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Correlated patterns of genetic diversity and differentiation across an avian family

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Abstract

Comparative studies of closely related taxa can provide insights into the evolutionary forces that shape genome evolution and the prevalence of convergent molecular evolution. We investigated patterns of genetic diversity and differentiation in stonechats (genus *Saxicola*), a widely distributed avian species complex with phenotypic variation in plumage, morphology and migratory behaviour, to ask whether similar genomic regions have become differentiated in independent, but closely related, taxa. We used whole-genome pooled sequencing of 262 individuals from five taxa and found that levels of genetic diversity and divergence are strongly correlated among different stonechat taxa. We then asked whether these patterns remain correlated at deeper evolutionary scales and found that homologous genomic regions have become differentiated in stonechats and the closely related *Ficedula* flycatchers. Such correlation across a range of evolutionary divergence and among phylogenetically independent comparisons suggests that similar processes may be driving the differentiation of these independently evolving lineages, which in turn may be the result of intrinsic properties of particular genomic regions (e.g. areas of low recombination). Consequently, studies employing genome scans to search for areas important for reproductive isolation or adaptation should account for corresponding regions of differentiation, as these regions may not necessarily represent speciation islands or evidence of local adaptation.

Keywords: differentiation peaks, *Ficedula*, genetic drift, genomic scan, linked selection, pooled sequencing, *Saxicola*

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Introduction

Evolutionary biologists seek to understand the genetic basis of speciation and the degree to which the divergence of lineages may involve independent changes on similar loci (Seehausen *et al.* 2014). Genomic sequencing has made it possible to examine patterns of differentiation across the genomes of organisms at different stages of divergence. Recent comparative studies of genome-wide patterns of variation, or 'genomic landscapes', have identified areas of the genome that are conspicuously differentiated relative to the genomic baseline among closely related taxa (Ellegren *et al.* 2012; Ruegg *et al.* 2014; Burri *et al.* 2015; Wang *et al.* 2016). It remains uncertain whether these regions are functionally important in speciation, and whether they typically arise during speciation-with-gene-flow or as a consequence of selection in allopatry.

Most empirical studies have used *F*-statistics (Wright 1965) and other measures that compare allele frequencies between two populations to infer the magnitude of

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differentiation across the genome. These statistics are influenced by levels of within-population genetic variation and are therefore classified as 'relative' measures of divergence (Hedrick 2005). Genomic outlier regions of high differentiation were first described as 'islands of speciation' in the face of gene flow (Turner *et al.* 2005) and hypothesized to harbour loci that were important for reproductive isolation (Nosil *et al.* 2009; Feder *et al.* 2012). However, subsequent studies have identified alternate mechanisms by which isolated genomic regions of elevated differentiation can be generated in allopatry, and thus independently of gene flow (e.g. Noor & Bennett 2009; Turner & Hahn 2010; White *et al.* 2010; Cruickshank & Hahn 2014). For example, postspeciation selective sweeps or background selection, especially in regions of reduced recombination, can drive the differentiation of these loci relative to the rest of the genome (Nachman & Payseur 2012; Cruickshank & Hahn 2014; Burri *et al.* 2015).

Selective sweeps bring beneficial alleles to high frequency in a population, greatly reducing genetic diversity at linked sites via 'hitchhiking' (Smith & Haigh 1974; Kaplan *et al.* 1989). The magnitude of the hitchhiking effect is influenced by recombination rate, in addition to the strength of selection, with areas of low recombination experiencing greater linkage and a commensurate reduction in diversity across larger sections of a chromosome (Begun & Aquadro 1993; Charlesworth *et al.* 1997; Nielsen 2005). This reduction in local within-population genetic diversity results in high differentiation as measured by F_{ST} (Charlesworth 1998; Keinan & Reich 2010; Cruickshank & Hahn 2014). Recurrent sweeps in similar genomic regions across independent populations may be caused by selection for different advantageous alleles at the same locus, or by selection on different but tightly linked loci. Alternatively, they could result from the adaptive introgression of globally advantageous mutations transmitted among populations by gene flow, followed by sweeps due to local adaptation (see Roesti *et al.* 2014; Delmore *et al.* 2015). A similar pattern could also arise from the increased establishment probability of beneficial mutations linked to selected sites in areas of low recombination (Yeaman *et al.* 2016). These related processes can result in corresponding areas of low genetic diversity and high differentiation in independent population comparisons.

Similarly, background (or purifying) selection purges deleterious alleles as they arise and may also independently generate similar genomic landscapes of diversity and differentiation across populations (Charlesworth 2013; Burri *et al.* 2015; Wang *et al.* 2016). Under this scenario, a neutral variant that emerges in a population will subsequently disappear if it is linked to a deleterious mutation, a process that reduces nucleotide diversity (Charlesworth *et al.* 1993, 1997; Stephan *et al.* 1998). As recombination rates decrease, linkage extends over larger genetic distances and the probability of a neutral variant associating with a deleterious mutation (and thus being purged) is higher. Therefore, areas of low recombination will generally exhibit a greater reduction in genetic diversity due to background selection (Charlesworth *et al.* 1993; Nordborg *et al.* 1996). If highly conserved genomic regions (e.g. of great functional importance) and/or areas of low recombination are similar across species, the effect of background selection may cause, or contribute to, parallel genomic landscapes of diversity and differentiation in comparisons of independently evolving lineages (Nordborg *et al.* 1996; Andolfatto 2001; Cruickshank & Hahn 2014).

The effects of background selection and selective sweeps on linked neutral loci have collectively been referred to as 'linked selection' (Turner & Hahn 2010; Cutter & Payseur 2013; Cruickshank & Hahn 2014). The frequency with which parallel signatures of linked selection occur in closely related taxa and the contributions of background selection and selective sweeps in shaping genomic landscapes remain actively debated (Keinan & Reich 2010; Burri *et al.* 2015). In addition, the degree to which this parallelism may extend beyond a few well-studied species complexes is currently unknown.

Genomewide scans of two independent groups of closely related bird species, *Ficedula* flycatchers and *Phylloscopus* warblers, have identified conspicuous peaks of relative divergence (i.e. genomic regions with very different allele frequencies) in pairwise comparisons of congeners that coincide with 'valleys' of absolute divergence (i.e. regions with few sequence differences, not influenced by within-population genetic diversity) (Burri *et al.* 2015; Irwin *et al.* 2016). This inverse relationship is inconsistent with the speciation-with-gene-flow paradigm, in which regions of high relative divergence are resistant to gene flow and therefore should show *high* absolute divergence (Noor & Bennett 2009; Nachman & Payseur 2012; Cruickshank & Hahn 2014). This suggests that postspeciation selection – not divergence-with-gene-flow – generates differentiation peaks in these systems. Within their respective species complexes, flycatchers and warblers show signatures of selection in similar genomic areas (Burri *et al.* 2015; Irwin *et al.* 2016), but neither the specific type of selection, nor their contribution to the speciation process, has been fully characterized. Furthermore, these studies primarily test the correspondence of divergent regions using correlation-based methods, which can be strongly affected by pseudoreplication due to linkage.

Here, we characterize the course of genomewide molecular evolution in a well-studied group of birds,

the *Saxicola* stonechats (Urquhart 2002; Collar 2016a,b). This genus began diversifying during the Late Miocene (8.2 million years ago; Illera *et al.* 2008) and currently comprises 15 recognized species (51 named taxa including subspecies; Gill & Donsker 2016). Some taxa are restricted to small islands, while others span continents, and they range from long-distance migrants to year-round residents (Baldwin *et al.* 2010). The well-documented evolutionary diversity in this clade makes *Saxicola* a powerful system for studying independently evolving lineages across a gradient of differentiation, phenotypic variation and life histories. We examine five stonechat taxa at disparate stages of divergence, including two that likely still exchange genes and two that diverged ~3.7 million years ago (Illera *et al.* 2008). These taxa show variation in morphology and behaviour (e.g. body size and migratory direction), and we survey both island and continental taxa, which are likely to have varied demographic histories.

Our primary research focus is to investigate the extent to which genome evolution is correlated in independently evolving, but closely related, taxa. We ask as follows: Have the same regions of the genome become differentiated over time in independent stonechat lineages? If so, what role has natural selection played in driving this correlated differentiation? We further ask whether evolution is correlated at a deeper scale, between stonechats and two *Ficedula* flycatchers (Sætre & Sæther 2010). Both genera belong to the family Muscicapidae. We posit that any loci that are differentiated in both genera are unlikely to arise from parallel ecological selection pressures and instead stem from intrinsic properties of those genomic regions. Finally, we examine the effects of life history and demography on the genome by comparing patterns of genetic diversity and differentiation between continental and island taxa. We hypothesize that an island taxon will show a weaker overall effect of selection on the genome, reflecting the theoretical prediction of increased drift with a smaller effective population size. Our goal is to shed light on the processes that influence the most conspicuous features – the high 'peaks' and low 'valleys' – of stonechat genomic landscapes. The results underscore the degree to which broad patterns of genetic diversity and differentiation are correlated across evolutionary time.

Methods

Study system and sampling

We included five stonechat taxa in this study: *Saxicola rubicola rubicola* from Austria (European stonechat); *S. rubicola hibernans* from Ireland (European stonechat); *S. torquatus axillaris* from Kenya (African stonechat); *S. maurus maurus* from Kazakhstan (Siberian stonechat); and *S. dacotiae dacotiae* from Fuerteventura Island, Spain (Canary Islands stonechat) (Gill & Donsker 2016). Using mitochondrial DNA, Illera *et al.* (2008) estimated that African stonechats diverged from the remaining four taxa about 3.7 mya, Siberian stonechats subsequently split from the remaining three about 2.5 mya, and Canary Islands stonechats diverged from European stonechats about 1.6 mya. Illera and colleagues could not distinguish Austrian and Irish stonechats using mitochondrial DNA. We expect that Canary Islands stonechats have diverged from other taxa without gene flow because this taxon occurs on an oceanic island, and the present-day ranges of Kenyan and Siberian stonechats lead us to expect no ongoing gene flow between these and the other taxa. Conversely, we expect that Austrian and Irish stonechats likely still exchange genes because of their close geographic proximity, lack of mitochondrial divergence, and evidence of breeding dispersal between the British Isles and continental Europe (Helm *et al.* 2006).

Most of the 262 stonechats included in this study originated from a common-garden experiment that Eberhard Gwinner initiated in 1981 at the Max Planck Institute in Andechs, Germany, except for Canary Islands stonechats, which were directly sampled in the wild (Table S1, Supporting information). For the other species, most birds were taken into captivity as nestlings, except for Irish stonechats (~50% captured on winter territories). The remaining sampled individuals were offspring of these captive stonechats, hatched between 1988 and 2006. Despite the inclusion of captive birds, relatedness within the pools was low (Table S1, Supporting information). Detailed descriptions of breeding and raising conditions are published elsewhere (Gwinner *et al.* 1987; Helm 2003; Helm *et al.* 2009).

The inclusion of second-generation progeny in our study could potentially lower measured levels of genetic diversity relative to a comparable sample of wild individuals. However, we find average genetic diversity (π) to be highest in Siberian stonechats, the species for which we incorporated the most captive-bred birds; this suggests that any putative bias is small and potentially negligible for the purposes of this study.

Draft reference genome

We assembled the genome of a male Siberian stonechat (*S. maurus*) collected in Kazakhstan (44.590° N, 76.609° E) and housed at the Burke Museum (UWBM# 46478). We generated one fragment library with insert sizes of 180 base pairs (bp) and two mate-pair libraries (insert sizes: 3 and 8 kilobases), and we sequenced each of them on one Illumina HiSeq 2500 lane (obtaining 101-

bp paired-end reads). We assembled the draft reference genome using the ALLPATHS-LG algorithm (Gnerre *et al.* 2011) and used HaploMerger (Huang *et al.* 2012) to improve the assembly by merging homologous scaffolds and removing those resulting from the erroneous split of two haplotypes into separate scaffolds. The final Siberian stonechat assembly comprised 2819 scaffolds, with a total scaffold length of 1.02 Gb and an N50 scaffold size of 10.0 Mb. Half of the final assembly is represented in 24 scaffolds, and 75% in 65 scaffolds. Ambiguous bases (N's) make up 4.4% of its total length.

We assembled scaffolds from our stonechat reference genome into draft chromosomes by mapping them to the *Ficedula albicollis* genome assembly, version 1.5 (RefSeq Accession no. GCF_000247815.1) (Kawakami *et al.* 2014) and used SatsumaSynteny (Grabherr *et al.* 2010) to align the *Saxicola* draft genome to the *F. albicollis* assembly. Because these species are phylogenetically close and synteny is relatively conserved among birds (Ellegren 2010), this method allowed us to position 97.1% of the stonechat reference genome in the presumed correct order. Inversions and other chromosomal rearrangements occur in birds (Backström *et al.* 2008), so it is possible that a small percentage of the genome may be ordered or oriented incorrectly.

Resequencing of five stonechat taxa

We extracted genomic DNA from stonechat blood or tissue samples using a salt extraction protocol and selected 49–56 individuals (n_{total} = 262, including both males and females) from each of the five stonechat taxa for sequencing (Table S1, Supporting information). We created five pooled libraries, one per taxon, from equimolar aliquots of DNA using the Illumina TruSeq Nano DNA kit and sequenced them on an Illumina NextSeq 500 (Table S2, Supporting information).

We used BWA-MEM (Li 2013) to align sequences to our reference genome and performed refinement and quality control steps with Picard (http://broadinstitute.github.io/picard/) and the Genome Analysis Toolkit (GATK) (McKenna *et al.* 2010), including filtering by mapping quality and removing duplicate sequences (Supporting information). Sequences mapped to the draft reference genome at a mean per-pool coverage between 13.8 and 26.1×, and mean mapping quality was between 45 and 46 for all taxa (Table S2, Supporting information). Although this level of coverage is insufficient to sequence every individual at every locus, the goal of our pooled sequencing strategy was to estimate population allele frequencies by sampling a subset of chromosomes in a pool. Gautier *et al.* (2013) found that allele frequencies of individual SNPs estimated with 10–50× pool coverage (pool size = 30) were strongly correlated with estimates derived from separate individual-based (n = 20) sequencing at 1–6× (r = 0.93) and 6–10× (r = 0.94) per individual. Additionally, the effects of pool-derived sampling error are greatly reduced in window-based analyses where variation and differentiation are summarized across groups of SNPs (Kofler *et al.* 2011a). Because we use a windowed approach and therefore do not rely on the frequencies of individual SNPs, we are confident that we can accurately assess and compare genomewide patterns of genetic variation with this level of coverage.

SNP-based phylogeny and intertaxa divergence

Although we used an existing mitochondrial phylogeny (e.g. Illera *et al.* 2008) as a basis for our study approach and design, we also constructed a phylogenetic tree for the five focal stonechat taxa using nuclear markers. This step was designed to confirm the mitochondrial findings and serve as a basis for phylogeny-based inference. We used Pied and collared flycatchers (*Ficedula hypoleuca* and *F. albicollis*) as outgroups. We constructed a maximum-likelihood tree with RAxML (Stamatakis 2014), using 16 876 859 fixed single-nucleotide polymorphisms (SNPs) from across the nuclear genome, of which 330 592 were polymorphic among the stonechat ingroup (Supporting information). We applied the Lewis correction, following the recommendation of Stamatakis (2014), for ascertainment bias resulting from the exclusion of invariant sites.

We generated mean genomewide F_{ST} and d_{XY} pairwise distance matrices for the five focal stonechat taxa and displayed them graphically using principal coordinate analyses performed with the APE package in R (Paradis *et al.* 2004).

Pooled population genomic analyses

We analysed sequence data with the software packages NPSTAT (Ferretti *et al.* 2013) and POPOOLATION2 (Kofler *et al.* 2011b), designed specifically for the analysis of pooled sequencing data. With NPSTAT we calculated: (i) Tajima's D, to test for rare variants as a signal of directional or purifying selection or large-scale demographic effects; (ii) π, an estimate of genetic diversity, which is derived from the number of pairwise sequence differences among members of a population; and (iii) Fay and Wu's H, a statistic related to Tajima's D but sensitive only to high-frequency-derived alleles, thus influenced by positive selection but not by background selection (Fay & Wu 2000). We polarized alleles using the collared flycatcher.

We then used POPOOLATION2 to calculate pairwise F_{ST} among all pairs of taxa. We also estimated d_{XY} (Nei &

Li 1979; Cruickshank & Hahn 2014), a measure of absolute divergence, as $A_XB_Y + A_YB_X$, where A and B are the frequencies of the two alleles at a locus and X and Y denote the two groups being compared.

For all analyses, we excluded bases within 5 bp of indels to reduce the probability of including erroneous genotypes due to misalignments. We calculated all metrics for 50-kb nonoverlapping windows (Supporting information), within which we only considered sites with minor allele counts ≥2 and coverage between half and three times that of the pool's average. We only retained windows in which at least 40% of bases (i.e. 20 kb) satisfied this coverage criterion. For d_{XY}, we calculated the windowed value by summing over the window and dividing by the total number of sites with sufficient coverage (variable or not). We calculated standardized nucleotide diversity for each taxon by dividing π by the maximum d_{XY} value from all pairwise comparisons involving that taxon (following Irwin *et al.* 2016).

Correlation analyses

We first quantified the similarity of genomewide patterns of genetic diversity and divergence using Spearman rank correlations. Although the *P*-values of these tests are affected by pseudoreplication due to the inclusion of genetically linked loci, they are nonetheless a valuable summary of genomewide similarity and provide a means to compare the results of this study with previous work.

Identification of genomic outlier regions

We identified regions of the stonechat genome showing consistently elevated or lowered values of Tajima's D, π, Fay and Wu's H, $F_{ST,}$ and d_{XY}, and that therefore may be important in the divergence of stonechat lineages. In particular, we wanted to determine whether any genomewide similarities revealed by the correlation analyses were driven by a relatively small number of genomic regions. We applied a kernel-based smoothing algorithm across 50-kb windows (box density with bandwidth of 30; see Supporting information) and compared this smoothed line with 25 000 smoothed lines obtained after permuting the order of the windows (see Ruegg *et al.* 2014). We called outlier locations where the observed smoothed line was more extreme than the most extreme smoothed value from the null (permutation) distribution. We merged outlier regions separated by four windows or fewer (i.e. by <200 kb). Because the effective population size of the Z chromosome is smaller than that of the autosomes, baseline levels of variation and differentiation are different from those of autosomes (Charlesworth 2001). We therefore permuted windows of the Z chromosome and autosomes separately (see Fig. S1, Supporting information).

Concordance of genomic outlier regions within Saxicola

Once outlier regions were identified, we assessed their overlap among stonechat taxa. For each pairwise comparison, we counted the number of outlier regions that showed any degree of overlap between the two data sets. By considering each region separately, we account for autocorrelation of their constituent windows due to linkage. Although this approach addresses the pseudoreplication that would have resulted from treating the multiple windows within the same outlier region as independent observations, it is important to note that it does not address the larger scale possibility that multiple outlier *regions* could be clustered together, for example due to a very large region of reduced recombination.

We then tested whether the observed number of overlapping regions was significantly greater than expected under the null hypothesis of no association in outlier positions between data sets, using a custom permutation test. While holding the number and size of outlier regions constant, we randomly permuted their locations across the genome 1000 times and measured the degree of overlap under these simulated scenarios. The *P*-value of the test was the proportion of simulations under which the number of overlapping outlier regions was equal to or greater than the observed value; we thus accounted for the varying number and size of outlier regions in each comparison. We applied a false discovery rate correction to each series of tests (Benjamini & Hochberg 1995) and considered tests with corrected *P*-values <0.05 to be statistically significant.

For each comparison, we calculate the proportion of outlier regions in one genomic landscape also present in the other (and vice versa) and report the greater of these two values. Thus, if landscape 1 shows 10 peaks and landscape 2 shows 50 peaks, and nine of 10 peaks in landscape 1 are also present in landscape 2, our outlier similarity score will be 9/10 = 0.90.

Previous studies have used scatterplots and correlation analyses as the primary manner of assessing association between outlier regions in independent comparisons (e.g. Burri *et al.* 2015; Irwin *et al.* 2016). However, these tests are affected by autocorrelation due to genetic linkage. By considering each outlier region as a single unit, our permutation approach overcomes this issue by treating each contiguous outlier region (instead of each 50-kb window) as an independent observation.

Correspondence of genomic landscapes between Saxicola and Ficedula

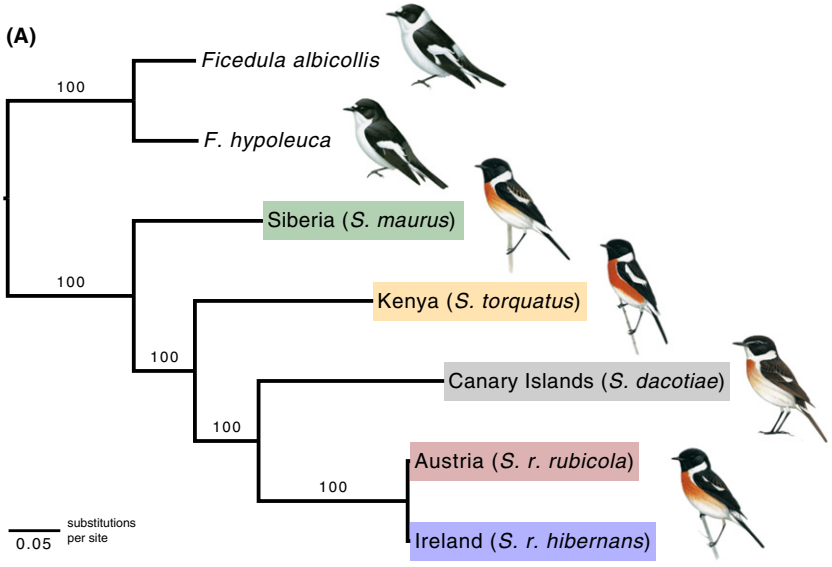
To test for conservation of genomic landscapes at a deeper level of divergence, we compared stonechat genomic landscapes to those of the genus *Ficedula*. We calculated F_{ST}, d_{XY} and π for pied flycatchers (*F. hypoleuca*) and collared flycatchers (*F. albicollis*). We downloaded reads from the Sequence Read Archive (project ERP007074; Accession no. PRJEB7359; http://www.ncbi.nlm.nih.gov/sra) for 10 individuals of each species (Smeds *et al.* 2015) (Table S3, Supporting information), and processed reads for quality as described for the stonechat analysis (Supporting information).

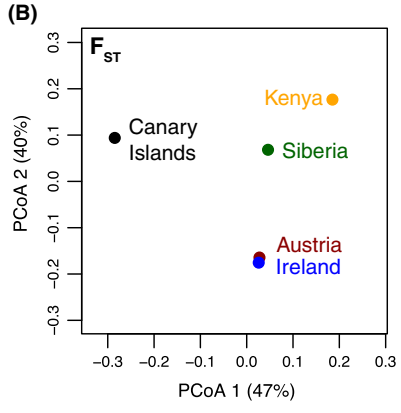
We filtered, trimmed and aligned *Ficedula* reads to the stonechat draft reference genome so that we could directly compare the locations of outlier regions between genera, using the same tools in GATK and Picard as for stonechat sequences (Supporting information). To calculate F_{ST}, we first generated a VCF file with UnifiedGenotyper from GATK and filtered raw variants with the VariantFiltration tool (settings: QD < 2.0 || FS > 60.0 || MQ < 40.0). We then calculated F_{ST} with VCFtools (Danecek *et al.* 2011) from the resulting SNPs across 50-kb nonoverlapping windows. We estimated d_{XY} from minor allele frequencies obtained in ANGSD (Korneliussen *et al.* 2014), using a custom script to calculate 50-kb windowed averages. We only included sites that had genotype calls for at least five of 10 individuals per species and retained windows for which at least 40% of bases satisfied this criterion.

Results

SNP-based phylogeny and intertaxon divergence

The maximum-likelihood (ML) phylogeny built on fixed nuclear sites showed high support for the placement of the Canary Islands stonechat as the sister taxon to the European stonechat (Austria and Ireland) (Fig. 1A). The





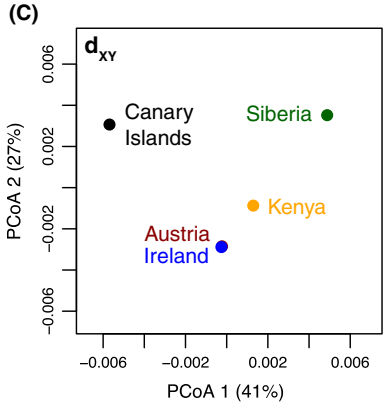


Fig. 1 (A) Maximum-likelihood phylogenetic tree constructed with RAxML from fixed sites across the stonechat nuclear genome, with two *Ficedula* species used as outgroups. Branch labels denote bootstrap support from 100 rapid bootstrap iterations. This topology places Siberian stonechat (*S. maurus*) as the sister lineage to the remaining taxa, in contrast to previous trees based on mitochondrial DNA. Canary Islands stonechats are most closely related to European stonechats. Illustrations (males shown) are reproduced with permission from Handbook of Birds of the World Alive (Collar 2016a,b). (B, C) Biplot of two principle coordinate axes derived from analyses of: (B) mean F_{ST} and (C) mean d_{XY}. Axes are labelled with per cent of variance explained.

clade comprising European, Canarian and Kenyan stonechats, to the exclusion of Siberian stonechats, was also strongly supported. This nuclear phylogeny contradicted the existing mtDNA topology. We verified that this result was not an artefact of sparse taxon sampling or choice of outgroup by constructing a ML tree with cytochrome-*b* consensus sequences obtained from Austrian, Irish, Kenyan and Siberian pools; not enough mitochondrial sequence was recoverable for Canarian stonechats. Here, Kenyan stonechats were placed as the sister lineage to the remaining stonechats, in agreement with past mitochondrial studies (not shown).

We calculated mean genomewide F_{ST} and d_{XY} to further examine relationships among stonechat taxa. The first two principal coordinate axes calculated from a distance matrix of mean pairwise F_{ST} (explaining a total of 87% of variance; 47% in first axis) revealed Siberian stonechats to be approximately equidistant from the other taxa in terms of overall allele frequency differentiation (Fig. 1B). Stonechats from Austria and Ireland were extremely similar (with only seven fixed differences of 10 164 331 sites with $F_{ST} > 0$, or $7 \times 10^{-5\%}$), reflecting their geographic proximity and common evolutionary history. In contrast, Kenyan and Canary Islands stonechats were most different (1 251 605 fixed differences of 12 401 462 sites with $F_{ST} > 0$, or 10.09%). Overall, Canary Islands stonechats were strikingly dissimilar to even their closest evolutionary relatives (Austria vs. Canary Islands: 782 967 fixed differences from 12 754 086 variable sites, or 6.14%). European stonechats were more similar genomewide to Siberian and Kenyan stonechats than to those from the Canary Islands, their sister lineage (Austria vs. Siberia: 244 623 fixed of 15 168 199 variable, or 1.61%; Austria vs. Kenya: 640 425 fixed of 12 032 148 variable, or 5.32%).

The principal coordinate analysis based on d_{XY} (68% of variance explained by first two axes; 41% by the first) was similar to the one based on F_{ST}, except that Kenyan stonechats were closer to European stonechats than to Siberian stonechats (Fig. 1C). This is consistent with the nuclear tree (Fig. 1A). Again, Austria and Irish stonechats were nearly identical. Canary Islands stonechats were distant from all stonechat taxa, but most similar to the European taxa.

Shared regions of high differentiation show low genetic diversity, except in Canary Islands stonechats

Measures of divergence were strongly correlated among stonechats. D_{XY} showed strong correlations across genomic windows (Fig. 2A–B), and d_{XY} outlier regions were highly similar (Fig. 2D; Figs S2 and S3, Supporting information); mean outlier similarity scores, averaged across all comparisons, were 0.85 for low-d_{XY} outliers and 0.79 for high d_{XY} outliers (Fig. S4, Supporting information). F_{ST} was also significantly correlated in all

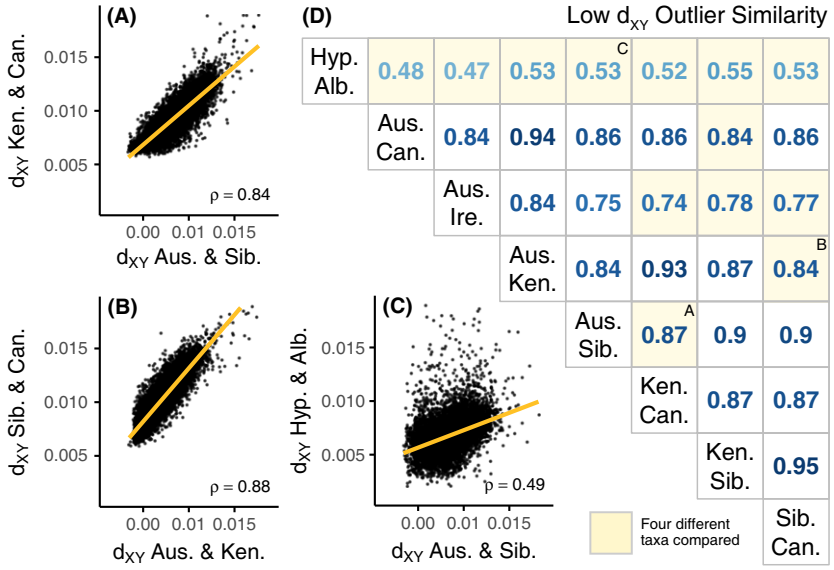


Fig. 2 Correlation of d_{XY} among stonechats and flycatchers (*Ficedula hypoleuca* and *albicollis*, or 'Hyp.' and 'Alb.'). (A, B, C) show scatterplots where each point represents one 50-Kb genomic window. Orange lines are best-fit lines, and the Spearman rank correlation rho (ρ) coefficient is given. (D) shows outlier similarity scores, which quantify the number of low-d_{XY} 'valleys' shared among different comparisons. Some comparisons including Irish stonechats are not shown because of their similarity to Austrian stonechats. All tests were significant after applying a false discovery rate correction. Cells with yellow backgrounds indicate that four independent taxa are being compared. Letters in the upper right of cells show which cells correspond to the scatterplots in sections A–C.

comparisons, but the strength of this correlation varied (Fig. 3A–D). The association was greatest in comparisons including Siberian stonechats (Fig. 3A), but F_{ST} was also correlated among independent comparisons (i.e. with no shared taxon) (Fig. 3B–C). Overall F_{ST} outlier similarity was lower than d_{XY} for both peaks and valleys (means of 0.31 and 0.24, respectively), indicating that approximately one-third of F_{ST} peaks were shared (Fig. 3 E and Fig. S5, Supporting information). Across all comparisons, windows with the lowest F_{ST} showed the most consistent associations. Of note, outlier regions showed significant overlap in several comparisons where the four taxa being compared were all different (Fig. 3 E), implicating common processes in independent stonechat lineages in the generation of differentiation landscapes.

Generally, regions of high F_{ST} showed low genetic diversity, both within (π) and between (d_{XY}) stonechat taxa. F_{ST} and d_{XY} were strongly negatively correlated, especially in comparisons including Siberian stonechats (Fig. 4A–B), and F_{ST} peaks overlapped strongly with d_{XY} valleys (Fig. 4A). F_{ST} valleys also overlapped with d_{XY} valleys in some comparisons. Regions of reduced absolute divergence also showed reduced nucleotide diversity (Fig. S6, Supporting information). Reductions in diversity occurred in the same genomic windows among stonechats, even after standardizing for levels of between-population diversity (Figs S7, S8 and S9, Supporting information). Note that, due to the high similarity between Austrian and Irish stonechats, we do not present comparisons of Irish stonechats with non-Austrian stonechats.

However, these patterns of genetic variation were often weaker, absent or even reversed for Canary Islands stonechats. Across the genome, F_{ST} and d_{XY} were *positively* correlated, despite the lowest F_{ST} windows showing high d_{XY} (Fig. 4C). Canary Islands stonechats showed the weakest associations in diversity correlations (Fig. S6A, D and S7B, D, Supporting information). Standardized nucleotide diversity (π/d_{XY}) was negatively correlated between Canary Islands and Siberian stonechats (Fig. S7D, Supporting information), indicating that the regions of the Siberian stonechat genome that showed the greatest diversity reductions were, in fact, relatively more diverse in Canary Islands stonechats than the rest of the genome (Fig. S9, Supporting information). No π/d_{XY} valleys regions were shared between Canarian stonechats and other stonechat taxa (Fig. S7E, Supporting information).

Evidence of selection and effects of demography

Among stonechats, genomic regions of high differentiation (F_{ST}), low absolute divergence (d_{XY}) and low genetic diversity (π) coincided with significant decreases in Tajima's D and Fay and Wu's H (Figs 5 and 6). Fay and Wu's H showed strong associations with F_{ST} only in

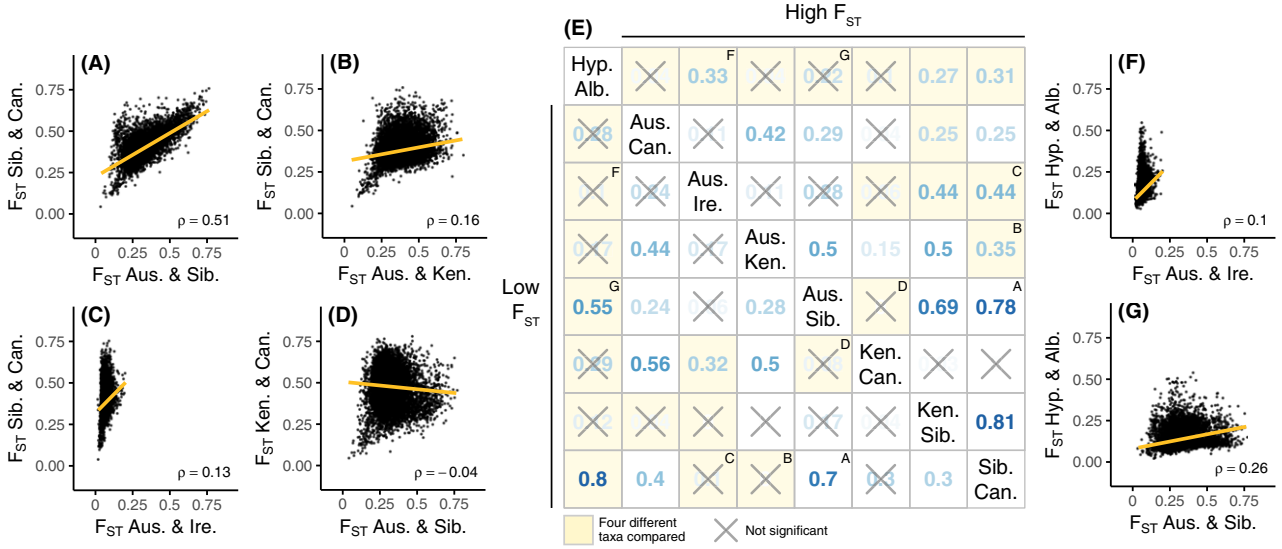


Fig. 3 Correlation of F_{ST} among stonechats and flycatchers (*Ficedula hypoleuca* and *albicollis*, or 'Hyp.' and 'Alb.'). (A, B, C, D, F, G) show scatterplots where each point represents one 50-Kb genomic window. Orange lines are best-fit lines, and the Spearman rank correlation rho (ρ) coefficient is also given. (E) shows outlier similarity scores, which quantify the number of high-F_{ST} 'peaks' shared among different comparisons (upper triangle of matrix) and the number of low-F_{ST} 'valleys' shared among different comparisons (lower triangle of matrix). Some comparisons including Irish stonechats are not shown because of their similarity to Austrian stonechats. Cells with an 'X' indicate tests that were not significant after applying a false discovery rate correction. Cells with yellow backgrounds indicate that four independent taxa are being compared. Letters in the upper right of cells show which cells correspond to the scatterplots in the other sections.

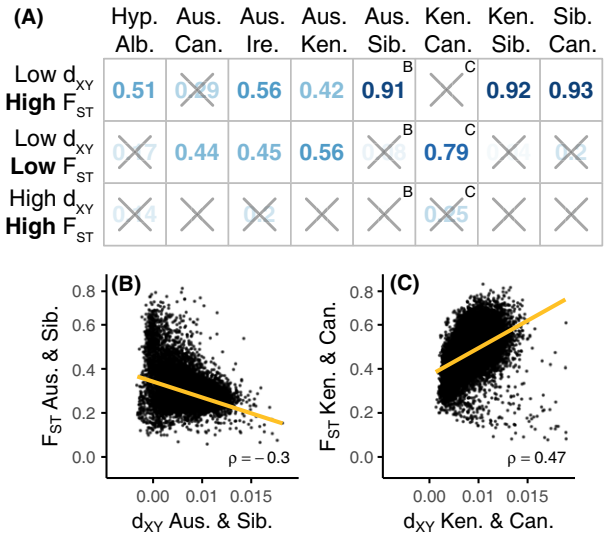


Fig. 4 Correlation of F_{ST} and d_{XY} among stonechats and flycatchers (*Ficedula hypoleuca* and *albicollis*, or 'Hyp.' and 'Alb.'). (A) shows outlier similarity scores, which quantify the number of low-d_{XY} 'valleys' that coincide with either high-F_{ST} 'peaks' (top row) or low-F_{ST} 'valleys' (bottom row). (B, C) show scatterplots where each point represents one 50-Kb genomic window. Refer to Figs 2 and 3 for details.

comparisons including Siberian stonechats. Fay and Wu's *H* outlier regions were relatively infrequent but coincided with low Tajima's *D* and π when they occurred, except in Canary Island stonechats (Fig. S10, Supporting information). Tajima's *D* outlier regions were generally shared across stonechats (Fig. S11, Supporting information). Some distinct low-*H* outlier regions occurred in only one taxon (e.g. chromosomes 4A and 6) (Fig. S12, Supporting information). Overall, in addition to lacking genetic diversity, outlier regions contained more low-frequency alleles than the rest of the genome, which is highly suggestive of a role of positive and/or background selection in shaping differentiation patterns.

The genomic baseline value of Tajima's *D* can be biased downward by demographic effects, particularly a population expansion. All stonechat taxa had median Tajima's *D* between −0.5 and −1.1, with the exception of Canary Islands stonechats, at −2.8 (Fig. S13, Supporting information). Negative values suggest that all five stonechat taxa have experienced past demographic expansion events, with the signal especially strong in the insular Canary Islands stonechats.

Correspondence of genomic landscapes between Saxicola and Ficedula

Genomewide patterns of genetic diversity and differentiation were also correlated between stonechats and flycatchers. Absolute divergence was correlated between the two genera (ρ = 0.37–0.49, Fig. 2C–D); d_{XY} outlier similarity was 0.48–0.52 between stonechats and flycatchers. Flycatchers and stonechats shared a significant number of F_{ST} peaks and valleys, but only for a subset of stonechat comparisons (Fig. 3E). Genomewide correlations of F_{ST} were significant but weak (Fig. 3F–G), and the strongest correlations occurred with Siberian stonechats. Finally, within-population genetic diversity (π) was strongly correlated between stonechat and flycatcher populations; some stonechat–flycatcher correlations were as strong as or stronger than stonechat–stonechat correlations (Fig. S7E–G, Supporting information). Overall, these results suggest that common processes are working independently and in parallel to influence genetic variation in similar regions of the genome in both genera, although the association between genera is weaker than within *Saxicola*.

Discussion

We examined patterns of genetic diversity and differentiation in an avian radiation and identified regions shared among stonechat taxa that were characterized by low within-population diversity, low absolute intertaxon divergence and high (or, in some cases, low) differentiation. These patterns are consistent with signatures of natural selection. We found that many stonechat outlier regions also appeared in the closely related genus *Ficedula*. In this genus, genomic regions of low genetic diversity and high differentiation are associated with infrequent recombination (Burri *et al.* 2015), which suggests that one possible explanation for the parallel patterns of differentiation in these genera is conserved (or convergently evolving) variation in recombination rate (see Singhal *et al.* 2015). Overall, our results are consistent with linked selection (positive selective sweeps and/or background selection) shaping large-scale patterns of genomic variation in Muscicapid birds. The presence of Fay and Wu's *H* valleys in differentiation outlier regions supports a role of positive selection in at least some cases. Despite a strong signal of similarity in genomic landscapes, we also found evidence for substantial lineage-specific evolution: Siberian stonechats appear to have experienced the strongest effects of selection, while drift may have shaped Canary Islands stonechats' genomes.

Discordance in nuclear and mitochondrial phylogenies

The phylogenetic tree constructed with SNPs from across the nuclear genome (Fig. 1A) was highly supported at all nodes, yet it is not fully concordant with previous trees constructed from mitochondrial DNA

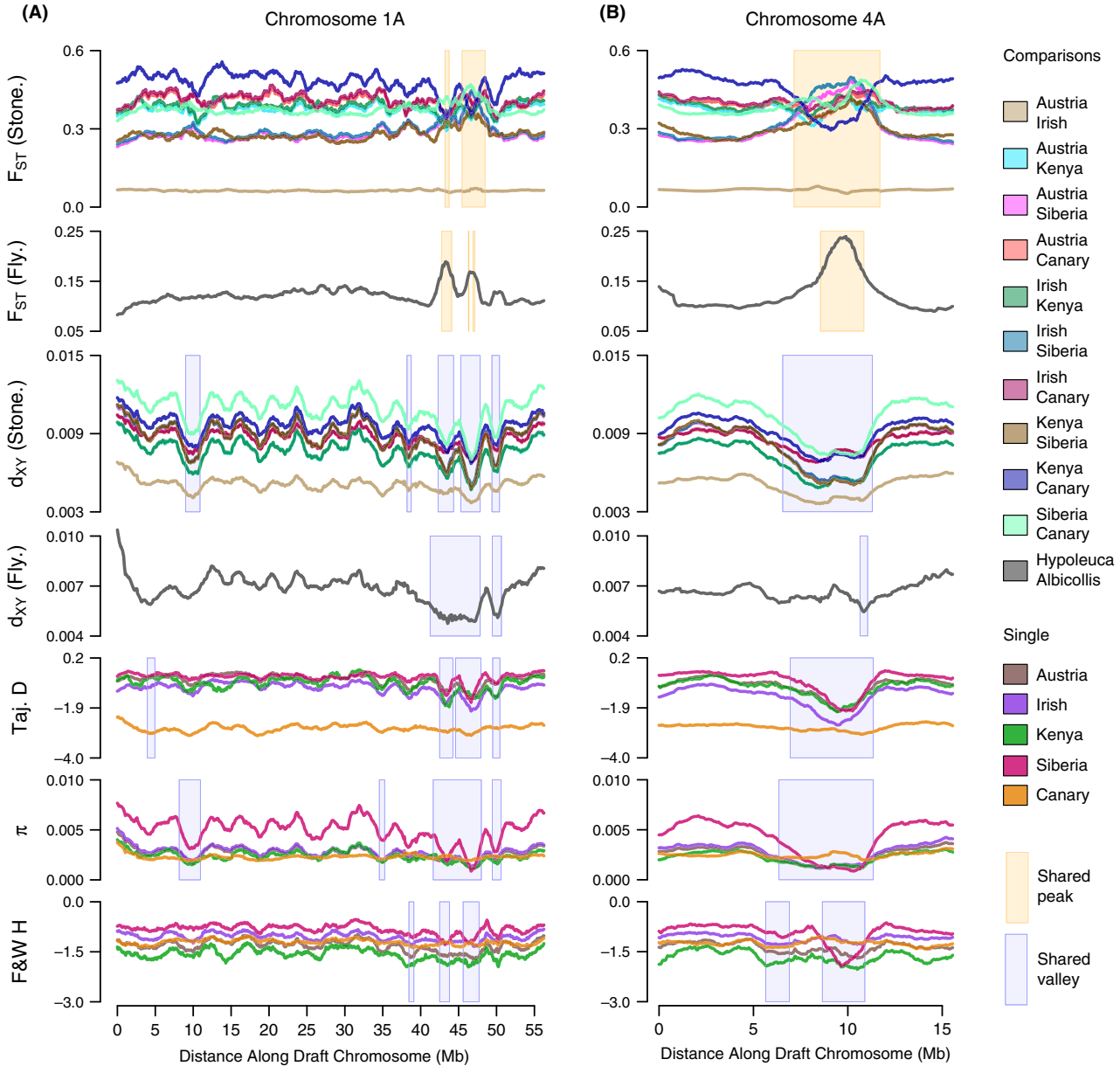


Fig. 5 Genomic statistics calculated across stonechat and flycatcher chromosomes 1A and 4A. Yellow and blue boxes indicate shared peaks and valleys, respectively. From top to bottom, the statistics and box details are as follows: F_{ST} among stonechats (peaks shared by three or more comparisons), F_{ST} between flycatchers (peaks that also overlap with shared stonechats peaks), d_{XY} among stonechats (valleys shared by three or more comparisons), d_{XY} among flycatchers (valleys that also overlap with shared stonechats valleys), Tajima's *D* (valleys shared by two or more taxa), nucleotide diversity (π) (valleys shared by two or more taxa) and Fay & Wu's *H* (valleys shared by two or more taxa).

sequences (Illera *et al.* 2008; Woog *et al.* 2008; Zink *et al.* 2009). These placed Kenyan stonechats (instead of Siberian, as in our reconstruction) as the sister lineage to the remaining stonechats. Branch support for a sister relationship of Siberian stonechats and the European/Canary Islands clade varied by study and tree-building algorithm. Mito-nuclear discordance could be a sign of past admixture, sex-biased gene flow or other biological phenomena (see Toews & Brelsford 2012). This well-resolved nuclear phylogeny serves as a basis for testing broader questions about genome-scale differentiation in this complex: for example, it helps explain why mean d_{XY} between European and Kenyan stonechats is relatively low compared to Siberian stonechats (Fig. 1C). Finally, although sparse taxon sampling (5 taxa) and choice of outgroup could potentially introduce biases (e.g. Stervander *et al.* 2015), our cytochrome-*b*-only tree (not shown) was consistent with previous mitochondrial

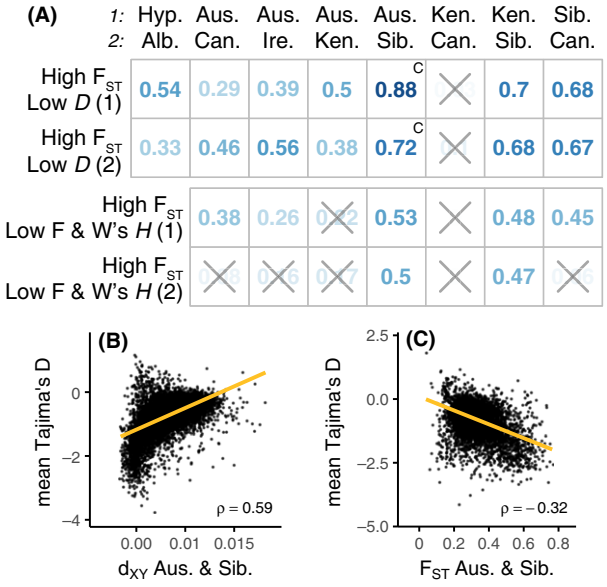


Fig. 6 Correlation of F_{ST} and d_{XY} with Tajima's D and Fay & Wu's H among stonechats and flycatchers (*Ficedula hypoleuca* and *albicollis*, or 'Hyp.' and 'Alb.'). (A) shows outlier similarity scores, which quantify the number of high-F_{ST} 'peaks' that coincide with low Tajima's D (top section) and low Fay & Wu's H (bottom section). Within each section, the top (No. 1) and bottom (No. 2) rows show the results for each of the two taxa being compared. This is necessary because Tajima's D and Fay & Wu's H are single-population statistics, while F_{ST} and d_{XY} compare two populations. All comparisons were significant after applying a false discovery rate correction. (B, C) show scatterplots where each point represents one 50-Kb genomic window. Refer to Figs 2 and 3 for details. The overlap between regions of low d_{XY} and low D was always high (ranging from 0.62 to 0.97) and statistically significant; these are not shown for simplicity.

studies, which achieved near-complete taxon sampling (e.g. Illera *et al.* 2008). The topology differences we find between mitochondrial and nuclear-based phylogenies are therefore unlikely to be artefacts of sampling. We note, however, that high bootstrap support does not always indicate a correct species tree (e.g. Suh 2016); further investigation into the larger *Saxicola* clade (e.g. using gene tree-based methods and demographic modelling; Nater *et al.* 2015) will be required to obtain a better understanding of their phylogenetic affinities.

Congruent genomic landscapes across a speciation continuum

Patterns of within- and between-population genetic diversity in stonechats show high levels of parallelism across multiple scales of evolutionary divergence. We found outliers in comparisons of highly similar taxa in the same regions as comparisons at deeper levels of divergence. The parallel reductions in d_{XY} are highly suggestive of selection before divergence (Cruickshank & Hahn 2014), and analogous patterns in standardized nucleotide diversity (π/d_{XY}) indicate that common selective forces have continued to reduce diversity on the branches leading to present-day taxa (see Irwin *et al.* 2016).

Reductions in Fay and Wu's H in some outlier regions suggest that positive selection has played a role in driving some of these regions of low genetic diversity and high differentiation. Some H outliers are present in multiple taxa, while others occur in only one (as in *Ficedula*, Burri *et al.* 2015), suggesting that localized selective sweeps may not have occurred in all groups, or that sweeps occurred too far in the past for detection using this method.

Pairwise comparisons that include Siberian stonechats show the most conspicuous F_{ST} peaks, which coincide with regions of low within-population diversity (π). Together, strongly reduced within-population genetic diversity in specific genomic regions and corresponding peaks of differentiation are consistent with Siberian stonechats experiencing the strongest effects of selection in outlier regions. Most of the larger outlier regions also showed significant decreases in Fay and Wu's H, suggesting that positive selective sweeps have contributed to this pattern. As temperate zone breeders and obligate long-distance migrants, Siberian stonechats are expected to generally show a faster pace of life, larger clutch sizes and higher metabolic rates, along with a range of specializations associated with a strongly migratory lifestyle (Wikelski *et al.* 2003; Tieleman *et al.* 2009; Baldwin *et al.* 2010; Robinson *et al.* 2010). It is possible that a combination of these factors has led to a strong footprint of selection on the Siberian stonechat genome.

Kenyan and Canarian stonechats showed the highest genomewide F_{ST}. Notably, however, we also observed conspicuous F_{ST} *valleys* in the same locations as the F_{ST} peaks of other pairwise taxon comparisons (Fig. 5 and Fig. S5, Supporting information). In other words, these taxa are differentiated across the vast majority of the genome, but they show low differentiation (F_{ST}) in regions of low absolute divergence (d_{XY}). This pattern is not unique to this comparison: Austrian and Irish stonechats, and occasionally others, show valleys in similar areas. F_{ST} valleys may occur where d_{XY} (between-group variation) is reduced but π (within-group variation) remains high, especially in Canary Islands stonechats, but more work is needed to understand this phenomenon.

Correlation of genomic variation between genera

Genomic landscapes of genetic diversity and differentiation in stonechats are significantly correlated with those

in Pied and collared flycatchers. These results contrast with recent findings in other passerine birds, for example greenish warblers (Irwin *et al.* 2016). Nucleotide diversity in greenish warblers is only weakly correlated with that in outgroup comparisons (π: Pearson's $r = 0.19$). We found a stronger association in nucleotide diversity between stonechats and flycatchers (π: Spearman's $\rho = 0.47$–0.60, excluding Canary Is.). *Saxicola* and *Ficedula* share certain aspects of their life history (e.g. they are insectivores, and the flycatchers and most stonechats are migratory), but the hypothesis that these parallel signatures of selection and differentiation are due to shared ecological selection pressures on the same loci appears unlikely. Burri *et al.* (2015) demonstrated a clear link between low recombination and areas of high differentiation in *Ficedula*, which suggests that low recombination might also contribute to shared differentiation outliers within *Saxicola*. Although initial evidence suggested that avian recombination landscapes change drastically over time (Backström *et al.* 2010), recent work has shown that recombination landscapes can be conserved in birds across millions of years of evolution (Singhal *et al.* 2015). It is therefore possible that coincident areas of low recombination, in combination with linked selection, may play a role in shaping the broad patterns of landscapes of genomic variation and differentiation across both closely related and deeply diverged taxa. However, direct measures of recombination rates in stonechats are needed to test this hypothesis. While recombination is reduced in close proximity to avian centromeres (Backström *et al.* 2010), centromeres do not explain the recombination deserts in the centres of acrocentric chromosomes (e.g. 4A, 9, 10, 11, 12, 13, and 18; Knief & Forstmeier 2016) (Kawakami *et al.* 2014; Burri *et al.* 2015). These regions frequently show high differentiation among flycatchers (Burri *et al.* 2015) and stonechats.

Decreases in Fay and Wu's H in a subset of outlier regions and a subset of taxa suggest that positive selection has also contributed to this convergent genomic evolution. Indeed, Irwin *et al.* (2016) favour positive selection as the likely driver of differentiation landscapes in greenish warblers, citing exceedingly low nucleotide diversity in differentiation peaks; in one comparison in that study, regions with $F_{ST} > 0.9$ showed just 6.7% the nucleotide diversity of regions with $F_{ST} < 0.6$. We found diversity reductions in stonechats and flycatchers to be less severe: between Austrian and Siberian stonechats, which show the greatest reduction in nucleotide diversity in F_{ST} peaks, π in regions with F_{ST} above the 95% percentile was reduced to 30–34% of that of regions with F_{ST} below the 50th percentile. In *Ficedula* flycatchers, this statistic was 43–50%. Therefore, we consider background selection, in concert with reduced recombination, to be an additional plausible driver of correlation in genomic landscapes.

Conserved variation in mutation rate is another possible driver of this correlation. Irwin *et al.* (2016) found weak correlations in d_{XY} between greenish warblers and more distant comparisons (Pearson's $r = 0.07$–0.14), which does not support this explanation. In contrast, we found reasonably strong correlations in d_{XY} between stonechat and flycatcher genera (Spearman's $\rho = 0.37$–0.49). Therefore, we cannot rule out a further contribution of conserved variation in mutation rate to these patterns.

Genomics and demography of Canary Islands stonechats

Canary Islands stonechats' genomic landscapes differed from those of the other stonechats. The valleys of standardized nucleotide diversity (π/d_{XY}) seen in other taxa were completely absent, suggesting that selection has not reduced diversity across the genome in a heterogeneous way. In fact, this ratio was elevated in the same regions in which it was reduced in the other taxa. Tajima's D was highly negative genomewide and showed lower variance than in other stonechats. Combined, these results are most consistent with a demographic history that included a severe population bottleneck (erasing existing patterns of variation), followed by a substantial population expansion. Previous research has found evidence of founder effects and/or bottlenecks in Canary Island birds (Barrientos *et al.* 2009, 2014; Spurgin *et al.* 2014). The evidence for a bottleneck and expansion and the marked homogeneity of genetic diversity across the genome in Canary Islands stonechats suggest that genetic drift has played a dominant role in its divergence from other stonechats, possibly overpowering selection (see Hansson *et al.* 2014; Spurgin *et al.* 2014; Gonzalez-Quevedo *et al.* 2015; Illera *et al.* 2016). The unusual pattern seen in standardized nucleotide diversity may be explained by this prevalence of drift over selection. Because selection has not reduced π in the outlier regions shared by other stonechats, this statistic shows little variation across the genome of Canary Islands stonechats. This unusual pattern therefore results from the lack of a reduction in within-population diversity (π) in areas where between-population diversity (d_{XY}) is still reduced, presumably due to selection in the ancestral stonechat.

Evidence of lineage-specific evolution

Despite striking similarities in the genomic landscapes of stonechats, we also find lineage-specific evolution. At the broadest levels of our analysis, in which we

compare genera, we identified conspicuous differentiation peaks that appear in *Ficedula* but not *Saxicola* (e.g. on chromosomes 3, 8, 10, 11, 12, 13 and 18; Fig. S14, Supporting information, shows chromosome 13), and vice versa (e.g. on chromosomes 6, 7, 17 and 20; Fig. S15, Supporting information shows chromosome 20). These outlier regions should be further examined from a functional perspective, as they appear to have resulted from evolutionary processes specific to a particular lineage. The most conspicuous outlier regions shared between these systems should likewise be examined (e.g. on chromosomes 1, 1A, 2, 3, 4 and 4A; Figs S16 and S17, Supporting information, show chromosomes 1A and 4A).

Conclusion

Few former studies (Burri *et al*. 2015; Lamichhaney *et al*. 2015; Irwin *et al*. 2016; Vijay *et al*. 2016) have examined genomewide patterns of differentiation in more than two avian taxa, yet comparative studies of closely related species have great potential to shed light on genome evolution (Cutter & Payseur 2013). We find parallel patterns of selection in the stonechat complex – likely occurring both before and after speciation – and evidence of demography potentially overwhelming signatures of selection in one species. In addition, this study suggests that parallel genomic processes are operating in independent evolutionary systems to drive the differentiation of similar genomic regions across genera. We hypothesize that linked selection coupled with areas of low recombination, which may be conserved across these taxa, have shaped these broad patterns. Whether concordant outlier regions actually contribute to reproductive isolation or are otherwise consequential in the speciation process is unknown. Therefore, we recommend that outlier markers obtained through genome scans and their relevance to speciation be interpreted with caution. Importantly, our comparative method also identified differentiation outlier regions that are not widely shared; these may harbour loci important in lineage-specific evolution and should be examined closely. As genomic comparisons among radiations accumulate, we will be able to compare the congruence in genomic landscapes and potentially reveal the phenomena that drive genomic differentiation over evolutionary time.

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B.V.D., M.L., L.C., I.J.L. and B.H. designed the research. B.H., M.L. and J.C.I. supplied samples. B.V.D. and L.C. performed all analyses and wrote the manuscript with input from all authors.

Data accessibility

The Siberian stonechat genome assembly is archived at the European Nucleotide Archive with Accession no. PRJEB19453. Pooled sequencing data from the five stonechat taxa are archived with Accession no. PRJEB19452.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Boxplots comparing F_{ST} and π between the Z chromosome and autosomes.

Fig. S2 Genome-wide landscape of d_{XY} for pairwise comparisons of stonechats and Pied and collared flycatchers (*Ficedula albicollis* and *F. hypoleuca*).

Fig. S3 D_{XY} across stonechat chromosome 1A.

Fig. S4 Correlation of high d_{XY} regions among stonechats and flycatchers (*Ficedula hypoleuca* and *albicollis*, or 'Hyp.' and 'Alb.').

Fig. S5 Genome-wide landscape of F_{ST} for pairwise comparisons of stonechats and Pied and collared flycatchers (*Ficedula albicollis* and *F. hypoleuca*).

Fig. S6 Correlation of nucleotide diversity (π) and d_{XY} among stonechats and flycatchers (*Ficedula hypoleuca* and *albicollis*, or 'Hyp.' and 'Alb.').

Fig. S7 Correlation of nucleotide diversity (π) and standardized nucleotide diversity (π/d_{XY}) among stonechats and flycatchers (*Ficedula hypoleuca* and *albicollis*, or 'Hyp.' and 'Alb.').

Fig. S8 Genome-wide landscape of π for five stonechat taxa. Canary Islands stonechats generally did not share the valleys present in the genomes of the other taxa.

Fig. S9 Genome-wide landscape of standardized nucleotide diversity (π/d_{XY}) for five stonechat and two flycatcher taxa.

Fig. S10 Correlation of nucleotide diversity (π) with Tajima's *D* and Fay & Wu's *H* stonechats.

Fig. S11 Genome-wide landscape of Tajima's *D* for five stonechat and two flycatcher taxa.

Fig. S12 Genomic landscape of Fay and Wu's *H* for each of five stonechat taxa.

Fig. S13 Boxplot of Tajima's *D* for five stonechat taxa. Each data point represents one 50-Kb window.

Fig. S14 F_{ST} across stonechat chromosome 13, including Pied and collared flycatchers (*Ficedula albicollis* and *F. hypoleuca*).

Fig. S15 F_{ST} across stonechat chromosome 20, including Pied and collared flycatchers (*Ficedula albicollis* and *F. hypoleuca*).

Fig. S16 F_{ST} across stonechat chromosome 4A, including Pied and collared flycatchers (*Ficedula albicollis* and *F. hypoleuca*).

Fig. S17 F_{ST} across stonechat chromosome 1A, including Pied and collared flycatchers (*Ficedula albicollis* and *F. hypoleuca*).

Table S1 Origin, sex, and relatedness information of stonechats included in this study.

Table S2 Summary of alignment of Illumina 150-bp reads from five stonechat taxa to the draft reference genome.

Table S3 *Ficedula* individuals included in the study [data from the Sequence Read Archive, or SRA, project ERP007074, published in Smeds *et al*. (2015)].

Appendix S1 Supplementary References.