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MtDNA genetic diversity and population history of a dwindling raptorial bird, the red kite (*Milvus milvus*)

S. Roques [a,*], Juan J. Negro [b]

[a] *Laboratory of Molecular Ecology, Estación Biológica Doñana, CSIC, Pabellón del Perú, Avda. María Luisa, s/n, 41013 Sevilla, Spain*
[b] *Department of Applied Biology, Estación Biológica Doñana, CSIC, Pabellón del Perú, Avda. María Luisa, s/n, 41013 Sevilla, Spain*

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Abstract

The red kite (*Milvus milvus*) occurs in a relatively small area in the southwestern Palearctic region, with population strongholds in Central Europe. Following strong human persecutions at the beginning of the 20th century, populations have receded, particularly in peripheral areas and islands. In order to describe and compare levels of genetic diversity and phylogeographic patterns throughout its entire distribution in Europe, sequence variation of a 357 bps part of the mitochondrial DNA control region was assessed in eight populations and 105 individuals. Overall, results indicate that population declines have affected red kite mtDNA variation. We found low levels of genetic diversity (values of nucleotide diversity ranging from 0 in Majorca island to 0.0062 in Central Europe), with only 10 distinct haplotypes, separated by low levels of genetic divergence (mean sequence divergence = 0.75%). Highest haplotype and nucleotide diversities match with demographic expectations, and were found in Central European and Central Spanish samples, where present strongholds occur, and lowest values in the declining southern Spanish and insular samples. Φst estimates indicated moderate gene flow between populations. Phylogeographic patterns and mismatch distributions analyses suggest central European regions may have been colonized from southern glacial refugia (in the Italian or Iberian peninsulas). Interspecific phylogenetic comparisons and divergence date estimates indicated the genetic split between the red kite and its closely related species, the black kite (*Milvus migrans*), might be relatively recent. The low level of genetic variation found in the red kite mitochondrial control region, compared to the black kite, is likely the result of relatively recent divergence (associated with founder events), successive bottlenecks and small population sizes. As there are several ongoing projects aimed at reinforcing populations in countries such as the United Kingdom, Italy or Spain, our results may prove useful for the genetic management of the species.

Keywords: *Milvus milvus*; Kite; Mitochondrial DNA; Genetic diversity; Phylogeography; Europe

1. Introduction

Birds of prey are prone to extinction, as most have traditionally been persecuted by humans. In addition, those living in agricultural landscapes may suffer from habitat destruction, population fragmentation and poisoning (Donázar et al., 2002). Among the main direct genetic consequences of population crashes are the loss of genetic diversity, population structuring, suppression of gene flow and inbreeding (Daniels and Walters, 2000; Groombridge et al., 2000; review in Frankham et al., 2002; Alonso et al., 2003). Quantifying and characterising avian genetic diversity should be a priority in conservation, to evaluate the effects of recent drastic population changes, to preserve present-day diversity and, eventually, to provide guidelines for future conservation plans (Negro and Torres, 1999).

Distributed in the southwestern Palearctic region, the red kite (*Milvus milvus*) has one of the smallest ranges amongst all Eurasian birds of prey, with almost its entire

* Corresponding author. Tel.: +34 954232340; fax: +34 954621125.
E-mail address: severine@ebd.csic.es (S. Roques).

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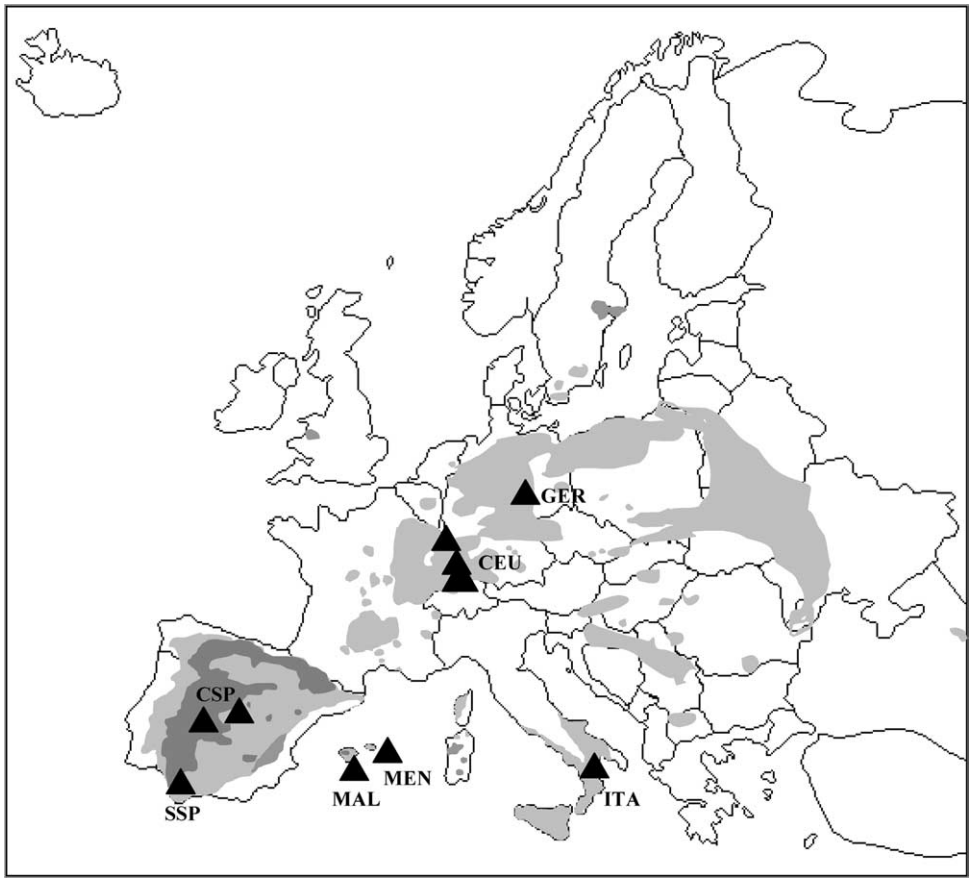

Fig. 1. Breeding distribution of the red kite, *Milvus milvus*, and sampling sites (▲). Light shade: Cramp and Simmons (1980); bold shade: only for Spain, reflecting recent range contraction, Viñuela et al., 1999). Sample codes: CSP (Central Spain: Caceres, Madrid), SSP (southern Spain: Doñana National Park), MAL (Majorca), MEN (Minorca), ITA (southern Italy, Italia Basilicata), GER (Germany, Häkel Forest), CEU (Central Europe: Switzerland, France, Luxemburg).

breeding population confined to Central Europe (Fig. 1). The species has declined drastically in most of its breeding range during the 20th century, mainly due to human pressure and changes in land use (Cramp and Simmons, 1980; Evans and Pienkowski, 1991). This resulted in the species becoming extinct in several countries (e.g., Canary Islands) following a marked long-term decrease in range and numbers (Evans and Pienkowski, 1991). Thus, remnant populations in western Asia and North Africa are virtually extinct, while populations in southern and eastern Europe are also declining and fragmented. It is only in northwestern and central Europe that numbers are stable or recovering. Recent and ongoing reintroduction programmes are carried out in Wales, Scotland, Italy and the Balearic islands, each with less than 100 wild pairs left, while in other parts of its distribution, management strategies still need to be implemented. Rough population estimates are available throughout the distribution range of the red kite, but we still lack precise data and information on present population trends in most regions (but see Evans and Pienkowski, 1991; Viñuela et al., 1999). Also, only a geographically restricted study in Wales has documented the genetic consequences, i.e., inbreeding and low variability – of a population bottleneck in this species (May et al., 1993).

Here, we will examine the patterns of mitochondrial control region sequence diversity in several geographic populations of the red kite in order to describe the species genetic variability and to infer relationships between populations, phylogeography, evolutionary patterns and past changes in population size. Diversity values will be compared with those obtained from a black kite (*Milvus migrans*) population in southern Spain. The latter is closely related to the red kite (Schreiber et al., 2000), whose distribution range is actually contained within the much larger breeding range of the black kite. At present, black kite populations seem to be very successful and may even be expanding. Such distributional and demographic differences lead us to predict a higher genetic variability in black kites. Given the availability of demographic information in some regions of the distribution area of the red kite, we will test the hypothesis that recent successive bottlenecks in the 20th century may have significantly reduced its genetic variability. In addition, we will discuss levels of gene flow and

phylogeographic patterns in relation to present day versus historical factors. Interspecific phylogenetic comparisons and divergence date estimations between both kite species may depict interesting patterns of their evolutionary history. These results will also be examined for conservation purposes, to provide appropriate genetic management for the species.

2. Methods

2.1. Sample collection and DNA extraction

Red kite samples ($n = 105$) were collected throughout the species' distribution range, including continental Spain, the Balearic islands, Germany, Italy, Switzerland, Luxemburg, and France. For population genetic analysis, samples were assigned to groups according to their geographic origin (Fig. 1). Samples ($n = 23$) of the population of black kite from Doñana National Park (southern Spain) were also collected, preserved and processed for comparative analyses using the same procedures. Blood and tissue samples were stored in 100% ethanol. Genomic DNA was extracted from blood stored in lysis buffer (Seutin et al., 1991) using a Lithium Chloride method adapted from Gemmell and Akiyama (1996). DNA from feather samples (Switzerland, France, Luxembourg) was extracted with DNeasy Tissue Kits (Qiagen) following manufacturer instructions, with previous washes in excess NTE (0.05 M Tris–HCl, 0.01 M NaCl, 0.02 M EDTA, pH 9.0) removing possible protease or PCR inhibitors (Hall et al., 1997).

2.2. PCR and sequencing

A large fragment (approximately 600 bps) of the red kite mitochondrial DNA control region was PCR amplified in a few samples using the external primers, tThrF and Dbox-R (Roques et al., 2004). Amplifications of a 357 bps fragment located in the left side of the domain I and including most of the polymorphic sites were performed in all the samples using the newly designed primers (program OLIGO 6.0, LifeScience Software Resource), Mil1/F (5′-ATAATGCACTATCATGGG-3′) and Mil2/R (5′-GGGCAAAGAATGGTC-3′). PCR was performed in 20 μl reaction volumes containing 1.25 mM $MgCl_2$, 0.25 mM dNTPs, 0.75 μM of each primer, 0.5 U Taq polymerase and approximately 10–50 ng of total DNA. Amplification reactions were performed in a MJ Research thermocycler, programmed for an initial denaturation cycle of 94 °C for 2 min, 31 cycles at 92 °C for 30 s, at 52 °C for 30 s and at 72 °C for 30 s. A final extension step at 72 °C for 5 min was performed. PCR products were cleaned through Microcon-PCR (Millipore Corp.) and sequenced on an automated DNA sequencer (ABI-310, Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit version 2.0 (Applied Biosystems, Inc.) following the manufacturer instructions. Both primers Mil1/F and Mil2/R were used for the sequencing reaction. Sequences were aligned using the program Sequencer™ 4.1.2 (Gene Codes Corporation) and improved manually.

2.3. MtDNA sequence analysis and phylogeny

Average and population haplotype diversity (h), nucleotide diversity (π), and mean number of pairwise sequence differences (D_{xy}) were calculated using DnaSP version 4.0 (Rozas and Rozas, 1999). The model of DNA substitution that best fitted the data was selected with the program MODELTEST, v. 3.06 (Posada and Crandall, 1998). The models HKY and Tr + N were selected by the hierarchical likelihood ratio test and the Akaike Information Index criterion, respectively. Phylogenetic relationships among the red and black kite haplotypes were analysed by distance methods in PAUP 4.0 (Swofford, 2002) assuming the models of evolution selected by MODELTEST. Neighbour-joining and Bootstrap analysis (1000 replicates) were used to build the phylogenetic tree. We also conducted a median-joining network (MJN) approach (Bandelt et al., 1999) to depict the relationships among the red kite haplotypes. This approach has proved to yield the best genealogies among other rooting and network procedures (Cassens et al., 2003) and it is also more convenient to represent relationships among closely related sequences. The median-joining network was estimated using the software NETWORK v. 3.1.1.1 (http://www.fluxus-engineering.com).

2.4. Demographic history

Signatures of population demographic changes (e.g., bottlenecks or expansions) in the red kite were examined by using two different approaches. First, the demographic history was investigated by comparing mismatch distributions in each geographic sample with those expected in stationary and expanding populations using DnaSP. The shape of the mismatch distribution of pairwise differences is usually multimodal in samples drawn from populations at demographic equilibrium, whereas a unimodal distribution is generally found in populations having passed through a recent demographic expansion (Rogers and Harpending, 1992; Harpending et al., 1998). It is important to note, however, that such a wave in the distribution could also be generated by a bottleneck, and that distinguishing between both demographic events remains difficult (Rogers and Harpending, 1992). The overall validity of the estimated demographic model is tested by obtaining the distribution of a test statistic SSD (the sum of squared differences) between the observed and the estimated mismatch distribution. A significant SSD value is taken

Table 1
Variable sites, numbers and frequency (*in italic*) of the 10 mtDNA control region haplotypes (H) found in the red kite in eight geographic samples

Position (in bps)											Samples (frequencies)							
	56	75	117	121	160	164	174	175	253	255	**CSP**	**SSP**	**MAL**	**MEN**	**ITA**	**GER**	**CEU**	**TOT**
H1	C	T	C	G	T	C	A	A	A	T	3 (*0.375*)	7 (*0.318*)	19 (*1*)	8 (*0.471*)	6 (*0.6*)	14 (*0.583*)	2 (*0.4*)	59 (*0.562*)
H2	.....	C	.....	.....	.....	.....	.....	.....	.....	.....	2 (*0.250*)	14 (*0.636*)		9 (*0.529*)	1 (*0.1*)	1 (*0.042*)		27 (*0.257*)
H3	.....	.....	.....	.....	.....	.....	.....	G	.....	.....	1 (*0.125*)	1 (*0.0454*)						2 (*0.019*)
H4	T	.....	.....	.....	.....	.....	.....	.....	.....	.....	1 (*0.125*)							1 (*0.0095*)
H5	.....	.....	.....	.....	.....	.....	.....	G	C	.....	1 (*0.125*)							1 (*0.0095*)
H6	.....	.....	C	.....	.....	.....	.....	.....	.....	.....					1 (*0.1*)			1 (*0.0095*)
H7	T	.....	C	.....	.....	.....	.....	.....	.....	.....					1 (*0.1*)			1 (*0.0095*)
H8	T	.....	T	.....	G	.....	.....	.....	.....	.....					1 (*0.1*)			1 (*0.0095*)
H9	T	.....	.....	.....	T	G	.....	.....	.....	.....						8 (*0.333*)	2 (*0.4*)	10 (*0.095*)
H10	.....	.....	A	.....	.....	.....	.....	.....	.....	.....						1 (*0.042*)	1 (*0.2*)	2 (*0.019*)

Samples codes are available in Fig. 1.

as evidence for departure from the estimated demographic model of sudden population expansion. Also, Tajima's D (Tajima, 1989) and Fu's F_S (Fu and Li, 1993; Fu, 1997) statistics were used to test whether CR data conform to expectations of neutrality, considering that departures from neutrality could also be due to factors other than selective effects, such as population bottleneck, expansion, or heterogeneity of mutation rates (see Aris-Brosou and Excoffier, 1996). F_S differences were tested for significance with a coalescent simulation program (1000 simulations), as implemented in the computer program ARLEQUIN version 2.000 (Schneider et al., 2000). Also, the parameter theta (τ) was estimated by maximum likelihood with the program FLUCTUATE 1.3 (Kuhner et al., 1995; Kuhner et al., 1998).

2.5. Population structure and gene flow

The partition of genetic diversity among and within population was studied by analysis of molecular variance (AMOVA) (Excoffier et al., 1992) using Φ estimators (Wright, 1965) as implemented in ARLEQUIN. Φst values between pairs of populations were obtained with and without the consideration of the molecular distance (Tamura–Nei) among haplotypes. Permutation tests (Raymond and Rousset, 1995) were also conducted to test for sample differentiation.

3. Results

3.1. Mitochondrial control region variability

Among the 105 red kite samples, 10 haplotypes were defined by 10 polymorphic sites (Table 1). As most haplotypes differ by only one or two mutations, the mean sequence divergence between haplotypes was low (0.75%) with a maximum of 1.41% (between H05 and either H08 or H09). Also, no transversion was found, indicating a recent polymorphism in the red kite. Some haplotypes were restricted to unique geographical populations: three haplotypes were sampled only in red kites from Italy and two in red kites from central Spain. Two haplotypes, H9 and H10, were restricted to central European samples GER and CEU, where they had relatively high frequencies (see Table 1). The two most common haplotypes H01 and H02 were shared by the majority of populations (86% of the samples) and H01 was fixed in MAL sample. Estimates of haplotype and nucleotide diversities for each group are shown in Table 2. Haplotype diversities vary across red kite populations (average $h = 0.61$ in the total sample), with the lowest values found in the southern Spanish and insular populations (SSP, MAL and MEN). Low nucleotide diversity (π) was found (0.32% from all individuals), but particularly at the periphery of its distribution (SSP, MAL, MEN) (see Table 2). Highest

Table 2
Gene diversity (h), nucleotide diversity (π), Tajima's (D), Fu's (F_S) and mistmatch distribution tests (τ, SSD), demographic data (number of breeding pairs, N_b and present status) in eight red kite samples

Sites	n	h	π	SSD (P value)	τ	D (P value)	F_S (P value)	No. of breeding pairs	Status
SSP	21	0.515 (0.081)	0.0016 (0.0015)	0.0151 (0.100)[a]	0.730	0.111 (0)	0.126 (0.414)	50–60	Declining
CSP	8	0.905 (0.107)	0.0045 (0.0030)	0.0166 (0.519)[a]	1.881	−1.02 (0.17)	−2.01 (0.030)y	400–500	Increasing
MAL	20	0.000	0.0000	NA	NA	NA	NA	<40	Declining
MEN	17	0.529 (0.045)	0.0015 (0.0014)	0.0292 (0.049)	0.827	1.52 (0)	1.389 (0.700)	<40	Declining
ITA	10	0.667 (0.163)	0.0037 (0.0028)	0.0058 (0.860)[a]	2.257	−1.03 (0.17)	−1.58 (0.047)y	110–140	Declining
CEU	5	0.800 (0.164)	0.0062 (0.0048)	0.0908 (0.23)[a]	3.742	0.95 (0)	0.803 (0.640)	2000–3000	Increasing
GER	24	0.569 (0.074)	0.0044 (0.0030)	0.1369 (0.03)	4.168	0.47 (0)	1.32 (0.793)	12,000–25,000	Increasing

Standard deviations for h and π are in brackets.
[a] Non-significant departure from the estimated demographic model of expansion; ψ = significant at the 5% level; NA = no possible analysis number of breeding pairs and status are taken from Evans and Pienkowski (1991).

values were found in Central Europe (GER, CEU) and in the population from Central Spain. For populations sampled in Doñana National Park, both mean sequence divergence and nucleotide diversity (π) (0.37% and 0.16%, respectively) were about half the values obtained in the black kite (0.79% and 0.29%, respectively).

3.2. Phylogenetic relationships

The NJ tree computed with Tamura–Nei distances revealed that the red and the black kite clustered in two monophyletic clades (95% bootstrap value, BP, Fig. 2). However, within the red kite group, low internal resolution and bootstrap support were found, with all populations sharing at least one haplotype with each other. Haplotypes H08 and H09, only present in central and eastern Europe (GER, CEU and ITA) form a cluster with moderate bootstrap support (BP = 62%). The shallow geographical structure of the red kite is supported by the medium-joining network (Fig. 3), which is star-like from the most common haplotype H01, but places also apart both H08 and H09.

In contrast to its sister species, the black kite cluster, representing only Doñana National Park samples ($n = 23$), includes five haplotypes and two clades with moderate bootstrap values (BP > 40%). Our comparison of red and black kite from Doñana National Park supports their recent divergence (only six point mutations and 2.5% of divergence in the control region). The coalescence time (estimated from the mean net number of substitutions per site of 0.02578) based on both a general rate for the control region of birds (20% per million years) and a more recent value of 4% per million years (Idaghdour et al., 2004) would fall between 128,900 years and 644,500 years, indicating they probably diverged as separate species during one of the last

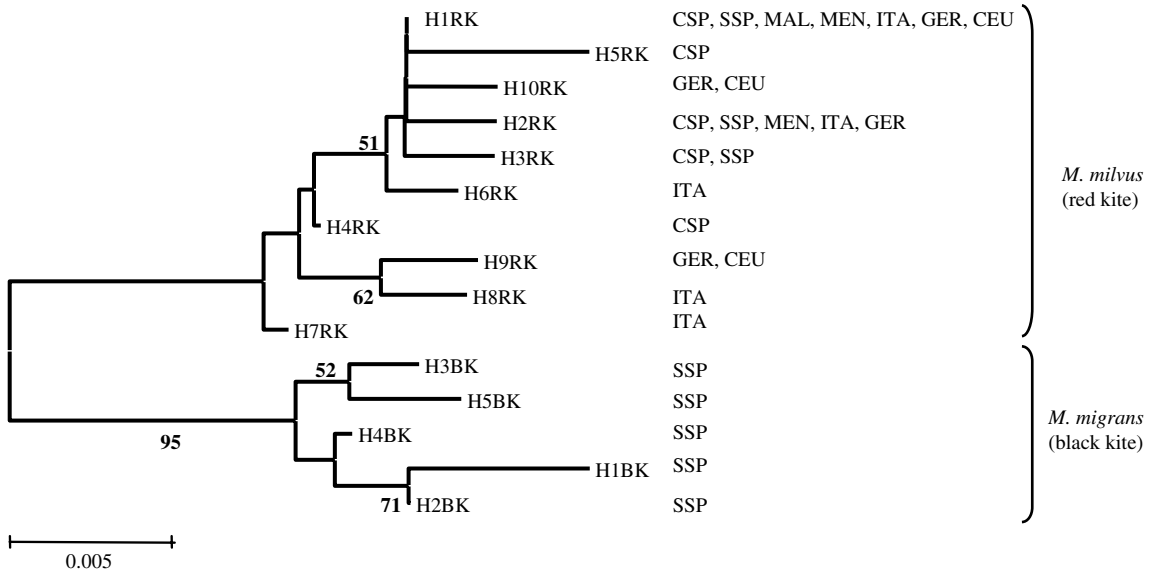

Fig. 2. Neighbour-joining tree computed by PAUP using Tamura and Nei's genetic distance among the mitochondrial control region haplotypes of the genus *Milvus*. Bootstrap percentage values >50% are indicated above the internodes. The Genbank assession codes of both species haplotypes are from AY62214 to AY62228.

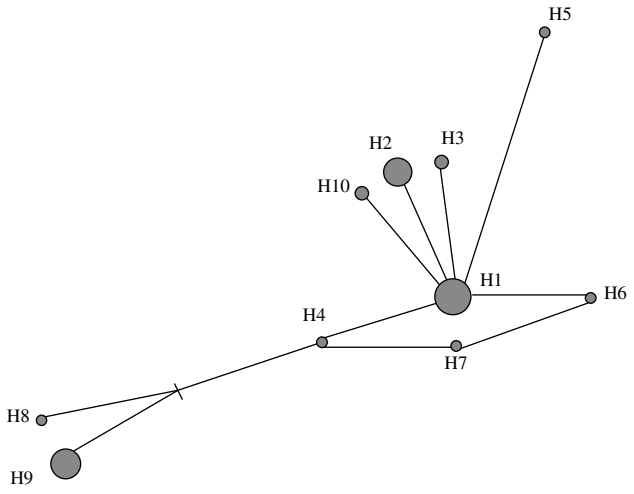

Fig. 3. Median-joining network of the 10 control region haplotypes of the red kite (*Milvus milvus*). The length of the line connecting two circles represents one or two mutations.

glaciations of the Pleistocene (Newton, 2003; Ferrer and Negro, 2004).

3.3. Population structure and gene flow

Results of AMOVA analyses indicated non-significant genetic differentiation with a 25% of the observed variation partitioned among populations, in both distance (Tamura–Nei) and frequency-based analyses (Table 3). 78% and 85% of variation occurs within populations, respectively. As a consequence of single-haplotype composition (see Table 1), MAL was strongly differentiated from all other samples (Table 4). As this sample may bias the tests and inflate the global value calculated from haplotype frequencies, we decided to remove MAL and rerun a AMOVA analysis. The amount of genetic variation attributable to differences among red kite populations dropped to 15% for the frequency-based analysis, as expected. Significant Φst values based on both methods however indicated differentiation between the eastern group (GER, CEU, ITA) and the south-western samples (MAL, SSP and MEN) (Table 4, 15.000 permutations). On the other hand, CSP and ITA were not significantly differentiated from GER and CEU, nor CSP from SSP, although they had moderate Φst valuevs. Φst values also indicated MEN and SSP were closely related. Exact tests of differentiation also significantly reject the null hypothesis of no differentiation between the south-western samples SSP, MEN, MAL and the eastern group (GER, CEU, ITA), but in contrast to Φst values, indicated significant differences between Germany (GER) and either Italy (ITA) and central Spain (CSP). Overall results indicated the occurrence of two main population units among red kite samples

Table 3
Results of AMOVA based on both haplotype frequency and distance (Tamura–Nei) methods

AMOVA	Source of variation	% of variation	
		Haplotype	Distance
1. All populations	Among populations (pop)	24.03	25.31
	Within pop	75.97*	74.69*
2. Six populations (excluding MAL)	Among pop	14.84	24.97
	Within pop	85.16*	75.03*
3. Eastern vs. South	Among groups	19.00*	26.60*
	Among pop within groups	1.90*	5.83*
	Within pop	79.10*	67.56*
4. Eastern vs. Western	Among	19.95*	30.33*
	Among pop within groups	1.25	3.15
	Within pop	78.80*	66.52*

Eastern: NEU, GER, ITA; South: SSP, MEN; Western: CSP, SSP, MEN.
* Significant at 0.5% level.

Table 4
Pairwise Φst values based on haplotype frequencies (above the diagonal) and on molecular distance (Tamura and Nei, below the diagonal) among haplotypes

	CSP	SSP	MA	MEN	ITA	GER	CEU
CSP		0.068	(0.4879**)	0.0114	0	0.0994	0.02232
SSP	0.1467		(0.6035**)	0	0.2275*	(0.3115**)	0.2980*
MA	(0.1807**)	(0.5582**)		(0.5167**)	0.2720**	(0.2879**)	0.6427**
MEN	0.0769	0	(0.5167**)		0.1073*	0.2179**	0.2226*
ITA	0	(0.2954**)	0.1479**	0.2147*		0.0494	0.0456
GER	0.1320	(0.3724**)	0.2524**	(0.3133**)	0.0822		0
CEU	0.1363	(0.4979**)	0.5391**	(0.4447**)	0.0876	0	

* and **: respectively significant at the 5% and 1% level, ( ) significant after sequential Bonferroni procedure.

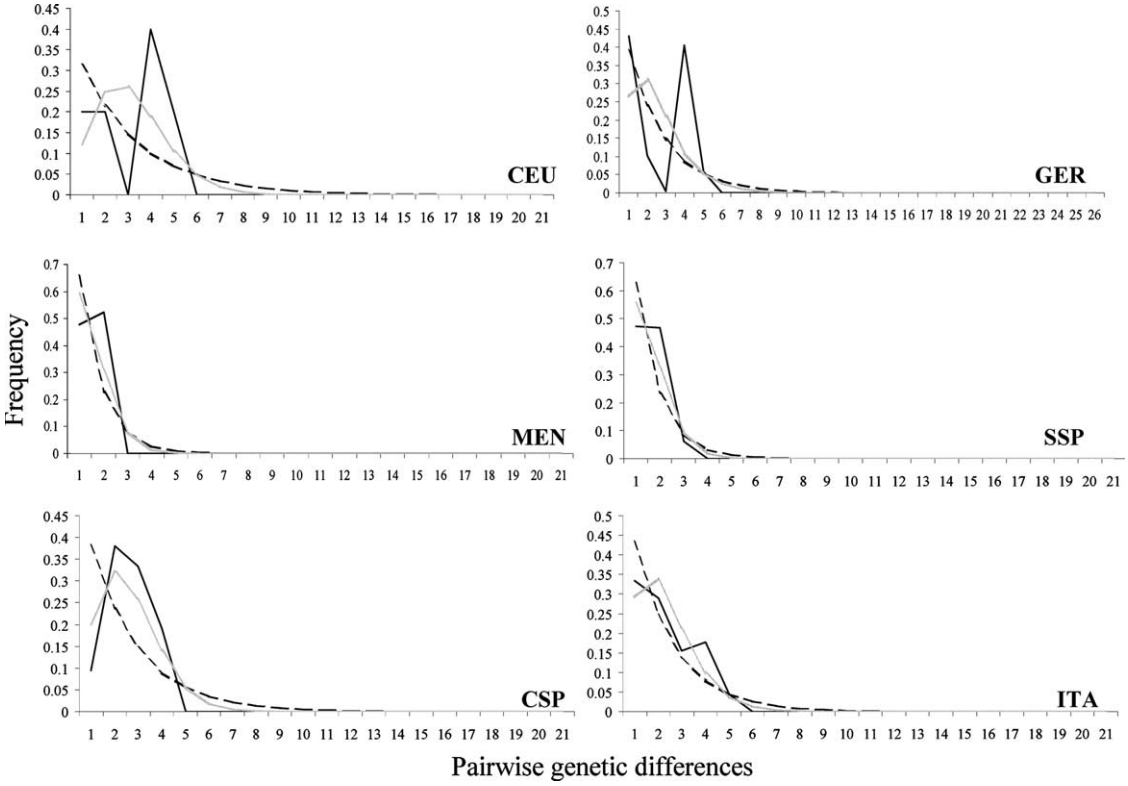

Fig. 4. Mismatch distributions observed in European red kite samples (——) and expected in either, stationary (▒▒▒▒) or expanding and/or bottleneck population (– – –) . Samples codes are given in Fig. 1.

throughout Europe, eastern (CEU, GER, ITA) and south-western (CSP, SSP, MEN) Europe, with Italian birds slightly differentiated from northern ones. The hierarchical analysis of variance AMOVA revealed these two groups are significantly different (P (random value $\geqslant$ observed value) $\geqslant 0.05$) with 33% and 20% of variation partitioned among groups for distance or frequency-based analysis, respectively.

3.4. Demographic analysis

The analysis of each geographical sample separately indicates different patterns among regions (Fig. 4). The shape of the function is unimodal for most samples, except for GER and CEU, presenting a bimodal distribution. These results probably reflect both the extremely low control region variability and strong bottlenecks in these samples. On the other hand, the distribution in CSP and ITA also conforms more closely to the distribution of expected values in expanding populations. Fu's tests and SSD values were also significant ($P \leqslant 0.05$) for these last samples (see Table 2). Tajima's test for selective neutrality was not significant for the total ($D = -1.02$, $P > 0.10$) nor for none of the geographic samples, although it had a negative sign for CSP ($D = -1.12$, $P = 0.17$) and ITA ($D = -1.03$, $P = 0.17$).

4. Discussion

4.1. Genetic and population history of red kite populations

With only 10 haplotypes detected, the red kite has one of the lowest mitochondrial control region diversities reported in birds of prey so far (e.g., five times lower than in the bearded vulture, *Gypaetus barbatus*, Godoy et al., 2004). Low genetic diversity has been already reported in other raptor species, generally associated to recent demographic crashes: the Californian condor, *Gymnogyps californianus* (Geyer et al., 1993), the Mauritius kestrel, *Falco punctatus* (Groombridge et al., 2000); the Norwegian peregrine falcon, *Falco peregrinus* (Lifjeld et al., 2002) and the critically endangered Spanish imperial eagle, *Aquila adalberti* (Martínez-Cruz et al., 2004). In this study, we found that the red kite and its sister species the black kite diverged recently, and also that the former may hold half the diversity compared to the latter, both for nucleotide and haplotype diversity values. The low mitochondrial DNA control region diversity in the red kite may therefore be not only a species-associated feature, but also the result of its recent evolutionary history and some contemporary events. Repeated bottlenecks and range contractions may have reduced genetic variability in red kite populations. Throughout Europe, red kite populations showed strong

declines during the 20th century, with a 20% decrease from 1970 to 1990 (Evans and Pienkowski, 1991). This decline has dramatically fragmented red kite populations and is still continuing in southwest and eastern Europe (Viñuela et al., 1999). In this study, the nucleotide diversity highly correlates with present-day population breeding size and status, with highest values found in central European regions and central Spain, where population strongholds occur. Germany sustains almost 60% of the worldwide population, and the Spanish population (mainly located in the central part of the county) is the second most abundant population in the whole red kite distribution. In contrast, lowest values are found in the Balearic Islands, where only a few tens of individuals remain (see Table 2). Also, values of τ closed to zero in SSP and MEN (Table 2) may reveal a very recent bottleneck event. The exception is Italy, with high diversity values, contrasting with data of low breeding size and population decline, pointing to a high pre-bottleneck genetic variation. Mismatch distributions are in agreement with demographic data, with bimodal distribution expected for populations at equilibrium only found in central European samples (CEU, GER). The results are consistent with the hypothesis that the recent population bottlenecks of the 20th century have led to a loss of genetic variation in the red kite in the southern populations, but that the main red kite populations remain genetically healthy.

In addition, our results suggest that the recent history may have shaped genetic patterns observed in the red kite. The phylogenetic revealed no deep structure among red kite haplotypes, thus suggesting recent divergence and low polymorphism. A rate of sequence divergence for the control region of 4–20% per one million years (Marshall and Baker, 1998; Idaghdour et al., 2004) gives a coalescence time of approximately between 19,000 and 95,000 years among red kite samples. This estimate matches the end of the last glaciations of the Pleistocene, which was an important period for the demographic history of birds (Kvist et al., 1999; Holder et al., 2000; Newton, 2003; Randi et al., 2003) and many other taxa in the Palaearctic, with culminated series of bottlenecks and expansions in population size and range (Hewitt, 1996, 1999; Avise and Walker, 1998; Taberlet et al., 1998). It may also have shaped the patterns of genetic variability in red kite populations. The current northern breeding range of the red kite (central Europe, Fig. 1) was covered by ice during part of this period (Webb and Bartlein, 1992), suggesting that the current species distribution in Europe may have resulted from an expansion northward. The low nucleotide diversity dominated by few common haplotypes and the lack of transversions (Table 1, Fig. 2), are all compatible with population bottlenecking and expansion in the red kite. During the coldest periods, the Iberian and Italian peninsulas, and the Balkans had relatively temperate climates (Bennet et al., 1991), from which populations of many taxa are known to have expanded northwards.

4.2. Population partitioning and gene flow

Overall results indicated a shallow genetic structure for the red kite throughout Europe, consistent with high dispersion capabilities. Inter-locality differences account for 25%, while a large amount of variation is among individuals of the same population. Also, a lack of phylogeographic structure was found, with two haplotypes being dominant in most samples (82%) and only a few haplotypes being private to a particular geographic region (Table 1). The significant hierarchical analyses of genetic substructure (AMOVA) indicate, however, two distinct groups throughout Europe, a South-western and Eastern one. Our results are consistent with life history and historical association. If the hypothesis of the colonisation northwards is true, patterns of differentiation and slight molecular separations observed in the red kite indicate recent subsequent isolation processes between both eastern and western regions. Phylopatry has often been invoked in large birds to explain genetic structuring. The red kite is considered as a partial migrant, as the northern and central populations migrate to more southern regions, especially in the Iberian Peninsula, whereas British and Mediterranean populations are sedentary (Cramp and Simmons, 1980). A study of the wintering population of red kite in Doñana National Park, indicated migratory and sedentary populations do not intermingle (Heredia et al., 1991). Life history traits may therefore explain in part the differences found between the northern populations (NEU and GER) and the southern sedentary ones (NSP, SSP). Alternatively, genetic diversity in the peripheral and/or insular southern populations (SSP, MAL, MEN) has suffered from genetic erosion, thus amplifying the effect of genetic drift and increasing the amount of genetic differences with central European samples. More sampling in central Europe and Spain would be needed to infer the relative importance of biological versus historical factors in the differences observed.

4.3. Implications for conservation

The red kite, once widespread in Europe, is now patchily distributed and globally threatened (May et al., 1993). It is one of the relatively few raptorial birds on the IUCNs Red list of endangered species. This species has already provided a prime example of population bottlenecking and inbreeding in the United Kingdom, where the population has recently been on the verge of extinction and has been supplemented with genetically more diverse individuals from continental Europe (Evans et al., 1998). In the Canary Islands, in the southernmost portion of its world distribution, the population got extinct in the

late 1980s . Our results indicate that the process of genetic erosion of red kite island populations still continues. In Minorca and Majorca islands, both with sedentary populations (De Pablo and Triay, 1996) and were a reintroduction plan has been proposed (De Pablo, 2005), the fixation of only the two most common mitochondrial haplotypes (i.e., H01 and H02) is alarming. The diversity loss by genetic drift, translating in the fixation of mitochondrial variants reflects the serious declines of the 20th century (Muntaner, 1981; Viada, 1996; Viñuela et al., 1999). Although recent demographic data indicate that the Balearic population may be have halted its decline (Viada, 1996; Viñuela et al., 1999), genetically impoverished local kites may have a reduced ability to adapt to changing environmental conditions. Now that direct poisoning, the main cause of mortality for adults, has been curtailed (De Pablo, 2005), management strategies should consider the reinforcement of local populations with individuals originating from the populations showing the closest genetic relatedness and with a much larger population and higher genetic variability (Table 2). Our AMOVA analysis indicates an absence of significant substructure within the Peninsula and also, between the Peninsula and Minorca, suggesting the introduction of red kites from the northern and central Spanish larger populations might be appropriate for the islands.

In addition, we found that the Doñana National Park population holds the lowest levels of genetic diversity among continental populations. The level of mtDNA polymorphism observed can be compared to that of other endangered raptor species that have also declined in the XXth century, such as the highly endangered Spanish imperial eagle, *Aquila adalbert* (Martínez-Cruz et al., 2004) and the bearded vulture (*Gypaetus barbatus*, L.) (Godoy et al., 2004). The red kite had a much larger range in the past and it has almost disappeared from most of southern and eastern Spain (Villafuerte et al., 1998; Viñuela et al., 1999). Moreover, this part of the Iberian Peninsula is on the edge of the range of the species, where declining populations are expected to be more vulnerable. Alternatively, the Doñana population is located in an atypical breeding habitat where red kites may only persist because of the exceptional productivity of the marshes, but where high densities cannot be reached due to interferences with the numerous members of the raptor guild (Seoane et al., 2003; Viñuela and Villafuerte, 2003. Conservation strategies may therefore aim to preserve the remnant diversity and avoid the occurrence of genetic inbreeding in this population. A lower genetic diversity in the genetically isolated PND compared to the northern populations has also been observed in the Spanish imperial eagle (Martínez-Cruz et al., 2004). However, the absence of population genetic structuring in the Iberian peninsula indicates that this population may be still connected with other breeding nuclei.

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