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***Research article***

# IDENTIFICATION AND CHARACTERIZATION OF DOPAMINE D4 RECEPTOR (*DRD4*) GENE SEQUENCE WITHIN AND AMONG NON MAMMALIAN SPECIES

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**ABSTRACT:** A number of polymorphic tandem repeats in human dopamine D4 receptor (DRD4) have been identified in the exons, including a 12-bp repeat in the first exon and a 48-bp repeat in exon III located in the third cytoplasmic loop. However, to determine whether the tandem repeats is specific to humans or not, we have identified and characterized dopamine receptor D4 (DRD4) Exon III tandem repeats in public available nucleotide sequences from 13 different non mammalian species. We found that the tandem repeat was composed of 21-bp modules in sequences from the *Mycobacterium smegmatis str. MC2 155*, *Salinibacter ruber DSM 13855*, *Danio rerio*, *Parus major*, *Corvus macrorhynchos*, and *Coturnix japonica.* A tandem repeat consisting of 30-bp modules was identified in sequence from *Melopsittacus undulates* while in the *Phalacrocorax capillatus* and *Numida meleagris* we identified tandem repeats composed of 3-bp modules. Tandem repeats could not be identified in sequences from *Carassius auratus*, *Phasianus colchicus* and *Gallus gallus.* To understand the evolutionary history of the Exon I region of DRD4—which in humans contains a polymorphic 12bp tandem duplication, a polymorphic 13bp deletion, and other rare variants—we examined the homologous exon in these different species. There was a low degree of similarity between the sequences of bacterial species and those from members of the piscean and avian and with human sequence. We identified transmembrane domain of DRD4 gene and signature of G-protein coupled receptors in the amino acid sequences. The number of transmembrane segments varied pronouncedly between species from 0 to 7 and signature of G-protein coupled receptors was found only in piscean species and was also identified in one avian species (*parus major)*. These findings suggest that an association between Drd4 gene polymorphisms and animal personality variation predates the divergence of the non mammalian and mammalian lineages. Furthermore, the analysis of Drd4 polymorphisms within and among populations may provide information for elucidating the phylogenetic relationship and such data may also provide a clue toward understanding the relation between the genetic variation and behavioral variation in animals.

**Keywords:** Dopamine D4 Receptor (*DRD4*), G-protein coupled receptors (GPCR), Transmembrane domain, Exon

## INTRODUCTION

The dopamine receptor $D_4$ is a 7-transmembrane helix, metabotropic G protein-coupled receptor encoded by the DRD4 gene. The $D_4$ is considered to be "$D_2$-like" dopamine receptors, found in the limbic system, frontal cortex, and other areas of the brain (Van Tol, '96) but is expressed at high levels in the prefrontal cortex, that is, a region of the brain associated with cognitive abilities (Oak et al. 2000; Tarazi and Baldessarini 1999). Studies reported over the past decade indicate that polymorphisms in the Drd4 gene may be associated with variation in measures of novelty-seeking behaviour in humans (Van Gestel & Van Broeckhoven 2003; Savitz & Ramesar 2004; Ebstein 2006).

Previous studies have been reported that polymorphisms in the Exon encoding the DRD4 receptor third intracellular loop are associated with personality variation among humans, monkeys and horses (Momozawa et al. 2005; Bailey et al. 2007). Exon I region of DRD4 gene exihibit polymorphism in human, primates, rat and mouse. This showed that recurrent, or parallel, mutations have occurred repeatedly in several primate lineages generating homoplasy in both repeat length and in amino acid substitutions. Moreover, a novel polymorphism was also found in Exon I of canine DRD4 based on 24 bp (8 amino acid) insertion and deletion.

Exon III of the DRD4 gene harbors a tandem repeat in several mammalian species. This variant codes for a 16– amino acid sequence located in the third intracellular loop of the D4 receptor protein, a region that is thought to interact with G-proteins and influence intracellular levels of cAMP. This tandem repeat is composed of 48-bp basic units in humans (which is highly polymorphic, exhibiting at least 25 alleles) (Lichter et al. 1993; Van Tol et al. 1992), nonhuman primates (Livak et al. 1995; Matsumoto et al. 1995), and prosimians (Inoue-Murayama et al. 1998) and the tandem repeat consisting of 18-bp or 36-bp basic units has been identified in domestic cow (Larsen et al., 2005) and in members of the horse family (Hasegawa et al. 2002). The repeat structure is complex in the dog family (Canidae) because it consists of basic units of different sizes, namely 39, 27, and 12 bp (Niimi et al. 2001) whereas, tandem repeats composed of 18-bp basic units were identified in other members of the mammalian order Carnivora, including domestic cat, polar bear, Asiatic bear, and common raccoon, implying that this repeat size is not restricted to hoofed animals (Larsen et al., 2005). A repeat structure has not been identified in the DRD4 gene of mouse (Fishburn et al. 1995). It is possible that there are functional implications of size variation of the tandem repeat in exon III in the DRD4 gene. Recent observations suggest that the length of the polymorphic repeat modulates the level of expression of the human DRD4 gene (Schoots and Van Tol 2003).

So far, studies of the tandem repeat in Exon I and III of the DRD4 gene have focused on primates, other terrestrial animals and cetaceans, while no attempts have been done to identify and characterize this tandem repeat in non mammalian species. This is surprising because in recent years, animal personalities, or 'behavioural syndromes', have become the subject of increasing scientific investigation (Groothuis & Carere 2005; Bell 2007). Aside from being of intrinsic interest, personality can influence how individual animals cope with both predictable and stochastic environmental variation and, consequently, how animal populations may adaptively evolve (Dingemanse et al. 2004; Both et al. 2005; Dingemanse & Re´ale 2005). To better understand the ecological and evolutionary significance of personality variation in natural, free-living animal populations, a greater understanding of the underlying molecular genetic mechanisms is needed. Furthermore, Drd4 gene polymorphisms have been reported for free-living species (Mogensen et al. 2006), but studies associating this variation with behaviour are absent. In this study, a tandem repeat in DRD4 Exon region of non mammalian species was identified and characterized.

## MATERIALS AND METHODS

### Sequences

We retrieved nucleotide and amino acid sequences of the *DRD4* exon III from Swissprot database and GenBank®. These sequences were derived from the *Mycobacterium smegmatis* str. MC2 155 (ABK75167), the *Salinibacter ruber* DSM 13855 (ABC46345), the Gold fish (*Carassius auratus*; ABR27744), the Zebra fish (*Danio rerio*; AAI08055, CAH68974, AAW80616, AAW80615, AAW80614), the Common carp (Cyprinus carpio; O42321_CYPCA and O42322_CYPCA), the Budgerigar (*Melopsittacus undulatus*; BAF34528 and BAF34527), the Great tit (*Parus major*; AAY56687), the Jungle crow (*Corvus macrorhynchos*; BAD18015), the (*Phalacrocorax capillatus*; BAD18019), the common pheasant (*Phasianus colchicus*; BAD18017), the chicken (*Gallus gallus*; BAD18014 and BAD18013), the Japanese quail (*Coturnix japonica*; BAD18012), the Helmeted guineafowl (*Numida meleagris*; Q75PL3_NUMME). The amino acid sequence of the 7R variant of the human *DRD4* exon III tandem repeat (*Homo sapien*; P21917) was also analyzed.

### Detection and characterization of tandem repeats in nucleic acid sequences

The Tandem Repeats Finder (TRF) program developed by Benson (1999) was used to identify and characterize tandem repeats in the nucleotide sequences. In this program the weight for match is _2 and cannot be varied, while weights for mismatch and indels are variable with three options, namely 3, 5, or 7 (interpreted as negative numbers). Lower numbers permit more mismatches or indels; higher numbers increase the stringency of the search conditions. If successful in identifying a tandem repeat, TRF characterizes it and reports the module size, consensus pattern of the repetitive modules and its percent GC. Also, percent of matches is calculated, that is, a measure of the identity between adjacent modules in a tandem repeat, not between the consensus pattern and the single modules. Occasionally, suggestions for different module sizes are provided. In these cases selection of the most appropriate is based upon the score values calculated by TRF. The program can be downloaded from the site: http://tandem.bu.edu/trf/trf.html. GC-compositional strand bias was calculated as (C _G)/ (C_G), where C and G denote the number of cytosine and guanine residues, respectively.

### Detection and characterization of repeats in protein sequences

The program Statistical Analysis of Protein Sequences (SAPS) was applied for detection of repeats in protein sequences. This program is capable of identifying simple tandem repeats as well as separated repeats. The SAPS program was developed in the group of Samuel Karlin at Stanford University, and is freely accessible for on-line analysis at http://www.isrec.isb-sib.ch/software/SAPS_form.html.

### Multiple Alignment analysis in protein sequences

The program: ClustalW2 was used for alignment analysis of protein sequences. Multiple alignments were edited and refined manually, and is freely accessible at http://www.ebi.ac.uk/Tools/clustalw2/.

### Detection of Transmembrane region in protein sequences

The program: Prediction of Transmembrane regions in proteins (PRED-TMR) is a novel method that predicts transmembrane domains in proteins using solely information contained in the sequence itself. The PRED-TMR program was developed in the group of Pasquier, C. at University of Athens, and is freely accessible for on-line analysis at http://athina.biol.uoa.gr/PRED-TMR/input.html.

### Prediction of signature of G-protein coupled receptors in protein sequences

Prediction of signature of G-protein coupled receptors in the amino acid sequences was carried out using Prosite: Database of protein domains, families and functional sites, and is freely accessible at http://www.expasy.org/prosite/.

## RESULTS

To access the diversity of DRD4 gene in non mammalian species, we examined the 20 nucleotide and amino acid sequences retrieved from Swissprot and GenBank, out of which 2 sequences were of bacteria, 8 were of Piscean species and 10 were of avian species. The piscean and avian species possess alleles of DRD4 gene of more than one size, alleles of four length were observed in *Danio rerio,* 2 different size of alleles were observed in *Cyprinus carpio*, *Melopsittacus undulates* and *Gallus gallus* whereas one alleles were observed in *Carassius auratus*, *Parus major*, *Corvus macrorhynchos*, *Phalacrocorax capillatus*, *Phasianus colchicus* and *Coturnix japonica.*

We attempted to detect *DRD4* Exon III tandem repeats in 20 nucleotide sequences and multiple suggestions for the module size of a tandem repeat were obtained. The most important results from these analyses are summarized in Table 1.

**Table 1. Detection of *DRD4* Exon III Tandem Repeats in Different non Mammalian Species**

| Species | Module size under diffferent stringencies[a] | |
|---|---|---|
| | Low | High |
| *Mycobacterium smegmatis str. MC2 155* | 30,21,26 | ND |
| *Salinibacter ruber DSM 13855* | 23,21 | ND |
| *Danio rerio* | 21,1 | 1 |
| *Cyprinus carpio* | 22,12 | ND |
| *Melopsittacus undulatus* | 30 | ND |
| *Parus major* | 21 | ND |
| *Corvus macrorhynchos* | 21 | ND |
| *Phalacrocorax capillatus* | 3 | 3 |
| *Coturnix japonica* | 21 | ND |
| *Numida meleagris* | 3 | ND |
| *Carassius auratus, Phasianus colchicus, Gallus gallus* | ND | ND |

[a]Stringencies of the alignment criteria were low (_2, _3, _5) and high (_2, _7, _7), where numbers refer to parameter settings for match, mismatch, and indel, respectively. If multiple suggestions for the module size are given they are listed in the order of decreasing score values. ND means that a tandem repeat was not detected

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We identified tandem repeats composed of 21-bp modules in the sequences from species of *Mycobacterium smegmatis str. MC2 155*, *Salinibacter ruber DSM 13855*, *Danio rerio*, *Parus major*, *Corvus macrorhynchos* and *Coturnix japonica*. In the nucleotide sequences from the *Phalacrocorax capillatus and Numida meleagris* a tandem repeat composed of 3-bp modules was found using permissive alignment conditions. A 30-bp module tandem repeat was identified in the *Melopsittacus undulates* (Table 2). Using permissive search conditions we identified tandem repeats composed of 12-bp modules in the sequences from the *Cyprinus carpio*. On increased stringency search conditions TRF reported the detection of 1 and 3-bp repeat structure in the *Danio rerio* and *Phalacrocorax capillatus* sequence respectively. Examination of sequences from the *Carassius auratus*, *Phasianus colchicus* and *Gallus gallus* did not unravel a tandem repeat, not even under permissive alignment conditions.

**Table 2. Characteristics of *DRD4* Exon III Tandem Repeats in Different non Mammals**

| Species | Nucleotide | | | | | | Amino acid |
|---|---|---|---|---|---|---|---|
| | Module size (bp) | Repetitive motif (consensus)a | Copy number | Percent matches[b] | Percent GC | GC-bias[c] | Repetitive motif (consensus)[d] |
| Mycobacterium smegmatis str. MC2 155 | 21 bp | GCGGGTGCCGGCGCCAGACCC | 3.5 | 53 | 86 | 0.36 | AAPV |
| Salinibacter ruber DSM 13855 | 21 bp | CCAGGCCGTAGTGCGGCGCCT | 4.9 | 54 | 75 | 0.06-0.19 | RLQAE |
| Carassius auratus | None | None | None | None | None | None | LAVA |
| Danio rerio | 21 bp | ATACAAAAAATAAAGATCTGA | 3.7 | 69 | 14 | 0.28 | LAVA |
| Cyprinus carpio | 12 bp | TCAACACCGTCA | 3.6 | 74 | 55 | 0.67 | FRKF |
| Melopsittacus undulatus | 30 bp | CATCCTGCTCATCGTGCGGGGCAACGGCCT | 2.5 | 72 | 66 | 0.03 | None |
| Parus major | 21 bp | CCTCGCCGTCCCCGACCTGCC | 2.5 | 64 | 72 | 0.41 | None |
| Corvus macrorhynchos | 21 bp | CCGGTCCACAGCATCGCCGCC | 2.7 | 70 | 79 | 0.34 | None |
| Phalacrocorax capillatus | 3 bp | CGC | 18.3 | 75 | 90 | 0.33 | None |
| Phasianus colchicus | None | None | None | None | None | None | None |
| Gallus gallus | None | None | None | None | None | None | None |
| Coturnix japonica | 21 bp | CCTCGCCCTCCCCGACCTGCC | 2.2 | 72 | 75 | 0.49 | None |
| Numida meleagris | 3 bp | CTC | 12.3 | 88 | 72 | 0.86 | None |

[a]Based upon low stringency search conditions (_2, _3, _5). The positions of some of the repeat starts were shifted relative to others, implying that their consensus patterns are not directly comparable. Codons are delimited by dots. If relevant consensus patterns were split into their basic units (listed in different rows one above another).
[b]Serves as a measure of the degree of identity between adjacent modules.
[c]GC-compositional strand bias was calculated as (C_G)/(C_G), where C and G denote the number of cytosine and guanine residues, respectively.
[d]Presence of repetitive motifs (separated or in tandem) consisting of at least four consecutive amino acids in the translated sequences.
[e]For the sake of simplicity the tandem repeats from both cetacean species were classified as 18-bp repeat structures.

A module size of 21 bp was shared by most of the species included in this study (Table 2). The number of modules identified in a tandem repeat was dependent upon the search stringency. The percent of matches was low in the tandem repeats from all animal species except the dog and domestic cat, reflecting a significant degree of sequence variation between adjacent modules. The GC-percent varied from 14 to 90 in the tandem repeats with a marked overrepresentation of C. This bias for C was in particular prominent in the tandem repeat from the *Cyprinus carpio* spp.and *Numida meleagris* spp. We were unable to detect repeat motifs in the deduced amino acid sequences from all the avian species (Table 2). In the sequences from the other species listed in Table 2, repeat motifs consisting of four to five conserved amino acids were detected.

Figure 1 gives the multiple alignment analysis of the amino acid sequence of DRD4 Exon I region for the 20 non mammalians alleles, retrieved from Swissprot and GenBank as well as that inferred from the previously published rat and mouse sequences (O'Malley et al., '92; Fishburn et al., '95). We compared the sequences among and within the species of the same as with the different class. For comparative purposes, sequence of DRD4 gene from human with the GenBank accession numbers P21917 was also analyzed. In bacteria, the sequences similarity between the two species was found to be 11 % and percent similarity with piscean and avian species was found to be from 9-3% and 4-16% respectively and with the human sequence, the *Mycobacterium sp.* showed 21% similarity while *Salinibacter sp*. showed 16% similarity. In pisceans, the similarity was found to be 53-99% within the species and the percent similarity was varied from 6 to 66 with the avian species, higher being with the *parus major* whereas in avians, similarity was found to be 76-100% within the species. Only one amino acid position, at codon 20, varies among the avians (A→T at codon 20 in *Coturnix*) (data not shown).

**Table 3. Detection of *DRD4* Transmembrane Region and Signature of G-Protein Coupled Receptors in Different non Mammalian Species.**

| *Species* | *Transmembrane region* | *Signature of g-protein coupled receptors* |
|---|---|---|
| *Mycobacterium smegmatis str. MC2 155* | 10 VVCLAAGGGVGLSVLLGAPAAL 31 | None |
| *Salinibacter ruber DSM 13855* | None | None |
| *Carassius auratus* | 6 VSLAVADLLLAVLVLPLFVYA 26<br>85 IVLLSATWILALAVASPVMFGI 106<br>130 VCSFFVPCPIMLLLYCGMF 148 | 53 ASIfNLCAISIDRFIaV 69 |
| *Danio rerio* | 20 LALICGVPLILIIILGNVLVCL 41<br>56 FIVSLAVADLLLAILVLPLYV 76<br>80 FLGGIWTLSMYICDALMTM 98<br>137 LALITATWVLSLGVASPVIFGL 158<br>182 VCSFFVPCPVMLFLYYWMF 200<br>346 VLPVVVGVFLACWTPFFVV 364<br>380LISVVTWLGYVNSAVNPIIYTAF 402 | 105 ASIlNLCAISVDRYIaV 121 |
| *Cyprinus carpio* | 19 LIFGILLIVIIICGNVLVCL 38<br>53 FIVSLAVADLLLAVLVLPLFV 73<br>134 IVLLSATWILASAVASPVMFGI 155<br>179 VCSFFVPCPIMLLLYCGMF 197<br>299 VLPVVVGAFLFCWTPFFVV 317<br>336 IVTWLGYVNSALNPVIYTVF 355 | 102 ASIfNLCAISIDRFIaV 118 |
| *Melopsittacus undulatus* | 12 IAALVLGIVLILLIVGG 28 | None |
| *Parus major* | 17 IAALVLGILLILLIVGG 33<br>54 FIVSLAVADLLLALLVLPLYV 74<br>92 ALMTMDVMLCTASIFNLCAISV 113<br>135 LILISTTWIFAFAVASPVIFGL 156<br>180 ICSFFIPCPVMLVLYCGMF 198<br>293 VLPVVVGAFLFCWTPFFVV 311 | 103 ASIfNLCAISVDRFIaV 119 |
| *Corvus macrorhynchos* | 11 IAALVLGILLILLIVGG 27 | None |
| *Phalacrocorax capillatus* | 16 VAALVLGIVLILLVVGG 32 | None |
| *Phasianus colchicus* | 15 IAALVLGIVLILLIVGG 31 | None |
| *Gallus gallus* | 13 IAALVLGIVLILLIVGG 29 | None |
| *Coturnix japonica* | 24 IAALVLGIVLILLIVGG 40 | None |
| *Numida meleagris* | 18 IAALVLGIVLILLIVGG 34 | None |

On comparative analysis with the human sequence, we found that the percent identity varied from 48 to 61 with avians and 49 to 57 with the piscean species (data not shown), with high values being observed for comparisons of human with *parus sp*. and *cyprinus sp*.. We found that all of the insertions/deletions and most of the amino acid substitutions occur in the first half of the Exon, corresponding to the amino-terminus extracellular tail. Many of the substitutions in the conserved region identified in the sequence of pisceans, avians and human, are semi conservative (e.g., K→T at codon 61, T→P at codon 68) and at least four sites (codons 40, 47, 50, and 60) have been repeatedly changed whereas demarcated mutation (Y→S) occured at codon 71 in human.. The Y→H→Q substitution in the pisces, aves and humans respectively, at amino acid 33 in the alignment occurs at the point where the peptide chain presumably enters the cellular membrane (Seeman et al., '94) (Figure 1).

As it has been proved that in humans, polymorphic region of Exon I consists of the repeat structure of 4 amino acid, GASA occurs near the junction of the extracellular domain of the protein and the first two transmembrane domain (Seeman et al., '94) but on the other hand, this repeat structure was not observed in *Mycobacterium sp., Salinibacter sp.*, piscean and avian species. Therefore, the polymorphic region was less homologus between the humans and these species compared to other region of Exon I. Moreover, the low homology between humans and these species was also observed in polymorphic region of Exon III.

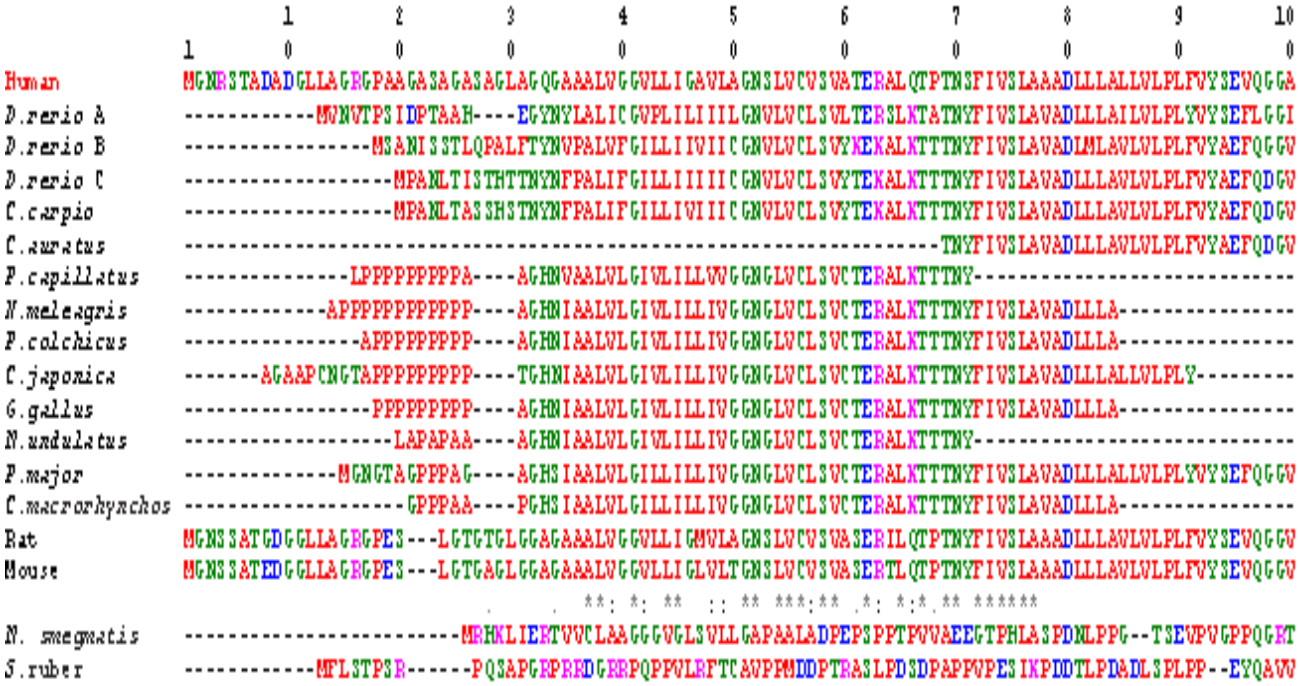


**Fig. 1. Amino acid sequence of Exon 1 in non mammalian species, human, rat and mouse. Dashes indicate deletions, star indicate identity to the human XL allele, single dot indicate ----, and colon indicate -----. Alignment in the duplication region is somewhat arbitrary, and furthermore, not meant to imply strict homology between aligned repeat units between species**

We predicted the seven-transmembrane topology characteristic and signature of G-protein-coupled receptors in the sequences of these species and we were unable to detect this region in only one species of bacteria, Salinibacter ruber DSM 13855, whereas in the sequences from the other species listed in Table 1, the number of transmembrane region was found to be one to seven. The signature of G-protein-coupled receptors was found only in sequences of *Carassius auratus, Danio rerio, Cyprinus carpio and Parus major* (Table 3).

## DISCUSSION

In this present study, we examined the variation of DRD4 gene in non mammalian species and found that DRD4 was highly polymorphic among these species as observed in humans. Several new findings were done. Evidence have been obtained, from a free-living bird species, supporting the hypothesis that Drd4 gene polymorphisms are associated with variation in the level of exploratory/ novelty-seeking behavior in vertebrates (Kempenaers et al., 2007) . Thus, different types of sequence polymorphisms (variable number tandem repeats, SNPs and indels), located in similar Drd4 gene regions (e.g. third intracellular loop coding region, promoter region), may be associated with variation in similar personality traits among taxonomically diverse vertebrates. Indeed, the results reported here suggest that a general association between Drd4 gene polymorphisms and animal personality variation predates the divergence of the non mammalian and mammalian lineages.

Previous studies have reported that most of the amino acid substitutions of Exon I in the hominids are found in the amino-terminus of the protein, corresponding to the extracellular domain - a region shown to be important for the binding of dopamine and signal transduction (Schoots et al., '96). In the polymorphic region of DRD4 Exon I gene in the non mammalian species, deletion/insertion of units was observed at the various positions, suggesting that it was these events which gave rise to the allelic variation of the DRD4 coding region. This apparent onset of relaxation of selection on the amino-terminus of this protein in these species seems to have occurred concomitantly with the Y→H→Q amino acid substitution at the junction of the extracellular and transmembrane domains.

Novel polymorphisms in DRD4 exon III tandem repeats were also detected and a more profound characterization of previously identified tandem repeats was provided. All identified tandem repeats were found to be GC-rich, a property typical of classical minisatellites (Vergnaud and Denoeud, 2000), but differed profoundly otherwise. A module of 48 bp was found in humans, but there occurred variation among the species which we have analyzed. Based upon analyses of tandem repeats, a module size of 21 bp was found in the majority of the species included in this study, including the *Mycobacterium smegmatis str. MC2 155*, *Salinibacter ruber DSM 13855*, *Danio rerio*, *Parus major*, *Corvus macrorhynchos*, and *Coturnix japonica*. The tandem repeats from the *Phalacrocorax capillatus* and *Numida meleagris* were composed of 3-bp modules and 30-bp in *Melopsittacus undulates.* A *DRD4* exon III tandem repeat has not been found in the *Carassius auratus*, *Phasianus colchicus* and *Gallus gallus*. Most likely, this reflects the accumulation of large numbers of nonsynonymous single nucleotide substitutions in the repeat modules, erasing the repeat structure in the polypeptide strands. A tandem repeat composed of 18-bp modules ranging from 3 to 9 in number has previously been reported in the equine species (Hasegawa *et al*., 2002).Similarly, the tandem repeat in the dolphin may have evolved by duplication of an 18-bp basic module. Based upon analyses of amino acid sequences an amino acid repeat motif was absent in the all the avian species.

In short, this is the first report to investigate the polymorphism on DRD4 gene of non mammalian species and in this, we have identified and characterized polymorphism in *DRD4* Exon I and III. The results suggested that there should exist a different evolutionary pathway in DRD4 of non mammalian species in comparison to humans and other species studied earlier. Our observations create a basis for future studies of the evolution of the *DRD4* Exon I and III tandem repeat, and they provide the useful information for understanding the origin of repetitive region and diversity of DRD4 gene.

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