

Evolution of Dopamine Receptor Genes of the D₁ Class in Vertebrates

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Abstract

The receptors of the dopamine neurotransmitter belong to two unrelated classes named D₁ and D₂. For the D₁ receptor class, only two subtypes are found in mammals, the D_{1A} and D_{1B} receptors, whereas additional subtypes, named D_{1C}, D_{1D}, and D_{1X}, have been found in other vertebrate species. Here, we analyzed molecular phylogeny, gene synteny, and gene expression pattern of the D₁ receptor subtypes in a large range of vertebrate species, which leads us to propose a new view of the evolution of D₁ dopamine receptor genes. First, we show that D_{1C} and D_{1D} receptor sequences are encoded by orthologous genes. Second, the previously identified Cypriniform D_{1X} sequence is a teleost-specific paralog of the D_{1B} sequences found in all groups of jawed vertebrates. Third, zebrafish and several sauropsid species possess an additional D₁-like gene, which is likely to form another orthology group of vertebrate ancestral genes, which we propose to name D_{1E}. Ancestral jawed vertebrates are thus likely to have possessed four classes of D₁ receptor genes—D_{1A}, D_{1B(X)}, D_{1C(D)}, and D_{1E}—which arose from large-scale gene duplications. The D_{1C} receptor gene would have been secondarily lost in the mammalian lineage, whereas the D_{1E} receptor gene would have been lost independently in several lineages of modern vertebrates. The D_{1A} receptors are well conserved throughout jawed vertebrates, whereas sauropsid D_{1C} receptors have rapidly diverged, to the point that they were misidentified as D_{1D}. The functional significance of the D_{1C} receptor loss is not known. It is possible that the function may have been substituted with D_{1A} or D_{1B} receptors in mammals, following the disappearance of D_{1C} receptors in these species.

Key words: dopamine receptor, gene duplication, phylogeny, telencephalon, tetrapod, teleost.

Introduction

Dopamine (DA) is a modulatory neurotransmitter acting in the central nervous system of vertebrates. In particular, this monoamine regulates a variety of brain functions including motor programming, reward-related behavior, reproduction, or learning and memory. The cellular effects of dopamine are mediated by receptors, which are integral membrane proteins belonging to the rhodopsin family (class I or A) of the G protein-coupled receptors (GPCR; <http://www.gpcr.org/7tm/proteinfamily/>, last accessed December 11, 2012; Ellis 2004).

Originally, two dopamine receptors, named D₁ and D₂, were identified on pharmacological grounds. D₁ receptor agonists stimulate the activity of adenylyl cyclase via G_s-like heterotrimeric G proteins, whereas D₂ receptor activation modulates several intracellular signaling pathways via Gi/Go-like proteins (Kebabian and Calne 1979; Missale et al. 1998). Subsequent molecular cloning of receptor-encoding genes revealed that the pharmacologically defined D₁ and D₂ receptors corresponded to two classes of DA receptor-encoding genes (named from D₁ to D₅ in mammals). Each of these receptor molecules was assigned either to the D₁ or to the D₂ receptor classes based on specific pharmacological

and biochemical profiles. The D₁ receptor class includes the D₁ and D₅ receptors with D₁-like biochemical properties. In non-human species, D₁ and D₅ receptors are called D_{1A} and D_{1B} (Monsma et al. 1990; Tiberi et al. 1991), and we use this latter nomenclature throughout the article. The D₂ receptor class includes the D₂, D₃, and D₄ receptors with D₂-like properties. Molecular cloning also brought structural support to the pharmacological classification of dopamine receptors. D_{1A} and D_{1B} receptors are encoded by intronless genes, exhibiting a short third cytoplasmic loop and a long C-terminal tail, whereas D₂, D₃, and D₄ receptor genes contains several introns, encoding a long third cytoplasmic loop and a short C-terminal tail (Bunzow et al. 1988; Sokoloff et al. 1990; Van Tol et al. 1991).

Interestingly, the D₁ class and D₂ class of dopamine receptors are not phylogenetically more related to each other than to the other class of monoamine receptors (Callier et al. 2003; Le Crom et al. 2003; fig. 1). This indicates that the ability to bind dopamine shared by the two receptor classes was acquired independently, by convergence during evolution. It is also worth mentioning that the receptor specificity for natural ligand is not very stringent among monoamine systems. Dopamine can bind to α_2 -adrenergic receptors in

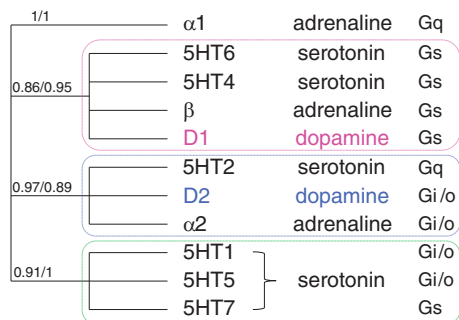


Fig. 1. Phylogenetic relationships of D_1 and D_2 classes of dopamine receptors compared with other monoamine receptors in vertebrates. The first branch support value corresponds to the bootstrap from Maximum-likelihood method, and the second value corresponds to the posterior probability from Bayesian method. Note that different class of dopaminergic, adrenergic, and serotonergic receptors are not more related to each other than to the other class of monoamine receptors. Instead, monoamine receptors coupled to the same class of G proteins tend to be clustered together.

birds and mammals (Cornil and Ball 2008). Amphioxus β -adrenergic-like receptor (Amphi D_1/β) binds dopamine (amphioxus has no noradrenaline or adrenaline), and it has pharmacological characteristics shared with both vertebrate β -adrenergic and D_1 dopamine receptors (Burman et al. 2009). The loose ligand-receptor specificity confers some flexibility, which may have favored the opportunistic recruitment of monoamine receptor genes in different physiological systems during evolution.

In mammals only two D_1 -like receptors, D_{1A} and D_{1B} receptors, are identified, but additional subtypes have been characterized in non-mammalian species. The D_{1C} receptor subtype has all the characteristics of the D_1 receptor class, but its sequence, pharmacological profile and resistance to desensitization make it significantly different from D_{1A} or D_{1B} receptors (Sugamori et al. 1994; Cardinaud et al. 1997; Le Crom et al. 2004). The D_{1C} receptor subtype was initially characterized in *Xenopus laevis* (Sugamori et al. 1994) and was also found in several teleost fishes such as eel (Cardinaud et al. 1997), carp (Hirano et al. 1998), and tilapia (Lamers et al. 1996). Another subtype of D_1 -like receptor was isolated from the chick genome. It displayed different pharmacological profiles from *Xenopus* D_{1C} receptor, with relatively low protein sequence identity. It was considered to be a separate subtype from the D_{1C} receptor, and named D_{1D} (Demchyshyn et al. 1995). More recently, a D_{1D} receptor gene was found in zebra finch as well (Kubikova et al. 2010), suggesting it may be specific to birds. Finally, a supernumerary D_{1X} receptor sequence, yet pharmacologically uncharacterized, was found in the carp retina. Although having D_1 -like receptor structure with no intron, it contains a unique amino acid sequence “RTCPLQHRAI,” which is not present in any other D_1 -like receptors (Hirano et al. 1998).

Phylogenetic analyses of the D_1 class dopamine receptors and a scenario of their evolution have been previously presented (Callier et al. 2003; Le Crom et al. 2003; Le Crom et al. 2004). The D_{1D} and D_{1X} receptor sequences fall outside

of the D_{1A} , D_{1B} , and D_{1C} receptor sequences, but the phylogenies were constructed with genomic data from a limited numbers of species only. Much larger data sets of genome sequences are now available from many vertebrate species, as well as a better knowledge of their chromosomal arrangement. Here, we analyzed the evolutionary relationships of D_1 -like receptor genes from a wide range of vertebrate species by phylogenetic analysis as well as by taking into consideration gene synteny. We also performed in situ hybridization of each D_1 class gene in the chick and *Xenopus* brains. Together with the current knowledge of the distribution of D_1 receptor in the brain of other vertebrate species, the results allow to better understand the functional consequences of the multiplicity of D_1 -related receptors in vertebrates.

Results

Phylogenetic Analysis

To study the relationship among the D_1 class of dopamine receptors in vertebrates, we performed phylogenetic analyses using protein and DNA sequences from variety of species. D_{1A} and D_{1B} receptor sequences formed distinct clades in both the protein (fig. 2A) and the DNA (fig. 2B) trees. D_1 -like receptor sequences of lampreys, which are jawless vertebrates, were located at the root of D_{1A} and D_{1B} receptor clades.

Although in the DNA tree, *Xenopus* D_{1C} , teleost D_{1C} -like, and sauropsid D_{1D} -like receptor sequences formed separate clades (fig. 2B), D_{1D} -like receptor sequences were in the same clade of D_{1C} receptor sequences in the protein tree (fig. 2A). Additionally, a close look at the amino acid sequences used for the analysis revealed a molecular signature conserved between D_{1C} and D_{1D} receptor sequences in the first extracellular loop between the second and third transmembrane domains (TM; supplementary fig. S1, Supplementary Material online), further suggesting a close relationship between D_{1C} and D_{1D} receptor sequences. An additional D_1 -like receptor sequence was found in some species, and the ones from turtle, turkey, and two species of lizards formed a clade in the DNA tree (fig. 2B), which we named D_{1E} .

In the protein tree, teleost D_1 -like sequences formed two subclades within the each D_{1A} , D_{1B} , and D_{1C} receptor groups. This would be accounted by the additional round of whole-genome duplication (indicated with arrows in fig. 2A) that occurred in the teleostean lineage (Aparicio 2000). In the D_{1B} clade, one of the subclades contained D_{1X} -like sequences. The zebrafish D_{1X} -like protein sequence contains the specific “RTCPLQHRAI” motif same as the carp D_{1X} receptor (Hirano et al. 1998), whereas other D_{1X} -like receptor sequences such as in stickleback did not. These results suggest that D_{1X} is a teleost-specific paralog of D_{1B} receptor genes.

The internal branches of sauropsid D_{1D} - and teleost D_{1X} -like receptor sequences are much longer than those of other D_1 receptor sequences, suggesting that they have diverged rapidly.

It is worth noting that the general topology of the protein trees was extremely unstable. For instance, the lamprey D_1 -like protein sequences, shown at the root of D_{1A} and D_{1B} receptor groups in the protein tree (fig. 2A), would be

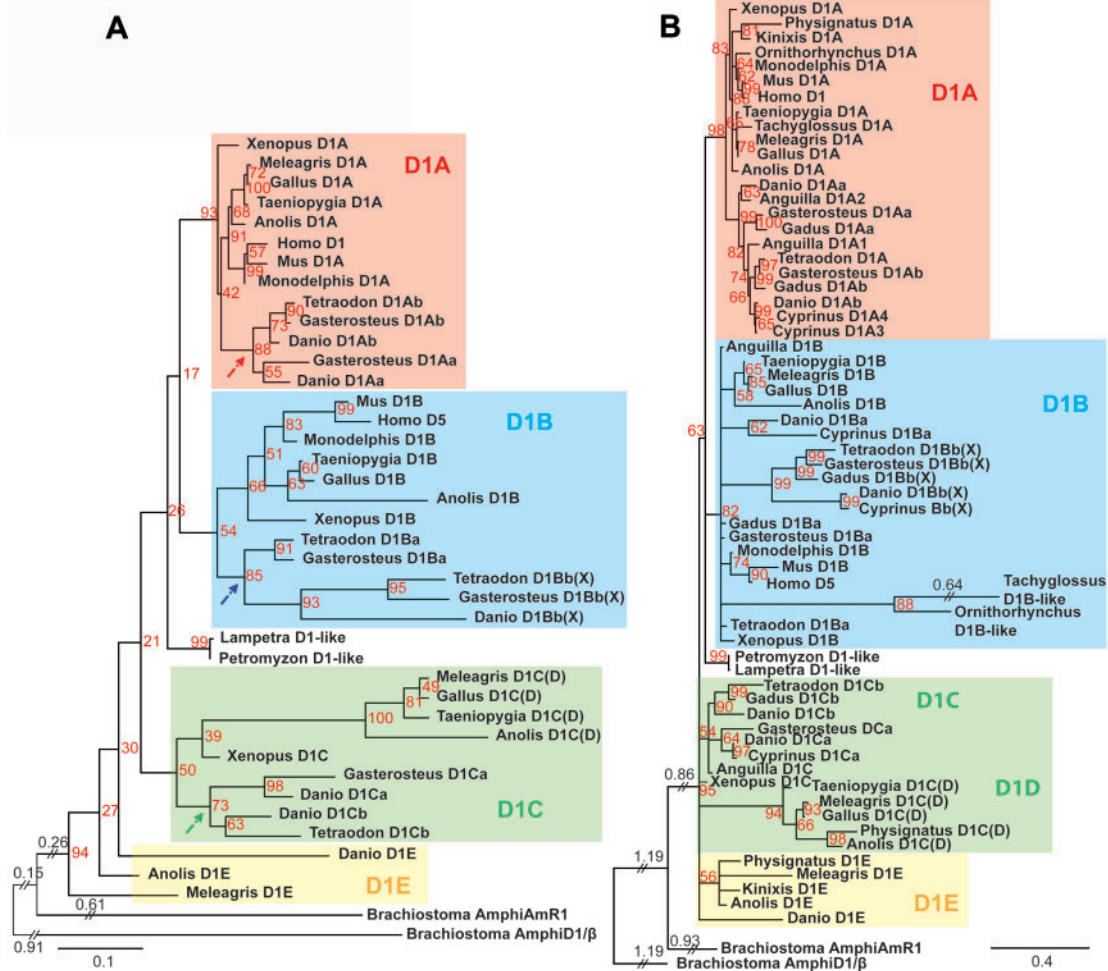


Fig. 2. A representative phylogenetic tree of the vertebrate D_1 receptors obtained using two different methods. (A) A phylogenetic tree obtained from maximum likelihood method using protein sequences, while (B) was obtained from Bayesian analysis using the first two codons of the corresponding nucleotide sequences. The trees were rooted on Amphioxus D_1 -like receptors. The branch support values (boot strap value in A, and posterior probabilities in B) are indicated in red, and the length of the horizontal lines is proportional to sequence divergence (scale bar: 0.1 arbitrary units in A and 0.4 arbitrary units in B), except those of Amphioxus (*Brachistoma AmphiAmR1* and $D_1\beta$); the scales are shown along the branch). The two trees provide complementary information, leading four major clades of D_1 -like dopamine receptors in jawed vertebrates. Arrows in (A) indicate teleost-specific genome duplication, which probably had born two copies of D_{1A} , D_{1B} , and D_{1C} .

clustered with D_{1C} receptor sequences when eel sequences were added to the analysis (data not shown). Since including incomplete sequences makes the tree even more unstable, we removed some of them from the analysis used in figure 2A. Bayesian analysis (Lartillot and Philippe 2004) using the corresponding DNA sequences (fig. 2B) did not completely enhance the resolution of the D_1 receptor phylogeny, nonetheless, we could obtain a consensus tree including the short sequences we did not use in the protein tree.

To verify the orthologous relationship between anamniote D_{1C} and sauropsid D_{1D} receptor genes, and among putative D_{1E} sequences, we also examined gene synteny of D_1 receptor genes in several vertebrate species.

Gene Synteny

Shared synteny is a reliable criterion for establishing orthology of genomic regions among different species. Gene synteny at the D_{1A} and D_{1B} receptor loci was conserved among different

vertebrate species (fig. 3; D_{1A} receptor loci in red, D_{1B} receptor loci in blue), in agreement with the result from phylogenetic analysis.

The comparison of the chromosomal organization of anamniote D_{1C} and sauropsid D_{1D} receptor gene revealed a striking conservation of their synteny (fig. 3, D_{1C} receptor loci in light green, D_{1D} receptor loci in dark green). This strongly suggests that anamniote D_{1C} receptor genes and sauropsid D_{1D} receptor genes are orthologs. Furthermore, similar arrangement of syntenic genes (*TNKS2*, *PPP1R3C*, and *HECTD2*) was found on the chromosomes of several mammalian species, but without D_1 -like gene at the locus. This suggests that the D_{1C} receptor gene was lost early in the mammalian lineage at a locus inherited at least from the common ancestors of gnathostomes. A chromosome breakage and inversion at one side of the D_{1C} receptor locus (*TBC1D12*, *NOC3L*, *PLCE1*, and *TMEM20*) in mammals may have accompanied the loss of the D_{1C} receptor gene.

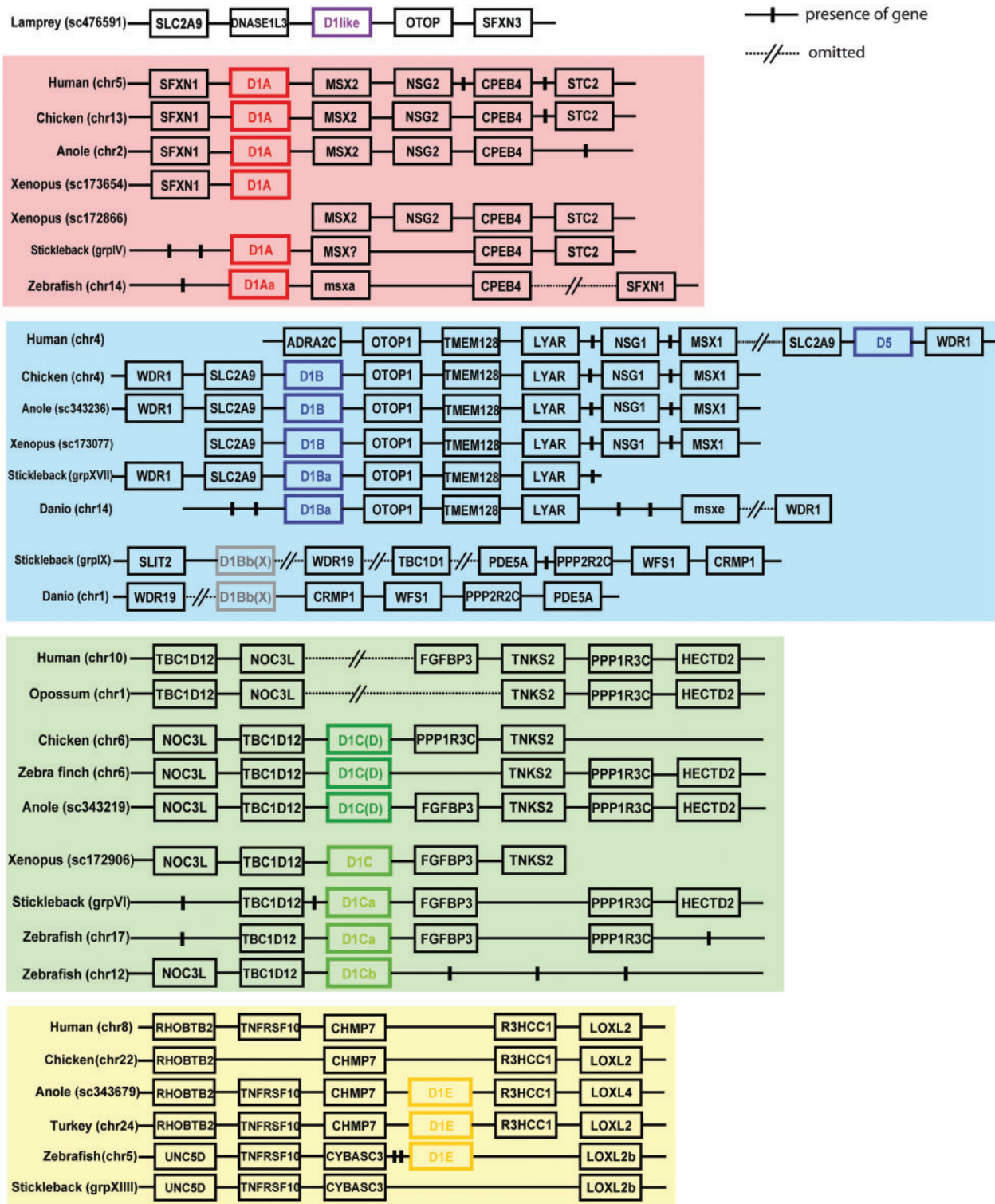


FIG. 3. Comparative gene synteny of the chromosome loci bearing the *D1*-like receptor genes in representative vertebrate species. Lamprey *D1*-like gene is illustrated in purple, putative *D1A* genes are illustrated in red, *D1B* genes in blue, and *D1X* in gray. The genes previously recognized to be *D1C* (found in anamniotes) are in light green, and the ones to be *D1D* (found in sauropsids) are in dark green. The shared synteny between *D1C* and *D1D* strongly suggests that they are orthologous genes. Note that the mammalian chromosomes lack the *D1C* or *D1D* gene at the loci but otherwise demonstrate the conserved synteny with other species. Newly identified genes found in anole lizard, turkey, and zebrafish are illustrated in yellow, named *D1E*. The comparison between turkey and anole lizard strongly suggests that they are orthologous genes, and the conserved synteny lacking the *D1E* loci on the chicken and mouse chromosomes indicates the secondary loss of *D1E* in these species. Some extent of conservation (e.g., *TNFRSF10* and *LOXL2*) is found on the zebrafish chromosome 5 and *D1E* neighbors of sauropsids, suggesting that the homologous loci may have been present before the divergence of jawed vertebrates.

The neighbors of the putative D_{1E} receptor genes (fig. 3, shown in yellow), were conserved in anole lizard and turkey. Two of the syntenic genes, *TNFRSF10* and *LoxL2*, were found surrounding the D_1 -like receptor gene of the zebrafish chromosome 5. Thus, they are likely to be orthologs. In species lacking the D_{1E} -like receptor genes, similar arrangement of syntenic genes were found without D_{1E} gene. This suggests that a secondary loss of the D_{1E} receptor gene has occurred independently in several animal groups during evolution.

Overall, the chromosomal organization of the D_{1A} , D_{1B} , D_{1C} , and D_{1E} receptor gene synteny was significantly different, allowing to easily distinguish orthologous loci from paralogous loci. Nonetheless, some degree of similarity was observed among paralogous loci. In particular, two paralogs of *MSX* and *NSG* genes were found as close neighbors of D_{1A} and D_{1B} receptor genes. In the lamprey *Petromyzon marinus*, D_1 -like receptor gene (purple in fig. 3) is surrounded by genes homologous to neighbors of both D_{1A} receptor (*SFXN*) and D_{1B} receptor (*SLC2A9* and *OTOP*) in jawed vertebrates, further suggesting that D_{1A} and D_{1B} receptor genes are closely related paralogs.

Zebrafish possesses two copies of D_{1A} , D_{1B} , and D_{1C} -like receptor genes, and in each case, one of the paralogous loci shared syntenic genes with tetrapods, but not the other (or in lesser extent). This suggests that the corresponding chromosome duplication was accompanied by major rearrangement on one of the duplicated locus. For example, although the D_{1A} receptor gene locus on chromosome 14 showed synteny shared with tetrapods (fig. 3 indicated as D_{1Aa}), the corresponding duplicated gene on chromosome 9 did not show any synteny conservation (data not shown). The sequence of the D_{1B} -like gene on the zebrafish chromosome 14 (fig. 3 indicated as D_{1Ba}) is not complete, and was not included in the phylogenetic tree of figure 2A. However, gene synteny clearly showed that it shared orthologous relationship with the D_{1Ba} receptor gene of other teleosts (e.g., stickleback shown in fig. 3) and tetrapod D_{1B} receptor genes. The other putative D_{1B} receptor gene paralog, the teleostean D_{1X} -like receptor genes (chromosome 1 D_{1X} in gray in fig. 3), did not display shared synteny with D_{1B} receptor gene when only close neighbors were taken into account. However, homologous genes of D_{1X} neighbors (*PPP2R2C*, *WFS1*, or *TBC1D1*) were found on the same chromosomes as D_{1B} receptor gene (human chromosome 4, mouse chromosome 5, or chicken chromosome 4). In addition, another copy of zebrafish *PPP2R2C* gene was found on the chromosome 14, where zebrafish D_{1Ba} receptor gene is located (data not shown). These observations are consistent with the results from the molecular phylogeny (fig. 2), suggesting a close relationship between D_{1B} and D_{1X} receptor genes.

Distribution of the D_1 Receptor Gene Transcripts in the Chick and *Xenopus* Brains

The expression profiles of D_1 receptor subtypes have been well studied in mammals (e.g., mouse, rat, and human) and also in the European eel, a teleost (Kapsimali et al., 2000).

To better understand the degree of conservation and divergence of dopaminergic areas in vertebrates, and to analyze it in the context of the organization of dopamine neurotransmission systems, we examined the expression of the D_1 receptor genes in the brain of *Xenopus* and chick by in situ hybridization.

Chick

We examined the localization of the transcripts of D_{1A} , D_{1B} , and D_{1C} (previously known as D_{1D}) dopamine receptor genes in 1-day-old chick brains. The overall expression pattern is consistent with that in older chicken (Sun and Reiner 2000; Kubikova et al. 2010). The most prominent expression of D_{1A} , D_{1B} and D_{1C} (D) receptor gene transcripts were found in the telencephalon (fig. 5). D_{1A} receptor mRNA was highly expressed in the striatum (fig. 5A). Weaker levels of expression were also found in the pallial areas, such as in the mesopallium and caudal nidopallium (fig. 5A). Remarkably, D_{1B} receptor transcripts were detected in the striatum and mesopallium (fig. 5B). Overall labeling intensity of $D_{1C(D)}$ was not as high as those of D_{1A} or D_{1B} receptor genes. The mesopallium and hippocampus contained the highest level of $D_{1C(D)}$ receptor gene transcripts, and weaker expression was found in the medial nidopallium (fig. 5C–E).

Xenopus

As for the case in amniotes, the amphibian striatum has been known to contain abundant dopaminergic terminals, but the presence of dopamine receptors has not been reported previously. Prominent levels of D_{1A} receptor mRNA was found in the striatum of the adult *Xenopus laevis*, and, to a lesser extent, in the lateral septum (LS; fig. 6A). In the telencephalon, the D_{1B} and D_{1C} receptor transcripts were weakly detected with a scattered pattern. D_{1B} receptor expression was found in the medial and lateral pallium, and also in the striatum and septum (fig. 6B). Faint levels of the D_{1C} receptor transcripts were found in the lateral (LS) and medial (MS) septal areas, in the medial pallium, as well as the in the striatum (fig. 6C). In contrast to its weak expression in the telencephalon, D_{1C} receptor transcripts were present at a high level in the posterior tuberculum (fig. 6D), where dopaminergic neurons are located (González and Smeets 1994). This suggests that D_{1C} receptor may act as an autoreceptor in this species.

Discussion

In this study, we combined molecular phylogeny and gene synteny analysis with an investigation of the expression pattern of the D_1 receptor subtypes in vertebrates, to get insight in the evolutionary history of this receptor class. Several salient features arose from our findings.

D_{1C}/D_{1D} Receptor Genes

We show here that the previously identified chicken D_{1D} receptor gene is the avian ortholog of the D_{1C} receptor subtype described in other vertebrate groups such as bony fish and amphibian. This contention is supported by both molecular phylogeny (fig. 2) and gene synteny (fig. 3). Thus, the avian

D_{1D} receptor gene should be renamed as D_{1C} and we use this nomenclature in the rest of the article.

The D_{1C} receptor genes display a strikingly conserved synteny in teleost fish, amphibians and non-mammalian amniotes. In placental (mouse and human for example) and marsupial (opossum) mammals, the same syntenic group exists but it conspicuously lacks D_1 -like genes, suggesting that the D_{1C} receptor gene was present in ancestral vertebrates, and secondarily lost in the mammalian lineage.

The D_{1C} receptor sequences in sauropsids have notably diverged much faster than those in anamniotes, as demonstrated by the longer internal branches of the sauropsid D_{1C} receptor sequences (shown as $D_{1C(D)}$ in fig. 2). This rapid divergence of the sauropsid D_{1C} (previously D_{1D}) receptor gene may account for the peculiarities of its pharmacological profile, which is significantly different from those of anamniote D_{1C} receptors (Demchyshyn et al. 1995; Le Crom et al. 2004).

D_{1B}/D_{1X} Receptor Genes

The D_{1X} receptor genes are exclusively found in teleosts and their sequences are located within the D_{1B} receptor clade in the phylogenetic trees. Therefore, they are likely to be teleost-specific paralogs of the D_{1B} receptor genes. In line with this hypothesis, several teleosts such as zebrafish possess two paralogs of the D_{1A} and D_{1C} receptor genes. They are probably all a consequence of the genome duplication that occurred early in the teleost lineage (Aparicio 2000). This is supported by the observation that homologous genes of the zebrafish D_{1X} neighbors (e.g., *WFS1* and *PPP2R2C* on chromosome 1) are also found on chromosome 14 as is the D_{1B} receptor gene. Thus, the teleost D_{1B} and D_{1X} receptor loci are duplicated from an ancestral chromosomal region. The shared synteny of the D_{1B} locus of zebrafish chromosome 14 with those of tetrapods suggests that significant reshuffling and recombination occurred on the zebrafish chromosome 1.

Based on this observation, we propose to rename the D_{1X} receptor as a D_{1B} receptor. We will name D_{1Ba} the zebrafish D_{1B} located on the chromosome 14, which displays conserved synteny with tetrapods D_{1B} receptor genes, and D_{1Bb} the zebrafish D_{1X} . Similarly, the zebrafish D_{1A} receptor gene on the chromosome 14, the synteny of which is conserved with tetrapod D_{1A} genes, will be named D_{1Aa} and the other (on the chromosome 9) will be named D_{1Ab} . For the D_{1C} receptor genes, the one on the chromosome 17 will be named D_{1Ca} and the one on the chromosome 12 will be D_{1Cb} receptor (fig. 3).

D_{1E} Receptor Gene

The wide survey of the vertebrate D_1 receptor genes we performed also led to the unexpected finding of a fourth subtype of D_1 receptor gene in zebrafish and several sauropsid species. We propose to name it D_{1E} to avoid any confusion with the previously known D_{1D} receptor (now the amniote D_{1C}). In amniotes, the D_{1E} receptor gene was not found in mammals and in some avians. Their presence in a wide range of

sauropsids, together with the overall gene synteny shared with mouse, chicken, zebra finch, turkey and anole lizard (fig. 3), strongly suggests that stem amniotes possessed a D_{1E} receptor gene and that it was independently lost in the mammalian lineage and in some avian species where it is lacking. Because there is no D_{1E} -like receptor gene found in any mammalian species so far, it was probably lost before the divergence of the therians. In contrast, the loss in the avian lineage seems to have happened much more recently. Chicken, in which D_{1E} receptor gene is absent, and turkey, in which D_{1E} receptor gene is present, both belong to the family of *Phasianidae*, in the order of Galliformes. Thus, the absence of the D_{1E} receptor gene in chicken may correspond to a gene loss after the separation of subfamilies. It is not known yet whether some species of Neoaves possess D_{1E} .

In teleosts, up to now, the D_{1E} receptor gene was only found in zebrafish (order Cypriniformes) and not in any of other teleost species including medaka (order Biloniformes), stickleback (order Gasterosteiformes), or Tetraodon (order Tetraodontiformes). Cypriniformes are phylogenetically basal in teleostean evolution. Thus, the loss of D_{1E} receptor in teleosts may have occurred before the divergence of other teleostean orders after the separation with Cypriniformes.

The phylogenetic analysis presented here demonstrated that the D_1 class of dopamine receptors comprises basically four subtypes in jawed vertebrates, and we propose to name these subtypes, D_{1A} , D_{1B} , D_{1C} and D_{1E} receptor genes.

Gene Duplications Timing

Overall, the gene duplications giving rise to D_1 receptor gene diversity should have occurred at an early stage of the vertebrate evolution, as only one D_1 -like gene (*AmphiAmR1*; Burman et al. 2007) is present in amphioxus. Amphioxus is the only protochordates for which we have D_1 receptor gene data, because D_1 -like receptor genes have been lost in urochordates (Kamesh et al. 2008). The high similarity of the four vertebrate D_1 receptor paralogs suggests that they were duplicated over a relatively short period of time.

Interestingly, paralogs around D_{1A} , D_{1B} , D_{1C} and D_{1E} receptor genes were found on four human chromosome regions (5q35, 4p16, 10q23, and 8p21, respectively), which are proposed to be a linkage group quadruplicated from an chromosomal region of stem chordates (Pébusque et al. 1998; Putnam et al. 2008). This suggests that the duplication of the dopamine D_1 class of receptor genes came along with the large-scale gene duplications that occurred in the early stage of chordate evolution.

The timing of the gene duplications giving rise to the molecular diversity of D_1 receptors in vertebrates is not easy to resolve. A recent phylogenetic analysis using 55 gene families including cyclostome genes favors the hypothesis that at least one round of the large scale duplications has occurred before the cyclostome-gnathostome split (Kuraku et al. 2009). In this hypothesis, there would be two paralogs of D_1 -like genes in lampreys, however, only one D_1 -like receptor gene has been found up to now in the genomes of two lamprey species. The completion of genome sequence of at least one lamprey species and one hagfish species is expected to know whether

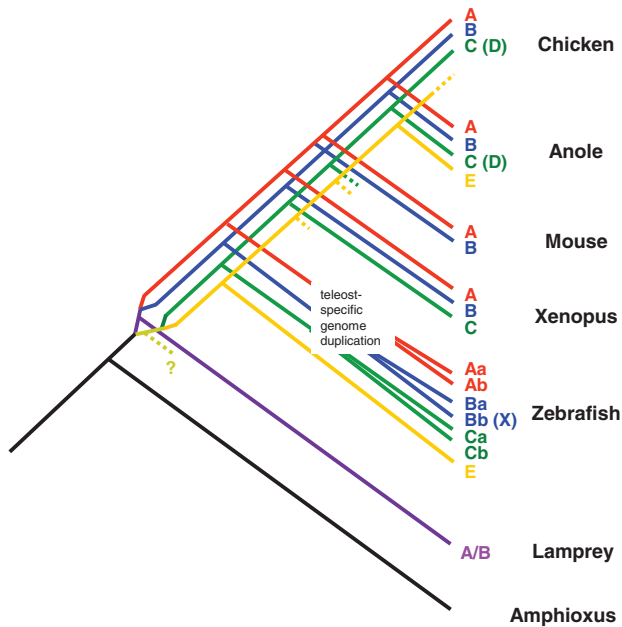


FIG. 4. A hypothetical evolutionary scenario of D_1 class dopamine receptor genes in chordates. The four D_1 -class genes, D_{1A} , D_{1B} (including teleostean D_{1X}), D_{1C} (including sauropsid D_{1D}), and D_{1E} are provably duplicated in the early stage of vertebrate evolution. The duplication process from one to four is not clear. Based on the hypothesis of large-scale gene/genome duplications, together with the phylogenetic position of lamprey D_1 -like gene, it is possible that ancestral D_1 gene was duplicated into ancestors of $D_{1A/B}$ and $D_{1C/E}$ before the separation of cyclostome and gnathostome, and then the two paralogs were further duplicated before the divergence of gnathostome lineage. D_{1C} and D_{1E} were secondarily lost in some lineages, resulting in only two paralogs in mammals, for example.

they have more than one gene in the D_1 receptor class. The phylogenetic analysis showed that lamprey D_1 -like gene is closely related to D_{1A} and D_{1B} . The conserved syntenic genes (*MSX1/2* and *NSG1/2*) between D_{1A} and D_{1B} also support the close relationship of D_{1A} and D_{1B} receptor genes. Thus, we hypothesize that a D_1 -like receptor gene in the stem chordate was firstly duplicated into $D_{1A/B}$ -like and $D_{1C/E}$ -like receptor genes before the separation of cyclostome and gnathostome lineages, then the two paralogs were further duplicated raising four paralogs before the divergence of gnathostomes (summarized in fig. 4).

Conservation of D_{1A} Receptor Gene

The sequence of the D_{1A} receptor gene is the most conserved among the subtype in the whole vertebrate phylum. This is easily visible in terms of coding sequences (as shown by short internal branches in fig. 2), as well as in terms of cellular effects of the D_{1A} receptor (strong adenylyl cyclase activation, fast desensitization, for example) in jawed vertebrates (Callier et al. 2003).

The conservation of D_{1A} receptor was also prominent by its expression pattern in the striatum (figs. 5A and 6A). In tetrapods, the neuronal pathways of the basal ganglia are well conserved, including the dopaminergic innervation from the substantia nigra to the striatum (Marin et al.

1998; Reiner et al. 1998) The similar expression pattern of D_{1A} receptor transcripts in the striatum of both chicken and *Xenopus* further supports the conserved feature of the tetrapod striatum. In the mammalian striatum, D_{1A} receptors are mostly expressed in substance P-positive medial spiny neurons, which project to the internal pallidum and substantia nigra pars reticulata, as part of the so-called direct output pathway (Albin et al. 1989; Gerfen 1992), which promotes voluntary movements. To confirm the conserved function of the D_{1A} receptor in this pathway, it would be interesting to determine whether D_{1A} receptor is indeed present in substance P-expressing neurons in the striatum of the species we have studied here.

A recent study suggested that the basic basal ganglia circuitry also exists in lampreys (Stephenson-Jones et al. 2011). We hypothesize that a lamprey D_1 -like receptor gene was present before the separation of vertebrate D_{1A} and D_{1B} receptors. If the lamprey D_1 -like receptor plays the same role as the D_{1A} receptor in the basal ganglia of other vertebrates, it would further indicate that the D_{1A} receptor preserved an ancestral function. At least, a relative high abundance of D_{1A} receptor transcripts in the striatum is consistently observed in species from most of the vertebrate classes (fig. 7). Additional anatomical data, especially in teleosts, which lack mesencephalic DA cells, are awaited to confirm the conservation of the basal ganglia circuitry throughout vertebrates.

Diversified Gene Expression Patterns in the Pallium

In contrast to the striatum, the expression patterns of D_1 receptor subtypes are much less conserved in the pallium of the vertebrates which have been studied so far. We observed that in the *Xenopus pallium*, the D_1 receptor transcripts were expressed in merely a scattered manner, whereas chick pallium showed prominent and discrete expression patterns. Interestingly, chicken D_{1C} receptor is highly expressed in the hippocampus, where in mammals (which does not possess D_{1C} receptor gene), the D_{1B} receptor is found (Tiberi et al. 1991). The expression of D_1 receptor genes in the mesopallium was more prominent in the chick brain as compared to zebra finch (Kubikova et al. 2010; fig. 5). One possibility is that they may play a role in imprinting, which is an important associative learning behavior in chick (Horn 1985) but not in zebra finch. In contrast, song nuclei of male zebra finch express D_{1A} or D_{1B} receptor genes (Kubikova et al. 2010), which is not the case in non-songbirds like chicken.

Dopamine is also known to play a critical role for working memory, and such similar cognitive functions in mammals and birds are considered to have evolved independently (Güntürkün 2005). Recent study demonstrated that the D_1 -like receptor gene expression is plastic according to different components of a working memory training in pigeon (Herold et al. 2012). It would be of interest to investigate whether the subfunctionalization or neofunctionalization of the paralogous genes encoding dopamine receptors contributed to the convergent evolution of cognitive functions in mammals and birds.

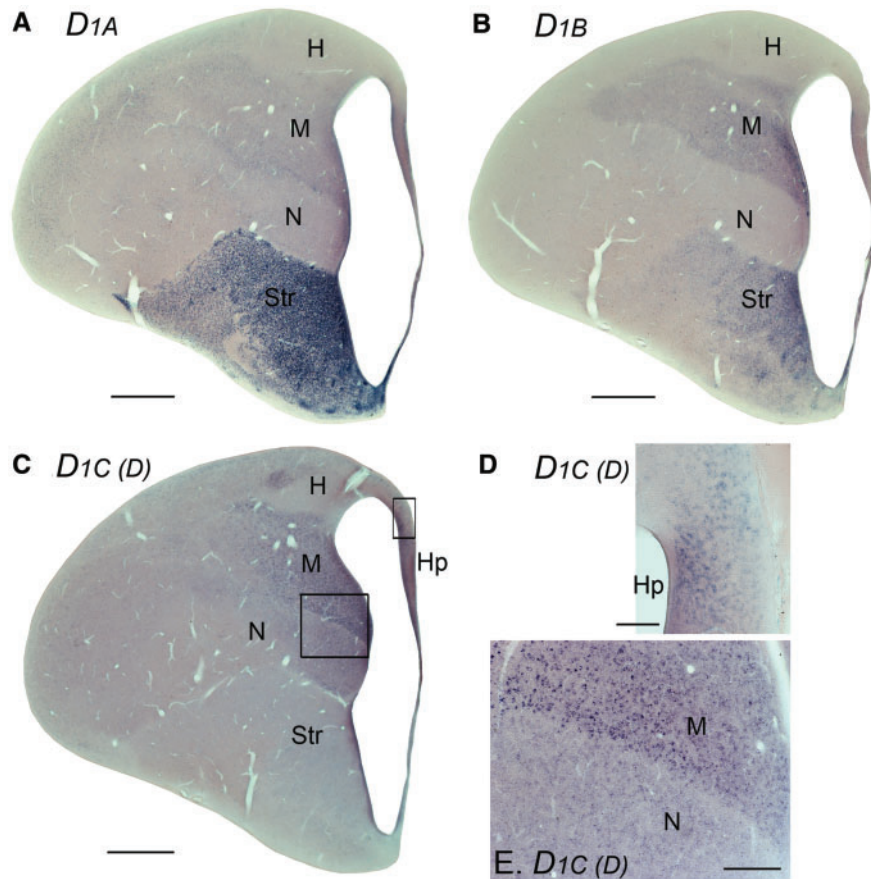


FIG. 5. In situ hybridization of D_{1A} , D_{1B} , and D_{1C} (previously known as D_{1D}) on transverse sections of chick telencephalon (midline to the right). The three genes are differentially expressed in the telencephalon. D_{1A} is abundant in the striatum (A), D_{1B} is in the striatum and the mesopallium (B), and $D_{1C(D)}$ is only in the pallium (C), notably in the mesopallium (E). The $D_{1C(D)}$ expression was found in the hippocampus as well (D). H, hyper pallium; Hp, hippocampus; M, mesopallium; N, nidopallium; Str, striatum. Scale bars = 1 mm for A, B, C, and 200 μ m for D, E.

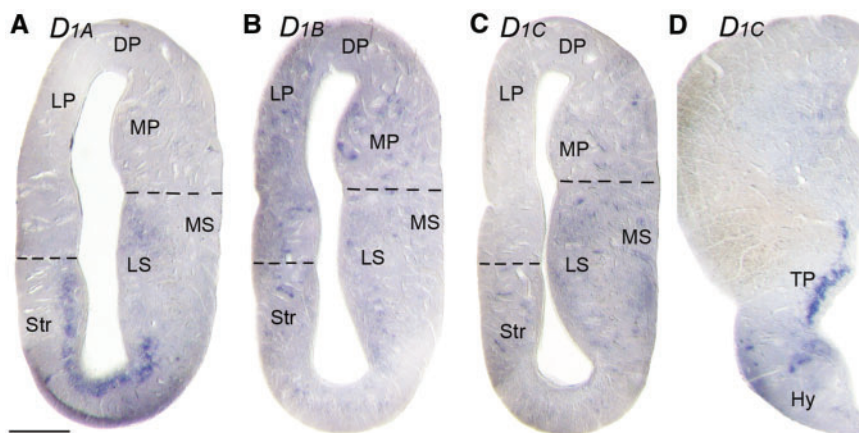


FIG. 6. In situ hybridization of D_{1A} (A), D_{1B} (B), and D_{1C} (C and D) on transverse sections of *Xenopus laevis* (midline to the right). Remarkable expression of D_{1A} was found in the striatum (A). D_{1B} was merely weakly expressed in the telencephalon (B). D_{1C} was also weakly expressed in the telencephalon (C), while highly expressed in the posterior tuberculum (D). DP, dorsal pallium; Hy, hypothalamus; LP, lateral pallium; MP, medial pallium; Str, striatum; TP, posterior tuberculum. Scale bar = 400 μ m.

To summarize, we found that the D_1 class dopamine receptor genes have been diversified in vertebrates, as a result of two large-scale gene duplications early in vertebrate evolution. Although one of the subtypes (D_{1A} receptor) has been

highly conserved throughout vertebrates, others have diverged significantly both in their coding and regulatory sequences. It can be hypothesized that this large diversity and loose specificity of receptor localization have promoted a

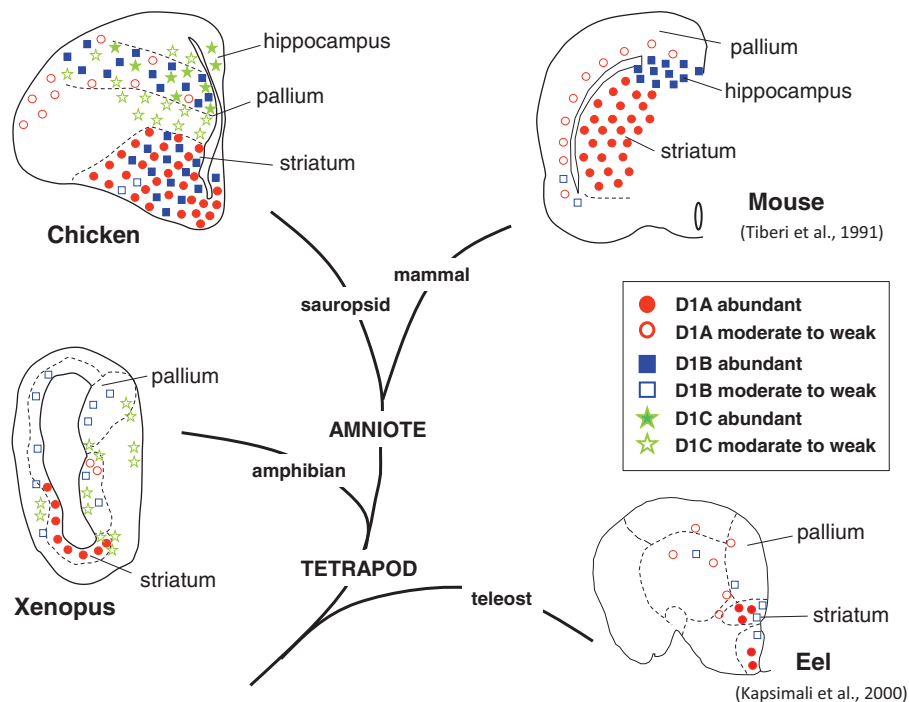


Fig. 7. Schematic drawing showing gene expression patterns of D_1 -class dopamine receptor genes in the telencephala of rodents, birds, and frogs. D_{1A} expression (red circles) in the striatum is well conserved in tetrapods. The expression pattern in the pallium, by contrast, is not much conserved. D_{1C} is selectively expressed in the avian pallium, whereas absent in mammals. Also note the prominent D_{1B} expression in the mammalian hippocampus, whereas D_{1C} is expressed in the avian hippocampus.

large degree of subfunctionalization or neofunctionalization of dopaminergic system, depending on the species, favoring thereby adaptation to different way of life.

Materials and Methods

Cloning D1 Class Dopamine Receptor Genes

As all known D_1 receptor genes are intronless, the sequences used in this study were cloned from genomic DNA extracted from blood samples of lamprey (*Lampetra fluviatilis*), Echidna (*Tachyglossus aculeatus*), and turtle (*Kinixys beliana*), *Xenopus laevis*, and from frozen tissue of Water Dragon (*Physignatus coccinus*). PCR amplification of cDNAs allowed to isolate fragments encompassing the transmembrane segments 3 to 6 of D_1 -like receptor sequences. Amplified DNA was subcloned in pBluescript vector and sequenced. Sequences were obtained by two different sets of degenerate oligos for D_1 receptor sequence, to increase the chance to find all the D_1 -related sequences in a given species. Full-length receptor sequence from *Lampetra fluviatilis* (accession number: JN618989) was then obtained by 5' and 3' extension of the sequence by the using the GenomeWalker Universal kit (Clontech, Saint-Germain-en-Laye, France) on genomic DNA according to the manufacturer protocol. Resulting DNA fragments were inserted in the PCR II TOPO vector (Invitrogen, Carlsbad, CA).

Phylogenetic Analyses

Protein and cDNA sequences similar to D1 class dopamine receptors were retrieved from the Ensembl genome browser (<http://www.ensembl.org/index.html>, last accessed

December 11, 2012) and GenBank (www.ncbi.nlm.nih.gov/genbank/, last accessed December 11, 2012). A broad range of species was considered so as to maximize sampling across the vertebrate tree. Animals and corresponding gene IDs for the sequences used in the analysis are given in [supplementary table S1, Supplementary Material](#) online.

For the maximum likelihood analysis using protein sequences (fig. 2A), the collected sequences were aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>, last accessed December 11, 2012). We removed from the alignment incomplete sequences and sites containing a majority of gaps, and then ran PhyML 3.0 (Guindon and Gascuel 2003; Guindon et al. 2010) through the phylogeny.fr interface system (Dereeper et al. 2008; fig. 2). Statistical branch support values were assessed using a Shimodaira-Hasegawa-like (SH-like) Likelihood Ratio Test (LRT) that is implemented in PhyML, and non-parametric bootstrap (100×) procedures. A representative tree visualized with the FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>, last accessed December 11, 2012) is shown in figure 2.

For constructing nucleotide phylogenetic trees (fig. 2B), we first wrote a script that replaces in the protein alignment each amino acid by the first two bases of each corresponding codon, using the cDNA sequence of each of the protein of the original alignment. The nucleic acid alignment was then manually curated to remove highly divergent sites. Bayesian phylogenetic analyses were then performed with PhyloBayes version 3.3b (Lartillot and Philippe 2004). The model that was used is the CAT + Γ_4 site heterogeneous mixture with a GTR

substitution model for the common relative exchange rates (options -cat -gtr -dgam 4). Four MCMC chains were run for 1,000 cycles, starting from a random topology. We discarded the first 200 points as burn-in. All four chains converged toward a similar phylogenetic tree, as assessed by the bpcomp evaluation of the differences between bipartitions (maxdiff < 0.3). Bayesian posterior probabilities were obtained with the 50% majority-rule consensus program bpcomp from the PhyloBayes package.

Gene Synteny

We examined the chromosomal localization of *D₁-like* genes and the neighboring genes in the available genomes of the Ensembl Genome Browser.

In Situ Hybridization

The probes for in situ hybridization were prepared using total RNA from brains of *Gallus gallus*, and reverse-transcribed using M-MLV reverse transcriptase with oligo dT and hexamer random primers (Promega, Madison, WI). Polymerase chain reaction (PCR) was run on a standard thermocycler using GoTaq Flexi kit (Promega) at annealing temperature of 55–65°C using specific primers (supplementary table S2, Supplementary Material online). Then, the PCR products were inserted into PCR II Vector (Invitrogen) and sequenced.

We used 1-day-old chick (*Gallus gallus*, 6 brains) and adult *Xenopus laevis* (5 brains) for examining expression patterns of each subtype of D1 receptors. All the animals were treated according the CNRS Animal Care and Use protocols. The 1-day-old chicks were anesthetized with a mixture of xylazine (Rompun; Bayer Healthcare, Leverkusen, Germany), ketamine (Imalgene; Virbac, Carros, France), and phosphate-buffered saline (PBS) at a ratio of 1:2:5, respectively. The chicks were transcardially perfused with 0.75% sodium chloride followed by 4% paraformaldehyde (PFA) in PBS (pH 7.4). The brains were removed and post-fixed in 4% PFA overnight at 4°C. Adult *Xenopus* were anesthetized with tricaine (MS-222; Sigma-Aldrich, St. Louis, MO), perfused with 0.9% saline and 4% PFA. The brains were embedded into albumin/gelatin mix (2:1) polymerized with 1.25% glutaraldehyde, and sectioned at 40–70 µm with a vibratome (Leica Microsystems, Wetzlar, Germany).

cRNA probes were synthesized by in vitro transcription by T7 or Sp6 RNA polymerase (Promega), labeled with digoxigenin (DIG)-UTP using DIG RNA Labeling Mix (Roche Diagnostics, Munich, Germany). Hybridization of the brain sections with DIG-labeled cRNA probe was performed at 60–65°C overnight, and was followed by stringent washes at 65°C. The tissues were then incubated with anti-DIG-alkaline phosphatase (DIG-AP; Roche Diagnostics) overnight at 4°C at a concentration of 1:5000 in blocking buffer containing 2% normal goat or sheep serum. The anti-DIG-AP was visualized by incubating with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate in Tris–NaCl buffer (Tris–HCl pH 9.5, 0.1 M NaCl in ddH₂O) for several hours to 48 h.

Supplementary Material

Supplementary tables S1 and S2 and figure S1 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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