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# Structure and genetic mapping of the Cytochrome P450 gene *(CYP1A5)* in the turkey *(Meleagris gallopavo)*

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**Abstract.** Cytochromes P450 (P450) are a superfamily of membrane-bound hemoproteins that oxidize a large number of endogenous and exogenous compounds. The recently cloned P450 gene *(CYP1A5)* encodes the primary protein responsible for epoxidation of aflatoxin $B_1$ ($AFB_1$) in the turkey, an animal extremely sensitive to this mycotoxin. Hypersensitivity of turkeys to $AFB_1$ was first demonstrated by association with 'Turkey X Disease' which caused widespread deaths of turkeys and other poultry throughout Europe in the 1960s, later shown to be caused by $AFB_1$-contaminated feed. In this study, comparative genomic approaches were used to selectively amplify and sequence the introns and 3′ flanking region of *CYP1A5*. The structure of the *CYP1A5* gene in the turkey is shown to be equivalent to that of the human *CYP1A* genes with seven exons of 38, 858, 127, 90, 124, 87 and 307 bp, respectively, and six introns. A single nucleotide polymorphism (SNP) in the 3′ UTR was used to assign *CYP1A5* to turkey linkage group M16 (equivalent to chicken chromosome 10). The results of this study provide the framework for identifying allelic variants of this biochemically important P450 gene in poultry.



Cytochromes P450 ('P450' for protein; 'CYP' for gene) are a superfamily of hemoproteins that catalyze the oxidation of a large number of endogenous and exogenous compounds, including steroids, eicosanoids, drugs, environmental pollutants, and dietary carcinogens (Parikh et al., 1997). Because P450 catalysis often results in the formation of reactive electrophilic intermediates responsible for toxicity and carcinogenicity, much research has focused on the role of these enzymes in human and animal health.

One important example of an environmental toxin that is oxidized by P450s is the mycotoxin aflatoxin $B_1$ ($AFB_1$), which is a potent hepatotoxin and hepatocarcinogen in humans and animals and is produced by strains of *Aspergillus flavus* and *A. parasiticus* (Coulombe, 1993). This mycotoxin was discovered in the early 1960s in feed contaminated with Brazilian peanut meal as the etiological agent of 'Turkey X Disease', responsible for widespread deaths of turkeys and other poultry throughout Europe (Asao et al., 1965). As exemplified by this episode, poultry are very sensitive to the toxic effects of $AFB_1$, and turkeys are the most sensitive poultry species (Giambrone et al., 1985; Klein et al., 2000). Hypersensitivity of turkeys to $AFB_1$ is associated with efficient P450 oxidation to form the electrophilic exo-$AFB_1$-8,9-epoxide (AFBO), which binds to DNA, proteins, and other critical cellular nucleophiles (Klein et al., 2000).

Turkeys are the most efficient $AFB_1$ epoxidators of any species studied thus far (Klein et al., 2000). As in humans and other animals, multiple P450s bioactivate $AFB_1$ in turkeys. Using specific P450-class inhibitors, it was determined

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**Table 1.** Summary of primers used to amplify *CYP1A5* fragments from turkey genomic DNA. For each fragment, primer sequences, sequence length in the chicken whole-genome sequence, observed product size in the turkey, GenBank accession number and the number of complete introns and exons contained within the fragments are given.

| Fragment | Forward | Reverse | Chicken | Turkey | GenBank | Intron/Exon |
|---|---|---|---|---|---|---|
| 1 | F1 – GTCCTGGAGCCTCATGTACC | R1 – GAAACGCTCTGGGTTGAAAG | 652 | 654 | DQ494207 | 3/2 |
| 2 | F2 – TACCTCCCCAGCCGCAAC | R2 – TACATGAGGCTCCAGGACAG | – | 907 | DQ494206 | 2/1 |
| 3 | F3 – CCTGACCTTCAGCACTGACAC | R3 – TCCAGAAATAAATCCATGTTGC | – | 399 | – | 0/1 |
| 4 | F4 – CTCTGCCCAGCTTCAAGG | R4 – TGCCCACCTGTACCATCAC | 244 | 235 | DQ494205 | 1/0 |
| 5 | F5 – AGCACTTTCAAGTCAAGAAACG | R5 – GGGGTCTGGTGTTATGGTTG | 818 | 993 | DQ494208 | 3′ UTR |

that turkey liver microsomes epoxidate $AFB_1$ primarily by proteins encoded by a *CYP1A* and to a lesser extent, a *CYP3A* homologue (Klein et al., 2000). Recently a full-length *CYP1A2* homologue was cloned, sequenced and heterologously expressed from turkey liver. This gene named *CYP1A5* (GenBank AY964644) encodes a protein, which like the microsomal form, has a high catalytic efficiency toward $AFB_1$ bioactivation (Yip and Coulombe, 2006). To our knowledge, this is the first functional *CYP* family gene from turkey to be cloned, and expressed. Because of the possible importance of *CYP1A5* in the hypersensitivity of turkeys to $AFB_1$, determining whether there are allelic variants of this P450 may assist in identifying possible resistance traits in turkeys. This study was designed to determine the genomic structure of the *CYP1A5* gene and identify single nucleotide polymorphisms (SNPs) to genetically map the gene in the turkey.

## Material and methods

Sequences with homology to the *CYP1A5* gene are located on chromosome 10 (GGA10) in the chicken. Although the genomic sequence in this region is incomplete (International Chicken Genome Sequencing Consortium, 2004), alignment of the *CYP1A5* cDNA from the turkey (Yip and Coulombe, 2006; GenBank AY964644) with the partial sequence was used as a starting point to identify intron/exon boundaries. In addition, the turkey *CYP1A5* cDNA sequence was aligned with the chicken *CYP1A5* (GenBank NM_205146) and *CYP1A4* (GenBank X99453) cDNA sequences (Gilday et al., 1996) to allow for design of primers specific to the *CYP1A5* gene (Fig. 1). Primers were designed with Primer V.3 software (Whitehead Institute for Biomedical Research, http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to amplify only *CYP1A5* products (Table 1).

Fragments were amplified from genomic DNA in PCR reactions containing approximately 25 ng DNA, 1.5 mM $MgCl_2$, 2.5 pmol each primer, 100 μM dNTP, and 0.35 U Taq polymerase (Qiagen, Inc). Primer annealing conditions were optimized for amplification of each DNA fragment in a Techne thermal cycler under the following conditions: 15 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at optimal annealing temperature, 1 min at 72°C; and a final extension of 5 min at 72°C. Amplified products were resolved on a 1% agarose gel, purified for sequencing with a Qiagen PCR clean up kit. Templates were sequenced in both directions with the original primers on an ABI automated sequencer at the Advanced Genetic Analysis Center, University of Minnesota.

Sequences for each fragment were obtained from multiple individuals in order to identify putative single nucleotide polymorphisms (SNPs) visualized as multiple peaks in the genomic DNA sequence. Our sample included the six $F_1$ individuals of the UMN/NTBF mapping families (Reed et al., 2003) and a single wild turkey sample from Indiana (eastern wild turkey, *M. gallopavo silvestris*). Sequences for each fragment were edited and contigs assembled with Sequencher software (Gene Codes Corp.).

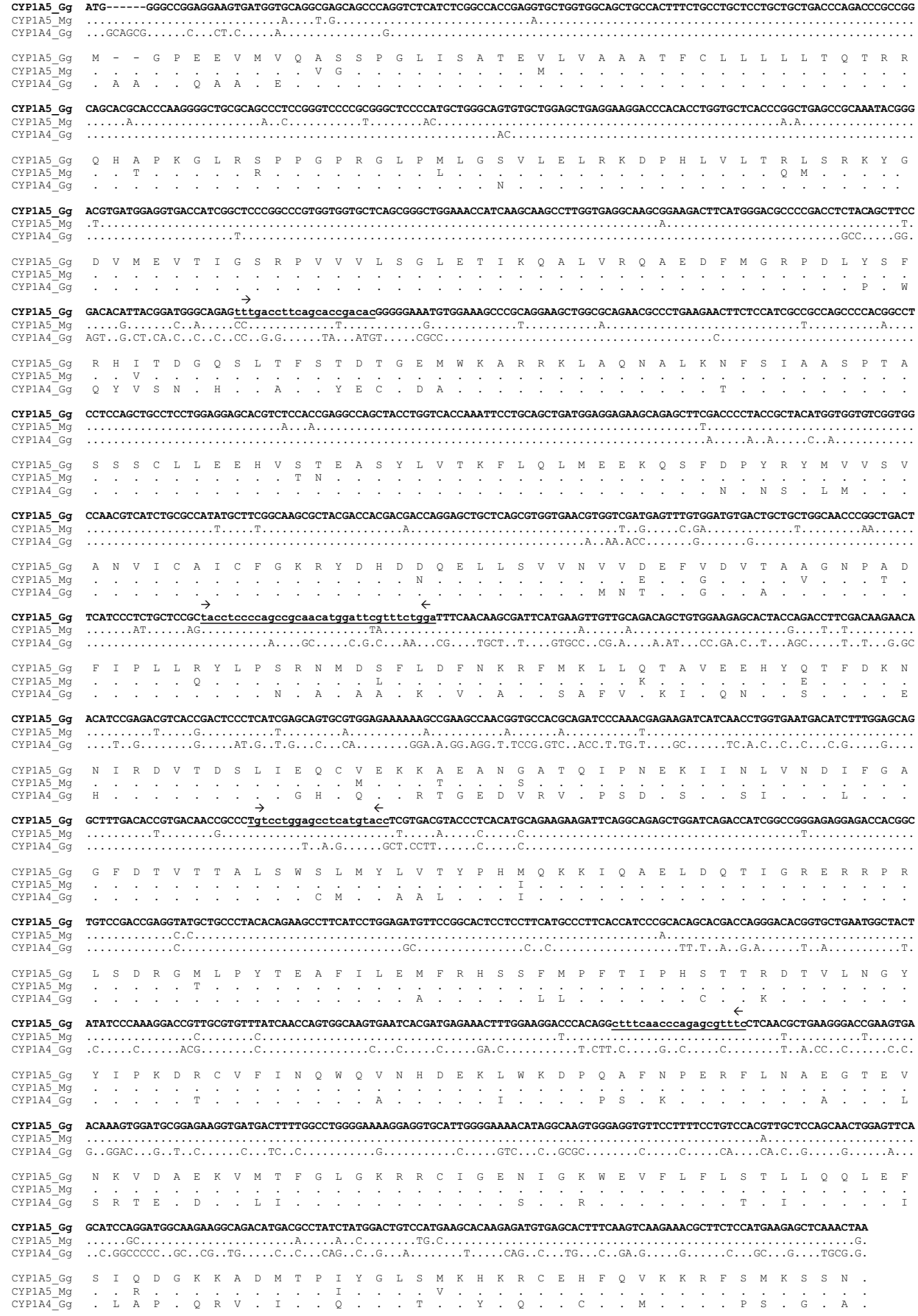
An SNP in the 3′ UTR of the *CYP1A5* gene was identified and allele-specific primers were designed to selectively amplify the two SNP alleles. Genotypes were obtained by PCR and verified by PCR/RFLP for the UMN/NTBF families and added to the UMN turkey genotype database for combined linkage analysis. Two-point linkage analysis was performed using Locusmap (Garbe and Da, 2003).

## Results

*Sequence structure of CYP1A5*

Investigation of the turkey *CYP1A5* gene started with a region completely represented in the chicken genome sequence. Primers anchored in putative exons 4 and 7 (1F and 1R) successfully amplified a 654-bp fragment from turkey genomic DNA (Fragment 1, Table 1 and Fig. 2). Alignment of this sequence (GenBank # DQ494207) with the turkey *CYP1A5* cDNA and chicken whole-genome sequence confirmed the presence of three introns and two exons within the amplicon. The three introns (4–6) were 84, 118 and 133 bp in length, respectively (Fig. 2). In comparison to the chicken genome sequence, intron sequences were highly conserved with seven nucleotide differences between the species in intron 4 (91.7% similarity), six differences in intron 5 (94.9%) and 11 differences in intron 6 (91.8%). With one exception, alignment of multiple turkey sequences did not indicate any nucleotide polymorphisms. In one male (D3804) an SNP (G/A) was observed three nucleotides downstream of the primer site in exon 4. This polymorphism would result in a synonymous codon substitution (GTG/GTA, common codon = GTG) and would not affect the amino acid sequence.

Because of the gap in the chicken genome sequence, the exon/intron boundaries upstream of exon 4 could not be determined from sequence alignments. Using the predicted human gene structure (Entrez Gene), a forward primer (F2) was designed corresponding to the hypothesized 3′ end of exon 2 for use in combination with a reverse primer (R2) complementary to the exon 4 forward primer (Fragment 2, Table 1). This fragment includes exon 3, which is the most



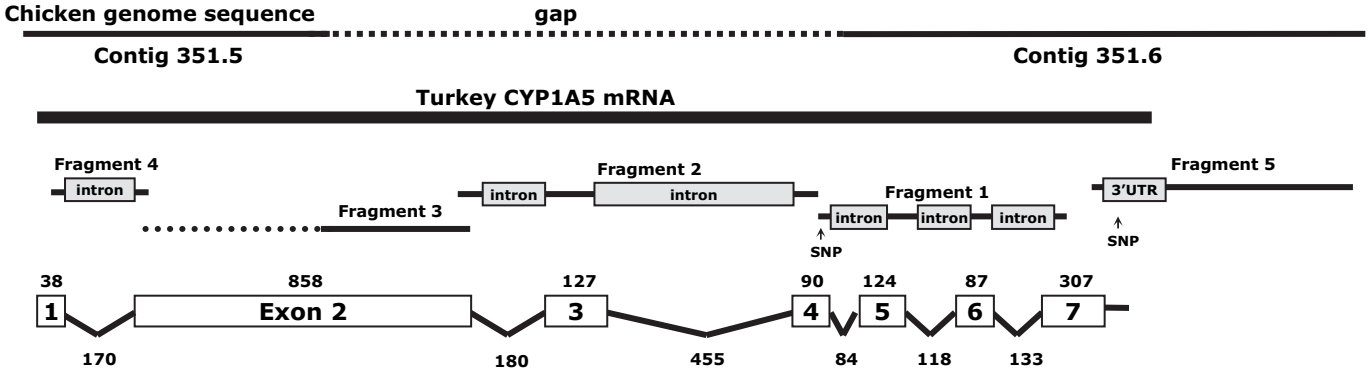



**Fig. 2.** Idiogram of *CYP1A5* gene indicating position of exons and introns within the amplified fragments. Location of gap in chicken genome sequence is indicated relative to the turkey cDNA alignment. Length in basepairs is given for each intron (below) and exon (above).

variable region between the *CYP1A5* and *1A4* genes in the chicken. PCR with these primers amplified a 907-bp fragment from turkey genomic DNA. Alignment of this sequence (GenBank # DQ494206) with the turkey *CYP1A5* cDNA indicated two introns and one exon within the amplicon. The two introns (2 and 3) were 180 and 455 bp in length, respectively (Fig. 2). Alignment of multiple turkey sequences did not reveal nucleotide polymorphisms within this fragment.

To confirm that no additional introns were present in the putative exon 2, primers were designed to amplify the remaining portion of the gene not present in the chicken genome sequence (Fig. 2). A forward primer (F3) anchored in exon 2 just upstream of the chicken genome sequence gap and a reverse primer (R3) partially overlapping F2, were designed to specifically amplify only *CYP1A5*. PCR with these primers amplified a 399-bp fragment from genomic DNA (Fragment 3, Fig. 2). The size of this amplicon and alignment of the resulting sequence with the turkey *CYP1A5* cDNA established the expected contiguous coding sequence of exon 2. This was also confirmed with an additional forward primer (F3–2), which amplified a 777-bp fragment from genomic DNA corresponding to the length predicted from the cDNA sequence. Affirming results were also obtained with amplification of chicken genomic DNA. No sequence variation in Fragment 3 was observed within our samples.

In humans, the 5′ UTR of *CYP1A* genes (1A1 and 1A2) include an intron; exon 1 is non-coding and the translation start codon is located in exon 2 (see Jaiswal et al., 1985). Alignment of the turkey cDNA sequence with the chicken genome indicated a similar condition in poultry. Primers anchored in exon 1 (4F) and exon 2 (4R) successfully amplified a 235-bp fragment from turkey genomic DNA (Fragment 4, Table 1 and Fig. 2). Alignment of this sequence (GenBank # DQ494205) with the turkey *CYP1A5* cDNA and chicken whole-genome sequence confirmed the presence of a single intron (170 bp in length) in the turkey (Fig. 2). In comparison to the chicken genome sequence, 15 single nucleotide differences and a 10-bp indel occurred between the species in intron 1 (90.1% similarity). Alignment of multiple turkey sequences found no variation among the turkey samples.

Finally, to obtain sequence downstream of *CYP1A5*, primers were designed to amplify the 3′ UTR and flanking sequence (Fragment 5, Fig. 2). A forward primer (F5) anchored in exon 7 was used in combination with a reverse primer (R5) designed from the chicken genome sequence (Table 1) and a 993-bp fragment was amplified by PCR from turkey genomic DNA. This fragment (GenBank # DQ494208) was longer than predicted from the chicken sequence due to a large 178-bp insertion. Excluding the insertion, the aligned turkey and chicken sequences included 79 nucleotide differences (91.5% similarity). Alignment of multiple turkey sequences identified a single SNP (G/A position 190, common allele = G) within the 3′ UTR in one F1 individual (D3804) from the UMN/NTBF mapping families. This SNP occurred within the recognition sequence ($GG\underline{T}GA(N)_8$) for the restriction endonuclease *Hph*I.

*Genetic mapping of CYP1A5*

Allele-specific primers were designed to independently amplify the two 3′ UTR SNP alleles for genotyping. Both primers (CYPUTRsnpF – TTGTTCACTTTCTAGTGTTTGTTC**g** and CYPUTRsnpR – TGCTGGGAAATGAAGAAGG**t**) utilized the variable site as their 3′ terminal nucleotide. All four primers (F5, R5, CYPUTRsnpF, and CYPUTRsnpR) were used in a single PCR cocktail (62°C annealing temperature) to amplify DNA of the UMN/NTBF families for which D3804 is the sire. Using this approach al-

**Fig. 1.** Sequences of turkey *CYP1A5*_Mg (GenBank AY964644) and chicken *CYP1A4*_Gg (GenBank X99453) cDNAs aligned with the mRNA sequence of the chicken *CYP1A5* gene (GenBank NM205146). Predicted amino acid sequences for each gene are given below the nucleotide sequence. Position (underlined lower case) and direction (arrows) of PCR primers are indicated.

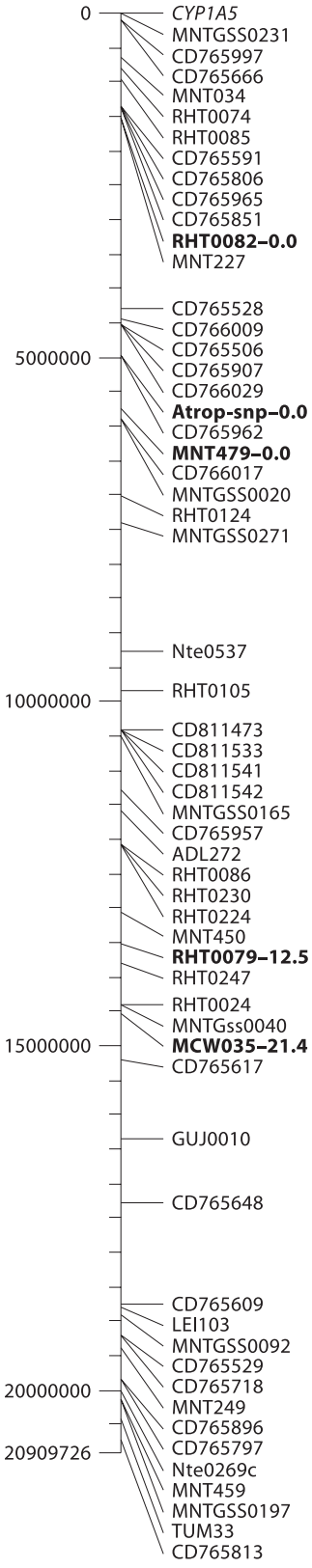



**Fig. 3.** Physical map of chicken chromosome 10 (GGA10) based on positions (determined by BLASTN) of turkey sequences aligned with the draft assembly of the chicken whole-genome sequence (units = bp). Distances in cM are given for each marker (bold) linked to the *CYP1A5* gene in the turkey.

leles were easily scored as individuals with the G/A genotype produced a single prominent 209-bp fragment and individuals with the G/G genotype produced a prominent 828-bp fragment. Some G/G homozygotes also produced the non-allele specific product at 209 bp evidenced as a faint band. PCR genotyping was confirmed in a subset of individuals by PCR/RFLP (digestion of Fragment 5 with *Hph*I). Restriction banding patterns were consistent with the genotypes determined by PCR and sequencing.

The current dataset for the UMN/NTBF families includes genotypes at over 400 markers (Reed et al., 2006 and unpubl.). Linkage analysis of *CYP1A5* genotypes (84 informative meioses) found significant two-point LOD scores (>3.0) between the *CYP1A5* SNP and five markers on turkey linkage group M16 (corresponding to GGA10). The microsatellites RHT079 and MCW035 were linked at 12.5 cM (LOD 9.6) and 21.4 cM (LOD 6.0), respectively. Three markers (RHT082, Atrop_snp and MNT479) had theta values of 0.00 (0.0 cM) and highly significant LOD scores (24.9, 16.6 and 24.9, respectively). Based on alignments to the chicken genome sequence, these markers are approximately 1.5, 5 and 6 Mbp, respectively from *CYP1A5* (Fig. 3). Previous comparison of the turkey genetic map with the chicken genome sequence indicated a ratio of 2.38 cM/Mbp within the UMN/NTBF mapping families (Reed et al., 2005). Assuming that these sequences are in a similar orientation in the turkey, this indicates a region of lower than average recombination.

## Discussion

Structure of the *CYP1A5* gene in the turkey is equivalent to that of the human *CYP1A* genes with seven exons and six intervening sequences. Exon 1 is non-translated with the start codon beginning six nucleotides into exon 2. Although mRNA sequence of the turkey *CYP1A5* (and bioactivity) more closely match human *CYP1A2* (Yip and Coulombe, 2006), intron size is more closely similar to the human 1A1 gene as opposed to 1A2 (a tandem duplication ancestral gene equivalent to 1A4 in chicken). Introns of the *CYP1A5* appear to be highly conserved in poultry with similarity values of aligned intron and UTR sequences exceeding 91%. In comparison, this value is not appreciably different than the 94.7% identity found between *CYP1A5* nucleic acid (cDNA) and protein sequences (Yip and Coulombe, 2006). Surprisingly, sequences from both commercial (UMN/NTBF birds) and a single wild turkey (Indiana sample) were virtually identical.

Sequence conservation in *CYP1A5* is also reflected in the significantly lower frequency of SNPs as opposed to previous studies of the UMN/NTBF families. In the present study, 1,896 bp of non-coding genomic sequence was surveyed with only a single SNP detected. In contrast, Reed et al. (2006) observed one SNP per 240 bp in EST-associated sequences and Chaves et al. (2003) found one SNP every 226 bp in muscle-associated gene introns within these same F1 individuals. Low polymorphism in this P450 gene could re-

flect selection pressure for maintaining specificity of these biochemically important genes. For example, a wide survey of SNP variation in the human *CYP1A2* gene found the pattern of DNA polymorphism to be consistent with the action of positive natural selection (Wooding et al., 2002). Gene conversion is also thought to play a role in evolution of the *CYP1A* gene subfamily (Goldstone and Stegeman, 2006).

In the turkey, *CYP1A5* is genetically linked to markers on linkage group M16, which is equivalent to GGA10. This is consistent with location of *CYP1A5* at the extreme end of GGA10 in the chicken genome sequence. The related gene *CYP1A4* is also located within the same supercontig on the opposite sequence strand of GGA10. In the human, *CYP1A1* and *1A2* are orientated head-to-head at a distance of 23 kb on HSA15 and induction appears to be simultaneously controlled through common regulatory elements (Corchero et al., 2001; Ueda et al., 2006). Based on the close similarity between the turkey and chicken genomes (Reed et al., 2005) it is likely that the *CYP1A4* gene is in a similar location as *CYP1A5* in the turkey genome.

Since multiple P450 genes occur in poultry, it is necessary to develop molecular tools to examine variation in individual P450 genes. Our comparative sequence analyses indicate that gene-specific (and allele-specific) primer sets can be used to amplify select regions of the *CYP1A5* gene despite the relatively high cDNA sequence similarity (~80%) that exists between this gene and the related *CYP1A4* (Gilday et al., 1996; Yip and Coulombe, 2006). In addition, based on the sequence alignments, three of the primer sets used in our study of turkeys are directly applicable to the chicken. With these tools, a wider survey of the genetic diversity in this largely uncharacterized P450 gene in poultry is now possible.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