing damages.

In total, 21 genotypes were detected by rub-tree sampling, 10 of which were males and 11 females (50% and 44% of all males and females, respectively, detected by all sampling methods). Out of 182 successfully genotyped samples collected at rub trees, 57 (31.3%) revealed to be replicates (i.e., >1 samples collected on the same RT and sampling occasion, and left by the same bear). Excluding these replicated samples, 80.2% (n=101) out of the remaining 126 rub-tree samples were left by 10 males, on average at a rate of 10 samples/male, whereas the remaining 19.8% (n=25) were left by 11 females (2.3 samples/female). Four (M11, M13, M12, M09) out of 10 detected males accounted for 75.2% (n=76) of the male samples, with 10 – 37 samples each, while the others males were sampled with 2-6 samples each (Fig. 11). Contrarily, only one

(HS028) out of 11 detected females accounted for 24% (n=6) of the female rub tree samples, while the others accounted for 1-3 samples each (Fig. 11).

Based on the 8 rub trees monitored by camera-traps for which we obtained video clips portraying bears (n=16), in 6 out of 8 rubbing event video-captured during the non invasive sampling period (June – September) we associated the collection of hair samples; these were collected from 0 to 5 days after the bear was filmed (Table 12). In 3 of such cases, the rubbing bear portrayed in the clip was individually recognized as a previously known (i.e., collared bear), and in all 3 cases the successively detected genotype matched the individual bear; the remaining 3 cases involved previously known bears but marked solely with eartags at the time of the clip so that we did not visually recognize them, although they were later revealed by genotype (Table 12).

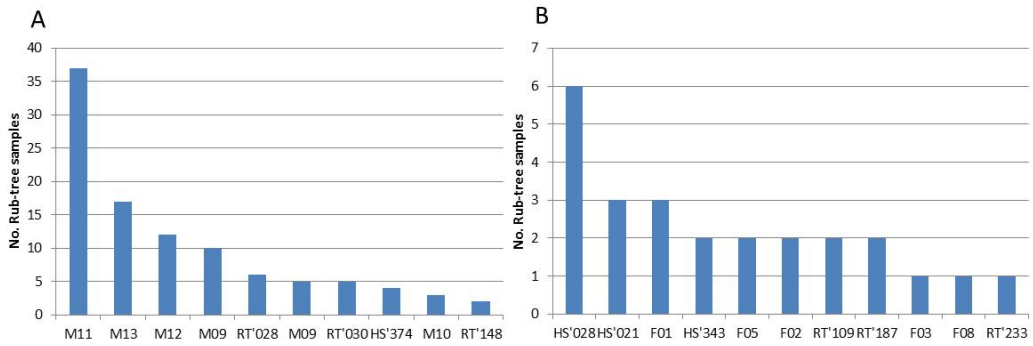

Figure 11. – Distribution of unduplicated rub-tree samples (n=126) collected from (A) 10 males (n=101) and (B) 11 females (n=25) based on the 21 detected genotypes (displayed on the x-axis) (PNALM, June – September 2011).

Rub tree code	Clip				Hair sample		Genotype
	date	hour	notes[a]		code	date	
RT15	7 Jul	09:10	Rubbing; unmarked bear with hairless patch (mange?) on rump		RT'174	8 Jul	M09
RT46	11 Jul	17:45	Investigating; eartag (unrecognized)		-	-	-
RT46	18 Jul	19:39	Rubbing, unmarked bear, dermatitis		RT'200	23 Jul	M13
RT75	9 Aug	04:18	Rubbing, unmarked bear		RT'241	13 Aug	M12
RT75	12 Aug	05:12	Investigating, unmarked bear		-	-	-
RT75	12 Aug	19:58	Investigating, unmarked bear		-	-	-
RT75	24 Aug	04:10	Passing by, unmarked bear		-	-	-
RT80	22 Apr	04:15	Rubbing, unmarked bear		-	-	-
RT80	24 Apr	07:11	Investigating, unmarked bear		-	-	-
RT83	18 Jul	06:29[b]	Rubbing, M11		RT'199	22 Jul	M11
RT83	1 Sept	00:27	Rubbing, eartag (unrecognized)		-	-	-
RT87	May	01:59	Rubbing, M10		-	-	-
RT87	June	12:16	Investigating, collared (unrecognized)		-	-	-
RT114	16 Jul	02:09	Rubbing, F08		RT'195	16 Jul	F08
RT114	18 Aug	19:49	Rubbing, M11		RT'280	23 Aug	M11
RT144	7 Aug	18:17[b]	Investigating, unmarked bear		-	-	-

[a]: unmarked bears include cases in which collars and/or eartags could have not been visible in the clip due to distance, light or perspective

[b]: 2 clips in succession

Table 12. – List of the 16 video clips (Multipir and Keep-guard IR cameras) portraying bears at 8 rub trees (PNALM, 22 April – 1 September 2011) and corresponding collection of hair samples for genotype identification.

Although the majority of positive rub trees were used by males only, trees were also simultaneously rubbed by both males and females, as well as by females only (Fig. 12). We detected samples left by 1 to 5 bears on the same rub tree and, although most rub trees (89%) were used by 1-2 bears only, 11% (n=6) were used by 3-5 bears (Fig. 13). Based on the samples we collected, males used proportionally more rub trees than females (Fig.

14). Each male we detected used from 2 to 21 different rub trees ($\bar{X}$ ±SD=6.6 ± 5 rub tree/male), whereas females used from 2 to 4 rub trees ($\bar{X}$ ±SD=1.7 ± 1 rub tree/female).

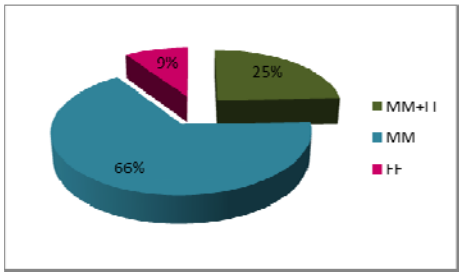

Figure 12. – Distribution of rub trees (n=56) used by bears according to gender (PNALM, June – September 2011).

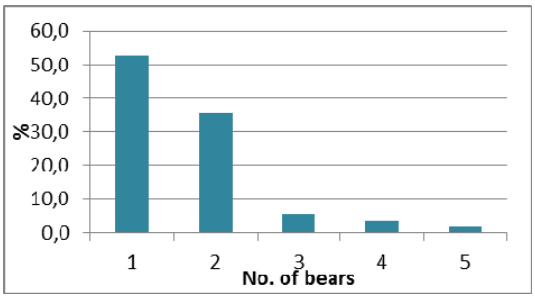

Figure 13. – Distribution of rub trees (n=56) according to the number of individual bears rubbing on them (PNALM, June – September 2011).

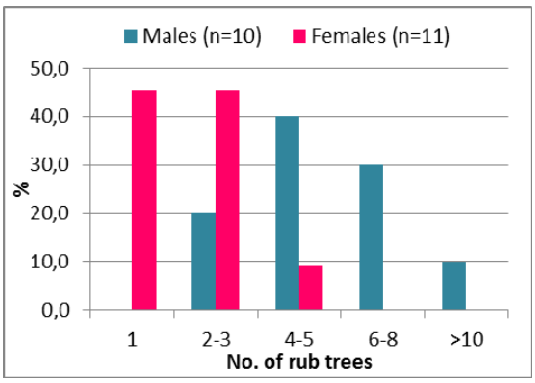

Figure 14. – Distribution of detected bears based on their gender and the number of rub trees (n=56) they used during the months of the survey (PNALM, June – September 2011). One single male (M11) was detected on 21 different rub trees.

Base on the number of bears detected during the survey period, standardized by the number of armed rub trees on a 15-day basis, rubbing extended from June – September in males, and from July – September in females (Fig. 15). Although the number of both males and females detected by rub tree sampling peaked during the second week of July, rubbing was common among males since the first week of June, and both sexes sharply decreased their rubbing activity by second week of August.

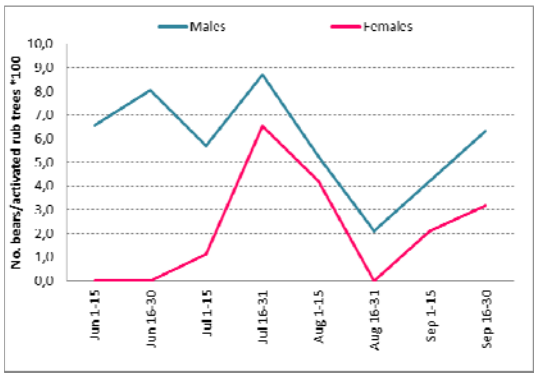

Figure 15. – Number of male and female bears detected by rub tree sampling, standardized by sampling effort (number of armed rub trees), on a bi-weekly basis (PNALM, June – September 2011).

Concerning incidental sampling, 47 (72.3%) of the samples were collected during verification of alleged damages caused by bears, 36 of which provided genotypes of 10 different bears (Table 13; Fig. 16). These included 5 previously marked and 5 previously unknown bears. The former included 3 bears known to cause recurrent damages (F01P, M08, M11). Whereas these results have relevant management implications, they also indicate that our final estimate of population size does comprise problem or 'management' bears.

Bear Code	No. samples	Sampling occasions	Note
M11	12	9	known problem bear
FP01	11	9	known problem bear
M08	4	3	known problem bear
M10	2	2	occasional depredation
ACC69	2	2	
ACC79	1	1	
M12	1	1	occasional depredation
HS021	1	1	
HS477	1	1	
RT233	1	1	

Table 13. – List of the 10 genotypes detected from hair samples collected during verification of damages to crops, beehives and livestock allegedly made by bears. However, out of 47 such samples, 11 (23.4%) did not prove adequate for DNA extraction (PNALM, June – September 2011). Six out of these 10 genotypes match with previously typed bears, 3 of which have been known as problematic (see also Fig. 16).

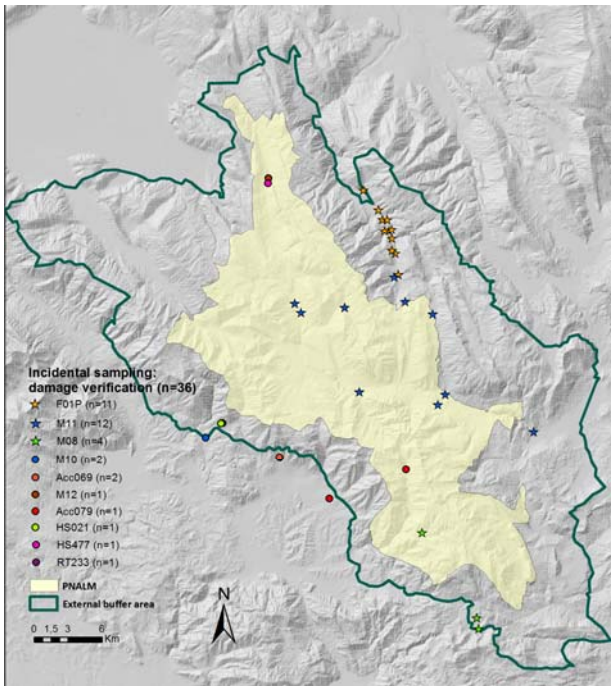

Figure 16. – Spatial distribution of bear samples (n=36) collected by incidental sampling during verification of alleged damages to crops, beehives and livestock by bears. Individual genotypes revealed 3 bears timely known to be problematic (F01P, M11, M08), although other 7 bears have been infrequently detected at damage sites (PNALM, June – September 2011).

3.3.5 Efficiency of detection by sampling method

In terms of sampling efficiency, and excluding inadequate samples, 5 out of every 7 collected samples yielded a successfully analyzed sample. However, due to the high re-sampling rates of some of the sampling methods, it took on average more than 13 collected samples through our integrated sampling strategy to detect one of all the 45 genotypes we revealed. Relative efficiency was lower for rub-tree sampling, with about 12 collected samples needed to detect one out of the 21 genotypes revealed by rub tree sampling (Table 8), although this figure does include most of intentionally collected back-up samples at the same RT and sampling occasion. Most efficient were hair snag and opportunistic sampling at buckthorn sites, with about 5 collected samples needed to detect one genotype.

We detected the highest number of different genotypes by hair-snagging (n=26), and both hair-snagging and opportunistic sampling at buckthorn sites accounted for the highest absolute number of previously unknown genotypes (n=15 for both; Table 8). These two sampling techniques also accounted for the highest number of genotypes exclusively sampled by a single sampling method (n=8 for both sampling methods), thereby providing precious information within the integrated data sources approach. Nevertheless, the proportion of unknown to known genotypes was higher for incidental sampling, possibly accounting for the lower absolute number of genotypes detected by this sampling technique. This is possibly due to the fact that many of the unknown genotypes were sampled rather marginally with respect to the core of the PNALM (Fig. 16), possibly due to a local prevalence of young and subordinated individuals with lower probability of detection with the other sampling techniques. However, incidental sampling ranked highest in terms of genotypes sampled only once (6 out of 10 detected genotypes), even though these cases reduced to only 4 genotypes out of 45 (8.9%) integrating all sampling methods.

Rub-tree sampling, including replicated samples, revealed proportionally less informative in terms of new genotypes (lowest proportion of unknown to known genotypes), even though it accounted for a comparable absolute number of genotypes detected with respect to the other sampling methods. The lower relative efficiency of rub-tree sampling was most apparent with respect to uniquely detected genotypes, with only 2 of the 20 uniquely detected genotypes by the other sampling methods, and about 129 collected samples needed to detect one of such genotypes (Table 8). Likewise, the high re-sampling rates of rub-tree sampling also

accounted for its lowest efficiency in terms of the costs of the genetic analyses (i.e., based on analyzed samples), corresponding to about 600 Euro per detected genotype (Table 8).

3.3.6 Comparison with previous ISPRA genotypes

Conversion factors computed by WGI to allow comparison with ISPRA scores (see Table 1) allowed us to compare individual multilocus genotypes based on the 10 markers in common to both labs. During this process, along with reliably matching genotypes, we detected some equivocal cases, essentially corresponding to 1, 2 and 3 MM-pairs or that had been scored by ISPRA at a lower number of loci. As these cases deserve further consideration prior to a quality consolidation of an overall Apennine bear genetic database, we provided elsewhere a case-by-case illustration that details such cases and suggests remedy actions (Ciucci et al. 2012).

In brief, by comparing the 25 tissue-scored genotypes, we assessed that 24 can be reliably matched, while 1 (WGI M09 vs. ISPRA Gen1.71) needs further evaluation to allow a reliable match (Table 14). This same process also highlighted two 1 MM-pairs in the previously scored ISPRA genotypes (Gen1.10 vs. Gen1.20, and Gen1.13 vs. Gen1.56) which would deserve further evaluation prior to final database consolidation. Concerning the other 30 genotypes that we non-invasively detected in 2011, these corresponded to (cf. Ciucci et al. 2012 for further details):

- 14 matches, including two equivocal cases (RAM072 vs. Gen1.43, and HS358 vs. Gen1.16) and one previously overlooked ISPRA match (Gen1.15 vs. Orsnec0108); the last three cases need further evaluation prior to database consolidation;
- 4 1 MM-pairs which would require further consideration prior to database consolidation;
- 14 2 MM-pairs;
- 2 3 MM-pairs between WGI- and ISPRA-scored genotypes.

WGI genotype (2011 survey)						**ISPRA genotype** (2000-2008 surveys)[a]			**Notes**
Code	No. samples	HS	RT	OPP	INC	Code	Sampled (from – to)	No. samples	
F01	10	2	4	4	-	Gen1.25	2002-2008	23	
F02	4	1	3	-	-	Gen1.56	2004-2008	6	Gen1.56 is 1 MM-pair to Gen1.13[b]
F03	3	1	2	-	-	Gen1.44	2004-2008	13	
F04	2	2	-	-	-	Gen1.12	2001-2008	42	
F05	14	-	2	12	-	Gen1.22	2002-2008	3	
F07	4	4	-	-	-	Gen1.23	2002-2008	19	
F08	3	-	1	2	-	Gen1.73	2008	1	
F10	1	-	-	1	-	Gen1.54	2004-2008	13	
FP01	16	4	-	-	12	Gen1.7	2001-2009	66	
M08	19	3	9	3	4	Gen1.60	2005-2008	9	
M09	19	6	13	-	-	Gen1.71[b]	2005-2007	10	Gen1.71 is 1 MM-pair to M09
M10	24	15	7	-	2	Gen1.10	2002-2005	19	Gen1.10 is 1 MM-pair to Gen1.20[b]
M11	70	-	57[c]	-	12	Gen1.72	2008-2010	21	
M12	23	5	15	2	1	Gen1.24	2002-2008	27	
M13	27	2	24	1	-	Gen1.66	2005-2008	5	

[a]: among those detected in previous surveys (2000 – 2008), only those matching non invasively sampled genotypes detected in 2011, and scored by WGI, are listed
[b]: matching of these genotypes requires further evaluation (cf. Ciucci et al. 2012: Tab. 2)
[c]: including 4 replicated samples

Table 14. – List of bears which were live-trapped in previous years (2005-2010) whose WGI-scored genotype (column I) matches previously ISPRA-scored genotype (column II). Number of non-invasive resampling occasions during the 2011 survey are reported according to each sampling method (HS: systematic hair-snagging; RT: rub tree sampling; OPP: opportunistic sampling at buckthorn patches; INC: incidental sampling). Comparison with ISPRA-scored genotypes is based on database ver. 24/07/2011.

Although, due to sampling considerations (i.e., replicates, dates), there are no reasons to believe that the 2 MM- and 3 MM-pairs above should be regarded as possibly mismatching individuals, few of the involved

genotypes revealed potentially equivocal as they had been extracted from scats and all typed with <11 loci. Also these genotypes deserve further evaluation (i.e., re-analysis or evaluation of their PCRs results) prior to any decision regarding their definitive inclusion into the overall Apennine bear dataset: their unjustified inclusion (or exclusion) from the dataset may in fact introduce relevant bias in the estimation of apparent survivorship by open population models (Ciucci et al. 2012).

WGI genotype (2011 survey)						**ISPRA genotype** (2000-2008 surveys)[a]			**Sex**	**Notes**
Code	No. samples	HS	RT	OPP	INC	Code	Sampled (from – to)	No. samples		
Acc069	2	-	-	-	2	-			M	
Acc079	2	-	-	-	2	Gen 1.2	2000-2004	5	F	
HS001	13	7	-	6	-	Gen 1.4	2000-2009	31	F	
HS021	22	6	3	10	1	Gen 1.84[b]	2010		F	also sampled by ARP Lazio
HS028	24	5	10	9		Gen 1.50	2004-2008	44	F	
HS037	7	1	-	6	-	Gen 1.59	2005-2008	7	F	
HS058	2	1	-	1	-	-			M	
HS330	3	2	-	1	-	-			F	
HS338	1	1	-	-	-	-			M	one sample only (see Appendix 2)
HS343	9	5	4	-	-	Gen 1.18	2002-2008	27	F	
HS349	4	4	-	-	-	-			M	
HS355	2	2	-	-	-	Gen 1.76[b]	209-2011		M	also sampled by ARP Lazio
HS358	10	10	-	-	-	Gen 1.16[b]	2002	1	F	
HS374	8	3	5	-	-	-			M	
HS451	3	3	-	-	-	-			M	
HS465	4	4	-	-	-	-			M	
HS477	4	3	-	-	1	-			F	
RAM011	11	-	-	11	-	Gen 1.58	2005-2008	16	F	
RAM024	1	-	-	1	-	-			M	one sample only (see Appendix 3)
RAM042	5	-	-	5	-	-			M	putative F10's cub (see §3.3.3)
RAM045	5	-	-	5	-	-			M	putative F10's cub (see §3.3.3)
RAM048	3	-	-	3	-	-			F	putative F10's cub (see §3.3.3)
RAM072	1	-	-	1	-	Gen 1.43[b]	204-2008	6	F	one sample only (see Appendix 3)
RAM118	5	-	-	5	-	Gen 1.85	2010		F	also sampled by ARP Lazio
RT028	9	-	8	-	1	-			M	
RT030	17	-	7	9	-	Gen 1.49	2004-2008	10	M	
RT109	4	-	3	1	-	-			F	
RT148	2	-	2	-	-	-			M	
RT187	2	-	2	-	-	Gen 1.37	2003-2007	10		
RT233	2	-	1	-	1	Gen 1.41	2003-2005	40		

[a]: among those detected in previous surveys (2000 – 2008), only those matching non invasively sampled genotypes detected in 2011, and scored by WGI, are listed
[b]: matching of these genotypes requires further evaluation (cf. Ciucci et al. 2012 for details)

Table 15. – List of the 30 WGI-scored genotypes detected as from the 2011 survey, and number of non-invasive samples collected by sampling method. These are considered the 'naïve' bears, that is those that were not live-trapped in previous years (M: male, F: female). Comparison with ISPRA-scored genotypes is based on database ver. 24/07/2011.

In conclusion, as already stated in § 3.3.3, 45 genotypes were detected from the 426 successfully analysed hair samples collected during the 2011 survey: 15 of these matched known bears live-trapped in previous years and

which were previously scored by ISPRA (Table 14); 14 matched other bears already non-invasively sampled in previous surveys (2002 - 2008), including 2 somewhat equivocal cases (see above); and the remaining 16 bears, never sampled before, which include bears that went undetected during previous surveys and new bears added to the population since the 2008 survey (Table 15). WGI multilocus genotype scores of the 30 non-invasively detected bears in 2011, based on the 14 markers used by WGI, are listed in Appendix 4, and the same genotypes converted in ISPRA scores, using the conversion factors provided by WGI for the 10 markers common to both labs, are listed in Appendix 5.

3.4 Modelling

3.4.1 Data sources and encounter history

Out of the 45 non invasively detected bears, 20 (44.4%) were sampled by only one sampling technique, 18 (40%) by two, 4 (8.9%) by three, and the remaining 3 (6.7%) by all four sampling techniques. In addition, 12 of the previously marked bears were also detected by sighting and/or camera-trapping, 3 of which were detected uniquely by these means; however, due to the relatively limited contribution of sightings and/or camera-trapping data to the overall individual encounter history, only non invasive sampling data have been considered for demographic modeling (Appendix 6).

3.4.2 Modeling and population estimate

The model selection procedure revealed a temporal variation in capture probability for the hair-snag sampling (Model 1 in Table 16). Such variation was best described by a negative trend, with capture probability decreasing from about 0.18 (95% CI = 0.11-0.28) in session 1 to about 0.09 (95% CI = 0.04-0.16) in session 5 (Fig. 17a). With the marginal exception of DFC, no other variables among the ones tested were significantly affecting capture probability by hair snag, suggesting that this sampling technique in 2011 was characterized by a rather low but relatively homogeneous capture probability within the bear population. Nevertheless, it should be noted that the lack of support provided by the data to variables such as gender or previous sampling history, otherwise evidenced in the data from the 2008 survey, could be also due to the low capture probability and associated statistical power of the 2011 hair-snag sampling.

The buckthorn sampling data also supported temporal variation in *p* (Table 16, Fig. 17b) which displayed a bell shape, with the highest *p* value in session 2; this strongly suggests that the development of the ripening season could be the underlying reason for such a trend. The use of an effort-based variable did not improve the performance of the model, suggesting that the temporal variation in capture probability was not likely to be due to sampling-related causes. However, a sex effect was indeed apparent in the data, with females showing a higher *p* than males (Fig. 17b); in fact, average capture probability for males at buckthorn sites was 0.16 (95% CI = 0.07 – 0.31), whereas for females was 0.27 (95% CI = 0.15 – 0.44).

A time effect was supported by the data also for the rub tree sampling, and using the cumulative effort per session (n. of rub tree nights per session) explained more variation in *p* than using a simple time effect or a trend effect. A strong support was provided to the interaction between gender and a previous hair-snag event. A model including this interaction had an AIC 11 points lower than a model excluding it. Capture probability estimates from the most supported model show that previously hair-snagged males (all older than 4 years) had a very high capture probability, on average equal to 0.79 (95% CI = 0.58 – 0.91), whereas never hair-snagged males (very likely to be younger than 4 years) had a much lower capture probability, on average equal to 0.10 (95% CI = 0.04 – 0.22) (Fig. 17c). Such a marked difference was instead not observed between younger and older females: we estimated an average rub tree capture probability of 0.12 (95% CI = 0.05 – 0.25) and 0.16 (95% CI = 0.08 – 0.32) for previously hair-snagged and never hair-snagged females, respectively (Fig. 17d). This confirms that the use of rub-trees was more frequent for males and in particular for males in reproductive age. A good support was also provided to the number of rub trees in each bear's "home range", allowing to model the additional individual heterogeneity, generated by the spatial and temporal variation in the RT sampling effort during the different sessions.

No variable was strongly supported by the data in affecting capture probability for the incidental sampling, likely due to the small sample size and low statistical power associated to this data source. Capture probability for this data source was in average 0.23 (95% CI = 0.13 – 0.36).

A graphical summary of the group-specific variation in capture probability along all the sampling sessions is depicted in Figure 18.

Among the most supported models, model 3 (Table 16) suggested the possibility of an effect of the distance from the grid edge on capture probability. Nevertheless, the beta estimate for this variable was quite imprecise (β = -0.157; 95% CI = -0.43; 0.12), thus preventing to assess in which direction this effect might influence capture probability. This, consistently with previous radio-telemetry data on the Apennine brown bear population (Gervasi et al. 2012), supports the hypothesis that closure violation of the population in the PNALM is a minor phenomenon.

The spatial heterogeneity analysis only provided a minor support for an effect of the distance from the grid center on the hair-snag capture probability (see Model 2 in Table 16), whereas this variable did not appear to affect the spatial variation in capture probability for the other data sources. The effect of DFC on the hair-snag capture probability was roughly a decrease of 0.01 for each 1 km increase in the distance from the grid center. Also the latitude*longitude effect was not supported by the data.

Based on these results, and performing a model averaging among all models, we produced a final population size estimate of 49 bears (95% CI = 47-61; CV = 7%), corresponding to 22 (95% CI = 22-28) males and 27 (95% CI = 26-33) females. The closure corrected density estimate, based on a previously estimated bear fidelity to the sampling grid of 95.1% (Gervasi et al. 2012), was 38.1 bears / 1000 km^2 (95% CI = 35.8 – 47.5).

When estimating population size based on a design including only buckthorn, rub tree, and incidental data (therefore excluding the hair-snag data), the resulting population estimate was 42 bears (95% CI = 38-60, CV = 10%), with a notable reduction of both the point estimate and its precision.

Model N.	Description	AICc	ΔAIC_c	Weight
1	HS(trend) OPP(time+sex) RT(effort+sex*prev.hs+nrub) INC(null)	550.87	0.00	0.243
2	HS(trend+DFC) OPP(time+sex) RT(effort+sex*prev.hs+nrub) INC(null)	550.62	0.76	0.166
3	HS(trend) OPP(time+sex) RT(effort+sex*prev.hs+nrub) INC(null) + DFE^2	551.69	0.82	0.161
4	HS(trend) OPP(time+sex) RT(effort+sex*prev.hs+nrub) INC(sex)	551.77	0.90	0.155
5	HS(trend) OPP(time+sex) RT(effort+sex*prev.hs+nrub) INC(null) + DFE	552.94	2.07	0.086
6	HS(trend) OPP(time+sex) RT(effort+sex*prev.hs+nrub) INC(null) + Log(DFE)	552.94	2.08	0.086
7	HS(trend) OPP(time+sex) RT(effort+sex*prev.hs+nrub+hs2011) INC(sex)	553.82	2.96	0.055
8	HS(trend) OPP(effort+sex) RT(effort+sex*prev.hs+nrub) INC(null)	554.35	3.48	0.015
9	HS(trend) OPP(time+sex) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	556.44	5.57	0.012
10	HS(trend) OPP(time*lat+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	556.77	5.90	0.007
11	HS(trend) OPP(time+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	557.80	6.93	0.005
12	HS(trend) OPP(time+lat+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	558.50	7.63	0.001
13	HS(time) OPP(time*lat+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	560.50	9.64	0.00
14	HS(time+sex) OPP(time*lat+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	562.67	11.80	0.00
15	HS(time+sex+prev.hs) OPP(time*lat+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	563.20	12.34	0.00
16	HS(time+sex+prev.hs+lt) OPP(time*lat+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	565.08	14.21	0.00
17	HS(time+sex*prev.hs+lt) OPP(time*lat+sex+prev.hs) RT(time+sex*prev.hs+nrub+hs2011) INC(sex)	567.27	16.40	0.00
18	HS(trend) OPP(time+sex) RT(effort+sex+prev.hs+nrub) INC(sex)	571.58	20.71	0.00

Table 16. –Model selection results for the Huggins closed population estimation, applied to the 2011 survey data of the Apennine brown bear population in the PNALM, Italy. Abbreviations for the data sources indicate hair-snag (HS), opportunistic sampling at buckthorn patches (OPP), rub-trees (RT), and incidental samples (INC). Parameter abbreviations indicate the number of active rub trees in each bear home range (*n. rub*), a previous hair-snag detection between 2003 and 2008 (*prev. hs*), and a hair-snag detection during 2011 sampling (*hs 2011*). The models shown are the 18 most supported ones, sorted by AIC_c values. Several other models have been fitted, which received a negligible support from the data (not listed).

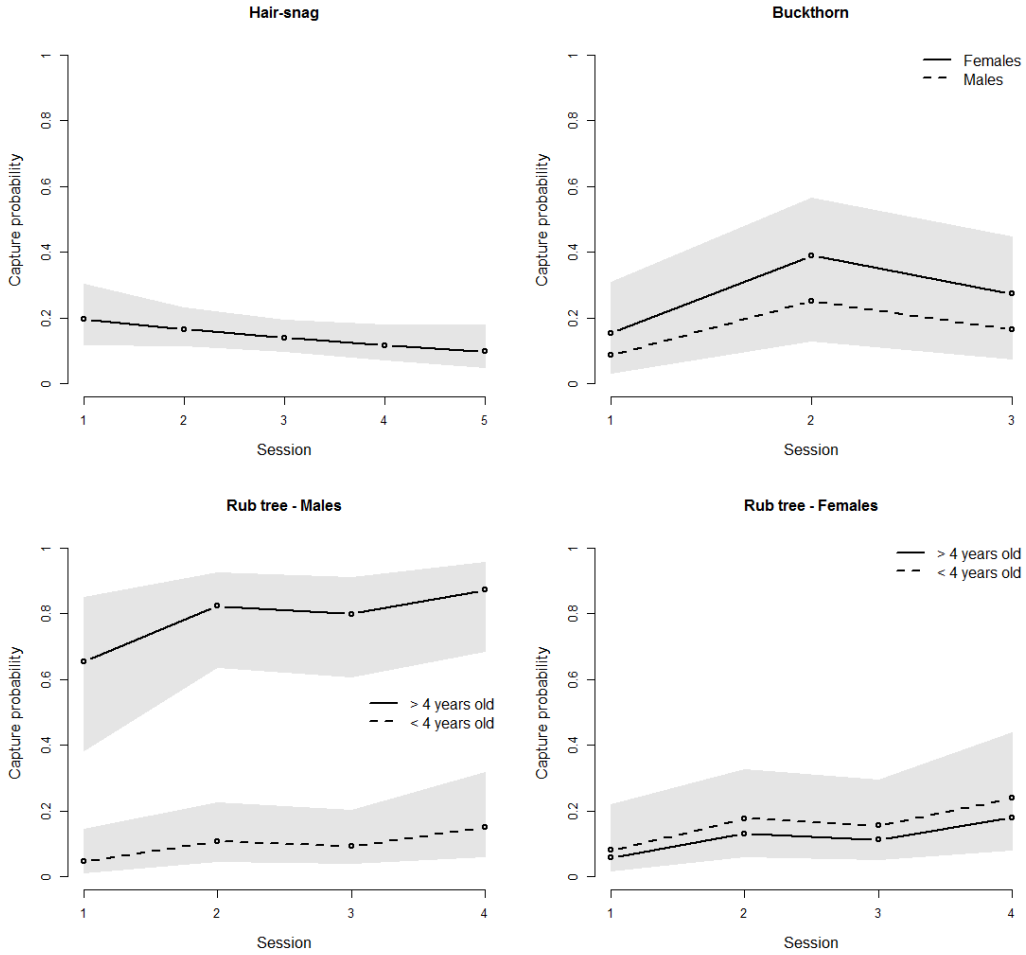

Figure 17. –Capture probability estimates for the hair-snag, buckthorn, and rub tree sampling (Apennine bear population survey in the PNALM, June – September 2011). Capture probability estimates are derived from the most supported model (model 1 in Table 1), and provided separately for the different sex and history of previous hair-snagging. Estimates are based on average values of the other covariates included in the model (effort, nrub). Grey areas represent 95% CIs of the estimates.

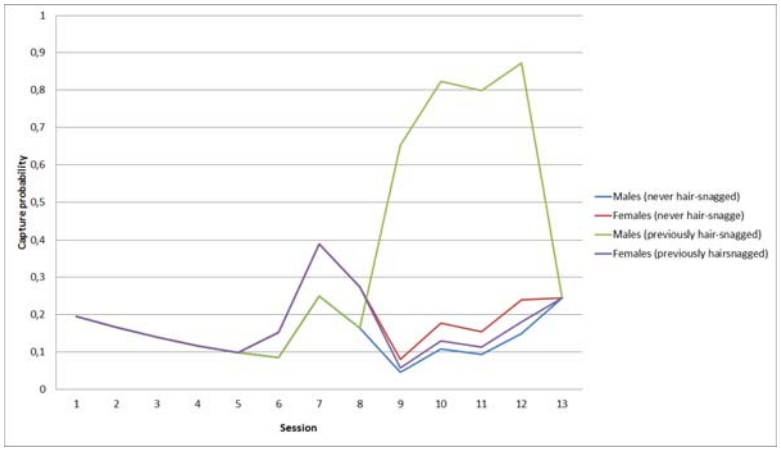

Figure 18. –Summary of capture probability estimates along all sampling sessions (Apennine bear population survey in the PNALM, June – September 2011). Sessions 1-5 refer to hair-snag sampling, 6-8 to buckthorn sampling, 9-12 to rub trees, and session 13 to incidental sampling. Capture probability estimates are derived from the most supported model (model 1, Table 1), and provided separately for the different sex and history of previous hair-snagging. Other covariates included in the model (effort, nrub) have been fixed at their average value.

4. DISCUSSION

4.1 Survey evaluation

4.1.1 Overall design and sampling techniques

The 2011 non invasive survey of the Apennine bear population was particularly effective in terms of samples collected and coverage of the various segments of the population. Through the adoption of complementary, non invasive sampling techniques, we managed to obtain a quite high overall capture probability of 0.91 (95% CI = 0.74 - 0.95), higher than that reported for the 2008 survey (p=0.82; 95% CI = 0.63 - 0.89). This reflects, however, not only the adoption of sampling techniques, namely buckthorn and rub tree sampling, that were not adopted in 2008, but also a higher number of sampling sessions (n=13) with respect to the 2008 survey (9 sessions). The higher number of sampling sessions in 2011 also explains why in this year we obtained a higher overall capture probability, even though the per-capita average probability per session (p=0.201; 95% CI = 0.172 - 0.230) was lower with respect to 2008 (p=0.311; 95% CI = 0.216 - 0.438). In this respect, there are two relevant issues that should be emphasized in comparing the 2008 vs the 2011 results. First, hair-snagging was much more efficient in 2008 than in 2011, possibly due to: (a) a habituation response by bears to lured hair-traps (Proctor et al. 2010), as this was the fourth hair-snagging application in 8 years on the same population, and/or (b) the 2-week delay of hair-snagging onset in 2011, that could have postponed hair-snagging to include summer weeks during which chances to attract bears to hair traps is on average lower than in spring or early summer. In any case, the lower efficiency of hair-snagging in 2011 is probably among the major determinants of the lower per capita capture probability we observed in 2011. Second, the adoption in 2008 of datasources such as sightings and live-trapping data might have contributed to significantly enhance average capture probability for those sessions; however, as these sampling techniques can be only applied to previously marked individuals, there might be the risk of overestimating the overall capture probability of unmarked bears and therefore underestimating their proportion in the population. In the 2011 application, through the adoption of both rub tree and buckthorn sampling, we significantly enhanced the detectability of all bears in the population, irrespective of their being marked or not, thereby increasing capture probability of unmarked bears with respect to the 2008 survey.

We therefore believe the 2011 design gains from a more sound theoretical basis over the one adopted in 2008 when, in addition to hair-snagging, sightings and live-trapping data were the only data sources available to be used within the integrated datasource approach. In 2011 we did not have to use sighting data of marked bears, potentially integrated by camera-traps detections, because the other sources of data corroborated individual encounter histories, preventing us from the risk of overestimating the capture probability of unmarked bears, or to stretch model assumptions (e.g., correlation between datasources). In 2011, for example, it would have been problematic to include both buckthorn sampling and sightings of previously marked bears, as direct observations of marked (and unmarked) bears are conducted at buckthorn aggregations. We therefore believe the sampling design used in 2011, although logistically more complex, is somewhat ideal for this small-sized bear population, as it increase sampling coverage, is theoretically more robust, and does not imply previous live-trapping and presence of marked bears in the population.

Each of the sampling methods we used in 2011 provided a substantial number of samples, contributing to increase the sample size available for CR analysis of this small bear population. In particular, whereas hair-snagging ensured a systematic and complete spatial coverage of the study area, the complementary, non-systematic sampling methods enhanced sampling coverage for those segments of the population hardly or more difficult to sample by hair-snagging alone. In particular, buckthorn sampling was particularly successful for females and family units, including cubs, and incidental sampling detected bears in more peripheral areas some of which prone to cause damage to farms and crops. Rub tree sampling, although it did not provide a comparable number of uniquely detected genotypes, enhanced recapture rates for both males and females, significantly contributing to increasing their capture probability and hence to the precision of the final population size estimate.

Although the number of cubs estimated to be present in the population by means of unduplicated counts was particularly low in 2011 (n=3, Ciucci et al. 2012b), sampling results confirm that cubs in our population have a nearly null probability of being sampled in early summer using traditional hair-snag traps; using hair traps with a double strand of barbed wire, placed at 15 and 30 cm of height, proved successful to sample cubs, and we had a fortuitous demonstration of this in sampling all three F10's cubs. Limiting this trap adjustment to buckthorn sites is logistically more feasible than extending it to all hair-snag traps, and it seems a reasonable

way to overcome this problem in future, non invasive surveys, especially if we can use a larger number of buckthorn patches for sampling.

Much can be done to further enhance effectiveness of each sampling strategy and, in particular, efficacy of rub tree sampling. This technique was used in 2011 for the first time and proved to be a very effective sampling method, even though it proved excessively costly compared to the other sampling methods (Euros per detected genotype; cf. Table 8). Not only spatial coverage of activated rub trees should be significantly improved in future non invasive surveys of the Apennine bear population, but also rub tree samples, and possibly rub trees per unit area, need to be more conservatively selected upon collection as to avoid unnecessary replicates. We'll address these and other sampling issues with simulation work as to design an optimal strategy to be implemented in the 2014 survey planned by the last year of the Life Arctos project.

4.1.2 Quality of the genetic dataset and ideal marker system

On average, 19.5% of the collected hair samples delivered for genetic analyses failed to produce reliable genotypes, whereas the remaining 80.5% (n=456) were scored using 14-locus data, detecting a total of 45 bears. In considering success rates, it is worth noting that WGI has an unusually low tolerance for samples with missing (i.e. low-confidence) data (Paetkau 2003). This is because such data are associated by definition with samples from which it is difficult to amplify all alleles, and thus where the risk of genotyping error (especially allelic dropout) is heightened. Furthermore, missing data increase match probabilities in ways that are difficult to quantify or control. For example, if we settle on a 12-locus system for individual identification, tolerating samples that have low-confidence data for up to 3 markers, we could encounter pairs of samples which are missing data for different subsets of 3 markers, leaving just 6 markers in common between samples for the purposes of deciding whether they came from the same individual. Clearly the associated increase in match probability would undermine the quality of the dataset.

Another consideration regarding success rates is that the relative invariability in the Apennine bear population makes it necessary to be unusually cautious with regards to potentially mixed samples. In a typical population, where one might see 8 or 9 alleles per marker, mixed samples stand out by amplifying 3 or 4 alleles at some markers. Those alleles might differ in strength if the mixture is uneven, but they are still noticeable in cases where the mixture is strong enough to create a risk of false individual identification. By contrast, when there are only 2 or 3 alleles per marker in the study population (cf. Table 1), we cannot rely on this method to identify mixed samples. Thus, we have to be particularly aware of cases where 1 allele in a heterozygous genotype is atypically strong, since this might be a mixed sample where 1 bear was homozygous and the other heterozygous. There is a meaningful risk that mixed samples like these could create chimeric genotypes that are identified incorrectly as unique individuals. Many of the low-confidence scores we revealed in this study represent such imbalances between alleles, and were accordingly culled from the analyses; we estimate that as many as 20% of culled samples may actually be mixed rather than weak samples.

The final set and number of markers which have been identified and used in this study appears to be optimal for the non invasive identification of single bears in this population, both because we introduced some new and informative markers with respect to the panel previously used By ISPRA, and because the 10 loci in common with the previous lab ensure the comparability of genotypes between labs. To this end, we also produced conversion factors using genotypes scored from blood samples and scored by both labs, and these allow the recombination of the ISPRA and WGI genetic dataset in a unique data base for this population, provided some suspicious cases are further investigated (Appendix 3; Ciucci et al. 2012).

For the future non invasive monitoring of this population, it is important to emphasize that we might have used more markers than strictly necessary to achieve a low match probability. In fact, we observed no 1MM-pairs and just a single 2MM-pair among the 55 individuals sampled in 2011 (25 from blood and hair samples, and 45 from hair samples), suggesting no realistic chance of having sampled any 0MM-pairs (cf. Fig. 9). It is obviously not desirable to analyze more markers than necessary (Waits and Leberg 2004, Paetkau 2004) but, on the other hand, it is also not advisable to decrease the number of marker beyond a threshold which would increase the chance of encountering false matches (0 MM-pairs). To this end, we did some simulation aimed to define the ideal set of markers for the Apennine bear population (cf. § 3.3.2), and concluded that: (a) all 10 markers in common to both labs should be retained; (b) other markers can be added among those we tested, and in particular those most variable in the population (cf. Table 1); (c) given the constraint to retain the 10 ISPRA markers to ensure a low match probability in comparisons between labs but, at the same time, the

recommendation of not using an excessive number of markers, our final suggestion is to remove both G10X and MSUT-2 for individual identification in future non invasive genetic surveys of the Apennine bear population, for a total of 11 markers plus gender.

4.2 Population size, trends and characteristics

Our estimate of the 2011 population size represents 49 bears (95% CI = 47 – 61), comprising 22 (95% CI =22 – 28) males and 27 (95% CI = 26 – 33) females. The overall sex-ratio based on the population estimate (1.23:1, FF:MM) is not dissimilar from the empirical sex-ratio based on the total number of genotypes sampled (1.25:1), confirming a slight preponderance of females in the population. The 2011 estimate, as well as the one from 2008, comprises all age cohorts, including cubs of the year that, in 2011, were estimated by means of unduplicated counts at a minimum of 3 in only one family group (Ciucci et al. 2012b).

Although the expected value of the 2011 survey results is the opportunity to compare the estimated population size in 2011 and 2014, it is useful to compare also previous population estimates obtained through formal methods (Gervasi et al. 2012), as this would provide the basis for a more meaningful comparison in 2014. To this end, although the confidence intervals of the 2008 and the 2011 estimates overlap considerably (40% and 42.9% for the 2008 and the 2011 95% CIs, respectively), the plain comparison between the two point estimates generates an annual increase of 7.5% (λ= 1.075). However, it should be emphasized that comparison of point estimates is an inherently faulty procedure, as it does not contemplate the potential contribution of the statistical uncertainty about the estimation process. Accordingly, comparing the 2008 and 2011 point estimates reflects an overly optimistic interpretation, apparently in slight disagreement with empirical data we produced from annual, unduplicated counts of females with cubs: from 2009 to 2011, a minimum of 13 cubs have been produced (Ciucci et al. 2012b), and by adding these to the estimated 40 bears in 2008, and subtracting the 7 bears reported dead from 2009 – 2011 (pre-survey data), results in 46 bears expected in the population in 2011, assuming 100% survivorship of all cubs detected in previous years and no unreported bear mortalities; thus, a more realistic figure should be lower than 46, unless immigration is also a factor to take into account. In any event, taking into full consideration the statistical uncertainty about the estimates, it is very unlikely that the population decreased in these 3 years, as the most negative scenario (i.e., upper 95% CI value for 2008 and lower for 2011) are reasonably close to each other (52 vs. 47 bears, respectively).

It is also important to note that the alleged increase by 2011 accounted mostly for males (λ= 1.120) rather than females (λ= 1.042), with regard to the 16 males and 24 females[2] estimated in the population in 2008. Accordingly, although we do not know the actual sex-ratio of the cubs produced after 2008 and their differential mortality patterns in the years following their initial observation, our empirical data suggest that female mortality was higher from 2008 to 2011, as among the 7 bears reported dead, 6 of which were sexed, 5 were females, comprising 3 in reproductive age (L. Gentile, com. pers.). Finally, with regard to the female segment of the population, we empirically estimated, by means of unduplicated annual counts of females with cubs, that at least 10-14 adult females resided in the PNALM bear population from 2008 to 2011; extrapolated to the total number of females as form the 2011 estimate (n=27), this reflects a proportion of 37-52% of adult females within the female segment, the others accounting for cubs, yearlings and non reproducing subadults, thereby providing a first, although very crude and subject to substantial variability, approximation of a critical demographic parameter. In any event, it should be emphasized that the projections above do not take into account the uncertainty about the 2008 and 2011 point estimates; in addition, it is also likely that we slightly underestimated population size in 2008, as the inclusion of live-trapping and observational data might have determined an overall overestimate of the capture probability of unmarked bears and therefore their underrepresentation in the final estimate (Gervasi et al. 2012). As we were not forced to use these two additional datasources in the 2011 survey (see § 4.1.1), the difference in population size between the 2 surveys might also partly account for the potential bias in the 2008 estimate.

Finally, it should be underlined that the 2011 population estimate does also include 'management' bears, or those somewhat habituated to humans and that recurrently create damages to poultry farms, cultivations, crops, and bee-hives. In particular, we non invasively detected 3 of these bears by incidental sampling during verification of alleged damages, one of which is an adult, reproductive female (FP01) timely known to

[2] 11 males and 18 females (1 year and older), plus 11 cubs; assuming a cub sex ratio of 50:50, this corresponds to 16 males and 24 females, including cubs.

represent a management bear. Conservation-wise, these bears should be soon subtracted from the number of effective bears composing the overall population, if their attitude will not be changed through pro-active, proper management interventions (e.g., sanitarization and negative conditioning). Until this is done, not only management and social problems will continuously undermine conservation efforts both within and outside the core distribution of the Apennine bear, but most importantly precious reproductive effort and genetic variants will be wasted and subtracted from the rest of the bear population.

In any event, we did not detect any negative trends from the 2008 estimate of population size, confirming that the relict Apennine bear population, at least within the core distribution, is still reproductively active, demographically capable of positive growth notwithstanding substantial levels of human mortality, and potentially able to support bear dispersers across a larger geographical scale. Although this tentative interpretation needs to be confirmed in the light of the 2014 estimate that will be produced within the Life Arctos project, it currently provides hope for a renewed conservation effort.

4.3 Prospects for the 2014 survey

Although we are currently doing simulation work based on the 2011 data to better assess the optimal sampling design for future surveys, some practical indications can be drawn in the prospect of the 2014 survey.

First of all, detecting 45 bears out of the 49 estimated in the population reveals a high coverage of the entire population, especially with regard to the 2004 (9 genotypes detected out of 43 estimated) and the 2008 (20 genotyped detected out of 40 estimated) surveys. This is an empirical indication that, provided hair snagging ensures a systematic and throughout sampling of the whole study area, the other sampling strategies appear to functionally complement hair snagging by significantly increasing the overall capture probability. However, the 2011 sampling design probably provided more samples than strictly necessary to the precision obtained, so that there might be some room for enhanced efficiency. This, which needs to be confirmed by simulation work, would entail revising both field methods and sampling intensity of some of the adopted sampling strategies.

For example, with reference to hair snagging, sampling efficiency could be improved by: (a) anticipating somewhat the onset of the first session, as it was originally done with increased sampling success in 2008; anticipating hair snagging by 10-14 days would perhaps corresponds to bears being more readily attracted by the lure, as there still is a less diversified availability of natural foods; (b) diversify complementary lures at each sessions (e.g., Kendall et al. 2008), and (c) possibly reducing the number of sessions.

Concerning rub tree sampling, we definitively need to increase the geographic coverage of the study area, especially in the external buffer area, but also to subsample more when collecting samples, both by reducing the number of samples collected at each single rub tree and by subsampling rub trees that may be visited by the same bear during the same movement route.

Incidental samples may and should be enhanced by stimulating a more throughout collection by park wardens in their patrolling activities, whereas their contribution to the overall genetic analysis need to be evaluated a posteriori based in the number of samples collected through the entire sampling period in each section of the study area.

Buckthorn sampling should also be maintained, but we may wish to increase the number of buckthorn area, especially in the most peripheral sections of the study area, and possibly reduce to 3 the effective sampling sessions.

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Appendix 1

List of 23 previously (2006-2010) live-trapped and marked bears (13 females and 10 males) potentially available to sampling during the 2011 survey. Eighteen have been re-sampled during the 2011 survey, either by non-invasive sampling or direct observation/camera trapping, whereas 5 were missing to any form of sampling.

Bear ID[a]	**Age class**[b]	**Last live-trapping event**	**Last evidence alive (year)**[c]	**2011 sampling**	
				non-invasive	other[d]
F01	adult	May 2006	2009	Yes	
F02	adult	Oct 2005	2009	Yes	Obs
F03	adult	July 2006	2008	Yes	
F04	elder	July 2006	2010	Yes	Obs
F05	adult	Sept 2006	2010	Yes	Obs
F06	adult	Nov 2006	2009		
F07	adult	May 2008	2011 (March)	Yes	Obs
F08	adult	Oct 2008	2010	Yes	Obs/CT
F09	adult	Sept 2009	2010		Obs
F10	adult	Sept 2009	2008	Yes	Obs
F11	subadult	Oct 2008	2010		
F13	adult	Oct 2009	2010		Obs
F01P	adult	Aug 2004	2011 (March)	Yes	Obs
M01	adult	May 2009*	2010		
M04	adult	Oct 2006	2009		Obs
M07	adult	Apr 2007	2007		
M08	adult	June 2009	2010	Yes	CT
M09	adult	June 2007	2010	Yes	
M10	adult	July 2008	2011 (March)	Yes	
M11	adult	June 2008	2010	Yes	Obs/CT
M12	adult	June 2008	2009	Yes	
M13	adult	Sept 2009	2010	Yes	
M15	subadult	Oct 2009	2010		

[a]: F: female; M: male
[b]: as estimated by tooth wear and body size at the time of first live-capture and projected up to 2011 (year of the survey): subadult: 3-4 years; adult: 4-19 years; elder: ≥ 20 years
[c]: before the 2011 survey
[d]: detection by sightings (OBS) and camera-traps (CT) during the survey period (June – September 2011)

Appendix 2

List of 9 previously (2006-2010) live-trapped and marked bears (3 females and 6 males) which have been not detected in the 2011 survey by any sampling method, either because death or missing.

Bear ID[a]	Age class[b]	Last live-trapping event	Last detection (year)	Known fate
F06	adult	Nov 2006	2009	missing
F11	adult	Oct 2008	2010	missing
F12	yearling	May 2009	2009	dead
M01	adult	May 2009	2010	missing
M02	adult	Oct 2005	2006	dead[c]
M06	adult	Apr 2007	2007	dead
M07	adult	Apr 2007	2007	missing
M14	yearling	Apr 2009	2009	dead
M15	yearling	Oct 2009	2010	missing

[a]: F: female; M: male
[b]: as estimated by tooth wear and body size at the time of first live-capture and projected up to year of last detection: yearling: 12-24 months; adult: 4-19 years; elder: ≥ 20 years
[c]: carcass retrieved in 2008

Appendix 3

Genotypes detected from hair samples collected during the 2011 survey of the Apennine bear population which revealed somewhat equivocal to post-control assessment and cross-checking, and were therefore re-analyzed by WGI to better assess their validity. The final decision as to how these genotypes should be handled for population assessment is reported in column IV.

Sample	Problem	Successive analysis	Remedy action
RAM024	- Unique sample for this genotype. Eight other samples have been collected at the same trap on the same day, providing genotypes RAM011 and HS028. The latter in particular has been sampled on the same day, on the same side of the trap, and on the barb adjacent to that of sample RAM024. - No sample material left, but WGI successively re-analyzed the sample to be certain there are no signs of mixture.	No signs of mixture, although DNA left did not allow to replicate al 14 markers	Keep it for the 2011 population assessment, but consider its deletion for future open population models as it represents a unique sample.
RAM072	- Unique sample for this genotype, although it matches to a previously captured bear (Gen1.43) so it has been somewhat replicated. The sample has been collected on the external portion of the trap (RAM), at about 30 cm from the ground. - No sample material left, but WGI successively re-analyzed this sample to be certain there are no signs of mixture.	No signs of mixture, although DNA did not allow to replicate al 14 markers.	Keep it for the 2011 population assessment, but consider its deletion for future open population models as it represents a unique sample.
RT167	Although there are 7 other replicates of this genotype (HS374), this sample was collected at RT no. 46 on 2 July 2011, when 2 other samples were collected on the same tree, very close to each other. One provided genotype for M13, the other was assigned to the individual HS374.	No DNA left for further extraction (single hair), but WGI validate this samples. RT167 is assigned to individual HS374 which was caught 8 times in total, so that this genotype is extremely unlikely to contain errors. It is unlikely that there was a second bear that mixed in with M13 in a way that was not detectable and that the sum total was exactly another bear in the dataset that wasn't present.	Retain the genotype
HS307	Sampled on 13 June at HS trap 166, which provided 26 hair samples, 14 of which were successfully genotyped and provided 3 genotypes: M10 (10 samples), M09 (3 samples), and HS343 (1 sample, HS307). The sample providing the genotype HS343 was collected on a different side of the trap than the others. It shares at least one allele with both M10 and M09, with the only exception of locus A06.	Sample HS307 did show a very slight amount of mixture at *MU11* that matches an allele found in both M10 and M09, and overall this sample was weak and required quite a large effort in terms of cleanup. If it hadn't been matching to an already known genotype one would have strongly considered culling this one at the first run of analysis. As there is no sample material left, exclusion of this sample would be the best approach if one wants to be cautious.	Culled
HS338	- Unique sample for this genotype, HS-collected one barb away from other 4 M10 samples (hair snag). - Since there is hair left over, WGI repeated a single hair extraction to see if mixture is a factor, even though the number of errors that would have to go into making this genotype from M10's is very unlikely (there would have to be allelic dropout at several of the markers and then slight mixture at several other markers)	Re-extraction confirms the genotype	Keep it for the 2011 population assessment, but consider its deletion for future open population models as it represents a unique sample.

Appendix 4

Multilocus (n=14 markers plus sex) genotypes, expressed in WGI scores, of the 45 bears non-invasively sampled during the 2011 survey (number of samples for each genotype in column II). Markers highlighted in red have been added by WGI to those previously used by ISPRA (see Appendix 5 for the same genotypes in expressed in ISPRA scores) (*Table continues in the next page*).

Genotype	Samples	G10B	G10C	G1D	G10L	G10P	MSUT-2	MU59	REN144A06	CXX20	MU50	MU51	G10X	MU05	MU11	Sex
Acc069	2	156.156	203.207	172.184	163.163	157.157	195.195	229.235	109.127	135.137	132.136	206.214	135.135	135.137	188.188	M
Acc079	2	140.140	207.207	172.186	163.163	157.157	199.203	229.235	127.129	137.139	132.136	206.206	129.135	137.137	192.192	F
F01	11	140.156	203.207	172.186	157.163	157.157	195.203	229.229	127.129	135.137	132.132	206.214	129.135	135.137	192.192	F
F02	5	156.156	203.207	172.172	157.163	145.157	195.203	229.229	109.109	137.137	132.132	206.212	129.135	135.135	188.192	F
F03	4	140.156	203.203	172.186	157.157	157.157	195.195	229.229	109.127	139.139	132.132	212.212	135.135	137.137	188.192	F
F04	3	140.156	203.203	172.184	157.163	145.157	195.203	229.235	109.127	137.137	132.136	206.212	129.135	137.137	188.192	F
F05	15	140.156	203.207	172.186	157.163	157.157	203.203	229.229	109.109	137.139	132.132	206.206	129.129	137.137	192.192	F
F07	5	140.156	203.207	172.186	157.163	145.157	195.195	229.235	109.127	137.139	132.136	212.212	135.135	135.137	188.192	F
F08	5	140.140	203.207	184.186	157.163	157.157	203.203	229.235	109.127	135.137	132.136	206.206	129.129	137.137	188.192	F
F10	2	140.156	203.207	172.172	157.163	157.157	203.203	229.235	109.127	137.137	136.136	206.206	129.129	135.135	188.188	F
FP01	17	140.156	203.203	172.172	163.163	145.157	195.203	229.235	109.109	135.137	132.136	206.214	129.135	137.137	188.192	F
HS001	13	140.140	197.203	172.186	163.163	145.157	195.199	235.235	127.129	137.139	136.136	206.206	135.135	135.137	188.192	F
HS021	22	156.156	203.207	186.186	163.163	157.157	203.203	235.235	109.127	135.139	132.132	206.206	129.129	137.137	192.192	F
HS028	24	140.156	207.207	172.184	163.163	157.157	203.203	235.235	109.129	135.139	132.136	214.214	129.129	137.137	192.192	F
HS037	7	156.156	203.207	172.184	157.163	157.157	203.203	229.235	109.129	135.137	132.136	206.214	129.129	137.137	192.192	F
HS058	2	140.156	203.203	172.172	163.163	157.157	203.203	229.235	127.127	137.139	132.132	206.206	129.129	137.137	192.192	M
HS330	3	140.156	203.203	172.172	163.163		195.195	229.235	127.127	135.137	132.136	206.214	135.135	135.135	192.192	F
HS338	1	140.156	203.207	186.186	163.163		203.203	229.229	109.109	135.139	132.132	206.214	129.129	135.137	192.192	M
HS343	9	140.140	203.207	172.172	163.163	157.157	195.195	229.235	109.129	137.137	132.136	206.206	135.135	137.137	188.188	F
HS349	4	154.156	203.207	172.186	157.163	145.157	195.195	229.229	109.129	137.137	132.136	206.206	135.135	135.137	192.192	M
HS355	2	156.156	207.207	172.172	157.163	157.157	195.203	229.229	127.129	135.137	132.132	206.206	129.135	135.135	188.192	M
HS358	10	140.156	203.207	172.184	157.157	157.157	203.203	229.229	109.129	135.137	132.136	206.206	129.129	135.137	192.192	F
HS374	8	140.140	203.207	172.186	157.163		203.203	229.229	127.127	139.139	132.136	206.206	129.129	135.137	188.192	M
HS451	3	140.156	203.207	184.186	163.163		203.203	235.235	127.127	135.137	132.132	214.214	129.129	137.137	188.192	M
HS465	4	140.140	203.207	172.186	163.163	157.157	203.203	229.235	109.127	135.137	132.132	206.206	129.129	137.137	192.192	M
HS477	4	140.140	203.203	172.184	163.163		195.195	229.235	109.127	135.137	132.132	212.214	135.135	137.137	192.192	F
M08	20	140.140	203.207	172.184	157.163	157.157	195.203	229.235	109.109	137.137	132.136	212.214	129.135	135.137	188.188	M
M09	20	140.156	203.207	172.186	163.163	157.157	195.203	229.229	109.109	137.137	132.132	206.212	129.135	135.137	188.192	M

Appendix 4

Genotype	Samples		G10B	G10C	G1D	G10L	G10P	MSUT-2	MU59	REN144A06	CXX20	MU50	MU51	G10X	MU05	MU11	Sex
M10	25		140.156	203.207	184.186	157.163	157.157	195.203	229.235	109.127	137.139	132.132	206.206	129.135	137.137	192.192	M
M11	71		140.156	203.203	172.172	157.163	157.157	203.203	229.229	109.127	137.139	132.132	206.206	129.129	135.137	188.192	M
M12	24		156.156	203.207	172.186	157.163	145.157	195.203	229.229	109.127	135.137	132.132	206.214	129.135	135.137	192.192	M
M13	28		140.156	203.207	172.186	157.157	157.157	195.203	229.235	109.127	137.139	132.132	206.212	129.135	137.137	188.192	M
RAM011	11		140.140	203.203	172.184	163.163	157.157	203.203	229.235	127.127	137.139	132.136	206.212	129.129	135.137	192.192	F
RAM024	1		156.156	203.207	172.172	157.163	157.157	195.195	229.235	127.129	135.139	132.136	206.214	135.135	135.135	188.192	M
RAM042	5		140.156	203.203	172.172	157.163	157.157	203.203	229.235	109.127	137.139	132.136	206.206	129.129	135.137	188.188	M
RAM045	5		140.140	203.203	172.186	157.163		203.203	229.235	127.127	137.137	132.136	206.212	129.129	135.137	188.188	M
RAM048	3		140.156	207.207	172.186	157.163		203.203	229.229	109.127	137.137	132.136	206.212	129.129	135.137	188.192	F
RAM072	1		140.156	203.207	172.184	163.163	157.157	195.195	229.235	127.129	137.139	132.136	206.212	135.135	135.137	192.192	F
RAM118	5		156.156	203.203	172.172	163.163	157.157	195.203	229.229	109.129	135.139	132.132	206.206	129.135	135.137	192.192	F
RT028	9		140.140	203.207	172.172	157.163		199.203	229.235	127.127	137.139	132.132	206.206	129.135	135.137	188.192	M
RT030	17		156.156	203.207	172.186	157.163	157.157	195.203	229.229	109.127	139.139	132.136	206.214	129.135	135.137	192.192	M
RT109	4		140.140	203.203	172.172	163.163	157.157	203.203	229.235	127.127	135.137	132.132	206.214	129.129	137.137	192.192	F
RT148	2		140.140	203.203	172.186	157.163		203.203	235.235	127.127	139.139	132.136	214.214	129.129	137.137	192.192	M
RT187	2		156.156	207.207	172.172	157.163	157.157	195.203	229.229	127.127	137.139	132.136	206.206	129.135	135.135	188.192	F
RT233	2		140.156	203.203	184.186	163.163	157.157	203.203	235.235	109.127	135.137	132.132	206.212	129.129	135.137	192.192	F

Appendix 5

Multilocus genotypes, based on the markers in common to both WGI and ISPRA labs (n=10 plus sex) expressed in ISPRA scores, of the 45 bears non-invasively sampled during the 2011 survey (see also Appendix 4). Genotypes in parentheses are those matching between the 2011 (WGI-scored) and previous (ISPRA-scored) surveys, with question marks indicating equivocal cases whose matching require further assessment of samples' quality (cf. Tables 11 and 12) (*Table continues in the next page*).

Genotype		*G10B*	*G10C*	*G1D*	*G10L*	*G10P*	*MU59*	*MU50*	*MU51*	*MU05*	*MU11*	Sex
Acc069		128.128	101.105	150.164	154.154	164.164	101.107	100.104	114.122	135.137	88.88	M
Acc079 (Gen 1.2)		112.112	105.105	150.166	154.154	164.164	101.107	100.104	114.114	137.137	92.92	F
F01 (Gen1.25)		112.128	101.105	150.166	148.154	164.164	101.101	100.100	114.122	135.137	92.92	F
F02 (Gen1.56)		128.128	101.105	150.150	148.154	152.164	101.101	100.100	114.120	135.135	88.92	F
F03 (Gen1.44)		112.128	101.101	150.166	148.148	164.164	101.101	100.100	120.120	137.137	88.92	F
F04 (Gen1.12)		112.128	101.101	150.164	148.154	152.164	101.107	100.104	114.120	137.137	88.92	F
F05 (Gen1.22)		112.128	101.105	150.166	148.154	164.164	101.101	100.100	114.114	137.137	92.92	F
F07 (Gen1.23)		112.128	101.105	150.166	148.154	152.164	101.107	100.104	120.120	135.137	88.92	F
F08 (Gen1.73)		112.112	101.105	164.166	148.154	164.164	101.107	100.104	114.114	137.137	88.92	F
F10 (Gen1.54)		112.128	101.105	150.150	148.154	164.164	101.107	104.104	114.114	135.135	88.88	F
FP01 (Gen1.7)		112.128	101.101	150.150	154.154	152.164	101.107	100.104	114.122	137.137	88.92	F
HS001 (Gen 1.4)		112.112	95.101	150.166	154.154	152.164	107.107	104.104	114.114	135.137	88.92	F
HS021 (Gen 1.84?)		128.128	101.105	166.166	154.154	164.164	107.107	100.100	114.114	137.137	92.92	F
HS028 (Gen 1.50)		112.128	105.105	150.164	154.154	164.164	107.107	100.104	122.122	137.137	92.92	F
HS037 (Gen 1.59)		128.128	101.105	150.164	148.154	164.164	101.107	100.104	114.122	137.137	92.92	F
HS058		112.128	101.101	150.150	154.154	164.164	101.107	100.100	114.114	137.137	92.92	M
HS330		112.128	101.101	150.150	154.154	.	101.107	100.104	114.122	135.135	92.92	F
HS338		112.128	101.105	166.166	154.154	.	101.101	100.100	114.122	135.137	92.92	M
HS343 (Gen 1.18)		112.112	101.105	150.150	154.154	164.164	101.107	100.104	114.114	137.137	88.88	F
HS349		126.128	101.105	150.166	148.154	152.164	101.101	100.104	114.114	135.137	92.92	M

Appendix 5

Genotype		G10B	G10C	G1D	G10L	G10P	MU59	MU50	MU51	MU05	MU11	Sex
HS355 (Gen 1.76?)		128.128	105.105	150.150	148.154	164.164	101.101	100.100	114.114	135.135	88.92	M
HS358 (Gen 1.16?)		112.128	101.105	150.164	148.148	164.164	101.101	100.104	114.114	135.137	92.92	F
HS374		112.112	101.105	150.166	148.154	.	101.101	100.104	114.114	135.137	88.92	M
HS451		112.128	101.105	164.166	154.154	.	107.107	100.100	122.122	137.137	88.92	M
HS465		112.112	101.105	150.166	154.154	164.164	101.107	100.100	114.114	137.137	92.92	M
HS477		112.112	101.101	150.164	154.154	.	101.107	100.100	120.122	137.137	92.92	F
M08 (Gen1.60)		112.112	101.105	150.164	148.154	164.164	101.107	100.104	120.122	135.137	88.88	M
M09 (Gen1.71?)		112.128	101.105	150.166	154.154	164.164	101.101	100.100	114.120	135.137	88.92	M
M10 (Gen1.10)		112.128	101.105	164.166	148.154	164.164	101.107	100.100	114.114	137.137	92.92	M
M11 (Gen1.72)		112.128	101.101	150.150	148.154	164.164	101.101	100.100	114.114	135.137	88.92	M
M12 (Gen1.24)		128.128	101.105	150.166	148.154	152.164	101.101	100.100	114.122	135.137	92.92	M
M13 (Gen1.66)		112.128	101.105	150.166	148.148	164.164	101.107	100.100	114.120	137.137	88.92	M
RAM011 (Gen 1.58)		112.112	101.101	150.164	154.154	164.164	101.107	100.104	114.120	135.137	92.92	F
RAM024		128.128	101.105	150.150	148.154	164.164	101.107	100.104	114.122	135.135	88.92	M
RAM042		112.128	101.101	150.150	148.154	164.164	101.107	100.104	114.114	135.137	88.88	M
RAM045		112.112	101.101	150.166	148.154	.	101.107	100.104	114.120	135.137	88.88	M
RAM048		112.128	105.105	150.166	148.154	.	101.101	100.104	114.120	135.137	88.92	F
RAM072 (Gen 1.43?)		112.128	101.105	150.164	154.154	164.164	101.107	100.104	114.120	135.137	92.92	F
RAM118 (Gen 1.85)		128.128	101.101	150.150	154.154	164.164	101.101	100.100	114.114	135.137	92.92	F
RT028		112.112	101.105	150.150	148.154	.	101.107	100.100	114.114	135.137	88.92	M
RT030 (Gen 1.49)		128.128	101.105	150.166	148.154	164.164	101.101	100.104	114.122	135.137	92.92	M
RT109		112.112	101.101	150.150	154.154	164.164	101.107	100.100	114.122	137.137	92.92	F
RT148		112.112	101.101	150.166	148.154	.	107.107	100.104	122.122	137.137	92.92	M
RT187 (Gen 1.37)		128.128	105.105	150.150	148.154	164.164	101.101	100.104	114.114	135.135	88.92	F
RT233 (Gen 1.41)		112.128	101.101	164.166	154.154	164.164	107.107	100.100	114.120	135.137	92.92	F

Appendix 6

Summary encounter histories for all 45 Apennine brown bears sampled in the 2011 survey in the PNALM, Italy (June – September). The individuals previously live-trapped are identified with their code (cf. Appendix 1), whereas all other bears are coded with the identification code of the first hair-sample through which they have been identified. For each individual (rows), columns III-XV report capture (1) or no capture (0) in each session by sampling method (HS: hair-snag, sessions 1-5; Opp: opportunistic sampling at buckthorn patches, sessions 6-8; RT: rub tree sampling, sessions 9-12; INC: incidental sampling, session 13).

Individual	Sex	HS_1	HS_2	HS_3	HS_4	HS_5	OPP_1	OPP_2	OPP_3	RT_1	RT_2	RT_3	RT_4	INC
M10	M	1	1	0	0	0	0	0	0	1	0	0	0	1
M12	M	1	0	0	0	0	0	1	0	1	1	0	1	1
FP01	F	0	0	1	0	1	0	0	0	0	0	0	0	1
HS'001	F	1	0	0	0	0	0	1	1	0	0	0	0	0
HS'021	F	1	1	0	0	0	0	1	1	0	0	1	1	1
HS'028	F	0	0	0	1	1	1	1	0	0	0	1	1	0
HS'037	F	0	0	1	0	0	1	0	0	0	0	0	0	0
HS'058	M	0	0	1	0	0	0	1	0	0	0	0	0	0
HS'330	F	1	0	0	0	0	1	0	0	0	0	0	0	0
HS'338	M	0	1	0	0	0	0	0	0	0	0	0	0	0
HS'343	F	1	0	1	0	0	0	0	0	0	0	0	1	0
HS'349	M	0	0	1	1	0	0	0	0	0	0	0	0	0
HS'355	M	1	0	0	0	0	0	0	0	0	0	0	0	0
HS'358	F	0	1	0	1	0	0	0	0	0	0	0	0	0
HS'374	M	0	1	0	0	0	0	0	0	1	1	0	1	0
HS'451	M	1	0	0	0	1	0	0	0	0	0	0	0	0
HS'465	M	1	0	0	0	0	0	0	0	0	0	0	0	0
HS'477	F	0	0	0	0	1	0	0	0	0	0	0	0	1
F04	F	0	0	0	0	1	0	0	0	0	0	0	0	0
F01	F	0	0	0	0	1	0	1	1	0	1	0	0	0
M08	M	1	0	0	0	0	0	1	0	1	1	1	1	1
F02	F	0	0	0	1	0	0	0	0	0	1	0	1	0
M09	M	1	0	0	0	0	0	0	0	1	1	0	1	0
F07	F	0	0	0	1	0	0	0	0	0	0	0	0	0
F03	F	0	0	1	0	0	0	0	0	0	0	1	0	0
M13	M	1	0	1	0	0	0	1	0	1	1	1	1	0
Acc'069	M	0	0	0	0	0	0	0	0	0	0	0	0	1
Acc'079	F	0	0	0	0	0	0	0	0	0	0	0	0	1
M11	M	0	0	0	0	0	0	0	0	1	1	1	1	1
RT'028	M	0	0	0	0	0	0	0	0	0	1	1	0	1
RT'030	M	0	0	0	0	0	1	1	0	0	1	1	1	1
RT'233	F	0	0	0	0	0	0	0	0	0	0	1	0	1
F05	F	0	0	0	0	0	0	1	1	0	1	0	0	0
F10	F	0	0	0	0	0	0	1	0	0	0	0	0	0
RAM'011	F	0	0	0	0	0	1	1	1	0	0	0	0	0
RAM'024	M	0	0	0	0	0	0	1	0	0	0	0	0	0
RAM'042	M	0	0	0	0	0	0	1	1	0	0	0	0	0
RAM'045	M	0	0	0	0	0	0	1	1	0	0	0	0	0
RAM'048	F	0	0	0	0	0	0	1	1	0	0	0	0	0
RAM'072	F	0	0	0	0	0	0	0	1	0	0	0	0	0
RAM'118	F	0	0	0	0	0	1	0	0	0	0	0	0	0
RT'109	F	0	0	0	0	0	0	0	1	0	1	0	1	0
F08	F	0	0	0	0	0	0	0	1	0	1	0	0	0
RT'148	M	0	0	0	0	0	0	0	0	0	0	0	1	0
RT'187	F	0	0	0	0	0	0	0	0	0	1	0	0	0