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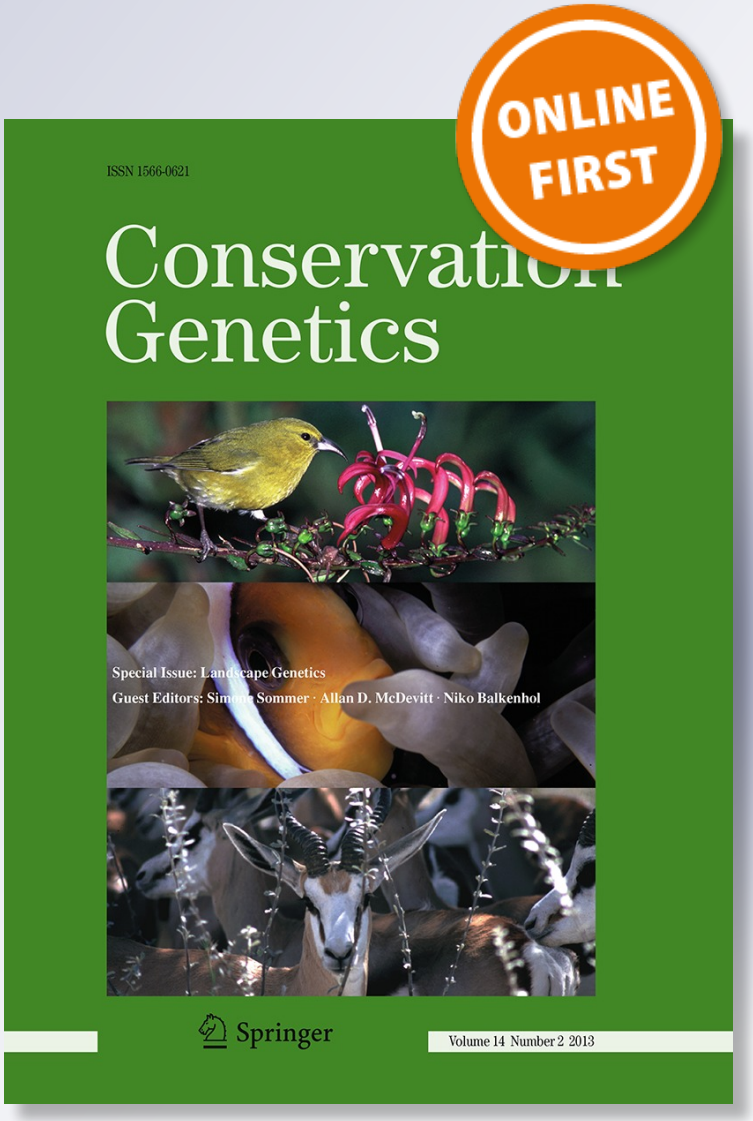
Conservation Genetics

ISSN 1566-0621

Conserv Genet
DOI 10.1007/s10592-013-0491-x



Springer

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RESEARCH ARTICLE

Genetic data confirm critical status of the reintroduced Dinaric population of Eurasian lynx

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Received: 12 February 2013 / Accepted: 23 April 2013

Abstract Eurasian lynx (*Lynx lynx*) reintroduction to the Dinaric Mountains is considered one of the most successful reintroductions of a large predator. Six reintroduced animals founded the population, which rapidly expanded from Slovenia, through Croatia, and all the way to Bosnia and Herzegovina. However, a decrease of the population size has been observed during the last 10–15 years. Considering that possible inbreeding depression would be additive to threats like poaching, traffic mortality and prey base depletion, another extinction of this species from the Dinaric Mountains is a real possibility. We analyzed 204 samples collected between 1979 and 2010 using twenty microsatellite loci and 900-bp mitochondrial DNA control region sequence to evaluate conservation genetics aspects of this endangered population. Both markers confirmed low genetic variability of the Dinaric lynx population, and considerable effective inbreeding (0.3) compared to the source Carpathian population. Our analysis of effective population size and microsatellite variability supported field observations of decreasing population number. As a natural recolonization is a very remote possibility, we recommend population augmentation from a large source population.

Keywords *Lynx lynx* · Microsatellite · Control region · Inbreeding · Effective population size

Introduction

Similar to other large carnivores, the Eurasian lynx (*Lynx lynx*) was one of the most heavily persecuted animal species in Europe. Severe human impact started as early as the sixteenth century and reached its peak in the nineteenth century (Schmidt et al. 2011), causing lynx extinctions all over the continent. The last refuges for the species were in Western Russia, Scandinavia, Carpathians and Balkan Peninsula (von Arx et al. 2004). The story was similar in the Dinaric Mountains, where the last Eurasian lynx were shot in Croatia in 1903 (Koritnik 1974), in Slovenia in 1908 (Kos 1928) and in Bosnia and Herzegovina in 1911 (Mirić 1978).

The 1970s brought the first lynx reintroductions, sourced mostly from the Carpathians (von Arx et al. 2004). One of these, the reintroduction to the Dinaric Mountains, is considered to be one of the most successful reintroductions of a large predator (Breitenmoser et al. 1998). On March 2nd 1973 three females and three males, live captured in 1971 and 1972 in Slovakian Carpathians, were released to Kočevje forests of Slovenia (Čop 1987; Koubek and Červený 1996). These lynx already included two pairs of related animals (mother and son; brother and sister) (Koubek and Červený 1996). The newly established population encountered favorable habitat with abundant prey base, and all three females produced offspring in the 1st year. The population rapidly expanded, with lynx appearing in Croatia almost immediately in 1974, and in Bosnia and Herzegovina in 1980 (Čop 1987; Frković 2001). Lynx were reintroduced with a desire to obtain a new game species

Electronic supplementary material The online version of this article (doi:10.1007/s10592-013-0491-x) contains supplementary material, which is available to authorized users.

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Published online: 05 May 2013

and already in 1978 the hunting started both in Slovenia and Croatia (Frković 2001). In 1984, 11 years after the reintroduction, the first lynx was killed in Bosnia and Herzegovina, 185 km from the release site (Čop and Frković 1998).

Vigorous population growth and range expansion during the initial 15 years was followed by what seemed like a period of stabilization in the late 80s and early 90s. However, in the last 10–15 years the trend of this success story turned downwards, with the population seemingly on the decrease (von Arx et al. 2004; Gomerčić et al. 2009; Gomerčić et al. 2010). Today the Dinaric lynx population is estimated at about 130 remaining animals and is strictly protected over its entire range (Sindičić et al. 2010). This population includes all lynx in Slovenia south of the Ljubljana—Trieste highway, lynx in Croatia, as well as lynx in western Bosnia and Herzegovina. A contact with the Alpine and the Balkan lynx populations has not been thoroughly researched yet, but given the geographic distances and landscape barriers the offspring of the six reintroduced animals were probably reproducing in isolation for the last 40 years.

In small and isolated populations like this, alleles are randomly fixed or lost due to genetic drift and deleterious mutations accumulate because of less effective selection (Lande 1995; Lynch et al. 1995). In the long term these processes erode quantitative genetic variation necessary for adaptive evolution (Lande 1995; Keller and Waller 2002). Additionally, recessive deleterious alleles become exposed phenotypically because of increasingly homozygous individuals. All this can lead to reduction of fitness termed inbreeding depression (Keller and Waller 2002). Inbreeding can have deleterious consequences on all aspects of reproduction and survival, and increases the risk of extinction (Frankham et al. 2002; Reed et al. 2003).

The Dinaric lynx population provides an interesting and rare case study of an isolated population that originated from a known number of founders and reproduced in isolation for four decades. Such studies can yield valuable insight into dynamics of genetic markers under constraints of demographic bottlenecks and subsequent changes in population size (Hundertmark and Van Daele 2010). Using microsatellite genotyping and analysis of mitochondrial DNA control region sequences we investigate the genetic diversity of the Dinaric lynx population and its fluctuations through the population's history. We were interested in dynamics of genetic diversity and inbreeding through time to (1) explore how severely the population is affected by inbreeding relative to the expected decline in heterozygosity caused by genetic drift, and (2) to detect any significant changes in allele frequencies and diversity indices that might point to a possibility of natural immigration events. We also (3) explored the changes in the effective population size through time and (4) discussed the implications of our findings for conservation of this endangered population.

Materials and methods

Sampling and DNA extraction

A total of 204 samples of the Dinaric lynx population from Slovenia (101), Croatia (101) and Bosnia and Herzegovina (2) (Fig. 1) were analyzed in this study, spanning the period between 1979 and 2010. Three types of samples were collected: tissue (16 muscle, 13 blood samples), historic (43 cut footpad, 40 drilled footpad, 25 samples of turbinal bone and septum from the nasal cavity of the trophy skulls, 3 tanned skin samples) and non-invasive samples (32 hair samples, 27 scat, 5 urine samples). Muscle samples were taken during necropsies of animals killed by traffic, poaching, or disease, while blood samples came from animals live-captured for radiotracking studies. Collection of historic samples is described in Polanc et al. (2011). Non-invasive samples were collected in regular monitoring programs.

DNA from tissue and non-invasive samples was extracted using commercial kits (GeneElute Mammalian Genomic DNA Miniprep Kit, Sigma; Taufkirchen, Germany and ChargeSwitch® Forensic DNA Purification Kits, Invitrogen, Life Technologies, Carlsbad, CA, USA). Extraction from historic samples is described in Polanc et al. (2011). All extracts were stored at –20 °C until used.

We used a dedicated laboratory for DNA extraction and PCR setup for noninvasive and historic samples, and enforced strict rules and procedures to prevent contamination. A negative control extraction was performed with each batch of 12 samples and later analyzed downstream

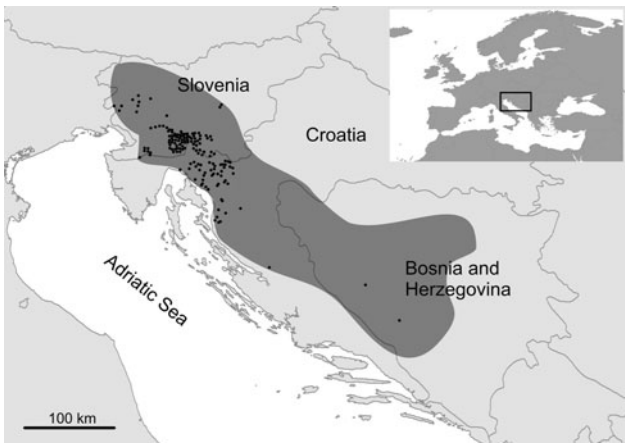

Fig. 1 The *shaded area* indicates the Dinaric lynx population range. *Dots* represent sample locations

with the samples. Three negative controls were used on each 96-well PCR plate to detect possible contamination.

Mitochondrial DNA amplification and analysis

Two non-overlapping fragments of mitochondrial DNA (mtDNA) control region (CR) were amplified by polymerase chain reaction (PCR). A 675 base pair (bp) fragment was amplified with primers L15997 (5′-CACCATTAGCACCC AAAGCT-3′) (Ward et al. 1991) and H16498 (5′-CCTGAA GTAAGAACCAGATG-3′) (Meyer et al. 1990), while primers F20-F (5′-ACTGTGGTGTCATGCATTTGG-3′) and F20-R (5′-GACTCATCTAGGCATTTTCAG-3′) (Wu et al. 2007) were used to amplify a 838 bp fragment. Polymerase chain reaction (PCR) conditions are described in Sindičić et al. (2012).

Sequence alignment was performed using Clustal W (Thompson et al. 1994) implemented in BioEdit software (Hall 1999) and alignments were manually proofed. Lynx control region sequences were aligned with the reference sequence of domestic cat (*Felis catus*) complete mitochondrial genome (Lopez et al. 1996). A 675 bp fragment corresponds to positions 16,284–16,958; while 838 bp fragment corresponds to positions 76–891 of the complete domestic cat mitochondrion (Lopez et al. 1996). Sequences were deposited in the GenBank under accession numbers JN084446–JN084451. For haplotype diversity inference, repetitive sequences were excluded from the analysis and a total of 900 bp was analyzed. Repetitive sequences were described in Sindičić et al. (2012). Obtained sequence was compared to twelve Eurasian lynx control region haplotypes with known origin retrieved from the GenBank database (accession numbers AY034813–AY034816, EU107389–EU107391, JQ928871–JQ928879). Due to various lengths of the sequences, analysis was based on two overlapping fragments of total length of 300 bp, corresponding to positions 16,299–16,455 (157 bp) and 16,799–16,941 (143 bp) of the complete domestic cat mitochondrion (Lopez et al. 1996).

Microsatellite genotyping

In total 20 microsatellite loci (FCA001, FCA82, FCA123, FCA132, FCA161, FCA201, FCA247, FCA293, FCA369, FCA391, FCA424, FCA559, FCA567, FCA650, F53, F115, FCA723, FCA742, HDZ700, Lc106) (Menotti-Raymond et al. 1999; Butler et al. 2002; Williamson et al. 2002; Carmichael et al. 2000) and the sex specific marker SRY were genotyped following the PCR protocol described in Polanc et al. (2011). Fragment analysis was run on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, California, USA) and interpreted using GeneMapper v.4.0. software (Applied Biosystems, Life Technologies, Carlsbad, California, USA). Each electropherogram was checked independently by two persons and we did not accept an allele in a consensus genotype until we saw it at least twice. We used a multitube-based genotyping procedure (Taberlet et al. 1996; Adams and Waits 2007), with a constraint that the maximum likelihood estimated reliability using program Reliotype (Miller et al. 2002) of the sample must be at least 0.95. Analysis of each sample was repeated at least twice, and up to eight times with regard to the estimated reliability. We conducted a two phase screening of the PCR products. In the first phase the samples were discarded if they did not produce any PCR products after two replications, and at the second phase the samples were discarded if after four replications less than seven loci were successfully amplified. We used the methods recommended by Broquet and Petit (2004) to estimate the frequency of allelic dropouts (ADO) and false alleles (FA), and calculated a quality index (QI) for each sample following the method described by Miquel et al. (2006). Samples with a quality index below 0.4 were discarded. Consensus genotypes were determined using an Access database application programmed by Skrbinšek (unpublished).

Individual inbreeding model (IIM) implemented in INEST program (Chybicki and Burczyk 2009) was used for estimation of null allele frequencies, to avoid upwardly biased estimates of null allele frequencies resulting from inbreeding (Van Oosterhout et al. 2006). Arlequin v.3.11. (Excoffier et al. 2005) was used to assess deviations from Hardy–Weinberg equilibrium (HWE) and to calculate pairwise linkage disequilibrium (LD), both with an adjusted p value corresponding to $\alpha = 0.05$ after the Holm–Bonferroni correction for multiple tests (Holm 1979). The probability of identity (PI), the probability of identity among sibs (PIsib) and cumulative PIsib over all loci were calculated according to Paetkau and Strobeck (1994) and Waits et al. (2001), using Gimlet v.1.3.1 (Valiére 2002).

Genetic diversity analysis

Genetic diversity analyses were performed using Genalex v.6 (Peakall and Smouse 2006) and Fstat (Goudet 1995, 2001). Nuclear genetic diversity was measured as the total number of alleles (n), mean number of alleles per locus (n_A), observed heterozygosity (Ho), expected heterozygosity (He) and the effective number of alleles (n_e).

The temporal dynamics of genetic diversity was analyzed using a traveling window approach with a temporal subpopulation of 30 individuals moving forward in time by one individual at each step. Diversity measures were calculated for each of the 58 subpopulations created in this manner. We also estimated deviations from HWE for each temporal subpopulation using program Arlequin, as

described above. Three samples were excluded from the analysis as the year of sampling was not available.

Population structure analysis

To test the (unlikely) hypothesis of population subdivision and possible immigrants we used program Structure v.2.3.3 (Pritchard et al. 2000) under the admixture model without a priori definition of populations and correlated allele frequencies. Ten independent runs were performed for each number of possible genetic clusters (K value from one to five), with the length of the burn-in period of 10^5 and followed by 10^5 Markov Chain Monte Carlo repetitions. The repetitions were run to see if there were deviations among the different runs for a fixed K and to calculate means and standard deviations. The most probable K was inferred from the mean log-likelihood values (Ln P(D)) according to the criteria by Pritchard et al. (2000). In addition, for values $K > 1$ we calculated ΔK as described by Evanno et al. (2005) in order to support our decision on the number of genetic clusters.

Inbreeding analysis

Using Fstat (Goudet 1995, 2001) we calculated the inbreeding coefficient F_{IS} (inbreeding as non-random mating within subpopulations) for each of the 58 resampled temporal subpopulations. The software package Coancestry (Wang 2011) was used to estimate individual inbreeding coefficients in each of the 58 subpopulations, implementing methods TrioML (Wang 2007) and DyadML (Milligan 2003). We also estimated the effective inbreeding coefficient of the Dinaric lynx in comparison with the source population in Carpathians as $F_e = 1 - \frac{H_D}{H_C}$ (Frankham 1998), where H_D is heterozygosity of the reintroduced population in the Dinaric mountains and H_C heterozygosity of the source population. Heterozygosity of the source Carpathian population was estimated by Breitenmoser-Würsten and Obexer-Ruff (2003) using 19 microsatellite loci and 20 samples as 0.61.

Estimating effective population size

We looked at the changes in the effective population size (N_e) through time using two methods, the linkage disequilibrium and approximate Bayesian computation. The linkage disequilibrium (LDNe) method (Hill 1981; Waples 2006) uses as a signal the expectation that in a finite population the loci that are otherwise unlinked will depart from linkage equilibrium. The method has been extensively tested through simulations and shown to be reasonably precise and unbiased already at sample sizes of approximately 25 individuals if the actual effective population size is small (Waples 2006; Waples and Do 2010), which is a reasonable assumption in our case. We applied the method to our data using program LDNe (Waples and Do 2008), and excluded rare alleles with frequencies below 0.03, a value between 1/S and 1/2S, where S equals sample size (Waples and Do 2010). The approximate Bayesian computation (ONeSAMP) method uses an approximate Bayesian computation procedure to estimate N_e by simulating a large number of populations and comparing eight summary statistics related to N_e to the same statistics in the studied population (Tallmon et al. 2008). While it has not been thoroughly evaluated (Luikart et al. 2010), several authors reported good performance of the method (Barker 2011; Phillipsen et al. 2011; Skrbinšek et al. 2012). We used 4 and 100 as limits for a uniform prior on N_e. The samples were divided into three periods each spanning approximately a decade of sampling: 1979–1990 ($N = 32$), 1991–2000 ($N = 27$) and 2001–2010 ($N = 28$). Three samples were excluded as they had missing data on five loci. Both methods assume discrete generations (no generation overlap), an assumption that is clearly violated. A reasonable conjecture is that if the number of cohorts represented in a sample is roughly equal to the generation length, the estimate from the LDNe method should roughly correspond to N_e for a generation (Waples and Do 2010). This has been recently confirmed by Robinson and Moyer (2012), and less than approximately 15 % downward bias of N_e estimates was found when only adult animals were sampled, which was mostly the case in our study. The 10-year periods in our study are longer than the generation time in lynx (approximately 4 years; Spong and Hellborg 2002), possibly causing an upward bias, however as the periods in our study are of equal length, this bias should be the same in each period making them comparable. On the other hand, the bottleneck should cause a downward bias in N_e estimates in the first couple of post-bottleneck generations (Waples 2006). The ONeSAMP method has not been explored in such detail, but has been applied to species with overlapping generations with plausible results (Tallmon et al. 2008; Phillipsen et al. 2011; Skrbinšek et al. 2012). We estimated N_e using both methods for each period, but also estimated the long-term harmonic mean N_e using the ONeSAMP method and all collected samples with enough data ($N = 86$), an approach was shown to provide reasonable long-term Ne estimates in some studies (Tallmon et al. 2008; Skrbinšek et al. 2012).

Results

Mitochondrial DNA

Mitochondrial DNA control region was successfully amplified and sequenced from 29 tissue and non-invasive samples (19 from Croatia, eight from Slovenia and two from Bosnia and Herzegovina). A single mtDNA haplotype was found, as no polymorphic sites were detected in either of analyzed fragments in total length of 900 bp. A 300 bp fragment was identical to the GenBank sequence AY034816 (haplotype named H4). This haplotype was first described by Hellborg et al. (2002) in Estonia and Latvia. Gugolz et al. (2008) identified it in Carpathian Mountains and reintroduced Alpine population, while Ratkiewicz et al. (2012) confirmed the presence of this haplotype in Carpathian and Baltic (Estonia and Latvia) populations, but also in lynx from northern Poland. As recent Dinaric and Alpine populations were both established by reintroduction from Carpathians, and all Carpathian lynx samples researched until now possessed exclusively the haplotype H4, it came as no surprise that the three populations share the same haplotype.

Microsatellite genotyping

Microsatellite analysis included 90 individual genotypes from Slovenia (44), Croatia (44) and Bosnia and Herzegovina (2) (30 females, 60 males) genotyped using 20 microsatellite loci. This is a "cleaned" dataset with usable genotypes; due to rigorous procedures 56 % of all collected samples were discarded and genotypes included in the analysis had low average allelic dropout (2.9 %) and false allele (0.3 %) rates per locus, and consequently average quality index was high (0.87) (Table 1). Samples were evenly distributed through time, with approximately 30 samples representing each decade in the 1979–2010 period.

Statistically significant ($p < 0.05$) deviations from Hardy–Weinberg expectations were found at four loci—Lc106 (due to higher than expected heterozygote frequencies), Fca161, Fca293 and Fca82 (due to lower frequencies of heterozygotes), but after Holm–Bonferroni correction for multiple tests significant deviation was confirmed only for locus Lc106. To avoid biased detection of null alleles caused by inbreeding, we implemented the method of Chybicki and Burczyk (2009) which provides an accurate estimate of null allele frequency regardless of the actual inbreeding coefficient. Estimate of null allele frequency under this model is not purely based on its absolute estimated value and should only be considered significant when the locus deviates from HWE expectations. In our data set locus Lc106 deviates from HWE and possible null-allele frequency indicated by the INEST program is very low (pij = 0,013, SE = 0,011). Even the other three loci (Fca161, Fca293 and Fca82) that showed deviations from HWE before the Holm–Bonferroni correction have relatively low frequencies of possible null alleles (from 0,081 to 0,082).

Out of total 190 pairwise comparisons, after Holm–Bonferroni correction for multiple testing, we found 11 (5.8 %) pairs of loci tested statistically significant for linkage disequilibrium (LD). Data about chromosomal location are available for 18 out of 20 microsatellites we genotyped, so we had data about chromosomal location for 153 locus-pairs. Two out of ten locus-pairs (20 %) located on the same chromosome and 8 out of 143 locus-pairs (5.6 %) located on different chromosomes had significant LD. For one locus pair with significant LD microsatellite chromosomal locations were not available. This LD can be explained by the low effective population size and strong founder effect (Frankham et al. 2002; Gillespie 2004; Bensch et al. 2006).

The information on multi-locus probability of identity is important for evaluating the resolution power of a combination of markers and their information content for genetic analyses. We omitted locus Lc106 from calculations because it deviates from HWE. The power of the entire panel of markers to accurately distinguish between closely related individuals was relatively high, PIsib = 4.9×10^{-5} (Online Resource 1). Locus Fca742 was the most informative, with probability of identity (PI) of 0.12 and probability of identity among sibs (PIsib) of 0.42. It should be noted that linkage disequilibrium violates the assumptions of the probability of identity calculations, possibly biasing the results, however we do not think that this bias would be important for the purpose of this study.

Table 1 Number of samples successfully genotyped for each sample type, mean quality index, mean per locus allelic dropout (ADO) and mean per locus false allele (FA) for 90 samples successfully genotyped on 20 microsatellite loci

Sample type	No. of samples successfully genotyped (%)	Mean quality index	Mean per locus ADO (%)	Mean per locus FA (%)
Historic	58 (52.3 %)	0.77	5.8	0.7
Invasive	26 (89.7 %)	0.89	1.2	0.1
Non-invasive	6 (9.4 %)	0.95	1.6	0.2
Average	90 (44.1 %)	0.87	2.9	0.3

Genetic diversity

Genetic diversity analyses were performed on 19 microsatellite loci, as we excluded locus Lc106 that deviated from HWE. All loci were polymorphic and yielded between two and five alleles each. A total of 59 different alleles were found among 90 samples from the Dinaric lynx population, with the mean number of alleles per locus $n_A = 3.11$. Seven alleles were relatively rare with frequencies of 0.06. Genetic variability for all 90 genotypes was estimated by observed (Ho = 0.471) and expected heterozygosity (He = 0.482), and the effective number of alleles ($n_e = 2.080$).

We can see increased homozygosity from Hardy–Weinberg expectations during the initial period up to the mean year of the subsample 1991 (Fig. 2), however none of the Hardy–Weinberg tests of travelling window subsamples proved statistically significant. Both He and Ho converge after that period and have a decreasing trend until the end of our study period.

Population structure

Analysis of subdivision within Dinaric population using program Structure suggested a single genetic cluster. The ΔK method of inferring the most likely K can not be used if $K = 1$, so we also applied the model for K values from 2 to 5, but clear peak was not obvious for any of the values.

Inbreeding

The traveling window analysis of inbreeding coefficients (Fig. 3) can be explained through the presumed demographic history of the population. Soon after the reintroduction the total number of animals was low, and direct mating between close relatives was unavoidable, as shown by increased Fis and individual inbreeding coefficients until mean year of subsample 1991. However, these results are not well supported as Hardy–Weinberg tests do not support departures from equilibrium. When the population expanded very rapidly (as is evident from hunting bag and monitoring records), these inbreeding coefficients declined. From 1991 to 2010 the mean individual inbreeding coefficient were 0.111 (TrioML) and 0,123 (DyadML), which is an indication that on average mating is equivalent to mating of half siblings, grandparent–grandchild, uncle-niece or double first cousins.

When compared to the source population, effective inbreeding coefficient using the first 30 samples collected between 1979 and 1990 is $F_e = 0.22$. By the last part of our study period, 1999–2010, inbreeding increased to $F_e = 0.30$.

Effective population size

Estimates of effective population size and its change through time are presented in Table 2. This parameter estimated using LDNe was stable in the population for the first two study periods, but dropped considerably after the year 2000 (Fig. 4). The ONeSAMP estimates, on the other hand, seem quite stable. Heterozygosity in a small population should drop by $1/2N_e$ per generation as a consequence of genetic drift (Crow and Kimura 1970), which fits well with the actual drop in heterozygosity observed in our

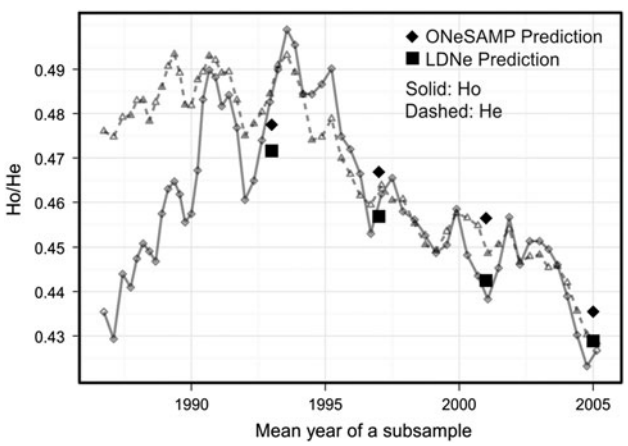

Fig. 2 Mean observed (Ho) and expected (He) heterozygosity of the 30-samples traveling window subsamples. *Black diamonds* predicted He using LDNe estimates of the effective population size, *black squares* predicted He using ONeSAMP estimates of *Ne*

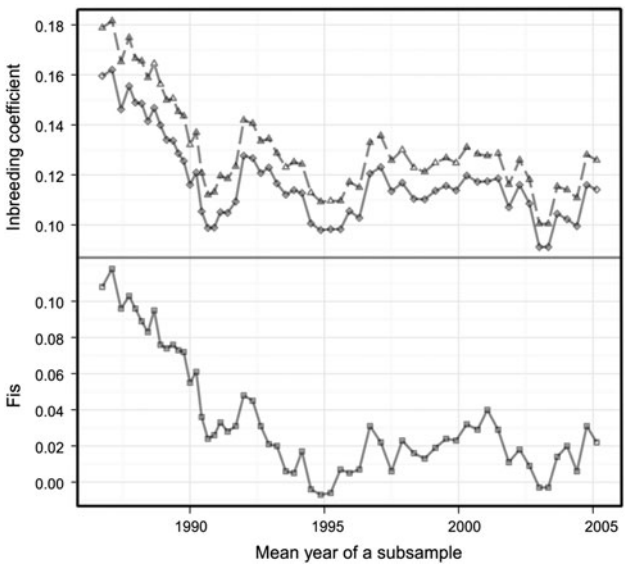

Fig. 3 Changes in average individual inbreeding coefficients (*upper graph*) and Fis (*lower graph*) through time in the Dinaric lynx population using the travelling time window subsampling (30 individuals in each subsample). *Upper graph dashed line* TrioML, *solid line* DyadML

Table 2 Estimates of effective population size for Dinaric lynx

Period	*N*	LDNe (95 % CI)	ONeSAMP (95 % CI)
1979–1990	32	22.9 (13.8–45.1)	14.8 (12.4–18.5)
1991–2000	27	22.4 (13.6–44)	16.0 (13.8–19.4)
2001–2010	28	10.9 (7.2–16.8)	16.2 (14.2–19.7)
1979–2010	87	–	21.2 (18.0–26.6)

LDNe; linkage disequilibrium method, *ONeSAMP*; approximate Bayesian computation method, *N*; number of samples

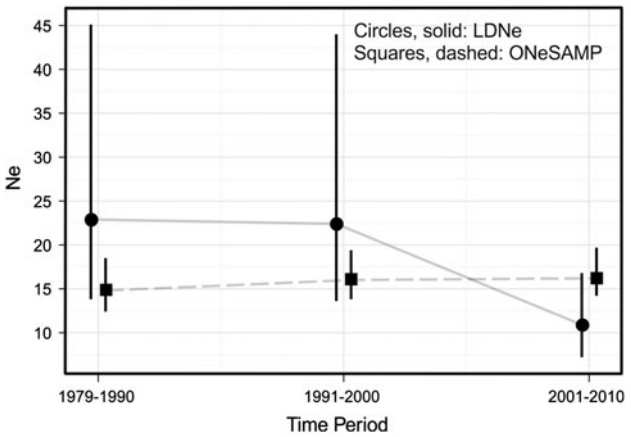

Fig. 4 Changes in the effective population size (*Ne*) in the Dinaric lynx popuolation through time. *Solid line*, *circles* LDNe, *dashed line*, *squares* ONeSAMP. *Vertical lines* show the 95 % confidence intervals of the estimates

data (Fig. 2). The ONeSAMP estimate of N_e over the entire time of the study provides a higher N_e estimate than the results obtained for each time period, in line with expectations of an upward bias when many generations are included in the estimate. Still, there is a considerable overlap of the confidence intervals.

Discussion

Out of all Eurasian lynx populations researched until now (Hellborg et al. 2002; Spong and Hellborg 2002; Rueness et al. 2003; Breitenmoser-Würsten and Obexer-Ruff 2003; Schmidt et al. 2009; Davoli et al. 2012), the Dinaric population has the lowest microsatellite diversity. The low heterozygosity we observed is only comparable to the heterozygosity in the reintroduced populations in Swiss Alps and Jura Mountains researched by Breitenmoser-Würsten and Obexer-Ruff (2003). Similarly low is the effective population size. As one of the most important concepts in conservation and evolutionary biology (Charlesworth 2009), effective population size summarizes in a single parameter both the population's evolutionary potential as well as its vulnerability to genetic stochasticity. As can be expected, N_e in the Dinaric lynx population is below the short-term inbreeding avoidance threshold of 50 (Franklin and Frankham 1998). The violations of model assumptions should be biasing the estimates high, meaning that the actual N_e of this population is probably even lower than what our estimates are showing. On the other hand, both methods we used provided comparable results, as did the ONeSAMP method applied to all samples. The drop in heterozygosity through genetic drift which we explored using the travelling window subsampling fits well with the loss predicted from the N_e estimates through the 1/2 N_e relationship, giving additional support to our results. There is a difference, though, between methods in the estimates for the last decade (2000–2010), where LDNe shows a rapid drop in N_e while ONeSAMP remains constant. LDNe, as applied in our study, provides approximate estimates of per-generation inbreeding effective population size (Robinson and Moyer 2012) while the estimate of the ONeSAMP method should be mostly influenced by the recent few generations, however some of the statistics the later method uses apply to even longer time frames (David Tallmon, personal communication). This makes LDNe more sensitive to rapid changes in N_e, and the results we obtained for the last decade seem to signify an actual drop in *Ne* in the 1996–2006 period (time period studied—one generation), which would be missed by ONeSAMP. This fits with the decrease in the census population size that has been observed from the field data within roughly the same time frame. A decrease in N_e increases the rate at which genetic diversity is being lost and inbreeding accumulates (Frankham et al. 2002), speeding up the deleterious processes for population viability and increasing the risk of its extinction.

We found an interesting relationship in the traveling time window subsampling analysis (Fig. 2): during the first decade following the reintroduction, He is higher than Ho and slowly converging, as Fis approaches zero (Fig. 3). For this Ho/He difference we can't confirm statistical significance with the amount of data we have available. A decreasing trend is observed in individual inbreeding coefficients during the same period (Fig. 3). A tempting explanation is that as the population size must have been very low and mating between close relatives must have been occurring, with chance in such small number of matings driving the Fis and the individual inbreeding values up. Another, more mundane explanation is that these older samples had more allelic dropout and hence higher observed homozygosity. However, as we applied the same strict error checking protocols to both historical and recent samples before a genotype was included in the analysis, this is less likely. As the population size increased, Ho and He remained approximately the same and drifted according

to the low *Ne* expectations, and individual inbreeding estimates stabilized around 0.1.

The effective inbreeding coefficient was 0.22 during the second decade following the reintroduction, and has grown to 0.30 by the 1999–2010 period. While many laboratory and natural populations show signs of inbreeding depression at even lower levels of inbreeding (Frankham 1998; Keller et al. 2012), the most relevant parallel can be drawn with another well-studied endangered population of a large cat, the Florida panther (*Puma concolor*, Johnson et al. 2010). This population shrunk to 20–25 adults, had lower genetic variation than other puma populations and many indications of inbreeding depression (cryptorchidism, low sperm quality, low fecundity, heart defects etc.). The population was on the verge of extinction, but was recovered through introduction of eight individuals from Texas. Effective inbreeding coefficient before this reintroduction (compared to the Texas population) was 0.47. If (in lack of better data) we speculate this as a reference point for severe inbreeding depression in felids, and considering that $H_t = \left(1 - \frac{1}{2N_e}\right)^t \cdot H_0$ (Crow and Kimura 1970) assuming $N_e = 16$, the Dinaric lynx population should reach this level of inbreeding in approximately 17 generations ($\sim$68 years). If we consider the more pessimistic LDNe estimate for 1999–2010 of $N_e = 10.9$, this same level of inbreeding should be reached in 12 generations ($\sim$48 years). Of course, any such inter-species extrapolation is risky and these estimates should by no means be taken at their face value, but we can presume that the Dinaric lynx population will reach a point where inbreeding depression becomes a real problem sooner rather than later.

Our results paint a grim, if expected, picture of the Dinaric lynx population. Low effective population size, considerable inbreeding, low genetic diversity and demographic stochasticity make long-term prospects of this population questionable. The signs of decreasing population size that we're observing over the last 10–15 years in the field and that are now supported by the low and possible decreasing N_e shown in this study may not necessarily be direct signs of the inbreeding depression; however, the possible reduction in fitness of individual animals caused by genetic factors is additive to other "traditional" threats like poaching, traffic mortality and prey base depletion, and can quite possibly lead to another extinction of this species from the Dinaric Mountains. Fragmentation by highways and geographic distance make natural recolonization from other large lynx populations a very remote possibility. We believe that besides activities aimed at dealing with the other "traditional" threats, the population is going to need direct assistance.

Since it is difficult to imagine the Dinaric lynx population to meet viability criteria in foreseeable future without human influence (i.e. $N_e > 50$ to avoid inbreeding, $N_e > 500$ to preserve evolutionary potential; Franklin and Frankham 1998), translocations will be required on a regular basis (e.g. 2 animals per year, or larger number of animals over longer periods if more practical) to ensure genetic viability and avoid inbreeding. A simulation study should be carried out to establish the optimal number of effective migrants per time period that would keep inbreeding at an acceptable level. As the translocated animals should be chosen from a population with low inbreeding and similar environment (Frankham 2009), the source of these animals should ideally be the nearest large and genetically diverse lynx population (e.g. Carpathian population). Apart from the translocations, establishing natural gene flow with other lynx populations (Carpathian population, reintroduced populations in the Alps) should also be seen as a long-term goal. Translocated animals should be monitored to assess their reproductive success, and population management, as well as management of the main prey species, should be adjusted to achieve the conservation goals.

Reintroductions are often controversial, especially if the species in question is a large carnivore like the Eurasian lynx. Socioeconomic and political issues of such translocations are extremely complex (Clark et al. 2002; MacDonald 2009) and will usually limit the number of animals considered for translocation. The human dimensions of such augmentation projects are critical and should be taken into account to avoid negative public attitudes towards lynx and ensure long-term coexistence with humans (MacDonald 2009). However, action should be taken sooner rather than later if we are still to have lynx in the Dinarics in the not-too-distant future.

Acknowledgments We are grateful to Vedran Slijepčević, Miha Krofel, Josip Tomaić, Josip Kusak, Franc Kljun, Marko Jonozovič, Hubert Potočnik and Ivan Kos for their help with obtaining the samples. Carlos Fernandes gave us valuable comments for data analysis. This research was in part supported by the Interreg IIIA project "Transboundary cooperation in management, conservation and research of the Dinaric lynx population". Further, it was supported by the Slovenian Research Agency Project L1-6484 and co-funded by the Environmental Agency of the Republic of Slovenia, the Ministry of Agriculture of the Republic of Slovenia and the Institute of the Republic of Slovenia for Nature Conservation. Also we are grateful for financial support provided by Croatian State Institute for Nature Protection, Croatian Environmental protection and energy efficiency fund, and Carlsberg Croatia.

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