

# Detecting hybridization between wild species and their domesticated relatives

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## Abstract

The widespread occurrence of free-ranging domestic or feral carnivores (dogs, cats) or ungulates (pigs, goats), and massive releases of captive-reproduced game stocks (galliforms, waterfowl) is raising fear that introgressive hybridization with wild populations might disrupt local adaptations, leading to population decline and loss of biodiversity. Detecting introgression through hybridization is problematic if the parental populations cannot be sampled (unlike in classical stable hybrid zones), or if hybridization is sporadic. However, the use of hypervariable DNA markers (microsatellites) and new statistical methods (Bayesian models), have dramatically improved the assessment of cryptic population structure, admixture analyses and individual assignment testing. In this paper, I summarize results of projects aimed to identify occurrence and extent of introgressive hybridization in European populations of wolves (*Canis lupus*), wildcats (*Felis silvestris*), rock partridges and red-legged partridges (*Alectoris graeca* and *Alectoris rufa*), using genetic methods. Results indicate that introgressive hybridization can be locally pervasive, and that conservation plans should be implemented to preserve the integrity of the gene pools of wild populations. Population genetic methods can be fruitfully used to identify introgressed individuals and hybridizing populations, providing data which allow evaluating risks of outbreeding depression. The diffusion in the wild of invasive feral animals, and massive restocking with captive-reproduced game species, should be carefully controlled to avoid loss of genetic diversity and disruption of local adaptations.

*Keywords:* admixture analysis, *Alectoris* partridges, *Canis lupus*, captive reproduction, domestic animals, *Felis silvestris*, hybridization, invasive and feral organisms, translocations, wildcat, wolf

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## Introduction

In 2006, the *Journal of Applied Ecology* published a paper (Sutherland *et al.* 2006) listing the 100 ecological questions of major concern which are of interest to policy makers in the UK. Those questions included most of the major issues concerning the conservation of biodiversity in environments that are deeply changing by human activities, such as the biological impacts of global climate change, or the consequences of forest management, farming and fisheries on ecosystem structure and functions. Two specific questions pointed out the risks of loss of biodiversity by hybridization between wild and domesticated animals.

The first question (n. 45) suggested to evaluate carefully the manifold effects of widespread invasive organisms, including ferals, on animal populations in rural and urban environments. The second question (n. 87) addressed the risk that the use of nonlocal populations in reintroduction programmes might cause losses of local genetic adaptation or outbreeding depression. The uncontrolled diffusion of feral and translocated organisms might have deeply impacts on the structure of local communities, leading to loss of genetic diversity and eventual local extinctions via introgressive hybridization (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). Introgressive hybridization between translocated or invasive organisms and local wild populations would enhance genetic homogenization, leading to disintegrate those components of genetic diversity which have been generated by divergent adaptation to heterogeneous habitats during the evolutionary process.

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In this paper, I will summarize results of ongoing research projects at the laboratory of genetics of the Italian Wildlife Institute (INFS), that have been designed to develop cost-effective genetic tools aiming to assess rates of introgressive hybridization between wild populations and their domesticated or captive-reproduced relatives. In particular, I will report findings concerning case-studies of intraspecific hybridization between wild and free-ranging populations of wolves (*Canis lupus*) and dogs, and between wild and domestic cats (*Felis silvestris*) in Italy and in other countries in Europe. I will also report an assessment of rates of interspecific hybridization between local wild and captive reproduce stocks (often hybrids) of *Alectoris* partridges, which are massively released in nature to restock over-hunted populations. In these studies, maternal mitochondrial DNA (mtDNA) and biparental microsatellite markers, were used to estimate allele and genotype frequencies in parental reference populations ('pure' wolves, dogs, wild or domestic cats, and three species of partridges). Those reference populations have then been used to identify putative inter- or intraspecific hybrids through principal component analyses (Benzécri 1973), or Bayesian clustering and admixture procedures (Pritchard *et al.* 2000; Falush *et al.* 2003). The power of small numbers of molecular markers to correctly identify hybrids was assessed by simulation (Vähä & Primmer 2006; Barilani *et al.* 2007b). The assessment of introgressive hybridization and the identification of admixed individuals are preliminary steps leading to evaluate (i) the risk of biodiversity losses due to genetic homogenization; (ii) the risk of outbreeding depression in local population in consequences of gene flow between domesticated and wild animals.

### Population genetic approaches for identifying hybrids

In these studies, we used DNA samples, individually stored at  $-20^{\circ}\text{C}$  in 95% ethanol, which were extracted using a guanidinium-silica protocol (Gerloff *et al.* 1995), and analysed by sequencing mtDNA control-region (CR), or genotyping a number of autosomal microsatellite loci that were specifically amplified by polymerase chain reaction (PCR). DNA sequences and microsatellite alleles were analysed in an ABI 3100 automated sequencer, using the programs GENESCAN 3.7 and GENOTYPER 2.1 for microsatellites, and SEQUENCING ANALYSIS 3.7 and NTNAVIGATOR for sequences.

#### Population genetic and multivariate analyses

Phylogenetic trees of mtDNA sequences were obtained using MEGA 2.1 (Kumar *et al.* 2004), with the neighbour-joining procedure (NJ; Saitou & Nei 1987) and Tamura-Nei's (TN93; 1993) genetic distance model, which is

appropriate to describe the evolution of CR sequences. Commonly used summary population genetic statistics and *F*-statistics (Weir & Cockerham 1984) were computed using GENETIX 4.04 (Belkhir *et al.* 2001; [www.univ-montp2.fr/~genetix/genetix.htm](http://www.univ-montp2.fr/~genetix/genetix.htm)). Patterns of differentiation were visualized by factorial correspondence analysis (FCA; Benzécri 1973) of individual multilocus scores computed using GENETIX.

#### Admixture analyses and identification of the hybrids

Maternal hybridization was assessed searching for mtDNA haplotypes discordant with morphologic classifications. Multilocus microsatellite genotypes were analysed using a Bayesian clustering procedure implemented in STRUCTURE (Pritchard *et al.* 2000; <http://pritch.bsd.uchicago.edu>). We used STRUCTURE (with five repetitions of  $10^5$  iterations following a burn-in period of  $10^4$  iterations) to identify the number of genetically distinct clusters that maximize the likelihood of the data, and to assign the individuals to the clusters, using only genetic information. Admixture analyses were performed with the 'admixture' model (each individual may have ancestry in more than one parental population), and, with linked loci, the 'linkage' model in STRUCTURE 2.1 (Falush *et al.* 2003), which accounts for the amount of linkage disequilibrium (LD) arising by admixture. The number of populations  $K$  was set at the value that maximized the increase in the posterior probability of the data  $\ln P(D)$  according to the formula  $[\ln P(D)_k - \ln P(D)_{k-1}]$ , as suggested by Garnier *et al.* (2004). For the selected  $K$  values, we assessed the average proportion of membership ( $Q_i$ ) of the sampled populations to the inferred clusters. Then, we assigned each individual to the inferred clusters, using a threshold  $q_i > 0.80$  or  $q_i > 0.90$  (according to results from simulations procedures; see Barilani *et al.* 2007a) for the assignment of individual genomes to one cluster, or, in the case of admixed individuals, jointly to two or more clusters, if the proportion of membership to each one was  $q_i < 0.80$  or  $q_i < 0.90$ .

The power of admixture analyses to detect  $F_1$ ,  $F_2$  hybrids and first-generation backcrosses, given the number of markers and the level of genetic differentiation between parental populations (estimated by  $F_{ST}$ ), was assessed by simulations. We randomly selected groups of 50 parental genotypes to generate 100 of each  $F_1$ ,  $F_2$  and backcross genotypes with the software HYBRIDLAB (Nielsen *et al.* 2001). Hybrid genotypes are created by random sampling alleles from their frequency distributions in the parental populations, assuming neutrality, linkage equilibrium and random mating. The parental genotypes were selected from populations located as far as possible from known area of hybridization. The simulated genotypes were used to carry out admixture analyses with STRUCTURE 2.1 using

the admixture model, and no prior population information. Results were used to compute the proportion of hybrid individuals that can be correctly identified as hybrids in the simulated data sets.

## Case studies

### *Population expansion, genetic divergence and hybridization between wolves and free-ranging dogs in Italy*

Wolves were presumably widespread almost everywhere in Eurasia throughout the Holocene. Human persecution, deforestation and the decrease of natural prey led wolf populations to strongly decline in Europe during the last centuries (Boitani 2003). Wolves in Italy were confined south of the Po River since the turn of the last century, continuing to decline until the 1970s, when *c.* 100 individuals ranged in two fragmented areas in central-southern Apennine (Zimen & Boitani 1975). Declining demographic trends quickly reversed in the 1980s, when wolves started to expand in Italy and in other European countries, as well. In Italy, wolves crossed the northern Apennine starting to recolonize the southwestern Alps since 1992 in France and 1996 in Switzerland (Fabbri *et al.* 2007). Nowadays, the Italian wolf population is guessed to number more than 600 individuals (Boitani 2003). However, wolves are expanding in presence of large numbers of free-ranging or feral dogs, which are widespread particularly in the central-southern Italian Apennine (Genovesi & Dupré 2000), raising risk of extensive introgressive hybridizations. Thus, the genetics of wolf expansion and colonization needs to be carefully monitored.

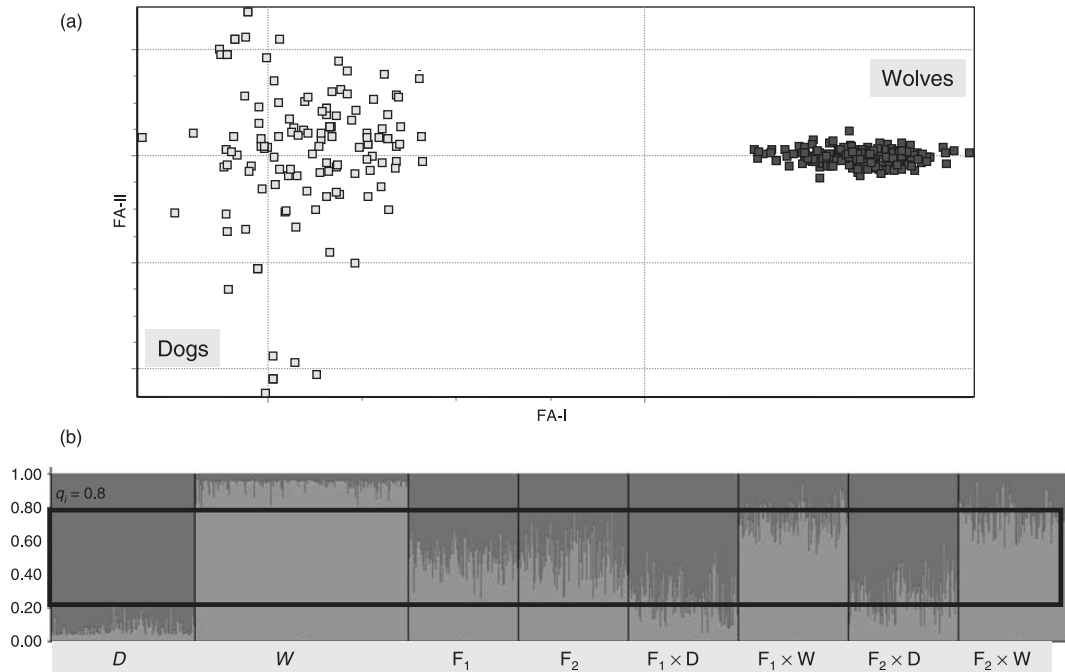
DNA samples were obtained from tissues collected from found-dead, shot or trapped wild-living wolves. Dogs were randomly sampled from different breeds from veterinary practices, and included some free-living animals, which were collected within the range of wolves in Italy. A few known captive-bred hybrid wolves were also included in these studies as reference genotypes for admixture analyses (Randi & Lucchini 2002). Additional wolf genotypes were identified from scat samples using noninvasive genetic procedures (Lucchini *et al.* 2002; Fabbri *et al.* 2007). The diagnostic mtDNA CR haplotype of Italian wolves was identified using laboratory protocols described by Randi *et al.* (2000). All wolves and dogs were genotyped using a panel of 18 unlinked canine microsatellite loci, which were assigned to different chromosomes (Neff *et al.* 1999), as described by Randi & Lucchini (2002), or by 16 microsatellites belonging to four different linkage groups from four different chromosomes (Verardi *et al.* 2006).

Results of genetic analyses showed that wolves in Italy have distinctive genetic traits, with one unique mtDNA

CR haplotype (named W14 by Randi *et al.* 2000), and microsatellite allele frequencies that are sharply different from any other wolf population and dog breed genotyped so far (Randi & Lucchini 2002). FCA and STRUCTURE clustering of microsatellite genotypes indicate sharp distinctions between wolves and dogs (Fig. 1a; average  $F_{ST} = 0.20$ ), which can be assigned to distinct clusters with high  $q_i$  values ( $q_i > 0.96$ ) and small 90% confidence intervals (dogs CI = 0.97–1.00; wolves CI = 0.99–1.00). Admixture analyses performed on unlinked microsatellites led to identify two samples (over 107 presumed wolves = 1.8%) which were jointly assigned to the dog and to the wolf clusters, showing admixed ancestry (Randi & Lucchini 2002). One of these two wolves showed an unusually dark coat ('black wolf'), and another one showed a spur in both hindlegs ('dewclawed wolf'), two phenotypic traits that are usually absent in European wolf populations, and that suggest hybridization. Three additional dewclawed wolves, sampled in Tuscany (central Italy, in 1993–2001) were assigned to the Italian wolf population with lower than usual  $q_i$  values (i.e.  $q_i = 0.95, 0.90$  and  $0.76$ , respectively) and larger 90% confidence intervals (CI = 0.58–1.00, 0.75–1.00 and 0.60–0.92, respectively), providing further evidence of their admixed ancestry (Ciucci *et al.* 2003). Further, 220 presumed Italian wolves, genotyped using 16 microsatellites belonging to four different linkage groups (plus four unlinked microsatellites), and analysed by modelling the gametic disequilibrium arising between linked loci during admixtures (a Bayesian procedure implemented in software STRUCTURE 2.1), indicated that linkage disequilibrium was higher in wolves than in dogs, and that 11 wolves (corresponding to 5.0% of the analysed sample) were likely admixed (Verardi *et al.* 2006). All wolves genotyped in Italy, including the admixed individuals, showed the Italian wolf mtDNA CR haplotype. All the admixed wolves identified so far also showed wolf Y-haplotypes (E. Fabbri, E. Randi, unpublished), indicating that they were not first-generation hybrids, but probably backcrosses. No hybrid wolves were found among 130 distinct wolf genotypes noninvasively identified in the Alps (Fabbri *et al.* 2007). STRUCTURE analyses of simulated genotypes generated by HYBRIDLAB suggested that all  $F_1$  and  $F_2$  genotypes could be detected, but that *c.* 20% of the first-generation backcrosses might be confused with wolf or dog parentals (Fig. 1b).

### *Very different introgressive hybridization rates between wild and free-ranging domestic cats in Italy and in Hungary*

The European wildcat (*Felis silvestris silvestris*), the Sardinian wildcat (*Felis silvestris libyca*) and the domestic cat (*Felis silvestris catus*) are distinct subspecies of the endangered European wildcat *Felis silvestris* (Wozencraft 1993; Randi

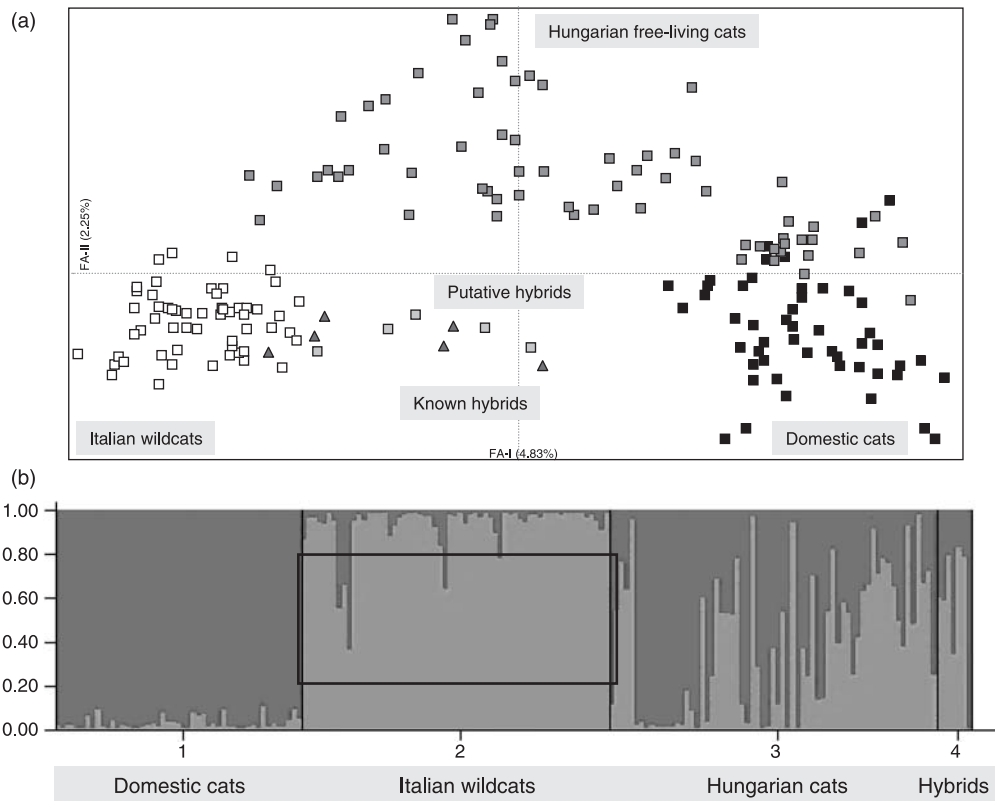


**Fig. 1** (a) Factorial correspondence analysis, computed using GENETIX, showing relationships among the multilocus genotypes of individual Italian wolves and dogs. FA-I and FA-II are the first and second principal factors of variability. (b) STRUCTURE analyses (performed assuming  $k = 2$  distinct genetic clusters) of observed and simulated genotypes that were generated by HYBRIDLAB ( $D$  = observed parental dog genotypes;  $W$  = observed parental wolf genotypes;  $F_1$  and  $F_2$  = first and second generation simulated hybrids;  $F_1 \times D$  = first generation simulated backcrosses with dogs;  $F_1 \times W$  = first generation simulated backcrosses with wolves;  $F_2 \times D$  = second generation simulated backcrosses with dogs;  $F_2 \times W$  = second generation simulated backcrosses with wolves). Admixed genotypes, at threshold  $q_i = 0.80$ , are evidenced.

*et al.* 2001), which is currently distributed in fragmented populations ranging from Russia to Portugal, and from Scotland to the Near East (Nowell & Jackson 1996). During the 18th and 19th centuries, wildcat populations declined, were locally eradicated and went fragmented because of deforestation, trapping and hunting throughout most of central and western Europe. Concomitantly, the anthropogenic diffusion of domestic cats throughout Europe originated widespread free-ranging populations in agricultural ecosystems, raising concerns about the genetic integrity of wildcats (McOrist & Kitchener 1994). The domestication process did not deeply change body size and shape of domestic cats (except for the obvious coat colour mutations that were recently selected by cat breeders), which made it difficult to identify 'pure' wildcat specimens.

DNA samples were obtained from tissues collected from found-dead or trapped wildcats, which were identified morphologically by collectors, mainly using coat colour traits. Domestic cats which were randomly sampled from different breeds and included free-living animals, were collected within the range of wildcats in Italy, and a few known reference captive-bred hybrids (Pierpaoli *et al.* 2003). Cats were genotyped using 12 unlinked microsatel-

lites (Menotti-Raymond *et al.* 1999), or 21 linked microsatellites, mapping in the domestic cat genetic linkage groups A1, A3, B1, C2 and D3 (Menotti-Raymond *et al.* 2003), and analysed using protocols described by Randi *et al.* (2001). A survey of genetic variation in 336 cats, that were sampled from nine European countries and genotyped using 12 unlinked microsatellite loci, indicated sharp genetic distinctions among phenotypically identified European wildcats, Sardinian wildcats and domestic cats. The average  $F_{ST} = 0.11$  and  $R_{ST} = 0.41$  among those taxonomic groups were significant ( $P < 0.001$ ), indicating that wild and domestic cats are subdivided into distinct gene pools in most of European countries (Randi *et al.* 2001; Pierpaoli *et al.* 2003). Admixture analyses identified some cryptic hybrids among wildcats in Italy ( $n = 2/64 = 3\%$ ), Bulgaria ( $1/6 = 17\%$ ) and Belgium ( $1/19 = 5\%$ ). Additional research revealed the presence of  $4/34 = 14\%$  hybrid wildcats in Portugal (Oliveira *et al.* 2007). However, much more extensive hybridization was detected between wild and domestic cats sampled in Hungary (Fig. 2; Pierpaoli *et al.* 2003). Cats in Hungary include a composite assemblage of variable phenotypes and genotypes, which, as it was documented also in Scotland (Beaumont *et al.* 2001), might



**Fig. 2** (a) Factorial correspondence analysis showing relationships among the multilocus genotypes of individual Italian wildcats, free-ranging Hungarian cats, domestic cats and putative or known hybrids. FA-I and FA-II are the first and second principal factors of variability. (b) STRUCTURE analyses (performed assuming  $k = 2$  distinct genetic clusters) of multilocus microsatellite genotypes of domestic cats, Italian wildcats, Hungarian free-ranging cats and captive-reproduced hybrids. Admixed genotypes, at threshold  $q_i = 0.80$ , are evidenced.

originate from long-lasting introgressive hybridization. Genetic admixture analyses in 182 European wild and domestic cats genotyped at 27 microsatellites, including 21 linked loci mapping on five distinct feline linkage groups and analysed with STRUCTURE 2.1, showed that domestic and wild cats sampled in Italy were split into two distinct clusters with average  $Q > 0.90$ , congruent with prior morphological identifications (Lecis *et al.* 2006). In contrast, free-living cats sampled in Hungary were assigned partly to the domestic and the wild cat clusters, with average  $Q < 0.50$ . Admixture analyses of individual genotypes identified  $5/61 = 8\%$  hybrids among the Italian wildcats, and  $16-20/65 = 25\%-31\%$  (according to different admixture thresholds) hybrids among the Hungarian free-living cats. The five Italian admixed wildcats were sampled in peripheral areas, thus suggesting that hybridization in Italy might be more frequent at geographical and ecological edges of the wildcat distribution. STRUCTURE analyses of simulated genotypes generated by HYBRIDLAB indicated that also in cats, 100% of the  $F_1$  and  $F_2$  genotypes could be detected, but that *c.* 20% of the first generation backcrosses might be confused with their parents.

#### *Hybridization between wild and released captive-reproduced Alectoris partridges*

The *Alectoris* partridges include seven closely related interfertile species distributed in Eurasia, China and southern Arabia (Johnsgard 1988), which can hybridize naturally in parapatric contact zones in the southern French Alps, in Thrace (Greece) and in central China (Barilani *et al.* 2007a). A red-legged (*A. rufa*)  $\times$  rock partridge (*A. graeca*) natural hybrid population, distributed over a narrow overlapping area along the southern edge of the French Alps, showed shorter than expected introgression of red-legged allozyme, microsatellite and mtDNA markers in Alpine rock partridge populations distributed up to *c.* 150 km from the contact zone (Randi & Bernard-Laurent 1998, 1999; Barilani *et al.* 2007a), suggesting that the diffusion of hybrid partridges in nature could be contrasted by outbreeding depression. Red-legged partridges and rock partridges are hunted and restocked throughout the entire species range in Iberia, France and Italy. Most of their natural populations declined in the second part of the last century because of habitat changes and over-hunting. Population

declines have been contrasted by massive releasing of captive-reproduced partridges, often using chukars (*A. chukar*) or hybrids with chukars. Published reports indicate that chukar mtDNA haplotypes are widespread in most of the red-legged partridge populations studied so far (Negro *et al.* 2001; Baratti *et al.* 2004; Barbanera *et al.* 2005). Released interspecific hybrids raise the risks of introgressive hybridization, and can contribute to further depress the fitness of native populations. Thus, the genetic integrity of red-legged and rock partridges is at risk of widespread introgressive hybridization.

Partridges were collected from nine geographical regions across the native distribution range of the red-legged partridge, rock partridge and chukar (see details in Barilani *et al.* 2007a, 2007b). Samples were assigned to one of the three *Alectoris* species, or were identified as hybrids, based on diagnostic morphological traits and geographical origin (Bernard-Laurent 1984; Johnsgard 1988). Results of genetic analyses in 671 red-legged partridges (collected in Iberia, France and Italy), rock partridges (collected in France and Italy), and chukar partridges and natural hybrids, genotyped at the mtDNA CR (Randi & Lucchini 1998) and eight nuclear microsatellites, revealed diffuse occurrences of hybridization (Barilani *et al.* 2007a, 2007b). Thirty-nine samples (6.2%) showed mtDNA haplotypes discordant with their phenotypes, indicating chukar mtDNA introgression in native red-legged and rock partridges collected in France and Italy. Admixture analyses of multilocus microsatellite genotypes further identified 32 additional rock partridges in France and Italy (5.1%) that were hybridized mainly with chukars. Moreover, 39 samples collected from the presumed natural red-legged  $\times$  rock partridge hybrid zone in the French Alps, surprisingly showed 28% birds with typical chukar mtDNA, indicating hybridization with introduced chukars or hybrids. Mitochondrial DNA CR sequences and Bayesian admixture analyses of multilocus microsatellite genotypes in 319 wild rock partridges collected in Greece, led to identify from four to 28 putative hybrids (according to different assignment criteria; Barilani *et al.* 2007b), corresponding to 1.2–8.8% of the samples. The hybrids were widespread throughout all the country. Power of admixture analyses, assessed using simulated hybrid genotypes (HYBRIDLAB), revealed that, despite very high  $F_{ST}$  values around 0.50, a small number of markers can detect all first and second generation hybrids ( $F_1$  and  $F_2$ ), but only up to 90% of the first generation backcrosses. Thus, the true proportion of recently introgressed rock partridges in Greece has been underestimated. These findings indicate that introgressive hybridization is widespread in *Alectoris*, and that released captive-bred partridges can reproduce and hybridize in nature, polluting the gene pool of wild red-legged and rock partridge populations throughout the entire species' ranges in Europe.

## Discussion

Introgressive hybridization among local wild populations and translocated or invasive organisms, together with habitat degradation and loss of ecological structure, unsustainable selective pressures for adaptation to global climate changes, overexploitation and loss of community structure, are acknowledged as the future emerging major threats to biodiversity preservation (various authors in this volume). Invasive species and translocated populations are threatening native populations by hybridization, raising risks of genetic extinction, loss of local adaptations or outbreeding depression (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). Research showed in this study indicates that introgressive hybridization can be locally pervasive in populations of carnivores (wolves and wildcats) and galliforms (*Alectoris* partridge), and that conservation plans should be enforced to preserve the integrity of their gene pools.

After centuries of human persecution and worldwide demographic declines, wolves are now expanding in several European countries and in North America. In some European countries, wolves are expanding in the presence of large numbers of free-ranging or feral dogs. Although population genetic studies did not reveal large-scale introgression of dog genes in European wolf populations, wolf  $\times$  dog hybrids have been detected in several countries in Europe (Vilà & Wayne 1999; Randi *et al.* 2000; Andersone *et al.* 2002; Randi & Lucchini 2002; Vilà *et al.* 2003). Moreover, the presence of anomalous morphological characters (i.e. black coat colour or dewclaws), have been observed in some wolves in Italy. Vestigial first toes (dewclaws) on the hind legs are common in some dog breeds, but were never detected in wolves. Black wolves are widespread in some North America populations, but they were never been observed in Europe. Both these traits could have been introduced in the Italian wolf population via hybridization with free-ranging domestic dogs.

Interestingly, but not unexpectedly, the admixed wolves were mostly confined to peripheral areas of the species' distribution range, sometime in areas of recent colonization, were wolves being rarer are more likely to crossbred with free-ranging dogs. Genetic data showed that, despite occasional hybridization, wolf and dogs remain genetically distinct, suggesting that introgression in nature might be strongly counteracted by selection or by ethological factors (Randi & Lucchini 2002). Often, but not always, admixed wolves showed morphological signals of hybridization. Therefore, both morphological and molecular traits could be used to identify wolf  $\times$  dog admixed individuals. Population assignment and genetic admixture analysis of genotypes identified using a limited number (18 loci) of microsatellites markers, might allow to identify  $F_1$  and  $F_2$  hybrids, and a majority of first or second generation backcrosses. These procedure is cost-effective, and could

be applied in long-term monitoring projects aimed to detect and limit the diffusion of hybrid wolves. However, the identification of past generation backcrosses need the use of many more markers.

The empirical evidence available so far suggests that wild and domestic cats hybridize sporadically in some European countries (i.e. Spain, France, Germany and Italy), while they are known to hybridize extensively in other parts of their distribution range, as in Scotland or in Hungary (Beaumont *et al.* 2001; Pierpaoli *et al.* 2003). Despite the widespread occurrence of free-ranging cats, wild and domestic cat populations are reproductively isolated in Italy. In contrast, wild and domestic cats in Hungary are deeply introgressed, suggesting that hybridization is ongoing, or that it has been frequent in the near past. We do not know what peculiar historical or ecological factors might have differentially affected the population genetic structure of wild-living cats in Scotland, Hungary, and in other regions in Europe. Declining demographic trends in wildcat populations, arising from the consequences of deforestation and human persecution, and the concomitant widespread diffusion of domestic cats, could have led to extensive waves of hybridization and deep introgression in the past. Alternatively, wild  $\times$  domestic cat hybrids could be fitted and survive as well as their parentals in particular landscapes, such as those present in countries with predominant traditional agriculture. For instance, the northeastern regions of Hungary are characterized by mosaics of forests patches and traditional agriculture with human settlements, a kind of landscape where encounters between free-ranging domestic cats and wildcats could be facilitated. Integrative eco-ethological and population genetic research could perhaps elucidate the origin and dynamics of introgression in wildcat populations, suggesting wise conservation strategies for this species.

Worldwide translocations of nonindigenous populations are threatening a number of species of game birds, in particular anseriforms (such as the New Zealand grey duck, *Anas superciliosa superciliosa*; Rhymer *et al.* 1994) and galliforms (such as the Italian subspecies of grey partridge, *Perdix perdix italica*; Liukkonen-Attila *et al.* 2002; wild populations of the common quail, *Coturnix c. coturnix*, threatened by restocking with domesticated Japanese quail, *Coturnix c. japonica*; Barilani *et al.* 2005). Our studies indicate that hybridization with chukars is widespread across the entire distribution range of the red-legged partridge and the rock partridge, suggesting that released captive-reproduced partridges can mate in nature, eventually hybridizing with local wild populations, and that interspecific hybrids are fertile. However, genetic data suggest also that, for instance, survival and diffusion of natural hybrids in Alpine habitats is constrained by natural selection, perhaps as consequences of differential selective and/or competitive pressures between hybrids and

their parental populations. Randi & Bernard-Laurent (1999) estimated that neutral clines generated after postglacial secondary contact between red-legged and rock partridges in the Alps should be about  $2 \times 10^3$  km wide, that is much wider than the observed clines of *c.*  $10^2$  km. If hybrids are unfit and their survival and dispersal are constrained by natural selection, we expect also negative impacts from the restocking of natural populations. Introgressive hybridization may disrupt local adaptations in natural red-legged partridge and rock partridge populations. Consequently, farming and restocking of these (and other) game species should be strictly controlled.

The small number of genetic markers used in these three research projects showed a limited power of hybrid detection after the first generation of backcrossing. STRUCTURE analyses of simulated genotypes in wolves, wildcats and partridges, concordantly revealed that less than 20 unlinked microsatellite loci underestimates the proportion of first backcrosses of *c.* 10% to 20%. Consequently, such a small number of markers would severely underestimate the proportion of past generation backcrosses. These limitations can, in principle, be overcome by increasing the number of microsatellite, or other markers [i.e. SNP (single nucleotide polymorphism); Morin *et al.* 2004]. Detecting past generation hybrids when parental allele frequencies are not fixed, or unknown, would require the use of more than 50–100 unlinked markers, which might be impractical. It is therefore important to define explicitly the assignment thresholds and rules, and assess the uncertainty of admixture analyses. A comparative evaluation of results obtained in the three case-studies discussed in this paper allows deducing the following guidelines for hybrid detection in wild populations: (i) the power of DNA markers in detecting  $F_1$ ,  $F_2$  and first generation backcrosses should be assessed by simulations; (ii) large numbers of unlinked microsatellite loci (> 20) should be typed to increase the probability to detect past generation backcrosses; (iii) mtDNA typing can be used to integrate biparental markers, also because discordant mtDNA haplotypes can detect past (maternal) introgression events; (iv) simulations and the use of reference known pure or hybrid genotypes should be used to estimate the range of variation of confidence interval (CI) of individual  $q_i$  values, which are expected to be larger in admixed individuals; (v) simulations and reference genotypes should be used also to set the thresholds of individual  $q_i$  values (i.e. 0.80 or 0.90) to identify admixed individuals; (vi) the advantages of linked loci in species where linkage maps are available should be carefully explored, because modelling the 'admixture linkage disequilibrium' promises more careful and deep detection of older admixture events (Falush *et al.* 2003), as suggested, for instance, by our recent research on hybridizing wildcats (Lecis *et al.* 2006), and wolves (Verardi *et al.* 2006).

## Conclusions

The uncontrolled diffusion of free-ranging dogs and cats, and the massive restocking of over-hunted game species with captive-reproduced partridge, is leading to introgressive hybridization of local natural populations in several European countries. In practical conservation, there is more than enough technical information available to control the diffusion of ferals and limit the restocking of game species. However, political pressures of stakeholders (i.e. hunters), and a number of legislative constraints (i.e. protection of domestic animals), might actually severely limit concrete actions. Thus, genetic monitoring and the dissemination of relevant empirical data should support conservation actions, which should include the implementation of officially accepted analytical protocols, aimed to identify genetically pure or hybrid populations (both in nature and in captivity), using DNA markers and admixture analyses. Hybridization assessment programmes could be used (i) to map the distribution of nonintrogressed natural populations, and support their conservation in the wild; (ii) to enforce strict controls of the genetic status of game stocks reproduced in captivity and used for restocking. These actions should help in preserving the genetic integrity of natural populations of carnivores and partridges in Europe.

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This study is part of a long-term project on population and conservation genetics of wolves, wildcats and partridges in Europe. E.R. is head of conservation biology and genetics at INFS.

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