POULTRY EVOLUTION

A CONCENTRATION ON NAG, CPSI, and the UREA CYCLE

by

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ABSTRACT

This study concentrates on the urea cycle of poultry and investigates the evolutionary process behind the absence of urea production in poultry. Previous genomic mapping studies of the genes encoding the urea cycle enzymes have detected all urea cycle genes except N-acetyl glutamate synthase (NAGS) and polymerase chain reaction (PCR) experiments revealed low levels of expression of the remaining enzymes. So, while this study began with the hypothesis that poultry did not contain the enzymes necessary to produce urea, genomic studies and sequence analysis shows that there is only one absent enzyme, NAGS, as well as one possibly deficient enzyme, carbamoyl phosphate synthase I (CPSI). It has been hypothesized that, by selecting against N-acetyl glutamate (NAG) production and thus eliminating synthesis of urea, poultry enabled the laying of land-based and hard-shelled eggs. CPSI, while found in the poultry genome, appears to be nonfunctional due to sequence analysis showing lack of a mitochondrial transit peptide. Why, then, is CPSI conserved in the chicken genome? This is potentially due to interactions between CPSI and Raf, a promoter of cell growth. While CPSI remains enzymatically nonfunctional in relation to urea cycle studies, it has been shown to interact with Raf (1). Phylogenetic tree analysis performed in this experiment also revealed that most of the urea cycle enzyme genes are in evolutionary agreement, suggesting that the genes are all intact, potentially serving some other purpose in the poultry genome. Finally, use of the Genomicus v66.01 gene search helped depict conserved syntanic relationships of CPSI with its surrounding enzymes across similar species. Meanwhile gene searching of NAGS

revealed much less conservation across the genomes of various organisms, with a notable absence of any NAGS locus in the anole. Closer analysis showing the deletion of the locus of NAGS in both lizards and birds makes it appear as though the deletion must have occurred basal to the divergence of these genuses, resulting in elimination of the urea cycle.

Chapter 1

INTRODUCTION

There exist three different classes of nitrogenous waste excretors: ureootelics—where nitrogenous waste is emitted in the form of urea via the urea cycle as shown in Figure 1 below, ammonotelics—where ammonia is the primary form of nitrogenous waste released, and uricotelics—where uric acid is the main form of nitrogenous waste produced (2). Poultry are manufacturers of hard-shelled eggs and excrete nitrogenous waste solely in the form of urate, also known as uric acid.

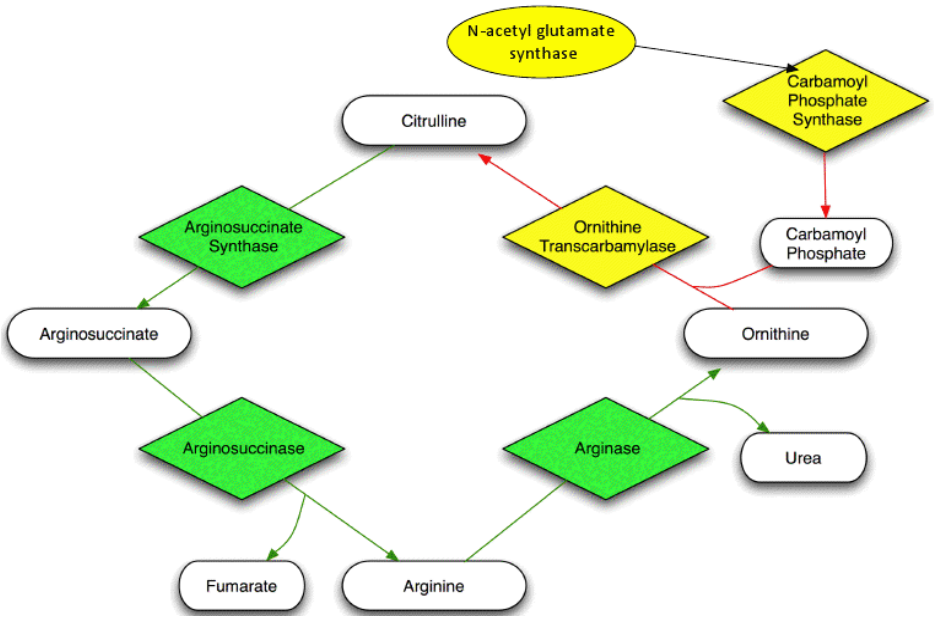


Figure 1 The urea cycle. Here the yellow colored enzymes and red path lines depict poultry's known inability to synthesize Arginine from Ornithine, while the green enzymes represent their alternate ability of creating Arginine from Citruline.

Similar to poultry, terrestrial turtles excrete nitrogenous waste mainly in the form of uric acid. These reptilians have also displayed significant liver arginase activities (28000 µmoles product/hr/g wet wt. liver**)** yet low levels of CPSI (10), ornithine transcarbamylase, OTC (276), and arginosuccinate synthase, ASS (10), a trend consistently in poultry, but varying greatly from other uricotelic animals showing low arginase activities in the liver (2). Despite the uricotelic functioning of terrestrial turtles, the ureotelic-type arginase functioning remains in small amounts compared to most ureotelics, suggesting that terrestrial turtles are intermediates in the evolutionary process toward uricotelism.

Previous studies in the lab of Dr. Carl Schmidt show that all urea cycle enzymes, aside from NAGS, are present in the poultry genome. T.Shimogiri has also shown the expression of ARG1 in the liver, ARG2 in extra hepatic tissues such as the kidney, and so on (3). More specifically, radiation hybrid (RH) mapping was performed, indicating the location of CPSI on GGA7 (Gallus Gallus Arm 7); OTC on GGA1, ASL onto GGA19 and ARG2 on GGA5. ASS however, could not be assigned to a specific chromosomal region, but it was proposed that it may be a marker of GGA17 (3). It has also been proposed that the deletion of even a single urea cycle enzyme would require a transition to uric acid metabolism (4).

This loss in urea cycle functioning coincides with the findings that poultry are incapable of forming arginine from ornithine, a pathway that could only possibly work if CPSI and OTC were active. Still, findings have shown citruline can be metabolized to arginine, supporting past findings of an active ASS liver enzyme (4). With the decrease in the functional urea cycle enzymes comes an increasing requirement for dietary arginine (2). Normally, ureotelics would use the urea cycle enzymes to

synthesize L-arginine from ornithine, but uricotelic poultry lack the active CPSI enzyme necessary for fixation of ammonia and require additional arginine from external sources. The amino acid requirement for arginine is essential for successful protein synthesis, growth, feathering, and other biological functions (5). While dietary arginine is essential and poultry certainly cannot produce arginine from ornithine, an alternate route of arginine production from citruline has proven successful, using arginosuccinate synthase followed by arginosuccinate lyase to yield arginine. The reduced arginase activity in poultry liver further prevents wasting of any arginine (6).

Also in relation to the presence of arginine in poultry, NAG synthase has undergone much evolutionary change going from prokaryotes to vertebrates and mammals. In prokaryotes, NAGS is inhibited by arginine and acts as a precursor to the arginine-biosynthesis pathway (7). In mammals, NAGS was originally stimulated by arginine and did not act as a precursor to arginine biosynthesis. According to Haskins though, the evolution from arginine inhibition of NAGS to activation was gradual, starting with complete inhibition in bacteria, partial in fish, and activation in frogs and mammals, further allowing the transition from aquatics to land (8).

Once NAGS no longer served as a precursor to arginine biosynthesis, mammals began synthesizing arginine via the enzymes of the urea cycle, instead using NAGS to supply CPSI with its allosteric activator N-Acetyl Glutamate (NAG). Binding NAG to CPSI creates a conformational change in the subunits of the enzyme and that without it the CPSI activity is undetected (7). Therefore, CPSI has been deemed the rate-limiting step, requiring allosteric binding with NAG in order to activate the urea-cycle (8)

Haskins explains that the appropriate transition of toxic ammonia into non-toxic metabolites was an evolutionary necessity permitting animals to transition from water-based environments into land-based environments (8). So, knowing that the urea cycle converts ammonia to urea in many animals, studies have suggested the urea cycle came into play at the start of metamorphosis (9) as a means of more efficiently excreting nitrogenous toxicities. The research of Cohen and Brown further explain the favorable free-energy changes of the cycle steps allowing for thermodynamic feasibility.

It has also been suggested that the evolution of uric acid synthetic enzymes would have allowed for an even greater freedom from aquatic environments during embryonic growth, allowing for less dependence on urea cycle enzymes and their functions. Presumably, this is what happened in the course of reptilian (9) and avian evolution. Had poultry maintained urea production through evolution, this very soluble and toxic waste would have been excreted during embryonic development, but would have no release or ability to escape the hard shell, thus killing its embryo. An evolutionary tree as designed by Brown and Cohen also proposed deletions as the main reason for the dysfunctional urea cycle in poultry. Here, Brown defines a deletion as reducing enzymatic activity so greatly that the urea cycle is no longer able to function at enough of a level to properly eliminate ammonia in the form of urea.

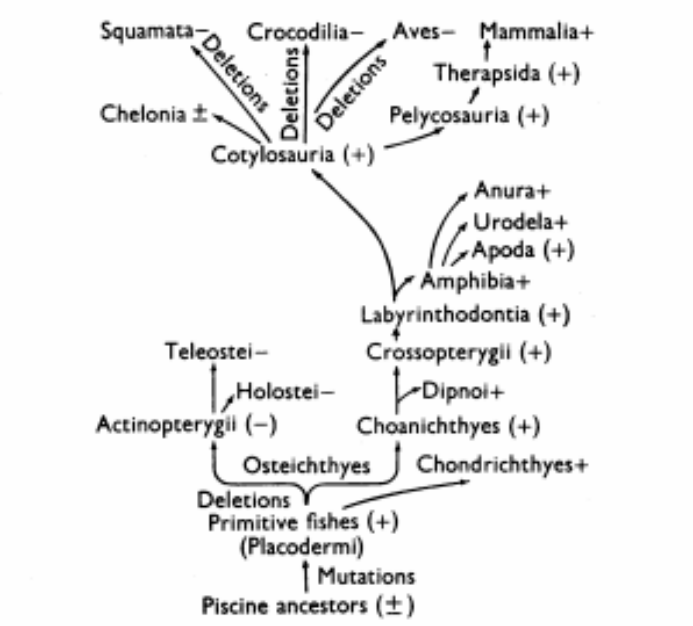


Figure 2 An evolutionary tree of vertebrates. Here, a (+) represents the postulated presence of a functioning urea cycle and (-) represents the absence of such. If the sign is not in parentheses then some members of the group are also known to either possess or lack the functioning complement of urea-cycle enzymes. "deletion" as labeled after cotylosauria indicates the proposed loss of activity of one or more enzymes, resulting in loss of urea cycle functioning (9)

Studies performed by Brown propose that the loss of even one urea cycle enzyme would require poultry to evolve toward uricotelism. One hypothesis in this project is that, by selecting against NAG production and eliminating urea production, poultry were able to lay land-based, hard-shelled eggs.

This proposed loss of urea cycle functioning coincides with the inability of poultry to produce arginine from ornithine while maintaining the ability to produce arginine from citruline (10). Liver activities of arginase in the vertebrates have a decreasing trend descending from sharks to frogs, turtles, and lizards, to snakes and

birds that have completely lost arginase functioning, supporting the observation of liver arginase activity loss through evolution.

This project proposes the investigation of the structure of poultry carbamoyl phosphate synthase I (CPSI), an enzyme that, in humans, is responsible for the proper flow of the urea cycle when activated by N-acetyl glutamate and is potentially functional in the promotion of cell growth when bound with the proto-oncogene Raf. This protein kinase is of significant importance in signal transduction, and is involved in the Ras/MAP-kinase pathway, cell proliferation, and in maintaining a differentiated cell phenotype (1). Raf exists in three different isoforms; A-Raf, B-Raf and C-Raf. Both A-Raf and C-Raf interact with CPSI but ASS only interacts specifically with A-Raf (1). Other CPSI functioning relations include pyrimidine synthesis from carbamyl aspartate, which have also been shown to require a supply of carbamyl phosphate (11). Exploring the presence or absence of certain binding sites on poultry CPSI compared to human CPSI allows for further determination of poultry CPSI functionality.

Looking at the structure of the CPSI enzyme, the presence or absence of any NAG binding site would first determine whether or not poultry evolved to prevent urea production via selection for loss of NAG binding. Kazuyo has demonstrated stimulation of urea formation by adding NAG and ornithine in perfused liver (12). So, should the NAG binding site remain, the urea cycle could potentially be activated in poultry by introducing cloned NAG into a poultry liver cell line. This would activate the urea cycle as shown in Figure 1. If the NAG binding site no longer remains, cloning and introducing a specialized human CPSI that maintains an NAG binding site along with cloned NAG may then result in poultry urea production, thus proving that there is expression of all enzymes involved in the urea cycle.

Chapter 2

METHODS

Protein Alignment of Human, Rat, and Chicken CPSI

A specific comparison was done in which the sequences of chicken, human, and rat CPSI were aligned using UniprotKB's alignment tool (13). The highlighting feature of this website's program allowed for notation of the allosteric activator as well as other sequence features.

Gene Expression of Urea Cycle Enzymes

As noted previously, the lab of Dr. Carl Schmidt had already determined the presence of various enzymes in the poultry genome. Multiple experiments had been carried out in order to determine the expression levels of these different enzymes and were recorded into the BIGBIRD database—a database created by the University of Delaware, Mississippi State University, and University of Arizona laboratories while working on mapping the poultry genome and performing heat stress studies. To investigate the various expression levels of the urea cell cycle enzymes, specific rpkm values—reads per kilobase of model, representing the number of transcripts per million transcripts of sample per kilobase (l4)—of each urea cycle enzyme were identified and the tissues with the highest levels of expression were selected for further analysis.

PCR of urea cycle enzymes

Primers were designed for each of the urea cycle enzymes by inputting FASTA formatted sequences from the NCBI database into the Primer3Plus web system. Using this system, the general settings consisted of the product size ranging from 75-150. From there, the task was set to the detection tab and the "pick primers" option selected for creation of primers. In each case the first designed primer was selected and sent to the Primer3Manager for ordering. The resulting primers of choice are displayed in Table 1 below.

Table 1 Primer-Pairs for CPSI, OTC, ASS, ASLI, ASL, and ARG

Enzyme Symbol	Sequence Reference	Forward primer Reverse primer	Tm
CPSI	NM_001045841.1	GTAGTGCCCTGGGTGAAAAA CCAACACCATCACTCCACAG	55.3 55.5
OTC	AF065629.1	ATCCTTGGCCATGATTTTTG GCCCAGGTGAATGTCTTGTT	55.6 55.7
ASS	NM_001013395.1	CTGAAGGAGCAGGGCTACAC ACACCTTCTTAGCCCCCAGT	57.6 58.3
ASLII	NM_001030714.1	GCCATTCAGACAAGCTCAC GGCTGATGCTCTTCAAGTCC	55.5 55.9
ASLII	NM_001030714.1	TGCCATTCAGACAAGCTCAC GGCTGATGCTCTTCAAGTCC	57.6 58.3
ASLI	NM_205501.1	TACCCTCCAGGTCAACAAGG GGCATTCCTTTACGAACCAA	56.3 53.2
ARG2	NM_001199704.1	GATGTTGATCGCCTTGGAAT GCATCGATGTCAAAGCTCAA	53.1 53.5

Once all primers were designed and ordered, all forward and reverse primers for each urea cycle enzyme were labeled with the appropriate identification stickers and diluted to a stock concentration of $1x10^{-5}$ M using tris-EDTA buffer at pH 8.0 (note: later primers were diluted using nuclease-free water, which is also acceptable).

Each stock solution was then diluted 10-fold using 10µL of stock solution and 90µL of tris EDTA pH 8.0 buffer solution.

The first three primers utilized were ASSI, ASL, and CPSI. In order to run the PCR, 25 microliter reactions were set-up consisting of 12.5µL of 2XGoTaq, 2.5 µL Forward primer, 2.5µL reverse primer, 1µL template DNA, and 6.5µL nuclease free DNA. The cDNA utilized as template for this particular run was from a Ross 708 (Ross poultry line, organism number 708) solution of concentration 1846 ng/µL which was diluted to 100 ng/µL before use. Each reaction was run using an annealing temperature 5 degrees lower than the lowest Tm—melting point at which 50% of the primer is liquid. Primer ARG2 had the lowest melting point at 53.1 °C and so the PCR annealing temperature was set specifically to 48.1 °C. The initial degradation was set to 2 minutes based off of the 1 minute per kb of extension expectancy and the number of cycles was set to 35 as a starting baseline.

PCR products were analyzed using a 1% agarose gel set up in 1X TAE buffer in order to run an electrophoresis of the products and determine any enzyme expression. A 100 base pair ladder (aka the marker) and a positive control, proliferating cell nuclear antigen (PCNA) gene, along with 2 µL samples of each PCR product. The gel was run at 80 V and 200 mA and was allowed to run for 30 minutes. Subsequently, the gel was removed and placed into an ethidium bromide treated buffer on a shaker for 15 minutes. Upon removal, the gel was read using the VisionWorks computer program. If said program is not available, a UV light bed can also be used to read the gel results. When using a UV light bed, make sure to take proper safety precautions and wear an eye shield before looking into the light.

The same protocol using a 25 µL reaction was used for the remaining urea cycle enzymes and the PCR run with an annealing temperature of 48.1 °C. Again, the results were analyzed on a 1% agarose gel for 30 min at 80 V and 200 mA and were compared to a 100 base pair ladder.

Real-time qRT-PCR of Urea Cycle Enzymes

Once it was clear the primers were working a qRT-PCR reaction was set up for each of the enzymes of the urea cycle. A master mix was created so that each reaction would have 6µL SYBR green, 4.5µL nuclease-free water, 0.5 µL of a forward and reverse primer mix—made by adding 10µL forward primer, 10 µL reverse primer, and 80 µL nuclease-free water—and 1 µL cDNA, making each reaction 12 µL total. This time, the cDNA used was CELCN1 standing for Chicken Embryo Liver Cells at Normal Temperature.

Each reaction of interest was set up three times (using three different wells) for the purpose of generating statistically sound results. Once all three reactions were prepared in the qPCR plate for each enzyme, the plate was spun down for 3 minutes at 4000 rpm. The PCR machine was then turned on and set to use the 7500 fast system software. A new run was created using the FASTcyber detector.

Another qPCR method was used for trials 3 and 4 in which cDNA was first diluted to 10 ng/µL and each reaction was set up separately as opposed to use of a mastermix. Specifically, each reaction used 6 µL SYBR green, 4.5 µL nuclease-free water, .5 µL of a pre-made mixture of 10 µL forward primer and 10 µL reverse primer, and 1 µL of the diluted cDNA. Once the plate was set up it was spun for 2 minutes at 4000 rpm and run in the qPCR machine using the same methods of the previous qPCR method.

Phylogenetic Tree Production for Urea Cycle Enzymes

To explore the phylogenetic relationships of the urea cycle pathway, a number of representative sequences from multiple species at each step in the pathway were needed for construction of an evolutionary tree. The organisms selected for in this particular case are described in Table 2. While not every single organism had a representative sequence for every enzyme of the urea cycle, those that were able to be found were included in the trees. ARG2 and both forms of ASL also included alternative enzymes and so two different tables were made and compared for these particular enzymes.

The protein sequences were identified by using the chicken protein sequences and the blast algorithm to identify ortholog sequences of other species. Next, the EMBL-EBI-ClustalW2 system was used by pasting sequences into the box and clicking submit (15). The resulting phylogenetic tree image was then saved. This procedure was repeated for each urea cycle enzyme. For reference to each tree, please see Appendix B.

Table 2 Scientific Names, Common Names, and Taxonomic IDs of all Organisms Included in the Phylogenetic studies

Scientific Name	Common Name	Taxonomic ID
Gallus gallus	Chicken	9031
Rattus norvegicus	Rat	10116
Homo sapien	Human	9606
Monodelphis domestica	Gray short-tailed opossum	13616
Xenopus tropicalis	Tree frog	8364
Danio rerio	Zebrafish	7955
Anolis carolinensis	Anole	28377
Drosophila annanassae	Fruit Fly	7217
Escherichia coli	E-coli	K12
Taeniopygia guttata	Zebra Finch	59729
Ornithorhynchus anatinus	Ductal Platypus	9258

General Bacterial Plate Preparation

Bacterial plates were made using 7.54 g Agar and 12.03 g Lysogeny Broth (LB) in 500mL ddH20 mixed and autoclaved for 50 minutes on the wet setting. The autoclaved solution was then placed in a water bath and 100mg/mL carbanicillin in 50% EtOH was added before pouring the plates.

Raf PCR Product Cloning

Once PCR product was attained for Raf using graduate student Janet DeMena's duodenal cDNA, attempts of cloning Raf's PCR product were made using a TOPO TA cloning Kit from QIAGEN. First, a chemical transformation reaction was set up using 2 µL fresh PCR product, 1 µL provided salt solution, 2 µL provided sterile water, and 1 µL provided TOPO vector, totaling a 6 µL reaction. The reaction tube was flicked a few times for mixing and then centrifuged lightly to gather the solution together before setting for 5 minutes at room temperature and placing on ice. Then, a One Shot Chemical Transformation took place in which the provided One

Shot E-coli ells were thawed on ice before adding 2 µL of the TOPO cloning reaction and mixing. This newly formed solution was incubated on ice for approximately 20 minutes and then heat shocked for 30 seconds at 42 °C without shaking. 250 µL Soc medium was added to the cells, the tubes were capped and shaken at 37 °C for one hour, and then 10 µL and 50 µL of the transformation was added to X-gal and carbanicillin containing LB plates. These plates were incubated overnight at 37 °C.

Following plate growth, 0.5 mL carbanicillin was added to 500 mL LB Broth and mixed. 2 mL of this carbanicillin/LB solution were added into 8 different sample tubes. Clear/white cultures from each bacterial plate were inoculated into the different sample tubes using yellow-tipped rods before labeling lids on the tubes. It is important to note that the tubes must NOT be sealed in order to allow for oxygen entry for optimum culture growth. These tubes were then placed, with the rack, into the heater/shaker at 37 °C over night. In the first trial it was found that the heater had been turned off, and so the heater was turned back on the next morning and the tubes allowed to shake for another 3.5 hours. Once the samples were ready, the solutions were spun down but no pellets were formed. Therefore, 1 mL from each tube was taken and put into new media. Furthermore, new cultures were inoculated including a blue colony as a control. Once more the cultures were allowed to set overnight.

The cloning procedure was repeated again using 4 µL of the old PCR product, 1 µL salt solution, and 1 µL topo vector in attempt to attain further cultures, but no growth was observed. Therefore, PCR product purification was performed using the QIAquick PCR Purification Kit. Seventy-five microliters of Buffer PB were added to 15 µL Raf PCR Product and placed into a QIA quick spin column that was added to a 2 mL collection tube. This was centrifuged for 1 minute and the flow through

discarded. The sample was then washed using 0.75 mL Buffer PE and centrifuging 1 minute before discarding the remaining flow-through and centrifuging an extra minute. The QIAquick column was then added to a new 1.5 mL micro centrifuge tube. In order to elute the DNA, 50 µL Buffer EB was added to the QIAquick membrane and the sample centrifuged for 1 minute. This purified PCR product was used for further cloning experiments along with freshly made cDNA from IOWA poultry RNA samples.

The cDNA from IOWA poultry RNA samples was produced using samples 198 IOWA (heat stress) concentration 879.92 ng/µL and 196 IOWA (normal) 343.82 ng/µL, both of which only 1ug was used for First-Strand Synthesis. In each case, the RNA sample was added along with 1 µL dNTP mix, 1µL Random hexamers, and additional ddH_2O to create a total of 10 µL samples. These solutions were incubated at 65 ℃ for 5 minutes and then placed on ice for a minimum of 1 minute. A 2X reaction mix was then created using 5 µL 10XRT buffer, 10 µL 25 mM MgCl2, 5 µL .1M DTT, and 2.5 µL RNaseOUT (40 U/µL). Nine microliters of this 2X reaction mix was added to each RNA/primer mixture, mixed gently, and collected by centrifugation before being incubated at room temperature for 2 minutes. Then 1 µL of SuperScript II RT was added to each tube and the solutions were incubated further at room temperature for another 10 minutes, then incubated another 50 minutes at 42 ℃. The reaction was then terminated at 70 ℃ for 15 minutes before being chilled on ice. Finally, the reaction was briefly centrifuged for collection and 1 µL RNase H was added to each tube. These final solutions were incubated for 20 minutes at 37 ℃, making them ready for use in PCR reactions.

Leghorn Male Hepatocarcinoma (LMH) Cell Growth and Splitting

LMH cell growth began by taking one 75 cm^2 flask, adding 5 mL attachment factor (AF) to each, and storing at 4 °C for a minimum of 10 minutes in order to coat the surface with gelatin for LMH cell surface binding. Some runs of cell growth proved difficult in maintaining properly functioning attachment factor in each flask, so it is important to ensure this full incubation time is achieved. The flasks were removed from the 4 degree Celsius refrigerator and the attachment factor (AF) removed. Then,10 mL of Waymouth's Media. complete with 1% FBS or 150 mL added per container of Waymouth's was added to each of the flasks and the flasks were stored at 37 °C, 5% CO_2 for 15 minutes. Next, 2.5mL of frozen LMH cells were taken and poured into the prewar med media of the gelatinated flask and placed in 37 °C, 5% CO_2 for storage. In order to maintain the cells, media was changed as needed every 2-3 days using 10 mL complete Waymouth's solution. After approximately 1-1.5 weeks of growth, the LMH cells were split.

In order to split the LMH cells, four new flasks treated with AF had to first be prepared as previously done to make room for new cell growth. Then, from the flask containing fully grown LMH cells, the old media was first removed. 5 mL 1X trypsin was then added and the flask rocked for 1 minute before discarding the trypsin solution. Again, 5 mL of fresh 1X trypsin was added and the flask kept at 37 °C, 5% CO_2 for 5 minutes before removing from the incubator and rocking to detach all cells. 5 mL of media was then added to the flask and 2.5 mL of the newly formed solution was taken and added to each of the four newly prepared LMH cell growth flasks. These flasks were all stored at 37 °C, 5% CO_2 and had their media changed as needed every 2-3 days, followed by cell splitting as needed every week or so. Please note the extreme importance in ensuring at least two different flasks of Waymouth's solution

are used to avoid complete experiment contamination. Also ensure that gloves are changed regularly, the hood of use is UV sterilized for a minimum of 15 minutes before use, and that all things being placed into the hood are sprayed with 70% ethanol beforehand. Such steps will help reduce risk of contaminating cells.

Other future studies remaining include LMH growth, splitting, and urea assaying in which baseline urea assays may be taken for various LMH cell colonies followed by introduction of cloned NAGS (and possibly CPSI depending on transit peptide existence) and further urea assay analysis. Depending on any expected urea production increase, a fusion protein experiment could then be carried out to explore NAGS interaction through the urea cycle as well as CPSI activities. Such research would certainly provide further information on the evolutionary purposes of urea cycle maintenance.

Genomicus Gene Search of CPSI and NAGS

Further research using Genomicus v66.01 was performed investigating the presence of any syntanic relationships surrounding CPSI across similar species as well as for enzyme NAGS. Knowing that syntanic relationships have tendencies of conservation across similar species, noting the presence or absence of such relationships for CPSI would hopefully provide support that we were indeed studying the proper, existing urea cycle enzyme. Similarly, further study of the NAGS locus aimed to reveal evolutionary relationships among various species, providing for a better understanding of the proposed elimination of NAGS from the poultry genome.

Chapter 3

GENE EXPRESSION OF UREA CYCLE ENZYMES

Results/Discussion

RPKM studies of urea cycle enzymes

Using the BIGBIRD database to explore the urea cycle enzymes rpkm values—reads per kilobase of model, or the number of transcripts per million transcripts of sample per kilobase (14)—it was found that there was zero NAGS expression, a small amount of CPSI expression in the duodenum and kidney, a very scarce amount of OTC expression in the liver, a high amount of ASS expression in the liver, ileum, and kidney, and then a small amount of expression in ARG1 in the liver. These results can be found in Table 3, which presents the rpkm values for those tissues representing some of the highest levels of expression. These same tissues, liver, duodenum, and intestinal, were all selected for use in the PCR reactions of this experiment.

Table 3 Enzyme expression levels as per BigBird database libraries

Enzyme	GeneID	Chromosome	Rpkm	animal	tissue
CPS1	428994	7	.93	Gallus	Liver
OTC	395735	1	0	Gallus	Liver
	426115		1.54	Gallus	Liver
ASS1	417185	17	47.77	Gallus	Liver
	280726	11	133.328	Gallus	Ileum
			648.702	Gallus	Kidney
ASL1	396498	19	9.51	Gallus	Duodenum
ASL2	417545	19	38.56	Gallus	Liver

PCR of urea cycle enzymes

Using the purified PCR product along with the newly produced IOWA cDNA samples, PCR was performed once more on the CPSI, Raf, and BRaf primers, but no further products were formed. Yet another duodenal RNA was used to produce cDNA but it too failed to yield any useable PCR products and so a new approach was taken.

In each case, it was clear that the primers were working and present based on the PCR gels performed. However, upon initial real-time qRT-PCR analysis, the expression levels seemed to be extremely low. The values attained are all presented in Table 4, which provides the sample enzyme, number of samples made, number of samples that yielded detectable results, the average count, standard deviation of counts attained, and the melting temperature of the primers used. Further qPCR trials also supported the idea that the enzymes were indeed present, but had extremely low levels of expression. These results are also provided in the tables below. As a result, it was expected that any assaying experimentation performed would not yield a significant result. Therefore, a new approach was taken in which phylogenetic trees were

designed to take a closer look at the evolutionary process behind each urea cycle enzyme.

Table 4 qPCR results for cDNA ROSSDuod [10 ng/µL]

Sample	#samples (determined)	Average Count	st.dev count	Tm (primer)
CPSI	3 (0)	x	x	56.2
OTC	3(2)	3.43477	0.120986	55.8
ARG2	3(1)	24.9068	x	558
ALS2	3(1)	3.34468	x	55.8
ALS1	3(1)	3.88888	x	55.8
ASL1	3(3)	3.082757	0.408014	55.8
ASS1	3(1)	3.3547	0.1372	55.8

Table 5 qPCR results for cDNA N3CELC (normal tissue)

Sample	#samples(determined)	count	st.dev count	Tm(primer)
CPS1	3(3)	30.549	0.3501	76.367
ALS2	3(0)	x	x	56
ASL2	3(1)	30.178	x	62,7
ASL1	3(3)	28.762	0.1546	79.3
ASS1	3(3)	28.977	0.0848	80

Table 6 qPCR results for HS4CELC (heat stress tissue)

Sample	#samples(determined)	count	st.dev count	Tm (primer)
CPS1	3(3)	30.252	0.8279	76.933
ALS2	3(1)	28.796	x	63.167
ASL2	3(1)	30.925	x	62.833
ASL1	3(3)	29.2595	0.023271	80
ASS1	3(3)	29.1922	0.04977	80

Table 7 qPCR results for Normal [9.9 ng/µL] (top) and Heat Shock [10.2 ng/µL] (bottom)

Sample	#samples(determined)	count	st.dev count	Tm (primer)
OTC	3(3)	35.232	0.2477	71.767
ARG2	3(1)	25.2496	x	76.7333

Sample	#samples(determined)	count	st.dev count	Tm (primer)
OTC	3(3)	35.909	0.2591	74.167
ARG2	3(3)	24.3541	0.31555	75.5667

Conclusion

As previously discussed, a series of evolutionary steps allowing poultry to transfer from aquatic to terrestrial living environments has led to the uricotelic preference of poultry, rendering the urea cycle inactive. A lack of urea production would seemingly imply that there is an absence of the necessary mechanisms for urea production, but studies have shown that all urea cycle enzymes aside from NAGS are present in the poultry genome. Exploration of the BigBird database RNA libraries created by the workers of Dr. Schmidt's lab has further shown a small amount of activity of CPSI, OTC, ASS and ASL, but not NAG or ARG, supporting the hypothesis that selection against NAGS led to the inability to activate CPSI and therefore halted the functioning of the urea cycle. Supporting evidence of the inactivity of the urea cycle enzymes has also been presented by a few qPCR runs yielding extremely low expression levels for the urea cycle enzyme primers described.

Chapter 4

PROTEIN ALIGNMENT OF HUMAN, RAT, AND CHICKEN CPSI

Results/Discussion

Specific alignment and modeling of this CPSI enzyme revealed an identical allosteric activator binding site in the rat, human, and chicken protein sequences. The TRP1410, GLN413, and PHE1445 were also present in all three sequences, indicating the preservation of the NAG binding site identified in Pekkala's study (16). This can be seen in Figure 3 below, with the allosteric site highlighted in green and the three markers in gray text.

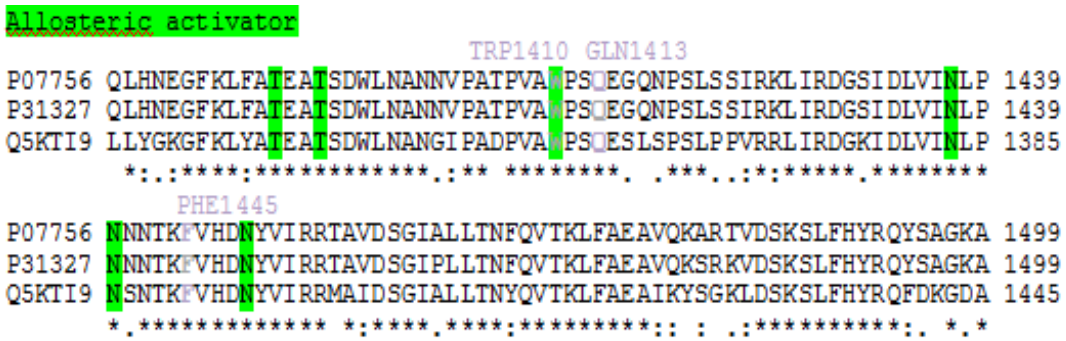


Figure 3 Preservation of the NAG binding site as per Pekkala's work. Note the perfect conservation of these residues across Rats (top row), Humans (middle row), and Poultry (bottom row).

During inspection of the CPSI sequence, it was noted that a transit peptide was missing when compared to Human and Rat CPSI. This implies that the transit peptide

is missing. This transit peptide is essential for CPSI to enter the mitochondria, thus blocking production of carbamoyl phosphate and eliminating urea production. Figure 4 depicts the missing transit peptide sequence, highlighting the transit peptides of rats and humans in gray. While it appears that this transit peptide is truly missing, later studies performed suggest this may also be an error in the sequence provided.

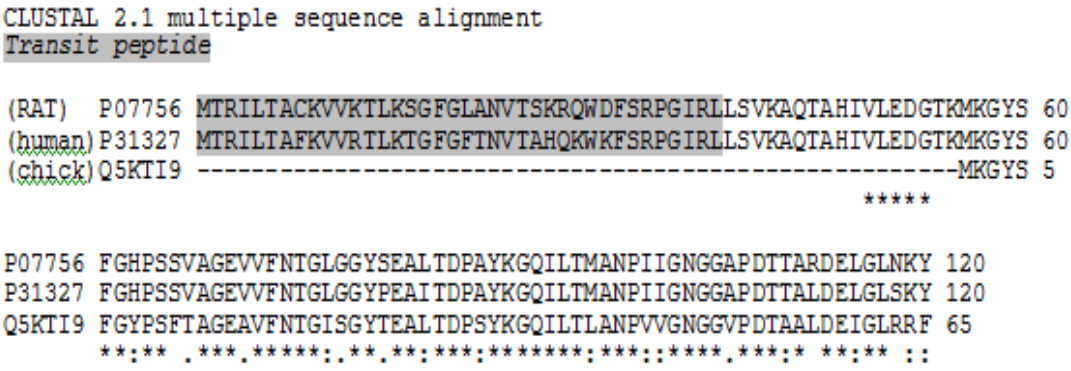


Figure 4 Missing transit peptide in poultry

Conclusion

Further bioinformatics studies exploring the structures of Rat, Human, and poultry CPSI showed that the NAG binding site was indeed preserved, but also revealed a potentially missing transit peptide in the CPSI sequence. Without this transit peptide, CPSI would not be able to enter the mitochondria for activation of the urea cycle. Still, a quick search of the zebra finch and turkey CPSI sequences shows the transit peptide is not missing in their sequences, leading to the belief that this suggested missing transit peptide in poultry CPSI is actually a sequencing error.

This lack of urea cycle functioning coincides with the label of arginine as an essential amino acid in the poultry diet and further explains the lack of ARG

functioning to break down arginine into urea. Time constraints prevented the intended cloning of NAG and proper CPSI, but future cloning experimentation could provide significant information on the interactions and functioning on urea cycle enzymes.

Chapter 5

PHYLOGENETIC TREE PRODUCTION OF UREA CYCLE ENZYMES

Results/Discussion

Phylogenetic trees for all components of the urea cycle were generated using the EMBL-EBI-ClustalW2 system. For this analysis we chose representative gene products from the species available as previously listed in Table 2.

Inactivation of CPSI Enzymatic Activity

If the sole function of the CPSI gene was to encode CPSI protein with enzymatic activity then it would be predicted that loss of urea cycle functioning, and thus reduction in enzymatic activity of CPSI, would relax evolutionary constraints of the gene sequence. Consistent with the natural theory of evolution, the CPSI gene in birds would then evolve faster than other genes that encode the urea cycle enzymes. However, inspection of phylogenetic trees indicates that the chicken CPSI is not evolving at a faster rate than other urea cycle gene products. This suggests that, despite the loss of activity, the chicken CPSI gene is still under evolutionary constraint.

One interesting occurrence experienced in the process of producing the phylogenetic tree of CPSI was the presence of both a large and small subunit of e-coli. While the small subunit was left out of the tree for sequence-size similarity purposes, it is important to note that literature has described the presence of two different subunits interacting with one another in the early forms of CPSI (17). It wasn't until

later down the evolutionary line that these subunits combined into present CPSI forms. Another interesting observation was made in regards to ASLI and ASLII. Locating ASLI and II on the evolutionary trees, it was observed that the two different enzymes were located directly next to each other on the tree for rats, humans, and poultry. This lack of distance between the enzymes reveals their homologous relationship, showing that ASLI and II are, indeed, functionally the same. To view and compare these evolutionary trees, see Appendix B. Also, to view and compare the various distances involved in each phylogenetic tree, see Appendix C.

Conclusion

In evolutionary terms, observations from phylogenetic trees designed for each urea cycle enzyme lead to the deduction that urea cycle enzymes have been evolving together as a whole, experiencing just a few inactivation mutations along the way, rendering the cycle dysfunctional.

Chapter 6

RAF PCR PRODUCT CLONING

Results/Discussion

An effort was made to perform Raf cloning as a means of introducing Raf into a cell-line of poultry cells to observe any potential interactions of this enzyme with urea cycle enzymes—CPSI specifically. In order to ensure experimental results were not affected by the potentially missing transit peptide described earlier, two different primer sets were created for CPSI. While PCR was performed involving both BRaf and Raf along with two forms of CPSI primer sets, most attempts yielded zero product. Figure 5 portrays the one and only PCR result that seemingly had Raf product. However, upon purification, bacterial plate growth, and the TOPO TA cloning methods described previously, the cloning process remained unsuccessful.

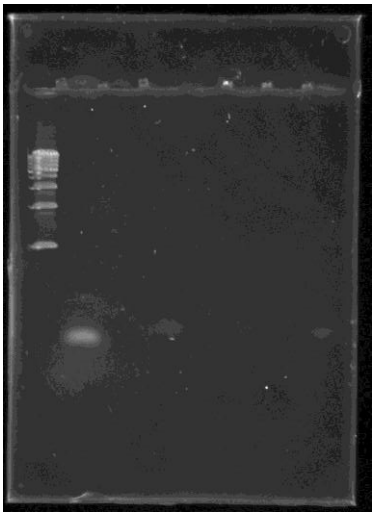
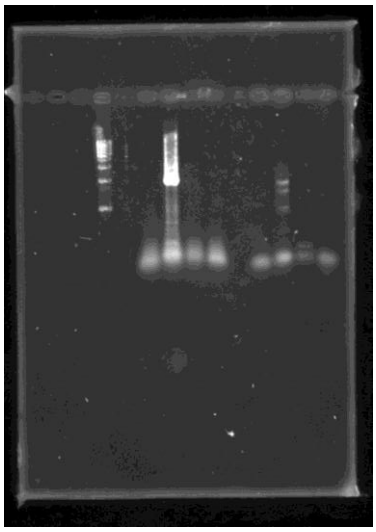
a.  b. 

Figure 5 PCR gel results for CPSRafPCR 1/10/12 (left) and CPSRafPCR2,3 1/11/12. Figure (a) top to bottom: CPS1a, CPS1b, Raf, B-Raf. Figure b top to bottom: CPS1a, CPS1b, Raf, B-Raf using 10ng H2CELC; CPS1a, CPS1b, Raf using D2duod4778, then B-Raf using D14Duod4947, 1kb DNA ladder. Note in figure (a) the lack of any significant PCR product compared to the base ladder versus the Raf products shown in Figure (b).

An attempt was made to clone the Raf products but no desirable colonies were obtained. Furthermore, upon product purification and latter PCR checking, no product was recovered. This is shown in Figure 6.

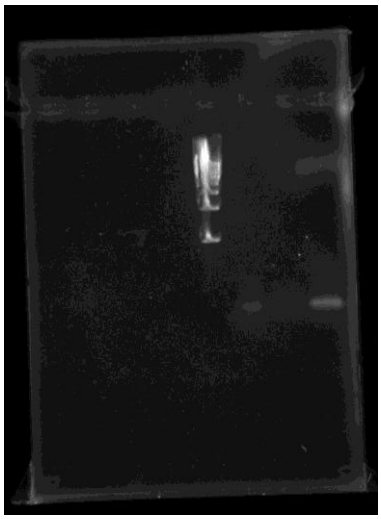
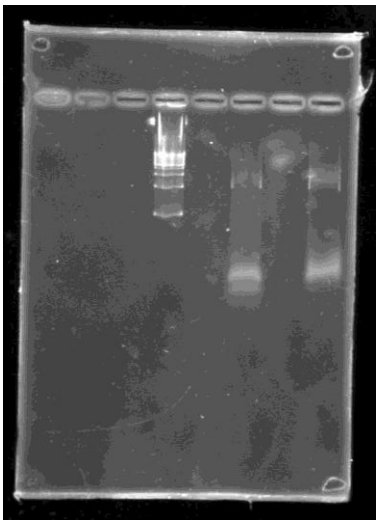
a.  b. 

Figure 6 PCR gel results for CPSRafPCR4 1/13/12 (left) RafcDNA198/196 1/26/12. Figure (a) top to bottom: CPSa, CPSb, B-Raf using D14 Duod 4947. Figure (b) top to bottom: Raf from IOWA198 heat-stressed poultry sample cDNA, Raf from IOWA196 normal cDNA, 1kb DNA ladder, purified Raf from D2Duod4778. Note the supposed Raf product from poultry numbers 196 and 198 versus the lack of actual Raf in the purified product from PCR using D2Duod4778.

The Raf products then obtained using IOWA198 heat-stressed poultry sample RNA and IOWA196 normal poultry sample RNA were further used in another attempt at cloning. Once again, no useful cloned colonies were attained. A final attempt was made to gain purposeful Raf product in which a spread of various poultry tissue RNA was used but still no product was yielded (Figure 7). Subsequently, due to time constraint, the decision was made to move on to a different approach of performing urea assays on LMH cells. While Raf cloning results were unattainable to-date, future repeat experimentation could lead to potentially valuable information related to Raf-CPSI interactions.

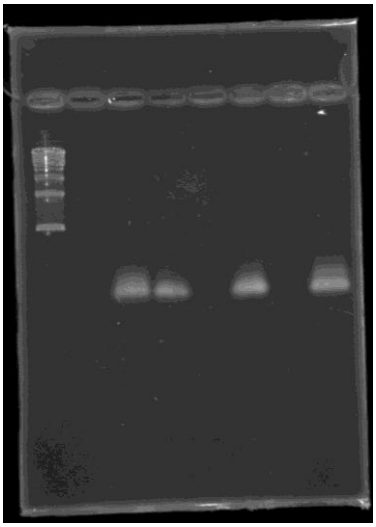
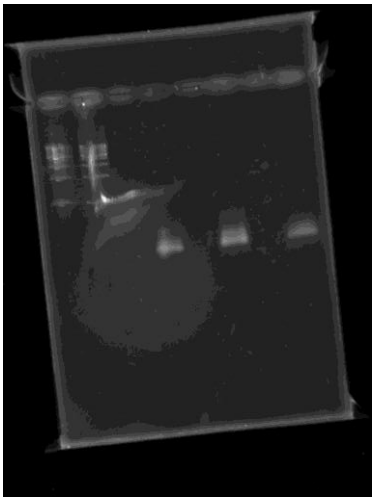
a.  b. 

Figure 7 PCR gel results for Rafspread 1/31/12 (left) and Rafspread part 2 2/5/12 (right). Figure (a) top to bottom: LMH cDNA combo, 653VLL Ross w7cDNA, cDNA198, cDNA 196, 1kb ladder. Figure (b) top to bottom: dilutions of cDNA 4932 starting from 100µL, 9µL, .9µL and then two wells of 1kb ladder. No useful products were attained for any of the cDNA types utilized.

Conclusion

Still, curiosity about the interactions between Raf and CPSI and the potentially alternative function of this urea cycle enzyme existence led to multiple Raf cloning attempts. While positive results have yet to be attained, the successful Raf cloning and CPSI-Raf interaction studies could serve extremely useful in future explanations of urea cycle enzyme maintenance in the poultry genome.

Chapter 7

GENOMICUS OF CPSI AND NAGS

Results/Discussion

Genomicus v66.01 was utilized in the investigation of any potential syntanic relationships involving CPSI among various species. Looking at Figure 8, it is clear that CPSI (lime green) is well conserved, locked in synteny with its surrounding region of enzymes, supporting the belief that we are indeed, correct in our hypothesis that CPSI is real and present in the poultry genome.

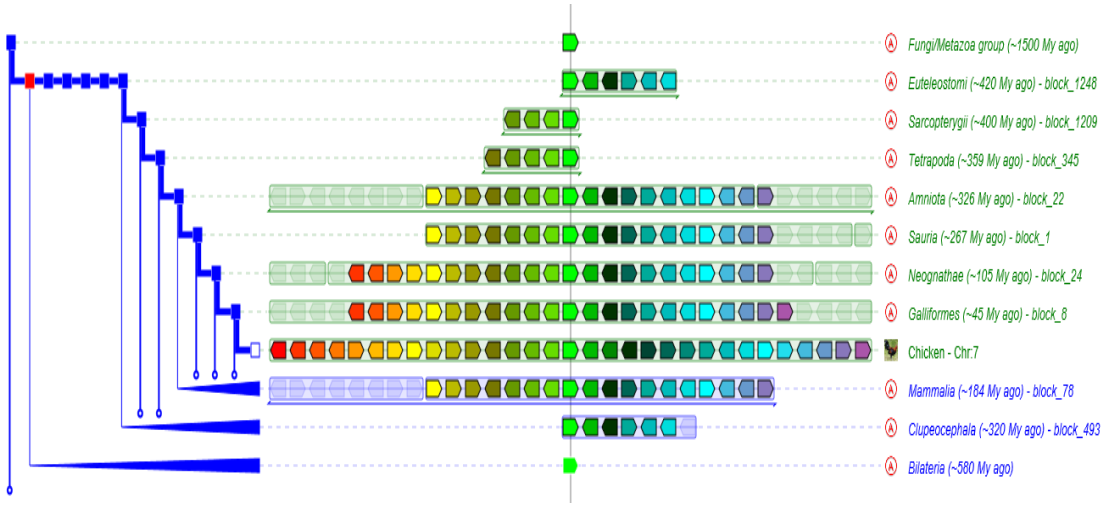


Figure 8 Genomicus representation of CPSI in various organisms

Furthermore, the Genomicus study of NAGS (lime green) in Figure 9 showed how NAGS is conserved all the way to the anole, where NAGS first disappears from

the genomic arrangement. Following the evolutionary progression, an increasing trend of rearrangement and loss of enzyme conservation is seen as each sequence approaches the poultry genome. This helps support the idea that either NAGS is actually missing from the poultry genome, or there has been some sequencing error. It is also important to note that the deletion of the NAGS locus is seen in both lizards (anole) as well as in birds, proposing that not only are poultry and lizards more closely related than poultry and humans, but that a deletion must have occurred basal to the divergence of lizards and birds, resulting in loss of the urea cycle. These conclusions coincide with the evolutionary diagram shown in Figure 2 of the introduction, which shows the relations between various organisms and proposed urea cycle deletions through evolution.

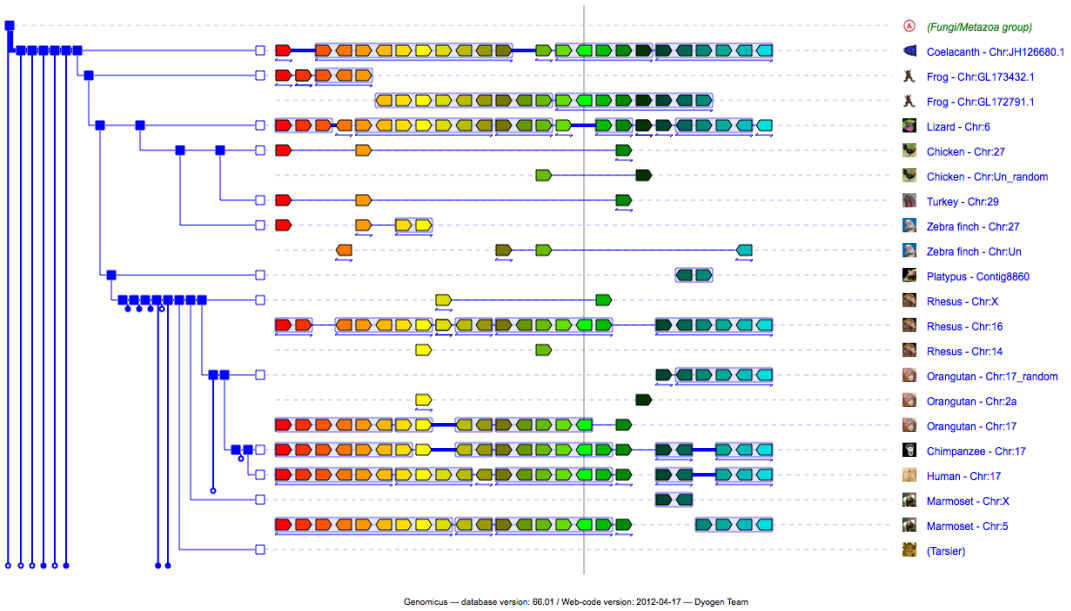


Figure 9 Genomicus representation of NAGS in various organisms

Conclusion

Using the Genomicus gene search it appears that the CPSI chromosomal region is conserved in poultry while the NAGS chromosomal region is not. The CPSI syntanic relations tend to be conserved across similar species, showing similar regions having less rearrangement in the genome. However, when looking at NAGs it appears that the locus has been deleted in both birds and lizards and that regions of the genome are much more scattered surrounding the areas where NAGS would be in the genome. This strongly corresponds with the hypothesis that, by selecting against NAG production, the urea cycle ceased function, enabling the laying of land-based and hard-shelled eggs.

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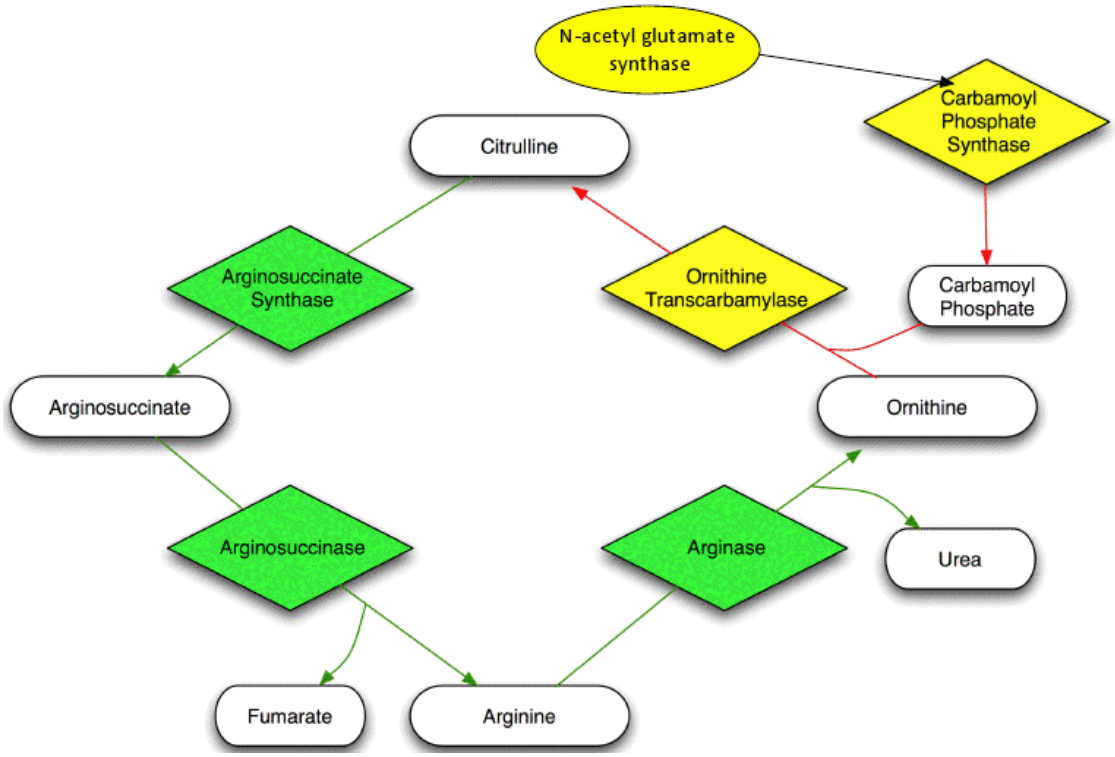
Appendix A

The Urea Cycle

Figure 10 The Urea Cycle



Appendix B

Phylogenetic Tree Images

Figure 11 CPSI

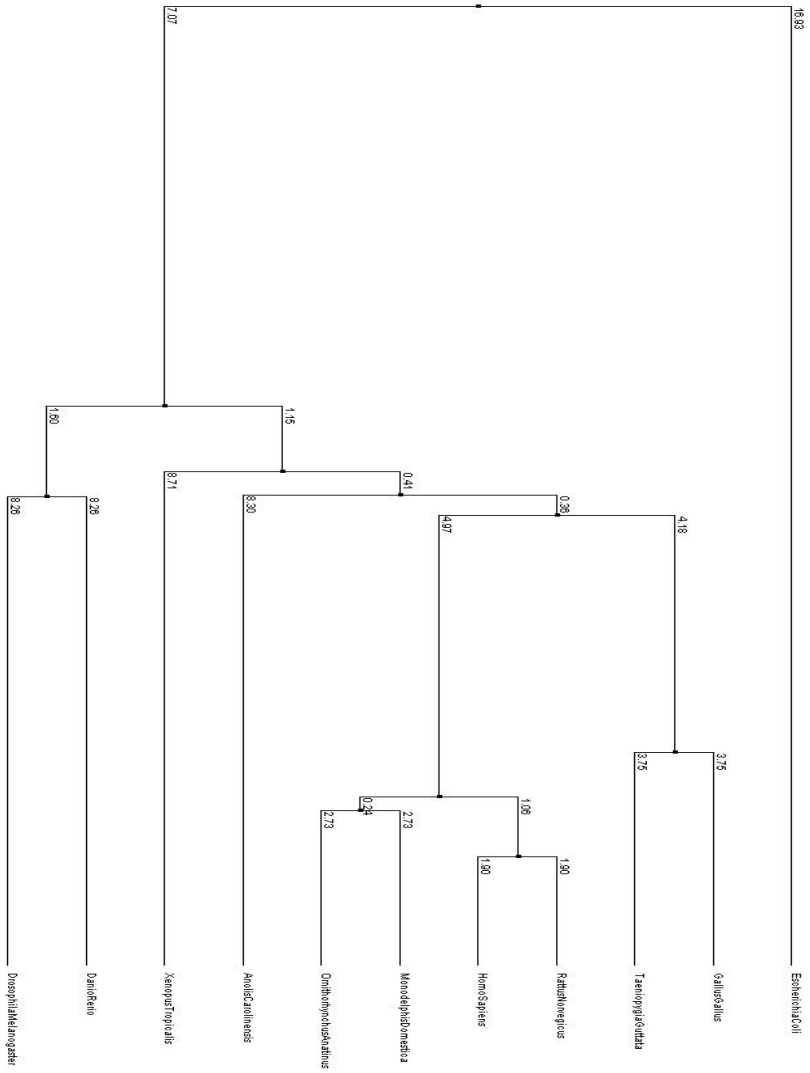


Figure 12 OTC

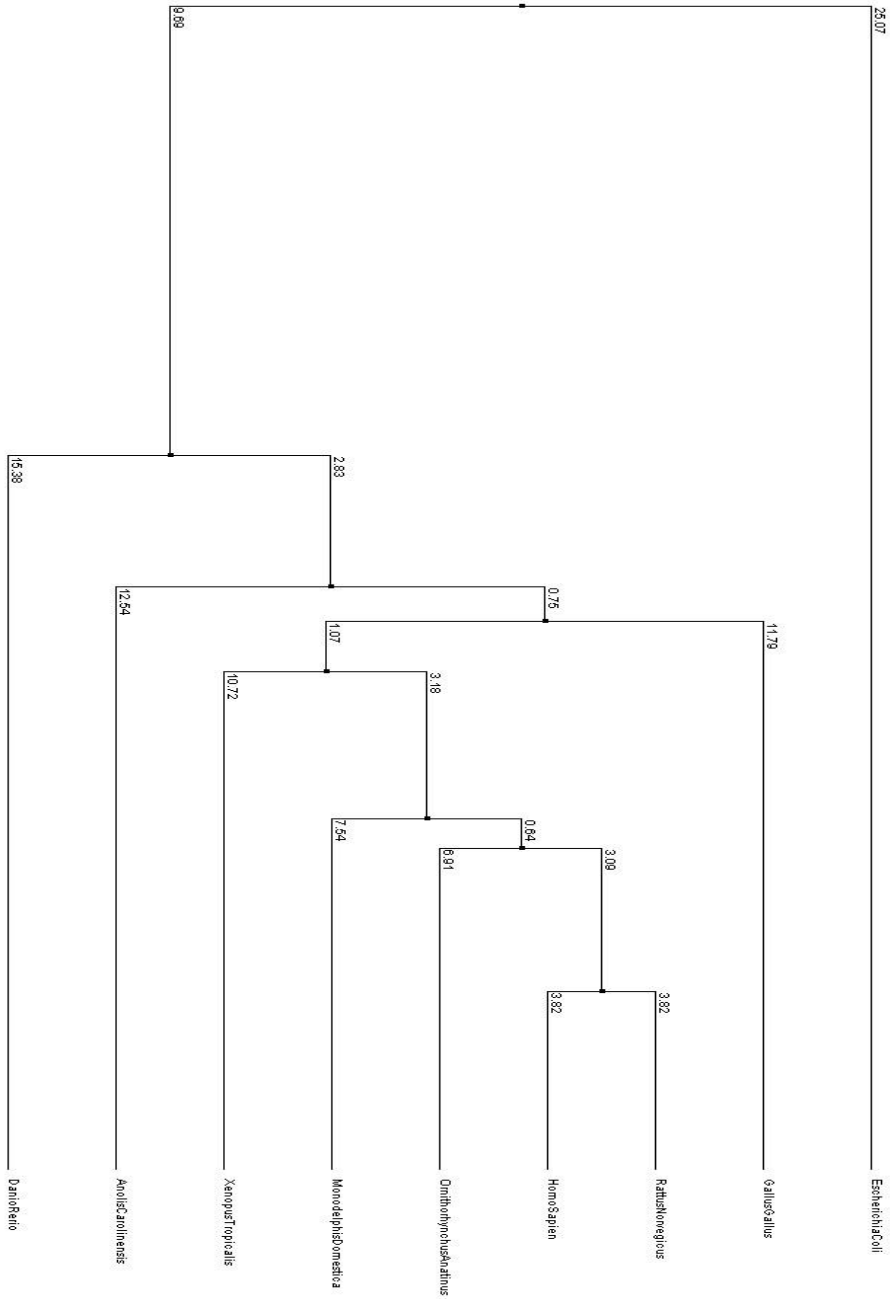


Figure 13 ARG2 (normal)

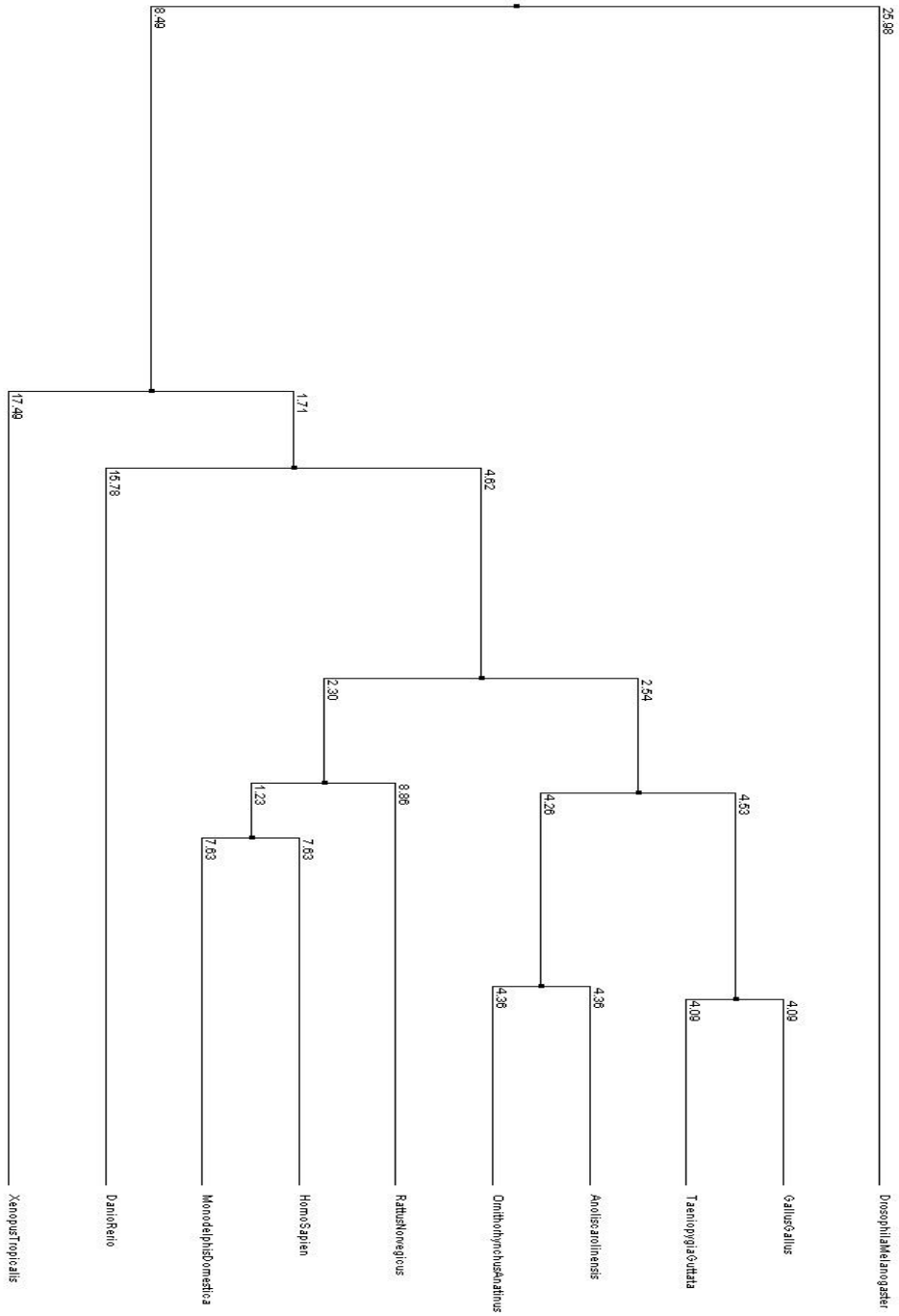


Figure 14 ARG2 (agmatase)

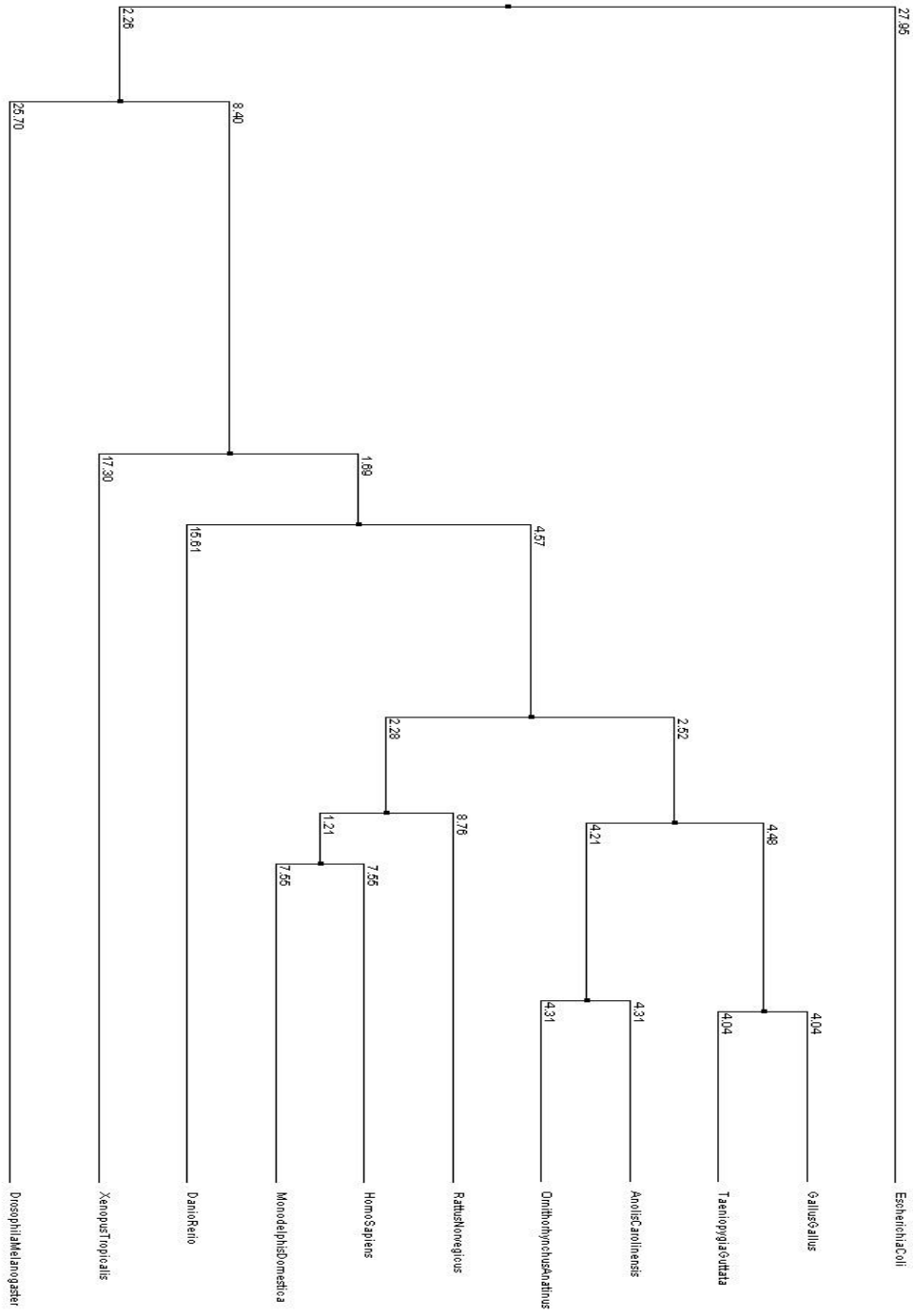


Figure 15 ASL (normal)

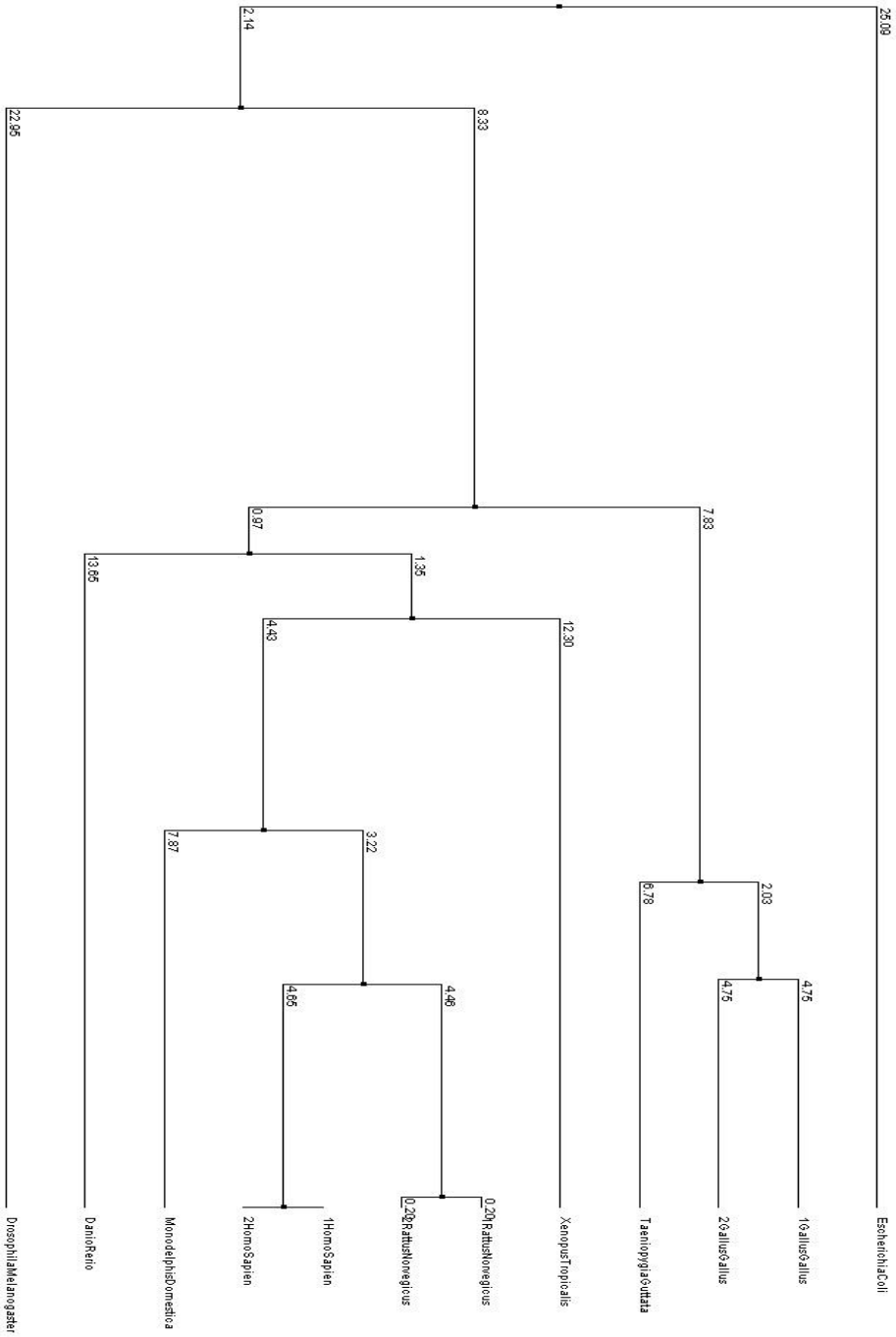


Figure 16 ASL (carnosine synthase)

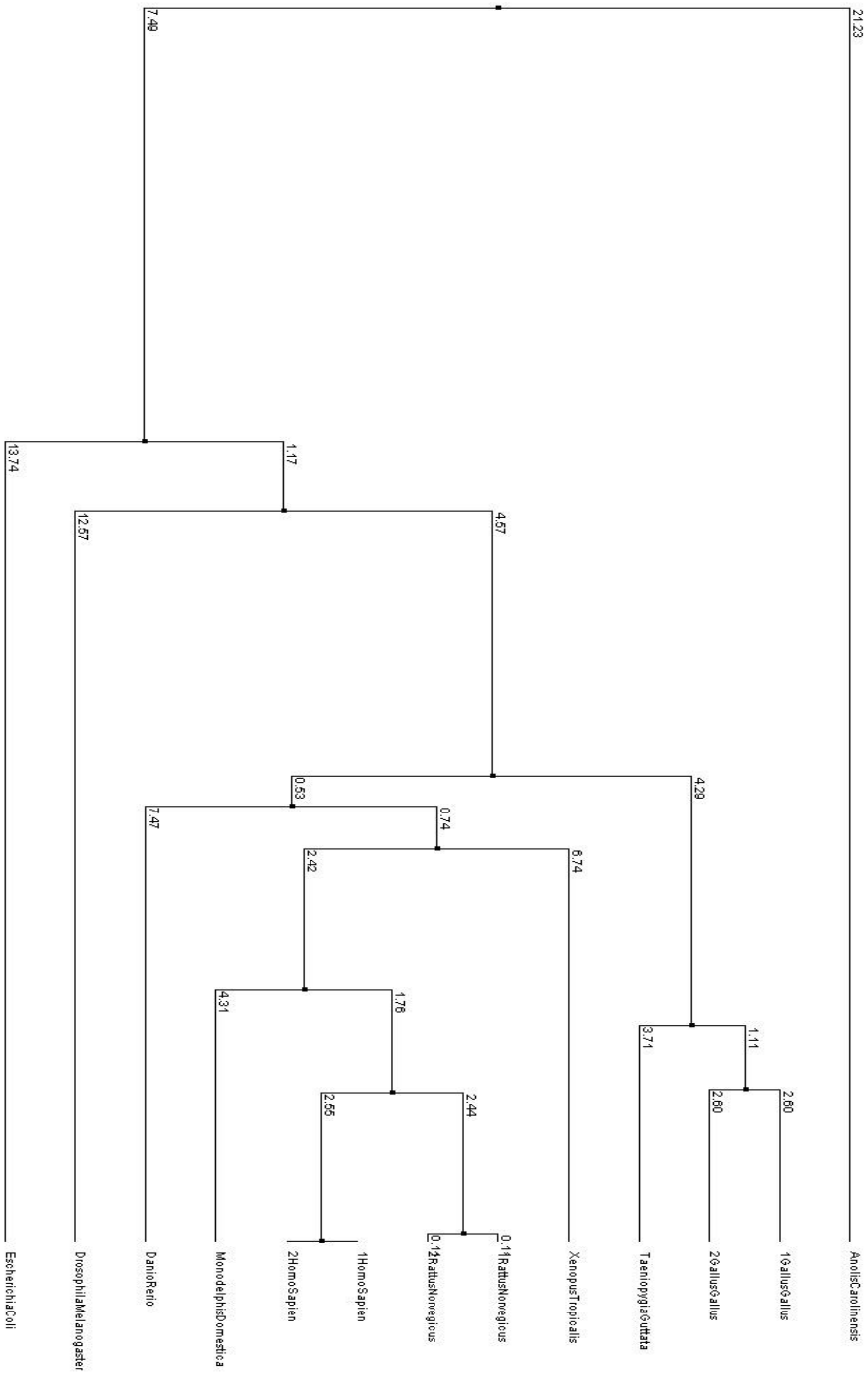
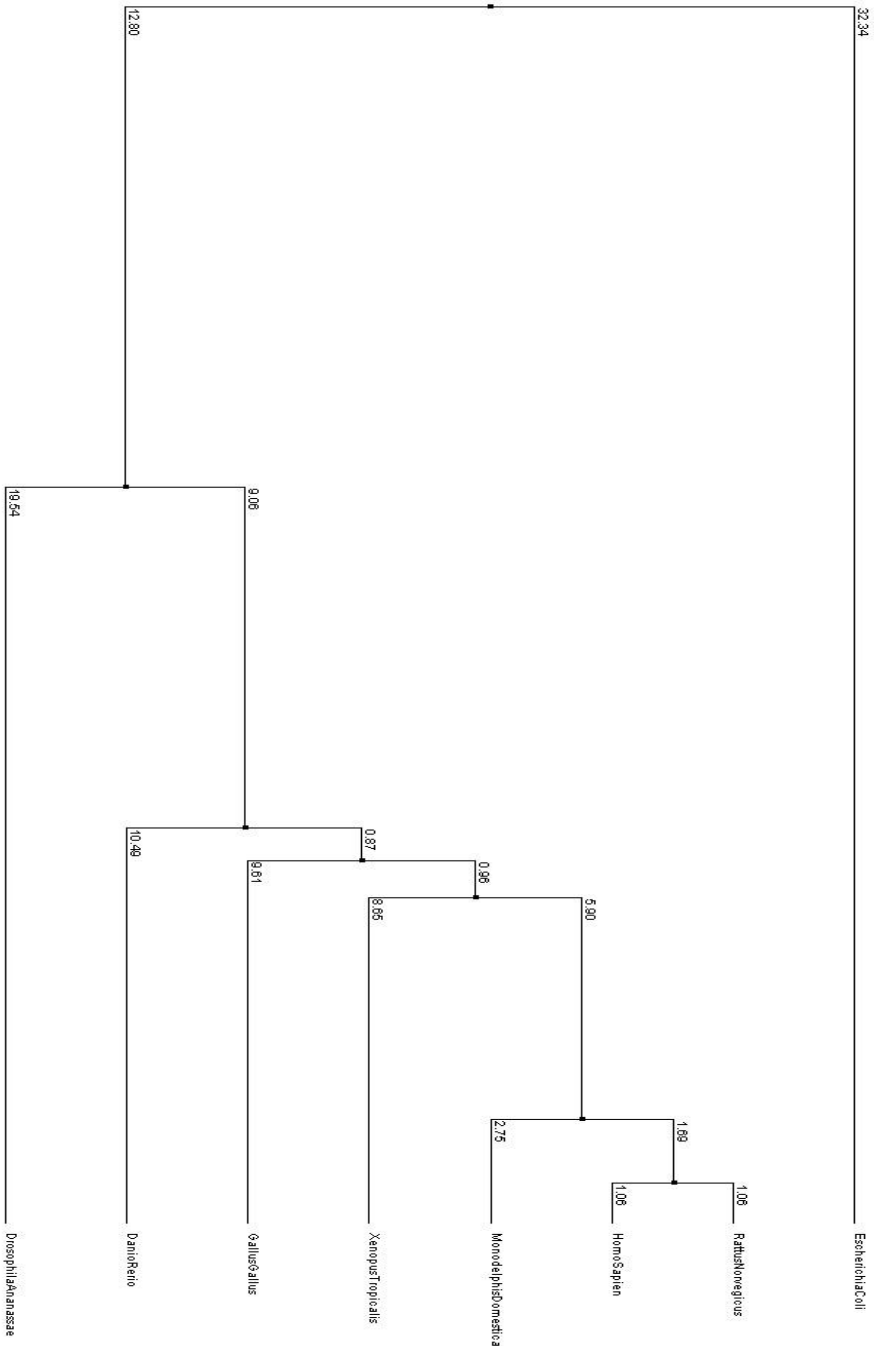


Figure 17 ASSI



Appendix C

Phylogenetic Tree Distance Tables

Table 8 CPSI

	poultry	rat	human	oppossum	platypus	zebra finch	ecoli	xenopus	zebra fish	anole	drosophila
Poultry	x	x	x	x	x	x	x	x	x	x	x
Rat	7.93	x	x	x	x	x	x	x	x	x	x
Human	7.93	1.9	x	x	x	x	x	x	x	x	x
Opposum	7.93	2.96	2.96	x	x	x	x	x	x	x	x
platypus	7.93	2.96	2.96	2.73	x	x	x	x	x	x	x
zebra finch	3.75	7.93	7.93	7.93	7.93	x	x	x	x	x	x
ecoli	16.93	16.93	16.93	16.93	16.93	16.93	x	x	x	x	x
xenopus	8.71	8.71	8.71	8.71	8.71	8.71	16.93	x	x	x	x
zebra fish	9.86	9.86	9.86	9.86	9.86	9.86	16.93	9.86	x	x	x
anole	8.3	8.3	8.3	8.3	8.3	8.3	16.93	8.71	9.86	x	x
drosophila	9.86	9.86	9.86	9.86	9.86	9.86	16.93	9.86	8.26	9.86	x

Table 9 OTC

	poultry	rat	human	oppossum	platypus	zebra finch	ecoli	xenopus	zebra fish	anole	drosophila
Poultry	x	x	x	x	x	x	x	x	x	x	x
Rat	11.79	x	x	x	x	x	x	x	x	x	x
Human	11.79	3.82	x	x	x	x	x	x	x	x	x
Opposum	11.79	7.54	7.54	x	x	x	x	x	x	x	x
platypus	11.79	6.91	6.91	7.54	x	x	x	x	x	x	x
zebra finch	-	-	-	-	-	x	x	x	x	x	x
ecoli	25.07	25.07	25.07	25.07	25.07	-	x	x	x	x	x
xenopus	11.79	10.72	10.72	10.72	10.72	-	25.07	x	x	x	x
zebra fish	15.38	15.38	15.38	15.38	15.38	-	25.07	15.38	x	x	x
anole	12.54	12.54	12.54	12.54	12.54	-	25.07	12.54	15.38	x	x
drosophila	-	-	-	-	-	-	-	-	-	-	x

Table 10 ARG2 (normal)

	poultry	rat	human	oppossum	platypus	zebra finch	ecoli	xenopus	zebra fish	anole	drosophila
Poultry	x	x	x	x	x	x	x	x	x	x	x
Rat	11.16	x	x	x	x	x	x	x	x	x	x
Human	11.16	8.86	x	x	x	x	x	x	x	x	x
Opposum	11.16	8.86	7.63	x	x	x	x	x	x	x	x
platypus	8.62	11.16	11.16	11.16	x	x	x	x	x	x	x
zebra finch	4.09	11.16	11.16	11.16	8.62	x	x	x	x	x	x
ecoli	-	-	-	-	-	-	x	x	x	x	x
xenopus	17.49	17.49	17.49	17.49	17.49	17.49	-	x	x	x	x
zebra fish	15.78	15.78	15.78	15.78	15.78	15.78	-	17.49	x	x	x
anole	8.62	11.16	11.16	15.78	4.36	8.62	-	17.49	15.78	x	x
drosophila	25.98	25.98	25.98	25.98	25.98	25.98	25.98	25.98	25.98	25.98	x

Table 11 ARG2 (agmatase)

	poultry	rat	human	oppossum	platypus	zebra finch	ecoli	xenopus	zebra fish	anole	drosophila
Poultry	x	x	x	x	x	x	x	x	x	x	x
Rat	11.04	x	x	x	x	x	x	x	x	x	x
Human	11.04	8.76	x	x	x	x	x	x	x	x	x
Opposum	11.04	8.76	7.55	x	x	x	x	x	x	x	x
platypus	8.52	11.04	11.04	11.04	x	x	x	x	x	x	x
zebra finch	4.04	15.61	11.04	11.04		x	x	x	x	x	x
ecoli	27.95	27.95	27.95	27.95	27.95	27.95	x	x	x	x	x
xenopus	17.3	17.3	17.3	17.3	17.3	17.3	27.95	x	x	x	x
zebra fish	15.61	15.61	15.61	15.61	15.61	15.61	27.95	17.3	x	x	x
anole	8.52	11.04	11.04	11.04	4.31	8.52	27.95	17.3	15.61	x	x
drosophila	25.7	25.7	25.7	25.7	25.7	25.7	27.95	25.7	25.7	25.7	x

Table 12 ASL (normal)

	poultry	rat	human	oppossum	platypus	zebra finch	ecoli	xenopus	zebra fish	anole	drosophila
Poultry	4.75	x	x	x	x	x	x	x	x	x	x
Rat	14.62	0.2	x	x	x	x	x	x	x	x	x
Human	14.62	4.65	0	x	x	x	x	x	x	x	x
Opposum	14.62	7.87	7.87	x	x	x	x	x	x	x	x
platypus	-	-	-	-	x	x	x	x	x	x	x
zebra finch	6.78	14.62	14.62	14.62	-	x	x	x	x	x	x
ecoli	26.09	26.09	26.09	26.09	-	26.09	x	x	x	x	x
xenopus	14.62	12.3	12.3	12.3	-	14.62	26.09	x	x	x	x
zebra fish	14.62	13.65	13.65	13.65	-	14.62	26.09	13.65	x	x	x
anole	-	-	-	-	-	-	-	-	-	x	x
drosophila	22.95	22.95	22.95	22.95	-	22.95	26.09	22.95	22.95	-	x

Table 13 ASL (carnosine)

	poultry	rat	human	oppossum	platypus	zebra finch	ecoli	xenopus	zebra fish	anole	drosophila
Poultry	2.6	x	x	x	x	x	x	x	x	x	x
Rat	8	0.11	x	x	x	x	x	x	x	x	x
Human	8	2.55	0	x	x	x	x	x	x	x	x
Opposum	8	4.31	4.31	x	x	x	x	x	x	x	x
platypus	-	-	-	-	x	x	x	x	x	x	x
zebra finch	3.71	8	8	8	-	x	x	x	x	x	x
ecoli	13.74	13.74	13.74	13.74	-	13.74	x	x	x	x	x
xenopus	8	6.73	6.73	6.73	-	8		x	x	x	x
zebra fish	8	7.47	7.47	7.47	-	8			x	x	x
anole	21.23	21.23	21.23	21.23	-	21.23	21.23	21.23	21.23	x	x
drosophila	12.57	12.57	12.57	12.57	-	12.57	12.57	12.57	12.57	21.23	x

Table 14 ASSI

	poultry	rat	human	oppossum	platypus	zebra finch	ecoli	xenopus	zebra fish	anole	drosophila
Poultry	x	x	x	x	x	x	x	x	x	x	x
Rat	9.61	x	x	x	x	x	x	x	x	x	x
Human	9.61	1.06	x	x	x	x	x	x	x	x	x
Opposum	9.61	2.75	2.75	x	x	x	x	x	x	x	x
platypus	-	-	-	-	x	x	x	x	x	x	x
zebra finch	-	-	-	-	-	x	x	x	x	x	x
ecoli	32.34	32.34	32.34	32.34	-	-	x	x	x	x	x
xenopus	9.61	8.65	8.65	8.65	-	-	32.34	x	x	x	x
zebra fish	10.49	10.49	10.49	10.49	-	-	32.34	10.49	x	x	x
anole	-	-	-	-	-	-	32.34	-	-	x	x
drosophila	19.54	19.54	19.54	19.54	-	-	32.34	19.54	19.54	-	x

Appendix D

Sequence Accession Numbers

Table 15 Accession Numbers for All Sequences Utilized

Enzyme	Symbol	Organism	Reference Number
Carbamoyl phosphate Synthase I	CPSI	Gallus gallus	F1N9P0 (tree) 113951670 (Raf experiments)
	CPSI	Homo sapien	P31327
	CPSI	Rattus norvegicus	P07756
		Monodelphis domestica	F7FS26
		Ornithorhynchus anatinus	345306951
		Taeniopygia guttata	224056010
		Eschericha coli	312944639
		Xenopus tropicalis	B2GU38
		Danio rerio	F6PBT6
		Anolis carolinenss	G1KK90
		Drosophila melanogaster	4337094
Ornithine Transcarbamylase	OTC	Gallus gallus	46048857
		Homo sapien	P00480
		Rattus norvegicus	P00481
		Monodelphis domestica	F6UHY3
		Ornithorhynchus anatinus	F6QRP1
		Taeniopygia guttata	-
		Eschericha coli	129264
		Xenopus tropicalis	Q6DJ41
		Danio rerio	E9QHD9
		Anolis carolinenss	G1KQ60
		Drosophila melanogaster	-
Arginosuccinate synthase	ASS	Gallus gallus	61657937
		Homo sapien	P00966

		Rattus norvegicus	P09034
		Monodelphis domestica	F7G4N7
		Ornithorhynchus anatinus	-
		Taeniopygia guttata	-
		Eschericha coli	170082708
		Xenopus tropicalis	Q5M8Z6
		Danio rerio	Q66I24
		Anolis carolinenss	-
		Drosophila melanogaster	B3M0W9
Arginosuccinate Lyase	I	Gallus gallus	45382827
	II	Gallus gallus	270288808
		Homo sapien	E7EMI0
		Homo sapien	P04424
	I	Rattus norvegicus	Q4QRB8
	II	Rattus norvegicus	P20673
		Monodelphis domestica	334324759
		Ornithorhynchus anatinus	-
		Taeniopygia guttata	224076287
		Eschericha coli	16131798
		Xenopus tropicalis	A9JTM9
		Danio rerio	Q7SY44
Carnosine Synthase		Anolis carolinenss	327260538
		Drosophila melanogaster	Q960C6
Arginase		Gallus gallus	340523133
		Homo sapien	P78540
		Rattus norvegicus	O08701
		Monodelphis domestica	F6Y7C7
		Ornithorhynchus anatinus	345316653
		Taeniopygia guttata	224051299
Agmatinase		Eschericha coli	209760196
		Xenopus tropicalis	Q6DIU5
		Danio rerio	Q6PH54
		Anolis carolinenss	327263953
		Drosophila melanogaster	10728356
v-Raf murine sarcoma viral oncogene homolog B1	BRaf	Gallus gallus	45384285
v-Raf-1 murine leukemia viral oncogene homolog 1	Raf1	Gallus gallus	45384313