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Association between DRD4 gene polymorphism and personality variation in great tits: a test across four wild populations

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Abstract

Polymorphisms in the dopamine receptor D4 gene (*DRD4*) have been related to individual variation in novelty-seeking or exploratory behaviour in a variety of animals, including humans. Recently, the human *DRD4* orthologue was sequenced in a wild bird, the great tit (*Parus major*) and a single nucleotide polymorphism in exon 3 of this gene (SNP830) was shown to be associated with variation in exploratory behaviour of lab-raised individuals originating from a single wild population. Here we test the generality of this finding in a large sample of free-living individuals from four European great tit populations, including the originally sampled population. We demonstrate that the association between SNP830 genotype and exploratory behaviour also exists in free-living birds from the original population. However, in the other three populations we found only limited evidence for an association: in two populations the association appeared absent; while in one there was a nonsignificant tendency. We could not confirm a previously demonstrated interaction with another *DRD4* polymorphism, a 15 bp indel in the promoter region (ID15). As yet unknown differences in genetic or environmental background could explain why the same genetic polymorphism (SNP830) has a substantial effect on exploratory behaviour in one population, explaining 4.5–5.8% of the total variance—a large effect for a single gene influencing a complex behavioural trait—but not in three others. The confirmation of an association between SNP830 genotype and personality-related behaviour in a wild bird population warrants further research into potential fitness effects of the polymorphism, while also the population differences in the strength of the association deserve further investigation. Another important future challenge is the identification of additional loci influencing avian personality traits in the wild.

Keywords: animal personality, candidate gene, dopamine receptor, *DRD4*, genetic association, exploratory behaviour, gene by environment interaction, great tit *Parus major*, novelty seeking, replication study, wild populations

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Introduction

In animals, consistent individual differences in suites of correlated behaviour occur, similar to personality variation in humans (Gosling 2001; Sih *et al.* 2004a,b; Réale *et al.* 2007). Historically, behavioural ecologists have often dismissed such behavioural variation as random noise around an adaptive mean (Wilson 1998). However, there is now growing interest in the idea that variation in behavioural phenotypes is adaptive, with different personalities having a selective advantage under different circumstances (Dall *et al.* 2004; Wolf *et al.* 2007, 2008; Biro & Stamps 2008; McNamara *et al.* 2009). Such personality-related behavioural variation could have important ecological and evolutionary consequences (Sih *et al.* 2004a,b; Dingemanse & Réale 2005; Réale *et al.* 2007) and may, for example, predict success in exploiting novel food sources, colonization of new habitats, or adaptation to environmental change (e.g. Greenberg 1990; Sol *et al.* 2002; Martin & Fitzgerald 2005; Shultz *et al.* 2005; Duckworth & Badyaev 2007; Harfmann Short & Petren 2008). Yet, the molecular genetic basis that may underlie part of this variation is almost completely unknown.

Both in humans and other animal species, personality variation has a substantial heritable component (McGue & Bouchard 1998; van Oers *et al.* 2005; Réale *et al.* 2007). It is largely unclear, however, what selective processes contribute to the maintenance of this genetic variation. The observed genetic variation may simply be the result of mutation–selection balance, but it is more likely that adaptive selective processes, such as frequency- and density-dependent selection for different personality types and/or balancing selection due to spatio-temporal environmental variation, contribute to its maintenance (Wilson 1998; Dall *et al.* 2004; Dingemanse *et al.* 2004; Réale *et al.* 2007; Quinn *et al.* 2009; see also Mitchell-Olds *et al.* 2007). Insight into the molecular genetic mechanisms underlying personality variation, specifically in wild populations, would help to improve our understanding of the evolutionary processes that promote and maintain such variation (van Oers *et al.* 2005; see also Ellegren & Sheldon 2008). For example, the identification of genetic loci underlying personality variation in wild populations opens the unique possibility of investigating how frequencies of specific variants of personality-related genes change in space and time in relation to environmental variation (i.e. to observe selection on these genes; Ellegren & Sheldon 2008). This would thus make it possible to directly link selection on the phenotypic level to changes in the observed genetic variation.

One of the most promising candidate genes related to personality variation in humans is the dopamine receptor D4 gene (*DRD4*; Savitz & Ramesar 2004). *DRD4* is expressed in the central nervous system as part of the dopaminergic system involved in motivational behaviours (Netter 2006), and has been associated with variation in novelty seeking (Munafò *et al.* 2003, 2008; Savitz & Ramesar 2004). Recently, Fidler *et al.* (2007) identified the orthologue of the human *DRD4* in a wild bird species—the great tit (*Parus major*)—that is a commonly used model in ecological field studies. Fidler and colleagues furthermore detected a single nucleotide polymorphism located in exon 3 of this orthologue (SNP830, with a C–T substitution) that associated with individual variation in exploratory behaviour, both in lines artificially selected for divergent exploratory behaviour and in wild-caught nestlings which were raised in the lab. In addition, Fidler *et al.* (2007) found that in the wild-caught birds there was a significant interaction of the SNP830 genotype with another *DRD4* polymorphism—a 15 bp indel (ID15) located 5′ to the putative transcription initiation site. Associations between polymorphisms of *DRD4* orthologues and novelty seeking-related behaviours have also been found in domestic chickens *Gallus gallus* (Flisikowski *et al.* 2009), domestic horses *Equus caballus* (Momozawa *et al.* 2005), domestic dogs *Canis familiaris* (Hejjas *et al.* 2007), and captive vervet monkeys *Chlorocebus aethiops* (Bailey *et al.* 2007; James *et al.* 2007). However, unlike the great tit, none of these species is suitable for large-scale field studies on wild populations.

The great tit has proven an excellent model in ecological and evolutionary research in general (e.g. Postma & van Noordwijk 2005; Charmantier *et al.* 2008) and is probably the best studied model for the ecology and evolution of personality variation in the wild (Sih *et al.* 2004a; Réale *et al.* 2007; see Groothuis & Carere 2005 for a review). Individual great tits differ consistently in their exploratory behaviour along an axis of fast versus slow exploration when measured in a standard test room representing a novel environment (Verbeek *et al.* 1994; Dingemanse *et al.* 2002). The variation in exploratory behaviour is phenotypically and genetically correlated with several other behaviours: faster explorers are relatively bolder towards novel objects, more aggressive towards conspecifics, take more risks, and form foraging routines more quickly (Verbeek *et al.* 1994, 1996; van Oers *et al.* 2004a,b). Both artificial selection experiments and field studies (*e.g.* using parent offspring regression as well as 'animal model' methodology) have shown a heritable basis for the variation in exploratory behaviour in multiple populations (h^2 = 0.22–0.54; Dingemanse *et al.* 2002; Drent *et al.* 2003; Quinn *et al.* 2009). Fidler *et al.* (2007) subsequently found that faster exploration was associated with the presence of the T

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allele for SNP830. Finally, field studies presented evidence for fitness effects of individual differences in exploratory behaviour through—often spatially and/or temporally varying or otherwise context-specific—associations with breeding success (Dingemanse *et al.* 2004; Both *et al.* 2005; Quinn *et al.* 2009), extra-pair mate choice (van Oers *et al.* 2008), and survival (Dingemanse *et al.* 2004).

In the present paper our overall aim is to test the generality of the association between the *DRD4* SNP830 C/T polymorphism and exploratory behaviour across different wild great tit populations. We specifically aimed to: (i) test if the association between the *DRD4* SNP830 genotype and exploratory behaviour found in captive, hand-raised great tits by Fidler *et al.* (2007) is also present in free-living individuals from the same wild population; (ii) test if the association between *DRD4* genotype and exploratory behaviour is present in three other wild great tit populations; (iii) confirm the previously reported effect on exploratory behaviour of the interaction of the SNP830 with the ID15 indel.

Materials and methods

Study populations and sample selection

The populations investigated for exploratory behaviour and *DRD4* SNP830 and ID15 genotypes are: Westerheide (WH; The Netherlands; $n = 79$), Lauwersmeer (LM; The Netherlands; $n = 196$), Boshoek (BH; Belgium; $n = 103$), and Wytham Woods (WW; United Kingdom; $n = 288$) (Fig. 1; Table S1, Supporting Information). For more details on these study populations see Dingemanse *et al.* (2002; WH), Nicolaus *et al.* (2009; LM), Hollander *et al.* (2008; BH), and McCleery *et al.* (2004; WW). The unselected captive individuals in which Fidler *et al.* (2007) found an association between polymorphisms of the DRD4 gene and exploratory behaviour originated from the Westerheide population where they were collected as nestlings (in the spring of 1998) and subsequently hand-raised and tested for exploratory behaviour when juveniles (for details on the test protocol see also Drent *et al.* 2003).

We only included first-year birds that had not yet bred and were caught during the nonbreeding season between July–March to reduce potential variation in behaviour among individuals due to differences in age and (breeding) experience. These criteria also kept our sample relatively comparable to the sample of hand-raised juvenile birds investigated by Fidler *et al.* (2007). Birds born in the study areas were all individually ringed as nestlings and their exact age was known; birds not born in the study areas (50.3% of the total sample of 666) were aged as first-year based on the colour of their primary wing coverts (Jenni & Winkler 1994). For the Lauwersmeer, Boshoek and Wytham Woods populations, only one offspring from each brood was included (where brood of origin was known), as well as all immigrants that entered the study population. In Lauwersmeer and Wytham Woods, data were collected in two and three nonbreeding seasons respectively (see Table S1, Supporting Information), and we only included one randomly chosen individual for each set of birds with a parent–offspring relationship. In summary, no individual had a known first-order family relationship to any other individual in the Lauwersmeer, Boshoek and Wytham Woods datasets. For

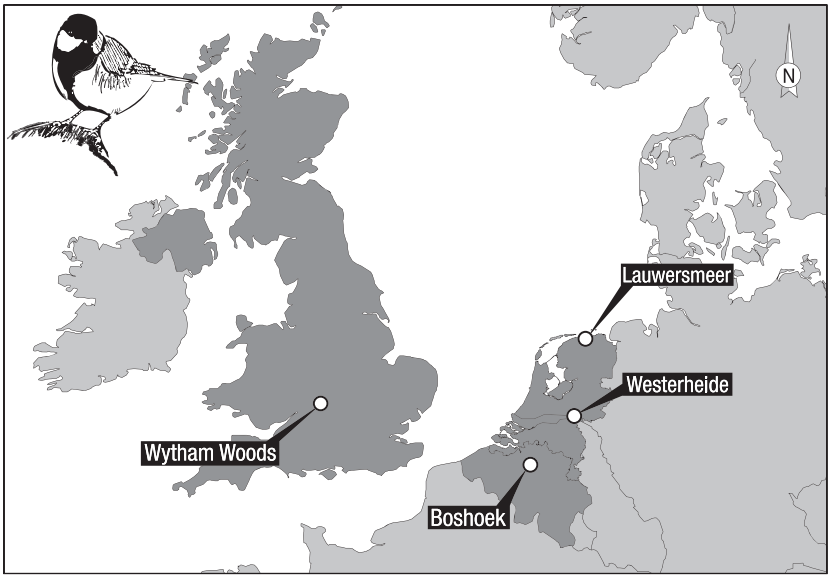

Fig. 1 Locations of four wild great tit populations investigated for associations between exploratory behaviour and *DRD4* SNP830 and ID15 polymorphisms. Populations are Westerheide (WH), Lauwersmeer (LM), Boshoek (BH) and Wytham Woods (WW).

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Westerheide multiple individuals from the same brood were included to obtain sufficient sample size. We took the relatedness of birds in the Westerheide data set into account in our statistical analyses (see below).

Measuring exploratory behaviour

The exploratory behaviour of individuals was measured following a well-established protocol using a standard test chamber with five artificial 'trees' as a novel environment (see Dingemanse *et al.* 2002). In summary, individuals were caught with mistnets near feeding stations or when roosting in nestboxes, and transported to the lab where they were kept overnight in individual cages adjacent to the test chamber. The next morning each individual was released from its cage directly into the test chamber by opening a sliding door. After release, the individual's behaviour was monitored for 2 min and all hops and flights between perches (e.g. branches of the artificial trees, sliding doors, the walls and floor) were recorded. Exploration scores were calculated by summing all recorded hops and flights per individual. Procedures were standardized between populations and the test chambers had similar design and dimensions in all four populations (WH, LM, BH: $4.0 \times 2.4 \times 2.3$ m; WW: $4.0 \times 3.3 \times 2.5$ m). In the Wytham Woods population, the observation protocol differed slightly in that hops *within* a single perch (e.g. branch of a tree or floor) were also included. These additional hops could not be excluded from the exploration score retrospectively due to the methodology of data collection (see also Quinn *et al.* 2009). It is unlikely that this difference between test protocols qualitatively affected our results and conclusions, because previous work has shown that small modifications of this type of behavioural test protocols give qualitatively similar results (Dingemanse *et al.* 2002; Quinn *et al.* 2009). The majority of tests were carried out between 8:00–13:00 h in all four populations. All tests included in our analyses are of birds that were tested for the first time during their lives, thus excluding any effects of habituation to the test as have been reported previously (Dingemanse *et al.* 2002).

Fidler *et al.* (2007) used a somewhat different protocol to assess exploratory behaviour. In short, birds were raised in the lab under standard conditions and tested between *ca.* 35–50 days after hatching (Drent *et al.* 2003). Fidler and colleagues furthermore used a compound measure for exploratory behaviour ('early exploratory behaviour' or 'EEB') that included the exploration of a novel test room (using a modified observation protocol compared to ours) and the approach to two different novel objects (for details see Verbeek *et al.* 1994; Drent *et al.* 2003). Our protocol for assessing exploratory behaviour was modified compared to the protocol used in Fidler *et al.*'s study to make it suitable for reliably testing wild-caught adults (following Dingemanse *et al.* 2002).

SNP830 and ID15 genotyping

We collected blood (WH, LM and WW) or feather samples (BH) for DNA extraction after the exploration test of each individual. Blood samples were stored in either Queen's lysis buffer (WH), or pure ethanol (LM, WW); feathers were stored in paper envelopes (BH). DNA was extracted from WH and LM blood samples using commercially available kits: the GFX Genomic Blood DNA Purification Kit (GE Healthcare, Freiburg, Germany) for samples in Queen's buffer (WH), and the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) for samples in ethanol (LM). DNA from WW samples was extracted using a standard Chelex extraction protocol (Walsh *et al.* 1991). Phenol-chloroform extraction was used to obtain DNA from the feathers (BH).

The genotyping for the SNP830 (*DRD4* C/T SNP830 of Genbank entry DQ006802) of BH, LM and WH samples was performed through a PCR based primer extension reaction and detection of the allele-specific extension products by matrix-associated laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Sequenom) at the Clinic for Psychiatry and Psychotherapy of the Technical University, Munich (Germany). WW samples were SNP830 genotyped at the Edward Grey Institute of the University of Oxford (UK) following the six-FAM labelled primer combination design and amplification protocol previously published (Fidler *et al.* 2007). A sample of 165 WW birds was independently genotyped for SNP830 in the lab in Munich, which showed a discrepancy in the assigned genotypes for only two individuals (1.2% of $n = 165$; these two individuals were both assigned C/T in Oxford, while they were assigned T/T in Munich); hence we believe the genotyping error rate to be low. A total of 655 birds were successfully genotyped for SNP830. Samples were genotyped for the ID15 insertion–deletion polymorphism (*DRD4* ID15 indel, ± 15 bp, coordinates 713–727 of Genbank entry DG006801) through amplification of the region using primers DR902R and DR634F with sequences GCC CCA AAG TTC CCT TAC TCT T and CCT CTG GAA GCA GAA TTT GAG GA, respectively (Fidler *et al.* 2007). Amplification products were subsequently resolved using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) along with a molecular size standard (GeneScan-500 LIZ, Applied Biosystems) and amplification product sizes were calculated using the

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commercial software GENESCAN 3.7 and GENOTYPER 3.6 (Applied Biosystems). The *DRD4* ID15+ and ID15- alleles generated amplification products of 269 and 254 bp, respectively. A total of 491 birds were successfully genotyped for ID15. All birds were sexed using Griffiths *et al.*'s (1998) P2 and P8 primers.

Statistical analysis

We used Chi-square tests to test for Hardy-Weinberg equilibrium in each of the populations and to test whether genotype frequencies differed between the populations. To investigate the association between the *DRD4* SNP830 genotype and exploratory behaviour we tested for an additive genetic effect of the SNP830 alleles ('additive genetic model') and for a dominance effect of the T allele ('dominant-T genetic model') following Fidler *et al.* (2007). Similarly for ID15, we tested for an additive genetic effect and a dominant ID15- effect (see Fidler *et al.* 2007). In addition, we tested if there was a significant effect of the SNP830 × ID15 interaction, as was previously found in the Westerheide population (Fidler *et al.* 2007). We used a general linear mixed model approach and took into account the family clusters in the Westerheide population by including brood of origin (brood ID) as a random factor. For direct comparison, we included the original data of the birds studied by Fidler *et al.* (2007) that were collected as nestlings in the Westerheide ($n = 91$) in some of our analyses; we also took into account the family clusters in this data set using the above approach. Significance (two-tailed $\alpha = 0.05$) was assessed by the increase in deviance (Δ deviance, which follows a χ^2 distribution) when a parameter was removed from the model. The association analyses were carried out with the MLwiN 2.02 software package, which is particularly suited for implementing multilevel and mixed models (Rasbash *et al.* 2004; estimation method was set to IGLS).

Exploration scores are known to vary within individuals with season (Dingemanse *et al.* 2002), and the slopes of these within-individual changes did not differ between the four populations tested (Bouwman *et al.* submitted). Therefore, we corrected the exploration scores for the seasonal trend for all four populations using the equation: 'season corrected exploration score' = 'measured exploration score' – (0.030 × 'July date') + 10, where 'July date' was the number of days from 1 July onwards. For details see Dingemanse *et al.* (2002) and Bouwman *et al.* (submitted).

After separate analyses for each population, we combined the data of the four populations to maximize the statistical power for detecting an overall association between *DRD4* genotype and exploratory behaviour, and to test for population differences in the strength of the association. As there were differences in the magnitude of exploration scores between the study populations (see Results), we standardized the scores for each population by subtracting the population mean and dividing by the population standard deviation before inclusion in the models. We also standardized Fidler *et al.*'s exploration scores following the same procedure before including these data in our models.

Results

None of the populations deviated from Hardy-Weinberg equilibrium for either the SNP830 (Fig. 2A) or ID15 (Fig. 2B) polymorphism (all $P > 0.47$; see Table S2, Supporting Information). The observed genotype frequencies did not differ between the populations, either for SNP830 ($\chi^2 = 4.986$, d.f. = 6, $n = 655$, $P = 0.55$) or for ID15 ($\chi^2 = 6.926$, d.f. = 6, $n = 491$, $P = 0.33$). In contrast, mean exploration scores differed significantly between populations (Fig. 3; Δ deviance = 64.434, d.f. = 3, $n = 666$, $P < 0.001$).

In the Westerheide population, exploratory behaviour was significantly associated with SNP830 genotype for the additive genetic model, while the dominant-T model showed an almost significant trend (Fig. 4A; Table 1), which is largely consistent with the findings of Fidler *et al.* (2007). In this population SNP830 genotype explained 5.8% and 4.5% of the total variance in exploration scores for the additive and dominant genetic models, respectively. In Lauwersmeer (Fig. 4B), a similar but weaker and marginally non-significant association occurred for the dominant-T model only (Table 1B). Exploratory behaviour was not significantly associated with SNP830 genotype in Boshoek or Wytham Woods (Fig. 4C, D; Table 1). The association between the SNP830 genotype and exploratory behaviour was independent of the sex of individuals, as sex and its interaction with SNP830 had no significant effect on exploratory behaviour in any of the populations (all $P > 0.17$; see Table S3, Supporting Information). Therefore, we pooled the sexes in further analyses.

Subsequent analyses showed that the association between SNP830 genotype and exploratory behaviour was similar for Fidler *et al.*'s (2007) and our data of the Westerheide. SNP830 genotype explained 5.6% and 6.0% of the total variance in exploratory behaviour in Fidler *et al.*'s dataset for the additive and dominant genetic models, respectively. Furthermore, there was no significant interaction between SNP830 genotype and the origin of the data (SNP830 genotype × Fidler *et al.*'s *vs.* our data; additive genetic model: $n = 168$, Δ deviance = 1.348, d.f. = 1, $P = 0.25$, dominant-T genetic model: $n = 168$, Δ deviance = 0.037, d.f. = 1, $P = 0.85$).

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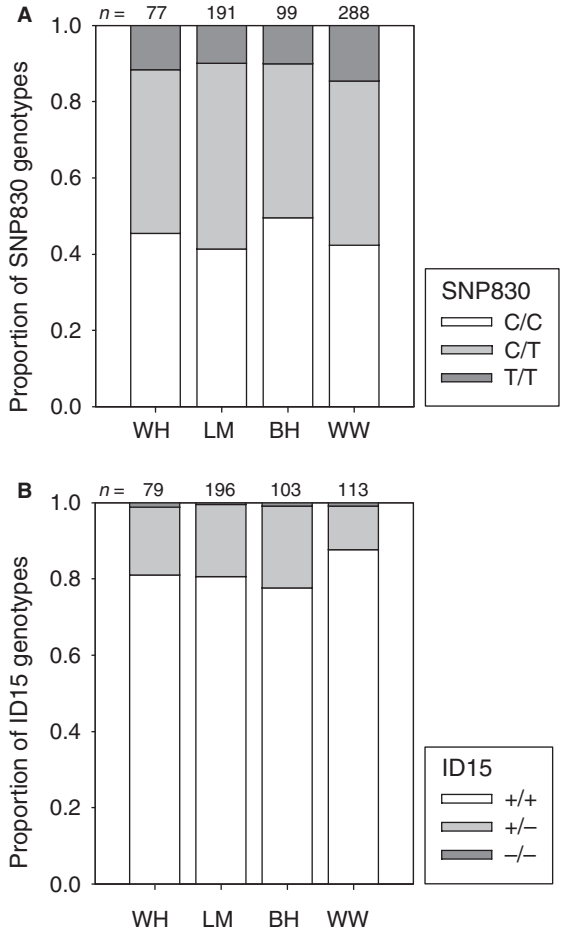

Fig. 2 Proportions of *DRD4* SNP830 (A) and ID15 (B) genotypes in four wild great tit populations: Westerheide (WH), Lauwersmeer (LM), Boshoek (BH) and Wytham Woods (WW).

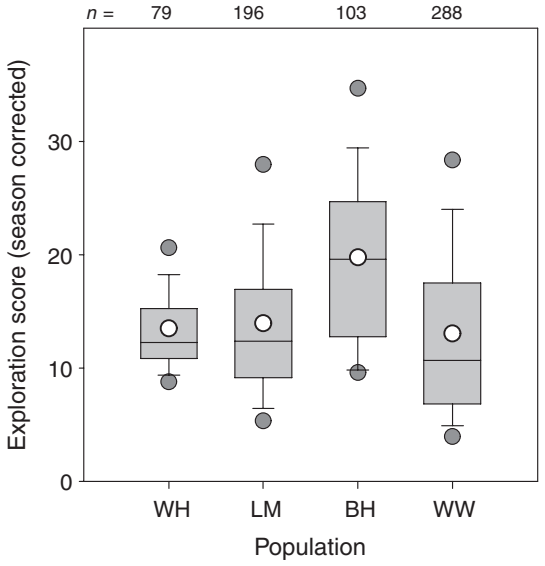

Fig. 3 Box plots of exploration scores (corrected for seasonal trend) of four wild great tit populations: Westerheide (WH), Wytham Woods (WW), Boshoek (BH), and Lauwersmeer (LM). Box plots indicate medians and the 10th, 25th, 75th and 90th percentiles; 5th and 95th percentiles are indicated by the filled circles; the open circles indicate the population means.

Given that the effect of SNP830 genotype was not different between Fidler *et al.*'s and our data for this population, we pooled the two datasets to increase the statistical power in further analyses.

A subsequent analysis combining the data from all four populations showed the SNP830 genotype × population interactions (population d.f. = 3) to be significant for both the additive and dominant-T genetic models (Table 2), but there was no significant overall main effect of SNP830 genotype on exploratory behaviour.

Exploratory behaviour was not significantly associated with ID15 genotype in any of the populations (all $P > 0.32$; see Table S4, Supporting Information). Although Fidler and colleagues also found no main effect of ID15, they reported a significant interaction effect of the SNP830 and ID15 genotypes. Therefore, we also tested for such an interaction in our data on the Westerheide for which there was a significant main effect of SNP830. As the homozygote ID15-/- genotype was extremely rare (Fig. 2), we pooled the ID+/- and ID-/- genotypes for this analysis (following Fidler *et al.* 2007; this equates to a dominant ID- genetic model). We could not confirm the previously reported SNP830 × ID15 interaction (dominant-T model for SNP830, following Fidler *et al.* (2007); Δ deviance < 0.001, d.f. = 1, $P = 0.99$; see Fig. S1, Supporting Information).

Discussion

We investigated the association between exploratory behaviour and polymorphisms in the *DRD4* orthologue in four wild great tit populations. To our knowledge, this is one of the most extensive studies of gene variants influencing personality-related traits in free-living animals to date (see also Trefilov *et al.* 2000; Krawczak *et al.* 2005), and the first to compare different wild populations. Fidler *et al.* (2007) recently identified the orthologue of the human *DRD4* gene in the great tit and discovered a significant association between the SNP830 in exon 3 of this gene and variation in exploratory behaviour, both in captive selection lines and hand-raised birds from a wild population (Westerheide, The Netherlands). Our study confirms this association in an independent sample of free-living individuals from the same population. Additional analyses showed that the strength of the association was not different between Fidler *et al.*'s hand-raised birds and the free-living birds from this population tested in our study. Separate analyses for three other wild great tit populations (Lauwers-

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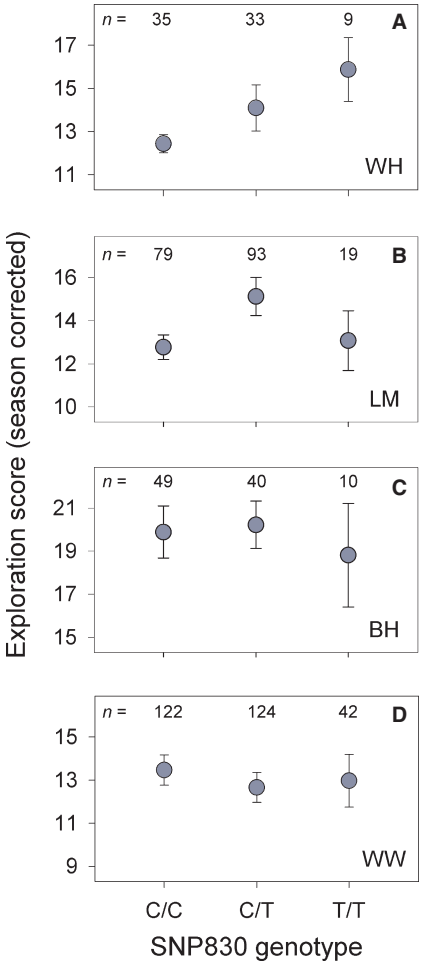

Fig. 4 Exploration scores (corrected for seasonal trend; means with standard errors) of wild great tits of four populations in relation to the *DRD4* SNP830 genotype. Exploration scores were significantly associated with SNP830 genotype in Westerheide (See Table 1).

Table 1 Associations between exploratory behaviour and *DRD4* SNP830 genotype in four wild great tit populations

Population	Δ deviance (χ^2_1)	*P*	Total variance explained
(A) Additive genetic model			
Westerheide (*n* = 77)	4.316	0.038	5.8%
Lauwersmeer (*n* = 191)	1.473	0.22	0.8%
Boshoek (*n* = 99)	0.036	0.85	0.04%
Wytham Woods (*n* = 288)	0.352	0.55	0.1%
(B) Dominant-T genetic model			
Westerheide (*n* = 77)	3.480	0.062	4.5%
Lauwersmeer (*n* = 191)	3.749	0.053	1.9%
Boshoek (*n* = 99)	0.001	0.97	0.001%
Wytham Woods (*n* = 288)	0.632	0.43	0.2%

meer, The Netherlands; Boshoek, Belgium; Wytham Woods, UK) indicated that here the association was absent or very weak and not significant. A difference in the strength of the association between SNP830 and exploratory behaviour between the Westerheide and the other populations was also indicated by a significant interaction between the SNP830 genotype and the origin of the data. Our data furthermore show that the association of the SNP830 genotype and exploratory behaviour is independent of the sex of individuals. We could not confirm the influence of the ID15 polymorphism on exploratory behaviour in interaction with the SNP830 as reported by Fidler *et al.* (2007). This suggests that the previously found effect may have been a false positive result, also because the biological interpretation of this interaction is currently unclear (see Fidler *et al.* 2007 for discussion).

Effect of SNP830 genotype

The SNP830 genotype explained 4.5–5.8% and 5.6–6.0% of the total variation in exploratory behaviour in our and Fidler *et al.*'s (2007) studies on the Westerheide population, respectively. From a statistical viewpoint these are small effects, which are difficult to detect and require large sample sizes to prevent strongly increased rates of false negative results (type II errors). From a genetic viewpoint, however, these are substantial effects, in particular for a genetic association of a complex trait like exploratory behaviour or personality (Munafò *et al.* 2008). Such complex traits are most likely to be influenced by a large number of genes which each have small effects (e.g. Gudbjartsson *et al.* 2008; Lettre *et al.* 2008; Weedon *et al.* 2008). In addition, environmental factors often have a strong influence on these types of trait, as is illustrated by the heritability values for exploratory behaviour in great tits which are between 0.22–0.54 (Dingemanse *et al.* 2002; Drent *et al.* 2003; Quinn *et al.* 2009). In line with these considerations, a recent meta-analysis of *DRD4* association studies in humans (Munafò *et al.* 2008) concluded that a SNP in the promoter region (C-521T) explained at most 3% of the variation in novelty seeking and impulsivity.

Although we found no significant associations between SNP830 and exploratory behaviour for the other three populations when separately analysed, we cannot yet exclude the possibility of small effects of SNP830 genotype in some of them. For example, for the Lauwersmeer population, the dominant-T model was marginally nonsignificant and explained 1.9% of the total variance in exploratory behaviour, which would be non-negligible as a genetic effect (e.g. Gudbjartsson *et al.* 2008; Lettre *et al.* 2008; Weedon *et al.* 2008). However, such small effects would make it impossible to

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Table 2 Associations between exploratory behaviour and the *DRD4* SNP830 genotype of wild great tits and its interaction with study population. This study's and Fidler *et al.*'s (2007) data for Westerheide were pooled; Lauwersmeer, Boshoek and Wytham Woods populations were included separately (population d.f. = 3; $n = 746$). Significance of the population and the SNP830 genotype × population terms was assessed by adding them sequentially to the model. Note that the main effects of population were non-significant, because exploration scores were standardized for each population

	SNP830		Population		SNP830 × Population	
Genetic model	Δ deviance (χ^2_1)	*P*	Δ deviance (χ^2_3)	*P*	Δ deviance (χ^2_3)	*P*
Additive	2.112	0.15	0.032	1.00	8.217	0.042
Dominant-T	3.202	0.074	0.037	1.00	9.156	0.027

use the SNP830 as a genetic marker for personality phenotype, at least for individual variation in exploration behaviour as we measured it, and will make it very challenging to detect current selection on the SNP830 gene variants in the wild. The identification of additional genes influencing personality variation in these populations would enable us to explain a larger proportion of the individual behavioural variation, and may further aid the investigation of the evolutionary processes maintaining this variation. A candidate gene approach may be a suitable method for identifying such additional loci in the great tit (Fitzpatrick *et al.* 2005; Steinmeyer *et al.* 2009), which will be greatly facilitated by the publication of the chicken (ICGSC 2004) and zebra finch genomes (http://www.songbirdgenome.org). Alternatively, a large number of SNPs could be used to construct a genetic linkage map for the great tit, and subsequently identify genome regions that associate with variation in exploratory behaviour (van Bers *et al.* in press).

Between-population differences in the strength of the associations are commonly found in genetic association studies, and may be due to a combination of type I and II errors, as well as genuine population-specific effects (Hirschhorn *et al.* 2002; Eisenberg *et al.* 2008). It is not unlikely that in different populations different underlying genetic mechanisms and gene variants contribute to the observed phenotypic variance as a result of, for example, divergent selective pressures or different mutations (Hedrick 2006). The *DRD4* SNP830 polymorphism may furthermore be interacting with variants of other genes that may occur at different frequencies in the different populations. Also environmental differences between populations may modify the genetic effects (*i.e.* gene-environment interaction), as was for example recently documented for the expression of various personality traits in sticklebacks in relation to the presence or absence of predators in their environment (Dingemanse *et al.* 2009).

At this stage the underlying genetic model of the influence of SNP830 on exploratory behaviour is speculative. Our data of the Westerheide population provide both support for an additive genetic model ($P = 0.038$; 5.8% of total variance explained; Table 1A) and a dominant-T model ($P = 0.062$; 4.5% of total variance explained; Table 1B). The association pattern in the Lauwersmeer population may even suggest an overdominant model with the C/T heterozygotes having the highest mean exploration scores (Fig. 4), although this model was not formally significant (Δ deviance = 5.098, d.f. = 2, $P = 0.078$). It remains uncertain how the effect of the SNP830 comes about, because the polymorphism is synonymous, not leading to a difference in protein structure (Fidler *et al.* 2007). However, there is growing evidence that synonymous polymorphisms can influence transcription, splicing, mRNA stability and translation, in general (Chamary *et al.* 2006), and in dopamine receptors, in particular (Duan *et al.* 2003). Alternatively, the SNP830 may be linked to another functional polymorphism (see Fidler *et al.* (2007) for a further discussion). For example, Flisikowski *et al.* (2009) recently showed the presence of linkage disequilibrium between a *DRD4* polymorphism and variants of a neighbouring gene involved in the regulation of the serotonergic system (*DEAF1*, encoding deformed epidermal auto regulatory factor one) in chickens. In case of linkage disequilibrium of the SNP830 polymorphism with another functional polymorphism, phase differences could potentially exist between populations, which could lead to population differences in the relationship between SNP830 genotype and exploratory behaviour. To further explore this possibility, additional sequencing of the flanking regions of the DRD4 gene for individuals from the different populations is needed.

The frequencies of the SNP830 and ID15 genotypes were very similar between the four populations investigated. One may have expected differences in genotype frequencies in the samples of the different populations due to spatio-temporal variation in the selection on certain personality types (Dingemanse *et al.* 2004; Both *et al.* 2005) and associated *DRD4* genotypes. Also the differences in magnitude of the exploration scores

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between the populations (Fig. 3) might have suggested population differences in SNP830 genotype frequencies. Nevertheless, genotype frequencies did not differ between the populations (in which birds were also measured in different years, see Table S1, Supporting Information). This might be a result of frequency-dependent selection on the different personality types, which could be driven by the outcome of social or ecological interactions between individuals of different personality type (Dingemanse & de Goede 2004; Both *et al.* 2005; van Oers *et al.* 2008). In case frequency-dependent selection is driven by the same social and ecological processes in the different populations, this could potentially stabilize the frequencies of *DRD4* variants at equal proportions in all populations. Alternatively, frequencies of *DRD4* genotypes may not differ between populations due to substantial gene flow between them. Obtaining more information on the genetic distance between the studied populations as well as the presence of genetic structure within them based on neutral genetic markers is important for resolving this issue. There is some evidence to suggest that European mainland great tit populations may be genetically relatively similar, while UK great tits may show limited genetic differentiation from the mainland populations, although there is probably also substantial gene flow from the mainland (Kvist *et al.* 2007). Genotype frequencies were also not significantly different between the Westerheide sample of Fidler *et al.* (2007) that was taken in 1998 (Drent *et al.* 2003) and our sample from 2007 (SNP830: $\chi^2 = 1.195$, d.f. = 2, $P = 0.55$; ID15: $\chi^2 = 4.885$, d.f. = 2, $P = 0.09$). This suggests that genotype frequencies remained similar over multiple generations in the Westerheide population.

Measuring exploratory behaviour

It is important to mention that our methods for measuring exploratory behaviour were different from the procedures followed by Fidler *et al.* (2007) in some respects. (i) Fidler and colleagues used a compound measure of exploratory behaviour which included the exploration of a novel test chamber and the approach to novel objects (for more details see Drent *et al.* 2003), whereas our measure only included exploration of a novel test chamber (following Dingemanse *et al.* 2002). (ii) The birds studied by Fidler and colleagues were raised under standard laboratory conditions, whereas the birds in our study were caught in the wild one day before being tested. (iii) The birds of Fidler and colleagues were tested at a maximum age of around 35–50 days after hatching (Drent *et al.* 2003), whereas we included individuals of up to 1 year old. The inclusion of older individuals that were not raised under standard conditions is expected to increase the environmental component of the variation in exploratory behaviour. Indeed, heritability estimates for personality variation measured in captive great tits bred under standard conditions (selection experiment: realized $h^2 = 0.54$; Drent *et al.* 2003) appeared somewhat higher than the estimates from great tits in the wild (parent–offspring regressions and sibling analysis: $h^2 = 0.22$–0.41; Dingemanse *et al.* 2002; 'animal model' methodology using pedigree information: $h^2 = 0.22$–0.28; Quinn *et al.* 2009). However, despite the methodological differences, our findings for the Westerheide population are remarkably similar to the results reported by Fidler and colleagues, both qualitatively and quantitatively (similar effect sizes; no significant interaction term for the two data sets). The observed personality variation in the Westerheide population, and its association with the SNP830 are apparently rather robust and not very much influenced by environmental factors.

Although we found no difference between populations in the frequencies of the *DRD4* genotypes, average exploratory scores differed significantly between populations (Fig. 3). These may be real biological differences, but it is also quite possible that slight methodological differences contribute to this effect (such as the slightly different behavioural test protocol for the Wytham Woods population). Even if slight differences in the measurement protocols contributed to differences in the magnitude of observed exploration scores between study populations, we do not expect this to have a major effect on the associations between *DRD4* genotype and exploratory behaviour within populations. The variation in the measured exploration scores can be seen as a proxy for between-individual variation in a suite of correlated functional behaviours (see Introduction), including exploration, aggression, risk taking, routine formation and dispersal (Dingemanse *et al.* 2003), and the between-individual variation in these broad behavioural phenotypes should be relatively robust to small modifications in the exact measurement protocol. As discussed above, local circumstances may also differ between populations and may lead to a different distribution of exploratory scores, as a result of phenotypic plasticity or local selection. Further population comparisons could shine light on this, but the most effective means would involve common environment experiments.

Conclusion

Our data on free-living great tits of the Westerheide study population confirm the findings of Fidler *et al.* (2007) who reported an association between the SNP830 in the DRD4 gene on exploratory behaviour in hand-raised great tits from the same wild population. This confirmation of an association between the SNP830 and exploratory behaviour in a wild great tit population

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warrants further research into the influence of this genetic polymorphism on personality variation in wild great tits and it may open the possibility to detect selection on the different SNP830 gene variants in this population. However, in only one of three other wild great tit populations that we investigated we found weak evidence for an association between exploratory behaviour and SNP830. This finding highlights the importance of studying genetic associations in different populations that may differ in their environmental and genetic background. An important future challenge is to gain a better understanding of population differences in the strength of such genetic associations. Furthermore, the identification of additional loci underlying personality variation in these wild populations is needed to explain a larger proportion of the observed behavioural variation, and may aid in further improving our understanding of the evolutionary processes maintaining such variation.

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Supporting information

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

Table S1 Wild great tit populations investigated for association of exploratory behaviour and *DRD4* SNP830 and ID15 genotype (n = 666 individuals)

Table S2 None of the four great tit populations deviated significantly from Hardy-Weinberg equilibrium for the *DRD4* SNP830 and ID15 polymorphisms

Table S3 No main effects of the sex of individuals or genotype × sex interaction effects on exploration scores in four populations of wild great tits. Significance of the sex and the SNP830 genotype × sex terms was assessed by adding them sequentially to the model

Table S4 Associations between exploratory behaviour and *DRD4* ID15 genotype in four wild great tit populations

Fig. S1 Exploration scores (corrected for seasonal trend; means with standard errors) of wild great tits of the Westerheide population in relation to the *DRD4* SNP830 and ID15 genotypes. Groups represent individuals either with or without the SNP830 T allele and either with or without the ID15- allele (following Fidler *et al.* 2007). The interaction between the SNP830 and the ID15 polymorphisms was not significant (Δ deviance < 0.001, d.f. = 1, P = 0.99).

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