

# The $\alpha^D$ -Globin Gene Originated via Duplication of an Embryonic $\alpha$ -Like Globin Gene in the Ancestor of Tetrapod Vertebrates

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Gene duplication is thought to play an important role in the co-option of existing protein functions to new physiological pathways. The globin superfamily of genes provides an excellent example of the kind of physiological versatility that can be attained through the functional and regulatory divergence of duplicated genes that encode different subunit polypeptides of the tetrameric hemoglobin protein. In contrast to prevailing views about the evolutionary history of the  $\alpha$ -globin gene family, here we present phylogenetic evidence that the  $\alpha^A$ - and  $\alpha^D$ -globin genes are not the product of a single, tandem duplication of an ancestral globin gene with adult function in the common ancestor of extant birds, reptiles, and mammals. Instead, our analysis reveals that the  $\alpha^D$ -globin gene of amniote vertebrates arose via duplication of an embryonic  $\alpha$ -like globin gene that predated the radiation of tetrapods. The important evolutionary implication is that the distinct biochemical properties of  $\alpha^D$ -hemoglobin (*HbD*) are not exclusively derived characters that can be attributed to a post-duplication process of neofunctionalization. Rather, many of the distinct biochemical properties of *HbD* are retained ancestral characters that reflect the fact that the  $\alpha^D$ -globin gene arose via duplication of a gene that had a larval/embryonic function. These insights into the evolutionary origin of *HbD* illustrate how adaptive modifications of physiological pathways may result from the retention and opportunistic co-option of ancestral protein functions.

## Introduction

Gene duplication can result in the acquisition of novel protein functions in cases where one duplicate copy retains the original function of the ancestral gene while the other copy accumulates new mutations that adapt the encoded protein to a new or modified physiological task (Ohno 1970; Kimura and Ohta 1974; Goodman et al. 1975; Ohta 1993, 1994; Hughes 1999). This functional specialization may entail changes in protein function as well as changes in the tissue specificity or developmental timing of gene expression. The globin superfamily of genes provides an excellent example of the kind of physiological versatility that can be attained through the functional and regulatory divergence of duplicated genes that encode different subunit polypeptides of the tetrameric hemoglobin protein (Goodman et al. 1975; Hardison 2001). In amniote vertebrates, hemoglobin has been optimized for oxygen transport under the vastly different physiological conditions encountered during the embryonic, fetal, and adult stages of development. For example, in eutherian mammals, regulatory switching between paralogous genes that encode functionally distinct subunit polypeptides can produce adult hemoglobins that are optimized for pulmonary/tissue oxygen transport, fetal hemoglobins that are optimized for placental/tissue oxygen transport, and embryonic hemoglobins that are optimized for diffusional oxygen scavenging from the amniotic fluid as well as oxygen transport during the transition from a vitelline to placental circulation (Hardison 1998, 2001; Nagel and Steinberg 2001; Brittain 2002).

In gnathostome vertebrates, the hemoglobin protein is a heterotetramer composed of two  $\alpha$ -chain and two  $\beta$ -chain subunits. In amniote vertebrates, each of the different subunit polypeptides is encoded by different sets of duplicated genes that are located on different chromosomes. In amphibians and ray-finned fishes, the  $\alpha$ - and  $\beta$ -like globin

genes are closely linked on the same chromosome, reflecting the fact that the progenitors of the  $\alpha$ - and  $\beta$ -globin gene families arose via tandem duplication of an ancestral globin gene approximately 450–500 mya, prior to the radiation of amniote vertebrates (Goodman et al. 1975; Czelusniak et al. 1982; Goodman et al. 1987).

Members of the  $\alpha$ - and  $\beta$ -globin gene families have diversified in both biochemical properties and developmental timing of expression. The genes in each cluster are generally organized in a 5'-3' sequence that is co-linear with the temporal sequence of expression during development. The archetypal arrangement of the tetrapod  $\alpha$ -globin gene family consists of two functional genes, each of which may be present in one or more copies: an  $\alpha$ -like globin gene expressed early in embryonic development,  $\alpha^E$ -globin (known as  $\alpha^L$ -globin in amphibians,  $\zeta$ -globin in mammals, and  $\pi$ -globin in birds), and the adult  $\alpha^A$ -globin gene. Birds, mammals, and reptiles possess an additional member of the family, the adult  $\alpha^D$ -globin gene, whereas the  $\theta$ -globin gene is found only in mammals. In nearly all mammals studied to date, the  $\alpha$ -chains of adult hemoglobin are encoded by duplicate copies of the  $\alpha^A$ -globin gene, which are almost always identical in sequence and therefore encode identical polypeptides (Zimmer et al. 1980; Higgs et al. 1989). The  $\alpha^D$ -globin gene was only recently identified in mammals (Goh et al. 2005; Hughes et al. 2005; Cooper et al. 2006), and although it is transcriptionally active, the product of this gene is not known to be assembled into functional hemoglobin tetramers. In birds and reptiles, by contrast, the  $\alpha$ -chains of adult hemoglobin are encoded by both the  $\alpha^D$ - and  $\alpha^A$ -globin genes. In most birds studied to date,  $\alpha^D$ -containing hemoglobin (*HbD*) constitutes the minor fraction of adult hemoglobin and  $\alpha^A$ -containing hemoglobin (*HbA*) constitutes the major fraction (Borgese and Bertles 1965; Brown and Ingram 1974; Hiebl et al. 1987). Thus, unlike the case in most mammals, the mature erythrocytes of adult birds and reptiles may contain a mixture of functionally distinct hemoglobin isoforms that have different biochemical properties. Specifically, *HbD* generally has a higher oxygen affinity and a higher cooperativity of oxygen binding than *HbA* (Ciotto and Geraci 1975; Baumann et al. 1984; Riggs 1998; Knapp et al. 1999). Thus, regulatory

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adjustments that alter the stoichiometric ratio of these two isoforms in circulating erythrocytes may modulate rates of oxygen flux in response to changes in metabolic demand (Hiebl et al. 1987; Hiebl et al. 1988).

It has traditionally been thought that the  $\alpha^D$ - and  $\alpha^A$ -globin genes arose via tandem duplication of an ancestral proto  $\alpha^A$ -globin gene with adult function in the common ancestor of birds and mammals (Czelusniak et al. 1982; Hardison 1998, 2001), and recent phylogenetic analyses lend support to this idea (Cooper et al. 2006). According to this scenario, biochemical properties of  $\alpha^D$ -globin that distinguish it from  $\alpha^A$ -globin may be derived characteristics that evolved under the influence of directional selection that favored a physiological division of labor between the two co-expressed gene duplicates. This scenario has intriguing evolutionary implications because functional studies have demonstrated striking similarities between *HbD* and embryonic hemoglobin (*HbE*) with respect to oxygen-binding affinity, cooperativity of oxygen binding, solubility, and responsiveness to chloride ions and other allosteric effectors (Ciroto and Geraci 1975; Baumann et al. 1984; Chapman et al. 1980; Chapman et al. 1982; Grigg et al. 1993; Knapp et al. 1999). If the  $\alpha^D$ - and  $\alpha^A$ -globins arose via tandem duplication of an ancestral proto  $\alpha^A$ -globin gene with adult function, then it would appear that  $\alpha^D$ -globin has convergently evolved a suite of biochemical properties that are characteristic of embryonic globins.

In contrast to prevailing views about the evolutionary history of the  $\alpha$ -globin gene family, here we present evidence that the  $\alpha^D$ - and  $\alpha^A$ -globins are not the product of a single, tandem duplication of an ancestral globin gene with adult function in a common ancestor to extant birds, reptiles, and mammals. Instead, results of our phylogenetic analyses reveal that the  $\alpha^D$ -globin of amniote vertebrates arose via duplication of a proto  $\alpha^E$ -globin gene that predated the radiation of tetrapods. The important evolutionary implication is that the distinct biochemical properties of *HbD* are not exclusively derived characters that evolved during a post-duplication process of neofunctionalization. Rather, many of the distinct biochemical properties of *HbD* are retained ancestral characters that reflect the fact that the  $\alpha^D$ -globin gene arose via duplication of a gene that had a larval/embryonic function in the ancestor of all tetrapods.

## Material and Methods

### DNA Sequence Data

We obtained DNA sequences for structural genes in the  $\alpha$ -globin gene family from either Genbank or Ensembl (release 37, February 2006). When possible, we focused on sequences from a single genomic contig, genomic scaffold, or full chromosome, depending on the nature of the available data. We also included sequences from shorter records based on genomic DNA or cDNA in order to attain a broad and balanced taxonomic coverage of the tetrapod phylogeny. This provision allowed us to include sequences from amphibians (*Xenopus* and *Pleurodeles*), reptiles (*Geochelone* and *Hydrophis*), birds (*Cairina*), as well as some additional mammalian taxa. The basic annotation was derived from the database records in most cases, but

we also identified globin genes in unannotated sequences by comparing known exon sequences to genomic contigs using the program BLAST 2 sequences (Version 2.2; Tatusova and Madden 1999), available from the NCBI website (<http://www.ncbi.nlm.nih.gov/blast/bl2seq>).

We restricted the phylogenetic analysis to functional copies of the four  $\alpha$ -like globin genes and we excluded redundant sequences. We obtained sequences from functional members of the  $\alpha$ -globin gene family in representatives from all tetrapod classes including amphibians, reptiles, birds, and mammals. The  $\alpha$ -globin sequences from zebra fish (*Danio rerio*) and puffer fish (*Sphoeroides nephelus*) were used as outgroups. Alignment was based on the amino acid translation, and was carried out in ClustalW (Thompson et al. 1994) as implemented in BioEdit (Hall 1999). Having confirmed orthologous relationships among the larval/embryonic  $\alpha$ -like globins among all vertebrate taxa (see Results), we use the name  $\alpha^E$ -globin to refer to the  $\alpha^L$ -globin of amphibians, the  $\pi$ -globin of birds, and the  $\zeta$ -globin of mammals.

### Amino Acid Sequence Data

Due to the limited amount of DNA sequence data available for the  $\alpha$ -like globin genes of reptiles, we also assembled a protein sequence data set to corroborate observations derived from the DNA data set. In the protein data set, we increased our representation of  $\alpha$ -like globin genes from amphibians and reptiles, and we included translated amino acid sequences from a subset of the avian and mammalian taxa that were included in the DNA analyses. Specifically, we included translated sequences of  $\alpha^{E-}$ ,  $\alpha^D$ , and  $\alpha^A$ -globin from two representatives of the class Aves (duck and chicken) and translated sequences of  $\alpha^{E-}$ ,  $\alpha^D$ ,  $\alpha^A$ , and  $\theta$ -globin from four representatives of the class Mammalia (an Australian marsupial, a New world marsupial, cow, and human). As with the DNA analysis, we included translated  $\alpha$ -globin sequences from zebra fish and puffer fish as outgroups.

### Phylogenetic Inference

We estimated phylogenetic relationships among the different  $\alpha$ -like globin DNA sequences in our data set using neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian approaches. Neighbor-joining and MP searches were conducted in PAUP version 4.b10 (Swofford 2002), ML analyses were implemented in Treefinder version May 2006 (Jobb et al. 2004), and Bayesian analyses were implemented in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). For the the NJ analyses, we selected the best-fit model of nucleotide substitution by using hierarchical Likelihood Ratio Tests as implemented in the program ModelTest version 3.7 (Posada and Crandall 1998). For the the MP analyses, all substitutions were equally weighted. In ML and Bayesian analyses, we allowed searches to jointly optimize branch lengths and parameter values for the best-fit model of nucleotide substitution. We then compared tree searches with and without site-specific rates for each of the three codon positions using an independent General-Time-Reversible model

**Table 1**  
**Results from the Parametric Bootstrap Test. We Simulated 1000 Sequence Data Sets Using the Tree and GTR +  $\Gamma$  Model of Nucleotide Substitution that Corresponded to the Null Hypothesis. The Difference in Likelihood Score Between the Null Hypothesis ML Topology and the Alternative Hypothesis ML Topology is given as  $\Delta = (-\ln L H_0) - (-\ln L H_{alt})$**

Topologies Compared	Mean $\Delta$ from Simulated Samples	Observed $\Delta$	Critical $\Delta$ values ( $\alpha = 0.01$ )	P-value
$H_0 = \text{Fig 2B } H_{alt} = \text{Fig 2A}$	1.79	-2.79	-1.5	$P < 0.003$
$H_0 = \text{Fig 2C } H_{alt} = \text{Fig 2A}$	22.11	-11.33	5.13	$P < 0.001$
$H_0 = \text{Fig 2D } H_{alt} = \text{Fig 2A}$	38.65	-12.93	13.24	$P < 0.001$

(GTR; Rodriguez et al. 1990) in which rate variation followed a discrete gamma distribution for each position. Support for nodes in NJ, MP, and ML analyses was evaluated using the bootstrap procedure. We employed 1000 pseudoreplicates in NJ and MP analyses, and 100 in ML analyses using the same parameter settings that were used in the phylogenetic searches. Bayesian analyses were run for  $5 \times 10^6$  iterations of a Markov Chain Monte Carlo algorithm, with samples taken every 1000 iterations. Support for the nodes and parameter estimates were derived from a majority rule consensus of the last 2,500 trees sampled after convergence. In the case of the protein sequence data, we used ProtTest version 1.3 (Abascal et al. 2005) to find the best-fitting model of amino acid substitution, and set ML searches in Treefinder to jointly estimate the phylogeny and parameter values for the selected model.

### Hypothesis Testing

To compare alternative tree topologies, we used the Shimodaira-Hasegawa topology test (Shimodaira and Hasegawa 1999), and the approximately unbiased test suggested by Shimodaira (2002). We computed site-specific likelihood scores for each site in the baseml program (Yang 1997), and used the CONSEL program (Shimodaira and Hasegawa 2001) to test whether differences among alternative topologies were statistically significant. Given that the hypotheses evaluated were mutually exclusive, we performed a SOWH test (Swofford et al. 1996), as suggested by Goldman et al. (2000). This test uses a parametric bootstrapping approach to test for differences in topology. First we constrained our searches to find the ML tree that was congruent with the null hypothesis and its associated model of nucleotide substitution, partitioning data into the three different codon positions. Next, we simulated 1,000 data sets based on the null hypothesis ML phylogram, and its corresponding model of nucleotide substitution. For each of the 1,000 simulated data sets we calculated the difference in likelihood score,  $\Delta$ , between the null hypothesis ML topology and the alternative hypothesis ML topology. Using an  $\alpha$ -level of 0.01, the null hypothesis ML topology is rejected if  $\geq 99\%$  of the simulation-based  $\Delta$ -values exceed the observed value.

### Results

#### Sequence Data

We collected 120 functional  $\alpha$ -like globin sequences from a diverse array of tetrapod taxa, including amphibians,

reptiles, birds, and mammals, in addition to two fish (zebra fish HBAA1 and puffer fish HBA2). The final data set consisted of a 429 bp alignment excluding stop codons (Supplementary Material, Table 1).

#### Phylogenetic Inference

On the basis of previous work, the following phylogenetic results are predicted: (i)  $\alpha^E$ -globins from mammals and birds,  $\alpha^D$ -globin, and  $\theta$ -globin should each form a monophyletic clade; (ii)  $\alpha^A$ -globin should be paraphyletic relative to  $\theta$ -globin; (iii) mammalian  $\alpha^A$ -globin should be sister to mammalian  $\theta$ -globin; and (iv) relationships within each set of orthologous sequences should match the expected phylogenetic relationships among species. The goals of our phylogenetic analysis were to resolve relationships among the four  $\alpha$ -like globin paralogs and to assess the robustness of the inferred tree in comparison with trees predicted by alternative hypotheses regarding the evolution of the  $\alpha$ -globin gene family. Neighbor-joining, MP, ML, and Bayesian phylogenetic analyses of our DNA alignment produced highly concordant results. Here, we present results from the ML analysis, with support from ML bootstrap (MLbs) and Bayesian posterior probabilities (BApp). Results of the NJ and MP analyses are available in the supplementary material (Supplementary Material, Figs 1 and 2). In all cases the sequences were grouped into three main lineages: fish  $\alpha$ -globins (which were sister to tetrapod  $\alpha$ -globins), tetrapod  $\alpha^E$ - and  $\alpha^D$ -globins, and tetrapod  $\alpha^A$ - and  $\theta$ -globins (Fig. 1). The ML analysis of the protein data set was consistent with results based on the DNA sequences (Supplementary Material, Fig. 3).

We found strong support for monophyly of the clade containing all  $\alpha^E$ -globin sequences (MLbs = 78%, BApp = 98%). Surprisingly, we also found support for a sister-group relationship between a clade that included all embryonic  $\alpha^E$ -globin sequences (amphibian  $\alpha^L$ -globin, avian  $\pi$ -globin, and mammalian  $\zeta$ -globin), and a clade that included all  $\alpha^D$ -globin sequences in addition to an  $\alpha^A$ -globin gene of the slender sea-snake (MLbs = 59%, and BApp = 99%). Bootstrap support for the monophyly of the clade that grouped all  $\alpha^D$ -globin genes with the slender sea-snake  $\alpha^A$ -globin was over 70% in ML, and the posterior probability was just below the 95% threshold set in the Bayesian analysis. Although the putative  $\alpha^D$ -globin gene of the slender sea-snake grouped with other  $\alpha^D$ -globin sequences in the phylogeny reconstruction, this sequence does not share a second-codon deletion that is shared by all other  $\alpha^D$ -globin genes. Instead, the second codon of the sea-snake sequence codes for valine, as is the case for all amniote

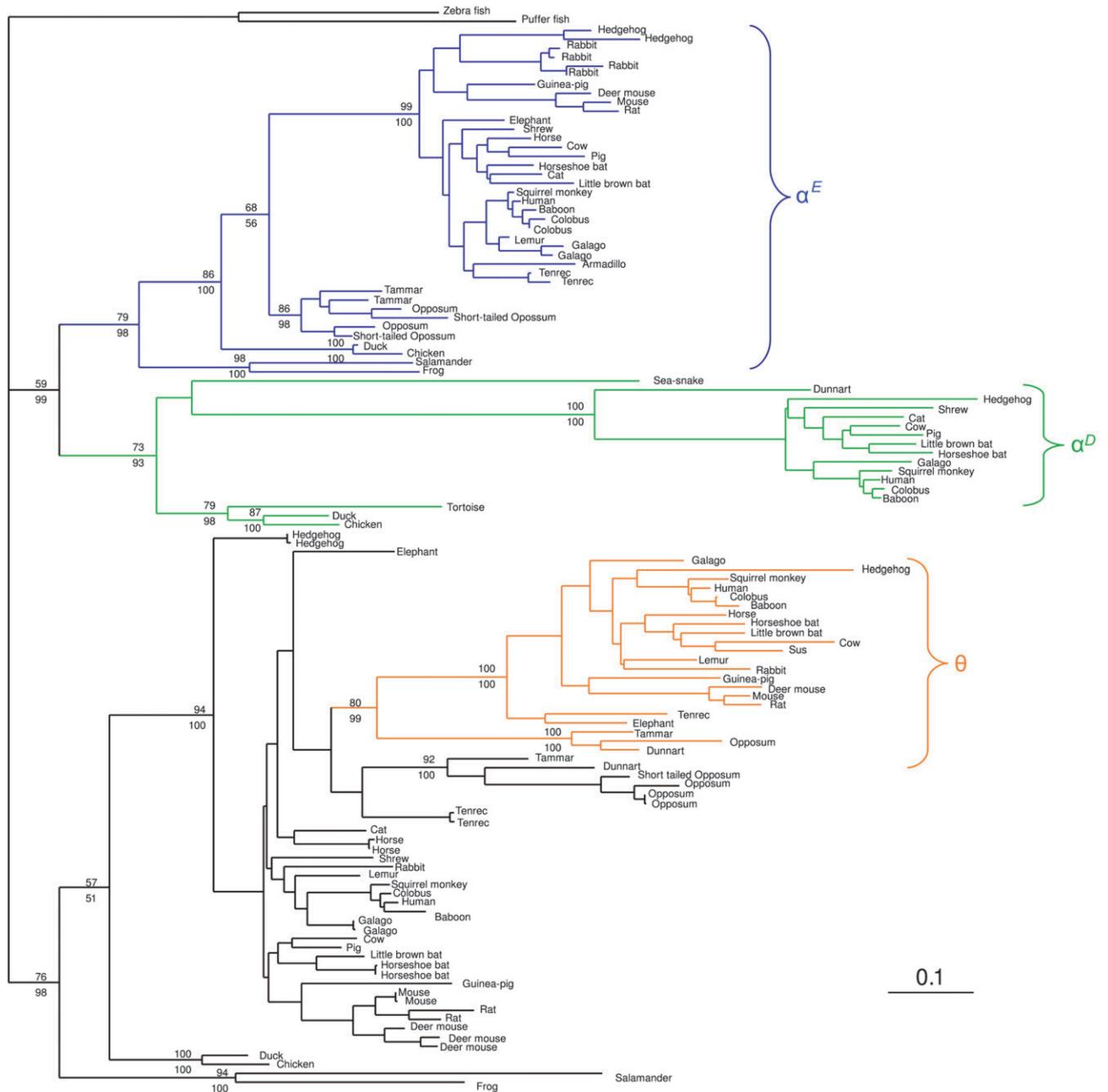


FIG. 1.—Maximum likelihood phylogram describing relationships among the  $\alpha$ -like globin genes of vertebrates, with  $\alpha^E$ -globin sequences (including amphibian  $\alpha^E$ -globin, avian  $\pi$ -globin, and mammalian  $\zeta$ -globin) shown in blue,  $\alpha^D$ -globin sequences shown in green,  $\alpha^A$ -globin sequences shown in black, and  $\theta$ -globin sequences shown in red. Two fish  $\alpha$ -globin sequences were used as outgroups. Searches were conducted in Treefinder version May 2006 (Jobb, von Haeseler, and Strimmer 2004) using an independent site-specific GTR+ $\Gamma$  model of nucleotide substitution for each codon position. Numbers above the nodes correspond to maximum likelihood bootstrap support values, and those below the nodes correspond to Bayesian posterior probabilities.

$\alpha^A$ -globin genes in our database. This suggests a history of gene conversion where the 5' end of the sea-snake  $\alpha^D$ -globin coding sequence has been converted by the  $\alpha^A$ -globin paralog. Further insights into the nature and prevalence of interparalog gene conversion between the  $\alpha^A$ - and  $\alpha^D$ -globin genes should be possible when genomic se-

quence data become available for snakes and other squamate reptiles.

We also found strong support for a clade that included amphibian, reptilian, and avian  $\alpha^A$ -globins, as well as the mammalian  $\alpha^A$ - and  $\theta$ -globins (MLbs = 76%, BApp = 98%). As expected, the  $\alpha^A$ -globin clade was found to be

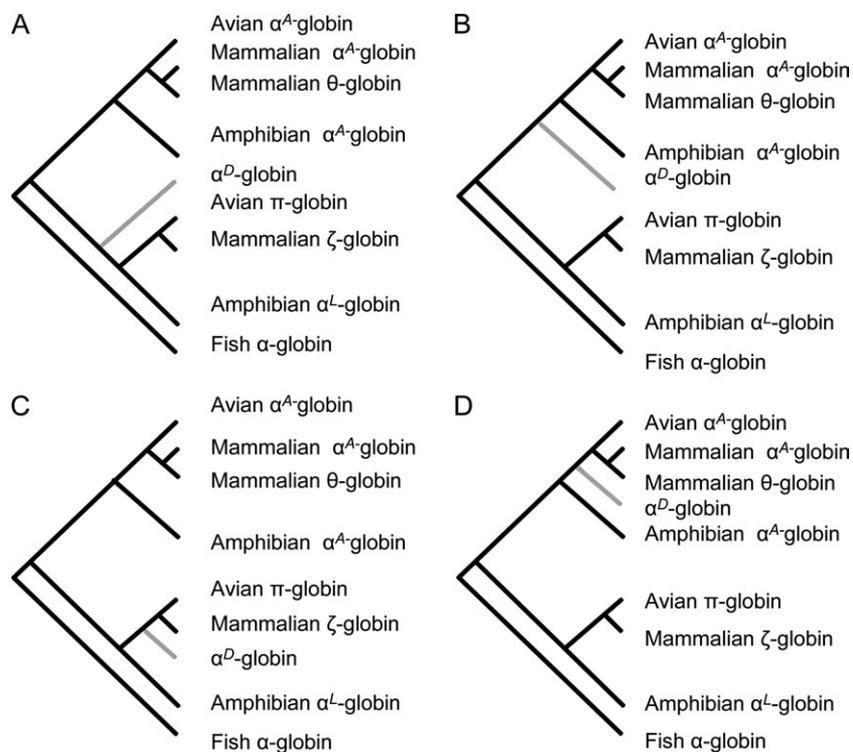


FIG. 2.—Schematic representations of alternative hypotheses regarding phylogenetic relationships among the  $\alpha$ -like globin genes of amniote vertebrates. In A,  $\alpha^D$ -globin arose via duplication of an ancestral embryonic  $\alpha^E$ -globin that predated the radiation of tetrapods. In B, the  $\alpha^D$ -globin arose via duplication of an ancestral adult  $\alpha^A$ -globin that predated the radiation of tetrapods. Our results support hypothesis A. In C,  $\alpha^D$ -globin arose via duplication of an ancestral embryonic  $\alpha^E$ -globin that occurred after the divergence of amphibians from the common ancestor of amniote vertebrates, whereas in D,  $\alpha^D$ -globin arose via duplication of an ancestral adult  $\alpha^A$ -globin that occurred after the divergence of amphibians from the common ancestor of amniote vertebrates.

paraphyletic relative to mammalian  $\theta$ -globin. The monophyly of the mammalian  $\theta$ -globins, in turn, was strongly supported (MLbs = 80%, BApp = 99%).

### Hypotheses Testing

The topology of the ML tree in Fig. 1 met a subset of our initial expectations regarding phylogenetic relationships within and among the different paralogs, although there were several minor discrepancies between the observed and expected relationships among mammalian taxa within each set of orthologous sequences. Eutherian and marsupial sequences were placed sister to each other in the case of  $\alpha^E$ -globin,  $\alpha^D$ -globin, and  $\theta$ -globin. Adult  $\alpha^A$ -globins, however, departed from this pattern. Here, the deepest nodes corresponded to the split of the hedgehog  $\alpha^A$ -globins from a clade that contained the remaining therian  $\alpha^A$ -globins and therian  $\theta$ -globins. Marsupial  $\alpha^A$ -globins were sister to the two tenrec paralogs, and this clade in turn was sister to the  $\theta$ -globin clade. Statistically significant support for the sister relationship between therian  $\theta$ -globins and the clade containing marsupial and tenrec  $\alpha^A$ -globins was only found in Bayesian analyses (BApp = 99%, MLbs < 50%). The presence of  $\theta$ -globin sequences in both marsupial and eutherian mammals indicates that a copy of this gene was present in the therian lineage prior to the divergence of marsupials from eutherians. The lack of support

for a sister relationship between the therian  $\alpha^A$ - and  $\theta$ -globin sequences is most likely attributable to lack of power and/or long branch attraction between marsupial  $\alpha^A$ -globins and therian  $\theta$ -globins.

The main discrepancy between our inferred tree and the tree predicted by the conventional view of  $\alpha$ -globin gene family evolution concerns the placement of the  $\alpha^D$ -globin lineage. Our inferred tree indicates that  $\alpha^D$ -globin is more closely related to embryonic  $\alpha^E$ -globin than to adult  $\alpha^A$ -globin, and that the duplication that gave rise to  $\alpha^D$ -globin preceded the radiation of extant tetrapods.

Given that our results contradict prevailing views of  $\alpha$ -globin gene family evolution, we used constrained searches to explore differences in likelihood scores among the alternative hypotheses (Fig. 2). Specifically, we explored alternative placements of the  $\alpha^D$ -globin sequences that are predicted under competing evolutionary hypotheses, and compared differences among the alternative tree topologies in a statistical framework. The conventional view is that the duplication that gave rise to  $\alpha^D$ -globin occurred after the divergence of amphibians from the common ancestor of all amniote vertebrates (Fig. 3A). By contrast, our results suggest that this duplication took place in the common ancestor of all extant tetrapods (Fig. 3B). Accordingly, we carried out ML phylogenetic reconstructions in which  $\alpha^D$ -globin divergence occurred before the split of either the  $\alpha^E$ - or  $\alpha^A$ -globins of amphibians (Fig. 2A and 2B), and compared these inferred trees to constrained trees in

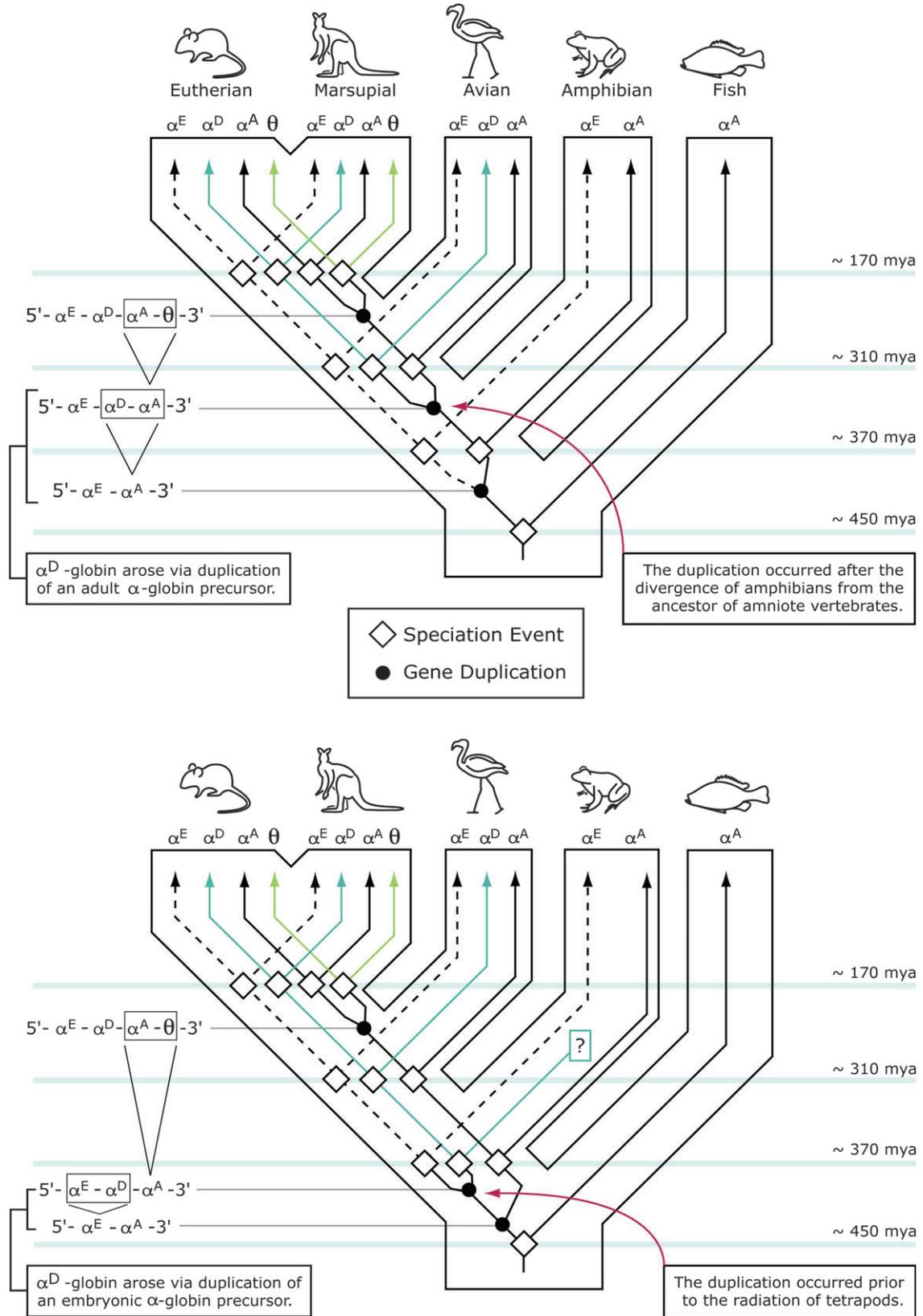


FIG. 3.—Two alternative hypotheses regarding the evolutionary history of the  $\alpha$ -globin gene family. Panel A summarizes the prevailing view, in which the  $\alpha^E$ -globin and  $\alpha^A$ -globin genes arose via tandem duplication of the  $\alpha$ -globin pro-ortholog in the ancestor of all extant tetrapods. This was then followed by a second duplication of the adult  $\alpha^A$ -globin gene which gave rise to the  $\alpha^D$ -globin gene in the common ancestor of all amniotes. Panel B shows the evolutionary scenario favored by our phylogenetic analyses. Under this scenario, the  $\alpha^D$ -globin gene arose via duplication of the  $\alpha^E$ -globin pro-ortholog in the common ancestor to all tetrapods.

which amphibian  $\alpha^E$ - or  $\alpha^A$  globins arose prior to the divergence of  $\alpha^D$ -globin (Fig. 2C and 2D). Differences in likelihood scores associated with each of the four alternative hypotheses were not statistically significant in the SH and AU tests. However, the parametric-bootstrapping SOWH test was able to distinguish the best ML tree (Fig. 2A) from each of the three alternative constrained trees with a high level of statistical significance ( $P < 0.005$ ; Table 1).

## Discussion

We used a large number of vertebrate genome sequences and recently developed phylogenetic methods to re-evaluate evolutionary relationships among members of the  $\alpha$ -globin gene family in vertebrates. Results of our phylogenetic analyses indicate that  $\alpha^D$ -globin is more closely related to embryonic  $\alpha^E$ -globin than to adult  $\alpha^A$ -globin, and that the tandem duplication that gave rise to  $\alpha^D$ -globin preceded the radiation of extant tetrapods. Our conclusion that the  $\alpha^D$ -globin gene arose via duplication of a proto  $\alpha^E$ -globin gene has important evolutionary implications for understanding the role of hemoglobin isoform differentiation in the oxygen-transport systems of birds and other archosaurs.

The prevailing view of  $\alpha$ -globin gene family evolution has been that the  $\alpha^E$ - and  $\alpha^A$ -globin genes arose via tandem duplication of the  $\alpha$ -globin pro-ortholog in the ancestor of all extant tetrapods (Czelusniak et al. 1982; Hardison 1991, 2001; Aguileta et al. 2006; Cooper et al. 2006; Fig. 2A). This was then followed by a second duplication of the adult  $\alpha^A$ -globin gene, which gave rise to  $\alpha^D$ -globin. This second duplication was initially thought to be restricted to modern reptiles and birds, but the recent discovery of a transcriptionally active copy of  $\alpha^D$ -globin in the  $\alpha$ -globin gene cluster of mammals (Goh et al. 2005; Hughes et al. 2005; Cooper et al. 2006) suggests that the duplication must have occurred in the common ancestor of amniote vertebrates. Several previous studies have presented phylogenetic reconstructions consistent with a sister relationship between the  $\alpha^D$ - and  $\alpha^E$ -globins (Goodman et al. 1982; Fushitani et al. 1996; Gorr et al. 1998; Wheeler et al. 2004), but the complete history of gene duplication and divergence was never reconstructed nor were the evolutionary implications explored.

If the duplication leading to  $\alpha^D$ -globin had occurred after the lineage leading to amniotes diverged from the stem lineage of amphibians (the conventional view), we would expect  $\alpha^D$ -globin sequences to be basal to either the amniote  $\alpha^E$ - or amniote  $\alpha^A$ -globin clade, and to branch after the divergence of the amphibian  $\alpha^E$ - or  $\alpha^A$ -globins (Fig. 2A). However, monophyly of the group that includes the  $\alpha^E$ -globins to the exclusion of  $\alpha^D$ -globins is strongly supported in both ML and Bayesian analyses, as is the monophyly of the clade that includes the  $\alpha^A$ -globins of all amniotes and the  $\theta$ -globins of mammals to the exclusion of  $\alpha^D$ -globins. We therefore conclude that the gene duplication that gave rise to  $\alpha^D$ -globin predated the deepest split within either the  $\alpha^E$ - or  $\alpha^A$ -globin clades, as illustrated in Fig 3B. This would imply that an  $\alpha^D$ -globin paralog was present in the ancestor of all tetrapods, regardless of whether  $\alpha^D$ -globin is allied to

embryonic  $\alpha^E$ - or adult  $\alpha^A$ -globins. To date, there is no evidence for the presence of an  $\alpha^D$ -globin gene in amphibians, which suggests that it may have been secondarily lost.

Our evolutionary hypothesis that  $\alpha^D$ -globin is the product of a duplication of the  $\alpha^E$ -globin pro-ortholog is supported by analyses of both DNA and amino acid sequences, which indicate a sister relationship between the  $\alpha^D$ - and  $\alpha^E$ -globins (Fig. 3B). Support for this relationship is strong in Bayesian analyses (BApp = 99%), and moderate in the ML analysis (MLbs = 59%). The sister relationship of  $\alpha^D$ - and  $\alpha^E$ -globins is also strongly supported by the parametric-bootstrapping SOWH test. This indicates that the  $\alpha^D$ -globin gene, which is expressed in adult birds and reptiles, shares a common ancestor with  $\alpha^E$ -globin, which is only expressed during the earliest stages of embryonic development. Our analyses also provide strong support for monophyly of the clade that includes all  $\alpha^E$ -globins (amphibian  $\alpha^E$ -globins, mammalian  $\zeta$ -globins, and avian  $\pi$ -globins) to the exclusion of all other  $\alpha$ -like globin sequences. This suggests an orthologous relationship among all  $\alpha$ -like globin genes that are expressed during the earliest stages embryonic development, and is consistent with previous studies that confirmed an orthologous relationship between avian  $\pi$ -globins and mammalian  $\zeta$ -globins (Czelusniak et al. 1982; Proudfoot et al. 1982).

In the common ancestor of amniote vertebrates, it may be that the ancestral  $\alpha^D$ -globin was expressed early in development, and was later recruited for expression in the mature erythrocytes of adult birds and reptiles, although the ancestral mode of embryonic expression has been retained in some avian species (Godovac-Zimmermann and Braunitzer 1984). In most birds, the  $\alpha^D$ -globin gene is expressed at lower levels than  $\alpha^A$ -globin, but the relative expression levels of the two genes vary widely among different species (Borgese and Bertles 1965; Godovac-Zimmermann and Braunitzer 1984, 1985; Hiebl et al. 1986; Hiebl et al. 1987; Hiebl et al. 1989; Nothum, Braunitzer et al. 1989; Nothum, Weber et al. 1989; Ikehara et al. 1997).

The functional differentiation of *HbA* and *HbD* isoforms appears to have played an important role in the evolution of hypoxia tolerance in a number of birds, including high-soaring vultures (Hiebl et al. 1988; Weber et al. 1988; Hiebl et al. 1989) and migratory ducks and geese (Hiebl et al. 1986; Hiebl et al. 1987). The graded oxygen affinities of  $\alpha^A$ - and  $\alpha^D$ -containing hemoglobin isoforms provides a 'cascade' mechanism for fine-tuning hemoglobin-oxygen affinity in response to variation in ambient oxygen tension. Under conditions of high-altitude hypoxia, high-affinity *HbD* isoforms ensure efficient pulmonary oxygen loading in the parabronchial lung whereas low-affinity *HbA* isoforms ensure the efficient unloading of oxygen to the cells of aerobically metabolizing tissues (Hiebl et al. 1988; Weber et al. 1988).

One of the most striking examples of the role of hemoglobin isoform differentiation in high-altitude respiration involves Ruppell's griffon (*Gyps rueppelli*), an African vulture that nests at sea-level and is known to fly at altitudes of over 11,000 m. As a result of tandem duplication and divergence of the  $\alpha^A$ - and  $\alpha^D$ -globin genes, the red blood cells of these birds contain a heterogeneous mixture of four functionally distinct hemoglobin

isoforms with the following rank-order of oxygen-binding affinities (low to high): *HbA*, *HbA'*, *HbD*, and *HbD'*. The graded oxygen affinities of these four hemoglobin isoforms permits a cascade mechanism of pulmonary/tissue oxygen transport, and appears to provide an important regulatory reserve of oxygen transport capacity (Hiebl et al. 1988; Weber, Hiebl, and Braunitzer 1988). The fact that the  $\alpha^D$ -globin gene arose via duplication of a precursor gene with an embryonic/larval function suggests that the encoded protein was 'pre-adapted' to the task of pulmonary oxygen loading at low partial pressures of oxygen. The protein was pre-adapted to this new task in the sense that the requisite biochemical properties were present in the ancestral condition, and could therefore be co-opted for a modified function when the possession of a high-affinity adult hemoglobin became advantageous. Given what we know about the role of *HbD* in the evolution of hypoxia tolerance in birds, and possibly in other archosaurs (Shishikura 2002; Stoeckelhuber, Gorr et al. 2002), our insights into the evolutionary origin of the  $\alpha^D$ -globin gene illustrate how adaptive modifications of physiological pathways may result from the retention and opportunistic co-option of ancestral protein functions.

### Supplementary Material

Supplementary Material is available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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