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Morphological consequences of range fragmentation and population decline on the endangered Iberian lynx (*Lynx pardinus*)

C. Pertoldi[1,2,3*], R. García-Perea[4*], J. A. Godoy[2], M. Delibes[2] & V. Loeschcke[1,5]

1 Department of Ecology and Genetics, Institute of Biological Sciences, University of Aarhus, Ny Munkegade, Aarhus C, Denmark
2 Department of Applied Biology, Estación Biológica de Doñana, CSIC. Pabellón del Perú, Seville, Spain
3 Department of Wildlife Ecology and Biodiversity, National Environmental Research Institute, Kalø Grenåvej, Rønde, Denmark
4 Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, Madrid, Spain
5 Institute of Advanced Study, La Trobe University, Vic., Australia

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Correspondence
Cino Pertoldi, Department of Ecology and Genetics, Institute of Biological Sciences, University of Aarhus, Ny Munkegade, building 540, 8000 Aarhus C, Denmark. Email: biocp@nf.au.dk
Rosa García-Perea, Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, CSIC. J. Gutierrez Abascal 2, 28006 Madrid, Spain. Email: mcng310@mncn.csic.es

*These authors contributed equally to this work.

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Abstract

The Iberian lynx *Lynx pardinus* is one of the world's most endangered felids and is vulnerable to human-induced mortality and habitat loss, which reduce population size and accelerate the loss of genetic variation. Twenty-five metric traits of Iberian lynx skulls have been measured on 95 skulls collected between 1872 and 2003. The skulls belong to three geographically distinct areas/populations, which have recently diverged from each other as a consequence of increased habitat fragmentation: Doñana area, Sierra Morena mountains and Montes de Toledo area. The morphometric study was undertaken using univariate, multivariate and admixture analysis approaches, and all three techniques provided evidence for morphometric differentiation, both in skull size and shape, among the three populations for both males and females. Environmental and genetic forces that may have shaped these patterns are discussed. The males of the population of the Doñana area showed a different degree of reduction in size in nine of the skull traits with time, which has been suggested to be partly because of worsened habitat conditions. However, the heterogeneity of the degree of mean size reduction and the relatively high degree of reduction of some of the skull traits investigated ($>4\%$), which have altered the original proportions between the skull variables, could also partly be attributed to inbreeding depression in the Doñana population. The phenotypic variability of the skull traits showed significant increases (two traits) or decreases (nine traits) with time, and this different pattern of change with time has been suggested to be because of a different number of genes controlling the traits with different degrees of dominance and epistatic interactions. The increased phenotypic variability of two of the traits has also been attributed to a possible decreased level of developmental stability, which can be produced by environmental and/or genetic stress. The findings of this investigation contribute to the discussion about the utility and the limits of quantitative genetics techniques for conservation purposes.

Introduction

Effect of inbreeding and population bottlenecks on genetic variability and fitness-related traits

Among populations, the consequences of isolation and small population size include genetic differentiation driven by genetic drift, whose effect is inversely related to the effective population size (N_e). Within the populations, loss of genetic variation and inbreeding depression can occur. Numerous studies conducted both in natural and in controlled conditions have shown a direct correlation between genetic diversity and measures of fitness (e.g. Koehn, Diehl & Scott, 1988; Reed & Frankham, 2003). One reason is the impact of inbreeding depression (Saccheri, Brakefield & Nichols, 1996; Keller & Waller, 2002). Inbreeding can contribute to a change of the morphometric traits because of the increased expression of genetic load, as well as reduced opportunities to express overdominance (see e.g. DeRose & Roff, 1999; Keller & Waller, 2002). However, there are few direct demonstrations of such phenomena in natural populations. A problem, which affects these correlational studies, is the lack of accurate knowledge of the past

and present demographic trends and distribution of the populations under study. Furthermore, environmental factors also can lead to variation in fitness components and morphometric traits, because of genotype–environment interactions (GXE) (Turelli, 1988). Thus, to find a link between the decline in a fitness component or morphometric trait and inbreeding depression can be difficult in natural populations (Keller & Waller, 2002). It is even more challenging to try to assess the contribution of inbreeding depression to the risk of population extinction. Reports from the field of conservation biology on populations which have suffered severe bottlenecks, but nevertheless currently prosper (Ellegren *et al*., 1993), could question that inbreeding depression is a severe threat for long-term survival of populations. However, these examples are isolated studies that are not unexpected because of the large variation in inbreeding depression between populations. Consequently, empirical studies where the species' demographic changes have been extensively studied are necessary, in order to draw stronger conclusions about the effects of habitat loss and population bottlenecks.

A case study: the Iberian lynx

The Iberian lynx *Lynx pardinus* (Temminck, 1827) is at present one of the rarest mammals on Earth, the only cat listed in Category 1 of vulnerability to extinction and considered as critically endangered (CE) by the IUCN (IUCN, 2003). The Iberian lynx is also a well-documented example of a carnivore suffering the consequences of human-induced mortality, scarcity of prey and habitat loss. Lynx habitat has been severely modified and reduced by extensive destruction (Delibes, Rodríguez & Ferreras, 2000). The wild population is believed to consist of less than 200 individuals (Guzmán *et al*., 2003). By the early years of the 20th century, the Iberian lynx had become very rare in northern Spain, although it was still common in central and southern Spain. By the 1960s, its range was essentially limited to the south-western quarter of the peninsula (Rodríguez & Delibes, 1992) (see Fig. 1).

The decline of the lynx population since the 1950s has been primarily caused by habitat loss and a decline of their main prey species, the European rabbit *Oryctolagus cuniculus*. In fact, there was a drastic population bottleneck during the 1950s and 1960s, when the myxomatosis viral disease hit the rabbit populations (Villafuerte *et al*., 1993). Recent estimates suggest that there are just two populations left, the Doñana (D) and the Sierra Morena (SM) populations, inhabiting an area larger than 2000 km^2 and separated by more than 300 km. The D population, with about 40–50 lynx, seems to have been isolated from the other surrounding and now extinct populations for more than 50 years, because of an expansion of croplands to the north and dense human settlements to the west (Rodriguez & Delibes, 1992). About 150 individuals remain at western Sierra Morena. The Montes de Toledo (TM) population, which is probably extinct, was near the SM population. The two small and isolated remaining populations of the Iberian lynx are

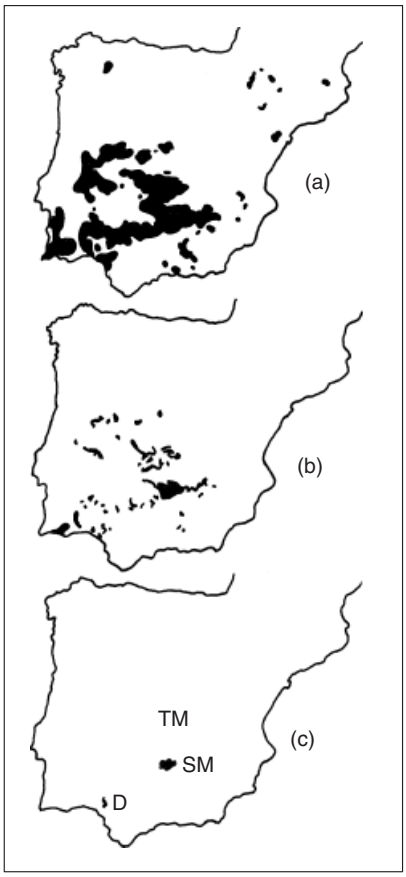

Figure 1 Three stages of the decline of the Iberian lynx *Lynx pardinus* populations in the second half of the 20th century: (a) estimated distribution in the 1960s (based on Rodríguez & Delibes, 1990); (b) estimated distribution in the 1990s (based on Rodríguez & Delibes, 1992); (c) breeding populations at present (based on Guzmán *et al.*, 2003). TM, location of the extinct Montes de Toledo population; SM, Sierra Morena population; D, Doñana population.

theoretically vulnerable to inbreeding and genetic drift, where alleles with low frequency are likely to disappear from the population's gene pool. Beltrán & Delibes (1993) found preliminary evidence for this occurrence in the D population. Three pelage patterns were present in the population before 1960, but now no animals exhibit the rarer fine-spotted pattern. Additionally, the genetic variation estimated in mtDNA genes and nuclear microsatellites was found to be reduced in the Iberian lynx relative to most other felid species, suggesting that they experienced a fairly severe demographic bottleneck and that the two investigated populations of D and SM were genetically differentiated at the genomic level and showed heterozygosity deficiency (Johnson *et al*., 2004).

Given the well-documented present and past demographic trends of the Iberian lynx, this investigation could constitute an empirical example of the morphometrical changes of a species which became endangered and fragmented within a relatively short time span.

Methodological approach and aim

Several methodological approaches have been applied to the study of the genetic consequences of habitat loss, habitat fragmentation and population bottlenecks. Studies on ecologically relevant traits, such as craniometrical investigation, have been applied to yield information on differences among populations and their structure (e.g. Huson & Page, 1980; Clutton-Brock, Kitchener & Lynch, 1994; Lynch & Hayden, 1995; Simonsen *et al.*, 2003). Some researchers believe that morphological similarity cannot be interpreted to indicate genomic similarity (Baverstock & Adams, 1987), as the selective pressures acting on molecular markers are different: the molecular markers are by definition considered neutral, and the adaptive traits non-neutral (Lynch, 1996). However, in polygenic characters (as most of the morphometric characters are), the forces of selection are distributed over a large number of loci, rendering the selective forces on specific loci sufficiently overwhelmed by random genetic drift. Furthermore, for a N_e smaller than few hundred individuals, the expected amount of variation for a quantitative character is nearly independent of the strength of selection and largely a result of mutation-drift balance (Lynch, 1996). In the small-sized lynx populations under study, the selective pressures should be overwhelmed by genetic drift effects, making all the traits and the genes selectively neutral, or nearly so. Hence, the eventual detection of morphometrical differences between the different populations studied is mainly a consequence of genetic drift and/or environmental variability.

The aim of this investigation was to test whether there is temporal and spatial variation (of the skull trait size and shape) among the three populations investigated, which have recently been isolated from each other.

Predictions

Isolation associated with small population size differences could have produced genetic divergence of the three populations studied (D, SM, TM), and the concomitant effect of inbreeding depression could have produced a change of the skull traits, altering the proportions between them. Size differences may reveal different habitat conditions the individuals collected have been exposed to. The detection of shape differences could indicate the populations under study are genetically differentiated (Simonsen *et al.*, 2003), as several studies in controlled conditions have shown that genes regulate the shape of the traits more tightly than they regulate size (Birdsall *et al.*, 2000; Workman *et al.*, 2002) and that more genes are involved in the regulation of shape than size (Workman *et al.*, 2002).

The detection of significant differences in trait size and trait shape between different periods of collection (before and after the potential population bottleneck happened in the D population in the 1950–1960s) could also reveal that environmental and/or genetic changes have occurred in this population. The detection of changes in the phenotypic variability (V_p) of the skull traits could also reveal genetic changes as in a constant environment (V_p) are roughly correlated to the additive genetic variance (V_a) in the absence of dominant and epistatic interactions and to N_e (Podolski, 2001). The loss of genetic variability could also have disrupted developmental stability (DS) and thereby increased the variability of quantitative traits (Hoelzel, 1999).

Materials and methods

Iberian lynx skulls were available from the scientific collections of the following Institutions: Estación Biológica de Doñana-CSIC, Seville, Spain; Museo Nacional de Ciencias Naturales-CSIC, Madrid, Spain; The Natural History Museum, London, United Kingdom; Naturhistorisches Museum Basel, Basel, Switzerland; American Museum of Natural History, New York, USA; National Museum of Natural History, Washington DC, USA; Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany; Zoologische Staatssammlung München, München, Germany; Universidad Complutense, Madrid, Spain. We studied 95 skulls, representing about 90% of the skulls of this species kept in scientific collections. These individuals were collected between 1872 and 2003 (Iberian lynx are protected by Spanish law since 1973; specimens collected after that date were found dead or were confiscated by conservation officers).

Twenty-five metric traits were measured on each skull. Thirteen of these traits are related to the cranium or mandible and 12 of these measurements are related to teeth (see Fig. 2 for description of the traits and abbreviations).

The skulls belong to three geographically distinct areas: D (58 specimens, 35 males, 23 females, collected between 1872 and 2003), SM (12 specimens, eight males, four females, collected between 1889 and 1997) and TM (25 specimens, 15 males and 10 females, collected between 1960 and 1985).

The skulls were of known sex or sex was estimated on the basis of a discriminant function. Skulls were identified as adults (fully grown) and subadults (not fully grown) based on tooth sections (following the method described by Zapata *et al.*, 1997), or by checking the ossification of cranial sutures (García-Perea, 1996).

Some skulls were damaged, but whenever possible all the 25 measurements were taken on each skull. All the measurements were taken with a digital calliper, to the nearest 0.1 mm, and are expressed in mm in the tables.

For the statistical treatment of measurements related to cranium and mandible, only adult skulls were included, since they show age variation (García-Perea, 1991). However, all the tooth measurements were done on both adults and subadults, as these measurements are not affected by the age of the individual (García-Perea, 1991).

Multivariate and univariate analyses have been performed contemporarily, in order to utilize all the measurements of the available data set. The sexes have been analysed separately, because of the sexual dimorphism of the lynx

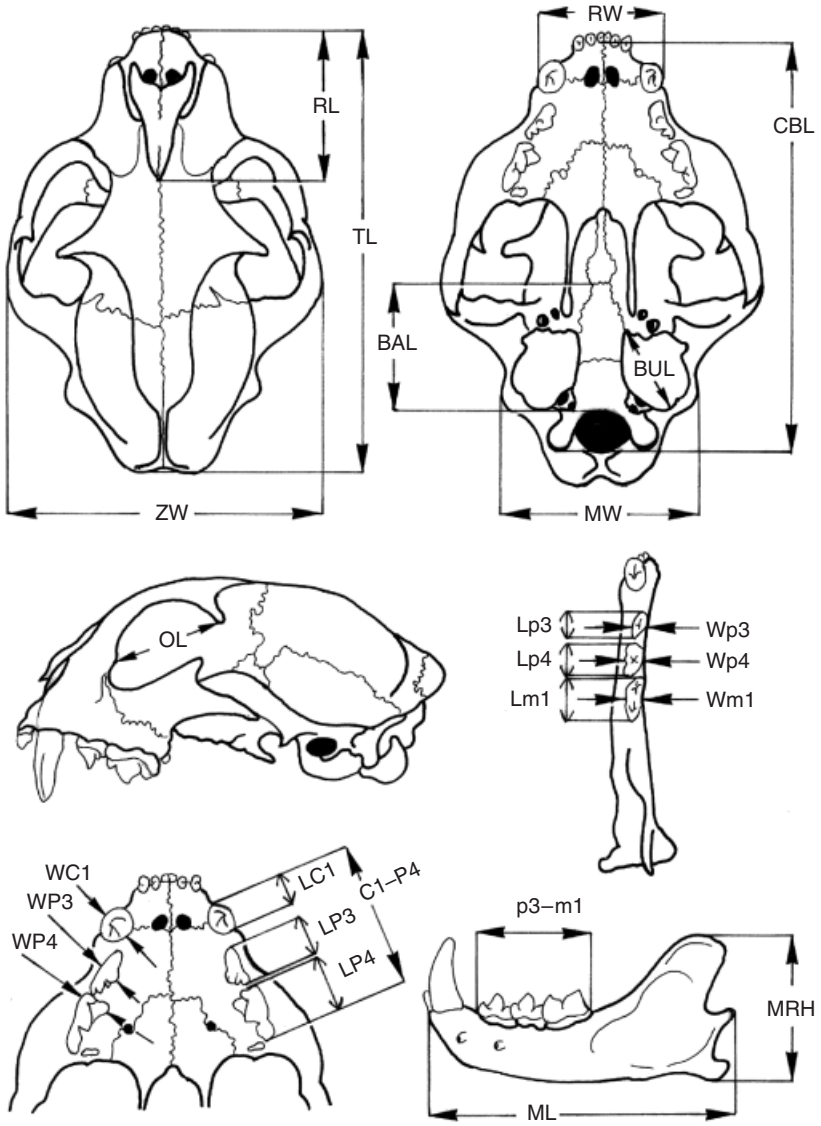

Figure 2 Description of the traits measured. Thirteen traits are related to the cranium or mandible: TL, total length of cranium; CBL, condylobasal length of cranium; BAL, basicranial axis length; RL, rostral length; OL, orbital length; ZW, zygomatic width; MW, mastoid width; RW, rostral width; BUL, bullar length; ML, mandible length; MRH, mandible ramus height; C1–P4, C1 to P4 length; p3–m1 = p3 to m1. Twelve measurements are related to teeth: Lp3, p3 maximum length; Wp3, p3 maximum width; Lp4, p4 maximum length; Wp4, p4 maximum width; Lm1, m1 maximum length; Wm1, m1 maximum width; LP3, P3 maximum length; WP3, P3 maximum width; LP4, P4 maximum length; WP4, P4 maximum width; LC1, C1 maximum length; WC1, C1 maximum width.

skull (García-Perea, Gisbert & Palacios, 1985; Beltrán & Delibes, 1993).

Admixture analysis

An admixture analysis was conducted pooling all the fully grown skulls (holding the sexes separated). This analysis was conducted in order to ascertain if the a priori subdivision of our data set into three separated geographic groups (D, SM and TM) was correct. The fitting of normal or *t*-component mixture models to multivariate data, using maximum likelihood via the EM algorithm, is well known and commonly used. However, for a set of data containing a group or groups of observations with longer than normal tails or atypical observations, the use of normal components may unduly affect the fit of the mixture model. Furthermore, this approach normally requires the initial specification of an initial estimate of the vector of unknown parameters, or, equivalently, of an initial classification of the data with respect to the components of the mixture model under fit. Therefore, a more robust approach by modelling the data by a mixture of *t* distributions is provided. The use of the ECM algorithm to fit this *t* mixture model is called EMMIX and is described in McLachlan & Krishnan (1997) and McLachlan & Peel (1998). Additionally, the algorithm utilized for this purpose does not require any specification of the above-mentioned parameters and automatically clusters each skull into its most probable group, and the a posteriori probability of an individual to belong to the cluster in which it has been assigned is estimated. The option of unrestricted component covariance matrices for the data set has been chosen. The significant level (*P*-value) is produced by the optional bootstrap analysis. By sequentially testing n, $n+1$, $n+2$, etc. and stopping when the step becomes insignificant, the number of components can be assessed.

Multivariate analysis

Since our collection is of limited size, we decided to employ resample statistics for our multivariate and univariate investigation, which utilizes 100% of the information available and is less sensitive towards small sample sizes and deviations from normality (Davison & Hinkley, 1997). A resample program was designed in order to compare the data samples. All the resample tests were conducted as Monte Carlo style permutation tests with replacement.

The multivariate analysis was conducted on fully grown skulls, and because multivariate methods do not allow observations to have incomplete data, the multivariate analysis was performed on a smaller subset of measurements (24 instead of 25 measurements, as the trait p3–m1 was excluded from the analysis) and specimens (30 females and 40 males). Skull specimens with more than five missing values were excluded from further multivariate analyses and missing values were estimated by means of a stepwise regression analysis (Zar, 1999).

A principal component analysis (PCA; Marcus, 1990) was carried out on the covariance matrix derived from the skull measurements. This analysis classifies phenotypic variation into independent components that can be used to dissect genetic networks regulating complex biological systems (Chase *et al*., 2002).

Thus, if size variation is present in the data and the coefficients of principal component (PC)1 are either all positive or all negative, then this PC can be said to summarize the within-sample size variation (Bookstein, 1989). Shape is thus defined as that subspace of dimensions one less than the number of measured variables and quantifies the variation that cannot be explained by size variation and allometric relationships. The expectation from theory is that functionally independent parts of the skull should vary independently among the loci controlling quantitative traits, and therefore should be associated with different PCs (Klingenberg & Leamy, 2001).

All the skulls were grouped into three distinct groups on the basis of their geographical origin. A non-parametric MANOVA (NPMANOVA) (5000 permutations) (Anderson, 2001) was conducted in order to test the significance of multivariate differentiation among the three geographic groups and a non-parametric ANOVA (NPANOVA) was conducted on the first three principal components (PC1–PC3).

Multiple comparison tests were made with a Scheffé's *F*-test (Zar, 1999), for comparing the differences between the PCs in the populations.

The D males' skulls were separated into two time periods, from 1872 to 1961, which is called the pre-bottleneck period, and from 1971 to 1998, which is called the post-bottleneck period. An NPMANOVA was made for testing multivariate differences between the two periods of collection. This analysis has not been performed on females because of the too small sample size of the skulls of this sex available from the pre-bottleneck period.

Univariate analysis

A non-parametric ANOVA (NPANOVA) was conducted in order to test the significance of each single trait differentiation among the three geographic groups, and the pairwise comparisons between the groups were made with a Scheffé's *F*-test. A resample *t*-test (1000 permutations), which takes into account deviation from normal distribution, inequality of sample size and variances among the groups tested, was conducted for each trait for testing differences in size between the two periods of collection of the males from the D population. The degree of change of mean and variance between the periods of collection have been estimated and expressed in per cent. The significance of the differences between variances has been tested by a non-parametric *F*-test (1000 permutations), which takes into account the non-normal distribution of the data and unequal sample size.

Because of the large number of tests that we have performed in this investigation, an overall Bonferroni correction (Rice, 1989) was applied to all the resample *t*- and *F*-tests to avoid significant results arising as a consequence of a large number of related tests. Following Miller's (1981) suggestions, we made a separate probability statement grouping all the traits with an isometric relationship, which had a correlation coefficient $R > 0.5$, both for the skull- and mandible-related traits group and the teeth traits group. This grouping ended up with three groups for the skull-related traits ($k = 3$; threshold $P = 0.167$) and five groups for the tooth traits ($k = 5$; threshold $P = 0.01$) (data on correlation coefficients and grouping of traits are available on request from the corresponding authors).

Results

Admixture analysis

The admixture analysis gave four clusters for the males skulls ($k = 4$, $P = 0.012$) and three clusters ($k = 3$, $P = 0.045$) for the female skulls. The percentage of specimens correctly assigned to the geographic group they belonged to was 100% for both males and females, with the exception of the comparison TM versus SM in females, which gave 88.89% of correctly classified individuals. The males from the D population were separated by the admixture program into two clusters ($k = 2$), which were coincident with the temporal a priori subdivisions (pre- and post-bottleneck periods), with the exception of two skulls assigned to the pre-bottleneck period but collected in the post-bottleneck period.

Multivariate analysis

The first three PCs explained 83.68% of the males and 82.79% of the females total variation; therefore, the multivariate statistics have been conducted on the first three PCs (see Table 1).

Table 1 Vectors of the principal components (PC1–PC3) and the per cent of variance explained by the single PC for males and females

Males			Females		
PC	Vector	% of variance explained	PC	Vector	% of variance explained
1	56.87	73.35	1	60.190	69.653
2	6.410	8.270	2	6.252	7.241
3	3.920	5.061	3	5.111	5.901

PC, principal component.

PC1 explained 73.35 and 69.65% of the total variability in males and females, respectively (see Table 1).

The skull traits total length of cranium and condylobasal length of cranium showed the highest loadings on PC1 in both sexes, whereas zygomatic width (ZW) showed the highest loading on PC2 and rostral length on PC3 in both sexes. The tooth traits showed relatively smaller loadings on all the three PCs as compared with the skull traits and showed a general tendency to have higher loadings on PC2 and PC3. The traits' loadings for the first three PCs for both males and females, computed from the pooled within-group variance–covariance matrix of traits' measurements, and the percentage of variation accounted for by each PC are available on request from the corresponding authors.

The NPMANOVA conducted in order to test the significance of multivariate differentiation among the three geographic groups was highly significant for both males and females (males: $F = 6.33$, $P = 0.0002$, females: $F = 3.39$, $P = 0.0058$).

The NPANOVAs conducted for testing whether the first three PCs were different among populations were significant for both sexes and for all three PCs (Table 2). Pairwise Scheffe's test showed a significantly bigger PC1 for males of the D population as compared with the SM population, that the PC2 of D was significantly bigger than TM's and the PC3 of SM was significantly bigger than that of TM (Table 2). In females, no significant pairwise differentiations were found for PC1; the PC2 of D was significantly bigger than that of SM and the PC2 of TM was significantly bigger than that of SM; finally, the PC3 of D was significantly bigger than those of TM and SM (Table 2).

The NPMANOVA that was performed for testing multivariate differences between males from the D population collected in the two periods was significant ($F = 3.94$, $P = 0.034$).

Univariate analysis

The NPANOVAs that were conducted in order to test for differences of every single trait among the three geographic groups were significant for 50% of the skull- and mandible-related traits in males and 38.5% in females, whereas for the tooth traits, the percentages of significant tests were 16.7 and 25% for males and females, respectively (Table 2).

The pairwise comparisons between the groups (Scheffe's test) showed a general tendency for the D population to have a bigger mean size of the skull traits in both sexes, followed by TM and finally SM, which had the smallest size. Scheffe's test conducted on the males' tooth traits showed that the traits WP3 of SM and TM were bigger than in the D population. The SM population also had bigger Lp4 than D. The females of D showed significantly bigger Wm1 and LC1 than that of TM, and both the D and TM populations showed a bigger Lp3 than SM (Table 2).

The test for differences in size between the two periods of collection of the male skulls from the D population with the resample *t*-test showed a significant reduction in size in the post-bottleneck period in 30.8% of the skull traits and in 25% of the tooth traits (Table 3). All the other traits also showed a reduction in size in the post-bottleneck period although this was not significant (with the only exception being trait Lm1). The mean reduction in the skull traits' size was 2.12% (range: 0.21–4.29%), whereas in the tooth traits

Table 2 Non-parametric ANOVA (NPANOVA) conducted for testing differences among the three populations (D, Doñana ; SM, Sierra Morena; TM, Toledo mountains) of the mean of the principal components (PC1–PC3)

		Males						Females					
PCs	Populations	*n*	Mean	SE	NPANOVA (*F*-value)	*P*	Scheffe's *F*-test	*n*	Mean	SE	NPANOVA (*F*-value)	*P*	Scheffe's *F*-test
PC1	D	26	253.70	1.53	6.85	0.0029	(D > SM)**	21	237.78	1.73	3.73	0.037	
	TM	10	249.02	1.00				6	229.39	2.48			
	SM	4	241.32	0.79				3	230.51	2.09			
PC2	D	26	1.51	0.42	15.94	0.0001	(D > TM)***	21	−13.32	0.46	5.79	0.0081	(D > SM)**, (TM > SM)*
	TM	10	−2.48	0.37				6	−13.17	0.65			
	SM	4	−0.16	0.65				3	−17.83	2.23			
PC3	D	26	−25.82	0.39	5.79	0.0065	(SM > TM)**	21	−33.07	0.48	6.35	0.0055	(D > TM)*, (D > SM)*
	TM	10	−27.22	0.42				6	−35.07	0.60			
	SM	4	−23.72	0.50				3	−36.23	0.57			

Pairwise test between the populations were performed with a Scheffe's *F*-test. Both tests were performed separately for the two sexes.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
NS, non-significant; PC, principal component.

Table 3 Non-parametric ANOVA (NPANOVA) conducted for testing differences among the three populations (D, Donana; SM, Sierra Morena; TM, Toledo mountains) of the mean of the skull, mandible and tooth traits (expressed in mm)

		Males						Females					
Traits	Populations	*n*	Mean	SE	NPANOVA (*F*-value)	*P*	Scheffe's *F*-test	*n*	Mean	SE	NPANOVA (*F*-value)	*P*	Scheffe's *F*-test
Skull and mandible traits													
TL	D	28	136.12	0.88	4.38	0.0188		20	127.36	1.06	2.35	0.1165	
	TM	12	135.18	0.82				6	123.30	1.22			
	SM	4	129.70	0.36				2	123.60	2.60			
CBL	D	25	122.25	0.85	2.58	0.0903		17	113.50	1.02	0.86	0.4371	
	TM	10	122.66	0.85				6	111.25	1.10			
	SM	3	117.27	0.13				1	114.90				
BAL	D	25	40.80	0.34	2.75	0.0783		18	37.16	0.52	0.17	0.8500	
	TM	9	39.82	0.47				6	36.717	0.305			
	SM	2	38.45	0.35				1	36.4				
RL	D	27	55.73	0.52	5.28	0.0095	(D > SM)*	21	51.49	0.68	2.75	0.0823	
	TM	10	53.84	0.68				5	48.14	1.12			
	SM	4	51.80	0.95				3	50.17	0.27			
OL	D	26	35.43	0.24	2.092	0.1386		22	34.33	0.36	0.16	0.8559	
	TM	10	35.81	0.18				6	34.58	0.77			
	SM	2	34.10	0.10				3	33.93	0.14			
ZW	D	27	97.30	0.57	7.931	0.0013	(D > SM)**, (D > TM)*	22	91.66	0.61	6.53	0.0045	(D > SM)*
	TM	11	94.85	0.60				7	88.64	1.43			
	SM	3	91.67	1.05				3	85.57	2.05			
MW	D	27	59.86	0.29	12.72	0.0001	(D > SM)***, (D > TM)*, (TM > SM)*	19	56.45	0.44	4.41	0.0234	(D > TM)*
	TM	11	58.38	0.37				6	53.83	0.77			
	SM	3	55.97	0.48				2	54.90	1.40			
RW	D	27	53.13	0.26	0.430	0.6554		22	50.77	0.36	0.38	0.6859	
	TM	9	53.56	0.35				7	50.14	0.58			
	SM	5	53.58	1.02				4	50.52	0.92			
BUL	D	24	28.19	0.41	11.85	0.0001	(D > TM)***	18	26.82	0.26	27.54	0.0001	(D > TM)***, (D > SM)***
	TM	9	24.76	0.39				4	22.67	0.86			
	SM	2	27.25	0.05				2	22.35	1.05			
ML	D	26	90.831	0.59	2.40	0.1051		20	84.96	0.78	2.20	0.1301	
	TM	10	89.79	0.65				7	82.77	0.67			
	SM	4	87.80	0.80				4	82.00	1.78			
MRH	D	26	39.11	0.32	9.49	0.0005	(D > SM)*, (D > TM)**	20	35.83	0.47	7.25	0.0029	(D > TM)*, (D > SM)*
	TM	10	37.07	0.30				7	33.04	0.69			
	SM	4	36.90	0.55				4	32.87	0.76			
P_3–M_1	The test could not be performed because no measurements were available for TM							18	28.84	0.250	1.93	0.176	
								1	28.10				
								1	30.81				
C1–P4	D	25	39.24	0.24	1.96	0.155		22	37.682	0.24	6.01	0.0067	(D > TM)**
	TM	10	38.62	0.29				6	35.817	0.402			
	SM	5	39.74	0.39				3	37	1.097			
Tooth traits													
Lp3	D	29	7.57	0.08	0.83	0.4435		18	7.256	0.084	4.56	0.0205	(D > SM)*, (TM > SM)*
	TM	11	7.74	0.11				7	7.329	0.087			
	SM	5	7.74	0.21				3	6.533	0.524			
Wp3	D	29	4.10	0.04	0.99	0.3806		18	3.95	0.04	1.17	0.3255	
	TM	11	4.21	0.06				7	3.90	0.07			
	SM	5	4.16	0.18				3	3.77	0.18			
Lp4	D	31	10.25	0.09	1.61	0.2117		21	9.87	0.09	0.339	0.7153	

Table 3 Continued.

		Males						Females					
Traits	Populations	*n*	Mean	SE	NPANOVA (*F*-value)	*P*	Scheffe's *F*-test	*n*	Mean	SE	NPANOVA (*F*-value)	*P*	Scheffe's *F*-test
	TM	11	10.34	0.11				6	9.77	0.20			
	SM	4	10.67	0.21				2	9.65	0.15			
Wp4	D	31	4.94	0.04	0.18	0.8376		21	4.76	0.05	2.77	0.081	
	TM	11	4.90	0.07				6	4.55	0.034			
	SM	4	4.95	0.06				2	4.7	0.2			
Lm1	D	33	12.79	0.08	3.06	0.0555		21	12.44	0.090	0.23	0.7993	
	TM	13	12.96	0.10				7	12.37	0.13			
	SM	7	13.26	0.21				3	12.27	0.49			
Wm1	D	33	5.68	0.04	2.51	0.0917		21	5.47	0.04	5.09	0.0128	(D > TM)*
	TM	13	5.52	0.05				7	5.20	0.03			
	SM	7	5.71	0.12				4	5.40	0.16			
LP3	D	29	10.12	0.09	1.68	0.1986		19	9.77	0.09	0.56	0.5787	
	TM	12	10.32	0.09				5	9.96	0.09			
	SM	4	10.45	0.06				2	9.85	0.15			
WP3	D	29	4.88	0.05	10.06	0.0003	(SM > D)**, (TM > D)*	19	4.60	0.05	0.47	0.6326	
	TM	12	5.12	0.05				5	4.68	0.12			
	SM	4	5.42	0.17				2	4.75	0.15			
LP4	D	33	14.96	0.09	5.75	0.0056	(SM > D)**	22	14.37	0.08	0.18	0.8331	
	TM	13	15.26	0.16				7	14.43	0.21			
	SM	8	15.61	0.14				4	14.52	0.45			
WP4	D	34	7.37	0.07	1.42	0.2514		22	6.78	0.08	0.89	0.4218	
	TM	13	7.15	0.11				7	6.63	0.14			
	SM	8	7.27	0.15				4	6.97	0.31			
LC1	D	34	7.46	0.06	2.47	0.0947		21	7.17	0.01	3.65	0.0395	(D > TM)*
	TM	13	7.72	0.12				6	6.67	0.12			
	SM	5	7.68	0.17				3	6.97	0.18			
WC1	D	33	6.10	0.06	1.70	0.1929		21	5.94	0.07	1.80	0.1843	
	TM	13	6.31	0.07				6	5.63	0.21			
	SM	5	6.10	0.18				3	5.87	0.18			

Pairwise test between the populations were performed with a Scheffe's *F*-test. Both tests were performed separately for the two sexes (males left, females right).

*$P<0.05$, **$P<0.01$, ***$P<0.001$.

TL, total length of cranium; CBL, condylobasal length of cranium; BAL, basicranial axis length; RL, rostral length; OL, orbital length; ZW, zygomatic width; MW, mastoid width; RW, rostral width; BUL, bullar length; ML, mandible length; MRH, mandible ramus height; C1–P4, C1 to P4 length; p3–m1 = p3 to m1. Lp3, p3 maximum length; Wp3, p3 maximum width; Lp4, p4 maximum length; Wp4, p4 maximum width; Lm1, m1 maximum length; Wm1, m1 maximum width; LP3, P3 maximum length; WP3, P3 maximum width; LP4, P4 maximum length; WP4, P4 maximum width; LC1, C1 maximum length; WC1, C1 maximum width.

the mean reduction was 2.57% (range: 0.32–5.4%) (Table 3).

The resample *F*-test showed a significant reduction of V_p of the skull traits in the post-bottleneck period compared with the pre-bottleneck period in 38.6%, and a significant increase in 7.6% (trait basicranial axis length) of the skull traits. In the teeth, a significant reduction of V_p was found in 33.3% of the traits, whereas a significant increase was found in 8.33% (trait WP3) of the traits (Tables 3 and 4).

Discussion

Despite the relatively small sample size of the skulls investigated and the relatively long time period of collection, certain size and shape differences were detected among the a priori defined geographic groups. This geographic subdivision was also supported by the admixture analysis, which has proven to be a useful tool for the detection of temporal and/or spatial patterns of morphometric differences. Morphological differences in size-related traits (PC1) were not significant, except when comparing males from D and SM populations. However, significant differences in shape-related traits (PC2, PC3) were found among the three populations. This similarity in size and the small shape differences in a sample covering a time span of almost one century suggest that no large size differences have ever existed among the three populations, although the slightly larger size of Doñana's lynx (especially before the hypothetical bottleneck had occurred) could reflect a peculiar characteristic of this population, which is most commonly

Table 4 A comparison of the mean size (in mm) and variance of the traits of skulls of males collected in the pre-bottleneck and in the post-bottleneck period in the Doñana area

Skull and mandible traits	Pre-bottleneck period (mean) (Var) (*n*)	Post-bottleneck period (mean) (Var) (*n*)	Resample *t*-test (*t*-value)	*P* *t*-test	Changes in % of the mean trait size	*P* resample *F*-test	Changes in % of the variance of the trait
TL	138.00 (17.63) (10)	135.08 (21.97) (18)	1.69	NS	(pre > post) 2.1	NS	(post > pre) 19
CBL	125.46 (12.34) (7)	121.01 (15.17) (18)	2.76	*	(pre > post) 3.5	NS	(post > pre) 18.65
BAL	41.67 (1.78) (7)	40.46 (3.13) (18)	1.85	NS	(pre > post) 2.47	*	(post > pre) 43.11
RL	57.38 (4.43) (9)	54.91 (7.16) (18)	2.61	*	(pre > post) 4.29	NS	(post > pre) 38.12
OL	35.66 (1.36) (8)	35.32 (1.66) (18)	0.66	NS	(pre > post) 0.95	NS	(post > pre) 18.07
ZW	98.08 (16.17) (9)	96.91 (5.27) (18)	0.81	NS	(pre > post) 1.19	**	(pre > post) 67.41
MW	59.94 (3.75) (10)	59.81 (1.55) (17)	0.18	NS	(pre > post) 0.21	*	(pre > post) 58.65
RW	53.84 (2.38) (10)	52.71 (1.22) (17)	2.035	NS	(pre > post) 2.1	*	(pre > post) 48.86
BUL	28.30 (5.51) (8)	28.14 (3.63) (16)	0.169	NS	(pre > post) 0.57	NS	(pre > post) 34.11
ML	91.94 (11.73) (10)	90.14 (6.54) (16)	1.43	NS	(pre > post) 1.96	*	(pre > post) 44.27
MRH	39.38 (3.81) (10)	38.94 (2.04) (16)	0.612	NS	(pre > post) 1.10	*	(pre > post) 46.65
p3–m1	31.04 (1.17) (9)	29.8 (0.71) (11)	2.82	*	(pre > post) 4.00	*,#	(pre > post) 39.48
C1–P4	40.10 (1.56) (8)	38.84 (0.91) (17)	2.52	**	(pre > post) 3.14	*,#	(pre > post) 41.67
Tooth traits							
Lp3	7.82 (0.12) (12)	7.39 (0.20) (17)	2.98	**	(pre > post) 5.40	*,#	(post > pre) 40
Wp3	10.47 (0.31) (8)	4.08 (0.03) (17)	0.76	NS	(pre > post) 1.21	NS	(pre > post) 19.05
Lp4	4.13 (0.04) (12)	10.26 (0.14) (16)	0.97	NS	(pre > post) 2.00	NS	(pre > post) 20.91
Wp4	4.96 (0.02) (10)	4.93 (0.06) (21)	0.38	NS	(pre > post) 0.61	NS	(pre > post) 59.75
Lm1	12.75 (0.32) (12)	12.82 (0.18) (21)	(−0.37)	NS	(post > pre) 0.53	*	(pre > post) 45.37
Wm1	5.73 (0.05) (12)	5.64 (0.05) (21)	1.13	NS	(pre > post) 1.57	NS	(post > pre) 3.40
LP3	10.42 (0.32) (10)	9.96 (0.12) (19)	2.35	*,#	(pre > post) 4.43	**	(pre > post) 63.58
WP3	4.89 (0.04) (10)	4.87 (0.09) (19)	0.17	NS	(pre > post) 0.32	*	(post > pre) 55.43
LP4	15.17 (0.24) (12)	14.84 (0.24) (21)	1.87	NS	(pre > post) 2.18	NS	(post > pre) 1.24
WP4	7.59 (0.08) (12)	7.24 (0.17) (22)	2.89	**	(pre > post) 4.57	**	(pre > post) 51.78
LC1	7.64 (0.15) (12)	7.36 (0.11) (22)	2.13	*,#	(pre > post) 3.63	NS	(post > pre) 27.39
WC1	6.27 (0.06) (12)	6.00 (0.14) (21)	2.56	**	(pre > post) 4.38	**	(pre > post) 56.20

The tests conducted are a resample *t*-test (1000 permutations), which takes into account unequal variances and a resample *F*-test (1000 permutations), which takes into account deviations from the normal distribution. The skull and mandible traits were measured only on adult individuals, tooth traits have been measured on both adults and sub-adults. The changes of the mean and variance of the traits between the two periods of collection have been estimated in per cent. An overall Bonferroni test, following Miller's suggestions (1981), was applied making a separate probability statement grouping all the traits that had a correlation coefficient >0.5 for both the skull related traits ($k=3$, threshold: $P=0.0167$) and tooth traits ($k=5$, threshold: $P=0.01$).

*$P<0.05$, **$P<0.01$.

NS, non-significant; TL, total length of cranium; CBL, condylobasal length of cranium; BAL, basicranial axis length; RL, rostral length; OL, orbital length; ZW, zygomatic width; MW, mastoid width; RW, rostral width; BUL, bullar length; ML, mandible length; MRH, mandible ramus height; C1–P4, C1 to P4 length; p3–m1 = p3 to m1. Twelve measurements are related to teeth: Lp3, p3 maximum length; Wp3, p3 maximum width; Lp4, p4 maximum length; Wp4, p4 maximum width; Lm1, m1 maximum length; Wm1, m1 maximum width; LP3, P3 maximum length; WP3, P3 maximum width; LP4, P4 maximum length; WP4, P4 maximum width; LC1, C1 maximum length; WC1, C1 maximum width.

#, result no longer significant after a sequential Bonferroni test.

reported to include ungulates in its diet (Delibes *et al.*, 1975; Delibes, 1980; Aymerich, 1982).

In recent times, we know that the Iberian lynx has a polygynous mating system, with males defending territories, which can include the breeding territories of several females (Ferreras *et al.*, 1997). In a preserved area like Doñana N.P., a large male size should be advantageous because of the increased probabilities of occupying and defending a territory. However, this advantage may decrease if high human-induced mortality reduces lynx density and thus the strength of competition for territories, as it likely happened in TM and SM. This could partly explain why geographical differences in size are larger for males than for females, and why males are larger in the D population.

Most morphological differences observed among the three populations are shape related (PC2, PC3), and concern the following measurements: ZW, mastoid width, bullar length and mandible ramus height (MRH). Two of these variables (ZW and MRH) are related to the origin and insertion of the main masticatory muscles, masseter and temporalis, which are also responsible for the strength of the lethal bite (Smith, 1993). Significantly higher values of these variables in the D population are likely related to the ability to hunt larger prey. Related to the tooth traits, although the

PCA revealed shape differences in carnassials (P4 and m1), Scheffe's test failed to find significant differences when making pairwise comparisons between populations, and the few differences identified did not reveal a clear pattern of variation. The differences found among the three populations appear not to be strongly linked to any ecological specialization nor have a specific anatomical-functional meaning. Therefore, considering that shape differences are more genetically determined than size differences (Atchley, Rutledge & Cowley, 1981; Klingenberg & Leamy, 2001; Workman *et al.*, 2002) and given that shape of morphological traits are in general assumed to be more stable than size and submitted to strong developmental constraints (Debat *et al.*, 2003), our results suggest that the three geographic groups investigated are also genetically differentiated. These data agree with the previous findings of a genetic differentiation between D and SM at the genomic level (Johnson *et al.*, 2004).

Related to TM and SM populations, Rodríguez & Delibes (1992) estimated that both populations were still connected in the 1980s, just before the probable extinction of the TM population. Our results indicate a genetic differentiation between TM and SM, suggesting that either Rodríguez & Delibes (1992) were too optimistic in their evaluation of the lynx situation, or that very low effective population sizes drove a fast genetic structuring of the populations. These two possibilities will be evaluated in an extended study of current and historical variation, now in progress.

Differences observed between D and TM populations (Table 2) are not consistent with data previously reported on both populations, as those of García-Perea (1991) on TM and of Beltrán & Delibes (1993) on D show. Values of similar traits measured by these authors indicated that TM males were larger than D males, while TM females were smaller than D females. This lack of congruence is probably a consequence of the small size of the D sample analysed, likely composed by a mixture of specimens collected in different periods (pre- and post-bottleneck). This fact reveals how important these kinds of studies are for species having suffered severe declines within relatively short time spans.

The pattern of morphometric differentiation was more or less consistent for both males and females, although males seem to have a higher phenotypic plasticity than females, as has been shown by the relatively bigger percentage of variance explained in PC1 and PC2 in males as compared with females. The changes in size and shape in male skulls of the D population can be because of several factors: spatial and temporal variation in habitat quality and population density can affect adult body size and skull traits. For example, fragmented habitats usually play the role of islands, recreating sometimes the effect of directional body size change observed for several mammal species on islands, referred to as the island rule (Van Valen, 1973). A small number of captive-reared, free-born Iberian lynx from Doñana and Sierra Morena have reached larger body sizes than their siblings reared in nature (I. Sánchez, pers. comm.), which suggests an important effect of the trophic conditions on adult size during the growing period. Therefore, a reduction of the lynx size in Doñana since the 1960s could be a consequence of the decrease in the number of rabbits, the lynx's main prey, following the arrival of myxomatosis and later the rabbit haemorrhagic disease (RHD). A similar effect could be expected to have occurred in SM. Lynx from TM had no chance to experience these changes because they were already extinct.

Some traits however showed stronger reduction in size as compared with others, even if the compared traits were isometrically related. There are therefore strong indications that the observed heterogeneity in the degree of mean size reduction and the relatively high degree of reduction of some of the skull traits investigated ($>4\%$) can also be attributed to inbreeding depression. The heterogeneity of the degree of mean size reduction (which has consequently altered the original proportions between the skull traits) could be caused by the different number of genes controlling the traits with different degree of dominance and epistatic interactions, conferring traits different susceptibility to environmental change. This hypothesis was supported by recent works which suggest that traits with different degree of dominance and epistatic interactions have different degrees of susceptibility to the environment (e.g. Podolski, 2001). We must keep in mind that inbreeding depression will only be observed if dominance interactions of traits are present (Lynch, 1996).

Under a strictly additive genetic architecture, all elements of the genetic covariance matrix for a set of traits are expected to shrink with the same factor, $1–1/(2N_e)$ (Wright, 1951; Lande, 1979). Thus, the genetic correlation and the mean trait size are not expected to change with drift. This is not what we found as both the mean trait size and trait proportions have changed. The complexity of the effects of inbreeding on trait means is further manifested as being environment specific. Interactions between inbreeding and the environment are especially investigated in conservation biology, where there is a growing awareness that it is the combined effect of genetic and environmental stress that threatens long-term population survival.

The V_p of the skull traits showed heterogeneity with respect to either an increase or a decrease with time for several traits. These findings reinforce the hypothesis that the different skull traits are controlled by several genes, where the additive interaction is more or less expressed.

The effect of inbreeding on variance components has also been studied (Fowler & Whitlock, 1999). Theoretical predictions under an additive model state that the additive part of the genetic variance (V_a) decreases within populations proportional to the inbreeding coefficient and to the amount of genetic drift. If V_p is roughly correlated to V_a, the reduced amount of V_p in the D population in the post-bottleneck period could be because of a loss of V_a. Environmental factors such as e.g. a GXE interaction or the reduction of the trophic niche breadth could also have contributed to a reduction of V_p (Van Valen & Grant, 1970), but the possibility of a GXE interaction can be excluded in this case, as it requires local adaptation, which is not compatible with the small lynx N_e as both mutation and drift counteract the possibility of the populations to adapt.

The increased V_p observed for some traits could also be partially because of a decreased level of DS, which is trait specific. Several studies have documented an increase of V_p in populations that have gone through a severe bottleneck or in which the environmental conditions have deteriorated (Hoelzel, 1999). Epistatic interactions can however also increase traits' V_p in populations which pass through a bottleneck enhancing V_a under inbreeding (Cheverud *et al.*, 1999).

In conclusion, the different degree of increase or reduction of V_p in the different traits can be attributed to the antagonistic and synergistic effects of the above-mentioned factors. However, the observed reduction of V_p for several traits is mainly because of the reduction of V_a, which seems to be the predominant factor counteracting the eventual increase of V_p because of a possible decrease of DS.

The males of the D population have been found to 'converge' to the TM population because of the skull-size related traits becoming smaller. However, given the different degrees of reduction and increase that the different size and shape traits show, it is easy to see that the D population does not converge to a common morphology as might be expected in traits with an isometric relationship if environmental plasticity should play a major role (Sparks & Jantz, 2002).

The finding that the D population was already differentiated in size in the pre-bottleneck period could be explained by more optimal habitat conditions, which are found in the D area (e.g. greater availability of food).

The signs of inbreeding depression on morphometric traits showed by the D population could have some implications on the population's average fitness. Traits affected by inbreeding may interact to reduce overall fitness substantially, even though there is no automatic connection between fitness reduction and trait size reduction (Mousseau & Roff, 1987; Roff & Mousseau, 1987). However, the inbreeding depression observed in the skull traits could be a 'soft reflection' of the real inbreeding depression affecting fitness of the D population, as morphometric traits are less affected by inbreeding depression than fitness-related traits.

That inbreeding does not affect all traits to the same degree has been suggested by several authors: primarily, traits closely associated with fitness, that is viability, fertility and disease resistance, are prone to inbreeding depression (Falconer & Mackay, 1996; DeRose & Roff, 1999). The mean values of morphological traits, such as adult body size or bristle number in *Drosophila,* are however often not changed significantly by inbreeding (DeRose & Roff, 1999), but if a trait is controlled by several genes, the eventual effect of dominance and epistatic interactions should be relatively small, unless there are pleiotropic effects (Lynch, 1996).

Conservation strategies

Despite the fact that morphometrical differences have been found among the three populations, suggesting a different genetic composition of the populations, it is recommendable to produce gene flow between them as the D population has shown signs of inbreeding depression. Inbreeding depression could have occurred because of the rapid decline of the lynx population, which did not allow genetic purging. In fact, population genetic models predict that the increased homozygosity resulting from inbreeding will expose recessive deleterious alleles to natural selection, and thereby purge the genetic load (Bijlsma, Bundgaard & Van Putten, 1999; Charlesworth & Charlesworth, 1999). However, purging only works when inbreeding occurs gradually and over several generations. If inbreeding is sudden and extreme, N_e is reduced and drift becomes stronger relative to selection, resulting in more random fixations. Consequently, because there are more generations and greater opportunity for selection to act before a given inbreeding coefficient is reached, lower rates of inbreeding are expected to be less deleterious than faster inbreeding for the same total level of inbreeding (Day, Bryant & Meffert, 2003).

A higher connectivity among the scattered populations will increase N_e, reduce the role of genetic drift, alleviate the inbreeding depression and increase the genetic diversity, which will increase the lynx' evolutionary potential: the so-called 'genetic rescue effect' (Richards, 2000; Tallmon, Luikart & Waples, 2004). We cannot ignore that rapid extreme environmental changes will place a premium on genetic variability and adaptability of many populations in fragmented environments during the coming centuries. Furthermore, an increased number of immigrants will increase numerical abundance, which will reduce demographic variation, the so-called 'rescue effect' (Brown & Kodric-Brown, 1977).

Since the current wild populations are separated by several hundreds of kilometres, an increase in connectivity should be considered not only for these populations, but also in the current captive breeding programme. In particular, we recommend the maximization of the genetic variability in the lynx captive population; in this way, future introductions of captive-born lynx will act as a kind of genetic link between the remaining natural populations.

The risk of outbreeding depression by admixing the populations is negligible as the GXE effect does not exist. The mutation-drift regimes governing the populations might in fact have counteracted any possible genetic adaptation. The risk of outbreeding depression is also minor relative to the risk of inbreeding depression, given the recent historical connectivity, the absence of obvious adaptive divergence and the prevalence of drift relative to selection in the recent past.

The comparisons between the genetic variability detected using neutral molecular markers (e.g. microsatellites) and the genetic variability detected in quantitative traits could make important contributions to the open debate among conservation biologists about the correlation between these two measures.

Molecular markers cannot identify the likelihood of loss of genetic variance in traits of ecological significance as the correlation between molecular diversity (heterozygosity) and quantitative genetic variation (e.g. heritability) is weak,

with molecular measures explaining only about 4% of the variation in quantitative traits (Reed & Frankham, 2001). Therefore, the potential for evolutionary response in quantitative traits cannot be predicted by the use of molecular markers, and thus remains a central issue for quantitative genetics analysis in the assessment of the extinction risk in conservation biology. We will therefore underline the potential utility that craniometrical investigations could have in the planning of future conservation strategies.

The main problem associated with the application of results from craniometrical studies to endangered species is the limited sample size, which has also afflicted this investigation. Limited sample size increases the possibility of committing type 2 errors when conducting statistical analyses. The possibility of using statistical packages, which allow bootstrapping (as the resample statistic), can at least limit the problems associated with small sample sizes.

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