Phylogeography of the Eurasian Green Woodpecker (Picus viridis)

Article *in* Journal of Biogeography · February 2011

DOI: 10.1111/j.1365-2699.2010.02401.x

4 authors:

Jean-Marc Pons
Muséum National d'Histoire Naturelle
107 PUBLICATIONS **2,314** CITATIONS
SEE PROFILE

Georges Olioso
Independent Researcher
21 PUBLICATIONS **152** CITATIONS
SEE PROFILE

Corinne Cruaud
603 PUBLICATIONS **32,933** CITATIONS
SEE PROFILE

Jérôme Fuchs
Muséum National d'Histoire Naturelle
131 PUBLICATIONS **2,192** CITATIONS
SEE PROFILE

Journal of Biogeography (J. Biogeogr.) (2011) 38, 311–325

ORIGINAL ARTICLE

Phylogeography of the Eurasian green woodpecker (*Picus viridis*)

J.-M. Pons[1,2*], G. Olioso[3], C. Cruaud[4] and J. Fuchs[5,6]

[1]*UMR7205 'Origine, Structure et Evolution de la Biodiversité', Département Systématique et Evolution, Muséum National d'Histoire Naturelle, 55 rue Buffon, C.P. 51, 75005 Paris, France,* [2]*Service Commun de Systématique Moléculaire, IFR CNRS 101, Muséum National d'Histoire Naturelle, 43 rue Cuvier, 75005 Paris, France,* [3]*190 rue de l'industrie, 11210 Port la Nouvelle, France,* [4]*Genoscope, Centre National de Séquençage, 2 rue Gaston Crémieux, CP5706, 91057 Evry Cedex, France,* [5]*DST/NRF Centre of Excellence at the Percy FitzPatrick Institute, University of Cape Town, Rondebosch 7701, Cape Town, South Africa,* [6]*Museum of Vertebrate Zoology and Department of Integrative Biology, 3101 Valley Life Science Building, University of California, Berkeley, CA 94720-3160, USA*

*Correspondence: J.-M. Pons, UMR7205 'Origine, Structure et Evolution de la Biodiversité', Département Systématique et Evolution, Muséum National d'Histoire Naturelle, 55 rue Buffon, C.P. 51, 75005 Paris, France.
E-mail: pons@mnhn.fr

ABSTRACT

Aim In this paper we investigate the evolutionary history of the Eurasian green woodpecker (*Picus viridis*) using molecular markers. We specifically focus on the respective roles of Pleistocene climatic oscillations and geographical barriers in shaping the current population genetics within this species. In addition, we discuss the validity of current species and subspecies limits.

Location Western Palaearctic: Europe to western Russia, and Africa north of the Sahara.

Methods We sequenced two mitochondrial genes and five nuclear introns for 17 Eurasian green woodpeckers. Multilocus phylogenetic analyses were conducted using maximum likelihood and Bayesian algorithms. In addition, we sequenced a fragment of the cytochrome *b* gene (cyt *b*, 427 bp) and of the Z-linked BRM intron 15 for 113 and 85 individuals, respectively. The latter data set was analysed using population genetic methods.

Results Our phylogenetic results support the monophyly of *Picus viridis* and suggest that this taxon comprises three allopatric/parapatric lineages distributed in North Africa, the Iberian Peninsula and Europe, respectively. The North African lineage split from the Iberian/European clade during the early Pleistocene (1.6–2.2 Ma). The divergence event between the Iberian and the European lineages occurred during the mid-Pleistocene (0.7–1.2 Ma). Our results also support a post-glacial range expansion of these two lineages from distinct refugia located in the Iberian Peninsula and possibly in eastern Europe or Anatolia, which led to the establishment of a secondary contact zone in southern France.

Main conclusions Our results emphasize the crucial role of both Pleistocene climatic oscillations and geographical barriers (Strait of Gibraltar, Pyrenees chain) in shaping the current genetic structure of the Eurasian green woodpecker. Our molecular data, in combination with diagnosable plumage characters, suggest that the North African green woodpecker (Levaillant's woodpecker) merits species rank as *Picus vaillantii* (Malherbe, 1847). The two European lineages could be distinguished by molecular and phenotypic characters over most of their respective geographical ranges, but they locally exchange genes in southern France. Consequently, we prefer to treat them as subspecies (*P. viridis viridis*, *P. viridis sharpei*) pending further studies.

Keywords

Climatic oscillations, geographical barriers, glacial refugia, Picidae, *Picus viridis*, Pleistocene, species limits, suture zones, Western Palaearctic, woodpeckers.

Journal of Biogeography

INTRODUCTION

The role of past climatic events and geographical barriers in shaping the current genetic structure and population history of temperate species in the Western Palaearctic has been the focus of major research programmes over the last decade (Taberlet *et al.*, 1998; Avise, 2009). As a consequence of Pleistocene climatic oscillations, temperate flora and fauna went through repeated expansions and range contractions that left signatures in the geographical distribution and genetic diversity of extant populations (e.g. Avise, 2000; Brito, 2005). Although phylogeographical patterns may vary greatly from one species to another, comparative genetic surveys and the reconstruction of palaeovegetation maps highlighted in Europe three main refugia (areas of suitable habitat where populations could survive and reproduce through time) not covered by Pleistocene ice masses, namely the Iberian peninsula, the Italian peninsula and the Balkan region (Taberlet *et al.*, 1998; Hewitt, 2000; Weiss & Ferrand, 2007; Avise, 2009). In many cases, Holocene range expansions from these distinct refugia brought divergent lineages into secondary contact at suture zones concentrated in specific areas (the Alps, the Pyrenees, Central Europe and Scandinavia, Taberlet *et al.*, 1998; Schmitt, 2007). Furthermore, some studies suggested that North Africa may also have acted as a refuge area for Palaearctic taxa (Hewitt, 2000; Leroy & Arpe, 2007), with the Strait of Gibraltar acting as a more or less permeable geographical barrier (Leroy & Arpe, 2007; García *et al.*, 2009; Juste *et al.*, 2009).

In this paper, we examine the influence of Plio-Pleistocene climatic oscillations and geographical barriers on the current genetic structure of the Eurasian green woodpecker (*Picus viridis* Linnaeus, 1758), a medium-sized non-migratory arboreal bird. The Eurasian green woodpecker is widely distributed throughout Europe and North Africa, but notably is absent from Mediterranean islands and Ireland (Fig. 1). The Eurasian green woodpecker has limited dispersal behaviour – movements above 20 km are unusual (Cramp, 1985) – is strongly associated with woodlands, and needs open foraging habitats such as lawns and meadows. Given the ecological characteristics of this species, in combination with its low dispersal capacity and the occurrence of potential biogeographical barriers, we should expect some genetic differentiation among populations across its range.

The genus *Picus* (15 species) is endemic to the Palaearctic and Indo-Malaya, with a centre of diversity in the Indo-Malayan biogeographical realm (containing 13 species, 12 of which are endemic). *Picus viridis* is a recent species within the genus and probably colonized the Western Palaearctic in recent geological times (Fuchs *et al.*, 2008). Some geographical variation in plumage pattern (e.g. colour of the malar stripe) and calls have been described within the species. Four subspecies are currently recognized throughout Eurasia (Winkler & Christie, 2002): (1) *viridis* occurs from Great Britain east to European Russia, (2) *karelini* is distributed over Italy, the Balkans, the Caucasus and south-west Turkmania, (3) *innominatus* is restricted to south-west Iran, and (4) *sharpei* occurs in Iberia and southern France (Cramp, 1985). Morphological differences among *innominatus*, *karelini* and *viridis* are very subtle (Cramp, 1985). In contrast, *sharpei* differs markedly from the other three subspecies in having a restricted amount of black on the side of the face, often limited to indistinct grey lines on the lores (Short, 1982; Cramp, 1985). Consequently, it has been suggested that Iberian birds may deserve a specific rank (Winkler & Christie, 2002). The taxonomic status of a fifth subspecies of *P. viridis*, *P. v. vaillantii*, endemic to northern Africa, has long been debated and it has been successively considered a subspecies of *P. viridis* (Winkler & Christie, 2002), a separate but closely related species (Vaurie,

Figure 1 Geographical distribution of the 427-bp cytochrome *b* fragment of *Picus viridis*. Symbol size matches the number of individuals sampled (small circle, $N < 5$; medium circle, $N < 11$; large circle, $N < 26$). *Picus viridis viridis/karelini* lineage, black circle; *P. v. sharpei*, white circle; *P. v. vaillantii*, grey circle. Exact localities are given in Appendix S1. The grey region corresponds to the known range of *P. viridis*.

1959; Voous, 1973; Short, 1982) and even an early offshoot that occupies a basal position among red-crowned *Picus* species (Voous, 1977). In a molecular phylogeny of the genus *Picus*, Fuchs *et al.* (2008) showed that the red-crowned species (10 species) form a monophyletic group and suggested a sister-group relationship between *P. v. viridis* and *P. v. sharpei*, but they were not able to include the North African *P. v. vaillantii* in their study.

From a biogeographical perspective, it is of particular interest to investigate whether the Iberian green woodpecker may be considered as an intermediate bridge between European and North African green woodpeckers as has been suggested on the basis of plumage coloration patterns (Winkler & Christie, 2002). Three biogeographical scenarios are predicted, depending on the branching order among the three putative lineages: (1) if the Iberian lineage is the closest relative of the North African lineage, the colonization of northern Africa would have been sequential from western Europe, and the Pyrenees may have acted as a biogeographical barrier before the Strait of Gibraltar; (2) if the European and Iberian lineages are sister lineages, then the Strait of Gibraltar would have acted as a barrier to gene flow before the Pyrenees chain did so; and (3) if the European and the North African lineages are sister groups, then the biogeographical scenario would be more complex, with an early isolation of the Iberian lineage and a later colonization of Africa from Europe.

Here we address the biogeographical history and systematics of *P. viridis* using DNA sequence data gathered from several loci. The use of multilocus data has become a fruitful approach for testing biogeographical hypotheses such as the existence of post-glacial refugia, population expansions, and colonization routes (Edwards & Bensch, 2009). Indeed, the use of multiple loci circumvent some problems associated with mitochondrial DNA (mtDNA), which may not always fulfil the basic assumptions (selective neutrality, evolutionary rate constancy, no hybridization) required to obtain reliable divergence date estimates or valid species delineations (Bellemain *et al.*, 2008; Galtier *et al.*, 2009; but see also Baker *et al.*, 2009).

MATERIALS AND METHODS

Sample collection and data analysis

We obtained tissue samples from 113 individuals, covering a large part of the Eurasian green woodpecker's distribution and belonging to 17 geographical populations (Fig. 1; see Appendix S1 in the Supporting Information for details of exact localities). Individuals were identified based on diagnostic characters (amount of black on side of face to differentiate between subspecies *sharpei* and *viridis*/*karelini*) or on geography (to differentiate between *karelini* and *viridis*).

Our analyses were performed at two levels. First, we addressed the phylogenetic relationships of the four main subspecies of *P. viridis* with respect to other red-crowned species. For this purpose, we selected 17 *P. viridis* individuals that were collected throughout Europe (nine of *P. v. viridis* and three of *P. v. karelini*), in Spain and southern France (three of *P. v. sharpei*) and Morocco (two of *P. v. vaillantii*), and six other red-crowned species from the *Picus* Clade 2 *sensu* Fuchs *et al.* (2008). Three more distantly related species, *Campethera nivosa*, *Colaptes melanochloros* and *Picus mentalis* (*Picus* Clade 1 *sensu* Fuchs *et al.*, 2008), were included as outgroups. Some of the individuals that we included in the analyses are length-variant heterozygotes for some loci (BRM for *C. nivosa*, GAPDH for *P. awokera* and *P. xanthopygaeus)* (Fuchs *et al.*, 2008). In these cases, we selected the allele that was not autapomorphic with respect to all other individuals. The list of taxa included in the phylogenetic analyses and GenBank numbers are reported in Appendix S2.

Second, we performed phylogeographic and population genetic analyses on a 427-bp fragment of the mitochondrial cytochrome *b* (cyt *b*) gene ($n = 113$ individuals) and on the Z-linked BRM intron 15 ($n = 85$ individuals) to see if the geographical distribution of mitochondrial lineages corresponded to that recovered by the Z-linked locus (paternally inherited).

DNA sequencing

We obtained sequence data from four autosomal introns [myoglobin intron 2 (MB), β-fibrinogen intron 7 (FGB), glyceraldehyde 3-phosphate dehydrogenase intron 11 (GAPDH), transforming growth factor β-2 intron 5 (TGFb2)], one Z-linked nuclear intron (BRM intron 15; BRM) and two mitochondrial protein-coding genes (a fragment of cyt *b* and ATP6). Sequence lengths are reported in Table 1. The phylogeographic analyses were based on the 427-bp cyt *b* fragment. Standard protocols described in Fuchs *et al.* (2007) were used to perform DNA extraction, polymerase chain reaction amplification and sequencing. All primers used for amplification and sequencing are reported in Fuchs *et al.* (2008).

Table 1 Length of the alignment and DNA substitution models selected for each individual locus using the decision-theoretic approach. The Trn + I model is not implemented in MrBayes. We thus used the GTR + I model for ATP6 and the mtDNA.

Locus	Alignment length (bp)	Model	Harmonic mean (−ln)
ATP6	684	Trn + I	3031.93
Cyt *b*	427	HKY + Γ	1761.36
mtDNA	1111	Trn + I	4756.28
MB	662	K80	1423.1
GAPDH	426	HKY + I	966.44
TGFb2	565	K80 + Γ	1246.45
FGB	697	HKY	1382.4
BRM	370	HKY	795.92
Nuclear DNA	2720		5503.14
Concatenated	3831		10582.87

MB, myoglobin intron 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase intron 11; TGFb2, transforming growth factor β-2 intron 5; FGB, β-fibrinogen intron 7; BRM, BRM intron 15.

Phylogenetic relationships

Molecular phylogenies were estimated using Bayesian inference (BI) and maximum likelihood (ML), as implemented in MrBayes 3.1 (Huelsenbeck & Ronquist, 2003; Ronquist & Huelsenbeck, 2003) and RaxMl 7.0.4 (Stamatakis, 2006; Stamatakis *et al.*, 2008). We selected the likelihood model using the decision-theoretic approach implemented in DT_Modsel (Minin *et al.*, 2003). Four Metropolis-coupled Markov chains (three hot and one cold) were run for five million generations with trees sampled every 100 generations (50,001 trees sampled). The first 100,000 generations (10,000 trees) were discarded (as an initial 'burn-in' period), and posterior probabilities (PPs) were estimated for the remaining sampled generations. Two independent Bayesian runs initiated from random starting trees were performed for each data set, and the log-likelihood values and posterior probabilities were compared to ascertain that the chains had reached the posterior distribution. For the concatenated data set (MB, GAPDH, FGB, TGFb2, BRM, ATP6, cyt *b*), we conducted the partitioned analyses for both BI and ML by leaving all parameters (base frequencies, rate matrix, shape parameter, proportion of invariable sites) except topology to vary among partitions (using the *unlink* and *prset* commands for the BI). We checked that the potential scale reduction factor (PSRF) approached 1.0 for all parameters and that the average standard deviation of split frequencies converged towards zero. We also used Tracer 1.4 (Rambaut & Drummond, 2007) to check that we reached convergence for the posterior distributions of the parameter estimates and that our effective sample size of the underlying posterior distribution was large enough for a meaningful estimation of parameters.

Incongruities between the individual gene trees were detected by comparing resulting topologies and nodal support. The criterion for incongruence was set at 0.95 for posterior probabilities (Huelsenbeck & Ronquist, 2003) and at 70% for bootstrap values.

Divergence times

We used Beast 1.4.8 (Drummond *et al.*, 2002, 2006; Drummond & Rambaut, 2007) to estimate divergence dates within the genus *Picus*. We assigned the best-fitting model, as estimated by DT_Modsel, to each of the seven partitions. We assumed a Yule speciation process for the tree prior and ran analyses assuming either an uncorrelated lognormal distribution for the molecular clock model or a strict molecular clock model (Ho, 2007). We estimated the divergence times among *Picus* lineages using two independent strategies: (1) a secondary calibration point; and (2) a recently estimated neutral molecular clock rate for fourfold-degenerated sites (Subramanian *et al.*, 2009).

For the calibration point, we used the split between *P. canus* and *P. viridis*, estimated to have occurred around 2.8 Ma (95% credibility interval: 1.8–4.1) (Fuchs *et al.*, 2006, 2007). This estimate was obtained by a previous study (Fuchs *et al.*, 2006) that used a primary calibration point of 4.5–5.5 Ma for the split between *Sasia ochracea* and *S. abnormis*. During that period, two important seaways (nearly 100 km wide) separated continental Asia (range of *S. ochracea*) from Borneo and the Thai-Malay peninsula south of the Isthmus of Kra (range of *S. abnormis*) (Woodruff, 2003). We previously hypothesized that this seaway promoted the differentiation between *S. abnormis* and *S. ochracea*, two species with poor inferred dispersal capacities (tiny size, short and rounded wings, short tail). The *P. canus*/*P. viridis* split was modelled either as a normal distribution, with the mean set to 2.8 Ma and the standard deviation to 0.6, so that the 95% credibility interval falls within the 1.8–3.8 interval, or as a lognormal distribution with a zero probability offset at 1.8 Ma, a lognormal mean of 0.01 and a standard deviation of 0.57, so that the 95% credibility interval is between 1.8 and 4.1 Ma with the mode of the distribution at 2.8 Ma. These different analyses were performed in order to assess the effect of prior choice on the Bayesian estimates of divergence times.

We compared the divergence time estimates obtained using the secondary calibration point with a newly proposed molecular clock rate estimated from fourfold-degenerated sites. Based on nearly complete mtDNA genomes of Adelie penguins (*Pygoscelis adeliae*), Subramanian *et al.* (2009) suggested that the synonymous substitution rate occurring on fourfold-degenerated sites remained constant over time, and estimated the rate of evolution at fourfold-degenerated sites of Adelie penguins (*Pygoscelis adeliae*) to be 0.073 substitutions site^{-1} Myr^{-1} [95% highest posterior density (HPD): 0.025–0.123]. We applied this mutation rate, and the associated uncertainty, to the fourfold-degenerated sites within our mitochondrial data set (246 sites). For these analyses, we used an uncorrelated lognormal molecular clock model, a coalescent tree prior with constant population size, and the HKY + Γ model of sequence evolution. These are the same parameters as used by Subramanian *et al.* (2009) to estimate the rate. The only exception is the substitution model, as some particular transversion types of the GTR model (the model used by Subramanian *et al.*, 2009) were not represented in our data set. We used default prior distributions for all other parameters and ran Markov chain Monte Carlo (MCMC) analyses for 20 million steps.

Testing for selection on the mitochondrial locus

To test for selection on the mitochondrial protein-coding genes, we used the McDonald–Kreitman test (M–K test) (McDonald & Kreitman, 1991) implemented in DnaSP 5.0 (Rozas *et al.*, 2003). The M–K test compares synonymous and non-synonymous variation within and between pairs of taxa (or groups of individuals). Under neutrality, the ratio of non-synonymous (d*NS*) to synonymous (d*S*) fixed substitutions between species should be the same as the ratio of non-synonymous (d*NS*) to synonymous (d*S*) polymorphisms within species. Significance was assessed using Fischer's exact test and a threshold of 0.05. The stop codon was not included

in the analyses. As an outgroup, we used the sequence of *Picus erythropygius*.

Median-joining network

Because many of the underlying assumptions of traditional tree-building methods (fully bifurcating trees, complete lineage sorting) could be violated when addressing intraspecific studies (Posada & Crandall, 2001), we also used networks to explore the phylogeographical structure within *P. viridis*. We used Network 4.2 (Bandelt *et al.*, 1999) to reconstruct the median-joining network based on 113 partial cyt *b* sequences.

Genetic variation

Standard diversity indices (haplotype diversity *H*, nucleotide diversity π, Watterson's theta) were calculated using Arlequin 3.1 (Excoffier *et al.*, 2005). We used Fu's F_S test (1000 replicates), implemented in Arlequin 3.1, to detect signatures of population expansion. In the absence of selection, significant negative values of Fu's F_S are indicative of population expansion (Nielsen, 2005). In addition, we tested whether our observed data fitted a sudden-expansion model using a test of goodness-of-fit derived from mismatch-distribution analyses implemented in Arlequin 3.1.

Intra- and inter-genetic differentiations among taxa and geographical populations were evaluated using Φ_{ST} based on the Tamura–Nei genetic distance, the closest model to the HKY model. The null distribution of pairwise Φ_{ST} values is obtained by performing 1000 random haplotype permutations among haplotypes between populations in Arlequin 3.1. The *P*-value of the test is the proportion of permutations leading to a Φ_{ST} value larger than or equal to the observed one.

Populations were defined according to the multilocus phylogeny, the haplotype network and sampling localities of individuals. Regarding *P. v. viridis*, we retained three populations: eastern Europe (Russia, Poland, $n = 7$), northern Europe (Sweden, $n = 6$) and western Europe (France, Austria, Switzerland, Great Britain, Italy, $n = 51$). Three geographical populations were also delineated for *P. v. sharpei*, namely central Spain ($n = 11$), north-east Spain ($n = 9$) and southern France ($n = 25$).

RESULTS

Phylogenetic relationships

The alignment length, model selected under the decision-theoretic approach, and the harmonic mean likelihood value of the posterior distribution are indicated in Table 1. As we obtained the same topology with maximum likelihood methods, bootstrap values are directly reported on the 50% majority consensus rule tree from the Bayesian analysis.

The mitochondrial tree (Fig. 2) highlights three divergent lineages within the *P. viridis* group: (1) one lineage that includes individuals collected from Great Britain to Russia that belong to *P. v. viridis* or *P. v. karelini*; (2) one Iberian lineage (corresponding to *P. v. sharpei*); and (3) one North African lineage (corresponding to *P. v. vaillantii*). The mitochondrial data set strongly supported a sister relationship between *P. v. viridis/karelini* and *P. v. sharpei* but could neither recover the monophyly of the *P. viridis* group nor resolve the phylogenetic relationships among *P. canus*, *P. v. vaillantii*, *P. v. karelini/viridis/sharpei* and *P. erythropygius*. The mean genetic distance varied from 7.1% between *P. v. viridis/karelini* and *P. v. vaillantii* to 3.4% between *P. v. viridis/karelini* and *P. v. sharpei*. Genetic distances within the three primary lineages (*vaillantii*, *viridis/karelini*, *sharpei*) did not exceed 0.6%.

Picus v. viridis/karelini, *P. v. sharpei* and *P. v. vaillantii* formed a well-supported monophyletic group in the concatenated nuclear tree (Fig. 3) and in four out of the five nuclear autosomal and Z-linked gene trees (see Figs S1, S3, S4 & S5 in Appendix S3). The GAPDH tree was mostly unresolved (see Fig. S2 in Appendix S3). In the MB tree (Fig. S3 in Appendix S3), *P. v. viridis/karelini* and *P. v. sharpei* were sister taxa but the relationships of this clade with *P. canus*, *P. v. vaillantii* could not be resolved. Individual gene trees failed to unravel the branching order among *P. canus*, *P. erythropygius* and the *P. viridis* group but provided support for the reciprocal monophyly of the three main lineages within the *P. viridis* group (*sharpei*, *vaillantii* and *viridis/karelini*).

Most nodes recovered from the concatenated data received strong support, except for the relationship between *P. viridis* and its closest relatives (*P. canus*, *P. erythropygius*), which obtained low support (Fig. 4). The North African green woodpecker was sister group to all European birds (*sharpei* and *viridis/karelini*). The monophyly of *P. viridis* was further supported by a 3-bp insertion in the BRM intron 15 and an 8-bp insertion in the FGB intron (Fig. 4). The two European lineages shared a 3-bp deletion in the FGB indel. *Picus viridis* is thus recovered as a monophyletic unit with strong support.

Divergence times

Our divergence time estimates were very similar across analyses: the choice of the molecular clock prior (strict clock or uncorrelated lognormal) or calibration point (normal or lognormal) had no effect on the absolute values of the estimates. The analyses performed using the molecular clock rate from the fourfold-degenerated sites yielded more recent divergence time estimates and broader 95% HPD intervals than the analyses conducted using the *canus–viridis* calibration point. However, the 95% HPDs among analyses were largely overlapping (Table 2). The divergence time analyses indicate that the North African lineage *vaillantii* probably diverged from the European lineage between 1.6 and 2.2 Ma (Pleistocene). The split between *sharpei* and *viridis/karelini* occurred about 0.7–1.2 Ma (Table 2). Hence, all analyses suggest that the *P. viridis* lineage diversified during the Early Pleistocene, although the 95% HPD includes the Pliocene in all analyses. However, assigning the speciation events to any particular

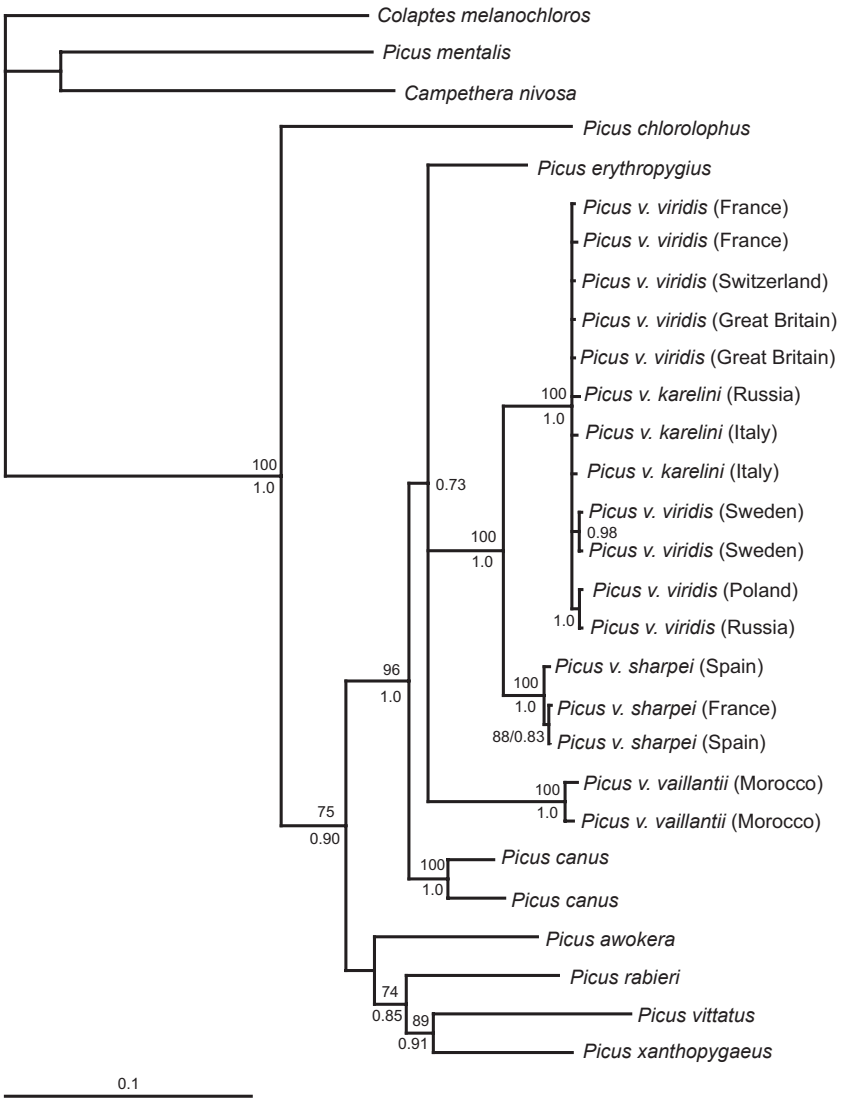

Figure 2 Fifty per cent majority rule consensus tree for *Picus viridis* and its relatives obtained from the Bayesian inference analyses of mitochondrial DNA (1111 bp). Numbers close to branches are posterior probabilities (greater than 0.70) and bootstrap values (greater than 70%). The scale bar represents the number of substitutions per nucleotide site.

events or climatic oscillation during that period appears too speculative, given the broad 95% HPD intervals and the assumptions of our dating analyses (choice of calibration points and/or molecular clock rate). We also note that 95% HPDs are larger in the analyses that used the neutral fourfold rate; we attribute this result to the fact that the prior distribution was larger than for the biogeographical calibration point.

Selection on the mitochondrial locus (cytochrome *b*)

Results of the MacDonald–Kreitman test when comparing *P. viridis* ($n = 111$) with *P. erythropygius* indicate no clear evidence of selection ($P = 0.57$).

Median-joining network

The median-joining network was based on cyt *b* sequences obtained from 109 Eurasian green woodpeckers distributed throughout Europe and from four North African individuals. Sixteen cyt *b* haplotypes were detected; these haplotypes clustered in three sub-networks separated from each other by respectively 10 and 25 substitutions (Fig. 5). The two sub-networks corresponding respectively to the European and Iberian lineages displayed the same 'star-like' relationship of haplotypes, with a common haplotype having a wide geographical distribution at the centre of the sub-network and rare variants radiating from the ancestral sequence. This is a common pattern observed in the case of recent range expansion of lineages. Within each sub-network, derived haplotypes were weakly differentiated from their respective ancestral haplotype by one to two mutation steps at most.

Despite its low genetic diversity, the *viridis/karelini* sub-network showed some geographical structure, as northern (Sweden) and eastern (Russia, Poland) haplotypes were not found in western Europe. On the other hand, all Italian and Caucasian green woodpeckers (*karelini*) hold the most common European haplotype. In the *sharpei* sub-network, it is worth noting that all derived haplotypes but one were from central Spain. A larger sample size covering a wider geographical

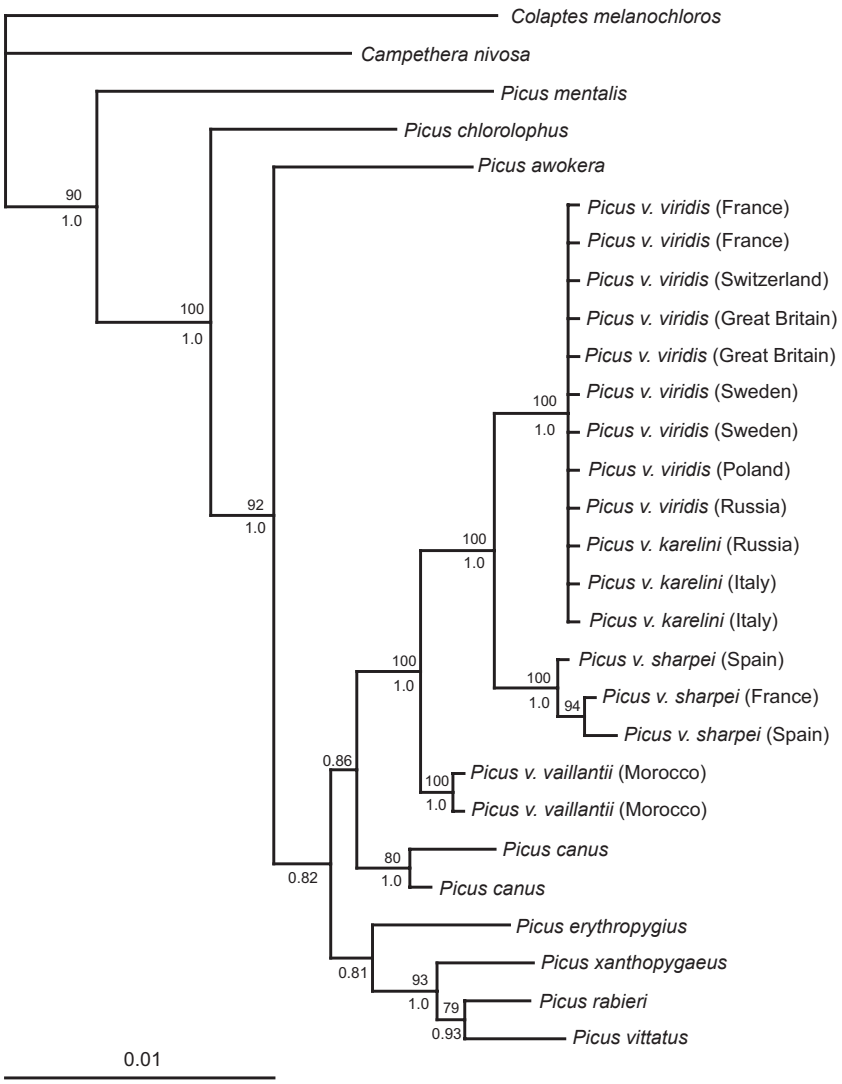

Figure 3 Fifty per cent majority rule consensus tree for *Picus viridis* and its relatives obtained from the Bayesian inference analyses of 2720 bp of nuclear DNA (autosomal introns: 2350 bp, Z-linked intron: 370 bp). Numbers close to branches are posterior probabilities (greater than 0.70) and bootstrap values (greater than 70%). The scale bar represents the number of substitutions per nucleotide site.

area up to the eastern Maghreb would be necessary to investigate the phylogeographical structure of North African populations.

Genetic diversity

Evidence of population expansion was detected in both *P. v. viridis/karelini* and *P. v. sharpei* lineages, for which we found significant negative Fu's F_S values (*viridis/karelini*, $n = 62$, Fu's $F_S = -3.76$, $P < 0.01$; *sharpei*, $n = 45$, Fu's $F_S = -6.44$, $P < 0.0001$). Mismatch distribution analyses supported the sudden expansion model (raggedness test, $P > 0.10$ for both lineages). Taken together, these results suggest, in accordance with their 'star-like' networks, that both *P. v. viridis/karelini* and *P. v. sharpei* experienced population expansions.

In Table 3, we report estimates of the genetic variability of the Z-linked intron BRM 15, which is paternally transmitted. We excluded birds from southern France and north-east Spain to prevent any bias owing to possible gene flow. The results clearly show that *sharpei* exhibits much more genetic variability at this nuclear locus than *viridis* does. Genetic diversity values indicate that *sharpei* probably maintained a larger effective population size than *viridis* (Watterson's theta values: *viridis/karelini*, $\theta = 0.00299$; *sharpei*, $\theta = 0.00375$). As for the maternal mtDNA, we found complete lineage sorting for the paternally inherited BRM intron 15 (three diagnostic mutations), despite its greater effective population size and the slower mutation rate of nuclear loci. This result favours an ancient divergence event between these two lineages.

Geographical structure of the genetic variation

Within the *viridis* lineage, population pairwise Φ_{ST} values ranged from 0.65 to 0.91, showing significant partitioning of the genetic variability among geographical populations. The percentage of the variance explained by differences between populations was much lower in the case of *P. v. sharpei*. We nevertheless found a significant partitioning of the genetic variability between central Spain and southern France populations (Table 4). Haplotype diversity estimates decreased sharply

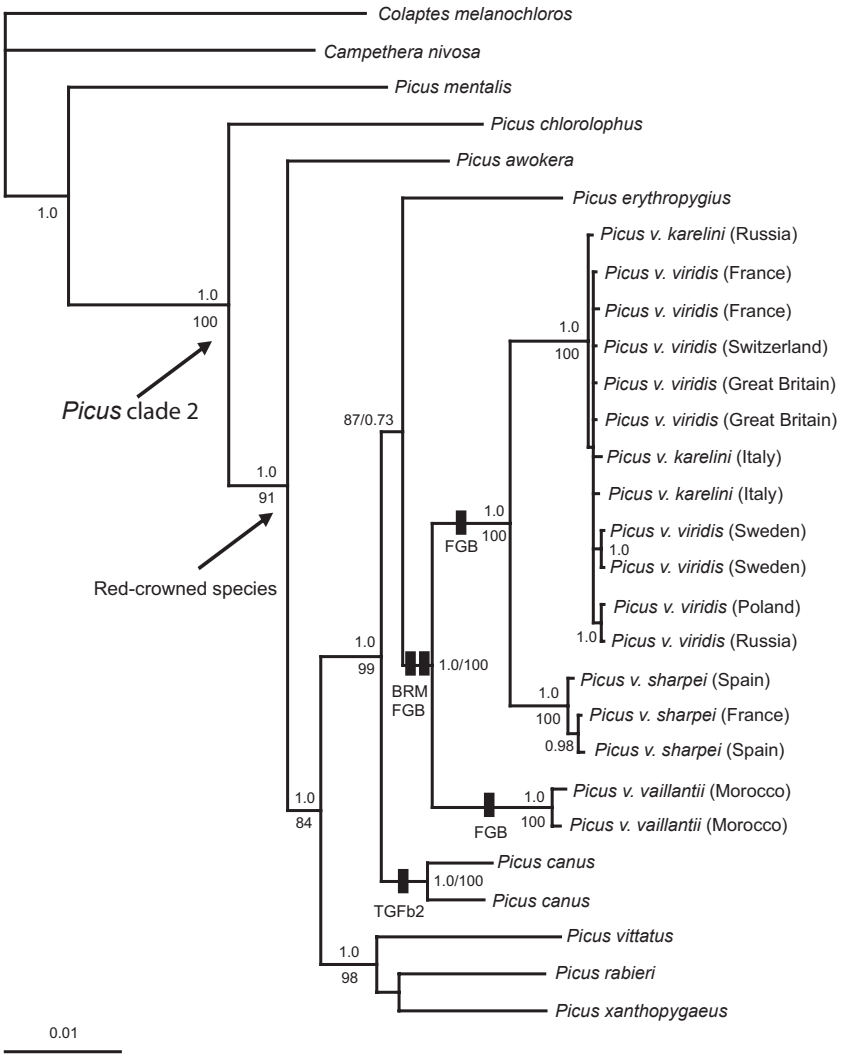

Figure 4 Fifty per cent majority rule consensus tree for *Picus viridis* and its relatives obtained from the Bayesian inference analyses of 3831 bp of nuclear (autosomal introns: 2350 bp, Z-linked intron: 370 bp) and mitochondrial (1111 bp) DNA. Numbers close to branches are posterior probabilities (greater than 0.70) and bootstrap values (greater than 70%). *Picus viridis* is recovered as monophyletic and comprises three divergent lineages. Indels that support the monophyly of *P. viridis* as well as the monophyly of two lineages within *P. viridis* are mapped onto the tree (see text for details on the nature of events). The scale bar represents the number of substitutions per nucleotide site. See Fuchs *et al.* (2008) for details on the *Picus* clade 2.

Table 2 Divergence times in Ma (95% highest posterior density) obtained using different priors for the molecular clock model [strict clock (columns 1 and 2) or uncorrelated lognormal (columns 3 and 4)] and for the distribution of the calibration point [normal (columns 1 and 3) or lognormal (columns 2 and 4) for the split between *Picus canus* and *P. viridis*]. Divergence times in the last column were obtained using a neutral fourfold-degenerated sites rate.

	Molecular clock	Molecular clock	Uncorrelated lognormal	Uncorrelated lognormal	Uncorrelated lognormal
Clade	Normal distribution	Lognormal distribution	Normal distribution	Lognormal distribution	Normal distribution
Picus clade 2	5.3 (3.9–6.9)	5.2 (3.6–7.2)	5.3 (3.7–7.0)	5.2 (3.4–7.4)	4.6 (1.2–9.7)
P. viridis sensu lato	2.2 (1.6–2.8)	2.2 (1.5–3.0)	2.1 (1.5–2.7)	2.1 (1.4–2.9)	1.6 (0.4–3.3)
P. v. viridis/karelini	0.13 (0.06–0.21)	0.13 (0.06–0.22)	0.14 (0.06–0.23)	0.14 (0.06–0.23)	0.18 (0.03–0.4)
P. v. sharpei	0.14 (0.04–0.25)	0.14 (0.04–0.25)	0.14 (0.04–0.26)	0.14 (0.04–0.26)	0.05 (0.04–0.15)
P. v. viridis/karelini–P. v. sharpei	1.2 (0.7–1.6)	1.2 (0.7–1.6)	1.1 (0.7–1.5)	1.1 (0.6–1.5)	0.7 (0.2–1.6)
P. v. vaillantii	0.14 (0.04–0.27)	0.15 (0.04–0.27)	0.15 (0.05–0.29)	0.15 (0.04–0.29)	0.17 (0.09–0.4)
P. canus–P. viridis	2.7 (2.1–3.3)	2.7 (2.0–3.6)	2.7 (2.1–3.3)	2.7 (2.0–3.6)	1.8 (0.5–3.6)
P. canus	1.2 (0.8–1.6)	1.2 (0.7–1.7)	1.2 (0.7–1.8)	1.2 (0.6–1.8)	0.6 (0.1–1.3)
P. rabieri–P. xanthopygaeus–P. vittatus	2.9 (2.1–3.8)	2.8 (1.9–4.0)	2.7 (1.8–3.7)	2.7 (1.6–3.9)	2.8 (0.9–5.8)

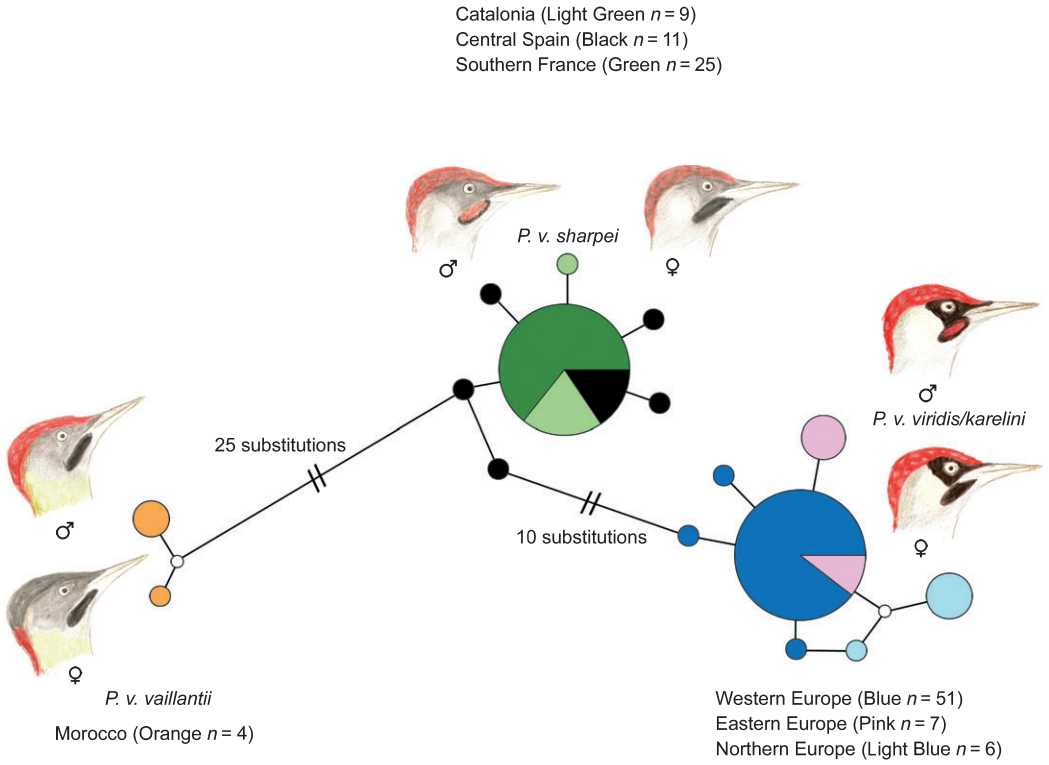

Figure 5 Median-joining cytochrome *b* haplotype network for *Picus viridis*. Each circle represents a unique haplotype, and the size of the circle is proportional to haplotype frequency. The small white circle corresponds to a missing (unsampled or extinct) haplotype. Western Europe: France, Austria, Switzerland, Great Britain, Italy; eastern Europe: Russia (Moscow), south-west Russia (Krasnodar), Poland; northern Europe: Sweden. Birds from Italy and south-west Russia (*P. v. karelini*) all possess the most common haplotype. The illustrations show that each lineage is further characterized by distinctive facial patterns. *Picus viridis viridis* and *P. v. karelini* belong to the same lineage and exhibit the same facial pattern characterized by a black mask. In *P. v. vaillantii* the male possesses a black moustache and the female a dark crown.

Table 3 Variability of the Z-linked BRM intron 15 in 50 allopatric individuals of *Picus viridis viridis* and *P. v. sharpei*.

	$N_{individuals}$			Heterozygote			
	Males	Females	Unknown	Males (%)	$N_{alleles}$	$N_{polymorphic\ sites}$	$N_{fixed\ mutations}$*
P. v. viridis†	10	8	22	0	1	0	3
P. v. sharpei (central Spain)	6	2	2	50	4 or 6‡	4	

*Number of diagnostic mutations existing between *P. v. viridis/P. v. karelini* and *P. v. sharpei*.
†Samples were from France (n = 25), Great Britain (n = 2), Switzerland (n = 2), Italy (n = 3), Sweden (n = 5), Poland (n = 1), Russia (n = 2).
‡We could not unambiguously find the exact number of alleles because one male had more than one polymorphic site.

Table 4 Genetic differentiation among geographical populations of *Picus viridis viridis–P. v. karelini* (n = 64) and geographical populations of *P. v. sharpei* (n = 45).

viridis/karelini	Western Europe	Eastern Europe	*sharpei*	Central Spain	North-east Spain
Northern Europe	0.91*	0.76*	South France	0.12*	0.12 n.s.
Eastern Europe	0.65*		North-east Spain	0.009 n.s.	

Population pairwise Φ_{ST} using Tamura–Nei distance. P-values: *$P < 0.001$; n.s. $P > 0.05$.

when moving from central Spain towards southern France (Fig. 6a). Concerning *P. v. viridis*, the western European populations are less variable than their northern and eastern counterparts (Fig. 6b). The same trends were observed for nucleotide diversity estimates, which were an order of magnitude lower in the western population of *viridis* than in eastern

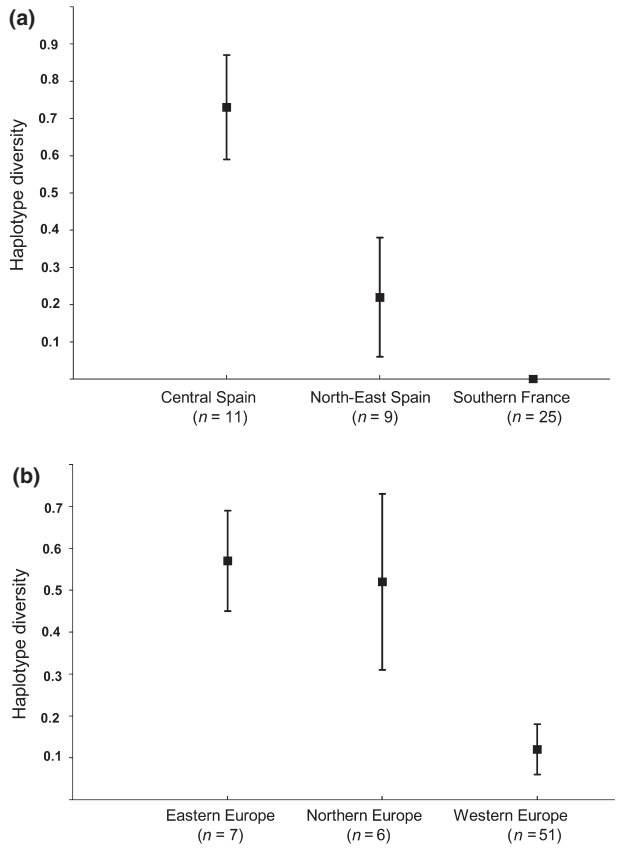

Figure 6 (a) Geographical variation in haplotype diversity within *Picus viridis sharpei*. The haplotype diversity sharply decreases from central Spain to southern France, where it equals zero. (b) Geographical variation in haplotype diversity within the *viridis* lineage (subspecies *viridis* and *karelini)*. The haplotype diversity sharply decreases from northern and eastern Europe to western Europe. Estimates are given with standard errors.

and northern *viridis*/*karelini* populations ($\pi_{\text{northern Europe}} = 0.0025 \pm 0.0021$; $\pi_{\text{eastern Europe}} = 0.0013 \pm 0.0014$; $\pi_{\text{western Europe}} = 0.00028 \pm 0.00050$). Nucleotide diversity values also decreased on a north/east axis for *sharpei* ($\pi_{\text{central Spain}} = 0.0029 \pm 0.0022$; $\pi_{\text{north Spain}} = 0.00052 \pm 0.00077$; $\pi_{\text{southern France}} = 0$).

Geographical distribution of cyt *b* and Z-linked BRM intron 15 in southern France

We sequenced the Z-linked BRM intron-15 locus and a partial fragment of the cyt *b* for 35 and 36 green woodpeckers from southern France (Languedoc-Roussillon, Midi-Pyrénées), respectively. We found a mixture of cyt *b* haplotypes belonging to *sharpei* (69%) or to *viridis* (31%), highlighting the parapatric distribution of these two subspecies in this region. Mitochondrial results were confirmed by the Z-linked BRM alleles found in the same localities, which belonged either to *sharpei* (66%) or to *viridis* (26%). Three males were heterozygous for fixed mutations in allopatric individuals ($n = 50$) and thus could not be assigned either to *viridis* or to *sharpei*. These individuals may represent hybrids or backcrosses. We never found *sharpei* cyt *b* haplotypes outside Languedoc-Roussillon and the Midi-Pyrénées in France ($n = 21$). Similarly, we never detected *viridis* haplotypes in Catalonia ($n = 10$). This result was also recovered with the Z-linked BRM. In summary, these results suggest that the contact zone is probably limited to southern France, although a larger sample size would be necessary to address this point fully.

DISCUSSION

Phylogenetic relationships

Based on the analysis of the multilocus data set, the monophyly of *P. viridis* was recovered with strong support (PP = 1.0, bootstrap percentage = 99%). The North African taxon was the sister group of a clade that comprised two divergent European lineages. This topology was further supported by three insertion/deletion events (Fig. 4). The closest relatives of *P. viridis* could not be recovered with high confidence. *Picus canus* was found to be sister to *P. viridis* in the nuclear data set, but only with moderate support. The mitochondrial data set could not recover the monophyly of *P. viridis* – neither could it resolve the relationships with its closest relatives. Such a lack of resolution may be explained by the nearly simultaneous origin of four lineages that differentiated into *P. erythropygius*, *P. canus*, *P. viridis*/*karelini*/*sharpei* and *P. v. vaillantii*. It is striking to note that a better resolution of this node was achieved with nuclear markers than with mtDNA, despite the number of variable characters in the nuclear data set being smaller (nuclear: 220 variable sites; mitochondrial: 367 variable sites). A better resolution of recent nodes is usually expected with mitochondrial data because of its higher mutation rate (Bellemain *et al.*, 2008; Zink & Barrowclough, 2008). We attribute the lower resolution of the mitochondrial tree when compared with the nuclear tree to homoplasy. Hence, our study underlines the fact that the use of multiple independent nuclear genes may help to resolve relationships among closely related species that differentiated recently.

Species and subspecies limits

Our results provide some new insights into the genetic structure within *P. viridis*. The geographical distribution of genetic discontinuities helps to soundly establish the species and subspecies limits that best reflect the evolutionary history of taxa (Avise, 2000; Baker, 2007; Price, 2008). The Eurasian green woodpecker, as currently defined, comprises three divergent lineages that occupy distinct geographical ranges in Europe, Spain/Portugal and North Africa, respectively. Each genetic lineage is further characterized by distinctive plumage characters.

The North African taxon diverged from its European counterparts during the Pleistocene (1.6–2.2 Ma), although

the 95% HPD of all analyses extended slightly into the Pliocene. Such a divergence, and lack of subsequent gene flow, is old enough to allow the reciprocal monophyly of the mitochondrial and nuclear loci we analysed. In addition to differentiation in neutral markers, the North African green woodpecker exhibits several diagnostic plumage characters (Cramp, 1985; Winkler & Christie, 2002). As this lineage differs in several functionally independent genetic and phenotypic traits, we think, in line with some previous authors (Peters, 1948; Vaurie, 1959; Voous, 1973), that species rank *Picus vaillantii* (Malherbe, 1847) is the best taxonomic treatment.

Our results also showed reciprocal monophyly in mtDNA and nuclear loci between the Iberian green woodpecker (*sharpei*) and all other green woodpeckers distributed across Europe (*viridis*, *karelini*). The Iberian taxon could also be distinguished from other Eurasian green woodpeckers by several discrete plumage characters (greyish ear-coverts and greyish area around eyes, non-barred undertail coverts; see Cramp, 1985; Winkler & Christie, 2002). The Iberian green woodpecker would clearly deserve a species status under the phylogenetic species concept (Cracraft, 1983; Helbig *et al.*, 2002). However, our genetic results show that *P. v. viridis* and *P. v. sharpei* are parapatric and meet in a secondary contact zone in southern France, where they form a narrow hybrid zone (Pons *et al.*, in prep.). Such a narrow hybrid zone may suggest the formation of a very recent contact zone or severe restriction in gene flow, and thus an advanced speciation process (Barton & Hewitt, 1985). We prefer not to change the taxonomic status of the Iberian form, however, until further ecological and genetic studies have been completed (Pons *et al.*, in prep.).

On the basis of slight differences in size and plumage, birds from Italy and the Caucasus have been assigned to the *karelini* subspecies (Winkler & Christie, 2002). Our genetic results do not reveal any divergence that may favour such a taxonomic treatment. All green woodpeckers currently assigned to *P. v. karelini* share with *P. v. viridis* the most common mtDNA haplotype found everywhere across Europe (except in the Iberian peninsula and Sweden). Assigning an inappropriate subspecies rank to a population may obscure our understanding of the evolutionary history of a species (Zink, 2004). We thus agree with Short (1982) and Vaurie (1959), who suggested that the subspecies *karelini* should be synonymized with *P. v. viridis*. By contrast, we found that eastern (Poland, Russia) and northern (Sweden) populations hold some private haplotypes that reveal a slight but significant genetic differentiation without any apparent differences in plumage characters. Given the low genetic divergence and lack of diagnosibility, these geographical populations seem not to deserve any subspecies status.

Although we were not able to include the Iranian *innominatus* subspecies in our sampling, we are confident in our phylogenetic results regarding the reciprocal monophyly of the three lineages highlighted here. Indeed, there is a good agreement between genetic discontinuities and plumage variation within the *P. viridis* complex. As mentioned above, each genetic lineage (*viridis/karelini*, *sharpei*, *vaillantii*) is further characterized by discrete plumage characters. As the Iranian birds are only slightly different in plumage from the *viridis* birds (head side and underparts are slightly paler and upperparts are less green) and do not exhibit any discrete differences in plumage characters (Winkler & Christie, 2002), there is little chance that the Iranian population belongs to a fourth deeply differentiated lineage. To obtain a complete picture of the intraspecific taxonomy of *P. viridis*, it would be necessary to include the south-west Iranian population in a molecular study.

Biogeographical aspects

Old separation between the European and North African lineages

The most parsimonious pattern supported by our results, in conjunction with the biogeographical history of the genus *Picus* (Fuchs *et al.*, 2008), consists of an ancient colonization dating back to the early Pleistocene of both Europe and North Africa by an eastern lineage. Following this epoch, the African and European lineages evolved independently without gene flow. The Gibraltar Strait, which is only 14 km of open sea, thus constituted an efficient geographical barrier that has prevented gene flow between North Africa and Europe since then. The lack of connectivity between populations on either side of the Strait of Gibraltar could be a result of the low dispersal abilities of the green woodpecker, a sedentary woodland species that is absent from Ireland and Mediterranean islands. Brito (2005) found a similar pattern for the tawny owl (*Strix aluco*), as North African and European individuals belonged to two distinct lineages. The Strait of Gibraltar may now also constitute a major barrier to gene flow for more vagile, but woodland- and forest-adapted bird species such as the pied flycatcher (*Ficedula hypoleuca*, Saetre *et al.*, 2001) and blue tit (*Parus caeruleus*, Salzburger *et al.*, 2002). This pattern is in complete contrast to what is described for arid-adapted birds, as several recent colonization events from North Africa to Spain (Thekla lark, *Galerida theklae*, Guillaumet *et al.*, 2006; Dupont's lark, *Chersophilus duponti*, García *et al.*, 2007; trumpeter finch, *Bucanetes githagineus*, Barrientos *et al.*, 2009) or from Spain to North Africa (crested lark, *Galerida cristata*, Guillaumet *et al.*, 2006) have been described. In a comparative study involving bats, which exhibit flight abilities comparable to birds, García *et al.* (2009) highlighted that the Strait of Gibraltar acted either as a bridge or as an efficient geographical barrier to bats' movements in accordance with several ecological and historical factors that interact in a complex way. It is thus difficult at present to draw any general conclusions about the degree of permeability of the Strait of Gibraltar with regard to birds or bats, although existing data might suggest that arid-adapted bird species are more prone to disperse across the Strait of Gibraltar.

Mid-Pleistocene divergence of the two European lineages

All our divergence date estimates suggest a mid-Pleistocene split between the two European lineages, of which one is restricted to the Iberian Peninsula and southern France and the other is found in the rest of Europe up to western Russia. Similar phylogeographical patterns, with two or three European lineages having differentiated during the Pleistocene in southern glacial refugia, have been described for a number of animal and plant species (Taberlet *et al.*, 1998; Hewitt, 2000, 2001; Schmitt, 2007). They almost certainly result from Pleistocene climatic oscillations, which led to successive expansions and contractions of species ranges, favouring allopatric divergence and in some cases speciation (Hewitt, 2000, 2001). With regard to the Eurasian green woodpecker, our results also support the Pleistocene climatic oscillations as the most probable biogeographical scenario that would explain its genetic diversification.

Pyrenean suture zone

The geographical distribution of cyt *b* and Z-linked BRM intron-15 alleles in southern France shows that the two European lineages came into secondary contact along the Pyrenees chain and in the Languedoc-Roussillon region. This secondary contact zone was probably established after the Last Glacial Maximum (LGM, 0.23–0.18 Ma), when the climate warmed and the ice retreated, allowing many temperate species to expand their range (Hewitt, 2004). Interestingly, this time interval is within the 95% HPD for the time to the most recent common ancestor (TMRCA) estimate for the *sharpei* and *viridis/karelini* populations (0.05–0.14 Ma and 0.13–0.18, respectively; Table 2). The Pyrenees chain has long been recognized as an important suture zone (Schmitt, 2007) for a diverse range of organisms (insects: *Chorthippus parallelus*, Taberlet *et al.*, 1998; plants: *Alnus glutinosa*, Hewitt, 2001; birds: *Phylloscopus collybita*, Helbig *et al.*, 2001). Brito (2007) described a more complex situation for the tawny owl (*Strix aluco*), as the location of the contact zone was dependent on the type of genetic marker analysed. In accordance with the predictions of population genetic theory, that contact zones tend to move towards natural barriers, where population densities are low and dispersal is limited (Barton & Hewitt, 1985), microsatellites placed the contact between the Iberian and the Balkan lineages close to the Pyrenees. In contrast, mtDNA haplotypes of tawny owls showed a steep transition further south from the Pyrenees, between the populations of Portugal and Madrid, where 65% of haplotypes belonged to the Balkan clade. Such a discrepancy in phylogeographical patterns among co-distributed species may be attributable to a number of factors, such as sex-biased dispersal, differences in location and size of the refugia, population density and stochastic factors (mutation and genetic drift). The recent development of methods that allow us to explicitly test the existence of common phylogeographical patterns among co-distributed species (Hickerson *et al.*, 2006) may help to further clarify the evolution of the Palaearctic communities through time and space.

Glacial refugia and colonization routes

Our results highlight the importance of the Iberian Peninsula as a major glacial refugium for the Eurasian green woodpecker. The important role of Iberia as a Pleistocene refugium for European temperate species has been underlined by a number of recent phylogeographical studies covering a wide range of organisms (reviewed by Weiss & Ferrand, 2007). The mismatch distribution of haplotypes and Fu's F_S test suggest that the Iberian population has undergone a rapid expansion. As the genetic diversity is much higher in central Spain than in Catalonia and southern France, we suggest, in line with the leading-edge expansion hypothesis (Hewitt, 2000), a northward colonization of the whole Iberian Peninsula by the *sharpei* lineage from permanent refugia located somewhere in southern or central Spain. The existence of restricted southern refugia within the Iberian Peninsula has recently been proposed for terrestrial mammals and other organisms (Weiss & Ferrand, 2007; Centeno-Cuadros *et al.*, 2009) but remains to be evaluated with regard to birds.

The 'star-like' network of the *viridis/karelini* lineage, with a common widespread haplotype at the centre of the network, and rare poorly differentiated variants radiating from this ancestral sequence, is typical of populations having experienced a recent expansion from a unique refugium. Overall, we found a low level of genetic differentiation within that lineage that dated back to the end of the LGM, although the 95% HPD for the TMRCA for the haplotypes is very large. By contrast, there was a fairly strong geographical structure of the genetic variation among three geographical clusters. The northern population from Sweden holds private haplotypes not found elsewhere in Europe. The eastern population from Poland and Russia comprises the most common haplotype and a recently derived haplotype that differs from the common one by only one mutation step. Hence these geographical populations harbour a higher genetic diversity than western populations from England, France and Italy (Fig. 6b), suggesting a westward colonization route. We could not identify with certainty the exact location of the eastern refugia: one possible refugium could be situated in the Carpathians, where potentially bank voles (*Clethrionomys glareolus*) survived during the LGM (Kotlík *et al.*, 2006). Unlike most studies that focused on the Palaearctic, our data do not favour the Italian peninsula as a possible glacial refugium for the Eurasian green woodpecker. Only the most common haplotype was found in Italy, a pattern not expected if Italy acted as a glacial refugium. Brito (2005) found a high genetic diversity in tawny owl individuals from northern Italy, which may have constituted a glacial refugium for this species. Eurasian green woodpeckers from southern Italy, an area known to harbour

endemic lineages of vertebrates (birds, Brito, 2007; Brambilla *et al.*, 2008; mammals and fish, Grill *et al.*, 2009), would nevertheless be necessary to definitively rule out the possibility that Italy was a refugium for the Eurasian green woodpecker during cooling Pleistocene periods.

It is worth pointing out that we included individuals sampled in the Caspian/Caucasian region of Krasnodar, where highly differentiated populations of birds have been described (Drovetski *et al.*, 2004; Hewitt, 2004; Pavlova *et al.*, 2005; Hansson *et al.*, 2008). However, in the present case, these individuals hold the most common haplotype and thus do not reveal any signs of endemism, a pattern also recovered for other birds (Zink *et al.*, 2002, 2006, 2009). Hence the bird community of the Caspian/Caucasian area is a mixture of species that have persisted there through time (e.g. *Motacilla alba*, *Troglodytes troglodytes*, *Sitta europaea*) and of species that recently recolonized the area (e.g *Picus viridis*, *Dendrocopos major*). In summary, our genetic results favour the existence of only one eastern refugium, from which the present range of the Eurasian green woodpecker (*viridis/karelini*) was recently recolonized. Further sampling including birds from the Balkans, Turkey and Anatolia would be necessary to precisely locate this eastern refugium and fully unravel the phylogeographical pattern of the Eurasian green woodpecker.

ACKNOWLEDGEMENTS

We are very grateful to S. Birks (University of Washington, Burke Museum, Seattle, UWBM), G. Boano (Museo Civico di Storia Naturale di Carmagnola, MCSN), A. Cibois (Museum of Natural History, Geneva, MNHG), G. Frisk and P.G.P. Ericson (Swedish Museum of Natural History, Stockholm, NRM), E. Garcia Franquesa (Museu de Ciències Naturals, Barcelona, MCN), O. Llama Palacios and I. Rey Fraile (Museo Nacional de Ciencias Naturales, Madrid, MNCN), R. Prys-Jones and M. Adams (Natural History Museum, Tring, UK, NHM) for tissue loans as well as to all contributors listed in Appendix S1. Laboratory help was provided by C. Bonillo, J. Lambourdière and A. Tillier at the Muséum National d'Histoire Naturelle, Paris (MNHN). We are also grateful to M.A. El Agbani (University Mohammed V), A. Qninba (CEMO) and Hamid Rguibi Idrissi (University Chouaib Doukkali) for hospitality and sample acquisition in Morocco. We thank D.A. Pons and D.V. Pons, who helped to draw Fig. 5. This work was supported by the Plan Pluriformation 'Evolution et Structure des Ecosystèmes', the 'Consortium National de Recherche en Génomique', and the 'Service de Systématique Moléculaire' of the Muséum National d'Histoire Naturelle (IFR 101). This study is part of agreement no. 2005/67 between the Genoscope and the Muséum National d'Histoire Naturelle on the project 'Macrophylogeny of life' directed by Guillaume Lecointre. J.F. was supported by a postdoctoral fellowship from the DST/NRF Centre of Excellence at the Percy FitzPatrick Institute.

REFERENCES

Avise, J.C. (2000) *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge, MA.

Avise, J.C. (2009) Phylogeography: retrospect and prospect. *Journal of Biogeography*, **36,** 3–15.

Baker, A.J. (2007) Molecular advances in the study of geographical variation and speciation in birds. *Ornithological Monographs*, **63,** 18–29.

Baker, A.J., Tavares, E.S. & Elbourne, R.F. (2009) Countering criticisms of single mitochondrial DNA gene barcoding in birds. *Molecular Ecology Resources*, **9,** 257–268.

Bandelt, H.J., Forster, P. & Rohl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16,** 37–48.

Barrientos, R., Kvist, L., Barbosa, A., Varela, F., López-Iborra, G.M. & Moreno, E. (2009) Colonization patterns and genetic structure of peripheral populations of the trumpeter finch (*Bucanetes githagineus*) from Northwest Africa, the Canary Islands and the Iberian Peninsula. *Journal of Biogeography*, **36,** 210–219.

Barton, N.H. & Hewitt, G.M. (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16,** 113–148.

Bellemain, E., Bermingham, E. & Ricklefs, R.E. (2008) The dynamic evolutionary history of the bananaquit (*Coereba flaveola*). *BMC Evolutionary Biology*, **8,** 240. doi: 10.1186/1471-2148-8-240.

Brambilla, M., Vitulano, S., Spina, F., Baccetti, N., Gargallo, G., Fabbri, E., Guidali, F. & Randi, E. (2008) A molecular phylogeny of the *Sylvia cantillans* complex: Cryptic species within the Mediterranean basin. *Molecular Phylogenetics and Evolution*, **48,** 461–472.

Brito, P.H. (2005) The influence of Pleistocene glacial refugia on tawny owl genetic diversity and phylogeography in western Europe. *Molecular Ecology*, **14,** 3077–3094.

Brito, P.H. (2007) Contrasting patterns of mitochondrial and microsatellite genetic structure among Western European populations of tawny owls (*Strix aluco*). *Molecular Ecology*, **16,** 3423–3437.

Centeno-Cuadros, A., Delibes, M. & Godoy, J.A. (2009) Phylogeography of southern water vole (*Arvicola sadipus*): evidence for refugia within the Iberian glacial refugium. *Molecular Ecology*, **18,** 3652–3667.

Cracraft, J. (1983) Species concepts and speciation analysis. *Current ornithology* (ed. by R.F. Johnston), pp. 159–187. Plenum Press, New York.

Cramp, S. (1985) *The birds of the Western Palearctic*, Vol. 4. Oxford University Press, Oxford.

Drovetski, S.V., Zink, R.M., Rohwer, S., Fadeev, I.V., Nesterov, E.V., Karagodin, I., Koblik, E.A. & Red'kin, Y.A. (2004) Complex biogeographic history of a Holarctic passerine. *Proceedings of the Royal Society B: Biological Sciences*, **271,** 545–551.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7,** 214.

Drummond, A.J., Nicholls, G.K., Rodrigo, A.G. & Solomon, W. (2002) Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data. *Genetics*, **161,** 1307–1320.

Drummond, A.J., Ho, S.Y.W., Phillips, M.J. & Rambaut, A. (2006) Relaxed phylogenetics and dating with confidence. *PLoS Biology*, **4,** 699–710.

Edwards, S. & Bensch, S. (2009) Looking forwards or looking backwards in avian phylogeography? A comment on Zink and Barrowclough 2008. *Molecular Ecology*, **18,** 2930–2933.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin version 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1,** 47–50.

Fuchs, J., Ohlson, J.I., Ericson, P.G.P. & Pasquet, E. (2006) Molecular phylogeny and biogeographic history of the piculets (Piciformes: Picumninae). *Journal of Avian Biology*, **37,** 487–496.

Fuchs, J., Ohlson, J.I., Ericson, P.G.P. & Pasquet, E. (2007) Synchronous intercontinental splits between assemblages of woodpeckers suggested by molecular data. *Zoologica Scripta*, **36,** 11–25.

Fuchs, J., Pons, J.-M., Ericson, P.G.P., Bonillo, C., Couloux, A. & Pasquet, E. (2008) Molecular support for a rapid cladogenesis of the woodpecker clade Malarpicini, with further insights into the genus *Picus* (Piciformes: Picinae). *Molecular Phylogenetics and Evolution*, **48,** 34–46.

Galtier, N., Nabholz, B., Glémin, S. & Hurst, G.D.D. (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology*, **18,** 4541–4550.

García, J.L., Ibánez, C. & Juste, J. (2009) The Straits of Gibraltar: barrier or bridge to Ibero-Moroccan bat diversity. *Biological Journal of the Linnean Society*, **96,** 434–450.

García, J.T., Suárez, F., Garza, V., Calero-Riestra, M., Hernández, J. & Pérez-Tris, J. (2007) Genetic and phenotypic variation among geographically isolated populations of the globally threatened Dupont's lark *Chersophilus dupontii*. *Molecular Phylogenetics and Evolution*, **46,** 237–251.

Grill, A., Amori, G., Aloise, G., Lisi, I., Tosi, G., Wauters, L.A. & Randi, E. (2009) Molecular phylogeography of European *Sciurus vulgaris*: refuge within refugia? *Molecular Ecology*, **18,** 2687–2699.

Guillaumet, A., Pons, J.-M., Godelle, B. & Crochet, P.-A. (2006) History of the Crested Lark in the Mediterranean region as revealed by mtDNA sequences and morphology. *Molecular Phylogenetics and Evolution*, **39,** 645–656.

Hansson, B., Hasselquist, D., Tarka, M., Zehtinjiev, P. & Bensch, S. (2008) Postglacial colonisation patterns and the role of isolation and expansion in driving diversification in a passerine bird. *PLoS ONE*, **3,** e2794. doi: 10.1371/journal.pone.00022794.

Helbig, A.J., Salomon, M., Bensch, S. & Seibold, I. (2001) Male-biased gene flow across an avian hybrid zone: evidence from mitochondrial and microsatellite DNA. *Journal of Evolutionary Biology*, **14,** 277–287.

Helbig, A.J., Knox, G., Parkin, T., Sangster, G. & Collison, M. (2002) Guidelines for assigning species rank. *Ibis*, **144,** 518–525.

Hewitt, G. (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405,** 907–913.

Hewitt, G.M. (2001) Speciation, hybrid zones and phylogeography – or seeing genes in space and time. *Molecular Ecology*, **10,** 537–549.

Hewitt, G.M. (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359,** 183–195.

Hickerson, M.J., Stahl, E.A., Lessios, H.A. & Crandall, K. (2006) Test for simultaneous divergence using approximate Bayesian computation. *Evolution*, **60,** 2435–2453.

Ho, S.Y.W. (2007) Calibrating molecular estimates of substitution rates and divergence times in birds. *Journal of Avian Biology*, **38,** 409–414.

Huelsenbeck, J.P. & Ronquist, F. (2003) *MRBAYES: a program for the Bayesian inference of phylogeny, version 3.0b4*. Available at: http://mrbayes.csit.fsu.edu/.

Juste, J., Bilgin, R., Muñoz, J. & Ibáñez, C. (2009) Mitochondrial DNA signatures at different spatial scales: from the effects of the Straits of Gibraltar to population structure in the meridional serotine bat (*Eptesicus isabellinus*). *Heredity*, **103,** 1–10.

Kotlík, P., Deffontaine, V., Mascheretti, S., Zima, J., Michaux, J.R. & Searle, J.B. (2006) A northern glacial refugium for bank voles (*Clethrionomys glareolus*). *Proceedings of the National Academy of Sciences USA*, **103,** 1860–1864.

Leroy, S. & Arpe, K. (2007) Glacial refugia for summer-green trees in Europe and south-west Asia as proposed by ECHAM3 time-slice atmospheric simulations. *Journal of Biogeography*, **34,** 2115–2128.

McDonald, J.H. & Kreitman, M. (1991) Adaptive protein evolution at the *Adh* locus in *Drosophila*. *Nature*, **351,** 652–654.

Minin, V., Abdo, Z., Joyce, P. & Sullivan, J. (2003) Performance-based selection of likelihood models for phylogeny estimation. *Systematic Biology*, **52,** 1–10.

Nielsen, R. (2005) Molecular signatures of natural selection. *Annual Review of Genetics*, **39,** 197–218.

Pavlova, A., Zink, R.M., Rohwer, S., Koblik, E.A., Red'kin, Y.A., Fadeev, I.V. & Nesterov, E.V. (2005) Mitochondrial DNA and plumage evolution in the white wagtail *Motacilla alba*. *Journal of Avian Biology*, **36,** 322–336.

Peters, J.L. (1948) *Check-list of birds of the world*, Vol. 6. Museum of Comparative Zoology, Harvard University Press, Cambridge, MA.

Posada, D. & Crandall, K.A. (2001) Intraspecific phylogenetics: trees grafting into networks. *Trends in Ecology and Evolution*, **16,** 37–45.

Price, T. (2008) *Speciation in birds*. Roberts and Company Publishers, Greenwood Village, CO.

Rambaut, A. & Drummond, A.J. (2007) *TRACER v1.4*. Available at: http://beast.bio.ed.uk/software/seal.

Ronquist, F. & Huelsenbeck, J.P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19,** 1572–1574.

Rozas, J., Sánchez-Del Barrio, C., Messeguer, X. & Rozas, R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19,** 2496–2497.

Saetre, G.P., Borge, T. & Moum, T. (2001) A new bird species? The taxonomic status of the ''Atlas Flycatcher'' assessed from DNA sequence analysis. *Ibis*, **143,** 494–497.

Salzburger, W., Martens, J. & Sturmbauer, C. (2002) Paraphyly of the blue tit (*Parus caeruleus*) suggested from cytochrome *b* sequences. *Molecular Phylogenetics and Evolution*, **24,** 19–25.

Schmitt, T. (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology*, **4,** 11. doi: 10.1186/1742-9994-4-11.

Short, L.L. (1982) *Woodpeckers of the world*. Delaware Museum of Natural History, Greenville, DE.

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, **22,** 2688–2690.

Stamatakis, A., Hoover, P. & Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML web-servers. *Systematic Biology*, **75,** 758–771.

Subramanian, S., Denver, D.R., Millar, C.D., Heupink, T., Aschrafi, A., Emslie, S.D., Baroni, C. & Lambert, D.M. (2009) High mitogenomic evolutionary rates and time dependency. *Trends in Genetics*, **25,** 482–486.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G. & Cosson, J.-F. (1998) Comparative phylogeography and post glacial colonization. *Molecular Ecology*, **7,** 453–464.

Vaurie, C. (1959) Systematic notes on Palearctic birds. No. 34. Picidae: the genera *Picus* and *Dryoscopus*. *American Museum Novitates*, **1945,** 20–21.

Voous, K.H. (1973) List of recent Holarctic bird species. Non-passerines. *Ibis*, **115,** 612–638.

Voous, K.H. (1977) *List of recent Holarctic bird species*. British Ornithologists' Union/Academic Press Ltd, London.

Weiss, S. & Ferrand, N. (2007) *The phylogeography of southern European refugia*. Springer, Dordrecht.

Winkler, H. & Christie, D.A. (2002) Family Picidae (woodpeckers). *Handbook of the birds of the world, Vol. 7. Jacamars to woodpeckers* (ed. by J. Del Hoyo, A. Elliot and J. Sargatal), pp. 296–555. Lynx Edicions, Barcelona.

Woodruff, D.S. (2003) Neogene marine transgressions, palaeogeography and biogeographic transitions on the Thai-Malay Peninsula. *Journal of Biogeography*, **30,** 551–567.

Zink, R.M. (2004) The role of subspecies in obscuring avian biological diversity and obscuring conservation policy. *Proceedings of the Royal Society B: Biological Sciences*, **272,** 71–78.

Zink, R.M. & Barrowclough, G.F. (2008) Mitochondrial DNA under siege in avian phylogeography. *Molecular Ecology*, **9,** 2107–2121.

Zink, R.M., Drovetski, S.V. & Rohwer, S. (2002) Phylogeographic patterns in the great spotted woodpecker *Dendrocopos major* across Eurasia. *Journal of Avian Biology*, **33,** 175–178.

Zink, R.M., Drovetski, S. & Rowher, S. (2006) Selective neutrality of mitochondrial ND2 sequences, phylogeography and species limits in *Sitta europaea*. *Molecular Phylogenetics and Evolution*, **40,** 679–686.

Zink, R.M., Pavlova, A., Drovetski, S., Wink, M. & Rohwer, S. (2009) Taxonomic status and evolutionary history of the *Saxicola torquata* complex. *Molecular Phylogenetics and Evolution*, **52,** 769–773.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 List of specimens of *Picus viridis* subspecies and localities included in the phylogeographic analyses. GenBank numbers are given for each cytochrome *b* haplotype and each BRM intron-15 sequence.

Appendix S2 List of taxa studied in the phylogenetic analyses, and GenBank accession numbers.

Appendix S3 Bayesian tree for each nuclear locus (β-fibrinogen intron 7, GAPDH intron 11, myoglobin intron 2, transforming growth factor β-2 intron 5 and BRM intron 15).

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

BIOSKETCH

Jean-Marc Pons is a researcher at the Museum of Natural History in Paris, France, and is interested mainly in the evolutionary history of birds and in the mechanisms of speciation.

Author contributions: J.M.P. performed some of the laboratory work, carried out the population genetic analyses and wrote the first draft of the manuscript; G.O. helped to collect tissue samples and commented on the manuscript; C.C. performed some of the laboratory work; and J.F. performed the phylogenetic and dating analyses and helped to draft the manuscript.

Editor: Malte Ebach