

Novel reptilian uncoupling proteins: molecular evolution and gene expression during cold acclimation

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Many animals upregulate metabolism in response to cold. Uncoupling proteins (UCPs) increase proton conductance across the mitochondrial membrane and can thereby alleviate damage from reactive oxygen species that may form as a result of metabolic upregulation. Our aim in this study was to determine whether reptiles (*Crocodylus porosus*) possess UCP genes. If so, we aimed to place reptilian UCP genes within a phylogenetic context and to determine whether the expression of UCP genes is increased during cold acclimation. We provide the first evidence that UCP2 and UCP3 genes are present in reptiles. Unlike in other vertebrates, UCP2 and UCP3 are expressed in liver and skeletal muscle of the crocodile, and both are upregulated in liver during cold acclimation but not in muscle. We identified two transcripts of UCP3, one of which produces a truncated protein similar to the UCP3S transcript in humans, and the resulting protein lacks the predicted nucleotide-binding regulatory domain. Our molecular phylogeny suggests that uncoupling protein 1 (UCP1) is ancestral and has been lost in archosaurs. In birds, UCP3 may have assumed a similar function as UCP1 in mammals, which has important ramifications for understanding endothermic heat production.

Keywords: metabolism; oxidative phosphorylation; reactive oxygen species; thermoregulation; temperature; crocodile

1. INTRODUCTION

Animal fitness depends on sufficient ATP production for housekeeping functions, such as maintaining membrane potentials, protein synthesis and gluconeogenesis (Hulbert & Else 2000), as well as for growth, reproduction and locomotion. Therefore, it is of selective advantage for animals to regulate metabolic pathways so that varying ATP concentrations do not constrain animal performance (Brand 1997). Physiological and biochemical rates are particularly sensitive to changes in temperature, and ectothermic organisms are potentially exposed to large fluctuations in rate functions as a result of environmental temperature fluctuations (Somero 1995; Guderley & Johnston 1996; Guderley 2004). Many ectotherms therefore regulate cellular rate functions to offset potentially detrimental thermodynamic effects (Guderley & St-Pierre 2002). Although terrestrial reptiles regulate their body temperature (Hertz *et al.* 1993), diurnal and seasonal variations in body temperature can be considerable (Seebacher *et al.* 1999, 2003a). Hence, both aquatic and terrestrial ectotherms upregulate glycolytic and oxidative pathways in cooler conditions, e.g. in winter, to achieve reaction rates that are similar to summer conditions despite 10–15°C differences in body temperature (Guderley & Johnston 1996; St-Pierre *et al.* 1998; Seebacher *et al.* 2003b; Glanville & Seebacher 2006).

Oxidative ATP generation encompasses electron transport between complexes I and IV in the inner mitochondrial membrane. In consequence, a proton gradient is

established across the inner mitochondrial membrane, and its release powers the phosphorylation of ADP to ATP in complex V (ATP synthase) of the electron transport chain. Protons can leak through the membrane, and this proton leak leads to oxygen consumption without ATP production and may constitute up to 20% of resting metabolic rate (Rolfe & Brand 1996). During cold exposure, the capacities of rate-limiting enzymes in oxidative pathways are upregulated so that the depressing effect of decreasing temperature on ATP production is ameliorated (Johnston *et al.* 1985; St-Pierre *et al.* 1998; Lucassen *et al.* 2003). A negative consequence of increasing metabolic capacities is that the rate of production of reactive oxygen species (ROS) may also increase (Skulachev 1998; Abele *et al.* 2002). In fact, the ROS production may set the upper limit to metabolic upregulation (Brand *et al.* 2004; Guderley 2004). The advantages of metabolic regulation during thermal acclimation are maximized if the damage caused by ROS is minimized. It would be advantageous, therefore, if the proton leak could be regulated.

Several intermembrane proteins can facilitate proton leak and transport fatty acid anions across the inner mitochondrial membrane (Kadenbach 2003; Schaefer & Staples 2006). Of these proteins, uncoupling protein 1 (UCP1) has the best known uncoupling function, and it facilitates non-shivering thermogenesis in brown adipose tissue of mammals (Argyropoulos & Harper 2002). Uncoupling proteins (UCPs) are evolutionarily ancient, and orthologues occur in many eukaryotes (Jastroch *et al.* 2004; Brand & Esteves 2005; Ito & Seymour 2005; Nogueira *et al.* 2005), although the function of UCP1 paralogues (e.g. UCP2 and UCP3) in uncoupling

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electron transport from ATP production is disputed (Nedergaard & Cannon 2003; Mozo *et al.* 2006; Schrauwen *et al.* 2006). UCP2 and UCP3 may protect mitochondrial membranes against ROS damage by a mild uncoupling of the electron transport chain, thereby reducing the energy contained in the proton gradient (Skulachev 1998), or by translocating fatty acid peroxides across the membrane (Goglia & Skulachev 2003). Avian uncoupling protein (avUCP) when expressed in yeast cells protects mitochondria against damage inflicted by ROS (Criscuolo *et al.* 2005). Cold exposure induces UCP expression in birds (Raimbault *et al.* 2001; Toyomizu *et al.* 2002), presumably owing to their protective function in response to superoxide production (Brand *et al.* 2004).

There may be a trade-off between the benefits of metabolic regulation during acclimation to different thermal environments and the ROS damage it incurs. Hence, here we test the hypothesis that UCPs are important in the metabolic acclimation of a thermoregulating ectotherm, the crocodile *Crocodylus porosus*. UCPs are not known for reptiles, but given their phylogenetic distribution they are likely to be present. Our aim was to determine whether reptiles possess UCP genes and, if so, to place them within a phylogenetic context, and to determine whether the expression of UCP genes is increased during cold acclimation.

2. MATERIAL AND METHODS

(a) Gene identification and phylogenetic analyses

Total RNA was isolated from 50 mg of snap-frozen tail muscle (*longissimus caudalis*) and liver tissue from *C. porosus* using TRIreagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. One microgram of total RNA was treated with the 3 U Dnase I (Sigma, USA) to degrade any residual DNA. RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Australia) using 200 U of SuperScript III reverse transcriptase, 250 μ M random hexamer primers and 2 U of RNase H according to the manufacturer's instructions.

Vertebrate UCP sequences in GenBank regions of the UCP were used to design semi-degenerate primers similar to the previously published primers to target UCP genes in *C. porosus*: UCP-reverse primer 5'-3': AACATNAY-BACGTTCCASGABCC, similar to Raimbault *et al.* (2001) and UCP-forward primer: CTTYCCNYTGGACACNGC-CAAAG, similar to Vianna *et al.* (2001). The PCR contained: 1X ImmoMix (Bioline, USA); 2.25 mM MgCl₂; 1.5 μ M forward degenerate primer; 0.35 μ M reverse degenerate primer; and 1 μ l cDNA from muscle. Cycle: 95°C for 7 min (hotstart); 15 cycles of: 94°C for 25 s, 47°C for 25 s, 60°C for 10 s, 65°C for 10 s, 72°C for 1 min; 25 cycles of: 94°C for 25 s, 52°C for 25 s, 65°C for 10 s, 72°C for 1 min; and 72°C for 3 min. The PCR products were excised from 2% low-melt gel, purified (Wizard SV Gel and PCR Clean-up System, Promega, USA) and TA-cloned (Topoisomerase T-A cloning Kit, Invitrogen). Twelve clones were sequenced (Macrogen, South Korea) and BLASTED in GenBank. Three sequences were identified as belonging to the UCP family: one similar to UCP2 and two similar to UCP3 (hereafter referred to as UCP3a and UCP3b). Cloned genes from *C. porosus* have been submitted to GenBank (accession numbers: EF623984; EF623985; and EF623986). We verified our new sequences

by amplifying the UCP3a and UCP2 genes from cDNA from four different individuals using our real-time PCR primers and sequencing the cleaned PCR products directly.

For the phylogenetic analyses, additional sequences were obtained through the searches of translated protein sequences on the databases GenBank, Ensembl and the Joint Genome Institute (accession numbers are given in figure 2), using TBLASTN searches with ancestral UCP1, UCP2 and UCP3 genes. Genes less than 300 bp in length were not informative enough to include in the phylogenetic analysis. We aligned our sequences using CLUSTAL W and MAFFT, and also examined them manually for errors. We examined our alignment for violation of the assumption of homogeneity of variance, using Bowker's test of symmetry (Bowker 1948). This showed that the alignment did not violate the assumption of homogeneity of variance, as only 5 out of 1770 tests were significant at the 0.05 level.

We used the Akaike information criterion to determine the log likelihood of 79 different substitution models for the analysis of our aligned protein sequences, respectively, using the program PROTTEST (Abascal *et al.* 2005). The optimal model was found to be JTT (Guindon & Gascuel 2003) and gamma distributed across-site rate variation, with a proportion of invariant sites. Alignments were analysed using maximum likelihood (ML) and the optimal model and parameters in the program PHYML v. 2.4.4 (Guindon & Gascuel 2003). ML bootstrap analyses were performed using 100 replicates. Alignments were also analysed using Bayesian inference using the same optimal model parameters, in the program MRBAYES (Huelsenbeck & Ronquist 2001). Generations were run until the standard deviation of split frequencies was less than 0.01, and sampled every 100 generations, discarding the results of the first 150 000 generations.

To evaluate whether both the UCP3a and the UCP3b were functional genes, we conducted pairwise tests for positive and purifying selection in MEGA v. 3.1 (Kumar *et al.* 2004) using the Z-test of selection with the Nei-Gojobori method (*p*-distance; Nei & Kumar 2000) on the UCP3a and UCP3b from the crocodile, UCP3 from pig and cow, and avUCP.

(b) Acclimation treatments

Thermal conditions for two acclimation treatments were chosen to resemble winter (mean body temperature 20.9°C, *N*=8 crocodiles; mean (\pm s.e.) mass=182.5 \pm 7.1 g) and summer (mean body temperature 29.0°C, *N*=9; mean (\pm s.e.) mass =171.8 \pm 12.6 g) in *C. porosus*' natural habitat in Australia, as described previously (Glanville & Seebacher 2006). Juvenile (1-yr old) crocodiles of undetermined sex were acclimated for 33 days, then euthanized (sodium pentobarbitone 200 mg kg⁻¹) and liver and tail muscle (*longissimus caudalis*) samples were collected for the following analyses.

(c) Gene expression analyses

Samples taken for gene expression analyses were immediately stored in RNAlater (Ambion, USA). RNA extraction, reverse transcription and qRT-PCR (using an Applied Biosystems 7500 qRT-PCR machine, Applied Biosystems, USA) were conducted according to published protocols (Seebacher & Murray 2007). New primers and probes for qRT-PCR were designed from the *C. porosus*-cloned sequences to amplify UCP2 specifically, and both UCP3a and UCP3b simultaneously and, as a housekeeping gene, 28S rDNA (see Seebacher & Murray (2007) for sequences of

the latter); we were not able to design primers that amplified UCP3a and UCP3b differentially. The new primer and probe sequences were in 5'-3' direction: UCP2, forward: ATAACCTCCCGTGCCACTTCA; UCP2 reverse: CTCACGGCGCTGCTGTACT; UCP2 dual-labelled probe: (DFAM)-CTTCCGCATTGGGGCTGGCTT-(DBH2); UCP3 forward: GACAGCACCAGTATCC-TCACTC; UCP3 reverse: CGGTCCCATTGTACTTC TTG; UCP3 dual-labelled probe: (DCY5)-CTCACGTGA CGCTGATGGACGG-(DBH2). Real-time PCRs for *C. porosus* genes contained: 1X ImmoMix (Bioline), 4–5 mM MgCl₂, 900 nM primers and 250 nM probe, 1X ROX (Invitrogen, USA) and 50 ng cDNA. The cycle consisted of: 95°C for 7 min; 45 cycles of: 95°C for 15 s; 58.5°C for 40 s; and 60°C for 20 s.

Gene expression data for muscle were obtained from eight crocodiles in each treatment, and in liver sample sizes were $N=9$ warm-acclimated crocodiles for UCP2, $N=7$ warm-acclimated UCP3, $N=6$ cold-acclimated UCP2 and $N=3$ cold-acclimated UCP3; all assays were performed in triplicate. Relative gene expression in different tissues was calculated according to Pfaffl (2001). For statistical comparisons between acclimation treatments within tissues, we performed *t*-tests on cycle threshold values of target UCP gene expression normalized with 28S threshold values (Su *et al.* 2007); all data were log transformed to meet assumptions of normality.

3. RESULTS

Results of the BLAST search identified one clone with 82% nucleotide sequence identity to *Sus scrofa* UCP2. Two other clones showed 85% nucleotide sequence identity to *S. scrofa* and *Bos taurus* UCP3 (hereafter referred to as UCP3a and UCP3b). Among the crocodile amino acid sequences, UCP2 showed 36–38% divergence from the UCP3 sequences, and the UCP3a and UCP3b had 7% divergence. Based on annotated sequences in GenBank (Solanes *et al.* 1997; Argypoulos *et al.* 1998), the *C. porosus* UCP sequences extend from the end of exon 2 to the beginning of exon 7. All three of the crocodile UCP sequences contained the three conserved proton carrier signals that define mitochondrial carrier proteins. The predicted UCP2 and UCP3b proteins contain the α -helix transmembrane domains 2–6, but UCP3a had a stop codon in exon 6 that truncates the protein so that it is missing the last transmembrane domain and the predicted purine-binding site identified in human UCP1, UCP2 and UCP3L (figure 1).

The UCP2 orthologue in *C. porosus* is ancestral to a clade consisting of the mammalian UCP2 sequences, with a ML bootstrap support value of 75% (figure 2).

The two similar UCP3a and UCP3b transcripts were the sister group to a clade of UCP3 from birds, with a support value of 73%. Together, the archosaurian UCP3 clade was ancestral to a clade of UCP3 genes from mammals, with 85% support. In general, the UCP2 and UCP3 genes from all vertebrates except fishes formed two well-defined sister clades and appeared to be more derived than the mammalian and fish UCP1 clade. A gene identified as a *Danio rerio* UCP3 orthologue (NM_200353), based on synteny, did not cluster in the mammalian/reptilian UCP3 clade. This sequence clustered in the broad UCP2/3 clade, as the sister group to another fish UCP sequence (*Lethenteron japonicum*), but within this the branches could not be

grouped in any particular clade. Vertebrate UCP1, UCP2 and UCP3 genes together formed a well-supported (92%) clade, separate to the invertebrate, plant and protist UCP genes, as well as the vertebrate and invertebrate UCP4 and UCP5 genes.

Pairwise comparisons for positive or purifying selection indicate purifying selection ($p \leq 0.026$) for all pairwise comparisons between the crocodile UCP3a and UCP3b, avUCP, pig and cow UCP3.

(a) Acclimation experiments

Gene expression of UCP2 and one or both of the UCP3 genes was significantly upregulated in the liver of the cold-acclimated crocodiles (UCP2: $t_{13} = -2.57$, $p < 0.03$; UCP3: $t_8 = -2.41$, $p < 0.05$). In contrast, transcription of UCP2 and the UCP3 genes in the muscle were not significantly affected by acclimation treatment (UCP2: $t_{14} = -0.63$, $p = 0.54$; UCP3: $t_{14} = 0.12$, $p = 0.91$; figure 3).

4. DISCUSSION

We present the first evidence of the presence and expression of UCP genes in non-avian reptiles. UCP1, UCP2 and UCP3 have been identified in fishes (Jastroch *et al.* 2005) and eutherian mammals (Boss *et al.* 1997; Fleury *et al.* 1997). Interestingly, we have found only UCP2 and UCP3 genes in the crocodile, and not UCP1. Our crocodilian UCP sequences allow us to verify the homology of the single UCP described in birds (avUCP), as birds are a sister group to crocodiles. The avUCP clearly clusters with reptilian and mammalian UCP3, and this gene is not closely related to either reptilian or mammalian UCP2 (Raimbault *et al.* 2001; Vianna *et al.* 2001). We designed our primers to amplify all UCP genes present, and our failure to detect UCP1 implies that this gene is absent in crocodiles, although to be certain this finding has to be verified by sequencing the genomic region where UCP1 is usually found in other vertebrates. Similarly, UCP1 is also not present in birds, and this pattern in archosaurs implies a sequential loss of UCP1 and then UCP2 genes (or at least their expression) during the evolution of the lineage leading from fishes to archosaurs.

Whether or not UCP2 and UCP3 function in uncoupling electron transport from oxidative phosphorylation has been in dispute since their discoveries (Boss *et al.* 1997; Fleury *et al.* 1997; Nedergaard & Cannon 2003). Although, UCP2 and UCP3 may not necessarily be regulated by the same mechanism as UCP1 (Nedergaard & Cannon 2003), a general consensus from studies on endotherms indicates that the former have the potential to decrease the proton gradient across the mitochondria membrane, thereby protecting the mitochondria from the production of ROS—a major cause of oxidative damage to the cell. For example, UCP3 can be activated by superoxides and lipid peroxides to reduce ROS production (Echtay *et al.* 2002). Cold exposure in mice caused a threefold increase in skeletal muscle UCP3 mRNA and protein (Simonyan *et al.* 2001), and mice without UCP3 have increased levels of oxidative damage to protein, lipids and DNA (Brand *et al.* 2002). Furthermore, cold exposure induces UCP expression in avian endotherms, presumably owing to their protective function in response to metabolic upregulation and superoxide production (Talbot *et al.* 2003). The superoxide-induced increase in proton conductance is inhibited by

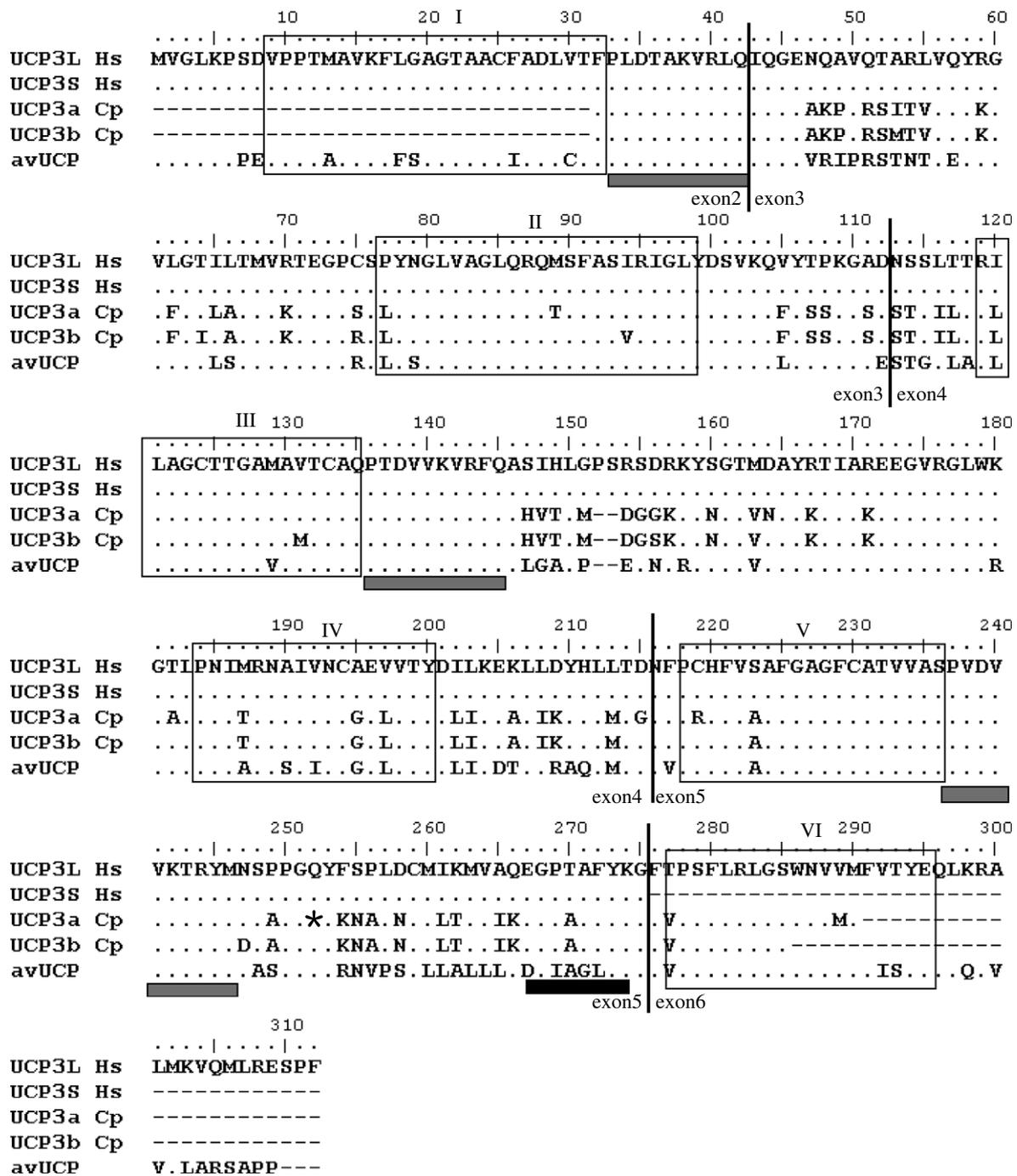


Figure 1. Amino acid alignment of the long and short human UCP3 (UCP3L and UCP3S), the newly identified crocodile UCP3a (asterisk indicates the stop codon) and UCP3b, and avUCP. Exon boundaries are defined and the six (I–VI) α -helix transmembrane domains are identified by a box. Mitochondrial carrier signals have a thick grey underline and the potential nucleotide-binding regulatory domain has a thick black underline.

GDP, which points towards an uncoupling role of avUCP (Talbot *et al.* 2003). In addition, the expression of avUCP in yeast cells protects the mitochondria against damage inflicted by ROS (Crisuolo *et al.* 2005).

Similarly, we show that expression of UCP2 and UCP3 increases in liver following chronic cold exposure, which indicates that uncoupling of the electron transport chain may be important during cold acclimation in ectothermic reptiles. Additionally, cold acclimation causes a significant upregulation in the activity of cytochrome *c* oxidase, the principal rate-limiting enzyme in electron transport, in liver but not in muscle (Glanville & Seebacher 2006). The significant increase in gene expression of UCP2 and UCP3 in liver may be related to this upregulation of

oxidative capacity following cold exposure in *C. porosus*. However, this suggested role of UCPs in crocodiles must be verified experimentally.

The expression and cold-induced upregulation of UCP3 in the liver of crocodile is unlike patterns in other vertebrates. While UCP2 is expressed ubiquitously (Fleury *et al.* 1997), transcription of UCP3 is known to occur only in skeletal muscle of fishes (Jastroch *et al.* 2005), and skeletal muscle, heart muscle and brown adipose tissue in mammals (Brand *et al.* 2002). In carp, UCP1 and UCP2, but not UCP3, are expressed in liver (Jastroch *et al.* 2005). Perhaps in the absence of the UCP1 gene in crocodiles, UCP3 plays the functional UCP1 role in the liver? To extrapolate further from this proposition,

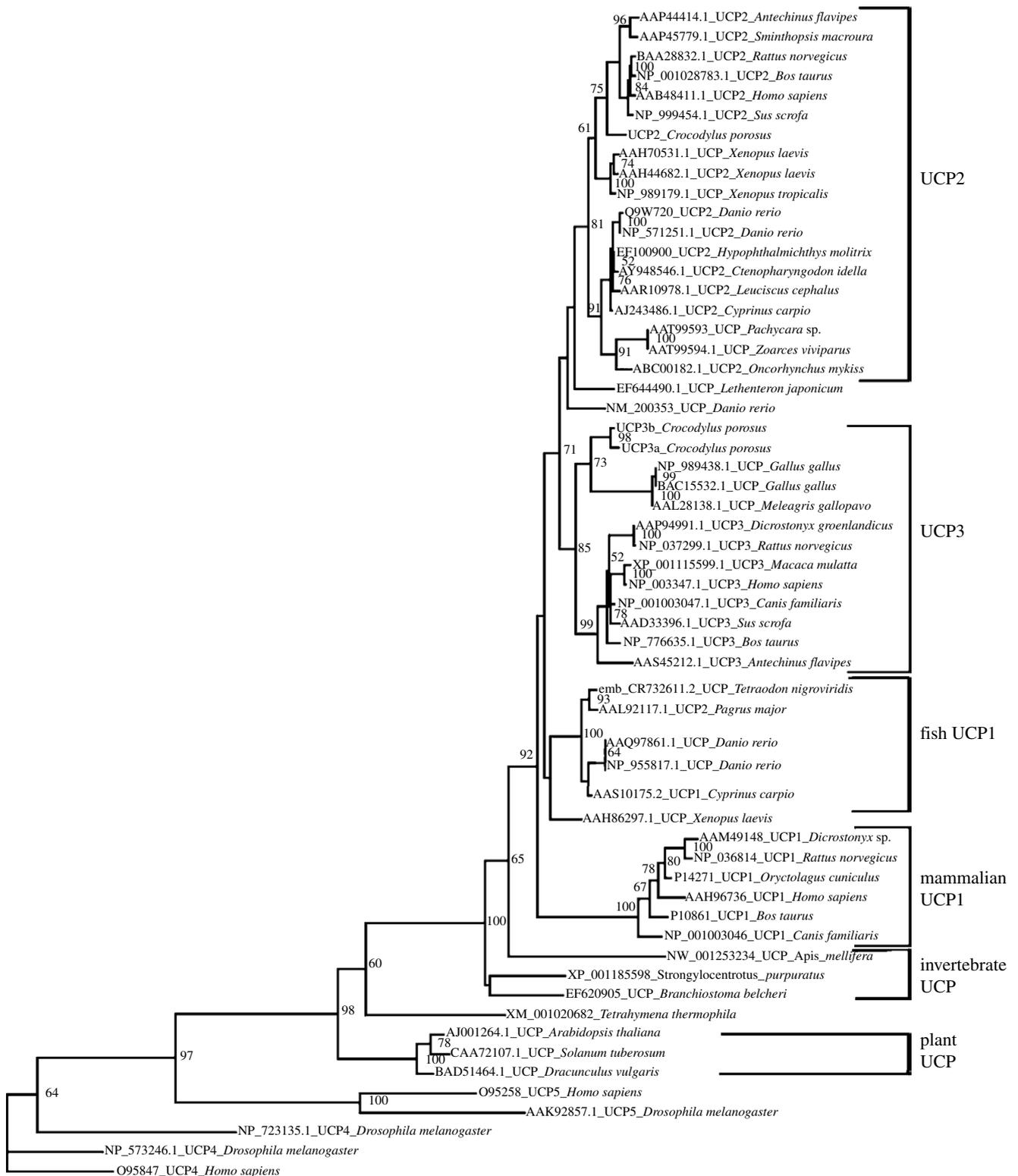


Figure 2. Results of the phylogenetic analysis of UCP homologues in vertebrates using ML and the substitution model JTT+I+G. The ML of the phylogeny = -6283.6. The values at nodes indicate maximum-likelihood bootstrap support values based on 100 replicates. Where support was less than 50%, values are not given.

in birds UCP3 may have assumed a similar function to UCP1 in mammals, and testing this hypothesis would significantly advance understanding of the evolution of endothermic heat production.

The two transcripts of UCP3 indicate a recent duplication event. UCP3a has a stop codon in the sixth exon that truncates the protein so that it lacks the sixth transmembrane domain, which is similar to the short version of the human UCP3 (UCP3S) protein. The UCP3S results from a

differential splicing event that produces an mRNA lacking the seventh exon, and a shortened protein (Solanes *et al.* 1997). In comparison, the crocodile UCP3a and UCP3b do not result from differential splicing, but are probably produced by different genes because nucleotide differences are found throughout their sequences, and are not restricted to a particular exon. Both genes were cloned from cDNA and were therefore being expressed, which indicates that both produce proteins. Furthermore, both are under

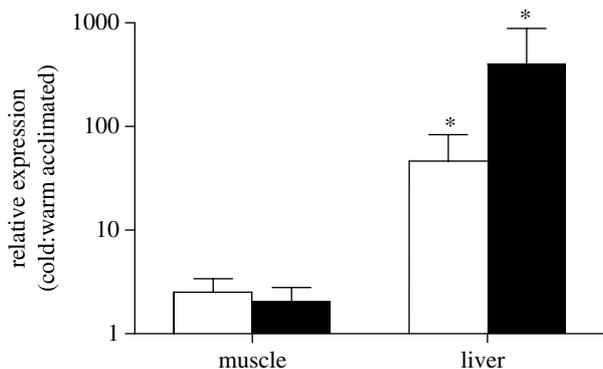


Figure 3. Gene expressions of the UCP2 and the UCP3 in muscle and liver. Gene expression of cold-acclimated animals is shown relative to warm-acclimated crocodiles. Expression of UCP2 and UCP3 genes was significantly greater in the liver of cold-acclimated animals, but there were no differences between acclimation treatments in muscle. Open bars, UCP2; filled bars, UCP3.

purifying selection (as well as UCP2), and no mutations leading to amino acid changes were found in regions that characterize functional mitochondrial carrier proteins, although there were amino acid differences in the α -helix domains (figure 1).

The functional role of UCP3S versus UCP3L has been questioned because UCP3S lacks the last intermembrane domain, which slows its insertion into the mitochondrial membrane, and is the region that contains the predicted regulatory nucleotide-binding domain (Solanes *et al.* 1997; Renold *et al.* 2000). This discovery of a similar truncated version of the UCP3 that has evolved independently (gene duplication versus differential splicing) provides intriguing potential for an adaptive function for this truncated protein lacking a regulatory domain. We were unable to differentiate the expression of UCP3a and UCP3b in our experiment, and it is not clear whether the two variants are differentially expressed in response to temperature. Considering the phylogenetic placement of reptiles in the evolution of vertebrates and endothermy, our data have important implications for the evolution of the UCP gene family and their functional proteins in the mitochondrial membrane of vertebrates. For example, do UCPs have a similar function in lepidosaurian reptiles that do not contain endothermic lineages, and which are not known to regulate metabolic capacity in response to environmental variability?

All experimental procedures were approved by the University of Sydney Animal Ethics Committee (approval no. L04/7-2003/3/3754).

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