

# Comparative Genomics Reveals Evolution of a Beak Morphology Locus in a High-Altitude Songbird

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## Abstract

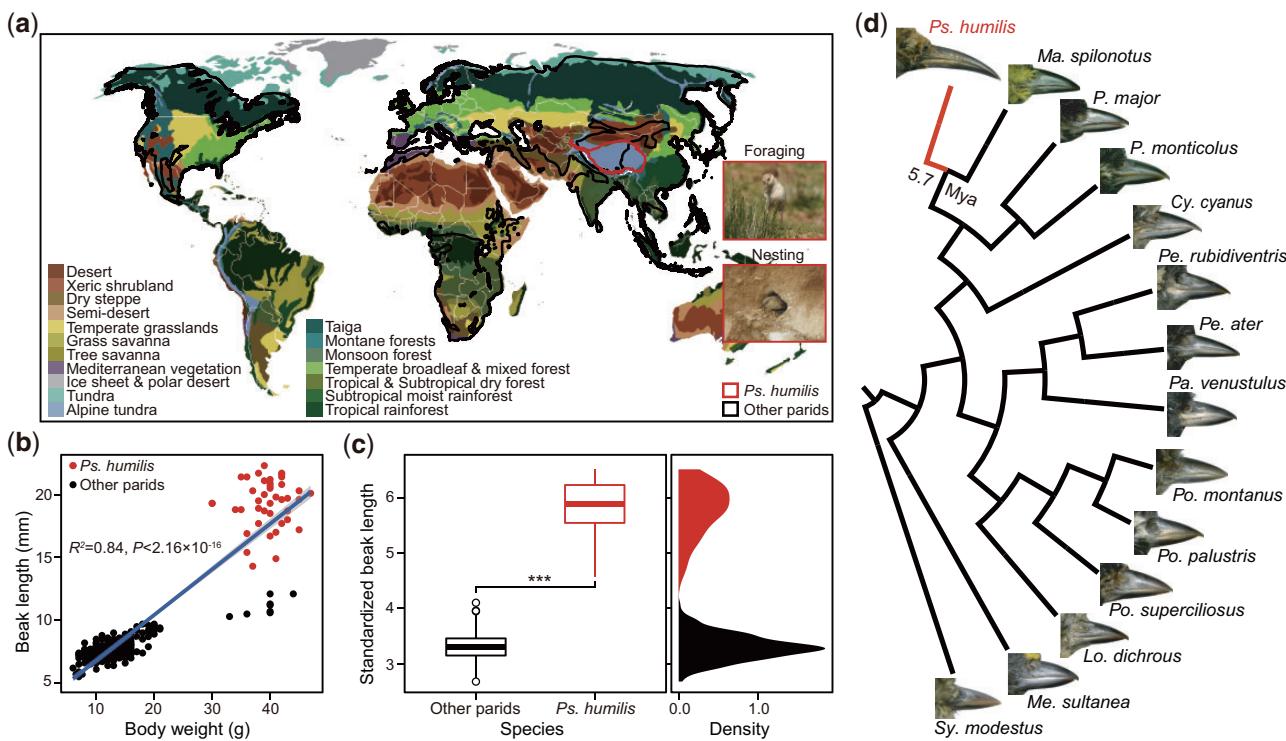
The Ground Tit (*Pseudopodoces humilis*) has lived on the Qinghai-Tibet Plateau for ~5.7 My and has the highest altitudinal distribution among all parids. This species has evolved an elongated beak in response to long-term selection imposed by ground-foraging and cavity-nesting habits, yet the genetic basis for beak elongation remains unknown. Here, we perform genome-wide analyses across 14 parid species and identify 25 highly divergent genomic regions that are significantly associated with beak length, finding seven candidate genes involved in bone morphogenesis and remolding. Neutrality tests indicate that a model allowing for a selective sweep in the highly conserved *COL27A1* gene best explains variation in beak length. We also identify two nonsynonymous fixed mutations in the collagen domain that are predicted to be functionally deleterious yet may have facilitated beak elongation. Our study provides evidence of adaptive alleles in *COL27A1* with major effects on beak elongation of *Ps. humilis*.

**Key words:** genomics, beak, adaptation, major effect, fixation.

The avian beak has a multitude of forms, which is the consequence of the variety of functions that beaks serve (e.g., foraging, preening, nest-building, cavity excavation) and the diversity of habitats where birds live. As a result, the beak is a highly evolvable structure that permits birds to rapidly respond to environmental changes (Grant and Grant 2011). However, the high plasticity and diversity of beak morphology, along with the fact that beak shape, like most quantitative phenotypes (Yang et al. 2011), is a polygenic trait (Bosse et al. 2017), presents challenges to our understanding of the genetic mechanisms underlying beak evolution. Under the polygenic model, natural selection often acts on many loci simultaneously (Pritchard et al. 2010), resulting in the combination of a few loci with major effects and many loci with small effects controlling adaptive changes in phenotypes (Orr 1998; Rockman 2012). Due to the characteristics of being more likely to be fixed by strong selective coefficients (Olson-Manning et al. 2012) and less susceptible to loss by genetic drift (Kimura 1962), major loci with high allele frequency may be more relevant to phenotypes and easier to be detected from the genome. As a result, previous studies have identified species-specific major genes affecting distinct beak morphology in chickens (Wu et al. 2004), Darwin's finches

(Abzhanov et al. 2004; Mallarino et al. 2011; Lamichhaney et al. 2016), and great tits (Bosse et al. 2017).

Around 5.7 Ma, the Ground Tit (*Pseudopodoces humilis*; Aves: Paridae) split from a common ancestor with genus *Machlolophus* and colonized the Qinghai-Tibet Plateau (QTP) (Qu et al. 2013; Cheng YL, unpublished data). As a result of millions of years of isolation in this sparsely vegetated and high-altitude environment (fig. 1a), *Ps. humilis* has evolved pale plumage, large body size, long tarsi, and a long-decurved beak (Qu et al. 2013; Shao et al. 2016; Gosler et al. 2019). Because of phenotypic similarities with ground jays, for over a century *Ps. humilis* was believed to be a member of the family Corvidae (Monroe and Sibley 1993; Hume 2008). After molecular systematics recognized it as the largest of parids (James et al. 2003), many studies have attempted to understand which processes underly this unique phenotype and how genetic changes control its development. Morphological analyses found significant correlations of tarsus length and beak morphology with altitude, suggesting that long tarsi and a long-decurved beak are the result of ground-living in open plateau habitats (Shao et al. 2016). Comparative transcriptomic analyses identified differential expression of genes involved in skeletal development that



**Fig. 1.** Long beaks in response to ground-living habits. (a) Vegetation types in the distribution ranges of tits and chickadees (Paridae). Only *Pseudopodoces humilis* lives in open steppe habitats of the QTP. (b) Significant correlation between beak length and body weight. (c) Standardized beak length of *Ps. humilis* is significantly longer than that of other parids. (d) Beak images of 14 parid species. These images are from Cheng et al. (2017), which shows the distinct beak morphology between *Ps. humilis* and other 13 parids. Mya indicates million years ago. The vegetation map was downloaded from Wikipedia (<https://en.wikipedia.org/wiki/Vegetation#/media/File:Vegetation-no-legend.PNG>, last accessed March 9, 2020). Distribution maps of parids downloaded from BirdLife International (<http://datazone.birdlife.org/>, last accessed April 16, 2020) were projected onto the vegetation map in ArcGIS v10.0 (ESRI, Redlands, CA).

probably affect beak morphogenesis (Cheng et al. 2017). However, to date, adaptive alleles at major loci responsible for the morphological transformation of the beak in *Ps. humilis* remain unknown. Here, we use whole-genome sequences of 14 parid species (supplementary table S1, Supplementary Material online), to compare the long-beaked *Ps. humilis* with short-beaked parids via the integration of multiple genome-wide scanning analyses. Furthermore, we assess the selection signals and predicted the function for mutations in candidate genes.

## Results and Discussion

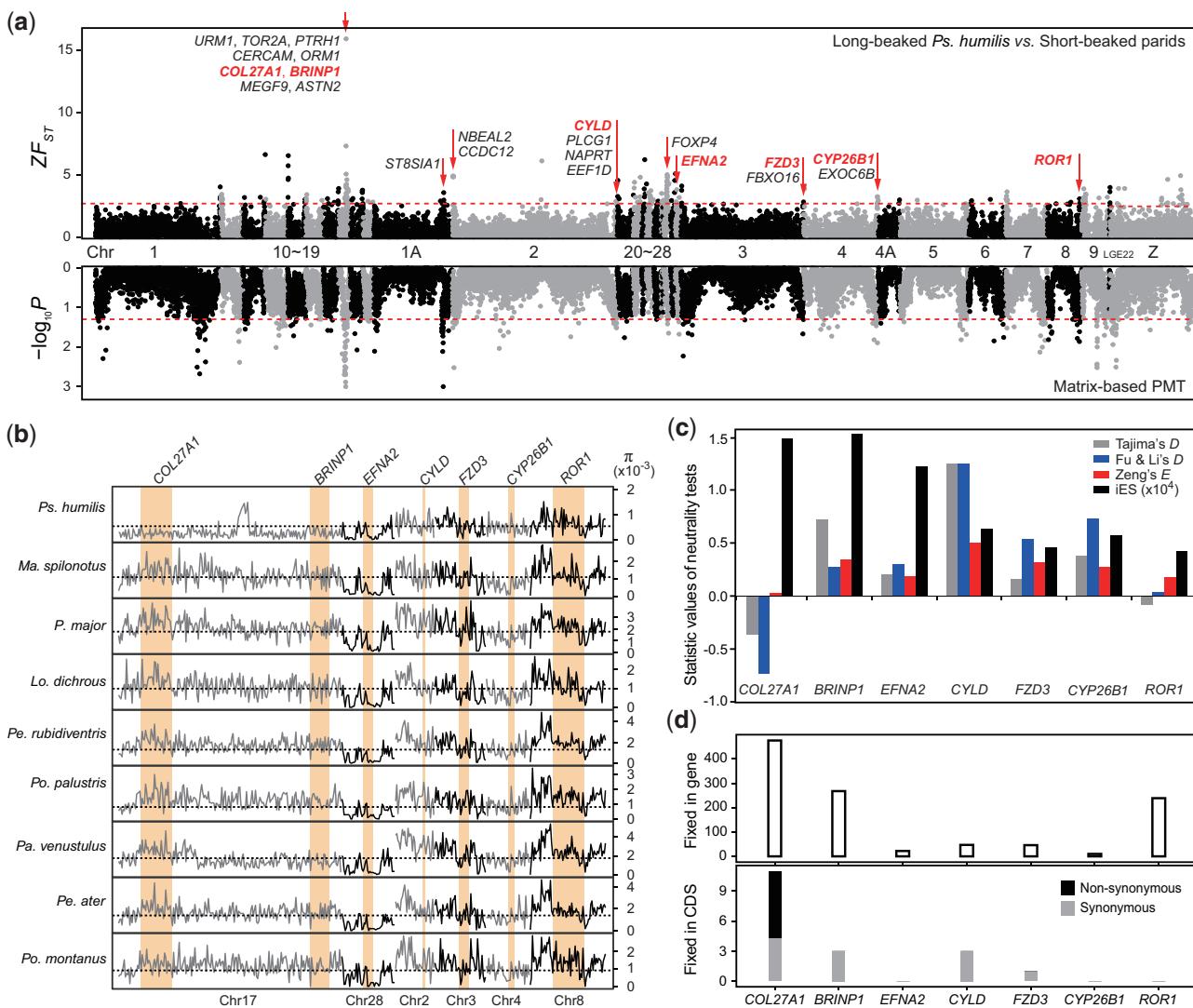
Morphological traits are commonly correlated with body size, which may veil adaptive variation. In 14 parids, we found that body length, wing length, tail length, tarsus length, and beak length were strongly correlated with body weight ( $r = 0.46\text{--}0.92$ ; fig. 1b; supplementary fig. S1a, Supplementary Material online). Among these measurements, both tarsus length and beak length are strikingly larger in *Ps. humilis* in relative to all other 13 parids (fig. 1b; supplementary fig. S1a, Supplementary Material online), even after standardizing for body weight differences (fig. 1c; supplementary fig. S1b, Supplementary Material online). In fact, after standardizing for body weight, the difference in beak length between *Ps. humilis* and the other parids was the most extreme of all of our measurements (Wilcoxon-rank sum = 0,

$P < 2.16 \times 10^{-16}$ ; fig. 1c; supplementary fig. S1b, Supplementary Material online), suggesting that the elongated beak may be the most adaptive morphological change in *Ps. humilis*, allowing for ground-foraging and burrow-nesting in the open steppe QTP habitat (fig. 1a and d).

### Candidate Genes Correlated with Beak Length

To identify genes controlling beak elongation, we calculated Z-transformed  $F_{ST}$  ( $ZF_{ST}$ ) values between the long-beaked *Ps. humilis* and the short-beaked group (the other 13 species) to identify highly divergent regions (supplementary table S2, Supplementary Material online). Mantel tests (Mantel 1967) identify outliers between two distance matrices and can identify nonneutral phenotypic evolution (Ho et al. 2017); here, we performed partial Mantel tests (PMT) to look for outliers between the  $F_{ST}$  matrix and the difference matrix of body weight-standardized beak lengths across these 14 species (supplementary fig. S2, Supplementary Material online).

The  $F_{ST}$  analysis identified 202 highly diverged windows ( $ZF_{ST} > 2.69$  for autosomes,  $ZF_{ST} > 2.63$  for chromosome Z), whereas the PMT identified 258 significantly correlated windows ( $-\log_{10}P > 1.30$ ) (fig. 2a), with 25 windows shared between the two sets (supplementary fig. S3, Supplementary Material online). We assume these 25 regions are most likely to be under divergent selection. We used SnpEff v4.3 (Cingolani et al. 2012) to annotate genes and predict gene

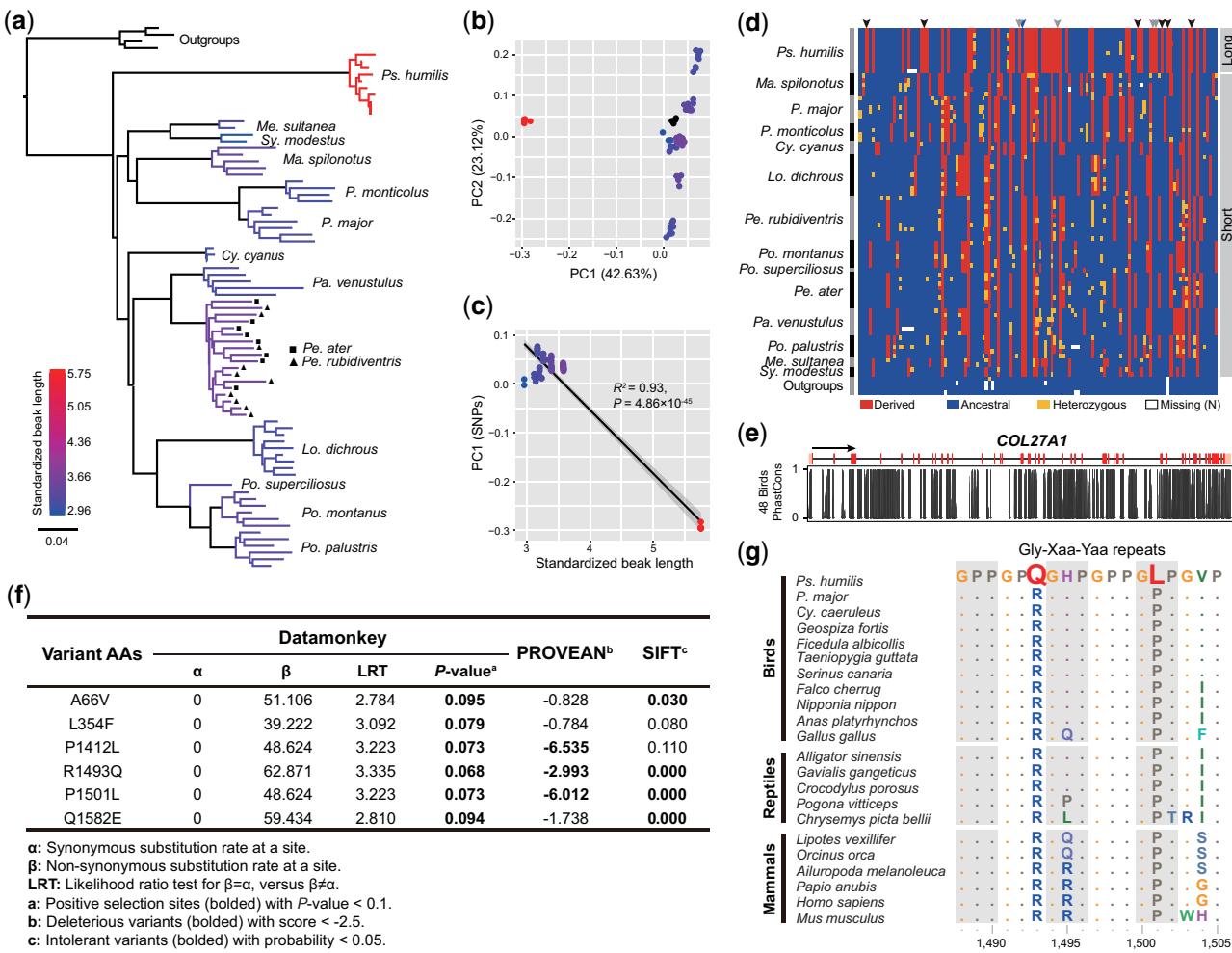


**Fig. 2.** Screening for candidate genes associated with beak length. **(a)** Genome-wide  $F_{ST}$  analysis between long-beaked *Pseudopodoces humilis* and short-beaked parids (upper panel), and partial Mantel tests (PMT) between pairwise  $F_{ST}$  matrix and a pairwise difference matrix of beak lengths (lower panel). Red dash lines represent cutoff values in both analyses. Red arrows indicate regions identified as outliers in both  $F_{ST}$  and PMT analyses. Candidate genes are in bold and red. **(b)** Nucleotide diversity of the seven candidate genes in species with sample sizes >5, orange shading covers the regions of these genes, and dashed lines show the mean nucleotide diversity of each species. **(c)** Neutrality tests for the seven candidate genes within *Ps. humilis*; negative Tajima's D and Fu & Li's D, nearly zero for Zeng's E, and high iES are signals of positive selection. **(d)** Count of fixed sites in the seven genes. COL27A1 had the most fixed sites between the two groups across the entire gene (introns and exons) and only the coding regions (CDS).

function in each of these. We found 23 genes in our windows, seven of which were functionally annotated as candidate genes involved in bone morphogenesis and remodeling (supplementary fig. S4, Supplementary Material online), including frizzled-3 (FZD3), receptor tyrosine kinase-like orphan receptor 1 (ROR1), BMP/retinoic acid-inducible neural-specific protein 1 (BRINP1), cytochrome P450 26B1 (CYP26B1), collagen alpha-1(XXVII) chain (COL27A1), ubiquitin carboxyl-terminal hydrolase CYLD, and ephrin-A2 (EFNA2) (fig. 2a; supplementary table S3, Supplementary Material online). Although these genes have not been found to be related to beak morphology in other birds (Lamichhaney et al. 2015, 2016; Bosse et al. 2017), their functional pathways play roles in beak development (Mallarino et al. 2011; Medio et al. 2012;

Elba et al. 2015). Our previous analysis also indicated that transcriptional changes in genes related to bone morphogenesis and remodeling affect beak development in embryos of *Ps. humilis* (Cheng et al. 2017).

FZD3 and ROR1 were associated with the most significant ontology term “Wnt-protein binding” (GO: 0017147) ( $P = 7.5 \times 10^{-4}$ ; supplementary fig. S4, Supplementary Material online) involved in the Wnt signaling pathway, which is known to promote osteoblast differentiation (Hartmann 2006) and which plays role in beak morphogenesis (Medio et al. 2012). Multiple studies have reported other Wnt-protein binding genes, such as FZD1 (Brugmann et al. 2010), DKK3 (Mallarino et al. 2011), FRZB1, and WIF1 (Cheng et al. 2017), as responsible for craniofacial or beak



**Fig. 3.** *COL27A1* genotypes and adaptive SNPs affect beak length. (a) A phylogenetic tree based on *COL27A1* coding region SNPs. Branches are colored by beak length. (b) PCA analysis of all *COL27A1* coding SNPs; *Pseudopodoces humilis* is separated from other parids. (c) Significant correlation between beak length and PC1. (d) Genotypes at all coding SNPs, showing striking difference between *Ps. humilis* and other parids. Fixed SNPs are indicated with arrows; black and gray arrows indicate nonsynonymous and synonymous derived sites, respectively, blue arrows indicate ancestral sites. Outgroup genotypes determined allele states of parids. (e) Conservation of nucleotide bases in *COL27A1* among 48 avian species based on PhastCons scores from Zhang et al. (2014). (f) Selection detection and function predictions for the six completely fixed nonsynonymous substitutions in *COL27A1*. All nonsynonymous mutations are under positive selection, and only two mutations are intolerant. (g) Conservation of the two deleterious amino acid sites among birds, reptiles, and mammals. These two mutations occur only in *Ps. humilis*.

development at the expression level. *BRINP1* and *CYP26B1* respond to retinoic acid, which is required for facial morphogenesis in chickens (Lee et al. 2001; Schneider et al. 2001). Mutations in *CYP26B1* result in craniofacial disorders in mice and zebra fish (Yashiro et al. 2004; Laue et al. 2011). *COL27A1*, located in one of the three most significant outlying regions (fig. 2a; supplementary table S2, Supplementary Material online), is a type XXVII collagen gene, and plays a role in the transition of cartilage to bone during bone morphogenesis (Hjorten et al. 2007). A homologous gene of *COL27A1*, *COL4A5* is associated with the elongated beak of *Parus major* as a response to supplementary feeding at bird feeders (Bosse et al. 2017). *CYLD* and *EFNA2* were also identified to be under likely divergent selection but they did not have the highest  $Z_{ST}$  values; however, they are associated with osteoclast differentiation participating bone resorption that negatively affects beak length (Ealba et al. 2015). Interestingly, *FGF13* and *ITGB3*—two genes associated with beak development that

we previously found to be differently expressed between *Ps. humilis* and *P. major* (Cheng et al. 2017)—were not identified as candidate genes in this analysis.

### Strong Selection on One Locus

To detect the strength of selection on the seven candidate genes, we calculated multiple statistics to test their evolutionary neutrality. Among the seven genes, only *COL27A1* had reduced nucleotide diversity in *Ps. humilis* and elevated diversity in other parids (fig. 2b). Furthermore, *COL27A1* had a long haplotype block (IES =  $1.49 \times 10^4$ ), whereas neutrality test values for Tajima's D ( $-0.36$ ) (Tajima 1989), Fu & Li's D ( $-0.73$ ) (Fu and Li 1993), and Zeng's E ( $0.02$ ) (Zeng et al. 2006) were consistent with strong positive selection (fig. 2c). Furthermore, *COL27A1* had the highest frequency of completely fixed sites in introns and exons (476) and in just exons (11) (fig. 2d). Phylogenetic analysis of *COL27A1* exonic SNPs shows reciprocal monophyly and deep divergence between *Ps. humilis* and other parids

(fig. 3a) but not in a data set of SNPs from both exons and introns (supplementary fig S5, Supplementary Material online). Collectively, these results suggest that COL27A1 underwent strong divergent selection in *Ps. humilis*, with most molecular evolution occurring in coding regions.

Principal component analysis (PCA) for COL27A1 coding region SNPs separated long-beaked *Ps. humilis* from the short-beaked parids (fig. 3b), and PC1 was significantly correlated with beak length ( $r = -0.96$ ,  $P = 4.86 \times 10^{-45}$ ; fig. 3c). Short branch lengths in the gene tree and low variation in PC values within *Ps. humilis* were associated with low genetic variation in COL27A1 (fig. 3a and b). We observed the highest level of derived homozygous genotypes in *Ps. humilis*, with only five heterozygous sites (fig. 3d), which is consistent with a selective sweep (Nielsen 2005). A selective sweep is also supported by the high ratio of fixed SNPs to all coding SNPs (11/120). All but one of the fixed SNPs were derived, as determined by comparison with outgroup sequences (fig. 3d). Among the ten derived fixed SNPs, six were nonsynonymous, and the remaining four were synonymous (fig. 3d; supplementary table S4, Supplementary Material online). Given the highly conserved nature of COL27A1 across 48 birds (Zhang et al. 2014) (fig. 3e), the high ratio of nonsynonymous to synonymous substitutions observed here (6/4) is likely to result in protein function change.

### Potential Adaptive Substitutions in COL27A1

To assess selection signals and predict function for the six nonsynonymous substitutions, we reconstructed COL27A1 coding sequences (CDS) for each species using our high-coverage SNP data (supplementary fig. S6, Supplementary Material online). We compared our constructed sequences with the *Ps. humilis* published sequence (XM\_014256225.1) and found high identity between them, especially at fixed sites, suggesting that our constructed CDS are accurate. We performed a selection analysis in Datammonkey (Weaver et al. 2018) on the COL27A1 alignment across all 14 parid species. All six nonsynonymous substitutions were identified as under positive selection (fig. 3f). Likewise, functional prediction from PROVEAN (Protein Variation Effect Analyzer) (Choi and Chan 2015) and SIFT (Sorting Intolerant From Tolerant) (Kumar et al. 2009), respectively, classed three and four substitutions as intolerant, including two substitutions (R1493Q and P1501L) identified as intolerant in both predictions (fig. 3f). These two mutations occur in a Gly-Xaa-Yaa repeat region of the collagen domain (supplementary fig. S7, Supplementary Material online), which determines the structure and property of type XXVII collagen (Pace et al. 2003). Mutations in this region would likely change the biological function of COL27A1 protein, leading to skeletal abnormalities, such as a long and oval-shaped face (Gonzaga-Jauregui et al. 2015), chondrodysplasia (Plumb et al. 2011), and scoliosis (Christiansen et al. 2009). These two amino acid sites show high conservation among birds, reptiles, and mammals, which supports the hypothesis that these two missense mutations in *Ps. humilis* may have caused a fundamental change in beak form (fig. 3g), ultimately allowing for adaptive elongation of the beak.

## Conclusion

The prominent beak of *Ps. humilis* is likely the result of adaptation for foraging and cavity nest excavation in open habitats of the QTP.  $F_{ST}$  and PMT analyses across 14 parid species identify 25 genomic regions under divergent selection that correlate to beak length and contain seven candidate genes known to be involved in bone morphogenesis and remodeling. Among these genes, only COL27A1 appears to have experienced a selective sweep in *Ps. humilis*. Additionally, COL27A1 has high fixation probability and high homozygosity, especially in the coding region. All non-synonymous fixed substitutions show evidence of positive selection, but being part of a single linkage group, we cannot determine whether all five were targets of selection. However, the two substitutions in the collagen domain are predicted to be deleterious; and likely lead to large changes in COL27A1 protein function. These findings suggest that COL27A1 has major effects on the extreme beak evolution in *Ps. humilis*.

## Materials and Methods

A complete description of our methods is provided in the Supplementary Material online.

## Supplementary Material

Supplementary data are available at Molecular Biology and Evolution online.

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## Author Contributions

F.L. and Y.C. designed this research. Y.C., M.J.M., and D.Z. analyzed the data. All authors wrote and approved the article.

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