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RESEARCH ARTICLE

An improved procedure to estimate wolf abundance using non-invasive genetic sampling and capture–recapture mixture models

Romolo Caniglia · Elena Fabbri · Sarah Cubaynes · Olivier Gimenez · Jean-Dominique Lebreton · Ettore Randi

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Abstract Non-invasive genetic sampling (NGS) is increasingly used to estimate the abundance of rare or elusive species such as the wolf (*Canis lupus*), which cannot be directly counted in forested mountain habitats. Wolf individual and familial home ranges are wide, potentially connected by long-range dispersers, and their populations are intrinsically open. Appropriate demographic estimators are needed, because the assumptions of homogeneous detection probability and demographic closeness are violated. We compiled the capture–recapture record of 418 individual wolf genotypes identified from ca. 4,900 non-invasive samples, collected in the northern Italian Apennines from January 2002 to June 2009. We analysed this dataset using novel capture–recapture multievent models for open populations that explicitly account for individual detection heterogeneity (IDH). Overall, the detection probability of the weakly detectable individuals, probably pups, juveniles and migrants ($P = 0.08$), was ca. six times lower than that of the highly detectable wolves ($P = 0.44$), probably adults and dominants. The apparent annual survival rate of weakly detectable individuals was lower ($\Phi = 0.66$) than those of highly detectable wolves ($\Phi = 0.75$). The population mean annual finite rate of increase was $\lambda = 1.05 \pm 0.11$, and the mean annual size ranged from $N = 117$ wolves in 2003 to $N = 233$ wolves in 2007. This procedure, combining large-scale NGS and multievent IDH demographic models, provides the first estimates of abundance, multi-annual trend and survival rates for an open large wolf population in the Apennines. These results contribute to deepen our understanding of wolf population ecology and dynamics, and provide new information to implement sound long-term conservation plans.

Keywords *Canis lupus* · Capture–recapture analyses · Individual detection heterogeneity · Multilocus genotypes · Non-invasive genetics · Population size estimation

R. Caniglia (✉) · E. Fabbri · E. Randi
Laboratorio di Genetica, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), Via Cà Fornacetta 9, 40064 Ozzano dell'Emilia, Bologna, Italy
e-mail: romolo.caniglia@gmail.com

S. Cubaynes · O. Gimenez · J.-D. Lebreton
Centre d'Ecologie Fonctionnelle et Evolutive, Campus CNRS, UMR 5175, 1919 Route de Mende, 34293 Montpellier Cedex 5, France

Introduction

After centuries of population decline and range contractions, some species of large carnivores, such as the wolf (*Canis lupus*), brown bear (*Ursus arctos*) and lynx (*Lynx lynx*), are now expanding in Europe (Breitenmoser 1998). Determining the rates and patterns of population expansion is crucial to design sound conservation strategies, and to efficiently manage the impact that top predators have on their prey, mainly wild and domestic ungulates (Luikart et al. 2010). However, estimating demographic parameters is not trivial (Mills et al. 2000). Top predators in Europe are distributed at low densities across large geographic areas, often in forested mountain regions, and their individual and familial home ranges are wide. After centuries of persecution they have a strong tendency to avoid any contact with humans and are difficult to observe directly (Breitenmoser 1998). Standard field methods, such as direct counts, track counts, wolf-howling, trapping and radio-tracking, are challenging or exceedingly expensive (Wilson and Delahay 2001). The recent developments of

non-invasive genetic sampling (NGS) and molecular identification of species, individuals and gender can offer solutions. In fact, despite their costs, molecular techniques can provide more exhaustive information than any other method (Lukacs and Burnham 2005; Waits and Paetkau 2005; Lukacs et al. 2007). Reliable individual genotypes (DNA fingerprints) can be obtained analysing DNA extracted from biological samples such as hair, faeces, urine and blood traces that are non-invasively collected, without any contact with the animals (Taberlet et al. 1999). The capture–recapture (CR) record of individual genotypes can be used to directly count the minimum population size (Ernest et al. 2000; Lucchini et al. 2002; Creel et al. 2003), and to estimate their abundance by appropriate demographic models (Kohn et al. 1999; Mills et al. 2000; Lukacs and Burnham 2005). For these reasons, NGS has been integrated in a number of large carnivore monitoring projects (Kohn and Wayne 1997; Fabbri et al. 2007; Marucco et al. 2009; Cubaynes et al. 2010).

A variety of demographic models have been recently developed to estimate abundance, apparent survival (the probability that an individual is alive and present in the study area), migration, movement or transition rates, fecundity and growth trends for both "closed" and "open" populations (Nichols 1992; Luikart et al. 2010). However, most of these models ignore the individual detection heterogeneity (IDH) in capture probability, with the risk to compute biased estimates, and in particular to underestimate abundance (Hwang and Huggins 2005; Cubaynes et al. 2010). In closed populations these threats can be minimized using CR models that assign individuals in classes with distinct detection probabilities (Pledger 2000), or incorporating individual covariates (McDonald and Amstrup 2001). Even in open populations, which do not assume population closure, underestimates of abundance can be avoided using individual covariates in CR models. However, it is difficult, if not impossible, to obtain covariate datasets within NGS projects, because the target individuals are never observed. Multievent models (Pradel 2005) now offer solutions under the form of CR models incorporating individual heterogeneity (Pledger et al. 2003; Pradel 2009).

In this study we aimed at estimating demographic parameters in an open wolf population that has been monitored for 8 years. Individuals were identified by genotyping 12 autosomal microsatellites in DNAs extracted from faecal and tissue samples, collected in a sector of the northern Italian Apennine ridge from 2002 to 2009. Samples were collected from an area of about 10,000 km^2, permanently used by at least 30 wolf packs, which are part of a larger wolf population living in the northern Italian Apennines (Emilia-Romagna, Tuscany and neighbouring regions; Fig. 1).

The genotypes' CR record was used to estimate the apparent survival, individual capture probabilities and abundance of the studied wolf population. We expected significant heterogeneity in individual detection probability, due to the social behaviour of wolves (territoriality and dominance; Mech 1970; Mech and Boitani 2003), and to the extensive sampling areas which prevented any local intensive sampling. Thus, we followed Cubaynes et al. (2010), who suggested that multievent CR models for open populations with IDH are the most appropriate. In their approach the classical Horvitz-Thompson type estimator of population size (Williams et al. 2002) used by Marucco et al. (2009) has been modified, and different detection probability classes of individuals are explicitly modelled, aiming to minimize population estimate biases (Pledger 2000; Pledger et al. 2003; Cubaynes et al. 2010). We investigated the effects of temporal (sampling time, particular years or seasons), biological (gender) and climate (cold and hot semesters) variables on survival and detection probabilities.

Materials and methods

Study area

The study area includes a large portion of the northern Apennine hills between Emilia-Romagna and Tuscany, covering a surface of about 10,000 km^2 (Fig. 1). Most of this region is higher than 700 m a.s.l., with mountains ranging from 1,407 (Mount Fumaiolo) to 2,165 (Mount Cimone) m a.s.l. These mountains are covered by temperate-sub-Mediterranean deciduous forests, and are populated by rich communities of wild ungulates: wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and fallow deer (*Dama dama*). The lowest hill ranges are characterized by pastures and livestock breeding, while slopes and valleys are cultivated. Some parts of the region are urbanized, with medium to small-sized villages and fairly dense road networks. About 25% of the study area is protected: there are two national and eight regional parks, which constitute the core areas of the wolf distribution (Fig. 1).

Sample collection and DNA extraction

Faeces were systematically collected along trails and country roads opportunistically chosen based on documented or presumed wolf presence. Sampling was carried out thanks to the collaboration of more than 150 volunteers and students, personnel of the Italian Forestry Service (CFS), national and local parks and provinces. The wide study area and long-term programme did not allow

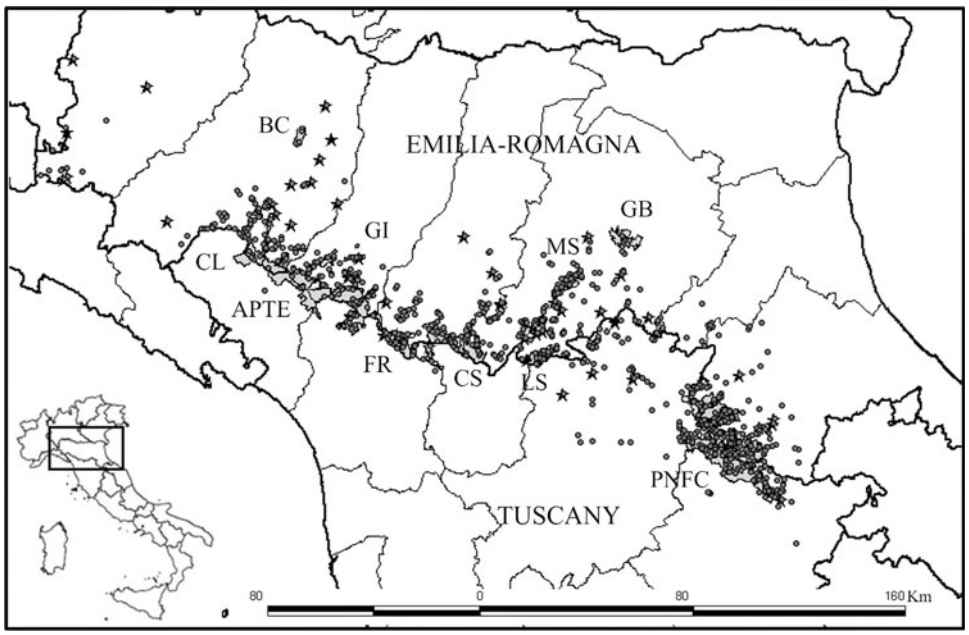

Fig. 1 The study area with sampling effort and distribution. *Dark circles* stand for the non-invasively collected samples, *black stars* for the tissues obtained from found-dead wolves. Protected areas are indicated in *grey* and include two national parks: *APTE* Appennino Tosco-Emiliano and *PNFC* Foreste Casentinesi, Monte Falterona, Campigna, and eight regional parks: *BC* Boschi di Carrega, *CL* Cento Laghi, *CS* Corno alle Scale, *FR* Frignano, *GB* Gessi Bolognesi e Calanchi dell'Abbadessa, *GI* Gigante, *LS* Laghi di Suviana e Brasimone and *MS* Monte Sole

standardizing sampling efforts in space and time. Thus, sampling was more intense in winter-spring seasons and in the protected areas. Collaborators were trained to collect only fresh samples, discarding scats judged older than 2 weeks (Santini et al. 2007). Scats and tissues were individually stored at −20°C in 10 volumes of 95% ethanol. DNA was extracted from a total of 4,898 samples collected between January 2002 and June 2009, including 4,839 presumed wolf scats and 59 tissues from found-dead wolves accidentally or illegally killed. DNA samples were automatically extracted using a MULTIPROBE II^{EX} Robotic Liquid Handling System (Perkin Elmer) and the QIAGEN stool and tissue extraction kits (Qiagen Inc, Hilden, Germany).

Molecular markers and PCR amplifications

DNAs were PCR-amplified and genotyped at 12 unlinked autosomal microsatellites: seven dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12; Fredholm and Wintero 1995; C09.250 and C20.253; Ostrander et al. 1993) and five tetranucleotides (FH2004, FH2079, FH2088, FH2096 and FH2137; Francisco et al. 1996), selected for their polymorphism in the Italian wolf population (Randi and Lucchini 2002). Gender was identified by PCR-RFLP of the ZFX/ZFY (zinc-finger protein) sequences (Lucchini et al. 2002). PCR-amplifications were carried out in 10 µl reactions, using respectively 1 or 2 µl of DNA solutions from tissue or scat extractions, plus 2 µg of BSA. PCR conditions were optimised for each primer and for tissue or scat samples, the number of cycles varied from 30 to 45. Faecal DNAs were extracted and amplified in separate rooms only dedicated to low DNA-content samples, and genotyped using a multiple-tube protocol as implemented by Lucchini et al. (2002) and Fabbri et al. (2007). DNA quality was initially screened by PCR-amplifying each DNA sample four times at two loci (FH2096 and FH2137). Only samples showing >50% positive PCRs were further amplified four times at the remaining 10 loci and sexed. Negative (no DNA in PCR) and positive (samples with known genotypes) controls were always used. PCR products were analysed in an ABI (Applied Biosystems) 3130XL automated sequencer (Foster City, CA), using the ABI software GeneMapper 4.0.

A reliability analysis was performed using RelioType (Miller et al. 2002). Unreliable genotypes (at threshold $R < 0.95$) were additionally replicated another four times. Samples that were not reliably typed at all loci after eight PCR replicates were definitively discarded. Consensus genotypes were reconstructed using Gimlet 1.3.3 (Valière 2002), accepting heterozygotes if the two alleles were seen at least in two replicates, and homozygotes if a single allele was seen at least in four replicates. Gimlet was also used to estimate PCR success and errors: allelic dropout (ADO), and false alleles (FA) following Broquet and Petit (2004). The probability of identity (PID) and the expected PID among full sib dyads (PIDsibs; Mills et al. 2000; Waits et al. 2001) were computed by GenAlEx 6.1 (Peakall and Smouse 2006).

Demographic analyses

Multilocus genotypes were used as CR records, and individual encounter tables were constructed subdividing the whole study period in 30 three-month capture occasions, starting from the earliest quarter in 2002. The first

detection of a genotype was considered as the initial capture (marking), and, assuming that matching genotypes belonged to the same individual, further detections were classified as recaptures.

Demographic analyses were carried out through CR models designed for open populations (Lebreton et al. 1992). In a first step, a goodness-of-fit (GOF) testing procedure was performed to identify a model that adequately fits the data (Pradel et al. 2005). We checked for significant deviations from a general model assuming that both recapture (P) and survival (Φ) probabilities are dependent on time. Following Cubaynes et al. (2010), we paid particular attention to the issue of heterogeneity in the detection probability, i.e. an excess of encounter histories with consecutive "captures" (i.e. runs of '1'), corresponding to highly detectable individuals, and consecutive "non-captures" (i.e., runs of '0'), corresponding to poorly detectable individuals. In particular, some of the runs of '0' occur at the end of the capture history, inducing both "transience" (i.e. lower chance of recapture of first-encountered individuals than already encountered ones) and "trap-shyness" (i.e. lower probability to encounter at time $t + 1$ the individuals encountered at time t than the individuals not encountered at time t but known to be alive because of previous and future recaptures). Here, trap-shyness might correspond to individuals belonging to a subordinate social status or to individuals avoiding defecating in areas that have been visited by humans, i.e., avoid areas near study transect lines. Using the software U-CARE 2.3 (Choquet et al. 2009a, b), we carried out specific tests (Pradel et al. 2005) to assess for transience (Test 3.SR) and/or trap-dependence (Test 2.Ct). Both tests are significant if detection heterogeneity occurs (Péron et al. 2010), and should be accounted for as explained in the next section.

Multievent model selection and population size estimation

Multievent models (Pradel 2005) are general CR models in which events (observations) are distinguished from the hidden states of variables (physiological or geographical) of biological interest. Models with heterogeneous recapture probabilities were initially proposed by Pledger et al. (2003), and then reformulated in the multievent framework by Pradel (2009). We assumed that individuals can be described by two events (observed, not-observed) and arranged in two fixed classes of capturability (an individual cannot change class over time) informed on the basis of their resampling frequencies obtained from the individual encounter table. Technically, we considered a proportion π of individuals having low capture probabilities (P_L) and a proportion $(1 - \pi)$ of individuals having high capture probabilities (P_H). The total number of individuals in the population at time t, N_t, consists of the sum of U_t new individuals and M_t individuals already present and still alive at time t. In the heterogeneity model, the newly detected ones are made up of $\pi \cdot U_t$ individuals in state L, and $(1 - \pi) \cdot U_t$ individuals in state H. We obtained an estimate $\hat{U}_t$ of the expected number of new individuals in the population using a Horvitz–Thompson type estimator accounting for heterogeneity and an estimate $\hat{M}_t$ of the expected number of already detected individuals still alive at time t applying the survival estimates to already detected individuals (see Cubaynes et al. 2010 for further details). Once the structure of the model was defined, we built a set of 28 biologically plausible models in which variations in both survival and detection probabilities were possibly explained by several temporal and individual effects and their combinations. Following Cubaynes et al. (2010), we considered year and seasonal effects to test if individual survival and detection could be influenced by particular years, because of differential mortality, or seasons, because of variable climate or sampling efforts. Variations in the detection process were also investigated accounting for a "climate effect", dividing each year in cold (October–March) and hot (April–September) 6-month periods, expecting a higher detection during winter due to a better DNA conservation. Additionally, to remove other eventual sources of heterogeneity, we tested for a gender effect on survival and detection probabilities.

To identify the best model explaining the data, in particular the most appropriate structure of the recapture rates which are required for estimating abundance, we used the Akaike's information criterion corrected for small sample sizes (AICc; Burnham and Anderson 2002). The model with the lowest AICc was considered the most parsimonious. A better interpretation of AICc comparison and the relative merits of competing models were facilitated using the Akaike weights (Burnham and Anderson 2002). We used a non-parametric bootstrap procedure (Davison and Hinkley 1997) to resample the individual encounter histories with 1,000 replicates in order to obtain 95% confidence intervals (CI) for population size (Cubaynes et al. 2010). Model fitting and selection were carried out using the program E-SURGE 1.6.0 (Choquet et al. 2009a, b). Finally, we estimated the mean annual finite rate of increase between two successive years (λ) as the ratio of their mean annual population sizes.

Results

Genetic analyses

The first quality-screening test was not passed by 1,837 non-invasive samples (38%), which were immediately

discarded. The remaining 3,002 samples (62%) were amplified at the other 10 loci and sexed. Other 846 samples (17%) showing <50% PCR success or $R < 0.95$, were further discarded. The remaining 2,156 samples (45%) obtained a complete genotype. After a regrouping procedure we identified 432 individual genotypes. The observed average error rates across loci were: ADO = 0.14 ± 0.03 and FA = 0.009 ± 0.002.

Post-process quality controls on these genotypes were performed through a mismatch analysis (software MM-Dist; Kalinowski et al. 2006) that identified 67 genotypes differing for one and 110 for two mismatches. Genotyping errors at one or two loci are more likely than multiple errors (Pompanon et al. 2005), thus these samples were genotyped again and 52 of them (12% of total) were further deleted because of high occurrence of ADO (67%) and FA (30%), or due to scoring errors or misinterpretation of the electropherograms (3%). We identified new genotypes in 38 (64%) of the 59 tissue samples. The others matched with genotypes already non-invasively sampled.

Finally, we identified 418 genotypes that were definitely accepted: 245 (59%) males and 173 (41%) females, with multilocus PID = 1.10×10^{-8}, and PIDsibs = 3.6×10^{-4}, meaning that only 3.6 wolves in 10,000 siblings are expected to share by chance an identical genotype, suggesting no "shadow effect" (all the genotypes identify distinct individuals; Mills et al. 2000), and that matching genotypes are recaptures of the same individual. Resampling frequencies were heterogeneous: 169 genotypes (40%) were sampled only once, while the other 249 (60%) were sampled from two to 56 times. The resampled individuals also showed highly heterogeneous permanence periods ranging from a few days to about 6 years.

Goodness-of-fit test

The overall GOF test was significant ($\chi^2_{118} = 201.64$; $P < 0.0001$) and the general model was rejected. In particular, both test 2.Ct ($\chi^2_{27} = 48.49$; $P < 0.001$) and test 3.SR ($\chi^2_{26} = 69.62$; $P < 0.0001$) were strongly significant, indicating strong signals of recapture heterogeneity among individuals (Péron et al. 2010), and suggesting that, in order to avoid bias in abundance estimation, we need more complex models accounting for IDH.

Model selection

Model selection was performed on a set of 28 biologically plausible models, characterized by: (1) survival depending on individual heterogeneity or homogeneity, and on time, sex and their interactive or additive effects; (2) detection depending on individual heterogeneity and its interactive or addictive effects with time, sex, season, climate and year. The best model (AICc value = 3,175.12) resulted that with individual heterogeneity in survival and an interaction effect of heterogeneity and season in detection (Table 1). This model was characterized by a low capture probability (P_L) depending on winter quarter effect against all the other seasons of the year, and a high capture probability (P_H) depending on an interaction effect of heterogeneity and time. The Akaike weight for this model was close to 1, much higher than that of all the others, confirming it was the best model supported by our data (Table 1). The second best model (AICc = 3,191.72) was characterized by individual heterogeneity in survival, and detection affected by an interaction effect of heterogeneity and time. The third (AICc = 3,191.89) and fourth (AICc = 3,192.17) best models showed survival depending, respectively, on individual heterogeneity and homogeneity, but they both showed detection depending on an interaction effect of heterogeneity with an additive effect of time and sex (Table 1). Individual heterogeneity was the predominant variable in survival and detection of most of the models. On the other hand, time, sex and their combinations had a minimal effect on survival (AICc ≤ 3,215.39) and were absent from the survival of the top six models. Season, climate and year had a minimal effect on detection probabilities (AICc ≤ 3,250.53) and were not included in the detection of the top ten models (Table 1).

Parameter estimates from the best model

The newly marked population was composed of 0.46 (95% CI: 0.34–0.56) of individuals in state *L* (low capture probability) and 0.54 (95% CI: 0.44–0.66) of individuals in state *H* (high capture probability). Annual survival (product of all 3-month survival probabilities) of wolves in state *L* was 0.66 (95% CI: 0.54–0.77) versus 0.75 (95% CI: 0.68–0.81) for wolves in state *H*. Detection probability strongly differed in the two classes of detectability (Fig. 2). The detection probability of wolves in state *H* (0.44) was 6–7 times greater than that of wolves in state *L* (0.08), on average during the whole study. For individuals in state *L*, detection probability was higher in winter (0.18; 95% CI = 0.09–0.27) than during the rest of the year (0.05; 95% CI = 0.02–0.09). Detection probability in state *H* showed marked seasonal variations, with lower values in summer than in other seasons, ranging from 0.15 (95% CI = 0.06–0.26) in summer 2006 to 0.76 (95% CI = 0.60–0.93) in winter 2007. Finally, survival and detection probabilities were positively correlated, having weakly detectable individuals a lower survival probability than highly detectable individuals (Fig. 2).

Table 1 List of the 28 models (sorted by increasing AICc values) incorporating heterogeneity

Model		np	Deviance	AICc	ΔAICc	AICc weight
Survival	Detection					
het	P_L (winter) P_H (het × t)	34	3,107.12	3,175.12	0.00	0.99
het	het × t	61	3,069.72	3,191.72	16.60	0.01
het	het × (t + g)	63	3,065.89	3,191.89	16.77	0.00
hom	het × (t + g)	62	3,068.17	3,192.17	17.06	0.00
het	het + t	33	3,127.28	3,193.28	18.16	0.00
hom	het × t	60	3,083.45	3,203.45	28.33	0.00
(t + g)	het × t	89	3,037.39	3,215.39	40.27	0.00
t	het × t	87	3,048.70	3,222.70	47.59	0.00
het	het × (t × g)	119	3,001.30	3,239.30	64.18	0.00
hom	het × (t × g)	118	3,007.11	3,243.11	67.99	0.00
hom	het × season	10	3,230.53	3,250.53	75.42	0.00
hom	het + season	7	3,237.26	3,251.26	76.14	0.00
het	het × season	11	3,229.93	3,251.93	76.81	0.00
het	het + season	8	3,236.46	3,252.46	77.34	0.00
(t × g)	het × t	116	3,025.00	3,257.00	81.88	0.00
g	het × t	61	3,145.31	3,274.41	99.29	0.00
het	het + climate	6	3,259.82	3,271.82	96.70	0.00
hom	het × climate	6	3,260.51	3,272.51	97.39	0.00
het	het × climate	7	3,259.81	3,273.81	98.69	0.00
hom	hom × winter	6	3,263.74	3,275.74	100.62	0.00
hom	hom × winter	5	3,266.12	3,276.12	101.00	0.00
het	hom × winter	7	3,262.88	3,276.88	101.76	0.00
het	het + winter	6	3,265.34	3,277.34	102.22	0.00
hom	het + year	11	3,278.62	3,300.62	125.50	0.00
het	het + year	12	3,277.32	3,301.32	126.20	0.00
het	het × year	19	3,273.12	3,311.12	136.00	0.00
hom	het × year	18	3,275.73	3,311.73	136.61	0.00
hom	het + climate	5	3,310.17	3,320.17	145.05	0.00

ΔAICc the differences between the AICc of a model and the lowest AICc, *np* the number of parameters, *Deviance* the minimum relative deviance, *AICc weight* the relative weigh of each tested model; *het and hom* heterogeneity and homogeneity effects, *g* and *t* indicate group (females and males) and time effects, *Climate* effect of the cold (October–March) vs. the hot (April–September) semester, *Season* seasonal effect, *Year* year effect, *Winter* effect of the winter semester vs. all the others, P_L and P_H capture probabilities for low and high detectable individuals; + additive effect of several variables; × interaction effect of several variables

Population size estimation

The total size of the investigated wolf population showed a clear seasonal trend, with a minimum of 54 (95% CI: 20–141) individuals in summer 2002 and a maximum of 381 (95% CI: 184–782) individuals in autumn 2002 (Table 2; Fig. 3). This seven fold increase between summer and autumn of the first year might be partially determined by the larger number of samples analysed in autumn (about five times larger than in spring) and by the consequent increase of the detected genotypes (Table 2).

The average annual population size ranged between a minimum of 117 (95% CI: 70–214), during year 2003, to a maximum of 233 (95% CI: 148–402) individuals, during year 2007 (Table 2). The mean annual population size showed an increasing trend with the exception of years 2003 and 2008 (Table 2). Temporal trends of population size varied regularly over all the study period, with *N* being the highest during the autumnal quarters, and strongly decreasing in winter, reaching the lowest rates (except for year 2005) during summer (Table 2; Fig. 3). Decreasing population size in winter might be consequent to lower detection rates, probably due to reduced recapture rates of cubs and sub-adults in winter because their mortality peak occurs in middle-late autumn (November/December; Mech and Boitani 2003; Lovari et al. 2007). Hence, sub-adults

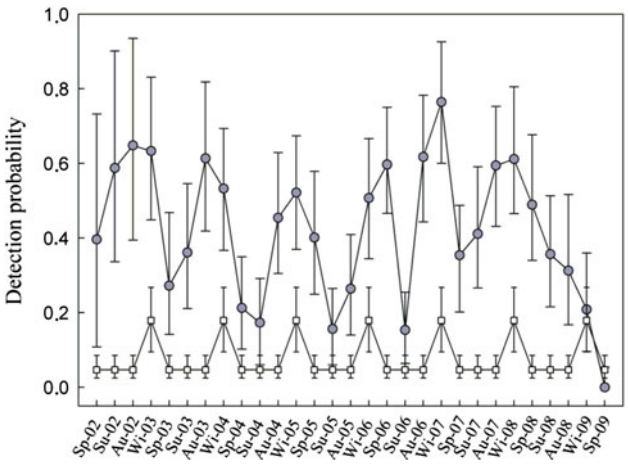

Fig. 2 Detection probability pattern. Time variation of detection probabilities of individuals in state *L* (*open-squares*) and individuals in state *H* (*filled-circles*) and their 95% confidence intervals (*vertical bars*) obtained by a bootstrap procedure

cannot be sampled anymore in winter. Low population size in summer might be due to the lowest faecal sample field detection (Table 2) because of reduced individual or pack mobility from the denning season until middle-late autumn or to dispersing yearlings, which leave their natal packs from January to May, just before summer (Fuller 1989). Yearlings in summer have lower survival rates than adults (Boyd and Pletscher 1999). In detail, the first year (2002) was the most variable one because it was characterized by the lowest (summer) and highest (autumn) population size values of the whole study period. Year 2003 was characterized by a decreasing population trend during winter, spring and summer, followed by an increasing autumnal value. Year 2004 showed comparable sizes during winter and spring, a deep decreasing during summer and a considerable increasing during autumn. Year 2005 was characterized by an anomalous trend with increasing sizes during winter, spring and summer, and a maximum during autumn. Year 2006 showed again decreasing sizes during winter, spring and summer, with a maximum during autumn. Both years 2007 and 2008 showed a regular pattern with increasing sizes during winter and spring, decreasing during summer and the highest peaks during autumn. The last year (2009) was characterized only by the usual decreasing size during winter, in fact we could not estimate population size during the last sampling occasion (spring 2009) because the detection probability of the individuals with high capture probability (P_H) was not identifiable during this quarter (Table 2; Fig. 3).

The mean annual finite rate of increase (λ) of the population was 1.05 ± 0.11. We estimated values of λ greater than one for years 2004 (1.41), 2005 (1.17), 2006 (1.15) and 2007 (1.05), and less than one only for years 2003 (0.67) and 2008 (0.87).

Discussion

In this study we obtained, for the first time, estimates of abundance, multi-annual population trend and survival rates in a sector of the northern Italian Apennine wolf population. After centuries of decline, wolves in Italy disappeared from the Alps and northern Apennines before the 1920s, and continued to decline throughout the country until the end of the Second World War. In the 1970s only about 100 individuals survived in secluded forest patches in the central and southern Apennines (Zimen and Boitani 1975). Then, socio-ecological changes in mountain areas led to a rapid growth of forests and wild ungulates, generating the conditions for a natural expansion of the Italian wolf population. Wolves expanded along the whole Apennine ridge, recolonizing parts of their historical range, and reached the south-western Alps in 1992 (Fabbri et al. 2007). The impact of wolf expansion on natural ungulate communities is important, as well as predations on livestock have been, in some cases, significant (Gazzola et al. 2008). However, despite national (Genovesi 2002) and European (Boitani 2000) conservation guidelines strongly recommending that wolf population dynamics to be actively monitored, only a few local short-term monitoring projects have been activated in European countries so far (Wabakken et al. 2001; Marucco et al. 2009; Caniglia et al. 2010; Cubaynes et al. 2010). Consequently, both global and local estimates of wolf abundance are still lacking. Informal expert opinions put the estimated wolf population in Italy at about 600 individuals in 2003 (Mech and Boitani 2003), although their current number could now be close to 900–1,000 individuals. These guessed estimates are not based on quantitative approaches or statistical inference, which makes it impossible to evaluate their precision and accuracy. Local studies described wolf packs of 2–7 individuals, with territories of 150–250 km^2, and estimated density from one to four wolves per 100 km^2 (Ciucci and Boitani 1999; Apollonio et al. 2004). However, these results are limited to a few case studies, and cannot be extrapolated to the entire species distribution range.

Here we show how an integrated NGS-CR design can be used to estimate the fundamental demographic parameters of a large open wolf population. We used a new CR procedure proposed by Cubaynes et al. (2010). Estimates of NGS-based population size have been reported in bear (*Ursus* spp., Woods et al. 1999), coyote (*Canis latrans*, Kohn et al. 1999), wolf (*Canis lupus*, Creel et al. 2003) and European badger (*Meles meles*, Wilson et al. 2003). Some of these estimates have been likely biased by both genotyping errors (such as in Creel et al. 2003), or by the use of inappropriate statistical methods (as highlighted by Lukacs and Burnham 2005). It is well known that genotyping errors can produce strongly biased estimates of population

Table 2 Number of analysed samples, number and percentage of discarded samples, detected multilocus genotypes, population size and corresponding lower and upper 95% confidential intervals (CI) estimated for each of the 3-month sampling occasions in which the study period was subdivided

Sampling occasion	Analysed samples	Discarded samples	% of Discarded samples	Detected genotypes	Estimated population size	Lower 95% CI	Upper 95% CI
2002							
Spring	33	12	36.36	13	90	36	232
Summer	48	22	45.83	14	54	20	141
Autumn	242	125	51.65	47	381	184	782
Mean	108	53	49.23	25	175	80	385
2003							
Winter	207	102	49.28	45	100	69	164
Spring	67	41	61.19	16	99	61	177
Summer	66	44	66.67	18	97	59	183
Autumn	186	115	61.83	35	172	92	332
Mean	132	76	57.41	29	117	70	214
2004							
Winter	271	158	58.30	51	137	100	207
Spring	90	58	64.44	18	144	89	260
Summer	47	30	63.83	10	82	61	132
Autumn	186	106	56.99	44	298	156	590
Mean	149	88	59.26	31	165	102	297
2005							
Winter	177	84	47.46	52	151	114	219
Spring	116	65	56.03	31	165	109	302
Summer	62	34	54.84	17	189	118	333
Autumn	122	68	55.74	34	270	168	492
Mean	119	63	52.62	34	194	127	337
2006							
Winter	390	223	57.18	70	211	159	334
Spring	251	153	60.96	51	201	141	345
Summer	90	65	72.22	18	197	126	332
Autumn	269	172	63.94	54	279	170	508
Mean	250	153	61.30	48	222	149	380
2007							
Winter	504	293	58.13	85	195	151	302
Spring	130	72	55.38	36	200	130	360
Summer	84	38	45.24	33	193	121	331
Autumn	216	100	46.30	60	341	189	614
Mean	234	126	53.85	54	233	148	402
2008							
Winter	427	230	53.86	73	194	141	297
Spring	186	99	53.23	45	222	139	389
Summer	75	36	48.00	29	144	96	251
Autumn	112	56	50.00	33	246	139	474
Mean	200	105	52.63	45	201	129	353
2009							
Winter	135	81	60.00	34	172	115	257

Because the capture–recapture likelihood is built conditional on first capture of individuals (Lebreton et al. 1992), detection probability was not estimated at the first sampling occasion. Consequently, population size was not estimated in winter 2002; population size was not identifiable even for the last sampling occasion, spring 2009, because of a too low detection probability of the individuals with high capture probability. Mean indicates the mean annual values for each of the described categories

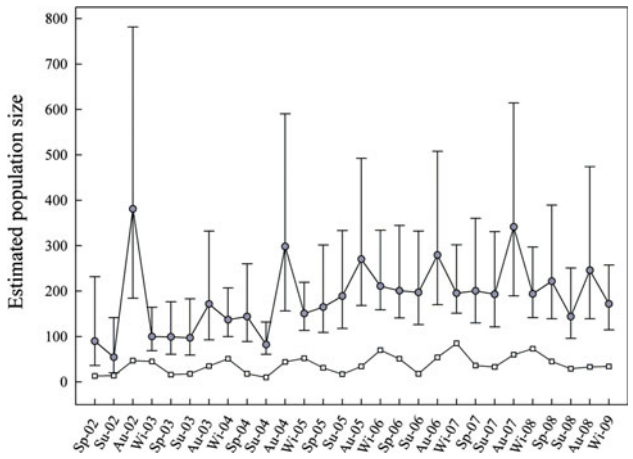

Fig. 3 Population size estimation. Total estimated wolf population size in the northern Italian Apennines from Spring 2002 to Winter 2009 (*filled-circles*) with corresponding 95% confidence intervals (*vertical bars*), and multilocus genotypes detected during the same 3-month sampling occasions (*open-squares*)

size (Waits and Leberg 2000), but the impact of these errors can be limited through accurate laboratory procedure and data quality controls (Pompanon et al. 2005). It is more difficult to avoid the biases which derive from an inefficient sampling and low capture probability in the dataset (Boulanger et al. 2006), or from IDH (Boulanger et al. 2008). Cubaynes et al. (2010) showed that models ignoring IDH produced population underestimates of about 27% on average.

In this study we used multievent CR models for open populations with two detection classes: a proportion of individuals having low capture probabilities and a proportion of individuals having high capture probabilities (Pledger et al. 2003; Pradel 2009). Results showed that, in contrast with Marucco et al. (2009), the trade-off of a large-scale NGS project was a significant IDH. The GOF tests indicated highly significant IDH among wolves, and the 19 top models incorporated IDH (Table 1). Detection heterogeneity can be produced by different factors (Ebert et al. 2010), such as genotyping errors (Lukacs and Burnham 2005), variable sampling efforts (Devineau et al. 2006), or intrinsic traits the species biology (Crespin et al. 2008). In our study, IDH was probably due to the wide study area, which: (a) was partially sampled (only the western side of the north Apennines was sampled), and that (b) is a natural corridor used by wolves in the ongoing colonization of the Alps (Fabbri et al. 2007). Hence, the detection probabilities of territorial wolves having their home ranges in the core vs. the edges of the sampling area, as well as of the transient vs. the territorial individuals, have been certainly variable during the whole sampling period. These findings confirm the need to use sophisticated demographic models incorporating IDH when dealing with CR experiments in open populations. As it was difficult to directly incorporate environmental heterogeneity in our demographic model, we focused on building models accounting for the effects that such heterogeneity could have had on sampling effort and detection probabilities. The impact of different sources of heterogeneity was thus tested analysing 28 biologically plausible models, accounting for temporal and individual effects and their interactions on both survival and detection probabilities. Results indicated minimal effects of sex, time and their interactions on survival rates: the six best models did not incorporate any gender or time effect, suggesting that differential mortality in males vs. females should not condition the data throughout the whole sampling period.

In contrast, sex plus time affected the individual detection probability in four of the ten top models, suggesting a significant role for differential faecal marking behaviour, which should be greater in territorial males in winter and spring than in females throughout the year (Peterson et al. 2002). Sex biased detection probability strongly interacted with time, highlighting the consequences of increasing sampling efforts and wolf population abundance during the study period. These findings indicate a weakness of our sampling strategy, which, being based on faecal samples collected along selected transects, could have led to missing an unknown number of subordinate females (female to male ratio was 0.71). This kind of bias could be reduced by additional sampling efforts during snow periods in winter seasons. The effects of season, climate (including winter) and year were not included in any of the top ten models, indicating that, in presence of strong IDH, these factors did not have major effects on survival and detection rates.

In the best-fitting model, the heterogeneous detection of weakly capturable individuals was conditioned by the winter season, whereas the detection of highly capturable individuals was conditioned by the interaction effect of individual heterogeneity and time. The studied wolf population increased over the eight years of the study, with the exception of years 2003 and 2008. The population grew with a mean annual finite rate of increase $\lambda = 1.05 \pm 0.11$, very similar to values found in the Alpine (Marucco et al. 2009; Cubaynes et al. 2010), Scandinavian (Wabakken et al. 2001) and north American (Mech and Boitani 2003) wolf populations. Each year was characterized by a clear population trend well reflecting the life cycle of the species, with the highest peaks after the breeding season, decreasing in winter with the lowest rates (except for year 2005) during summer, consistent with mortality and dispersion cycles reported by Chapron et al. (2003) in western Europe. The described wolf Apennine trends were concordant with data showing lower abundance during late winters (February-May) in the western Alps (Marucco et al. 2009; Cubaynes et al. 2010), and were similar to results obtained in north American wolf populations

(Pletscher et al. 1997). The P_L individuals (low capturability) showing higher detection probability in winter, could be pups, yearlings and subordinates, whose winter survival is usually much higher than during spring and summer, and that can be sampled only after the breeding period, or migrants that can generally disperse after the winter, about 10 months after their births (Fuller 1989; Boyd and Pletscher 1999; Chapron et al. 2003; Mech and Boitani 2003; Marucco et al. 2009). This hypothesis is further confirmed by the high percentage (64%) of carcasses belonging to previously unsampled individuals, and concurs with data about wolf mortality already reported in the northern Apennines (Scandura et al. 2011). In contrast, the P_H individuals (high capturability), corresponding to individuals detected throughout all the year, could be territorial adults and dominants that frequently mark their home ranges (Vila et al. 1994; Mech and Boitani 2003). Higher winter detection probabilities for both classes of detectability could be due to a larger number of samples analyzed during winters rather than to a better DNA conservation. In contrast with other NGS studies (Lucchini et al. 2002) we did not find significant differences ($P = 0.48$, χ^2 test; see also Table 2) in genotyping success between cold and hot semesters, probably because in the Apennines seasonal temperature variations are not so marked as in the Alps and snow cover is generally less extensive. Moreover, in winter the detection probability is not so dependent on the social role of individuals, because on the snow it is possible to find scats of all individuals of a pack (Mech and Boitani 2003). Additionally, weakly detected individuals showed an annual survival rate (0.66) significantly less than highly detected individuals (0.75), which could be explained by the higher mortality of pups and yearlings (Chapron et al. 2003; Mech and Boitani 2003; Scandura et al. 2011). These estimates are concordant with wolf survival rates in the western French Alps (Cubaynes et al. 2010). Annual survival rates of weakly detected individuals were also concordant with north American survival values described by Pletscher et al. (1997) for dispersing wolves (0.66), and similar to what estimated for pups by Fuller (1989), who reported values ranging from 0.48 to 0.89, but they were significantly higher than pup survival described by Mech (1970) which ranged from 0.06 to 0.43. Annual survival rates of highly detected individuals were also similar to the annual adult survival values found by Pletscher et al. (1997) among resident adult wolves (0.80–0.84).

The average annual Apennine wolf population size ranged between a minimum of 117 individuals (in 2003) to a maximum of 233 individuals (in 2007). These estimates roughly compare with the population size indirectly estimated from the number of permanent packs which have been inferred in the study area using both NGS and field monitoring methods (mainly snow-tracking and wolf-howling sessions; Caniglia et al. 2010). In the study area, we mapped ca. 30 distinct packs, which, assuming an average of four-six wolves per pack (Ciucci and Boitani 1999; Apollonio et al. 2004), correspond to a minimum of 120–180 wolves permanently present in the study area, excluding transient and dispersers. However, both these estimates highlighted that during this NGS monitoring project a large proportion of the investigated wolf population went undetected. Only 15–25% of the estimated population was annually sampled, as shown by the number of the identified wolf genotypes. Therefore, sampling designs should be well-planned in future NGS large-scale wolf monitoring programs (Crespin et al. 2008; Schwartz and McKelvey 2008; Ebert et al. 2010).

Conclusions

Reliable estimates of population size and dynamics are crucial to understand the biology and ecology of large carnivores and ensure their long-term conservation. In this study we show that integrated NGS-CR programmes can allow estimation of basic demographic parameters and monitor their trends through time with reasonable precision also in large open populations distributed across wide territories. The population we monitored for 8 years represents a portion of the Italian Apennine wolf population, which played a fundamental role in the recent recolonization of western Alps. This population continued to grow during the last decades, expanding in both remote and urbanized areas, close to villages and towns, raising conflicts with local communities and human activities, mainly with hunters (which are supposed to compete for the same ungulate prey) and livestock breeders (for the economical losses caused by predations on domestic herds). Thus, monitoring population structure and demographic trends could help wildlife managers and authorities to implement sound conservation actions, which should favour the coexistence of wolves and humans.

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