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Genetic structure among remnant populations of a migratory passerine, the Northern Wheatear *Oenanthe oenanthe*

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Continuous animal populations often become fragmented due to anthropogenic habitat alterations. These small, fragmented populations are fragile due to demographic and genetic factors, whereas immigration can enhance their long-term viability. Previously, we showed that high philopatry affected the local dynamics of three small and remnant subpopulations of Northern Wheatears in The Netherlands. Here, we show that these three populations together with an additional larger population in the European lowlands are highly genetically differentiated based on 22 microsatellite markers. In contrast, we found no evidence for differentiation using two mitochondrial DNA markers. An IMa2 analysis indicates that gene flow has occurred regularly among our sampled populations. As immigration of colour-ringed birds among our sampled populations is rare at best, our results suggest that the populations have recently become isolated from one another. Low dispersal rates in highly mobile birds may occur when suitable habitat becomes highly fragmented, and will accentuate stochastic demographic processes and inbreeding, both reducing population viability. As dispersal rates are low among populations of Northern Wheatears in The Netherlands, there is only a small probability of recolonization of habitat patches where populations have become locally extinct.

Keywords: F_{ST}, gene flow, genetic drift, genetic structuring, heterozygosity, IMa2, microsatellite, mtDNA, philopatry, songbird.

Due to a variety of anthropogenic influences, most prominently habitat destruction, populations once distributed continuously may become fragmented, persisting in isolation of each other. These populations are at risk of extinction because of demographic and genetic factors (Gilpin & Soulé 1986). Exchange between populations is important for the long-term persistence of small populations (Hanski & Gilpin 1997, Hanski 1998): it buffers against stochastic population fluctuations and against loss of genetic diversity through genetic drift and inbreeding (Brown & Kodric-Brown 1977, Slatkin 1987, Frankham 1996). Connectivity of populations can be determined both demographically and genetically, with each method having its own merits and shortcomings (Lowe & Allendorf 2010). Demographic connectivity, defined as the degree to which population growth and vital rates are influenced by exchange between populations, is a function of the relative contribution of immigration to population growth. Genetic

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connectivity, defined as 'the degree to which gene flow influences evolutionary processes in populations' (Lowe & Allendorf 2010), is mainly a function of the absolute number of effective immigrants (Waples & Gaggiotti 2006), defined as breeding individuals that contribute offspring to the next generation. For conservation purposes we need information on both demographic and genetic connectivity to make inferences about the potential persistence of populations.

Previously we showed, using an Integrated Population Model, that the small relict populations of Northern Wheatear *Oenanthe oenanthe* in The Netherlands have high philopatry, and that subpopulation dynamics were to a varying degree determined by local population processes (Van Oosten *et al.* 2015). These local relict populations constitute an estimated 50% of the breeding Northern Wheatears in The Netherlands, and intensive colour-ringing allowed estimation of all demographic parameters with high accuracy. The estimated and observed low levels of immigration and small population sizes may quickly lead to genetic differentiation as a result of genetic drift and rapid fixation of alternate alleles among isolated populations.

In The Netherlands, Northern Wheatears had a large breeding range 25 years ago, with a large continuous population in the coastal dunes and a more isolated population inland (Teixeira 1979, Sovon 2002; Fig. 1). Dispersal was probably more frequent (as is often the case in more continuous populations; Wiens 1995) and demographic isolation may not have resulted in genetic structuring, although the possibility exists that the coastal and inland populations were separated to some extent. At present, limited immigration may still maintain sufficient gene flow between seemingly isolated populations of this declining species, as has been shown for migratory and philopatric passerines that show little genetic structuring among contemporary fragmented populations (Cerulean Warbler *Setophaga cerulea*, Veit *et al.* 2005, Golden-cheeked Warbler *Setophaga chrysoparia*, Lindsay *et al.* 2008). Other long-distance migratory species with large ranges (e.g. European Pied Flycatcher *Ficedula hypoleuca*, Lehtonen *et al.* 2009, Barn Swallow *Hirundo rustica*, Santure *et al.* 2010, Eurasian Reed Warbler *Acrocephalus scirpaceus*, Prochazka *et al.* 2011) show little genetic differentiation over most of their breeding ranges, suggesting appreciable levels of dispersal in these highly mobile species.

This paper aims to describe the level of genetic differentiation between four discrete remnant populations of Northern Wheatears. We use microsatellite and mitochondrial DNA (mtDNA)

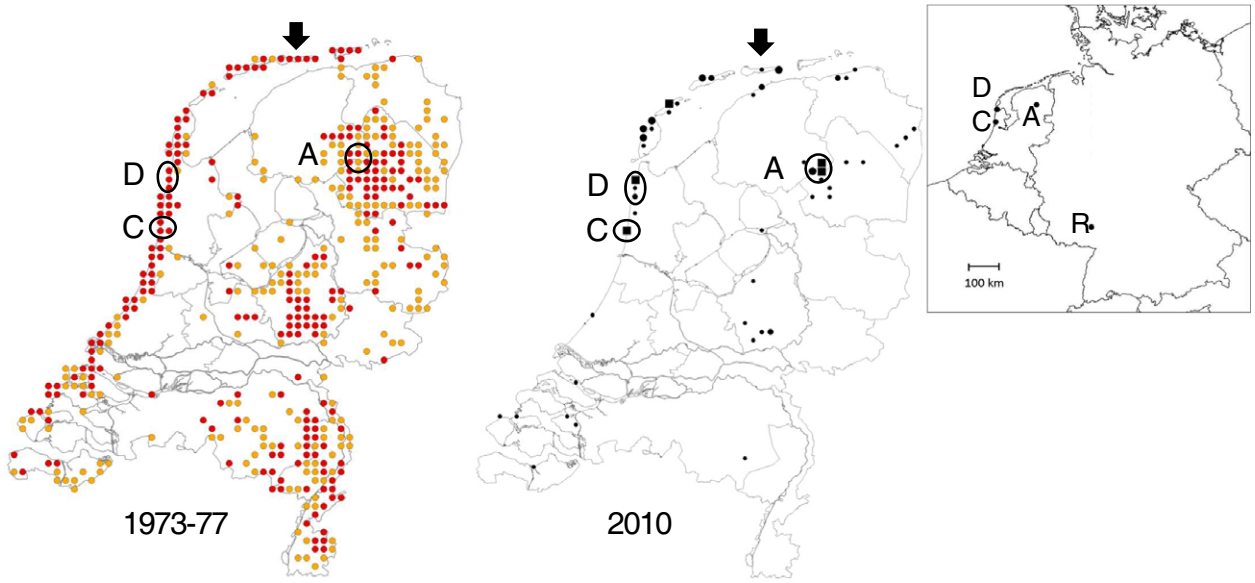

Figure 1. Breeding distribution of Northern Wheatears in the Netherlands in 1973–77 (left; Teixeira 1979) and 2010 (right; Boele *et al.* 2012). The three research sites are denoted with capital letters (see text) and the arrow indicates the island of Ameland from where additional DNA was sampled for mtDNA analysis. Dots and squares refer to different number of territories per 5-km square. The inset shows the location of the four sites in Northwestern Europe. Maps courtesy of Sovon.

marker data to test three alternative predictions about genetic variation.

1 Geographically isolated Northern Wheatear populations show no genetic structure, because there is sufficient gene flow or because the separation is too recent. Genetic variation is similar across the populations.
2 Recent isolation and genetic drift have already resulted in the development of population genetic structure. In this case, we predict that populations differ with respect to their mtDNA but not (yet) nuclear microsatellites, because effective population size of mtDNA markers is only one-quarter that of the nuclear microsatellite markers. Due to drift processes, genetic variation may be lower in the smaller populations compared with a larger German population in the Rhineland-Palatinate.
3 Past selection on birds breeding in different habitats (see below) has led to more ancient isolation and the formation of genetically widely differing sub-populations. In this case, we expect strong population structure, both in microsatellites and in mtDNA, with a likely separation between the coastal and inland populations.

To differentiate between these hypotheses, we analysed 22 nuclear microsatellite markers and two mtDNA markers. The mtDNA markers have been used to distinguish between (sub)species within the genus *Oenanthe* (Aliabadian *et al.* 2007) and should therefore be suitable for investigating potential differentiation between coastal and inland populations.

METHODS

Study species and study sites

Northern Wheatears are insectivorous long-distance migrants that breed from eastern Canada across Eurasia to western Alaska (Glutz von Blotzheim & Bauer 1988). The species is in decline in Europe, where the breeding population decreased by 60% between 1980 and 2011 (PECBMS 2013). In the Netherlands, Northern Wheatears were once widely distributed, but declined by 87% between 1990 and 2010 (Boele *et al.* 2012, Fig. 1). In the western European lowlands, the species occurs in sandy, oligotrophic grasslands in coastal dunes and heathlands where they often breed in the burrows of Rabbits *Oryctolagus cuniculus* or in holes in the trunks of felled trees. Individuals forage mostly on the ground, hunting for beetles, beetle larvae and caterpillars (Van Oosten *et al.* 2014). Large expanses of breeding habitat were lost due to eutrophication and acidification, which stimulated growth of tall grasses (Bobbink *et al.* 2010). This rendered suitable short grown grasslands into inaccessible stands of tall grasses, including species such as Wood Small-reed *Calamagrostis epigejos*, Sand Sedge *Carex arenaria* and Marram Grass *Ammophila arenaria*.

We collected DNA of Northern Wheatear nestlings at four sites: three in the Netherlands (Castricum (C), Den Helder (D), Aekingerzand (A)) and one in Germany (Rhineland-Palatinate (R)) (Fig. 1) between 2006 and 2013. The coastal population Castricum (site C, 7–24 breeding pairs 2007–2013, also present more than 200 years ago; Nozeman 1789) is separated by 35 km from the coastal population Den Helder (site D, 47–74 breeding pairs 2007–2013). The inland population at Aekingerzand (site A, 11–47 breeding pairs 2007–2013) is 110 km from the Castricum population and 125 km from the Den Helder population. Individuals from populations C and D breed in coastal dunes within 1 km of the sea, whereas individuals from population A breed in heathland with drift sands. The German population near Bad Dürkheim, Rhineland-Palatinate (site R, 100 breeding pairs), consists of individuals that breed mostly in vineyards (Buchmann 2001) and is approximately 400 km from the three Dutch populations. This population is one of eight spatially separated German populations that have been studied intensively since 1995 over a total area of 72 km^2 (Buchmann 2001). Northern Wheatears are threatened with extinction in Germany (Südbeck *et al.* 2008).

DNA extraction, genotyping and sequencing

All Dutch DNA samples stem from growing feathers of nestlings collected when colour-ringing the birds. The German DNA samples stem from blood obtained by brachial venipuncture of nestlings at the time of ringing. DNA from feathers was extracted using the QuickExtract DNA Extraction Solution (Epicentre, Madison, WI, USA), with the following particulars: 90 μL QE solution, two cut down growing feathers, 1 h of incubation at 65 °C, vortexing every 30 min, heat denaturation for 3 min at 95 °C and down-spinning twice after

denaturation. DNA from blood was extracted using the QIAamp blood extraction kit (Qiagen, Hilden, Germany), following the protocol provided by the manufacturer.

Microsatellites

In total we used DNA of 194 putatively unrelated birds across the four sites (C: 72, A: 58, D: 13, R: 51; one random nestling per nest). Individuals were genotyped with 22 microsatellite markers (Supporting Information Tables S1 and S2). Microsatellite PCR-amplifications and sexing were performed in multiplexed PCRs using the Qiagen Type-it Microsatellite PCR Kit (Qiagen) and primer mixes containing four to seven primer pairs (M1–M6, Table S1). Forward primers were labelled at their 5′ end with fluorescent dyes from Life Technologies GmbH (Darmstadt, Germany). Each 10-μL multiplex PCR contained 1 μL DNA, 5 μL of the 2X Qiagen Type-it PCR Master Mix and 1 μL of one of the six primer mixes (Table S1). Cycling conditions were: 5 min initial denaturation at 95 °C, followed by 31 cycles of 30 s at 94 °C, 30 s at the annealing temperature given in Table S1, 1 min at 72 °C; followed by 30 min to complete the final extension at 60 °C. PCR product (1.5 μL) was mixed with formamide containing the GeneScan 500LIZ size standard, heat-denatured and resolved in POP4 polymer on an ABI 3130xl Genetic Analyzer (all Life Technologies GmbH). GeneMapper 4.0 (Applied Biosystems, Carlsbad, CA, USA) was used to score allele sizes.

Mitochondrial DNA

We collected DNA from nine individuals from sites C and D, with additional samples from the island of Ameland (Fig. 1, collectively denoted as 'coastal'), and a further 10 individuals from site A and 10 from site R, which were chosen randomly. All individuals were juveniles and, except for the Ameland birds, all were also used for the microsatellite analyses.

We sequenced 695 base pairs (bp) of the mitochondrial cytochrome oxidase subunit 1 (COX1) gene, using the primers BirdF1 TTCTCCAACCA CAAAGACATTGGCAC and BirdR1 ACGTGG GAGATAATTCCAAATCCTG (Hebert *et al.* 2004). Additionally, we sequenced 569 bp of the mitochondrial 16SrRNA gene using the primers 16SA-L CGCCTGTTTATCAAAAACAT and 16SB-H CCGGTCTGAACTCAGATCACGT (Aliabadian *et al.* 2007). The PCR amplifications were performed in 50-μL reaction volumes consisting of 1× PCR buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 200 μM dNTP, 1.25 U Taq DNA Polymerase (Fisher Scientific Germany GmbH, Schwerte, Germany), 1 μL genomic DNA and 0.5 μM of each forward and reverse primer. Cycling conditions were: 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 at 55 °C for COX1 or 50 °C for 16SrRNA, 1 min at 72 °C; and a final extension at 72 °C for 1 min. PCR products (10 μL) were visualized on an agarose gel. Two separate volumes of 20 μL were treated for 15 min at 37 °C with 5 U Exonuclease I and 10 U FastAP Thermosensitive Alkaline Phosphatase (both Fisher Scientific Germany GmbH), followed by a 15-min heat denaturation of the enzymes at 85 °C. Subsequently, the PCR products were premixed with 20 pmol primer and sent to an external contractor for sequencing (Eurofins MWG GmbH, Ebersberg, Germany).

Data analyses

Within- and between-population genetic variation was determined by calculating the mean number of alleles per locus, observed and expected heterozygosity and F_{ST} values using microsatellite genotype data for 22 loci in Genepop 3.4 (Raymond & Rousset 1995). For 192 of 194 individuals all 22 markers were scored, for one individual four markers were not scored, and for one individual one marker was not scored. Allelic richness, the number of alleles per site corrected for different sample sizes among sites, was calculated using fstat 2.9.3.2 (Goudet 2002, accessed 15 June 2015). Smallest sample size was 13 individuals (site D). The significance of pairwise F_{ST}-values was calculated in GenAlEx 6.5 (Peakall & Smouse 2006). To assess analytical problems due to possible null alleles we investigated whether deviations from the Hardy–Weinberg equilibrium occurred, and we tested for linkage disequilibrium among all marker pairs in Genepop 3.4 (Raymond & Rousset 1995). We conducted an analysis of variance using spss 21.0 (IBM Corp. 2012, Armonk, NY, USA) to investigate whether differences in allelic richness or observed heterozygosity occur among sites.

Using structure v. 2.3.4 (Pritchard *et al.* 2000) we estimated the most likely number of genetic clusters (K) present in our sample and the cluster affiliations of each individual. The structure program was run with K ranging from one to seven, with 12 replicate runs per K-value. Simulations

were run with a burn-in period of 100 000 followed by an additional 500 000 steps. Individuals could have mixed ancestry in the analyses (admixture model) and allele frequencies were allowed to correlate among populations. In addition, the most likely number of genetic clusters was also determined by calculating the ΔK statistic, as suggested by Evanno *et al.* (2005). Results from STRUCTURE were used as input in the program STRUCTURE HARVESTER (Earl & vonHoldt 2012) to calculate the ΔK statistic.

The number of haplotypes of the COX1 and 16SrRNA regions of the mtDNA was determined per site locality. Due to mtDNA being inherited as a single locus, the COX1 and 16SrRNA haplotypes of an individual bird were combined to produce an overall haplotype. These data were used to test for population differences using a chi-square test.

We performed a coalescence analysis with the program IMa2 (Hey 2010), using the M-mode and L-mode modules, which uses Bayesian inference and an MCMC method for generating posterior probabilities for complex demographic population models. We ran the program with a scenario based on the four populations. We assumed a mutation rate of 1×10^{-5} for 15 microsatellite loci (Ellegren 2000) and fitted a stepwise mutation model (SSM). We excluded seven loci because their allele variation did not appear to follow a stepwise mutation model. Based on multiple exploratory runs the following priors were chosen: effective population size = 20, splitting time = 5 and migration rate = 10. We used heating with a geometric model, 25 chains and 0.975 for the first and 0.8 for the second heating parameter. Analysis was run for 4 988 983 MCMC steps. The burn-in period was specified as −b1.0, which means that there was 1 h between the writing of each burn-in file. Adequate parameter mixing was checked from plots of parameter trends, comparison of set0 and set1 values, effective population sizes (ESS) and autocorrelation analyses provided by IMa2. Parameter estimates for divergence times, effective population sizes and migration rates were inferred from the posterior density distributions.

RESULTS

Genetic diversity

Microsatellites

Our analyses recovered between two and 17 alleles per locus among all individuals surveyed (Table S1). Allelic richness varied among sites (Table 1; $F_{3,87} = 2.817$, $P = 0.044$). Tukey *post-hoc* comparisons of the four groups indicate that allelic richness was higher at site R than at site A ($P = 0.044$). All other pairwise comparisons were non-significant. Observed heterozygosity averaged across loci was similar among populations (Table 1; $F_{3,87} = 0.550$, $P = 0.65$). After controlling for multiple tests using sequential Bonferroni correction (Rice 1989; critical $P = 0.0023$), no loci showed significant departure from Hardy–Weinberg equilibrium. All loci were independent of each other when testing for linkage disequilibrium.

Table 1. Summary of genetic diversity for the four sites sampled based on 22 microsatellite loci. Parameters used to measure genetic diversity per site are: average number of alleles per locus (A), allelic richness (A_R), expected heterozygosity (H_E) and observed heterozygosity (H_O).

Site	Sample size	A	A_R	H_E	H_O
C	72	6.00	4.77	0.62	0.62
A	58	5.05	4.36	0.63	0.65
D	13	5.50	5.50	0.67	0.68
R	51	8.14	6.12	0.70	0.69

Mitochondrial DNA

Within the 695 bp of the mitochondrial COX1 region sequences, we detected nine polymorphic sites (Table 2a) representing four haplotypes. Two of the four haplotypes (H3 and H5) were recovered by Aliabadian *et al.* (2007) and occurred in 25 of 27 sampled birds. One coastal bird and one bird from site R each had a different haplotype (Table 2a). The coastal bird differed from haplotype H3 by four transitions and from H5 by two transitions, and was assigned haplotype H6. The Rhineland-Palatinate bird differed in seven transitions from haplotype H3 and in one transition from haplotype H5, and was assigned haplotype H7 (Table 2a). One major haplotype (H3 of 16SrRNA) was missing for site A. Within the 569 bp of the mitochondrial 16SrRNA region sequences, two polymorphic sites were recovered representing two haplotypes (Table 2b): both haplotypes were previously recovered by Aliabadian *et al.* (2007). We recovered five haplotypes when combining the COX1 and 16SrRNA fragments.

Population differentiation

F_{ST}-values were moderately high (mean $F_{ST} = 0.10$), and all pairwise comparisons were

Table 2. Summary of mtDNA genotyping of four Wheatear populations. (a) Haplotypes found within the 695 bp of the COX1 mtDNA region, and their frequency per site. Sequence positions are given with respect to Northern Wheatear sequences from Aliabadian *et al.* (2007). New haplotypes H6 (GenBank accession number KM891725) and H7 (GenBank accession number KM891726) were not found in that study. (b) Haplotypes found in the 569 bp of the mitochondrial 16SrRNA region and their frequency per site. Sequence positions are given with respect to Northern Wheatear sequences from Aliabadian *et al.* (2007). 'Coastal' refers to populations C, D and Ameland.

Haplotype ID	Position in alignment									Site			
(a) COX1	255	294	336	366	456	483	519	618	672	A	Coastal	R	Total
H3	A	T	G	C	C	T	T	C	A	1	2	3	6
H5	G	T	A	G	T	T	C	C	G	7	6	6	19
H6	A	T	G	G	T	T	C	C	G	0	1	0	1
H7	G	C	A	G	T	T	C	C	G	0	0	1	1
Sample size										8	9	10	27

Haplotype ID	Position in alignment		Site			
(b) 16SrRNA	306	370	A	Coastal	R	Total
H2	A	G	9	7	7	23
H3	G	G	0	3	3	6
Sample size			9	10	10	29

Table 3. Population pair-wise F_{ST}-values (below diagonal) and corresponding *P*-values for each population pair (above diagonal).

Site	C	A	D	R
C	–	< 0.001	< 0.001	< 0.001
A	0.10	–	< 0.001	< 0.001
D	0.10	0.12	–	< 0.001
R	0.13	0.10	0.06	–

significant (Table 3). The number of pairwise significant loci ranged between 13 and 22, from 22 loci. The STRUCTURE analysis using the 22 non-linked microsatellite loci revealed strong clustering of the populations, in line with the high F_{ST}-values. The model with $K = 4$ genetic clusters is most likely (Supporting Information Fig. S1) because Ln P(D) values start to plateau at higher K and variation per K increases. The delta K method (Evanno *et al.* 2005) also suggested greatest support for $K = 4$ genetic clusters (Supporting Information Fig. S2). The clusters show high congruence with the four populations C, A, D and R (Fig. 2). Admixed or immigrant individuals appear to be rare, except at site C, where 24 of the 72 individuals sampled had more than a *c.* 50% probability of being assigned to site D rather than site C where the individuals were sampled (Fig. 2). mtDNA haplotypes based on five unique COX1 plus 16SrRNA combinations per individual did not differ among the populations 'coastal', A and R ($\chi^2 = 8.84$, df = 8, $P = 0.36$).

The coalescent analyses for population history using IMa2 resulted in divergence times (expressed in tu (t = number of generations, u = neutral mutation rate)) of 0.0225 (95% highest posterior density (HPD): 0.0125–0.0675) between sites C and D, 0.0425 (0.0225–0.0925) between C + D and A, and 1.093 (0.8225–1.282) between C + D + A and R, indicating a relatively recent origin for the Dutch populations. The German site R had the greatest population size (expressed in 4Nu) of 1.21 (95% HPD: 0.95–1.51), followed by D (0.43, 95% HPD: 0.25–0.67), C (0.31, 95% HPD: 0.17–0.53) and A (0.17, 95% HPD: 0.09–0.37). The rate of gene flow between the Dutch populations ranged from about one to eight individuals per generation. Gene flow from C to A was 6.455 (95% HPD: 4.645–8.745) and 7.765 (95% HPD: 5.845–9.995) in the other direction. Gene flow from D to A was 0.715 (95% HPD: 0.105–2.385) and 7.135 (95% HPD: 3.425–9.845) in the other direction. Gene flow from D to C was 6.625 (95% HPD: 4.625–9.685) and 6.175 (95% HPD: 4.685–9.95) in the other direction. We also found evidence for gene flow between Germany and the Dutch populations, but the power of the microsatellite dataset was insufficient confidently

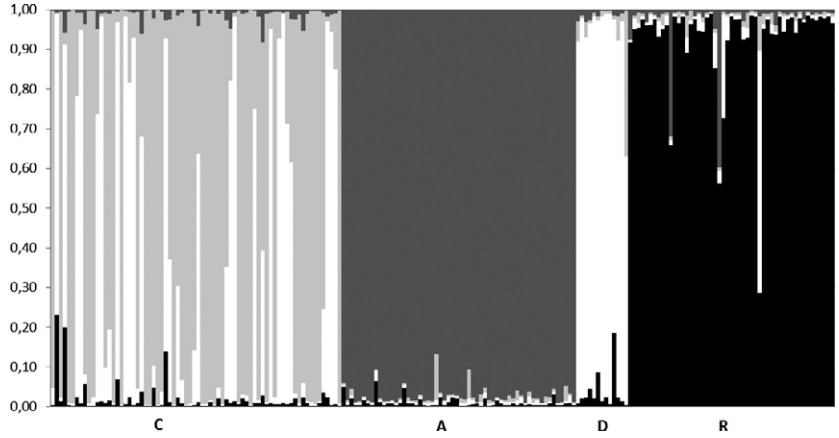

Figure 2. Assignment of individuals to each of $K = 4$ genetic clusters without prior population information. Each bar represents the estimated membership coefficient for each individual bird in each cluster. Birds are placed *a posteriori* into their sampling populations by vertical lines.

to assess the number of individuals exchanged per generation.

DISCUSSION

Northern Wheatears have declined significantly across the lowland habitats of Europe (Cramp 1988, Glutz von Blotzheim & Bauer 1988) and today often occur at small and seemingly isolated sites. Demographic analyses using mark–recapture data have demonstrated low dispersal rates among sub-populations (Van Oosten *et al.* 2015), and here we show that this once widespread species at present consists of genetically differentiated sub-populations that are likely to be of recent origin, a consequence of habitat fragmentation. The results do not support our first prediction because of strong genetic structuring. Prediction 3 states that past selection on birds breeding in different habitats has led to more ancient isolation. This is also rejected because mtDNA does not differ between populations and because the IMA2 analysis suggested gene flow continues to occur sporadically among populations. Prediction 2 garnered the most support, that recent isolation and genetic drift have already resulted in population genetic structuring.

Population structure

The degree of genetic structure (F_{ST}-values), based on microsatellite markers, among our sub-populations of Northern Wheatears is high compared with those reported for other migratory songbirds, especially given the small geographical distances between our populations (mean $F_{ST} = 0.10$, range 0.06–0.13). For common and widespread species, F_{ST}-values are typically low and no structuring is reported (e.g. European Pied Flycatchers: mean $F_{ST} = 0.01$, range 0–0.052, $n = 16$ sites covering most of their breeding range, Lehtonen *et al.* 2009, Eurasian Reed Warblers: mean $F_{ST} = 0.013$, range 0–0.064, $n = 31$ sites, Prochazka *et al.* 2011). Interestingly, this is also true for some strongly declining passerines in rather isolated populations (e.g. Cerulean Warbler: mean $F_{ST} = 0.01$, range 0–0.051, $n = 14$ sites, Veit *et al.* 2005, Black-capped Vireo *Vireo atricapilla*: mean $F_{ST} = 0.021$, Barr *et al.* 2008, Golden-cheeked Warbler: mean $F_{ST} = 0.008$, range 0–0.026, $n = 7$ sites, Lindsay *et al.* 2008). The difference between these studies and our study may be due to the very small sub-population sizes in the Northern Wheatears we studied, and to their potentially higher philopatry. The F_{ST}-values we found for the Northern Wheatear are even higher than those typically reported for different subspecies (e.g. seven subspecies of the Bluethroat *Luscinia svecica*: mean $F_{ST} = 0.042$, range 0–0.17, $n = 7$ subspecies samples at $n = 11$ sites, Johnsen *et al.* 2006, three subspecies of the Common Reed Bunting *Emberiza schoeniclus*: mean $F_{ST} = 0.043$, range 0–0.11, $n = 3$ subspecies at $n = 13$ sites, Kvist *et al.* 2011).

Consistent with the relatively high and significant F_{ST}-values, indicating population differentiation, individual Northern Wheatears were

genetically assigned to four clusters which closely matched the four sampled populations (Fig. 2). Therefore these populations seem to be close to genetic isolation, except for recent immigration of birds from population D into population C, but not vice versa based on results from a mark–recapture study (Van Oosten *et al.* 2015). During 2007–2011 we observed the immigration of six birds (five males, one female) that were colour-ringed in population D into population C, and birds born in population C were never observed in population D, despite intensive fieldwork and equally high resighting probabilities ($P = 0.97$ for adults and 0.95 for returning first-year birds; Van Oosten *et al.* 2015). Notwithstanding this limited dispersal, populations C and D differed genetically (Table 3), suggesting that either the number of yearly immigrants is too small to maintain genetic panmixia or that the immigrants did not contribute to the gene pool because of low reproductive success. The IMa2 analysis suggests that some gene flow continues to occur among all four populations, although dispersal of colour-ringed individuals into different breeding populations was rare or absent (Van Oosten *et al.* 2015). Although IMa2-based analyses attempt to determine whether shared alleles among populations are a consequence of recurrent gene flow or the retention of ancestral polymorphism, this remains a difficult problem if gene flow has only recently ceased. Thus, when we consider our estimate of immigration from colour-ringed individuals (Van Oosten *et al.* 2015), it seems most logical to conclude that immigration of individuals into other populations may well have occurred until very recently when populations in the Netherlands were much larger and more continuous, but that gene flow is now greatly reduced, a result consistent with the relatively high F_{ST}-values recovered and the STRUCTURE analyses. Furthermore, the four study populations did not differ with respect to their mtDNA sequences. This suggests that population segregation is a recent phenomenon. We postulate that the genetic structuring has developed due to high philopatry and genetic drift during the last 25–35 years, when Dutch breeding numbers declined. Together, these data provide strong support for the hypothesis that recent isolation and genetic drift has resulted in a population genetic structure, as shown in the microsatellite data.

The combination of high philopatry and genetic drift in small populations may have resulted in the observed structuring, but raises the question of why philopatry is so high. Philopatry may be a recent phenomenon, resulting from population fragmentation. Indeed, philopatry typically increases as a response to decreasing population sizes and isolation (Weatherhead & Forbes 1994, Heino & Hanski 2001). Currently, suitable breeding sites for Northern Wheatears are probably rare, and often > 10 km from the natal site, such that they may not easily be discovered by individuals prospecting for breeding sites. Alternatively, strong natal and breeding philopatry may be an inherent trait of Northern Wheatears, as similar survival rates (observed return rate corrected by resighting probability, White & Burnham 1999) have also been reported for other populations (Arlt *et al.* 2008, Seward *et al.* 2013). Of more than 2500 resighted Northern Wheatears since 1995 in eight populations of the Rhineland-Palatinate (in total 300 breeding pairs, Buchmann 2001), only 1.6% ($n = 40$ individuals) were observed in a different population to their natal or breeding site (M. Buchmann pers. comm.). This is indicative of strong philopatry in the Rhineland-Palatinate populations as well.

To distinguish between the two possibilities that strong philopatry is a recent phenomenon or that it is inherent to the species, it would be instructive to conduct a similar study in a large, continuous population, as may perhaps be found in the Scandinavian uplands.

Genetic diversity

We did not observe strong differences in heterozygosity among populations in spite of varying population sizes in combination with reduced gene flow (as shown by the strong population structuring). Nevertheless, we found that allelic richness was lower at site A than in the large German population, suggesting recent loss of alleles in population A. Despite small population sizes, overall observed heterozygosity was still in the range of heterozygosities reported in other studies on songbirds (e.g. Brewer's Sparrow *Spizella breweri*: mean $H = 0.77$, Croteau *et al.* 2007, Golden-cheeked Warbler: mean $H = 0.75$, Lindsay *et al.* 2008, European Pied Flycatcher: $H = 0.76$–0.82, Lehtonen *et al.* 2009 and Common Reed Warbler: $H = 0.55$–0.74, Prochazka *et al.* 2011).

Conservation implications

Inbreeding and genetic drift eventually deteriorate the viability of small populations (Soulé & Mills 1998, Westemeier *et al.* 1998, Spielman *et al.* 2004). Little contemporary immigration into the breeding population may not be sufficient to counteract the loss of allelic diversity, and therefore the potential for long-term evolutionary adaptation (Lande & Barrowclough 1987).

Another worrying outcome of our study is that it supports the low probability of natural recolonization events, as reported in Van Oosten *et al.* (2015) based on a capture–mark–recapture study, in otherwise suitable sites or restored ecosystems due to high natal and breeding philopatry. Occasional dispersal was only observed along the coast: we observed two different males in 2 years, ringed as nestlings in C, which established territories in a dune area where Northern Wheatears were extinct (25 km from C). Both males remained unpaired. Furthermore, we observed a male defending a territory on the island of Ameland, 130 km from C where it was born. In spite of these examples of occasional dispersal into abandoned areas (interestingly, all by males, which contrasts with the general notion that in birds females are the more dispersive sex, e.g. Ribeiro *et al.* 2012), odds are small that pairs settle and breed in these areas, given high philopatry.

Low dispersal and immigration may be typical not only for Northern Wheatears, but also for other declining species in isolated populations (Weatherhead & Forbes 1994, Heino & Hanski 2001, Hansson *et al.* 2002). Philopatry may increasingly have been selected for, as birds may not easily find suitable breeding sites due to the scattered occurrence of the breeding habitat. As dispersal has been shown to be heritable in other passerines (Doligez *et al.* 2009, Duckworth & Kruuk 2009) and can be genetically associated with other flexible behaviours (Dingemanse *et al.* 2004, Duckworth & Kruuk 2009), selection for genetic philopatry may hamper the possible recovery even if new suitable habitat patches become available. We hypothesize that habitat fragmentation can lead to a self-reinforcing process, with dispersing individuals not establishing and thereby exerting selection for philopatry among individuals that are less able to adapt flexibly to new situations. Such a process would lead to isolated populations that cannot easily recolonize new suitable patches and finally reach sizes where stochastic demographic and genetic processes result in local extinction. Therefore, conservation of just a few large populations may not be sufficient.

Practical conservation efforts should be directed to establishing suitable breeding habitat between populations to connect larger populations. For the Dutch coastal dunes, suitable habitat characteristics and food densities are available for C, which may serve as a baseline for establishing suitable habitat (Van Oosten *et al.* 2014).

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SUPPORTING INFORMATION

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

Figure S1. Estimated log-likelihood (± sd) of each number of tested clusters, K = 1–7, with 12 iterations per value of K. Based on calculations using Structure 2.3.4.

Figure S2. Estimated delta K for each number of tested clusters, K = 1–7, with 12 iterations per K.

Table S1. List of 22 microsatellite loci and a sexing primer used for genotyping *Oenanthe oenanthe*.

Table S2. List of raw microsatellite genotypes and GenBank sequence accession numbers for the COX1 and 16SrRNA mtDNA.