Conserv Genet (2008) 9:1–11
DOI 10.1007/s10592-007-9297-z

RESEARCH ARTICLE

Molecular analysis of hybridisation between wild and domestic cats (*Felis silvestris*) in Portugal: implications for conservation

Rita Oliveira · Raquel Godinho · Ettore Randi · Nuno Ferrand · Paulo Célio Alves

Received: 9 August 2006 / Accepted: 3 February 2007 / Published online: 18 April 2007

Abstract The endangered European wildcat (*Felis silvestris silvestris*) is represented, today, by fragmented and declining populations whose genetic integrity is considered to be seriously threatened by crossbreeding with widespread free-ranging domestic cats. Extensive and recent hybridisation has been described in Hungary and Scotland, in contrast with rare introgression of domestic alleles in Italy and Germany. In Portugal, the wildcat is now listed as VULNERABLE in the Red Book of Portuguese Vertebrates. Nevertheless, genetic diversity of populations and the eventual interbreeding with domestic cats remain poorly studied. We surveyed genetic variation at 12 autosomal microsatellites for 34 wild and 64 domestic cats collected across Portugal. Wild and domestic cats were significantly differentiated both at allele frequencies and sizes (F_{ST}=0.11, R_{ST} = 0.18, $P < 0.001$). Population structure and admixture analyses performed using Bayesian approaches also showed evidence of two discrete groups clustering wild and domestic populations. Results did not show significant genetic divergence among Northern, Central and Southern wildcats. Six morphologically identified wildcats were significantly assigned to the domestic cluster, revealing some discrepancy between phenotypic and genetic identifications. We detected four hybrids (approximately 14%) using a consensus analysis of different Bayesian model-based software. These hybrids were identified throughout all sampled areas, suggesting that hybridisation is of major concern for the appropriate implementation of wildcat conservation strategies in Portugal.

Keywords wildcat · domestic cat · hybridisation · microsatellites · admixture analysis · Bayesian clustering · conservation genetics

R. Oliveira (✉) · R. Godinho · N. Ferrand · P. C. Alves
CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairao, Portugal
e-mail: ritaoliveira@mail.icav.up.pt

R. Oliveira · N. Ferrand · P. C. Alves
Departamento de Zoologia e Antropologia, Faculdade de Ciências da Universidade do Porto, 4099-002 Porto, Portugal

E. Randi
INFS, Istituto Nazionale per la Fauna Selvatica, Via Cà Fornacetta 9, 40064 Ozzano dell'Emilia, Bologna, Italy

Introduction

Although globally distributed across Europe and South-western Asia, the European wildcat (*Felis silvestris silvestris*) is currently represented by fragmented and declining populations. Even though legally protected by important Directives (as Habitat Directive, Bern Convention and CITES) in most European countries, wildcat populations are considerably threatened mainly due to the concomitant habitat destruction and fragmentation, poison and road kills, proliferation of viral diseases and hybridisation with its domestic counterpart (Stahl and Artois 1994; Nowel and Jackson 1996; Beaumont et al. 2001; Randi et al. 2001).

Crossbreeding with widespread free-ranging domestic cats is one of the main threats for wildcat survival underlined by the European Council (Stahl and Artois

1994). Therefore, it became imperative to study differentiation between wild, domestic cats and cryptic hybrids and to evaluate the rate and impact of hybridisation. The problematic definition of morphological criteria allowing unambiguous distinction between the three forms, along with the particularly challenging identification of hybrids beyond first generation (Daniels et al. 1998; Allendorf et al. 2001), prompted the initiation of genetic studies into diagnostic molecular traits. A number of European wildcat studies have used microsatellites with much more accurate results than former works using mitochondrial DNA (Hubbard et al. 1992) and allozymes (Randi and Ragni 1991), especially when combining highly polymorphic markers and recently developed Bayesian clustering models. Among European populations, results suggest variable rates of domestic genes introgression, with wide and recently hybridising populations in Hungary and Scotland (Beaumont et al. 2001; Daniels et al. 2001; Pierpaoli et al. 2003; Lecis et al. 2006) contrasting with a low admixture scenario in Italy and Germany (Randi et al. 2001; Pierpaoli et al. 2003; Eckert and Hartl 2005; Lecis et al. 2006). Although reasons for the observed variability remain unidentified, the anthropogenic-mediated dispersion of domestic cats throughout wildcat distribution and the unknown effects of long-term simpatry raised a global concern regarding both genetic and taxonomic status of the European wildcat (McOrist and Kitchener 1996; Daniels et al. 1998).

With the exception of littoral areas, wildcats were formerly widespread in Portugal (Nowell and Jackson 1996). However, its present distribution appears to be considerably smaller. Similarly to other European populations, massive habitat loss and landscape fragmentation, progressive and invasive urbanization, and scarce availability of prey (as a result of the severe decrease of wild rabbit, the main natural prey in Mediterranean landscapes, Gil-Sánchez et al. 1999; Lozano et al. 2003) may have led to population decline and, eventually, promoted reproductive interactions with domestic cats. A few ecological studies were implemented in Portuguese protected areas (Sarmento 1996; Fernandes 1996; Monterroso et al. 2005; Ferreira et al. 2005), documenting an evident versatility in food ecology and habitat selection. Nevertheless, ecological, ethological and, particularly, genetic features of the wildcat population are still poorly explored. A first molecular approach was performed by Fernandes (1996); however, the analysis of a small number of samples and loci prevented obtaining consistent results. More recently, Pierpaoli et al. (2003), in a broad European study, identified one individual with hybrid ancestry among 13 Portuguese wildcats. Nevertheless, frequency, extension and impact of domestic genes introgression remain unknown.

In this study, we present the first integrated approach combining the use of highly polymorphic loci and Bayesian statistical approaches to (i) investigate the extend of genetic variation and differentiation in Portuguese wild and domestic cat populations; (ii) pinpoint hybridisation and evaluate introgression of domestic alleles, (iii) provide new insights and critical guidelines to the regional and global conservation of this threatened feline. This work represents a first-step to clarify central population-level questions for wildcat management and long-term protection in Portugal, producing reference molecular data for future studies on historical and recent patterns of genetic diversity and for monitoring populations' demography, gene flow and genetic structure.

Materials and methods

Sampling and DNA extraction

We analysed a total of 98 tissue, blood and swab samples comprising 34 wild and 64 domestic cats (of which 16 are purebred and 48 are mutt/feral individuals). Wildcat samples were provided by BTVS-ICN (Wild Animal Tissue Bank, Portuguese Conservation Institute), and were distributed across the North (4), the Centre (4) and the South (26) of Portugal (Fig. 1). The low population density of wildcats in Portugal associated with their elusive behaviour difficult obtaining larger sample sizes from this feline. Wildcats were taxonomically identified by collectors according to their coat-colour pattern (Ragni and Possenti 1996), biometrics (Schauenberg 1977) and geographical location, independently from any genetic information. In order to survey potentially divergent domestic cat gene pools and obtain a representative sampling of the domestic subspecies, we collected samples from Northwest and South-east of Portugal (Fig. 1). We directed sampling effort to areas where human settlements are known to overlap with wildcat distribution. We extracted total genomic DNA using salting-out and phenol–chloroform procedures, both adapted from Sambrook et al. (1989).

Microsatellites typing and data analysis

Individual genotyping

We assessed individual multilocus genotypes using 12 neutral unlinked microsatellites, formerly isolated and characterized in domestic cat (Menotti-Raymond and

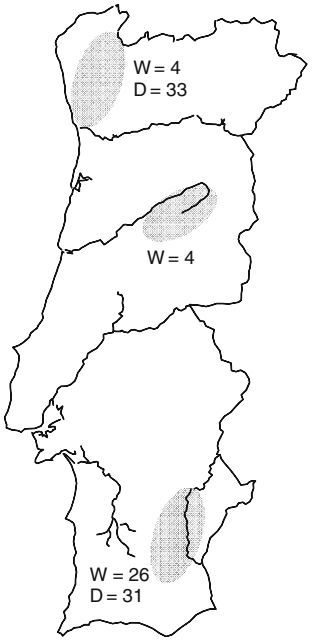

Fig. 1 Geographical location and number of sampled individuals (W, wildcats; D, domestic cats)

O'Brien 1995; Menotti-Raymond et al. 1999). Specific choice of this battery is justified by its prior successful and informative use in recent wildcat studies (Beaumont et al. 2001; Daniels et al. 2001, Randi et al. 2001; Pierpaoli et al. 2003; Eckert and Hartl 2005; Lecis et al. 2006). Polymerase chain reaction (PCR) amplifications of individual microsatellites followed Randi et al. (2001). PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel and visualized by silver staining.

Analysis of genetic diversity

Allele frequencies, standard diversity indices and observed (H_O) and expected (H_E) heterozigosities for each locus and population were calculated using GENETIX 4.05 (Belkhir et al. 1996–2004). We estimated allelic richness (AR) using FSTAT 2.9.3.2 (Goudet 2001). Guo and Thompson's (1992) Markov chain method (MCMC) was implemented in GENEPOP 3.4 (Raymond and Rousset 1995) to evaluate significant deviations from Hardy–Weinberg Equilibrium (HWE) for all locus-population combinations and statistically infer pairwise Linkage Equilibra (LE) among loci. We adjusted significance levels using sequential Bonferroni correction for multiple comparisons in the same data set (Rice 1989). GENEPOP 3.4 and FSTAT 2.9.3.2 were used to compute single and multilocus F (Weir and Cockerham 1984) and R statistics (Slatkin 1995), accounting for variation in population sizes. We estimated the genetic relationship between wild and domestic populations through a hierarchical Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992), implemented in ARLEQUIN 3.01 (Excoffier et al. 2005) using Φ_{ST} and R_{ST}. We used the same analysis to estimate the genetic differentiation between geographically separated cats sampled across Portugal, within both wild and domestic populations. In order to increase the number of individuals per wildcat geographical group, we assembled cats from North and Centre and compared with the ones from South. The significance of genetic differentiation was tested by random permutation, under the null hypothesis that all individuals belong to a single global population. Using FSTAT 2.9.3.2, we computed Wilcoxon signed rank test to evaluate differences in allelic diversity (AD), allelic richness (AR) and H_E between pairs of geographical groups.

Population structure and admixture analyses using multilocus genotype data

Population structure, individual assignments and admixture proportions were estimated through different Bayesian-based statistical techniques using: (i) the clustering procedure described by Pritchard et al. (2000) and recently updated in STRUCTURE 2.1 (Falush et al. 2003); (ii) the method developed by Anderson and Thompson (2002) and performed in NEWHYBRIDS and (iii) a model-based software described by Wilson and Rannala (2003) and carried out in BAYESASS 1.2. Both STRUCTURE and NEWHYBRIDS were implemented providing prior non-genetic classification for all known domestic cats, since we had the confident reference that all domestic individuals were true domestic without any recent ancestry in the wild population. The use of this type of information frequently results in Bayesian inference improvement and is strongly supported by STRUCTURE's authors in cases of unequivocal pre-classification (Pritchard and Wen 2003). We included or not that information for the wildcats and the putative hybrids detected without non-genetic information. NEWHYBRIDS was used to achieve a more detailed analysis of admixture proportions and hybrids ancestry, by inferring the posterior probability assignment (Q) of each sampled individual to six genotype frequency classes: Pure I; Pure II; F1; F2; Backcross I and Backcross II. We also used BAYESASS to estimate recent migration rates between wild and domestic populations. In this software results are presented as

the number of times each individual is assigned to each population and were transformed, in this study, into probabilistic values.

We assessed the power of admixture analysis to detect parentals and F1, F2 and backcross hybrids by simulation of parental and hybrid genotypes in the program HYBRIDLAB (Nielsen et al. 2006), as recently described by Barilani et al. (2006). Briefly, in our original dataset, we selected a subset of 20 wild and 40 domestic cats that revealed, in STRUCTURE, an individual proportion of membership (qi) > 0.90 to their parental cluster, in order to exclude possible hybrids. Starting from this sampling, we simulated 100 genotypes of each parental and hybrid classes, procedure that was repeated 10 times. The simulated genotypes were then used in STRUCTURE with $K = 2$ and no prior population information, in order to evaluate the efficiency of admixture analysis to study our population and define the appropriate threshold value that should be used for the individual assignment to one single population cluster or hybrid class. Following simulations data, we defined a threshold of 0.80 (see ''Results'') for all methods and each genotype was assigned to each group based on its qi. In the case of STRUCTURE, we also evaluated the 90% credibility intervals (CI) of individual's qi. According to each model features and their previous use in population structure analyses, we computed all programs using the profiles described in Table 1.

Results

A first exploratory Bayesian analysis revealed that six morphologically pre-classified wildcats, named Fs2, Fs6, Fs9, Fs10, Fs21 and Fs23, were significantly assigned to the domestic cluster according to their multilocus genotypes (e.g. $q_1 > 0.94$ in STRUCTURE; $P \leq 0.80$). Based on these results and on documented errors for the unequivocal phenotypic distinction between European wildcats, tabby domestic cats and their hybrids (Ragni 1993), wrong morphological identification was considered the most plausible explanation for this incongruence. In fact, Fs2 was identified as a ''strange'' colour pattern wildcat by the collector and Fs21 and Fs23 were found particularly damaged in the field, preventing a complete analysis of morphological traits or hiding some phenotypic signs of domestication. Consequently, these six individuals were excluded from the analysis and the new sampling profile became constituted of 28 wildcats, 21 from the South, four from the Centre and three from the North of Portugal.

Analysis of genetic diversity

All loci were polymorphic in both wild and domestic cats, showing from seven (Fca077) to 16 (Fca026) alleles per locus. Although differential frequency distribution of alleles was the most significant parameter of distinction between both populations, we found a total of 12 private alleles, six in wild and six in domestic cats (in order to prevent sampling and/or genotyping errors we only considered alleles with frequency higher than 5%; Table 2). A significant deficit of heterozygotes was detected in domestic cats ($F_{IS} = 0.09$; $P < 0.05$). None of the combinations between pairs of loci disclosed a significant deviation from Linkage Equilibra (LE) ($P < 0.0041$, Bonferroni-corrected for 12 independent replications). A significant departure from HWE was observed in only two over 24 possible locus-population combinations, Fca126 in wildcats ($P = 0.0004$; $F_{IS} = 0.21$) and Fca088 in domestic cats ($P = 0.0000$; $F_{IS} = 0.38$; Table 3).

Table 1 Programs profiles defined to analyse population structure using three Bayesian clustering methods

Profile	Program		
	STRUCTURE	NEWHYBRIDS	BAYESASS[c]
MCMC iterations	10^5	10^5	3.0×10^6
Burn-in period	10^4	10^4	10^6
Inference of K (populations)	MAXPOP = 1–5		
Others	Independent runs = 5 Model = *Admixture model*[a]	*Uniform priors*[b]	Sampling frequency = 2,000

[a] Allows individuals to have mixed ancestry and was performed using two model options: correlated (F model) and independent (I model) allele frequencies between populations

[b] *Uniform priors* consider that at least one copy of each allele has been found in both populations. This approach reduces the influence of low frequency alleles, preventing sampling and genotyping errors in closely related populations

[c] Convergence of MCMC algorithm was first confirmed using different initial values of migration and inbreeding levels (0.10 and 0.15 for both parameters)

Table 2 Allelic frequencies at 12 polymorphic microsatellites among Portuguese wild and domestic cat populations

Locus	*N*	Population	Allelic frequencies															
Allele size (bp)			123	125	131	135	137	139	141	143	145	147	149					
Fca008	63	Domestic	0.01	0.15	0.01	0.02	0.22	0.07	0.11	0.15	0.23	0.01	0.02					
	28	Wild	0.00	0.02	0.00	0.00	0.02	0.11	0.29	0.46	0.07	0.03	0.00					
Allele size (bp)			132	134	136	138	140	142	144	146	148	150	152	154				
Fca023	63	Domestic	0.01	0.13	0.00	0.02	0.59	0.05	0.05*	0.01	0.02	0.07*	0.03	0.02				
	28	Wild	0.42	0.27	0.07*	0.00	0.12	0.03	0.00	0.00	0.02	0.00	0.02	0.05				
Allele size (bp)			130	132	134	138	140	142	144	146	148	150	152	154	156	158	160	162
Fca026	64	Domestic	0.05	0.00	0.00	0.01	0.03	0.00	0.02	0.16	0.09	0.27	0.18	0.07	0.05	0.02	0.04	0.01
	28	Wild	0.04	0.02	0.02	0.00	0.00	0.07*	0.12	0.16	0.18	0.07	0.05	0.14	0.13	0.00	0.00	0.00
Allele size (bp)			118	120	122	124	126	128	130	132	134	138	142	148	150			
Fca043	64	Domestic	0.02	0.02	0.32	0.05	0.06*	0.37	0.08	0.00	0.02	0.01	0.01	0.03	0.01			
	28	Wild	0.07	0.09	0.63	0.05	0.00	0.05	0.04	0.05*	0.00	0.02	0.00	0.00	0.00			
Allele size (bp)			147	151	152	153	154	155	156	157	158	159	160	161	162	164		
Fca045	64	Domestic	0.04	0.02	0.01	0.21	0.08	0.24	0.00	0.01	0.04	0.28	0.01	0.06	0.00	0.01		
	28	Wild	0.03	0.00	0.05	0.07	0.04	0.04	0.05*	0.02	0.23	0.07	0.27	0.09	0.04	0.00		
Allele size (bp)			211	219	221	223	225	227	229	231	233	235						
Fca058	64	Domestic	0.00	0.02	0.01	0.23	0.08	0.04	0.06	0.51	0.02	0.03						
	28	Wild	0.02	0.04	0.05	0.07	0.05	0.11	0.41	0.25	0.00	0.00						
Allele size (bp)			143	145	147	149	151	153	155									
Fca077	61	Domestic	0.01	0.39	0.05	0.22	0.16	0.15	0.02									
	28	Wild	0.00	0.20	0.14	0.13	0.21	0.32	0.00									
Allele size (bp)			111	113	115	117	119	121	123	125	127	129						
Fca088	62	Domestic	0.01	0.04	0.22	0.08	0.17	0.06	0.16	0.24*	0.00	0.02						
	28	Wild	0.00	0.00	0.02	0.20	0.32	0.18	0.26	0.00	0.02	0.00						
Allele size (bp)			185	209	211	213	215	217	219	221	223	225	227	229	231	233	237	
Fca096	61	Domestic	0.02	0.03	0.67	0.03	0.01	0.01	0.10	0.02	0.08	0.00	0.00	0.02	0.00	0.01	0.00	
	28	Wild	0.03	0.02	0.14	0.05	0.07	0.04	0.05	0.20	0.05	0.04	0.05	0.04	0.20*	0.00	0.02	
Allele size (bp)			137	139	141	143	145	147	149	151	153	155	161					
Fca126	64	Domestic	0.00	0.03	0.10	0.38	0.09	0.20*	0.04	0.12	0.02	0.01	0.01					
	27	Wild	0.35*	0.07	0.17	0.20	0.15	0.00	0.00	0.04	0.02	0.00	0.00					
Allele size (bp)			138	140	142	144	146	148	150	152	154	156	158	160				
Fca132	60	Domestic	0.24	0.17	0.03	0.01	0.04	0.01	0.10	0.24	0.03	0.11	0.01	0.01				
	28	Wild	0.04	0.02	0.09	0.11	0.02	0.00	0.12	0.28	0.23	0.09	0.00	0.00				
Allele size (bp)			122	124	128	130	132	134	138									
Fca149	61	Domestic	0.00	0.23	0.33	0.17	0.17	0.10*	0.00									
	28	Wild	0.02	0.02	0.09	0.32	0.42	0.00	0.13*									

* Private alleles ($P \geq 0.05$)

An average $F_{ST} = 0.11$ over all loci revealed a significant genetic differentiation between wild and domestic Portuguese populations ($P < 0.001$; Table 3). Multilocus R_{ST} was also highly significant ($R_{ST} = 0.18$; $P < 0.001$; Table 3). These results reflect distinct gene pools for both groups, differing simultaneously in allele frequencies and sizes, and suggest that new mutations are also contributing to the allelic diversity found in both populations.

The hierarchical AMOVA among different geographical groups revealed a non-significant differentiation between localities (North + Centre vs. South), with 96.33% of genetic diversity explained by interindividual differences within groups ($\Phi_{ST} = 0.04$; $P \leq 0.05$; Table 4). Partition of microsatellites variability between Northern and Southern domestic cats also disclosed a non-significant value ($\Phi_{ST} = 0.02$; $P \leq 0.05$; Table 4). According to R_{ST} statistic, allelic size variation is also not significantly partitioned among wild and domestic cat groups ($R_{ST} = -0.03$ and $R_{ST} = 0.02$, respectively; $P \leq 0.05$). Moreover, Wilcoxon signed rank tests corroborated these results, showing no significant differences in H_E, allelic richness (AR) and allelic diversity (AD) between pairs of geographical sites. These results encouraged the analysis of Portuguese wild and domestic cats as two global clusters, each one comprising all geographically separated individuals from each subspecies.

Bayesian inference of population structure and admixture analyses

We first used STRUCTURE to identify the best performing model for admixture analysis and, as a result, we defined allele frequencies correlated model (*F* model) as the one that better explains the observed

Table 3 Summary of diversity indices for each locus-population combination: allelic diversity (AD), allelic richness (AR), observed (H_O) and expected heterozygosities (H_E) and inbreeding coefficient (F_{IS})

Locus	Domestic cats					Wildcats					F_{ST}	R_{ST}
	AD	AR	H_O	H_E	F_{IS}	AD	AR	H_O	H_E	F_{IS}		
Fca008	11	8.88	0.78	0.83	0.08	7	6.93	0.61	0.68	0.13	0.11	0.12
Fca023	11	9.10	0.54	0.62	0.14	8	7.93	0.61	0.73	0.19	0.22	0.30
Fca026	13	11.10	0.82	0.85	0.03	11	10.93	0.86	0.88	0.04	0.04	0.01
Fca043	12	9.30	0.73	0.74	0.01	8	7.97	0.68	0.59	–0.14	0.12	0.20
Fca045	12	9.22	0.78	0.80	0.04	12	11.97	0.75	0.85	0.13	0.11	0.13
Fca058	9	7.60	0.61	0.67	0.10	8	7.97	0.71	0.75	0.06	0.13	0.00
Fca077	7	6.11	0.77	0.75	–0.02	5	5.00	0.86	0.78	–0.09	0.04	0.06
Fca088	9	8.19	0.52	0.83	0.38*	6	5.93	0.71	0.75	0.07	0.08	–0.02
Fca096	11	8.28	0.42	0.53	0.20	14	13.93	0.96	0.88	–0.08	0.21	0.42
Fca126	10	8.49	0.66	0.78	0.17	7	7.00	0.63	0.78	0.21*	0.11	0.39
Fca132	12	9.58	0.80	0.83	0.04	9	8.93	0.96	0.82	–0.16	0.06	0.13
Fca149	5	5.00	0.77	0.77	–0.01	6	5.93	0.89	0.69	–0.28	0.12	0.25
Average (SE)	10.08	8.40	0.69 (0.13)	0.75 (0.10)	0.09	8.41	8.37	0.77 (0.13)	0.76 (0.09)	0.01	0.11	0.18

F_{ST} (coefficient of genetic differentiation) and R_{ST} (F_{ST} analogue accounting for allelic size variation) estimations between wild and domestic populations are also presented for each locus

SE, standard error

*Significant departures from HWE ($P < 0.0041$; Bonferroni-corrected for 12 independent comparisons)

Table 4 Hierarchical analysis of molecular variance (AMOVA) for wild (North + Centre and South) and domestic cat (North and South) geographical groups computed in ARLEQUIN, using Φ_{ST}

	Source of variation	Variance	% of variation	Φ_{ST}
Wildcat	Among groups	0.169	3.67	0.037 ($P \leq 0.05$)*
	Within groups	4.445	96.33	
	Total	4.614		
Domestic cat	Among groups	0.077	1.91	0.019 ($P \leq 0.05$)*
	Within groups	3.946	98.09	
	Total	4.023		

*P = significance level, after 15,000 permutations

population structure, providing the most accurate assignment of all unequivocally pre-classified domestic cats. This model also provided a better assignment in other European studies (Pierpaoli et al. 2003; Lecis et al. 2006) and is frequently more efficient to detect genetic structure in closely related groups (Pritchard and Wen 2003). Using *F* model without any prior non-genetic information, we inferred the most probable number of genetic clusters (K) presented in the sample by estimating *Ln* posterior probabilities of the data and choosing the smallest value of K that captures the major structure in the data set (Pritchard et al. 2000). Maximum increase in *Ln* posterior probabilities was observed for $K = 2$ and the conversion of likelihood values obtained for all inferred K (1–6) into probabilities following Pritchard and Wen (2003) revealed an approximately 100% probability of having two distinct clusters in the dataset against almost 0.00% for all other values of K. These results suggest that pooled individuals might be subdivided in two genetically discrete populations. With $K = 2$ and using only genetic information, we estimated the average membership proportions (Q) of each predefined group (wild and domestic cats) into both clusters inferred by the program. Results showed a clear partition of both predefined populations, by the separation of two distinct genetic clusters grouping domestic (Cluster I; $Q_I = 0.89$) and wild (Cluster II; $Q_{II} = 0.97$) individuals. Nevertheless, a q value of 0.11 in Cluster II coming from the domestic population predicted domestic genes introgression into wildcat population.

The admixture analysis performed on simulated genotypes was able to efficiently recognise 100% of the parental individuals at a threshold of $qi = 0.80$ (the minimum q_I value was 0.802) and all the F1 hybrids were correctly identified as admixed cats. However, 12 F2 (12%) and 20 backcross (20%) genotypes showed a $qi > 0.80$ to one single cluster and could not be dis-

tinguished from parental individuals. All hybrids detected by simulations revealed very wide 90% CI, ranging between 0.18 and 0.80.

Using STRUCTURE with a cutpoint of 80% and without information on wildcats' prior identification, all domestic cats were grouped in Population I (average membership coefficient of individuals (q_I) = 0.99) and Population II grouped approximately 86% of the pre-classified wildcats (24/28) with average q_{II} = 0.98 and 90% CI between 0.81 and 1.00. Putative wildcats Fs4, Fs7, Fs11 and Fs33 were genetically identified as hybrids, demonstrating cumulative individual $q_{II} < 0.80$ distributed between the two sampled groups (Fig. 2), and with 90% CI in cluster I and II ranging between 0.21 and 0.79 (Table 5). In a second performance of the model using prior morphological identification for all sampled wildcats (USEPOPINFO = 1) and including or not that information for the putative hybrids formerly identified (POPFLAG = 0 or 1), all posterior probabilities were congruent: the four admixed cats also revealed a wild assignment and 90% CI < 0.80, disclosing significant values of domestic ancestry. The ancestral class of individuals whose genotypes indicate a hybrid ancestry can be assessed, either in current or first and second past generations. However, none of these individuals presented posterior probabilities >0.80 for only one of the past hybrid generations. Even though Fs4 presented a considerably superior probability of being an F1 hybrid, his membership proportion was lower than the 80% threshold considered in this study (q_{F1} = 0.72).

Using prior individual non-genetic classification for all domestic cats in NEWHYBRIDS, we obtained a sharp distinction between individual membership proportions of domestic and wild individuals. All domestic cats were probabilistically assigned to the same genotype frequency class, Pure I (average Q = 0.98), while Pure II class grouped approximately 82% of the wildcats (23/28), with an average posterior probability of 0.95. Five phenotypically identified wildcats—Fs4, Fs5, Fs7, Fs11 and Fs33—were only partially assigned to the wild population (individual $Q < 0.80$) and revealed posterior probabilities clearly distributed among different hybrid frequency classes. Similar results were achieved for all but Fs5 when prior non-genetic information was included for all domestic and wildcats or when excluding that information for the five putative hybrids. A detailed analysis of individual membership revealed that none of the genetically admixed cats was assigned to a single hybrid class with $Q > 0.80$, hindering the clear definition of their admixed ancestry (Fig. 3).

The estimation of recent migration rates in BAYEASS revealed a potential introgression of domestic alleles in wildcat population (m = 0.064; SD = 0.027) corresponding to a migration proportion of 4.1 ± 1.73 individuals per generation. A negligible migration of 0.14 ± 0.14 wildcats was detected into the domestic population (m = 0.005; SD = 0.005). According to the probability distributions of individual migrant ancestries in three possible states—non-migrant, migrant or offspring of a migrant and a non-migrant—all domestic cats were correctly assigned to the domestic cluster with posterior probabilities higher then 99%. Among the 28 analysed wildcats, 82% (23/28) were significantly allocated to the wildcat cluster ($P > 0.86$). In agreement with inferences made with Pritchard et al's (2000) and Anderson and Thompson's (2002) approaches, putative wildcats Fs4, Fs7, Fs11 and Fs33 were assigned to both wild and domestic clusters, disclosing a significant posterior probability of being a second gen-

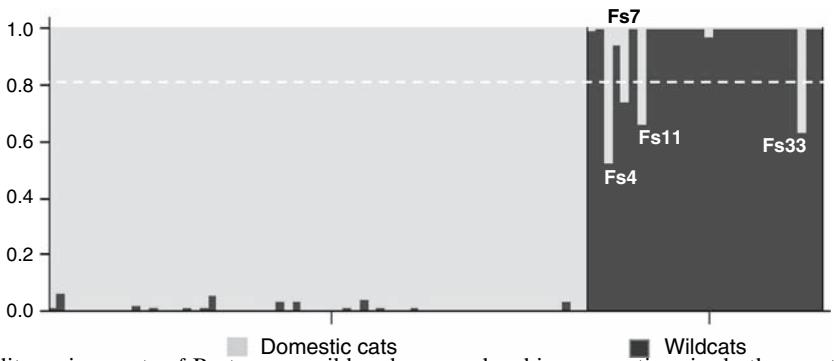

Fig. 2 Posterior probability assignments of Portuguese wild and domestic cats, using prior non-genetic information for all domestic individuals. Each cat is represented by a vertical bar fragmented in K sections of specific length, according to their membership proportion in both genetic clusters inferred by STRUCTURE. Fs4, Fs7, Fs11 and Fs33 revealed a significant ancestry in the domestic cluster and are most likely admixed (horizontal white line = Q threshold)

Table 5 Individual proportions membership (q) of the four putative hybrids Fs4, Fs7, Fs11 and Fs33 using prior non-genetic information for all domestic individuals, both in STRUCTURE and NEWHYBRIDS

Cat	STRUCTURE		NEWHYBRIDS					
	Domestic	Wild	Domestic	Wild	F1	F2	Bx I	Bx II
Fs4	0.526 (0.383–0.765)	0.474 (0.235–0.617)	0.099	0.033	0.678	0.083	0.04	0.067
Fs7	0.303 (0.213–0.591)	0.697 (0.409–0.787)	0.288	0.073	0.224	0.197	0.078	0.140
Fs11	0.392 (0.307–0.603)	0.608 (0.397–0.693)	0.632	0.039	0.070	0.097	0.014	0.149
Fs33	0.366 (0.244–0.729)	0.634 (0.271–0.756)	0.223	0.209	0.165	0.200	0.083	0.119

In STRUCTURE, individuals were assigned into two clusters corresponding to the domestic and wild groups and, in NEWHYBRIDS, into different genotype classes: pure domestic cat, pure wildcat, F1, F2, Bx I (backcross with domestic cat) and Bx II (backcross with wildcat). STRUCTURE 90% credibility intervals (CI) are shown in brackets

eration migrant (0.91, 0.95, 0.87, 0.91, respectively). As observed in NEWHYBRIDS estimates without incorporating prior population information, wildcat Fs5 revealed a significant posterior probability of having hybrid ancestry, however, a consensus analysis of all procedures did not allow this individual to be identified as an admixed cat, considering that five out of seven methodological options performed in this study resulted in its significant wild assignment ($P > 0.87$). A consensus evaluation of all Bayesian analyses consistently identified four individuals with hybrid ancestry among the 28 putative wildcats analysed (Table 5). The admixed cats where collected throughout the sampling area: one in North (Fs33), one in Centre (Fs7) and two in South of Portugal (Fs4 and Fs11).

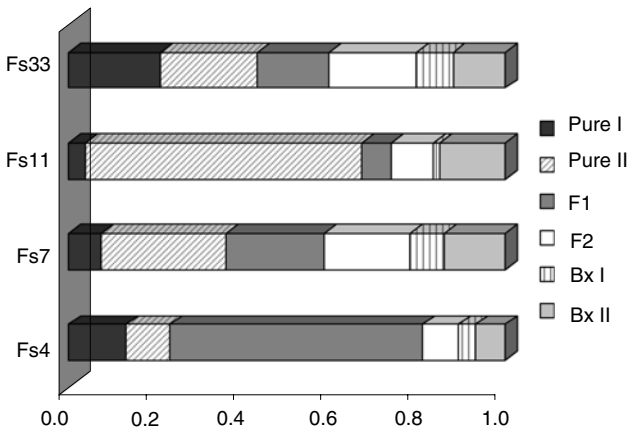

Fig. 3 Posterior probabilities of genotype frequency classes performed in NEWHYBRIDS for the four admixed cats (Fs4, Fs7, Fs11 and Fs33), inferred without including their prior phenotypic information. Each cat corresponds to a horizontal bar divided in six segments representing the probability of each individual into the different genotype classes: Pure I (pure domestic cat), Pure II (pure wildcat), F1, F2, Bx I (backcross with domestic cat) and Bx II (backcross with wildcat)

Discussion

Phenotypic vs. genetic identifications

Six morphologically pre-classified wildcats were identified as domestic according to genetic data, showing some discrepancy between phenotypic and molecular identifications. We know that morphometric identification is often difficult when characters are close to indicative thresholds, especially under the uncertain definition of diagnostic traits and the possibility of resemblance between wild, domestic and hybrid cats due to natural variation (Daniels et al. 1998). Furthermore, some of the analysed samples were found extremely deteriorated in the field disabling a detailed identification and their sympatric location to wildcat populations certainly complicated their classification. Individuals considered to be erroneously pre-identified were collected in protected areas where wildcats inhabit, which confirm an effective overlap between wild and domestic cats in these areas. Considering it is expected that wildlife protection actively occurs in natural parks, control of free-ranging domestic cats should be questioned in these regions. Incongruence between phenotypic and genetic classifications supports the idea that genetic identifications are essential tools in conservation issues, especially in cases where morphological identifications are dubious. Accordingly, one important feature of this work is the construction of reference genetic compositions for Portuguese wild and domestic cats, which, based on genetic clustering comparisons, will allow the future allocation of unidentified samples of this endangered species.

Genetic diversity

Differential allele frequencies, private alleles and significant F_{ST} and R_{ST} values reveal a clear genetic

distinction between Portuguese wild and domestic cats (Table 3). Similarly, high genetic variability disclosed by allelic diversity (AD), allelic richness (AR) and H_E (Table 3) is in concordance with published data in genetically viable wildcat populations, such as Italian, German and Slovenian ones (Randi et al. 2001; Pierpaoli et al. 2003; Lecis et al. 2006). In contrast, genetic diversity observed in this study clearly opposes results obtained in highly admixed populations from Hungary and Scotland (Beaumont et al. 2001; Daniels et al. 2001; Pierpaoli et al. 2003; Lecis et al. 2006). Accordingly, we may infer that Portuguese population of European wildcat maintains its genetic identity, despite some recent introgression of domestic genes.

Analyses of Molecular Variance performed among wild and domestic cat geographical groups suggest the absence of genetic substructure in both subspecies (Table 4), which coincide with the low genetic differentiation observed in domestic populations across Europe ($F_{ST} \approx 3\%$ and $R_{ST} \approx 1\%$; Beaumont et al. 2001; Pierpaoli et al. 2003; Eckert and Hartl 2005). This genetic continuity is certainly related to the anthropogenic character of domestic reproduction, which hinders the definition of isolated and panmictic populations. On the other hand, low genetic divergence between geographically separated wildcats opposes documented values for other European populations, such as German ones, where Western and Eastern populations disclosed a $F_{ST} = 0.19$ (Eckert and Hartl 2005). Although our results should be taken with caution due to the low number of samples from Northern and Central Portugal, they indicate that widely separated Portuguese wildcats might have maintained gene flow in the past. However, the increasing habitat fragmentation and the destruction of important ecological corridors might lead to a considerable geographic isolation and differentiation in the future.

Population structure of wild and domestic populations and admixture analysis

Sample partition obtained using STRUCTURE has an obvious biological sense, since it corresponds to the split of wild and domestic cats in two discrete genetic clusters. Among the 28 putative wildcats analysed in this study, we identified four genetically admixed individuals through a consensus evaluation of all model-based Bayesian approaches and specific methodological options. However, hybrids ancestry remained undisclosed since the global analysis of all clustering methods did not statistically define a single hybrid class assignment for any of the admixed cats. Analysis of the simulation results revealed that the 12 microsatellites used in this study are able to detect 100% of parentals and F1 hybrids using a threshold of 80%, while only 88% of F2 and 80% of backcrosses were detected. These simulated hybrids revealed wide ranges of 90% CI, which are known to occur in admixed genotypes (Pritchard et al. 2000; Barilani et al. 2006). In our population, while most of the 90% CI ranged between 0.80 and 1.00 in wildcats, the four putative hybrids showed values ranging from 0.21 to 0.79, as expected. These findings suggest that our analyses are reliable in the identification of the four admixed cats, but might represent an underestimation of the true number of existing hybrids, since a few F2 and backcross genotypes can remain undetected. The cutpoint of 80% selected in our study is in agreement with previous works focusing on wild and domestic cat hybridisation (Pierpaoli et al. 2003; Lecis et al. 2006). At the same time, high performance has been attributed to this q-value for the accurate detection of purebred and hybrid groups in both STRUCTURE and NEWHYBRIDS, when using 12 loci to study populations with $F_{ST} \approx 0.12$ (for details see Barilani et al. 2006; Vähä and Primer 2006). The uncertainties in the detection of past generations admixture and in the definition of hybrid classes highlight the inherent difficulty to deal with closely related (sub)species and might be explained by the need of a strong genetic differentiation and an increased number and type of loci for the clear allocation of wild × domestic cat hybrids to a single genotypic class (Wilson and Rannala 2003; Lecis et al. 2006). In fact, at least 48 unlinked loci might be needed to detect hybrids beyond first generation, even in cases of clearly divergent parental populations (Vähä and Primer 2006). Even though improving admixture analysis with linked loci did not significantly improve its power in population studies of Italian and Hungarian cats (Lecis et al. 2006), genotyping a large number of unlinked and linked microsatellites, combined with novel molecular markers, may enable better statistical estimates of hybridisation further back in the past (Falush et al. 2003; Lecis et al. 2006). Although we should carefully interpret our results, Fs4 might be an F1 hybrid, even though its association to this class was not statistically supported by the threshold used in this study. Fs7, Fs11 and Fs33 may have a more ancient ancestry in the domestic population. Wild and domestic cat populations revealed asymmetrical migration rates, suggesting only a possible introgression of domestic alleles into the wildcat population ($m = 0.064$) and not a bidirectional gene flow.

Implications for conservation and management

The endangered European wildcat has a central importance in Portuguese wildlife protection, since it might be the only resident wild feline after the probable extinction of reproductive populations of Iberian-lynx (Pires and Fernandes 2003). Accordingly, results of this molecular study should be used as guidelines by Portuguese conservation authorities, in order to effectively preserve and monitor the long-term genetic integrity of wildcat populations. Even though we found no genetic evidence for a constant and generalized gene flow between sympatric populations of wild and domestic cats, at least in most recent generations, admixture analysis revealed a significant proportion of hybrids (around 14%), distributed in all regions analysed, and migration rates documented an effective negative impact on wildcats' genetic composition caused by hybridisation. The extensive geographical distribution of admixed cats reveals domestic introgression clearly not restricted to a particular area, while alerts for a possible decrease in differentiation between Portuguese wild and domestic cats. Accordingly, we suggest that regional and global management strategies should recognize the prevention of crossbreeding between European wildcat and domestic cat as high conservation priority (Randi et al. 2001; Wolf et al. 2001). To avoid the risk of genetic admixture, outbreeding depression, reduced fitness and lowered genetic variability three main actions should be promoted, including: (i) public campaigns to inform authorities and local human populations on Portuguese wildcat status and threats; (ii) the legal control of domestic cats by capturing and neutering free-ranging animals; (iii) and the effective protection of large suitable habitats, mainly preventing the creation of environmental obstacles for wildcat dispersal (Stahl and Artois 1994).

Acknowledgements We thank Armando Loureiro from BTVS-ICN (Wild Animal Tissue Bank, Portuguese Conservation Institute), Pedro Monterroso and Dra Maria Antonieta Ferreira for providing samples. We also thank to all veterinarians and biologists that assisted in sample collection. We are grateful to Massimo Pierpaoli for helpful comments on data analysis. Rita Oliveira is supported by Fundação para a Ciência e a Tecnologia (FCT) through a PhD grant SFRH/BD/24361/2005.

References

Allendorf FW, Leary RF, Spruell P, Wenburg JK (2001) The problems with hybrids: setting conservation guidelines. Trends Ecol Evol 16:613–622

Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160:1217–1229

Barilani M, Sfougaris A, Giannakopoulos A, Mucci N, Tabarroni C, Randi E (2006) Detecting introgressive hybridisation in rock partridge populations (*Alectoris graeca*) in Greece through Bayesian admixture analyses of multilocus genotypes. Conserv Genet. DOI: 10.1007/s10592-006-9174-1

Beaumont M, Barratt EM, Gottelli D, Ktchener AC, Daniels MJ, Pritchard JK, Bruford MW (2001) Genetic diversity and introgression in the Scottish wildcat. Mol Ecol 10:319–336

Belkhir K, Borsout P, Goudet J, Chikhi L, Bonhomme F (1996–2004) Genetix, logiciel sous Windows^{TM} pour la génétique des populations. Laboratoire Génome et Population, CNRS UPR 9060, Université de Montpellier II, Montpellier, France

Daniels MJ, Balharry D, Hirst D, Kitchener AC, Aspinall RJ (1998) Morphological and pelage characteristics of wild living cats in Scotland: implications for defining the 'wildcat'. J Zool 244:231–247

Daniels MJ, Beaumont MA, Johnson PJ, Balharry D, Macdonald DW, Barratt E (2001) Ecology and genetics of wild-living cats in the north-east of Scotland and the implications for the conservation of the wildcat. J Appl Ecol 38:146–161

Eckert I, Hartl GB (2005) Conservation genetics of the European wildcat (Felis silvestris silvestris) in Germany. In: Poster presentation in symposium of biology and conservation of the European wildcat (Felis silvestris silvestris). Germany, January 21st–23rd 2005

Excoffier L, Simouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitocondrial DNA restriction data. Genetics 131:479–491

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Falush D, Stephens M, Pritchard J (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

Fernandes M (1996) Estudo genético do gato-bravo europeu: abordagem ao problema da hibridação. Dissertação de mestrado em Conservação da Diversidade Animal. Faculdade de Ciências da Universidade de Lisboa, Portugal

Ferreira PJ, Pereira I, Marques JT, Grilo C, Baltazar C, Santos-Reis M (2005) Modelling wild cat (Felis silvestris) distribution in a Portuguese Natura 2000 Site (SPA Moura Barrancos, SE Portugal). In: Poster presentation in symposium of biology and conservation of the European wildcat (Felis silvestris silvestris). Germany, January 21st–23rd 2005

Gil-Sánchez JM, Valenzuela G, Sánchez JF (1999) Iberian wild cat Felis silvestris tartessia predation on rabbit Oryctolagus cuniculus: functional response and age selection. Acta Theriol 44:421–428

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). http://www.unil.ch/izea/softwares/fstat.html

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics 48:361–372

Hubbard AL, McOrist S, Jones TW, Boid R, Scott R, Easterbee N (1992) Is survival of European wildcats in Britain threatened by interbreeding with domestic cats? Biol Conserv 61:203–208

Lecis R, Pierpaoli M, Birò S, Szemethy L, Ragni B, Vercillo F, Randi E (2006) Bayesian analyses of admixture in wild and domestic cats (Felis silvestris) using linked microsatellite loci. Mol Ecol 15:119–131

Lozano J, Virgos E, Malo A, Huertas DL, Casanovas JG (2003) Importance of scrub-pastureland mosaics on wildliving cats

occurrence in a Mediterranean area: implications for the conservation of the wildcat (Felis silvestris). Biodivers Conserv 12(5):921–935
McOrist S, Kitchener AC (1996) Current threats to the European wildcat in Scotland. Biol Conserv 76(2):212
Mennoti-Raymond MA, ÓBrien S (1995) Evolutionary conservation of ten microssatellites loci in four species of FELIDAE. J Hered 86:319–322
Menotti-Raymond MA, David VA, Lyons LA et al (1999) A genetic linkage map of microsatellite in the domestic cat (Felis catus). Genomics 57:9–23
Monterroso P, Sarmento P, Ferreras P, Alves PC (2005) Spacial distribution of the European wildcat (Felis silvestris) in Vale do Guadiana Natural Park, South Portugal. In: Poster presentation in symposium of biology and conservation of the European wildcat (Felis silvestris silvestris). Germany, January 21st–23rd 2005
Nielsen EE, Bach LA, Kotlicki P (2006) Hybridlab (version 1.0): a program for generating simulated hybrids from population samples. Mol Ecol Notes 6(4):971–973
Nowell K, Jackson P (1996) The wild cats: status survey and conservation action plan. International Union for Nature Conservation/Cat Specialist Group, Gland, Switzerland
Pierpaoli M, Birò ZS, Herrmann M, Hupe K, Fernandes M, Ragni B, Szemethy L, Randi E (2003) Genetic distinction of wildcat (Felis silvestris) populations in Europe, and hybridization with domestic cats in Hungary. Mol Ecol 12:2585–2598
Pires AE, Fernandes ML (2003) Last lynxes in Portugal? Molecular approaches in a pre-extinction scenario. Conserv Genet 4:525–532
Pritchard J, Wen W (2003) Documentation for structure software: version 2. Department of Human genetics, University of Chicago, USA
Pritchard J, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959
Ragni B (1993) The crucial problem of in vivo identification of wildcat and recognition of hybrids with domestic cats. Institute of Zoology, Peruglia University, Italy
Ragni B, Possenti M (1996) Variability of coat-colour and markings system in Felis silvestris. Ital J Zool 63:285–292
Randi E, Ragni B (1991) Genetic variability and biochemical systematics of domestic and wild cat populations (Felis silvestris: FELIDAE). J Mammol 72(1):79–88
Randi E, Pierpaoli M, Beaumont M, Ragni B (2001) Genetic identification of wild and domestic cats (Felis silvestris) and their hybrids using Bayesian Clustering Methods. Mol Biol Evol 18(9):1679–1693
Raymond M, Rousset F (1995) GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248–249
Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225
Sambrook E, Fritsch F, Maniatis T (1989) Molecular cloning. Cold Spring Harbour Press, Cold Spring Harbour, New York
Sarmento P (1996) Feeding ecology of the European wildcat Felis silvestris in Portugal. Acta Theriol 41:409–414
Schauenberg P (1977) La stature du Chat forestiere Felis silvestris Schreber, 1777, et la variabilité morphologique de l'espèce. Rev Suisse Zool 84:323–337
Stahl P, Artois M (1994) Status and conservation of the wildcat (Felis silvestris) in Europe and around the Mediterranean rim. Convention on the conservation of European wildlife and natural habitats standing committee. Council of Europe Nature and Environment Series 69, pp 1–76
Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462
Vähä JPK, Primmer CR (2006) Detecting hybridization between individuals of closely related populations – a simulation study to assess the efficiency of model-based Bayesian methods to detect hybrid individuals. Mol Ecol 15:63–72
Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370
Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. Genetics 163:1177–1191
Wolf DE, Takebayashi N, Riesberg LH (2001) Predicting the risk of extinction through hybridization. Conserv Biol 15:1039–1053