

Gene Turnover in the Avian Globin Gene Families and Evolutionary Changes in Hemoglobin Isoform Expression

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Abstract

The apparent stasis in the evolution of avian chromosomes suggests that birds may have experienced relatively low rates of gene gain and loss in multigene families. To investigate this possibility and to explore the phenotypic consequences of variation in gene copy number, we examined evolutionary changes in the families of genes that encode the α - and β -type subunits of hemoglobin (Hb), the tetrameric $\alpha_2\beta_2$ protein responsible for blood-O₂ transport. A comparative genomic analysis of 52 bird species revealed that the size and membership composition of the α - and β -globin gene families have remained remarkably constant during approximately 100 My of avian evolution. Most interspecific variation in gene content is attributable to multiple independent inactivations of the α^D -globin gene, which encodes the α -chain subunit of a functionally distinct Hb isoform (HbD) that is expressed in both embryonic and definitive erythrocytes. Due to consistent differences in O₂-binding properties between HbD and the major adult-expressed Hb isoform, HbA (which incorporates products of the α^A -globin gene), recurrent losses of α^D -globin contribute to among-species variation in blood-O₂ affinity. Analysis of HbA/HbD expression levels in the red blood cells of 122 bird species revealed high variability among lineages and strong phylogenetic signal. In comparison with the homologous gene clusters in mammals, the low retention rate for lineage-specific gene duplicates in the avian globin gene clusters suggests that the developmental regulation of Hb synthesis in birds may be more highly conserved, with orthologous genes having similar stage-specific expression profiles and similar functional properties in disparate taxa.

Key words: avian genomics, gene deletion, gene duplication, gene family evolution, hemoglobin, protein evolution.

Introduction

Relative to the genomes of mammals, avian genomes are characterized by lower rates of chromosomal evolution and lower levels of structural variation within and among species (Burt et al. 1999; Shetty et al. 1999; Derjusheva et al. 2004; Griffin et al. 2007, 2008; Backstrom et al. 2008; Ellegren 2010, 2013; Zhang et al. 2014). At the nucleotide sequence level, birds have lower substitution rates relative to mammals and squamate reptiles, but they have somewhat higher rates than crocodylians (the sister group to birds, representing the only other extant lineage of archosaurs) and turtles, the sister group to archosaurs (Mindell et al. 1996; Nam et al. 2010; Shaffer et al. 2013; Green et al. 2014). The high level of conserved synteny among avian genomes suggests that birds may have experienced relatively low rates of gene turnover in multigene families. This hypothesis can be tested by characterizing rates and patterns of gene turnover in well-characterized multigene families—such as the globin gene

superfamily—that share a high degree of conserved synteny across all amniote lineages.

The α - and β -globin genes encode subunits of the tetrameric $\alpha_2\beta_2$ hemoglobin (Hb) protein, which is responsible for blood-O₂ transport in the red blood cells of jawed vertebrates (gnathostomes). The α - and β -globin genes were tandemly linked in the last common ancestor of gnathostomes, a chromosomal arrangement that has been retained in some lineages of ray-finned fishes, lobe-finned fishes, and amphibians (Jeffreys et al. 1980; Hosbach et al. 1983; Gillemans et al. 2003; Fuchs et al. 2006; Opazo et al. 2013; Schwarze and Burmester 2013). In amniote vertebrates, the α - and β -globin genes are located on different chromosomes due to transposition of the proto β -globin gene(s) to a new chromosomal location after the ancestor of amniotes diverged from amphibians (Hardison 2008; Patel et al. 2008; Hoffmann, Storz, et al. 2010; Hoffmann, Opazo, Storz, et al. 2012).

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Because the vertebrate Hb genes comprise one of the most intensively studied multigene families from a functional standpoint (Hardison 2001, 2012; Hoffmann, Storz, et al. 2010; Storz, Opazo, et al. 2011; Hoffmann, Opazo, Storz, 2012; Storz et al. 2013; Burmester and Hankeln 2014), the examination of globin gene family evolution provides an opportunity to assess the phenotypic consequences of changes in gene content. Specifically, changes in gene copy number and divergence between paralogs can promote Hb isoform differentiation, which can result in physiologically important modifications of blood-O₂ transport and aerobic energy metabolism. Changes in the size and membership composition of the α - and β -globin gene families may produce changes in the developmental regulation of Hb synthesis and may therefore constrain or potentiate functional divergence between Hb isoforms that incorporate the products of paralogous globin genes (Berenbrink et al. 2005; Berenbrink 2007; Opazo et al. 2008a, 2008b; Runck et al. 2009; Hoffmann, Storz, et al. 2010; Storz, Hoffmann, et al. 2011; Storz, Opazo, et al. 2011; Grispo et al. 2012; Damsgaard et al. 2013; Opazo et al. 2013; Storz et al. 2013). Indeed, over a deeper timescale of animal evolution, the co-option and functional divergence of more ancient members of the globin gene superfamily have played a well-documented role in evolutionary innovation (Hoffmann, Opazo, et al. 2010; Blank et al. 2011; Hoffmann, Opazo, Hoogewijs, et al. 2012; Hoffmann, Opazo, Storz, 2012; Hoogewijs et al. 2012; Schwarze and Burmester 2013; Schwarze et al. 2014).

All gnathostome taxa that have been examined to date express structurally and functionally distinct Hb isoforms during different stages of prenatal development (Hardison 2001), and some tetrapod groups coexpress different Hb isoforms during postnatal life. The majority of birds and non-avian reptiles coexpress two functionally distinct Hb isoforms in definitive erythrocytes: HbA (with α -chain subunits encoded by the α^A -globin gene) and HbD (with α -chain subunits encoded by the α^D -globin gene; fig. 1). In adult birds, HbA and HbD represent major and minor isoforms, respectively, where HbD typically accounts for 10–40% of total Hb (Grispo et al. 2012).

Most bird species have retained each of three tandemly linked α -type globin genes that were present in the last common ancestor of tetrapod vertebrates: 5'- α^E - α^D - α^A -3' (Hoffmann and Storz 2007; Hoffmann, Storz, et al. 2010; Grispo et al. 2012). The proto α^E - and α^A -globin genes originated via tandem duplication of an ancestral proto α -globin gene, and α^D -globin originated subsequently via duplication of the proto α^E -globin gene (Hoffmann and Storz 2007; Hoffmann, Storz, et al. 2010). Both duplication events occurred in the stem lineage of tetrapods after divergence from the ancestor of lobe-finned fishes approximately 370–430 Ma (Hoffmann and Storz 2007). In all tetrapods that have been examined to date, the linkage order of the three α -type globin genes reflects their temporal order of expression during development: α^E -globin is exclusively expressed in larval/embryonic erythroid cells derived from the yolk sac, α^D -globin is expressed in both primitive and definitive erythroid cells, and α^A -globin is expressed in definitive erythroid

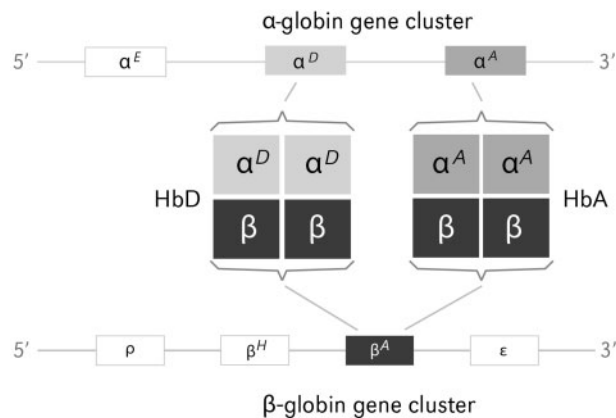


Fig. 1. Postnatally expressed Hb isoforms in avian red blood cells. The major isoform, HbA ($\alpha^A_2\beta_2$), has α -type subunits encoded by the α^A -globin gene, and the minor isoform, HbD ($\alpha^D_2\beta_2$), has α -type subunits encoded by the α^D -globin gene. Both isoforms share identical β -type subunits encoded by the β^A -globin gene. The remaining members of the α - and β -globin gene families (α^E , ρ , β^H , and ϵ -globin) are not expressed at appreciable levels in the definitive erythrocytes of adult birds. Within each gene cluster, the intergenic spacing is not drawn to scale.

cells during later stages of prenatal development and postnatal life (Ciroto et al. 1987; Ikehara et al. 1997; Alev et al. 2008, 2009; Storz, Hoffmann, et al. 2011).

Most variation in the size of the avian α -globin gene family is attributable to multiple independent deletions or inactivations of the α^D -globin gene (Zhang et al. 2014). Due to consistent differences in O₂-binding properties between the HbA and HbD isoforms (Grispo et al. 2012), losses of the α^D -globin gene likely contribute to among-species variation in blood-O₂ affinity, which has important physiological consequences. In all bird species that have been examined to date, HbD is characterized by a higher O₂-binding affinity relative to HbA (Grispo et al. 2012), and it also exhibits other unique properties, such as a propensity to self-associate upon deoxygenation (Cobb et al. 1992; Knapp et al. 1999; Rana and Riggs 2011) which may enhance the cooperativity of O₂ binding (Riggs 1998).

Comparative studies of endothermic vertebrates have documented a positive scaling relationship between body mass and blood-O₂ affinity (Schmidt-Nielsen and Larimer 1958; Lutz et al. 1974; Bunn 1980), although the pattern is not as clear in birds as it is in mammals (Baumann and Baumann 1977; Lutz 1980). Available data suggest that, in general, the higher the mass-specific metabolic rate of an animal, the higher the partial pressure of O₂ (PO₂) at which the blood releases O₂ to the cells of respiring tissues. This high unloading tension preserves a steep O₂ diffusion gradient between capillary blood and perfused tissue, thereby enhancing O₂ flux to the tissue mitochondria. Given that relative HbA/HbD expression levels should be an important determinant of blood-O₂ affinity, it is of interest to assess whether such expression levels are phylogenetically correlated with body mass. One clear prediction is that avian taxa with especially high mass-specific metabolic rates, like hummingbirds and passerines,

have evolved a reduced HbD expression level, which (all else being equal) would produce a reduced blood-O₂ affinity.

The main objectives of the present study were to assess the phenotypic consequences of changes in gene copy number, and to assess relationships between rates of gene retention, expression levels, and functional constraint. The specific aims were 1) to characterize patterns of gene turnover and interparalog gene conversion in the α - and β -globin gene clusters of birds, 2) to estimate the number of independent inactivations/deletions of the α^D -globin gene during the diversification of birds, 3) to characterize among-species variation in the relative expression of HbA and HbD isoforms, 4) to reconstruct evolutionary changes in HbA/HbD expression levels during the diversification of birds, and 5) to characterize variation in functional constraint among paralogous globin genes. To achieve these aims, we conducted a comparative genomic analysis of the avian α - and β -globin gene clusters in 52 species (supplementary table S1, Supplementary Material online), building on our previous analysis of high-coverage genome assemblies for 22 species (Zhang et al. 2014). We conducted molecular evolution analyses based on globin sequences from a phylogenetically diverse set of 83 bird species, including 50 newly generated sequences from species for which full genome sequences were not available. Finally, using experimental measures of protein expression for 122 bird species, we conducted a phylogenetic comparative analysis to reconstruct evolutionary changes in HbA/HbD isoform abundance.

Results and Discussion

Genomic Structure of the Avian α - and β -Globin Gene Families

To characterize the genomic structure of the avian globin gene clusters, we analyzed contigs from a set of 24 bird species that contained the full complement of α - and β -type globin genes. Our comparative genomic analysis of these 24 species revealed that the size and membership composition of the globin gene families have remained remarkably constant, as most bird species have retained an identical complement of three tandemly linked α -type globin genes (5'- α^E - α^D - α^A -3') and four tandemly linked β -type globin genes (5'- ρ - β^H - β^A - ϵ -3'; fig. 2). In eutherian mammals, in contrast, the α - and β -globin gene clusters have experienced high rates of gene turnover due to lineage-specific duplication and deletion events, resulting in functionally significant variation in globin gene repertoires among contemporary species (Hoffmann et al. 2008a, 2008b; Opazo et al. 2008a; Opazo et al. 2009; Gaudry et al. 2014). In rodents alone, the number of functional α -type globin genes ranges from 2 to 8 (Hoffmann et al. 2008b; Storz et al. 2008) and the number of functional β -type globin genes ranges from 3 to 7 (Hoffmann et al. 2008a). To quantify this apparent difference in the tempo of gene family evolution in birds and mammals, we used a stochastic birth–death model to estimate rates of gene turnover (λ) in the globin gene clusters of 24 bird species and 22 species of eutherian mammals that span a similar range of divergence times. Results of this analysis revealed that the rate

of gene turnover in the avian globin gene clusters is roughly two times lower than in eutherian mammals ($\lambda = 0.0013$ vs. 0.0023). Available data suggest that the α - and β -globin gene clusters of squamate reptiles have also undergone a high rate of gene turnover relative to homologous gene clusters in birds (Hoffmann, Storz, et al. 2010; Storz, Hoffmann, et al. 2011).

Inference of Orthologous Relationships and Detection of Interparalog Gene Conversion

To reconstruct phylogenetic relationships and to examine patterns of molecular evolution, we analyzed all available α - and β -type globin sequences from a phylogenetically diverse set of 83 bird species. Phylogeny reconstructions based on coding sequence confirmed that the paralogous α -type globin genes grouped into three well-supported clades and clearly identifiable orthologs of each gene are present in other amniote taxa (fig. 3). Similarly, the phylogeny reconstruction of β -type genes recovered four clades representing the ρ -, β^H -, β^A -, and ϵ -globin paralogs (fig. 4). The four avian β -type paralogs are reciprocally monophyletic relative to the β -type globins of other amniotes, indicating that they represent the products of at least three successive rounds of tandem duplication in the stem lineage of birds. The phylogeny revealed several cases of interparalog gene conversion between the embryonic ρ - and ϵ -globin genes, as documented previously in analyses of the chicken and zebra finch β -globin gene clusters (Reitman et al. 1993; Hoffmann, Storz, et al. 2010). A history of interparalog gene conversion between ρ - and ϵ -globin is evident in several cases where sequences from genes that are positional homologs of ρ -globin are nested within the clade of ϵ -globin sequences, and vice versa (fig. 4). For example, ρ - and ϵ -globin coding sequences of downy woodpecker (*Picoides pubescens*) were placed sister to one another in what is otherwise a clade of ρ -globin sequences (fig. 4), indicating that the ϵ -globin gene of this species has been completely overwritten by $\rho \rightarrow \epsilon$ gene conversion. Additional examples of interparalog gene conversion between the ρ - and ϵ -globin genes are evident in phylogeny reconstructions with more extensive taxon sampling (supplementary fig. S1, Supplementary Material online).

In both chicken and zebra finch, the β^H -globin gene is expressed at scarcely detectable levels in definitive erythrocytes (Alev et al. 2008, 2009). But whereas the β^H -globin gene is quiescent in the primitive erythrocytes of the developing chick embryo (Alev et al. 2008), it is highly expressed during the analogous stage of embryogenesis in zebra finch (Alev et al. 2009). Analyses of chicken β -type genes by Reitman et al. (1993) suggested that the two embryonic genes (ρ and ϵ) form a reciprocally monophyletic group relative to β^H and β^A , although recent analyses based on more extensive sequence data recovered the topology ($\beta^H(\beta^A(\rho, \epsilon))$) (Alev et al. 2009; Hoffmann, Storz, et al. 2010). Results of the Bayesian analysis shown in figure 4 are consistent with these more recent analyses, although posterior probabilities for key nodes varied markedly depending on which alignments were used. Maximum-likelihood analyses did not provide better resolution, as bootstrap support values for key nodes were

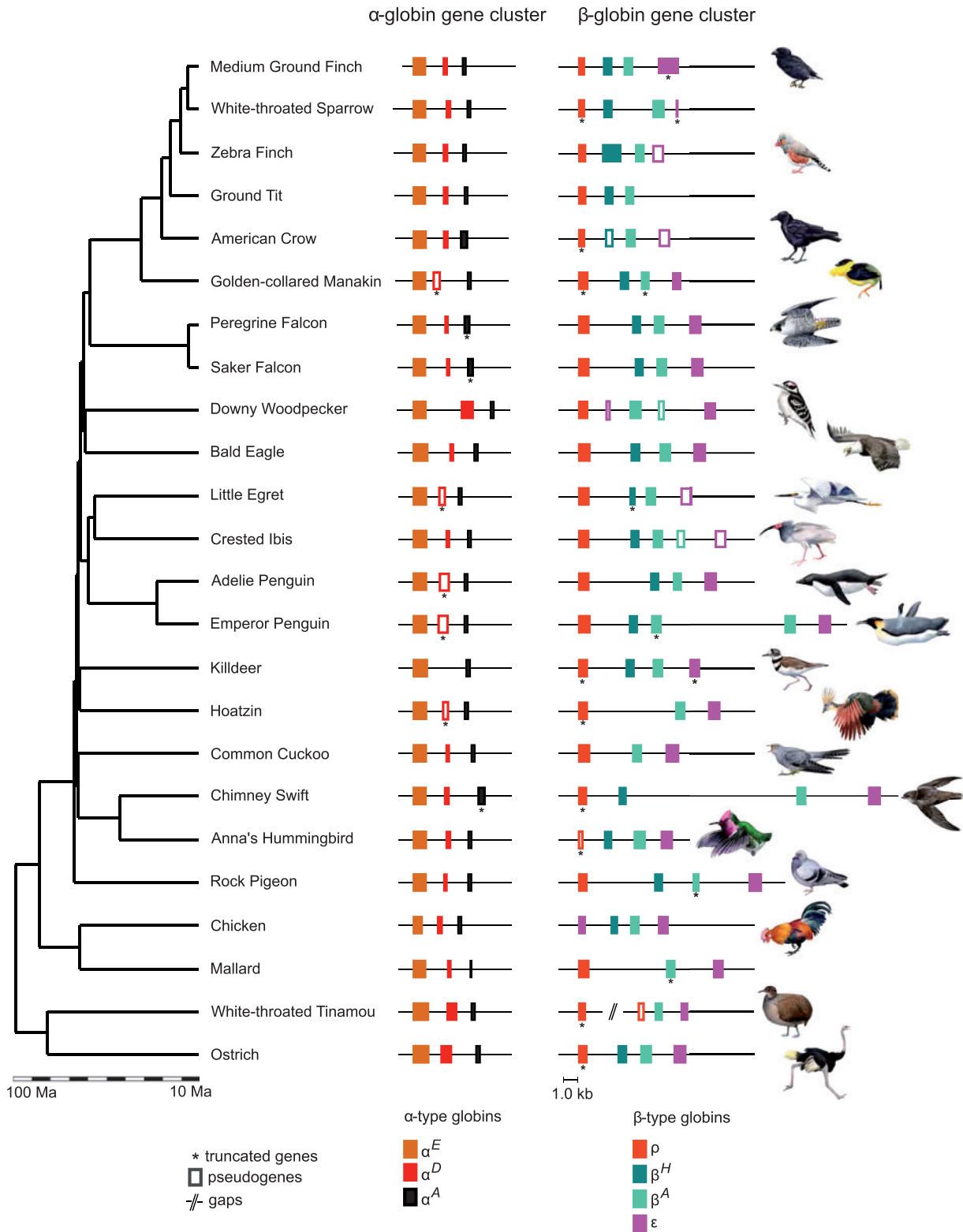


FIG. 2. Genomic structure of the α - and β -globin gene clusters in 24 bird species. The phylogeny depicts a time-calibrated supertree (see Materials and Methods for details). The 24 species represented in the figure are those for which we have accounted for the full repertoire of α - and β -type globin genes. Truncated genes may have been produced by partial deletion events, but in some cases the apparently truncated coding sequences may represent assembly artifacts.

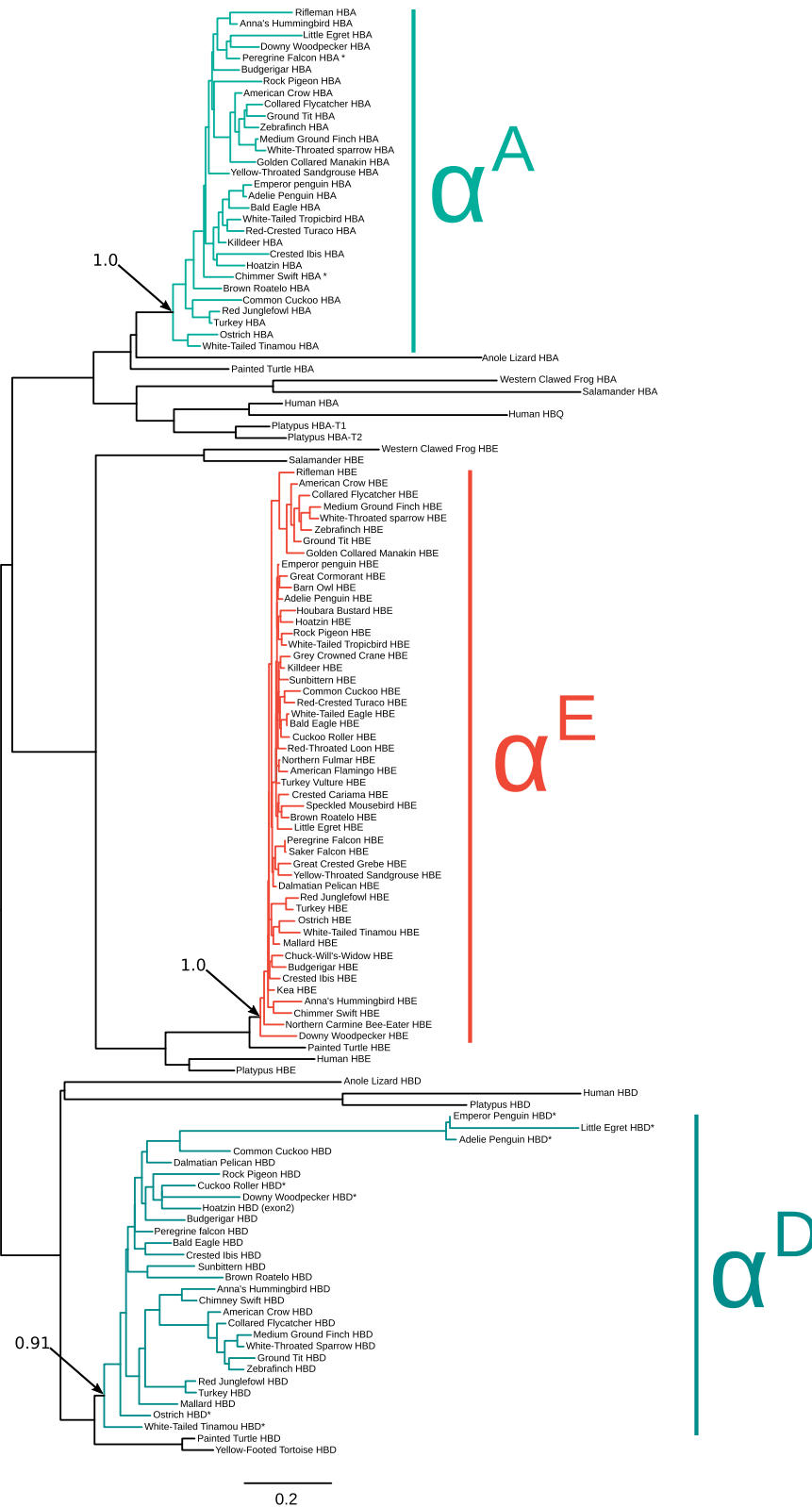


FIG. 3. Bayesian phylogeny depicting relationships among avian α -type globin genes based on nucleotide sequence data. Sequences of α -type globin genes from other tetrapod vertebrates (axolotl salamander [*Ambystoma mexicanum*], western clawed frog [*Xenopus tropicalis*], anole lizard [*Anolis carolinensis*], painted turtle [*Chrysemys picta*], platypus [*Ornithorhynchus anatinus*], and human [*Homo sapiens*]) were used as outgroups. Bayesian posterior probabilities are shown for the ancestral nodes of each clade of orthologous avian genes.

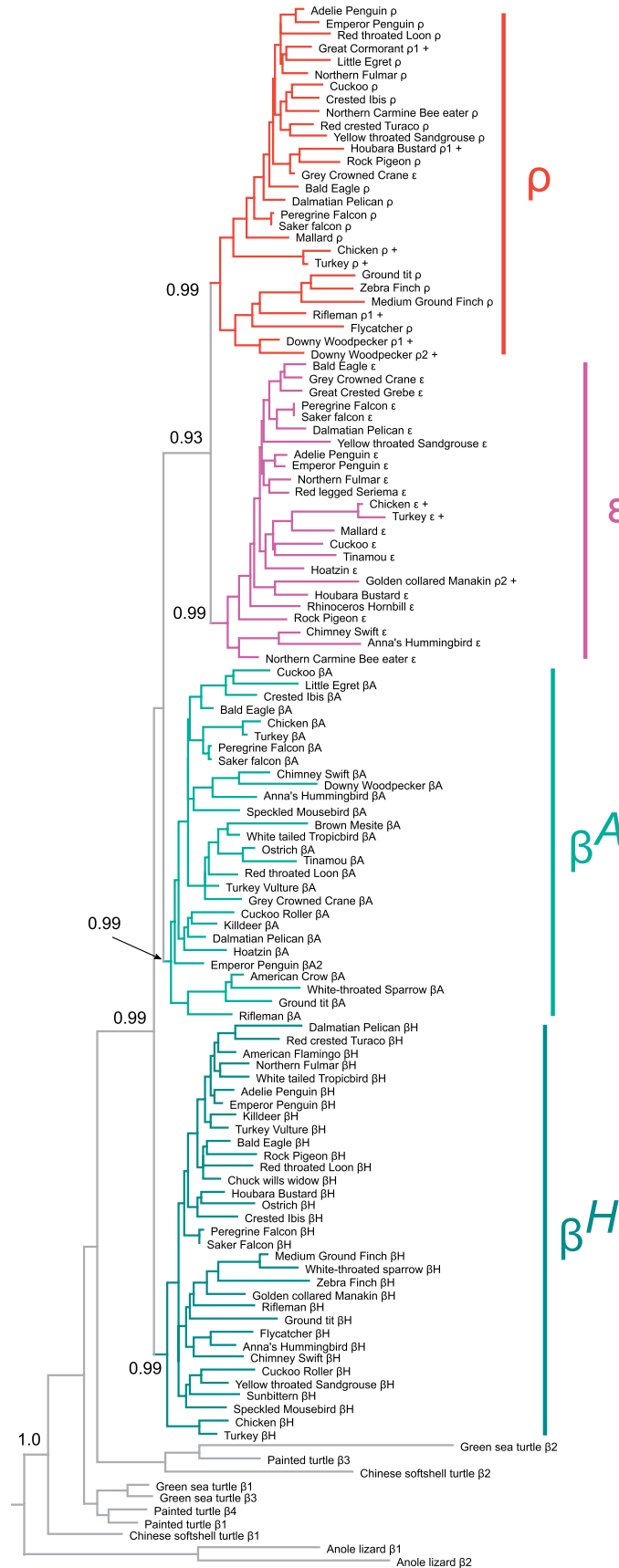


Fig. 4. Bayesian phylogeny depicting relationships among avian β -type globin genes based on nucleotide sequence data. Sequences of β -type globin genes from other tetrapod vertebrates (anole lizard [*Anolis carolinensis*], Chinese softshell turtle [*Pelodiscus sinensis*], green sea turtle [*Chelonia mydas*], and painted turtle [*Chrysemys picta*]) were used as outgroups. Bayesian posterior probabilities are shown for the ancestral nodes of each clade of orthologous avian genes, and for select nodes uniting clades of paralogous genes. Branch-tip labels with '+' symbols denote genes that have been partially overwritten by interparalog gene conversion (see text for details).

consistently less than 50%. Likelihood-based topology tests comparing the two best-supported arrangements ((β^H, β^A) (ρ, ϵ)) and $(\beta^H (\beta^A (\rho, \epsilon)))$ were not statistically significant (supplementary table S2, Supplementary Material online). Based on available data, we regard the relationship among β^H , β^A , and the two embryonic β -type globins (ρ, ϵ) as an unresolved trichotomy.

Gene Turnover in the Avian α - and β -Globin Gene Families

The comparative genomic analysis revealed that most variation in the size of the avian globin gene families is attributable to deletions or inactivations of individual genes from the ancestral repertoire of α - and β -type globins. We documented lineage-specific duplications of the β^A -globin gene in several species, including downy woodpecker, crested ibis (*Nipponia nippon*), and emperor penguin (*Aptenodytes forsteri*). In both downy woodpecker and crested ibis, the 3'- β^A gene copies are clearly pseudogenes (fig. 2). We also identified putatively pseudogenized copies of duplicated ρ - and ϵ -globin genes in several species (fig. 2).

We found no evidence for lineage-specific gene gains via tandem duplication in the α -globin gene family, although analyses of Hb isoform diversity at the protein level suggest that duplications of postnatally expressed α -type globin genes have occurred in at least two species of Old World vultures: Griffon vulture, *Gyps fulvus* (Grispo et al. 2012) and Rüppell's griffon, *G. rueppellii* (Hiebl et al. 1988). Most variation in gene content in the avian β -globin gene family is attributable to occasional lineage-specific deletions or inactivations of the β^H , ρ -, and ϵ -globin genes (fig. 2).

In the avian α -globin gene family, most interspecific variation in gene content is attributable to independent deletions or inactivations of the α^D -globin gene. In the sample of 24 species for which we have contigs containing complete α -globin gene clusters, parsimony suggests that there were multiple independent inactivations of α^D -globin (fig. 2). Specifically, the α^D -globin gene appears to have been deleted or otherwise pseudogenized in golden-collared manakin (*Manacus vitellinus*), little egret (*Egretta gazetta*), Adelle penguin (*Pygoscelis adeliae*), emperor penguin (*A. forsteri*), killdeer (*Charadrius vociferus*), and hoatzin (*Opisthocomus hoazin*). Partial deletions of the α^D -globin gene in both penguin species were likely inherited from a common ancestor, consistent with evidence that penguins do not express HbD during postnatal life (fig. 5; supplementary table S3, Supplementary Material online). The α^D -globin appears to have been deleted in killdeer and pseudogenized in hoatzin. Killdeer (representing Charadriiformes + Gruiformes) and hoatzin (Opisthocomiformes) are sister taxa in our tree, which is based on the total-evidence phylogenomic time-tree of Jarvis et al. (2014). Although this suggests that inactivation of α^D -globin occurred in the common ancestor of killdeer and hoatzin, these taxa diverged during a rapid post-KPg radiation and their phylogenetic positions are still poorly resolved even with whole-genome data (Jarvis et al. 2014). Furthermore, the period during which killdeer and

hoatzin shared an exclusive common ancestor would have been exceedingly brief, supporting the hypothesis of independent inactivation in each lineage. In light of this uncertainty, parsimony suggests either four or five independent losses of the α^D -globin gene in the set of species included in our comparative analysis.

The repeated inactivations of α^D -globin in multiple avian lineages are reflective of a broader macroevolutionary trend among tetrapods. Genomic evidence indicates that the α^D -globin gene has been independently inactivated or deleted in amphibians and mammals (Cooper et al. 2006; Fuchs et al. 2006; Hoffmann and Storz 2007; Hoffmann et al. 2008b; Hoffmann, Storz, et al. 2010; Hoffmann et al. 2011). Some mammalian lineages have retained an intact, transcriptionally active copy of α^D -globin (Goh et al. 2005), but there is no evidence that the product of this seemingly defunct gene is assembled into functionally intact Hbs at any stage of development. The HbD isoform is not expressed in the definitive erythrocytes of crocodylians (Weber and White 1986, 1994; Grigg et al. 1993; Weber et al. 2013), suggesting that the α^D -globin gene has been inactivated or deleted in the extant sister group of birds. Alternatively, the crocodylian α^D ortholog (if present) may only be expressed during embryonic development, in which case the HbD isoform may have simply gone undetected in studies of red blood cells from adult animals. The fact that the α^D -globin gene has been deleted or inactivated multiple times in birds may simply reflect the fact that it encodes the α -type subunit of a minor Hb isoform; it may therefore be subject to less stringent functional constraints than the paralogous α^E - and α^A -globin genes that encode subunits of major (less dispensable) Hb isoforms that are expressed during embryonic and postnatal development, respectively.

Evolutionary Changes in HbA/HbD Isoform Expression

The assembly of $\alpha_2\beta_2$ Hb tetramers is governed by electrostatic interactions between α - and β -chain monomers and between $\alpha\beta$ dimers (Bunn and McDonald 1983; Mrabet et al. 1986; Bunn 1987). For this reason, and also because of possible interparalog variation in mRNA stability, the relative transcript abundance of coexpressed α - and β -type globin genes in hematopoietic cells may not accurately predict the proportional representation of the encoded subunit isoforms in functionally intact, tetrameric Hbs. We therefore directly measured the relative abundance of HbA and HbD isoforms in red cell lysates rather than relying on measures of α^A/α^D transcript abundance as proxy measures of protein abundance.

Our survey of HbA/HbD expression levels in 122 avian species revealed high variability among lineages (supplementary table S3, Supplementary Material online). HbD accounted for approximately 20–40% of total Hb in the majority of species, but this isoform was altogether absent in all or most representatives of five major avian clades: Aequornithia (core waterbirds; represented by one species of stork, two herons, two pelicans, one cormorant, and two penguins), Columbiformes (represented by six species of

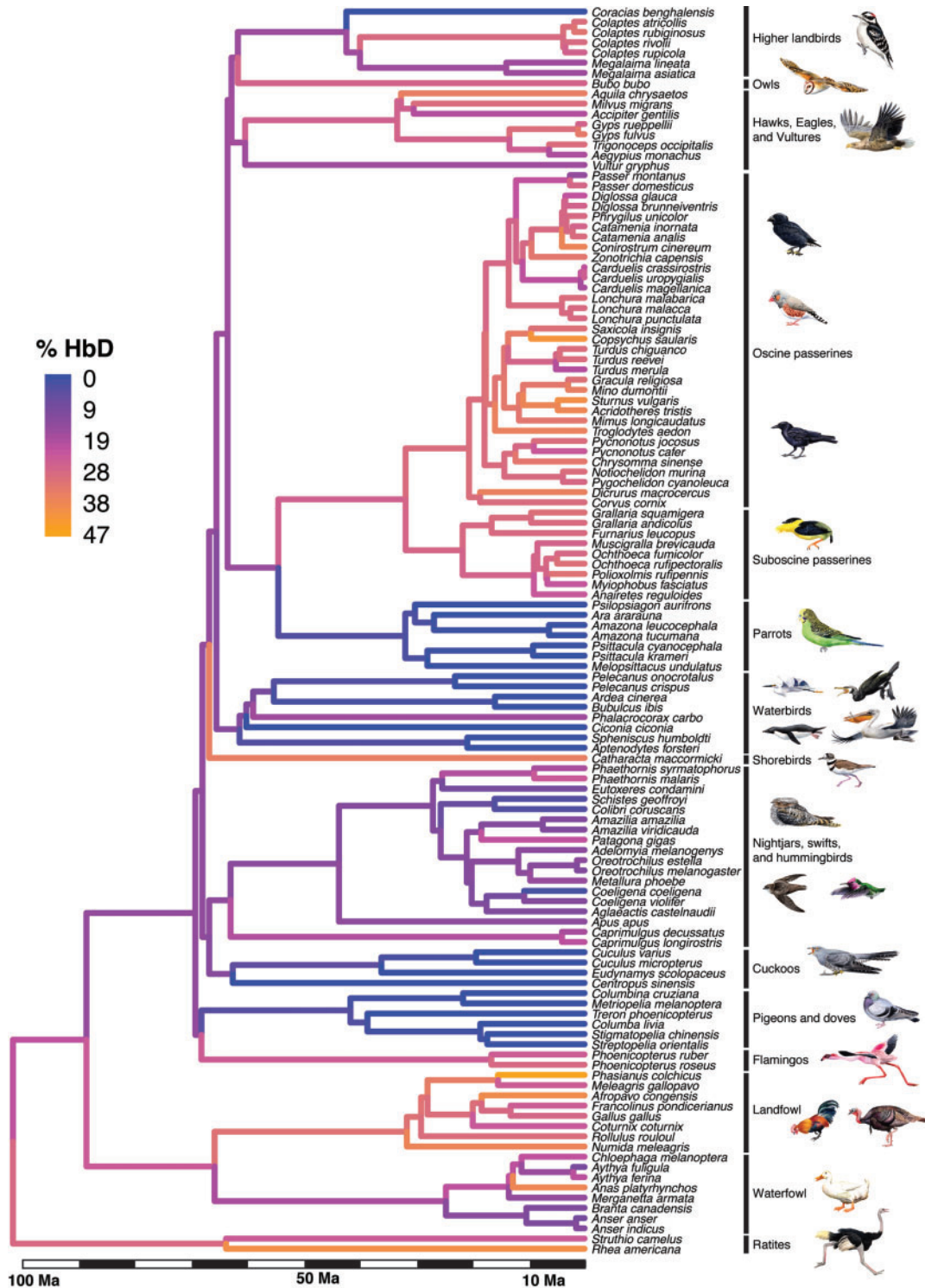


FIG. 5. Inferred evolutionary changes in relative expression level of the HbD isoform (% of total Hb) during the diversification of birds. Expression data are based on experimental measures of protein abundance in the definitive red blood cells of 122 bird species ($n = 1-30$ individuals per species, 267 specimens in total). Terminal branches are color-coded according to the measured HbD expression level of each species, and internal branches are color-coded according to maximum-likelihood estimates of ancestral character states. Branch lengths are proportional to time. See Materials and Methods for details regarding the construction of the time-calibrated supertree.

pigeons and doves), Coraciiformes (represented by one species of roller), Cuculiformes (represented by three species of cuckoo and one coucal), and Psittaciformes (represented by seven species of parrot from the families Psittacidae and Psittaculidae). On average, HbD expression was also

quite low in swifts and hummingbirds (Apodiformes), not exceeding 25% of total Hb in any species. HbD was expressed at very low levels in several species of hummingbirds (mean \pm 1 SD): $3.4\% \pm 1.2$ in the wedge-billed hummingbird, *Schistes geoffroyi* ($n = 7$ individuals), $3.2\% \pm 0.7$ in the

sparkling violetear, *Colibri coruscans* ($n = 7$), and $1.6\% \pm 2.0$ in the bronzy Inca, *Coeligena coeligena* ($n = 7$; supplementary table S3, Supplementary Material online). In contrast, HbD seldom accounted for less than 25% of total Hb in 42 examined species of passerines (supplementary table S3, Supplementary Material online).

The relative expression level of HbD exhibited strong phylogenetic inertia (fig. 5). Pagel's lambda (Pagel 1999), which varies from zero (no phylogenetic inertia) to 1.0 (strong phylogenetic inertia) was 0.981 for the percentage of HbD across the avian phylogeny. Blomberg's K was 0.861 (significantly greater than zero, $P < 0.001$), indicating that there was less trait similarity among species than expected for a trait evolving under perfect Brownian motion ($K = 1$). These results indicate that measured HbD expression levels exhibit a statistically significant pattern of phylogenetic conservatism, although the deviation from expectations under Brownian motion could be partly attributable to measurement error or sampling error (Blomberg et al. 2003; Garland et al. 2005).

Body mass exhibited even stronger phylogenetic inertia than HbD expression in the same set of taxa (Pagel's lambda = 0.999; Blomberg's $K = 3.80$). Regression analysis using a phylogenetic generalized least-squares model revealed no evidence for an association between body mass and HbD expression ($R^2 = 0.01$, $P = 0.62$). Thus, if blood- O_2 affinity scales inversely with mass-specific metabolic rate in birds, as suggested by Lutz et al. (1974), results of our analysis suggest that the scaling relationship is not attributable to evolutionary changes in HbA/HbD expression levels. Instead, any such relationship must be attributable to evolutionary changes in the inherent oxygenation properties of Hb isoforms and/or changes in intraerythrocytic concentrations of cellular cofactors that allosterically regulate Hb- O_2 affinity.

Variation in Functional Constraint among Paralogs

To infer relative levels of functional constraint among the different globin paralogs, we used a codon-based maximum-likelihood approach to measure variation in the ratio of nonsynonymous-to-synonymous substitution rates ($\omega = d_N/d_S$). For the α -type genes, the model that provided the best fit to the data permitted four independent ω -values: one for each avian paralog and one for all avian outgroup branches (table 1). If rates of gene retention and relative expression levels are positively correlated with the degree of functional constraint, then the clear prediction is that the α^D -globin clade will be characterized by a higher ω -value than the α^A -globin clade. Consistent with expectations, the estimated ω -value for the α^D -globin gene was appreciably higher (0.281 vs. 0.237, respectively; table 1). The exclusively embryonic α^E -globin gene was characterized by the lowest ω -value (0.127; table 1). In the case of the β -type globin genes, the model that provided the best fit to the data permitted three independent ω -values for 1) β^H and β^A , 2) ρ and ϵ , and 3) all nonavian branches (table 2). Similar to the pattern observed for the α -type genes, the embryonic ρ - and ϵ -globin genes were characterized by lower ω -values relative to β^H - and β^A -globin (0.084 vs. 0.100; table 2). Observed patterns for both the α - and β -type globins are

consistent with the idea that genes expressed early in development are generally subject to more stringent functional constraints relative to later-expressed members of the same gene family (Goodman 1963).

Tests of Positive Selection

Likelihood ratio tests (LRTs) revealed significant variation in ω -values among sites in the alignments of each of the avian α -type paralogs (table 1), and results of Bayes empirical Bayes (BEB) analyses suggested evidence for a history of positive selection at several sites, as indicated by site-specific ω -values greater than 1.0 (table 1). Statistical evidence for positive selection at α^A64 and α^A115 is especially intriguing in light of evidence from site-directed mutagenesis experiments that charge-changing mutations at both sites produce significant changes in the O_2 affinity of mouse Hb (Natarajan et al. 2013). LRTs also revealed significant variation in ω -values among sites in the alignment of avian β^A -globin sequences, and results of the BEB analysis suggested evidence for a history of positive selection at two sites, $\beta4$ and $\beta55$ (table 2). Statistical evidence for positive selection at both sites is intriguing in light of evidence from protein-engineering experiments. Site-directed mutagenesis experiments involving mammalian Hbs have documented affinity-altering effects of substitutions at $\beta4$ and the adjacent $\beta5$ residue position that perturb the secondary structure of the β -chain A-helix (Fronticelli et al. 1995; Tufts et al. 2015). Likewise, site-directed mutagenesis experiments involving recombinant human Hb have documented affinity-enhancing effects of substitutions at $\beta55$ that eliminate intradimer ($\alpha1\beta1$) atomic contacts (Jessen et al. 1991; Weber et al. 1993). Protein-engineering experiments involving recombinant avian Hbs are needed to probe the functional effects of amino acid substitutions at each of these candidate sites for positive selection.

Physiological Implications

Due to the fact that the HbD isoform (which incorporates products of α^D -globin) has a consistently higher O_2 -affinity than HbA (which incorporates products of α^A -globin; [Crispo et al. 2012]), repeated inactivations of the α^D -globin gene can be expected to contribute to among-species variation in blood- O_2 affinity. To evaluate the phenotypic consequences of α^D -globin inactivation in definitive erythrocytes, we compiled standardized data on the oxygenation properties of purified HbA and HbD isoforms from 11 bird species (Crispo et al. 2012; Projecto-Garcia et al. 2013; Cheviron et al. 2014; table 3). In all 11 species, HbD exhibited higher O_2 -affinity in the presence of allosteric modulators of Hb- O_2 affinity: Cl^- ions (added as 0.1 M KCl) and inositol hexaphosphate (IHP [a chemical analog of inositol pentaphosphate that is endogenously produced in avian red blood cells], present at saturating or near-saturating concentrations). We compiled in vitro measurements of O_2 -affinity on purified HbA and HbD isoforms under the same "KCl+IHP" treatment, and we calculated the expected O_2 -affinity of the composite mixture of HbA and HbD using a weighted average of

Table 1. Maximum-Likelihood Analysis of the Ratio of Nonsynonymous-to-Synonymous Substitution Rates, ω ($=d_N/d_S$), in the α -Type Globin Genes of Birds.

Models	ln L	No. Parameters	Parameter Estimates	Candidate Sites for Positive Selection	Test
Site models—α^E-globin					
M0	-3887.294	98	$\omega_0 = 0.154$	NA	
M1a	-3759.181	99	$\omega_0 = 0.047$ ($p_0 = 0.84$) $\omega_1 = 1.000$ ($p_1 = 0.16$)	NA	
M2	-3758.104	101	$\omega_0 = 0.047$ ($p_0 = 0.84$) $\omega_1 = 1.000$ ($p_1 = 0.15$) $\omega_2 = 2.107$ ($p_2 = 0.01$)	53 A ($\omega = 1.901$, $P = 0.894$)	M2 vs. M1a Not Significant
M3	-3737.840	102	$\omega_0 = 0.016$ ($p_0 = 0.71$) $\omega_1 = 0.325$ ($p_1 = 0.24$) $\omega_2 = 1.387$ ($p_2 = 0.05$)		M3 vs. M0 $P << 10^{-3}$
Site models—α^D-globin					
M0	-5666.162	96	$\omega_0 = 0.236$	NA	
M1a	-5377.678	97	$\omega_0 = 0.080$ ($p_0 = 0.66$) $\omega_1 = 1.000$ ($p_1 = 0.34$)	NA	
M2	-5358.625	99	$\omega_0 = 0.083$ ($p_0 = 0.64$) $\omega_1 = 1.000$ ($p_1 = 0.30$) $\omega_2 = 3.253$ ($p_2 = 0.06$)	12 P ($\omega = 2.393$, $P = 0.576$) 17 G ($\omega = 2.987$, $P = 0.788$) 23 L ($\omega = 3.236$, $P = 0.880$) 29 D ($\omega = 3.585$, $P = 1.000^{**}$) 48 H ($\omega = 3.580$, $P = 0.998^{**}$) 49 P ($\omega = 3.569$, $P = 0.994^{**}$) 63 A ($\omega = 2.581$, $P = 0.639$)	M2 vs. M1a $P << 10^{-3}$
M3	-5320.501	100	$\omega_0 = 0.016$ ($p_0 = 0.44$) $\omega_1 = 0.292$ ($p_1 = 0.40$) $\omega_2 = 1.306$ ($p_2 = 0.16$)		M3 vs. M0 $P << 10^{-3}$
Site models—α^A-globin					
M0	-5166.185	112	$\omega_0 = 0.214$	NA	
M1a	-4867.244	113	$\omega_0 = 0.050$ ($p_0 = 0.74$) $\omega_1 = 1.000$ ($p_1 = 0.26$)	NA	
M2	-4837.546	115	$\omega_0 = 0.049$ ($p_0 = 0.73$) $\omega_1 = 1.000$ ($p_1 = 0.24$) $\omega_2 = 4.862$ ($p_2 = 0.03$)	5 N ($\omega = 4.457$, $P = 0.872$) 49 H ($\omega = 5.271$, $P = 0.788$) 64 A ($\omega = 4.911$, $P = 0.880$) 115 S ($\omega = 5.263$, $P = 1.000^{**}$)	M2 vs. M1a $P << 10^{-3}$
M3	-4830.789	116	$\omega_0 = 0.021$ ($p_0 = 0.65$) $\omega_1 = 0.374$ ($p_1 = 0.21$) $\omega_2 = 1.216$ ($p_2 = 0.14$)		M3 vs. M0 $P << 10^{-3}$
Branch models					
1 ω	-19867.046	341	$\omega_{\text{all branches}} = 0.200$	NA	
2 ω	-19861.435	342	$\omega_{\alpha\text{-type genes}} = 0.214$ $\omega_{\text{other branches}} = 0.157$	NA	1 ω vs. 2 ω $P << 10^{-3}$
4 ω	-19831.984	344	$\omega_{\alpha^E \text{ total group}} = 0.127$ $\omega_{\alpha^D \text{ total group}} = 0.281$ $\omega_{\alpha^A \text{ total group}} = 0.237$ $\omega_{\text{other branches}} = 0.158$	NA	2 ω vs. 4 ω $P << 10^{-3}$
7 ω	-19831.607	347	$\omega_{\alpha^E \text{ stem}} = 0.097$ $\omega_{\alpha^E \text{ crown}} = 0.128$ $\omega_{\alpha^D \text{ stem}} = 0.483$ $\omega_{\alpha^D \text{ crown}} = 0.280$ $\omega_{\alpha^A \text{ stem}} = 0.152$ $\omega_{\alpha^A \text{ crown}} = 0.239$ $\omega_{\text{other branches}} = 0.159$	NA	4 ω vs. 7 ω Not Significant

isoform-specific P_{50} values (the partial pressure of O_2 at which heme is 50% saturated) based on the naturally occurring relative concentrations of the two Hb isoforms in each species. The assumption that the avian HbA and HbD isoforms have additive effects on blood- O_2 affinity is validated by experimental studies on the oxygenation properties of isolated isoforms and composite hemolysates (Weber et al. 2004; Grispo

et al. 2012). We calculated the expected increase in blood P_{50} (i.e., reduction in blood- O_2 affinity) caused by the complete loss of HbD expression by comparing the predicted P_{50} of the HbA+HbD composite mixture and the measured P_{50} of HbA in isolation. Although the P_{50} of a composite "HbA+HbD" hemolysate is not expected to be identical to the P_{50} of whole blood (Berenbrink 2006), the loss of HbD

Table 2. Maximum-Likelihood Analysis of the Ratio of Nonsynonymous-to-Synonymous Substitution Rates, ω ($=d_N/d_S$), in the β -Type Globin Genes of Birds.

Models	ln L	No. Parameters	Parameter Estimates	Candidate Sites for Positive Selection	Test
Site models					
M0	-13679.145	313	$\omega_0 = 0.102$	NA	
M1a	-13223.916	314	$\omega_0 = 0.070$ ($p_0 = 0.905$) $\omega_1 = 1.000$ ($p_1 = 0.095$)	NA	
M2	-13221.227	316	$\omega_0 = 0.071$ ($p_0 = 0.905$) $\omega_1 = 1.000$ ($p_1 = 0.079$) $\omega_2 = 1.69$ ($p_2 = 0.016$)	4 S ($\omega = 1.89$, $P = 0.927$) 43 A ($\omega = 1.28$, $P = 0.553$) 55 I ($\omega = 1.35$, $P = 0.675$)	M2 vs. M1 Not significant
M3	-13018.598	317	$\omega_0 = 0.022$ ($p_0 = 0.608$) $\omega_1 = 0.153$ ($p_1 = 0.304$) $\omega_2 = 0.697$ ($p_2 = 0.088$)		M3 vs. M0 $P < < 10^{-3}$
Branch models					
1 ω	-13679.145	313	$\omega_0 = 0.103$	NA	
3 ω	-13659.727	316	$\omega_0 = 0.192$ $\omega_{\rho} = \omega_{\epsilon} = 0.084$ $\omega_{\beta^H} = \omega_{\beta^A} = 0.100$	NA	1 ω vs. 3 ω $P < < 0.001$
5 ω	-13659.190	318	$\omega_0 = 0.192$ $\omega_{\rho} = 0.082$ $\omega_{\epsilon} = 0.086$ $\omega_{\beta^H} = 0.106$ $\omega_{\beta^A} = 0.094$		3 ω vs. 5 ω Not significant
9 ω	-13653.580	322	$\omega_0 = 0.188$ $\omega_{\rho} \text{ stem} = 0.244$ $\omega_{\epsilon} \text{ stem} = 999$ $\omega_{\beta^H} \text{ stem} = 0.575$ $\omega_{\beta^A} \text{ stem} = 361$ $\omega_{\rho} \text{ crown} = 0.080$ $\omega_{\epsilon} \text{ crown} = 0.083$ $\omega_{\beta^H} \text{ crown} = 0.093$ $\omega_{\beta^A} \text{ crown} = 0.105$		5 ω vs. 9 ω $P < 0.05$ 3 ω vs 9 ω Not significant
Site models—β^A-globin					
M0	-4248.522	123	$\omega_0 = 0.979$		
M1	-4117.445	124	$\omega_0 = 0.053$ ($p_0 = 0.877$) $\omega_1 = 1.000$ ($p_1 = 0.123$)		
M2	-4113.220	126	$\omega_0 = 0.055$ ($p_0 = 0.876$) $\omega_1 = 1.000$ ($p_1 = 0.109$) $\omega_2 = 3.149$ ($p_2 = 0.014$)	4 T ($\omega = 3.414$, $P = 0.954$) 55 L ($\omega = 3.251$, $P = 0.952$)	M2 vs. M1 $P < 0.05$
M3	-4072.267	127	$\omega_0 = 0.022$ ($p_0 = 0.752$) $\omega_1 = 0.316$ ($p_1 = 0.226$) $\omega_2 = 2.080$ ($p_2 = 0.021$)		

expression should produce a commensurate increase in P_{50} in both cases.

In hummingbirds, which typically have very low HbD expression (table 2 and fig. 5, supplementary table S3, Supplementary Material online), loss of HbD is predicted to produce a modest increase in blood P_{50} , ranging from +1.3% for the violet-throated starfrontlet, *Coeligena violifer*, to +8.6% for the giant hummingbird, *Patagona gigas*. In contrast, in passerines (which typically have much higher HbD expression levels; table 2 and fig. 5, supplementary table S3, Supplementary Material online), the predicted increase in blood P_{50} was +15.4% for house wren, *Troglodytes aedon*, and +18.0% for rufous-collared sparrow, *Zonotrichia capensis*. The calculations for passerines provide an indication of the magnitude of change in blood- O_2 affinity that must have accompanied the quantitative reduction or whole-sale loss of HbD expression in other avian lineages (fig. 5). These results

illustrate the potentially dramatic physiological consequences of inactivating the α^D -globin gene. Because HbD is also expressed at high levels during prenatal development (Ciroto et al. 1987; Alev et al. 2008, 2009), inactivation or deletion of α^D -globin could affect O_2 -delivery to the developing embryo. Intriguingly, representatives of several taxa (families Cuculidae [cuckoos] and Columbidae [pigeons and doves] and the superfamily Psittacoidea [parrots]) do not appear to express HbD during postnatal life (fig. 5, supplementary table S3, Supplementary Material online), but they have retained α^D -globin genes with intact reading frames. In such taxa, HbD may still be expressed during embryonic development, as documented in domestic pigeon (Ikehara et al. 1997).

Conclusions

Our results indicate that recurrent deletions and inactivations of the α^D -globin gene entailed significant reductions in blood-

Table 3. O₂ Affinities (P_{50} , torr) and Cooperativity Coefficients (n_{50}) of Purified HbA and HbD Isoforms from 11 Bird Species.

Species	Hb Isoform	Abundance (%)	$P_{50(KCl+IHP)}$ (torr)	n_{50}
Accipitriformes				
Griffon vulture, <i>Gyps fulvus</i>	HbA	66	28.84	1.98
	HbD	34	26.61	1.99
	HbA+HbD		28.08	1.98
Anseriformes				
Graylag goose, <i>Anser anser</i>	HbA	92	43.95 ^a	3.00 ^a
	HbD	8	29.79 ^a	2.51 ^a
	HbA+HbD		42.68	2.96
Apodiformes				
Amazilia hummingbird, <i>Amazilia amazilia</i>	HbA	88	29.84	2.42
	HbD	12	23.20	2.40
	HbA+HbD		29.04	2.42
Green-and-white hummingbird, <i>Amazilia viridicauda</i>	HbA	89	24.24	2.07
	HbD	11	20.36	2.29
	HbA+HbD		23.81	2.09
Violet-throated starfrontlet, <i>Coeligena violifer</i>	HbA	88	19.12	1.70
	HbD	12	17.01	2.46
	HbA+HbD		18.87	1.79
Giant hummingbird, <i>Patagona gigas</i>	HbA	78	25.86	2.49
	HbD	22	16.56	2.56
	HbA+HbD		23.81	2.51
Great-billed hermit, <i>Phaethornis malaris</i>	HbA	76	28.13	2.04
	HbD	24	24.92	2.72
	HbA+HbD		27.36	2.20
Galliformes				
Ring-necked pheasant, <i>Phasianus colchicus</i>	HbA	54	29.51	2.55
	HbD	46	24.24 ^a	2.46 ^a
	HbA+HbD		27.09	2.51
Passeriformes				
House wren, <i>Troglodytes aedon</i>	HbA	64	25.87	2.11
	HbD	36	16.28	2.36
	HbA+HbD		22.42	2.20
Rufous-collared sparrow, <i>Zonotrichia capensis</i>	HbA	67	38.13	2.29
	HbD	33	20.49	2.40
	HbA+HbD		32.31	2.32
Struthioformes				
Ostrich, <i>Struthio camelus</i>	HbA	79	32.73 ^a	2.85
	HbD	21	22.90 ^a	2.44
	HbA+HbD		30.67	2.76

NOTE.—O₂ equilibria were measured in 0.1 mM HEPES buffer at pH 7.4 (± 0.01) and 37 °C in the presence of allosteric effectors ([Cl⁻], 0.1 M; [HEPES], 0.1 M; IHP/Hb tetramer ratio, 2.0 or >20, as indicated; [heme], 0.30–1.00 mM (for details of experimental conditions, see Grispo et al. [37], Projecto-Garcia et al. [80], and Cheviron et al. [81]). P_{50} and n_{50} values were derived from single O₂ equilibrium curves, where each value was interpolated from linear Hill plots (correlation coefficient $r > 0.995$) based on four or more equilibrium steps between 25% and 75% saturation. The reported P_{50} for the HbA+HbD composite hemolysate is the weighted mean P_{50} of both isoforms in their naturally occurring relative concentrations.

^aSaturating IHP/Hb4 ratio (>20).

O₂ affinity in numerous avian lineages—changes that may have been compensated by functional changes in the remaining pre- or postnatally expressed globins. These results provide a concrete example of evolutionary changes in a physiologically important phenotype that were caused by recurrent gene losses.

In mammals, evolutionary changes in globin gene content have given rise to variation in the functional diversity of Hb isoforms that are expressed at different stages of prenatal development (Opazo et al. 2008a, 2008b). For example, duplicate copies of different β -type globin genes have been independently co-opted for fetal expression in anthropoid primates and artiodactyls (Storz, Opazo, et al. 2011; Storz et al. 2013), which demonstrates

how gene duplication may provide increased scope for evolutionary innovation because redundant gene copies are able to take on new functions or divide up ancestral functions. In birds, the relative stasis in globin gene family evolution suggests that the developmental regulation of Hb synthesis may be more highly conserved, with orthologous genes having similar stage-specific expression profiles and carrying out similar functions in disparate taxa. In comparison with the globin gene clusters of mammals and squamate reptiles, the general absence of redundant gene duplicates in the avian gene clusters suggests less opportunity for the divergence of paralogs to facilitate the acquisition of novel protein functions and/or expression patterns.

Materials and Methods

Analysis of Globin Gene Family Evolution

The 52 avian genome assemblies that we analyzed were derived from multiple sources (Hillier et al. 2004; Dalloul et al. 2010; Huang et al. 2013; Zhan et al. 2013; Jarvis et al. 2014; Zhang et al. 2014). Details regarding all examined genome assemblies and scaffolds are provided in [supplementary table S1, Supplementary Material](#) online. We identified contigs containing α - and β -globin genes in the genome assembly of each species by using BLAST (Altschul et al. 1990) to make comparisons with the α - and β -globin genes of chicken. We then annotated globin genes within the identified gene clusters of each species by using GENSCAN (Burge and Karlin 1997) in combination with BLAST2 (Tatusova and Madden 1999) to make comparisons with known exon sequences, following Hoffmann et al. (Hoffmann, Storz, et al. 2010; Hoffmann et al. 2011). Due to incomplete sequence coverage in the genome assemblies of several species, there were some cases where it was not possible to ascertain the full extent of conserved synteny.

To estimate rates of gene turnover in the globin gene clusters of birds and mammals, we used a stochastic birth–death model of gene family evolution (Hahn et al. 2005), as implemented in the program CAFE v3.1 (Han et al. 2013). As input for the program we used ultrametric trees based on well-resolved phylogenies of birds (Jarvis et al. 2014) and eutherian mammals (Meredith et al. 2011). The analysis was based on data for 24 bird species for which we had complete sequence coverage of the α - and β -globin gene clusters. For comparison, we used genome sequence assemblies for 22 mammal species representing each of the major eutherian lineages, as reported in Zhang et al. (2014). These sets of birds and eutherian mammals span a similar range of divergence times, and therefore provide a good basis for comparing lineage-specific rates of gene family evolution.

Sample Collection

We preserved blood and tissue samples from 250 voucher specimens representing 68 bird species that were collected from numerous localities in South America ([supplementary table S4, Supplementary Material](#) online). In addition to the 250 wild-caught, museum-vouchered specimens, we also obtained blood samples from captive individuals representing 17 species. Our proteomic analysis of Hb isoform composition was based on blood samples from a total of 267 specimens (see below).

All wild-caught birds were captured in mist-nets and were then bled and euthanized in accordance with guidelines of the Ornithological Council (Fair and Paul 2010), and protocols approved by the University of New Mexico IACUC (Protocol number 08UNM033-TR-100117; Animal Welfare Assurance number A4023-01). Acquisition and study of Peruvian samples was carried out under permits issued by the management authorities of Peru (76-2006-INRENA-IFFS-DCB, 087-2007-INRENA-IFFS-DCB, 135-2009-AG-DGFFS-DGEFFS, 0199-

2012-AG-DGFFS-DGEFFS, and 006-2013-MINAGRI-DGFFS/DGEFFS). For each individual bird, we collected whole blood from the brachial or ulnar vein using heparinized microcapillary tubes. Red blood cells were separated from the plasma fraction by centrifugation, and the packed red cells were then snap-frozen in liquid nitrogen and were stored at -80°C . We collected liver and pectoral muscle from each specimen as sources of genomic DNA and globin mRNA, respectively. Muscle samples were snap-frozen or preserved using RNAlater and were subsequently stored at -80°C prior to RNA isolation. Voucher specimens were preserved along with ancillary data and were deposited in the collections of the Museum of Southwestern Biology of the University of New Mexico and the Centro de Ornitología y Biodiversidad (CORBIDI), Lima, Peru. Complete specimen data are available via the ARCTOS online database ([supplementary table S4, Supplementary Material](#) online).

Molecular Cloning and Sequencing

To augment the set of globin sequences derived from the genome assemblies and other public databases, we cloned and sequenced the adult globin genes (α^A -, α^D -, and β^A -globin) from 34 additional bird species. We used the RNeasy Mini Kit (Qiagen, Valencia, CA) to isolate total RNA from blood or pectoral muscle. Using a few representative species from each order, we used 5'- and 3'-RACE (rapid amplification of cDNA ends [Invitrogen Life Technologies, Carlsbad, CA]) to obtain cDNA sequence for the 5'- and 3'-untranslated regions (UTRs) of each adult-expressed globin gene. We then aligned the 5'- and 3'-UTRs of each sequenced gene and designed paralog-specific primers. This strategy enabled us to obtain complete cDNA sequences for each globin paralog using the OneStep RT-PCR kit (Qiagen, Valencia, CA). We cloned gel-purified RT-PCR products into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen Life Technologies, Grand Island, NY), and we then sequenced at least 4–6 colonies per individual. All sequences were deposited in GenBank under the accession numbers KM522867–KM522916.

Phylogenetic Analysis and Assignment of Orthologous Relationships

We used phylogenetic reconstructions to resolve orthology for all annotated globin genes, including truncated sequences. The α - and β -type globin sequences were aligned separately and, when possible, amino acid sequence alignments were based on conceptual translations of nucleotide sequence. All alignments were performed using the L-INS-i strategy from MAFFT v. 6.8 (Katoh and Toh 2008), and phylogenies were estimated using both maximum-likelihood and Bayesian approaches. We used Treefinder (v. March 2011; [Jobb et al. 2004]) for the maximum-likelihood analyses, and MrBayes (Ronquist and Huelsenbeck 2003) for Bayesian estimates of each phylogeny. In the maximum-likelihood analyses, we used the “propose model” routine of Treefinder to select the best-fitting models of nucleotide substitution, with an

independent model for each codon position in analyses based on nucleotide sequences. Model selection was based on the Akaike information criterion with correction for small sample size. In the case of MrBayes, we ran six simultaneous chains for 2×10^7 generations, sampling every 2.5×10^3 generations, using default priors. A given run was considered to have reached convergence once the likelihood scores reached an asymptotic value and the average standard deviation of split frequencies remained less than 0.01. We discarded all trees that were sampled prior to convergence, and we evaluated support for the nodes and parameter estimates from a majority rule consensus of the last 2,500 trees.

For the β -type globin genes, we analyzed four separate sets of sequences: 1) an inclusive data set that included all genes, apparent pseudogenes, and truncated sequences (containing at least one full-length exon) that were derived from the genome assemblies, 2) a data set restricted to genes with full-length coding sequences and intact reading frames, and 3) the set of full-length coding sequences plus 34 β^A -globin cDNA sequences from avian taxa that do not currently have fully sequenced genomes. We used each of these data sets to perform likelihood-based topology tests (Shimodaira 2002).

For the β -type globin genes, we inferred orthologous relationships by using an approach that combined the “orthology-by-content” and “orthology-by-context” criteria (Song et al. 2011, 2012). We applied the orthology-by-content criterion to assign genes to the β^H , β^A , or $\rho+\epsilon$ groups (based on phylogenetic relationships), and we then used the orthology-by-context criterion to assign genes in the latter group as ρ - or ϵ -globin (based on positional homology).

Molecular Evolution Analysis

To measure variation in functional constraint among the avian α - and β -type globin genes and to test for evidence of positive selection, we estimated ω ($=d_N/d_S$, the ratio of the rate of nonsynonymous substitution per nonsynonymous site [d_N] to the rate of synonymous substitution per synonymous site [d_S] site) using a maximum-likelihood approach (Goldman and Yang 1994) implemented in the CODEML program, v. 4.8 (Yang 2007). In all cases, we used LRTs to compare nested sets of models (Yang 1998). For each alignment, we first compared models that allow ω to vary among codons (M0 vs. M3, M1a vs. M2a). The latter LRT constitutes a test of positive selection, as the alternative model (M2a) allows a subset of sites to have $\omega > 1$, in contrast to the null model (M1a) that only includes site classes with $\omega < 1$. These analyses revealed statistically significant variation in ω among sites in the alignment of β^A -globin sequences (table 2), so we then used the BEB approach (Yang et al. 2005) to calculate the posterior probability that a given site belongs to the site class with $\omega > 1$, which suggests a possible history of positive selection.

Characterizing Hb Isoform Composition of Avian Red Blood Cells

We used isoelectric focusing (IEF; PhastSystem, GE Healthcare Bio-Sciences, Piscataway, NJ) to characterize Hb isoform composition in red cell lysates from each of the 267 bird

specimens representing 68 species ($n = 1\text{--}30$ individuals per species). After separating native Hbs using precast IEF gels (pH 3–9), red (heme-containing) bands were excised from the gel and digested with trypsin. The resultant peptides were then identified by means of tandem mass spectrometry (MS/MS). Database searches of the resultant MS/MS spectra were performed using Mascot (Matrix Science, v1.9.0, London, UK), whereby peptide mass fingerprints were used to query a custom database of avian α - and β -chain sequences. After separating the HbA and HbD isoforms by native gel IEF and identifying each of the constituent subunits by MS/MS, the relative abundance of the different isoforms in the hemolysates of each individual was quantified densitometrically using Image J (Abramoff et al. 2004).

Supertree Construction

We constructed a time-calibrated supertree of all study species by starting with a backbone provided by a dated, total-evidence phylogeny from Jarvis et al. (2014). This phylogeny was based on nucleotide sequence data derived from 48 whole avian genomes and 19 fossil calibrations. Using recent avian taxonomic classifications, we were able to unambiguously assign each study species to its appropriate branch in the Jarvis et al. (2014) backbone tree. Subtrees for each branch were obtained from the dated supertree of Jetz et al. (2012), which was constructed using the Hackett et al. (2008) backbone. Divergence times in trees from Jetz et al. tended to be older than the ones from Jarvis et al. (2014); we resolved topological or branch-length conflicts by favoring the Jarvis et al. (2014) tree because it was based on far more sequence data. Before grafting time-calibrated subtrees onto the backbone, we uniformly rescaled the subtree branch-lengths so that shared nodes were the same age as they were in the backbone (following Sibly et al. 2012).

Comparative Analysis of HbA/HbD Expression Levels

For the comparative analysis of HbA/HbD expression levels, we combined our experimental data for 68 species with published data for an additional 54 species (supplementary table S3, Supplementary Material online), yielding data for a phylogenetically diverse set of 122 species in total. We used the final supertree to conduct comparative analyses under a Brownian motion model of evolution in R v. 2.15.1. Maximum-likelihood estimates of ancestral states for percent expression of HbD were generated using APE (Paradis et al. 2004). Phylogenetic inertia in expression level (percent) of HbD was quantified using Blomberg’s K (Blomberg et al. 2003) and Pagel’s Λ (Pagel 1999), calculated using GEIGER (Harmon et al. 2008). We compiled body mass data for each species from Museum of Southwestern Biology specimens and Dunning (Dunning 2008), and we tested for an association between body mass and expression level of HbD using a phylogenetic generalized least-squares model as implemented in APE (Paradis et al. 2004). We log-transformed body mass and arcsin-squareroot-transformed HbD percent prior to all analyses.

Supplementary Material

Supplementary figure 1 and tables S1–S4 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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