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Applications of Variable Number Tandem Repeat Genotyping in the Validation of an Animal Medical Model and Gene Flow Studies in Threatened Populations of Reptiles

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APPLICATIONS OF VARIABLE NUMBER TANDEM REPEAT GENOTYPING IN THE VALIDATION OF AN ANIMAL MEDICAL MODEL AND GENE FLOW STUDIES IN THREATENED POPULATIONS OF REPTILES

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ABSTRACT

We used variable number tandem repeats (VNTR) to validate the chicken as a human medical model for Pulmonary Arterial Hypertension. We identified seven regions on four chromosomes and interrogated for VNTR markers that significantly associate with Pulmonary Hypertension Syndrome/ascites. In those regions, we identified 7 candidate genes; AGTR1, ACE, p38MAPK, SST, 5HT2B, NET1, and CALM3 for further analysis as significantly contributing QTL for ascites/PHS. We also used variable number tandem repeats to measure gene flow and gather evidence for multiple paternity in a population of Timber rattlesnakes, *Crotalus horridus*. We were able to verify 1 VNTR that can be used to assess gene flow in and among our populations. We were also able to use this VNTR to provide evidence of multiple paternity in the Timber rattlesnake.

This dissertation is approved for
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DEDICATION

I feel privileged to have had the pleasure of working with Dr. Douglas Rhoads. The tools with which I leave the Rhoads' lab include more than technique. The level of guidance and mentoring provided to me by Doug extends far beyond the bench and I am a better scientist and person for it. He has provided me the foundation for a lifetime of seeking complete answers.

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INTRODUCTION

Publication of the full genome sequences of multiple organisms has allowed the development of multiple avenues by which the genome can be analyzed. Variable number tandem repeat (VNTR) genotyping is one such tool. Long stretches of short, repetitive sequences can serve as biomarkers to track gene flow, identify mutation rates, locate genes contributing to the pathology of a disease, and more. This work presents the application of VNTR genotyping as a tool for identification of genes contributing to disease and as a tool for the analysis of the gene flow within and among a population of threatened animals.

Chapter 1: Genetic Analysis of Chromosomal Regions Associated with Ascites in the Chicken: Literature Review

Ascites and Pulmonary Arterial Hypertension

Ascites is an industry term for a metabolic condition of the cardiopulmonary system of broiler chickens that is associated with a fast growth rate and mortality in older birds (Pavlidis 2007). Ascites is more accurately known as pulmonary arterial hypertension (PAH) or Pulmonary Hypertension Syndrome (PHS) which are diseases shown to affect rats, pigs, and humans (Naeiji 2007, Robbins 2004). The genes involved in resistance and susceptibility to PAH are not yet fully described. Pulmonary arterial hypertension is also observed in the human population. In humans, it is comprised of a group of disorders with varying etiologies and underlying conditions. It is known as both a symptom of disease (secondary pulmonary hypertension) and a disease of its own right (primary pulmonary hypertension). Our work addresses this polygenic trait through quantitative trait locus (QTL) analysis to identify candidate genes that contribute to the disease.

Ascites/PAH is a multi-symptom disease, affecting both the heart muscle and the vasculature of the bird (Wideman 2006). Pulmonary arterial hypertension is caused by an insufficient cardiopulmonary response to rapid growth of muscle mass. Initially, when subjected to pulmonary challenge, blood pressure rises as the cardiovascular system constricts vessels to increase flow to tissues. When this response is sustained over time, increased total blood volume, blood pressure, and red blood cell count (polycythemia) to supply the increased tissue mass with oxygen are observed. This uptick in blood volume results in right heart growth (hypertrophy) which further increases the pulmonary pressure and can result in hepatic damage and plasma accumulation to the abdomen. Abdominal

fluid resulting from hepatic damage is referred to as “ascites” which has become the name of PAH/PHS in poultry.

Currently, it is estimated that ascites-related mortality costs the poultry industry worldwide more than $500 billion per year (Katanbaf 2009). The poultry industry offsets the problems associated with PAH/ascites with protocols that alter growth rate and improve air quality. Feed restriction, used to control growth rate and good ventilation, used to reduce the respiratory strain on the cardiopulmonary system (Ozkan 2006) are effective tools, yet PAH remains a concern for the poultry industry. The possibility of genetic selection to eliminate PAH development in production birds is a tantalizing prospect that would permit an alternative and more cost effective control strategy for the rapid growth of broilers.

The poultry industry has a specific interest in the genetics of PAH/ascites because broiler birds are susceptible to the disease. Broilers are genetically selected for high growth rates and efficient feed conversion to allow for the rapid accumulation of body mass. Rapid increase in body mass without a concomitant increase in cardio-pulmonary output can lead to hypoxia in the bird, triggering an increase in cardio-pulmonary pressure to meet oxygen requirements of the tissues (Julian 2000). Ascites affects the poultry industry worldwide, but is more prevalent at high altitudes and regions where ventilation of bird houses is problematic (McMullin 2004). Ascites averages a 1-5% morbidity rate and a 1-2% mortality rate that can increase up to 30% at high altitude. Currently, ascites is managed in commercial flocks by feed restriction, but with this practice comes limited growth rate; consequently, feed intake must be carefully monitored and imposed upon flocks at strategic points along their life-span to allow concomitant maximum growth rate and lowered ascites

incidence (Ballay 1992, Yu 1992). The selection of breeding lines based on genetic markers is known as marker-assisted selection. The use of marker-assisted selection in tandem with phenotypic selection against susceptibility to ascites would allow for more streamlined breeding practices and presents the possibility for intervention at an earlier life stage in the animals.

The vascular system in chickens is made of resistance-sensitive vessels that respond to changes in pressure and air quality with constriction, dilation, or structural change (Gibbons and Dzau 1994). There are three layers to each of these vessels: the intima, made of endothelial cells; the media, made of smooth muscle cells; and the adventitia which is made of connective tissue elements and nerve tissues. The increase and decrease of vascular pressure leads to an increase or decrease of shear stress within the vessels which serves as a physical signal to the endothelial cells to begin proliferation and modification of vessel structure, specifically, the proliferation of smooth muscle cells. Proliferation in the vessels is an essential response to stimuli and is mandated by the need for vessels to maintain a dynamic response to an increase in pressure with a thickening of their walls to contain blood. Tissue modification in response to the dynamics of blood pressure is referred to as vascular remodeling which requires at least four cellular processes: cell growth, apoptosis, cell migration, and the restructuring (degradation and production) of the extracellular matrix.

The respiratory system of the chicken is heterogeneous and functionally segregated, with roughly 90% of the respiratory system of a bird being comprised of air sacs, which do not have much capacity for expansion beyond their normal inflation and act as a bellows to ventilate the lungs (Whittow 2000). The lungs are fixed and rigid with little capacity for

expansion to accommodate any rapid increase in blood volume. The parabronchi, which contain the air capillaries where gas exchange takes place, utilize an efficient cross-current model juxtaposing ventilation and perfusion at a right angle (Powell 2004). When compared to its progenitor, the Red Jungle Fowl (Moiseyeva 2003), the domestic broiler chicken has reduced lung volume and a thickened blood-gas tissue barrier, both of which present a challenge for efficient oxygen supply to tissues (Vidyadaran 1990). Research comparing broilers and layers has shown that there is a significant association between ascites and decreased relative size and function of lung, heart, and thoracic cavity. Broilers have greatly reduced cardiothoracic capacity as a percentage of weight when compared to leghorns (Hassanzadeh 2005, Martinez-Lemus 1998). Therefore, the consequences of selective breeding practices have led to a whole line of birds (broilers) whose oxygen requirements are only marginally met by their anatomy.

The pathobiology of PAH involves complex biochemical pathways for growth and vascular tone regulation. The growth of cardiac muscle and development of hypertrophy requires proliferation of smooth muscle and connective tissue; these processes necessitate growth factors that must be up-regulated for quick growth. Fibroblast growth factor I (FGF1) has been shown to induce angiogenesis and proliferation of capillary endothelial cells *in vivo* (Montesano 1986). Transforming growth factor beta (TGFβ) has been shown to induce pathologic myocardial fibrosis (Campbell 1997). Platelet derived growth factor (PDGF) has been implicated as a modulator of myogenesis (Yablonka-Reuveni 2008). If oxygen availability to an animal is significantly reduced, there is an immediate contraction of the pulmonary arterioles, presumably in an effort to preferentially provide vital organs with oxygen and an increase in blood volume to carry it. The immediate result is elevated

pulmonary arterial pressure. Elevated pressure, if protracted, induces a proliferative effect that results in the heart walls thickening and the ventricles becoming larger. Hypertrophy and persistent elevated pressure can often lead to valvular insufficiency which allows back flow of blood in the heart. Unlike striated muscle, there is no stem cell population to allow an adult heart to regenerate myocytes (Mima 1995). Sustained elevated pressure results in a parallel addition of sarcomeres in an effort to increase heart wall thickness (Hunter and Chien, 1999). Increased blood volume results in sequential addition of sarcomeres to lengthen myocytes and increase the size of the chamber (Julian 2007). Lack of oxygen results in myocyte death and the remaining myocytes are stretched ultimately leading to hypertrophy of the stressed right heart. Simply, there is an increase in heart muscle density but not contractile ability which can lead to the heart not emptying completely when it contracts, continued myocyte death, and significant congestion. The growth of the heart in response to elevated pressure and increasing blood volume further exacerbates the stress on the cardiopulmonary system. Growth factors play an intrinsic role in allowing this growth to occur in both smooth muscle cells and connective tissues, but they are not fully responsible for the damage done by PAH.

Platelet dysfunction is often observed in tandem with PAH. Platelets appear to be activated in the progress of PAH, aggregate at the site of endothelial damage in the vessels due to elevated pressure, and exacerbate vasoconstriction by depositing serotonin into an already constricted system of vessels (Chaouat 1996, Humbert 2004, Eddahibi 2001). A study in 2004 compared the relative balance of vasodilators (nitric oxide, prostacyclin, and vascular endothelial growth factor) and vasoconstrictors (thromboxane A, serotonin, and endothelin-1) in broilers challenged with lipopolysaccharide (Wideman 2004). They found

that the hypertensive response is modulated when more vasodilators than vasoconstrictors were generated by the animal. Accordingly, they posited that innate differences in chemical response by the animal may have a key role in the response to vascular pulmonary challenge and/or pulmonary hypertension. The vascular response and homeostatic balance of these vasoactive substances is well studied in birds (Stebel 2008, Chapman 2006 and 2008).

In humans, PAH is divided into several different forms by the American Lung Association: associated, familial, and idiopathic pulmonary arterial hypertension (APAH, FPAH, and IPAH, respectively), PAH associated with venous or capillary compromise, and PAH of the newly born (Simonneau 2004). Secondary pulmonary hypertension includes FPAH and APAH which are often observed in patients with chronic obstructive pulmonary disease (COPD), congenital heart disease, AIDS, lupus, and other hypoventilation disorders. The familial form of PAH applies to cases where a family history of PAH can be established. Idiopathic PAH is also known as primary pulmonary hypertension (PPH), which is an exclusionary term that is applied to any case of pulmonary hypertension with no known cause (Rich 2000).

All forms of PAH in humans display right ventricular hypertrophy, elevated pulmonary pressure, fatigue, chest pain, shortness of breath, plexiform lesions, and eventually, right ventricular failure (RVF) (Tuder 1998, Rubin 1997). Plexiform lesions are conspicuous complex vascular lesions within the lungs. The lesions show signs of disordered endothelial proliferation. As PAH progresses, more and more plexiform lesions are observed, leading to successive lobe failure. There is currently no cure for PAH and patients face a poor prognosis, with a mortality rate around 50% at five years after

diagnosis (NHLBI Index 2009). The current treatments include anticoagulants, vasodilators, and lung transplantation, (Fukumoto 2007, Rubin 1990, Ghofrani 2003, Farber 2004) but none of these works to counteract the underlying cause, namely, cardiac hypertrophy and diminished lung capacity.

We propose the chicken as a medical model for human IPAH. The fawn-hooded rat is commonly used as a model for PAH (Muramatsu 2001). The rat is a well established as a model organism. However, the chicken is economical, can be selected for phenotype, has a completed genome, and ascites/PAH is easily induced. Moreover, PAH is a commercial concern for the poultry industry as breeding practices for meat birds have exacerbated susceptibility for PAH. The chicken, in contrast to the rat, has been shown to spontaneously develop lesions indistinguishable from plexiform lesions observed in the human lung (Wideman 2009, Hamal 2009). Additionally, leukocyte recruitment studies suggest that the immune response in the chicken may more closely mirror the human response to pulmonary challenge than that shown in the rat (Tuder 1994). Specifically, in rats, a high number of macrophages and a few lymphocytes localize to the site of inflammation upon micro-particle injection (Cook 1989, Cook 1990). Alternatively, when injected into chickens, micro-particles induce inflammation and recruitment of high numbers of lymphocytes and some macrophages. This lymphocyte population closely mirrors the populations observed when human plexiform lesions were analyzed (Tuder 1994).

While it is of concern that the chicken and the human diverged somewhere between 471-315 million years ago (Grauer 2004) and the lung of the chicken is anatomically different than that of mammals, there is also significant similarity in both the biochemical

regulation of pulmonary pressure and the anatomy of the heart (Whittow 2000). The genes presented in this work are functionally relevant to vascular regulation in both mammals and avian species.

The underlying physiology of PAH in the chicken appears to be similar to the disorder in humans. PAH is easily induced in the broiler by elevated growth rate, lipopolysaccharide injection, microparticle injection, high altitude, poor ventilation, or growth in a hypobaric chamber (Chapman 2005, Balog 2000). The ease with which disease can be induced, the ease of breeding for a trait, a similar genome to the human (Groenen 2000), and spontaneous lesion formation (Wideman 2009) all make the chicken an ideal model organism for study of the genetics and subsequently the molecular physiology of PAH/IPAH in humans

Molecular analysis of PAH in humans presents a challenge due to the various forms of the condition. Identification of candidate genes is typically conducted in an ontological manner, with the function of known genes related to characteristics of the disease meriting investigation first. PAH involves many tissues, chemical signals, and processes. It has therefore been challenging to ontologically assign candidate genes for analysis, though some QTL are known in humans (Lifton 2001, Chung 2009). However, all forms of PAH involve remodeling of smooth muscle and pulmonary arteries and in many forms of the disease involve endothelial cell proliferation, inflammatory response, and platelet dysfunction (Humbert 2004). Recently, the study of single nucleotide polymorphisms (SNPs) in IPAH patients has revealed that the genes for angiotensin type II receptor I (AGTR1) are associated with PAH (Chung 2009). Chung *et al* observed that specific variants of the AGTR1 gene were significantly associated with late-onset PAH which

suggests a role for the renin-angiotensin-aldosterone system as an involved pathway in the progression of PAH. Additionally, the role of serotonin and its receptors in PAH have been extensively studied in recent years. A review published in 2009 indicated association of particular alleles of serotonin and its receptors 5HT2b and 5HT1b with PAH (MacLean 2009). Specifically, over-expression of serotonin receptors is associated with heightened vasoconstriction and aggravated PAH symptoms.

Genetic Mapping of PAH in the Chicken

Surveying genomes to identify genes involved in disease is one of the most prevalent applications of modern molecular genetics today. One of the simplest forms of genome mapping is the utilization of variable number tandem repeats (VNTRs) as heritable markers for genetic variation (Mousseau 1998). VNTRs are stretches of repeats typically 1-6 base pairs repeated in tandem arrays for as many as hundreds of copies. The repeat number of these nucleotides can vary due to recombination or errors in replication. Variants for the repeat (different "alleles") can be used to distinguish chromosomes based on the alleles inherited (genotype). Additionally, VNTRs serve as excellent markers for a genotype as they are linked to and inherited with genes in close proximity. Polymerase chain reaction (PCR) allows for simple and straight-forward amplification of the repeats. PCR products are easily resolved on polyacrylamide gels or by capillary electrophoresis. Fluorescent tags on the PCR primers allow for high-sensitivity detection, high-throughput genotyping of many individuals. Subsequent comparison of genotype to phenotype profiles allows elucidation of VNTR alleles or genotypes that are significantly associated with a phenotype of interest.

Quantitative genetics defines quantitative trait loci as contributors to a polygenic trait. For most traits, many loci contribute small effects that lead to a particular phenotype (Jansen 1994). Inbred lines of animals have been extensively mapped for QTL. These plants and animals can exhibit pronounced phenotypes that are readily quantified. Within an inbred population, examination of a trait, such as flower color, allows for identification of loci for that trait and obvious phenotypic change if that locus (loci) is manipulated (Lynch and Walsh 1998). Within a species, this mapping technique comparing phenotype

to genotype is especially effective. But there are currently few examples of homologous QTL between two organisms (Flint 2009).

While a single locus can often be attributed to a trait, the power of QTL analysis lies in the identification of multiple loci contributing to the effect. A study in 2004 evaluated the effect of multi-locus involvement of 13 polymorphisms of 8 candidate genes, including genes for angiotensin II type 1 receptor and angiotensin converting enzyme (ACE) when compared to single gene effects (Williams 2004). The study supported a model that required multiple pathway involvement to predispose an individual to hypertension. A multi-locus approach is especially important in diseases where multiple biochemical pathways contribute to symptoms and pathology, such as hypertension. As such, it is necessary to categorize PAH as a QTL disease and to approach candidate gene analyses from a polygenic perspective.

We have utilized resistant and susceptible sub-populations from a susceptible line of birds reared in a hypobaric chamber (Pavlidis 2007). The divergent selection used to develop this susceptible line was also used to develop a resistant line, distinguishable from the susceptible line by divergent ascites development, livability, right ventricle: total ventricle weight comparison (RV:TV), and body weight at 42 days. Our study aims to identify molecular markers linked to resistance and susceptibility between resistant and susceptible animals within the susceptible sub-population.

For this portion of the dissertation, we present the evidence of the evaluation of 7 chromosomal regions previously identified as possibly associated with ascites susceptibility and cardiac hypertrophy. These regions were identified in a whole genome SNP analysis of an F1·F2 population from a cross between an ascites resistant and ascites

susceptible line. We developed VNTR markers for those chromosomal regions and genotyped susceptible and resistant birds from the susceptible line. This work confirmed 5 regions on 4 chromosomes as associated with ascites susceptibility.

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Chapter 2: Genetic Analysis of Chromosomal Regions Associated with Ascites in the Chicken.

ABSTRACT We used a genome-wide single nucleotide polymorphism survey to identify 7 chromosomal regions that showed linkage disequilibrium with respect to ascites susceptibility and ventricular hypertrophy. We then used variable number tandem repeats to obtain genotype data for these 7 regions in a separate, larger population sample segregating for ascites susceptibility. We analyzed the data for allele and genotype frequencies, and, where applicable, combined multiple loci to derive haplotypes associated with ascites. We validated two regions on Gga9 and one region each on Gga1 and Gga27 for involvement in ascites. Probable candidate genes have been identified within these regions that also relate to pulmonary arterial hypertension in humans. This work further validates the chicken as a medical model for human pulmonary arterial hypertension and identifies potential loci for selection against ascites in the chicken.

INTRODUCTION

Idiopathic pulmonary arterial hypertension (IPAH) is one of five types of pulmonary hypertension recognized by the American Lung Association. It is characterized in humans by right ventricular hypertrophy, elevated pulmonary arterial pressure, fatigue, chest pain, shortness of breath, and ultimately, right ventricular failure (RVF). Our group has been investigating the genetics of ascites susceptibility and whether ascites approximates IPAH in humans. Ascites is a commercial concern for the poultry broiler industry and the ability to genetically select populations based on resistance to the disease is of interest to breeders. Selection for meat production through higher feed conversion efficiency and rapid

production of body mass has increased demands on the cardiovascular system to support the growing tissues (Wideman 2001). Application of genetic analysis of Pulmonary Hypertension Syndrome (PHS) in the chicken will result in better selection processes for the poultry industry and complementary molecular data for better screening, treatment, and evaluation of the chicken as a model for IPAH in humans. Here, we present data confirming multiple chromosomal regions for linkage disequilibrium of segregation relative to ascites resistance or susceptibility in this population.

METHODS

Experimental Population: Resistant and susceptible birds from the susceptible line (Pavlidis 2007) were reared under ideal conditions of lighting, temperature, and ventilation and received *ad libitum* food unless compromised by ascites development (Wideman 2003). Birds were rescued with furosemide and/or feed restriction upon onset of PHS. All birds were evaluated for resistance or susceptibility to ascites using right ventricle to total ventricle (RV/TV) weight and electrocardiogram evaluation. Lead II ECGs were used to evaluate all birds for onset of PHS. Birds with relatively small and equal R and S wave amplitudes were considered to be clinically healthy. Birds with a small or non-existent R wave amplitude combined with a large, deeply negative S wave amplitude were considered to be pre-ascitic. Birds that developed clinical PHS/ascites were rescued by diuretic furosemide, skip-a-day feeding, and light restriction. RV/TV ratios were done post-mortem to confirm right heart hypertrophy in the ascitic birds.

Isolation of DNA: A lancet was used to puncture a wing vein. Twenty µl of blood was pipetted into 400 µl of STM (64 mM sucrose, 20 mM Tris Cl, pH 7.5, 10 mM MgCl2, and 0.5% Triton X-100) for DNA isolation by the method of Bailes 2007.

Primer Design: VNTR (variable number tandem repeat) sequences were identified using the UCSC genome browser. PCR primer pairs were designed using Primer3 (http://frodo.wi.mit.edu/) or PrimerSelect (DNAStar ver 6.0). Primer pairs were designed to satisfy the following criteria: 18-30 nucleotides in length, 40-60% GC content, 40-72° C Tm, and product length of approximately100-300 base pairs. Primer pairs were synthesized (MWG Biotech) and fluorescently labeled on the 5' end of the forward primer with Cy3, Cy5, FAM, or HEX.

Polymerase Chain Reaction: PCR was performed in 96 well plates using either an MJ Research PTC-100 thermocycler or an Eppendorf Mastercycler gradient. Reactions (20ul) contained 1X Taq Buffer (50 mM Tris-Cl (pH 8.3), 1mM $MgCl_2$, 30ug/ml BSA), 0.2 mM dNTPs, 0.4 uM each forward and reverse primers, 2.5 units Taq polymerase, and 2 µl of DNA (approx. 50 ng). PCR conditions were an initial denaturation at 90°C for 1 minute followed by 40-45 cycles of 90°C for 25 s, 30 s anneal temperature (Table 1), 72°C for 60 s, and a final extension at 72°C for 3 minutes.

Gel Electrophoresis: PCR products were resolved in 6% denaturing polyacrylamide gels (38:2 acrylamide:MBA, 50% urea) in 1xTEB (100 mM Tris, 10 mM boric acid, 2 mM EDTA, pH 9.2). PCR product (2ul) was mixed with 5ul loading buffer (95% formamide, 1X TEB, 0.02% bromophenol blue). Samples were heat denatured at 90° C for 5 minutes, quick chilled on ice, and then 1-2 µl was loaded per lane. CXR ladder (Promega Corp.) was used as size marker.

Gel Imaging: Gels were scanned with a Model 9600 Typhoon imager (GE Health Care) and images analyzed using ImageQuant software.

Statistical Methods: Genotype and allele counts were analyzed in MS Excel. Expected genotype counts were calculated according to standard Hardy-Weinberg distributions. Expected allele frequencies for a sub population were calculated based on the allele frequency observed in the entire population. For each allele, genotype, or haplotype we calculated P-values using the chi-test function to determine where observed differed from expected for either or both of the resistant and susceptible.

RESULTS

A genome wide single nucleotide polymorphism (SNP) panel was conducted in 2006 (Cheng 2006). We were able to include 183 DNAs representing F1 and F2 birds from a cross between our ascites resistant and susceptible lines (Pavlidis 2007). For our samples, 970 SNPs were uninformative while 1763 were polymorphic. We analyzed SNP frequencies with respect to low vs. high RV/TV (hypertrophy) and resistance vs. susceptibility to ascites. The data was analyzed by Chi square to detect deviation of observed from expected for both ascites and RV/TV. We then selected regions where successive SNPs deviated ($P<0.01$) for both traits with minor allele frequencies greater than 0.20. This analysis identified at least 7 regions on 4 chromosomes; 0.64-1.05 Mbp, 18.38-21.48 Mbp, and 127.01-128.3 Mbp on Gga1; 13.5-15 Mbp and 15.5-16.28 Mbp on Gga9; 2.04-2.32 Mbp on Gga27; and 47.12-48.98 Mbp on GgaZ as containing probable QTL affecting RV/TV and ascites. We designed PCR primers to analyze one or more VNTRs in each of these 7 regions for further linkage analyses.

We generated a new test population consisting of 35 resistant females, 40 susceptible females, 56 resistant males, and 54 susceptible males from our susceptible line. Six

resistant birds of unknown sex were also included. All individuals were genotyped for all 7 regions identified from the original SNP panel using 13 different primer pairs (Table 1).

The region on Gga9 from 13.5-15.0 Mbp was interrogated with three primer pairs, PHS015, PHS009, and PHS010. PHS009 and PHS015 amplified three alleles each with PHS009 amplifying one allele of 275bp that was significantly under-represented in the resistant and over-represented in the susceptible sub-population (35.0 v.s. 24.5%, P=0.055) (Table 2). PHS010 amplified two alleles at approximately the same frequencies between the two sub-populations with the 161 bp allele being the most prevalent over all. Discrimination approaching significance for this VNTR was present at the genotypic level (Table 3) with the 151/151 homozygote being most associated with susceptibility (8.4 v.s. 4.7%, P<0.03). The 161/151 genotype was nearly equally distributed, as was the 160/160. Owing to the low number of alleles and the overabundance of allele 161 at locus PHS010, we combined the data for the three markers in this region into haplotypes combining PHS015 with PHS009 and PHS009 with PHS010. We combined PHS015 with PHS009 and generated haplotypes for 49 resistant 61 susceptible animals. There was no significant difference between the sub-populations for PHS015+PHS009. We were able to combine PHS009 and PHS010 for probable haplotypes for 68 resistant and 76 susceptible animals. This combination yielded one significant haplotype and one haplotype that approached significance. The 277-151 haplotype approaches significance and is over-represented in the susceptible sub-population and under-represented in the resistant sub-population (7.9 v.s. 2.9%, P=0.075). The 275-161 haplotype is over-represented in the resistant and under-represented in the susceptible sub-populations (38.2 v.s. 23.7%, P<0.026). The combination of all three markers PHS009+PHS015+PHS010 did not yield any haplotypes

that discriminated the sub-populations. However, the combination of PHS009 and PHS010 is sufficient to differentiate our sub-populations.

The region on Gga9 from 15.5-16.28 Mbp was evaluated with two primer pairs, PHS152 and PHS001. PHS152 amplified two alleles of 182 and 198 bp that did not differ significantly from expected frequencies for either the resistant or susceptible populations (Table 2). However, the 198/198 genotype was over-represented in the resistants (29 v.s. 17%; $P<0.04$). PHS001 amplified 5 alleles between 139-157 bp approximating expected allele frequencies. The 155/155 genotype was observed nearly twice as often in the susceptible birds as in the resistant (21 v.s. 12%; $P<0.04$). Conversely, the 147/139 genotype was observed more than twice as often in the resistant sub-population (21.1 v.s. 10.4%; $P<0.005$). We could combine these two markers for 64 resistant and 66 susceptible animals. For PHS152-PHS001, the largest difference between resistant and susceptible haplotypes was 182-155 (19.5 v.s. 30.3%) which approaches significance ($P=0.082$). Thus, the use of two VNTRs did not allow us to identify haplotypes related to ascites in this region. Analysis with additional markers or for more DNA samples could provide significance for this region on Gga9.

The region on Gga1 between 0.5 Mbp and 2.1 Mbp was interrogated with PHS150 and PHS135. PHS150 amplified 3 alleles between 175-195 bp. The 175 allele was a rare allele present only in the resistant sub-population. The 195 bp allele was the major allele at 80.72% of the population. The 179 bp allele was present at 18% and was also equally represented in the resistant and susceptible sub-populations. All genotypes were represented equally between the sub-populations. PHS135 amplified 4 alleles between 220-256 bp. The 228 and 224 bp alleles were the most frequent with the 228 allele being

slightly over-represented in the susceptible sub-population. The 256, 224, and 220 bp alleles were all represented at equal frequencies in both populations. There were no genotypes that showed a significant difference between the resistant and susceptible sub-populations. Unambiguous haplotypes could be assigned for 73 resistant and 89 susceptible individuals. No haplotypes showed any significant difference between resistant and susceptible sub-populations.

The region on Gga1 from 18.38-21.48 Mbp was evaluated with PHS118 and PHS126. PHS118 amplified 6 alleles with the 242 bp allele being the most prevalent (33%). The 262 bp allele was present only in the resistant sub-population. Genotypic analysis showed the 274/242 heterozygote approached significant over-representation in the susceptible sub-population (6.7 vs. 15.2%, $P=0.06$) and the 246/246 and 242/242 homozygotes were over-represented in the resistant sub-populations ($P<0.03$, 0.04, respectively). PHS126 had no significant deviation in allele frequency between the sub-populations, but did have an over-representation of the 259/244 heterozygote in the susceptible sub-population (4.5 vs. 14%, $P<0.05$). We combined the markers for haplotype analysis for 52 resistant and 52 susceptible animals. The 242-244 haplotype was significantly over-represented in the susceptible sub-population (13.5 vs. 2.9%, $P<0.02$). We are therefore able to assign a haplotype in this region associated with susceptibility to ascites.

The region on Gga1 from 127.01 to 128.31 Mbp was interrogated with PHS144 which amplified 9 alleles from 248 to 323 bp with similar frequencies for the resistant and susceptible sub-populations (Table 2). The 303/263, 263/263, and 303/283 genotypes were over-represented in the susceptible sub-population ($P<0.015$, 0.00005, 0.05, respectively) (Table 3). The 288/283 heterozygote was over-represented in both sub-populations

compared to the expected counts (P<0.02). The 323/283 heterozygote was under-represented in both sub-populations with respect to expected frequencies (P<0.028).

The region on Gga27 between 1.4-3.1 Mbp was interrogated with two primer pairs, PHS051 and PHS094. PHS051 amplified two alleles of 145 and 151 bp with no significant deviation in either allele or genotype frequencies although the heterozygote 151/145 approached significance (P=0.0847) (Table 2). PHS094 amplified three alleles between 180-192 bp at expected frequencies. The 186/186 homozygote was over-represented in the susceptible sub-population (20 v.s. 13%; P<.007) as was the 192/192 homozygote (7 v.s. 3%; P<.054) (Table 3). The 186/180 heterozygote was over-represented in both the resistant and susceptible sub-populations but was significantly more frequent in the resistant sub-population (56 v.s. 43%; P<0.000001). The 180/180 heterozygote was under-represented in both sub-populations (P≤0.002). Because of the low diversity at both loci, we combined the markers for haplotype analysis. We could unambiguously assign haplotypes for 92 individuals (49 resistant, 43 susceptible) (Table 4). The PHS051-PHS094 haplotype 151-192 was nearly 3x more frequent in the susceptible when compared to the resistant population (15.1 v.s. 5.1%, P<0.04).

The region on GgaZ from 47.12 to 48.98 Mbp was evaluated with PHS091 which amplified 3 alleles from 275 to 295 bp (Table 2). The 295 and 282 bp alleles represented over 95% of the population. The female chicken is heterogametic and so hemizygous for GgaZ (Table 3). The 282 allele is significantly over-represented in the female susceptible sub-population as compared to the resistant (9 v.s. 91%, p<0.03). We were unable to develop another polymorphic marker in this region. Therefore, no haplotype analysis could be performed.

DISCUSSION

VNTR analysis of the 7 regions identified by the whole genome SNP analysis identified 5 regions of significant association with respect to ascites/PHS in the chicken at the allele, genotype, and/or haplotype level of analysis. Low numbers of alleles and/or low allele diversity at many of our VNTR loci and the low numbers of some genotypes required that we genotype for more than one VNTR per region. Combining genotypes into haplotypes proved informative for some of these regions. There is a notable absence of single allele markers for ascites in the chicken which is expected in light of the complex pathology of the disease and the involvement of many QTLs. None of our VNTR markers has the power to detect significant association with susceptibility at all levels of analysis. PHS009, PHS010, PHS150, and PHS118 were significant at either the allele or genotype level when used for analysis independently but were far more informative when combined into respective haplotypes. There were statistically significant but rare genotypes for PHS144. Therefore, the use of any of our VNTR markers alone proved insufficient for informative analysis of our populations. Our results demonstrate that multiple VNTRs can be required to discern QTL regions. Indeed, the analysis of the 15 Mbp region of Gga9 with PHS001 and PHS152 only approaches significance. However, we believe this region is involved in ascites/PHS due to the presence of a gene for a serotonin receptor, 5HT2B. Serotonin is a key mediator of susceptibility to ascites/PHS based on i.v. microparticle injection method (Wideman 2006). Over-expression of 5HT2B has been shown to lead to hypertrophic cardiomyopathy in mice. In humans, high levels of serotonin and increased uptake of serotonin in vascular tissue of patients with active PAH implicate serotonin transporters as contributors to vascular remodeling and hypertrophy (Eddahibi, 2001, MacLean 2009). A

polymorphism in the 5' translated region of the human serotonin receptor 5HT2B has been shown to result in increased expression and appears to be linked to an increase in hypoxemia, structural lung changes, inflammation, and a decrease in compensatory response to pulmonary challenge (Bianchi 2005 and Eddahibi 2003). The presence of genotypic evidence associating this region to resistance or susceptibility to ascites and the haplotype data that approaches significance warrants further investigation of this region.

The region investigated on GgaZ while insufficient for sub-population analysis at this time, remains of interest in that females appear to be physiologically and anatomically predisposed to early development of ascites symptoms (N. Anthony, unpublished results). This information, combined with our preliminary evidence of a significant allele at this locus, will drive future investigation of GgaZ.

The regions on Gga9:13, Gga27:2, and Gga1:20, contain genes related to the angiotensin signaling pathway. On Gga9, PHS009, PHS010, and PHS015 are 40KB upstream of AGTR1, an Angiotensin II type 1 receptor. In humans, a SNP in the 3'-UTR of AGTR1 disrupts a miR155 target site. This SNP is associated with early onset of PAH in humans (Chung 2009 and Martin 2006). The chicken gene for angiotensin-converting enzyme (ACE) is on Gga27 at 2.58 Mbp. Polymorphisms of ACE are associated with development of severe IPAH in humans (Abraham 2003). MAPK11, on Gga1 at 21.81 Mbp, also known as p38MAPK, is implicated as a downstream effector of inflammatory pathways from AGTR1/angiotensin II activation. There is evidence that cross talk between p38MAPK and c-Jun N-terminal kinases leads to myocardial cell growth and hypertrophy through the integrin-FAK-Src-Ras pathway (Clerk 2006). Additionally, p38MAPK induces

iNOS production, a known vasoactive chemical messenger (Mehta 2007, LaPointe 1998; Aikawa 2001, Clerk 2006).

In summary, we have shown 4 regions on three chromosomes in the chicken that are linked to the development of PHS/ascites. The role of serotonin and serotonin receptors in pulmonary disease is well established and well characterized pharmacologically and histologically. We provide genetic evidence that the serotonin transporter 5HT2B is involved in the pathology of PHS/ascites in the chicken. The emerging model for angiotensin II receptor 1 as a polymorphic biomarker in humans for early onset IPAH is supported by our finding that the regions containing the angiotensin receptor AGTR1 and the angiotensin-converting enzyme ACE are linked to the development of PHS/ascites in the chicken. Our evidence suggests that the chicken could provide an excellent model for human PAH research on disease development and analysis of the underlying genetics of PAH. In addition, identification of these regions as contributing to ascites susceptibility will provide biomarkers for use in the poultry industry for selection strategies to reduce the incidence of ascites.

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Primer Name	Locus	Sequence (5'-3')	VNTR	# Cycles	Annealing Temperature	# Alleles	Size Range (bp)
PHS150	Gga1:0.895	ATCTGTAACCAGAGCACATA CTTTTAGGACTTCCTTCTTG	$(AAAC)_8$	43	43	3	175-195
PHS135	Gga1:0.991	CCCCAAGAGGACTCAAAGAAACA AACCGCAATGAACAGGGACACC	$(TCCA)_9$	43	55	4	220-256
PHS118	Gga1:20.28	TGCTCAATCTAGTGGGTGATTT CCCAAGAAGTGGTGGAAAAG	$(CTTT)_{32}$	43	50	6	242-274
PHS126	Gga1:20.56	TCACGTGGGGAATTAACACA AAGATGGAGTTCCCAAGCAG	$(GGATA)_{20}$	45	50	4	239-259
PHS144	Gga1:127.85	CCGCTCCCTGTTTATCCTTA TCCATTTATTGGGGAGTGAAA	$(TTCTC)_{20}$	45	50	9	248-323
PHS015	Gga9:13.111	TTTTTCTTGTGTATATGGCTAAT GCAATGCAAAAATGTGAAA	$(GA)_{13}$	43	45	3	151-167
PHS009	Gga9:13.42	ATTGTTCATAGCACTTCATTTCT AGCAGTCATAAGCCTACATTTTG	$(GA)_{13}$	40	52	3	267-277
PHS010	Gga9:13.54	ACAGAATTAGGGTGGGTTTTTG GCGGTGTGCCTGCTTTCT	$(AC)_{16}$	43	50	2	161,151
PHS152	Gga9:15.882	GTGACATTCATACTGGAAAC GCTATCTTGTTTGCTAATCT	$(AAAG)_{12}$	45	43	2	198,182
PHS001	Gga9:15.96	GAGGCTCCATCGCTATTGA AGTTTTTGTCTTCCCAGTCTCC	$(CA)_{17}$	43	50	5	139-157
PHS051	Gga27:2.285	CATCCCTGAGCCCACCCCAACTG CCACGTGCCCGGAGGAGGAAATAA	$(CA)_{25}$	43	60	2	145,151
PHS094	Gga27:2.860	GAACACAGTGAGGCACGAGA GTGTGCTGTGCTCTGATGCT	$(CA)_{17}$	43	45	3	180-192
PHS091	GgaZ:48.61	TGCATACAAGCTCATACAAATCTG AAAAATCCACTTCCCAAATCTCT	$(GAAA)_{25}$	43	50	3	270-295

Table 1. All PHS project primer pairs listed with location, sequence (Forward over Reverse), VNTR, number of PCR cycles, optimized annealing temperature (°C), number of alleles amplified, and the size range of those alleles (bp).

Primer	Allele (bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value	Primer	Allele (bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS015	167	17(16)	16(17)	0.730	PHS126	259	24(27)	42(39)	0.230
	161	101(104)	113(110)	0.699		249	75(72)	103(106)	0.640
	151	62(60)	62(64)	0.741		244	36(34)	47(49)	0.099
PHS009	277	88(94)	111(105)	0.374		239	1(3)	6(4)	0.822
	275*	63(53)	49(59)	0.055	PHS051	151	59(61)	53(51)	0.681
	267	29(33)	40(36)	0.374		145	83(81)	65(67)	0.720
PHS010	161	142(137)	147(152)	0.581	PHS094	192	28(33)	42(37)	0.189
	151	30(35)	43(38)	0.272		186	89(91)	102(100)	0.729
PHS152	198	85(79)	82(88)	0.375		180	61(53)	50(58)	0.134
	182	93(99)	115(109)	0.426	PHS144	323	9(7)	5(7)	0.207
PHS001	157	10(12)	14(12)	0.510		313	11(12)	15(14)	0.599
	155	60(66)	76(70)	0.319		303	29(32)	38(35)	0.494
	153	13(16)	20(17)	0.301		293	20(20)	22(22)	0.983
	147	54(50)	50(54)	0.471		288	11(15)	20(16)	0.182
	139	43(36)	32(39)	0.121		283	30(24)	21(27)	0.104
PHS135	256	3(2)	2(3)	0.571		278	28(26)	26(28)	0.518
	228	104(111)	130(123)	0.375		263	26(29)	36(33)	0.384
	224	64(71)	64(71)	0.627		248	4(3)	3(4)	0.608
	220	2(2)	2(2)	0.256			**Female**	**Female**	
PHS150	195	134(134)	154(154)	0.972	PHS091	295	18(16)	22(27)	0.295
	179	28(30)	36(34)	0.644		*282	1(3)	10(5)	0.016
	*175	4(2)	0(2)	0.032		275	0(0)	0(0)	0.534
PHS118	274	40(38)	40(42)	0.637					
	270	26(26)	28(28)	0.909			**Male**	**Male**	
	266	19(20)	24(23)	0.676		295	85(86)	84(81)	0.742
	*262	5(2)	0(3)	0.018		282	17(17)	14(16)	0.623
	246	34(34)	37(37)	0.930		275	2(1)	0(1)	0.106
	242	56(60)	71(67)	0.460					

Table 2. Allele count for Resistant (Res) vs Susceptible (Sus) birds for each locus where observed (Obs) versus expected (Exp) significantly deviated (P=0.055) are indicated (*).

Locus	Genotype	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS010	151/151	4(3)	8(4)	0.033
PHS152	198/198	16(18)	17(19)	0.039
PHS001	155/155	11(12)	20(13)	0.043
	147/139	19(10)	10(11)	0.005
PHS118	266/262	2(0)	0(0)	0.001
	246/246	7(3)	5(3)	0.020
	242/242	17(10)	12/11	0.027
PHS094	192/192	3(3)	7(3)	0.054
	186/186	12(23)	19(26)	0.007
	186/180	50(27)	42(30)	0.000
	180/180	2(8)	1(9)	0.001
PHS144	263/263	3(2)	11(3)	0.000
	303/283	3(5)	8(4)	0.042
	288/283	5(2)	4(2)	0.014
PHS135	224/220	0(1)	2(1)	0.041
PHS091 (Female)	282	1(3)	10(5)	0.016

Table 3. Genotypes in the Resistant (Res) and Susceptible birds (Sus) where observed (Obs) versus expected (Exp) significantly deviated (represented >1% of the total population and $P \leq 0.055$).

Haplotype	%Resistant	%Susceptible	P-value	Haplotype	%Resistant	%Susceptibl	P-value
PHS015+PHS009				**PHS118+126**			
151-277	9.2	13.1	0.390	242-239	1.0	1.0	1.000
167-277	3.1	1.6	0.487	*242-244	2.9	13.5	0.008
161-275	42.9	48.4	0.549	242-249	21.2	16.3	0.423
167-275	2.0	0.0	0.115	242-259	15.4	8.7	0.162
161-275	11.2	7.4	0.347	246-244	2.9	1.9	0.655
151-275	18.4	12.3	0.248	246-249	14.4	14.4	1.000
151-267	1.0	4.1	0.169	246-259	1.9	1.9	1.000
167-267	0.0	2.5	0.121	266-244	2.9	4.8	0.480
161-267	12.2	10.7	0.728	266-249	5.8	8.7	0.439
PHS009+PHS010				266-249	1.0	0.0	0.317
277-161	47.1	52.0	0.555	266-259	0.0	1.0	0.317
277-151	2.9	7.9	0.075	266-259	1.0	0.0	0.317
*275-161	38.2	23.7	0.026	270-244	1.0	1.9	0.564
275-151	2.2	3.3	0.582	270-249	2.9	7.7	0.132
267-161	3.7	7.9	0.141	270-259	1.9	0.0	0.157
267-151	5.9	5.3	0.824	274-239	0.0	1.9	0.157
PHS051+094				274-244	4.8	2.9	0.480
*151-192	5.1	15.1	0.030	274-249	17.3	12.5	0.369
145-186	30.6	33.7	0.710	274-259	1.9	1.0	0.564
151-186	21.4	22.1	0.923				
145-192	12.2	10.5	0.721				
151-180	12.2	7.0	0.254	**PHS152+PHS001**			
145-180	18.4	11.6	0.242	182-139	10.9	8.3	0.498
PHS150+PHS135				182-147	14.8	15.2	0.949
195-228	50.7	55.1	0.591	182-153	4.7	6.1	0.633
195-224	32.9	28.1	0.436	182-155	19.5	30.3	0.082
179-256	0.7	0.6	0.888	182-157	3.1	5.3	0.393
179-228	11.6	12.4	0.853	198-139	10.2	6.1	0.245
179-224	2.1	3.4	0.479	198-147	14.1	11.4	0.541
179-220	0.7	0.6	0.888	198-153	3.9	2.3	0.453
175-228	0.7	0.0	0.270	198-155	18.0	13.6	0.379
175-224	0.7	0.0	0.270	198-157	0.8	1.5	0.582

Table 4. Percentages for haplotypes where two or more VNTR markers could be combined ($P \leq 0.05$). The percentage of the sub-populations possessing a haplotype (% Resistant or % Susceptible) was compared using a Chi test (P-value).

Chapter 3: Genetic Analysis of Chromosomal Regions Associated with Ascites in the Chicken: Candidate Genes

In 2006, a genome wide SNP analysis of chickens was conducted by a consortium of researchers that analyzed SNPs at approximately 10KB spacing for a total of 2733 SNPs of which 1763 were informative (Cheng 2006). The association of SNPs with right ventricle/ whole ventricle weight and resistance or susceptibility to ascites was evaluated in the population of birds. From those results, we identified 7 chromosomal regions on 4 chromosomes (Figure 1) that were associated with right ventricle/whole ventricle weight and ascites and designed primers to amplify proximal VNTR (Table 1). We used these markers to evaluate 2 sub-populations (resistant and susceptible to PAH) from a susceptible line of chickens. We found loci significantly associated with PAH at an allele, genotype, or haplotype level of analysis. We subsequently interrogated the regions identified by our markers for genes that could be relevant to ascites and PAH.

We analyzed our data in a hierarchal order from allele to genotype to combined haplotype level. At the allele level, we found several loci that showed significant association to susceptibility to PAH. However, the majority of our VNTR markers displayed low allele diversity, typically having 3-5 alleles with 2 major alleles and other alleles present at very low frequency. We subsequently combined alleles for genotypes and analyzed the sub-populations for genotype and probable haplotype. We found several genotypes at multiple loci that appear to be associated with resistance and susceptibility to PAH (Tables 2-8). The genotype analysis yielded adequate results for breeding experiments to commence, but the combination of genotypes into probable haplotypes proved much more informative (Tables 9-13).

A review of the genes located within the regions we have demonstrated to be associated with PAH in the chicken identified 10 candidate genes for possibly mediating the QTL effects.

When we combined genotypes for 3 markers between 13.1-13.5 Mbp, we identified 2 haplotypes significantly associated with either resistance or susceptibility to ascites. These haplotypes combined represent 36% of the total population for which haplotypes could be assigned. This locus, 13.1-13.9 Mbp, contains the gene for angiotensin II type 1 receptor (AGTR1). Based on current models for angiotensin's role in vascular tension, we are confident that this gene is an excellent candidate for sequence analysis to identify polymorphisms associated with variation in pulmonary response between our resistant and susceptible sub-populations. Angiotensin II-type receptor 1(AGTR1) is located at 13.49 Mbp on chromosome 9 in the chicken (Gga9). Recently published work indicates that a polymorphism in the 3'-untranslated region in the *AGTR1* gene in humans is associated with early onset IPAH (Chung 2009). This 3' UTR SNP may alter the target sequence for a miRNA which would imply a dysregulation of AGTR1 expression. The vasoconstrictor angiotensin is regulated in humans by angiotensin-converting enzyme (ACE) which is required to cleave the precursor angiotensinogen into angiotensins I and II. The chicken ACE homolog is found on Chromosome 27 at 2.58Mbp. Only angiotensin II is known to be biologically active (Chung 2009, Campbell 1987). Activation of AGTR1 mediates an up-regulation of a mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway in vascular smooth muscle cells that is highly indicated in ventricular hypertrophy (Kagiyama 2002). Furthermore, when we combined two markers at the locus on Gga27 at 2.2-2.9 Mbp, we found a significant haplotype associated with susceptibility to

PAH. The gene for angiotensin converting enzyme (ACE) is located at Gga27:2.58 Mbp in the chicken. This gene is responsible for converting angiotensinogen into biologically active angiotensin II. The relative interaction between these two loci, one on Gga9 and one on Gga27 is an obvious nod to the angiotensin biochemical pathway in vascular response to pressure changes, and one that has clear implications in PAH.

Integrin beta-3 (ITGB3) is located on Gga27 at 2.2Mbp. Thrombocytes are involved in the serotonin response to elevated pulmonary arterial pressure. Integrins are necessary for platelets (the thrombocyte homolog) to bind fibrinogen and function properly (Di Castelnuovo 2005). Therefore the ITGB3 gene remains on the list of candidate genes for PAH.

HRAS is located on Gga9 of the chicken at 14.25Mbp. Ras is a well established GTPase switch that is activated by myriad extracellular stimuli and consequently activates the Raf-MEK-ERK kinase pathway (Shields 2000). The activation of Ras and its subsequent downstream effects are still not well characterized, but its role as an activator of intracellular pathways is well known. The activation of the ERK pathway and subsequent MAPKs and the possible implications where inflammation and tissue proliferation are concerned should not be ignored in light of the important role that inflammation, the immune system, and MAP kinases play in the response to respiratory demands put on chickens in pulmonary challenge, and potentially, in humans living with IPAH.

Costello syndrome, a rare condition that is hallmarked by cardiac hypertrophy and other abnormalities, was addressed by a consortium of investigators in 2005 with the intention of finding genotypic evidence of genes linked to the particular symptoms or phenotypes of the syndrome (Gripp 2005). Their results found that germ line HRAS mutations lead to

Costello syndrome in most patients. Ras serves as a highly regulated GTPase switch that can be turned on by a highly varied number of signals. Costello syndrome and IPAH/PHS are highly related in physiological implications, and the validated involvement of HRAS mutations represents an ideal candidate gene possibility for further investigation.

The third locus that yielded significant haplotype association with PAH was located on Gga1:20.2-22.5 Mbp. This locus yielded one haplotype significantly associated with susceptibility which was present in 13% of the susceptible sub-population. One megabase upstream of the two markers combined for haplotype is the gene for mitogen activated protein kinase 11 (MAPK11), also known as p38MAPK. p38MAPK has been used as a target for pharmacological intervention in hypertension patients for some time, and has been implicated for involvement in hypertension in rats possessing divergent expression levels in hypertensive versus normotensive animals (Ravingerova 2003, Buermans 2005).

MAPK11, also known as p38MAPK, is located on Gga1 at 21.8 Mbp. p38MAPK is implicated as a downstream effector from AGTR1/angiotensin II activation that leads to myocardial cell growth and hypertrophy through the integrin-FAK-Src-Ras pathway (Mehta 2007, LaPointe 1998). The primary target of p38MAPK activation if MAPKMAPK-2, which is a protein kinase that subsequently activates a heat shock protein, promoting polymerization of actin filaments (Ranvingerova 2003). p38MAPK has been indicated as a target of many inflammatory stimuli indicating an essential role in cell inflammatory responses (Raingeaud 1995, Han 1997). Importantly, p38MAPK has also been shown to regulate iNOS expression at the transcriptional level (Da Silva 1997). These various levels of vasoactive regulation that have been shown to be dependent upon p38MAPK implicate it as a candidate gene for involvement in the progression of PAH.

There was a fourth region that approached significance when analyzed for haplotype. This region is at 15.8 Mbp on Gga9 and is directly downstream from the gene for serotonin receptor 2B (5HT2B). The combination of PHS152 and PHS001 provide a haplotype that is associated with susceptibility and present in 30% of the susceptible sub-population. We are currently developing more markers for this region due to the very clear involvement of serotonin in the response to PAH. Serotonin is a well characterized vasoconstrictor with an established role in pathologies of the cardiopulmonary system in both chickens and humans.

Serotonin receptor 5HT2B is located at 16.2Mbp on Gga9. Serotonin (5HT) is arguably the most potent vasoconstrictor known in poultry and is actively carried by thrombocytes which are the avian equivalent to human platelets (Kunicki 1985, Chapman 2002). If administered intravenously, 5HT is capable of causing extensive pulmonary arterial constriction leading to suffocation if careful administration isn't observed. A study of IPAH and FPAH patients and serotonin transporter [5HT2B] divergent alleles found a correlation between age of diagnosis of PAH and a polymorphism in 5HT2B (Willers 2005). In a related study, it was found that the presence of any amine transporter in combination with serotonin-specific receptors was sufficient to induce hypertrophy independently of serotonin concentrations (Bianchi 2005). While either mechanism, receptor independent or dependent can lead to hypertrophy, there is a cumulative effect noted with an abundance of both amine transporters *and* 5HT2B receptor leading to perceptibly greater hypertrophy. In short, much weight is placed upon the serotonin transporter in PAH development, with less emphasis put upon the plasma serotonin concentrations when determining susceptibility to PAH. The presence of both 5HT and

5HT2B leads to the downstream activation of mitogen activated protein kinase (MAPK) driving the transcription of genes highly implicated in cell proliferation (Machado 2006). Serotonin has also been shown to interact synergistically with Urotensin II leading to the induction of proliferation via a G-protein coupled receptor/MAPK pathway leading to vascular remodeling (Watanabe 2001).

Somatostatin (SST) is located on Gga9 at 15.9Mbp. Somatostatin is a pleiotropic chemical signal that acts through a set of 6 G-protein coupled receptors known as SSTR1-SSTR5 to carry out many biological functions that may be summarized under two basic functions: inhibition of secretion or inhibition of proliferation. In some secreting cells, somatostatin is released by increasing cytosolic Ca2+ concentration and/or membrane depolarization. In a feedback loop, SST serves as an inhibitor of cAMP and Ca2+ release upon ligand binding. Free Ca2+ has been shown to be elevated in hypertensive rats and humans (Bruschi 1985, Zhang 2005). Somatostatin is also indicated in the inhibition of proliferation in several cell types by MAPK path regulation. SST release can also be stimulated by a variety of cytokines, growth factors, corticotrophin-releasing hormone and the inflammatory cytokines IL-1, TNFα, and IL-6 (Bronstein-Sitton 2006). Immunological response to respiratory challenge could indicate that the signal by inflammatory cytokines to release somatostatin is an important upstream regulator of PAH. Inhibition of MAPK signaled smooth muscle proliferation ties SST directly into PAH pathogenesis.

Fibroblast growth factor-12 (FGF12) is located on Gga9 at 14.52Mbp. It has been established that inhibition of FGF by FGF-specific antibody can inhibit the proliferation of smooth muscle cell after vascular injury and that nitric oxide (NO), in a primary response to vascular injury, amplifies the FGF stimulated proliferation of smooth muscle cells

(Lindner 1991, Janssens 1998). Nitric oxide is thought to be one of the most important chemical messengers and vasodilators involved in pulmonary hypertension syndrome in chickens. It is involved in many processes ranging from cardiovascular to immune in nature and has been established as a potential human QTL in echocardiographic [hypertensive] phenotypes (Kraja 2008). NO is synthesized by a breakdown of l-arginine in an enzymatic process carried out by an inducible nitric oxide synthase (iNOS) or a constitutive endothelial nitric oxide synthase (eNOS) (Wideman 2006). iNOS binds Ca2+/ Calmodulin to produce massive amounts of nitric oxide, a common chemical response to pulmonary occlusion or elevated pulmonary pressure (Wideman et al 2004). Taken together, these facts support a nitric oxide mediated response to vascular challenge that results in an effect upon the various growth factors, including FGF12, in the vascular system. Fibroblast Growth Factor 12 is a well established growth factor responsible for the induction of granulation tissues and concordant fibrosis, collagen production, and angiogenesis (Roberts 1986). The pathobiology of PAH requires the involvement of growth factors and angiogenesis appears to be a consistent response to occlusion of the pulmonary vasculature as an effort to bypass the blockage. It is therefore logical that FGF12 could play a role as a QTL and serve as a marker for genetic selection for breeding purposes.

When we combined the genotypes for PHA150 and PHS135, located between 0.895 and 0.991 Mbp on Gga1, we got no significant association with PAH. However, the addition of markers in this region may show an association not clearly defined with only two primer pairs. In this region are two potential candidate genes; calmodulin-3 (CALM-3) and neuroendothelial transforming gene (NET1).

Calmodulin-3 (CALM-3) is located on Gga1 at 1.0Mbp and may be highly implicated in the nitric oxide synthesis pathway. Two variations on the theme of nitric oxide synthesis may play a role in the response of the pulmonary vascular system to respiratory challenge or pressure changes. There are currently two accepted forms of nitric oxide synthase, eNOS (the constitutively expressed endothelial nitric oxide synthase) and the macrophage carried inducible iNOS. eNOS binds Ca2+ in a readily reversible way and so exhibits a more transient activation whereas iNOS binds Ca2+/Calmodulin tightly even at very low Ca2+ concentrations (Korhonen 2005). iNOS is highly regulated by a calcium/calmodulin-dependent protein kinase II in vascular smooth muscle; based on the role that iNOS plays in PAH and as the highly important upstream regulator of chemical signals for nitric oxide, it is reasonable to include CALM-3 on the list of potential candidate genes involved in PAH.

Neuroendothelial transforming gene-1 (Net1) is located on Gga1 at 1.01Mbp. NET1 is a guanine exchange factor for the small G protein RhoA. RhoA is recognized as a major regulator of arterial tone, smooth muscle contraction and proliferation, and actin stress fiber formation (Sauzeau 2001). Rho family proteins are key regulators of cytoskeletal organization and are often involved in oncogenic transformation; importantly, the Rho GTPases comprise a main branch of the Ras superfamily of biomolecular switches involved in the activation of many downstream effectors including relative vasoactive substances such as urotensin and somatostatin. Translocation of NET1 to the cytoplasm of a cell results in the formation of actin stress fibers and cellular transformation (Qin 2005). NET-1 has also been shown to be an important oncogene and guanine exchange factor (GEF) involved in the activation of RhoA leading to increased proliferation,

reorganization, and migration of epithelial cells (Qin 2005, Murray 2008). The increased expression of NET-1 therefore may be involved in the increase of active RhoA. Increased activity of other biomolecular switches like Rho GTPases would actively propagate signals by interacting with downstream effector proteins. A hallmark of all forms of PAH remodeling is the distal extension of smooth muscle into small peripheral arteries (Humbert 2004) which could involve NET 1 and RhoA cytoskeleton remodeling.

GgaZ was interrogated for a single VNTR. PHS091 was used to analyze the resistant and susceptible sub-populations. The female chicken is heterogametic and possesses only one copy of the Z chromosome. As such, we identified a hemizygous genotype in the female sub-population associated with susceptibility to ascites. Further investigation with additional markers may identify ascites as a sex-linked disease. There are currently no candidate genes for ascites on GgaZ.

The region Gga1:127 Mbp was interrogated with PHS144. This primer pair was polymorphic but non-significant when analyzed for association with susceptibility or resistance to ascites. The gene for a Rho-GTPase is very proximal to our PHS144 and with additional markers, may be considered as a candidate gene for PAH/ascites.

Studies to characterize genes involved in hypertension in humans have yielded complex gene systems, thought to be a result of redundant biochemical pathways contributing to the regulation of blood pressure (Bull 2007). These studies can be resolved when complex interaction between the renin-angiotensin system, nitric oxide dependent and independent regulation of the vascular system, and sodium balance systems are considered. Quantitative traits often have redundant biochemical pathways contributing to an overall characteristic. In the case of PAH, multiple pathways for the regulation of vascular tone and response to

pulmonary challenge exist and interact with one another. In this work, we find four potential candidate genes for involvement with PAH, all directly involved in vasotension regulation and/or hypertrophy of cardiac tissue.

After evaluating each locus independently and with respect to proximal markers, we find AGTR1, ACE, 5HT2B, and p38MAPK are attractive candidates as QTL for PAH/ascites. All of these genes have been investigated in human medicine. AGTR1 and 5HT2B have polymorphism studies linking age of diagnosis of PAH and a divergent genotype profile (Chung 2009, Willers 2005). Functional polymorphisms somewhere between intron 18 and the 3'UTR as well as one in the 5'UTR have been indicated as potentially interacting with other risk factors for PAH (Sayed-Tabatabaei 2006). p38MAPK has been well studied as a potential therapeutic target for PAH patients (Shirai 2007, MacLean 2009).

Our work is very easily applicable to agriculture where marker-assisted selection of breeding stocks is an attractive prospect in light of the economical impact of the ability to impose selection upon animals before expending resources rearing them. In human medicine, biomarkers for PAH may lead to better characterization of the biochemical pathways involved in the disease, potentially point the way for pharmaceutical intervention, and provide earlier detection through genetic screens. Our identification of significantly associated genes already implicated in human IPAH in a population of ascites/ PAH-susceptible chickens provides evidence that the chicken is an excellent animal model for IPAH research. The application of VNTR genetic mapping may pave the way for a better chicken as both food and medical model.

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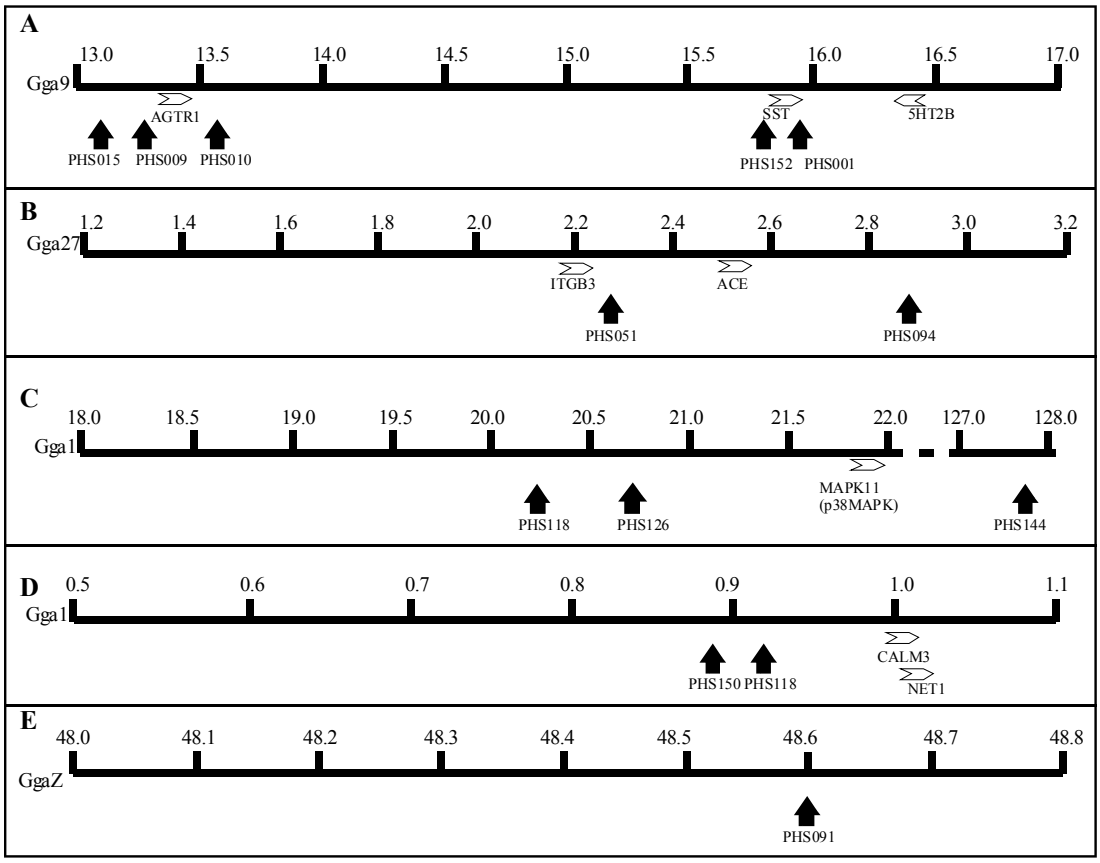

Figure 1. Schematic representation of chicken chromosomes (Gga9, 1, 27, and Z) with VNTR primer pairs (↑) and candidate genes (↔) noted at approximate locations (Mbp).

Primer Name	Locus	Sequence (5'-3')	VNTR	# Cycles	Annealing Temperature	# Alleles	Size Range (bp)
PHS150	Gga1:0.895	ATCTGTAACCAGAGCACATA CTTTTAGGACTTCCTTCTTG	$(AAAC)_8$	43	43	3	175-195
PHS135	Gga1:0.991	CCCCAAGAGGACTCAAAGAAACA AACCGCAATGAACAGGGACACC	$(TCCA)_9$	43	55	4	220-256
PHS118	Gga1:20.28	TGCTCAATCTAGTGGGTGATTT CCCAAGAAGTGGTGGAAAAG	$(CTTT)_{32}$	43	50	6	242-274
PHS126	Gga1:20.56	TCACGTGGGGAATTAACACA AAGATGGAGTTCCCAAGCAG	$(GGATA)_{20}$	45	50	4	239-259
PHS144	Gga1:127.85	CCGCTCCCTGTTTATCCTTA TCCATTTATTGGGGAGTGAAA	$(TTCTC)_{20}$	45	50	9	248-323
PHS015	Gga9:13.111	TTTTTCTTGTGTATATGGCTAAT GCAATGCAAAAATGTGAAA	$(GA)_{13}$	43	45	3	151-167
PHS009	Gga9:13.42	ATTGTTCATAGCACTTCATTTCT AGCAGTCATAAGCCTACATTTTG	$(GA)_{13}$	40	52	3	267-277
PHS010	Gga9:13.54	ACAGAATTAGGGTGGGTTTTTG GCGGTGTGCCTGCTTTCT	$(AC)_{16}$	43	50	2	161,151
PHS152	Gga9:15.882	GTGACATTCATACTGGAAAC GCTATCTTGTTTGCTAATCT	$(AAAG)_{12}$	45	43	2	198,182
PHS001	Gga9:15.96	GAGGCTCCATCGCTATTGA AGTTTTTGTCTTCCCAGTCTCC	$(CA)_{17}$	43	50	5	139-157
PHS051	Gga27:2.285	CATCCCTGAGCCCACCCCAACTG CCACGTGCCCGGAGGAGGAAATAA	$(CA)_{25}$	43	60	2	145,151
PHS094	Gga27:2.860	GAACACAGTGAGGCACGAGA GTGTGCTGTGCTCTGATGCT	$(CA)_{17}$	43	45	3	180-192
PHS091	GgaZ:48.61	TGCATACAAGCTCATACAAATCTG AAAAATCCACTTCCCAAATCTCT	$(GAAA)_{25}$	43	50	3	270-295

Table 1. All PHS project primer pairs listed with location, sequence (Forward over Reverse), VNTR, number of PCR cycles, optimized annealing temperature (°C), number of alleles amplified, and the size range of those alleles (bp).

Primer	Locus	Allele(bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value	Genotypes	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS150	Gga1:0.895	195	134(134)	154(154)	0.972	195/195	56(54)	65(62)	0.671
		179	28(30)	36(34)	0.644	195/179	20(24)	24(28)	0.276
		175*	4(2)	0(2)	0.032	195/175	2(2)	0(2)	0.170
						179/179	4(2)	6(3)	0.064
PHS135	Gga1:0.991	256	3(2)	2(3)	0.571	228/228	31(35)	47(38)	0.129
		228	104(111)	130(123)	0.375	224/220*	0(1)	2(1)	0.041
		224	64(71)	64(71)	0.256	256/224	1(1)	1(1)	0.864
		220	2(2)	2(2)	0.627	228/220	1(1)	0(1)	0.318
						156/228	2(2)	1(2)	0.515
						224/224	15(11)	13(13)	0.269
						228/224	39(40)	35(44)	0.176

Table 2. Combined data for Gga1:0.8 Mbp (Locus) that was interrogated with PHS150 and PHS135 (Primer). Allele (bp) counts for resistant (Res) v.s. susceptible (Sus) birds for each locus where observed (Obs) versus expected (Exp) significantly deviated ($P \leq 0.055$) are indicated (*). Genotypes in the resistant (Res) and susceptible (Sus) birds were scored and where observed (Obs) versus expected (Exp) significantly deviated (representing >1% of the total population and $P \leq 0.055$) is noted.

Primer	Locus	Allele(bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value	Genotypes	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS118	Gga1:2.028	274	40(38)	40(42)	0.637	274/274	7(4)	5(4)	0.125
		270	26(26)	28(28)	0.909	274/270	9(5)	8(6)	0.076
		266	19(20)	24(23)	0.676	274/266	5(4)	3(4)	0.389
		262*	5(2)	0(3)	0.018	274/262	1(0)	0(0)	0.305
		246	34(34)	37(37)	0.930	274/246	5(7)	4(8)	0.117
		242	56(60)	71(67)	0.460	274/242	6(13)	15(14)	0.058
						270/270	1(2)	1(2)	0.352
						270/266	4(3)	4(3)	0.427
						270/262	1(0)	0(0)	0.195
						270/246	5(5)	4(5)	0.577
						270/242	5(9)	10(9)	0.219
						266/266	2(1)	2(1)	0.306
						266/262*	2(0)	0(0)	0.001
						266/246	1(4)	5(4)	0.136
						266/242	3(7)	8(7)	0.141
						262/262	0(0)	0(0)	0.856
						262/246	1(0)	0(0)	0.276
						262/242	0(1)	0(0)	0.197
						246/246*	7(3)	5(3)	0.020
						246/242	8(11)	13(12)	0.326
						242/242*	17(10)	12/11	0.027
PHS126	Gga1:20.56	259	24(27)	42(39)	0.244	259/259	1(3)	7(5)	0.092
		249	75(72)	103(106)	0.670	249/249	20(19)	28(34)	0.538
		244	36(34)	47(49)	0.105	244/244	1(4)	9(7)	0.392
		239	1(3)	6(4)	1.000	239/239	0(0)	0(0)	0.799
						259/249	12(14)	27(25)	0.137
						259/244*	10(7)	8(12)	0.039
						259/239	0(0)	1(2)	0.360
						249/244	23(18)	36(32)	0.252
						249/239	0(2)	5(6)	0.367
						244/239	1(1)	0(3)	0.150

Table 3. Allele and genotype data for PHS118 and PHS126 on Gga1. Columns and analyses are as indicated in Table 2.

Primer	Locus	Allele(bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value	Genotypes	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS144	Gga1:127.85	323	9(7)	5(7)	0.207	313/313	0(0)	1(1)	0.432
		313	11(12)	15(14)	0.599	303/303	3(5)	4(5)	0.093
		303	29(32)	38(35)	0.494	293/293	1(2)	1(1)	0.431
		293	20(20)	22(22)	0.983	288/288	0(0)	1(4)	0.004
		288	11(15)	20(16)	0.182	283/283	3(4)	1(1)	0.409
		283	30(24)	21(27	0.104	278/278	2(4)	2(2)	0.272
		278	28(26)	26(28)	0.518	263/263*	2(3)	3(11)	0.000
		263	26(29)	36(33)	0.384	248/248	0(0)	0(1)	0.000
		248	4(3)	3(4)	0.608	323/313	1(1)	0(0)	0.406
						323/303	2(3)	1(1)	0.246
						323/293	1(1)	1(0)	0.440
						323/288	1(0)	1(0)	0.288
						323/283*	2(3)	1(2)	0.028
						323/278	2(0)	1(1)	0.202
						323/263	1(1)	1(1)	0.738
						313/303	2(0)	3(3)	0.168
						313/293	1(1)	2(4)	0.090
						313/288	1(0)	2(0)	0.127
						313/283	2(1)	2(1)	0.384
						313/278	2(3)	2(3)	0.287
						313/263	2(4)	3(2)	0.066
						313/248	0(1)	0(0)	0.128
						303/293	3(2)	4(6)	0.291
						303/288	2(0)	4(2)	0.085
						303/283*	5(3)	4(8)	0.042
						202/278	5(2)	5(7)	0.138
						303/263*	5(6)	7(1)	0.014
						303/248	1(3)	1(0)	0.004
						293/288	1(3)	2(1)	0.085
						293/283	4(2)	2(1)	0.209
						293278	3(4)	3(5)	0.247
						293/263	3(3)	4(3)	0.540
						288/283*	2(5)	2(4)	0.014
						288/278	2(2)	3(2)	0.623
						288/263	2(1)	4(3)	0.486
						283/278	5(6)	3(2)	0.480
						283/263*	5(2)	4(1)	0.051
						283/248	1(0)	0(0)	0.305
						278/263	4(3)	5(2)	0.135
						278/248	1(0)	0(0)	0.297
						263/248	1(0)	1(1)	0.337

Table 4. Allele and genotype data for PHS144 on Gga1:127.85 Mbp. Columns and analyses are as indicated in Table 2.

.

Primer	Locus	Allele(bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value	Genotypes	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS015	Gga9:13.111	167	17(16)	16(17)	0.730	167/167	0(1)	1(1)	0.373
		161	101(104)	113(110)	0.699	167/161	10(9)	11(10)	0.636
		151	62(60)	62(64)	0.741	167/151	7(5)	3(6)	0.186
						161/161	26(30)	31(32)	0.466
						161/151	39(35)	39(37)	0.408
						151/151	8(10)	10(11)	0.500
PHS009	Gga9:13.42	277	88(94)	111(105)	0.374	277/277	22(25)	32(27)	0.304
		*275	63(53)	49(59)	0.055	277/275	29(28)	26(31)	0.365
		267	29(33)	40(36)	0.374	277/267	15(17)	21(19)	0.494
						275/275	13(8)	8(9)	0.062
						275/267	8(10)	8(9)	0.212
						267/267	3(3)	6(3)	0.137
PHS010	Gga9:13.54	161	142(138)	147(152)	0.580	161/161	59(55)	60(61)	0.570
		151	30(35)	43(38)	0.270	151/151	4(4)	8(4)	0.030
						161/151*	23(28)	27(31)	0.270

Table 5. Allele and genotype data for PHS015, PHS009, and PHS010 on Gga9:13 Mbp. Columns and analyses are as indicated in Table 2.

Primer	Locus	Allele(bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value	Genotypes	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS152	Gga9:15.882	198	85(79)	82(88)	0.375	198/198*	26(18)	17(23)	0.039
		182	93(99)	115(109)	0.426	198/182	3(44)	47(43)	0.096
						182/182	30(27)	34(30)	0.389
PHS001	Gga9:15.96	157	10(12)	14(12)	0.510	157/157	0(0)	1(0)	0.258
		155	60(66)	76(70)	0.319	155/155*	11(12)	20(13)	0.043
		153	13(16)	20(17)	0.301	153/153	0(1)	3(1)	0.007
		147	54(50)	50(54)	0.471	147/147	4(7)	10(8)	0.144
		139	43(36)	32(39)	0.121	139/139	4(4)	1(4)	0.139
						157/155	2(4)	4(5)	0.264
						157/153	1(1)	3(1)	0.070
						157/147	4(3)	2(3)	0.373
						157/139	3(2)	3(2)	0.592
						155/153	6(6)	4(6)	0.371
						155/147	18(18)	17(20)	0.549
						155/139	12(13)	11(14)	0.364
						153/147	5(4)	1(5)	0.081
						153/139	1(3)	6(3)	0.063
						147/139*	19(10)	10(11)	0.005

Table 6. Allele and genotype data for PHS152 and PHS001 on Gga9:15 Mbp. Columns and analyses are as indicated in Table 2.

Primer	Locus	Allele(bp)	Res Obs(Exp)	Sus Obs(Exp)	P-value	Genotypes	Res Obs(Exp)	Sus Obs(Exp)	P-value
PHS051	Gga27:2.285	151	59(61)	53(51)	0.681	151/151	16(13)	15(11)	0.147
		145	83(81)	65(67)	0.720	151/145	27(35)	27(29)	0.085
						145/145	28(23)	21(19)	0.260
PHS094	Gga27:2.86	192	28(33)	42(37)	0.189	192/192*	3(3)	7(3)	0.054
		186	89(91)	102(100)	0.729	192/186	15(17)	22(19)	0.358
		180	61(53)	50(58)	0.134	192/180	7(10)	6(11)	0.079
						186/186	12(23)	19(26)	0.007
						186/180*	50(27)	42(30)	0.000
						180/180*	2(8)	1(9)	0.001

Table 7. Allele and genotype data for PHS051 and PHS094 on Gga27:2. Columns and analyses are as indicated in Table 2.

Primer	Locus	Allele(bp)	Female Res. Obs(Exp)	Female Sus. Obs(Exp)	P-value	Male Res. Obs(Exp)	Male Sus. Obs(Exp)	P-value
PHS091	GgaZ:48.61	295	28(32)	28(35)	0.171	85(87)	84(82)	0.757
		282*	10(8)	14(8)	0.031	17(16)	14(15)	0.709
		275*	3(1)	1(1)	0.017	2(1)	0(1)	0.170
		270	0(0)	1(0)	0.025	0(0)	0(0)	
Genotype	Female Res. Obs(Exp)	Female Sus. Obs(Exp)	Chi Test		Male Res. Obs(Exp)	Male Sus. Obs(Exp)	Chi Test	
295	18(20)	22(25)	0.468					
282*	1(9)	10(11)	0.010					
275	0(1)	0(2)	0.074					
270	0(0)	0(0)	0.371					
295-295	0(0)	0(0)			36(36)	36(34)	0.766	
295-282	8(0)	4(0)			11(13)	12(13)	0.506	
295-275	2(0)	1(0)			2(1)	0(1)	0.128	
282-282	0(0)	0(0)			3(1)	1(1)	0.107	
282/275	1(0)	0(0)						
295/270	0(0)	1(0)						

Table 8. Allele and genotype data for PHS091 on GgaZ: 48 Mbp. Columns and analyses are as indicated in Table 2.

Haplotype	Res Obs(Exp)	Susc. Obs(Exp)	ChiTest
175-224	1(0)	0(1)	0.27
175-228	1(0)	0(1)	0.27
179-220	1(1)	1(1)	0.89
179-224	3(4)	6(5)	0.48
179-228	17(18)	22(21)	0.85
179-256	1(1)	1(1)	0.89
195-225	48(44)	50(54)	0.44
195-228	74(78)	98(94)	0.59

Table 9. Alleles for homozygous individuals were combined into probable haplotypes for PHS150 and PHS135 at Gga1:0.8 Mbp. The counts of observed (Obs) versus expected (Exp) haplotypes for the resistant (Res) and susceptible (Sus) sub-populations were compiled. Haplotypes where significant deviation between resistant and susceptible sub-populations are noted (*).

Haplotype	Res Obs(Exp)	Susc. Obs(Exp)	ChiTest
242-244*	3(9)	14(9)	0.01
270-249	3(6)	8(6)	0.13
266-249	6(8)	9(8)	0.44
266-244	3(4)	5(4)	0.48
274-239	0(1)	2(1)	0.16
270-244	1(2)	2(2)	0.56
266-259	0(1)	1(1)	0.32
246-259	2(2)	2(2)	1.00
246-249	15(15)	15(15)	1.00
242-239	1(1)	1(1)	1.00
246-244	3(3)	2(3)	0.65
274-259	2(2)	1(2)	0.56
262-259	1(1)	0(1)	0.32
262-249	1(1)	0(1)	0.32
270-259	2(1)	0(1)	0.16
274-244	5(4)	3(4)	0.48
274-249	18(16)	13(16)	0.37
242-249	22(20)	17(20)	0.42
242-259	16(13)	9(13)	0.16

Table 10. Alleles for homozygous individuals were combined into probable haplotypes for PHS118 and PHS126 at Gga1:20 Mbp. Abbreviations and columns are as in the legend in Table 9.

Haplotype	Res Obs(Exp)	Sus Obs(Exp)	Chitest
161-277	42(45)	59(56)	0.55
151-277	9(11)	16(14)	0.39
151-267	1(3)	5(3)	0.17
167-267	0(1)	3(2)	0.12
167-277	3(2)	2(3)	0.49
161-267	12(11)	13(14)	0.73
167-275	2(1)	0(1)	0.11
161-275	11(9)	9(11)	0.35
151-275	18(15)	15(18)	0.25

Table 11. Alleles for homozygous individuals were combined into probable haplotypes for PHS015 and PHS009.Abbreviations and columns are as in the legend to Table 9.

Haplotype	Res Obs(Exp)	Susc Obs(Exp)	Chitest
277-161	64(68)	79(75)	0.55
277-151	4(8)	12(8)	0.07
275-161	52(42)	36(46)	0.03
275-151	3(4)	5(4)	0.58
267-161	5(8)	12(9)	0.14
267-151	8(8)	8(8)	0.82

Table 12. Alleles for homozygous individuals were combined into probable haplotypes for PHS009 and PHS010. Abbreviations and columns are as in the legend to Table 9.

Haplotype	Res Obs(Exp)	Susc. Obs(Exp)	ChiTest
180-155*	25(32)	40(33)	0.08
180-157	4(5)	7(6)	0.39
180-153	6(7)	8(7)	0.63
196-157	1(1)	2(2)	0.58
180-147	19(19)	20(20)	0.95
196-153	5(4)	3(4)	0.45
180-139	14(12)	11(13)	0.50
196-147	18(16)	15(17)	0.54
196-139	13(10)	8(11)	0.25
196-155	23(20)	18(21)	0.38

Table 13. Alleles for homozygous individuals were combined into probable haplotypes for PHS152 and PHS001. Abbreviations and columns are as in the legend in Table 9.

Haplotype	Res Obs(Exp)	Susc. Obs(Exp)	ChiTest
146-181	18(15)	10(13)	0.24
146-187	30(31)	29(28)	0.71
146-191	12(11)	9(10)	0.72
150-181	12(10)	6(8)	0.25
150-187	21(21)	19(19)	0.92
150-191*	5(10)	13(8)	0.03

Table 14. Alleles for homozygous individuals were combined into probable haplotypes for PHS094 and PHS051. Abbreviations and columns are as in the legend in Table 9.

Chapter 4: Using VNTRs to examine gene flow and multiple paternity in the Timber rattlesnake *Crotalus horridus*

INTRODUCTION

In addition to serving as biomarkers for disease, the use of VNTRs for gene flow and isolation studies in threatened and endangered species is becoming increasingly prevalent in the conservation biologist's tool kit. Scientists can use VNTRs to identify familial relationships among capture and release populations of animals, survey the genetic variability of an area, and identify multiple paternity (Selkoe 2006, McDowall 2008, Narain 2000, Hoekert 2002). The guidelines by which policy is made to protect threatened and endangered animals can be better defined with higher quality population genetic information for the animals under focus.

The Timber rattlesnake, *Crotalus horridus,* is found widely across the Eastern half of the United States and historically ranges from lower Ontario and Minnesota into Florida and Texas (Brown 1993). Populations are becoming increasingly fragmented due in large part to human encroachment, captures, and den compromises (A.M. Clark 2003, Reinert 1999). The Timber rattlesnake is reported to be an animal of rigorous den fidelity, not straying much farther than 2 or 3 miles from a den site and returning to the same den each season for communal hibernation (Shine 2008). Males tend to forage in wider areas than females for both food and mates but typically remain inside a range (Shine 2008, Bonnet 1998). These viviparous animals typically live 20-25 years and pay a high cost for reproduction (Shine 2008, Gardner-Santana 2009). The snakes are especially vulnerable in the spring as they emerge from hibernation with major negative affects being den destruction and adult capture (Bonnet 1998). The Timber rattlesnake has been extensively studied in the Northeastern

region of the United States due to its increasingly endangered status (Brown 1982, Aldridge 1995, Reinert 1999, Furman 2007). It is listed as declining or extirpated in all New England states and is generally considered to be in decline in all states where it can be found (Clark 2008). The Timber rattlesnake can be found from the plains of Kansas, (Fitch 2004) to the Appalachian mountains, (Martin 1993) to the Northeastern region of the United States (Reinert 1999). Suitable habitats for Timber rattlesnakes are being rapidly encroached upon by urbanization (Laidig 2004) and hunting for sport or out of fear remains problematic (Furman 2007). The remaining habitat areas are being fragmented, which may be problematic for these philopatric snakes (Clark 2007). The Timber rattlesnake is known to forage widely for food, but is predominantly an ambush predator. Males are also commonly mobile over wide areas while seeking mates and indeed have been linked to gene flow among den populations (Clark 2008). Aside from the summer months when mates are being sought and years when food availability is limited, timber rattlesnakes are often somewhat invisible to the human population. There is some consensus that males are far more mobile than females (Shine 2008, Bonnet 1998). Moreover, the inconsistent and often sparse movement of these animals leads to little human interaction and typically results in sampling of groups of animals found in overwintering and communal basking sites (Shine 2008). Directed molecular analysis of large populations of snakes could be effective in determining the microevolutionary processes and trends of gene flow between and among dens (Manier 2006, Storfer 1998, Blouin-Demers 2002, Holycross 2002, Clark 2008).

In 2008, 373 timber rattlesnakes from 14 hibernacula in New York were genotyped using nine microsatellite (VNTR) loci (Clark 2008). Genotype data were used to gauge gene flow between and among the adjacent hibernacula in the study (average distance between

hibernacula was 3.2 km). The results suggest that individual movements were more important for genetic structure than geography and elucidated the importance of movement corridors for the animals in active seasons of the year. Bushar *et al.* (1998) used VNTR genotypes to implicate population structure over kilometer distances as critical determinants of basking sites and communal dens. VNTR analyses of the Massasauga rattlesnake in the northeast United States and Ontario identified fine-scale population structure among populations <50 kilometers apart (Gibbs 1997). A population of northern water snakes in Ontario was analyzed with VNTRs in 1999 (Prosser 1999). Microgeographic genetic structure between populations <2 km apart was identified using deviation from expected heterozygote frequencies.

Genetically motivated study of the Timber rattlesnake may lead to more informed conservation efforts and "nuisance animal" strategies. Currently, wildlife management personnel utilize short distance wildlife translocation as a humane alternative to exterminating wildlife that impedes human activity (Brown 2009). Brown *et al* conducted a year-long study on Western rattlesnakes in an effort to determine if short distance (500m) relocation of the animals had detrimental effects. They concluded there was increased overall activity and movement in the relocated animals, but those relocated to "high-quality" habitats suffered no adverse effects. In a conflicting report, there is evidence that "capture and release" is ineffectual as a relocation practice for Timber rattlesnakes when they are encountered by humans (Reinert 1999). Reinert *et al* found that the snakes in their study had difficulty establishing in a new hibernacula and displayed abnormally high overwintering mortality rates.

We have been studying a set of capture areas that range over approximately 5,666 ha at the Bear Hollow Natural Area, the Ozark Natural Science Center, and the Madison County Wildlife Management Area (WMA) (Gardner-Santana 2009). The geography of the WMA is such that animals are not geographically isolated and are able to travel from one site to another, but distances from the opposite ends of the sample area could be considered limiting and may represent boundaries for gene flow. Characterization of the normal gene flow and population structure for Timber rattlesnakes may aid in identification of more successful ways to preserve, handle, and reintroduce these animals to protected lands.

The use of VNTR interrogation of our population of snakes, particularly in litters of captive born animals along with mothers, also allowed us to use the VNTR loci to examine whether there is multiple paternity within a single litter. Timber rattlesnake females are known to store sperm over the winter (Aldridge 1995). Copulation with multiple males is also commonly observed (Gibbs 2001). Thus these snakes may exhibit multiple paternity in a litter. Multiple paternity is well documented in snakes, (McCracken 1999, King 2001, Garner 2002), tortoises (Johnston 2006), lizards (Gullberg 1997), turtles (Pearse 2002),and salamanders (Adams 2005) but no reports of multiple paternity have been made for the Timber rattlesnake. We provide evidence here of multiple paternity at two of our three VNTR loci.

METHODS

Sampling and Populations: A population of 186 animals in the WMA was divided into 8 sub-groups: 6 sub-groups within the wildlife management area (Groups A1-D2), 1 group of 35 animals from outside the management area but within the state of Arkansas (AR), and

one group of 22 diverse samples from Mississippi, Illinois, and Virginia (US) (Figure 1, Table 1).

For multiple paternity, all litters were born in captivity. We collected blood for maternal alleles on 3 groups, 4 groups have no sampled mother. All were sampled via shed skin or blood isolation method.

Isolation of DNA from Shed Skin: Skin samples were homogenized in 5 ml TE (10 mM TrisCl, 1mM EDTA, pH 7.5) per gram of tissue using a Tissue-Tearor tissue homogenizer. The homogenate was brought to 0.5% SDS, and 100µg/mL each RNAseA and Pronase E. After incubation for 30 minutes at 37°C, the solution was extracted once with phenol:chloroform:isoamyl alcohol (125:24:1), then once with chloroform:IAA (24:1). DNA was precipitated with ethanol and rehydrated. All DNAs were dissolved in Te (10 mM TrisCl, 0.1mM EDTA, pH 7.5) and stored frozen at -20°C.

Isolation of DNA from Blood: DNA isolation followed the method of Bailes 2007.

Polymerase Chain Reaction: PCR was typically performed in 96 well plates using either an MJ Research PTC-100 Programmable Thermal Controller Thermocycler or an Eppendorf Mastercycler Gradient. Reactions of 20ul contained 1X Taq Buffer (50 mM Tris-Cl, pH 8.3, 1 mM $MgCl_2$, 30µg/ml BSA), 0.2 mM dNTPs, 0.4 µM each forward and reverse primers, 2.5 units Taq polymerase, and 2 µl of DNA (approx. 50 ng). PCR conditions were an initial denaturation at 90° C for 1 minute followed by 40-45 cycles of 90° C for 25 s, 30 s anneal at 56, 53, or 50° C (Table 2), 72° C for 60 s, and a final extension at 72° C for 3 minutes.

Gel Electrophoresis: PCR products were resolved in 6% denaturing polyacrylamide gels (38:2 acrylamide:MBA, 50% urea) in 1xTEB (100 mM Tris, 10 mM boric acid, 2 mM

EDTA, pH9.2). PCR product (2µl) was mixed with 5ul loading buffer (95% Formamide, 1X TEB, 0.02% Bromophenol Blue). Samples were heat denatured at 90° C for 5 minutes, quick-chilled on ice then 1-2 µl was loaded to a lane. CXR ladder (Promega Corp.) was used as size marker.

Gel Imaging: Gels were scanned with a Model 9600 Typhoon imager (GE Health Care) and images analyzed using ImageQuant software.

Statistical Analysis: Genotype data was assembled in groups as specified above and analyzed with Genepop (www.genepop.curtin.edu.au) for allele diversity, genotype diversity, and Fst based on geographic versus genetic distance.

RESULTS

For our gene flow study, we used three established primer pairs, 5-183, CwB6, and CwD15 (Villareal 1996, Holycross et al, 2002). All primer pairs were polymorphic, with 5-183 amplifying 5 alleles of 126-142 bp, CwB6 amplifying 11 alleles of 96-116 bp, and CwD15 amplifying 3 alleles of 124-133 bp (Table 3). We analyzed the sub-groups and the out-groups in a pair-wise manner to compare the ability of our VNTR markers to differentiate populations. Each population was analyzed for adherence to Hardy-Weinberg equilibrium (HWE) by calculation of Chi-square values comparing observed allele frequencies to expected allele frequencies for each locus for each sub-group (Table 4). The entire Arkansas population (A1-D2) was analyzed in paired sub-groups for allele and genotype differences. We also compared each sub-group to two out-groups, AR and US (Tables 5-10).

In the whole population, primer pair 5-183 amplified 5 alleles from 179 individuals with allele sizes ranging from 128 to 142 bp (Table 3). The heterozygote frequency was 0.64

(Table 2). The allele frequencies for all groups, when analyzed independently, were in equilibrium except for the B and the US groups (Table 4). We analyzed our samples for allelic differentiation between each sub-group and the two out-groups. Primer pair 5-183 showed significant difference between the US out-group and all but two sub-groups; A1 and C ($P \leq 0.03$) (Table 5). Sub-group B was different from D1, D2, AR, and US sub-groups ($P \leq 0.04$). Sub-group A2 was significantly different than groups D2 and US ($P \leq 0.03$). Sub-group A1 was significantly different from groups B and D1 ($P \leq 0.02$). We also analyzed each sub-group for genotypic difference when compared to all other sub-groups (Table 6). The US sub-group was significantly different from the B, D1, and AR sub-groups ($P \leq 0.03$). The sub-group A1 was significantly different than the B, D1, and D2 groups ($P \leq 0.03$).

Primer pair CwB6 amplified 11 alleles from 217 individuals. Heterozygote frequency was 0.59 and allele sizes ranged from 96 to 128 bp (Tables 2 and 3). This primer pair amplified alleles that did not conform to HWE for 6 of 8 sub-groups (Table 4). When analyzed for allelic difference between sub-groups (Table 7), this locus showed a significant difference between D1 and A1 ($P=0.02$). There was a significant difference between the AR sub-group and the A2 and B sub-groups ($P \leq 0.0009$). We also analyzed the sub-groups for genotypic difference (Table 8). The B and AR sub-groups differed significantly ($P=0.04$).

CwD15 amplified 3 alleles from only 104 individuals with allele sizes of 124-133 bp (Table 3). The heterozygote frequency was 0.40 (Table 2). All subgroups were within HWE for CwD15 (Table 4). For allelic frequencies (Table 9) sub-group A2 was significantly different when compared to sub-groups A1, B, D1, and AR ($P \leq 0.02$). There

was also a significant difference between sub-group B when compared to C and to the US out-group ($P \leq 0.05$). This locus was also analyzed for genotypic difference between sub-groups (Table 10). Sub-group A2 significantly differed from the B, AR, and US sub-groups ($P \leq 0.05$).

In an effort to determine the power of these 3 primer pairs to amplify significant and unique alleles across our sub-groups, we performed an Fst pairwise analysis (Weir 1983). When computed relative to geographic distance, we found no significant difference for any pair of sub-groups ($P=0.99$) (Data not shown).

For investigation of multiple paternity, we analyzed 7 separate litters of snakes whose location within or outside the WMA are known (Table 11); for three litters we know the mother and she is in our dataset. For four litters, the mother is not known. Of the groups with a sampled mother, two are from Arkansas, but outside the WMA, while one is from within the WMA. Of the four litters of siblings with no mother, 3 are from within the WMA and one is from Arkansas, outside the WMA. We genotyped all litters for these three loci and determined how many alleles were present in each litter for each locus.

For litter 1, primer pair 5-183 two alleles were amplified, 136 bp and 128 bp. The mother was heterozygous for these two alleles, 128/136. Two offspring were homozygous, one 128/128 and one 136/136. The other 3 offspring were heterozygous, 128/136. Four alleles were amplified with primer pair CwB6, (110, 108, 106, and 104 bp). The mother has a genotype 110/108. All five offspring were heterozygous (110/104 or 108/106). For locus CwD15 only one allele was amplified (124 bp).

For litter 2, locus 5-183, four alleles were detected, 128, 130, 136, and 142 bp. The mother was heterozygous 136/128; four offspring were heterozygous with genotypes of

142/136, 136/128, 136/130, or 130/128. Five offspring were homozygous; 128/128 or 136/136. CwB6 amplified five alleles (114 bp, 110 bp, 108 bp, 106 bp, and 104 bp). All offspring at this locus were heterozygous (110/108, 110/104, or 110/106). The mother was heterozygous (110/108). All individuals were homozygous for the 124 bp allele for locus CwD15. At locus CwB6, this group of animals had 4 individuals that could not be correlated with either maternal allele, likely due to genotypes that included artifacts. So this data set has been excluded until the mother and offspring can be resolved unambiguously.

For litter 3, all individuals contained at least one maternal allele for each locus. Primer pair 5-183 amplified 2 alleles (128 bp, 136 bp). Four offspring of were heterozygous (136/128) and one each was homozygous (128/128 and 136/136). Primer pair CwB6 amplified 4 alleles for litter 3 (110, 108, 106, and 104 bp). The mother was heterozygous 106/104. Five offspring were heterozygous (108/104, 110/106, or 110/104) and 1 was homozygous (106/106). CwD15 was inconclusive as several individuals failed to amplify in multiple PCRs.

For sibling group 1 (mother unknown), primer pair 5-183 amplified 2 alleles (136 bp, 128 bp) for 4 out of 5 siblings. Three were heterozygous (136/128) and one was homozygous (128/128). Primer pair CwB6 amplified 3 alleles for 4 of 5 animals (112 bp, 110 bp, 102 bp). All animals were heterozygous (110/102, 112/110, or 110/102). We were able to genotype only one animal in this group with CwD15.

For sibling group 2, primer pair 5-183 amplified 2 alleles (136 bp and 128 bp). Three animals were homozygous (136/136) and one was heterozygous (136/128). Primer pair CwB6 amplified two alleles. All individuals were heterozygous 130/114 at this locus.

CwD15 amplified two alleles in this group (124 bp and 130 bp). One animal was heterozygous; the other three were homozygous (124/124).

For sibling group 3, primer pair 5-183 amplified 3 alleles (128 bp, 130 bp, and 136 bp). Two individuals were heterozygous for genotype 136/128, one was heterozygous for 136/130, and one was homozygous 128/128. CwB6 amplified 4 alleles (118 bp, 114 bp, 110 bp, and 102 bp). All individuals were heterozygous (118/114, 114/102, 114/110, 114/112). CwD15 was monomorphic; all individuals were homozygous 124/124.

For sibling group 4, primer pair 5-183 amplified 2 alleles (128 bp and 136 bp). Both individuals were homozygous, one with the genotype 136/136 and one with 128/128. CwB6 amplified 2 alleles, 114 bp and 112 bp. Both individuals were heterozygous with a genotype 114/112. CwD15 also amplified 2 alleles, 130 bp and 124 bp. Both individuals were heterozygous with the genotype 130/124.

DISCUSSION

Gene Flow

The use of VNTR analysis to determine population structure and gene flow is increasingly popular in conservation ecology (Selkoe 2006). Genetic analysis can complement field studies and allow for better conservation practices and management plans (Clark 2007, Ciofi 1999 and 2002, Calsbeek 2007, Prior 1997, Dever 2002). We applied VNTR analysis to our population in an effort to distinguish population structure and elucidate any patterns in gene flow.

The 18 animals from the US group from outside the state that could be genotyped were significantly different from every sub-group in Arkansas except the A1, A2, and the C groups which represent only 11 of the 179 individuals that could be genotyped for this

locus. This difference between groups may indicate that 5-183 is a useful marker for testing geographic isolation and gene flow. Furthermore, locus 5-183 amplified alleles that displayed sufficient diversity and maintained HWE in a Hardy Weinberg Exact test (Guo 1992), suggesting adequate sampling and normal gene flow within the populations. Our analysis of the Arkansas population, when compared to the animals from outside the state, showed a significant difference in the frequency of alleles and genotypes at locus 5-183. Primer pair CwB6, while amplifying more alleles than 5-183, was not in HWE for nearly every sub-group. This could be sampling bias or more likely evidence that some of the alleles may be artifactual PCR products. CwD15 is consistently in HWE, but lacks diversity sufficient to distinguish populations. All primer pairs were able to differentiate sub-groups by allele and/or genotype comparison, especially when comparing the Arkansas animals to those from outside Arkansas. However, within the WMA population, these loci fail to detect any finer population structure. This could mean that there is adequate gene flow within the WMA population, or that three loci with a small number of alleles are insufficient to detect subpopulations. In a study of the Galápagos tortoise on the Galápagos islands, VNTR analysis was used to describe genetic diversity and structure among and between the islands (Ciofi 2002). Our study aims to describe the diversity among our Arkansas population and compare it to populations of known geographic origin outside Arkansas and to a geographically separate population inside Arkansas. A finer analysis of this population of animals with respect to gene flow and population structure could be possible using more loci possibly with greater allelic diversity. The majority of VNTR analyses that well-define population structure appear to address the population at multiple polymorphic loci (Clark 2007, Dever 2002, Ciofi 1999 and 2002). In a study comparing

hibernacula of black rat snakes, 14 randomly amplified polymorphic DNA (RAPD) regions were used to determine population structure (Prior 2000). F_{st} analysis was able to show genetic variation was highest between individual animals in the study, though some significance was shown between their sub-populations. In a more robust study, microsatellite [VNTR] analysis revealed an important link between seasonal male dispersal and gene flow between hibernacula of Timber rattlesnakes in the Northeastern United States (Clark 2007). Few studies have been undertaken for population structure in the Timber rattlesnake. Our populations' high individual count, well characterized den sites, and frequent recapture will allow us to learn much about the way genes are disseminated in the Arkansas population of Timber rattlesnakes in the Ozark Mountains.

Multiple Paternity

Multiple paternity occurs due to mating with multiple males within single reproductive cycle or mating with one or multiple males across multiple reproductive cycles and storing sperm (Uller 2008). Multiple paternity is frequently reported in reptiles and is particularly common in snakes and lizards (Uller 2008). Many methods have been employed for multiple paternity verification in reptiles; microsatellite [VNTR] analysis, DNA fingerprinting, and RFLP amplification (Uller 2008). Our analysis for multiple paternity used VNTR genotyping and was based almost exclusively on genotypes at locus CwB6. Locus CwD15 amplified only two alleles and did so sporadically. Our analysis of captive-born families allows us to confidently eliminate maternal alleles from the genotypes of the offspring and accurately (within the power of our marker) assign alleles to a prospective father or fathers.

For mother known litter 1, when genotyped with 5-183 there is no evidence of more than two paternal alleles, 128 and 136. For CwB6our data identifies only two paternal alleles, 104 and 106. No conclusions can be drawn for CwD15.

For mother known litter 2, paternal alleles of 142 bp and 130 bp can be assigned for primer pair 5-183. For CwB6, we have evidence of multiple paternity with the presence of three alleles (108 bp, 106 bp, and 104 bp) that cannot be contributed by the mother. No conclusions can be drawn from CwD15.

For mother known group #3, no conclusions can be drawn for 5-183 or CwD15. CwB6 amplified three alleles (110 bp, 108 bp, and 106 bp) that cannot be contributed by the mother. These findings support multiple paternity in our group of Timber rattlesnakes.

Sibling groups #1 through #4 show no evidence of multiple paternity.

There is currently no record in the literature of multiple paternity being reported in Timber rattlesnakes. As such, we will replicate these data both biologically and methodologically to ensure our report is complete. To that end, we will also interrogate more loci for multiple paternity testing. Indeed, in 2000 a study showed that the use of 6 polymorphic microsatellites [VNTR] had the power of 159 AFLP when determining paternity (Gerber 2000).

In summary, our data support the use of locus 5-183 for population genetics studies of Timber rattlesnakes, but with limited ability to identify discrete populations within a small geographic area. Locus CwB6, while reported to be highly polymorphic and useful for analysis of small geographic areas, proved insufficient in our analysis. CwD15 has insufficient diversity for use as a marker in gene flow analysis for isolated populations.

The primer pair CwB6 was useful for multiple paternity studies as the level of polymorphism allows successful identification of maternal alleles. However, due to its lack of conformation to Hardy-Weinberg Equilibrium and the presence of artifactual alleles, these findings require further investigation. Our continued efforts will utilize 5-183 and CwB6 to genotype and analyze more captivity-born families. We are currently optimizing published VNTR sites for further analysis.

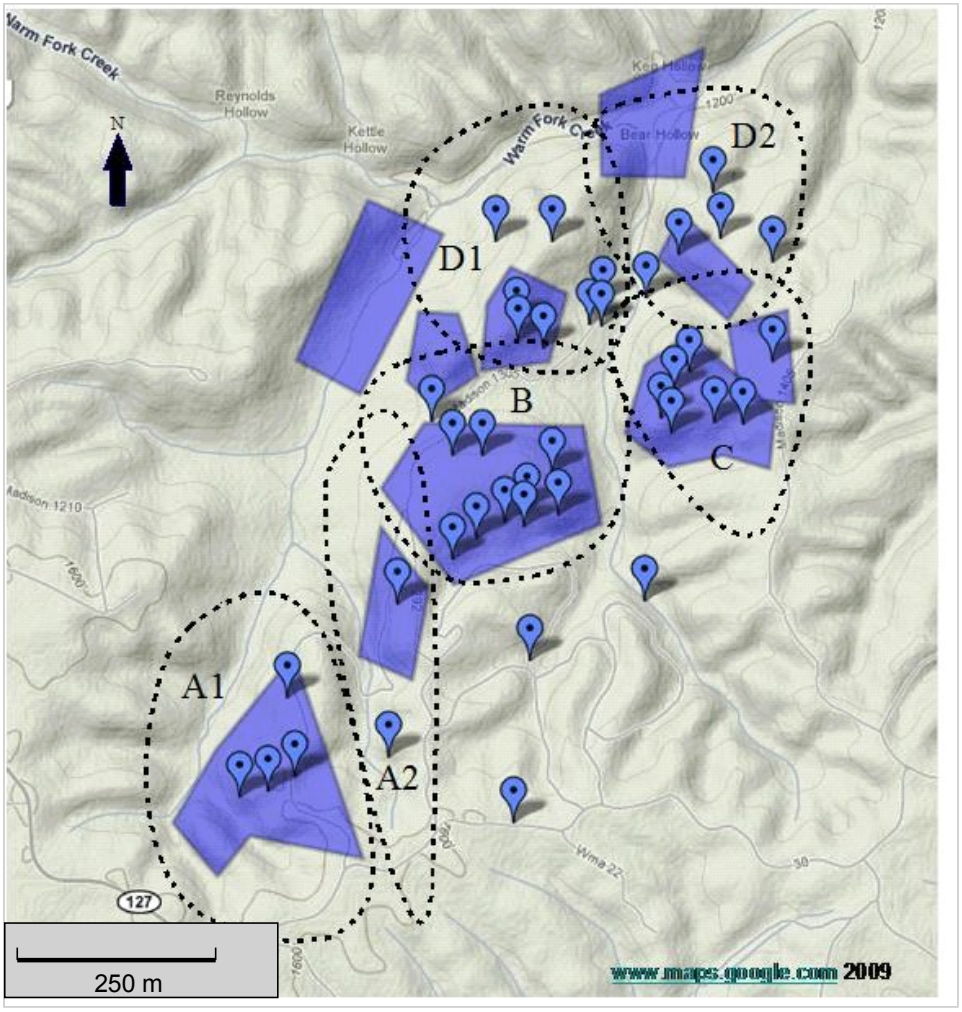

Figure 1. Capture sites in the Northwest Arkansas Wildlife Management Area. Areas grouped for our gene flow analysis are designated by a dashed line (A1-D2). Capture group areas are indicated with shaded areas and pins indicate catch sites.

	A2	B	C	D1	D2
A1	247.8	459.6	1052.8	1115.0	1362.5
A2		371.6	758.6	867.2	1130.2
B			507.8	459.6	805.0
C				433.4	464.5
D1					396.2

Table 1. Geographic distances between the centers of capture regions of sub-groups inside the WMA in meters.

Locus	Allele (bp)	A1	A2	B	C	D1	D2	AR	US
5-183	142	0(3)	0(1)	11(7)	3(2)	11(8)	0(1)	4(5)	1(3)
	140	4(6)	3(2)	25(16)	5(5)	15(17)	2(2)	7(10)	2(6)
	136	18(11)	5(4)	22(30)	10(9)	31(31)	4(4)	19(18)	10(11)
	130	3(6)	2(2)	10(16)	4(5)	23(17)	5(2)	14(10)	2(6)
	126	11(10)	2(3)	26(26)	8(8)	20(27)	1(3)	14(16)	21(10)
CwB6	116	8(6)	5(2)	18(14)	1(5)	15(15)	2(2)	3(7)	4(5)
	112	7(5)	1(2)	7(12)	5(5)	14(13)	1(2)	8(7)	6(4)
	110	10(11)	1(4)	25(26)	12(10)	32(28)	1(3)	16(14)	9(9)
	106	1(3)	0(1)	4(8)	8(3)	9(8)	0(1)	5(4)	4(3)
	118	0(2)	1(1)	8(5)	2(2)	4(5)	1(1)	2(3)	3(2)
	114	6(5)	4(2)	16(13)	2(5)	14(13)	3(2)	4(7)	2(5)
	108	11(9)	4(3)	23(20)	6(8)	16(21)	2(3)	10(11)	9(7)
	104	1(2)	0(1)	3(5)	4(2)	5(6)	1(1)	8(3)	0(2)
	102	4(2)	0(1)	2(5)	2(2)	7(5)	1(1)	4(3)	1(2)
	128	0(1)	0(0)	4(1)	2(1)	0(2)	0(0)	0(1)	0(1)
	96	0(1)	0(0)	1(2)	0(1)	2(2)	2(0)	0(1)	2(1)
CwD15	133	4(3)	2(0)	2(5)	3(1)	2(5)	1(1)	2(2)	4(3)
	130	3(4)	2(1)	6(9)	3(2)	9(8)	1(1)	4(3)	7(5)
	124	19(19)	0(3)	48(41)	8(10)	39(37)	6(6)	16(15)	19(22)

Table 2. Allele counts for all sub-groups. The alleles (bp) amplified by each primer pair (Locus) were tabulated for each sub-group (A1-US) and observed (Obs) versus expected (Exp) counts were compared.

Primer	Sequence (5' to 3')	VNTR	# Alleles	Size (bp)	Annealing T (C°)	Heterozygote frequency
5-183	TTGTTGTAACCAGTGTGTGTGAT CTGCAGACACTTATTATTATACC	$(CA)_{11}$	5	128-142	50	0.64
CwB6	CTCTTTTACGCCCACCACTTTA CCCCGCTAACCTTTGCTCAG	$(GA)_{19}$	11	96-128	56	0.59
CwD15	TAATGTTGTAAGCCACCTAGAAT TTCTTCAAAGCACATAACACATC	$(CAT)(TAT)(CAT)_{14}$	3	124-133	53	0.40

Table 3. VNTR primer pairs: 5-183 (Villareal et al., 1996), CwB6 and CwD15 (Holycross et al, 2002) with sequence information, the repeat amplified (VNTR), number of alleles amplified (bp), optimized annealing temperature, and the calculated heterozygote frequency.

	5-183	**CwB6**	**CwD15**
A1	0.07	0.00	0.17
A2	0.47	0.20	1.00
B	0.00	0.00	0.25
C	0.08	0.01	0.52
D1	0.35	0.00	0.32
D2	0.36	0.54	1.00
AR	0.15	0.00	0.66
US	0.05	0.01	0.68

Table 4. Hardy-Weinberg Equilibrium Probability Test for each group (A1-OTHER) at each locus (5-183, CwB6, and CwD15).

	N	A2	B	C	D1	D2	AR	US
A1	**18**	0.460	*0.01	0.260	*0.02	0.260	0.120	0.100
A2	**6**		0.510	0.810	0.740	*0.03	0.690	*0.03
B	**47**			0.720	*0.04	*0.04	*0.04	*0.003
C	**5**				0.820	0.280	0.790	0.070
D1	**50**					0.560	0.890	*0.0003
D2	**6**						0.600	*0.0002
AR	**18**							*0.01
US	**29**							

Table 5. Allelic differentiation between groups at locus 5-183. Sub-group size is noted (N). P-values of P ≤0.05 for significantly different combinations of sub-groups within the WMA are indicated (*).

	N	A2	B	C	D1	D2	AR	US
A1	**18**	0.490	*0.02	0.200	*0.02	*0.03	0.090	0.090
A2	**6**		0.570	0.240	0.620	0.580	0.680	0.060
B	**47**			0.850	0.100	0.120	0.130	*0.02
C	**5**				0.840	0.250	0.230	0.150
D1	**50**					0.480	0.910	*0.001
D2	**6**						0.560	0.006
AR	**18**							*0.03
US	**29**							

Table 6. Genotype differentiation between all sub-groups for marker 5-183. The size of the sub-groups are given (N). P-values indicating significant differences between sub-groups are indicated (*).

	N	A2	B	C	D1	D2	AR	US
A1	**24**	0.340	0.240	0.500	*0.02	0.500	0.310	0.290
A2	**8**		0.730	0.190	0.120	0.500	*0.0009	0.120
B	**54**			0.090	0.180	0.450	*0.0006	0.290
C	**19**				0.790	0.510	0.160	0.920
D1	**57**					0.410	0.590	0.071
D2	**6**						0.220	0.021
AR	**19**							0.150
US	**30**							

Table 7. Allelic differentiation between groups at locus CwB6. Columns and labels are as in Table 4.

	N	A2	B	C	D1	D2	AR	US
A1	**24**	0.380	0.350	0.770	0.560	0.780	0.520	0.460
A2	**8**		0.770	0.240	0.260	0.960	0.070	0.240
B	**54**			0.280	0.340	0.850	*0.04	0.500
C	**19**				0.760	0.530	0.230	0.980
D1	**57**					0.850	0.710	0.790
D2	**6**						0.730	0.500
AR	**19**							0.320
US	**30**							

Table 8. Genotypic differentiation between all sub-groups for marker CwB6. Columns and labels are as in Table 5.

	N	A2	B	C	D1	D2	AR	US
A1	**13**	*0.01	0.130	0.550	0.220	0.550	0.720	0.052
A2	**2**		*0.0004	0.140	*0.0003	1.000	*0.02	*0.04
B	**28**			*0.02	0.560	0.490	0.190	*0.05
C	**7**				0.090	1.000	0.700	0.810
D1	**25**					0.570	0.580	0.260
D2	**4**						1.000	0.850
AR	**15**							1.000
US	**10**							

Table 9. Allelic differentiation between groups at locus CwD15. Columns and labels are as in Table 4.

	N	A2	B	C	D1	D2	AR	US
A1	**13**	0.060	0.220	0.660	0.240	1.000	0.770	0.460
A2	**2**		*0.006	0.250	0.960	0.130	*0.05	*0.05
B	**28**			0.080	0.660	0.700	0.470	0.070
C	**7**				0.180	0.700	0.740	0.770
D1	**25**					1.000	0.710	0.260
D2	**4**						1.000	0.820
AR	**15**							0.900
US	**10**							

Table 10. Genotypic differentiation between all groups for marker CwD15. Columns and labels are as in Table 5.

ID	SITE	5-183		CwB6		CwD15	
Group 1							
MOM#1	AR-O	136	128	110	108	124	124
OS1-1	AR-O	136	128	108	106	124	124
OS1-1	AR-O	136	128	110	104	124	124
OS3-1	AR-O	136	128	108	106	124	124
OS4-1	AR-O	136	136	108	106	124	124
OS5-1	AR-O	128	128	110	104	124	124
Group 2							
MOM#2	AR-O	136	128	114	110	?	?
OS1-2	AR-O	142	136	110	108	130	124
OS2-2	AR-O	136	136	104	102	130	124
OS3-2	AR-O	136	128	110	104	124	124
OS4-2	AR-O	128	128	104	102	124	124
OS5-2	AR-O	136	130	110	106	124	124
OS6-2	AR-O	136	136	110	104	?	?
OS7-2	AR-O	128	128	104	104	130	124
OS8-2	AR-O	128	128	110	108	?	?
OS9-2	AR-O	130	128	118	106	124	124
Group 3							
MOM#3	C	136	128	106	104	?	?
OS1-3	C	136	128	108	104	130	124
OS2-3	C	136	128	110	106	?	?
OS3-3	C	136	128	110	104	?	?
OS4-3	C	136	136	110	104	?	?
OS5-3	C	136	128	106	106	?	?
OS6-3	C	128	128	110	106	?	?
Group 4							
SIB1-1	AR-O	136	128	110	102	124	124
SIB2-1	AR-O	128	128	112	110	?	?
SIB3-1	AR-O	136	128	110	102	?	?
SIB4-1	AR-O	136	128	?	?	?	?
SIB5-1	AR-O	?	?	112	110	?	?
Group 5							
SIB1-2	B	136	136	130	114	124	124
SIB2-2	B	136	136	130	114	130	124
SIB3-2	B	136	128	130	114	124	124
SIB4-2	B	136	136	130	114	124	124
Group 6							
SIB1-3	D1	136	130	118	114	124	124
SIB2-3	D1	128	128	114	102	124	124
SIB3-3	D1	136	128	114	110	124	124
SIB4-3	D1	136	128	114	112	124	124
Group 7							
SIB1-4	D1	136	136	114	112	130	124
SIB2-4	D1	128	128	114	112	130	124

Table 11. Familial analysis for multiple paternity at three VNTR loci (5-183, CwB6, and CwD15) for 3 maternal-inclusive groups (ID). OS#-# indicates offspring. Four groups of siblings only are represented (SIB#-#).

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CONCLUSION

It is useful to utilize VNTR genotyping as a tool to point the way toward regions on chromosomes are important to the biology of an organism. While seldom contributive to a phenotype, these biomarkers are often road signs to allow for successful description of complex systems or the evolution of a species.