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Sperm specific expression of temperature-sensitive ion channel TRPM8 correlates with vertebrate evolution

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Abstract

Transient Receptor Potential subfamily Melastatin member 8 (TRPM8) is involved in detection of cold temperature and different noxious compounds, execute thermo- as well as chemo-sensitive responses at cellular levels. Here we explored the molecular evolution of TRPM8 by analyzing sequences from different species. We elucidate that different regions of TRPM8 had different levels of selection pressure and the 4-5th transmembrane regions remain highly conserved. Synteny analysis suggests that since vertebrate origin, TRPM8 gene is linked with SPP2, a bone morphogen. We found 16656 TRPM8 variants in 1092 human genomes with top variations are SNPs, insertions and deletions. 692 missense mutations are also mapped to human TRPM8 protein. TRPM8 expresses endogenously in sperm cells of different vertebrates ranging from fish to human. We conclude that TRPM8 has emerged during vertebrate evolution (ca 450 MYA) and sperm-specific expression has guided its molecular evolution. These understandings may have medical importance as well.

Key words: Cold-sensitivity, TRP ion channel, molecular evolution, sperm cells, Ca^{2+}-signaling, vertebrate evolution

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Introduction:

Precise thermo-sensitivity allowing animals to discriminate very minute temperature changes ranging from warm to hot, cool to extremely cold temperatures is an ability which is highly conserved in the entire animal kingdom [1]. Though the thermosensitivity in the animal kingdom is well established, the key molecules involved in such responses and the molecular mechanisms behind such responses are not clear [2-4]. Recently, TRPM8 has been reported to be an important molecular thermo-sensor as it can be activated by low temperature [5-8]. TRPM8 is a well-established non-selective cation channel and so far has been reported from several species yet exclusively in vertebrates. In most cases TRPM8 is involved in different sensory processes and physiological functions such as detection of cold temperature (between 22-27^{0}C). In addition, TRPM8 can be modulated by certain natural compounds like menthol, ethanol, icilin, eucalyptol, 1,8-cineole and peppermint oil which provides a cool sensation [5, 9-12]. Often, the cooling sensation mediated by these natural compounds is species specific and context dependent [9]. For example, TRPM8-specific agonist-mediated cation influx via TRPM8 is pH-dependent. The Ca^{2+}-influx induced by menthol and icilin through murine TRPM8 can be completely inhibited or reduced at pH 6.3 and 8.0 respectively when compared to the response at pH 7.5 [13]. In agreement with its precise sensory functions, TRPM8 is expressed in a subpopulation of sensory neurons in Dorsal Root Ganglia (DRG), Trigeminal Ganglia (TG) and in taste papillae of higher animals [14-16].

So far obtained reports suggest expression and functional repercussion of TRPM8 is restricted within vertebrates only and the primary function of TRPM8 is conducting Ca^{2+}-influx in response to temperature changes, a function which remains conserved across different species [17]. In case of human and lower species such as *Xenopus*, activation of TRPM8 by low temperature increases intracellular Ca^{2+} concentration within the sensory neurons [15]. TRPM8 is also involved in immune response [18-19]. In addition, TRPM8 is involved in body temperature regulation, and activation of TRPM8 causes hyperthermia [20-25]. TRPM8 present in brown adipocytes is involved in the regulation of body temperature and is relevant for the survival of newborns and hibernating mammals [26]. Cold-seeking behavior during systemic inflammation is also regulated by TRPM8 [24]. TRPM8 also contributes to the cold allodynia and neuropathic pain [27]. As TRPM8 is involved in the detection and further avoidance (or preference) of certain environment rich in TRPM8-specific physical (such as low temperature) and chemical (such as natural compounds) stimuli, activation of TRPM8 poses immense importance on the animal sensory behavior and animal physiology. Sensitivity against both temperature and natural compounds rendered TRPM8as an important molecular factor for detection of suitable habitat, ecological

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niche formation, adaptation, further speciation and evolution; especially in response to certain selection pressure where TRPM8-mediated sensory processes are involved [28-30].

In spite of the involvement of TRPM8 in precise thermo detection, the actual mechanism of TRPM8 is activated by temperature is not clear. Exchanging the entire C-terminus of TRPM8 with that of TRPV1 (which act as a hot channel) reverses hot and cold-sensitivity of the chimeric channels confirming that distinct and smaller part located in its C-terminus of TRPM8 can act as the molecular-sensors for low temperature [4, 31]. TRPM8 also act as a voltage gated ion channel which becomes activated upon membrane depolarization [32-34]. Indeed, a small yet highly conserved (in many ion channels) region located in the transmembrane region 3 (TM3) of TRPM8 can act as a "voltage-sensor" [35]. Similarly, a small double cysteine motif is important for TRPM8 channel functions [36]. These reports indicate the involvement of specific small regions rather than the entire TRPM8 channel in different specific functions which are fairly conserved throughout the evolution. Involvement of such small regions in specific functions is also apparent from sequence homology. TRPM8 of different species shares modest sequence identity and homology. *Xenopus* TRPM8 shares approximately 70% identity with rat TRPM8 [17]. Therefore, these small regions shed light on the mechanistic details and the molecular evolution of this channel. In particular, these small regions can be used as good indicators to understand how these thermosensitive channels detect temperature and conduct Ca^{2+}-influx upon activation in different species. Molecular characterization of TRPM8 mutations, particularly in the voltage-sensor regions and different transmembrane regions has also revealed plausible mechanism/s by which thermal and chemical stimuli can modulate this channel [33-34].

As TRPM8 is involved in the thermosensation and Ca^{2+}-signaling, in this work we have used TRPM8 sequences from different species and have critically analyzed the molecular evolution of this channel. Our results unravel that TRPM8 is conserved throughout vertebrate evolution. We also demonstrate for the first time that molecular evolution of TRPM8 correlates well with the sperm specific expression in vertebrates.

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Results

TRPM8 is a highly conserved protein evolved during vertebrate evolution ca 390 MYA

We explored the molecular evolution of TRPM8 in details and therefore retrieved full-length or partial TRPM8 sequences from different databases (**Tab 1**). Full-length protein sequences were used for the establishment of bayesian phylogenetic history, which depicts that there is a single copy of TRPM8 which is conserved in different vertebrates with high statistical support [37] (**Fig 1a**). Furthermore, we calculated the evolutionary age and pattern of TRPM8. For that purpose we estimated the number of amino acid changes per 100 sites among different TRPM8 sequences (**Fig 1b**) as reported previously [38-40]. This analysis also indicates that TRPM8 has evolved during the Devonian era (approximately 390 MYA) when amphibians stared evolving from fishes. The evolutionary slope of the TRPM8 indicates that it went different level of selection pressure in different era. Apparently, in the first 50 million years (since its origin to till middle of the carboniferous era), TRPM8 had not acquired many changes. However, since late half of the carboniferous era, it acquired many changes rapidly indicating that these changes were probably coinciding with the development of birds from reptiles (warm blooded animals from cold blooded animal). Subsequently in the cretaceous era, TRPM8 incorporated few changes indicating towards a molecular stabilization process coinciding with the radiation of mammals. During the tertiary era, TRPM8 incorporated only very few changes supporting the notion that TRPM8 structure-function relationship is fairly stabilized during the primate evolution.

Emergence of TRPM8 co-relates with vertebrate evolution

In order to understand the conservation of TRPM8in the genome of different species throughout the evolution, we performed synteny analysis (**Fig 1c**). Interestingly, TRPM8 gene has been detected in coelacanth (*Latimeria chalumnae*, a fish close to mammals and reptiles). Human TRPM8 shares 80.2%, 73.5%, 81.5% and 52.8% identities with its orthologs in chicken, frog, lizard and coelacanth, respectively (**Tab 2**). To evaluate whether invertebrates have TRPM8, we carried out extensive survey of TRPM8 gene using BLAST suite. Majority of hits picked up were hypothetical or without proper annotation and hence we picked up sequences from few selected invertebrates only. We report that TRPM-like genes exist in invertebrate genomes which are not genuine TRPM8 orthologs (**Tab S1**). These TRPM-like genes from hydra to lancelet share 22.3-27.1% of identities with human TRPM8 (**Tab 3**). A comparative phylogentic tree also demonstrates that genuine TRPM8 orthologs are not present in invertebrates, but these species possess distinct homologs such as TRPM2 or TRPM3-like genes (**Fig S1**).

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We found that since ~400 MYA (a time relevant for vertebrate evolution), TRPM8 has shared close relationship with a 24 kDa protein named secreted phosphoprotein 2 (SPP2). To evaluate the importance of TRPM8 in vertebrate evolution in more details, we mapped chromosomal locus of these two genes (TRPM8 and SPP2) in the representative vertebrate genomes. We found that TRPM8 and SPP2 genes are juxtaposed in a head to tail orientation on the human chromosome 2, flanked by a tetrad of autophagy related 16-like 1 (ATG16L1), ubiquitin specific peptidase 40 (USP40), HEAT repeat containing 7B1 (HEATR7B1), Holliday junction recognition protein (HJURP) on the one side. On the other side, we found a dyad of ADP-ribosylation factor-like 4C (ARL4C), ArfGAP with GTPase domain, ankyrin repeat and PH domain 1 (AGAP1) as conserved arrangements. Notably, this locus is conserved in all mammals tested, i.e. in human (chromosome 2), mouse (chromosome 1), rat (chromosome 9) and oppossum (chromosome 2) (**Fig 1c**). Furthermore, we identified the same locus in chicken, zebra finch and turkey where these two genes are present on the chromosome 7. In reptiles also, this locus seem to be maintained. Amphibians, namely *Xenopus* has this locus on scaffold GL172651.1. Interestingly, coelacanth (*Latimeria chalumnae*), a fish close to mammals and reptiles, has this locus maintained. But this locus is not found in any other known fish genomes available so far. However, Cod, stickleback, tilapia and zebrafish possess SPP2 at another locus flanking by a dryad of zinc finger CCCH-type containing 13 (ZC3H13), ribosomal protein L31 (RPL31) ribosomal protein L31 (RPL31) and carbohydrate sulfotransferase 10 (CHST10) on the one side while other side a dyad of carboxypeptidase B2 (CPB2) and asparagine-linked glycosylation 11, alpha-1,2-mannosyltransferase homolog (ALG11) is conserved. This altered locus is present in Fugu, platyfish and tetraodon, but does not contain SPP2 gene. Overall, adjunct chromosomal location of TRPM8 and SPP2 from coelacanth to mammals suggests the importance of these two genes in vertebrate evolution. Though the other fishes have no TRPM8 gene and corresponding locus; SPP2 gene is present in selected fishes on other locus. This probably suggests that TRPM8 gene was lost specifically in ray-finned fishes after separation from basal fishes at about 450 MYA. As SPP2 is a morphogen involved in bone formation and the conservation of these two genes in the same genomic locus for last 390 million years strongly suggests the importance of these gene products for some common functions.

Different regions of TRPM8 have evolved with different selection pressure

Next we tested the conservation in different motifs and domains of TRPM8 (**Fig 2, Tab 4**)[41-47]. We noted that all four TRPM homology regions (MHR) are well conserved in throughout evolution. This is in full agreement with the previous analysis which suggested that MHR regions are conserved in all members of the TRPM family [41]. The MHR4 is most conserved followed by MHR1, MHR2 and MHR3. Among all the TM regions, TM-4, TM-5 and TM-6 reveal highest level of conservation

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respectively. TM-2 is also well conserved and this agrees well with the fact that this region is involved in the interaction with specific agonist such as menthol [48]. However, TM1 and TM3 show more variability compared to all other TM regions suggesting that these two TM regions have less functional importance than the TM-4, TM-5 and TM-6. Among all the loops, the loop-4 is highly conserved and loop-1 as well as loop-3 are least conserved. Loop-2 is also less conserved when compared with loop-4. Interestingly, pore loop is less conserved than loop-4, suggesting an important functional contribution of loop-4 in the channel gating. The amino acid region 40-86 (located at the N-terminus and required for channel localization and tetramerization) is not well conserved [41]. This may suggest that the importance of this region in the context of channel localization and function is limited to higher mammalians only. Notably, the 40 amino acids (AA 1007-1047) located at the C-terminus (has been described as important for self interaction) is not well conserved [43]. However, report suggests that this region is functionally important as R1008Q mutant (human TRPM8) is defective in desensitization [49]. This may suggest the importance of this region in the higher mammals only. Coiled coil region located at the N-terminus (AA 594–628) is more conserved when compared to the coiled coil region located at the C-terminus (AA 1064-1104) [41, 43, 47]. The TRP-domain regions (AA 990-1025 and AA 993-1016) as described by two different groups is less conserved than the TRP-box region (AA 993-998) located within this region [41, 46]. Notably the TRP-box is highly conserved region indicating the functional importance of this region in channel function. This conservation accords well with the documented report of PIP_2 interaction with TRPM8 in this region [46]. The voltage-sensing region (AA 842-856) seems to be totally conserved across all the species indicating the functional importance of this region [33]. In the similar manner, a small region (AA 799-805) required for channel gating is conserved in TRPM8 throughout evolution indicating its importance in the channel function [33, 44-45].

Catalog of genetic variation in TRPM8 using 1092 human genomes

While TRPM8 remain conserved in most of the mammals, in human it has a large number of variants. We computed variations in the TRPM8 gene in 1092 human genomes as obtained from 14 different populations and details of these studies are provided (**Fig 3**). Majority of these are SNPs (85.8%). There are total 16656 variations ins observed in 16 variant types, with top 5 variant types were intron variants (10116), Nonsense-mediated mRNA decay (NMD) transcript variants (3558), downstream gene variants (2409), upstream gene variants (1747) and non-coding (NC) transcript variant (1556). Top four variant classes are single-nucleotide polymorphism (SNP) variants (85.8%), insertions (6%), deletions (3.6%) and single nucleotide variant (SNV) (3.35%). We further examined 692 missense variations to examine what are the critical changes, i.e. changes which can alter both TRPM8 amino acid sequence and thus secondary structural elements (compiled in **Tab S2**). The N-terminal of TRPM8

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portion has more variants compared to the C-terminal region (**Fig S2 and Tab S2**). Various structural elements of TRPM8 have at least 1 genetic variant (summarized in **Tab S2)**. In general, we noted that in case of the positions that have multiple variations, the variations are not random and a clear biasness for certain amino acids is prominent (**Fig S2**).

TRPM8 is a sperm specific protein and expressed in sperm cells throughout vertebrate evolution

Recently expression and functional involvement of TRPM8 in higher mammals, namely in human and mouse sperm cells and prostate have been described [50-53]. Since our *in silico* analysis indicated that TRPM8 is a well conserved channel involved in thermosensation, we hypothesized that TRPM8 might be present in the sperm cells of different species, especially because sperm cells are highly responsive to temperature fluctuations. To explore these aspects, we checked the endogenous expression of TRPM8 in the sperm cells from different species ranging from lower vertebrates to higher mammals by indirect immunofluorescence analysis. By using specific antibody we confirmed that TRPM8 is expressed in the sperm cells in these representative species (ranging from fish, amphibians, reptiles, avian and mammals) (**Fig 4**). These TRPM8-specific immunoreactivities were abolished / reduced when we used a specific blocking peptide. Our *in silico* analysis also confirms that the epitope sequence (SDVDGTTYDFAHC) recognized by the TRPM8-specific antibody is highly conserved throughout the vertebrates (**Fig S3a**). In addition, the same sequence is absent in other TRPM sequences (**Fig S3b**). Taken together, the sperm-specific expression of TRPM8 strongly correlates with its importance throughout the vertebrate evolution.

Overall, our results strongly suggest that TRPM8 has evolved nearly 400 million years ago and is an evolutionary conserved ion channel. We conclude that sperm cell specific expression is a major aspect that has guided the molecular evolution of TRPM8.

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Discussion

In this work we have explored the nature of conservation of a thermosensitive ion channel TRPM8 and indicate that the molecular evolution of TRPM8 strongly correlates with vertebrate evolution.

TRPM8 in the context of evolutionary conserved physiological functions

So far it has been proposed that TRPM8 is involved in the detection of cold temperature and thus involved in thermosensation, especially in endotherms. Interestingly, it seems that amphibian genome contains two open reading frames for TRPM8 that are homologous to mammalian TRPM8 [54]. These two copies in *Xenopus* i.e. xlTRPM8 and xlTRPM8b display 65% and 70% identity to the rat TRPM8 protein sequence, respectively. Out of these two, only the xlTRPM8 is responsive to cold and menthol [17]. Moreover, in comparison to the TRPM8 channels of homeothermic animals (i.e. TRPM8 in mammals and birds, which gets activated at low temperatures like 25^0C), their poikilothermic counterparts (such as frogs) require substantially lower temperatures to get activated. It is important to note that both ecological niches as well as body temperatures of amphibians are lower than the core body temperature of mammals and birds [17].

As activation temperature for TRPM8 is different for different species, it is tempting to speculate that these changes in the activation temperature correlate with the different environmental temperatures and ecological niches. This is supported by the fact that the trajectory of TRPM8 evolution reveals different slopes in different era (**Fig 1b**). For example, during the radiation of non-human primates, changes are at minimum while changes are higher during radiation of mammals (**Fig 1b**). In the same manner, TRPM8 underwent more changes during evolution of warm blooded animals from cold blooded animals such as evolution of birds from reptiles which occurred in150 MYA.

Involvement of TRP channels in evolution and presence of TRPM8 in fish

We have analyzed the molecular evolution of TRPM8 and the conservation of its different domains and motifs. In addition, we demonstrate that TRPM8 is expressed endogenously in sperm cells from all vertebrates tested so far suggesting strongly that functional importance of TRPM8 remain conserved in all vertebrate sperm cells. Our data shows that TRPM8 is well conserved throughout evolution (p value $\leq$ 0.0001, based on the comparison of TRPM8 protein sequence among total 24 different species, ranging from human to lower vertebrates (**Fig 1 and Fig 2**). Notably, based on the zebra-fish genome sequence, it has been described that TRPM8 is absent in the fish lineages but two divergent copies of TRPM8 are present in *Xenopus* [53]. Based on this observation, previously it has been suggested that TRPM8 might have evolved with these ectotherms. As TRPM8 is present in the Coelacanth (the oldest known living lineage of lobed-finned fish which appeared approximately 400

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MYA, Ensembl accession id ENSLACP00000016045), but not present in zebra fish, it needs more detailed studies including different fish species. Unavailability of the complete TRPM8 sequences at any of the query databases, especially for few selected species (such as in other fishes and reptiles) make it difficult to predict the exact evolutionary history and trajectory. However, the presence and absence of TRPM8 in different fish lineages suggest that it is probably evolved during evolution of fishes, but lost in certain lineages subsequently. Notably, in this work we demonstrate that sperm cell from *Labio rohita* contains TRPM8 and this may suggest an evolutionary conserved role of TRPM8 in thermosensation. In this study we have also demonstrated that TRPM8 orthologs are missing in invertebrates. There are four TRPM-like genes in the *C. elegans* genome, namely gon-2 (abnormal gonad development), gtl-1 (gon-two like 1), gtl-2 (gon-two like 2), and ced-11(cell death abnormal) [55-56]. Additionally, Ciona has also TRPM-like genes [55]. This also supports that TRPM-like genes are present in invertebrates but these species lack genuine TRPM8 ortholog.

TRPM8 in the context of conserved cellular functions

This work and other previous works have confirmed the presence of TRPM8 in many vertebrates tested so far, both at the genomic as well as in the protein level. This work also suggests that involvement of TRPM8 is conserved in the context of sperm cells and possibly also in the bone cells. We provide evidence supporting the endogenous expression of TRPM8 in sperm of all representative vertebrates. This is in line with the recent reports demonstrating the expression of TRPM channels in human, mouse sperm cells as well as in rat spermatogenic cells [50-52]. It is important to mention that recently a protein (Cysteine-rich secretory protein 4) which regulates sperm function has been characterized as an endogenous inhibitor of TRPM8 [53]. All these in general suggest the involvement of TRPM8 in the critical reproductive functions such as sperm development, sperm movement and fertility in vertebrates. Notably, all these factors are dependent on temperature and seasonality.

Detection of TRPM8 in the sperm cells of early vertebrates is intriguing. The sperm cell specific expression of TRPM8 suggests that TRPM8 may have played an important role in the adaptation (in response to temperature) of warm blooded (homeothermic animals) and cold blooded (poikilothermic) animals in different ecological niche, especially in animals (such as in fish and amphibians) where fertilization is exogenous in nature. Though we have detected TRPM8 expression in sperm cells from all the vertebrates that we have tested so far, an interesting pattern of TRPM8 localization is worth mentioning. In our analysis we noted that the localization of TRPM8 is mainly restricted in the tail region in case of homeothermic animals (such as mammals and avian) with warm blood and having internal fertilization. In contrast, poikilothermic animals with cold blood (such as fish, amphibians and reptiles) the localization of TRPM8 is mainly restricted in the neck region which contains mitochondria. Never-

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the-less, conserved expression of TRPM8 in the vertebrate sperm cells strongly suggests the evolutionary conserved role of TRPM8 and may also explain the thermosensitivity observed in these motile cells.

We noted that the TRPM8 share same genomic locus with secreted phosphoprotein 2 (SPP2) throughout the vertebrate evolution. As SPP2 is directly involved in the BMP-signaling and bone development, close association of TRPM8 with SPP2 is intriguing [57]. Though speculative, yet this close relationship of both TRPM8 and SPP2 for 400 MYA (since vertebrate evolution started), is an indicative for the involvement of these two genes in the context of bone formation and may argues for a possible co-evolution at molecular level. This is in full agreement with reports suggesting the presence of TRPM8 in bone cells [58]. This association also correlates well with the Ca^{2+}-signaling involved in the bone formation [59]. For example, Menthol and its metabolites have been shown to inhibit bone resorption when fed to the rats [60-61]. Similarly, TRPM8 also express in human odontoblasts and co-localize with dentine sialophosphoprotein (DSPP) [62]. Although presence of TRPM8 in bone cells has not been reported yet, the relationship between TRPM8 and bone formation is intriguing and important in the context of notochord formation, the prime feature of vertebrates. However, if and how TRPM8 and SPP2 share molecular interaction relevant for the bone development is not clear and further studies are needed.

Overall, our studies indicate that sperm specific expression is an important factor that has contributed in the molecular evolution of TRPM8 which correlates well with vertebrate evolution.

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Materials and Methods:

Sequence retrieval and alignment: The TRPM8 sequences were retrieved from the Ensembl release 73 (Sept 2013) [63-64] and NCBI database [65-66]. Details of each gene as well as protein are enlisted (**Tab 1 and Table S1**). The sequence alignment was done by using MUSCLE alignment software [67-68] with its default values. Sequences for Histone H4 (highly conserved protein) and Cytochrome-C (a semi-conserved protein) from different species was downloaded from the Ensembl (release 73) and also from different databases (Sept 2013) [38, 63-64]. The Histone and Cytochrome-C protein sequences used for this work have been described before [39].

Construction of the phylogenetic tree: The complete sequences of TRPM8 from different speceis were retreived from NCBI database and their accuracy were confirmed from Uniprot and Ensembl databases (**Tab 1**). MUSCLE alignment program was used to align the amino acid sequences of TRPM8 for the purpose of phylogenetic analysis [67-68]. The phylogenetic tree was constructed by the Bayesian approach (5 runs, 7500,000 generations, 25% burn-in-period, WAG matrix-based model in the MrBayes 3.2 program) [37].

Calculation of evolutionary time: In order to explore the molecular evolution of TRPM8, the sequences among different classes were compared and number of changes of amino acids per 100 amino acids was calculated by comparing birds with reptiles, fish with reptiles and reptiles with mammals for different available TRPM8 sequences [38-39]. Human TRPM8 sequence has been considered as the most recent one and therefore, the evolutionary time reference of human TRPM8 is considered as zero million year. The average changes were calculated and radiations of mammalian TRPM8 were plotted against million years. While calculating radiation of non-human primates, Gibbson, Gorilla, and Olive Baboon were compared with human TRPM8 sequence. For calculating total mammalian radiation, Dog, Guinea Pig, Pig, and Rat sequences were compared with human TRPM8. In these cases, the average value representing the amino acid change/100 amino acids were considered. For calculation of birds with amphibians, Zebra Finch and Chicken TRPM8 were compared with *Xenopus* (Silurana) *tropicalis* TRPM8. Amphibian and mammals were compared by using TRPM8 from *Xenopus* (Silurana) *tropicalis* and Human. For similar analysis, we used histone-4 as a highly conserved protein and Cytochrome-C as a semi-conserved protein [38-40].

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Fragmentation of TRPM8 in different domains and motifs: In order to analyze the degree of conservation of the different domains present in TRPM8, its various structural regions, domains and motifs were analyzed separately [41-47] (**Tab 4**). The N- and C-terminal as well as TM regions and the respective loop regions were considered as mentioned before [41-42, 46]. In addition, conservation of specific functional regions such as region involved in self interaction, trafficking and assembly were also explored [43]. A specific region involved in tetramerization was also analyzed [41]. In all cases, the human TRPM8 sequence was used as the template. Specific domain and motif sequences described for other species were used as querry in order to find the corresponding regions present in the human TRPM8 and also in TRPM8 sequences from different species.

MUSCLE software was used to align and find out the respective regions present in other species [67-68]. The aligned data were subsequently imported into "R" statistical tool for statistical analysis. As the complete TRPM8 sequences from certain species are not available (mostly due to sequencing errors at certain regions), the analysis aimed to understand the conservation of different domains and motifs of TRPM8 were conducted with the available full-length sequences only (**Tab 1**). We omitted incomplete sequences in cases where full-length sequences are needed. In all cases, the distance matrix generation and statistical tests were performed as described before [39, 69-70].

Synteny analysis of TRPM8 genes: We utilized Ensembl genome browser for building synteny of TRPM8 gene loci from selected vertebrate genomes [71]. Additionally, we examined *X. tropicalis* genome using JGI genome browser (accessed on 28 March 2012, Webpage, http://genome.jgi.doe.gov/help/browser_main.jsf).

Collection and isolation of sperm cells: Freshly ejaculated sperms from bovine (*Bos indicus*) were collected from healthy bulls after at least 48 hours of sexual abstinence by trained professionals (at the Frozen Semen Bank, Cuttack) by means of artificial vagina. For collection of avian sperm, chicken (*Gallus gallus domesticus*) testis were collected (n = 4) from the slaughter house and bought to the laboratory within 15 minutes. After removing the tunica albuginea (outer covering membrane), the testis was chopped into pieces, smeared and then immediately fixed in 4% PFA. For collection of sperm from reptiles, we used house lizard (*Hemidactylus leschenaultii*). Sexually mature males (n = 3) were collected from institutional campus and sacrificed by cervical dislocation. Testes was dissected out and immediately fixed in 4% PFA. For collection of sperm from amphibians, we used a common toad (*Duttaphrynus melanostictus*). Sexually mature male toads (n = 3) were collected from institutional campus and sacrificed by cervical dislocation. Testes were dissected out and immediately fixed in 4% PFA. In case of chicken, lizard and common toad, the testis were smeared and centrifuged at 1000 RPM

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for 30s. The supernatants containing the sperm cells were collected for further analysis. Sperm pellet was obtained by centrifugation at 6000 RPM for 5min. Mature sperm from Rohu fish (*Labeo rohita)* were collected as described before [72]. In all cases, extreme care was taken to minimize the sufferings and the number of animals used. All experiments were done according to the approval from institutional animal ethics committee of NISER (NISER-IAEC/SBS-AH/07/13/10).

Immunofluorescence analysis and microscopy: Immunocytochemical analysis of sperm cells were performed as described previously [72]. In brief, immediately after collection, sperm cells were fixed with 2% paraformaldehyde (PFA) and were permeabilized with 0.1% Triton X-100 in PBS (5 min). Subsequently, the cells were blocked with 5% bovine serum albumin for 1 hour. Rabbit polyclonal anti-TRPM8 antibody (Alomone Lab, directed against the pore loop i.e. amino acid residues 917-929) of human TRPM8 and corresponding peptide (SDVDGTTYDFAHC, Alomone Lab) have been used. All primary antibodies were incubated for overnight at 4°C in PBST buffer (PBS supplemented with 0.1% Tween-20). AlexaFluor-488 labeled anti rabbit (Molecular probes) were used as secondary antibodies (1:1000 dilutions). All images were taken on a confocal laser-scanning microscope (LSM-780, Zeiss) with a 63X-objective and analyzed with the Zeiss LSM image examiner software and Adobe Photoshop

Creating the catalogue of TRPM8 variants in 1092 human genomes: TRPM8 variants were generated from 1092 human genomes (14 different populations) available in 1000 genomes project [73]. Sorting Intolerant From Tolerant (SIFT) is a software tool that predicts whether an amino acid substitution affects protein function and it helps in prioritize substitutions for further study [74]. Polymorphism Phenotyping v2 (PolyPhen-v2) is a tool that predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations [75]. Evaluation of the TRPM8 variant impact on human protein was performed using these two methods. We combined impact of these TRPM8 variants by using SIFT [74] and PolyPhen V2 [75] tools (**Tab S 2).**

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Figure legends:

Figure 1: Molecular evolution of TRPM8. a. Bayesian phylogeny of TRPM8 illustrates that there is a single copy of this gene which is conserved across different vertebrates. Bayesian phylogenetic tree of TRPM8 proteins from mammals (green) and birds (red) was generated using MrBayes 3.2 [37]. Percentage posterior probabilities are marked at the node of the branches while mean branch length is marked in decimal on the respective branch. TRPC7 gene from *Nematostella vectensis* (JGI acc. id - estExt_fgenesh1_pg.C_6220005) served as out-group in this phylogenetic tree. **b.** Conservation analysis with TRPM8 in comparison to histone H4 (a conserved protein) and Cytochrome-C (a semi-conservative protein) are shown here. The values for histone-H4 and Cytochorome-C are plotted as best-fit while values for TRPM8 are plotted according to the original values. **c.** Synteny analysis of TRPM8 indicates that TRPM8 and SP2 are juxtaposed throughout the vertebrate evolution. [In case of turtle and lizard, the presence of SPP2 is not confirmed yet (indicated by a question mark) as genome assembly of lizard and turtle are still in draft conditions].

Figure 2: Different domains, motifs and interacting sites of TRPM8 had different evolutionary selection pressure. The lower value indicates more conservation and higher value indicates less conservation. Different regions of the TRPM8 are indicated by different colors. MHR1: TRPM homology regions; TM: Transmembrane region; L: Loop region; Pore; Pore loop; TRP-box: Signature motif for TRP-box; Localization: regions required for channel localization and tetramerization; Coiled coil-Nt: Coiled coil region at the N-terminus; channel gating: Critical region for channel gating; Voltage-sensor: Region important for voltage sensing; TRP domain: TRP domain of TRPM8; self-interacting sites: regions required for self interaction; coiled coil-Ct: Coiled coil region at the C-terminus; N-terminus: N-terminal cytoplasmic domain; Middle: Middle portion containing all the TM and loop regions; C-terminus: C-terminal cytoplasmic domain of TRPM8; FL: Full-length; Histone 4: Histone 4. TRP-domain and coiled-coil-Ct (indicated in black and blue script) represent TRP-domain of TRPM8 and Coiled coil region at the C-terminus which are over-lapping but different sequences as described in **table 5**. The schematic drawing of TRPM8 (below) represents different domains and motifs (not according to the scale). All values are significant ($P < 0.0001$, Kruskal-Wallis test).

Figure 3: TRPM8 variant analysis from human genome. Human TRPM8 variants computed from 1092 genomes of 14 human populations demonstrates that SNPs are predominant variant class.

Figure 4: TRPM8 is present in vertebrate sperm cells. Confocal images demonstrating the presence of TRPM8 in mammalian (**a**, bovine), avian (**b**, duck), reptilian (**c**, house lizard), amphibian (**d**, common toad) and piscean (**e**, rahu fish) sperm are shown. Cluster of sperm cells were immunostained for TRPM8-specific antibody in presence or absence of a specific blocking peptide. Fluorescence images representing TRPM8 (green) and DNA (blue) were merged with DIC images. In right panel, confocal images of TRPM8 expression and localization in single sperm from different species are shown. An enlarged view of the same cell is shown in extreme right. In mammals TRPM8 is exclusively localized in the neck and tail regions. In avian sperm, it is exclusively localized in the tail regions and mostly excluded from head and neck regions. In reptilian and amphibian sperm, TRPM8 is exclusively localized in the neck regions. In piscean, weak yet specific TRPM8 immunoreactivity (restricted in the neck regions) is observed in a some but not in all mature sperm cells.

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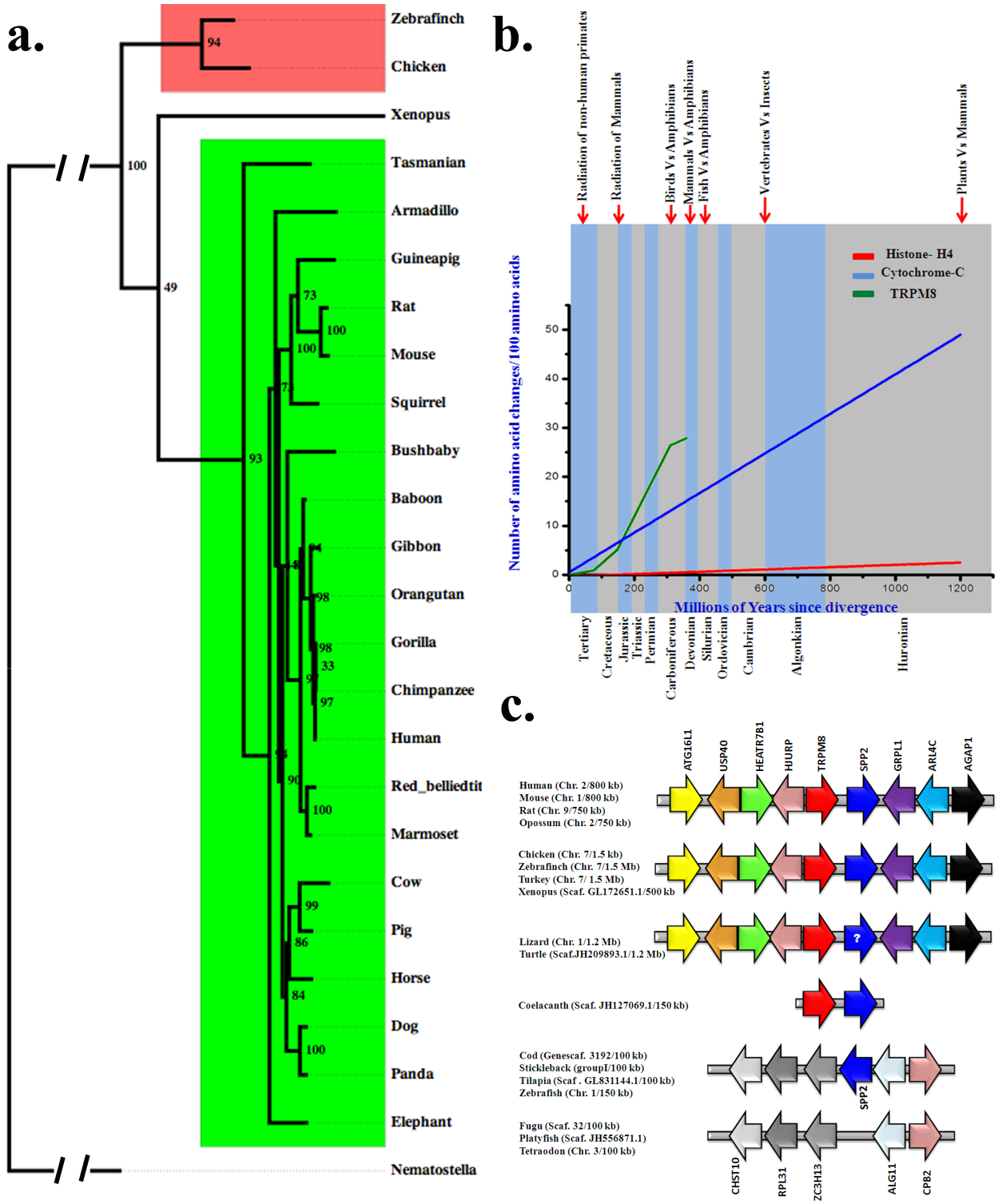

Figure 1:

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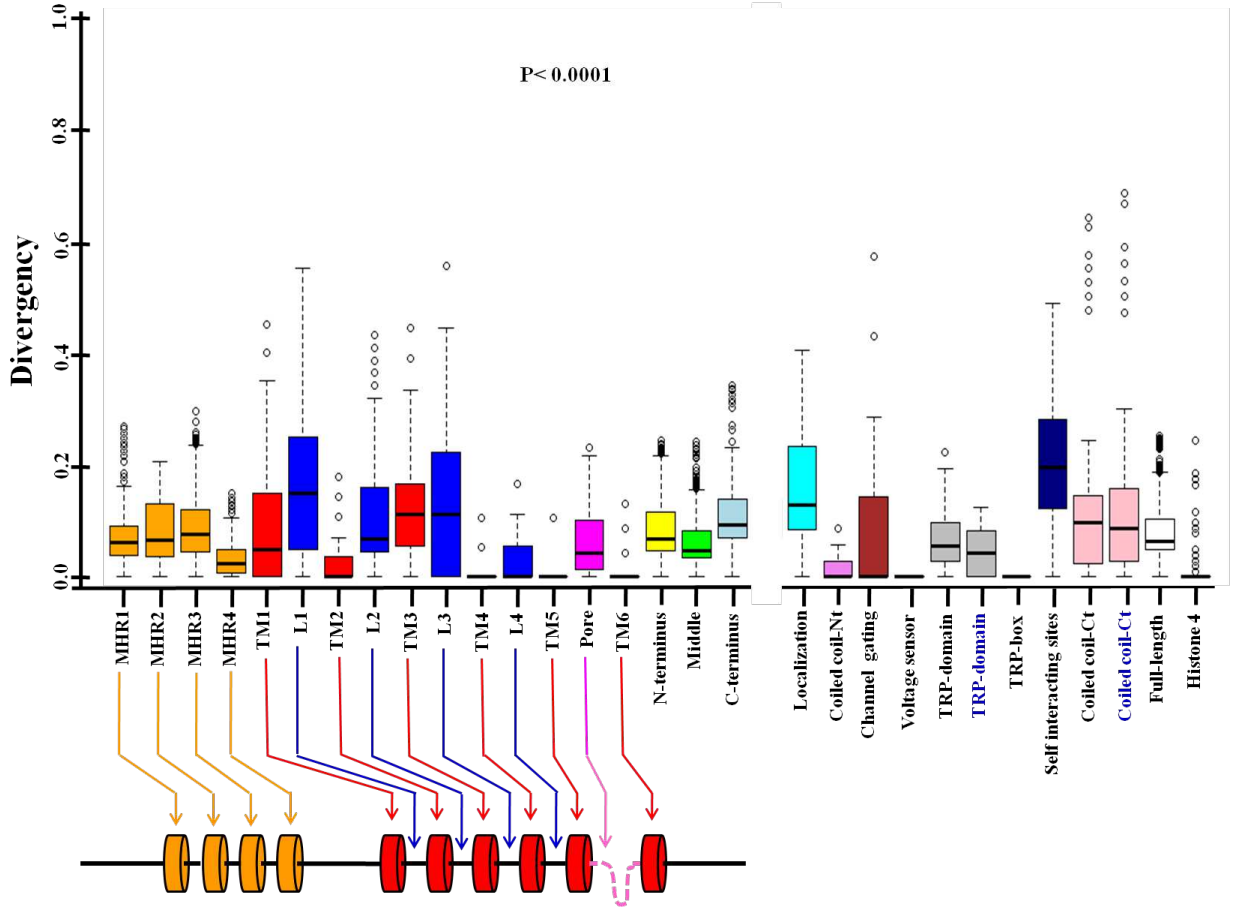

Figure 2:

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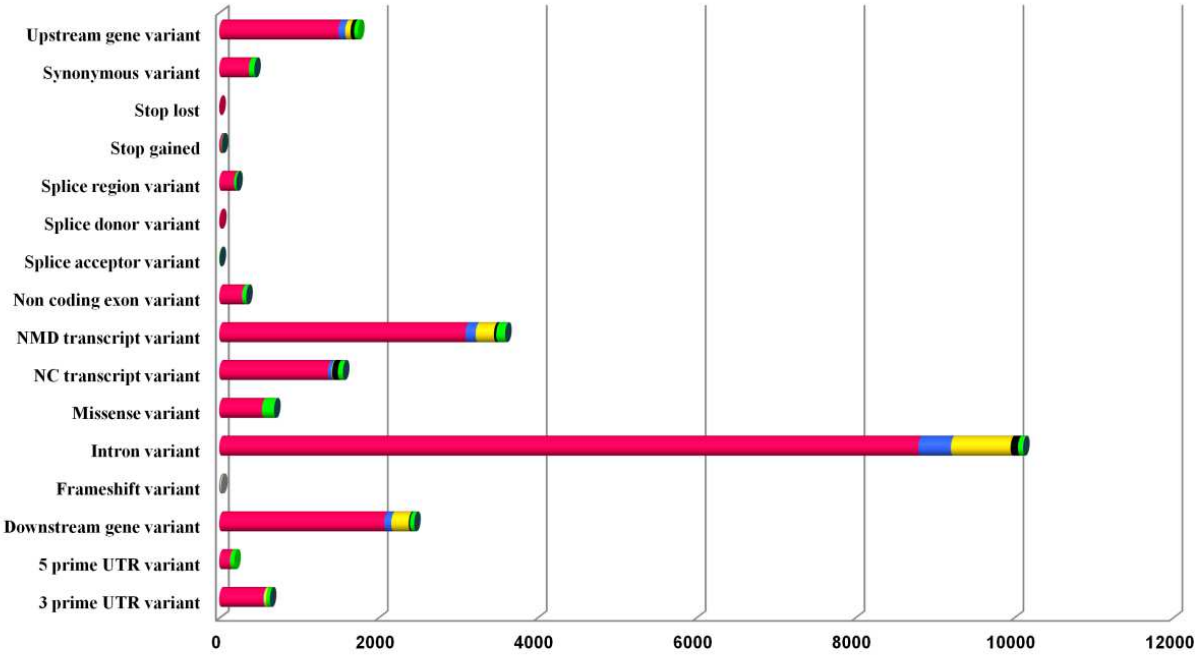

	3 prime UTR variant	5 prime UTR variant	Downstream gene variant	Frameshift variant	Intron variant	Missense variant	NC transcript variant	NMD transcript variant	Non coding exon variant	Splice acceptor variant	Splice donor variant	Splice region variant	Stop gained	Stop lost	Synonymous variant	Upstream gene variant
SNP	551	136	2077	0	8798	538	1368	3099	286	0	12	186	24	2	373	1501
Deletion	10	1	89	13	411	0	46	129	5	0	0	0	0	0	0	85
Insertion	30	3	219	11	751	0	8	230	2	0	0	0	0	0	0	67
Seq. alteration	0	0	16	0	93	0	72	30	0	0	0	0	0	0	0	45
Somatic SNV	47	51	52	0	60	152	61	109	46	5	0	29	9	0	69	49
somatic insertion	0	0	1	1	4	0	2	1	1	0	0	0	0	0	0	0
Substittution	1	0	0	0	3	2	1	2	1	0	0	0	0	0	0	0

Figure 3.

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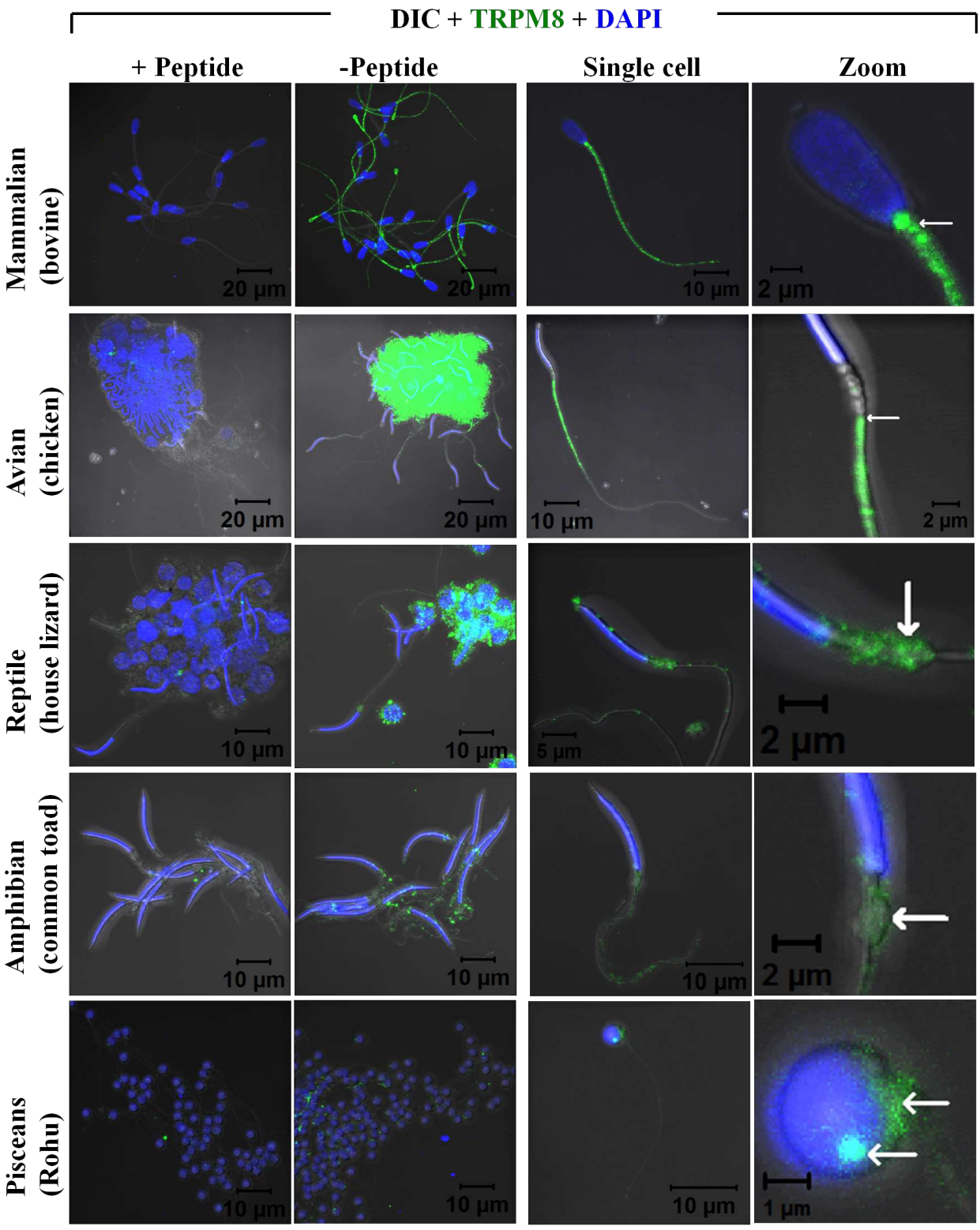

Figure 4

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Table 1: List of TRPM8 sequences

List of species having complete sequences					
	Name of species	**Scientific name**	**Protein id**	**Length**	**Source**
1	Human	*Homo sapiens*	NP_076985.4	1104	NCBI
2	Chimpanzee	*Pan troglodytes*	ENSPTRP00000022348	1052	Ensembl
3	Gorilla	*Gorilla gorilla gorilla*	ENSGGOP00000008197	1105	Ensembl
4	Orangutan	*Pongo abelii*	XP_002813060.1	1104	NCBI
5	Gibbon	*Nomascus leucogenys*	XP_003278601.1	1104	NCBI
6	Olive baboon	*Papio anubis*	ABX89284.1	1104	GenBank
7	Red-bellied titi	*Callicebus moloch*	ACA57875.1	1104	GenBank
8	Common marmoset	*Callithrix jacchus*	ABY79104.1	1104	GenBank
9	Bushbaby	*Otolemur garnettii*	ENSOGAP00000012043	1088	Ensembl
10	Dog	*Canis lupus familiaris*	NP_001104239.1	1104	NCBI
11	Panda	*Ailuropoda melanoleuca*	XP_002917995.1	1104	NCBI
12	Horse	*Equus caballus*	XP_001499536.1	1104	NCBI
13	Cow	*Bos taurus*	ENSBTAP00000019509	1104	Ensembl
14	Pig	*Sus scrofa*	XP_003133798.1	1104	NCBI
15	Squirrel	*Spermophilus tridecemlineatus*	ENSSTOP00000013235	1104	Ensembl
16	Guineapig	*Cavia porcellus*	ACU30144.1	1104	GenBank
17	Rat	*Rattus norvegicus*	NP_599198.2	1104	NCBI
18	Mouse	*Mus musculus*	NP_599013.1	1104	NCBI
19	African elephant	*Loxodonta africana*	XP_003417951.1	1108	NCBI
20	Nine-banded armadillo	*Dasypus novemcinctus*	ACO88994.1	1104	GenBank
21	Tasmanian devil	*Sarcophilus harrisii*	ENSSHAP00000003620	1103	Ensembl
22	Zebra finch	*Taeniopygia guttata*	ENSTGUP00000003679	1087	Ensembl
23	Chicken	*Gallus gallus*	ENSGALP00000039026	1106	Ensembl
24	Xenopus	*Xenopus laevis*	NP_001155066.1	1139	NCBI
List of species with incomplete/fragmented sequences					
	Species name	**Scientific name**	**Protein id**	**Length**	**Source**
1.	Wallaby	*Macropus eugenii*	ENSMEUP00000004623	1050	Ensembl
2.	Turkey	*Meleagris gallopavo*	ENSMGAP00000002375	1069	Ensembl
3.	Tree shrew	*Tupaia belangeri*	ENSTBEP00000008744	1104	Ensembl
4.	Tarsier	*Tarsius syrichta*	ENSTSYP00000009832	1064	Ensembl
5.	Sloth	*Choloepus hoffmanni*	ENSCHOP00000006168	1048	Ensembl
6.	Platypus	*Ornithorhynchus anatinus*	ENSOANP00000020169	1096	Ensembl
7.	Pika	*Ochotona princeps*	ENSOPRP00000001346	1103	Ensembl
8.	Oppossum	*Monodelphis domestica*	ENSMODP00000010005	1096	Ensembl
9.	Mouse lemur	*Microcebus murinus*	ENSMICP00000013415	1090	Ensembl
10.	Megabat	*Pteropus vampyrus*	ENSPVAP00000005934	1092	Ensembl
11.	Monkey	*Macaca mulatta*	ENSMMUP00000027950	192	Ensembl
12.	Dolphin	*Tursiops truncatus*	ENSTTRP00000004154	1103	Ensembl
13.	Coelacanth	*Latimeria chalumnae*	ENSLACP00000016045	1044	Ensembl
14.	Anole lizard	*Anolis carolinensis*	ENSACAP00000010017	1103	Ensembl
15.	Alpaca	*Vicugna pacos*	ENSVPAP00000002122	1059	Ensembl

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Table 2: Comparison of TRPM8 proteins for selected vertebrates using percentage identity

	Chimpanzee	Chicken	Xenopus	Turtle	Lizard	Coelacanth
Human	95.1	80.8	73.5	80.5	81.5	52.8
Chimpanzee		76.5	69.8	76.1	77.6	49.2
Chicken			73.4	84.3	84.1	53.7
Xenopus				74.9	73.9	52.4
Turtle					84.5	51.9
Lizard						53.6

Table 3: Comparison of sequence identities of invertebrate TRPM-like sequences with human TRPM8

	Capitella	Branchiostoma	Trichoplax	Aplysia	Strongylocentrotus	Hydra	Bombyx	Acyrthosiphon	Crassostrea	Danaus	Ceratitis
Human	**25.9**	**27.1**	**25.5**	**23.8**	**22.3**	**22.5**	**25.7**	**24.1**	**24.1**	**21.6**	**23.9**
Capitella		35.4	31.2	21.1	22.3	25.9	24.2	26.3	28.5	23.5	26.1
Branchiostoma			29	21.9	20.2	26	23.4	27.3	31.2	21.5	25.6
Trichoplax				30.1	32.5	25.6	28	26.8	24.9	24.8	27.9
Aplysia					24.2	23.7	22.1	24.5	22.5	21.3	24.2
Strongylocentrotus						23.4	22.6	24.3	21.4	19.9	24.9
Hydra							26.9	26.3	23.2	23.2	26
Bombyx								65.9	24.2	68.4	67.6
Acyrthosiphon									23.5	52	63.1
Crassostrea										18.4	23.4
Danaus											56.5

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Table 4: Description of different domains and motifs

Region	Amino acids	Refs
MHR1	117-245	[41]
MHR2	246-352	[41]
MHR3	353-550	[41]
MHR4	551-692	[41]
TM-1	691-710	[42]
Loop-1	711-730	[42]
TM-2	731-758	[42]
Loop-2	759-802	[42]
TM-3	803-820	[42]
Loop-3	821-829	[42]
TM-4	830-848	[42]
Loop-4	849-866	[42]
TM-5	867-885	[42]
Pore region	886-954	[42]
TM-6	955-977	[42]
N-terminal	1-692	[41]
TM and loop region	693-989	[41]
C-terminal	978-1104	[42]
Regions required for channel localization and tetramerization	40-86	[41]
Coiled coil region at the N-terminus	594–628	[43]
Critical region for channel gating	799-805	[50] [44] [45]
Region important for voltage sensing	842-856	[33]
TRP-domain	990-1025	[41]
TRP-domain	993-1016	[46]
TRP-Box	993-998	[46]
Self-interacting sites	1007-1047	[43]
Coiled coil region at the C-terminus	1064-1104	[47]
Coiled coil region at the C-terminus	1070-1104	[41]

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