Comparative study of the *P2X* gene family in animals and plants

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Abstract P2X receptors are ligand-gated ion channels that can bind with the adenosine triphosphate (ATP) and have diverse functional roles in neuropathic pain, inflammation, special sense, and so on. In this study, 180 putative *P2X* genes, including 176 members in 32 animal species and 4 members in 3 species of lower plants, were identified. These genes were divided into 13 groups, including 7 groups in vertebrates and 6 groups in invertebrates and lower plants, through phylogenetic analysis. Their gene organization and motif composition are conserved in most predicted P2X members, while group-specific features were also found. Moreover, synteny relationships of the putative *P2X* genes in vertebrates are conserved while simultaneously experiencing a series of gene insertion, inversion, and transposition. Recombination signals were detected in almost all of the vertebrates and invertebrates, suggesting that intragenic recombination may play a significant role in the evolution of *P2X* genes. Selection analysis also identified some positively selected sites that acted on the evolution of most of the predicted P2X proteins. The phenomenon of alternative splicing occurred commonly in the putative *P2X* genes of vertebrates. This article explored in depth the evolutionary relationship among different subtypes of *P2X*

genes in animal and plants and might serve as a solid foundation for deciphering their functions in further studies.

Keywords P2X · Evolution · Phylogenetic analysis · Alternative splicing

Introduction

Ligand-gated ion channels (LGICs) are a kind of transmembrane ion channels activated in response to the binding of ligands and are involved in neurotransmission on synapses. Based on their structure, LGICs can be divided into several categories: pentameric channels (e.g., GABAA, nAChRs), tetrameric channels (e.g., AMPA, Kainate, NMDA), and trimeric channels (e.g., P2X receptor) [1].

The first P2X cDNAs was cloned in 1994 from rat vas deferens [2]. From then, P2X receptors have been found in several vertebrates and invertebrate species [3, 4]. Specially, mammalian species have seven P2X subtypes which are named from P2X1 to P2X7. The family of seven P2X genes was recently evolved after splitting between vertebrates and invertebrates [5]. In invertebrates and plants, P2X receptors were detected in some species such as *Dictyostelium discoideum*, *Ostreococcus tauri*, and, interestingly, *Hypsibius dujardini* [3, 5–7]. Though these primitive P2X receptors share low sequence homology with vertebrate P2X receptors, they still maintain functions as ATP activated ion channels [8]. P2X receptors have not been found in prokaryotic species so far [3, 9].

P2X receptors are composed of three subunits, which can form functional homomeric or heteromeric receptors. Only P2X6 cannot form a functional homomeric receptor, whereas only P2X7, on the contrary, cannot form a functional heteromeric receptor [10, 11]. Regarding other heteromeric

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receptors, P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/5, P2X2/6, and P2X4/6 were also reported so far [12, 13]. A common topology is shared by all subtypes—two transmembrane (TM) domains, a large cysteine-rich extracellular loop, an intracellular variable C-terminus, and a N-terminus [5, 13, 14]. The crystal structure of zebrafish P2X4 receptor revealed a trimer in the shape of a chalice, and each subunit adopted a dolphin-like shape with two TM domains and an extracellular loop resembling the fluke and body, respectively [15, 16]. In primitive P2X receptors, the degree of ectodomain cysteine conservation varied greatly, while the trimer formation is still conserved suggested by previous work in *Dictyostelium* [7, 8].

P2X receptors have a large number of physiological and pathophysiological roles. Most P2X subtypes are expressed in different regions of the central nervous system (CNS). P2X3, P2X2, P2X4, and P2X6 receptors are reported to be involved in neurotransmission and neuromodulation [17, 18]. Several P2X subtypes, such as P2X2, P2X3, P2X4, and P2X7, are involved in the reflex activities of guts in rats [19, 20]. The expression of P2X1, P2X3, P2X4, P2X5, and P2X6 in the heart affects the modulation of cardiac contractility of mice [21]. Moreover, P2X receptors also mediate the sensory functions. For example, P2X1, P2X2, and P2X3 mediate taste sensation and pain in the tongue of rats [22, 23]. As for pathophysiology roles of P2X receptors, many P2X subtypes were reported to mediate neuropathic pain. P2X3 receptors were suggested to be involved in mechanosensory transduction of mice—the base of inflammatory pain [24]. Furthermore, P2X2, P2X4, and P2X7 receptors are expressed in dorsal horn neurons delivering nociceptive information in mice [25]. In addition, other roles of P2X have been identified: P2X7 are associated with inflammation, immunomodulation [26], and apoptosis of cancer cells [27]; P2X4 and P2X1 are involved in cardiovascular diseases in rats and humans [28]; P2X2 and P2X3 are involved in special sense [29, 30].

Previous work has provided some information on the arrangements of exon and intron of *P2X* genes in several species, which are mainly focused on the size, number, similarity, and gain and loss of *P2X* exon [31]. However, thorough study about the evolutionary relationship of *P2X* genes among more species, especially in primitive species, has not been involved. Moreover, further work on the evolutionary feature of special structure-related or subtype-specific residues has not been implemented.

In this article, we identified 180 *P2X* genes from animals and plants and explored their evolutionary relationship from different perspectives such as phylogenetic analyses, subtype-specific motifs and site-specific selection assessment, intragenic recombination, and alternative splicing. With these comprehensive analyses, we hope to be able establish the relationship between the P2X receptor function as ATP-gated ion channels and the evolutionary features of the critical structures.

Material and method

Sequence retrieval and identification of putative P2X proteins

To obtain complete data of putative P2X proteins in different species, seven P2X sequences (P2X1–P2X7) in *Homo sapiens* were used as the input sequences in BLAST searches in several online databases. Ensembl (<http://asia.ensembl.org/index.html>) [32] and Metazome v3.0 (<http://www.metazome.net/>) were used to search for putative P2X genes in animals. The same queries were also used to search against Phytozome v10 (<http://phytozome.jgi.doe.gov/pz/portal.2.html>) for putative *P2X* genes in plants. The NCBI Conserved Domain Search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) [33] was employed to confirm if the candidates belong to the P2X family. Molecular weights and isoelectric point (pI) of proteins were predicted by ProtParam tool (<http://web.expasy.org/protparam/>) [34]. Subcellular localizations were predicted by Cello v2.5 (<http://cello.life.nctu.edu.tw>) [35].

Sequence alignment and phylogenetic analyses

Multiple sequence alignment was carried out on the predicted amino acid sequences by the muscle method with the default settings in MEGA 6.06 [36]. The output sequences were analyzed with a neighbor-joining method using MEGA 6.06, a Bayesian inference method in MrBayes 3.2.5 [37], and a maximum likelihood (ML) method using PhyML 3.1 [38]. The neighbor-joining (NJ) tree was constructed with 1000 bootstrap replications. The substitution model was p-distance and the treatment for gaps data is pairwise deletion. As for Bayesian analyses, we first asked the software to sample across ten built-in fixed-rate amino acid models and the chain sampled each model according to its probability. Then, two independent runs were carried out with a single chain per analysis. The analyses were run for one million generations and parameters were sampled every 1000 generations in order to get 1000 samples from the posterior probability distribution. The first 25 % of the samples were discarded and the consensus trees were constructed. ML analysis on amino acid data was carried out with LG substitution model and four substitution rate categories. The initial tree is BioNJ and the method for tree topology search is NNIs. This analysis was run with 100 bootstrap replicates. The topology depicted in Fig. 1 was generated by neighbor-joining method.

Identification of conserved Motifs in these predicted P2X proteins

To identify conserved motifs within the predicted P2X proteins in animals and plants, sequences were analyzed by

Multiple Expectation Maximization for Motif Elicitation (MEME) program (<http://meme.nbcr.net>) [39] with 35 as the maximum motif number. The following settings were employed in the analysis: normal mode; zero or one occurrence of motifs per sequence; the minimum and maximum width of motifs is 6 and 50, respectively.

Synteny analysis of the P2X genes in vertebrates

Genomicus v78.01 (<http://www.genomicus.biologie.ens.fr/genomicus-78.01/cgi-bin/search.pl>) [40] was employed to search for orthologous and paralogous copies of the putative *P2X* genes in vertebrates. Seven subtypes of *P2X* genes of *H. sapiens* were used as queries. The data in Genomicus are from the Ensembl and only genes annotated in Ensembl were compared in this analysis.

Site-specific selection assessment of putative P2X proteins

As the result of evolution, some sites remain conserved due to their functional significance, while others are highly variable [41]. Such variability is likely to be advantageous to the organism [42]. Positive and negative selection pressure at each site was estimated by the ratio of nonsynonymous (K_a) substitutions to synonymous substitutions (K_s), namely the K_a/K_s ratio. The K_a/K_s ratio of positive-selection sites is greater than 1, which indicates that these sites change rapidly during evolution. In order to analyze the changing rate of amino acid in each subtype, the CDS sequences in each subtype were submitted together to the SELECTON Server (<http://selecton.tau.ac.il/>) [43] using three different evolutionary models: M8 (beta + w>=1), M5, MEC with default value. Then, codon K_a/K_s scores of the reference sequence were downloaded and the table was summarized after calculating the average K_a/K_s scores and filtering positive-selection sites. These models use different biological assumptions testing the model that better fits the data. The models are Bayesian models that assume a statistical distribution to account for heterogeneous K_a/K_s values among sites and the number of categories for the distribution is 8 as the default value [42].

Detection of recombination events in P2X genes

Intragenic recombination is a kind of evolutionary event, which plays an important role in generating genetic diversity [44]. Recombination Detection Program (RDP v4.43) [45] was used to identify potential recombination events through different models. In this study, three methods were used to analyze the sequence: RDP [46], GENECONV [47], and MaxChi [48]. The highest

acceptable p value is 0.05 with 100 times of permutation and shuffled alignment columns.

Collecting of alternative splicing

Enabling limited coding genes to generate much more functional proteins by including or excluding particular exons, alternative splicing (AS) is a common posttranscriptional gene regulation phenomenon [49]. All the putative splicing variants were downloaded from the Ensembl. Exons and introns were identified and the patterns of alternative splicing were recognized and illustrated in the figure.

Result and discussion

Identification of putative P2X genes in animals and plants

To identify putative genes of *P2X* family of vertebrates, invertebrates, and plants, we first performed blast search in the Ensembl, Phytozome, and Metazome databases by using the seven human *P2X* genes as query sequences. As a result, a total of 180 putative *P2X* genes (144 members in vertebrates, 32 in invertebrates, and 4 in lower plants) were identified. Interestingly, none of any putative *P2X* genes were found in the species of insects, nematodes, and higher plants. However, four putative *P2X* genes in three species of Chlorophyta were identified, which are *Micromonas pusilla* CCMP1545, *Micromonas pusilla* RCC299, and *Ostreococcus lucimarinus*. Unlike the absence in insects, putative *P2X* genes were found in Arachnida such as *Ixodes scapularis*. Almost all the species in mammals have seven subtypes of putative *P2X* genes except *Monodelphis domestica* which lacks the *P2X6* gene. Only *Meleagris gallopavo* in the nonmammalian vertebrates have seven subtypes of *P2X* genes. Putative *P2X* genes are encoded for proteins ranging from 81 to 1117 amino acids in length with the predicted isoelectric point (pI) ranging from 4.50 to 10.14 (Table S1). Out of 180, 149 predicted *P2X* proteins from vertebrates, invertebrates, and lower plants have the pI larger than 7. The subcellular localization of all the predicted *P2X* proteins predicted by Cello [35] show that 84.4 % (152/180) of predicted *P2X* proteins are localized in the plasma membrane. This result is in accord with the basic function of *P2X* receptors as ligand-gated ion channels which open to allow ions such as Na^+ , Ca^{2+} to pass through the membrane [14]. However, 28 out of 180 predicted proteins are predicted to populate the membranes of intracellular organelles (cytoplasmic, chloroplast, mitochondrial, nuclear), supporting recent study which has showed that the *P2X* receptors populating the contractile vacuole mediate calcium release and osmoregulation in *Dictyostelium discoideum* [7, 50, 51]. We also found that 149 predicted *P2X* proteins have 2 TM domains, while 25 have only one TM domain, which

mostly exist in invertebrates. This incomplete TM structure has an influence on the properties of ion flow in P2X receptors making these predicted P2X receptors of invertebrate species functional different from other P2X receptors of vertebrates.

Phylogenetic analysis of the putative P2X genes

To evaluate the evolutionary relationship of putative P2X genes among animals and plants, we performed phylogenetic analyses of P2X proteins based on a neighbor-joining method using MEGA 6.06 [36], a Bayesian inference method in MrBayes 3.2.5 [37] and a maximum likelihood method using PhyML 3.1 [38]. These distinct methods showed similar tree topologies and values, and we used the NJ tree as an example to carry on the following analysis. The 144 predicted proteins in vertebrates were divided into seven groups based on sequence similarity, resulting in a result similar to previous classifications [14], and 36 predicted proteins of invertebrates and plants were classified into six groups (Fig. 1). The number of each clade varied from 19 to 21 in vertebrates and 2 to 7 in invertebrates and plants. We designated the clades in invertebrates and plants from P2XA to P2XF. Some novel uncategorized P2X proteins of vertebrates were classified into different subtypes. For example, ENSCJAT00000014021, a predicted protein from *Callithrix jacchus*, was classified into P2X5 subtypes. Seven vertebrate species have more than one gene encoding different proteins for the same P2X subtype.

The clades of vertebrates branched distinctly with lower animals and plants and evolved into seven subtypes. Identification of the P2X genes in unicellular green algae *Ostreococcus tauri* indicated that the existence of P2X receptors antecede the start of multicellular organisms [3, 8]. This suggests that the P2X protein in lower animals and lower plants were an ancestral form of the P2X protein found in vertebrates. In addition, we found some interesting evolutionary relationships within the seven subtypes of P2X proteins in vertebrates. According to the topology of the phylogenetic tree, it is likely that P2X4 and P2X7 share the same ancestral gene and emerged after gene duplication. This phenomenon also occurred between P2X5 and P2X6, P2X2, and P2X3, which are consistent with the study of the Loera-Valencia R et al. [31]. Furthermore, an interesting finding is that the P2X7 genes locate right next to the P2X4 genes on chromosomes. Recent studies showed that they have similar tissue distribution and a close physical and functional interaction especially with kidney functions supporting their close relationship [52].

Gene structure and motif analysis of the P2X subtypes

The gain and loss of intron is a common event which enhances the diversity of gene structure [53]. To further determine the evolutionary divergences among P2X subtypes, we compared the exon-intron structures of predicted P2X proteins among all

Fig. 1 Phylogenetic relationships, exon–intron organization and motif distribution of the putative P2X genes in animals and lower plants. The phylogeny tree on the left was constructed by MEGA 6.06 with NJ method. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. Different clades were designated with the name from P2X1 to P2X7 and P2XA to P2XF in the background of different colors. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of NJ method, ML method, and posterior probabilities for Bayesian analyses are shown next to the branches. Values are given for the following order: bootstrap in NJ (%) / posterior probability / bootstrap in ML (%). When a clade is not recovered by the analysis, it is indicated with “-”. The 0, 1, and 2 phase introns are marked with blue, orange, and red triangles, respectively, and the name of introns are shown beside. The right one was the 35 motifs identified by MEME program

species. A diagram of distribution, position, and phase of introns of P2X proteins is illustrated in Fig. 1. In general, distribution and phase of introns of P2X are well conserved in vertebrates, supporting previous work on the exon-intron organization of P2X genes in several species [31]. For the convenience of explanation, we designated the introns from Ia to Ix to demonstrate the feature of exon-intron evolution of P2X. Introns Ia to Ij are conserved among almost all the putative P2X genes in vertebrates, which range across N-terminus, TM1, the extracellular loop, and TM2. In other words, variations of the introns which distinguish the subtypes of P2X receptors exist mostly at the C-terminus of each sequence. For instance, the subtype-specific introns of P2X4 are Ip and Iq, and Io, It, Ix introns only exist in P2X7. As for invertebrates and plants, P2XB and P2XC have similar conserved introns. Furthermore, the P2XC is the only group of invertebrates which has the conserved intron Ii. It is noteworthy that there are some exceptions which may be due to intron loss in evolution. One interesting example is the disappearance of Ij intron of P2X5 receptor in primates (Mmu, Ggg, Cja, Hsa, Ptr). Lost of an exon (and adjacent Ij intron) of P2X5 in primates, which partially constitutes TM2 domain, usually leads to nonfunctional proteins [1, 54, 55].

We also employed the MEME (<http://meme.nbcr.net>) [39] to detect conserved motifs among predicted P2X proteins. As a result, 35 distinct motifs were identified in these proteins. 19 motifs (motifs 11, 18, 14, 12, 10, 2, 17, 15, 6, 1, 26, 16, 7, 3, 9, 8, 5, 4, and 13) are conserved among almost all the sequences (Fig. 1). However, subtype-specific motifs are mainly located in the C-terminus, such as motifs 27 and 35 in mammalian P2X6, which is thought to be involved in desensitization [56]. A series of P2X7-specific motifs (motifs 19, 24, 23, 20, 16, and 21) was found, which can constitute a longer TM2 domain enabling P2X7 receptors to provide a pathway that allows the passage of larger organic cations [57, 58]. In addition, some primitive species (Aqu-p2xa, Sko-p2xa, Nve-p2xa) share a series of conserved motifs with advanced species, while some homologous short motifs were also detected in

other primitive species (Olu-p2xb, Hma-p2xa), revealing the evolution of *P2X* genes.

Conserved motifs 18 and 14 compose the TM1 domain in most the subtypes while P2X7 is composed of motif 33, which

lost one amino acid in G48 (Dre-P2X4a numbering) when compared with the other TM1 domain. As for TM2, this domain consists of motif 4 and motif 13 (motif 4 and motif 30 in P2X7). When it comes to other special sites of the P2X receptor, ATP binding sites (motifs 12, 1, 8, and 5) and five disulfide bonds in extracellular domains are quite conserved among all the vertebrates and invertebrates. Although the sites directly participating in ATP binding are conserved among subtypes, the affinities for ATP are quite distinct among different subtypes [59]. Based on mutagenesis studies on P2X7 receptors, residues around the ATP binding sites are likely to contribute to the sensitivity to ATP [60, 61]. Our study showed that the positions analogous to K145 and R276 in Rno-p2x7 are conserved within subtypes (Table 1). The properties of these sites (e.g., charged or noncharged, the side chain volume) may affect the difference of affinities among seven subtypes. However, ion gate residues are subtype-specific (Table 2). The difference of these amino acid sites may lead to a difference of feature of ion permeability such as Ca^{2+} selectivity among subtypes [62, 63]. For instance, the homologues of T336 in different subtypes are hydrophilic except the alanine in P2X3, and this can partially explain the low Ca^{2+} permeability of the P2X3 receptors [63, 64].

Synteny analysis of the putative *P2X* genes in vertebrates

Synteny analysis is a useful tool to explore the evolutionary and functional relationships between genes. Previous work indicated the syntenic relationship between *P2X* genes of mouse and human [31]. In this study, we made further efforts using the Genomicus to carry out the synteny analysis in vertebrates. Putative *P2X* genes are generally well conserved among the vertebrate species (Fig. S1). The synteny analysis of *P2X3* subfamily is taken as an example to demonstrate more detailed evolutionary relationship between species

Table 1 Subtype-specific residues around the *P2X* ATP binding sites

Site ^a	145	276
P2X1	Q	H
P2X2	N	R
P2X3	G	T
P2X4	N	R
P2X5	N/S	S/N
P2X6	H/H	Q
P2X7	K	R

^a The sites are corresponding sites in Rno-p2x7

Table 2 Subtype-specific *P2X* ion gate residues of animals and plants

Site ^a	332	336	339
P2X1	T	S	G
P2X2	I	T	T
P2X3	I	A	T
P2X4	I	S	A
P2X5	I	S	A
P2X6	I/V	T	A
P2X7	V	S	S
Invertebrates and plants	L	S	A/G

^a The sites are corresponding sites in Rno-p2x2

(Fig. 2a). Gene insertion and gene deletion exist commonly in the neighboring genes of the *P2X3* subfamily. Gene insertion (*RP11-872D17.8*) occurred between *PRG2* and *SLC43A3* in *H. sapiens*, while *Canis familiaris* may experience the loss of *PRG2*. In addition, we also found that some genes such as *OR5M10* and *OR5M1* only exist in *H. sapiens* and *Pan troglodytes* resulting from evolution divergence.

An interesting pattern was found throughout the evolution: the neighboring genes of *P2X3* experienced a series of gene insertion, inversion and transposition (Fig. 2b). Based on the time of emergence, we divided the genes into several segments. The primordial pattern was composed of segments A (*SSRP1*, *P2X3*) and B (start from *TMX2* to *SERPING1*), and segment B was in the upstream of segment A. *Danio rerio* and *Taeniopygia guttata* both adopt this pattern. In *Meleagris gallopavo*, segment B experienced gene transposition and was moved to the downstream of segment A. However, when it comes to *Anolis carolinensis*, segment B was inversed and segment C was inserted between A and B. Besides, segment D was inserted upstream to segment A. The elements of segments C and D also evolved gradually. For instance, segment C was comprised of genes from *UBE2L6* to *SLC43A3* (from upstream to downstream) in *Anolis carolinensis*; *PRG2* was inserted in the upstream of gene *UBE2L6* in the advanced species *Monodelphis domestica*; Moreover, *PRG3* was inserted upstream to *PRG2* in *Bos Taurus*. In primates, segment E was finally inserted upstream to segment D, which includes genes coding for olfactory receptors.

Detection of intragenic recombination events in the putative *P2X* genes

To understand the evolutionary feature of *P2X* genes, recombination points were investigated using the RDP

Fig. 2 Synteny analysis of vertebrate *P2X3* genes. The figure shows the position of *P2X3* genes and neighboring genes on chromosomes. The shapes of arrow in the same color are the same gene type. The

P2X3 genes are in the middle with the color of green. Irrelevant genes are not shown in the figure

software with the RDP [46], Geneconv [47], and MaxChi [48] methods. As shown in Table 3, a total of 116 intragenic recombination events were identified, 64 identified using RDP, 10 using GENECONV, and 42 using MaxChi. *D. rerio* experienced the most frequent intragenic recombination with 15 recombination events indicating its evolutionary variability. However, it was found that some species such as *Ornithorhynchus anatinus* had no recombination signals, suggesting their functional and evolutionary conservation. *P2X1*, *P2X4*, and *P2X7* are subtypes which are usually involved in intragenic recombination. Few recombination events were detected in invertebrates suggesting lower gene polymorphism in invertebrate species. For example, the RDP analysis showed that recombination events occurred in Mga-P2X4 with major and minor parents being Mga-P2X1 and Mga-P2X5, respectively (Fig. 3) (p value: RDP 6.061×10^{-11} , Geneconv 2.551×10^{-1} , MaxChi 4.663×10^{-9}), indicating that Mga-P2X4 demonstrate high pairwise identity with Mga-P2X1 and Mga-P2X5 in some parts.

Variable selective pressures among amino acid sites of the P2X

Analyzing and detecting amino acid site under selective pressure is critical for understanding protein function and structure [41]. Past studies have shown that duplicated genes undergo three different kinds of fates after duplication events: neo-functionalization (gain new functions), sub-functionalization (subdivide the

functions), and pseudogenization (loss of function) [65–67]. These neo-functionalized genes are under positive selection, while those sub-functionalization genes are supposed to be under purifying selection pressure. In the phylogenetic analysis, we found out that three duplication events happened in the P2X family in vertebrates generating seven subtypes. In order to explore how these P2X subtypes evolve after duplication events and the amino acid substitutions of these subtypes by selective pressures, we further investigated variable selective pressures among amino acid sites of the P2X. Putative CDS sequences of each group were submitted to the SELECTON Server (<http://selecton.tau.ac.il/>) [43]. We used three evolutionary models [M8 (beta + w>=1), M5 (gamma), MEC] to perform the tests. For vertebrates, M8 (beta + w>=1) and MEC model predicted some positive-selection sites in P2X proteins, while M5 was not.

From the result, we found that even the K_a/K_s values of the putative sequences from the subtypes which may share the same ancestor before gene duplication is quite different. Generally, the K_a/K_s are similar in the subtypes of P2X5 and P2X6 (both near 0.28). While for P2X4 and P2X7, the subtypes which maybe emerged after gene duplication concluded in the phylogenetic analysis (Fig. 1), the K_a/K_s are relatively higher in P2X7 than that in P2X4, indicating that two subtypes evolved differently after gene duplication. This result also occurred between the K_a/K_s values of P2X2 and P2X3. The K_a/K_s values of invertebrate species are higher than the K_a/K_s values of vertebrates, implying a

Table 3 Intragenic recombination events among *P2X* genes of animals and plants

Species	Recombination methods			Genes undergone recombination events
	RDP	GENECONV	MaxChi	
<i>Equus caballus</i>	5	0	3	<i>P2X1, P2X2, P2X5, P2X6A, P2X6B, P2X6C</i>
<i>Bos taurus</i>	1	0	3	<i>P2X1, P2X4, P2X5, P2X7</i>
<i>Callithrix jacchus</i>	1	0	3	<i>P2X1, P2X2, P2X4, P2X5, P2X7</i>
<i>Canis familiaris</i>	6	0	1	<i>P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7</i>
<i>Homo sapiens</i>	2	0	3	<i>P2X1, P2X2, P2X4, P2X5, P2X7</i>
<i>Macaca mulatta</i>	1	0	0	<i>P2X2, P2X4</i>
<i>Monodelphis domestica</i>	0	1	0	<i>P2X4, P2X5</i>
<i>Ailuropoda melanoleuca</i>	2	1	2	<i>P2X1, P2X4, P2X5, P2X6A, P2X7</i>
<i>Mus musculus</i>	3	0	1	<i>P2X1, P2X4, P2X5, P2X7</i>
<i>Ornithorhynchus anatinus</i>	0	0	0	-
<i>Oryctolagus cuniculus</i>	4	0	1	<i>P2X1, P2X2, P2X3, P2X5, P2X7</i>
<i>Pan troglodytes</i>	0	1	6	<i>P2X1, P2X2, P2X3, P2X4, P2X5, P2X7</i>
<i>Rattus norvegicus</i>	4	1	3	<i>P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7</i>
<i>Sus scrofa</i>	5	1	4	<i>P2X1A, P2X1B, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7</i>
<i>Gorilla gorilla gorilla</i>	2	1	0	<i>P2X1, P2X4, P2X6, P2X7</i>
<i>Meleagris gallopavo</i>	3	0	0	<i>P2X1, P2X2, P2X3, P2X4, P2X5</i>
<i>Taeniopygia guttata</i>	5	2	1	<i>P2X1A, P2X1B, P2X3, P2X4B, P2X5A, P2X5B</i>
<i>Xenopus tropicalis</i>	6	0	1	<i>P2X1, P2X2, P2X4, P2X5, P2X7</i>
<i>Danio rerio</i>	9	0	6	<i>P2X1, P2X2, P2X3A, P2X3B, P2X4A, P2X4B, P2X5A, P2X5B, P2X7</i>
<i>Anolis carolinensis</i>	1	0	1	<i>P2X3, P2X6, P2X7</i>
<i>Branchiostoma floridae</i>	1	2	0	<i>P2XA, P2XB, P2XC</i>
<i>Hydra magnipapillata</i>	0	0	0	–
<i>Ixodes scapularis</i>	0	0	0	–
<i>Saccoglossus kowalevskii</i>	3	0	0	<i>P2XA, P2XB, P2XE</i>
<i>Schistosoma mansoni</i>	0	0	2	<i>P2XD, P2XC</i>
<i>Strongylocentrotus purpuratus</i>	0	0	1	<i>P2XD, P2XC</i>

faster changing rate in invertebrates than in vertebrates (Table 4). Despite these differences in K_a/K_s , all the values are lower than 1, indicating that the P2X sequences are under purifying selection pressure.

However, some positively selected sites were also found in the analysis. For instance, 14 sites were found under positive selection in vertebrate putative P2X4 proteins, as predicted by M8 model. Among them, three positive sites were predicted in

Fig. 3 Intragenic recombination events among P2X4, P2X1, and P2X5 of *Meleagris gallopavo*. The plot display of recombination events was detected by the RDP method

Table 4 Likelihood values and parameter estimates of the selection pressure for P2X proteins

Gene branches	Selection model	K_a/K_s	Log-likelihood	Positive-selection sites
p2x1	M8(beta + w>=1)	0.1583	−10508.8	Not found
	M8a	0.1573	−10507.9	Not found
	M7(beta)	0.1529	−10507.8	Not found
p2x2	M8(beta + w>=1)	0.2119	−12908.8	371, 372, 374, 386, 390
	M8a	0.2011	−12913.7	Not found
	M7(beta)	0.1862	−12925.6	Not found
p2x3	M8(beta + w>=1)	0.1159	−9597.9	Not found
	M8a	0.1167	−9597.97	Not found
	M7(beta)	0.1070	−9597.53	Not found
p2x4	M8(beta + w>=1)	0.2170	−11832.2	6, 8, 126, 127, 133, 136, 153, 170, 171, 305, 307, 382, 383, 385
	M8a	0.1939	−11840.4	Not found
	M7(beta)	0.1805	−11854	Not found
	M8(beta + w>=1)	0.3013	−16681.6	125, 302, 371, 375, 377, 379, 381, 384, 386, 387, 388
p2x5	M8a	0.2929	−16684.4	Not found
	M7(beta)	0.2866	−16691.7	Not found
	M8(beta + w>=1)	0.2925	−10499.9	2, 20, 21, 177, 230, 236, 252, 385, 389, 392, 399, 401, 404, 409, 412, 414, 415
p2x6	M8a	0.2795	−10502.6	Not found
	M7(beta)	0.2701	−10512.4	Not found
	M8(beta + w>=1)	0.2950	−18564.8	Not found
	M8a	0.2911	−18566.3	Not found
p2x7	M7(beta)	0.2857	−18568.1	Not found
	M8(beta + w>=1)	0.4530	−5352.63	Not found
	M8a	0.4551	−5352.5	Not found
p2xA	M7(beta)	0.4242	−5350.81	Not found
	M8(beta + w>=1)	0.2830	−5111.64	Not found
	M8a	0.2669	−5108.09	Not found
p2xB	M7(beta)	0.2807	−5113.69	Not found
	M8(beta + w>=1)	0.2215	−7771.64	Not found
	M8a	0.2032	−7768.83	Not found
p2xC	M7(beta)	0.2103	−7772.27	Not found
	M8(beta + w>=1)	0.4059	−7497.45	Not found
	M8a	0.4007	−7497.61	Not found
p2xD	M7(beta)	0.3881	−7496.81	Not found
	M8(beta + w>=1)	0.4552	−12187.6	Not found
	M8a	0.4498	−12186.4	Not found
p2xE	M7(beta)	0.4426	−12185.9	Not found

TM1 domain, while no positive sites were found on TM2 domain. This result supports recent study which has showed that TM1 domain plays a less significant role in ion conduction pathway than TM2 domain [63]. In other words, TM1 is more variable than TM2 during evolution. Furthermore, none of the positive sites lie in other key sites of the P2X receptors—the disulfide bonds, ATP binding sites, and ion gate residues [63]. A large number of positive sites exist in the C-

terminus of P2X proteins, suggesting that these sites might change the function of P2X proteins and lead to the divergence of P2X subtypes.

Alternative splicing of the *P2X* genes in vertebrate

Alternative splicing (AS) is a posttranscriptional gene regulation phenomenon enabling limited coding genes to generate

Table 5 Alternative variants of *P2X* genes in vertebrates

Species	Abbreviation	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6	P2X7
<i>Homo sapiens</i>	<i>Hsa</i>	—	8	2	5	7	2	—
<i>Gorilla gorilla gorilla</i>	<i>Ggg</i>	—	2	—	—	2	—	—
<i>Macaca mulatta</i>	<i>Mmu</i>	—	6	—	—	2	—	5
<i>Callithrix jacchus</i>	<i>Cja</i>	—	3	2	—	—	2	6
<i>Mus musculus</i>	<i>Mmu</i>	2	2	2	4	3	2	4
<i>Rattus norvegicus</i>	<i>Rno</i>	—	2	—	—	—	—	—
<i>Canis familiaris</i>	<i>Cfa</i>	—	—	—	—	2	2	—
<i>Equus caballus</i>	<i>Eca</i>	—	—	—	—	2	—	—
<i>Ornithorhynchus anatinus</i>	<i>Oan</i>	—	—	—	—	—	2	—
<i>Meleagris gallopavo</i>	<i>Mga</i>	—	—	—	—	—	—	2
<i>Danio rerio</i>	<i>Dre</i>	2	—	2	4	2	—	—

much more functional proteins by including or excluding particular exons, which can largely expand the biodiversity and tissue specificity in species [49]. It is reported that this phenomenon take place in more than 90 % of the multi-exons of *H. sapiens* [68]. Based on different splicing patterns, AS events can be categorized into several types: exon skipping, intron retention, mutually exclusive exon, alternative 5' splice sites, alternative 3' splice sites, alternative promoters, and alternative poly-A sites [69]. The process of alternative splicing is carried out by spliceosome and regulated through *trans*-acting factors and *cis*-acting sites [70].

The phenomenon of alternative splicing occurs commonly in the putative *P2X* genes of vertebrates (Fig. S2). However, we could not find any alternative variants of putative *P2X* genes in invertebrate species or plants. Among all the genes, the AS events of *P2X5* involve the largest number of species and only two species have alternative variants of *P2X1*. Alternative splicing events occur in almost every subtype of *P2X* receptors of the primate species (Table 5). Advanced

species tend to have more alternative variants than lower species and new variants are likely to emerge from tandem exon duplication [71]. However, in some subtypes (*P2X1*, *P2X3*, and *P2X6*), all the species have the same numbers of alternative variants, indicating that these AS patterns have not changed during evolution.

Taking the AS phenomenon in *P2X2* subtypes as an example, many alternative variants were found among muridae and primate species (Fig. 4). Two typical AS events happen on Ej and the exons ranging from Eb to Ee. The former, which is a kind of intron retention and generates three types of variants, exists in the all the *P2X2* genes except the *P2X2* gene of *Rattus norvegicus*. Besides, mutually exclusive exon events in the fore part generate four kinds of variants in Humans and Macaques. Surprisingly, for Gorillas and Chimpanzees, which have much closer phylogenetic relationships [72], these AS patterns have not been found, indicating that humans and macaques may obtain this AS pattern separately or that gorillas and chimpanzees lost this pattern during evolution.

Fig. 4 Alternative splicing among *P2X2* genes of muridae and primate species. The figure was constructed based on the gene data from Ensembl. Solid blocks are exons, and hollow blocks are UTR regions. Different patterns of AS are shown in different kinds of lines above the blocks

Conclusions

In this article, we systematically compared the orthologous and paralogous genes of P2X receptors. A total of 180 putative *P2X* genes were identified and classified into 13 groups. Most of the predicted proteins are located on the plasma membrane, as according to their role as ligand-gated ion channels. In phylogenetic analysis, we demonstrated the evolutionary history of seven mammalian P2X subtypes and found that some of them may share the same ancestral gene. Analysis of exon–intron structure implies that introns showed a conserved distribution in the sequence region of the P2X proteins, except C-terminus. Moreover, 35 distinct motifs were identified and subtype-specific motifs are mainly located in the C-terminus. Some subtype-specific residues were found around the ATP binding sites and the putative position of the ion gate. The analysis of *P2X3* subfamily illustrates that *P2X* genes have strong synteny conservation among all vertebrates. An interesting finding is that from primitive *P2X* genes to advanced ones, gene segments near *P2X3* experienced a series of gene insertion, inversion, and transposition. Recombination signals were detected in almost all the vertebrates and invertebrates, involving different subtypes of P2X proteins, thusly highlighting the significant role that intragenic recombination plays. Analysis of selective pressure demonstrated that none of the positive sites were located in the function key sites of P2X receptors such as ATP binding sites, ion gate residues, and TM2 domain. This suggests that these critical functions of P2X receptors are conserved even after long period of molecular evolution. Alternative splicing is a quite common phenomenon in putative vertebrate *P2X* genes, indicating that AS may play an important role to increase the genetic and functional diversity of P2X receptors. After collecting a large amount of predicted P2X protein sequences from animals and plants, we compared and analyzed the evolutionary relationship among different P2X subtypes providing useful information about how the P2X receptors family has evolved and contributed to establishing a foundation for further research about their structures and functions.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

This article does not contain any studies with animals performed by any of the authors.

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