Pronounced genetic structure and low genetic diversity in European red-billed chough (*Pyrrhocorax pyrrhocorax*) populations

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Abstract

The red-billed chough (*Pyrrhocorax pyrrhocorax*) is of conservation concern in the British Isles and continental Europe, with historically declining populations and a highly fragmented distribution. We quantified the distribution of genetic variation within and among European populations to identify isolated populations that may need to be managed as demographically independent units, and assess whether individual populations are denuded of genetic diversity and so may show reduced viability. We genotyped 326 choughs from ten wild populations and 22 from one captive population at 16 nuclear microsatellite loci, and sequenced 34 individuals across three mitochondrial regions to quantify genetic structure, diversity and phylogeography. Microsatellite diversity was low (often less than 4 alleles per locus), but pairwise population differentiation was high (often $D_{est} > 0.1$), with a signature of isolation-by-distance. Bayesian-inferred *a posteriori* genetic clusters coincided with *a priori* populations, supporting strong genetic structure. Microsatellites also allowed us to identify the probable origin of the captive choughs and one recently founded wild population. Mitochondrial DNA sequence diversity was low ($\pi = 0.00103$). Phylogeographic structure was consequently poorly resolved, but indicated that sampled continental-European populations are ancestral to British Isles populations, which comprised a single clade. Our data suggest that British Isles chough populations are relatively isolated with infrequent gene flow and relatively genetically depauperate, potentially requiring genetic management. These findings should be integrated into conservation management policy to ensure long-term viability of chough populations.

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Introduction

Primary goals of conservation genetics are to quantify demographic and genetic connectivity among and genetic diversity within populations of conservation concern, consider the consequences for population viability and apply appropriate management action (Frankham, 1995, 2010a). Small, isolated populations can have increased extinction risk due to demographic, environmental and genetic stochasticity, whereas frequent dispersal and gene flow can counteract these stochas-

tic effects and decrease extinction risk (Lande, 1998; Tallmon et al, 2004). Management intervention may consequently be required to alleviate stochastic loss of genetic diversity and increase long-term adaptive potential in small, isolated populations (Reed and Frankham, 2003; Frankham, 2005, 2010b). Appropriate translocation of wild individuals, or introduction of captive-bred individuals, can successfully increase population viability in such cases (reviewed by Fischer and Lindenmayer, 2000; Frankham, 2005). In this context, quantifying the pattern and degree of population connectivity and genetic diversity can identify the populations and spatial scales on which conservation management may need to focus.

Connectivity can be inferred from patterns of genetic structure and diversity within and among populations, assuming that weak genetic structure and near parity in genetic diversity primarily reflect the homogenising effect of gene flow (e.g. Nichols et al, 2001; Segelbacher et al, 2003; Funk et al, 2007; Techow et al, 2010). Genetic structure and diversity are influenced by both recent and historic processes, so comprehensive characterisation of demographic interactions and evolutionary relationships requires consideration of multiple temporal and spatial scales. The distribution of variation in neutral nuclear markers, such as microsatellite length polymorphisms, indicates genetic structure and diversity arising from contemporary connectivity (Balloux and Lugon-Moulin, 2002). These patterns can be used to consider the need to translocate individuals among wild or captive-bred populations and identify appropriate source populations and the origin of recent natural colonisation events (IUCN, 1998; Frankham, 2008, 2010a). In contrast, genetic structure inferred from mitochondrial DNA sequence variation reflects long-term demographic processes associated with historic geological events such as tectonic movement of land masses, floods or glaciation (Taberlet et al, 1998; Hewitt, 2000). Phylogeographic analysis of mitochondrial sequence variation (Avise et al, 1987) can elucidate evolutionary heritage among populations, clarify taxonomic uncertainties and identify evolutionarily significant units (ESUs; Moritz, 1994) for the management of evolutionary diversity in cryptic species complexes, subspecies and ecologically isolated populations (e.g. Burbrink et al, 2000; Hebert et al, 2004; Segelbacher and Piertney, 2007).

The red-billed chough (*Pyrrhocorax pyrrhocorax*, Corvidae) is a Species of European Conservation Concern with "amber status" (second most critical status) in the United Kingdom (Eaton et al, 2009) due to declining population sizes and contracting European distributions, particularly in the British Isles during the 19th and early 20th centuries (Holloway and Gibbons, 1996). Its current Western European distribution is fragmented and restricted to coastal areas of the British Isles (the Scottish islands of Islay and Colonsay, the Isle of Man, Wales, Cornwall and Ireland) and Brittany, and to parts of the Alps, Spain and Portugal (Monaghan, 1988; Carter et al, 2003; Johnstone et al, 2011). Current published taxonomy recognises a nominate Atlantic coast subspecies *P. p. pyrrhocorax* (British Isles and Brittany) and a Continental European subspecies *P. p. erythrorhamphos* (Vaurie, 1954; Monaghan, 1988), although this distinction was based on few morphological data from unverified museum specimens. The closely-related Alpine chough *Pyrrhocorax graculus* occurs in mountain regions in Southern and Central Europe, particularly the Alps (Delestrade and Stoyanov, 1995).

Multiple censuses of red-billed chough populations were conducted across the British Isles and Brittany from 1963 to 2002 (Johnstone et al, 2007 and references therein). These suggested slight increases in most population sizes after severe decreases prior to the 1950s (Holloway and Gibbons, 1996). Nevertheless, most populations remained small in 2002: Ireland held the largest population (445–838 breeding pairs), followed by Wales (228–262 pairs), Isle of Man (128–150 pairs), Scotland (71–83 pairs, including 56–64 on Islay), Brittany (48–58 pairs) and England (Cornwall) and Northern Ireland (Rathlin) with only one pair each. Since the last UK-wide census in 2002, the population on Islay declined to c. 45 breeding pairs (Reid et al, 2011). These small and decreasing population sizes are causing heightened conservation concern (Kerbiriou et al, 2005; Johnstone et al, 2007).

Most European populations are the focus of some degree of conservation action and demographic study, involving monitoring of breeding success, survival and movements of colour-ring marked individuals. This work has identified intrinsic and extrinsic constraints on population growth rate (e.g. Blanco et al, 1998a; Kerbiriou et al, 2006; Reid et al, 2004, 2006, 2008), and highlighted the key role of human impacts in the chough's decline, involving historic persecution (Monaghan, 1988; Carter et al, 2003), contemporary tourism pressure (Kerbiriou et al, 2009) and agricultural land-

use change (Blanco et al, 1998b; Whitehead et al, 2005; Kerbiriou et al, 2006).

Colour-ring resightings also indicate that choughs in northwestern Europe are typically sedentary and philopatric as long-distance dispersal between populations is very rarely observed (Carter et al, 2003; Reid et al, 2003, 2008; Moore, 2008). Nevertheless, occasional long-distance movements are observed, most notably between North Wales and the Isle of Man during 1997–2004 (c. 100 km; Moore, 2006, 2008). Furthermore, unringed choughs of unknown origin recolonised Cornwall in 2001 after the chough had been extinct there since at least 1973 (Carter et al, 2003). Aided by nest protection and habitat management, this small population has persisted since and comprised five breeding pairs in 2011 (Johnstone et al, 2011). The colonisers are speculated to have originated from the nearest wild populations in Wales or Brittany (Carter et al, 2003). This has not been proven, but is of considerable interest in the context of future genetic management of the small Cornish population (Johnstone et al, 2011).

Overall, it remains unclear whether long-distance dispersal is as rare as suggested by ringing studies, or occurs more frequently but goes undetected by direct observation. The low observed dispersal rates among the small remaining chough populations raise the possibility that many or all remaining populations have low and declining genetic diversity, potentially constituting an additional threat to population persistence that conservation management has not yet identified and integrated into priorities. Genetic diversity has not been comprehensively quantified across all relevant chough populations and molecular markers, with only two previous small-scales studies (Monaghan, 1988; Kocijan and Bruford, 2011). If genetic diversity within the British Isles populations is indeed low, translocation of individuals among populations, or release of captive-bred individuals, may need to be considered (Burgess et al, in press), taking into account genetic compatibility between source and target population (Frankham, 2010a). For this potential purpose, a captive chough population has been sustained in Paradise Park Wildlife Sanctuary, Cornwall (hereafter: "Paradise Park") since the late 1970s (Burgess et al, in review). Documentation and anecdote suggest that at least some ancestors of the captive population came from North Wales (Burgess et al, in press). However, some uncertainty remains over their origin and therefore suitability for release into wild populations (IUCN, 1998; Frankham, 1995, 2010a).

To provide the genetic information required to inform chough conservation management policy, we conducted a large-scale analysis of genetic structure, genetic diversity and phylogeography across British Isles chough populations and a sample of populations from Continental Southwestern Europe. Our objectives were to 1) quantify genetic differentiation among and genetic diversity within populations using microsatellite loci (Wenzel et al, 2011); 2) infer the phylogeographic structure of the sampled populations from nucleotide variation across mitochondrial DNA regions; and 3) identify the likely origins of the choughs that recently recolonised Cornwall and of the ancestors of the captive Paradise Park population.

Materials and Methods

Sample collection

A total of 327 DNA samples were collected from wild red-billed chough populations at eleven locations across the British Isles and Continental Europe, including a single sample from the sole breeding pair in Northern Ireland (Figure 1). This single sample is not useful for estimation of genetic diversity and differentiation for Northern Ireland, but inclusion in phylogeographic analysis can indicate evolutionary relationships with other populations and inform management decisions. In addition, 22 samples were collected from the captive population at Paradise Park. Finally, one sample each was also collected from Alpine choughs (*P. graculus*) in the French Alps and Corsica to use as a phylogeographic outgroup.

Samples were obtained non-invasively and opportunistically from moulted feathers, bones, legs or liver samples from choughs found dead, or remnant egg-shells and membranes from nests, avoiding sampling of known full siblings. The Alpine choughs were blood sampled. Samples were collected over several years for most populations (Table 1).

DNA extraction

DNA was extracted from a 3-5 mm clipping of the lower feather calamus, or scrapings of bone/leg tissue, shreds of liver tissue, fragments of egg-shell and membrane, or 50 µl of well-mixed blood, using Proteinase K diges-

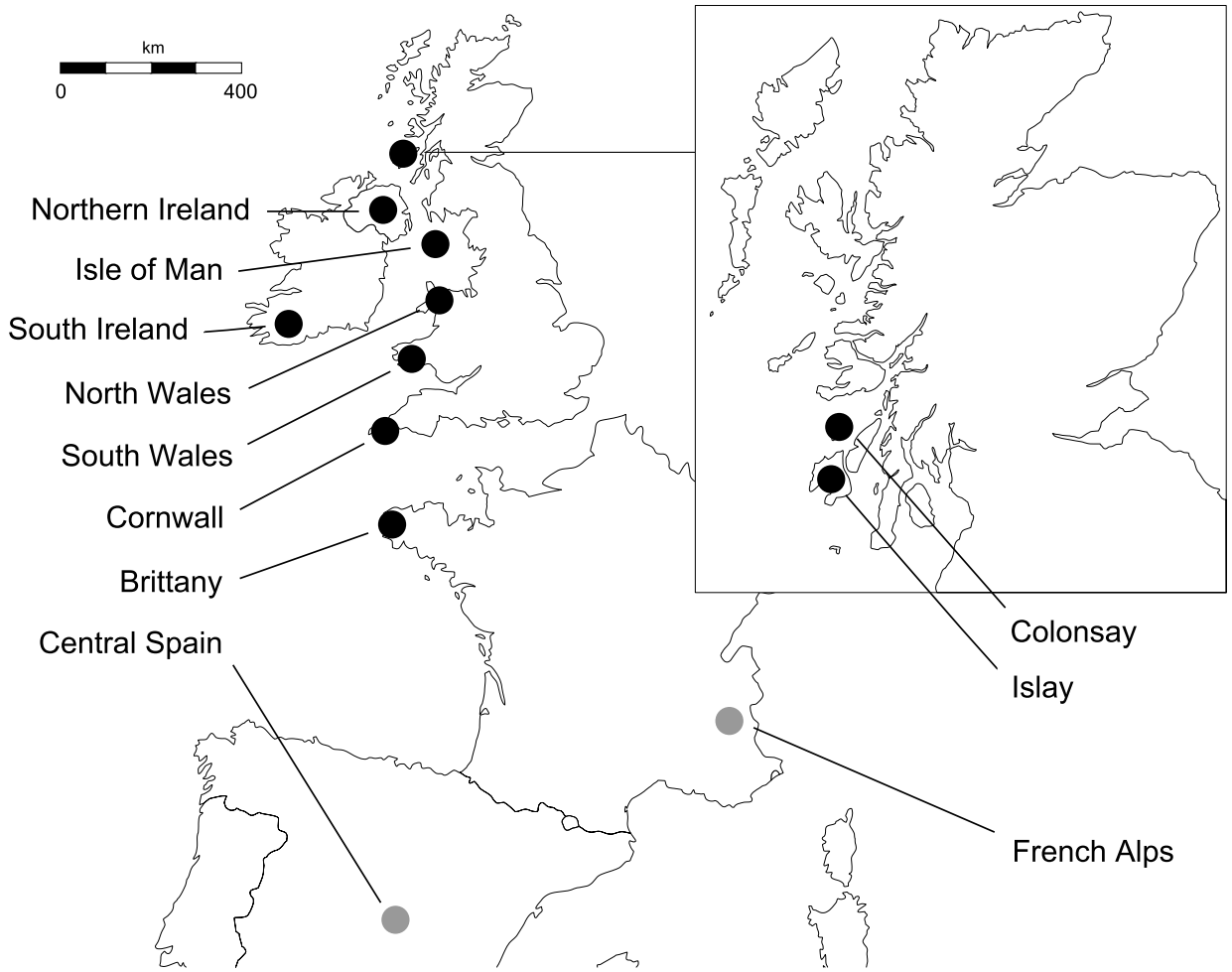

Figure 1: Sampling locations for red-billed chough populations classified by published taxonomy as nominate Atlantic coast subspecies *Pyrrhocorax pyrrhocorax pyrrhocorax* (black circles). For comparison, two Continental European populations (subspecies *P. p. erythrorhamphos*; grey circles) and a captive population at Paradise Park Wildlife Sanctuary, Cornwall, were also sampled.

tion, ammonium acetate precipitation of cell debris and DNA recovery by ethanol precipitation as described in Hogan et al (2008). DNA quality and quantity were assessed with a NanoDrop ND-1000 spectrophotometer.

Molecular sexing

In order to test whether DNA was of sufficient quality for genotyping PCR (Hogan et al, 2008), PCR-based sex determination was attempted for all individuals, using the P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCCAAGGATGAGRAAYTG-3') primers (Griffiths et al, 1998). PCRs were performed in an MJ Research PTC-100 or Thermo Hybaid Px2 thermocycler. The total reaction volume was 20 µl and contained 2.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.2 mM of each nucleotide, 0.5 µM of each primer, 0.5 U of Taq DNA polymerase (Bioline or Sigma-Aldrich) and 20-500 ng of template DNA. An initial denaturation step at 95 °C for 5 min was followed by 30 cycles of annealing at 49 °C for 30 s, elongation at 72 °C for 30 s and denaturation at 95 °C for 30 s, a final annealing step at 49 °C for 1 min and a final elongation step at 72 °C for 5 min. PCR products were checked and scored on 3 % agarose-TBE gels run at 6 V cm^{-1} and stained with WebGreen DNA stain.

Microsatellite genotyping

All individuals were genotyped at 16 microsatellite loci (Ppy-001 to Ppy-016) developed specifically for red-billed chough (Wenzel et al, 2011). A subset of 31 individuals, selected to cover the entire sampled geographic range and as many different alleles as possible, was genotyped twice to estimate genotyping error rates. PCRs were performed in simplex following Wenzel et al (2011), but using TouchDown gradients from

Table 1: Collection years and total and genetically sexed (male, female or unknown) sample sizes of presumed *a priori* red-billed chough populations.

Population	Collection years	Total	Male	Female	Unknown
Colonsay	2005–2011	40	19	15	6
Islay	2004–2011	77	35	29	13
Isle of Man	2004–2011	41	15	23	3
Northern Ireland	2010	1	1	–	–
South Ireland [a]	2010	26	12	9	5
North Wales	2009–2011	73	39	29	5
South Wales	2011	11	5	6	–
Cornwall (wild)	2003–2011	9	3	1	5
Brittany	2005–2010	18	9	7	2
French Alps	2008–2010	14	7	1	6
Spain	2010	17	11	4	2
Paradise Park (captive)	2003–2011	22	9	11	2
Total		349	165	135	49

[a] Beara and south coast; hereafter "Ireland"

60 °C to 50 °C for all loci except for locus Ppy-007, where a 55 °C to 45 °C gradient was used. The 5' end of each forward primer was fluorescently labelled with either 6-FAM, HEX, NED or PET, and genotypes were resolved on an automatic ABI 3730 Capillary DNA sequencer (DNA Sequencing & Services, MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk).

Genotypes were scored by eye using GENEMARKER 1.4 (SoftGenetics). The dataset was checked for genotyping errors and to estimate null-allele frequencies per population using MICROCHECKER 2.2.3 (van Oosterhout et al, 2004). GIMLET 1.3.3 (Valiere, 2002) was used to calculate the unbiased probability that two unrelated individuals drawn at random from each population (or the overall dataset) will have the same genotype (probability of identity P_{ID}; Waits et al, 2001). These probabilities were used to screen the dataset for duplicate samples from the same individual (genotype-grouping function in GIMLET), which were removed.

Observed (H_O) and expected (H_E) heterozygosity at each locus were calculated in GENALEX 6.4 (Peakall and Smouse, 2006). Using an MCMC approach (1000 dememorisations, 100 batches, 1000 iterations), GENEPOP 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008) was used to test for deviations from Hardy-Weinberg equilibrium per locus by performing global χ^2 tests across population-specific F_{IS} (Wright, 1951) estimates (Fisher's method) and to test for linkage disequilibrium between each of 120 locus combinations ($\frac{1}{2} \cdot 16 \cdot 15$) in each of 11 population (= 1320 tests).

Genetic differentiation

Global and pairwise genetic differentiation among eleven *a priori* red-billed chough populations (including Paradise Park but excluding the single Northern Ireland sample) was estimated using the statistics D (Jost, 2008) and F_{ST} (Wright, 1951). The software SPADE (Chao and Shen, 2010) was used to calculate an adjusted estimator for global and pairwise D (D_{est}) with 95 % confidence intervals constructed from 1,000 bootstrap replicates using a percentile method and re-centering (Chao and Shen, 2010). Global and pairwise F_{ST} estimates (Weir and Cockerham, 1984) were calculated in FSTAT 2.9.3.2 (Goudet, 1995, 2002) with a 95 % CI for global F_{ST} constructed from 15,000 bootstrap replicates over loci and significance tests for pairwise F_{ST} performed by randomising multi-locus genotypes between each population pair (1100 permutations; strict Bonferroni-corrected significance level $\alpha = 0.00091$).

Both D_{est} and F_{ST} pairwise estimates of population differentiation (excluding Paradise Park) were then used to test for isolation by distance (Wright, 1943; Slatkin, 1993) using the software IBD 1.52 (Bohonak, 2002). A Mantel test with 1,000 randomisations was performed to test for correlation between D_{est} or $F_{ST}/(1–F_{ST})$ and logarithmic Euclidean geographic distance as proposed for two-dimensional habitat (Rousset, 1997). A linear regression line was constructed using a Reduced Major Axis (RMA) method (Hellberg, 1994).

Bayesian inference of genetic structure

The software STRUCTURE 2.3.3 (Pritchard et al, 2000; Falush et al, 2003) was used to implement Bayesian Markov Chain Monte Carlo (MCMC) inference of *a posteriori* genetic clusters to detect any cryptic genetic structure unidentified by the assumed *a priori* populations (Mank and Avise, 2004). The number of assumed genetic clusters (K) was set from 1 to 11, and 15 runs were performed for each K with 200,000 MCMC iterations (a precursory burn-in of 10,000 iterations was found sufficient) using the admixture ancestry model with correlated allele frequencies. The full analysis was then repeated with the same parameters, but also including *a priori* sampling locations as prior information (LOCPRIOR setting) to detect any further structure unidentified by the standard model (Hubisz et al, 2009; Barlow et al, 2011). To test for spurious results caused by individuals with missing genotype data, all analyses were repeated after excluding individuals with partially missing data.

STRUCTURE HARVESTER 0.6.7 (Earl, 2011) was used to collate the results and infer the statistically best supported K using the ΔK statistic (Evanno et al, 2005). Replicate runs for each K were aligned and averaged in CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007), using the *Greedy* alignment algorithm with 10 randomised input orders, and visualised using DISTRUCT 1.1 (Rosenberg, 2004).

Genetic diversity

Genetic diversity was calculated per population using a variety of statistics. Mean allele numbers and allelic richness (allele numbers rarefacted to a minimum sample size of 4 across all populations in the dataset; Mousadik and Petit, 1996) were calculated in FSTAT. Allele frequencies as calculated by FSTAT were used to count private alleles and to calculate the effective number of alleles per population (Kimura and Crow, 1964; Jost, 2008). Observed (H_O) and expected (H_E) heterozygosity were calculated in GENALEX.

F_{IS} was calculated per population and tested for statistical significance by randomising alleles within populations (3520 randomisations; strict Bonferroni-corrected significance level $\alpha = 0.00028$) in FSTAT in order to identify deviations from Hardy-Weinberg equilibrium and potential substructuring within populations (Wahlund, 1928).

Mitochondrial DNA sequencing

A 1,205 bp segment of the mitochondrial control region was amplified in three individuals per population (chosen to represent a broad geographic area within populations) using the primers JCR03 (Saunders and Edwards, 2000) and H1248 (Tarr, 1995). The single individual from Northern Ireland was included, as were two Alpine choughs as an outgroup.

PCRs were performed in a G-Storm GS1 or MJ Research PTC-100 thermocycler. The total reaction volume was 25 µl and contained 2.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.2 mM of each nucleotide, 0.5 µM of each primer, 0.625 U of Taq DNA polymerase (Bioline or Sigma-Aldrich) and 50-200 ng of template DNA. A denaturation step at 95 °C for 2 min was followed by 20 TouchDown cycles from 60 °C to 50 °C in 0.5 °C decrements (denaturation at 95 °C for 45 s, annealing for 45 s, elongation at 72 °C for 1 min), 15 standard cycles (denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s, elongation at 72 °C for 1 min) and a final elongation step at 72 °C for 10 min. PCR products were checked on 1 % agarose-TBE gels stained with WebGreen DNA stain (run at 9 V cm^{-1}) and purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Using the same primers, DNA sequencing was performed by Eurofins MWG GmbH, Ebersberg, Germany or Beckman Coulter Genomics, Takeley, UK.

In addition, two mitochondrial protein coding regions were PCR amplified using primers designed in PRIMER3 (Rozen and Skaletsky, 2000) based on conserved regions of the consensus sequence of three mitochondrial genomes of species closely related to red-billed chough (retrieved from GENBANK using the Basic Local Alignment Search Tool BLAST [www.ncbi.nlm.nih.gov/blast/]: rook *Corvus frugilegus* accession Y18522, Hume's ground-tit *Pseudopodoces humilis* accession HM535648, and Eastern Orphean warbler *Sylvia crassirostris* accession NC_010229). Fragment CHMT06 corresponded to a 922 bp segment of the NADH1 gene; and fragment CHMT17 contained the final 612 bp of the NADH5 gene, a 9 bp non-coding segment and the first 607 bp of the CYTB gene. PCR amplification conditions were the same as described above, but with different TouchDown temperature gradients (Appendix Table 5).

Inference of phylogeography

Sequences were checked by eye and then aligned in MEGA4. Resolved haplotypes were deposited in GENBANK for each fragment separately. The ingroup sequences of the three fragments were concatenated into one aligned dataset for phylogeographic analyses. Overall haplotype diversity (h) and nucleotide diversity (π) were calculated in DNASP v5 (Librado and Rozas, 2009). A statistical parsimony haplotype network was constructed using TCS v1.21 (Clement et al, 2000).

The software JMODELTEST 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) was used to find the optimal of 88 models of nucleotide evolution for the sequence data (including outgroup sequences) using the Akaike information criterion (AIC; Akaike, 1974). The optimal model (ln likelihood = –5632.84; AIC = 11415.67) was defined as HKY+G (Hasegawa-Kishino-Yano + gamma rate distribution) with base frequencies A = 0.2977, C = 0.2889, G = 0.1339 and T = 0.2795, transition/transversion ratio = 6.8537 and gamma shape = 0.0140. This model was used for a Maximum Likelihood analysis implemented in PAUP* 4.0b10 (Swofford, 2000), using a heuristic search with tree bisection and reconnection (TBR) as the branch-swapping algorithm. Bootstrapping was performed 10,000 times using the Neighbour-Joining method on the same evolutionary model.

Results

Characterisation of microsatellite loci

The number of alleles per microsatellite locus ranged from three (locus Ppy-015) to 14 (loci Ppy-010) (Appendix Table 6). Observed (H_O) and expected (H_E) heterozygosity ranged from 0.05 to 0.66 and 0.07 to 0.71, respectively. Significant deviations from Hardy-Weinberg equilibrium ($\alpha = 0.05$) based on pooled population-specific F_{IS} estimates were found in loci Ppy-003, Ppy-005, Ppy-008, Ppy-012 and Ppy-016 (Appendix Table 6). Heterozygote deficiency identified by MICROCHECKER suggested that null alleles might be present at some of these loci (Appendix Table 6; negative null-allele frequencies are a software artefact and can be interpreted as zero). However, this was not consistent across populations for any locus, suggesting that heterozygote deficiency was not due to null-alleles. Significant linkage disequilibrium ($\alpha = 0.05$) was detected for 147 out of 1320 loci combinations in 11 populations, but in no case was any combination out of equilibrium consistently across all populations, suggesting no physical linkage of loci (results not shown).

Evidence was found for allelic drop-out at some loci from replicate genotyping of 31 individuals. Of 496 replicated genotypes (31·16 loci), seven cases (= 1.4 %) occurred where either the original or the replicate genotype was heterozygous whereas the other was homozygous. In these cases, the heterozygote genotype was retained. Occurrence of allelic drop-out was not systematic for particular loci or populations and restricted to individuals where PCR quality was low overall, probably caused by contamination of the template DNA extract as apparent from a low spectrophotometric 260 nm : 230 nm ratio in these cases.

The probability of identity (P_{ID}) for two individuals drawn at random from the final dataset (348 individuals) decreased from $9.04 \cdot 10^{-2}$ (most informative locus Ppy-011) to $2.53 \cdot 10^{-10}$ (all 16 loci), indicating a high power to discriminate between individuals. Within populations, the highest P_{ID} was observed in Colonsay and decreased from $1.96 \cdot 10^{-1}$ to $6.60 \cdot 10^{-6}$.

Genetic differentiation

Global genetic differentiation among all eleven red-billed chough populations was $D_{est} = 0.241$ (95 % CI: 0.222, 0.259) and $F_{ST} = 0.208$ (95 % CI: 0.179, 0.245). Pairwise D_{est} and F_{ST} estimates were highly significantly correlated ($r = 0.70$; $p < 0.001$) and were greater than 0.10 for most population pairs (Table 2). Cases of weak differentiation were Islay vs. Colonsay ($D_{est} = 0.017$; $F_{ST} = 0.047$), Cornwall vs. Ireland ($D_{est} = 0.020$; $F_{ST} = 0.053$) and Paradise Park vs. North Wales ($D_{est} = 0.065$; $F_{ST} = 0.069$). The only non-significant D_{est} estimate was Cornwall vs. Ireland (95 % CI: 0.000, 0.080; bounded by zero). All F_{ST} estimates were significant at the 5 %-level, but some estimates involving populations with small sample sizes were not significant after strict Bonferroni correction (Table 2).

There was a highly significant correlation between geographic distance and genetic differentiation both for D_{est} ($r = 0.81$; $p < 0.001$) and F_{ST} ($r = 0.59$; $p < 0.001$). The RMA regression lines for D_{est} and F_{ST} explained 65.0 % and 35.3 % of the variation respectively (Figure 2). When the Continental European populations Spain and French Alps were removed, the corre-

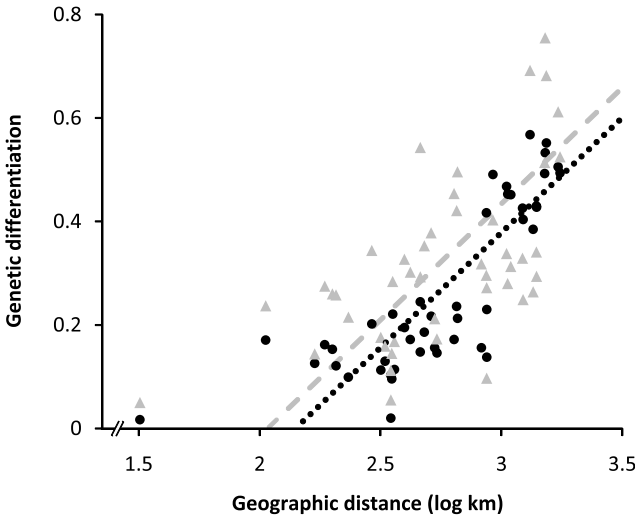

Figure 2: Relationships between geographic distance and genetic differentiation (isolation by distance), using D_{est} (dots, dotted line) and $F_{ST}/(1–F_{ST})$ (triangles, dashed line).

lations for both D_{est} ($r = 0.50$; $p = 0.007$) and F_{ST} ($r = 0.52$; $p = 0.002$) were still significant and the regression lines explained 25.0 % and 27.0 % respectively.

Bayesian inference of genetic structure

Based on the ΔK statistic, the best supported number of a *posteriori* genetic clusters was K = 3 for the standard admixture model and K = 2 for the LOCPRIOR model (ΔK = 73 and 83 respectively; Appendix Table 7). For K = 3, the first cluster comprised Spain and the French Alps, the second cluster comprised Ireland, Wales, Cornwall, Brittany and Paradise Park, and the third cluster comprised Scotland and the Isle of Man (Figure 3).

However, the Spain and French Alps populations (subspecies *P. p. erythrorhamphos sensu* Vaurie, 1954) were so strongly differentiated from all other populations (subspecies *P. p. pyrrhocorax sensu* Vaurie, 1954) that more subtle genetic structure among these other populations may not have been detected. When Spain and the French Alps were excluded from the analysis to clarify genetic structure within the remaining nine populations (running K = 1 to 9), the best supported number of clusters was K = 2 (ΔK = 534 and 108 respectively; Appendix Table 7), but with a strong secondary peak at K = 4 (ΔK = 180 and 46 respectively; Appendix Table 7). The two main clusters divided the geographic range into a northern group (Scotland and the Isle of Man) and a southern group (Ireland, Wales, Cornwall, Brittany and Paradise Park). At K = 4, Isle of Man became separated from Scotland, and the southern group became subdivided into Ireland, Cornwall and Brittany versus Wales and Paradise Park (Figure 3). At K = 5, Brittany became separated from Ireland and Cornwall. At higher K, the delineation of genetic clusters coincided well with the *a priori* populations.

A small number of individuals were assigned to a different cluster to that of most other individuals in their *a priori* population, using the standard admixture model. However, most of these cases were not apparent in the LOCPRIOR models. Overall, no differences in cluster distribution at any K or the best supported number of clusters were observed when individuals with partially missing genotypes were excluded.

Genetic diversity

Table 3 summarises the genetic diversity statistics for each *a priori* population. The Continental European populations Spain and French Alps had highest diversity and the northernmost populations Colonsay, Islay and the Isle of Man had lowest diversity. Ireland and Wales had highest diversity in the British Isles. Deviations from Hardy-Weinberg equilibrium (heterozygote deficiency) were apparent in Colonsay (F_{IS} = 0.131), Ireland (F_{IS} = 0.130) and the French Alps (F_{IS} = 0.167) at the 5 % level, but only the latter value remained significant after strict Bonferroni correction (Table 3).

Phylogeography

A total of 3,355 base pairs could be resolved unambiguously across three PCR amplicons in ingroup sequences. Of these, 19 sites were polymorphic with only two transversions: G↔T at site 403 (control region) and T↔A at site 1,474 (NADH1). The polymorphic sites defined ten haplotypes, with haplotype diversity h = 0.750 ± 0.068 SD and overall nucleotide diversity π = 0.00103 ± 0.00019 SD (Table 4). These haplotypes are stored in GENBANK at accessions JQ924832–JQ924841 (control region), JQ924842–JQ924851 (CHMT06) and JQ924852–JQ924861 (CHMT17). The resolved Alpine chough outgroup sequences for the three fragments

Table 2: Pairwise genetic differentiation among eleven *a priori* red-billed chough populations based on 16 microsatellite loci. Jost's D_{est} with 95 % confidence intervals is given below the diagonal, Wright's F_{ST} with annotated significance at the 5 % level (*) and strict Bonferroni-corrected level ($\alpha = 0.00091$**) is given above the diagonal.

	Colonsay	Islay	Isle of Man	Ireland	North Wales	South Wales	Cornwall	Brittany	French Alps	Spain	Paradise Park
Colonsay		0.047**	0.177**	0.232**	0.144**	0.274**	0.332**	0.214**	0.406**	0.344**	0.270**
Islay	0.017		0.205**	0.227**	0.150**	0.261**	0.312**	0.241**	0.430**	0.380**	0.281**
	(0.003, 0.031)										
Isle of Man	0.099	0.121		0.247**	0.191**	0.256*	0.352*	0.296**	0.409**	0.339**	0.284**
	(0.073, 0.130)	(0.097, 0.147)									
Ireland	0.172	0.148	0.195		0.101**	0.137*	0.053*	0.175**	0.254**	0.209**	0.184**
	(0.132, 0.213)	(0.114, 0.189)	(0.154, 0.239)								
North Wales	0.114	0.113	0.171	0.103		0.126*	0.127**	0.148**	0.248**	0.228**	0.069**
	(0.089, 0.142)	(0.091, 0.136)	(0.139, 0.205)	(0.071, 0.139)							
South Wales	0.217	0.186	0.202	0.130	0.126		0.215*	0.221*	0.239*	0.199*	0.146*
	(0.155, 0.281)	(0.129, 0.250)	(0.138, 0.268)	(0.074, 0.195)	(0.075, 0.181)						
Cornwall	0.213	0.172	0.245	0.020	0.096	0.162		0.206*	0.252*	0.219*	0.212**
	(0.141, 0.284)	(0.104, 0.239)	(0.178, 0.322)	(0.000, 0.080)[a]	(0.044, 0.157)	(0.079, 0.251)					
Brittany	0.138	0.156	0.236	0.156	0.146	0.221	0.153		0.287*	0.228**	0.193**
	(0.096, 0.180)	(0.113, 0.199)	(0.190, 0.288)	(0.105, 0.209)	(0.109, 0.189)	(0.150, 0.294)	(0.078, 0.238)				
French Alps	0.552	0.533	0.568	0.430	0.426	0.452	0.468	0.491		0.088**	0.240**
	(0.480, 0.621)	(0.460, 0.605)	(0.498, 0.637)	(0.352, 0.504)	(0.355, 0.495)	(0.365, 0.535)	(0.374, 0.561)	(0.409, 0.567)			
Spain	0.494	0.506	0.493	0.385	0.428	0.404	0.453	0.417	0.230		0.217 **
	(0.434, 0.553)	(0.450, 0.565)	(0.434, 0.549)	(0.322, 0.451)	(0.371, 0.485)	(0.318, 0.490)	(0.359, 0.541)	(0.349, 0.486)	(0.150, 0.309)		
Paradise Park	0.218	0.219	0.244	0.191	0.065	0.141	0.191	0.184	0.434	0.430	
	(0.172, 0.266)	(0.171, 0.267)	(0.194, 0.293)	(0.141, 0.244)	(0.034, 0.103)	(0.081, 0.214)	(0.125, 0.265)	(0.132, 0.240)	(0.357, 0.506)	(0.362, 0.496)	

[a] interval bounded by zero

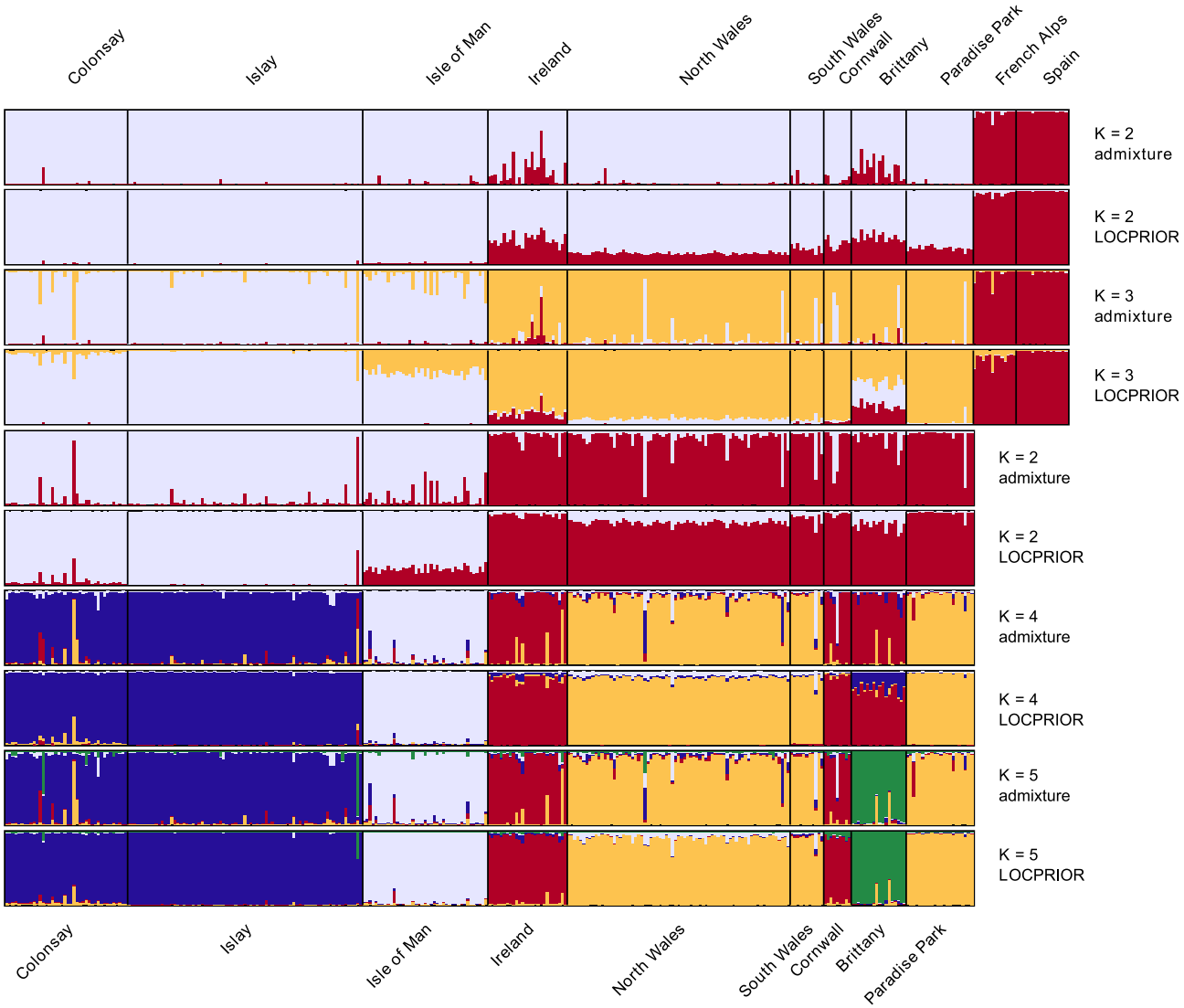

Figure 3: Individual membership coefficients derived from Bayesian inference of genetic structure across all eleven red-billed chough populations (top four plots) and Atlantic coast populations only (bottom six plots). Each individual is represented by a single vertical line. Black lines demarcate *a priori* populations. Coefficients are averaged across 15 replicate runs or from the single most likely replicate for K = 5, due to multiple solutions among replicates, using the standard admixture model or including sampling locations as prior information (LOCPRIOR).

are stored at JQ963890–JQ963892 (French Alps) and JQ963893–JQ963895 (Corsica).

A statistical parsimony network of ingroup haplotypes illustrates two major haplotype groups: Continental Europe (Spain, French Alps and Brittany) and the British Isles, diverged by five transitions (Figure 4). A Maximum Likelihood phylogram with Alpine chough as outgroup defined two clades, separating the Continental European populations Spain, French Alps and Brittany from all populations in the British Isles (Figure 5). Within the British Isles, a further lineage was apparent, consisting of Ireland, Cornwall and South Wales (two individuals only). None of these major groups were bootstrap supported, reflecting low overall levels of polymorphism.

Discussion

We quantified genetic structure, genetic diversity and phylogeography among red-billed chough populations across the British Isles in comparison to a sample of Continental European populations, in order to infer population connectivity, identify management units and assess the potential need for management intervention to increase genetic diversity. Our microsatellite loci were robust and provided a dataset with high resolution to identify individuals within populations and detect significant genetic differentiation among *a priori*

Table 3: Genetic diversity statistics (means ± 1 SD) derived from 16 microsatellite loci across 348 red-billed choughs from eleven populations. Population size (n) is given alongside the average percentage of missing genotype data, number of alleles (n_a), allelic richness (a_r), effective number of alleles (n_e), number of private alleles (n_p), observed heterozygosity (H_O), expected heterozygosity (H_E) and Wright's F_{IS} with significance indicated at the 5 % level (*) and strict Bonferroni-corrected level ($\alpha = 0.00028$**).

Population	n	Missing data (%)	n_a	a_r	n_e	n_p	H_O	H_E	F_{IS}
Colonsay	40	4.85 ± 9.01	2.88 ± 1.15	1.95 ± 0.58	1.60 ± 0.52	1	0.30 ± 0.05	0.33 ± 0.06	0.131*
Islay	77	6.51 ± 10.63	3.13 ± 1.31	1.97 ± 0.67	1.62 ± 0.62	2	0.40 ± 0.04	0.49 ± 0.05	0.024
Isle of Man	41	5.95 ± 10.85	3.13 ± 1.50	1.98 ± 0.67	1.63 ± 0.55	4	0.44 ± 0.05	0.58 ± 0.04	0.019
Ireland	26	6.31 ± 10.30	3.63 ± 1.50	2.53 ± 0.89	2.16 ± 0.95	1	0.52 ± 0.03	0.65 ± 0.04	0.130*
North Wales	73	7.18 ± 12.48	3.38 ± 1.54	2.46 ± 0.79	2.29 ± 0.83	1	0.40 ± 0.05	0.52 ± 0.05	–0.009
South Wales	11	6.55 ± 6.27	2.69 ± 1.01	2.34 ± 0.70	2.03 ± 0.63	0	0.49 ± 0.06	0.51 ± 0.06	–0.078
Cornwall	9	19.78 ± 25.04	2.25 ± 0.77	2.05 ± 0.62	1.75 ± 0.55	0	0.45 ± 0.06	0.45 ± 0.06	–0.031
Brittany	18	3.67 ± 4.67	2.81 ± 1.17	2.23 ± 0.70	1.96 ± 0.74	1	0.45 ± 0.06	0.47 ± 0.06	–0.040
French Alps	14	13.07 ± 18.43	4.88 ± 1.67	3.55 ± 0.86	3.33 ± 1.30	5	0.49 ± 0.06	0.46 ± 0.06	0.167**
Spain	17	4.35 ± 10.22	6.38 ± 2.55	4.11 ± 1.17	4.51 ± 1.92	28	0.42 ± 0.07	0.45 ± 0.06	–0.038
Paradise Park	22	3.86 ± 8.77	2.81 ± 1.05	2.38 ± 0.67	2.15 ± 0.61	0	0.37 ± 0.06	0.39 ± 0.06	–0.063
Total	348	6.57 ± 11.58	–	–	–	–	–	–	–

populations. Sequencing large portions of three mitochondrial regions provided good characterisation of mitochondrial polymorphism and hence phylogeographic structure. We demonstrate strong genetic differentiation among most populations, low nuclear and mitochondrial genetic diversity, and weak phylogeographic structure across the sampled populations.

Genetic structure and dispersal

Genetic differentiation is generally deemed moderately high when D_{est} or F_{ST} is greater than 0.10–0.15 (Balloux and Lugon-Moulin, 2002). The observed differentiation among most red-billed chough population pairs, separated by up to 1,700 km, exceeded 0.10. This is high compared to recent avian studies. Barlow et al (2011) report weak differentiation among philopatric European shag *Phalacrocorax aristotelis* populations (global $D_{est} = 0.066$ compared to $D_{est} = 0.241$ in choughs). Segelbacher et al (2003) report moderate differentiation among fragmented European capercaillie *Tetrao urogallus* populations (global $F_{ST} = 0.102$ compared to $F_{ST} = 0.208$ in choughs). However, genetic differentiation similar to that observed in choughs has been reported in house sparrow *Passer domesticus* with pairwise D_{est} of 0.07–0.33 among European populations (Schrey et al, 2011). Stronger differentiation has also been reported at very large spatial scales, e.g. pairwise $F_{ST} = 0.362$ in snowy plover *Charadrius alexandrinus* across 4,000 km (Funk et al, 2007) and pairwise $D_{est} = 0.260$ in giant petrel (*Macronectes* spp.) across 7,000 km (Techow et al, 2010). Overall, differentiation among red-billed chough populations was therefore notably high and demonstrates strong genetic structure.

Genetic differentiation between population pairs was strongly correlated with geographic distance; the latter explained 25–65 % of the variation in the former. Geographic distance rarely explains more than 20 % of variation in genetic differentiation in bird populations (e.g. Johnson et al, 2003; Funk et al, 2007; Techow et al, 2010). Notable exceptions include 27 % in European shags (Barlow et al, 2011) and 38.4 % in orange-crowned warblers *Vermivora celata* in Canada and Alaska across a large spatial scale of up to 4,000 km (Bull et al, 2010). Genetic structure among chough populations was apparent even on a relatively small geographic scale. The North and South Wales populations were considerably and significantly differentiated, even though they are not separated by sea. The Scottish islands of Colonsay and Islay are only 10 km apart, yet there was detectable small genetic differentiation between them. The strong genetic structure among chough populations was therefore at least partially explicable by geographic distance and implies very low rates of successful long-distance dispersal and gene flow across the British Isles, even among relatively proximate populations.

This conclusion concurs with ringing data. Only six ringed individuals have been observed to disperse between Islay and Colonsay in over twenty years (although Colonsay was probably colonised from Islay in the late 1960s, Reid et al, 2003, 2008). Nevertheless, field observations show that choughs do oc-

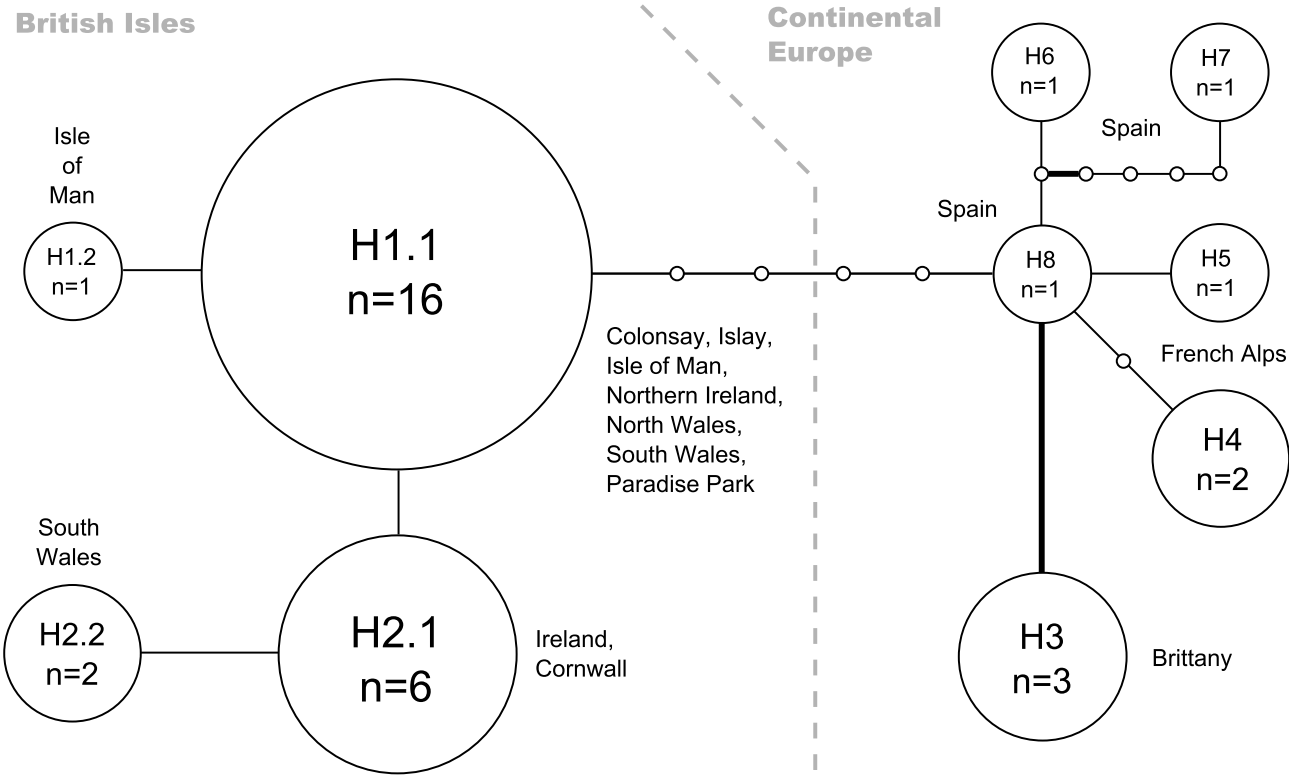

Figure 4: Statistical parsimony network of ten resolved haplotypes in 34 red-billed choughs from twelve locations. Haplotype names (e.g. H1.1) and frequencies (n) are given within circles. Circle areas are proportional to haplotype frequencies. Empty circles represent inferred, unsampled haplotypes. Transversion mutations are indicated by bold lines. Branch lengths are arbitrary.

casionally disperse over long distances. At least nine choughs moved between North Wales and the Isle of Man (c. 100 km) during 1997–2004, and two of them were proven to have bred (Moore, 2006, 2008). Furthermore, the recolonisation of Cornwall in 2001 is assumed to reflect natural long-distance dispersal from other wild populations (Johnstone et al, 2011). The colonisers are speculated to have originated in Brittany or South Wales (Carter et al, 2003). However, our genetic data show that the colonisers do not match these populations, or the local captive population in Paradise Park, but suggest they probably originated in Ireland. Although inference is constrained by the small sample size (9 individuals), the only case of non-significant genetic differentiation was Ireland vs. Cornwall. These populations also shared a mitochondrial haplotype and an *a posteriori* genetic cluster. Assuming that this recolonisation was unassisted, the genetic date therefore show that successful long-distance dispersal can occur.

Some individuals were initially assigned to different *a posteriori* genetic clusters than most other individuals from the same *a priori* population, implying some dispersal among Wales, Ireland, Scotland and Brittany. However, most such assignments were no longer apparent when sampling location was incorporated as prior information. They may therefore be erroneous initial assignments due to partially missing genotype data, small population size or local violation of the Hardy-Weinberg equilibrium assumption rather than true long-distance migrants (Pritchard et al, 2000; Evanno et al, 2005; Latch et al, 2006).

Phylogeography

Phylogeographic structure within the British Isles was poorly resolved due to low mitochondrial DNA sequence polymorphism. Observed polymorphism suggested weak diversification of haplotypes sampled in the British Isles from those sampled in Continental Europe. The phylogeographic tree placed the Continental European populations in Spain, French Alps and Brittany ancestral to all British populations, which is consistent with a classic northward pattern of postglacial recolonisation from refugia in southern Europe (Taberlet et al, 1998; Hewitt, 2000). No evidence for colonisation by more than one lineage (e.g. Celtic fringe scenario; Searle et al, 2009) was found, as all British populations formed a single clade. The single sample

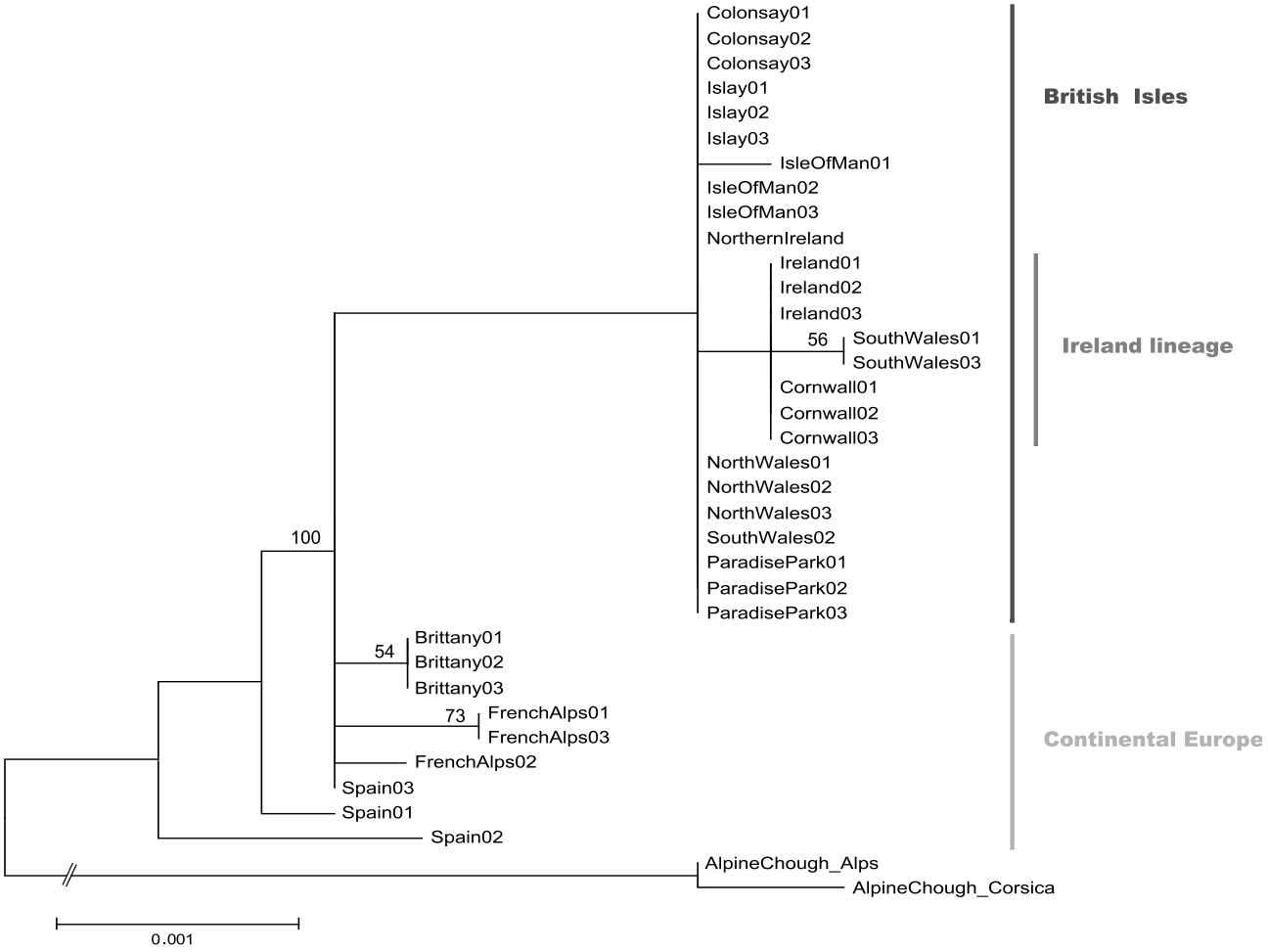

Figure 5: Maximum Likelihood phylogram of 34 red-billed choughs based on sequencing of three mitochondrial regions. Two Alpine choughs were used as an outgroup (branch clipped to clarify ingroup branching). The scale bar represents 0.001 nucleotide substitutions per site. The values on nodes are bootstrap support values (only $> 50\,\%$ are shown) derived from 10,000 iterations using the Neighbour-Joining construction method.

from Northern Ireland did not share the same haplotype and clade as Ireland and was more similar to the UK populations.

Weak mitochondrial genetic structure contrasted with strong nuclear genetic structure. Whilst microsatellite genotypes showed genetic differentiation even between Colonsay and Islay, almost the entire UK population shared a single mitochondrial haplotype. Such discrepancies in genetic structure are frequently reported for avian species (e.g. Johnson et al, 2003; Caparroz et al, 2009; Hefti-Gautschi et al, 2009) and are often attributed to sex-biased dispersal where a weaker mitochondrial structure would indicate female-biased dispersal. This is unlikely to be the case in choughs. Although females disperse slightly further than males within individual populations (Reid et al, 2006; Moore, 2008), long-distance dispersal is rarely observed in either sex. A more likely explanation is increased propensity to genetic stochasticity in mitochondrial DNA, caused by a smaller effective population size of mitochondrial versus nuclear DNA (Avise et al, 1987; Birky et al, 1989). Higher mutation rates in nuclear microsatellite loci are likely to amplify this discrepancy (Balloux and Lugon-Moulin, 2002). These explanations comply with the known decline of chough populations during the 18th–20th centuries and consequent bottlenecks (Holloway and Gibbons, 1996).

Our current aim was to link the phylogeography of chough populations in the British Isles with sampled Continental European populations, rather than to compile a full Continental European phylogeography. Sampling was therefore restricted to only one location in Spain and two locations in France. While including relatively few samples per location is not unusual (e.g. Taberlet et al, 1998; Questiau et al, 1999), future analyses could compile the full chough phylogeography by sampling a greater range of populations.

Table 4: Polymorphic nucleotide sites and defined haplotypes in mitochondrial DNA sequences of 34 red-billed choughs. Dots denote the same nucleotide as the reference sequence. Nucleotide positions are given for each of three sequence fragments separately as well as combined. GENBANK accessions are given for each fragment separately.

		Control region							CHMT06 (NADH1)								CHMT17 (NADH5/CYTB)					
									25	269	335	347	380	563	860		360	614	681	966	1197	
Haplotype	Accessions	47	137	296	352	403	1040	1049	1230	1474	1540	1552	1585	1768	2011		2487	2741	2808	3093	3324	Individual
H1.1	JQ924832; JQ924842; JQ924852	C	A	C	G	G	G	A	C	T	A	G	T	G	C		C	T	T	T	A	Colonsay01, Colonsay02, Colonsay03, Islay01, I
H1.2	JQ924833; JQ924843; JQ924853	.	.	T	.	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	IsleOfMan01
H2.1	JQ924834; JQ924844; JQ924854	.	.	.	.	.	.	.	.	.	.	.	C	.	.		.	.	.	.	.	Ireland01, Ireland02, Ireland03, Cornwall01, Co
H2.2	JQ924835; JQ924845; JQ924855	.	.	.	.	.	.	.	.	.	.	.	C	.	.		.	C	.	.	.	SouthWales01, SouthWales03
H3	JQ924836; JQ924846; JQ924856	.	.	.	A	.	.	.	.	A	G	A	.	.	T		T	.	.	.	.	Brittany01, Brittany02, Brittany03
H4	JQ924837; JQ924847; JQ924857	T	.	.	A	.	.	.	.	.	G	A	.	.	T		T	.	C	.	.	FrenchAlps01, FrenchAlps03
H5	JQ924838; JQ924848; JQ924858	.	.	.	A	.	.	.	T	.	G	A	.	.	T		T	.	.	.	.	FrenchAlps02
H6	JQ924839; JQ924849; JQ924859	.	.	.	A	.	A	.	.	.	G	A	.	A	T		T	.	.	.	.	Spain01
H7	JQ924840; JQ924850; JQ924860	.	G	.	A	T	A	G	.	.	G	A	.	.	T		T	.	.	C	G	Spain02
H8	JQ924841; JQ924851; JQ924861	.	.	.	A	.	.	.	.	.	G	A	.	.	T		T	.	.	.	.	Spain03

Genetic diversity

Neutral genetic diversity is expected to be reduced in small, isolated populations due to stochastic loss of alleles. The observed strong genetic structure among small chough populations indicates low population connectivity and consequently predicts low within-population genetic diversity.

Most British Isles chough populations had fewer than 4.0 alleles per locus, whereas the sampled Continental European populations had slightly higher diversity (c. 5.0–7.0 alleles). Observed heterozygosity was also low, ranging from 0.30 to 0.52. Colonsay, Ireland and French Alps were significantly deficient in heterozygote genotypes (positive F_{IS}), which might indicate some within-population sub-structuring caused by wrongly delineated *a priori* populations (Wahlund, 1928). However, *a posteriori* genetic clusters did not show sub-structuring in these populations, suggesting that heterozygote deficiency is not due to a Wahlund effect.

Threatened bird populations that are known to have experienced population bottlenecks typically have less than 3.0–4.0 alleles per locus, for example 4.0 in golden eagle *Aquila chrysaetos* (Bourke et al, 2010), 3.0 in Galapagos penguin *Spheniscus mendiculus* (Nims et al, 2008) and 1.9 in Madagascar fish-eagle *Haliaeetus vociferoides* (Johnson et al, 2009). Similarly, heterozygosity is typically below 0.50, for example 0.44 in capercaillie *Tetrao urogallus* (Segelbacher et al, 2003), 0.20 in black robin *Petroica traversi* (Ardern and Lambert, 1997) and 0.10 in Mauritius kestrel *Falco punctatus* (Nichols et al, 2001). At the other end of the spectrum are widely-dispersed, high-abundance species such as house sparrow *Passer domesticus* with 13.6 alleles per locus and heterozygosity of 0.83 (Schrey et al, 2011). In comparison, all chough populations had relatively low genetic diversity.

Within the British Isles, the northerly populations Colonsay, Islay and Isle of Man had lower genetic diversity than the more southerly populations. The new population in Cornwall had lower genetic diversity than its most likely source population in Ireland, which is not surprising because there were only 3–7 founders (Carter et al, 2003; Johnstone et al, 2011). The low genetic diversity in the north might be a consequence of founder effects during post-glacial south-north colonisation events, but lack of resolution within the phylogeographic tree precludes assessment of colonisation

routes within the British Isles. Furthermore, as there are no historic nuclear genetic diversity data available to compare to contemporary diversity, it is not possible to ascertain whether the observed patterns of genetic diversity reflect more recent population contraction and isolation. Notwithstanding the underlying causes, nuclear genetic diversity in most chough populations was notably low.

Compared with recent avian studies, mitochondrial genetic diversity was also low, even in the hypervariable control region (e.g. Piertney et al, 2001; Segelbacher and Piertney, 2007; Barbanera et al, 2009). A recent study that quantified mitochondrial genetic diversity in choughs did not find any polymorphism in a 365 bp control region segment among 23 extant Welsh choughs and 19 museum specimens from across the British Isles, and concluded that all extant choughs in the UK form a single matrilineage (Kocijan and Bruford, 2011). We confirm overall low mitochondrial diversity and that North Wales is monomorphic across 3,355 bp, but we resolved an additional haplotype in South Wales. We resolved four haplotypes across the British Isles overall, although one haplotype was much commoner than the other three. Low mitochondrial diversity is not unusual (e.g. Waits et al, 2003; Roques and Negro, 2005; Cadahia et al, 2007). Given the decline in chough population size and range during the 18th–20th centuries, bottlenecks in the early 20th probably caused losses of mitochondrial as well as nuclear genetic diversity (Holloway and Gibbons, 1996).

Implications for conservation management

Current published chough taxonomy (Vaurie, 1954) was based on morphology and has not been verified genetically. Subspecies taxonomy based on morphology alone may be misleading if phenotypic variation does not reflect evolutionary splits (e.g. Burbrink et al, 2000; Piertney et al, 2001; Segelbacher and Piertney, 2007). Microsatellite-based genetic differentiation and *a posteriori* genetic clusters matched current published taxonomy in that the Brittany population clustered with the British Isles population (equating to the nominate subspecies *P. p. pyrrhocorax sensu* Vaurie, 1954). However, the haplotype network and phylogeographic tree suggested that the Brittany population is more closely related to the Continental European populations (equating to *P. p. erythrorhamphos sensu* Vaurie, 1954). Strict application of the phylogenetic species concept based on reciprocal monophyly (Donoghue, 1985) would classify Brittany's choughs as part of the Continental European subspecies. However, given the weak statistical support for the phylogeographic groups, the microsatellite data may provide a more credible structure and therefore concur with Vaurie's taxonomy.

Similarly, if evolutionarily significant units (ESUs) are based solely on reciprocal monophyly (Moritz, 1994), the weakly supported chough phylogeography divides the sampled populations into three broad units: the Continental European populations in Spain, the French Alps (and possibly Brittany); the populations in Ireland, Cornwall and South Wales; and all other British Isles populations. However, given the high microsatellite differentiation among populations within these three units (Moritz, 1994), each population may need to be managed separately as each is to some extent a distinct genetic unit. The individual populations within the British Isles are already monitored and managed largely separately (Finney and Jardine, 2003; Gray et al, 2003; Kerbiriou et al, 2005; Whitehead et al, 2005; Moore, 2008; Johnstone et al, 2011). Our data suggest that this is an appropriate strategy to conserve genetic diversity and evolutionary potential.

There is growing evidence that reduced genetic diversity can increase long-term extinction risk (Reed and Frankham, 2003; Frankham, 2005, 2010b), even when reduced fitness is not immediately apparent (e.g. Jamieson et al, 2006; Johnson et al, 2009). Genetic diversity was comparatively low in all chough populations, indicating that concern over individual fitness, evolutionary potential and population persistence may be warranted, particularly for the Colonsay, Islay and Isle of Man populations. However, genetic diversity in neutral microsatellite markers may not be a good measure of adaptive genetic diversity (Moss et al, 2003). In fact, as microsatellite loci evolve faster than single nucleotide polymorphisms (SNPs) in genes, neutral genetic diversity may overestimate genome-wide adaptive genetic diversity (Väli et al, 2008). If adaptive diversity in choughs is low, as suggested by neutral diversity, concern over long-term adaptability may be justified and consideration of translocations to increase genetic diversity in particularly depauperate and isolated populations may be warranted.

Translocation can aid population recovery, as demonstrated for example in adders *Vipera berus* and

gray wolves *Canis lupus* (reviewed in Tallmon et al, 2004; Frankham, 2005), but many such projects fail (Fischer and Lindenmayer, 2000; Tallmon et al, 2004). Successful translocation programmes require considerable planning and effort to satisfy IUCN guidelines (IUCN, 1998). The source population must be genetically similar to the target population to avoid outbreeding depression, although Frankham et al (2011) argue that concerns over outbreeding depression may be exaggerated for populations that became fragmented relatively recently. The chough populations in Ireland and North Wales hold the greatest genetic diversity and are only moderately differentiated from the northern populations. They may therefore be suitable sources for translocations. The genetic data confirmed that the ancestors of the captive choughs in Paradise Park most probably originated from North Wales (Burgess et al, in press). They may be suitable for reintroduction, but are more substantially differentiated from the northern populations. In any case, given the very small census sizes of some populations, thorough evaluation of the consequences of removing individuals from these populations will be necessary. Not least, appropriate habitat management and restoration will be required before any useful translocations could take place. Indeed, improved habitat quality might even facilitate natural dispersal and hence genetic connectivity among populations (Johnstone et al, 2011).

Appendix

See tables 5, 6 and 7.

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Table 5: Characterisation of three primer pairs to amplify mitochondrial DNA regions in red-billed chough. Fragment sizes are given alongside PCR TouchDown annealing temperature gradients (T_a) and GENBANK accessions of resolved haplotypes.

Locus	Primer name	Primer sequence (5'–3')	Fragment size (bp)	T_a (°C)	GENBANK accessions
Control region	JCR03	CCCCCCCATGTTTTTACR	1205	60→50	JQ924832–JQ924841
	H1248	CATCTTCAGTGTCATGCT			
NADH1	CHMT06-F	AGGTTCAAATCCTCTCCCTAGC	922	65→55	JQ924842–JQ924851
	CHMT06-R	AACCATCATTTTCGGGGTATG			
NADH5/CYTB	CHMT17-F	AACCTAGCCCTAATAGGAAC	1228	55→45	JQ924852–JQ924861
	CHMT17-R	AGTAGTATGGGTGGAATGG			

Table 6: Characterisation of 16 microsatellite loci for red-billed chough. Statistics (± 1 SD) were calculated from 348 individuals in eleven populations. The microsatellite repeat unit is given alongside TouchDown annealing temperature gradient (T_a), number of alleles (n_a), allele range (bp), observed (H_O) and expected (H_E) heterozygosity, the probability of Hardy-Weinberg equilibrium (p_{HWE}) and null allele frequency (van Oosterhout et al, 2004). See Wenzel et al (2011) for full characterisation.

Locus name	Repeat unit	T_a	n_a	Allele range	H_O	H_E	p_{HWE}	Null-allele frequency
Ppy-001	TACA	$60 \rightarrow 50$	4	151–179	0.46 ± 0.06	0.45 ± 0.04	0.148	-0.04 ± 0.13
Ppy-002	ATCT	$60 \rightarrow 50$	4	151–179	0.33 ± 0.05	0.36 ± 0.06	0.993	-0.05 ± 0.12
Ppy-003	AGAT	$60 \rightarrow 50$	11	292–344	0.50 ± 0.04	0.58 ± 0.04	< 0.001	0.01 ± 0.11
Ppy-004	AGAT	$60 \rightarrow 50$	8	173–239	0.40 ± 0.03	0.46 ± 0.02	0.183	-0.04 ± 0.13
Ppy-005	TATC	$60 \rightarrow 50$	7	226–250	0.25 ± 0.04	0.30 ± 0.06	0.028	-0.02 ± 0.14
Ppy-006	CATC	$60 \rightarrow 50$	8	139–175	0.05 ± 0.03	0.11 ± 0.06	0.729	0.00 ± 0.05
Ppy-007	GATA	$55 \rightarrow 45$	9	161–193	0.61 ± 0.03	0.69 ± 0.02	0.425	0.00 ± 0.08
Ppy-008	GATA	$60 \rightarrow 50$	10	221–265	0.55 ± 0.03	0.66 ± 0.02	0.018	-0.02 ± 0.17
Ppy-009	AAGT	$60 \rightarrow 50$	6	222–242	0.58 ± 0.05	0.59 ± 0.02	0.420	-0.06 ± 0.11
Ppy-010	CA	$60 \rightarrow 50$	14	108–146	0.51 ± 0.05	0.50 ± 0.04	0.187	-0.11 ± 0.17
Ppy-011	TAGA	$60 \rightarrow 50$	10	163–191	0.66 ± 0.05	0.71 ± 0.02	0.190	-0.08 ± 0.13
Ppy-012	TAGA	$60 \rightarrow 50$	13	210–266	0.46 ± 0.07	0.61 ± 0.03	< 0.001	0.00 ± 0.23
Ppy-013	GATA	$60 \rightarrow 50$	10	197–221	0.58 ± 0.02	0.68 ± 0.02	0.493	0.01 ± 0.07
Ppy-014[a]	GATG	$60 \rightarrow 50$	5	239–275	0.34 ± 0.03	0.36 ± 0.02	0.615	0.02 ± 0.08
Ppy-015[a]	TATG	$60 \rightarrow 50$	3	152–158	0.06 ± 0.04	0.07 ± 0.04	0.120	-0.04 ± 0.15
Ppy-016	GGAT	$60 \rightarrow 50$	13	200–244	0.52 ± 0.03	0.60 ± 0.04	0.022	0.02 ± 0.07

[a] locus also isolated by Jaari et al (2008)

Table 7: Likelihood statistics of Bayesian inference of genetic clusters in STRUCTURE. The mean logarithmic likelihood (± SD) of 15 runs at each K is given alongside the ΔK statistic by Evanno et al (2005). Peak values for ΔK are indicated in bold.

	All populations				Atlantic coast populations only			
	Std. admixture		LOCPRIOR		Std. admixture		LOCPRIOR	
K	LnP(K)	ΔK	LnP(K)	ΔK	LnP(K)	ΔK	LnP(K)	ΔK
1	-11024 ± 1	–	-11024 ± 0	–	-8470 ± 0	–	-8470 ± 0	–
2	-10049 ± 5	68	-9960 ± 5	**83**	-7817 ± 1	**534**	-7787 ± 3	**108**
3	-9411 ± 4	**73**	-9339 ± 6	50	-7494 ± 21	0	-7456 ± 10	2
4	-9071 ± 19	4	-9011 ± 6	8	-7168 ± 1	**180**	-7141 ± 4	**46**
5	-8814 ± 230	0	-8733 ± 11	16	-7041 ± 21	1	-7021 ± 22	2
6	-8626 ± 22	3	-8633 ± 42	1	-6936 ± 30	1	-6947 ± 73	1
7	-8511 ± 20	1	-8558 ± 79	0	-6848 ± 49	1	-6911 ± 64	1
8	-8411 ± 36	1	-8484 ± 57	0	-6795 ± 103	0	-6798 ± 48	2
9	-8346 ± 52	0	-8425 ± 78	1	-6714 ± 52	–	-6783 ± 87	–
10	-8273 ± 11	7	-8465 ± 153	1	–	–	–	–
11	-8281 ± 127	–	-8378 ± 106	–	–	–	–	–

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