

Hypothesis

Evolution of mitochondrial uncoupling proteins: novel invertebrate UCP homologues suggest early evolutionary divergence of the UCP family

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Abstract Current hypothesis about the evolution of uncoupling proteins (UCPs) proposed by Hanak and Jezek (2001) [FEBS Lett. 495, 137–141] suggests that UCP4 is the earliest form of UCP ancestral to all other UCP orthologues. However, this hypothesis is difficult to reconcile with a narrow tissue distribution of UCP4 (which is a brain-specific isoform), suggesting highly specialized rather than ancestral function for this protein. We searched for UCP2, UCP3, and UCP5 homologues in invertebrate genomes using amplification with degenerate primers designed against UCP2-specific conserved sequences and/or BLASTP search with stringent ad hoc criteria to distinguish between homologues and orthologues of different UCPs. Our study identified invertebrate UCP homologues similar to UCP2 and 3 (which we termed UCP6) and an invertebrate homologue of UCP5. Phylogenetic analysis indicates that there are at least three clades of UCPs in invertebrates, which are closely related to vertebrate UCP1-3, UCP4, and UCP5, respectively, and shows early evolutionary divergence of UCPs, which pre-dates the divergence of protostomes and deuterostomes. It also suggests that the newly identified UCP6 proteins from invertebrates are ancestral to the vertebrate UCP1, UCP2, and UCP3, and that divergence of these three vertebrate orthologues occurred late in evolution of the vertebrates. This study refutes the hypothesis of Hanak and Jezek (2001) that UCP4 is an ancestral form for all UCPs, and shows early evolutionary diversification of this protein family, which corresponds to their proposed functional diversity in regulation of proton leak, antioxidant defense and apoptosis.

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1. Introduction

Uncoupling proteins (UCPs) belong to the mitochondrial anion carrier gene family and, as suggested by their name, can uncouple ATP production from mitochondrial respiration by causing proton leak [1,2]. The uncoupling proteins were best characterized in mammals where five different orthologues were identified – UCP1-4 and UCP5, or BMCP1 [2]. These proteins are expressed in different tissues and play

different roles in cellular metabolism. UCP1 is expressed exclusively in brown adipose tissue (BAT) and responsible for thermogenesis in hibernating mammals and mammal neonates [1,3]. UCP3 expression is mostly restricted to skeletal muscle in mammals, while UCP2 is widely distributed in all tissue types studied so far [2]. UCP4 and UCP5 are predominantly found in brain [3]. Specific physiological function in non-shivering thermogenesis is only well established for UCP1, whereas cellular functions of other UCPs are still a matter of debate. Current research demonstrated that all UCPs act as physiological uncouplers in vitro and in vivo, but failed to attribute a major portion of mitochondrial proton leak in tissues other than BAT to a single UCP [1,3]. It has also been shown that UCPs may play a role in antioxidant defense of mitochondrial matrix by causing “mild” uncoupling, which dissipates the protonmotive force and decreases production of reactive oxygen species [4]. This function was in particular suggested for UCP2, which has tissue-wide expression [5]. Brain-specific UCP4 and UCP5 has been suggested to play a role in apoptosis in the brain [6,7].

Given an important role of UCPs in proton leak, antioxidant defense, and apoptosis, one may expect a wide phylogenetic distribution of these proteins. Indeed, UCPs were described in poikilothermic vertebrates (i.e., fish) and plants [8,9]. Early genome-wide search did not reveal any homologues of UCPs in genomes of *Caenorhabditis elegans*, *Drosophila melanogaster* or *Saccharomyces cerevisiae* [10]. In contrast, a recent study by Hanak and Jezek [11] using presumptive UCP signature motifs (i.e., common sequence motifs that are found in UCPs but not in other mitochondrial carrier proteins) identified several putative proteins in *D. melanogaster* and *C. elegans* genomes. The phylogenetic analysis performed by the authors led them to a suggestion that invertebrate UCPs are most closely related to the vertebrate UCP4, but distant from UCP1, 2, and 3, and thus that UCP4 is an ancestral form of UCP. Other UCPs were thought to represent novel evolutionary acquisitions, which appeared following the divergence of protostomes and deuterostomes and independently in plants [11]. However, this hypothesis is difficult to reconcile with the predominant expression of UCP4 in mammalian brains and virtual absence of this form from other tissues, suggesting a specialized function for this form of UCP as compared to the ubiquitous isoform UCP2, which is likely to be a functional generalist and thus an evolutionary early form.

In contrast to Hanak and Jezek [11], we hypothesize that the divergence of the UCP family predates the divergence of

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protostomes and deuterostomes, and that UCP2 homologues could be found early on the phylogenetic tree of animals. Because the UCP signatures approach used earlier by Hanak and Jezek [11] failed to identify UCP2 homologues in invertebrate genomes, we used an alternative approach: we designed degenerate primers based on highly conserved amino acid motifs of UCP2 and used them to amplify a fragment of putative UCP2 homologue from genome and transcriptome of the eastern oyster *Crassostrea virginica*. We then submitted the partial UCP sequence from the oyster to BLASTP to search for other invertebrate homologues of UCP2. In a similar way, we used BLASTP to identify UCP3 and UCP5 homologues from invertebrate genomes using vertebrate proteins as a query. Finally, we performed two phylogenetic analyses using partial and full UCP sequences from vertebrates and invertebrates, which included newly identified UCPs from insects and the oyster and known UCPs from *Drosophila*, fish and mammals. Our study clearly demonstrates that the divergence of uncoupling proteins is an early evolutionary event predating the divergence of protostomes and deuterostomes and suggests an early functional diversification of this important protein family. It also suggests that novel invertebrate UCP6 is ancestral to UCP1, 2, and 3 from vertebrates, and that diversification of this subfamily of UCPs occurred after the evolutionary divergence of protostomes and deuterostomes.

2. Materials and methods

2.1. Sample collection and isolation of nucleic acids

The eastern oysters *C. virginica* Gmelin were collected from Wilmington, NC. DNA was extracted from gill tissue of individual oysters as described by Sokolov [12]. Total RNA was extracted using TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. mRNA was extracted from 150 to 200 µg of total RNA using Oligotex mRNA Mini Kit (QIAGEN, Valencia CA, USA).

2.2. Primer design and amplification of the putative UCP

Degenerate primers were designed by reverse translation of highly conserved amino acid sequences based on the sequence alignment of UCP2 proteins from a fish (*Danio rerio*), a mouse (*Mus musculus*), and a plant (*Arabidopsis thaliana*) (NCBI Accession Nos. AAH56737, AAH12967, and NP_568894, respectively). The primer sequences were:

UCP-2F 5' CCA CTG GAC ACN GCN AAR GTN AG 3'

UCP-2R 5' AGC CTG CAC CTT CAC NAC RTC NGT NGG 3'

We performed 50 µl PCRs each containing 5 µl of 10× Taq polymerase buffer and 1 U of Taq DNA polymerase (Promega, Madison WI, USA), 2.5 mM MgCl₂, 100 µM of each dNTP, 0.2 µM of each primer, and 50–80 ng of genomic DNA. Amplification was performed under the following conditions: initial denaturation at 95 °C, 5 min; 35 cycles of 45 s at 95 °C, 45 s at 50 °C, 45 s at 72 °C; final extension step of 45 s at 95 °C, 45 s at 50 °C, 5 min at 72 °C using Mastercycler thermal cycler (Brinkmann, Westbury NY, USA). Fragments were resolved on 1.5% agarose gel stained with ethidium bromide. Bands corresponding to ca. 650 bp fragment which amplified consistently from all five studied individuals were cut out, purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia CA, USA) and cloned with Perfectly Blunt Cloning Kit (Novagen, San Diego CA, USA). Three insert-positive clones were randomly selected and sequenced with a reverse and forward primer (Sequetech, Mountain View CA, USA). Sequences were aligned using Clustal W version 1.81 (<http://clustalw.genome.jp>) and found to be identical. To determine whether this UCP homologue is expressed in oyster tissues, we designed specific primers to the sequences of the putative exons based on the obtained genomic sequence. The primer sequences were:

UCP3-6F262 5' CCA AAA CAA TGA AGG TGG GCG TCC 3'

UCP3-6R574 5' CAG TGG TCA CTC CCG CGA AGA CA 5'

RT-PCR from *C. virginica* mRNA was performed using OneStep RT-PCR kit (QIAGEN, Valencia CA, USA) according to the manufacturer's protocol. Target fragments were amplified under the following conditions: reverse transcription step of 30 min at 50 °C; initial PCR activation step of 15 min at 95 °C; 35 cycles of 45 s at 95 °C, 45 s at 50 °C, and 45 s at 72 °C; final extension step of 45 s at 95 °C, 45 s at 50 °C, and 10 min at 72 °C. To check for possible DNA contamination of the mRNA samples, we performed RT-PCR with the same reaction mixture omitting the reverse transcription step. No product was obtained indicating that our samples were not contaminated with DNA (data not shown). Amplified fragments were gel-purified, cloned, and sequenced as described above.

The genomic UCP sequence from *C. virginica* was used for homology search in the protein databases using BLAST X 2.2.2 program [13]. Translation of the nucleotide sequence to the protein sequence was done using the universal codon table. Positions of putative intron and exons were determined based on the presence of stop codons and BLAST X alignments, and post hoc verified for the presence of splice donor and acceptor sequences (GT and AG, respectively) and by comparison with the mRNA sequence (AY736103–736106). Inferred UCP protein sequence from *C. virginica* was submitted to BLASTP search against the NCBI protein database [13].

Phylogenetic analysis was performed using the maximum likelihood with molecular clocks algorithms as implemented by PHYLIP 3.62 [14]. Amino acid sequences of UCP proteins from vertebrates and insects were obtained from the NCBI database using the following Accession Nos.: UCP1 – AAH69556, UCP2 – AAB48411, UCP3 – AAC18822, UCP4 – O95847, and UCP5 (BMCP) – O95258 from human; UCP2 – Q9W720, UCP3 – AAQ97861, and UCP5 – BI474135 from *D. rerio*; UCP6 – XP_320838.1 from *Anopheles gambiae*; UCP4a – NP_573246, UCP4b – NP_723135.1, UCP4c – NP_608976, and UCP5 – AAK92857.1 from *D. melanogaster*. A multiple alignment of all UCP protein sequences and the translated sequence of the putative UCP from *C. virginica* was performed using Clustal W version 1.81 (<http://clustalw.genome.ad.jp/>), and the sequences from the vertebrates and insects were truncated to cut off the upstream and downstream portions of the proteins, which were not represented in the oyster fragment. The final fragments were 108–124 amino acids long and represented >30% of the total protein length. Fragment alignments were used as an input file to generate a consensus tree and bootstrap values in PHYLIP 3.62. Graphic representation of the resulting phylogenetic tree was performed in TreeView v. 1.6.6. program [15]. In order to corroborate the phylogenetic relationships between different UCPs from protostomes and deuterostomes, we have also performed a phylogenetic analysis using full protein sequences from vertebrates and invertebrates (omitting UCP6 from *C. virginica* and UCP5 from *D. rerio*, for which the full sequences are not known) as described above.

3. Results

Degenerate primers consistently amplified a ca. 650 bp fragment from oyster genomic DNA (NCBI Accession No. AY736103), which demonstrated high similarity to uncoupling proteins 2 and 3 (*E* values 10⁻¹¹–10⁻¹⁰, 45–49% identity with vertebrate proteins) and was termed UCP6. RT-PCR from mRNA using specific primers designed against exons of the genomic sequence confirmed that this is a transcribed sequence and not a pseudogene (NCBI Accession No. AY136106). BLAST P search also identified a putative UCP6 from *A. gambiae* (NCBI No. XP_320838.1), which has high similarity with the oyster UCP6 fragment and vertebrate UCP2 and 3 (Figs. 1 and 2). Equally high similarity of the newly identified UCP6 from oyster and insect with UCP2 and UCP3 of the vertebrates suggests that these proteins may be homologous to an ancestral UCP, which later diverged into UCP2 and UCP3 orthologues in vertebrates.

We also used BLASTP to perform homology search of invertebrate homologues of UCP3 and UCP5. As an ad hoc

Uncoupling protein 2

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hsUCP2 PLDTAKVRLQIQGE-----SQGPVRATASAQYRGVMGTILTMVR
agUCP6 .....QPVRTVALAPATINTSASLKLNPST....QHV....LV...T.IT.
cvUCP6 .....VTRQWSSFALHG-----VFTRVQNNNEGGRPK....IR.LRI.CQ

hsUCP2 TEGPRSLYNGLVAGLQRQMSFASVRIGLYDSVKQFY----TKGSE---HASIGSRLLAGS
agUCP6 Q..F.T.....S.....LC.C.I.....T..T..GSLLENENA---GLQ..T.V...L
cvUCP6 E..M.G..T..TPAIH..LG..T.K..C..NT.HL.TKLR.YR.E.EPSYCPVCI.VF..V

hsUCP2 TTGALAVAVAQPTDVVKVR
aUCP26...GA..MI.....
cvUCP6 .....S.SL.....Q
    
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Fig. 1. Multiple alignments of translated sequences of the UCP fragment from *C. virginica* (NCBI No. AY736103), a putative UCP6 from *A. gambiae* (NCBI No. XP_320838.1) and UCP2 from human (NCBI No. AAB48411) using Clustal W version 1.81 (<http://clustalw.genome.ad.jp/>). Amino acids, which are identical with the human UCP2 sequence, are substituted by dots. Dashes indicate gaps.

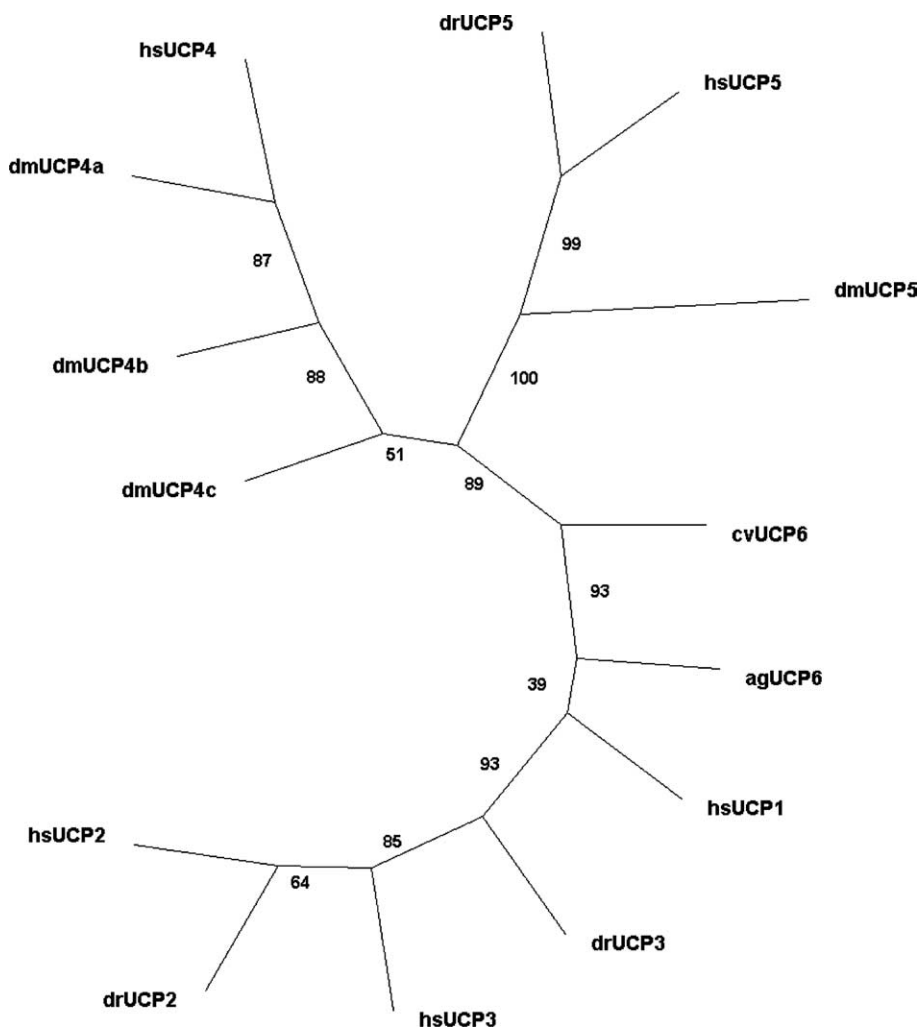


Fig. 2. Unrooted maximum likelihood tree as implemented by PHYLIP 3.6 [14] based on translated sequences of the putative UCP gene from *C. virginica* and partial sequences of UCP proteins from GenBank. The bootstrap values on the tree were generated by Phylip 3.62 based on 100 iterations and are shown next to the respective branches. Protein sequences: hsUCP1, hsUCP2, hsUCP3, hsUCP4, hsUCP5 – UCP1, UCP2, UCP3, UCP4, and UCP5, respectively, from *H. sapiens*, drUCP2, drUCP3, drUCP5 – UCP2, UCP3, and UCP5, respectively, from *D. rerio*, dmUCP4a, dmUCP4b, dmUCP4c, dmUCP4d – putative UCPS from *D. melanogaster*, agUCP6 – UCP from *A. gambiae*, cvUCP6 – translated sequence of the putative UCP gene from *C. virginica*. NCBI Accession Numbers for all sequences are given in Section 2.

criterion of homology rather than orthology, we required that similarity between the putative invertebrate homologue and the vertebrate query was higher than between two orthologues

from the same species (e.g., between different UCPS from the human). Using this criterion, we identified a putative UCP5 from *D. melanogaster* (NCBI No. AAK92857.1), which had

considerably higher similarity to human UCP5 (E value of 10^{-86}) than different human UCP orthologues have to each other (E value of 10^{-54} – 10^{-51}). BLASTP search with the same criterion failed to identify an invertebrate UCP3 homologue. The closest invertebrate homologue to the human UCP3 was UCP6 from *A. gambiae* (see above), but the similarity between the human UCP3 and the mosquito UCP was much lower (E values of 10^{-70}) than a similarity between human UCP1, 2, and 3 (E values of 10^{-124} – 10^{-96}). This supports our earlier conclusion that invertebrate UCP6 may be close to an ancestral UCP form, which later diversified into UCP1, 2, and 3 within the vertebrates (see below Figs. 2 and 3).

In the light of these new findings, we re-evaluated the evolutionary relationships between different vertebrate and invertebrate UCPs to test the hypothesis of Hanak and Jezek [11] that UCP4 is an ancestral form of the UCPs in vertebrates and invertebrates. For this analysis, we used putative UCP4 homologues from *D. melanogaster* from their work (dmUCP4a, b, c), UCP6 from the oyster and the mosquito, and UCP5 from *D. melanogaster* identified in this study. A phylogenetic analysis of partial UCP sequences from vertebrates and invertebrates unequivocally indicates three separate clades of vertebrate and invertebrate UCPs supported by high bootstrap values (Fig. 2). It is worth noting that bootstrap values >80% supporting a particular clade on a consensus tree are considered to be a strong evidence that proteins included into this clade are more similar (and thus more closely related) to each other than to any other protein on this tree [14]. Our analysis indicates that UCP6 from *A. gambiae* and *C. virginica* are significantly more similar to UCPs 1, 2, and 3 from vertebrates than to other vertebrate and invertebrate UCPs (bootstrap values of 84%). Invertebrate UCP6 proteins occupy a more basal position on the UCP1–3 branch, suggesting that they represent primitive, close to ancestral forms from which vertebrate UCP1, 2, and 3 have diverged. Two of the *Drosophila* UCP orthologues (UCP4a and UCP4b) are more closely related to the vertebrate UCP4 than to any other vertebrate of invertebrate UCP (bootstrap values of 87–88%). Phylogenetic position of UCP4c from *D. melanogaster* was not clearly resolved on this tree (51% bootstrap). And finally, putative UCP5 from *D. melanogaster* clusters with UCP5 from vertebrates, and the bootstrap value for its inclusion into the UCP5 clade is 100%, indicating much higher similarity between vertebrate and invertebrate UCP5 than between them and any other UCP isoform. A phylogenetic analysis based on the available full UCP sequences from vertebrates and invertebrates corroborates this conclusion and supports an early divergence of the UCP family of proteins into the same three major clades corresponding to UCP1–3 + UCP6 group, UCP4 and UCP5 with bootstrap values of 100%, 84%, and 100%, respectively (data not shown). Analysis of full sequences also improved resolution within the UCP1–3 branch and indicated that vertebrate UCP2 and 3 are more similar to each other than to UCP1 and UCP6 (bootstrap value of 95%), and that UCP2 from human and fish are more similar to each other than to UCP3 (bootstrap value of 100%).

4. Discussion

Phylogenetic analysis of Hanak and Jezek [11] suggested that putative UCPs from *Drosophila* and *Caenorhabditis* are more closely related to the human UCP4 than to other UCP

forms based on the fact that they cluster together on the inferred phylogenetic tree (Fig. 2 in [11]). In contrast, putative UCP4 genes from plants and a protozoan *Dictyostelium discoideum* were included in a separate cluster together with other vertebrate UCPs (1, 2, 3, and 5) [11]. The authors did not provide bootstrap values for their inferred phylogenetic tree, therefore it is difficult to determine how robust are the clades in their analysis. Based on these data, Hanak and Jezek [11] suggested that UCP4 most probably represents the ancestral UCP type from which the other invertebrate, mammalian, and plant uncoupling proteins diverged.

The results of this study contradict this hypothesis and show that uncoupling proteins diverged into at least three genetically (and likely also functionally) distinct forms early in the evolution. These three forms correspond to the three clades identified by the phylogenetic analysis and supported by high bootstrap values (84–100%): clade 1 containing vertebrate UCP 1, 2, and 3 genes and the newly identified UCP6 from *C. virginica* and *A. gambiae*; clade 2 containing vertebrate UCP5 and an UCP5 homologue from *D. melanogaster*; and clade 3 including UCP4 from mammals and UCP4a and UCP4b from *D. melanogaster*. UCP6 from invertebrates appear to be ancestral to the vertebrate UCP1, 2, and 3 (Fig. 3).

What are the possible ancestral functions of invertebrate UCP homologues? In vertebrates, UCP2 and 3 have the widest tissue distribution of all UCP isoforms and are more likely candidates for retaining less specialized, ancestral functions than highly specialized brown fat-specific UCP1 involved in non-shivering thermogenesis. It has been suggested that UCP2 and 3 may be involved in the regulation of energy expenditure, thermogenesis and body weight regulation in mammals through regulation of physiological proton leak [9]. Recent studies have confirmed that UCP2 and 3 act as uncouplers in vivo and in vitro, but failed to ascribe significant thermogenic function to any of these proteins [1,3] or demonstrate their role in body weight control [16]. On the other hand, UCP3 knockout mice models showed increased production of reactive oxygen species and oxidative stress, indicating that UCP3 may be involved in antioxidant defense [5]. There is also evidence that mammalian UCP2 is involved in the regulation of ROS production and ROS sensing [2,4,5]. This suggests that the primary function of UCP2 and UCP3 may be protection against excessive build-up of reactive oxygen species (ROS) in mitochondria by causing mild uncoupling [4,5]. Our analysis supports this idea and shows that UCP2 and 3 and their invertebrate homologue UCP6 are expressed in poikilothermic invertebrates as well as in fish, which cannot maintain their body temperature by endothermic heat generation. This excludes thermogenesis as a primary function of UCP2 and 3 homologues in these animals. We suggest that regulation of ROS formation may be an ancestral function of UCP2/3 homologues shared by invertebrates and vertebrates. Since this function involves regulation of proton leak, which is also crucial for thermogenesis, thermogenic function of UCP1 in mammals may have evolved on the basis of this ancestral function.

Early evolutionary occurrence of UCP4 and UCP5 homologues in invertebrates also suggests that these proteins carry out some basal functions, which are similar in vertebrates and invertebrates. In vertebrates, UCP4 and UCP5 are predominantly expressed in brain tissue and were suggested to play an important role in apoptosis, neuronal differentiation and synaptic plasticity in the brain [6,7]. Therefore, the ancestral function

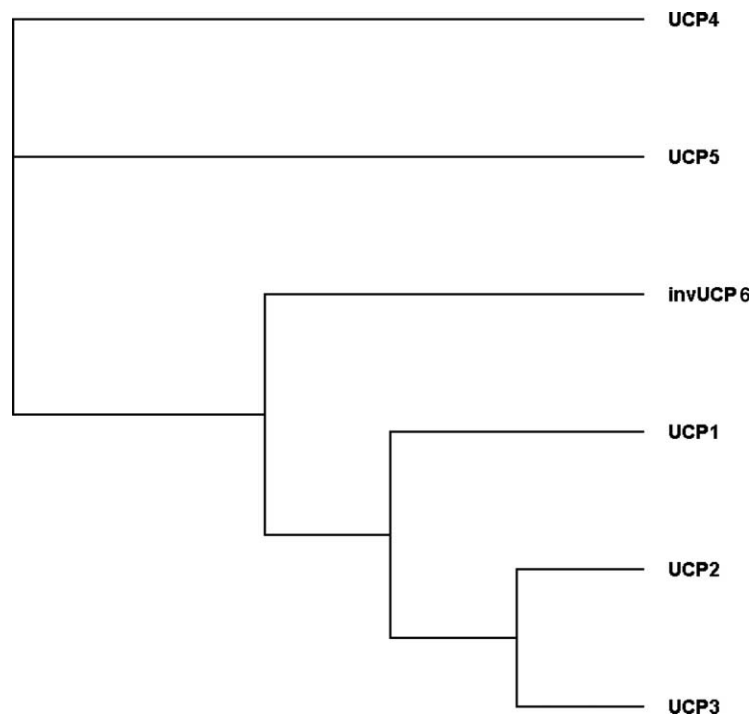


Fig. 3. A schematic diagram of the proposed hypothesis of evolution of animal UCPs. We hypothesize that animal UCPs diverged into three major branches early in evolution before the divergence of protostomes and deuterostomes. The trichotomy at the base of the hypothetical tree reflects insufficient resolution of the analysis to determine the order of evolutionary appearance of each UCP branch. Two of these branches gave rise to vertebrate and invertebrate UCP4, and vertebrate and invertebrate UCP5, respectively. The third branch gave rise to invertebrate UCP6 and an ancestral vertebrate UCP, which has later diverged into UCP1, 2, and 3.

of these proteins may be regulation of apoptosis during development of the central nervous system and/or protection from excessive apoptosis during stress in neural tissue.

As a corollary, our research refutes the currently accepted notion that UCP4 is ancestral form for all UCPs and demonstrates that genetic divergence of UCP family of proteins occurred very early in evolution before the divergence of protostomes and deuterostomes. This early genetic divergence corresponds to the diversity of key cellular functions carried out by these proteins, including regulation of physiological proton leak, mitochondrial ROS production and apoptosis. Thermogenic functions of UCPs (with UCP1 as an extreme example) probably developed later in the evolution through the enhancement of basal uncoupling properties shared by these proteins.

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