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Genomic Organization of Purinergic P2X Receptors

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Abstract

Purinergic P2X receptors are a family of ligand-gated cationic channels activated by extracellular ATP. P2X subunit protein sequences are highly conserved between vertebrate species. However, they can generate a great diversity of coding splicing variants to fulfill several roles in mammalian physiology. Despite intensive research in P2X expression in both central and peripheral nervous system, there is little information about their homology, genomic structure and other key features that can help to develop selective drugs or regulatory strategies of pharmacological value which are lacking today. In order to obtain clues on mammalian P2X diversity, we have performed a bioinformatics analysis of the coding regions and introns of the seven P2X subunits present in human, simian, dog, mouse, rat and zebrafish. Here we report the arrangements of exon and intron sequences, considering its number, size, phase and placement; proposing some ideas about the gain and loss of exons and retention of introns. Taken together, these evidences show traits that can be used to gain insight into the evolutionary history of vertebrate P2X receptors and better understand the diversity of subunits coding the purinergic signaling in mammals.

Keywords

Alternative Splicing, Intron, Genomic Organization, P2X, Purinergic Signalling

1. Introduction

Purinergic P2X receptors are a family of ligand-gated cationic channels activated by extracellular ATP [1].

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Seven subunits have been identified so far in mammalian species ($P2X_{1-7}$), and they are involved in numerous physiological roles like peristalsis, platelet aggregation, pain sensation, immune response and development [1]-[5]. To form a functional channel, P2X subunits assemble as homo or heteromeric trimers [6]. The pharmacological properties of the assembled P2X receptor vary in function to subunit composition [7] [8]. The subunit stoichiometry has a different arrangement among tissue in a given organism, and different composition among species, for example, the enteric nervous system of the rat, mouse and guinea pig expresses $P2X_2/P2X_3$ heteromeric receptors [6] [9] while sensory ganglia and heart of rodents and humans express homomeric $P2X_3$ receptors [10] [11]. In addition, P2X receptors can assemble from tissue specific splicing variants of its messenger RNA [1].

The physiological role of P2X receptors seems to be the same for different species of mammalians: purinergic neurotransmission. Even when the population of P2X subunits in a tissue between two species may vary [12] [13], the P2X subunit protein sequences are highly conserved between vertebrate species [14]. Sequences correspond to cysteine allowing disulfide bonds, and transmembrane domains I and II and a YXXXK motif in the c-terminus of each protein are specially conserved among species [1].

Despite the high conservation of P2X subunits between vertebrates, the analysis of completely sequenced genomes of non-vertebrate model organisms like *Drosophila melanogaster*, *Caenorhabditis elegans* and *Apis melifera* show no homologues to P2X receptors [14] [15]. Previous works have hypothesized that ATP is a very early neurotransmitter in evolution of vertebrates with a single P2X receptor as ancestor [16]. Phylogeny suggests that diversification of seven P2X subunits presented in mammalians is an evolutionary event subsequent to the split between vertebrates and invertebrates [17]. There are evidences showing that non-vertebrates like *Schistosoma mansoni* have P2X homologues [18], so it's been proposed that arthropods and nematodes lose their P2X homologues later in their own evolution [17].

The increase of genomic data is available from unicellular, and simple-celled organisms have substantially improved our knowledge about the evolutionary path of purinergic transmission and P2X receptors [14], however, to date there exist no selective agonist or modulator for the P2X family with few exceptions currently under testing [19]. Because of this, we have performed a bioinformatics analysis of the coding regions and introns of the seven P2X genes being presented in human, simian, dog, mouse, rat and zebrafish. Here we report the arrangements of exon and intron sequences, considering its number, size, phase and placement; proposing some ideas about the gain and loss of exons and retention of introns. We expect that these evidences show traits, which can be used to gain insight into the primary structure of vertebrate P2X receptors and help design selective pharmacological drugs and single-subunit regulatory strategies.

2. Materials and Methods

2.1. Analysis of Genomic Sequences of P2X Receptors

Several genomic cDNA sequences encoding P2X receptors of *Homo sapiens*, *Pan troglodytes*, *Rattus novergicus*, *Mus musculus*, *Canis lupus familiaris*, *Danio rerio* and *Anolis carolinensis* for $P2X_6$ (given the absence of *Danio rerio*'s $P2X_6$ receptor) were obtained from the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov) database and only a few of them from Ensembl database (www.ensembl.org). Each of the P2X receptors sequence and Gen Bank accession no. of each organism are shown in Supplementary **Table S1**.

All the P2X genes of the organisms mentioned above were analyzed for the determination of genomic organization, including the size, gain and loss of exons, as well as intron number, size, loss, retention, placement and phase. The exon-intron organization was obtained from the analysis of the information available in the NCBI database.

Pairwise alignments were conducted in order to establish exon and intron sequence identities among species using the Needleman-Wunsch (global) and Smith-Waterman (local) alignment programs at the EBI (European Bioinformatics Institute, Cambridge, UK; http://www.ebi.ac.uk) database. Microsynteny between P2X receptor genes from the different organisms was assembled using the information present in the NCBI database chromosome image (http://www.ncbi.nlm.nih.gov/gene). Prediction of possible transposable element sequences within the P2X genes was performed using Blastn algorithm of the NCBI database.

2.2. Molecular Phylogenetic Analysis

The aminoacid sequences of the P2X receptors were aligned and the respective phylogenetic tree constructed

Table 1. Gene information of P2X subunits used in the gene structure and phylogeny analyses.

P2X Gene	Gene lenght (bp)	Gene without UTR (bp)	mRNA (bp)	Messenger without UTR (bp)	Protein (aa)	mRNA Accession	Protein Accession	Status	Chromosome
$P2X_1$									
MUS	16,053	14,812	2441	1200	399	NM_008771.3	NP_032797.3	validated	11
RNO	15,053	13,782	2539	1200	399	NM_012997.2	NP_037129.1	provisional	10
HSA	20,076	18,412	2910	1200	399	NM_002558.2	NP_002549.1	reviewed	17
CAF	NE	13,559		1200	399	XM_548344.1	XP_548344.1	predicted	9
MMU	21,173	20,436	1937	1200	399	XM_001092205.2	XP_001092205.1	predicted	16
DAR	NE	27,735		1197	398	ENSDART00000011544	ENSDARP00000010823	provisional	5
$P2X_2$									
MUS	3224	2763	1712	1251	416	NM_001164833.1	NP_001158305.1	validated	5
RNO	3195	2782	1625	1212	403	Y10473	CAA71499.1	provisional	12
HSA	3570	3156	1629	1215	404	NM_174873.1	NP_777362.1	reviewed	12
CAF	NE	2757		1185	394	XM_851798.1	XP_856891.1	predicted	26
MMU	3485	3071	1626	1212	403	XM_001082602.2	XP_001082602.2	predicted	11
DAR	14,522	14,433	1292	1203	400	NM_198983.1	NP_945334.1	provisional	5
$P2X_3$									
MUS	39,283	36,287	4190	1194	397	NM_145526.2	NP_663501.2	validated	2
RNO	42,707	40,151	3773	1194	397	NM_031075.1	NP_112337.1	provisional	3
HSA	31,601	31,446	1349	1194	397	NM_002559.2	NP_002550.2	reviewed	11
CAF	NE	25,834		1194	397	XM_540614.1	XP_540614.1	predicted	18
PTR	33,271	32,483	1982	1194	397	XM_001136930.1	XP_001136930.1	predicted	11
DARa	15,454	14,977	1724	1233	410	NM_131623.1	NP_571698.1	provisional	14
DARb	12,235	10,544	2920	1239	412	NM_198986.2	NP_945337.2	provisional	1
$P2X_4$									
MUS	21,488	20,660	1995	1167	388	NM_011026.2	NP_035156.2	validated	5
RNO	17,652	16,846	1997	1167	388	NM_031594.1	NP_113782.1	provisional	12
HSA	24,246	23,385	2043	1167	388	NM_002560.2	NP_002551.2	reviewed	12
CAF	16,697	16,185	1679	1167	388	XM_543389.2	XP_543389.1	predicted	26
PTR	25,836	24,971	2032	1167	388	XM_509437.2	XP_509437.2	predicted	12
DARa	9193	9046	1330	1170	389	NM_153653.1	NP_705939.1	provisional	21
DARb	7918	7883	1243	1206	401	NM_198987.1	NP_945338.1	provisional	8
$P2X_5$									
MUS	12,158	11,238	2293	1368	455	NM_033321.3	NP_201578.2	validated	11
RNO	11,610	10,569	2436	1368	455	NM_080780.2	NP_542958.2	provisional	10
HSA	23,063	22,138	2206	1269	422	NM_002561.2	NP_002552.2	reviewed	17
CAF	13,312	12,894	1705	1287	428	XM_548343.2	XP_548343.2	predicted	9
PTR	26,312	20,930	2195	1269	422	XM_511272.2	XP_511272.2	predicted	17
DAR	23,322	22,426	2367	1443	481	NM_194413.1	NP_919394.1	provisional	5
$P2X_6$									
MUS	10,128	8952	2362	1170	389	NM_011028.2	NP_035158.2	validated	16
RNO	10,037	8919	2331	1170	389	NM_012721.2	NP_036853.2	validated	11
HSA	12,839	11,443	2754	1326	441	NM_005446.3	NP_005437.2	reviewed	22

Continued

CAF	NE	8980		1230	410	ENSECAFT00000023919	ENSCAFP00000022204	predicted	26
MMU	NE	12,957		1317	438	XM_001084368.2	XP_001084368.1	predicted	10
ACA	NE	4774		1167	389	ENSACAT00000011065	ENSACAP00000010841	predicted	NE
$P2X_7$									
MUS	40,374	37,231	4931	1788	595	NM_011027.2	NP_035157.2	validated	5
RNO	43,128	41,366	3540	1788	595	NM_019256.1	NP_062129.1	provisional	12
HSA	53,724	51,832	3680	1788	595	NM_002562.5	NP_002553.3	reviewed	12
CAF	NE	42,756		1788	595	NM_001113456.1	NP_001106927.1	provisional	26
MMU	55,453	54,038	3203	1788	595	XM_001092531.2	XP_001092531.1	predicted	11
DAR	20,914	20,847	1860	1791	596	NM_198984.1	NP_945335.1	provisional	8
$P2X_8$									
DAR	27,994	27,989	1178	1173	390	NM_198985.1	NP_945336.1	provisional	15

Table 2. Percent identity of mouse P2X paralogous (clustal W).

		1	2	3	4	5	6	7
1	**musP2X$_1$**	x	32.3	37.5	47.4	36.1	39.3	31.8
2	**musP2X$_2$**		x	41.3	39.4	40.1	35	28.1
3	**musP2X$_3$**			x	40.5	38.3	35.2	30.5
4	**musP2X$_4$**				x	45.9	41	40.5
5	**musP2X$_5$**					x	44.7	27.3
6	**musP2X$_6$**						x	29
7	**musP2X$_7$**							x

using the software MEGA version 4.0 with the maximum parsimonia method (500 bootstrap).

3. Results and Discussion

The seven P2X subunits (1 - 7) in mammals are diverse in size and gene organization. $P2X_2$ is the smallest of the subunits, with a 2.78 Kb transcript. The longest is $P2X_7$ with 37.23 Kb (see **Table 1**). However, the ORF size of the seven subunits from the species analyzed in this work has an average of 1.3 Kb without untranslated sequences—P2X4 has the smallest transcript with 1.16 Kb and $P2X_7$ possess the longest transcript of 1.78 Kb, their aminoacid sequences are 388 and 595 residues respectively. Mainly, the difference in size between P2X subunits is related to the size of their C-terminus domains.

3.1. Genomic Organization of P2X Genes of the Mouse

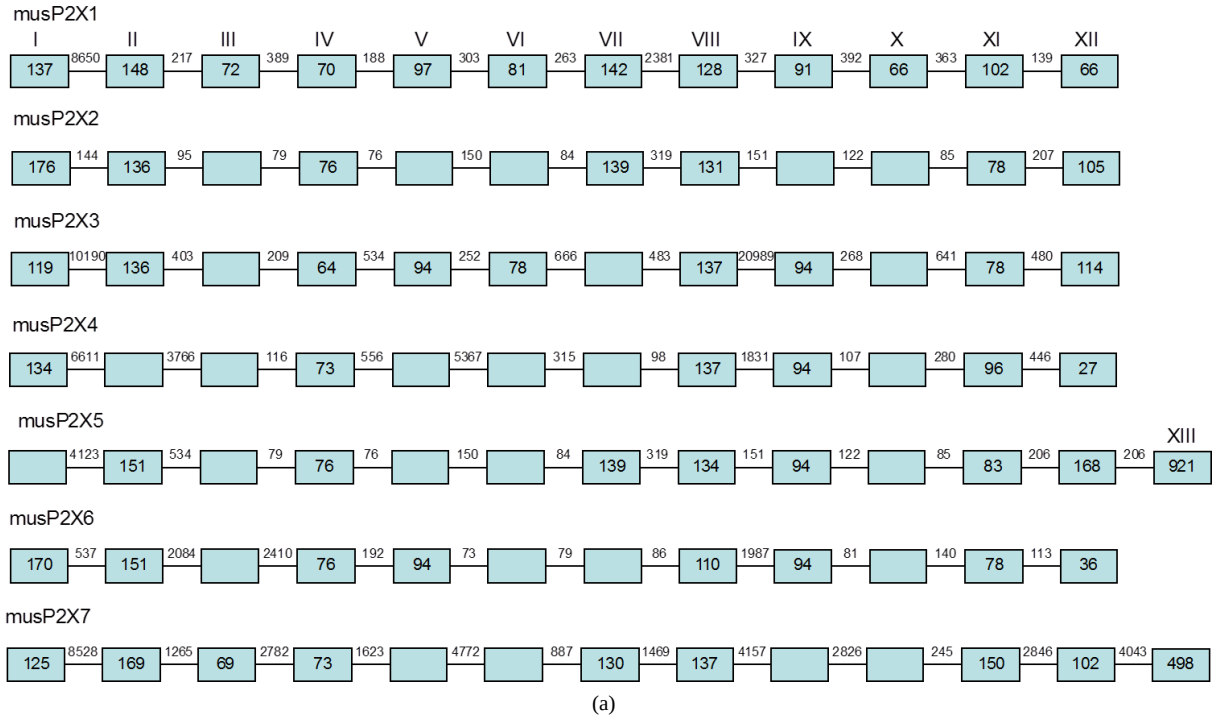
The P2X subunits of mouse consist of 12 to 13 exons and 11 to 12 introns according to the reported sequences of Gene Bank (**Figure 1(a)**). In detail, subunits $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_6$ have 12 exons and 11 introns, whereas $P2X_5$ and $P2X_7$ have an arrangement of 13 exons and 12 introns.

Despite the differences of aminoacid sequences among P2X subunits (below 50% identity, **Table 2**), exon size trends to be conserved from Exons III to X, which is the middle portion of the ORF; with exons III and X as the most conserved (most of them are 72 and 66 nucleotides long respectively), whereas the last exons are the most variable in size (**Figure 1(a)**). In counterpart, the introns have a high variability in size and sequence, this could be the cause of identity differences at genomic DNA level between P2X genes in mice. The conservation of exon size and high variability of intron length has been previously described in other gene families in previous works [20], which are evidence of evolutionary mechanisms affecting their gene structure.

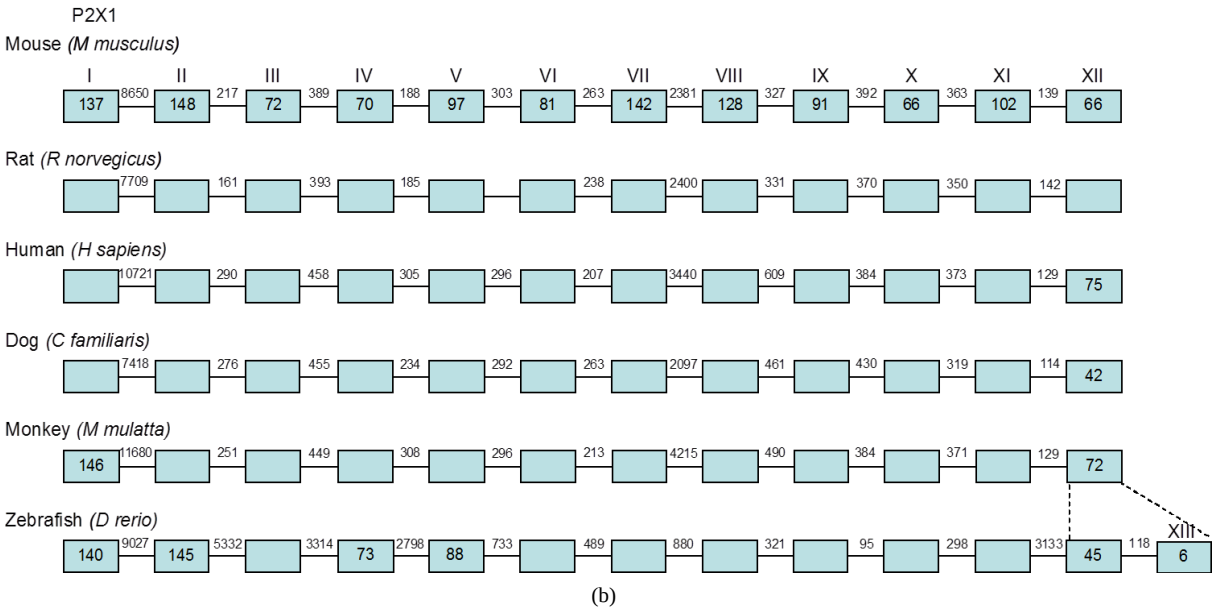
Exon III codes for 24 aminoacids located in the extracellular loop, and exon X forms the first half of the transmembrane region II, part of the channel pore [21]. In counterpart, the most variable regions of the exons of P2X subunits are those involved in traffic, receptor desensitization, cytoskeleton binding, receptor-receptor in-

teraction and regulatory proteins, which contribute to the diversity of function of the assembled P2X receptors.

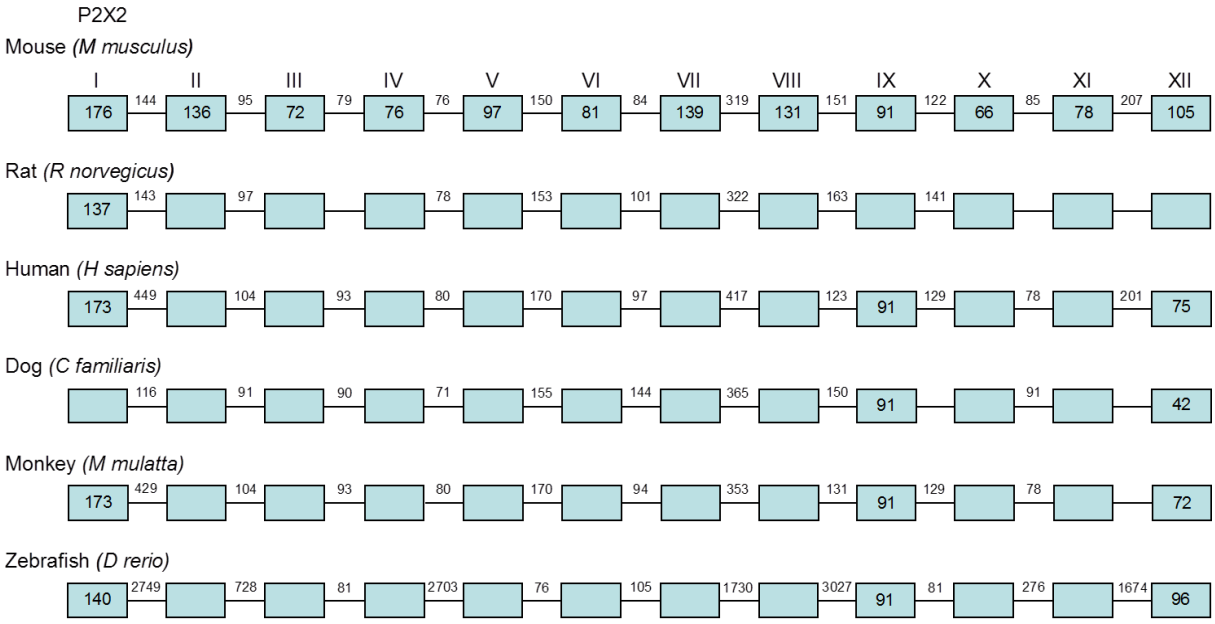
Intron size among P2X subunits in mouse is highly variable, with $P2X_2$ as the gen with the shortest introns (76 to 319 bp). Previous works showed shorter introns in constitutive genes compared to those of low expression [22] [23]. This is explained by the naturally selected gene compression since transcription and mRNA processing are slow and energetically costly processes [22] [24]. The small intron size of P2X2 correlates with its high prevalence in cells responsive to purinergic signaling like neurons of the peripheral nervous system [25]. On the other hand, subunits like $P2X_3$ seem to be present mostly at early stages of development and scarcely found in adult neurons according to other works [5] [26]-[28] and to our single cell PCR results performed in myenteric neurons of mice [29]. These results correlate with the longer size of $P2X_3$ introns in a gene that is not as widely expressed by neural cell types and does not need high efficiency transcription rates.



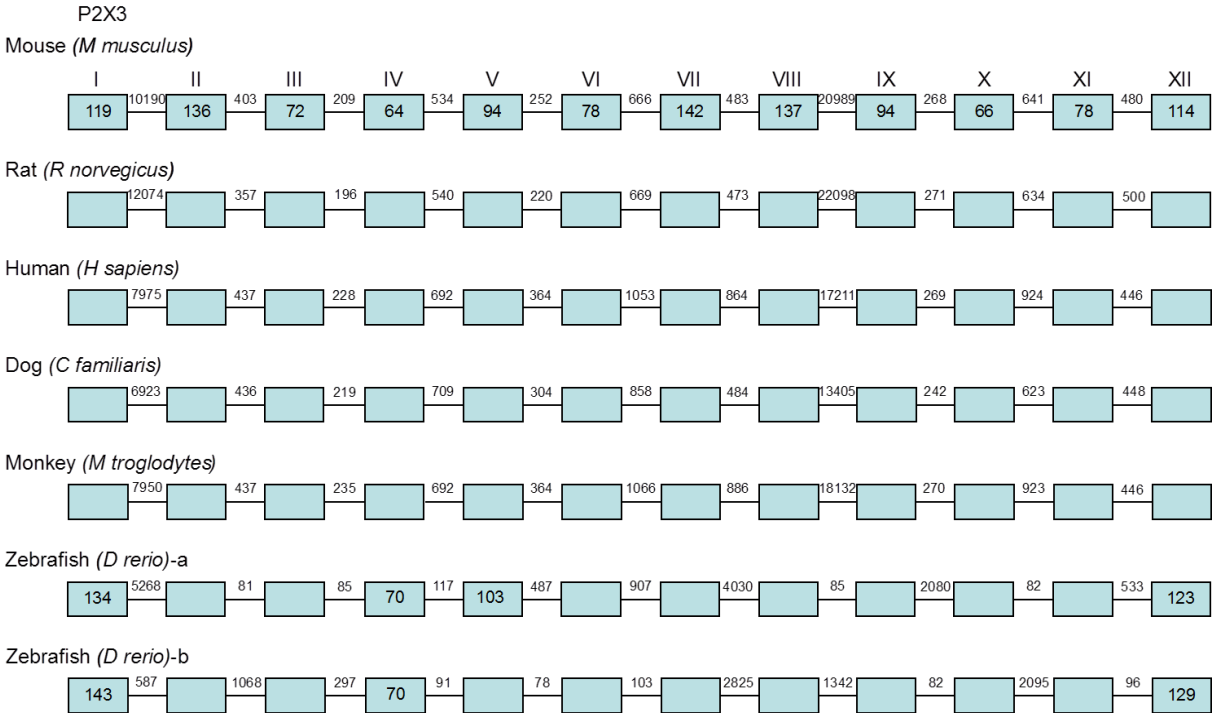
(a)



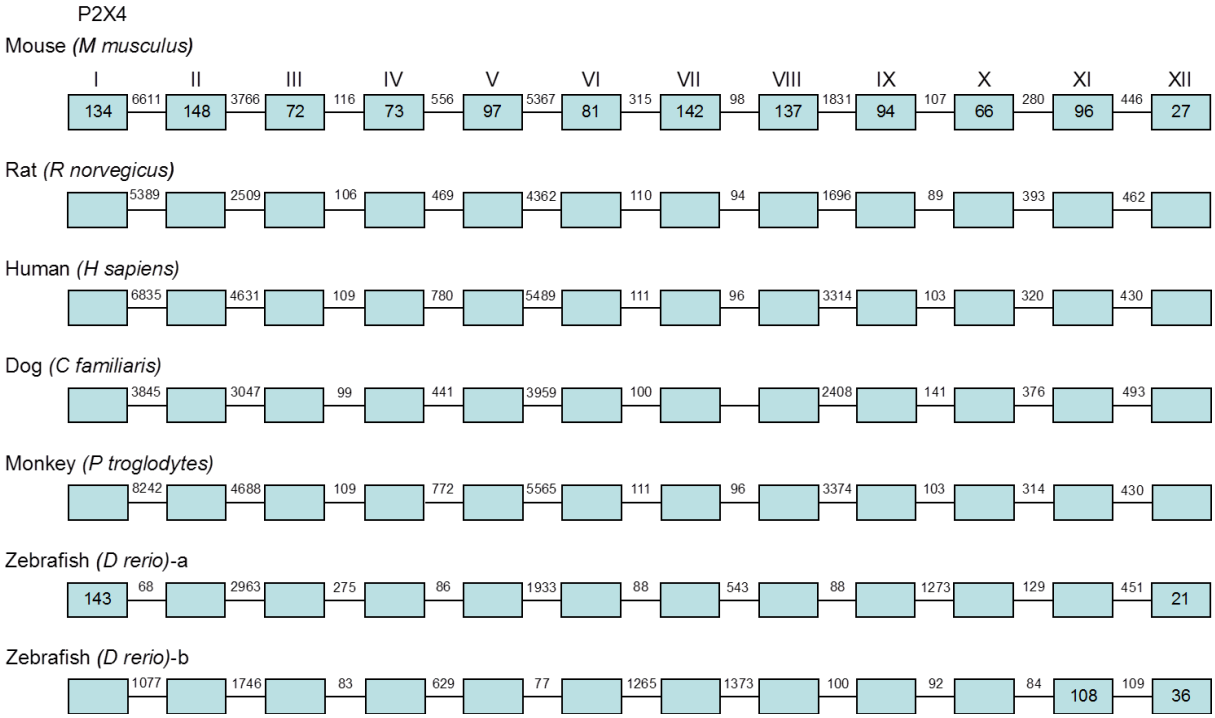
(b)


P2X2
Mouse (M musculus)
I II III IV V VI VII VIII IX X XI XII
176 144 136 95 72 79 76 76 97 150 81 84 139 319 131 151 91 122 66 85 78 207 105
Rat (R norvegicus)
137 143 97 78 153 101 322 163 141
Human (H sapiens)
173 449 104 93 80 170 97 417 123 91 129 78 201 75
Dog (C familiaris)
116 91 90 71 155 144 365 150 91 91 42
Monkey (M mulatta)
173 429 104 93 80 170 94 353 131 91 129 78 72
Zebrafish (D rerio)
140 2749 728 81 2703 76 105 1730 3027 91 81 276 1674 96

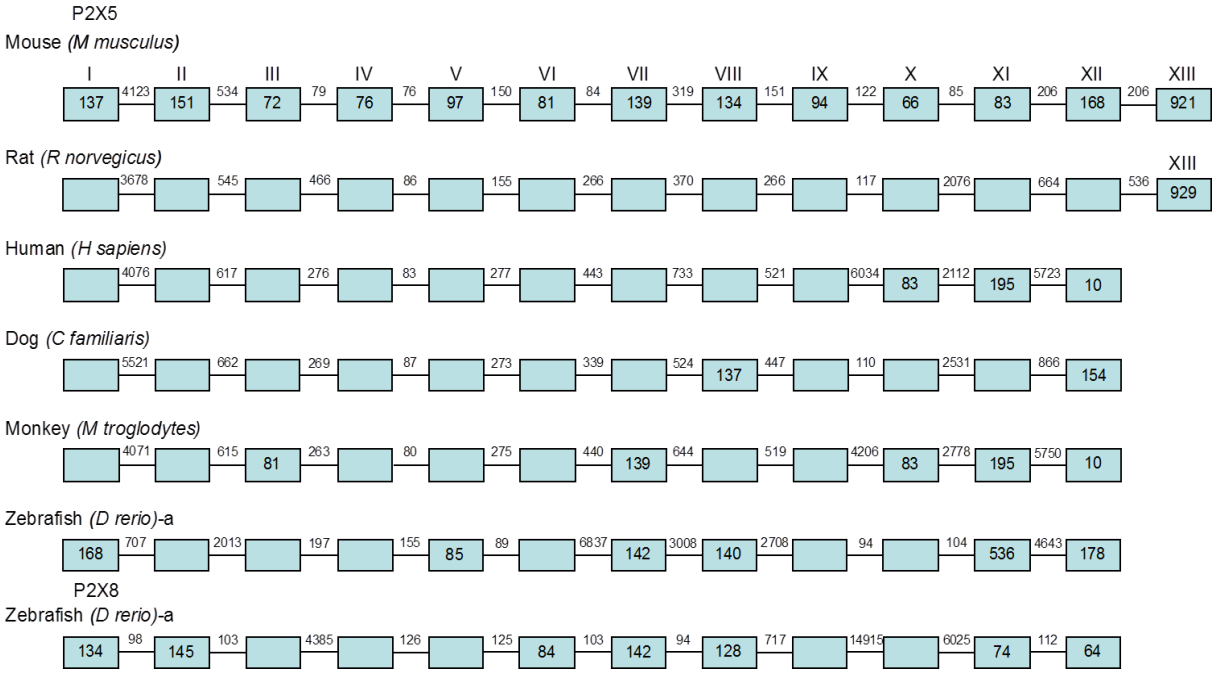
(c)


P2X3
Mouse (M musculus)
I II III IV V VI VII VIII IX X XI XII
119 10190 136 403 72 209 64 534 94 252 78 666 142 483 137 20989 94 268 66 641 78 480 114
Rat (R norvegicus)
12074 357 196 540 220 669 473 22098 271 634 500
Human (H sapiens)
7975 437 228 692 364 1053 864 17211 269 924 446
Dog (C familiaris)
6923 436 219 709 304 858 484 13405 242 623 448
Monkey (M troglodytes)
7950 437 235 692 364 1066 886 18132 270 923 446
Zebrafish (D rerio)-a
134 5268 81 85 70 117 103 487 907 4030 85 2080 82 533 123
Zebrafish (D rerio)-b
143 587 1068 297 70 91 78 103 2825 1342 82 2095 96 129

(d)


P2X4
Mouse (M musculus)
I II III IV V VI VII VIII IX X XI XII
134 6611 148 3766 72 116 73 556 97 5367 81 315 142 98 137 1831 94 107 66 280 96 446 27
Rat (R norvegicus)
5389 2509 106 469 4362 110 94 1696 89 393 462
Human (H sapiens)
6835 4631 109 780 5489 111 96 3314 103 320 430
Dog (C familiaris)
3845 3047 99 441 3959 100 2408 141 376 493
Monkey (P troglodytes)
8242 4688 109 772 5565 111 96 3374 103 314 430
Zebrafish (D rerio)-a
143 68 2963 275 86 1933 88 543 88 1273 129 451 21
Zebrafish (D rerio)-b
1077 1746 83 629 77 1265 1373 100 92 84 108 109 36

(e)


P2X5
Mouse (M musculus)
I II III IV V VI VII VIII IX X XI XII XIII
137 4123 151 534 72 79 76 76 97 150 81 84 139 319 134 151 94 122 66 85 83 206 168 206 921
Rat (R norvegicus)
XIII
3678 545 466 86 155 266 370 266 117 2076 664 536 929
Human (H sapiens)
4076 617 276 83 277 443 733 521 6034 83 2112 195 5723 10
Dog (C familiaris)
5521 662 269 87 273 339 524 137 447 110 2531 866 154
Monkey (M troglodytes)
4071 615 81 263 80 275 440 139 644 519 4206 83 2778 195 5750 10
Zebrafish (D rerio)-a
168 707 2013 197 155 85 89 6837 142 3008 140 2708 94 104 536 4643 178
P2X8
Zebrafish (D rerio)-a
134 98 145 103 4385 126 125 84 103 142 94 128 717 14915 6025 74 112 64

(f)

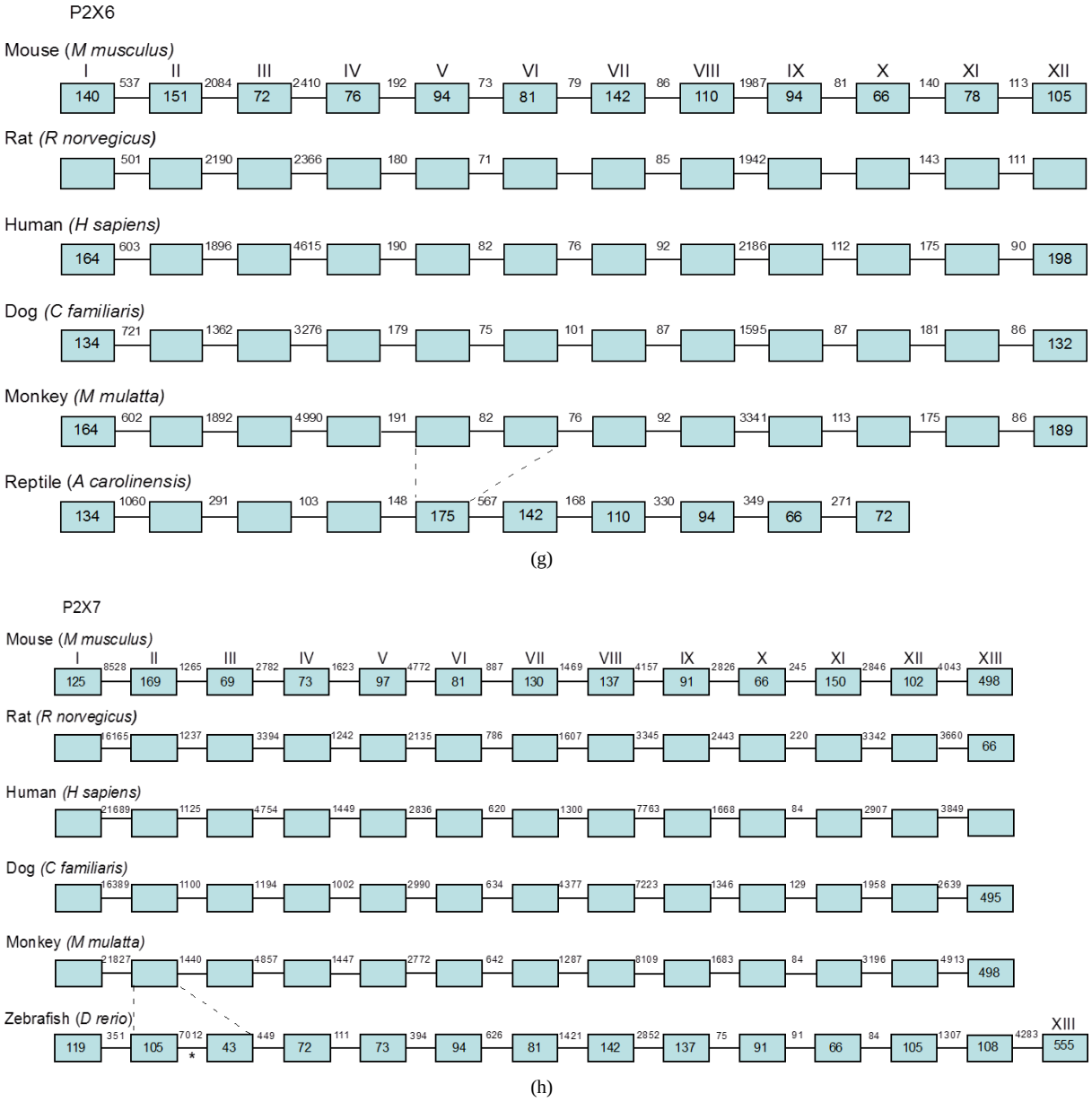


Figure 1. Schematic representation of the genomic organization of P2X receptors in several organisms. Exons are depicted as boxes with roman numbers on top, while introns are represented as solid lines. Numbers inside exons indicate the size in base pairs (bp) as well as number on top of the solid lines represent the intron size in bp. The first gene belongs to mouse in all cases and the lack of label below a given exon represents the conservation of size between homologous genes. (a) Genomic organization of the seven P2X subunits of mouse (Mus musculus); (b)-(h) Genomic organization of the P2X subunits compared between orthologous. When indicated, a dashed line points to exon fusion or exon separation between orthologous P2X genes.

Intron I is often referred to contain expression enhancers and other regulatory elements in mammals [30]-[32], this is the case of the purine nucleoside phosphorylase, where short portions of the intron 1 (around 170 bp) provided enhanced transcription in mammalian cell culture expression systems. In P2X orthologs (**Figures 1(b)-(h)**), intron I size and sequence it's also conserved, possibly pointing to unidentified regulatory elements in P2X genes.

3.2. Genomic Organization of P2X Orthologous Genes

To determine how P2X gene orthologous have been conserved in evolution among species, we analyzed the ge-

nomic organization of the seven P2X subunits of different mammalian species, including mouse (musP2X), rat (rnoP2X), primates (mmuP2X for Macacamulata and ptrP2X for Pan troglodytes), human (hsaP2X) and dog (cafP2X). Additionally, we included in this study the zebrafish (darP2X for *Dario rerio*) from the family Cyprinidae from the class Actinopterygii, as ancestral species of mammals. In zebrafish, nine P2X genes have been reported: $P2X_1$ to $P2X_5$ and $P2X_7$ which are orthologous to mammal subunits. Another two paralogous of $P2X_3$ and $P2X_4$ named $P2X_3$b and $P2X_4$b, and $P2X_8$ from which there are no reported orthologous in mammals [33]-[35]. From our phylogenetic tree, we could propose that $P2X_8$ is an orthologous gene to $P2X_5$, although further functional and pharmacological evidence could uncover more similarities between these two subunits.

When we analyzed the exon size of P2X gene orthologous, we found a high conservation among the different species of mammals, whit some exceptions in the first and last exon (**Figure 1**). Also the zebrafish showed variability in the size of some exons compared to P2X subunits in mammals. On the contrary, intron size is variable in each ortholog of P2X subunits, however, the size of introns trend to be better conserved between the orthologous of a specific P2X subunit (**Figures 1(a)-(h)**). The zebrafish again presents the most variable arrangement of introns compared to the other species. For most introns, the identity percentage was not significant, but in rare events the identity rate was up to 70% (**Table 3**). Additionally, we discovered that P2X subunits have a conserved intron phase among musP2X paralogs (**Table 4**). Among orthologous, P2X genes conserve their intron phase as well, however, some changes do occur in $P2X_5$ and $P2X_7$, overall in the 3' region of introns (**Table 4**). Thus, we found that, in general, introns I, V, VI and VIII are in phase two, whereas exons II, III, VII, IX, X, XI and XII end up in phase zero. Only exon IV appear in phase one.

Some P2X paralogous are found in the same chromosome, like $P2X_1$ and $P2X_5$; $P2X_2$, $P2X_4$ and $P2X_7$ showing syntenic traits. On **Figure 2** we show the blocks of syntenic P2X genes in mouse and human. The comparison with all the analyzed species is shown in Supplementary **Table S1**. The genes $P2X_1$ and $P2X_5$ conform a block of syntenic genes between mouse and human with opposite orientation. In a similar way, $P2X_4$ and $P2X_7$ are syntenic between all orthologs. In the zebrafish case, with two different $P2X_4$ genes, only $P2X_4$b keeps synteny with $P2X_7$ (Supplementary **Table S1**), and the $P2X_4$a is different than b and other orthologous regarding its chromosome location. We also found synteny for $P2X_2$ orthologs but these are located further away from $P2X_4$ and $P2X_7$ (**Figure 2**). In the case of $P2X_3$, the genes in positions 1, 2, -1 and -2 are conserved completely in the mouse and human, however, the orientation is inversed. In zebrafish, $P2X_3$a and b keep the same microsynteny than their orthologous. Genes that keep synteny with $P2X_6$ conserve order as well as orientation.

The ortholog genes $P2X_{1\text{-}7}$ are much conserved at the protein level, most of all between rat and mouse or between human and primate (**Table 3**). The identity percentages (Clustal W, Slow/Accurate, Gonnet) between rat and mouse range from 85% ($P2X_7$) to 99% ($P2X_3$). On a similar way, between human and primate, identities range between 97% ($P2X_4$) and 100% ($P2X_7$). The lowest identity percentages are found between mammals and zebrafish, with values around 50% in most cases. These results are in accordance with the phylogenetic tree (**Figure 3**) where rodents and primates are closer to each other and farthest from zebrafish.

It has been previously described that orthologous genes trend to conserve their intron position compared with non-orthologous genes, even when orthologous sequence identity is low [36]-[38]. We found low percentage of protein identity between $P2X_{1\text{-}7}$ from zebra fish regarding their respective mammalian orthologous. However, even when zebrafish is an evolutionary distant organism, it trended to conserve certain characteristics such as exon-intron organization and intron position with its mammalian counterparts, which makes evident the sharing of common ancestry in P2X evolution (**Figure 3**). The main difference between fish P2X genes and mammalian was centered in the size of introns, indicating some re-organization in exon-intron position after mammalian divergence.

In zebrafish, two paralogous genes for $P2X_3$ and $P2X_4$ have been reported with distinctive localization and genomic organization. Several lines of evidence have suggested that whole genome duplications occurred before the vertebrate/ascidian divergence [39] and, later on, in the lineage of teleostheus after tetrapod divergence where only one set of these duplicated genes were maintained [40]-[43]. This is supported by the existence of several duplicated segments in zebrafish chromosomes [44]. The two genes darP2X$_3$a and b seem to be the result of this duplication, since they are located in a cluster of duplicated genes found in different chromosomes and conserves synteny with their orthologous (Supplementary **Table S1**). In darP2X$_4$a and b there is no conservation of gene duplicates, each $P2X_4$ is located in a different chromosome near single copy genes. $P2X_4$b is close in position to darP2X$_7$, but that is not the case of darP2X$_4$a, therefore, it is possible that $P2X_4$a was origin-

Table 3. Global (needle) and local (water) alignments of P2X subunits gene orthologous in ebi's align software.

$P2X_1$	Mouse vs X (needle/water)					
	Rat	Human	Dog	Monkey	Zebrafish	
Intron I	69.9/69.8	44.8/43.9	45.7/45.3	44.4/43.6	42.3/41.1	
II	66.5/67	52.8/53.2	54/54	57.7/59.2	2.8/42.1	
III	87.6/87.6	58.2/55.5	52.4/49.3	55.7/54.3	6.9/39.7	
IV	86.8/86.8	44/44.4	47.8/47.3	42.9/43.7	4/42.9	
V	78.3/78.3	56.9/57.4	57/56.3	57.1/57	21.2/40.2	
VI	78/78.7	50.7/50.2	54.5/53.2	52/50.5	28.8/36.8	
VII	81.5/80.9	40.9/40	42.3/42.3	33.4/34	21.2/41.1	
VIII	81.3/83.7	33/50.2	46.2/48.3	21.7/47.1	40.9/44.7	
IX	79.8/79.5	60/58.6	54.3/52.2	58.8/59.1	14.7/45.8	
X	86.3/86.5	63.4/62.2	54.9/53.1	62.7/58.4	38.8/36	
XI	84.5/84.5	54.5/59.1	54.4/53.6	57/57.2	2.7/46.3	
$P2X_2$	Mouse vs X (needle/water)					
	Rat	Human	Dog	Monkey	Zebrafish	
Intron I	89/90.3	21.3/46.5	54.8./57.8	22/53	3.6/30.5	
II	92.9/94.8	55.9/55.9	48.7/49.6	56.8/57.8	7.2/48.1	
III	87.7/89.9	53.9/50.9	51.1/48.1	51.9/50	43.5/48.6	
IV	90/92.3	52.1/52.8	40.8/55.7	53.7/55.7	2.1/35.5	
V	82.4/83.4	42.1/49.7	44.9/45.4	47.8/48.5	31/54.7	
VI	67.6/68.9	43.1/49.1	36.2/44.4	43.9/49.5	37.5/51.8	
VII	87.3/87.9	46/46.5	50.5/52.1	50.4/50.6	10.6/48.2	
VIII	75.8/76.7	38.6/49.7	40.3/42.1	44.4/53.6	3.5/37.2	
IX	75.5/76.6	47.2/47.2	37.4/52.6	49.3/49.3	33.6/43.2	
X	82.8/84.7	57.6/58.8	55/57.1	58.5/61.2	21.1/46.5	
XI	93.8/95.6	69.4/70.6	51.6/52.8	70.1/70.8	8/32.8	
$P2X_3$	Mouse vs X (needle/water)					
	Rat	Human	Dog	Monkey	$Zebrafish_a$	$Zebrafish_b$
Intron I	56.7/61	40.2/48.7	35.5/42.9	40.9/48.9	28.7/37.7	3.2/38.4
II	75.6/75.8	52.6/52.7	34.1/54.4	53.3/56.5	13.7/34.1	23.7/33.3
III	81.5/85.9	58.9/63.8	23.8/35.6	54.1/58.3	18.3/48.4	14.1/29.9
IV	79.7/80.4	49.1/49.4	44.6/44.6	49.6/49.9	13.2/46.2	8.8/36.7
V	78/78.3	48.9/48.9	44.5/49.2	48.9/48.9	26.4/40.1	20.5/47.8
VI	85.5/85.7	41.3/41.3	48.9/50.6	41.2/41.2	38.4/38.5	9.6/39.3
VII	80.1/80.3	36.2/52.5	45/47.4	38/48.6	7.9/38.8	10.8/40.1
VIII	68.7/68.8	43.7/48.2	39.4/43.7	40.8/44.8	0.3/58.2	4.1/38.6
IX	90.5/90.8	65.1/65.1	51.4/51.6	0.7/51.4	6.9/37.5	15.1/40
X	82.5/82.6	44.8/45.4	51.1/52.3	43.4/46.5	9.1/44.5	19.6/35.5
XI	74.6/74.8	55.5/55.5	50.2/50.2	55.1/55.1	40.9/44.5	12.5/45.5
$P2X_4$	Mouse vs X (needle/water)					
	Rat	Human	Dog	Monkey	$Zebrafish_a$	$Zebrafish_b$
Intron I	55.2/54.6	40.4/46.1	34.5/43.7	41.2/40.3	0.7/46.7	9.7/43.3
II	41.1/41.6	29/40	42.7/44.1	30/39.7	43.3/43.5	39.7/41.6
III	71.2/71.2	63.9/63.6	55.6/56	59.3/55.8	25.7/39.6	41.9/48.4
IV	69.3/69.8	42.5/43.9	49.7/50.1	43.4/42.6	9.6/43.2	44.5/44.5
V	62.2/61.4	39.6/46.6	42.0/42.0	39.4/43.2	22.4/35.5	0.9/40.0
VI	24.9/30	23.9/45.9	22.9/37.4	22.7/46	16.6/39.6	17.3/33.6
VII	65.2/66.1	54.1/56.8	55.4/56.4	52.5/54.2	12.1/34.2	4.5/53.0

Continued

VIII	59.4/62.4	34.4/36.8	44.4/44.7	35.5/36.3	3.0/43.7	3.8/48.9
IX	72.5/72.5	51.9/49.6	46.6/48.6	53.2/51.9	6.0/42.7	50.5/50.9
X	54.7/54.6	50.3/50	45.6/45.9	52.9/50.1	27.3/39.3	18.0/42.6
XI	77.2/77	49.2/52.2	51.2/54.4	71.4/48.7	44.6/44.8	16.0/41.1
$P2X_5$	Mouse vs X (needle/water)					
	Rat	Human	Dog	Monkey	Zebrafish	P2X8 Zebrafish
Intron I	65.1/64.5	47/44.4	42.3/41.3	44.5/44.5	10.5/40	1.8/37.4
II	83.5/83.3	57.7/56.8	53.6/51.9	56.9/56.9	16.7/68.4	13.0/36.0
III	69.6/69	30.3/52.7	29.3/38.1	29.4/54.4	17.8/40.9	8.0/34.0
IV	84.9/84.9	68.5/68.5	63.2/64.2	68.5/68.5	27/66.7	28.6/38.4
V	80.7/80.7	38.9/42.8	40.5/50.2	43/42.8	32/35.8	27.0/35.7
VI	84.9/84.9	41.7/42.8	54.1/50.7	40.7/41.3	2.4/31.9	22.8/34.1
VII	71.5/74.1	42.2/42.3	50.8/48.3	47.2/46.1	9.1/37.3	13,1/36,8
VIII	76.4/76	33.5/53	30.8/41.3	33.5/46.6	20.2/45.5	21.5/37.2
IX	87.1/87.1	1.3/50.3	2.7/56.7	2.1/45.5	2.8/36.4	0.6/37.7
X	70.4/70.2	43.1/41	4.4/39.6	33.3/39.7	2.5/59.8	22.4/38.9
XI	68.5/68.7	7.0/38	43.3/45.8	6.9/38.1	10.8/48.1	10.2/43.9
XII	83.1/83.1	NA	NA	NA	6.6/40.5	
$P2X_6$	Mouse vs X (needle/water)					
	Rat	Human	Dog	Monkey	Zebrafish	Rat
Intron I	78.6/79.7	52.2/52.5	48/47.4	52.5/50.4	31.9/33.8	78.6/79.7
II	67.8/67.9	46.9/46.8	40.5/39.7	47.3/46.2	8.7/38	67.8/67.9
III	81/80.8	36.9/37.3	45.7/44.9	32.4/36.6	2.9/48.5	81/80.8
IV	77/78.9	66.5/64.1	50.5/57.1	62.6/58.7	50.5/46.4	77/78.9
V	86.5/86.5	55.1/54.7	46.7/56.6	53.4/54.1	-	86.5/86.5
VI	88.6/88.6	55.3/56.2	43.4/59.5	52.6/52.9	10/43	88.6/88.6
VII	86.2/86.2	65.6/70.5	64.1/65.1	64.9/70.5	29.1/50.6	86.2/86.2
VIII	63.8/63.6	46.6/42.3	44.8/42.8	39.4/38.6	10.2/42.8	63.8/63.6
IX	88.9/88.9	49.6/52.4	62.9/62.9	49.1/53.3	11.4/45.8	88.9/88.9
X	68.8/75.9	55.9/57.6	55.2/52.5	58.9/58.2	35.1/44.5	68.8/75.9
XI	81/80.5	57.3/72.2	53.8/54.7	55.6/70	-	81/80.5
$P2X_7$	Mouse vs X (needle/water)					
	Rat	Human	Dog	Monkey	Zebrafish	Rat
Intron I	-	-	54.2/60.2	-	2.8/41.8	-
II	83.1/83.3	56.2/62.4	25.5/48.9	47.4/50.8	20.8/39.4	83.1/83.3
III	63.7/63.9	35.8/45.3	39/39.7	35.7/46.9	2.6/53.9	63.7/63.9
IV	54.7/54.7	41.6/41.6	32.2/42.6	45.6/42.2	14.1/36.6	54.7/54.7
V	40.7/41.4	35.2/35.9	42.9/42.9	34.2/35.1	23.4/36.9	40.7/41.4
VI	70.2/70.2	45.9/45.9	22.4/33.6	47.6/43.5	36.5/38.4	70.2/70.2
VII	68.7/69	46.6/46.6	38/38	49.4/46.2	30.8/39.4	68.7/69
VIII	59.2/59.2	20.2/40	27.3/44.9	28.5/44.4	1.3/46.2	59.2/59.2
IX	59/59	29.2/45.9	25/43.8	29.3/45.4	2.1/39.3	59/59
X	69/69	21.1/60.4	38.1/40.1	23.8/52.8	18.4/46.6	69/69
XI	50.8/53.5	43/40.8	40/40.1	37/34.9	28.9/34.5	50.8/53.5
XII	64.1/63.8	44.6/41.9	39.9/41.6	43.7/41.7	22.2/36.7	64.1/63.8

Table 4. Intron phase of P2X paralogous of mouse.

Intron #	Intron Phase (at the end of intron)						
	musP2X$_1$	musP2X$_2$	musP2X$_3$	musP2X$_4$	musP2X$_5$	musP2X$_6$	musP2X$_7$
1	2	2	2	2	2/0[a]	2	2
2	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
4	1	1	1	1	1	1	1
5	2	2	2	2	2	2	2
6	2	2	2	2	2	2	2
7	0	0	0	0	0	0	0
8	2	2	2	2	2	2	2
9	0	0	0	0	0	0	0
10	0	0	0	0	0/2[b]	0	0/1[e]
11	0	0	0	0	2	0	0/1[e]
12					2		

[a]Phase zero only in darP2X$_5$. [b]Phase two only in hsaP2X$_5$ and ptrP2X$_5$. Phase 1 in darP2X$_7$.

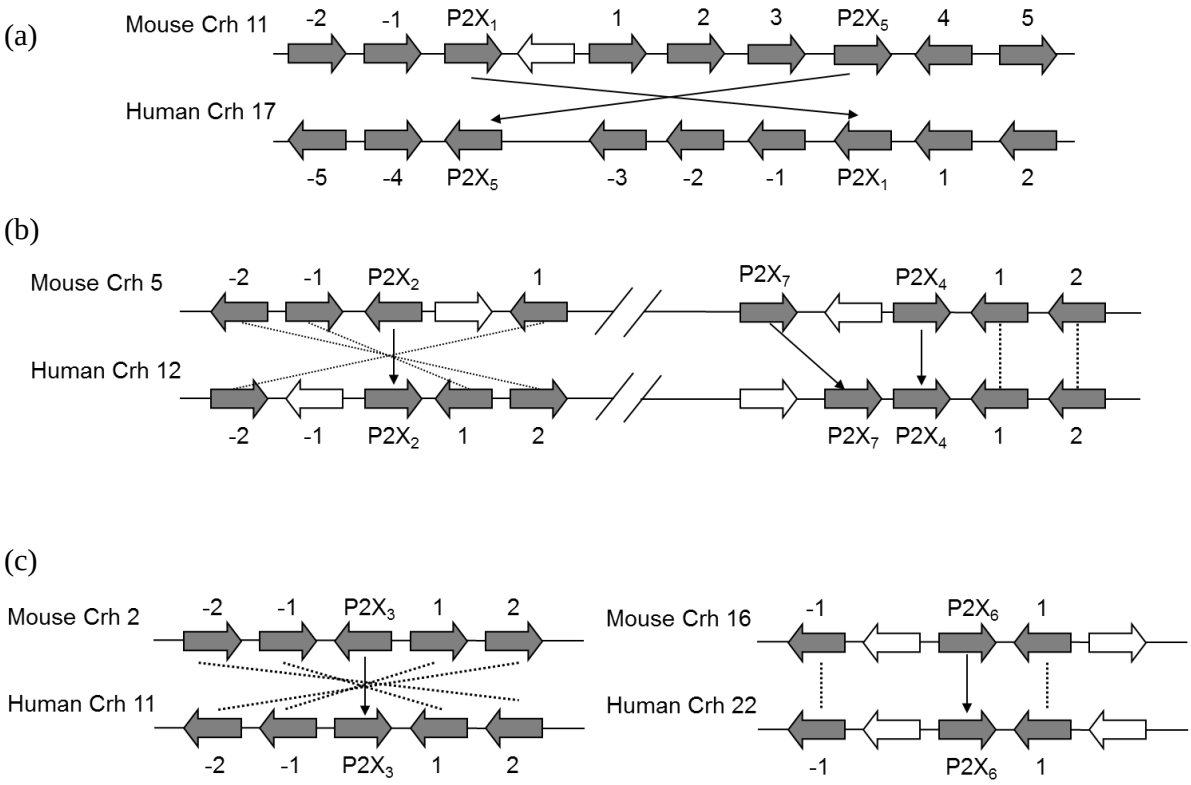


Figure 2. Microsynteny of P2X genes between mouse and human. The coding genes in chromosomes are depicted as filled arrows, while white filled arrows represent non-syntenic genes between mouse and human. The mouse was used as reference to catalog neighbor genes either upstream (negative numbers) or downstream (positive numbers) of the first P2X subunit found in the Watson DNA chain. Arrowhead lines represent the changes in P2X positions and dashed lines shows the changes in position of the neighbor genes. Gene Bank names for neighbor genes are shown in Supplementary **Table S1**. (a) Microsynteny of P2X$_1$ and P2X$_5$ genes. Schematic representation of the location of P2X$_1$ and P2X$_5$ in human and murine chromosomes respect to their chromosomic environment. Change in chromosome localization, sense of transcription and microsynteny of two genes for P2X$_1$ and five genes for P2X$_5$ can be observed; (b) Microsynteny of P2X$_2$, P2X$_4$ and P2X$_7$. Inversion of transcription sense and conservation of upstream genes -1 and -2 is shown, whereas genomic context is highly conserved for P2X$_4$ and P2X$_7$; (c) Microsynteny of P2X$_3$ and P2X$_6$. For P2X$_3$ inversion is observed in the whole chromosome context of the four neighbor genes. For P2X$_6$ genomic context is highly conserved for two genes on both sides.

nated in an independent duplication event explaining the lack of synteny in this gene.

We also analyzed the P2X orthologous intron phase to look for clues about the common ancestor of these genes as has been done elsewhere [45]. We found that intron phase is conserved in paralogous as well as in orthologous, with the zero phase as the most common, followed by phase two and phase one. Phase zero is the most common between mammalian orthologous and it's frequently found at the 3' region of a given gene [46]-[48]. Phase two is often referred as least common in gene arrangements; however P2X genes present this phase with a significant frequency. The implications of this phase conservation can be directly related to the allowance of functional variability. This is also correlated with the presence of phase zero in the conserved regions coding for transmembrane domains and C-terminus, which play significant roles in function and regulatory activity. Higher variability in exons coding for the extracellular domains could allow the evolution of regions affecting ligand affinity and gating.

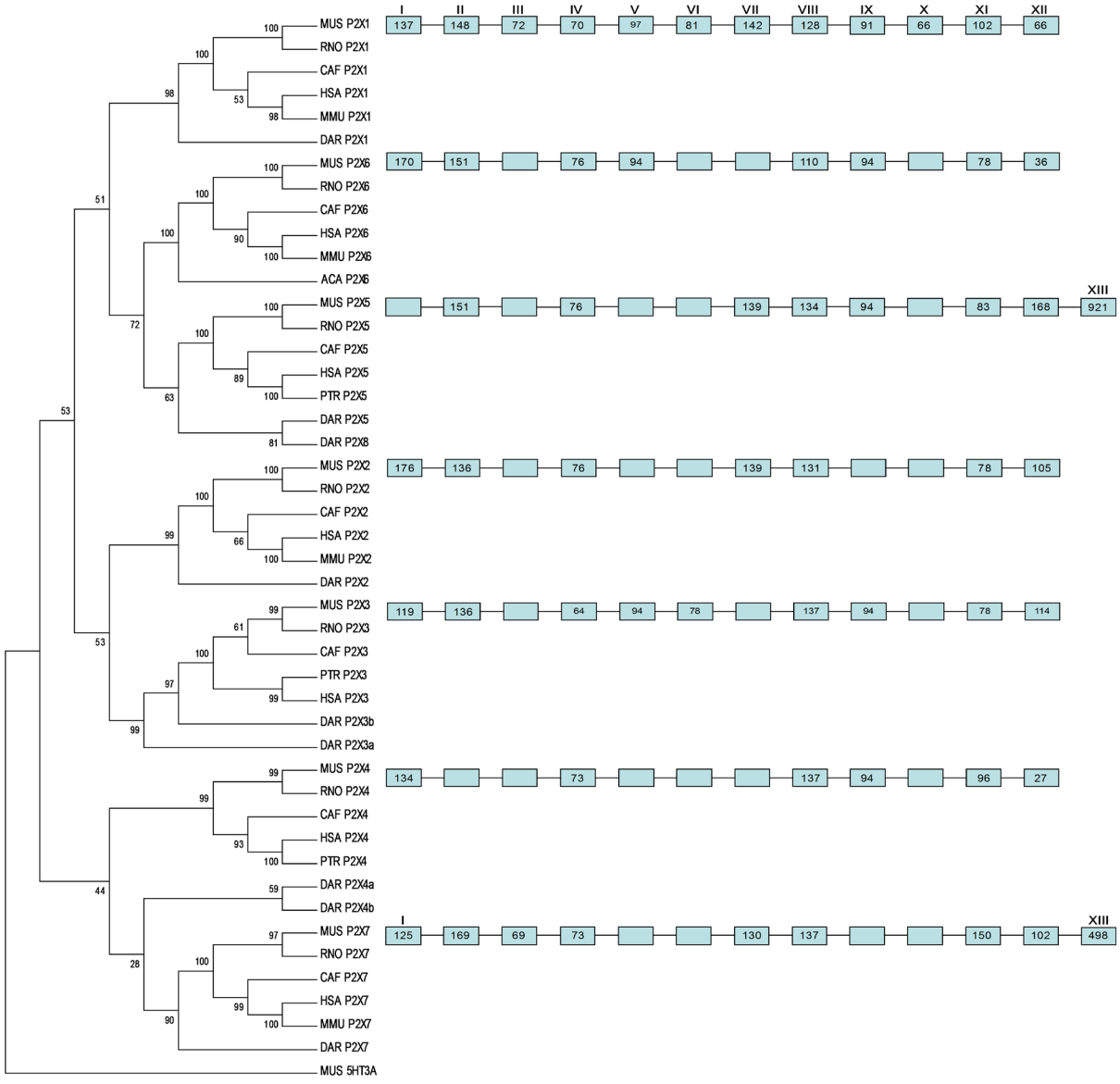


Figure 3. Phylogenetic tree representing P2X subunits from different organisms. Mus musculus (MUS), Rattusnorvegicus (RNO), Cannisfamiliaris (CAF), Homo sapiens (HSA), Macaccamulata (MMU), Danio rerio (DAR), Pan troglodytes (PTR). 5HT3 receptor from mouse was used as external gene to perform the alignment using the MEGA software version 4.0 with the maximum Parsimonia method (500 bootstrap). Numbers on the branches shows evolutionary distance represented as number of substitutions per residue. On the first branch of every clade the corresponding P2X subunit (1 to 7) gene is depicted.

In the next section, we describe particular characteristics of genomic organization for every P2X gene.

3.2.1. $P2X_1$

Mouse $P2X_1$ gene has a size of 16.05 Kb and mRNA of 1200 bp, which produces a protein of 399 aminoacids. The gen is organized in 12 exons and 11 introns. This organization is conserved among its mammals orthologous (rat, human, dog and primate) and differs with zebrafish organization, which has 13 exons and 12 introns (**Figure 1(b)**).

The size of $P2X_1$ exons is fully conserved in mammals, while conservations is sound only with exons III, VI, VII, IX and X of zebrafish (**Figure 1(b)**). An additional exon is present in $darP2X_1$ (exon XIII) with only 6 bp, from which only one aminoacid is coded together with the STOP codon.

$P2X_1$ introns are more divergent in size as well as in identity between the analyzed sequences. The first intron is the largest with >7400 bp in all the analyzed species. Using the Align algorithm (European Bioinformatics Institute) in its global (needle) and local (water) configuration we found identity values shown in **Table 3**. We showed that rat and mouse introns have the higher global identity (>66%) and intron IV has the highest unitary identity (87.6% in both needle/water modes). The zebrafish $P2X_1$ introns had the lower identity percentage (below 42.3% needle).

3.2.2. $P2X_2$

The $P2X_2$ gene of mouse is located in chromosome 5 and is characterized for being the shorter of mammal P2X genes (around 3 Kb, **Table 1**), mRNA without untranslated regions is 1248 bp long coding for a 416 aminoacid protein. $P2X_2$ gene was originally described by Brandle in 1997 with an organization of 11 exons and 10 introns, according to the NCBI reference $P2X_{2\text{-}1}$ (NM_053656). In previous work from our group we reported that $P2X_{2\text{-}2}$ isoform is actually the primary $P2X_2$ transcript and not the $P2X_{2\text{-}1}$ subunit as initially assumed. Based on this report we established the genomic arrangement of guinea pig $P2X_2$ as formed by 12 exons and 11 introns.

Since $P2X_{2\text{-}2}$ isoform is expressed in all mammals where splicing studies have been done (namely, mouse, rat and human), we have extrapolated the guinea pig model to the rest of species and confronted it with the genomic arrangement of zebrafish $P2X_2$ (**Figure 1(c)**). The addition of an exon in this 12 exon-11 intron arrangement is given by the separation of the last exon into two new exons (XI and XII) separated by an intron. To identify the donor and acceptor sites in this intron we use Net Gene2 algorithm (www.cbs.dtu.dk/services/NetGene2/) with all the analyzed $P2X_2$ gene sequences. In all genes we found a donor site with high confidence level at the beginning of the site where intron 11 is located. In the same way, we found an acceptor site in the same intron in human and guinea pig. For mouse and rat the site could be easily identified using the GT/AG rule. These sites support the existence of the intron 11 between exons XI and XII with a size of 91 or 206 bp, depending on the species (**Figure 1(c)**).

Phylogenetically, $P2X_2$ is closer to $P2X_3$ (**Figure 3**), which is reflected also in the conservation of the genomic arrangement of 12 exons and 11 introns between these paralogous. The same order is maintained among the orthologous of $P2X_2$, even with the more distant zebrafish (**Figure 1(c)**). With the exception of exons I, IX and XII, all exons conserve their size, including exon XI of 78 bp shared entirely by $P2X_3$ (**Figure 2**).

On its part, $P2X_2$ introns are less conserved and are characterized for their small size, however, introns size in zebrafish $P2X_2$ are variable, ranging from small (3, 5 and 9 of 81, 76 and 81 respectively) to large introns (intron 8 is 3027 bp). This contrasts with mammalian $P2X_2$ genes with no intron larger than 450 bp. In nucleotide sequence, the better conserved, both globally and locally regarding mouse are rat's introns 1 (89/90.3), intron 2 (92.9/94.8), intron 4 (90/92.3) and intron 11 (93.8/95.6). With the rest of orthologous the identity are equal or lower than 70%.

We have suggested that genomic organization of $P2X_2$ in mammals is composed of 12 exons and 11 introns, such as it's been displayed in Ensembl and fast DB databases. This model is based in the evidence that $P2X_{2\text{-}2}$ or $P2X_2b$ is the only mammal homologous to zebrafish $P2X_2$, which have P2X duplications rather than reductions in gene number. Additionally we observed that $P2X_{2\text{-}2}$ genomic arrangement is conserved between mammalian orthologous and the distant zebrafish. In the same way, this isoform is more close to the paralogous $P2X_3$, indicating our proposed genomic organization has a better evolutionary meaning than the previously proposed model.

We also observed that mammalian $P2X_2$ size is smaller than zebrafish $P2X_2$, showing a large variation in in-

tron size. This suggests that $P2X_2$ went over a shortening of introns that could have conferred a regulatory function in a similar way to some constitutive genes [22] [24]. All the intron phases are conserved in $P2X_2$ orthologous, suggesting that no major genomic re-arrangements have occurred. This is supported by functional evidence of our laboratory, where $P2X_2$ expression is sustained in myenteric neurons during embryonic development and to adulthood, implying the functionality of the subunit in a range of physiological events.

3.2.3. $P2X_3$

The murine $P2X_3$ receptor has a genomic size of 39.2 Kb and a mRNA of 1.4 Kb, coding for a 397 aminoacids protein. Its chromosomal localization is shown in **Table 1**. The genomic organization of $P2X_3$ is shown in Fig. 1D with an arrangement of 12 exons and 11 introns, which is conserved among orthologous.

The aminoacidic identities of $P2X_3$ between mouse and its orthologous were the highest of all P2X genes (higher than 93%). From the two $P2X_3$ genes in zebrafish, $darP2X_3b$ had the highest identity with mammalian $P2X_3$ (68% with Clustal W), while $darP2X_3a$ had 57% identity. When we compared $P2X_3a$ with $P2X_3b$, we found 58% of identity. These results are in agreement with the phylogenetic tree shown in **Figure 3**, where $darP2X_3b$ is closer to mammalian $P2X_3$ genes.

Figure 1(d) shows the high conservation between the mammalian orthologous, only some differences appear in exons I, IV, V and XII of zebrafish $P2X_3$ compared to mouse. Intron sequences between mouse and rat are highly identical; in intron 9 the conservation is 90.5/90.8% needle/water, intron 11 has a 74.6% needle identity, the same intron has up to 55.5% global identity compared to other mammals. As expected, the zebrafish has shorter and less conserved introns compared to other $P2X_3$ mammalian genes.

The evidence on $P2X_3$ suggests, together with other P2X subunits sequences, that zebrafish had a common ancestor with mammals. The divergence of these two lineages can be inferred with the accumulation of genetic material in mammal introns. In mammals, intron 8 has a larger size than zebrafish sequences. When we performed a PSI-BLAST analysis in this intron, we observed the presence of several elements similar to dSpmZea mays transposons, suggesting the increase in mammalian intronic sequences could be due to transposon insertion [37] [49] [50]. The phylogenetic analysis shows $darP2X_3a$ isoform diverging before the separation of mammalian clade (**Figure 3**), which leads us to propose that mammalian $P2X_3$ sequences are derived from $darP2X_3b$ found in the zebrafish ancestor.

3.2.4. $P2X_4$

Mouse $P2X_4$ receptor is located close to $P2X_7$ in chromosome 5, it has a 21.488 Kb size and mRNA of 1,995 bp, coding for a protein of 388 aminoacids (see **Table 1** and **Figure 2**). Analyzing genomic organization of $P2X_4$ (**Figure 1(e)**) we can see it's comprised of 12 exons and 11 introns. The phylogenetic tree shows it closer to $P2X_7$, however they do not share the same exon-intron arrangement. In zebrafish, $P2X_4$ paralogous (a and b) are kept in the same clade in the tree and have an identity of 57% between both proteins (**Figure 3**). Comparing $musP2X_4$ with the two zebrafish isoforms $darP2X_4a$ and $darP2X_4b$ using Clustal W, we encountered identities of 58% and 52% respectively (**Table 5**). Exon size is completely conserved among $P2X_4$ orthologous in mammals (**Figure 1(e)**). In zebrafish, the two $P2X_4$ genes conserve exon size compared to mouse; $darP2X_4a$ differs only in the first and last exon, whilst $darP2X_4b$ differs in the last two.

The size of introns is variable among $P2X_4$ orthologous; with sizes ranging from 99bp to 8 Kb. Introns 1 and 5 are the largest while intron 7 and 9 are the smallest. Comparing intron identity between orthologous we found the highest identities again between mouse and rat, particularly in introns 3, 4, 9 and 11, with 71/71%, 69/70%, 72/72% and 77/77% needle/water identity respectively (**Table 3**).

We observed that even when $darP2X_4a$ has a higher global identity with their orthologous than $darP2X_4b$, synteny occurs with $P2X_4b$ and $P2X_7$ suggesting that $P2X_4b$ was prior to genome duplication events that happened in zebrafish after mammalian divergence and therefore, originated the mammalian $P2X_4$ genes.

3.2.5. $P2X_5$

In mouse, $P2X_5$ receptor is located in chromosome 11 and has a size of 12.16 Kb with a mRNA of 2.293 Kb after the editing of their 13 exons and 12 introns. The $P2X_5$ subunit has 455 aminoacids. The genomic organization of mouse $P2X_5$ is quite unique, since it is conserved with the rat, but it's different to the other mammals analyzed, which present an organization of 12 exons and 11 introns.

Looking the phylogenetic tree on **Figure 2**, we can see that $darP2X_8$ is grouped in the same clade than $P2X_5$,

Table 5. Percent identity of mouse P2X orthologous genes (clustal W).

$P2X_1$	MUS	RNO	HSA	MMU	CAF	DAR	
MUS	***	97.5	89.5	90	89.2	55.2	
RNO		***	89	89.5	88.5	54.9	
HSA		12	***	97.5	90.7	54.4	
MMU				***	90.7	54.9	
CAF					***	56.2	
DAR						***	
$P2X_2$	MUS	RNO	HSA	MMU	CAF	DAR	
MUS	***	97.5	89.5	90	89.2	55.2	
RNO		***	89	89.5	88.5	54.9	
HSA		12	***	97.5	90.7	54.4	
MMU				***	90.7	54.9	
CAF					***	56.2	
DAR						***	
$P2X_3$	MUS	RNO	HSA	PTR	CAF	DARa	DARb
MUS	***	99	93.7	94	94.5	57.5	68
RNO		***	93.7	94	94.5	57.2	68
HSA			***	99.7	94.7	58	68.3
PTR				***	95	58	68.3
CAF					***	57.2	67.8
DARa						***	58.2
DARb							***
$P2X_4$	MUS	RNO	HSA	PTR	CAF	DARa	DARb
MUS	***	94.6	87.4	87.4	86.1	57.8	51.7
RNO		***	87.1	87.1	85.3	59.3	51.9
HSA			***	100	89.9	58.3	53.2
PTR				***	89.9	58.3	53.2
CAF					***	56.5	52.2
DARa						***	57.1
DARb							***
$P2X_5$	MUS	RNO	HSA	PTR	CAF	DAR	darP2X8
MUS	***	94.7	69.2	69	70	50.2	43.5
RNO		***	69	68.8	70.2	49.6	44
HSA			***	99.5	76.8	48.9	44
PTR				***	77	48.9	44
CAF					***	53	46.2
DAR						***	51
$P2X_6$	MUS	RNO	HSA	MMU	CAF	DAR	
MUS	***	94.7	69.2	69	70	60.2	
RNO		***	69	68.8	70.2	60.2	
HSA			***	99.5	76.8	58.5	
MMU				***	77	58	
CAF					***	59.6	
DAR						***	
$P2X_7$	MUS	RNO	HSA	MMU	CAF	DAR	
MUS	***	85	80.8	80.5	76.6	45.2	
RNO		***	80.3	80.2	76.4	44.5	
HSA			***	97.1	86.2	46.2	
MMU				***	85.5	46.2	
CAF					***	46.4	
DAR						***	

suggesting an evolutionary relationship between these two sequences. The gene darP2X$_8$ has 44% identity with musP2X$_5$, which is the same identity of darP2X$_5$. On the other hand the tree shows P2X$_5$ close to P2X$_6$ but their genomic organization is not conserved.

There is conservation in exon size between mammalian P2X$_5$ genes, overall among exons I to V (**Figure 1(g)**). With zebrafish orthologous P2X$_5$ and P2X$_8$ the size of exons is more variable. Intron sequence is the most similar between rat and mice with introns 3, 9 and 11, which have global identities superior to 70%.

As shown in **Figure 1(g)**, introns 2, 7, 8 and 11 are longer in zebrafish than in the other organisms analyzed, thus is probable that loss of genetic material could give some advantage in P2X$_5$ expression in mammals [51] [52].

The receptor darP2X$_8$ is grouped with P2X$_5$ in the phylogenetic tree; therefore we compared the nucleotide sequence and observed certain similarity between them. For example, the highest identity of 66.3% occurred for Exon VI (needle, data not shown). This is evidence of distant divergence between P2X$_5$ and P2X$_8$. However this is the only case where zebrafish has the same intron phase than the mammalian P2X$_5$ genes. This is additional evidence to the previously suggested evolutionary relationship between P2X$_5$ and P2X$_8$ in chicken (Gallus gallus) [53].

3.2.6. P2X$_6$

The murine P2X$_6$ gene has a size of 10.13 Kb and a mRNA of 1170 bp, generating a product of 389 aminoacids. The P2X$_6$ is organized in 12 exons and 11 introns; this organization is conserved among mammalian orthologous. Since zebrafish seems to lack P2X$_6$, we choose a reptile (*Anolis carolinensis*) as a possible distant species to compare gene sequences. In the case of *A. carolinensis* P2X$_6$ gene (acaP2X$_6$), its organization has 11 exons and 10 introns (**Figure 1(g)**).

Comparing exon size of mouse P2X$_6$ with its orthologous we observed that is conserved in all the mammalian species, with the exception of the first and last exons, as with other P2X analyzed. However, exon V of acaP2X$_6$ is 175 bp long, which is equivalent to the sum of the individual size of exons V (94 bp) and VI (81 bp) from mouse P2X$_6$. Reptilian exons from VI to X conserve the size with exons VII to XI of musP2X$_6$, respectively.

Introns present the higher divergence in size and identity among the analyzed sequences. Introns 2, 3 and 8 are the largest (more than 1200 bp) in mammals; while in reptile intron 1 had the larger size with 1060 bp (**Figure 1(g)**). Comparing intron sequence of mouse P2X$_6$ against its orthologs, as observed in **Table 3**, we found that rat and mouse have the highest identity (above 63%), with intron 9 the highest in score (88.9/88.9% needle/water). Introns from reptile P2X$_6$ had the lower identity percentage (below 50.5%).

Our analysis of P2X$_6$ sequences between mammals and reptile suggest that P2X genes were present in a common ancestor. We encountered the accumulation of genetic material in the case of some mammalian P2X$_6$ introns, including the presence of an intron between exons V and VI of mammals that is not observed in reptile. Mammalian exons V and VI match exactly in size with reptilian exon V, with identities of 60.8 and 73.2% respectively when aligned locally (data not shown). This explains the presence of only 11 exons in the reptile compared to the 12 exons in mammalian P2X$_6$. The presence of the same genetic structure in all of mammalians points that the intron present between exons V and VI was acquired more recently after reptilian and mammalian divergence through insertion. It has been proposed recently that the increased number of introns in an organism is related to less efficient expression. The insertion of this intron can contribute, along with other multiple regulatory mechanisms, to the in vivo behavior of P2X$_6$ receptors.

3.2.7. P2X$_7$

The murine gene coding for P2X$_7$ is the largest of the P2X family. In mice it has 37.2 Kb with a transcript of 1785 bp, giving a protein of 595 aminoacids. The gene organization of P2X$_7$ consists of 13 exons and 12 introns in mammalians and 14 exons and 13 introns in zebrafish (dar P2X$_7$, **Figure 1(h)**). This genomic organization is different to what is seen for other P2X genes (**Figure 1(a)**).

The P2X$_7$ subunit is notable for its longer C-terminus, with 230 aminoacids for mouse, compared to the shorter C-terminus of musP2X$_6$ with only 25 aminoacids and musP2X$_5$ with 94 aminoacids (second largest). Protein size is identical in the five mammalian species (**Table 1**), which is also reflected in the high conservation of the exon size. The only differences we found were in exons VII and XII of dog (**Figure 1(h)**).

The introns of P2X$_7$ are in general long, overall intron 1 which has more than 21000 bp in both human and mouse, contrasting with intron 10 with 84 to 245 bp. Intron 2 is very well conserved among species, with identities as high as 83% local/global between rat and mouse. Intron phase is conserved among mammalian species,

however, darP2X$_7$ (fish) have a shift in phases due to an insertion of an intron in exon II. Also the last three introns of the zebrafish uses phase one instead the phase zero of mammals.

The main difference between the P2X$_7$ of mammalians is their large size compared to the one of zebrafish (**Table 1**). Also exon-intron organization of the zebrafish is different to the mammalian genes, since it consists of a large intron of 7012 bp with a translated sequence corresponding to the reverse transcriptase of a retrotrasposon (Accession No.: XP_694080). This evidence suggests the insertion of the intronic sequence in exon 2 after the divergence of mammalian and fish lineages, generating the new exons II and III in darP2X$_7$ only. This suggests an evolutionary story where several insertions occurred in the lineage of zebrafish, elongating the introns of P2X$_7$ and conserved until know possibly to an advantage in expression regulation.

4. Concluding Remarks

The evolutionary origin of P2X receptors is still unclear; however, ancestral organisms diverging as far as 1 billion years ago have a single P2X receptor that has pharmacological and biophysical properties that resemble those of the seven P2X subunits in vertebrates [14] [17] [18] [54] [55]. As we have shown in this work, there is a high conservation of the gene structure among P2X receptors in the different organisms analyzed, even in the distant species of fish and reptile. This is additional evidence pairs with previous reports proposing that a single gene in a common ancestor very recently originates the current diversity of P2X subunits in vertebrates [14] [17]. After vertebrate divergence, P2X genes underwent duplications, gain of intron sequences and exon rearrangements that give the seven genes coding for P2X subunits a complexity underlying an important portion of the purinergic signaling in mammals.

Our phylogenic tree shows P2X$_4$ and P2X$_7$ as members of a more related clade. This is in agreement with previous hypothesis suggesting their origin from gene duplication [56]. Their joint evolution can be driven by the selective pressure generated by their functional role in the central nervous system, where these two subunits are mainly responsible for the activation of the inflammasome after injury [57]. In a similar way, the localization of P2X$_2$ and P2X3 in a clade with a recent common ancestor correlates with their high rate of appearance as heteromers in sensory neurons [9]. More importantly, an increasing amount of works have proven that P2X represents important therapeutic targets in pathologies as important as chronic pain in cancer and inflammation [58] [59]. With only few selective antagonists available [19], new strategies such as gene therapy can be the more effective choice when it comes to selectively regulate heteromeric P2X activation in cells [60]. In this work we provide a comprehensive depiction of the genomic organization of P2X receptors in the major model species of mammals. We expect our results will help to better understand phenomena at the transcription level such as splicing variants of P2X receptors and also to provide easy to access reference about the differences of P2X subunits at the nucleotide level, thus allowing to better design future strategies in basic science and therapeutics of P2X physiology.

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Supplementary

Table S1. Microsynteny of P2X subunit genes between mouse and human.

Mouse				Human	
	-2	Zzef1	zinc finger, ZZ-type with EF hand domain 1	Zzef1	2
	-1	Atp2a3	ATPase, Ca++ transporting, ubiquitous	Atp2a3	1
p2x1		P2rx1	purinergic receptor P2X, ligand-gated ion channel, 1	P2rx1	
	1	Camkk1	calcium/calmodulin-dependent protein kinase kinase 1, alpha	Camkk1	-1
		><		><	
	2	Itgae	integrin alpha E, epithelial-associated	Itgae	-2
p2x5		P2rx5	purinergic receptor P2X, ligand-gated ion channel, 5	P2rx5	
	4	Tmem93	transmembrane protein 93	Tmem93	-4
	5	Tax1bp3	Tax1 (human T-cell leukemia virus type I) binding protein 3	Tax1bp3	-5
	-2	Pxmp2	peroxisomal membrane protein 2	Pxmp2	2
	-1	Pole	polymerase (DNA directed), epsilon	Pole	1
p2x2		P2rx2	purinergic receptor P2X, ligand-gated ion channel, 2	P2rx2	
		><		><	
	1	Fbrsl1	fibrosin-like 1	Fbrsl1	-1
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		><		><	
p2x7		P2rx7	purinergic receptor P2X, ligand-gated ion channel, 7	P2rx7	
		><		><	
p2x4		P2rx4	purinergic receptor P2X, ligand-gated ion channel, 4	P2rx4	
	1	Camkk2	calcium/calmodulin-dependent protein kinase kinase 2, beta	Camkk2	1
	2	Anapc5	anaphase-promoting complex subunit 5	Anapc5	2
	-2	Prg2	proteoglycan 2, bone marrow	Prg2	2
	-1	Prg3	proteoglycan 3	Prg3	1
p2x3		P2rx3	purinergic receptor P2X, ligand-gated ion channel, 3	P2rx3	
	1	Ssrp1	structure specific recognition protein 1	Ssrp1	-1
	2	Tnks1bp1	tankyrase 1 binding protein 1	Tnks1bp1	-2
	-1	Thap7	THAP domain containing 7	Thap7	-1
		><		><	
p2x6		P2rx6	purinergic receptor P2X, ligand-gated ion channel, 1	P2rx6	
	1	Slc7a4	solute carrier family 7 (cationic amino acid transporter, y+ system), member 4	Slc7a4	1
		><		><	