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Original Article

Genomic analysis of Tibetan ground tits identifies molecular adaptations associated with cooperative breeding

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Abstract

Cooperative breeding is a sophisticated altruistic social behavior that helps social animals to adapt to harsh environments. The Tibetan ground tit, *Pseudopodoces humilis*, is a high-altitude bird endemic to the Tibetan plateau. Recently, it has become an exciting system for studying the evolution of facultative cooperative breeding. To test for molecular adaptations associated with cooperative breeding, we resequenced the whole genome of ground tits from 6 wild populations that display remarkable variation in the frequency of cooperative breeding. Population structure analyses showed that the 6 populations were divided into 4 lineages, which is congruent with the major geographical distribution of the sampling sites. Using genome-wide selective sweep analysis, we identified putative positively selected genes (PSGs) in groups of tits that displayed high and low cooperative breeding rates. The total number of PSGs varied from 146 to 722 in high cooperative breeding rate populations, and from 272 to 752 in low cooperative breeding rate populations. Functional enrichment analysis of these PSGs identified several significantly enriched ontologies related to oxytocin signaling, estrogen signaling, and insulin secretion. PSGs involved in these functional ontologies suggest that molecular adaptations in hormonal regulation may have played important roles in shaping the evolution of cooperative breeding in the ground tit. Taken together, our study provides candidate genes and functional ontologies involved in molecular adaptations associated with cooperative breeding in Tibetan ground tits, and calls for a better understanding of the genetic roles in the evolution of cooperative breeding.

Key words: cooperative breeding, ground tit, population genomics, social behavior

Social behaviors, which involve interactions among individuals within a society, are crucial for social animals, including humans. The complexity and diversity of social behaviors have inspired numerous theoretical and empirical studies focusing on the origin and evolution of these traits (Anderson 1984; Maynard Smith and Szathmary 1995; Koenig and Dickinson 2004). However, the genetic basis of social behaviors is largely unknown for several reasons. First, complex social behaviors could be regulated by dissimilar nervous systems and various molecular pathways across the animal kingdom. Second, many subtle and indirect effects of neural regulation influence social behavior over dynamic temporal and spatial scales. Third, genotype–environment interactions impact social behaviors, hence a natural context must be considered in behavioral studies (Robinson et al. 2008). Despite these challenges, a handful of genes associated with social behaviors have been identified in animals, ranging from insects to mammals (Robinson et al. 2008). At the expression level, for example, *egr1*, encoding a transcription factor important in neural plasticity, has been widely investigated in neural activation associated with animal behaviors. For example, in cichlid fish, *egr1* expression was induced by social opportunity associated with male dominance (Burmeister et al. 2005); in zebra finches, the rapid increase of *egr1* mRNA level was detected after the presentation of tape-recorded songs (Mello et al. 1992); and in rat, *egr1* was associated with maternal behavior by affecting promoter binding state of glucocorticoid receptor (Weaver et al. 2004). The expression level of vasopressin receptor gene *v1aR* has been shown to be involved in mating preferences and altered social behavior in voles (Lim et al. 2004; Hammock and Young 2005). By contrast, on the genetic level, *GP-9*, encoding a pheromone-binding protein, accounts for a remarkable form of social polymorphism in fire ants with the presence of 1 or more colony queens (Krieger and Ross 2002).

Cooperative breeding, where adults other than the father–mother pair help to care for the young (Skutch 1935), is a social behavior known to occur in some species of birds, mammals, fish, insects, spiders, and shrimp (Brockmann 1997; Koenig and Dickinson 2004; Solomon and French

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2007; Wong and Balshine 2011). In contrast to sociality that has been widely recorded in animals (over 15,000 ant species and 5% of mammal species), cooperative breeding behavior has been rarely reported (Rubenstein and Abbot 2017). This behavior was found in birds (9% of birds, over 100 families), and it is even rarer in other vertebrate lineages (Rubenstein and Abbot 2017). For example, it was observed in less than 0.1% of approximately 32,700 known fish species (Rubenstein and Abbot 2017) and around 2% of mammalian species (Eggert 2014). Nevertheless, it is an intriguing trait, since the altruism of helpers is incompatible with maximizing individual fitness (Solomon and French 2007). First documented in birds 80 years ago (Skutch 1935), this behavior has been extensively studied over the past 5 decades, after the publication of Hamilton's inclusive fitness theory (Hamilton 1964a, b). The attempts toward understanding the evolution of cooperative breeding have stimulated the development of 4 key hypotheses: (1) kin selection; (2) pay to stay; (3) group augmentation; and (4) signals of prestige (Wong and Balshine 2011). The kin selection hypothesis, established by Hamilton (Hamilton 1964a, 1964b), specifies that helpers tend to help related individuals to gain their inclusive fitness when altruism behavior occurs. The pay-to-stay hypothesis suggests that helping could be a form of payment for allowance to stay, with the threat of ejection by dominant breeders (Gaston 1978).

The group augmentation hypothesis, developed by Woolfenden, suggests that helping behavior may improve helpers' fitness by augmenting group size (Woolfenden 1975). Zahavi proposed the signals of prestige hypothesis, in which helping behavior was suggested to serve as an advertisement of individual quality, with helpers gaining higher social prestige (Zahavi 1995). However, these hypotheses cannot explain the full range of variation in cooperative breeding behaviors across the animal kingdom, since additional factors could have also played roles (Eggert 2014). With 3 exceptions linked to the role of genetics, the extensive variations in cooperative breeding within and between species have been exclusively linked in the literature to the role of ecology (Arnold and Owens 1999), although both roles of genetics and ecology are not mutually exclusive. The first exception was documented in a wild population of western bluebirds *Sialia mexicana* (Charmantier et al. 2007), the second was our previous work in Tibetan ground tits *Pseudopodoces humilis* (Wang and Lu 2018), and the third was a recently published cross-fostering experiment about banded mongooses *Mungos mungo* (Nichols et al. 2021). These studies provided the first 3 pieces of evidence for heritable variation in cooperative breeding behavior, suggesting a critical role of genetics.

The Tibetan ground tit (*Pseudopodoces humilis*, also known as *Parus humilis*), is a high-altitude bird endemic to the Tibetan plateau. It has become an exciting system for studying the evolution of cooperative breeding (Du and Lu 2009; Johannessen et al. 2011; Lu et al. 2011; Li et al. 2015b). Cooperative breeding behavior of ground tit was first documented in 2007 (Gosler and Clement 2007), and was soon confirmed by molecular parentage assessment (Du and Lu 2009). Cooperative breeding behavior in the ground tit is not present in all nests; hence some breeders have helpers while others do not. Moreover, the frequency of cooperative breeding behavior can differ significantly between ground tit populations. For example, 1 population in the Gahai National Nature Reserve (hereafter referred to as GH population) on the eastern Tibetan plateau (Figure 1) was recorded to have 15 of 172 nests with helpers (8.7%) in a 5-year field survey (Wang and Lu 2014). However, another population in Tianjun county (hereafter referred to as TJ population) on the northeastern Tibetan plateau (Figure 1) was found to have 88 of 187 nests with helpers (47.1%) during 5 breeding seasons (Li et al. 2015b). In addition to genetic differences, ecological constraints such as habitat saturation, mate shortage, and insufficient food may have played roles causing differences in the rate of cooperative breeding between populations. Although the ecological constraint hypothesis has been long proposed, specific ecological factors play the key role in driving the evolution of cooperative breeding remains controversial.

To test for molecular adaptations associated with the evolution of cooperative breeding, we undertook whole-genome resequencing of ground tits from 6 wild populations that display remarkable variation in the frequency of cooperative breeding. We classified the ground tit populations into 2 groups—high cooperative breeding rate (i.e., high cobreeding rate) and low cobreeding rate. We conducted genome-wide selective sweep analysis to identify putative positively selected genes (PSGs) in both cobreeding rate groups, with the aim of detecting molecular adaptations of specific genes and general functional categories that are associated with the evolution of cooperative breeding.

Materials and Methods

Study sites and field work

This work was part of a long-term monitoring project of the ground tit *P. humilis*, which is distributed in the alpine meadows at elevations from 2,500 to 5,500 m across the Tibetan plateau (del Hoyo et al. 1992; Wang and Lu 2011). Gahai (abbreviated as GH), Tianjun (TJ), and Dangxiong (DX) populations of the ground tit (Figure 1) have been extensively surveyed since 2004 (Du and Lu 2009; Johannessen et al. 2011; Wang and Lu 2011; Li et al. 2015b; Wang and Lu 2018). Our study sites covered broad regions of the Tibetan plateau and the 3 main distribution areas of the ground tit, including (1) the northern edge area, represented by Tianjun County, Qinghai; (2) the eastern margin of the Tibetan plateau, represented by Xiahe County and the Gahai National Nature Reserve, southern Gansu, and Hongyuan County, northwestern Sichuan; (3) the Tibetan plateau, represented by Dangxiong County and Changdu City, Tibet (Figure 1). Elevation, annual average air temperature, as well as longitude and latitude at the study site of each population are presented in Table 1. At each study site, all ground tit nests were located by observing adult breeding activities. The cobreeding rate for each population was calculated by the number of cobreeding nests divided by the number of total nests observed in our long-term fieldwork. During every breeding season, cooperative breeding behavior was recorded when more than 2 adults were involved in parental care at 1 nest. Numbers of cooperative breeding nests in Changdu (CD), Xiahe (XH), and Hongyuan (HY) were recorded in this study, whereas those in GH, TJ, and DX were published (Tables 1 and S5) (Wang and Lu 2014; Li et al. 2015b; Tang et al. 2017). Of these, XH and HY only recorded nests for 1 year (Tables 1 and S5). Each individual was marked and blood sampled as previously described (Johannessen et al. 2011; Li et al. 2015b).

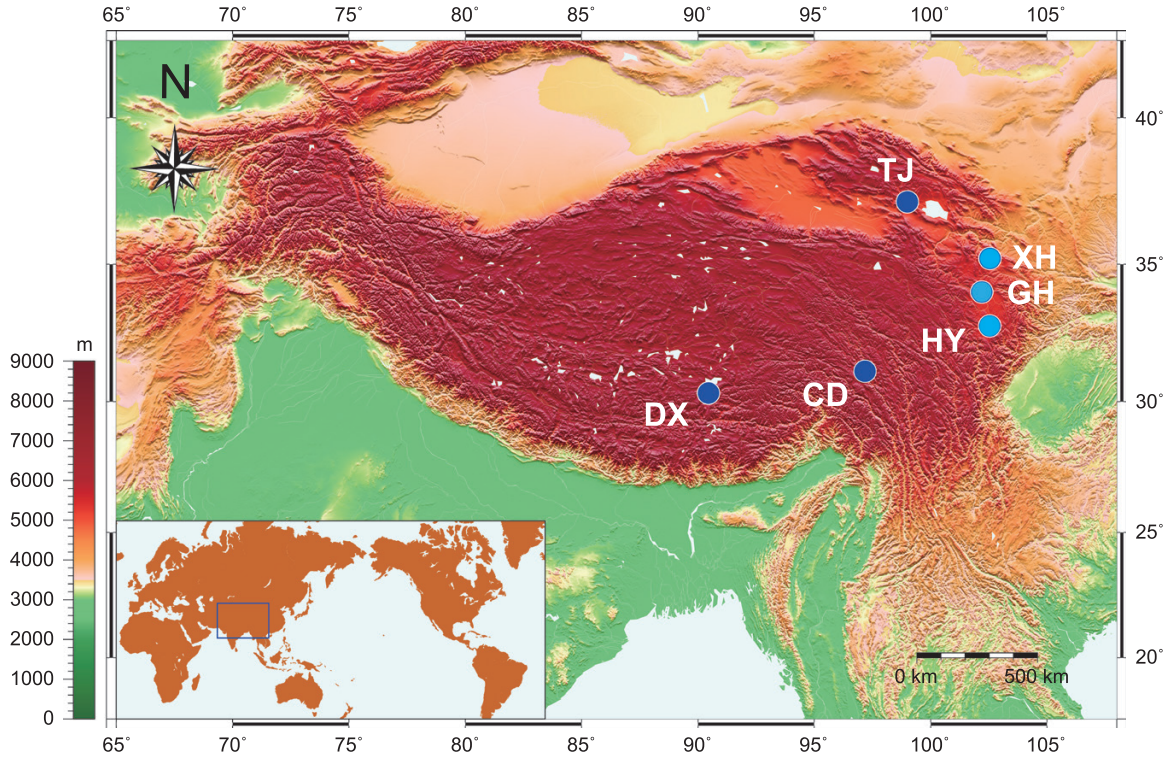

Figure 1. Geographic distribution of the six sampling sites. A total of 60 individuals of Tibetan ground tits were sampled and resequenced, representing 6 populations. Elevation is indicated by color, from green (low) to red (high). Blue dots indicate sampling sites. The depth of color indicates the frequency of cooperative breeding behavior: Dark blue indicates high cobreeding rate, whereas light blue indicates low cobreeding rate. CD: Changdu City; DX: Dangxiong County; GH: Gahai National Nature Reserve; HY: Hongyuan County; TJ: Tianjun County; XH: Xiahe county. This figure was made using the ETOPO1 Global Relief Model (Amante 2009), which only contained global topography and bathymetry datasets. Various colors only represent variations in topography. Elevation is indicated from green (low) to red (high), whereas rivers and lakes are shown in white. This figure did not include any national borders (see online version for color figure).

Table 1. Sampling site information and percentages of cooperatively breeding nests for each population

Population	Tibet		Gansu-Sichuan (GS)			Qinghai
	CD	DX	GH	XH	HY	TJ
Sampling site	Changdu	Dangxiong	Gahai	Xiahe	Hongyuan	Tianjun
Coordinates[a]	E 97°09′–97°13′ N 30°54′–30°55′	E 90°45′–90°46′ N 30°31′–30°32′	E 102°16′–102°22′ N 34°14′–35°15′	E 102°54′–102°56′ N 34°32′–34°33′	E 101°51′–101°52′ N 31°50 '–31°51'	E 98°58′–99°01′ N 37°17′–37°18′
Altitude	4,333–4,517 m	4,257–4,320 m	3,411–3,529 m	3,190–3,273 m	3,465–3,539 m	3,389–3,422 m
Temperature[b]	4–12°C	−1 to 9°C	−1 to 9°C	1–12°C	1–9°C	−9 to −2°C
Precipitation[c]	423 mm	431 mm	782 mm	533 mm	788 mm	345 mm
Food availability[d]	Poor	Poor	Rich	Rich	Rich	Poor
Cobreeding rate[e]	46.9% (114/243)	26.5% (133/502)	8.7% (15/172)	0.0% (0/12)	6.9% (2/29)	38.5% (69/179)

[a]Longitude and latitude coordinates for each sampling site, corresponding to each blue dot in Figure 1.
[b]Annual mean temperature.
[c]Annual precipitation.
[d]Food availability was roughly estimated in the field by the total weight of worms near the nests.
[e]Percentage of cooperatively breeding nests; Numbers in the brackets are cooperative breeding nests/all nests observed: Data in CD, XH, and HY were recorded in this study, whereas data in DX, GH, and TJ were published elsewhere (Wang and Lu 2014; Li et al. 2015b; Tang et al. 2017).

Sample collection and genome sequencing

Tibetan ground tits are facultative cooperative breeders, all individuals of which are potential helpers depending on factors such as environmental conditions and mate availability (Emlen 1982; Cornwallis 2018). The frequency of cooperative breeders in each population remains similar across years based on our long-term observation (Wang and Lu 2018). Thus, we consider the populations to have a stable frequency of cooperative breeding. This study was designed to examine the genetic basis underlying differences in the rate of cooperative breeding between populations. As a result, we did not consider the difference among individuals from 1 population. We thus sampled 60 individuals of ground tits *P. humilis*, randomly selected from 6 study sites (10 individuals from each population) that covered the main distribution of this species. All individuals were sampled from different nests, including

both cooperative and bi-parental breeding members in each population. The blood sample was drawn from venules (small veins) with 3–5 μL of fresh blood for each sample, and we set 20 g as the lowest weight of the sampling individual, to avoid irreversible damage to the newborn birds. Each sampled individual would be checked the next day, to ensure that it did not get injured by the blood drawing. The whole sampling procedure was reviewed and approved by the ethics committee of Wuhan University (WHU-TB-0009). Genomic DNA was extracted from blood samples using the DNeasy Blood and Tissue Kit (Qiagen, USA). Libraries were generated with an insert size of 500 bp, using the Illumina TruSeq DNA Sample Preparation Kit. Genome sequencing libraries were prepared as previously described (Li et al. 2015a; Tian et al. 2020), and were subsequently sequenced with 125-bp paired-end reads on an Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). In total, 816.8 Gb of raw reads were produced from the 60 libraries (Table S1).

Genome mapping and variation calling

After removing adapters, PCR duplicates, and low-quality reads, high-quality data were aligned to the reference ground tit genome assembly PseHum1.0 (GenBank assembly: GCA_000331425.1, coverage of 96.25×, scaffold N50 of 16337k, contig N50 of 165k) using Burrows-Wheeler Aligner (Li and Durbin 2009). The best alignments were built by SAMtools (Li et al. 2009) with the "rmdup" option. After genome mapping, single nucleotide polymorphism (SNP) calling was conducted using SAMtools for the 60 individually sequenced ground tits. The low-quality SNPs were trimmed using the Perl script vcfutils.pl in the BCFtools package (Li et al. 2009). We identified high-quality SNPs for further analysis with the following criteria (Liu et al. 2018): (1) coverage depth greater than 200 but lower than 3,000; (2) root-mean-square mapping quality greater than 10; (3) distance of adjacent SNPs greater than 5 bp. The posterior probability of each possible genotype in a candidate SNP position was estimated, and the genotype with the highest probability was selected (Li et al. 2015a). The Genome Analysis ToolKit (GATK) (McKenna et al. 2010) was employed to detect small insertions/deletions (InDels) using the Binary Alignment/Map (BAM) files generated by SAMtools. To limit the bias of sequence read misalignment in the InDel detection, we realigned InDel positions with overlapping reads using GATK, and validated each InDel until the realigned dataset did not include novel InDels (Wang et al. 2014). SNPs fitted with Hardy–Weinberg equilibrium within the 6 populations were retained using Vcftools with option "--hwe 0.001" and used to conduct the following analysis.

Genetic diversity

Genetic diversity was calculated with a 10-kb window with 5-kb step size across the genome with VCFtools (Danecek et al. 2011). Segregating sites (θ_ω) in every window were calculated by the Watterson method (WATTERSON 1975) and processed with Perl scripts; nucleotide diversity (θ_Π) was calculated with VCFtools. The mean theta values between populations were tested with a Mann–Whitney *U* test, and diversity was checked with a Wilcoxon test, with a *P*-value of $<2.2 \times 10^{-16}$ considered as significant. Linkage disequilibrium (LD) patterns were calculated within populations using the program Beagle (version 4.0) (Browning and Browning 2007). The correlation coefficient (r^2) between any 2 loci with a minor allele frequency greater than 0.05 was used as an indicator of the LD level, and average r^2 was calculated using VCFtools with the default parameters.

Phylogenetic Tree

TreeBeST (http://treesoft.sourceforge.net/treebest.shtml) was used to generate a Neighbor-Joining (NJ) to evaluate the phylogenetic relationships based on the nucleotide *p*-distances, with filtered SNPs with at least 10-kb intervals. The great tit (*Parus major*, SRA accession: SRR3087471) was used as the outgroup, and the reliability of the tree was assessed by 1,000 bootstrap resampling tests.

Principal component analysis and population structuring

To investigate the population structuring, principal component analysis (PCA) and population structure analysis were conducted using the ipyrad toolkit (Eaton and Overcast 2020). We demonstrated the individual eigenvalue for the top 4 principal components to reflect the real structure of the 60 individuals. A 3-way PCA was plotted using plotly (https://plot.ly/). Structure v2.3.4 was used to examine population genetic structuring based on allele frequencies (Falush et al. 2003). We used the "hdf" format file as the input file, which was produced in ipyrad. The parameter "burnin" was set to 5,000, and "numreps" to 10,000. The parameter of hypothetical genetic clusters was assumed from $K = 2$ to $K = 6$, and we observed that the result of $K = 4$ is consistent with the PCA clustering.

Genome-wide selective sweep test

To identify putatively PSGs, we performed selection analysis between populations with diverse cooperatively breeding behavior rates (also known as cobreeding rate). The cooperative breeding rate of each population has been stable based on our long-term field work (Ke and Lu 2009). Thus, we assume the cooperative breeding rate of each population as a fixed phenotype. In this work, we employed a well-established method to detect selective sweeps and study molecular adaptations associated with this phenotype of this trait. This method has been widely used in population genomic analysis of wild animals, such as honey bees (Chen et al. 2016), yaks (Qiu et al. 2015), and snub-nosed monkeys (Zhou et al. 2016). We calculated the genome-wide distribution of fixation index (F_{ST}) (Weir and Cockerham 1984) and nucleotide diversity (θ_Π) with a 10-kb sliding window and 5-kb step size. The F_{ST} values were Z-transformed, and the θ_Π ratios were $\log_2$-transformed. Windows with high F_{ST} values (top 5%) and low nucleotide diversity (bottom 5%) were recognized as putatively positively selected regions, and genes located in these regions were considered as PSGs. Based on the frequency of cooperative breeding behavior, we classified populations into 2o groups: high cooperative breeding rate (i.e., high cobreeding rate)—CD, DX, TJ, and H3 (a combination of CD, DX, and TJ); or low cooperative breeding rate—GH, HY, XH, and L3 (a combination of GH, HY, and XH). The threshold of a low cobreeding rate population was based on our current data that are available to us. When the cobreeding rate within a population is lower than 10%, we directly defined it as a low cobreeding rate population.

To cover all potential mechanisms and eliminate the signals other than selective sweeps, we compared each of the 4 high cobreeding rate populations (CD/DX/TJ/H3) with each of the 4 low cobreeding rate populations (GH/HY/XH/L3),

respectively. Eventually, we made 16 pairs of comparisons in total, and analyzed all the 32 results generated by 16 pairs of selective sweep tests. We estimated F_{ST} and θ_{Π} values with the 10-kb windows and 5-kb sliding steps in each comparison. Putative PSGs were identified by screening genomic regions that show low diversity (measured by θ_{Π} ratio) in 1 population but high divergence (measured by fixation index, F_{ST}) between the 2 populations being compared. PSGs can be identified from both high and low cobreeding rate populations.

Noise control

To examine the reliability of selection tests, we subjected screened regions to noise control analysis. We built a rigorous pipeline to remove potential background interferences and precisely identify genetic signatures related to the divergent cobreeding rates. We applied noise control analysis to all populations. We described the pipeline using CD as an example as follows. First, we obtained putative noise regions by identifying selected regions that are not related to cooperative breeding, which was completed by comparing the high cobreeding rate population CD with the other 2 high cobreeding rate populations (DX and TJ). Second, we removed noise regions in each pairwise comparison between CD and low cobreeding populations (GH, HY, XH, and L3). Third, we annotated selected regions with GenBank ID and Gene ID, using published genome annotation (Qu et al. 2013). Finally, we matched each Gene ID with corresponding UniProt IDs and conducted the enrichment analysis with all genes of *P. humilis* genome as background. Taken together, we applied the above procedure to each population, obtained 6 sets of PSGs after noise control analysis, divided them into high and low groups with divergent cooperative breeding rates, and finally identified potential molecular adaptations underlying the evolution of cobreeding behavior.

Functional enrichment analysis of positively selected genes

Enrichment and functional annotation analyses of PSGs were performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Dennis et al. 2003). We converted the gene IDs of the reference genome (*P. humilis*; GenBank assembly: GCA_000331425.1) to UniProt IDs (annotated with chicken, rat, mouse, and human), and submitted these PSGs to DAVID for Gene Ontology (GO) enrichment analysis using the whole gene set of *P. humilis* as a background. Results with *P*-values <0.1 (EASE score, modified Fisher's exact test) were identified and considered as significantly enriched (Hosack et al. 2003). To cover all the possible genetic mechanisms underlying cooperative breeding, we obtained all 32 groups. To reduce the background noise of our genomic data, we plotted the enriched GO terms that occur at least 3 times in these 32 paired comparisons. Following the KEGG pathway annotation (Kanehisa et al. 2017), we illustrated the pathway plot.

Results

Genome sequencing

We performed whole-genome sequencing for all 60 individuals, which generated 816.77 Gb of genomic data, for a total effective sequencing depth of 576× (Table S1). The generated data for each individual (genome size ~1.04 Gb; Cai et al. 2013) ranged from 10.1 to 18.8 Gb, corresponding to sequencing depths of 9.7–18.0× (Table S1). The average unique mapping rate of these sequencing reads was 98.22%, and the average effective coverage of the genome for an individual was 90.89% (Table S1). After aligning the short reads to the reference genome (Cai et al. 2013), we identified a total of 10.18 million high-quality SNPs for further analysis, with careful quality control and stringent filtering.

Population structuring

Whole-genome genetic diversity for each individual, estimated by Tajima's θ, was significantly lower in the CD population (mean $\theta_{\Pi} = 1.28 \times 10^{-3}$, mean $\theta_{\omega} = 1.07 \times 10^{-3}$) than in any other population ($P < 2.2 \times 10^{-16}$, Mann–Whitney U test) (Figure S1). Meanwhile, compared to the other 5 populations, CD showed a higher level of LD and a slower decay rate (Figure S2). This result appears to be consistent with the lower genetic diversity in CD (Figure S1), which also suggests that this population may have a small effective population size (Slatkin 2008).

To recover the phylogenetic relationships of the 60 individuals, we constructed an NJ tree based on the pairwise genetic distances using the 10-kb interval SNP data (Figure 2A). The NJ tree clearly identified 6 populations and 3 genetic groups: TJ, CD-DX, and GH-HY-XH, corresponding to the main distribution of ground tit habitat: the northern edge area, the platform of the plateau, and the southeastern margin of the plateau, respectively (Figure 1). PCA was performed to infer the genetic structure of the 6 populations. When plotting the first 3 components, CD, DX, and TJ were distinctly separated, while populations from the southeastern margin of the plateau (GH, HY, and XH) clustered together (Figures 2B and S3). However, HY and XH were divided by GH when plotting the first 4 components (Figure 2C), which is in accordance with the geographical distribution of the 3 sampling sites (Figure 1). A maximum-likelihood analysis of population structure also revealed 4 groups: CD, DX, TJ, and GS (a combination of GH, XH, and HY) (Figures 2D and S5), which is consistent with the PCA results (Figure 2B, C).

PSGs in the oxytocin signaling pathway

To dissect the molecular adaptations associated with cooperative breeding, we conducted genome-wide selective sweep analysis using the top 5% F_{ST} values and $\log_2(\theta_{\Pi}$ ratio) as cutoffs (Figure S6) to identify putatively PSGs. The total number of PSGs varied from 146 to 722 in high cobreeding rate populations, and from 272 to 752 in low cobreeding rate populations (Table S2). Our GO enrichment analysis of these PSGs detected several significantly enriched terms related to oxytocin signaling, estrogen signaling, and insulin secretion (Figure 3). As a result, PSGs involved in these functional ontologies suggest that molecular adaptations in hormonal and behavioral regulation may have played important roles in shaping the evolution of cooperative breeding in the ground tit.

Among all the enriched GO terms, the oxytocin (OXT) signaling pathway was most prominent, occurring 9 and 2 times in the low and high cobreeding populations, respectively (Figure 3 and Table S3). A total of 20 PSGs within the OXT signaling pathway were identified (Figure 4). To test the reliability of this finding, we conducted noise control analysis on the genomic regions with signatures of positive selection identified by our genome-wide selective sweep analysis (Figure S4). This analysis showed that the oxytocin signaling

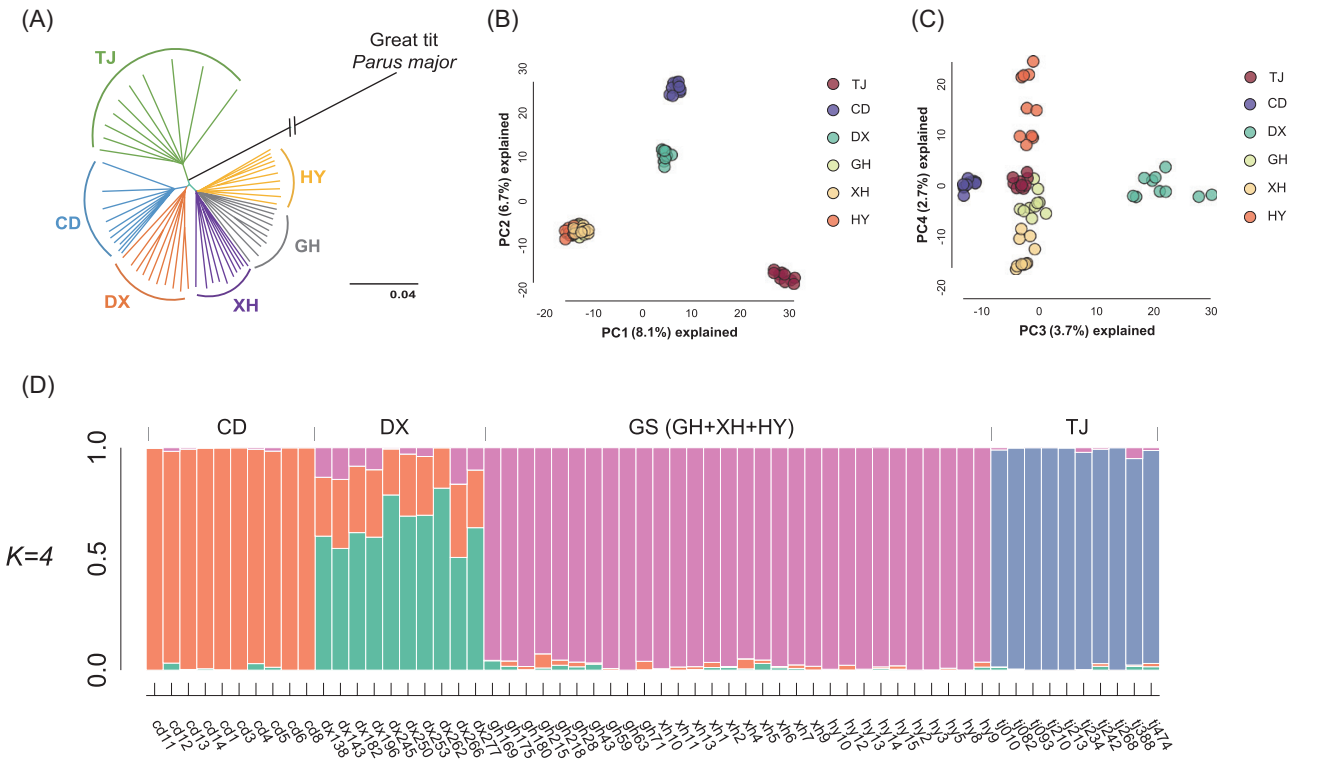

Figure 2. Population genetic analyses of Tibetan ground tits. (A) Phylogenetic tree of 60 individuals based on the NJ method. The great tit *Parus major* is used as the outgroup. The scale bar represents the evolutionary distances measured by *p*-distance. (B) PCA in dimensions PC1 and PC2. (C) PCA in dimensions PC3 and PC4. (D) Genetic structuring of the ground tits with $K = 4$ (ancestral populations). The *x* axis indicates sample codes for each individual, whereas *y* axis quantifies the proportion of an individual's genome inferred from ancestral populations.

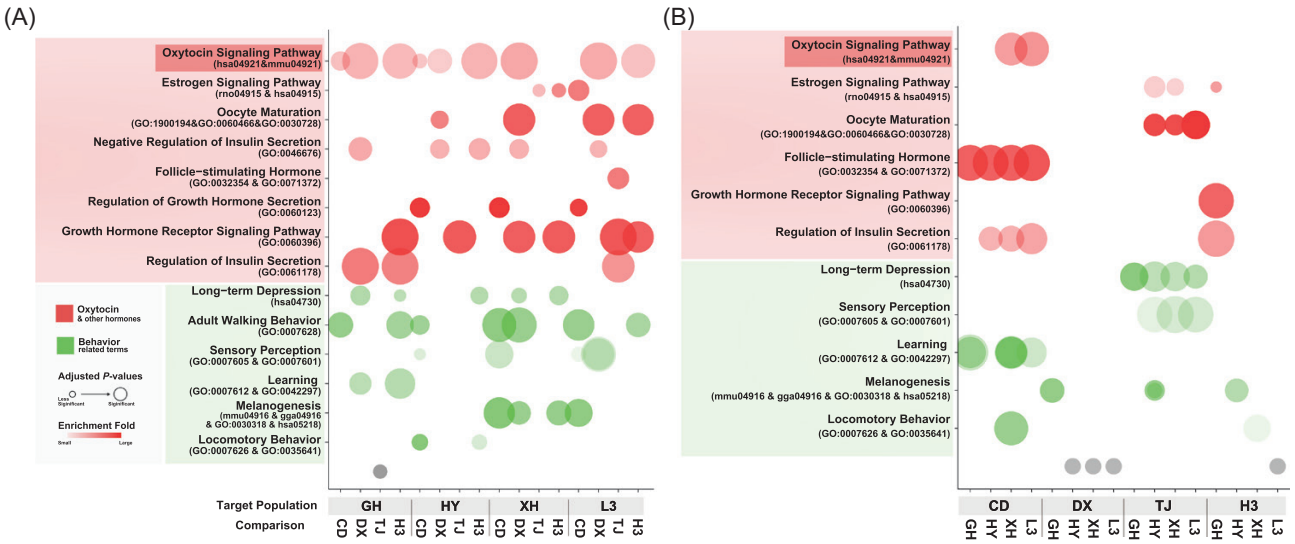

Figure 3. Functional enrichment analysis of putatively PSGs in (A) low cobreeding rate populations and (B) high cobreeding rate populations. The opacity and size of a circle represent the enrichment fold and *P*-value of each term, respectively. Distinct colors indicate different functional categories. The oxytocin signaling pathway is highlighted with dark red. Gray circles represent enriched GO terms that are not listed in the figure. Enriched GO terms detected by at least three independent pairwise comparisons are shown (see online version for color figure).

pathway remained significantly enriched in 3 low cobreeding populations, but not in high cobreeding populations (Table S4). Although genetic changes in oxytocin signaling pathway could be detected in both high and low cobreeding rate populations, it is still unknown how these genetic changes affect oxytocin functions.

PSGs related to other hormones

Apart from the oxytocin signaling pathway, PSGs were also found to be enriched in other hormone-related terms (Figure 3). For example, the estrogen signaling pathway was detected 6 times, 3 of which were in low cobreeding populations and 3e in high cobreeding populations (Figure 3 and Table S3). Estrogen is a sex hormone, mainly involved in the regulation

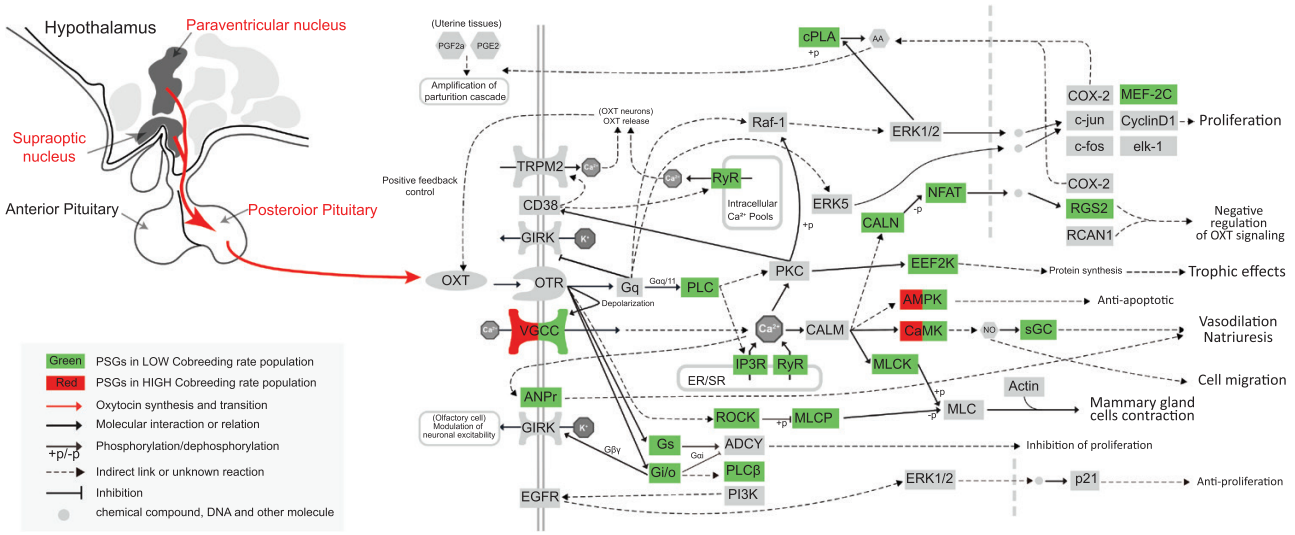

Figure 4. Positive selection in the oxytocin signaling pathway. Putatively PSGs in the oxytocin signaling pathway. Genes identified in high and low cobreeding rate groups are colored with red and green, respectively. Annotations, abbreviations, and connections are in accordance with KEGG standards. Molecular interaction or relation is indicated by solid lines; the dashed line shows the indirect link (see online version for color figure).

of sexual behavior and reproduction (Mhaouty-Kodja et al. 2018), as well as the development and regulation of vertebrate social behaviors, such as social learning and social recognition (Laredo et al. 2014; Ervin et al. 2015). Estrogen has been studied in birds, and is known to play important roles in various sexual behaviors (Balthazart et al. 2009). Moreover, 2 growth hormone–related GO terms (GO:0060123 and GO:0060396) were enriched 10 times, 9 of which were in low cobreeding populations (Figure 3). It is important to note that growth hormone was found to induce ovulation and has been used to stimulate the ovary to improve outcomes of in vitro fertilization cycles (Duffy et al. 2010). GO terms GO:0071372 (cellular response to follicle-stimulating hormone stimulus) and GO:0032354 (response to follicle-stimulating hormone) occurred multiple times, although not as frequently as oxytocin and estrogen—those terms were probably caused by its unique impact on the process of ovulation by yielding adaptations in hormones balance (Schwartz NB, et al. 1975; Johnson, et al. 2015). In addition, several candidate terms were involved in oocyte maturation and ovulation, which were downstream regulated by sex hormones (Rimon-Dahari et al. 2016).

Terms related to insulin secretion (GO:0061179, GO:0032024, GO:0061178, GO:0050796) and pathway hsa04911 (Insulin secretion) were detected 13 times in our enrichment analysis (Figure 3). In addition to its widely known function in controlling glucose homeostasis, insulin also affects ovulation by balancing sex hormones (Cataldo et al. 2001); excess insulin promotes testosterone production over estrogen production, which then causes insulin resistance (Sakumoto et al. 2010). In this condition, cells do not respond properly to insulin's signal and will cause delayed maturation of oocytes, leading to smaller eggs (Jungheim et al. 2010; Crofts et al. 2015). Insulin resistance mainly affects reproduction in humans by associating with polycystic ovary syndrome (PCOS), the most common endocrine disorder in females (Niu et al. 2014). Thus, we hypothesize that insulin may influence cooperative breeding behavior by regulating reproduction, which should be tested in the future.

PSGs related to behavior

Terms related to melanogenesis (hsa05218, mmu04916, GO:0030318) occurred 7 times in our enrichment analysis (Figure 3). Melanogenesis is the complex process by which the pigment melanin is produced in melanosomes by melanocytes (D'Mello et al. 2016). Due to the considerable variation in pigment patterns, cutaneous melanin pigment could be involved in social communication by exerting functions such as concealment and signaling of genotypic quality to conspecifics.

Other enriched terms related to behavior were concentrated in sensory perception (GO:0007601, GO:0060119, GO:0007605), learning (GO:0007612, GO:0042297), and memory (GO:0007616) (Figure 3). Visual and sound perceptions are typically associated with vocal and visual adaptations, respectively. Those sensory abilities may have helped ground tits to survive underground and could be critical for mate choice and parental care. Adaptations in learning and long-term memory could have conferred the ground tit's abilities to conduct helping behavior between generations. Parental care may not only be determined by genetic factors, but the social environment may also play a role. Sensory perception, learning from adult, and long-term memory may be vital reasons for helping behavior formation from birth to adulthood.

Discussion

In this study, we sequenced whole genomes of Tibetan ground tits from 6 populations with remarkable variation in the frequency of cooperative breeding. We identified putative PSGs in both higher and lower cobreeding rate groups using genome-wide selective sweep analysis. Our study revealed molecular adaptations in hormonal and behavioral regulation that may have played important roles in shaping the evolution of cooperative breeding.

Apart from ecological constraints, genetic variation must have played a key role during the evolution of cooperative breeding behavior, because the propensity to help was clearly demonstrated to be heritable in Tibetan ground tits and

western bluebirds based on pedigree analysis (Charmantier et al. 2007; Wang and Lu 2018). We found that putative PSGs were enriched in multiple pathways, such as oxytocin, estrogen, and insulin pathways, suggesting that the evolution of cooperative breeding behavior in Tibetan ground tits was driven by many factors. After comparing genetic variations between populations, we proposed that the selection on hormonal signaling pathways is the major factor during the evolution of cobreeding behavior in Tibetan ground tits.

Hormone-related terms were significantly enriched in Tibetan ground tit populations with both higher and lower levels of cooperative breeding, especially for oxytocin signaling pathway (Figure 3), which might be the key pathway associated with the cobreeding behavior evolution. In birds, there is an oxytocin-like hormone called mesotocin (MT) (Hyodo 2016), which differs from oxytocin in humans with a single substitution, Leu to Ile (Acher 2004). Because MT in birds is not as well studied as oxytocin in humans, the MT signaling pathway has not been well resolved thus far, to the best of our knowledge (Wu et al. 2019). Despite this, multiple studies have showed that MT and oxytocin play a similar functional role in other social behaviors. For example, in zebra finches, an oxytocin antagonist was able to impair pair bond formation, whereas MT increased the preference to associate with a larger social group (Goodson et al. 2009; Pedersen and Tomaszycki 2012).

Genetic changes in oxytocin signaling pathway were detected in both high and low cobreeding rate populations (Figure 3). OXT is a neuropeptide hormone synthesized in the hypothalamus and released from the pituitary gland, and plays important roles in sexual reproduction, childbirth, and social bonding (Yang et al. 2013). OXT signaling transduction starts at the hypothalamic paraventricular nucleus and supraoptic nucleus, and then is transported to the posterior pituitary and released into the bloodstream (Jurek and Neumann 2018). Besides its roles in milk let-down and uterine contraction during labor, oxytocin is also involved in responses to social stimuli by adaptively modifying neural circuits for social interactions and enhancing affiliative prosocial behaviors by attenuating stress (Kumsta and Heinrichs 2013; Marlin and Froemke 2017). In nonmammalian vertebrates, MT and isotocin are homologous to mammalian oxytocin in birds and fish, respectively. Interestingly, MT was experimentally shown to play an important role in facilitating prosocial behaviors in birds, and isotocin was proved to modulate social behavior in a cooperative breeder, which is a highly social cichlid fish (Reddon et al. 2012; Duque et al. 2018).

In our pairwise comparisons, we repeatedly detected 20 PSGs in oxytocin signaling pathway (Figure 4), suggesting that in addition to modulating social behaviors such as empathy, trust, and conflict (Shamay-Tsoory and Abu-Akel 2016), this category of hormones may also be a key regulator of cooperative breeding behavior. Three of these PSGs were detected in both low and high cobreeding populations (Figure 4). Specifically, *VGCC* (voltage-gated Ca^{2+} channel), which regulates Ca^{2+} influx, contributes to the full response of OXT receptor (OXTR) activation after depolarization (Sanborn et al. 1998; Sanborn 2007). The other 2 genes (*AMPK*, *CaMK*) encode the AMP-activated protein kinase and Ca^{2+}/calmodulin-dependent protein kinase, both of which are central regulators of cellular metabolism that can be activated by OXT in skeletal muscle cells and involved in the regulation of cardiovascular fear response, respectively (Lee et al. 2008; Florian et al. 2010; Viviani et al. 2011). The remaining 17 PSGs were all found in low cobreeding populations (Figure 4), which were located around Ca^{2+}-dependent cascades and downstream of the OXTR signaling cascades. Given that more PSGs involved in oxytocin signaling were found in low cobreeding populations, we speculated that populations with low cooperative breeding rate might have evolved from an ancestral population with a high cooperative breeding rate at some point during their evolution.

Apart from the oxytocin signaling pathway, several other pathways related to estrogen and ovulation were detected, which appear to have interactions with growth hormone that specifically leads to the induction of ovulation and stimulation of ovaries (Duffy et al. 2010), suggesting that molecular adaptation of sex and growth hormone regulation may have impacts on the ovulation and mate choice of female birds. Normally, hormone levels fluctuate at the stage of oocyte maturation (Mehlmann 2005). As a result, we speculate that in ground tit populations, the divergence of helping behavior rate might have led to adaptations in hormone secretion during the ovulatory cycle, in order to cope with selective pressures posed by cooperative breeding. We also found selective sweeps on several insulin-related terms (Figure 3). Indeed, previous studies have showed that insulin can balance sex hormones by promoting testosterone production, leading to insulin resistance (Sakumoto et al. 2010). In this condition, cells do not respond properly to insulin's signal, and would cause delayed maturation of oocytes, leading to smaller eggs (Jungheim et al. 2010; Crofts et al. 2015). Insulin resistance mainly affects reproduction in humans by associating with PCOS, the most common endocrine disorder in women (Niu et al. 2014). Thus, we hypothesize that insulin may influence cooperative breeding behavior by regulating reproduction, which should be tested in the future. Moreover, we speculate that the insulin level might fluctuate between helpers and nonhelpers, which might be a reason why helpers give up their own opportunities of independent breeding. Detection of various pathways under selection suggested that molecular adaptations on ovulation process were associated with the evolution of cooperative breeding behavior. In contrast to an earlier transcriptome study focusing on conserved genetic toolkit in the context of social behavior response across mouse, stickleback fish, and honey bee (Rittschof et al. 2014), our study focused on genetic differences at the whole-genome level within 1 bird species. Interestingly, both transcriptome and genome studies identified PSGs enriched in GO terms related to hormone signaling, but we did not find the conserved genetic toolkit in the transcriptome study to be overlapped with those genes in our study, suggesting that various genetic mechanisms underlying behavior evolution could be found in different clades of animals.

Of note, our analytic methods appear to have limitations. For instance, although we used all possible comparisons between high and low cobreeding populations, we cannot fully rule out the possibilities that other factors except cooperative breeding rate could affect our results. Even if cooperative breeding rate is the only variable that impacts genetic variation between populations, we are only able to identify a correlation that does not imply causation. Second, although the method of estimating F_{ST} (Weir and Cockerham 1984) and θ_{π} (Tajima 1983) is widely used for detecting selective sweeps in wild animals (Qiu et al. 2015; Chen et al. 2016; Zhou et al. 2016), we cannot completely avoid confounding factors such as recombination rate, GC content, population structure, and coding sequence density (Castellano et al. 2019). Third, in

addition to genetic variation, gene expression and epigenetic factors must play a role in the evolution of cooperative breeding behavior (Palumbo et al. 2018).

To the best of our knowledge, this study is the first attempt to explore the genetic variation in populations with distinct propensity of cooperative breeding behavior in birds, which will help to uncover the genetic basis of cooperative breeding of animals in the future. Our findings of putatively PSGs suggest important roles of hormonal and behavioral regulation in the evolution of cooperative breeding in this species. However, these candidate genes require functional validation in the future, because we could not fully rule out the possibilities of background noise in the genomic data. Furthermore, we could secure validations from future population-level studies on cooperative breeding behavior in other species. Although cooperative breeding behavior may have independently evolved among different species, an overlapping of genes or pathways under positive selection should give us a better understanding of genetic mechanism behind this complex social behavior. Taken together, our study offers insights into the genetic mechanisms of adaptations associated with cooperative breeding in Tibetan ground tits, and calls for a better understanding of the genetic roles in the evolution of cooperative breeding.

Data and materials availability

The resequencing data from the Tibetan ground tit, *Parus humilis*, has been deposited in the Genome Warehouse in the BIG Data Center (Zhang et al. 2020), Beijing Institute of Genomics (China National Center for Bioinformation), Chinese Academy of Sciences, under the accession number CRA004745.

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Author contributions

H.Z. designed the research; Y.W., C.W., W.H., S.T., Q.L., H.J., and N.Z. performed the research; Y.W., W.H., S.T., B.J.W., X.L., and H.Z. analyzed the data; and Y. W. and H.Z. wrote the paper.

Conflicts of Interest

All authors declare no conflict of interest.

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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