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A high density SNP chip for genotyping great tit (*Parus major*) populations and its application to studying the genetic architecture of exploration behaviour

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Abstract

High density SNP microarrays ('SNP chips') are a rapid, accurate and efficient method for genotyping several hundred thousand polymorphisms in large numbers of individuals. While SNP chips are routinely used in human genetics and in animal and plant breeding, they are less widely used in evolutionary and ecological research. In this paper we describe the development and application of a high density Affymetrix Axiom chip with around 500 000 SNPs, designed to perform genomics studies of great tit (*Parus major*) populations. We demonstrate that the per-SNP genotype error rate is well below 1% and that the chip can also be used to identify structural or copy number variation (CNVs). The chip is used to explore the genetic architecture of exploration behaviour (EB), a personality trait that has been widely studied in great tits and other species. No SNPs reached genome-wide significance, including at *DRD4*, a candidate gene. However, EB is heritable and appears to have a polygenic architecture. Researchers developing similar SNP chips may note: (i) SNPs previously typed on alternative platforms are more likely to be converted to working assays, (ii) detecting SNPs by more than one pipeline, and in independent datasets, ensures a high proportion of working assays, (iii) allele frequency ascertainment bias is minimised by performing SNP discovery in individuals from multiple populations and (iv) samples with the lowest call rates tend to also have the greatest genotyping error rates.

Introduction

It is now becoming commonplace to sequence and assemble the genomes of organisms that have been the focus of ecological research but are not classical genetic model organisms (Brawand *et al.* 2014; Colbourne *et al.* 2011; Ellegren *et al.* 2012; Hu *et al.* 2011; Jones *et al.* 2012; Lamichhaney *et al.* 2015; Soria-Carrasco *et al.* 2014). While assembled genomes are undoubtedly essential tools for understanding topics in evolutionary and ecological genetics, in taxa with moderate to large genomes the cost of sequencing the full genomes of hundreds or thousands of individuals remains prohibitive for the majority of laboratories, and beyond the budget of even very large grants. Thus, analytical techniques that require large sample sizes, such as quantitative trait locus (QTL) linkage mapping / genome-wide association studies (GWAS) (Visscher *et al.* 2017), molecular quantitative genetics (Gienapp *et al.* 2017a; Jensen *et al.* 2014) and studies that utilise realised relatedness / inbreeding coefficients (Powell *et al.* 2010) are reliant on alternative technologies. Broadly, these can be categorised into two approaches; (i) genotyping-by-sequencing (GBS) methods (Davey *et al.* 2011) such as restriction-site associated sequencing (RAD-seq) (Hohenlohe *et al.* 2010) and double-digest RAD-seq (ddRAD-seq) (Peterson *et al.* 2012) and (ii) SNP microarray ('SNP chip') methods (Spencer *et al.* 2009; Syvanen 2001), where a set of known SNPs are probed on chips manufactured by providers such as Illumina (Shen *et al.* 2005) and Affymetrix (Matsuzaki *et al.* 2004).

GBS-approaches while perhaps cheaper, are more technically demanding, both in terms of laboratory work, and in post-sequencing processing of NGS data (Bajgain *et al.* 2016; Miller *et al.* 2012; Robledo *et al.* 2017). Furthermore, the sites that are typed are typically not known in advance, and call rates can vary widely between different SNPs. SNP chips are more expensive, but tend to have higher call rates per SNP, and specific target SNPs can be included in chip design. In addition, the same SNPs are typed in every individual, which is not the case for GBS approaches (Bajgain *et al.* 2016). A disadvantage of SNP chips is ascertainment bias (Bajgain *et al.* 2016; Miller *et al.* 2012). Because SNPs have to be discovered before they are designed to be on a chip, there is usually bias towards the inclusion of SNPs with higher minor allele frequencies (MAF) on the chip. For some types of

analyses (e.g. GWAS) this is not necessarily a disadvantage, because statistical power is greater for SNPs with higher MAF. However, ascertainment bias is clearly a problem for tests that require an accurate description of the site frequency spectrum in different genomic regions (Albrechtsen *et al.* 2010) e.g. tests that aim to detect signatures of selection such as Tajima's D. Thus, the optimal method for genotyping many individuals can depend on the question being addressed, the laboratory and bioinformatics experience of the user and the laboratory budget.

The great tit (*Parus major*) is a model vertebrate system in evolutionary ecology because this passerine bird readily breeds in nest boxes (making it possible to identify parents and offspring and thus build pedigrees), it has a short generation time and large broods, and it is widely distributed across Europe, Western Asia and parts of the Middle East (Perrins 1979). Longitudinal studies (Kluijver 1951; Lack 1964) of great tits have informed researchers about classic topics in evolutionary and behavioural ecology (Lack 1968) including mating systems and reproductive decisions (Smith *et al.* 1989), the frequency (Harvey *et al.* 1979) and importance of dispersal (Garant *et al.* 2005; Postma & van Noordwijk 2005), adaptation to climate change (Charmantier *et al.* 2008; Nussey *et al.* 2005; Visser *et al.* 1998), the study of personality traits (Dingemanse *et al.* 2004; Groothuis & Carere 2005; Van Oers & Naguib 2013), innovativeness and cognition (Cole *et al.* 2012; Quinn *et al.* 2016; Titulaer *et al.* 2012), social learning (Aplin *et al.* 2015; Aplin *et al.* 2012), and understanding how quantitative genetic variation is maintained in natural populations (McCleery *et al.* 2004). In more recent years, great tits have become the focus of molecular genetic studies exploring the genetic architecture of quantitative traits (Gienapp *et al.* 2017b; Robinson *et al.* 2013; Santure *et al.* 2013; Santure *et al.* 2015), phylogeography (Kvist *et al.* 2003; Lemoine *et al.* 2016), fine-scale genetic structure and dispersal (Garroway *et al.* 2013; Radersma *et al.* 2017), the efficacy, nature and relative occurrence of positive and purifying selection (Corcoran *et al.* 2017; Gossmann *et al.* 2014) and immunogenetics (Sepil *et al.* 2013; Sepil *et al.* 2012). Much of this work has been facilitated by a SNP chip containing probes for around 10,000 SNPs, of which around 6,000 are polymorphic and reliably scoreable (Van Bers *et al.* 2012). This '10K chip' has been used in QTL and GWAS mapping studies and to construct a great tit linkage map (van Oers *et al.* 2014) which led to insights into the nature of sex-differences in

recombination rate (heterochiasmy). The linkage map was in turn used to help assemble the great tit genome (Laine *et al.* 2016).

While the 10K SNP chip has helped provide insight into the architecture of some quantitative traits, it also suffers from some important limitations (Santure *et al.* 2015). The most important of these is that the marker density (~ 1 SNP per 20Kbp) is too low for most of the genome to be adequately 'tagged' by typed SNPs that are in strong linkage disequilibrium (LD) with untyped sites. Furthermore, molecular quantitative genetic approaches such as chromosome partitioning (Yang *et al.* 2011) or regional heritability mapping (Nagamine *et al.* 2012), where markers are used to measure between-individual relatedness in specific genomic regions, typically require a much higher marker density than is afforded by the 10K chip (Berenos *et al.* 2014).

To overcome the low power of the 10K chip, and to provide better resolution in association studies, outlier detection tests and molecular quantitative genetic analyses we have developed a high density (HD) chip with probes for over 600 000 SNPs. In this paper we describe the development of this great tit HD SNP chip. The chip can also be used to detect the presence of structural variation or copy number variants (McCarroll & Altshuler 2007) in the great tit genome. We demonstrate an application of the HD chip, using a behavioural trait, to showcase how the genetic architecture of phenotypic variation can be estimated. It is hoped that the methods and lessons described in this paper will serve as a useful guide to researchers developing high density SNP chips in other organisms.

Methods

DNA sequencing

To identify SNPs to include on the chip, whole genome resequencing was performed on 30 birds. Ten of the birds were from the long term study population at Wytham Woods, Oxford, UK (51°46' N, 1°20' W), and the remaining 20 were from locations across a wide area of Europe (Fig. S1), collected as part of the Great Tit HapMap Project. The sequencing is described elsewhere (Laine *et al.* 2016), but briefly, samples were sequenced on an Illumina HiSeq 2000 platform at The Genome Institute, Washington University. Sequencing was paired-end, with insert sizes 300 bp and a read length of 100 bp. Each bird was sequenced to ~10x coverage. Note that one of the samples used in this paper, from near to Zurich in Switzerland (population #27 in Fig. S1), was not used in the genome assembly paper (Laine *et al.* 2016), because coverage was lower than for other samples (~5x). The Zurich sample is included in the NCBI sequence read archive submission (SRP066678).

SNP Discovery

SNP discovery was performed in several steps, with the aim of identifying markers that are polymorphic across multiple great tit populations, with minimal ascertainment bias towards populations where the SNPs were initially discovered. Paired-end reads were filtered and trimmed with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) using a length of 80 bp and quality score of 20 as minimum cut-off scores to remove low-quality reads. The remaining reads from each individual were mapped onto the great tit reference genome v1.03 with the MEM algorithm of the Burrows-Wheeler Aligner (Li & Durbin 2009). The aligned sequence reads on the genome were stored as individual BAM files. Using VCFtools (Danecek *et al.* 2011), the BAM files were filtered to a minimum quality score of 20 and read depth of 5.

Following alignment of reads to the great tit genome, a combination of different SNP discovery algorithms and different strata of the dataset were used, summarised in Figure 1.

SNPs were independently called using the ANGSD v0.549 (Korneliussen *et al.* 2014), SAMTools v0.1.19 (Li 2011; Li *et al.* 2009) and GATK v2.4 (DePristo *et al.* 2011; McKenna *et al.* 2010) packages. Parameter settings are reported in Table S1. SNPs were called either from the 10 UK birds, the 20 mainland European birds, or the combined dataset of 30 birds. SNPs called from the different software/datasets were then compared (Figure 1) and a set of ~1.4M SNPs that were common to all SNP discovery softwares and all datasets were considered for inclusion on the SNP chip. VCFtools was used to filter out SNPs with minor allele frequency (MAF) less than 0.05 and call quality less than 50. SNPs that were predicted to be within 30 bp of each other were filtered out because it was likely that the presence of one SNP would adversely affect the ability to successfully genotype the other(s), due to inefficient or biased hybridisation of allele-specific oligonucleotides. SNPs prone to this form of possible typing error are known as Off Target Variants (OTV) in the Affymetrix genotype calling workflow (see below). A total of 1,213,160 SNPs passed all of these filtering criteria (Figure 1).

SNP selection

The SNP discovery phase of the work identified more SNPs than could be included on the chip. To prioritise which SNPs to use on the chip, the following criteria were used:

1) 'Top priority' SNPs were those that had been successfully typed on the lower density 10K chip described in earlier work (Van Bers *et al.* 2012) or had been discovered in the SNP discovery pipeline described above *and* were discovered during the construction of the earlier 10K chip but not included on it (Santure *et al.* 2011; van Bers *et al.* 2010). 6,773 SNPs that were typed on the original chip and a further 9,713 SNPs that were discovered but not included on the 10K chip were included in the 'Top priority' set.
2) A list of candidate genes were identified that could potentially explain variation in ecologically relevant traits such as personality traits (Fidler *et al.* 2007; van Oers *et al.* 2004) and timing of breeding (Visser *et al.* 2003). A list of candidate genes and putatively associated traits is provided in Table S2. At the time the chip was being designed, the great tit genome was not annotated. Therefore, to identify the location

of the candidate genes on the great tit genome, the cDNA sequence of the candidate gene in zebra finch (*Taeniopygia guttata*), another passerine, chicken *(Gallus gallus*), or if none of those were available, human or mouse, was downloaded from NCBI and the location on the great tit genome was identified by BLAST search. The start and end point of the gene was identified and SNPs were considered for inclusion if they were within any part of the gene. 654 (of which 28 were also 'Top Priority' SNPs) from 110 genes were chosen for inclusion on the chip.

3) The remaining SNPs were selected based on how likely they were to be convertible to a working and scoreable assay on the chip. The list of SNPs and their flanking sequences were sent to the Affymetrix bioinformatics team who used their *in silico* design tool to model the probability (termed the 'P convert design score') of the SNP converting to a working assay. The software uses the SNP bases and its flanking sequence, and considers factors such as GC content and the predicted amount of non-specific hybridisation to other (non-target) genomic regions. Following this process, SNPs with a P convert design score >0.69 were retained for inclusion on the chip. This threshold compares favourably to those used in the design of HD chips for chicken (Kranis *et al.* 2013), catfish (Liu *et al.* 2014), and water buffalo (Iamartino *et al.* 2017), where thresholds of 0.20, 0.50 and 0.60 were used respectively.

An Axiom myDesign high density chip was manufactured by Affymetrix. A total of 610 970 SNPs were included on the final design, of which 17 122 were from criteria 1 or 2 and the remainder were from criteria 3. The genomic distribution of attempted SNPs are described in Table S3 and Fig. S1.

Genotyping

Genotyping was performed on a Gene Titan platform at Edinburgh Genomics. A total of 21 plates, each with up to 96 samples, were typed (2016 available slots). Across the 21 plates, 9 negative controls were included. All plates contained at least one duplicate sample to aid with estimation of error rate. 1073 typed samples were from the Wytham Woods population. The remainder of the total 2007 birds came from a number of study sites (Table 1, Fig. S1) from across the species range in Europe and Asia, and were provided by members of the

Great Tit HapMap Consortium, either as pre-extracted DNA, or more usually as blood samples in Queen's storage buffer or ethanol. DNA was extracted using an ammonium acetate precipitation method (Bruford *et al.* 1998) and DNA quality and quantity measured using picogreen on a fluorometer. 1,696 samples were at a concentration exceeding 50ng/ul, while 89 were at concentrations lower than 20ng/ul. All except 33 samples passed the manufacturer's recommendation of 200ng of DNA. 13 Japanese tit (*Parus minor*) birds were genotyped, as well as 9 putative *P. major / P. minor* hybrids. Abel, the male used as the reference bird for the great tit genome assembly (Laine *et al.* 2016), was typed four times (two replicates on two different plates). SNP genotype calling was performed using the Ps_Metrics and Ps_Classification functions within the Affymetrix Axiom Analysis Suite 1.1.0.616. Samples with dish QC < 0.82 or call rates <0.95 were discarded, as were SNPs with call rates <0.97 or those identified as containing Off-Target Variants (OTVs).

Quantifying Genotyping Error Rate

Genotyping errors were estimated in two ways. First, the replicated samples meant that the proportion of inconsistent genotypes between different typing attempts of the same bird could be estimated. The error rate was obtained from the Z2 score - the proportion of SNPs at which two individuals (replicates) share both alleles identically-by-descent - reported by the --genome command in Plink 1.9 (Chang *et al.* 2015). Second, genotypes from the SNP chip were compared with the whole genome resequencing SNP calls for 28 birds that were successfully genotyped and sequenced to ~10x coverage (Laine *et al.* 2016). Note that discrepancies between chip and resequencing SNP genotypes can arise either because the SNP chip genotype is wrong, or because the SNP call from the resequencing is wrong. Therefore, comparison between the resequencing and the SNP chip genotyping provides an upper limit on the genotyping error on the SNP chip. Concordance between the chip and the resequencing data was determined using the GenotypeConcordance tool implemented within GATK, after SNPs with Genotype Quality Scores <30 were filtered from the resequencing dataset.

Copy number variant (CNV) detection

CNVs were detected using the PennCNV software (Wang *et al.* 2007). PennCNV input files of the 996 birds from the Wytham Woods population were prepared using the Axiom Analysis Suite's CNVTool and probe intensities from all SNPs. PennCNV uses two parameters from the SNP genotyping, the logR ratio and the B allele frequency, to identify genomic segments containing SNPs indicative of copy number variation. The logR ratio is a measure of signal intensity. SNP assays in individuals with extra copies of a genomic region (duplications) should generate higher intensity signals, while SNPs in individuals with fewer than two copies of a genomic segment (deletions) should generate lower intensity signals. The B allele frequency measures the relative signal intensity of the two possible alleles at each SNP. Ratios that are inconsistent with allele call ratios of 2:0 (i.e. A allele homozygote), 1:1 (i.e. heterozygote) or 0:2 (i.e. B allele homozygote) are indicative of departures from two copies of that nucleotide (i.e. the normal diploid state) being present in the sample. For example, an individual with a duplication at a CNV site on one chromosome, would have three copies in total, meaning the ratios of alleles A:B could be 1:2 or 2:1, which is impossible when two copies are present. CNVs called by PennCNV were retained and converted to Plink format using the perl script penncnv_to_plink (www.openbioinformatics.org/penncnv/download/penncnv_to_plink.pl). The plink commands --cfile --cnv-overlap and --cnv-seglist were used to generate a list of all CNVs, identify overlapping CNVs, estimate CNV frequencies and summarise the CNVs present in each individual (.cnv.indiv file).

Additional CNV analyses included (i) an examination of two replicates of the reference genome bird, Abel, and (ii) CNV calling using nine father-mother-offspring trios from the Wytham Woods population. As with the analysis of all Wytham Woods birds, the PennCNV command detect_cnv_pl was used, only with the -trio argument included. In principle, detected CNVs are more likely to be reliable calls if they are observed to be inherited in a Mendelian fashion.

Genetic architecture of a personality trait

The chip was used to explore the genetic architecture of Exploration Behaviour in a novel environment (EB), a personality trait linked to aggression, risk-taking and dispersal in great

tits (Quinn *et al.* 2009). EB is known to be heritable (Dingemanse *et al.* 2004; Drent *et al.* 2003; Quinn *et al.* 2009; Santure *et al.* 2015) and it has also been the focus of candidate gene studies, especially at the Dopamine D4 receptor (*DRD4*) gene (Fidler *et al.* 2007; Korsten *et al.* 2010), following the first report that *DRD4* could affect novelty-seeking behaviour in humans (Ebstein *et al.* 1996). The protocol for measuring EB is described in detail elsewhere (Cole & Quinn 2014; Quinn *et al.* 2009). Briefly, wild birds were captured during February-March (2005) or September-March (2006-2009) and assayed in a novel environment room at Wytham Woods field station. For the purposes of the downstream genetic analyses we used the same measure of EB as that used in previous studies. Briefly, the first principal component (PC1) of 12 behavioural measures was treated as the EB score. PC1 was square-root transformed prior to genetic analysis and a single value for each individual was obtained by fitting a linear mixed model with the terms ID, year, days after September 1^{st}, and assay number of that individual all included as predictors. Details are described elsewhere (Quinn *et al.* 2009). Several aspects of EB genetics were explored. First, we performed a genome-wide association study (GWAS) using the Grammar method (Aulchenko *et al.* 2007a), implemented in GenABEL (Aulchenko *et al.* 2007b). Grammar accounts for the possibility of test statistic inflation caused by relatives in the dataset by fitting a realised genome-wide relationship matrix estimated from the SNP data as a random effect. The residual from the random model was used as the phenotype. In addition, genomic correction was performed by estimating lambda, the slope of observed chi square values on expected chi square values, and dividing all tests statistics by lambda before estimating nominal P-value. Genome-wide statistical significance was estimated by permutation test, using the GenABEL mmscore command and 1000 permutations of the data. The GWAS was performed on a total of 415 birds from Wytham Woods. All Z-linked SNPs and any autosomal SNPs with MAF <0.05 or significant departures from Hardy-Weinberg Equilibrium ($P < 1x10^{-5}$) were filtered from the dataset leaving a total of 459 502 autosomal SNPs.

In addition to the GWAS, an additional analysis of the same dataset fitted all SNPs simultaneously, in one model. Here, the objective was to estimate the proportion of phenotypic variation explained by each SNP, in order to understand aspects of the trait architecture such as the heritability, the number of SNPs in linkage disequilibrium with causal variants and the distribution of effect sizes of those SNPs. The BayesR method (Erbe *et al.*

2012), whereby it is assumed that the SNPs causing phenotypic variance are drawn from a mixture of different effect size distributions, was used to model the genetic architecture of EB. The BayesR package (Moser *et al.* 2015) was used to run the analyses, with default settings of 4 distributions, with mean effect sizes of 0.01, 0.001, 0.0001 or 0 of the phenotypic variation. The program was run for 50 000 iterations of an MCMC chain, with the first 20 000 iterations treated as burn-in, and every 10^{th} chain after that being sampled, giving a total of 3000 samples of the chain. Priors for V_A and V_E were specified using an inverted chi-squared distribution with scale parameters of 0.033 and 0.117 respectively, each with 4 degrees of freedom. These values give a prior heritability of around 0.20 which is consistent with pedigree-based estimates of EB in the Wytham Woods population (Quinn *et al.* 2009; Santure *et al.* 2015). Note that setting the priors so that V_A and V_E were identical (i.e. the heritability was 0.5) gave almost identical posterior estimates, so the genetic architecture does not appear to be sensitive to the priors.

Results

Summary Statistics

Following genotype calling and quality control steps, a total of 1 846 samples typed at 502 685 SNPs were retained for analysis. A summary of the different types of SNP category is provided in Table 2. Samples that contained less than the recommended 200ng of DNA were more likely to fail than those with >200ng of DNA; 9/33 failures versus 140/1962 failures (Fisher's Exact Test: Odds ratio = 4.87, 95% CI 1.95-11.12, P = 0.0005). However, among samples that passed quality control, there was no relationship between the call rate and the amount of DNA present in the sample ($F_{1,1844}$ = 0.942, P = 0.33). SNPs that had been previously typed on the 10K chip were more likely to be converted to a successfully typed SNP, and to pass QC checks. For previously typed SNPs the conversion rate was 5924/6773 (0.87) compared to 496 826 / 604 197 (0.82) for unvalidated SNPs; Fisher's Exact Test odds ratio 1.51, 95% CI = 1.40-1.62, P = 0.0006. However, SNPs that were discovered during both the construction of the 10K chip and of the HD chip but were not typed on the 10K chip actually had a lower conversion success rate, 7807/9713 (0.80), than SNPs that were only discovered during HD chip construction, 489 019 / 594 484 (0.82); Fisher's Exact Test: Odds ratio = 0.88, 95% CI = 0.84-0.93, P = 2.0 x 10^{-6}. Thus, the untyped SNPs from the low density chip were less reliable than the newly discovered SNPs.

Genotyping Error Rate

Among 30 individuals (resulting in 65 pairwise comparisons, due to some birds being typed >2 times) that were repeat genotyped on the SNP chip, there was a per SNP genotyping error rate of 0.004. If comparisons were restricted to the 56 comparisons where both samples had call rates >0.98, the error rate was 0.002, indicating that individuals with lower call rates tended to be more error prone. The discordance in SNP calls between the chip and the resequenced data was ~0.01, although this was apparently mostly driven by errors in the sequencing data, because the degree of discordance is negatively correlated with the depth of the genome coverage, which varies between 4.5x and 13.8x (see Fig. S3).

Resequencing data predict SNP chip allele frequencies

The minor allele frequencies (MAFs) of each SNP estimated from the 30 resequenced birds were compared to the MAFs estimated from the 996 birds genotyped in the Wytham Woods population. Notably, there was a very strong positive relationship between the minor allele frequencies in the two datasets (Fig. S4A; HD Chip MAF = 0.016 + 0.918*ReSeq MAF, $F_{1,480756}$ = 1.65*10^6, r^2 = 0.77, P < 2.2x10^{-16}). Thus, the MAFs estimated from the resequencing data from 30 birds sampled across Europe are a reliable predictor of the MAFs obtained by typing a much larger sample from a single population on the HD chip. Similar analyses using genotyped birds from two randomly selected mainland European populations showed the same pattern (Fig. S4B, S4C); Montpellier, HD Chip MAF = 0.023 + 0.867*ReSeq MAF, $F_{1,480756}$ = 8.16*10^5, r^2 = 0.63, P < 2.2x10^{-16}, 50 individuals; Gotland, HD Chip MAF = 0.022 + 0.874*ReSeq MAF, $F_{1,480756}$ = 8.69*10^5, r^2 = 0.64, P < 2.2x10^{-16}, 47 individuals. The relationship was stronger for the Wytham Woods birds than the two other populations, but this is largely because the HD chip MAFs were estimated from more birds in the Wytham Woods dataset, and are therefore presumably estimated more accurately. A similar analysis conducted on 50 randomly chosen birds from Wytham Woods produced a relationship that was only slightly stronger than that seen in the Montpellier and Gotland populations (Fig. S4D; HD Chip MAF = 0.023 + 0.879*ReSeq MAF, $F_{1,480756}$ = 9.76*10^5, r^2 = 0.67, P < 2.2x10^{-16}). Thus, the strong relationship between SNP chip MAF and resequencing is not simply an artefact of 10 of the 30 resequenced birds being from Wytham Woods. The mean minor allele frequencies were very similar in the three populations (Wytham 0.280, Montpellier 0.273, Gotland 0.274).

CNV analysis

A total of 41 526 putative CNVs (34,947 with PennCNV confidence scores >5) were discovered in 996 birds from Wytham Woods. The great majority (37 419 or 90.1%) of CNVs were single copy duplications. Birds had a mean (SD) of 41.9 (160.9) CNVs each, spanning a mean (SD) distance of 3.19 (16.22) Mbp. However, there was a strong positive relationship between the amount of CNV in a bird's genome and the Axiom Analysis Suite parameter cluster_distance_SD (Figure 2). Cluster_distance_SD is a per-sample measure, defined as the standard deviation of the distance to the cluster centre, estimated from all of the individual's called genotypes. Samples with high values of cluster_distance_SD are typically indicative of individuals whose genotypes are difficult to call, perhaps because the sample was of low quality or quantity. Restricting the analysis to those individuals with cluster_distance_SD

<0.65 (n = 701), resulted in far fewer CNVs. In total there were 8139 CNVs observed, of which 1523 (18.7%) were a deletion of two copies (i.e. the segment was missing from both chromosomes), 1 424 (17.5%) were single copy deletions, 5,176 were single copy duplications (63.6%) and 16 (0.2%) were double copy duplications. The retained birds had a mean (SD) of 11.6 (6.8) CNVs spanning a mean (SD) total distance of 0.34 (0.40) Mbp. The distributions of the number and total distance spanned of CNVs in the full dataset were far more skewed (Fig. 3A, 3B) than in the restricted dataset (Fig. 3C, 3D). The skewedness of the number and total distance of CNVs in the full dataset was 10.98 and 10.93 respectively, while equivalent values in the restricted dataset were 2.71 and 5.14. Plink estimated there were 1397 distinct non-overlapping CNVs, of which 1204 were at a frequency < 0.01. However, a small number of CNVs were at a frequency approaching 0.15. For an example of a large CNV identified in multiple individuals see Fig. S5.

PennCNV analysis of two replicates of the reference genome bird, Abel, revealed there were fewer CNVs than in the Wytham Woods population. For one replicate, the cluster_distance_SD score was sufficiently low (0.59) to retain the sample in the filtered dataset. No CNVs were detected, which is perhaps not surprising as CNV regions may not have been possible to assemble when the genome was being assembled. The other replicate had a cluster_distance_SD score of 0.69, and contained a total of six possible CNVs (although four of them had confidence scores <5), with a total length of 104 Kbp. Some, perhaps all, of these CNVs are likely to be false positives, but even with their inclusion, the reference bird contains less CNV regions than the mean of the Wytham Woods dataset (mean summed CNVs = 3.19 Mbp in the unfiltered dataset, 0.34 Mbp in the filtered dataset).

An analysis of nine father-mother-offspring trios from Wytham Woods (Table S4) identified 103 possible CNVs, of which 98 showed Mendelian inheritance, suggesting they were likely to be correct calls. 71 CNVs involved insertions, 27 involved deletions and 5 had both insertions and deletions segregating at the same location. The ratio of insertions: deletions is similar to that described in the analyses of all Wytham Woods samples.

Genetic architecture of Exploration Behaviour

The GWAS of EB did not identify any SNPs that were significant at the genome-wide level (Figure 4A). The QQ plots indicated that the distribution of p values was very close to that expected under the null distribution if none of the SNPs explain variation in EB (Figure 4B), and lambda was estimated as 1.018 (SE $1.7x10^{-5}$). Thus the effects of population genetic structure seem to be adequately accounted for. However, one SNP approached genome-wide significance (P = 0.136; Table S5), and is worthy of mention. SNP AX-100303447 at 49.67Mbp on Chromosome 3 is located approximately 3.5 Kbp downstream of interleukin 22 receptor subunit alpha 2 *IL22RA2* (Figure 4C). This gene is notable for being implicated in the regulation of alcohol drinking in alcohol-preferring laboratory rats; experimental interference of *IL22RA2* expression results in reduced alcohol intake (Franklin *et al.* 2015). There is no evidence that the *DRD4* gene explains variation in exploration behaviour in the Wytham Woods population (Figure 4D).

The BayesR analysis of EB was consistent with a highly polygenic genetic architecture. The heritability estimate was modest and had a very large 95% credible interval (Table 3), although it was very similar to previous estimates from pedigree-based quantitative genetic analyses. It was estimated that a large number of SNPs contributed to trait variation, and that much of the additive genetic variance (V_A) was caused by SNPs in the smaller effect size distributions (Table 3).

Discussion

In this study, we generated a high density SNP chip and showed that the majority of target SNPs could be genotyped reliably and accurately and across multiple great tit populations. A total of approximately 900 million SNP genotypes were generated with considerably less than 1% typing error. Similar chips are routinely used in studies of humans (Frazer *et al.* 2007; Simonson *et al.* 2010), model organisms (Yang *et al.* 2009), companion animals (Hayward *et al.* 2016) and agriculturally important species (Rincon *et al.* 2011; Winfield *et al.* 2016), but their application in wild vertebrate populations remains rare – although there are some examples using 40-50K SNP chips, e.g. in Soay sheep (Johnston *et al.* 2013), collared flycatchers (Kawakami *et al.* 2014; Silva *et al.* 2017) and house sparrows (Silva *et al.* 2017). We found the cost of genotyping to be relatively low (approximately £0.0003 per SNP genotype per individual).

Several lessons were learned that may be useful to researchers considering designing their own HD chips. First, we attempted to type some samples that were of marginal quality relative to the manufacturer's recommendations. Although many of them were successfully typed, the pass rate was lower than the remaining samples. Second, our chip included some SNPs that had already been successfully typed on a smaller 10K Illumina SNP chip. These SNPs did perform better than those which were unproven prior to the HD chip manufacture. Thus, we recommend using SNPs that have been previously validated, even if prior testing was performed on an alternative platform. Third, in addition to sequencing 10 birds from Wytham Woods, we sequenced 20 birds from multiple other populations during the SNP discovery and there is little evidence that the chip is biased towards SNPs that are more polymorphic in the Wytham Woods population. If the discovery had relied on sequencing a single population it is likely that there would have been a greater ascertainment bias towards SNPs that have high minor allele frequencies in that population. Perhaps, most importantly, our relatively high success rate (~82% of attempted assays were converted to QC-passed, polymorphic SNPs) is at least partially attributable to performing SNP calling with different datasets and different callers and then using consensus SNPs for the chip design.

There was a strong positive correlation between the SNP MAFs predicted from the 30 resequenced birds during the discovery phase, and the chip MAFs estimated from almost

1000 genotyped birds from the Wytham Woods population. During SNP discovery there will be a tendency to assign higher confidence scores to SNPs with higher MAFs, because the rare allele will be identified in multiple individuals. Thus, the site frequency spectrum of the SNP chip cannot be expected to be representative of the whole genome, but for many applications, a chip with relatively high MAFs can be beneficial. This is most obviously the case in GWAS or linkage mapping studies where the power to detect linkage is partially a function of MAF. The chip has already been used to detect regions of the genome responsible for adaptive evolution of bill length in European great tits (Bosse *et al.* 2017).

We used the chip to examine the genetic architecture of Exploration Behaviour, a widely-studied behavioural trait in great tits (Fidler *et al.* 2007; Korsten *et al.* 2010; Mueller *et al.* 2013) and other bird species (Edwards *et al.* 2015). No SNP reached genome-wide significance, although this is perhaps unsurprising given that the sample size was fairly modest (~400) and the trait was shown to have a reasonably low heritability in this dataset. These findings are similar to a previous study using the lower density 10K chip, where heritability of EB was also modest (h^2 = 0.26, SE = 0.08) and no SNPs were significant at the genome-wide level in a GWAS (Santure *et al.* 2015).

Previous candidate gene studies of personality traits in great tits and other birds have focused mainly on dopamine receptor D4 (*DRD4*), and there is convincing evidence that it explains a small but significant amount of variation in great tit EB in a population in the Netherlands (Fidler *et al.* 2007). With this in mind, *DRD4* was chosen as a candidate gene during the SNP construction and the region was over-represented on the chip. However, there was very little evidence that *DRD4* explained significant variation in the Wytham Woods population. This is consistent with earlier studies (Korsten *et al.* 2010; Mueller *et al.* 2013) that failed to find an association in Wytham Woods and elsewhere. It is probably prudent to be cautious about most associations between *DRD4* and exploration behaviour in bird species, unless genome-wide data are available. This is because single locus studies are unable to reveal the extent to which test statistic inflation due to population structure or covariance between environmental and additive genetic variance is driving false positive results; see for example Knowler *et al.* (1988), discussed in Lynch & Walsh (1998). Of course,

485 this potential form of bias applies to any candidate gene study that lacks comparable data
486 from numerous non-candidate genomic regions.

487

488 High density SNP chips have been used to identify structural or copy-number variation (CNVs)
489 in other organisms (Wang *et al.* 2013; Wu *et al.* 2015; Zhang *et al.* 2014). We used the
490 PennCNV software to identify putative CNVs in the great tit genome. CNVs tended to be at
491 low frequency, which made validation hard because relatively few cases of each putative CNV
492 are present. Furthermore, it was clear that lower quality samples were prone to false positive
493 CNV calls. An additional complexity is that identifying the exact start and end points of each
494 CNV is non-trivial, so when CNVs in different birds partially overlap, it is not straightforward
495 to determine whether they are the same CNV or not. That CNVs have lower minor allele
496 frequencies than SNPs is not surprising because (i) they may be under stronger purifying
497 selection if they have bigger phenotypic effects and (ii) the chip was biased in favour of the
498 inclusion of SNPs with moderately high minor allele frequencies and designed completely
499 blind to the existence of CNVs. While CNVs are not a main focus of this study, it is clear that
500 some CNV calls were repeatable across different birds, and that the extent and effects on
501 phenotypic variation of CNVs are legitimate follow-up questions. Future CNV analyses should
502 ideally include replication from different methodologies (e.g. qPCR or sequencing-based
503 methods).

504

505 High density chips provide a straightforward method for typing several hundred thousand
506 SNPs. It is also the case that HD chips are relatively robust to low yield or highly degraded
507 DNA, whereas the DNA requirements for sequencing, especially long-read sequencing
508 technologies, tend to be more demanding. Whole genome sequencing remains more
509 expensive than SNP typing on a per individual basis, but that will not be the case for much
510 longer. Indeed, the HD chip era may be relatively short. Sequencing strategies that involve
511 sequencing a few individuals' genomes at high coverage, which are then used to impute the
512 genomes of many more individuals sequenced at ~1x coverage or lower, may already be as
513 cheap an alternative, and will yield more data (Gorjanc *et al.* 2015; Li *et al.* 2011; Pasaniuc *et*
514 *al.* 2012). At present low coverage whole genome sequencing results in data that are harder

to process, although the challenges of low coverage assembly, SNP calling and imputation are becoming more straightforward. Ecological genomics studies that use low-coverage sequencing of many individuals are not yet common, but there are a few notable examples e.g. a population genomic analysis of walking-stick insects *Timema* genomes (Soria-Carrasco *et al.* 2014) and a phylogeography study of *Menidia menidia,* the Atlantic silverside fish, (Therkildsen & Palumbi 2017)

In summary, high density SNP chips are a relatively straightforward approach for investigating a diverse range of evolutionary genomics topics such as genetic architecture, adaptive evolution, phylogeography, and inbreeding depression. Ultimately HD chips will be replaced by whole genome sequencing, but they are likely to be used for a few more years, especially in population genetic studies of organisms with very large genome sizes such as pines (Neale *et al.* 2014; Nystedt *et al.* 2013) or salamanders (Nowoshilow *et al.* 2018), where sequencing remains a relatively expensive option. We hope that the methodologies, lessons learned and downstream applications described in this paper will be useful to other researchers considering developing a similar chip to address evolutionary or ecological questions in their favourite study organism. The chip described in this paper is available to other users from Thermo Fisher Scientific (the company that acquired Affymetrix in 2016). In great tits, the chip has already been used to detect signatures of selection (Bosse *et al.* 2017), to perform genomewide association studies on morphological (Bosse *et al.* 2017) and phenological (Gienapp *et al.* 2017b) traits, and to carry out detailed analysis of the role of CNVs on genomic architecture (da Silva *et al.* In Press).

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Author Contributions

Designed the chip (J-MK, AS, KvO, MAMG, JS), performed the CNV analysis (HB, JS), designed and performed the exploration behaviour assays (JQ, EC), coordinated the long term data and blood sample collection (MV, BS), performed the genetic architecture analyses (JS), collected field and DNA sample data (Great Tit HapMap consortium), wrote the paper (JS, with contributions from all authors), conceived the study (JS, BS, MAMG, KvO, MV).

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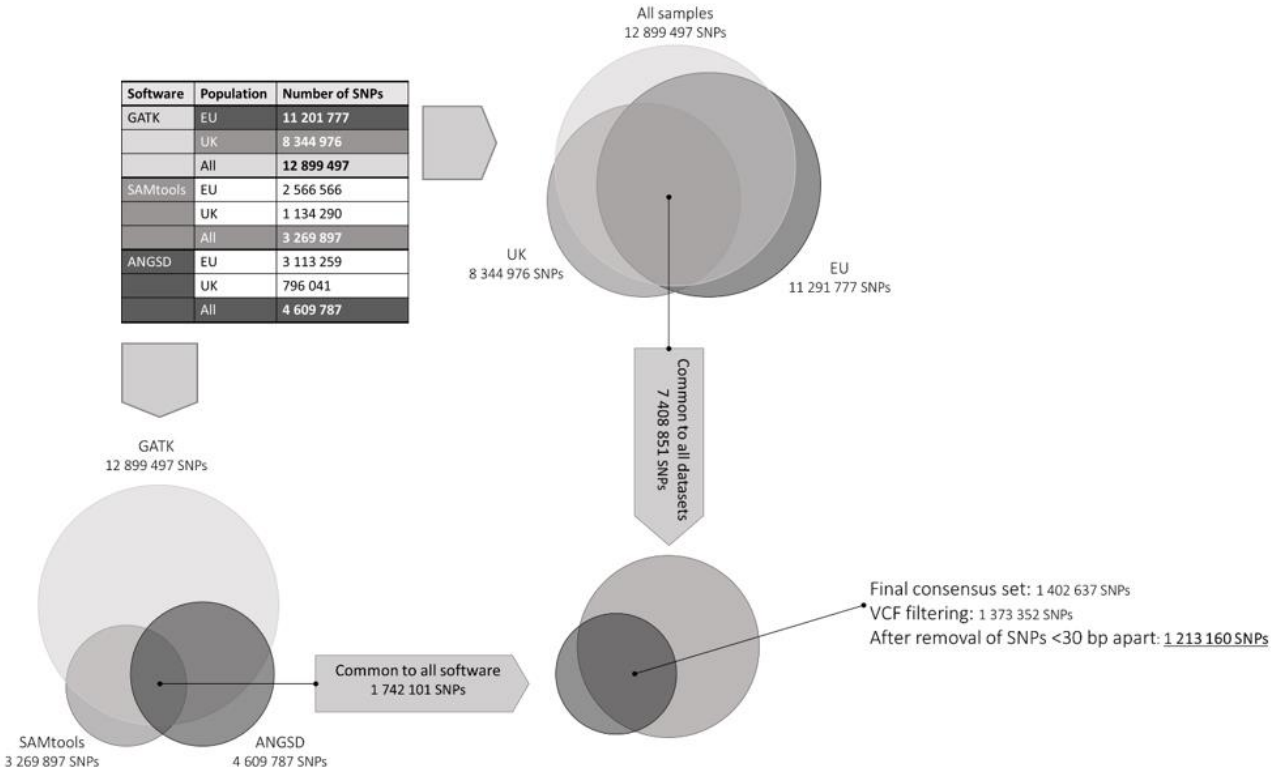
Data deposition

Genotype and phenotype data are deposited as Plink files on Dryad under the provisional record doi:10.5061/dryad.7d467b6. All SNPs included on the chip are reported on the European Variation Archive (https://www.ebi.ac.uk/eva/) under accession number PRJEB24964. SNP discovery was performed on 30 resequenced birds, whose genomes are reported on the NCBI sequence read archive under project ID SRP066678.

The Authors have no conflicts of interest.

Figure 1: The pipeline for SNP discovery. The top right part of the figure identifies SNPs that were found in the UK birds (n= 10), the mainland Europe birds (n=20) and in all birds (n=30) with the software GATK. The bottom left part of the figure identified SNPs that were found when all 30 birds were analysed with three software packages - GATK, SAMtools and ANGSD. The intersection of these discovery pipelines, i.e. SNPs that were detected in all populations by all software packages, were considered for inclusion on the chip. After filtering for MAF > 0.05 and removal of SNPs located within 30bp of each other, a final list of 1 213 160 SNPs remained.



828 Figure 2: Individuals with higher standard deviation (SD) in their cluster distance, indicating samples
829 whose genotypes are difficult to call, tend to have a greater proportion of their genomes called as
830 CNVs. The assembled great tit genome is approximately 1020Mbp long.

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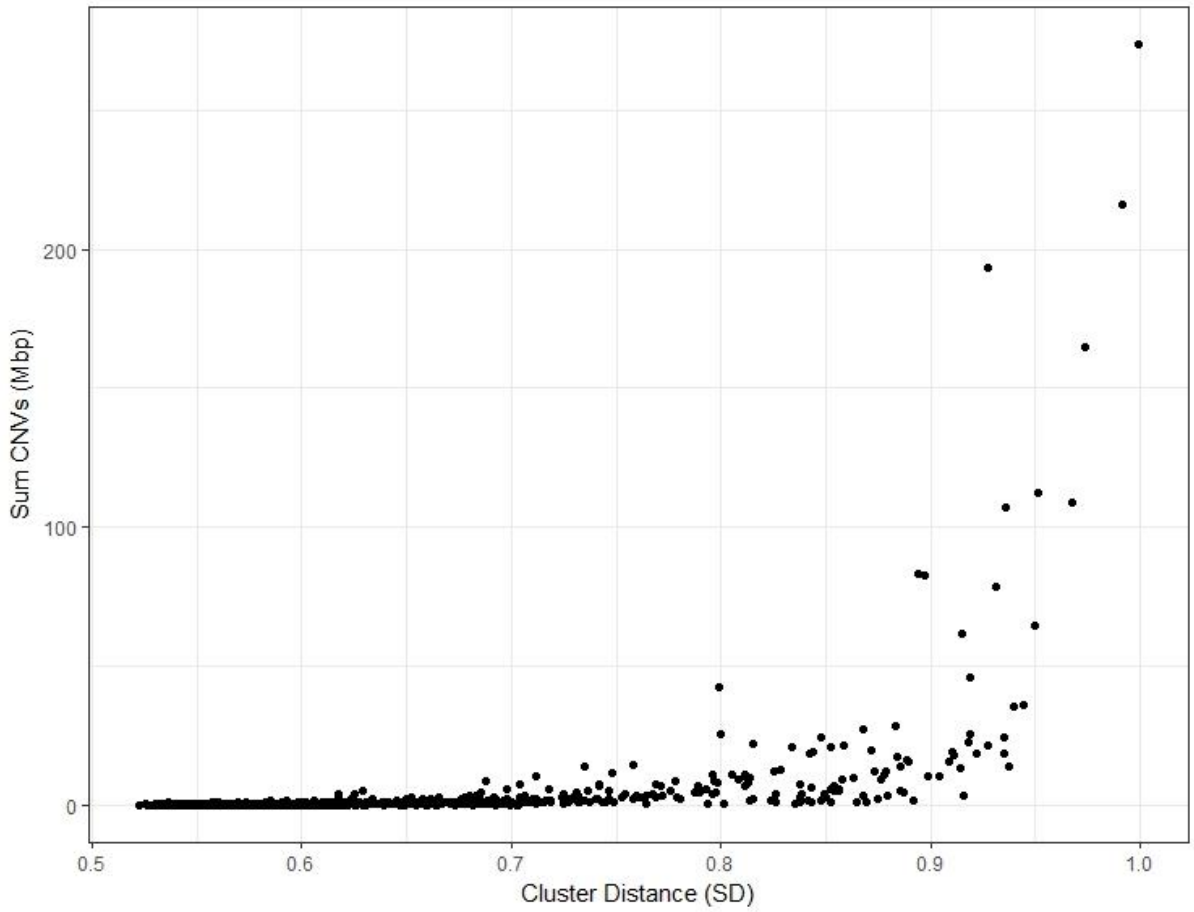

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835 Figure 3: Distribution of the number and total distance spanned of CNVs in 996 Wytham Woods birds
836 (top panels) and the remaining 701 Wytham Woods birds after filtering on cluster_distance_SD <0.65
837 (bottom panels); i.e. after removing samples whose genotypes are difficult to call.

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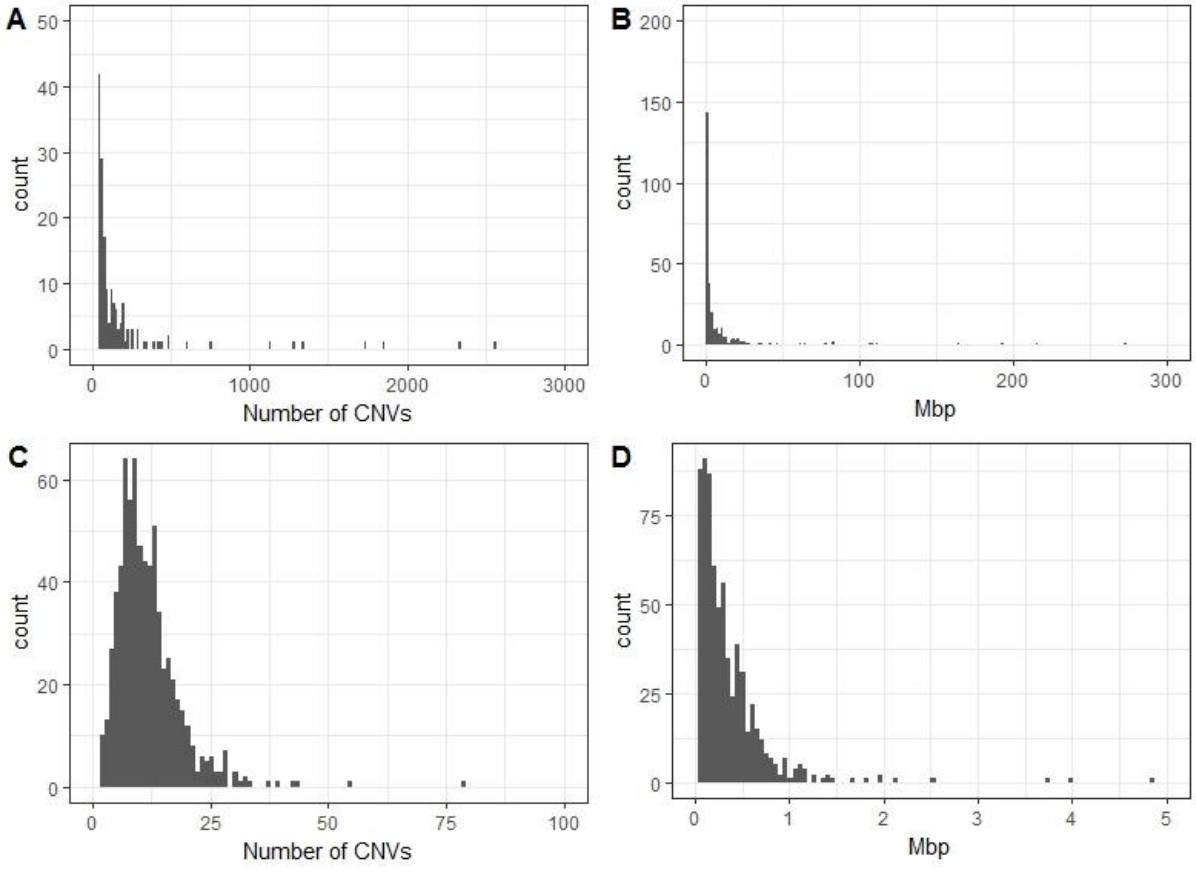

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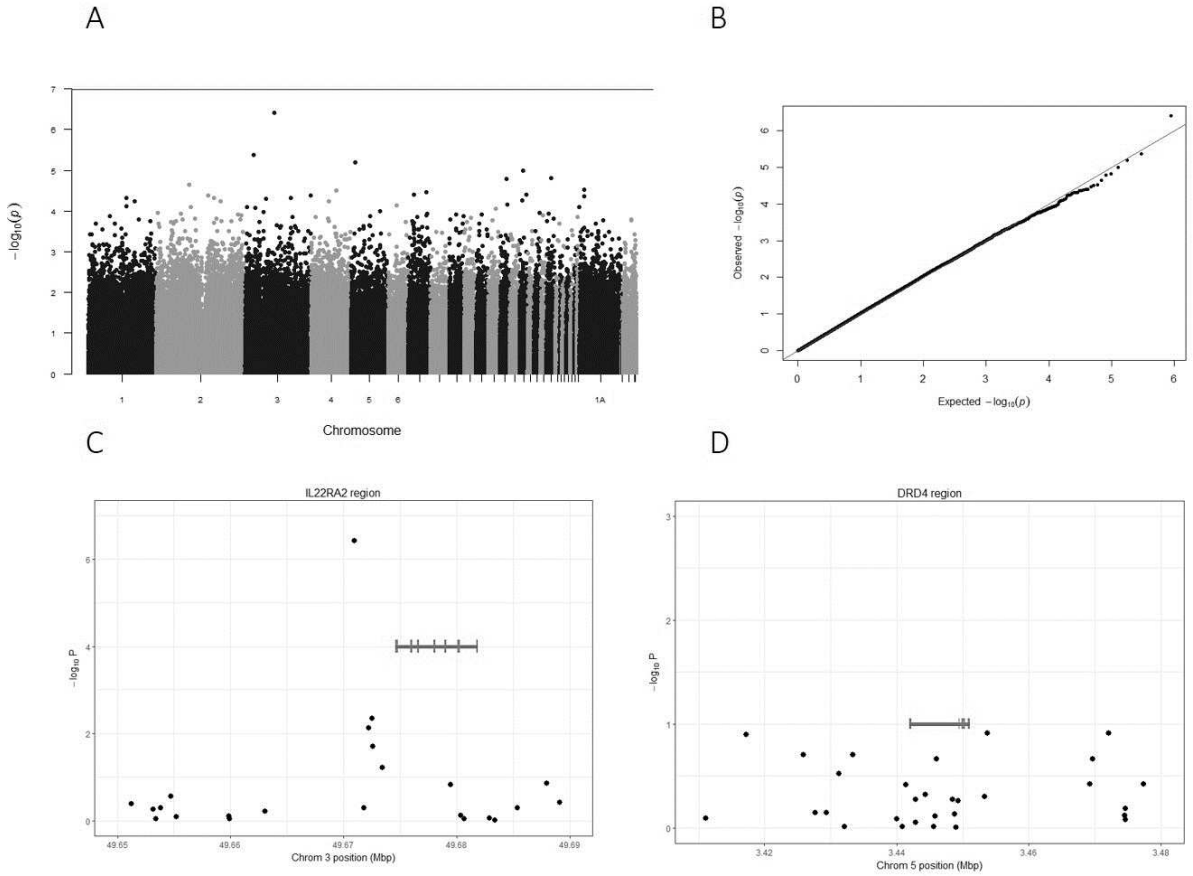
Figure 4: (A) Manhattan plot of a GWAS for Exploration Behaviour on 415 individuals for 459 502 autosomal markers. Chromosomes are ordered numerically from 1-15, 17-24, 25LG1, 25LG2, 26-28, 1A, 4A, LG22 and chromosome unknown. Horizontal line = genomewide significance. (B) QQ plot of onserved versus expected -log10 transformed P values. Lambda = 1.018 (SE $1.7x10^{-7}$). (C) and (D) Zoomed in plots of GWAS results close to the *IL22RA2* and *DRD4* genes. Horizontal lines represent location of genes. Note that the y-axis scale differs between plots.



851 Table 1: Great tit populations where genotyping was attempted (see also Fig. S1).

Population	Population Code	Coordinates (N, E) in decimal degrees	Birds typed	Birds passing QC
Amur, Russia[1]	1	50.62, 131.37	72	63
Antwerp, Belgium [3]	2	51.13, 4.53	36	30
Cambridge, UK[3]	3	52.40, -0.23	35	34
Font Roja, Spain[3]	4	38.66, -0.54	30	29
Gotland, Sweden[3]	5	57.14, 18.33	50	47
Groblas, Poland[3]	6	52.28, 17.90	4	4
Harjavalta, Finland	7	61.33, 22.17	44	44
Hoge Veluwe, Netherlands	8	52.07, 5.84	38	36
Israel	9	32.62, 35.24	1	1
La Rouviere, France[3]	10	43.66, 3.67	31	27
Loch Lomond, Scotland[3]	11	56.13, -4.62	43	41
Mariola, Spain[3]	12	38.73, -0.55	33	33
Montpellier, France[3]	13	43.61, 3.87	50	50
Oulu, Finland[3]	14	65.13, 25.88	50	45
Pilis Mountains, Hungary[3]	15	47.72, 19.02	36	34
Pirio and Muro, Corsica[3]	16	42.37, 8.75	30	27
Radolfzell, Germany	17	47.74, 8.98	30	27
Sakhalin Island, Russia[2]	18	50.52, 143.11	13	13
Seewisen, Germany[3]	19	47.97, 8.98	50	46
Tartu, Estonia[3]	20	58.17, 25.08	43	42
Tomakomai, Japan[2]	21	42.67, 141.60	10	9
Velky Kosir, Czech Republic[3]	22	49.53, 17.07	36	33
Vienna, Austria[3]	23	48.21, 16.26	38	31
Vlieland, Netherlands	24	53.28, 5.01	30	21
Westerheide, Netherlands	25	52.00, 5.83	39	35
Wytham Woods, UK[3]	26	51.77, -1.33	1073	996
Zurich, Switzerland[3]	27	47.39, 8.57	30	29
Zvenigorod, Russia	28	55.73, 36.85	20	19
Total			2007	1846

852 [1] Sample contains 63 *Parus* major and 9 putative *P. major/P. minor* hybrids

853 [2] *Parus minor* populations

854 [3] Population included in the 30 resequenced genomes dataset

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Table 2: Summary of SNP genotype calling, by Affymetrix Axiom Analysis Suite category. The Conversion Type columns uses the Affymetrix terminology but can be summarised as follows: PolyHighResolution = SNP that is polymorphic and can be reliably scored due to the different genotypes forming resolvable, discrete clusters; NoMinorHom = similar to a PolyHighResolution, but where the minor allele homozygote is not observed, presumably due to a low genotype frequency; MonoHighResolution = a monomorphic SNP that can be reliably scored because it forms a single cluster; CallRateBelowThreshold = a SNP with the expected number of clusters (usually 3, one for each possible genotype), but where the proportion of samples scored at the SNP falls below a user-defined threshold. Here the threshold was 0.97; Off-target variant = SNPs, where additional (i.e. more than 3) clusters are observed, making genotype calling ambiguous; Other = all other unresolvable SNPs.

Conversion Type	Count	Percentage	Retained for analysis
PolyHighResolution	498 036	81.5	497 972
NoMinorHom	4048	0.7	4047
MonoHighResolution	666	0.1	666
CallRateBelowThreshold	40 499	6.6	0
Off Target Variant (OTV)	9545	1.6	0
Other	58 176	9.5	0
Sum	610 970		502 685

871 Table 3: Genetic architecture of exploration behaviour

Parameter	Estimate (95% credible interval)
Heritability	0.161 (<0.001-0.671)
Number of SNPs	3,253 (315-8,499)
PGE_0.0001	0.41 (0.01-0.89)
PGE_0.001	0.26 (<0.01-0.80)
PGE_0.01	0.33 (<0.01-0.90)

872 Heritability is the total heritability captured by the genotyped SNPs (often termed "SNP heritability" or
873 "chip heritability"). Number of SNPs is the number of SNPs inferred as explaining some (non-zero)
874 trait variation. PGE is the proportion of SNP heritability explained by SNPs in the 0.001, 0.001 and 0.01
875 effect size distributions.

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