THE MOLECULAR EVOLUTION OF THE SEROTONIN SYSTEM IN MACAQUES (*MACACA*): A DETAILED SURVEY OF FOUR SEROTONIN-RELATED GENES.

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Anthropology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

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ABSTRACT

Serotonin, a hormone produced in the brain, has long been implicated in the regulation of critical behaviors, such as those related to aggression or impulse control. However, most research on serotonin has focused on the proximate connection to behavior, and little is known about its evolution. This is unfortunate, since the serotonin system has great potential to inform our understanding of behavioral evolution. I seek to address this gap in knowledge by investigating the molecular evolution of the serotonin system in macaques (genus *Macaca*).

The macaque genus represents a useful model for understanding behavioral evolution. Comprised of approximately 19 species, macaques display a wide range of behaviors. It is likely that behavioral differences are caused by differences in neuroendocrinology. Therefore, the serotonin system provides one potential mechanism through which evolution may act to shape macaque behavior. In this dissertation, I sequence four genes that are known to influence serotonin functioning and behavior: *HTR1A*, *HTR1B*, *TPH2*, and *SLC6A4*. I examine the pattern of genetic variation within and between several species of macaque, and, using an approach based on molecular evolutionary theory, discern which evolutionary force – positive selection, balancing selection, purifying selection, or random genetic drift – is most likely to have acted on these genes.

Three out of the four genes (*HTR1B*, *TPH2*, and *SLC6A4*), show a low level of overall genetic variation within the coding regions, suggesting that purifying selection is the predominate force acting on these genes. Within non-coding regions, the patterns of genetic variation found are consistent with genetic drift. Thus, positive selection does not seem to be affecting these genes. The genetic variation for these genes may contribute to the behavioral

variation found in macaques; however, any effect that these genes have on behavior is likely due to non-adaptive evolutionary forces.

In contrast to the other genes, *HTR1A* shows a pattern that is clearly distinct. *HTR1A* displays an unusually high level of interspecific variation, which is consistent with positive selection. Moreover, a subset of macaque species share a codon loss, an extremely rare event in gene evolution, and analyses of the coding region indicate a significant elevation of protein evolution among certain sites of the gene. These results suggest that positive selection has played a significant role in the evolution of the serotonin system and it is likely that the effects of positive selection on *HTR1A* contributed to macaque behavioral evolution. This research provides an important first step towards gaining a more thorough understanding of the mechanisms underlying the evolution of behavior.

ACKNOWLEDGEMENTS

This research is the result of a lot of hard work and support from many different people: educators, mentors, collaborators, friends, and family. First and foremost, I thank my advisor, Ripan Malhi, who was willing to take me on as a student when I was searching for direction. He exhibits incredible generosity, patience, and perhaps more than anything, bravery. He always showed an upbeat and optimistic attitude, even through times of incredible hardship and suffering. As a mentor, he was invaluable and I could not have done this without him.

I thank the members of my dissertation committee: Charles Roseman, Steve Leigh, and Lyle Konigsberg. They provided helpful guidance in a number of capacities throughout my time here at Illinois. Not only did they work with me on numerous drafts of my NSF proposal, provide a steady stream of letters of recommendation, and give dead-on critiques of my work, but they also made courses intellectually challenging and thoroughly enjoyable. I am a better scholar because of them. I also thank the people who served on earlier versions of my committee, when I was still undecided on a project. Paul Garber acted as my original advisor and gave me my start in graduate school, and his influence still remains even after I made the move from behavior to genetics. Dario Maestripieri provided me with feedback early on in this project and served on my preliminary committee; his question for my written preliminary exam was in some ways the most difficult and it helped me to more fully grasp the workings of the serotonin system. Rebecca Stumpf served on my intellectual committee and I have been fortunate to take several classes with her, including behavioral endocrinology, which fueled my fascination with hormones, the brain, and behavior. I have also collaborated with Becky on two papers that were exciting and challenging and reminded me why I love anthropology.

Other faculty members in the department have also played a role in my education. Both John Polk and Laura Shackelford (along with Lyle) taught me anatomy. I took one of my very first classes in graduate school with John and the first time I was a TA for ANTH102, John walked me through it. Laura was incredibly patient as a teacher and willing to walk through dozens of demonstrations of the workings of the digestive system the day before an exam. She also understands my obsession with my dog and has willingly made him part of her home when I was away. The wonderful anthropology office staff – Julia Spitz, Karla Harmon, and the always helpful and sunny Liz Spears – were invaluable in helping me navigate through graduate school.

Of course, much of my training here at Illinois happened outside of the classroom. So I must express my great appreciation of Raul Tito, an extremely talented and competent geneticist, who worked with me throughout much of this project, helped me to develop my methods, and taught me almost everything I know about a genetics lab. My thanks to Barb Christensen, who patiently answered my questions, always showed a great enthusiasm, and who took time out of her day to watch me defend this dissertation. My appreciation also goes towards my fellow lab-mates: Aly, Yan, Sylvia, and of course, Jesse, who was with me from practically the beginning. We shared in the frustration of failed cloning experiments, bad sequences data, and all that can wrong in the lab. What I did not learn from Raul or Barb, I learned from them. I am truly grateful that I was able to spend so much time in a lab full of funny, interesting, and intelligent people, which includes a score of undergraduate students (Christine, Jason, Vanessa, Dena, Mary, Tiffani, Kelsey, Caroline, Anish, and I am sure I am forgetting some); everyone who has come through that lab has made it a bright and pleasant place, despite being in a basement.

I would of course also like to thank all of my collaborators on this project: David Smith, Jessica Satkoski Trask, Amos Deinard, and Don Melnick. They generously shared their DNA

samples with me and offered insight and feedback on my work. In addition, Rasmus Nielsen and Alison Bell both took the time to meet with me to discuss this project, and gave me a fresh perspective at times when I was becoming overwhelmed in the details.

My thanks to everyone in The Dissertation Writing Group – Petra Jelinek, Mark Grabowski, Krista Milich, and Scott Williams – who read over my chapters and provided thoughtful critiques. It was reassuring to have others that could empathizes with the painstaking and stressful process of putting together a coherent sentence.

My thanks to my family, and my mother especially, who always believed in me, encouraged my curiosity, and supported me in everything I did. She provided me with a place to live and listened as I called to complain about all of my problems. She taught me the value of patience and sympathy, two characteristics that have brought me far in life. I do not think I will ever meet a kinder of more generous person.

I am especially grateful for two people who have been a great source of support and friendship. Krista Milich has a passion for life that is infectious; she has motivated me to do things I did not think I was capable of (such as running 7 miles in a marathon relay). She is a friend who will fight for you, who will go above and beyond what is asked of her. I am lucky to have met her. And Scott Williams, who never lets a day go by without making me smile. He puts everyone else ahead of him, is generous with his time, is an amazing scholar, and I am exceedingly proud of all that he has accomplished. He is my best friend and has been by far the most rewarding part of my entire experience at Illinois.

Finally, I want to thank the National Science Foundation and the Beckman Institute for Advance Science and Technology for providing me with the funds to carry out this project.

To Scott and Oliver,
whose presence granted me sanctuary.

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CHAPTER 1

INTRODUCTION: A NEW APPROACH TO THE STUDY OF BEHAVIOR

OBJECTIVES

This dissertation is an investigation into the molecular evolution of four genes related to serotonin functioning in macaques (genus *Macaca*), with the objective of deepening understanding of the evolutionary history of the serotonin system and its implications for behavior. Using DNA from several macaque individuals spanning 11 different species, I obtained sequence data on all four genes, including both coding and non-coding regions, and examined patterns of genetic variation within and between species of macaques. I applied multiple tests designed to detect and distinguish between different evolutionary forces – namely balancing selection, purifying selection, positive selection, and random genetic drift – in order to deduce the evolutionary history of the serotonin system in macaques. The methods employed in this research specifically address the molecular evolution of these four genes; as such, the results of this study most directly contribute to understanding of macaque and serotonin evolution at the genetic level. However, the implications of this dissertation research go beyond this. The serotonin system has been widely connected with a number of important behaviors including those related to aggression, dispersal, stress response, parenting and reproduction. Therefore, detailed knowledge of the evolution of the genes underlying serotonin functioning provides a critical step in understanding the mechanisms underlying behavioral evolution in primates and animals in general.

THE STUDY OF BEHAVIOR

Among the many pursuits of biology, the study of the biological basis of behavior presents an especially formidable challenge. Behavior, as an attribute, is both conceptual and transient; it cannot be measured in a concrete, physical way. Therefore, unlike studies based on the morphology or molecular attributes of an organism, behavioral studies must rely on indirect measures that are always somewhat subjective and which can change depending on a variety of circumstances that we do not yet fully understand. Despite such challenges, the study of behavior remains an interesting field of study with high potential. Many advances have been made over the last several decades that have granted us insights into the chemical underpinnings of a variety of behaviors such as depression, parental care, sexuality, and aggression.

Much of the study of behavior is motivated by its implication for human societies. By understanding the proximate causes of behaviors, it may become possible to minimize those activities that we deem detrimental, such as those related to aggression or violence. On this note, there have been extensive efforts in clinical psychology, neurology, and endocrinology, among others, to understand some of the biological factors that induce dysfunctional behavior, and the results of these studies have largely been used to help treat a number of psychopathologies. Closely linked to these studies, genetics has contributed to the understanding of the biology of behavior by pinpointing areas of the genome that are associated with increased likelihood of certain behavioral abnormalities. In most of these areas of study, the emphasis has been on understanding the biological risks of psychopathologies. However, the information runs both ways. Not only is research in neurology applied to psychology, for example, but the study of aberrant behaviors and their treatments are often what give clues as to what areas of the brain are important for regulating behaviors (e.g., Asberg et al., 1976). Likewise, geneticists have largely

been aided by concentrating on individuals who exhibit extreme behaviors (e.g., Brunner et al., 1993a, b). While these studies have been essential in providing an increased understanding in how neurological mechanisms contribute to behavior, in general they have been very narrow in focus; that is, studies are largely restricted to a few model species (such as rats, mice, humans, or fruit flies), usually in a laboratory setting, with an emphasis on abnormal behaviors.

Outside of clinical psychology, genetics, and other fields primarily interested in the immediate causes of behavior, other disciplines seek to place behavioral strategies in an evolutionary context. At this level, scientists try to explain behaviors in terms of the advantages they confer to the actor or its relatives. Here the behaviors addressed are not abnormalities to be remedied, but rather those that are part of an adaptive strategy to be explained in terms of ecology, life history, demographics, and phylogeny. Primatology has traditionally used this approach and has been especially useful in explaining the evolution of behaviors such as aggression and sociality. In particular, the socioecological model (Wrangham, 1980; Van Schaik, 1989; Sterck et al., 1997) has been pervasive in its influence on research in the field. In the socioecological model, the balance between aggression and cooperation in primate societies is largely dictated by the spacing and availability of their food sources. Thus, differences between species in their overall disposition are explained primarily by the ecological niches they inhabit. Unlike studies that focus on the proximate causes of behavior, evolutionary studies of behavior look at a broad range of taxonomic groups and are largely based on observational studies carried out in the field. However, even though the ultimate goal of these studies is to understand behavioral evolution, this approach tends to emphasize the adaptive value of a behavior and is limited in its ability to address other forces of evolution such as drift, despite evidence that these might play a significant role in behavioral evolution (Spuhler and Jorde, 1975; de Queiroz and

Wimberger, 1993; Di Fiore and Rendall, 1994; Chan, 1996; Owens, 2006; Rendall and Di Fiore, 2007).

Thus, studies of behavior might be divided into two groups: those that look for proximate explanations for behavior (where the focus is often on extreme or maladaptive behaviors) and those that seek explanations as to why certain behavioral patterns evolved (where the focus is on a normal or "healthy" range of behaviors). Both of these approaches have limitations, and both have largely remained independent of each other (Robinson et al., 2005). Incorporating knowledge of both areas of research could address these limitations and provide a more sophisticated framework in which to examine the biology of behavior (Robinson et al., 2005).

In this dissertation, I have begun to address this gap in the studies of behavior by examining the molecular evolution of the serotonin system in macaques (*Macaca*). The macaque genus represents a critical taxon for studying behavior, since it consists of several closely related species, all of which display wide behavioral variation. In addition, the serotonin system plays a significant role in regulating behaviors in a variety of animal species, including humans and macaques. Therefore, it is an important biological system to consider when studying the evolution of behaviors. Here, I sequence a set of genes that are known to influence serotonin function and analyze the sequence data using tests designed to detect and distinguish multiple evolutionary forces. This study thus takes a neurological system, researched primarily for its role in regulating behavior, and places it in an evolutionary context. Unlike behavioral evolution studies that rely exclusively on observations made in the field or the lab, examining genetic variation provides a way to statistically test for the role of various evolutionary forces such as balancing selection, positive selection, and purifying selection, as well as non-adaptive evolutionary forces such as genetic drift. In addition, by focusing on a few candidate genes, a

detailed description of the genetic variation within and between species can be obtained, laying the groundwork for future studies examining the connection between genetic and phenotypic variation. For example, genetic variation within the regulatory regions of a gene (non-coding) is likely to underlie differences in expression levels; thus, comprehensive knowledge of sequence variation in all regions of a gene, both coding and non-coding, is necessary to understand phenotypic differences associated with a gene.

The approach used here is not without its limitations. By using a candidate gene approach, practical considerations, including cost and time, restrict the number of genes that can be sequenced; therefore, this dissertation examined only a small portion of genes underlying the serotonin system. Additional studies looking at other genes are needed to fully understand the evolution of the genetic architecture underlying serotonin and behavior. In addition, by focusing exclusively on genetic evolution, little can immediately be said about phenotypic evolution. Many assumptions are made about the connection between the genetic evolution outlined in this dissertation and behavioral evolution.; indeed, the serotonin system in incredibly complex, consisting of several different components and interacting with other important neurological systems (outlined in Chapter 2). Thus, genetic variation can result in phenotypic effects at several different "levels." For example, a genetic mutation in a serotonin gene could affect: expression levels; serotonin turnover; development; plasticity; other parts of the brain with which serotonin interacts, such as the amygdala; epistasis; hormone levels; or behavior. Because these different phenotypic "levels" are not independent of each other, genetic variation can act on any number of these levels simultaneously. Therefore, although the focus and impetus for this study is behavioral evolution, the genetic evolution investigated here could be related to any number of

different phenotypes, which I have subsumed, for the sake of simplicity, under the general category of "serotonin functioning."

Despite these restrictions, this study fills a critical gap in serotonin studies, which have been preoccupied with defining serotonin's proximate role in behavior without addressing its evolution. Furthermore, while this study greatly contributes to evolutionary theory on serotonin and has intellectual merit in its own right, research is never meant to be understood in isolation. To truly understand the evolution of behavior, investigations at all phenotypic levels are needed (Konopka and Geschwind, 2010; Bell and Robinson, 2011); this study represents one such contribution. While this method alone may not directly explain how a given behavior, such as aggression, evolved, when applied with what is known from other studies on behavior, neurology, and development, it deepens our understanding of the biological bases of behavioral evolution, stepping beyond and complementing observational studies of behavior.

A Genetic Approach to Evolution

When selection occurs on an area of a genome, it leaves a distinctive pattern of variation on the sequence (a signature of selection). The nature of this pattern varies depending on both the type of selection occurring (i.e., positive, negative, or balancing) and the timing of the selection event (Nielsen, 2005). Various methods are available for detecting selection at the sequence level and most can differentiate between the types of selection occurring (reviewed in Nielsen, 2005). Therefore, if sufficient sequence data are available, DNA sequences (either individual genes or the entire genome) can be examined for signatures of selection.

Most of the methods for examining selection in the genome are based on two influential concepts: the neutral theory (and nearly neutral theory) and selective sweeps. The neutral theory

(Kimura, 1968, 1985; King and Jukes, 1969) posits that most new mutations are extremely deleterious and are therefore instantly removed from the population. Those mutations that remain are almost always neutral, with beneficial mutations being extremely rare. Thus, most genetic variation seen within a population should be the result of stochastic processes, which is referred to as genetic drift. New mutations will most likely remain at low frequencies and will eventually be lost from the population through genetic drift, while some will gradually reach fixation (frequency of 1) in the population. An expansion on the neutral theory, the nearly neutral theory (Ohta, 1992), states that most mutations that remain in the population are not entirely neutral, but are either slightly deleterious or slightly advantageous. However, given a small enough population size, these mutations will effectively act like neutral mutations because the effects of genetic drift will overwhelm the effects of selection (Ohta, 1992). While the relative contributions of selection and drift to evolution is unclear (see, for example, Gillespie, 1989, 2000a, b), the benefit of the neutral theory is that it provides a straightforward model for what is expected in the absence of selection.

The second concept that has had a major influence in molecular evolutionary theory is that of selective sweeps. When a gene is being selected for, its frequency in the population will quickly increase. However, segments of the DNA that surround the selected gene will also rise in frequency due to the linkage between the loci, an effect known as genetic hitchhiking (Smith and Haigh, 1974). If the selected gene reaches fixation (and subsequently fixes the surrounding genetic area) this is known as a selective sweep. Genetic hitchhiking and selective sweeps are predicted to drastically lower the levels of polymorphism in the areas surrounding the selected locus (Smith and Haigh, 1974), particularly where selection is strong and recombination is infrequent (Kaplan et al., 1989), providing a strong signal of selection.

Thus, both the neutral theory and the concept of selective sweeps provide a way to examine genetic data for signatures of selection. There are a number of different analyses that can be employed, and these differ primarily in which aspect of the genetic data that they use. For example, some are designed specifically for coding regions, or require comparison between species, while other analyses can be applied to any part of the genome and rely on intraspecific data. Each type of analysis has its strengths and limitations. One of the major shortcomings of many tests of selection is that they often cannot differentiate between selective events and demographic events. For example, in many ways a population expansion is expected to mimic the effects of selection because both processes involve an increase in reproductive output. The main difference between these two processes is that, with selection, only a small subset of the population – those carrying the favored allele – will experience an increase in reproductive success. In contrast, in a general population expansion, roughly all members of a population are expected to contribute to the increase in population size. Because of this complication, these tests often assume that there is constant population size, no migration, no population subdivision, and no recombination. Violation of these assumptions can either weaken the power of these tests to detect selection or increase the rate of false positives (detect selection when in fact there is no selection occurring), depending on the level of analysis. Therefore, the use of multiple tests that utilize different aspects of the genetic data are usually required to confirm the presence of selection.

To examine the role of selection in the evolutionary history of serotonin, I have employed six different tests of selection. Analyses used in this dissertation can be categorized into three major types: those that examine the ratio of synonymous and nonsynonymous mutations, which include the **ω** test, the McDonald-Kreitman test, and Phylogenetic Analyses using Maximum

Likelihood (PAML); those that look at the frequency spectrum, which includes Tajima's D and Fay and Wu's H; and those that examine the ratio of within- versus between-species variation, which here includes a single test, the HKA test. These tests are described and compared here.

The Ratio of Nonsynonymous to Synonymous Mutations

One straightforward test of selection is to examine the rate of synonymous versus nonsynonymous evolution between species. Within a gene, synonymous mutations are those that do not affect the structure of the protein for which the gene is coding; as a result, they are predicted to be neutral. In contrast, nonsynonymous mutations cause a change in protein structure and are thus more likely to be exposed to selection. At the most basic level, these selection tests look at the ratio of nonsynonymous to synonymous mutations between species (**ω**) (Kimura, 1977). Where **ω** < 1, this indicates purifying selection (selection against new mutations); where **ω** > 1, this indicates positive selection (selection favoring new mutations); where **ω** = 1, this indicates neutrality (Kimura, 1977). The significance of this can be calculated using a Z-test:

$$Z = \frac{(d_N - d_S)}{\sqrt{(Var(d_N) - Var(d_S))}},$$

where d_N is the number of nonsynonymous mutations per nonsynonymous site, d_S is the number of synonymous mutations per synonymous site, and $Var(d_N)$ and $Var(d_S)$ are their respective variances. The main advantage of the **ω** test is that it is robust to demographic assumptions and other non-selective processes. This is because nonsynonymous and synonymous sites are interspersed with each other, so that there is no reason to think that recombination or demographic events such as population expansion or migration, would affect nonsynonymous sites to greater degree than nonsynonymous sites (McDonald and Kreitman, 1991). However, the **ω** test is also quite conservative. This is because there may be many reasons why there may be a

relatively low number of nonsynonymous mutations, even if there is positive selection acting on the gene. For example, a functional gene might experience selective restraints such that most nonsynonymous mutations are deleterious. This will produce a low **ω** even if there were several nonsynonymous mutations that experienced positive selection. Additionally a single (or even a few) beneficial mutations would not register for this type of test. It would take multiple beneficial mutations to achieve a significant result. For this reason, **ω** tests are generally only useful for genes that would be expected to undergo multiple selective events, such as those involved in an "arms race" (e.g., genes involved in immunity or reproduction). This might also occur where a loss of function is selected for or where there is a heterozygous advantage.

Because of these restrictions, additional tests have been developed that are based on the basic premise of examining **ω**, but are far less stringent. One of these is the McDonald-Kreitman test (McDonald and Kreitman, 1991). McDonald-Kreitman compares the ratio of synonymous to nonsynonymous mutations that occur within a species to those that occur between species. Under neutral evolution, **ω** should be the same whether looking at variation within or between species (McDonald and Kreitman, 1991). However, positive selection is expected to increase the rate of fixation of new mutations, elevating the rate of nonsynonymous differences between species relative to the polymorphisms found within species (McDonald and Kreitman, 1991). Thus, if a significantly different **ω** is found within species than between, this generally indicates positive selection. Like the basic **ω** test, McDonald-Kreitman is robust to most demographic assumptions, although in certain usages the presence of recombination can increase the rate of false positives (Andolfatto, 2008). Compared to the **ω** test, McDonald-Kreitman in far less conservative since the overall value of **ω** does not have to be greater than 1 to achieve a significant result.

Another way to increase the power of the **ω** test is to examine evolution in a phylogenetic context. Phylogenetic Analyses using Maximum Liklihood (PAML) is a program that generates the likelihood values of different models of evolution within a phylogenetic framework. Models that incorporate selection can be compared to those that assume neutrality and likelihood values can be compared to determine which model best fits the data. Two types of selection tests based on **ω** can be used in PAML. In the first, **ω** values along different branches in the phylogenetic tree are compared to each other to see if they are significantly different from each other. An elevated value of **ω** along one branch indicates positive selection in that lineage. Because **ω** does not have to be greater than 1 but simply elevated compared to other lineages of the macaque phylogeny, this test is less conservative than the basic **ω** test. In the second, **ω** among different sites in the gene are compared. Because most sites on a functional gene are expected to experience strong purifying selection, the overall **ω** value of the gene is expected to be less than 1, even if a small portion of the gene is under positive selection. However, by allowing **ω** to vary among sites, PAML can detect elevations in **ω** occurring on just a portion of the gene. Like all tests based on **ω**, analyses using PAML should be robust to demography, but moderate levels of recombination can increase the rate of false positives, at least for analyses that examine different sites within a gene (Anisimova et al., 2003).

The Frequency Spectrum

The frequency spectrum is a measure of the allele frequencies in a population. Under neutrality, there should be a large proportion of low-frequency alleles, with higher-frequency alleles becoming increasingly rarer. Balancing selection, by definition, will maintain alleles at intermediate frequencies, creating a deviation from the expected frequency spectrum. In contrast, both negative selection and selective sweeps will tend to create an excess of rare genetic variants

(Charlesworth et al., 1993; Braverman et al., 1995). In this case of negative selection, this occurs because selection acts to keep new mutations at low frequencies. In the case of a selective sweep, the selected allele and linked loci will quickly rise in frequency, lowering variation around the selected locus. Once the selected allele has reached fixation, new mutations will appear, but at low frequency; thus, both positive and negative selection are expected to have similar effects on rare genetic variants. However, during a selective sweep, when the selected allele has not quite reached fixation, there should also be an increase of high-frequency variants (that is, the selected genetic variant and those linked to it) (Fay and Wu, 2000), which is not expected in negative selection. Based on these expectations, various tests have been designed that examine changes in the frequency spectrum (Ewens, 1972; Watterson, 1978; Tajima, 1989b; Fu and Li, 1993; Fu, 1996, 1997; Fay and Wu, 2000). Unlike analyses based on **ω**, which are restricted to the coding region, tests based on the frequency spectrum can examine evolution on any portion of the genome, including potential regulatory regions. This dissertation makes used of two of these tests: Tajima's D and Fay and Wu's H. Tajima's D (Tajima, 1989b) compares two different estimates of the parameter theta ($\theta = 4N\mu$, where N is the effective population size and μ is the mutation rate): θ_S (Watterson, 1975) and θ_π (Tajima, 1983). The estimate θ_S is determined by the number of segregating sites, which is more heavily affected by low frequency variants; the estimate θ_π is determine by the average number of pairwise differences, which is most affected variants at intermediate frequency. When there is an excess of rare alleles, Tajima's D is significantly negative ($\theta_S > \theta_\pi$), which is consistent with positive selection (or selection against slightly deleterious mutations) (Tajima, 1989b; Charlesworth et al., 1995). When there is an excess of intermediate-frequency alleles, Tajima's D is significantly positive ($\theta_S < \theta_\pi$), which is consistent with balancing selection. The main drawback of Tajima's D is that it is highly

sensitive to past demographic events, such as population bottlenecks and expansions (Tajima, 1989a). For example, a population bottleneck will tend to eliminate rare alleles, creating an effect similar to balancing selection. It is therefore difficult to differentiate selection from demographic events. However, Fay and Wu's H is much more robust to demographic assumptions (Fay and Wu, 2000). Fay and Wu's H is similar to Tajima's D, except that the two parameters that are compared are θ_π and θ_H. The parameter θ_H is most heavily affected by derived genetic variants (as determined by an outgroup) at high frequency. In this case, an excess of high frequency alleles will produce a significantly negative H-value ($\theta_\pi < \theta_H$), indicating positive selection. The advantage of Fay and Wu's H is that it can differentiate between positive and negative selection. In addition, because it relies on derived mutations, it should be less likely to be affected by past demographic events (Fay and Wu, 2000; but see Przeworski, 2002). For example, a population bottleneck, followed by a population expansion, might be expected to create an excess of high-frequency variants. However, because bottlenecks are expected to eliminate rare genetic variants (i.e., new, derived mutations), such a demographic event is not expected to create an excess of high-frequency *derived* variants. The main limitation of Fay and Wu's H is that there is a very narrow time frame in which it can detect selection; once a selective sweep is complete and the selected allele has reached fixation, then genetic variation is eliminated (Przeworski, 2002).

Comparison Within and Between Species

Neutral theory predicts that the mutation rate at a locus drives the level of variation both within and between species. Although different loci will have different mutation rates, the ratio of variation within to variation between species should be the same across loci if they are evolving neutrally. Departure from this expectation indicates that selection is occurring (Hudson

et al., 1987). This pattern can be tested using the HKA test (Hudson et al., 1987). For this test, the gene of interest is compared with other loci, preferably ones that are not expected to have experienced selection (i.e., are neutral). A significant p-value is obtained when the ratio of variation within to variation between species varies across loci. Specifically, if departure is due to a reduction of within species polymorphism in the candidate gene, this may be interpreted as the result of a selective sweep. If within species polymorphisms are increased, this is consistent with balancing selection. Like tests based on the frequency spectrum, the HKA test is advantageous because it is not limited to coding regions. In addition, because demographic events are expected to affect all loci in the genome, an approach that compares multiple loci, such as the HKA, should be unaffected by demography (but see Nielsen, 2001). However, the presence of recombination can make it more difficult for this test to detect selection (Hudson et al., 1987).

Because all of the analyses used in this research examine slightly different aspects of genetic data, they are not expected to yield identical results. For example, if the population size of the species examined has not remained constant, then those tests that are more sensitive to demography are likely to present different results from those that are not. Similarly, the presence of recombination will affect tests such as PAML differently than the HKA. Indeed, contrasting results will be informative as the likely cause of evolutionary change. For instance, if tests based on ω were all non-significant, but HKA indicated positive selection, this could mean that selection is acting primarily in the non-coding, regulatory regions of the gene. However, unless multiple tests confirm the presence of selection, results should be interpreted with caution. It is with these statistical tools that I address the evolution of the serotonin system in macaques.

OVERVIEW OF DISSERTATION

This dissertation is organized into a series of manuscripts that are largely designed to be read and understood independently. Therefore, some repetition of background and methodology exists. Chapter 2 provides background information on macaques, the serotonin system, and behavior. For each of Chapters 3, 4, and 5, I examine the genetic variation for a gene or set of genes within and between several species of macaques. I apply statistical tests of selection to determine the role of positive selection on each gene and discuss their implications. In Chapter 3, I look at both *HTR1A* and *HTR1B*, and find strong evidence for selection for *HTR1A*, but not *HTR1B*. In Chapter 4, I focus on *TPH2*. In particular, I emphasize the genetic variation identified for this gene as very few studies to date have looked at this in macaques. I find that, overall, *TPH2* seems to be evolving neutrally. In Chapter 5, I examine the evolution of *SLC6A4*. As with *TPH2* and *HTR1B*, I find that selection is not occurring on this gene. However, I did find a low level of linkage between the promoter region and the gene itself. Because I focused on the genetic variation within and immediately around *SLC6A4* and not the promoter region, it is possible that the promoter region has a distinct evolutionary history from the rest of *SLC6A4*. In Chapter 6, I summarize the results of these studies and discuss the implications of my findings and their contribution to evolutionary studies. Finally, I outline the next steps in research that I plan to take.

DATA ACCESS

The bulk of this dissertation consists of three chapters (Chapters 3-5) that outline the methodologies used and results obtained. These chapters are written in the format of scientific papers, to be submitted for individual publication with minimal modification. As such, these

chapters focus primarily on the most pertinent results, with detailed description of redundant or non-informative analyses removed. The appendices provide a more complete description of the results; any additional information not provided here is available at the request of the author.

All of the sequence information obtained in this research will be deposited into GenBank, and will be available to the public upon publication of the respective chapters in scientific journals. At the time of deposit of this dissertation, the submission process of sequence data to GenBank has been started, but no accession numbers have yet been assigned. However, the sequence data will be associated with the author's name (M.R. Shattuck) and a publication title similar to the chapter titles, and can thus be found through a search in the NCBI database (http://www.ncbi.nlm.nih.gov/). Readers interested in obtaining the sequence data before they are made public should contact the author (mshattuc@gmail.com).

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CHAPTER 2

MACAQUES, THE SEROTONIN SYSTEM, AND BEHAVIOR: A REVIEW

OVERVIEW

In this chapter, I will provide some of the background information for this project. First, a general overview of the macaques and their social system is provided, and an argument is made for their importance in behavioral evolution and molecular studies. This is followed by a brief review of the history of serotonin study and the mechanisms of the serotonin pathway. The bulk of this chapter will consist of an extensive review of the evidence for the relationship of the serotonin system to behavior in primates, with a particular focus on humans and macaques. Studies on the serotonin system and behavior are divided into three parts: population level studies, clinical studies, and genetic studies. In particular, the review of genetic studies will identify key genes that show great promise for their ability to predict behavior and will therefore be used as the focus of study in the following chapters. In addition, as it relates to this project, I will discuss any evidence available for the possible role of selection on these genes.

It should be noted that, to date, the vast majority of studies on serotonin have concentrated on a suite of behaviors that include, but are not limited to, exploration, alcoholism, dispersal, arson, murder, obsessive compulsive disorders (OCD), and anxiety. The general framework under which these behaviors are discussed treats them as manifestations of a decrease in impulse control. Of course, many of these behaviors, such as arson, are unique to humans, or at least rarely seen among nonhuman primates. Because we are more limited in the types of behaviors that can be observed in nonhuman primates, discussion for nonhuman primates frequently centers on impulsivity as it relates to aggression. Thus, this review will almost

exclusively discuss the connection between serotonin, aggression, and impulsivity. However, it should also be noted that this focus is narrow, and studies have increasingly expanded to include behaviors related to sociality (Samochowiec et al., 1999; Caspi et al., 2002; Anstey et al., 2009; Crockett et al., 2010), sex (Dominguez and Hull, 2010; Liu et al., 2011), and cognition (Borg, 2008; Ogren et al., 2008). A new framework is emerging in which these behaviors are seen as outcomes of increased sensitivity to environmental cues (Homberg and Lesch, 2010), and the genes underlying these behaviors viewed as "plasticity" genes (Belsky et al., 2009). I will therefore briefly review the role of environment in these genetic studies and discuss its relationship to evolution. Finally, I will address some of the limitations and challenges of the current research on behavior, biology, and genetics.

MACAQUES

This dissertation examines the evolution of the serotonin system within the macaque genus (*Macaca*), with the goal of adding to the understanding of behavioral evolution within and between species. Several features make the macaque genus useful for the study of the evolution of behavior, namely 1) a well studied phylogeny with a large number of species, 2) a wide range of ecological habitats, and 3) a diverse set of species specific behaviors. These three features allow for the testing of multiple hypotheses about behavioral evolution. In particular, they permit the examination of the influence of both ecology and phylogeny on the expression of behaviors.

The macaque genus is comprised of around 19-22 different species, depending on the classification used (see Fa, 1989 for a review); the relationship of these species with each other, as reconstructed from both morphology and genetics, is fairly well understood (Fooden, 1976; Hoelzer and Melnick, 1996). These species span an exceptionally large geographical range

(Lindburg, 1991); the rhesus macaque (*M. mulatta*) in particular inhabits a variety of habitat types, dwelling in both primary forests and alongside humans in urban settings (Figure 2.1). In fact, outside of humans, macaques have the widest distribution of any living primate. In addition, the macaque genus is notable for exhibiting a wide range of behavioral attributes. All macaque species share the same basic social structure (reviewed in Thierry, 2000). They form large multimale-multifemale groups where the females are philopatric and matrilines form the basis of hierarchies that are relatively stable. In contrast, the males migrate at sexual maturity and their rank fluctuates through time. In terms of aggressive and reconciliatory behavior, macaque species range from a fairly tolerant social structure (e.g., *Macaca tonkeana*) to more aggressive groups with strict hierarchies (e.g., *M. mulatta*) (Thierry, 1985, 2000; Caldecott, 1986; De Waal and Luttrell, 1989; Clarke and Lindburg, 1993; Aureli et al., 1997; Petit et al., 1997). Initially, the behavioral differences among macaque species were interpreted as the result of varied ecological conditions and mating strategies (Caldecott, 1986; De Waal and Luttrell, 1989; Clarke and Lindburg, 1993; Aureli et al., 1997). That is, certain species of macaques were argued to behave more aggressively toward group members because food resources were scarcer and external threats (i.e., predators and other conspecifics groups) were relatively rare. However, more recent research has emphasized the influence of phylogeny (Di Fiore and Rendall, 1994; Chan, 1996; Petit et al., 1997; Thierry, 2000; Thierry et al., 2000; Rendall and Di Fiore, 2007). Thierry (2000) proposed categorizing macaque species according to a 4-grade scale based on behavior, with grade 1 being the most aggressive and grade 4 being the least aggressive. He showed that these categories map well onto macaque phylogenies, with little homoplasy necessary to explain its distribution (Figure 2.2) (Thierry, 2000; Thierry et al., 2000). Based on this observation, Wendland et al. (2006) suggested that genetic differences, rather than

environmental differences, contributed the behavioral variation seen in this genus (although see Chakraborty et al., 2010). Thus, this genus presents a useful model for examining the genetic basis of interspecific behavioral variation.

In addition to these features, macaques are an important focus of study because they share many features that parallel humans; consequently, knowledge of behavioral evolution in this group can grant insight into human behavioral evolution as well. This is especially true for rhesus macaques (*M. mulatta*). For example, rhesus macaques have a wide geographic range (Figure 2.1), they do particularly well in human inhabited regions (Richard et al., 1989), and they possess many behavioral characteristics similar to those of humans, such as individualism and competition for power (Maestripieri, 2007). Moreover, they are frequently used as animal models in biomedical research, reflecting many of the physiological similarities they share with humans; these similarities extend to the serotonin system, which is especially evident when examining serotonin related genes. Several genetic variants have been identified within macaques, particularly rhesus macaques, which are similar to those found in humans (e.g., Lesch et al., 1997). The genetic variants found in macaques affect serotonin functioning and behavior in a manner analogous to those found in humans. However, these genetic variants are not identical to those in humans and are likely to have evolved independently (Lesch et al., 1997). This raises the possibility that similar selective pressures led to parallel evolution of the serotonin system in humans and rhesus macaques. If this is so, future comparative studies between humans and macaques may highlight important factors that influence behavior, allowing for testing of hypotheses of behavioral evolution. Moreover, the genetic parallels present between macaques and humans highlight the serotonin system as an important focus of study.

SEROTONIN

In 1953, an attempt to improve the current treatment of tuberculosis resulted in the creation of iproniazid (Fox and Gibas, 1953; Pletscher, 1991). Patients treated with iproniazid often expressed states of euphoria as a side effect and later the drug began to be used on depressed patients (Pletscher, 1991). At the same time, Zeller and colleagues (1952) discovered that iproniazid acted to inhibit monoamine oxidase (MAO), an enzyme that acts to breakdown serotonin. In a similar manner, during the early 1950's, research on antihistamines led to the development of imipramine, a tricyclic antidepressant that works in part by blocking reuptake of serotonin (Pletscher, 1991). The actions of these drugs, combined with the knowledge that another new drug, LSD, interacted with the serotonin system, pointed to serotonin as a key factor in moderating behavior. Based in large part upon the research of these early drugs, and others like them, studies were conducted to determine if inherent differences in serotonin levels could explain the presence of certain psychiatric conditions. These studies indicated lower serotonin levels in depressed patients (Ashcroft and Sharman, 1960; Ashcroft et al., 1966; Dencker et al., 1966; Coppen, 1972; Asberg et al., 1976) as well as in suicide victims (Shaw et al., 1967; Bourne et al., 1968) when compared to controls. These early studies opened the doorway for an abundance of research on serotonin. In the years to follow, knowledge of the neurotransmitter's influence would expand to include a wide range of behaviors, including aggression, sexual behavior, sociality, dispersal, stress response, and impulsivity.

Overview of the Serotonin Pathway

Serotonin is a phylogenetically old, well conserved system that is influential in many animal taxa, including both vertebrates and invertebrates. Its organization is similar across vertebrate taxa including birds, reptiles, and mammals (reviewed in Challet et al., 1996). Within

both vertebrates and invertebrates, serotonin plays a critical role in modulating behaviors. While the exact relationship between serotonin and behavior seems to differ between vertebrates and invertebrates, serotonin has been shown to play a role in behaviors in crickets, crustaceans, and fruit flies (reviewed in Kravitz and Huber, 2003), as well as reptiles (Deckel, 1996; Matter et al., 1998; Summers et al., 2005), birds (Ison et al., 1996; Sperry et al., 2003, 2005), fish (Winberg et al., 1993; Overli et al., 1999; Perreault et al., 2003; Clotfelter et al., 2007), and mammals (see below).

Serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter that is produced in the raphé nuclei of the brainstem and released to various parts of the brain (Figure 2.3). 5-HT is synthesized from tryptophan, an amino acid acquired through diet. Tryptophan is converted to 5-HT via a metabolic pathway that includes the enzymes tryptophan hydroxylase and amino acid decarboxylase (Figure 2.4). Serotonin is broken down by monoamine oxidase (MAO), which has two forms: form A (MAOA) and form B (MAOB). The breakdown of serotonin by MAO converts 5-HT into its metabolite 5-hydroxyindoleacetic acid (5-HIAA). 5-HIAA is relatively easily measured in cerebral spinal fluid (CSF), urine, or other bodily fluids and its levels are often used as an indication of overall serotonin activity (Murphy, 1990).

The basic schematic of the serotonin pathway is provided in Figure 2.5. 5-HT is produced in the neuron and released into the postsynaptic space. There, 5-HT can bind with numerous receptor types. There are 17 different receptor types that are classified based upon their function (Bradley et al., 1986). Most of these receptors are G-protein coupled receptors; the only exception to this is the 5-HT_3 receptor, which is a ligand-gated ion channel (Bradley et al., 1986; Gaddum and Picarelli, 1997; Barnes and Sharp, 1999; for a full review of the structure and functions of each receptor type, see Barnes and Sharp, 1999). While most receptor types are

located on the postsynaptic neurons, receptor types 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} are located both postsynaptically (where they are termed heteroreceptors) and on the neurons themselves (presynaptically, or autoreceptors). These three receptor types seem to act as inhibitors when they are presynaptic. When activated they prevent the neuron from firing, and thus provide a mechanism for self-regulation for the serotonin system (reviewed in Barnes and Sharp, 1999).

In addition to these three receptor types, the serotonin transporter (5-HTT) also works to regulate serotonin levels in the brain. By moving serotonin molecules from out of the post-synaptic space and back into the neuron, the serotonin transporter helps to limit the effect of serotonin by restricting its contact with receptors. However, as a feedback mechanism, the serotonin transporter appears to do more than just act as a doorway for 5-HT. When serotonin reuptake is reduced (for example, by means of selective serotonin reuptake inhibitors, SSRIs), there are multiple consequences. As expected, it leads to an increase in 5-HT levels in the synaptic spaces throughout the brain (Adell and Artigas, 1991; Bel and Artigas, 1992; Fuller, 1994). However, at least in the short term, it also leads to an overall decrease in serotonin turnover. Less tryptophan is converted into 5-HT (Carlsson and Lindqvist, 1978), there is a decrease in the levels of 5-HIAA in the body (Sheline et al., 1997), and the serotonergic neurons fire less often (de Montigny et al., 1990).

Once serotonin is produced, it is released to various parts of the brain where it acts to modulate the activity of these neurological systems (Figure 2.3). Two important neurological structures with which serotonin interacts are the prefrontal cortex and the amygdala (Figure 2.3). The prefrontal cortex controls decision making, including the choice of appropriate behavior given a particular the social and environmental context (Miller et al., 2002); the amygdala is involved in emotional response, particularly related to negative stimuli and fear (LeDoux, 2000,

2003, 2007). Both are likely regulated by serotonin (Mann et al., 1996; Hariri et al., 2002, 2005; Clarke et al., 2004, 2007; Furmark et al., 2004; Bertolino et al., 2005; Brown et al., 2005; Canli et al., 2005a, b; Heinz et al., 2005; Pezawas et al., 2005) and serotonin's role in regulating behavior is likely mediated, in part, through these structures.

THE ASSOCIATION BETWEEN SEROTONIN AND BEHAVIOR

Research on serotonin can be divided into three groups: population studies, clinical studies, and genetic studies. Population studies examine how natural variation in serotonin functioning is related to differences in behaviors. Clinical studies artificially manipulate components of the serotonin system, usually through pharmaceutical intervention, in order to determine the exact mechanisms underlying serotonin's role in regulating behavior. Finally, genetic studies examine variation in genes connected with serotonin to determine the role these genes play on behavioral variation. A review of these studies is presented here.

Population Studies

Researchers often examine how the natural variation in serotonin activity is correlated with various behaviors in a population. Overall serotonin activity is typically measured in two ways. The most common way is to look at the level of the serotonin metabolite (5-HIAA) in bodily fluids such as cerebral spinal fluid (CSF), urine, or blood. In this case, a higher concentration of 5-HIAA implies a higher level of serotonin activity. Another commonly used method is to measure the levels of certain hormones (particularly prolactin) following administration of a 5-HT agonist, such as fenfluramine. These agonists excite the serotonin system, which stimulates the pituitary gland to release hormones (Quattrone et al., 1983). The greater the neuroendocrine response to a 5-HT agonist “challenge”, the greater the inferred

sensitivity of the serotonin system (Quattrone et al., 1983; Yatham and Steiner, 1993). However, this particular method tends to work best in males, as differing results may be found in women depending on their current menstrual phase (McBride et al., 1990; Manuck et al., 1998; Steiner et al., 1999).

Highlighted here are just a few of the foundational studies that have examined serotonin activity and behavior at the population level, with an emphasis on aggressive and impulsive behavior. Since these studies were initially carried out, most of the results have been replicated in hundreds of additional studies, at least within humans (although there are some inconsistencies; see, for example, Fishbein et al., 1989; Wetzler et al., 1991; Castellanos et al., 1994; Zhou et al., 2006). Thus, the overall trends outlined here are quite robust, even if the exact relationship between serotonin and behavior is not clearly understood (see **Clinical Studies**).

Humans

The general trend found in humans is the higher the degree of aggression and impulsivity, the lower the overall levels of serotonin activity. Brown and colleagues (1979) found a negative correlation between CSF 5-HIAA and aggressive behavior in military men diagnosed with various personality disorders. In addition, those subjects that had a history of suicide attempts had significantly lower CSF 5-HIAA than those without a history of suicide (Brown et al., 1979). Lidberg et al. (1985) also found lower CSF 5-HIAA in men who attempted suicide and in men who murdered sexual partners, but not in other types of murderers. In this study, impulsiveness rather than aggression itself seemed to be the important factor. Similarly, males who commit impulsive versus premeditated murders have a lower CSF 5-HIAA (Linnoila et al., 1983), as do impulsive male arsonists (Virkkunen et al., 1987) and impulsive alcoholics (Virkkunen et al., 1994). In children and adolescents with disruptive behavior disorders, CSF 5-HIAA negatively

correlates with aggression towards other people (Kruesi et al., 1990). On the opposite side of the spectrum, there is some evidence that people with obsessive-compulsive disorder (OCD) have higher concentrations of CSF 5-HIAA than controls (Thoren et al., 1980; Insel et al., 1985), although the difference may not be significant (Thoren et al., 1980). Since patients with OCD are often characterized by the inability to act on aggressive impulses (Insel et al., 1990), a higher rate of serotonin turnover fits in with general trend found with serotonin, aggression, and impulsivity. While these general findings have been repeated in numerous studies, the main problem with this research is the tendency to focus on people with pathologies (Haller et al., 2005), particularly males, rather than the general population. It is therefore less clear whether more "adaptive" forms of aggression (Ferrari et al., 2005; Table 2.1), which may have more relevance for evolutionary questions, have a similar relationship with serotonin.

Studies done in “normal” populations are both rare and less consistent. Mannuck and colleagues (1998) found a negative correlation between prolactin response in a fenfluramine challenge and various measures of aggression and impulsivity, but only in males. In women, a similar trend was found only when considering postmenopausal subjects. This may be due in part to the method used to measure serotonin activity since fenfluramine challenges are more consistent in men. Roy, Adinoff, and Linnoila (1988) used CSF 5-HIAA and found a significant negative correlation between CSF 5-HIAA and the “urge to act out hostility”. However, when factoring out the influence of sex, this trend became non-significant. More studies are needed to verify the link between serotonin and aggression/impulsivity in the general population and in women in particular, but it is likely that the weakened correlation seen between genetic variation and behavior in may be due to intervening environmental effects (see **SEROTONIN GENES AND PLASTICITY** below).

Nonhuman primates

As with humans, a negative relationship between serotonin functioning and impulsivity is found in nonhuman primates. In rhesus macaques (*M. mulatta*), both males and females show a negative correlation between CSF 5-HIAA and the frequency of long leaps made at dangerous heights in the forest canopy (Mehlman et al., 1994; Westergaard et al., 2003b). Male vervet monkeys (*Chlorocebus aethiops*) with lower CSF 5-HIAA are more likely to approach a stranger (Fairbanks et al., 2001); likewise female cynomolgus monkeys (*M. fascicularis*) are more likely to approach strangers if they have lower serotonin turnover as measured by a fenfluramine challenge (Manuck et al., 2003). CSF 5-HIAA is also negatively correlated with age at emigration in male macaques (Mehlman et al., 1995).

In regards to aggression, the association with serotonin in nonhuman primates is typically only seen when examining *severe* aggression rather than overall rates of aggression. For example, in male rhesus macaques, low CSF 5-HIAA corresponds with high levels of aggression, but not overall rates of aggression (Mehlman et al., 1994; Higley et al., 1996a, c; Howell et al., 2007). In addition, young rhesus macaques with low CSF 5-HIAA are less likely to survive to adulthood (Higley et al., 1996b; Westergaard et al., 2003a; Howell et al., 2007), and the increase in mortality is at least partially related to aggression (Higley et al., 1996b). Thus, rather than finding an association between serotonin and more ritualized or adaptive forms of aggression (e.g., mounting, eye contact, etc.), it is more commonly found with aggression that can potentially have severe consequences for the actor and may even be maladaptive (but see Eaton et al., 1999). In this regard, the association found in nonhuman primates may be analogous to that found in humans with psychopathologies. That is, a correlation may be strongest in primates that show abnormal levels of aggression, but is weaker in "healthy" subjects that do not

display severe forms of violence. Also similar to humans, this association in nonhuman primates may be more robust in males. Westergaard and colleagues (2003b) found a negative correlation between CSF 5-HIAA and low levels of aggression in female rhesus macaques, but not for high levels of aggression. They attributed this finding to differences in female sociality where relatives can act as "social buffers." However, the link between serotonin function and aggression seems to be largely generalizable to both sexes.

Clinical Studies

In contrast to population studies that look at natural variation in serotonin activity, clinical studies chemically manipulate various parts of the serotonin system to see the effect it has on behavior. While these studies are probably not very useful for explaining how serotonin influences behavior in more natural settings, and therefore how it may have been influenced by evolution, they do help to determine the exact mechanisms by which serotonin influences behavior. Also in contrast to population studies, which show a straightforward relationship between serotonin and behavior, clinical studies demonstrate that the mechanisms behind this relationship are exceedingly complicated and minimally understood; a general consensus as to how the serotonin system works to influence behavior has not been reached. Nevertheless, an overview of what has been discovered to date is warranted here.

Because of the association found between low CSF 5-HIAA levels and aggression, the general paradigm put forward is that serotonin helps to increase moods and to control aggressive behaviors, in particular those related to impulse control (Soubrie, 1986). Various clinical studies seem to support this model, particularly for those studies based on the effects of antidepressants. Prolonged treatment of selective serotonin reuptake inhibitors (SSRI's), a form of antidepressant, is thought to increase 5-HT transmission. Although initial treatment of an SSRI causes a

decrease in neuron firing activity, after two weeks of continued treatment, firing activity of the neurons is restored (Blier et al., 1990). This supports the observation that antidepressants typically take a couple of weeks to become effective. This reactivation is thought to occur through a desensitization of serotonin autoreceptors, which normally act to inhibit firing activity (de Montigny et al., 1990). The same appears to be true for MAOA inhibiters (reviewed in Blier and de Montigny, 1994). Tricyclic antidepressants, however, appear to work by increasing sensitivity of postsynaptic receptors (Chaput et al., 1991). The mechanisms by which these drugs act all point to an increase in serotonin transmission as decreasing the symptoms of clinical depression.

However, the general idea that decreased serotonin signaling increases aggression and impulsivity may be oversimplified. Various studies indicate that the activation of the 5-$HT_{1A/1B}$ receptor types, as well as inactivation of 5-$HT_{2A/C}$ receptor types, leads to a reduction of aggressive behaviors (de Boer and Koolhaas, 2005 and sources therein). However, many of these drugs also cause a reduction of overall motor activity, making it unclear whether they affect aggression *per se* (de Boer and Koolhaas, 2005). Nevertheless, certain pharmaceuticals (termed serenics) are capable of reducing aggression without affecting other behaviors, particularly those that act as agonists for 5-$HT_{1A/1B}$ (Bell and Hobson, 1994; Olivier et al., 1995; de Boer et al., 1999). One major problem with these agonists, though, is that 5-$HT_{1A/1B}$ receptors can be found on both postsynaptic neurons and on the serotonin neurons themselves (see Figure 2.5). Therefore, 5-$HT_{1A/1B}$ agonists can either activate the autoreceptors, which inhibit serotonin neuron firing, or they can activate the heteroreceptors, which increases serotonin signaling, or both. Studies which remove the autoreceptors via specific neurotoxins, have found that 5-HT_1 agonists are still effective in reducing aggression, indicating that it is the postsynaptic receptors

that are responsible for this effect (Sijbesma et al., 1991; Sanchez and Hyttel, 1994; de Almeida et al., 2001). However, de Boer and Koolhaas (2005) have pointed out that these neurotoxins can affect the level of aggression regardless of administration of 5-HT_1 receptor agonists and they can trigger alterations of neuronal activity in other parts of the brain (e.g., Sijbesma et al., 1991). In contrast, S-15535 is a drug that acts as both a postsynaptic receptor antagonist and an autoreceptor agonist, which decreases serotonin neuron firing and acts effectively to reduce aggression (Millan et al., 1993; de Boer and Koolhaas, 2005). Collectively, these studies suggest that serenics act by reducing serotonin transmission rather than enhancing it.

Furthermore, there are differences in the kinds of aggression that an animal may display (Table 2.1). For example, there is a distinct difference between an aggressive *state* (a situational or temporary emotional state) and an aggressive *trait* (a stable personality trait where aggression is either chronic or more easily triggered). These different types of aggression may be influenced by slightly different neuronal mechanisms (de Boer and Koolhaas, 2005; Ferrari et al., 2005; Haller et al., 2005; Olivier and van Oorschot, 2005). For example, the results found when looking at immediate changes in serotonin levels in individuals during aggressive episodes are different from what is found when looking at overall differences between individuals (state versus trait). Some studies have found a decrease in 5-HT in the prefrontal cortex in rats during aggressive episodes, as would be expected given what is found in population studies (van Erp and Miczek, 2000; Ferrari et al., 2003). However, van der Vegt and colleagues (2003a) showed that while 5-HT decreased, the ratio of 5-HIAA to 5-HT increased, indicating enhanced serotonin turnover. Moreover, there is an increase in neuron firing following an aggressive encounter (van der Vegt et al., 2003b). These studies indicate that, at least for temporary displays of aggression, serotonin may enhance aggression, rather than inhibit it.

The majority of the studies mentioned in this section have been conducted in rats, and therefore their applicability to primates may be questionable (van der Vegt et al., 2003a). In fact, in contrast to the general findings in primates, van der Vegt et al. (2003a) found a positive correlation between CSF 5-HIAA and levels of aggression. However, as mentioned above, the evidence for decreased serotonin function in aggressive animals holds best for abnormal levels of aggression. Rats, more than primates, may benefit from increased aggression, and as such, displays of severe aggression may not be considered pathological (de Boer and Koolhaas, 2005; Ferrari et al., 2005). Only when abnormally aggressive rats are considered can a significant negative correlation between extra-cellular levels of 5-HT and aggression be found (de Boer and Koolhaas, 2005). It has been proposed, therefore, that aggression in general is in part the result of temporary increases in serotonin transmission; however, for those individuals with abnormally low baseline 5-HT levels, this increase has much more dramatic effects and results in pathological behavior (de Boer and Koolhaas, 2005). The validity of this theory waits further testing.

Genetics

In addition to the population studies and clinical research reviewed here, a number of polymorphisms in genes known to influence the serotonin system and behavior have been found, adding to the evidence for serotonin's role in behavior. One gene in particular – the serotonin transporter gene (*SLC6A4*) – is especially promising in its ability to predict behavior and has been the subject of a large body of research. A second gene, *HTR1A*, which codes for the 5-HT_{1A} receptor, has been the focus many behavioral genetic studies in humans, but has largely been neglected in macaque studies. Two other genes – the gene for the 5-HT_{1B} receptor (*HTR1B*) and the gene for the second isoform of the enzyme tryptophan hydroxylase (*TPH2*) – have only

recently become the focus of research, but seem to have a significant effect on behavior. These four genes (*SLC6A4*, *HTR1A*, *HTR1B*, and *TPH2*) are the focus of research for this dissertation, and their connection with behavior are reviewed here.

The serotonin transporter gene: SLC6A4

The serotonin transporter is a key element in the process of serotonin regulation (Figure 2.5). Polymorphisms in the gene that codes for the transporter – *SLC6A4* – are a source of great interest for their potential to predict behavioral abnormalities. In humans, the promoter region of *SLC6A4* shows variation in the number of repeats, leading to a 44 base pair difference (Heils et al., 1996), and these polymorphisms are termed "long" and "short" alleles. The short allele shows decreased transcriptional activity and decreased 5-HT uptake in lymphoblasts (Lesch et al., 1996; Heils et al., 1997; Greenberg et al., 1999), which carry transporters identical to those found in the brain. This in turn may lead to a decrease in serotonin turnover as indicated by 5-HIAA levels (Williams et al., 2001; Manuck et al., 2004; Smith et al., 2004), although results are highly inconsistent (Jonsson et al., 1998; Williams et al., 2003). In addition, the short allele has been linked with an increase in anxiety (Lesch et al., 1996), inability to cope with stressful life events (Caspi et al., 2003), aggression (Beitchman et al., 2006), and violence (Retz et al., 2004), to name only a few behaviors. This result is interesting since a decrease in reuptake of serotonin might be expected to increase the effects of serotonin by increasing the available 5-HT levels in the synaptic cleft. In addition, SSRI's, which in theory mimic the effects of the short allele (i.e., decrease reuptake), tend to have the opposite results. However, because 5-HT uptake is critical for development in embryos (Shuey et al., 1992), the short allele might cause other neuronal changes that influence these behaviors (Bethea et al., 2004).

Homologous polymorphisms have also been found in the rhesus macaque (*M. mulatta*) (Lesch et al., 1997; Rogers et al., 2006). The insertion/deletion is smaller than the polymorphism in humans (21 base pairs) and it occurs in a different area of the promoter region (Lesch et al., 1997). However, most studies indicate that it acts in a very similar manner to the human variant. Again, the short allele has lower transcriptional activity (Bennett et al., 2002) and associations with behaviors such as anxiety (Bethea et al., 2004), alcoholism (Barr et al., 2003), aggression (Bennett et al., 1998), age at dispersal (Trefilov et al., 2000), and infant disposition (Champoux et al., 2002). As with humans, the short allele in rhesus macaques has a tendency to be related to decreased CSF 5-HIAA and overall reduced serotonin functioning (Bennett et al., 2002).

Serotonin receptor 5-HT_{1A}: HTR1A

As discussed in the overview of clinical studies, the serotonin receptor 5-HT_{1A} is an important target for many behavior altering pharmaceutics. Thus, variation in its underlying gene, *HTR1A*, is expected to contribute to behavioral variation. In one of the first studies that looked at the effects of *HTR1A* and behavior, Ramboz et al. (1998) showed that knockout mice lacking *HTR1A* demonstrated increased anxiety and decreased exploratory behavior.

Shortly thereafter, researchers began to examine the genetic variation with humans (e.g., Kawanishi et al., 1998; Wu and Comings, 1999), but it was Lemonde and colleagues (2003) that first found a definitive link between a polymorphism in *HTR1A* and behavior. Specifically, they found that a single nucleotide polymorphism (SNP) in the promoter region of this gene (termed C-1019G) affected transcriptional activity and binding properties (Lemonde et al., 2003; Albert and Lemonde, 2004), and individuals who possessed the G allele were at a higher risk for suicide and major depression (Lemonde et al., 2003). Since this study, the vast majority of research on *HTR1A* has focused on this promoter SNP. In addition to depression and suicide (Lemonde et al.,

2003; Wasserman et al., 2006; Lenze et al., 2008) it has been shown to affect neuroticism (Strobel et al., 2003), harm avoidance (Strobel et al., 2003), impulse control (Benko et al., 2010), panic disorder, schizophrenia, and substance abuse (Huang et al., 2004).

Unfortunately, very little of this work has been repeated in primates. Bethea et al. (2005) examined the expression levels of *HTR1A* in the dorsal raphé of ***M. fascicularis*** individuals and found a non-significant trend towards decreased expression in less stress resistant individuals; a similar study examining expression in the hypothalamus, however, did not repeat these results (Centeno et al., 2007). While important, these studies did not look at the actual genetic variations. Thus, *HTR1A* remains an interesting avenue of future research in macaques.

Serotonin receptor 5-HT$_{1B}$ and tryptophan hydroxylase: HTR1B *and* TPH2

Two further genes of interest for their possible relationship to behavior are the 5-HT$_{1B}$ receptor gene (*HTR1B*) and the gene coding for the second isoform of tryptophan hydroxylase (*TPH2*). Both are involved in the serotonin system, and therefore may influence behavior. However, both these genes have been minimally studied compared to *SLC6A4* and *HTR1A* and therefore less is known about them.

Similar in structure to 5-HT$_{1A}$, the serotonin receptor 5-HT$_{1B}$ (also referred to as 5-HT$_{1D\beta}$ in humans) is another critical component of the serotonin system. Evidence for 5-HT$_{1B}$'s role in aggressive and impulsive behavior comes two main sources. First, mice that have had *HTR1B* silenced have shown a decrease in aggressive behaviors (Saudou et al., 1994; Brunner and Hen, 1997; Zhuang et al., 1999; Bouwknecht et al., 2001), particularly in relation to impulsivity (Bouwknecht et al., 2001). (Note that this is the opposite of what occurs in *HTR1A* knockout mice.) Secondly, several pharmacological studies indicate that 5-HT$_{1B}$ has a specific effect on aggression; several drugs that act to reduce aggressive behavior are agonists that are specific to

the 5-HT_{1B} receptor (Millan et al., 1993; Bell et al., 1995; Fish et al., 1999; de Almeida et al., 2001; Miczek and de Almeida, 2001; de Boer and Koolhaas, 2005; Olivier and van Oorschot, 2005).

Like *HTR1A*, *HTR1B* contains no introns, and several variants have been found in humans that may be linked to variation in behavior. One of the most widely studied mutations is G861C. This polymorphism has been linked to suicide attempts (New et al., 2001), alcoholism (Lappalainen et al., 1998; Fehr et al., 2000) and substance abuse (Huang et al., 2003), ADHD (Hawi et al., 2002; Quist et al., 2003), OCD (Mundo et al., 2002), major depression (Huang et al., 2003), and pervasive aggression in children (Davidge et al., 2004). However, the alleles that are associated with these conditions are not always consistent (Lappalainen et al., 1998; Fehr et al., 2000). Another allele of interest is A161T, which may be part of the promoter region of this gene and has been found (in certain combinations with other nearby mutations) to affect expression levels (Sun et al., 2002; Duan et al., 2003). A161T has been associated with both impulsive aggressive behavior and suicide (Zouk et al., 2007). While the manner in which this gene affects the phenotype is unclear, these studies all seem to indicate at least a minimal role in behavior.

Tryptophan hydroxylase (TPH), is an enzyme that is used in the process of converting tryptophan into 5-HT (Figure 2.4). Defects in this enzyme can thus limit the availability of serotonin in the brain. There are two genes that each code for the two isoforms of TPH: *TPH1* and *TPH2*. While polymorphisms found in *TPH1* have been linked to suicide (Nielsen et al., 1994, 1998; Buresi et al., 1997; Mann et al., 1997; Manuck et al., 1999), knockout mice with a complete deficiency in *TPH1* still exhibit normal levels of 5-HT in the brain and are behaviorally indistinguishable from wildtype mice (Walther and Bader, 2003). This is because while *TPH1* is

expressed mainly in peripheral tissue, *TPH2* is expressed almost exclusively in the brain (Walther and Bader, 2003; Zill et al., 2004b). Therefore, variations in this gene are more likely to confer a behavioral effect than *TPH1*. In humans, multiple polymorphisms in *TPH2* have been linked with major depression and affective disorders (Harvey et al., 2004; Zill et al., 2004a; Zhang et al., 2005; Zhou et al., 2005), a history of suicide attempts (Zill et al., 2004c; Zhou et al., 2005), autism (Coon et al., 2005), and ADHD (Sheehan et al., 2005; Walitza et al., 2005). Zhou and colleagues (Zhou et al., 2005) identified a haplotype (a set of polymorphisms on a chromosome that are inherited together) of *TPH2*, termed the yin haplotype, that was associated with suicidal behavior and depression. In addition, they also found that those homozygous for the yin haplotype had lower CSF 5-HIAA than those who lacked the yin haplotype completely. Recently, Chen and colleagues (2006) identified several polymorphisms in rhesus macaques (*M. mulatta*) that are associated with differences in hypothalamus-pituitary-adrenal (HPA) axis and with expression level differences. They further found that these polymorphisms are predictive of levels of self-injurious behavior, and thus *TPH2* seems to be connected to stress response in macaques (Chen et al., 2010). However, to date these are the only studies that have looked at *TPH2* and behaviors in macaques. Because *TPH2* has only been identified very recently, this gene awaits a great deal of future research.

EVOLUTIONARY THEORIES ON SEROTONIN AND PARTICULARLY *SLC6A4*

The extent to which variation in serotonin functioning affects fitness has received limited attention. Some studies have attempted to discern whether or not selection has acted on this system by examining the costs and benefits associated with differences in serotonin turnover. From a genetic perspective, discussion on serotonin evolution has been almost exclusively

centered on the promoter region variation of *SLC6A4*. Here, I review some of the evolutionary theories surrounding serotonin and *SLC6A4*, and make some predictions as to how each of these theories would be expected to affect genetic variation. (For a more detailed description for how different evolutionary forces affect genetic variation, see Chapter 1.)

In general, it is unclear to what degree variation is serotonin functioning affects fitness. Some studies have indicated that individuals with increased serotonin functioning should fare better than those lowered serotonin functioning. For example, in primates it is not always the most aggressive males that are dominant (Higley et al., 1996d; Takahata et al., 1999; Fairbanks et al., 2001). Furthermore, low serotonin levels are associated with lower rank (Higley et al., 1996d; Higley and Linnoila, 1997a, b; Fairbanks et al., 2001), higher risk of injury (Mehlman et al., 1994; Higley et al., 1996a), and decreased survival rates (Higley et al., 1996b; Westergaard et al., 2003a; Howell et al., 2007). In terms of reproduction, high CSF 5-HIAA is associated with increased time spent in courtship, mounting, and insemination in rhesus macaques (Mehlman et al., 1997). Gerald et al. (2002) examined rhesus macaque male pairs who were present during the time an offspring was conceived and found that the male with the higher CSF 5-HIAA is usually the sire. Female macaques with lower CSF 5-HIAA are less likely to give birth (Cleveland et al., 2004). In specific relation to the promoter region of *SLC6A4*, female macaques that carry the short allele are significantly less likely to exhibit ovulatory cycles (Hoffman et al., 2007). Taken together, these studies would indicate that selection is acting against genetic variants that are more likely to decrease overall serotonin functioning. In this respect, new mutations are less likely to be favored, particularly those that occur in coding regions, as these generally result in a loss of function (Kimura, 1985). Accordingly, purifying selection is likely to be the predominate

force acting on selection, and analyses of genetic variation should reflect this. That is, the level of overall genetic variation should be low, particularly at nonsynonymous sites (see Chapter 1).

However, in both rhesus macaques (*M. mulatta*) and humans, the short allele of *SLC6A4*, which is associated with lower serotonin levels, is present in high frequency [Humans: 43% (Lesch et al., 1996); Indian rhesus macaques: 40% (Chapter 5); Chinese rhesus macaques: 58% (Chapter 5)]. In addition, not all studies indicate that the short allele, or low serotonin functioning, is maladaptive. Within humans, Chiao and Blizinsky (2010) theorize that in areas where pathogen levels have been historically high, "collectivist" values are favored over "individualistic" values, creating a cultural environment where the short allele might thrive, or at least not be selected against (see **SEROTONIN GENES AND PLASTICITY**). This theory might be extended to primate studies, although this is admittedly problematic. While Howell and colleagues (2007) found that male macaques with low CSF 5-HIAA were at greater risk of premature death, once they emigrated to a new group they were more likely to achieve high rank. Other studies have found a negative correlation between CSF 5-HIAA and rank (Yodyingyuad et al., 1985; Kaplan et al., 2002; Fairbanks et al., 2004a). Another factor to consider is a species' ability to exploit various habitats. Macaque species can be divided into two different groups depending on their ability to live in areas affected by humans (Richard et al., 1989). Rhesus macaques in particular do very well in human inhabited areas and this may in part be due to an aggressive/impulsive nature (Richard et al., 1989; Thierry et al., 2000), which may be mediated through the serotonin system. Given the expansion of humans, there may be a selective advantage to tolerating humans. In fact, the ability to expand and exploit a variety of habitats may be related to serotonin functioning (Chapter 3), and thus lowered serotonin turnover may be favored. If this is so, then positive selection is predicted to shape genetic variation. Specifically,

positive selection is expected to increase the rate of protein evolution, increase interspecific genetic differences, and increase the number of high- and low-frequency genetic variants (Chapter 1).

Alternatively, balancing selection may be operating to keep genetic polymorphisms such as the short allele at high frequency in the population. Suomi (2006) suggested that because both humans and rhesus macaques show variation in genes related to serotonin, and because they are both the most widely distributed primate species, that it is genetic diversity that has allowed these species to adapt to a variety of habitats (see also Chakraborty et al., 2010). Gerald and Higley (2002) also suggested that certain alleles might be advantageous in specific environments, but detrimental in others. Trefilov et al. (2000) proposed that males that are heterozygous for *SLC6A4* might have an advantage over homozygotes. Furthermore, since rank is inherited and stable in females, but acquired and unstable in males, selection may be acting differently in males and females. Indeed, different results have been achieved in males and females when linking CSF-5HIAA with fitness (e.g., Trefilov et al., 2000; Westergaard et al., 2003b; Fairbanks et al., 2004a, b; Howell et al., 2007). Therefore, rather than positively selecting for one allele, balancing selection may be acting to maintain variation. This would be reflected by an increase in intraspecific variation and an excess of alleles at intermediate frequency (Chapter 1).

SEROTONIN GENES AND PLASTICITY

It is clear from these studies that selection's role in serotonin evolution is not clearly understood. Further complicating the matter is the fact that in both macaques and humans there appears to be a significant genotype by environment interaction. In humans, the association

between the short allele and lowered serotonin functioning (as measured by a fenfluramine challenge) is only seen in individuals with low socio-economic status; those individuals with high socio-economic status had high serotonin turnover regardless of genotype (Manuck et al., 2004). Likewise, a macaque's rearing condition (mother- or peer-raised) will influence the genotypic expression of *SLC6A4* on CSF 5-HIAA and behavior (Bennett et al., 2002; Champoux et al., 2002). Wasserman et al. (2006) found an association between the C -1019G polymorphism of *HTR1A* and suicide, but only when examining subject with a history of traumatic life event. Furthermore, there is evidence that both gender and race can influence genotype expression (Williams et al., 2003). In fact, many of the studies outlined indicate that the effect of genetic variants on behavior is best seen when paired with a poor environment.

There is a tendency to discuss genetic variants as risk factors, particularly within the field of psychology, and certain genotypes are assumed to increase an individual's vulnerability to environmental influences. However, several authors have recently argued that these genetic variants are better thought of as plasticity genes, rather than vulnerability genes, with certain genetic variants conferring more or less plasticity (Belsky et al., 2009; reviewed in Homberg and Lesch, 2010). In this context, plasticity can be defined as the degree of the gene by environment interaction. Accordingly, individuals who possess certain genetic variants ("plastic" alleles) show more variation across environments – for better or for worse – whereas those who do not possess such "plastic" alleles display phenotypes that are more consistent across environments (Figure 2.6). For example, Caspi et al. (2003) were among the first to demonstrate a significant gene by environment interaction for *SLC6A4* when they found that individuals with the short promoter allele were more likely to develop depression or contemplate suicide, but only if they had experienced two or more stressful life events. Among people who had not experienced stressful

life events, carriers of the short allele were actually less likely to develop these behaviors than carriers of the long allele. Thus, carriers of the short allele do both better and worse (i.e., are more plastic) than carriers of the long allele depending on the environment.

This viewpoint adds a layer of complexity to evaluating the evolution of the serotonin system. To begin with, we must discern whether the target of potential selection is a specific set of behaviors, plasticity itself, or some combination of the two. If the capacity to be plastic is the adaptation, then our expectations for the types of signatures of selection we would find in looking at these genes would not really differ. However, if plasticity is *not* the sole target of selection, then finding any evidence of selection on these genes becomes increasingly difficult. This is because the increased plasticity that some of these genetic variants impart would weaken the connection between specific types of behaviors and the underlying genetic structure. Thus, even if there was strong selection for a behavior, there might be very little corresponding genetic change. If so, random genetic drift would be expected to be the predominant evolutionary force driving genetic variation. These are factors that must be considered when interpreting data.

LIMITATIONS, CHALLENGES, AND CONCLUSIONS

Most of the information on the relationship between serotonin and behavior has come from three sources. The first of these is population level studies that look at how people afflicted with various behavioral abnormalities (particularly men) differ from control populations in their serotonin levels. The second source of information comes from clinical studies that artificially manipulate serotonin level (or manipulate certain aspects of the serotonin pathway) to see the affect on behavior. The third source of information comes from genetic studies that look at

associations between behaviors and variations of genes related to serotonin. All three of these sources are problematic.

In the first case, the emphasis on psychopathologies (and on men) limits the ability to extend findings to the general population. While these studies are, of course, useful for the field of psychology and the treatment of pathologies, it is difficult to make definite conclusions about how serotonin may play a role in behaviors that fall within the normal range. Furthermore, while these studies may help to frame hypotheses about how abnormal behaviors (by definition, non-adaptive behaviors; see Table 2.1) evolve and remain in a population, they add little to the theories about the evolution of adaptive behaviors. Unfortunately, the studies conducted thus far on "normal" populations of people are inconclusive. This may be due in part to the relatively small numbers of studies that have explicitly looked at this, but it is also likely due to a gene by environment interaction that makes the connection between genotype and phenotype less straightforward (e.g., Bennett et al., 2002; Champoux et al., 2002; Caspi et al., 2003; Wasserman et al., 2006). Within nonhuman primates, there is no way to "diagnose" psychopathologies in the same way that is done in humans. Therefore, studies conducted on nonhuman primates are, at least in theory, limited to "normal" populations. These studies overall indicate that the relationship between serotonin and aggression/impulsivity do hold for more adaptive forms of aggression, as do studies outside of primates (not reviewed here, but see for example Winberg et al., 1993; Matter et al., 1998; Kravitz and Huber, 2003; Clotfelter et al., 2007). However, as with humans, nonhuman primates show the strongest relationship between serotonin and extreme forms of impulsive aggression that may fall outside of the adaptive range.

The second source of information, clinical studies, is important for determining the exact mechanisms through which serotonin influences behavior. While the population level studies

make it clear that serotonin has a role in shaping behavior, clinical studies demonstrate that *how* this happens is complex and far from understood. The predominant paradigm has been that a lack of serotonin promotes aggressive and impulsive behavior; it is now appears that it is better to think of any imbalance of serotonin leading to aggressive behavior. However, the main problem with clinical studies is the artificial nature of the environment to which these animals are subjected. As mentioned previously, factors such as socio-economic status, cultural influences, and rearing conditions all affect both serotonin levels and the expression of aggressive and impulsive behaviors. Therefore, the laboratory environment in itself is likely to be influencing the outcome of these studies. Many of the methods employed in these studies (such as microdialysis or the application of neurotoxins) are likely to be stressful to the animals and may alter other components of the brain that interact with the serotonin system. These factors make clinical studies difficult to apply to questions of evolution.

Finally, genetic analyses are also used in the study of serotonin and behavior. Like population level studies, genetic studies tend to concentrate on abnormal behavior. In addition, some of the genetic studies have revealed an important environmental component to the expression of these genes, on both a physiological and behavioral level (Bennett et al., 2002; Champoux et al., 2002; Williams et al., 2003; Caspi et al., 2003; Manuck et al., 2004; Newman et al., 2005). While this is to be expected for a trait like behavior, these studies rarely take these factors into account, despite the fact that these environmental factors may help to explain any discrepancies found between studies. Particularly for *SLC6A4*, which has been the most widely studied, it seems that a good environment (i.e., high socio-economic status, a non-abusive childhood, or a strong social network) acts as a buffer against some of the negative effects of polymorphisms associated with behavioral pathologies (Champoux et al., 2002; Caspi et al.,

2003; Westergaard et al., 2003b; Manuck et al., 2004). It may be that the effects of some of these polymorphisms are only exposed under rare conditions, and therefore have little opportunity for selection to act on them. However, until there are more studies that examine the effects of these genes in the general population and that explicitly look at a variety of environmental conditions, it is uncertain how strongly or how often these genes may play a significant role in the evolution of behavior.

Despite these limitations, there is overwhelming evidence that serotonin acts to moderate behavior, even if some factors – such as the degree of its effect in general populations and the exact mechanisms behind this effect – are not yet fully understood. Therefore, it is perhaps the best neurological system to investigate in order to understand the biological basis of behavior within macaques. Furthermore, if we are to gain a deeper understanding of the evolution of behavior, we have to understand the evolution of its underlying biological mechanisms.

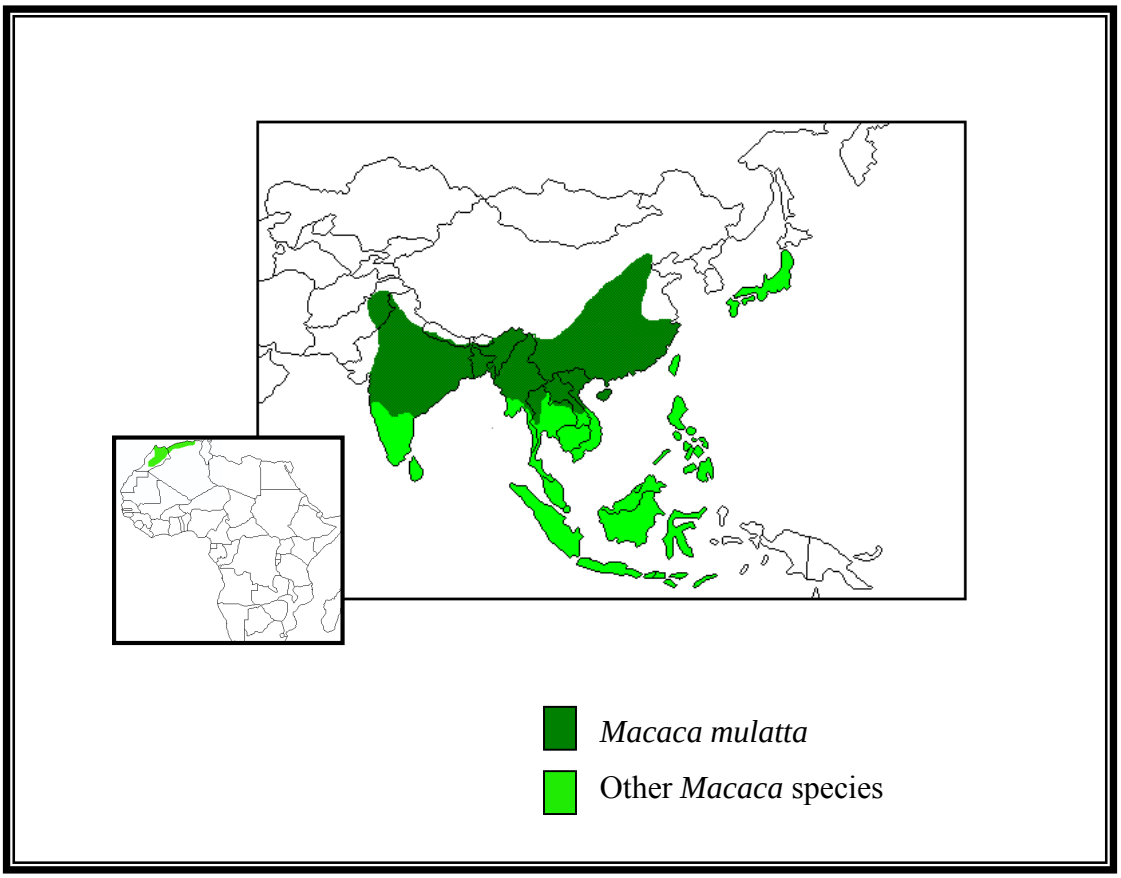

Figure 2.1. A map of the distribution of the macaque genus (all shades of green), with a special emphasis on the rhesus macaque (*Macaca mulatta*: dark green).

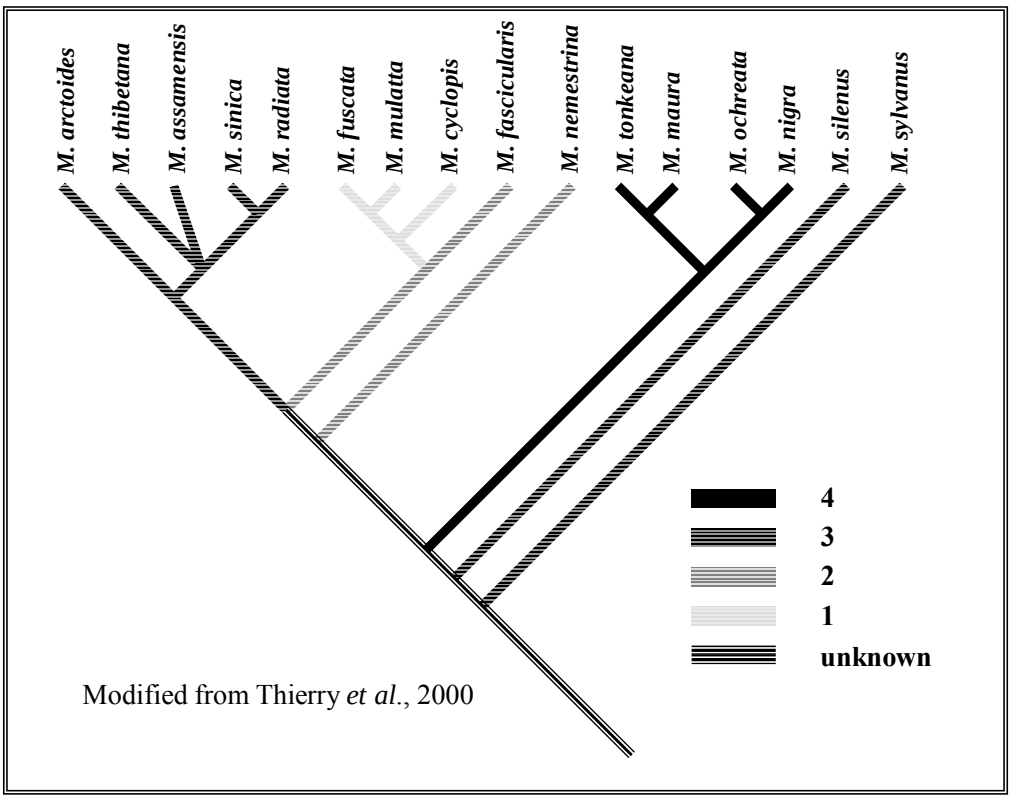

Figure 2.2. Behavioral categories of Thierry (2000) applied to a phylogeny of macaque species. Modified from Thierry et al. (2000). Category 1 describes the most aggressive species, while category 4 describes the least aggressive. Categories 2 and 3 are intermediate.

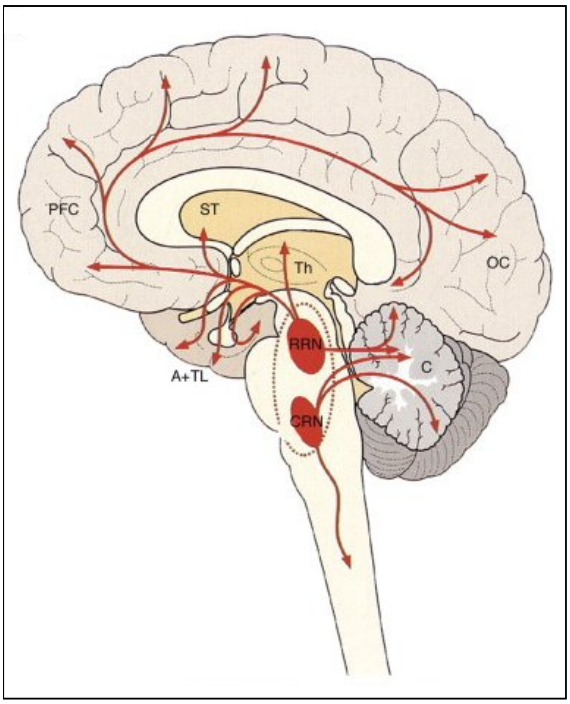

Figure 2.3. A schematic of how the serotonin system interacts with the human brain. Serotonin is produced in the caudal raphé nuclei (CRN) and one of the rostal raphé nuclei (RRN) and is projected to various regions of the brain. C, cerebellum; Th, thalamus; A, amygdala; TL, temporal lobe; ST, striatum; PFC, prefrontal cortex; OC, occipital cortex. Reprinted with permission from Cools et al. (2008).

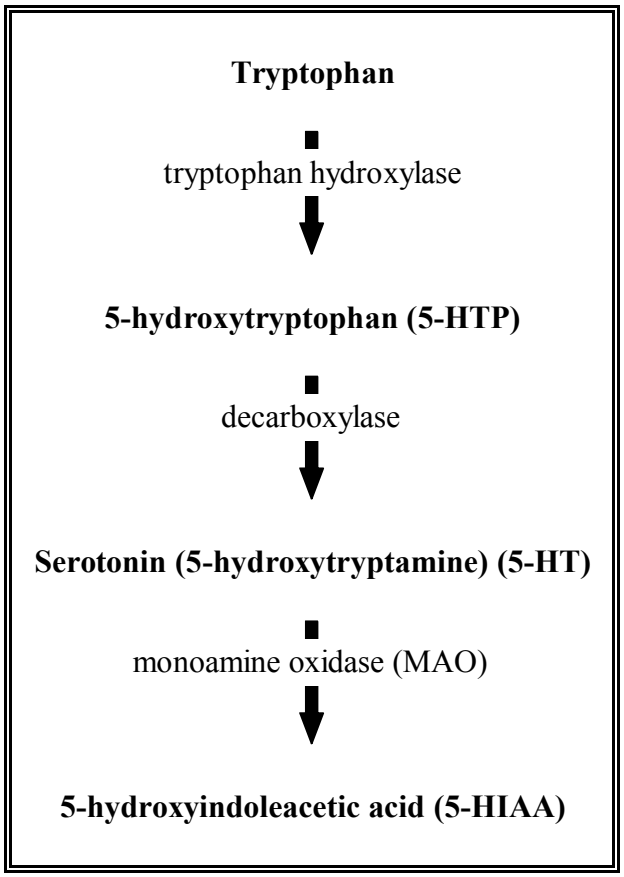

Figure 2.4. Overview of the different steps of serotonin synthesis. Tryptophan is acquired through the diet and becomes serotonin using the enzymes tryptophan hydroxylase and decarboxylase. Serotonin is eventually broken down using the enzyme monoamine oxidase.

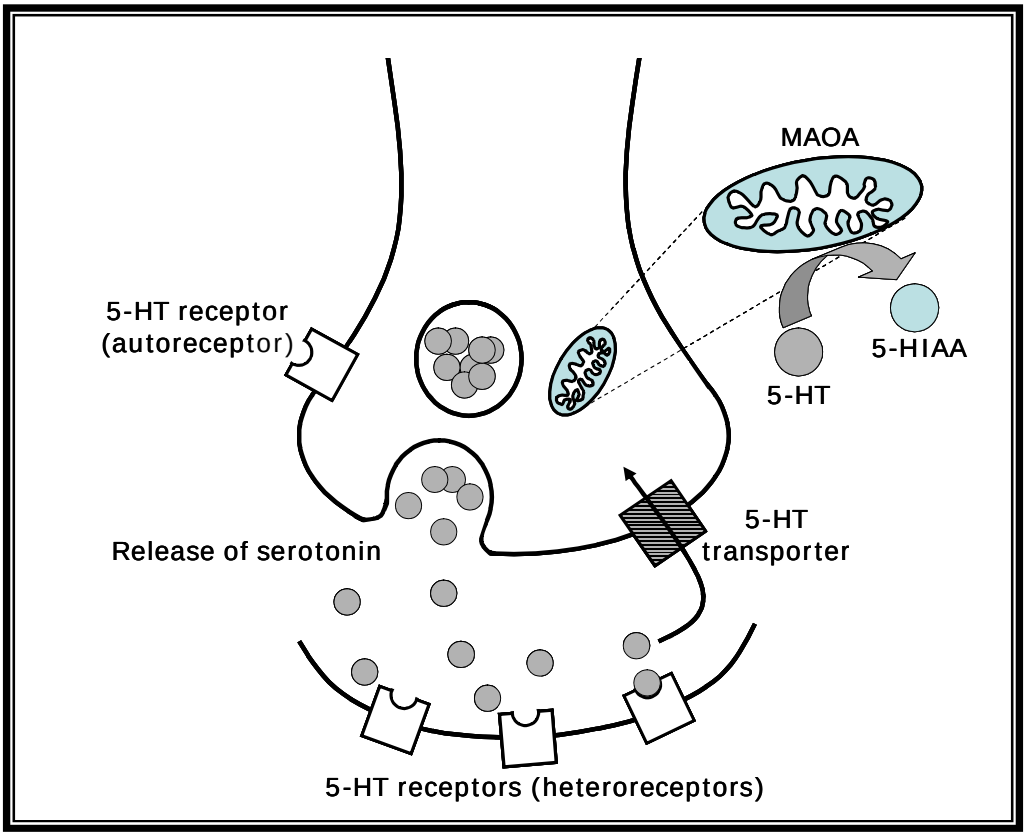

Figure 2.5. Basic schematic of the serotonin system. Serotonin is released from the serotonin neuron where it is free to bind with multiple receptor types. The serotonin transporter moves serotonin from the postsynaptic space and back into the serotonin neuron. MAOA breaks down serotonin (5-HT) into its metabolite 5-HIAA.

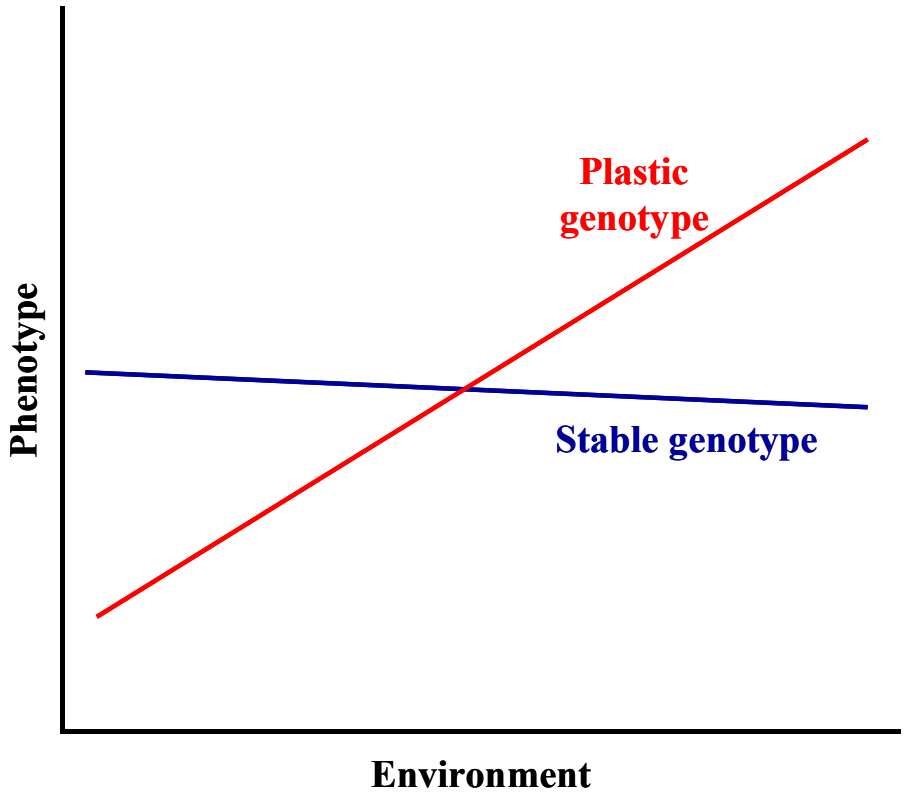

Figure 2.6. Graphical demonstration of variation in plasticity. All genetic variants are expected to show some phenotypic variation across environments (plasticity), but not all genetic variants will be affected to the same degree. Certain genetic variants ("plastic" genotypes, symbolized as the red line) will show a large amount of phenotypic variation across environments. In contrast, other genotypes ("stable" genotypes, symbolized as the dark blue line) will show less phenotypic variation across environments.

Table 2.1. A comparison of the different types of aggression.

State (or situational) a temporary emotional state	**Trait** A stable personality trait, where agression is either chronic or more easily triggered
Hyperarousal Aggression caused by "excessive autonomic arousal." Characterized by "outbursts."	**Hypoarousal** Associated with low arousal. This is more of the "calculated" or "cold-blooded" violence
Competitive Agression to increase resources. Also thought of as "proactive" or "offensive"	**Protective** Agression to protect oneself. Also thought of as "reactive" or "defensive"
Adaptive Agression within a normal range that helps to increase an individual's fitness	**Abnormal** Agression beyond the normal range that is actually maladaptive

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CHAPTER 3

EVIDENCE FOR SELECTION IS FOUND IN *HTR1A*, BUT NOT IN *HTR1B*

ABSTRACT

Research has increasingly highlighted the role of serotonin in behavior. However, relatively few researchers have examined serotonin in an evolutionary context, even though such research could provide insight into the evolution of important behaviors. The genus *Macaca* represents a useful model for studying the evolution of serotonin. It is comprised of 19 morphologically and behaviorally diverse species and this diversity correlates with phylogeny. In addition, many genetic features of the macaque serotonin system parallel those of the human lineage and evidence of positive selection on genes related to serotonin has been demonstrated in humans. If parallel evolution is occurring in macaques and humans, and selection is similarly shaping behavioral patterns in both primates, we should expect to see evidence of positive selection in macaques as well.

I examine the role of selection in the macaque serotonin system by comparing two genes that code for two types of serotonin receptors – *HTR1A* and *HTR1B* – across five species of macaques. The pattern of evolution is significantly different for *HTR1A* compared to *HTR1B*. Specifically, there is an increase in between-species variation compared to within-species variation for *HTR1A*, consistent with positive selection. This high level of divergence resulting from natural selection likely contributes to the behavioral diversity among species in the macaque genus, is potentially related to dispersal, and may parallel selection acting on similar behaviors in humans.

INTRODUCTION

Over the past 50 years, research has increasingly highlighted the role of the serotonin system in shaping behavior (for reviews see Pihl and LeMarquand, 1998; Munafo et al., 2003; Cools et al., 2008; Caspi et al., 2010). However, despite the large amount of research devoted to this system, it is rarely placed in an evolutionary context. This is surprising given the potential to explain the evolution of important behavioral strategies. For example, genetic, pharmaceutical, and hormonal studies have shown that serotonin influences age at dispersal (Trefilov et al., 2000; Krackow and König, 2008), social behavior (Samochowiec et al., 1999; Caspi et al., 2002; Anstey et al., 2009; Crockett et al., 2010), exploratory behavior (Ramboz et al., 1998), and aggressive behavior (Manuck et al., 2000; Newman et al., 2005; Filby et al., 2010; Vermeire et al., 2010) in animals. These characteristics have been used to define the "behavioral syndromes" (*sensu* Sih and Bell, 2008) of invasive animals (Duckworth and Badyaev, 2007; Sih and Bell, 2008; Cote et al., 2010) and may contribute to an individual's ability to expand its habitat and successfully compete with other individuals occupying similar niches. Thus, alteration of the serotonin system provides one possible mechanism through which selection can influence behavior, and may explain how and why similar behaviors evolve across species.

The genus *Macaca* represents a useful model for addressing the evolution of the serotonin system. The genus is composed of approximately 19 species (Fooden, 1976, 1980), which, while closely related, exhibit diverse social behaviors, including age at dispersal, frequency of reconciliation, degree of counter-aggression, and social play patterns (Thierry et al., 2000, 2008). These differences among species are significantly influenced by phylogeny (Thierry et al., 2000, 2008), suggesting that genetic differences in neurological functioning may underlie the behavioral differences in this genus (Wendland et al., 2006). Macaques also share

interesting parallels with humans. Specifically, they occupy a diverse set of habitats, making macaques – particularly the rhesus macaque (*Macaca mulatta*) – the most widely distributed of nonhuman primates. Much like invasive species, both humans and macaques, especially *M. mulatta*, are successful at expanding territory and competing with individuals from other species in new habitats. It has been hypothesized that the exploitation of a large part of the world by both humans and rhesus macaques was the result of the evolution of similar behavioral strategies, such as high between-group aggression, in both species (Suomi, 2006; Maestripieri, 2007). In line with this theory, several similar, though independently evolved, genetic variants related to serotonin functioning have been identified in both rhesus macaques and humans (e.g., Lesch et al., 1997). In both species, these uniquely derived genetic variants show similar connections to behavior (e.g., Lesch et al., 1996, 1997; Bennett et al., 2002; Barr et al., 2003; Caspi et al., 2003; Bethea et al., 2004; Beitchman et al., 2006). This suggests that similar behavioral features of these two species may be the result of selection acting on the same system in these species. The repeated recruitment of the same neurological system to produce similar behaviors, what I call here parallel evolution *sensu* Haldane (1932), may explain behavioral similarities across a wide range of animals (Fitzpatrick et al., 2005; Robinson et al., 2008). Recently, Claw and colleagues (2010) inferred that selection likely acted on the serotonin system in humans. If parallel evolution is occurring, then positive selection may also have influenced the serotonin system in macaques.

To examine the evolution of the serotonin system in macaques, I compared the genetic variation of two genes, *HTR1A* and *HTR1B*, within and among five macaque species. The two genes code for serotonin receptor types 1A and 1B, and are located on chromosomes 4 and 6 of the macaque genome, respectively (Rhesus Macaque Genome Sequencing and Analysis

Consortium et al., 2007). They are similar in structure, having just one exon (1269 and 1173 base pairs for *HTR1A* and *HTR1B*, respectively) and no introns; both receptor types are associated with various behaviors (e.g., Ramboz et al., 1996a,b, 1998; Davidge et al., 2004; de Boer and Koolhaas, 2005; Centenaro et al., 2008). I sequenced both genes and their flanking regions, including potential regulatory regions, and applied several tests designed to detect selection. These tests include Tajima's D, **ω**, McDonald Kreitman, Phylogenetic Analyses by Maximum Likelihood (PAML), and the HKA test (see Methods for explanation of these tests). While both receptors are similar in structure and exhibit an overlap in function (Barnes and Sharp, 1999), experiments with knockout mice indicate that these two receptors modulate behavior in opposite ways (Ramboz et al., 1996b; Ramboz et al., 1998), potentially due to the different distributions of the receptors within the brain (Ghavami et al., 1999). Knockout mice lacking *HTR1A* show increased anxiety and decreased exploratory behavior compared to the wildtype (Ramboz et al., 1998); in contrast, knockout mice lacking *HTR1B* are more impulsive, less anxious, and more aggressive than the wildtype (Ramboz et al., 1996b). Differences occurring in the coding and regulatory regions of these receptor genes may have contributed to the diverse behaviors macaque species exhibit today. Here, I investigate whether positive selection affects the pattern of genetic variation of *HTR1A* and *HTR1B* in the macaque genus.

METHODS

Subjects

DNA samples from 20 *Macaca mulatta* (11 from India, 9 from China), 11 *M. fascicularis*, 11 *M. fuscata*, 6 *M. nemestrina*, and 4 *M. sylvanus* were used in this study (Table A.1). Previous studies (Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2003;

Smith, 2005; Hernandez et al., 2007; Satkoski et al., 2008) have shown that *M. mulatta* consists of two groups that are genetically distinct, roughly split between those of Indian and Chinese origin. Because the substructure of this species could influence analyses, I analyzed each population separately as well as the species as a whole. In addition to the previously mentioned samples, one sample each from *Macaca assamensis*, *M. cyclopis*, *M. nigra*, *M. silenus*, and *Chlorocebus aethiops* (Table A.1) were used in PAML analyses (see below) to help place the results in an evolutionary framework. Because of unusual results obtained for *M. nigra*, for *post hoc* analyses I obtained 2 additional *M. nigra* samples, 1 *M. maura*, and 1 *M. tonkeana*. In total, 61 DNA samples were sequenced. The 11 different macaque species analyzed span the range of behavioral variation found in the genus. The *C. aethiops* (vervet) sample was used as an outgroup for the macaque species.

PCR and Sequencing

I amplified the coding and flanking regions, including potential regulatory regions, of *HTR1A* and *HTR1B* (see Table A.2 for primers) using both previously published (Cigler et al., 2001) and newly designed primers. The new primers were designed based on the *Macaca mulatta* draft assembly (Rhesus Macaque Genome Sequencing and Analysis Consortium et al., 2007) using Primer3 (Rozen and Skaletsky, 2000) and GeneRunner (generunner.net). In addition to *HTR1A* and *HTR1B*, five non-coding, presumably neutral regions were amplified in all of the samples (Table A.3) and used for the HKA test (see below) (Hudson et al., 1987). I designed these non-coding regions to be at least 20,000 base pairs from the nearest coding region (Satkoski Trask et al., 2011). The PCR protocols differed for each of the regions amplified and are available upon request of the author.

I used the ExoSAP-IT protocol (usb.com) to clean up the PCR product and submitted it to the W.M. Keck Center for Comparative and Functional Genomics, UIUC for Sanger sequencing. As with the PCR primers, the sequencing primers used were a combination of published and newly designed primers. Efforts were made to design primers that would provide substantial overlap with each other so that any one region being analyzed would have multiple reads from different primers, ensuring the accuracy and quality of the sequence.

Once received, sequences were aligned and edited manually using Sequencher (www.genecodes.com). Each heterozygote base pair was confirmed visually by identifying clear double peaks in the chromatogram. All SNPs (single nucleotide polymorphisms) and indels (insertions/deletions) were identified. Where indels involved more than one sequential nucleotide, the entire deleted region was treated as a single mutation. For example, the loss of a codon, which includes three nucleotides, was treated as a single mutational event rather than three separate events. Haplotypes were determined using the program Phase v.1 (Stephens et al., 2001; Li and Stephens, 2003; Stephens and Donnelly, 2003). In order to visualize the genetic variation and relationships among the haplotypes of each gene, I constructed a haplotype tree using the reduced median method in Network v4.5 (fluxus-engineering.com).

Analysis

With the exception of the likelihood ratio tests and the haplotype networks, all analyses were conducted on samples of five species: *M. mulatta*, *M. fuscata*, *M. fascicularis*, *M. nemestrina*, and *M. sylvanus*. Several indices of molecular diversity were calculated for each of the five species using Arlequin (Excoffier et al., 2005). These included two different estimates of theta ($\theta = 4N\mu$, where N is the effective population size and μ is the mutation rate): θ_S (Watterson, 1975) and θ_π (Tajima, 1983), which were used to estimate within-species diversity.

Nucleotide diversity (averaged over all loci) (Tajima, 1983) was used to estimate within- and among- species diversity.

In order to determine whether selection has acted on either gene, I used multiple approaches. For all selection tests, I used a significance level of 0.05. First, I applied the HKA test, which compares the ratio of within- versus among-species variation in a gene of interest with that of several unlinked, neutral loci. A gene that is evolving neutrally should not have a ratio that varies significantly from that of the other loci considered. I used the five non-coding regions described above and in Table A.3 for comparison to the serotonin genes in the HKA test. Because the HKA test requires a comparison between two species, I conducted pairwise comparisons for all five species using software provided by J. Hey (http://genfaculty.rutgers.edu/hey/software).

Second, I examined the ratio of nonsynonymous to synonymous mutations (ω) in the coding regions of the genes. Positive selection increases the relative rate of nonsynonymous substitutions, whereas purifying selection decreases it (Kimura, 1977). For this approach, I used multiple tests. First, I used the program Mega v.4 (Tamura et al., 2007) to determine whether ω between any two species significantly differs from 1.0 (Kimura, 1977; Yang and Nielsen, 2000). If $\omega > 1.0$, this indicates positive selection, while $\omega < 1.0$ indicates purifying selection. Second, I employed the McDonald-Kreitman (MK) test (McDonald and Kreitman, 1991), which compares synonymous and nonsynonymous ratios within and among two species, using the program DNAsp (Rozas et al., 2003). This ratio should remain constant for genes evolving neutrally. Like the HKA test, calculation of ω and the MK test require a comparison among two species. As with HKA, I made pairwise comparisons among all five species. Finally, I conducted likelihood ratio tests (LRT) on all of the samples available using the program PAML (Phylogenetic

Analysis using Maximum Likelihood; Yang, 1997; Yang, 2007). PAML determines the maximum likelihood values for different models of evolution within a phylogenetic framework. Models that incorporate positive selection can be compared to null models and the LRT used to determine which model best fits the data. Because PAML allows only one sequence per species, I included all ten species in the LRT using the phylogeny seen in Figure 3.2 (Vos, 2006). For this study, I examined two types of evolutionary models. First, I tested a variable branch model, which allows **ω** to vary among each of the branches of the macaque phylogeny, to see if selection has occurred on a specific lineage (Yang, 1998). Tested in this way, **ω** does not have to be greater than one, but simply elevated compared to other lineages of the macaque phylogeny. Second, I compared several sites models using PAML (M1 vs. M2, M7 vs. M8, and M8a vs. M8; see Yang et al. (2000) and Swanson et al. (2003) for explanation of models). These models allow **ω** to vary among different sites on the gene using different parameters (Nielsen and Yang, 1998; Yang et al., 2000; Swanson et al., 2003). Most sites on a gene are under strong purifying selection, which reduces the overall **ω** for a gene and can hide signals of positive selection occurring only in a small portion of the gene. Testing the sites models in PAML can determine if a portion of a gene shows signs of positive selection, even if the average **ω** ratio over the entire gene is low.

Finally, using Arlequin, I conducted a Tajima's D test, which detects skews in the frequency spectrum of alleles by comparing two different estimates of theta, θ_S and θ_π (Tajima, 1989). An excess of rare alleles ($\theta_S > \theta_\pi$, leading to a significantly negative Tajima's D) is consistent with positive selection, while an excess of intermediate-frequency alleles ($\theta_S < \theta_\pi$, leading to a significantly positive Tajima's D) is consistent with balancing selection.

Many of the tests that I employed assume absence of recombination, which can affect the results. Specifically, violation of this assumption can increase false positives in the MK test (Andolfatto, 2008) and in the LRT of the sites models using PAML (Anisimova et al., 2003). In contrast, the assumption of no recombination is conservative for the HKA test (Hudson et al., 1987). I used the SITES program of J. Hey (http://genfaculty.rutgers.edu/hey/software) to obtain an estimate of the recombination parameter *C*, where $C = 2Nc$, and c is the rate of recombination per generation per base pair and N is the effective population size (Hey and Wakeley, 1997). Because the LRT test only examines the coding region, whereas the HKA test can analyze both coding and non-coding regions, I obtained estimates of *C* based on the entire regions sequenced, and on the coding region alone. Presence of recombination indicates that a significant result for the MK test and the LRT of the sites models should be treated with caution, but should not affect the interpretation of a significant result for HKA.

RESULTS

Molecular Diversity

Overall, indices of genetic diversity are comparable to those reported for other areas of the macaque genome (Table 3.1) (Stevison and Kohn, 2009). Several studies have found a major split in the *M. mulatta* lineage between Chinese and Indian populations (Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2003; Smith, 2005; Hernandez et al., 2007; Satkoski et al., 2008). Because the presence of population substructure might have an affect on analyses, I examined representatives of each branch of this species separately. The values for all genetic diversity indices (Table 3.1) are very similar for both lineages, and for all analyses separate

examination of the two *M. mulatta* branches did not affect results. Therefore, for the remainder of this manuscript, I only report the results for the species as a whole.

A comparison of the two genes shows that genetic distances between species for *HTR1A* are generally high compared to *HTR1B* (Table 3.1). The only exception to this is between *M. mulatta* and *M. fuscata*, where the divergence is actually lower in *HTR1A* than *HTR1B*. These trends are also reflected in the gene trees by both the wider spacing of the haplotypes in *HTR1A* than in *HTR1B* and the tight clustering of *M. mulatta*, *M. fuscata*, and *M. cyclopis* in *HTR1A* (Figure 3.1). Because these three species are members of a monophyletic group that excludes the other macaque species studied (Figure 3.2), for ease of future discussion, *M. mulatta*, *M. fuscata*, and *M. cyclopis* will be referred to as the *mulatta* group (see Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2003).

While members of the same species tend to cluster together in the *HTR1A* haplotype network, one *M. fascicularis* individual provides an exception to this pattern by showing greater similarity to a clade with *M. nemestrina*, *M. assamensis*, and *M. silenus* individuals than to the other *M. fascicularis* individuals (Figure 3.1). It shares none of the SNPs that distinguish the other *M. fascicularis* individuals and possesses several SNPs that are unique to this individual. I repeated sequencing on this individual in order to rule out a PCR or sequencing error. This individual likely represents either substructure within *M. fascicularis* or introgression. It was not clear how this individual would affect the results, so I report here the results of analyses of *HTR1A* with and without this outlier (Tables 3.1, 3.2, and 3.3). With the exception of the HKA test, inclusion or exclusion of the outlier did not significantly change the test results (see below and Appendix A). Because this individual was largely undifferentiated in *HTR1B*, its inclusion had no effect on the results; therefore, only the original dataset, including the outlier, is reported.

Evidence for Selection

For the first set of tests, HKA, I obtained significant results for some among-species comparisons of *HTR1A*, but not for any comparisons of *HTR1B* (Table 3.3). These results were mainly driven by a high level of among-species divergence in *HTR1A* (Figure 3.1), and provide evidence for positive selection occurring in macaques. HKA results were not significant for any comparison with *M. sylvanus*, nor for the comparison between *M. mulatta* and *M. fuscata*. All other between-species comparisons are significant for *HTR1A* when the outlying *M. fascicularis* individual is excluded. When the outlier is included, only comparisons between *M. nemestrina* and the *mulatta* group (*M. mulatta* and *M. fuscata*) remain significant.

The second set of tests focused on the ratio of synonymous to nonsynonymous substitutions (**ω**). The overall ratios for both genes (Table 3.3) were almost all either significantly lower than one or else approaching significance; this is consistent with previous studies of the serotonin system that have shown the system to be evolutionarily conserved (Andres et al., 2007; Anbazhagan et al., 2010). However, results for the likelihood ratio tests (LRT) comparing various sites models in PAML showed significant results for *HTR1A*, but not for *HTR1B* (Table 3.4), indicating that at least some portions of *HTR1A* are under selection even though the overall **ω** was low. The LRT using the variable branch models were non-significant for both genes (not shown); that is, no selection was detected on a specific lineage. In addition, I also conducted McDonald-Kreitman (MK) tests on both genes. Due to the low number of nonsynonymous polymorphisms in several cases, both within and among species, the MK test could not be calculated. Where p-values could be computed for the MK test, results for both genes were non-significant (results not shown).

Finally, I calculated the Tajima's D values for both *HTR1A* and *HTR1B*, which are shown in Table 3.1. The Tajima's D values were non-significant for both genes for all species analyzed.

The Placement of *M. nigra*

Although not closely related to the *mulatta* group, the haplotype network of *HTR1A* shows that *M. nigra* clusters with these species, sharing several mutations unique to the *mulatta* group (Figures 3.1 and 3.2). Notably, all four species – *M. mulatta*, *M. fuscata*, *M. cyclopis*, and *M. nigra* – share both a codon loss and a nonsynonymous substitution that no other species exhibit. Based on models of the human serotonin receptor (Kobilka et al., 1987), both mutations affect the extracellular amino terminal region of the receptor. The effect of the codon loss is unknown; however, the nonsynonymous substitution is identical to a polymorphism identified in humans, Gly22Ser (Nakhai et al., 1995). While this polymorphism has not been associated with behavior, possibly due to its low frequency in humans (Nakhai et al., 1995; Bergen et al., 1996), it does show a different pharmacological response than the wildtype (Rotondo et al., 1997).

Because it is unexpected for both the *mulatta* group and *M. nigra* to share these unusual mutations, especially the loss of a codon, to the exclusion of other, more closely related species (e.g., *M. nemestrina*), I verified these results by sequencing two additional *M. nigra* samples, as well as a sample from *M. maura* and *M. tonkeana*, using the same methods outlined below. These three species are located on Sulawesi and are closely related to each other (Tosi et al., 2003). All four additional samples had the same codon loss and nonsynonymous mutation, and all cluster with the *mulatta* group (Figure 3.1).

Recombination

I estimated the recombination rate as $C = 0.002$ for *HTR1A* and $C < 0.001$ for *HTR1B* using the methods of Hey and Wakeley (1997). For *HTR1A*, these estimates seem to be largely

the result of recombination outside of the coding region for *HTR1A* because when I only included the coding region, $C = 0$. In other words, the data suggest no recombination occurring within the coding region for HTR1A (see Figure A.1 in Appendix A).

DISCUSSION

Although behaviors are complex and no explanation of behavior can be reduced to a single gene or gene product, it is increasingly recognized that genetic variation plays an important role in mediating social behavior (Fitzpatrick et al., 2005; Robinson et al., 2008; Anstey et al., 2009). In addition, because evolution is ultimately a genetic process, understanding behavioral evolution entails understanding the evolution of neurological systems and the genes that underlie them. The serotonin system represents one such genetic/neurological system. Its connection to behavior has been well established and may play a role in macaque behavioral evolution. A window on the evolution of the serotonin system in macaques is provided here by examining the evolutionary history of two serotonin related genes: *HTR1A* and *HTR1B*.

The overall difference in the evolution of *HTR1A* and *HTR1B* is most easily demonstrated by comparing the two gene trees presented in Figure 3.1. Unlike *HTR1A*, *HTR1B* shows little genetic differentiation among macaque species. All of the species *HTR1B* haplotypes cluster tightly together, with the exception of the vervet, which was purposely included to provide an outgroup. In contrast, *HTR1A* among-species variation is much higher than in *HTR1B*, and the vervet does not form a distinct outgroup. Instead, three main groups of haplotypes can be discerned: one including *M. sylvanus*, *M. nemestrina*, *M. silenus*, and *M. assamensis* (with the outlying *M. fascicularis* individual), a second one consisting of *M. fascicularis*, and third one which includes the *mulatta* group and the Sulawesi macaques. The

difference between the two genes can also be seen in nucleotide diversity among species, which is 4 to 5 times higher in *HTR1A* than in *HTR1B*. These two genes have clearly experienced distinct evolutionary histories.

To determine if the observed patterns of variability in *HTR1A* and *HTR1B* result from different selective events, I employed several tests designed to detect departures from neutrality. Phylogenetic analyses using maximum likelihood provided evidence that selection is acting on *HTR1A*, but not *HTR1B*. Although the overall **ω** for both genes was low, suggesting that purifying selection is the dominant force acting on the coding regions of these genes, the maximum likelihood analyses indicate that at least some portions of *HTR1A* are under positive selection. That is, there is evidence that selection is acting to alter the structure of the protein itself, which is consistent with the finding of both a codon loss and a nonsynonymous substitution in a subset of the macaque species.

The HKA test also shows that *HTR1A*, but not *HTR1B*, is evolving in a non-neutral manner. When the outlying *M. fascicularis* individual was removed, several among-species comparisons were significant for HKA due to the high level of among-species divergence in *HTR1A* compared to within-species variation. However, the HKA results were not significant for any comparison with *M. sylvanus*, or between *M. mulatta* and *M. fuscata*. *M. mulatta* and *M. fuscata* exhibit a low degree of differentiation from each other (Figure 3.1) due to their close phylogenetic relationship, which explains this non-significant result. *M. sylvanus* is the outgroup to all Asian macaque species, being an African relict species and the first to diverge from the other macaque species in any phylogenetic analysis (e.g., Tosi et al., 2003; Vos, 2006; Li et al., 2009). The non-significant results for comparison to *M. sylvanus* may indicate that the selective sweeps occurred after the macaque radiation diverged from its African congeners and began to

spread across Eurasia. If the outlier is included, comparisons with *M. fascicularis* become non-significant because the estimate of within species variation for this species increases dramatically (although see Appendix A for further discussion). Even with the inclusion of the outlier, however, significant results still remain between *M. nemestrina* and the *mulatta* group, suggesting that *HTR1A* experienced at least one instance of positive selection in the macaque lineage. Because the significant results for *HTR1A* are driven by the high level of among-species divergence in this gene and the vast majority of polymorphisms occur in the non-coding region, the HKA results are likely due to selective events occurring in the regulatory region of the gene. Given the constraint on changes in the gene itself, demonstrated by the low **ω** found, this is not surprising. Thus, this study provides evidence for positive selection acting on both the structure and regulation of *HTR1A*.

While several non-selective forces, such as demography, increased mutation rate, and recombination may influence some of these tests, this is unlikely given that I obtained significant results from two different types of tests. That is, none of these factors alone could explain the significant results seen in both PAML and HKA (see Appendix A for further discussion).

Until future studies can determine the phenotypic effect of the polymorphisms identified here, the exact cause of selection on *HTR1A* remains speculative. However, I propose here that one potential explanation may be related to dispersal. Previous studies on the serotonin system have shown that it is connected with behaviors characteristic of invasive species (Ramboz et al., 1998; Samochowiec et al., 1999; Manuck et al., 2000; Trefilov et al., 2000; Caspi et al., 2002; Newman et al., 2005; Duckworth and Badyaev, 2007; Krackow and König, 2008; Sih and Bell, 2008; Anstey et al., 2009; Cote et al., 2010; Crockett et al., 2010; Filby et al., 2010; Vermeire et al., 2010), and certain genetic variants of serotonin may allow a species to exploit more habitats

through increased plasticity (Belsky et al., 2009; Chakraborty et al., 2010). Outside of humans, *M. mulatta* has the widest geographic distribution of all primates, and their fossil record indicates a long history of expansion (Delson, 1980). It is therefore possible that the divergent *HTR1A* haplotype characteristic of *M. mulatta* actually enabled their increase in range. While *M. fuscata* and *M. cyclopis* do not have large ranges and are limited to small islands, their close phylogenetic relationship with Chinese *M. mulatta* (Smith et al., 2007) and likely dispersal from the mainland *mulatta* range during recent glacial maxima (Melnick et al., 1993) is sufficient to explain the presence of this "high-dispersal" haplotype.

In a similar manner, the geographic location of the Sulawesi macaques is of interest in addressing the possible influence of *HTR1A* on dispersal. The island of Sulawesi has been separated from land masses to the west for 50 million years (Hall, 1996, 1998); the sharp distinction in the faunal composition between Sulawesi and Java/Borneo (what is known as Wallace's line) reflects this isolation. Yet the Sulawesi macaques provide a notable primate exception to this distinction by managing to cross the Makassar Straits between Borneo and Sulawesi, and thus Wallace line, at least once, and possibly twice (Evans et al., 1999). Therefore, although these species are restricted to a small island, it would not be unexpected for them to have reached Sulawesi because of a propensity to expand and explore new habitats.

The existence of a haplotype that contributes to "high-dispersal" behavior has the potential to explain both the signatures of selection and the unusual grouping of the Sulawesi and *mulatta* species in *HTR1A*. When a gene tree is incongruent with a species tree, there are two likely explanations. The first is introgression. This seems unlikely based on the geographic distribution of the *mulatta* species and the Sulawesi species (Figure 3.3). Furthermore, previous studies examining mitochondrial, Y-chromosomal, and autosomal DNA have not shown

evidence for introgression between these two groups (Morales and Melnick, 1998; Tosi et al., 2003). The second possibility, differential lineage sorting, is a more likely explanation. While differential linage sorting is usually discussed as a random process, certain circumstances exist where it is not. These include natural selection and a process termed "spatial sorting." Spatial sorting is the tendency for genes related to dispersal to sort geographically, which are most likely to accumulate at the edges of expanding ranges (reviewed in Shine et al., 2011). Because of this phenomenon, it is likely for genes related to high dispersal to show different genealogies from other loci, in much the same way that Y-chromosome data might be expected to differ from mitochondrial data in a species that shows sex-biased dispersal (e.g., Tosi et al., 2003). Spatial sorting and natural selection are deeply connected (Shine et al., 2011) and would explain the presence of a "high-dispersal" haplotype on islands on the outskirts of the macaque range.

The only other species examined here whose range expands as far eastward as the Sulawesi macaques is *M. fascicularis*, which inhabits mainland Southeast Asia as well as the islands of Borneo, Sumatra, Java, and the Philippines (Figure 3.3). This species is similar to the *mulatta* and Sulawesi macaques in that, relative to *M. sylvanus*, it forms a derived haplotype group for *HTR1A* (excluding the outlier). Two substitutions that separate this species from the others are a G/C and a C/A mutation at positions 985 and 986 of the exon, respectively, resulting in the substitution of Ala for His at amino acid 329, which is part of the third intracellular loop of the receptor (Kobilka et al., 1987). Mutations in this part of the receptor are known to affect transductional properties (Bruss et al., 2005). Whether this and the other substitutions defining this group lead to a "high-dispersal" phenotype is again speculative, but if so, this species has clearly achieved this through a different path than the *mulatta* or Sulawesi species.

CONCLUSION

The patterns of variation found in the two genes examined, *HTR1A* and *HTR1B*, show that each gene was influenced by different evolutionary forces and that, for *HTR1A*, positive selection probably played an important role. This selection has led to a high level of divergence among species and may explain some of the species differences in behavior, although further studies on the phenotypic effect of the sequenced polymorphisms are needed to confirm this. A previous study showed that selection has acted on the serotonin system in humans as well (Claw et al., 2010). Parallel evolution acting on the serotonin system of both macaques and humans may have contributed to the evolution of similar behavioral strategies that resulted in the successful colonization and exploitation of a diverse set of habitats over large regions of the world by both species. Behaviors such as aggressiveness and likelihood to disperse, both of which are related to serotonin functioning (Manuck et al., 2000; Trefilov et al., 2000; Newman et al., 2005; Krackow and König, 2008; Filby et al., 2010; Vermeire et al., 2010), are hypothesized to comprise a behavioral syndrome characteristic of invasive species (Duckworth and Badyaev, 2007; Sih and Bell, 2008; Cote et al., 2010) such as mosquitofish (*Gambusa affinis*: Cote et al., 2010) and exotic signal crayfish (*Pacifatasus leniusculus*: Pintor et al., 2009). Selection acting on this behavioral syndrome through the recruitment of the serotonin system may be a widespread phenomenon among invasive species, including humans and macaques. The study of the serotonin system and analysis of patterns of variation of genes associated with this system in invasive species will contribute to our understanding of the evolution of a range of behaviors.

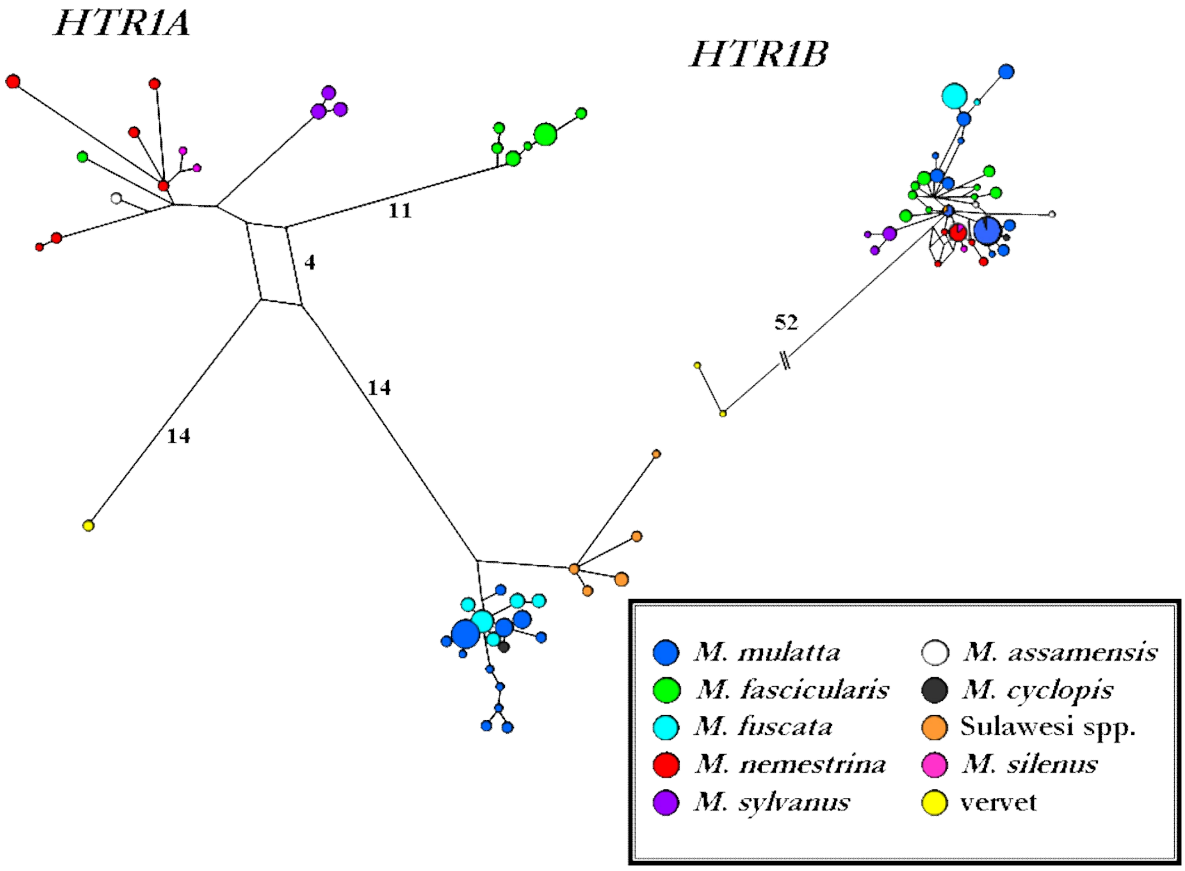

Figure 3.1. Shortest unrooted haplotype networks for *HTR1A* (left) and *HTR1B* (right). The networks represent all areas sequenced: coding regions and the areas flanking either side of the gene, including potential regulatory regions. Each circle represents a haplotype whose size is proportional to the frequency of the haplotype. The lengths of the lines connecting the circles are proportional to the number of mutations that separate each haplotype. To provide scale, a few of the lines are labeled to show the number of mutations between haplotypes. Because of the larger number of mutations separating the vervet from the macaques in *HTR1B*, this line is not drawn to scale.

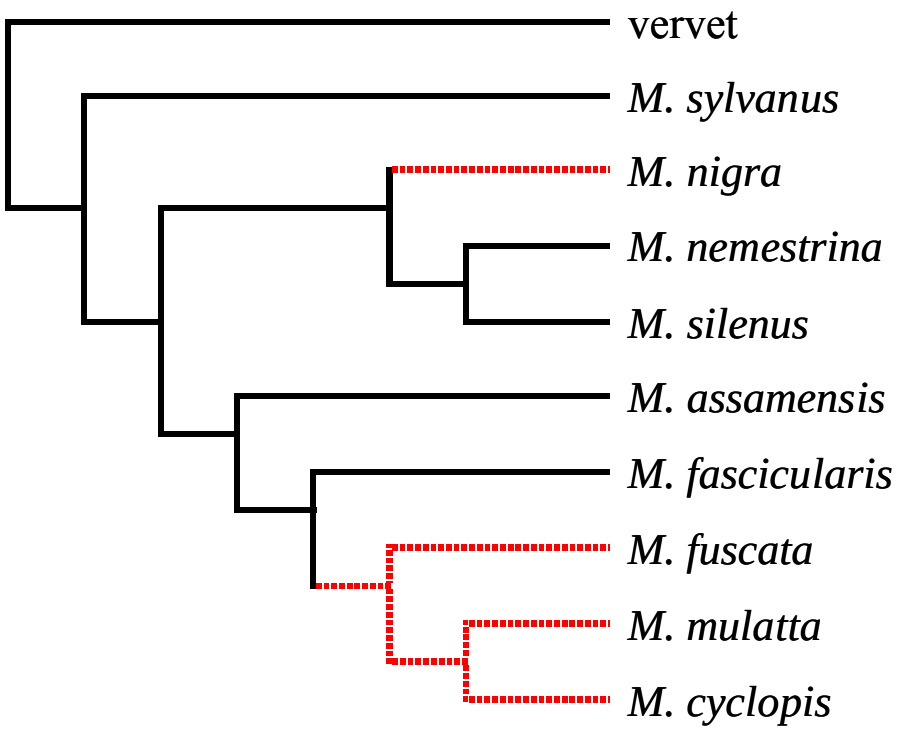

Figure 3.2. Phylogeny used to run analyses in PAML. The red dashed lines indicate where the loss of a codon occurred. Phylogeny based on Vos (2006).

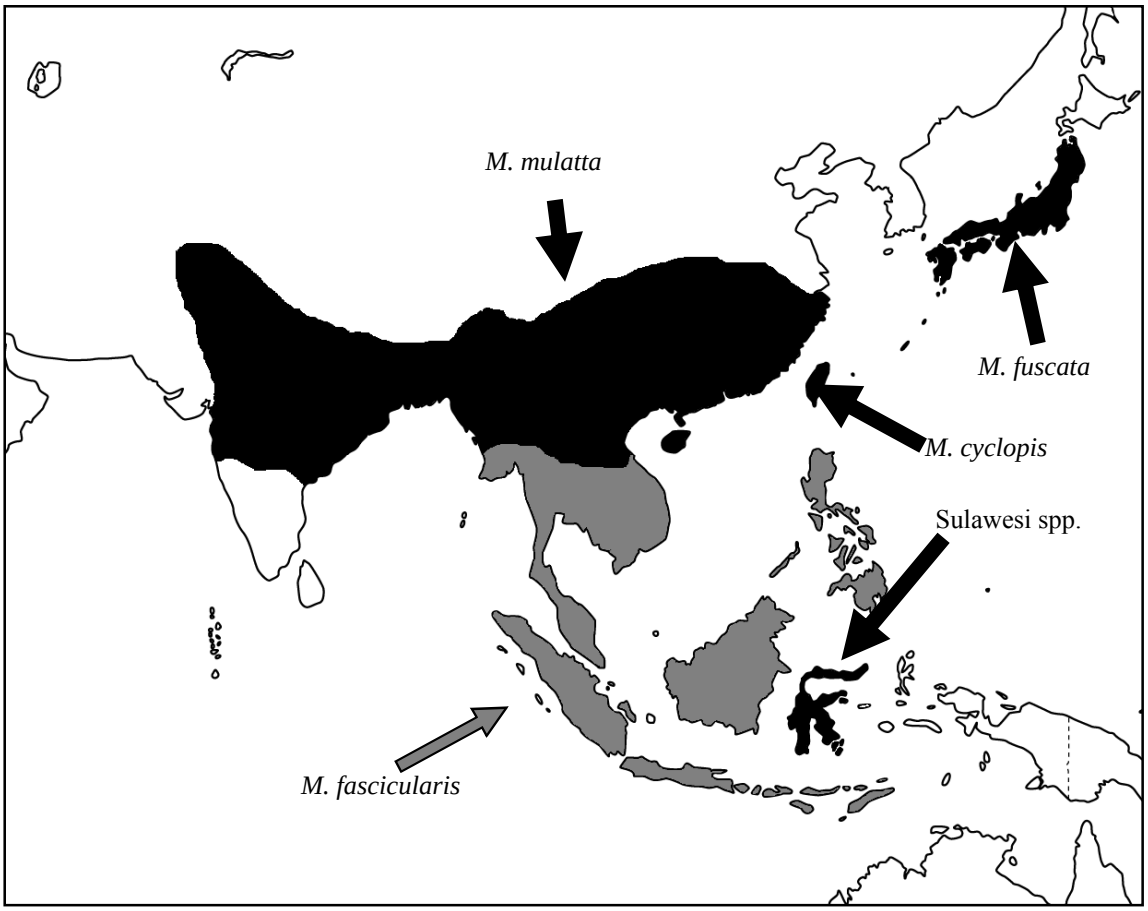

Figure 3.3. Map showing the distribution of *M. mulatta*, *M. fascicularis*, *M. fuscata*, *M. cyclopis*, and the Sulawesi macaques.

Table 3.1. Indices of within-species genetic diversity found in *HTR1A* and *HTR1B* for five species of macaque. Indices include the number of polymorphisms found within each species, two estimates of theta, and Tajima's D. The polymorphisms found within each species are separated into two types: SNPs and indels. In addition, the number of nonsynonymous (NS) polymorphisms is indicated. For *Macaca mulatta*, indices for both the Chinese and the Indian populations are shown separately, as well as indices for the species as a whole. For *M. fascicularis*, indices for *HTR1A* are shown both with and without the outlier.

HTR1A	*Macaca mulatta*			*Macaca fascicularis*		*Macaca*	*Macaca*	*Macaca*
	China	India	Total	Original	No Outlier	*fuscata*	*nemestrina*	*sylvanus*
Polymorphisms	18	15	18	28	7	6	27	2
SNP	16	13	16	26	7	6	25	2
Indel	2	2	2	2	0	0	2	0
Nonsynonymous	0	0	0	2	0	1	1	0
Haplotypes	8	7	11	7	6	6	6	3
Theta (S)	4.65	3.57	3.76	7.13	1.97	1.65	8.28	0.77
Theta (π)	5.72	3.28	4.65	5.82	2.27	1.82	9.95	1.07
Tajima's D	-0.78	0.28	0.25	-0.88	0.49	0.33	0.46	1.03

HTR1B	*Macaca mulatta*			*Macaca*	*Macaca*	*Macaca*	*Macaca*
	China	India	Total	*fascicularis*	*fuscata*	*nemestrina*	*sylvanus*
Polymorphisms	8	10	11	12	4	5	2
SNP	7	10	10	11	4	5	2
Indel	1	0	1	1	0	0	0
Nonsynonymous	0	0	0	0	2	1	0
Haplotype	6	5	10	9	3	5	3
Theta (S)	2.04	2.74	2.35	3.02	1.10	1.66	0.77
Theta (π)	2.25	3.77	3.20	3.51	1.74	1.35	0.68
Tajima's D	-0.24	1.27	0.84	0.28	1.65	-0.68	-0.45

Table 3.2. Genetic distance within and among species for *HTR1A* and *HTR1B*. The diagonal elements show the nucleotide diversity within species and the off-diagonal elements show the nucleotide diversity among species. Nucleotide diversity was averaged over all loci. Mul = *M. mulatta*; Fas = *M. fascicularis*; Fus = *M. fuscata*; Nem = *M. nemestrina*; Syl = *M. sylvanus*. See Table A.4 for similar comparisons in the non-coding regions sequenced.

HTR1A

	Mul	Fas	Fus	Nem	Syl
Mul	0.0015				
Fas	0.0125	0.0019			
Fus	0.0013	0.0120	0.0006		
Nem	0.0122	0.0068	0.0121	0.0032	
Syl	0.0115	0.0083	0.0115	0.0060	0.0003

***HTR1A* (outlier removed)**

	Mul	Fas	Fus	Nem	Syl
Mul	0.0015				
Fas	0.0125	0.0007			
Fus	0.0013	0.0121	0.0006		
Nem	0.0122	0.0071	0.0121	0.0032	
Syl	0.0115	0.0085	0.0115	0.0060	0.0003

HTR1B

	Mul	Fas	Fus	Nem	Syl
Mul	0.0015				
Fas	0.0021	0.0017			
Fus	0.0032	0.0036	0.0008		
Nem	0.0018	0.0020	0.0038	0.0006	
Syl	0.0028	0.0028	0.0047	0.0023	0.0003

Table 3.3. Results of two selection tests for *HTR1A* and *HTR1B*. For the **ω** test, the numbers represent the ratio calculated for each species comparison. The numbers for the HKA test represent the sum of deviations calculated in the HKA program. See Table 3.2 for list of species. *Results are significant ($p < 0.05$).

HTR1A

ω

	Mul	Fas	Fus	Nem
Fas	0.242*			
Fus	0.046*	0.252*		
Nem	0.152*	0.423	0.162*	
Syl	0.121*	0.269	0.129*	0.043*

HKA (sum of deviations)

	Mul	Fas	Fus	Nem
Fas	13.93			
Fus	3.65	10.72		
Nem	22.15*	10.23	18.89*	
Syl	14.82	5.84	6.93	6.22

***HTR1A* (outlier removed)**

ω

	Mul	Fas	Fus	Nem
Fas	0.253*			
Fus	0.046*	0.263		
Nem	0.152*	0.460	0.162*	
Syl	0.121*	0.296*	0.129*	0.043*

HKA (sum of deviations)

	Mul	Fas	Fus	Nem
Fas	23.04*			
Fus	3.65	22.69*		
Nem	22.15*	19.06*	18.89*	
Syl	14.82	13.84	6.93	6.22

HTR1B

ω

	Mul	Fas	Fus	Nem
Fas	0.000*			
Fus	0.128*	0.180		
Nem	0.039*	0.076*	0.189	
Syl	0.000*	0.000*	0.113*	0.031*

HKA (sum of deviations)

	Mul	Fas	Fus	Nem
Fas	1.75			
Fus	5.25	2.21		
Nem	4.73	7.58	9.86	
Syl	14.53	10.41	8.78	9.34

Table 3.4. Results of the PAML analyses comparing various sites models for *HTR1A* and *HTR1B*. Models M2 and M8 represent null models (no selection), whereas Models M1, M7, and M8a allow for selection at some sites within the gene. See (Yang and Nielsen, 2000; Swanson et al., 2003) for detailed explanation of models.

Models compared		**p-values**	
Null	*Selection*	*HTR1A*	*HTR1B*
M1	M2	0.0007	0.9909
M7	M8	0.0007	0.9160
M8a	M8	0.0001	0.8911

LITERATURE CITED

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CHAPTER 4

INTRA- AND INTERSPECIFIC VARIATION IN *TPH2* AND THE ROLE OF DEMOGRAPHIC HISTORY IN MACAQUE BEHAVIORAL EVOLUTION

ABSTRACT

The macaque genus (*Macaca*) represents a key area of research in the evolution of behavior. Comprised of 19 species, the macaques are notable for displaying a wide range of behavioral strategies across species. One biological system that has been suggested to play a role in creating this behavioral variation is the serotonin system. Heavily investigated for its connection to behavior, the serotonin system is a likely candidate for selection to act on in order to shape behaviors. However, little is known about its evolutionary history in these species. Here, I examine *TPH2*, a gene necessary for the production of serotonin in the brain.

Because *TPH2* was only recently discovered, studies in nonhuman primates are severely limited for this gene. In this chapter I examine the level of variation within and between several species of macaques and show that very few polymorphisms are shared across species. I also highlight how the presence of population structure can impact the analyses of molecular variation; the presence of two distinct populations of *M. mulatta* has a significant effect on results. Finally, I place this gene in an evolutionary context by examining the role of selection in its evolution. While individuals from Chinese populations of *M. mulatta* show signatures of elevated evolution by the presence of derived alleles at high frequencies, this is likely a result demographic effects, as most tests of selection indicate neutral evolution. Therefore, behaviors related to *TPH2* may be shaped by neutral forces and may not be adaptive. Alternatively, the presence of environmental and genetic "buffers" may limit the effect of selection on this gene.

INTRODUCTION

The evolution and proximate causes of behavior are of central concern to primatology and evolutionary biology in general. In this regard, the macaque genus (*Macaca*) represents a vital area of research. Several features make this genus an incredibly useful model for addressing questions surrounding the interactions between behavior, genetics, neurology, environment, and evolution. Because of the frequent use of macaques as animal models in biomedical studies, much is known about their biology. Furthermore, the genus is comprised of several species (approximately 19: Fooden, 1976, 1980) that span an exceptionally large geographic range (Lindburg, 1991). The rhesus macaque (*M. mulatta*), in particular, inhabits a diverse set of habitats, from the northern parts of Pakistan and India through eastern China, where they dwell in primary forests and alongside humans in urban settings. Most importantly, the macaque genus is notable for exhibiting a wide range of behaviors and social systems, ranging from groups with fairly tolerant social structures to more aggressive groups with strict hierarchies (Thierry, 1985, 2000; Caldecott, 1986; De Waal and Luttrell, 1989; Clarke and Lindburg, 1993; Aureli et al., 1997; Petit et al., 1997). These behavioral variations correlate strongly with phylogeny (Thierry et al., 2000, 2008), suggesting a common underlying neurological mechanism for behavioral differences (Wendland et al., 2006). Thus, one approach to understanding the evolution of different behavioral strategies is to examine the evolution of the macaque brain.

A key neurological system that has been repeatedly highlighted in studies on the biological bases of behavior is serotonin (5-HT). Across multiple taxa, including primates, serotonin has been shown to play a role in aggression (Newman et al., 2005), dispersal (Trefilov et al., 2000), social behavior (Anstey et al., 2009; Crockett et al., 2010), sexual behavior (Dominguez and Hull, 2010; Liu et al., 2011), stress response (Chen et al., 2010b), and

exploration (Ramboz et al., 1998). Likewise, investigations into variation for genes that underlie the serotonin system have identified several genetic features that are correlated with these behaviors (e.g., Brunner et al., 1993a, b; Lesch et al., 1996; Caspi et al., 2002, 2003; Davidge et al., 2004; Zill et al., 2004a, b; Chen et al., 2010a). Interestingly, similar, independently evolved genetic variants have been found in both macaques and humans (e.g., Lesch et al., 1997; Newman et al., 2005), suggesting parallel evolution between the two taxa. However, although a large amount of research has been devoted to investigating the proximate links between serotonin and behavior, little is known about its evolutionary history (but see Soeby et al., 2005; Andres et al., 2007). And yet, knowledge of the evolution of serotonin is likely to grant insight into behavioral evolution. For example, given the strong link established between serotonin and behavior, it has been argued that this system provides a promising mechanism for explaining behavioral differences between macaque species (Wendland et al., 2006; Chapter 3). Accordingly, genes related to serotonin functioning are predicted to vary between species and will result in behavioral differences. Furthermore, if the behavioral differences found between species are the result of selection, we should expect to find signatures of selection in the patterns of variation of these genes in one or more species. Indeed, previous studies examining various components of the serotonin system in humans (Claw et al., 2010) and macaques (Chapter 3) have found evidence for selection occurring in these lineages. Here, I expand on those studies by examining the gene *TPH2* in macaques.

Serotonin is produced though a two-step process that converts tryptophan into 5-HT, with tryptophan hydroxylase (TPH) acting as a rate-limiting enzyme in this process. *TPH2* codes for the second isoform of TPH and is expressed exclusively in brain tissue (Walther and Bader, 2003), making it a crucial gene of study for behavioral genetics. Although this gene was only

described in 2003, numerous studies have already demonstrated a connection between *TPH2* and behavior (e.g., Harvey et al., 2004; Zill et al., 2004a, b; Coon et al., 2005; Walitza et al., 2005; Zhang et al., 2005; Zhou et al., 2005). These studies have largely been limited to humans and little has been done to determine the genetic variation of this gene in nonhuman primates. A notable exception to this is Chen et al. (2006, 2010b), who both described the genetic variation within rhesus macaques (*Macaca mulatta*) and demonstrated that variations in the regulatory regions of this gene had a significant impact on stress response, as measured by HPA (hypothalamic-pituitary-adrenal) axis activity and levels of self-injurious behavior. However, these studies were restricted to only *M. mulatta* of Indian origin, and it is unclear to what extent these results may apply to other populations of *M. mulatta* or to other macaque species. Information on a wider range of taxa is critical because pharmacological and psychological studies often use different species of macaques, so knowledge of genetic differences between species may be needed to explain inconsistent results. Furthermore, if we are to address the hypothesis that differences in *TPH2*, and the serotonin system in general, explain the behavioral variation observed in macaques, a broader sampling of genetic data is needed. Consequently, in this study, I quantify the genetic variation of *TPH2* within and between several species of macaques. I also begin to place *TPH2* in an evolutionary context by testing the influence of selection on the evolutionary history of this gene.

METHODS

Subjects

DNA samples from 27 *Macaca mulatta* (13 from India, 14 from China), 10 *M. fascicularis*, 11 *M. fuscata*, 6 *M. nemestrina*, and 5 *M. sylvanus* were used in this study (Table

4.1). These species formed the focus of all analyses. Past studies have shown that *M. mulatta* individuals from China and India exhibit moderate levels of population differentiation (Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2003; Smith, 2005; Hernandez et al., 2007; Satkoski et al., 2008) a factor that can influence analyses. Therefore, I conducted an analysis of molecular variance (AMOVA) (Weir and Cockerham, 1984; Excoffier et al., 1992; Weir, 1996) using Arlequin (Excoffier et al., 2005) to estimate the degree of population differentiation. I also performed separate analyses on the Chinese and Indian samples as well as on *M. mulatta* as a whole. In addition to the previously mentioned species, one sample each from *Macaca assamensis*, *M. cyclopis*, *M. nigra*, *M. silenus*, and *Chlorocebus aethiops* were included (Table 4.1). These additional samples were primarily used for two purposes: 1) to generate an estimate of the level of between-species genetic variation and 2) for use in PAML analyses (see below) to place the results in a phylogenetic framework. Unless otherwise noted, they were not included in any other analyses described below. In total, 64 DNA samples were sequenced. The nine different macaque species analyzed span the range of behavioral variation found in the genus, and three of the four *Macaca* "species groups" are represented in this study (*sylvanus*-*silenus*, *fascicularis*, and *sinica*: Fooden, 1976, 1980). The *C. aethiops* (vervet) sample is used as the outgroup to the macaque species. Specifically, it was used as the outgroup for HKA, **ω**, McDonald-Kreitman, and Fay and Wu's H (described below).

PCR and Sequencing

TPH2 is incredibly large, consisting of 11 exons spread over a 90 kilobase (kb) region. In order to efficiently examine *TPH2* in a large number of samples, I focused on selected regions of the gene. I amplified four large segments of *TPH2*, which include i) the 5'UTR, Exon 1, and flanking regions (1527 baspairs (bp)), ii) Exon 2 and flanking regions (1212 bp), iii) Exons 3-4

and flanking regions (1409 bp), and iv) Exons 10-11, the 3'UTR, and flanking regions (2186/2345 bp) (Figure 4.1). In total I successfully sequenced 6352 bp, including 849 bp of coding regions. The sequenced regions provide an overview of variation present across the gene, particularly in non-coding, potentially regulatory regions where selection is less likely to limit variation. In addition, by focusing on both ends of the gene, an accurate sense of the level of recombination experienced by *THP2* is obtained. Because these sections of the genes are so widely separated, and because there was a chance of recombination between the regions, I analyzed each of these areas separately. However, in order to increase power, these regions were combined into one large haplotype and analyzed as a whole.

Amplification of the *TPH2* fragments was carried out using both previously published and newly designed primers (Table B.1 in Appendix B). The new primers were designed based on the *Macaca mulatta* draft assembly (Rhesus Macaque Genome Sequencing and Analysis Consortium et al., 2007) using Primer3 (Rozen and Skaletsky, 2000) and GeneRunner (generunner.net). In addition to *TPH2*, five additional non-coding regions were selected for amplification (Table A.3 in Appendix A); these regions were at least 20,000 base pairs from any feature with a known function (Satkoski Trask et al., 2011) and provide presumably neutral loci for use in the HKA test (see below) (Hudson et al., 1987). The PCR protocols varied for all regions amplified and are available upon request of the author.

I used the ExoSAP-IT protocol (usb.com) to clean up the PCR product and submitted it to the W.M. Keck Center for Comparative and Functional Genomics, UIUC for Sanger sequencing. As with the primers used for amplification, I used a combination of published and newly designed primers for sequencing (Table B.1). Efforts were made to design primers that would

provide substantial overlap with each other so that any one region being analyzed would have multiple reads from different primers, ensuring the accuracy and quality of the sequence.

Once received, sequences were aligned and edited manually using Sequencher (www.genecodes.com). Each heterozygote base pair was confirmed visually by identifying clear double peaks in the chromatogram. All SNPs (single nucleotide polymorphisms) and indels (insertions/deletions) were identified. Where indels involved more than one sequential nucleotide, the entire deleted region was treated as a single mutational event. For example, the presence of an 11-bp indel would be treated as a single polymorphic site rather than 11 polymorphic sites. Haplotypes were determined using the program Phase v.1 (Stephens et al., 2001; Li and Stephens, 2003; Stephens and Donnelly, 2003). In order to visualize the genetic variation and relationships among the haplotypes of each gene, I constructed a haplotype tree using the reduced median method in Network v4.6 (fluxus-engineering.com). Because of the large number of base pairs sequenced and the presence of recombination (see results), a minimum spanning tree was also generated, which presents the simplest relationship between haplotypes. The minimum spanning tree was generated in Arlequin (Excoffier et al., 2005) and graphically modified using HapStar (Teacher and Griffiths, 2011) and Adobe Illustrator®.

Analysis

With the exception of the likelihood ratio tests and the haplotype networks, all analyses were conducted on the five species for which I had more than one sample: *M. mulatta*, *M. fuscata*, *M. fascicularis*, *M. nemestrina*, and *M. sylvanus*. Several indices of molecular diversity were calculated for each of the five species using Arlequin (Excoffier et al., 2005). These included two different estimates of theta, θ_S (Watterson, 1975) and θ_π (Tajima, 1983), which

were used to estimate within-species diversity, and the nucleotide diversity (Tajima, 1983), which was used to estimate within- and among- species diversity.

In order to determine the role of selection on patterns of variation in *TPH2*, I used multiple approaches that each utilizes different aspects of genetic data. For all selection tests, I used a significance level of 0.05. First, I applied the HKA test on the five species of macaques. The HKA test examines the ratio of within- versus among-species variation in a gene of interest and compares it to that of several unlinked, neutral loci (Hudson et al., 1987). A gene under balancing selection will have a high level of within-species variation compared to the other loci, while one that has undergone positive selection will have a high level of between-species variation. I used the five non-coding regions described above for the comparison with *TPH2* (Table A.3). For all macaque species tested, *C. aethiops* was used as the comparison species. HKA was conducted on software provided by J. Hey (http://genfaculty.rutgers.edu/hey/software).

Next, I analyzed the ratio of nonsynonymous to synonymous mutations (**ω**) in the coding regions of the genes to detect signatures of selection. Because synonymous mutations do not affect protein structure, they are assumed to evolve neutrally. In contrast, selection is more likely to act on nonsynonymous mutations. Positive selection is expected to increase the relative rate of nonsynonymous substitutions, whereas purifying selection is expected to decrease it (Kimura, 1977). I determined whether **ω** between *C. aethiops* and the five species of macaques significantly differs from 1.0 (Kimura, 1977; Yang and Nielsen, 2000). A **ω** > 1.0 indicates positive selection, while **ω** < 1.0 indicates purifying selection. The significance of this is calculated using a Z-test:

$$Z = \frac{(d_N - d_S)}{\sqrt{(Var(d_N) - Var(d_S))}},$$

where d_N is the number of nonsynonymous mutations per nonsynonymous site, d_S is the number of synonymous mutations per synonymous site, and $Var(d_N)$ and $Var(d_S)$ are their respective variances. Using the program MEGA v.4 (Tamura et al., 2007), estimates of d_N and d_S were obtained using the Nei-Gojobori method (Nei and Gojobori, 1986) and variances were determined using the bootstrap method (Nei and Kumar, 2000).

However, this method of testing for positive selection is very conservative, so two additional sets of tests were employed. First, the McDonald-Kreitman (MK) test (McDonald and Kreitman, 1991) compares synonymous and nonsynonymous ratios within and between two species, using the program DNAsp (Rozas et al., 2003). This ratio should remain constant for genes evolving neutrally, but **ω** is expected to be higher between species if there is positive selection occurring. This test is more sensitive to selection since **ω** does not have to be greater than one to produce a significant result. As with HKA, I made pairwise comparisons between *C. aethiops* and the five macaque species. Second, I conducted likelihood ratio tests (LRT) on all of the samples available using the program PAML (Phylogenetic Analysis using Maximum Likelihood: Yang, 1997, 2007). PAML determines the maximum likelihood values for different models of evolution within a phylogenetic framework. Models that incorporate positive selection can be compared to null models and LRT used to determine which model best fits the data. Because PAML allows only one sequence per species, I included all ten species using the phylogeny seen in Figure 3.2 (Tosi et al., 2003; Vos, 2006; Li et al., 2009). Haplotypes were determined based on fixed differences between species and I did not perform separate analyses for Indian and Chinese macaques. I tested a variable branch model, which allows **ω** to vary among each of the branches of the macaque phylogeny, to see if selection has occurred on a specific lineage (Yang, 1998). Like MK, **ω** does not have to be greater than one, but simply

elevated compared to other lineages of the macaque phylogeny. I also compared several sites models using PAML (M1 vs. M2, M7 vs. M8, and M8a vs. M8: see Yang et al., 2000; Swanson et al., 2003). These models allow **ω** to vary among different sites on the gene using different parameters (Nielsen and Yang, 1998; Yang et al., 2000; Swanson et al., 2003). Most sites on a gene are under strong purifying selection, which reduces the overall **ω** for a gene and can hide signals of positive selection occurring only in a small portion of the gene. Testing the sites models in PAML can determine if a portion of a gene shows signs of positive selection, even if the average **ω** ratio over the entire gene is low.

Finally, I carried out two tests designed to detect skews in the frequency spectrum of alleles: Tajima's D (Tajima, 1989b) and Fay and Wu's H (Fay and Wu, 2000). Tajima's D compares the theta estimators θ_π (Tajima, 1983) and θ_S (Watterson, 1975); intermediate-frequency alleles contribute the most to θ_π, while θ_S is more affected by low-frequency alleles. An excess of rare alleles (significantly negative Tajima's D) is consistent with positive selection, while an excess of intermediate-frequency alleles (significantly positive Tajima's D) is consistent with balancing selection. The value and significance of Tajima's D was determined using Arlequin (Excoffier et al., 2005). Fay and Wu's H compares the theta estimators θ_π and θ_H. In this case, high-frequency, derived alleles contribute the most to θ_H, so that positive selection is inferred from an excess of high frequency alleles (significantly negative H). To calculate H, an outgroup is needed to estimate which alleles are derived; here I use the *C. aethiops* sample. Significance for Fay and Wu's H was determined by running coalescent simulations using an estimate of the recombination rate and theta (θ_π) as parameters (Fay and Wu, 2000). The value and significance of Fay and Wu's H was determined using DNAsp (Rozas et al., 2003).

Because of the large size of *TPH2*, recombination is likely (Chen et al., 2006). The presence of recombination can increase false positives in the MK test (Andolfatto, 2008) and the LRT of the sites models using PAML (Anisimova et al., 2003); for HKA and Fay and Wu's H, its presence makes positive selection more difficult to detect (Hudson et al., 1987; Fay and Wu, 2000). I used the SITES program of J. Hey (http://genfaculty.rutgers.edu/hey/software) to obtain an estimate of the recombination parameter *C*, where $C = 4Nc$, *c* is the rate of recombination per generation per base pair, and *N* is the effective population size (Hey and Wakeley, 1997). Because the LRT only examines the coding region, whereas the HKA test can analyze both coding and non-coding regions, I obtained estimates of *C* based on the entire regions sequenced and on the coding region alone. I was able to account for recombination in determining the significance of Fay and Wu's H by using it as a parameter in coalescent simulations, but not in the other tests employed here. Presence of high levels of recombination indicates that a significant result for the MK test and the LRT of the sites models should be treated with caution, but should not affect the interpretation of a significant result for HKA. In addition, in order to visualize the level of linkage disequilibrium (LD) an LD plot between all polymorphic sites within *M. mulatta* was generated using the program JLIN (Carter et al., 2006), which calculates LD based on genotypic, rather than haplotypic, data.

RESULTS

Substructure and Recombination in Macaque Species

The *M. mulatta* samples show significant differentiation between the Chinese and Indian populations ($p<0.0001$), with 11.54% of the total genetic variance in *M. mulatta* attributed to

between-population differences. I therefore discuss the results of the two populations separately, as well as discuss the results for the species as a whole.

I also found evidence for recombination. The recombination estimate of $C = 0.002$ (per base pair) is moderate, although there is no evidence of recombination in the coding regions alone ($C = 0$). Furthermore, the linkage disequilibrium plot shows a tendency for polymorphisms in the 3'UTR region to have low LD with the other regions sequenced, as might be expected (Figure 4.2) based on the physical distance separating it from other regions of the gene. However, results did not differ in a meaningful way, in the context of evolutionary history, between analyses that considered the regions amplified separately and those that examined all regions together. Consequently, only the results for the combined regions are discussed here.

Molecular Diversity

Measures of molecular diversity are shown in Tables 4.2 and 4.3, a list of polymorphic sites identified is provided in Table 4.4, and the minimum spanning tree is shown in Figure 4.3. (See also Table B.2 for a list of interspecific substitutions.) For clarity, I use the term "polymorphism" when referring to intraspecific differences, the term "substitution" when referring to interspecific differences, and the term "mutation" when referring to variation either within or between species. For ease of discussion and comparison with previous studies, I repeat the system for designating polymorphic sites utilized by Chen et al. (2006). Within the regions sequenced, most of the polymorphisms previously identified in *M. mulatta* (Chen et al., 2006) were confirmed in my samples, including a 159-bp insertion in the 3'UTR (2128S>L). I also identified several additional polymorphic sites in *M. mulatta* that were not previously described, including an 11-bp insertion in Intron 9 (IVS9-156del>in). This indel is in complete linkage with 1503A>G. As with Chen et al. (2006), I found 1503A>G (and IVS9-156del>in) to be in

complete linkage with 2128S>L, but only for the Indian *M. mulatta*; I did not find the 2128S>L polymorphism in Chinese *M. mulatta*, although this population is polymorphic for the two other sites (1503A>G and IVS9-156del>in).

In addition to *M. mulatta*, I describe the level of intraspecific variation and identify polymorphic sites in each of four additional macaque species: *M. fascicularis*, *M. fuscata*, *M. nemestrina*, and *M. sylvanus* (Table 4.4). Most polymorphisms identified were not shared by multiple species. In one instance, a polymorphic site was shared by both *M. mulatta* and *M. nemestrina*; however, the actual polymorphism differed between the species, as *M. nemestrina* possessed a T/C polymorphism rather than a T/G polymorphism. Across all macaque species examined, only one nonsynonymous polymorphism was identified (25Pro>His), and this was only in a small proportion of the Indian *M. mulatta* samples (minor allele frequency = 0.077). Comparing substitutions among species, only one nonsynonymous mutation was found (54Lys>Arg); this occurred between *C. aethiops* and all of the macaque species (Table B.2).

Notably, *M. sylvanus* was highly unusual in showing almost no intraspecific variation, especially compared to the other macaque species (Tables 4.2 and 4.3). It is possible that these results are due to allelic dropout. That is, the primers used in this study preferentially amplified only one allele in this species. Allelic dropout effectively decreases sample size for this species and makes any measure of intraspecific variation unreliable. Consequently, this species was dropped from all subsequent analyses except for PAML.

Evidence for Selection

Results for the HKA tests were non-significant (Table 4.5). For **ω**, all the values were significantly less than 1 (Table 4.5), consistent with purifying selection. Because of the low number of nonsynonymous mutations, McDonald-Kreitman could not be calculated except for in

M. mulatta – this was non-significant (Table 4.5). Likewise, the results for the LRTs in PAML were all non-significant (results not shown).

The results of Tajima's D and Fay and Wu's H are shown in Table 4.2 and 4.5. While none of the Tajima's D values were significant, the Chinese population of *M. mulatta* approached significance (D = -1.24, p = 0.08). For Fay and Wu's H, only *M. mulatta* showed significant results (H = -6.22, p = 0.03). An examination of the separate populations of *M. mulatta* show that it is likely that the Chinese population is driving these results. While the Indian population does not have a significant H-value (H = -1.81, p = 0.21), the Chinese population's H-value is significant (H = -4.98, p = 0.05). Thus, this population of *M. mulatta* possesses derived alleles at a higher frequency than would be expected under neutrality.

DISCUSSION

For this study, I provide a detailed description of the genetic variation within and among various species of macaques on *TPH2*, a gene known to play an important role in regulating behavior (Harvey et al., 2004; Zill et al., 2004a, b; Coon et al., 2005; Sheehan et al., 2005; Walitza et al., 2005; Zhang et al., 2005; Zhou et al., 2005). Previous studies (Chen et al., 2006, 2010b) identified a number of polymorphisms present in *M. mulatta* and showed a link between regulatory polymorphisms, HPA activity, and self-injurious behavior. However, this work was limited to *M. mulatta* individuals of Indian origin, so it is unclear 1) if the polymorphic sites identified are informative for other populations of *M. mulatta* or other macaque species, and 2) if the behavioral link established extends outside Indian *M. mulatta*. Addressing these questions is critical for biomedicine, which frequently uses macaques of various origins in research, because the presence of interpopulation and interspecific genetic differences may confound results.

Additionally, primatologists would benefit from the establishment of reliable genetic markers for field studies in behavior. In this study, I focus on the first of these issues by providing a description of the genetic variation within and among various species of macaques for *TPH2*. I also begin to frame this gene in an evolutionary context by examining the possible role of natural selection on *TPH2*.

I was able to confirm the polymorphisms identified by Chen et al. (2006) as well as identify several additional polymorphisms, including an 11-bp insertion. Future studies that examine the potential effect of this insertion and other polymorphisms are needed to determine their significance. Importantly, I found that polymorphic sites were rarely shared across multiple species. Even among the three most heavily sampled and most closely related species, *M. mulatta*, *M. fuscata*, and *M. fascicularis*, there were only four shared polymorphisms: three between *M. mulatta* and *M. fascicularis* and one between *M. fuscata* and *M. fascicularis*. This is in sharp distinction from previous research that found a substantial overlap in polymorphic sites (~31%) for *M. mulatta* and *M. fascicularis* when looking across the genome (Malhi et al., 2011). Furthermore, of the 52 polymorphisms identified in *M. mulatta*, 28 were unique to either the Indian or the Chinese populations (Table 4.4). It is possible that some of these polymorphisms do exist across groups at lower frequencies and larger sample sizes are needed to detect them. However, in general the results indicate knowledge of *M. mulatta* genetic variation alone is not very informative for identifying polymorphisms in other species or other *M. mulatta* populations, at least for *THP2*, potentially due to the effects of purifying selection. This point is important for both behavioral studies looking for potential polymorphisms to explain variation in behavioral strategies and pharmacological studies that may find inconsistent results across populations or species.

This study also highlights the need to look at species structure when examining genetic variation in an evolutionary context. The presence of structure within *M. mulatta* – namely, the division of this species into two distinct populations – influenced the results of the tests used here, discussed in more detail below. In addition, the inclusion of samples from China seems to explain at least some of the results that differ from past examinations of polymorphisms in *M. mulatta*. Whereas Chen et al. (2006) found complete linkage between the 159-bp insertion and 1503A>G in the 3'UTR region, I found complete linkage only for the Indian samples. Therefore, increased sampling from a wide variety of source populations is necessary for an accurate description of the molecular variation of this species.

The genetic variation found within and among species is the result of a number of evolutionary forces including drift, gene flow, mutation, demographics, and selection. Evidence of selection in genomic regions is often the focus of research because it highlights potential features that are important to an individual's fitness; however all evolutionary forces shape the patterns of genomic diversity of a population and species. Most analyses indicate that there was no significant positive selection acting on *TPH2* in macaques. Instead, the incredibly low number of nonsynonymous mutations suggests purifying selection.

The two tests that examine the allele frequency spectrum, Tajima's D and Fay and Wu's H, are suggestive of selection in the Chinese *M. mulatta* population. However, these results are likely a result of demographic history rather than the result of selection. In the case of Tajima's D, while the Chinese population has a low p-value ($p = 0.08$), it does not reach significance. Furthermore, Tajima's D is known to be highly sensitive to past demographic events, especially population expansions (Tajima, 1989a). A previous study showed that the Chinese population of *M. mulatta* underwent a 3-fold population expansion approximately 160 thousand years ago

(Hernandez et al., 2007). It is therefore very likely that the Tajima's D value reflects this past population event rather than selection. The results for Fay and Wu's H are moderately more robust. The H-value for *M. mulatta* was significant ($p = 0.03$) and a breakdown of the species into its two main populations (Chinese and Indian) show that this result is largely driven by the Chinese population. In contrast to Tajima's D, Fay and Wu's H is not expected to be affect by population expansions (Fay and Wu, 2000). This is because high-frequency, derived alleles contribute most heavily to H, whereas population expansions are expected to produce derived variants at low frequencies (Tajima, 1989a; Fay and Wu, 2000).

It is therefore tempting to propose that, taken together with the suggestive results of Tajima's D, the results of Fay and Wu's H indicate that *TPH2* underwent positive selection in the Chinese population of *M. mulatta*. However, I hesitate to support this interpretation for several reasons. Firstly, these results are not consistent with any of the other tests preformed. A strong case for selection is only made when it can be confirmed with multiple approaches that draw on different sources of genetic data. Secondly, the p-values obtained are only marginally significant, particularly in the case of the Chinese *M. mulatta*. Finally, while Fay and Wu's test is robust to most demographic assumptions, certain situations, such as population substructure, can affect the results (Fay and Wu, 2000; Przeworski, 2002). Because of the known substructure of *M. mulatta*, this likely explains the significant results found for this species. Although I have attempted to account for this structure by separating out Indian and Chinese populations, recent studies have indicated population structure within the Chinese population itself (Li et al., 2011), further weakening the argument for selection. So, while it is evident that Chinese *M. mulatta* has undergone significant evolution compared to the other macaque species, I argue that the rapid

increase in derived alleles that is observed in this group is due to a history of demographic expansion and population subdivision.

Despite the lack of evidence for selection on *TPH2*, it still remains a promising candidate gene for understanding the interactions between genetics, neurobiology, the environment, and behavior in macaques. The evidence connecting *TPH2* to various behaviors in humans (Harvey et al., 2004; Zill et al., 2004a, b; Coon et al., 2005; Sheehan et al., 2005; Walitza et al., 2005; Zhang et al., 2005; Zhou et al., 2005), macaques (Chen et al., 2010b), and other mammals (e.g., Liu et al., 2011) is strong and demonstrates that the variation found in this gene has a significant influence on behavior. What this study indicates is that the genetic variation in *TPH2* is the result of demographic processes and purifying selection, rather than positive selection, in macaques. There is evidence for a higher rate of evolution in Chinese *M. mulatta*, as indicated by Fay and Wu's H, which may have led to behavioral evolution. If so, these results suggest that the behaviors influenced by *TPH2*, such as sexual behavior (Liu et al., 2011) or the ability to handle stress (Chen et al., 2010a), might have evolved through neutral forces in this group; thus, there may not be an adaptive explanation for some of behavioral differences seen between populations or species. Alternatively, while selection may indeed be acting through the serotonin system to shape behaviors, it may be that both external environmental factors (such as nutrition, social interactions, and rearing conditions) and the internal, genomic environment (i.e., the presence of other genes that interact with *TPH2* and neurochemistry) act as "buffers" against selection by severely limiting the conditions under which deleterious behavioral strategies might manifest. For example, the self-injurious behavior that has been linked to variation in *TPH2* is certainly deleterious. However this behavior is most likely an artifact of captivity, rarely seen in more natural settings where selection could act to eliminate the alleles most likely to lead to such

behaviors. Instead, selection on behavior related to serotonin regulation might act through other serotonin-related genes (Chapter 3) or it could act indirectly on *TPH2* through genes that interact with it. Thus, one potential line of future inquiry would be to examine the evolution and proximate mechanisms of the genetic "buffers" themselves.

In summary, I have demonstrated that a broad sampling within and across species is needed to understand the variation at *TPH2* in the macaque genus and that, for *M. mulatta*, the presence of two genetically distinct populations always needs to be accounted for in studies of molecular evolution. I have identified several polymorphisms in four species of macaques, providing a useful list of possible variation that may influence behavior and differential response to drugs in pharmacological studies. Finally, I demonstrated that selection is unlikely to have shaped the variation within and among species of macaques in *TPH2*. This is not to say that *TPH2* is unimportant in the regulation of critical macaque behaviors; instead, I suggest that the evolution of behaviors regulated through serotonin can be mediated through neutral forces. Where selection does occur, it may be acting more strongly on different components of the serotonin system, or acting only indirectly on *TPH2* by shaping the genomic environment in which *TPH2* acts. It is only through a more comprehensive understanding of the genetic architecture through which the neurological systems act that we can begin to more thoroughly understand the evolution of behavior.

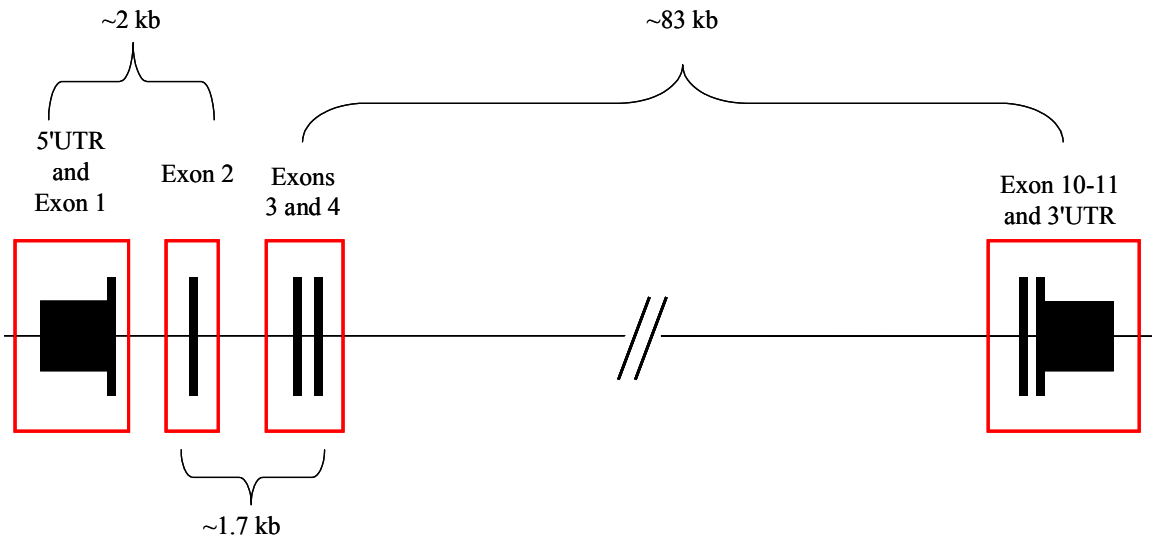

Figure 4.1. A schematic of the *TPH2* gene. The vertical lines represent exons and the solid back boxes represent the 5'UTR and 3'UTR region. The red boxes outline the areas sequenced for this study.

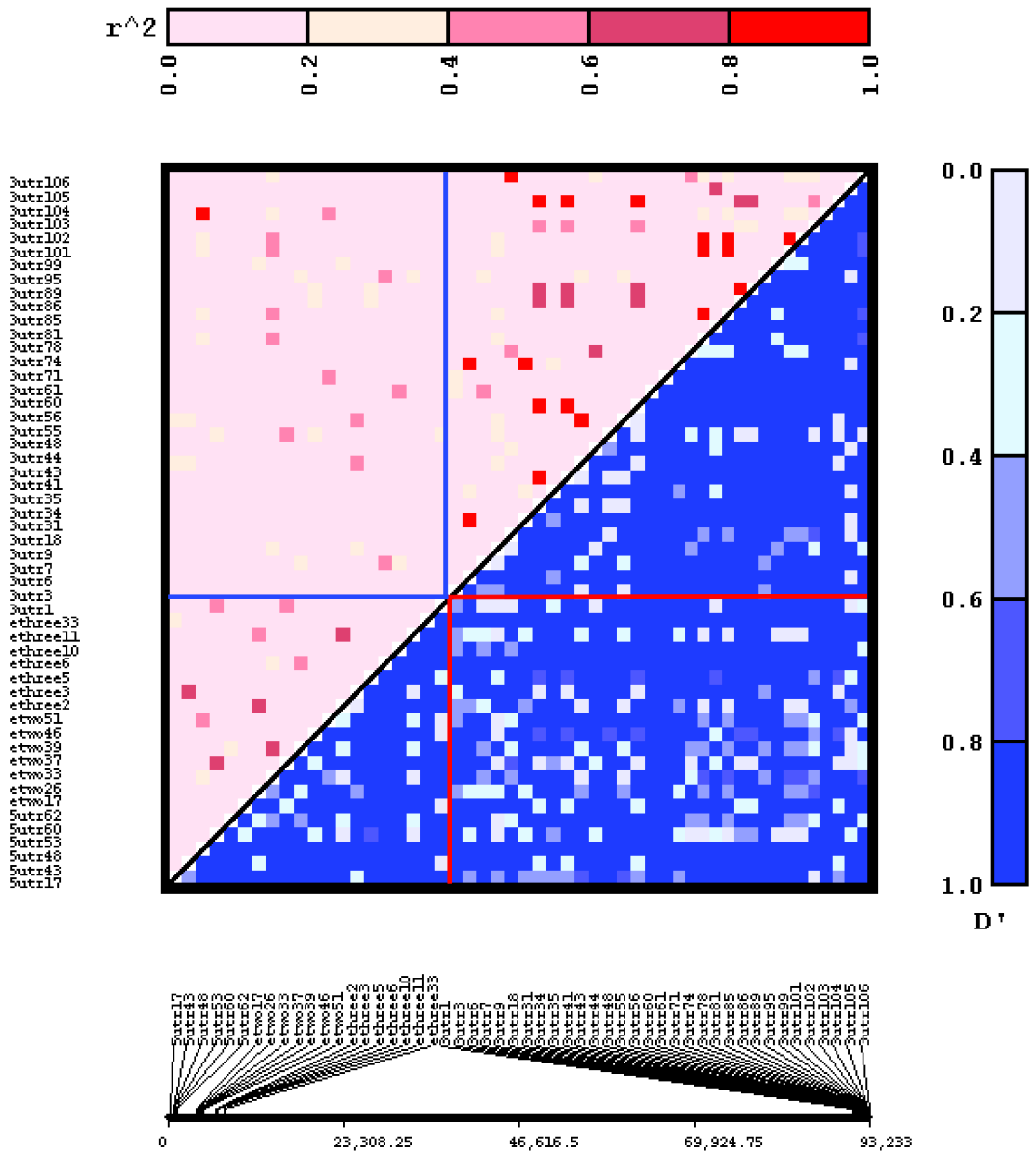

Figure 4.2. A plot showing linkage disequilibrium (LD) between different polymorphic sites in *M. mulatta*. Top left: R^2. Bottom right: |D'|. The X-axis shows the physical distance, in base pairs, between polymorphic sites. The red and blue lines indicate the separation between upstream areas of the gene sequenced and the down stream region sequenced. Both measures of LD are based on genotypic data, so LD estimates shown here are lower than would be generated based on the phased haplotypes.

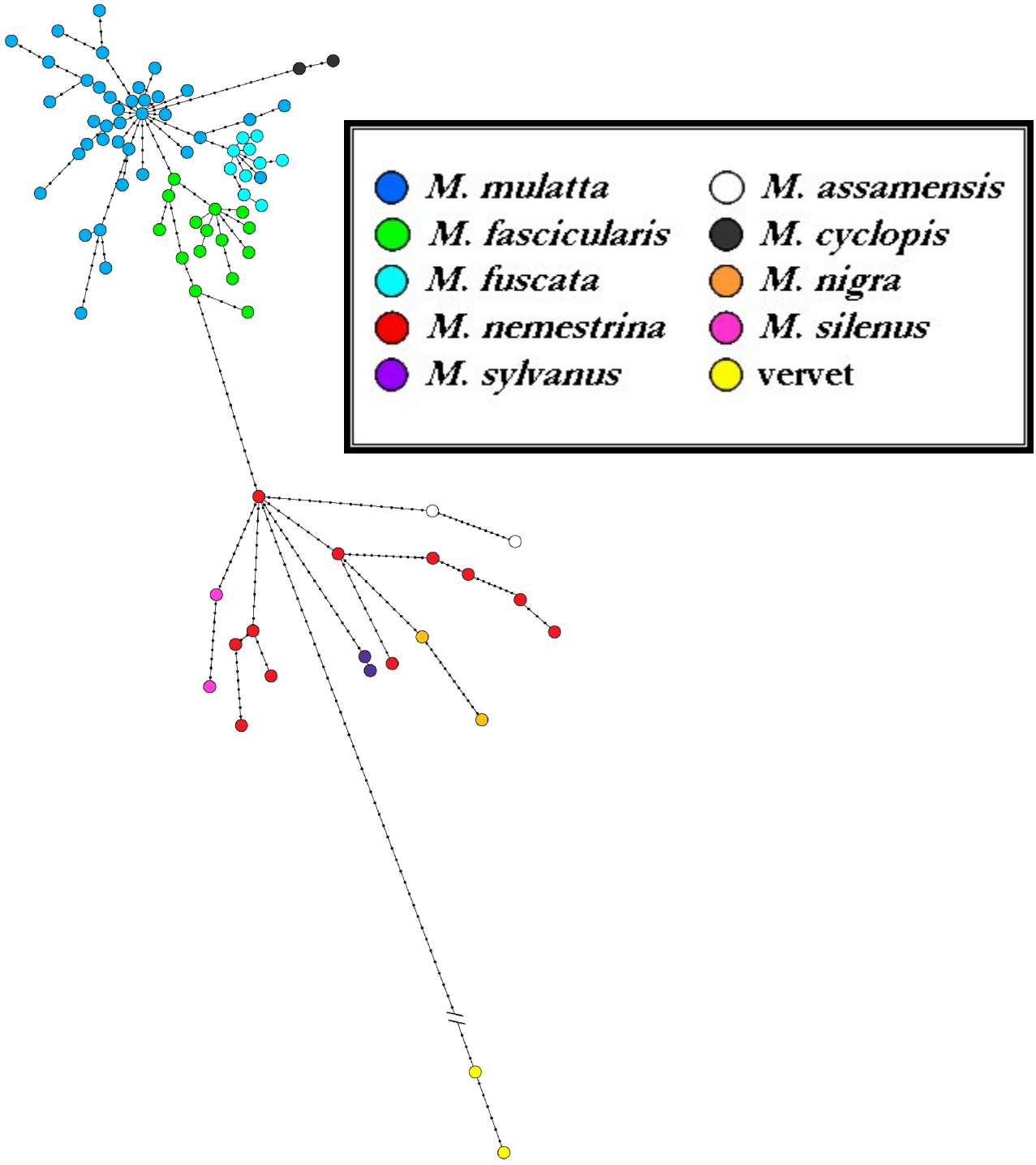

Figure 4.3. Minimum spanning tree of haplotypes. The open circles represent haplotypes, color coded by species. These circles are not proportional to the frequency of the haplotype. Solid black dots represent the number of mutations separating haplotypes.

Table 4.1. List of species used and sample sizes. For *M. mulatta*, samples from two different populations were used: India and China. For more information on the sources of these samples, see Table A.1 in Appendix A.

Species	N
M. mulatta	27
Indian	13
Chinese	14
M. fascicularis	10
M. fuscata	11
M. nemestrina	6
M. sylvanus	5
M. assamensis	1
M. cyclopis	1
M. nigra	1
M. silenus	1
C. aethiops	1
Total	64

Table 4.2. Indices of within-species genetic diversity found in *TPH2* for five species of macaque. Indices include the number of polymorphisms found within each species, two estimates of theta, and Tajima's D. The polymorphisms found within each species are separated into two types: SNPs and indels. In addition, the number of nonsynonymous (NS) polymorphisms is indicated. For *Macaca mulatta*, indices for both the Chinese and the Indian populations are shown separately, as well as indices for the species as a whole.

	M. mulatta			*M.*	*M.*	*M.*	*M.*
	China	India	Total	*fascicularis*	*fuscata*	*nemestrina*	*sylvanus*
Polymorphic sites	43	33	**52**	**22**	**12**	**36**	**1**
SNP	38	30	**46**	**20**	**11**	**33**	**1**
Indel	5	3	**6**	**2**	**1**	**2**	**0**
NS polymorphisms	0	1	**1**	**0**	**0**	**0**	**0**
Theta (S)	9.76	7.86	**10.09**	**5.64**	**3.02**	**10.93**	**0.35**
Theta (Pi)	7.53	8.32	**8.43**	**5.75**	**3.18**	**14.52**	**0.20**
Tajima's D	-1.24	-0.29	**-0.93**	**-0.34**	**0.08**	**1.35**	**-1.11**
Tajima's D, p-value	0.082	0.399	**0.194**	**0.407**	**0.565**	**0.941**	**0.194**

Table 4.3. Genetic distance within and among species for *TPH2*. The diagonal elements show the average number of pairwise differences within species and the off-diagonal elements show the average number of pairwise differences among species.

	M. mulatta	Indian	Chinese	*M. fascicularis*	*M. fuscata*	*M. nemestrina*	*M. sylvanus*
M. mulatta	0.00133						
Indian	N/A	0.00131					
Chinese	N/A	0.00141	0.00119				
M. fascicularis	0.00170	0.00165	0.00174	0.00091			
M. fuscata	0.00179	0.00186	0.00173	0.00209	0.00050		
M. nemestrina	0.00406	0.00411	0.00402	0.00378	0.00420	0.00237	
M. sylvanus	0.00444	0.00450	0.00439	0.00419	0.00457	0.00404	0.00003

Table 4.4. A list of polymorphic sites in five species of macaques. For ease of comparison with previous studies, I repeat the system of Chen et al. (2006) for designating sites. The site number lists the location of the polymorphism relative to the start of the coding region, with +1 marking the first nucleotide of the start codon. EH: Expected Heterozygosity.

Species	Site	Designation	Location	EH	Population	Notes
M. mulatta	-942	-942C>A	5'UTR	0.10692	China	
M. mulatta	-508	-508G>A	5'UTR	0.07268	China	
M. mulatta	-413	-413T>C	5'UTR	0.03704	India	
M. mulatta	-363	-363T>G	5'UTR	0.28302	Both	
M. mulatta	-111	-111G>A	5'UTR	0.07268	China	
M. mulatta	74	74C>A	Exon 1	0.07268	India	25Pro>His
M. mulatta	2566	IVS1-28G>A	Intron 1	0.30748	Both	
M. mulatta	2859	IVS2+116G>A	Intron 2	0.10692	Both	
M. mulatta	3026	IVS2+283C>T	Intron 2	0.20126	India	
M. mulatta	3084	IVS2+341G>A	Intron 2	0.07268	China	
M. mulatta	3141	IVS2+398C>A	Intron 2	0.13976	India	
M. mulatta	3315	IVS2+572T>C	Intron 2	0.07268	Both	
M. mulatta	3398	IVS2+655C>T	Intron 2	0.28302	Both	
M. mulatta	5243	IVS2-175T>Del	Intron 2	0.10692	China	1 bp indel
M. mulatta	5252	IVS2-166A>C	Intron 2	0.49825	Both	
M. mulatta	5277	IVS2-141G>A	Intron 2	0.03704	China	

Table 4.4 (cont).

Species	Site	Designation	Location	EH	Population	Notes
M. mulatta	5294	IVS2-124AA>Del	Intron 2	0.07268	China	2 bp indel
M. mulatta	5375	IVS2-43A>Del	Intron 2	0.20126	China	1 bp indel
M. mulatta	5385	IVS2-33G>A	Intron 2	0.03704	China	
M. mulatta	6208	IVS4+398T>C	Intron 4	0.35220	Both	
M. mulatta	89755	IVS9-729C>T	Intron 9	0.10692	China	
M. mulatta	89798	IVS9-686G>A	Intron 9	0.07268	China	
M. mulatta	89834	IVS9-650A>G	Intron 9	0.07268	China	
M. mulatta	89838	IVS9-646T>G	Intron 9	0.33054	Both	
M. mulatta	89879	IVS9-605T>C	Intron 9	0.20126	Both	
M. mulatta	90042	IVS9-442C>T	Intron 9	0.07268	China	
M. mulatta	90256	IVS9-228C>T	Intron 9	0.28302	Both	
M. mulatta	90304	IVS9-180C>T	Intron 9	0.07268	Both	
M. mulatta	90328	IVS9-156Del>Ins	Intron 9	0.33054	Both	11 bp indel
M. mulatta	90428	IVS9-56C>T	Intron 9	0.17121	China	
M. mulatta	90460	IVS9-24C>T	Intron 9	0.07268	India	
M. mulatta	90525	1206A>G	Exon 10	0.10692	India	NS
M. mulatta	90705	IVS10-40G>A	Intron 10	0.17121	China	
M. mulatta	90949	1503G>A	3'UTR	0.28302	Both	
M. mulatta	90959	1513G>A	3'UTR	0.03704	China	
M. mulatta	91072	1626A>G	3'UTR	0.03704	China	
M. mulatta	91127	1681G>T	3'UTR	0.07268	China	
M. mulatta	91337	1891G>A	3'UTR	0.10692	Both	
M. mulatta	91459	2013G>T	3'UTR	0.10692	Both	
M. mulatta	91497	2051A>C	3'UTR	0.30748	Both	
M. mulatta	91553	2107T>G	3'UTR	0.10692	Both	
M. mulatta	91574	2128S>L	3'UTR	0.22991	India	159 bp indel
M. mulatta	91732	2286A>G	3'UTR	0.22991	India	
M. mulatta	91763	2317C>T	3'UTR	0.07268	China	
M. mulatta	91838	2392A>T	3'UTR	0.10692	Both	
M. mulatta	91877	2431T>C	3'UTR	0.10692	Both	
M. mulatta	91939	2493G>A	3'UTR	0.10692	Both	
M. mulatta	91945	2499T>G	3'UTR	0.43955	Both	
M. mulatta	91950	2504G>T	3'UTR	0.03704	India	
M. mulatta	91956	2510G>T	3'UTR	0.28302	Both	
M. mulatta	91961	2515G>A	3'UTR	0.39133	Both	
M. mulatta	91965	2519Del>CTA	3'UTR	0.17121	Both	3 bp indel

Table 4.4 (cont).

Species	Site	Designation	Location	EH	Population	Notes
M. fascicularis	-1130	-1130A>C	5'UTR	0.18947		
M. fascicularis	-894	-894C>T	5'UTR	0.52105		
M. fascicularis	-435	-453C>G	5'UTR	0.26842		
M. fascicularis	-424	-424G>A	5'UTR	0.18947		
M. fascicularis	-370	-370C>Del	5'UTR	0.52105		1 bp indel
M. fascicularis	116	IVS1+11C>A	Intron 1	0.39474		
M. fascicularis	3158	IVS2+415A>T	Intron 2	0.18947		
M. fascicularis	3240	IVS2+497C>T	Intron 2	0.18947		
M. fascicularis	5861	IVS4+51T>C	Intron 4	0.33684		
M. fascicularis	6208	IVS4+398T>C	Intron 4	0.33684		
M. fascicularis	6334	IVS4+524C>T	Intron 4	0.10000		
M. fascicularis	90035	IVS9-449C>T	Intron 9	0.10000		
M. fascicularis	90054	IVS9-430T>G	Intron 9	0.47895		
M. fascicularis	90058	IVS9-426T>G	Intron 9	0.18947		
M. fascicularis	90130	IVS9-354G>A	Intron 9	0.18947		
M. fascicularis	90154	IVS9-330A>C	Intron 9	0.39474		
M. fascicularis	90291	IVS9-193Del>CAAA	Intron 9	0.10000		4 bp indel
M. fascicularis	90799	1353G>A	Exon 11	0.18947		NS
M. fascicularis	91072	1626A>G	3'UTR	0.18947		
M. fascicularis	91573	2127T>G	3'UTR	0.47895		
M. fascicularis	91945	2499G>T	3'UTR	0.10000		
M. fascicularis	91956	2510T>G	3'UTR	0.10000		
M. fuscata	-525	-525A>C	5'UTR	0.17316		
M. fuscata	120	IVS1+15C>A	Intron 1	0.24675		
M. fuscata	2319	IVS1-275TCAG>Del	Intron 1	0.09091		4 bp indel
M. fuscata	2539	IVS1-55G>T	Intron 1	0.24675		
M. fuscata	2763	IVS2+20A>T	Intron 2	0.45455		
M. fuscata	3150	IVS2+407T>A	Intron 2	0.24675		
M. fuscata	5897	IVS4+87C>T	Intron 4	0.31169		
M. fuscata	89900	IVS9-584T>C	Intron 9	0.36797		
M. fuscata	90035	IVS9-449C>T	Intron 9	0.09091		
M. fuscata	91031	1585A>G	3'UTR	0.24675		
M. fuscata	91170	1724G>T	3'UTR	0.24675		
M. fuscata	92044	2598T>C	3'UTR	0.45455		
M. nemestrina	-1255	-1255T>C	5'UTR	0.53030		
M. nemestrina	-1089	-1089T>C	5'UTR	0.53030		
M. nemestrina	-983	-983C>T	5'UTR	0.30303		
M. nemestrina	-845	-845T>C	5'UTR	0.53030		
M. nemestrina	-795	-795C>T	5'UTR	0.54545		

Table 4.4 (cont).

Species	Site	Designation	Location	EH	Population	Notes
M. nemestrina	-735	-735T>C	5'UTR	0.53030		
M. nemestrina	-640	-640G>A	5'UTR	0.53030		
M. nemestrina	-363	-363T>C	5'UTR	0.53030		
M. nemestrina	-356	-356A>G	5'UTR	0.48485		
M. nemestrina	-154	-154A>G	5'UTR	0.40909		
M. nemestrina	137	IVS1+32G>A	Intron 1	0.53030		
M. nemestrina	2319	IVS1-275TCAG>Del	Intron 1	0.16667		4 bp indel
M. nemestrina	2342	IVS1-252C>T	Intron 1	0.48485		
M. nemestrina	2441	IVS1-153G>T	Intron 1	0.48485		
M. nemestrina	2671	183C>T	Exon 2	0.40909		
M. nemestrina	2851	IVS2+108A>G	Intron 2	0.54545		
M. nemestrina	2858	IVS2+115T>C	Intron 2	0.30303		
M. nemestrina	3291	IVS2+548G>A	Intron 2	0.16667		
M. nemestrina	5912	IVS4+102A>C	Intron 4	0.53030		
M. nemestrina	6049	IVS4+239A>G	Intron 4	0.48485		
M. nemestrina	6180	IVS4+370T>A	Intron 4	0.30303		
M. nemestrina	6317	IVS4+507T>G	Intron 4	0.30303		
M. nemestrina	89785	IVS9-699T>C	Intron 9	0.53030		
M. nemestrina	89803	IVS9-681C>T	Intron 9	0.53030		
M. nemestrina	89838	IVS9-646G>T	Intron 9	0.30303		
M. nemestrina	89919	IVS9-565T>G	Intron 9	0.53030		
M. nemestrina	90089	IVS9-395G>T	Intron 9	0.40909		
M. nemestrina	90291	IVS9-193Del>CAAA	Intron 9	0.16667		4 bp indel
M. nemestrina	90369	IVS9-115A>C	Intron 9	0.48485		
M. nemestrina	90416	IVS9-68C>A	Intron 9	0.48485		
M. nemestrina	91459	2013G>T	3'UTR	0.30303		
M. nemestrina	91573	2127T>G	3'UTR	0.48485		
M. nemestrina	91754	2308A>G	3'UTR	0.30303		
M. nemestrina	91961	2515G>A	3'UTR	0.16667		
M. nemestrina	92016	2570C>T	3'UTR	0.30303		
M. nemestrina	92019	2573G>A	3'UTR	0.40909		
M. sylvanus	3291	IVS2+548G>A	Intron 2	0.20000		

Table 4.5. Results of four selection tests for *TPH2*. MK: McDonald-Kreitman test. The number for MK represents the G-value obtained by the trapezoidal method of numerical integration in DNAsp. The numbers for the HKA test represent the sum of deviations calculated in the HKA program.

Species	Fay and Wu's H	p-value	MK	p-value	ω	p-value	HKA	p-value
M. mulatta	-6.222	0.033	0.129	0.719	0.040	0.004	3.574	0.893
Indian	-1.809	0.209	0.399	0.528	0.042	0.004	6.192	0.626
Chinese	-4.979	0.050	NA	---	0.039	0.005	4.010	0.856
M. fascicularis	-1.537	0.197	NA	---	0.040	0.005	9.244	0.322
M. fuscata	0.745	0.620	NA	---	0.039	0.004	4.545	0.805
M. nemestrina	1.091	0.567	NA	---	0.041	0.004	8.093	0.424

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CHAPTER 5

THE EVOLUTIONARY HISTORY OF *SLC6A4* AND THE ROLE OF PLASTICITY

ABSTRACT

Serotonin has repeatedly been indicated as a biological marker of behavior. In particular, the serotonin transporter gene, *SLC6A4*, has been the focus of a large body of research. Interestingly, both rhesus macaques (*Macaca mulatta*) and humans have independently evolved a number of shared polymorphisms for this gene, which is indicative of parallel evolution between the two species. However, very little is known about the evolution of this gene, particularly within macaques. While there are several hypotheses as to the adaptive values of various polymorphisms, few authors have gone beyond theoretical discussion. Here, I examine the genetic variation of *SLC6A4* within and between several species of macaques and investigate whether selection has played a significant role in its evolutionary history. In addition, I assay the polymorphic region of the promoter region, which is known to play a significant role in regulating both serotonin turnover and behavior.

In examining the distribution of the promoter region polymorphism, I identified significant differences between Indian and Chinese populations of *M. mulatta*; furthermore, I discovered its presence in *M. cyclopis*, which has not been described before. In regards to the evolutionary history of *SLC6A4*, I found little evidence for selection and conclude that *SLC6A4* largely evolved through neutral processes, possibly due to its potential role in regulating behavioral plasticity. However, I also found very low levels of linkage between the gene and the promoter region. Because I limited analyses to the gene itself, it is possible that the promoter region shows a distinct evolutionary history from *SLC6A4*.

INTRODUCTION

In the search for the biological bases of behavior, one hormone, serotonin, has repeatedly made its way into much scientific research. For a variety of behaviors or psychological conditions, such as alcoholism (Virkkunen et al., 1994), novelty seeking (Heck et al., 2009), depression (Caspi et al., 2003), impulse control (Linnoila et al., 1993; Mehlman et al., 1994; Westergaard et al., 2003b), obsessive compulsive disorder (Denys et al., 2006), or antisocialism (Flory et al., 2007), indices of serotonin levels and serotonin turnover are consistently among the best biological markers for predicting behavioral patterns. Consequently, there is a vested interest in a detailed understanding of the serotonin system, particularly within the fields of psychology and medicine, but also in related fields such as evolutionary biology. Research of serotonin not only paves the pathway for improved pharmaceutical interventions for psychological conditions, it can also increase our ability to identify individuals with a biological predisposition for these disorders. Furthermore, the connection of serotonin with behavior provides a possible mechanism through which behavioral evolution can occur. To that end, much work has concentrated on examining the genetic variation of the serotonin system.

Perhaps the most widely studied of serotonin genes is the serotonin transporter gene (*SLC6A4*, also referred to as *5-HTT* or *SERT*). In particular, the promoter region of this gene, which lies approximately 15 kilobases (kb) upstream of *SLC6A4*, possesses a 44-basepair (bp) insertion/deletion (indel) polymorphism in humans, dubbed the "long" and "short" alleles (Heils et al., 1996; Lesch et al., 1996). This polymorphism influences transcriptional activity and serotonin turnover (Lesch et al., 1996; Heils et al., 1997; Greenberg et al., 1999; Williams et al., 2001; Manuck et al., 2004; Smith et al., 2004), and the short allele seems to increase susceptibility to a wide variety of psychological conditions (see for review Hariri and Holmes,

2006; Serretti et al., 2006; Canli and Lesch, 2007; Caspi et al., 2010; Chapter 2). A very similar polymorphism exists in rhesus macaques (*Macaca mulatta*) (Lesch et al., 1997; Rogers et al., 2006), a frequently used animal model in studies of medicine. In macaques, the polymorphism consists of a 21-bp indel and, although independently evolved (Lesch et al., 1997), it appears to regulate serotonin functioning (Bennett et al., 2002) and behavior (e.g., Bennett et al., 1998; Trefilov et al., 2000; Champoux et al., 2002; Barr et al., 2003; Bethea et al., 2004) in a manner similar to humans. Furthermore, additional polymorphisms in *SLC6A4* and other serotonin related genes have been identified in rhesus macaques that are also analogous to those found in humans (e.g., Newman et al., 2005; Vallender et al., 2008). This suggests that parallel evolution of the serotonin system is occurring between the two species (Vallender et al., 2008). However, despite its potential to provide a greater understanding of behavioral evolution, the evolutionary history of *SLC6A4* is not very well understood.

The very fact that these polymorphisms evolved and are maintained in both humans and macaques would indicate that they are advantageous in some way – that is, that there is positive selection acting on *SLC6A4* and its promoter regions. However, most of the research carried out to date seems to contradict this prediction. For example, in macaques lower serotonin levels have been shown to negatively affect rank (Higley et al., 1996b; Higley and Linnoila, 1997), survival (Higley et al., 1996a; Westergaard et al., 2003a; Howell et al., 2007), and reproductive success (Mehlman et al., 1997; Gerald et al., 2002; Cleveland et al., 2004; Hoffman et al., 2007). Therefore, any polymorphism that tends to decrease serotonin turnover, such as the short allele in the promoter region of *SLC6A4*, would be expected to be selected against; and yet, the short allele remains in high frequencies in both humans (Lesch et al., 1996) and macaques (Trefilov et al., 2000; Wendland et al., 2006).

This has led to a variety of hypotheses to explain the presence of polymorphisms that as a whole appear to be negative. Many of these hypotheses center around the idea of balancing selection. For example, Trefilov (2000) suggested that heterozygous advantage might explain the presence of both the long and short alleles in macaques. Other authors have suggested that behaviors that are negative in one set of circumstances might be positive in another; thus, balancing selection can occur through changes in selective pressures throughout an individual's lifetime (Gerald and Higley, 2002; Howell et al., 2007), between sexes (Westergaard et al., 2003b), in different social settings (Gerald and Higley, 2002), and in different habitats (Suomi, 2006; Chakraborty et al., 2010).

Alternatively, others have argued that polymorphisms in *SLC6A4*, which increase the risk of behavioral disorders in bad environments, also increase the ability to thrive in good environments (Belsky et al., 2009; Homberg and Lesch, 2010). As such, these genes are best viewed as plasticity genes, with certain polymorphisms conferring an increased sensitivity to environmental cues, good or bad (see Chapter 2; Figure 2.6). Note that this is slightly different from other, "traditional" hypotheses based on balancing selection. In the case of the "traditional" hypotheses, the behavior remains the same, but the adaptive value of the behavior changes across circumstances. These hypotheses are based on a more straightforward genotype-phenotype relationship. In the case of plasticity genes, the adaptive value of a behavior remains the same, but the circumstances under which it manifests varies across environments. While this *could* lead to balancing selection as well, if plasticity itself were under selection, which has been argued for in the case of humans (e.g., Potts, 1996), then positive selection for genetic variants such as the short allele in the *SLC6A4* promoter region would explain both their presence at high frequency and their independent evolution in humans and macaques. However, if the target of selection is a

specific behavioral pattern, rather than the capacity to be more or less plastic, then the diminished correlation between genotype and phenotype created by a gene by environment interaction would make it difficult to detect selection at a genetic level, and the gene might be expected to evolve according to neutral evolutionary forces.

Thus, there are several potential evolutionary scenarios for serotonin related genes. However, despite widespread interest in the potential selective advantages and disadvantages for variants of genes such as *SLC6A4*, most studies have been limited to theoretical discussions and so remain speculative. In this study, I use an empirical approach to understanding the evolutionary history of the serotonin system by exploring the genetic variation of *SLC6A4* within and between several species of macaques. I apply several tests of neutrality to determine whether selection – either positive, purifying, or balancing – played a significant role in shaping genetic variation. Using a similar molecular evolutionary approach, Claw and colleagues (2010) recently examined both *SLC6A4* and *HTR2A* (which codes for the serotonin receptor type 2A) in humans and found evidence of positive selection occurring within the UTRs of both genes. If the serotonin system of rhesus macaques is evolving in parallel with humans, then we would expect to see similar evidence of positive selection for this species as well.

METHODS

Subjects

For this study, I used DNA extracted from 27 *M. mulatta*, 12 *M. fascicularis*, 11 *M. fuscata*, 6 *M. nemestrina*, 6 *M. sylvanus*, and one individual each from *M. assamensis*, *M. cyclopis*, *M. nigra*, *M. silenus*, and *Chlorocebus aethiops* (a vervet). See Table A.1 in Appendix A for complete description of the source of these samples. The *M. mulatta* samples come from

two distinct areas within Asia: China and India. Previous studies have indicated a significant amount of differentiation between these two populations of *M. mulatta* (Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2003; Smith, 2005; Hernandez et al., 2007; Satkoski et al., 2008). Because the presence of population structure can have a significant influence on several of the tests employed here, an analysis of molecular variation (AMOVA) (Weir and Cockerham, 1984; Excoffier et al., 1992; Weir, 1996) was carried out to estimate the amount of population differentiation seen in this gene using the program Arlequin (Excoffier et al., 2005). If the AMOVA was significant, I applied separate analyses to each of the *M. mulatta* populations.

Previously (Chapter 4), I concentrated on the 5 species for which there were multiple samples (*M. mulatta*, *M. fascicularis*, *M. fuscata*, *M. nemestrina*, and *M. sylvanus*) and used the vervet as the outgroup for several of the analyses. However, for this study I was unable to obtain a complete sequence for the vervet and only obtained complete sequence data for one *M. sylvanus* individual (see **RESULTS**, Table 5.1). Therefore, this study focuses on only four macaque species: *M. mulatta*, *M. fascicularis*, *M. fuscata*, and *M. nemestrina*. Where sequence data for *C. aethiops* was available, I used this as the outgroup; but since this data was not available for all regions sequenced, I also used *M. sylvanus* as an outgroup since it is the sister species to all of the Asian macaques and the first to diverge in phylogenetic analyses (Tosi et al., 2003; Vos, 2006; Li et al., 2009; Figure 3.2). As with the previous studies, the other species were used primarily to place the focus species within a phylogenetic framework. Namely, these were used in the formation of haplotype networks and in PAML (Phylogenetic Analysis using Maximum Likelihood).

PCR and Sequencing

The total size of *SLC6A4* is approximately 25,000 base pairs (25 kb), with 13 exons and 1,890 bp of coding regions. Although this is a moderate sized gene compared to other serotonin genes, such as *TPH2* or *MAOA* (approximately 90 kb each), *SLC6A4* is incredibly complex, possessing numerous features – such as repeats and transposable elements – that make cost efficient sequencing difficult. Therefore, I limited sequencing to three regions dispersed across the gene: i) the 5'UTR, Exon 1, and flanking regions (982 bp), ii) Exons 2-6 and flanking regions (3,292 bp), and iii) Exon 13, the 3'UTR, and flanking regions (761 bp) (Figure 5.1). Hereafter, these areas shall be referred to as the 5' region, the "exonic" region, and the 3' region, respectively. In total, I sequenced 5,032 bp of the gene, including 1,157 bp of exon sequences. In addition, I assayed all individuals for the promoter region polymorphism. By obtaining sequence data from both ends of the gene (the 5' and 3' regions), a general estimate of the level of recombination and the level of variation within the gene is obtained.

This study extends on the work carried out for a pilot study, and therefore the methods for PCR and sequencing differ slightly between the regions analyzed and from those of previous studies (see Chapters 3 and 4). For the 5' and 3' regions, PCR and sequencing followed the same protocol as in Chapters 3 and 4. A list of primers used for these regions can be found in Appendix C (Table C.1) and details of the PCR protocol are available upon request of the author. The "exonic" region was amplified through a two-step process. In the first step, I used the PCR Extender System (www.5prime.com), which is designed for high fidelity, long-range amplification of complex genetic regions, to amplify approximately 6 kb of the target region. For the second step, I used nested primers to re-amplify two smaller, overlapping sections of the region (Figure 5.2). Samples were then cleaned up using the ExoSAP-IT protocol (usb.com) and

sent to the W.M. Keck Center for Comparative and Functional Genomics, UIUC for Sanger sequencing. This method was chosen to optimize the quality of sequence reads. However, because of the increased cost compared to other methods, I chose the minimum number of sequencing primers necessary to cover the "exonic" region. Therefore, most of this region was covered by only a single read. Furthermore, the multiple amplifications used in the methods increased the likelihood of allelic dropout, which occurs when PCR preferentially amplifies only one chromosome. To address these issues, any sample that appeared to be homozygous at all sites across the "exonic" region was re-amplified, beginning with the original DNA sample, using a different set of primers. Additionally, a small subset of the samples were cloned and sequenced, and checked against the original data obtained. A list of all primers used is provided in Table C.1, and detailed protocols used for both PCR and cloning can be obtained from the author.

Once received, sequences from all three regions (5', "exonic," and 3') were aligned and edited manually using Sequencher (www.genecodes.com). I used the same protocols for identifying polymorphic sites as in previous studies (see Chapters 3 and 4). Haplotypes for each of the regions were determined using the program Phase v.1 (Stephens et al., 2001; Li and Stephens, 2003; Stephens and Donnelly, 2003). In the case of the "exonic" region, the haplotypes generated by Phase were checked against the haplotypes obtained from the small subset of cloned samples. As with previous studies, an indel that included more than one base pair was treated as a single polymorphism.

In addition to the three regions sequenced, I also genotyped all *M. mulatta* individuals for the promoter region polymorphism. In order to do so, approximately 400 bp surrounding the polymorphism was amplified (see Table C.1 for primers). I then ran the PCR product through a

2% agarose gel for approximately 2 hours at 250V, which separates DNA bands based on their size. The gels were soaked in ethidium bromide and visualized under UV transillumination. The presence of the long or short allele was determined visually; a heterozygous individual showed two distinct bands, while those homozygous for the long or short allele showed a single band at specified levels in the gel (Figure 5.3). As this part of the research was carried out in the pilot study, the genotyping was carried out on a much larger sample of *M. mulatta* (N = 70). This larger sample size is used to ascertain the frequency of the promoter alleles within and across *M. mulatta* populations. Although the promoter polymorphism is unknown in the other species used for this study, a small subset of non-*M. mulatta* individuals were genotyped for this polymorphism as well, as part of another study not directly connected to this dissertation. However, because of the small sample size for the non-*M. mulatta* species, any analyses of the promoter region were restricted to the *M. mulatta* samples.

Finally, five additional non-coding regions were amplified and sequenced for use in the HKA test. These are the same regions that were used in previous studies (see Chapters 3 and 4), and are described in Table A.3 in Appendix A.

In order to visualize the relationship between the haplotypes, a network was created using the reduced median method in Network (fluxus-engineering.com). Additionally, data for a minimum spanning tree was generated in Arlequin (Excoffier et al., 2005). The tree itself was created in HapStar (Teacher and Griffiths, 2011) and graphically modified in Adobe Illustrator®.

Analyses

The regions sequenced are widely separated from each other. In particular, there are approximately 15 kb separating the promoter region from the 5' region, and about 18 kb separating the "exonic" region from the 3' region. Although analyzing a single gene, the large

size of *SLC6A4* makes recombination very likely, a factor which can affect certain analyses (see Chapters 3 and 4). To account for this possibility, I performed separate analyses on each of the three regions sequenced, as well as on the gene as a whole. To determine the level of recombination, I implemented the method of Hey and Wakeley (1997) to obtain an estimate of the parameter *C* (= 4Nc) for each of the regions sequenced, the gene as a whole, and the coding region as a whole (see Chapters 3 and 4 for a more detailed description of *C*). I obtained this estimate for both the *M. mulatta* samples and across the entire genus. I also conducted an exact test of linkage disequilibrium (LD) between all pairs of polymorphic loci to determine which pairs of loci are in significant LD, implemented in the program Arelquin (Lewontin and Kojima, 1960; Slatkin, 1994; Slatkin and Excoffier, 1996; Excoffier et al., 2005). To visualize the level of linkage disequilibrium, a linkage map was created using the program JLIN (Carter et al., 2006). In particular, these programs were used to determine the degree of LD between the promoter region and the rest of the gene.

General indices of molecular variation were calculated using in the program Arlequin (Excoffier et al., 2005). The indices generated are the same as those used in previous studies (Chapters 3 and 4), and include two estimates of the parameter theta: θ_S (Watterson, 1975) and θ_π (Tajima, 1983). For the promoter polymorphisms, a chi-square test of independence (Pearson, 1900)was used to determine if the populations were in Hardy-Weinburg equilibrium (HWE) and to see if there was a significant difference between the Chinese and Indian populations.

In order to determine the role of selection on the genetic variation of *SLC6A4*, I applied several types of selection tests that use different aspects of the sequence data. Since several of these tests are sensitive to non-selective evolutionary forces such as demographics and recombination, a single significant result, without confirmation from other tests, should be

treated with caution. However if significant results are obtained from multiple tests, evidence of selection is much more robust. Here, I briefly outline the tests employed and the parameters used for each one. These tests are the same as those used in previous studies (Chapters 3 an 4) and are described in greater detail there.

I used the HKA test (Hudson et al., 1987) to examine the ratio of genetic variation within and between species across several loci, to determine of *SLC6A4* showed a distinct pattern from other loci. Specifically, an increase in between-species variation for *SLC6A4* would indicate positive selection, while an increase in within-species variation would indicate balancing selection (Chapter 1). For this test, I used the gene of interest, *SLC6A4*, and five additional, presumably neutral loci, described in Table A.3. To determine the level of between-species variation, both *C. aethiops* and *M. sylvanus* were used as outgroups. This was carried out using the program HKA, provided by J. Hey (http://genfaculty.rutgers.edu/hey/software).

I also examined the ratio of nonsynonymous to synonymous substitutions (**ω**) (Kimura, 1977) by using a z-test to determine whether **ω** was significantly different that one (indicative of neutral evolution; see Chapter 1). A ratio greater than 1 would indicate positive selection, while a ratio lower than 1 would indicate purifying selection. The **ω** was calculated using the Nei-Gojobori method (Nei and Gojobori, 1986) and variance was estimated using the bootstrap method (Nei and Kumar, 2000). These analyses were carried out using the program MEGA v.4 (Tamura et al., 2007). In addition, I used McDonald-Kreitman test (McDonald and Kreitman, 1991) to look at **ω** within and between species using DNAsp (Rozas et al., 2003). For both of these tests, *C. aethiops* and *M. sylvanus* were used as outgroups.

Maximum likelihood methods were carried out using the codeml program in PAML (Phylogenetic Analysis using Maximum Likelihood; Yang, 2007). PAML compares different

models of evolution in a phylogenetic framework and determines which model best fits the data. Because PAML uses phylogenetic data, it requires the comparison of multiple species. I included all nine macaque species using the phylogeny seen in Figure 3.2 (Tosi et al., 2003; Vos, 2006; Li et al., 2009), excluding the *C. aethiops* sample since the sequence data for the coding regions of this sample were incomplete. PAML allows only one sequence per species, so haplotypes were determined based on fixed differences between species and I did not perform separate analyses for Indian and Chinese macaques. Furthermore, since the codeml program employed here only uses information from coding regions, I pooled the data from the three regions sequenced and did not do separate analyses for each region. PAML was used to determine if 1) positive selection has occurred on a specific lineage, as indicated by an elevated **ω** (Yang, 1998), and 2) positive selection had occurred on specific sites within the gene (Nielsen and Yang, 1998; Yang et al., 2000; Swanson et al., 2003).

Finally, both Tajima's D (Tajima, 1989) and Fay and Wu's H (Fay and Wu, 2000) were calculated to detect skews in the frequency spectrum not expected under neutrality (see Chapter 1). For these tests, a significantly positive value would indicate balancing selection while a significantly negative value would be consistent with positive selection. Fay and Wu's H requires an outgroup to ascertain which alleles in a population are derived and which are ancestral. Again, I used both *C. aethiops* and *M. sylvanus* as outgroups. Significance for Fay and Wu's H was determined by running coalescent simulations using an estimate of the recombination rate and theta (θ_π) as parameters (Fay and Wu, 2000). DNAsp (Rozas et al., 2003) was used to determine the value and significance of Fay and Wu's H. The value and significance of Tajima's D was determined using Arlequin (Excoffier et al., 2005).

RESULTS

Sequencing, Substructure, and Recombination

For the 3' region, I was able to obtain sequencing information for all samples used. However, 6 of the *M. mulatta* individuals did not amplify for the 5' region, and 14 of non-*M. mulatta* samples produced poor sequencing reads for the "exonic" region (Table 5.1). In particular, for the "exonic" region, I could not obtain sufficient sequence data for the *C. aethiops* sample, a large number of *M. fascicularis* samples, and all but one *M. sylvanus* individual (Table 5.1). For analyses of the "exonic" region and of the gene as a whole, I therefore could not use *C. aethiops* as an outgroup, and focus the discussion to results obtained from examining samples in reference to *M. sylvanus* alone.

An analysis of molecular variation (AMOVA) showed significant population differences between Chinese and Indian *M. mulatta* populations ($p<0.0001$), with 17.9% of the total genetic variation for this species being between groups. This is similar to results obtained for other serotonin genes examined (Chapters 3 and 4). I therefore present the results for each of these populations separately, as well as the results obtained for the species as a whole.

Results for recombination are shown in Table 5.2. In general, these estimates of recombination are moderately high, particularly compared to other serotonin genes sequenced in previous studies (Chapters 3 and 4). Recombination is high even within regions; this is particularly true for the "exonic" region, where the highest estimates of C were obtained. The most notable exceptions to these patterns are for the 5' region, which had no recombination, and the coding region. Although the examination of recombination levels across the gene indicates that there is recombination occurring between the coding regions, the overall low level of mutations that occurred in exons led to an estimate of $C = 0$.

The results for the recombination analyses are reflected visually in the linkage disequilibrium (LD) plot shown in Figure 5.4. The red and blue lines in the map outline the division between the three regions sequenced and the promoter region. This plot shows that there are low levels of linkage between the three sequenced regions. Notably, within the "exonic" region there is a low level of LD. The first third of the "exonic" region actually shows complete linkage between the polymorphic sites (labeled "ex1"-"ex40" on the LD plot), which corresponds to the area of the gene that includes part of Intron 1, Exon 2, and most of Intron 2. However, from Exon 3 until the end of the "exonic" region, there is low LD and it is presumably within this area that the high level of recombination is occurring.

Also of note, the promoter polymorphism shows a very low level of linkage with the gene itself. This is shown visually in the LD plot. Of the 59 polymorphic loci found in *M. mulatta*, the promoter only showed significant LD with 8 of these loci. This is in contrast to humans, which generally show more moderate levels of linkage between the promoter polymorphism and the gene itself (Claw et al., 2010).

Despite the high level of recombination seen, most results did not differ between regions, so for ease of discussion, I concentrate on results for the entire gene, and only discuss the results of separate regions where they differ. Appendix C provides more detailed results for each of the separate regions sequenced.

Molecular Diversity

General indices of molecular diversity are shown in Table 5.3 and Table 5.4. Like other serotonin genes, there are very few nonsynonymous polymorphisms; in fact, there are very few mutations within the coding region in general. Instead, most mutations, both within and between species, occurred either within the introns, at an average rate of 1 mutation per 22 bp, or in the

UTR regions, at an average rate of 1 mutation per 29 bp. In contrast, mutations within the coding region only occurred once per 129 bp. Compared to other serotonin genes previously examined, the nucleotide diversity for *M. mulatta*, averaged over all base pairs, is somewhat high, and approaches the nucleotide diversity of *M. nemestrina*, which normally gives much higher estimates of diversity than *M. mulatta*, despite the smaller sample size (see Chapters 3 and 4). A minimum spanning tree showing the relationship between all haplotypes is shown in Figure 5.5.

I ascertained the frequency of the promoter allele across *M. mulatta* populations using a much larger sample size (N = 70). In total I genotyped 29 individuals from India, 37 individuals from China, and 4 individuals of unknown origin. All of the individuals of unknown origin were homozygous for the long (L) allele. The frequency of the alleles and genotypes for the Chinese and Indian populations are shown in Figure 5.6. The Chinese population has a higher frequency of the short allele (S) than the Indian population, and this was significant ($p = 0.035$). Both populations are in HWE. Although analyses of the promoter polymorphism were limited to *M. mulatta*, a few samples from other macaque species were also genotyped. Notably, the *M. cyclopis* individual appeared to be heterozygous at the promoter region. That is, it possessed both a long and a short allele. To my knowledge, this is the first time that this polymorphism has been reported in this species, although the sample awaits cloning and sequencing for confirmation.

Selection

In order to ascertain the role of selection in shaping the genetic variation of this gene, I applied several tests of selection. The results of these tests when applied to all three sequenced regions combined are shown in Table 5.5. All of these tests are non-significant. Even **ω** was non-significant; while nonsynonymous mutations were rare, there was a low level of synonymous mutations as well so that **ω** was not significantly lower than 1.

Because of the overall low level of mutation in the coding regions, separate analyses on each of these regions for McDonald-Kreitman and **ω** could not be carried out. For all remaining tests, separate analyses of each of the regions largely replicated the results found for the combined regions (Appendix C). However, analyses of the 5' region alone did give significantly different results for Fay and Wu's H. In *M. mulatta*, the H-value calculated approached significance (Table 5.6). Separate applications of this test on the two *M. mulatta* populations show that the H-value for the Indian population is very significant ($p = 0.008$), while it is not significant for the Chinese population ($p = 0.246$). A look at the haplotype network for this region shows the presence of a derived haplotype at a high frequency in Indian rhesus macaques, but rare in Chinese macaques (Figure 5.7); this haplotype is most likely contributing to the significant H-value obtained for this population. The haplotype makes up 88.5% of the Indian population and is defined by the presence of two indels in Intron 1. The first indel is a 46-bp insertion; the second is a 12-bp deletion. These two indels are shared by another, closely related haplotype that is found exclusively in the Indian samples (Figure 5.7). Thus, these two polymorphic sites are present in 96.2% of the Indian samples. By contrast, it is found in only 31.5% of the Chinese population. A chi-square test of independence shows this difference between populations is highly significant ($p<0.0001$).

DISCUSSION

The gene for the serotonin transporter, *SLC6A4*, has been widely studied for its connection to behavior, particularly in humans and macaques. However, the evolutionary history of this gene is not well understood. Various authors have suggested a range of evolutionary scenarios for this gene, but to date very few studies have examined the molecular variation of the

gene itself in an evolutionary context. Here, I examined *SLC6A4* to determine the level of genetic variation within and between macaque species and to explore the possible role of selection on the evolutionary history of this gene.

One of the most heavily investigated polymorphisms for *SLC6A4* is 22-bp the promoter region polymorphism. Until very recently, the promoter region polymorphism was only known within *M. mulatta*, although it is now known to be present in *M. radiata* and the recently named species *M. munzala* (Sinha et al., 2005; Chakraborty et al., 2010). The analyses presented here indicate the presence of this polymorphism in *M. cyclopis* as well. *M. cyclopis* and *M. mulatta* are very closely related (see Figure 3.2), having only diverged from each other approximately 276 thousand years ago (Vos, 2006), so the presence of this polymorphism is not surprising. However, this sample awaits sequencing to verify the exact nature of this polymorphism.

Within *M. mulatta*, there is a significant difference in the distribution of the short and long alleles across populations. Specifically, the short allele was present in much higher frequency in the Chinese population than in the Indian population (Figure 5.6). This finding is consistent with studies on Chinese and Indian macaque behavior, which have generally found significant differences in temperament. For example, a previous study on the temperament of *M. mulatta* neonates showed that Chinese-Indian hybrids were more likely to score lower on tests of orientation and sustained attention and were more reactive and irritable than neonates that were Indian-derived (Champoux et al., 1994); in a similar manner, indices of serotonin turnover differed between the two groups (Champoux et al., 1997). Thus, differences in the prevalence of the short allele may explain some of the behavioral differences seen between these two populations.

Importantly for evolutionary studies on the *SLC6A4* promoter region, there is a very low level of linkage between the promoter polymorphism and *SLC6A4* itself. This is in contrast to the results of Claw and colleagues (2010), which showed higher indices of linkage looking at the analogous promoter polymorphism in humans. Given the population history of humans, a higher level of LD might be expected (Pritchard and Przeworski, 2001), and it increases the likelihood that the promoter region and the gene share similar evolutionary histories. For macaques, however, this low level of LD means that the promoter region and the gene itself could have distinct evolutionary histories, and that the results obtained from the sequencing data cannot meaningfully be extended to the promoter region.

In fact, in examining *SLC6A4*, I found very little evidence for selection acting on this gene. Even **ω**, which is typically low in a functional gene due to purifying selection (see Chapters 3 and 4), was not significantly different than 1. This is not to say that purifying selection does not occur on this gene – the overall low levels of mutations that occurred in exons compared to introns or the UTR would suggest otherwise. Rather, it indicates that the synonymous sites are not completely neutral and have also been subjected to purifying selection.

The most notable exception to the general results was found in the separate examination of the 5' region. For this region, which included the 5'UTR, Exon 1, and flanking areas, I found a borderline significant Fay and Wu's H-value for *M. mulatta*. This result seems to be driven by the Indian population, as the Chinese population had a non-significant H-value. In a previous study (Chapter 4), I found a significant H-value when examining *TPH2* in *M. mulatta*. As with this study, a breakdown of this species into a two-group comparison showed that only one population was contributing to the significant result. However, in the case of *TPH2*, it was the Chinese population that produced a significant result and the Indian population that did not. For

TPH2, I ruled out selection as the main cause of these results for three main reasons: 1) the p-values tended to be only marginally significant, 2) Fay and Wu's H is known to be affected by population structure (Fay and Wu, 2000; Przeworski, 2002), which may be a factor even when considering the Chinese population alone (Li et al., 2011), and 3) all other selection tests indicate neutrality. In the case of *SLC6A4*, the p-value for the Indian population is much more robust ($p = 0.008$). However, I cannot completely rule out the possibility that, like Chinese populations, there exists population structure in Indian rhesus macaques. Alternatively, this could be the result of a bottleneck, which is known to occur in the recent history of Indian *M. mulatta* (Hernandez et al., 2007), although bottlenecks are not necessarily expected to increase the frequency of derived haplotypes, as they tend to eliminate rare (i.e., new or derived) haplotypes. Moreover, this was the only test that indicated that positive selection was occurring on *SLC6A4*, and was only positive when considered in isolation from the other areas sequenced. Therefore, evidence of selection is weak, and an interpretation of neutrality is a stronger fit for the data. Regardless of the role of selection on this region, this test highlighted two polymorphisms that are derived and almost entirely define the Indian *M. mulatta* samples. Both of these polymorphisms are indels located in Intron 1. For human *SLC6A4*, polymorphic regions within introns are known to have a significant affect on serotonin functioning (Fiskerstrand et al., 1999) and behavior (Ogilvie et al., 1996; Vormfelde et al., 2006). While it is unknown at this time whether the polymorphisms highlighted by Fay and Wu's H have a similar affect on macaques, they remain an interesting source of potential future research. The presence of these indels may potentially contribute to behavioral differences seen between these two populations, or between *M. mulatta* and other macaque species.

Results of the 5' region notwithstanding, analyses indicate that genetic drift and other non-selective evolutionary forces are the predominant mechanisms shaping genetic variation for *SLC6A4*. This does not rule out *SLC6A4*'s adaptive importance. Indeed, the low level of linkage between this gene and its promoter region leaves open the possibility that selection has acted primarily through regulatory mechanisms. It is feasible that a separate examination of sequence data surrounding the promoter region would yield different results. While selection might be expected to increase LD between the gene and the promoter region (Sabeti et al., 2002), analyses show that recombination is high for this area of the genome when compared to other serotonin related genes. Thus, unless the selection occurred very recently, recombination would quickly break down any LD (Przeworski, 2002). Therefore, the results and interpretation of the analyses presented here are largely limited to *SLC6A4* itself, and cannot be applied to other components of the genome that may be interacting with this gene (see Chapter 4 for further discussion).

It has been argued that genes such as *SLC6A4* might be best viewed as plasticity genes (Belsky et al., 2009); that is, different alleles for *SLC6A4* have varying levels of gene by environment interaction and make individuals more or less affected by environmental circumstances (see Chapter 2; Figure 2.6). Regarded in this way, the expectations for selection on *SLC6A4* are not very straightforward. If behavioral plasticity itself was the target of selection, then we might make clear predictions about the role of positive selection on *SLC6A4*, and the focus of study should be on the degree that an individual responds to environmental variability (see, for example, Bell and Robinson, 2011). However, if plasticity is not being selected, then the possible impact of selection would depend very much on the history of environmental circumstances. For example, if the probability of being born into a poor social environment is persistently high, selection might be expected to act efficiently against alleles that make

individuals more vulnerable to such an environment; in contrast a persistently high probability of positive social interactions might favor these same alleles, as individuals possessing them are more likely to thrive (Belsky et al., 2009; Chiao and Blizinsky, 2010). However, if past environmental circumstances were widely variable, as might be expected in the course of macaque evolution (Delson, 1980; Jablonski et al., 2000), and the primary target of selection was not plasticity itself, then there would be a poor correlation between genotype and phenotype. As such, selection will be ineffective at producing genetic evolution, and the gene in question would largely evolve in a neutral fashion. The results shown here support this interpretation and contrast with other hypotheses about the evolution of *SLC6A4*, such as heterozygous advantage (Trefilov et al., 2000) or selection for maintenance of genetic diversity due to variable environments (e.g., Suomi, 2006), which focus more on specific behavioral outcomes rather than plasticity in general. In these latter hypotheses, balancing selection is hypothesized to be the primary evolutionary force for *SLC6A4*. None of the tests carried out here indicate balancing selection. (However, it should be noted, again, that many of these hypotheses were made with reference to the promoter region in particular and so I cannot definitively rule them out.)

In summary, I have shown that the promoter region polymorphism of *SLC6A4* is significantly variable across *M. mulatta* populations and I have furthermore discovered its presence in *M. cyclopis*, which has not been described before. I also demonstrate that the promoter region is not linked to *SLC6A4*, and is therefore likely to have a distinct evolutionary history from the gene itself. In examining *SLC6A4*, I found almost no evidence of selection. However, I did find two polymorphisms in the first intron that clearly separate Indian *M. mulatta* from Chinese *M. mulatta* and other macaque species, highlighting this region as a source of future research. Finally, I argue that the neutral evolution seen in this gene supports the idea that

SLC6A4 is best viewed as a gene that influences the level of plasticity shown in an individual. As such, the evolutionary focus for this gene should not be on specific behaviors, but on behavioral plasticity in general. This shift in focus could help to explain inconsistent results seen across study populations (Belsky et al., 2009; Homberg and Lesch, 2010), and will lead to a more nuanced understanding of behavioral evolution.

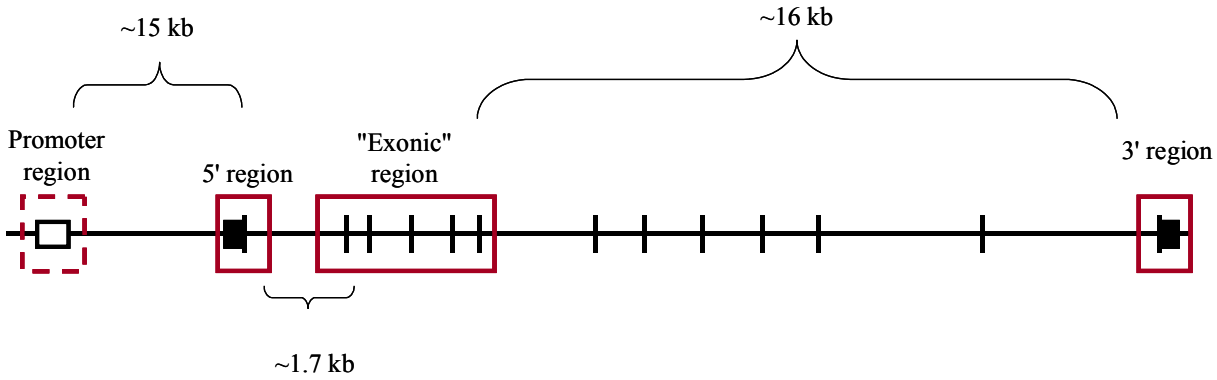

Figure 5.1. A schematic of the *SLC6A4* gene. The vertical lines represent exons, the open box represents the promoter regions, and the solid back boxes represent the 5'UTR and 3'UTR region. The solid red boxes outline the areas sequenced for this study. Dashed red box outlines the promoter region that was assayed.

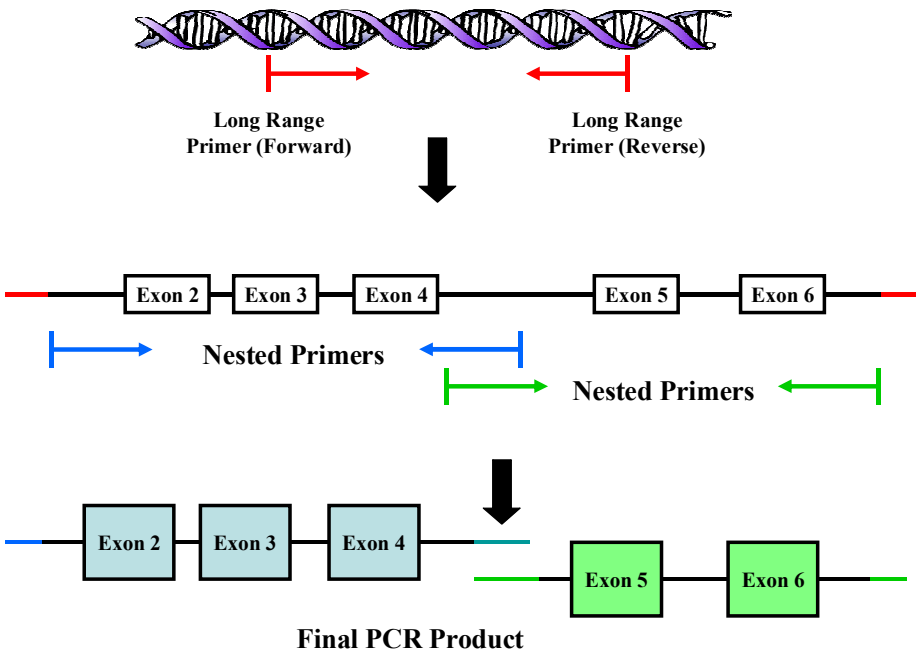

Figure 5.2. Schematic of the methods used to amplify the "exonic" region of *SLC6A4*. Long range primers were used to amplify approximate 2kb around Exons 2-6. Nested primers were then used to further amplify two over lapping segments of DNA.

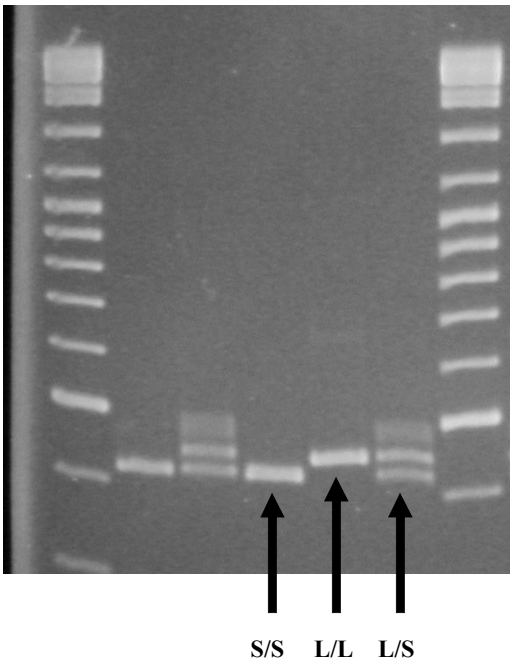

Figure 5.3. Example of a gel used to genotype the polymorphic region of the promoter. S/S: individual homozygous for the short allele. L/L: individual homozygous for the long allele. L/S: heterozygous individual.

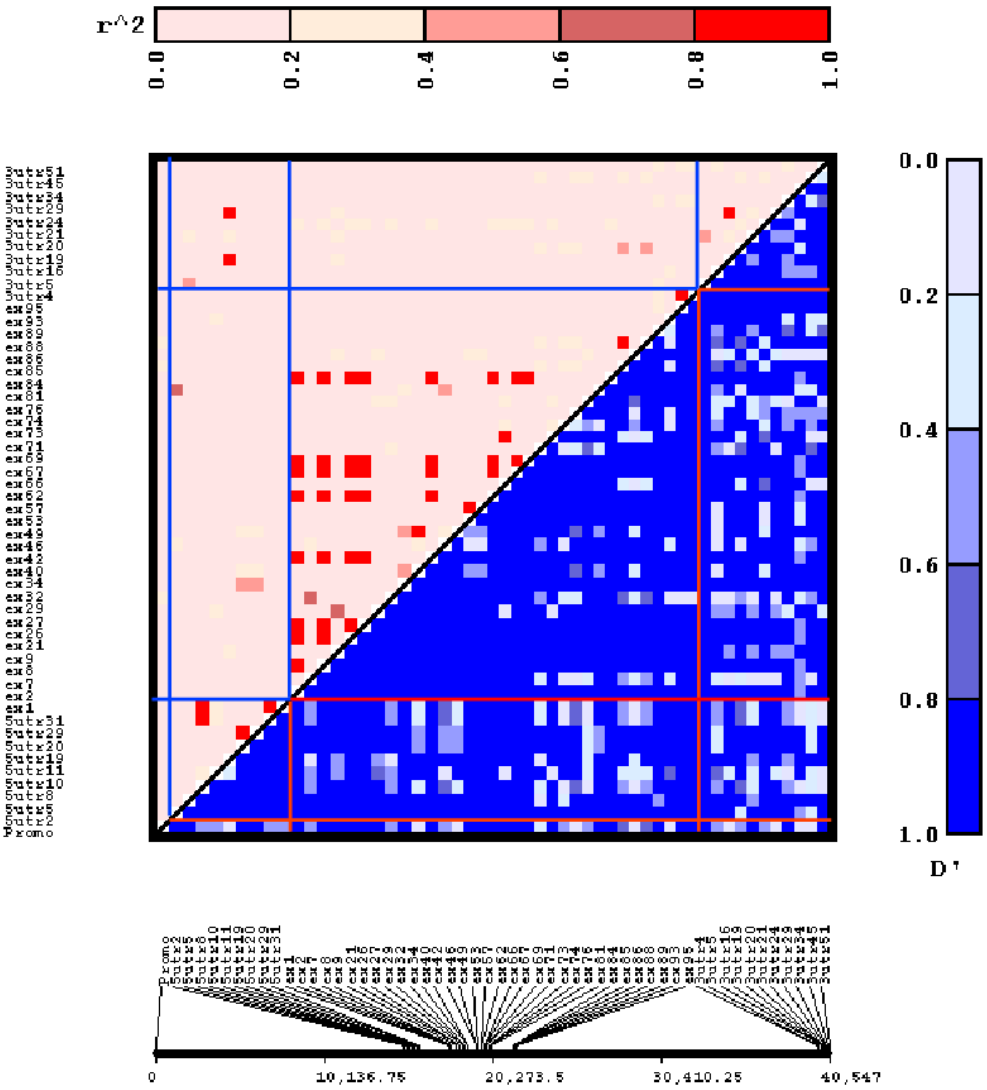

Figure 5.4. A plot showing linkage disequilibrium (LD) between different polymorphic sites in *M. mulatta*. Top left: R^2. Bottom right: |D'|. The X-axis shows the physical distance, in base pairs, between polymorphic sites. The red and blue lines indicate the separation between different regions sequenced and the promoter region. Both measures of LD are based on genotypic data, so LD estimates shown here are lower than would be generated based on the phased haplotypes.

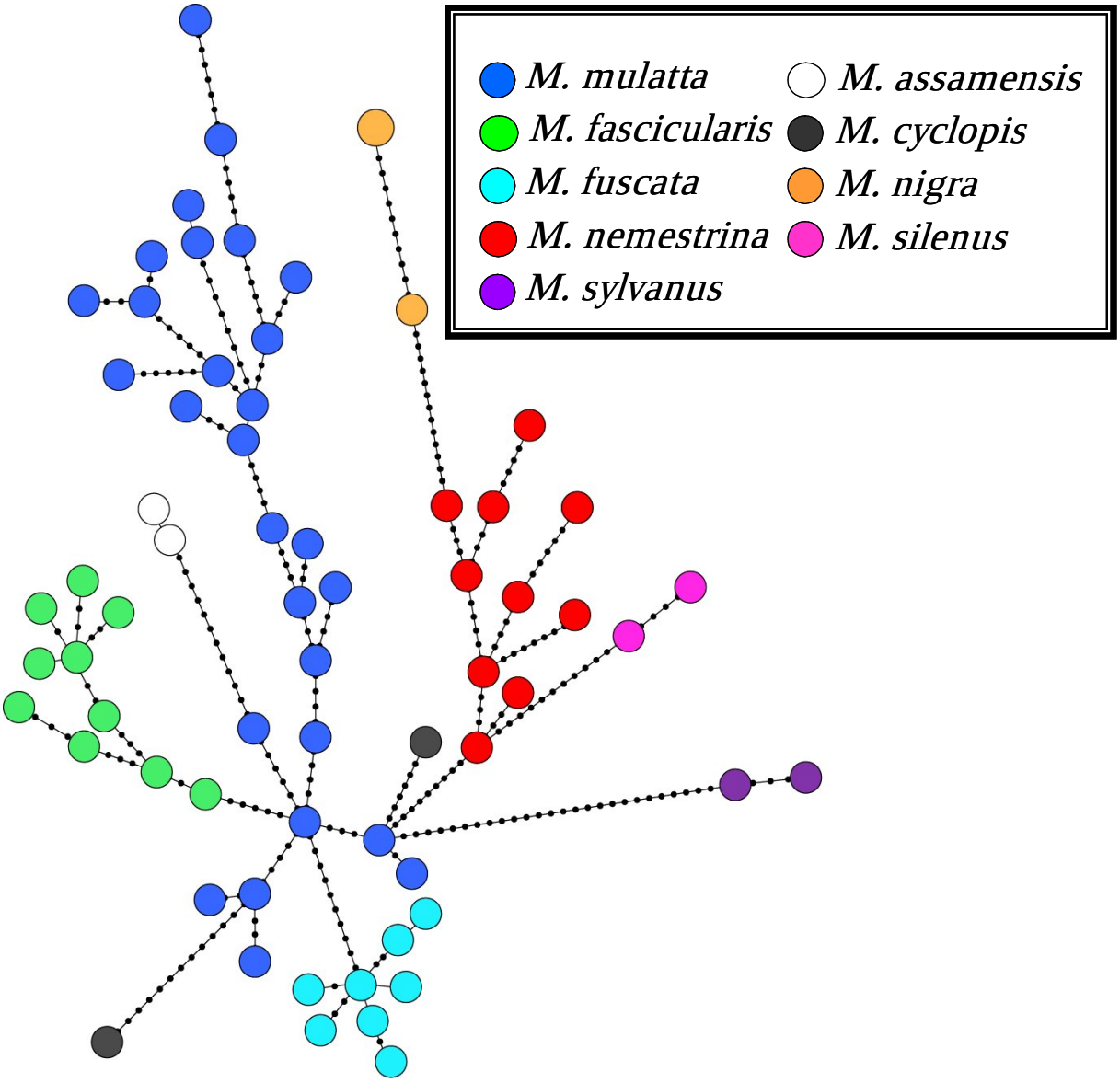

Figure 5.5. Minimum spanning tree of haplotypes. The open circles represent haplotypes, color coded by species. These circles are not proportional to the frequency of the haplotype. Solid black dots represent the number of mutations separating haplotypes.

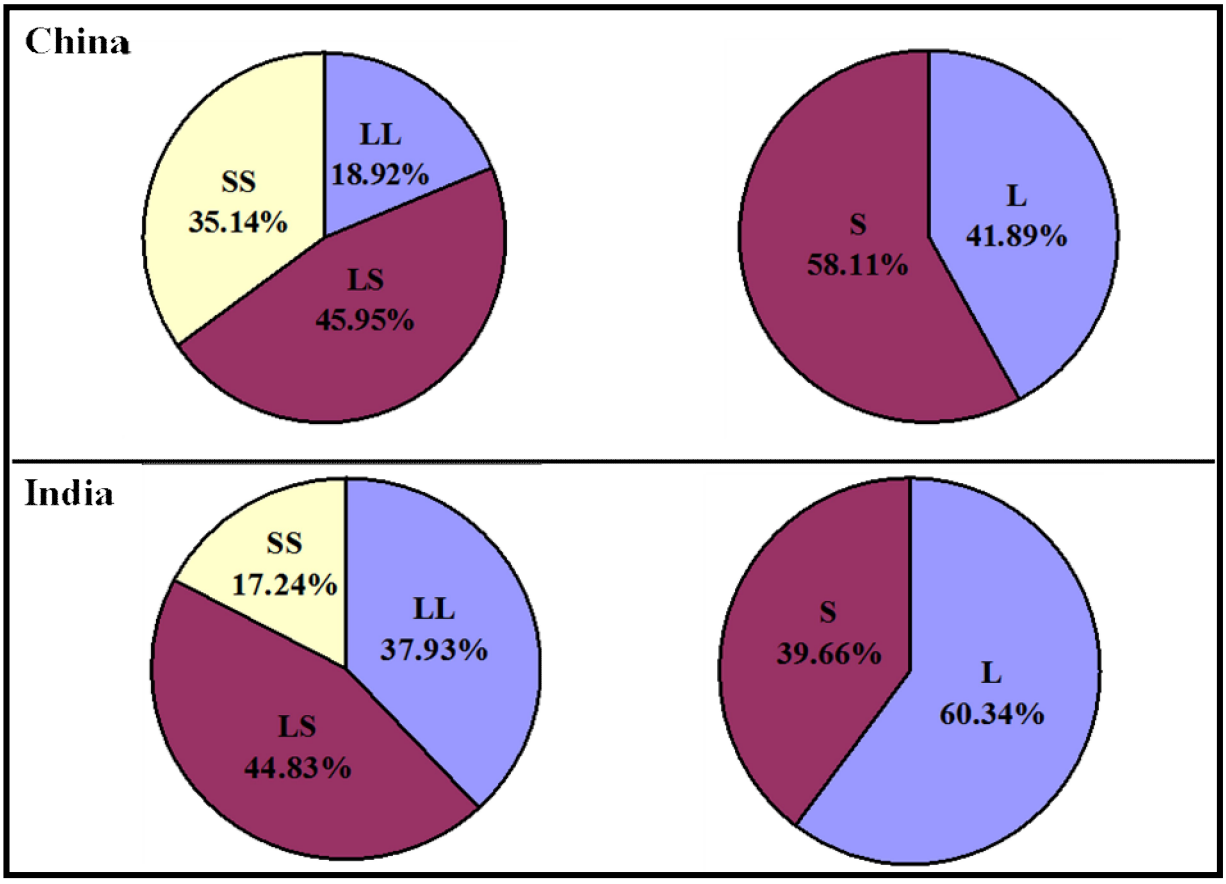

Figure 5.6. Genotypic (left) and allele (right) frequency for the polymorphic region of the promoter in two populations of *M. mulatta*. L: long allele; S: short allele.

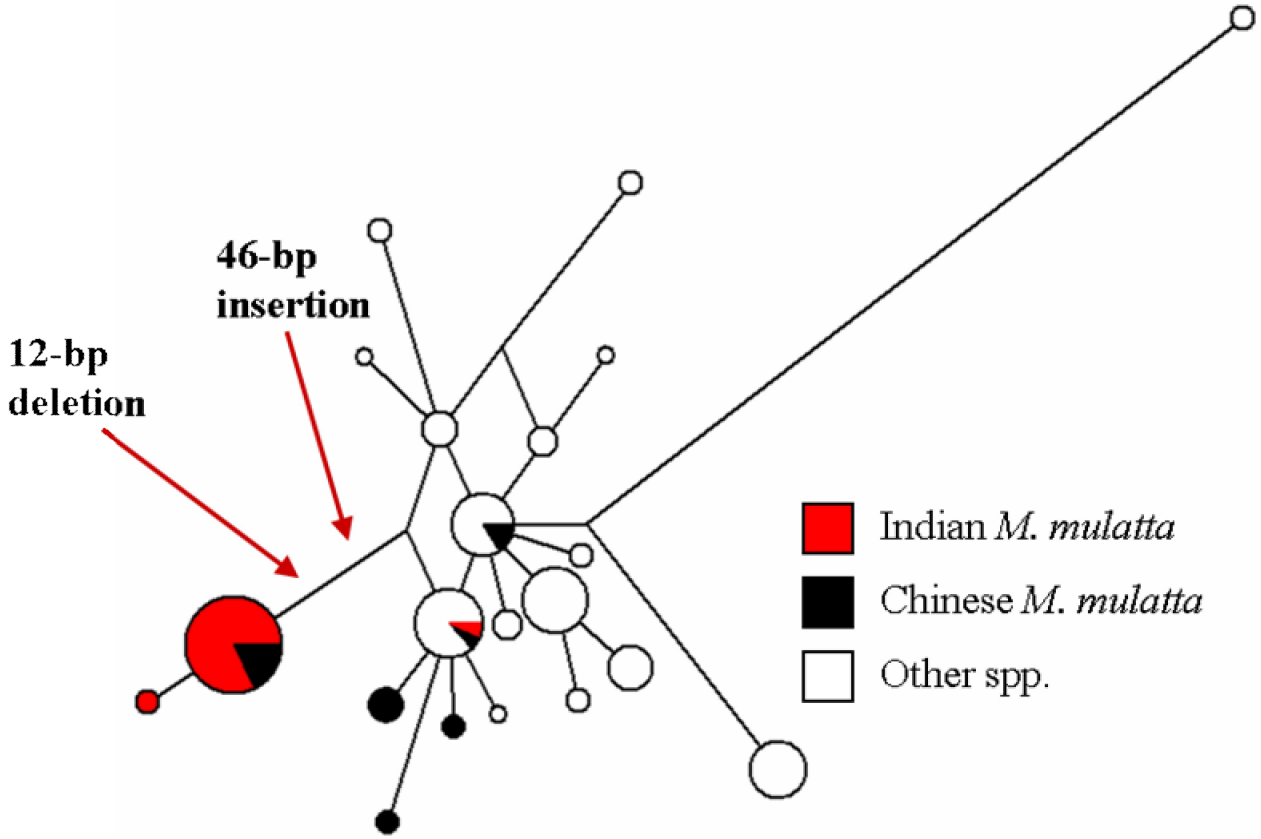

Figure 5.7. Haplotype network for the 5' region of *SLC6A4*. Each circle represents a haplotype whose size is proportional to the frequency of the haplotype. The lengths of the lines connecting the circles are proportional to the number of mutations that separate each haplotype. Two indels that separate most Indian *M. mulatta* from the other species are indicated.

Table 5.1. List of species used and sample size, along with the number of samples successfully sequenced for region examined. For *M. mulatta*, samples from two different populations were used: India and China. For more information on the sources of these samples, see Table A.1 in Appendix A.

		Succsessfully Sequenced			
Species	**N**	**5'**	**Exonic**	**3'**	**Total**
M. mulatta	27	21	26	27	20
Indian	13	13	12	13	12
Chinese	14	8	14	14	8
M. fascicularis	12	11	7	12	7
M. fuscata	11	11	9	11	9
M. nemestrina	6	6	5	6	5
M. sylvanus	6	5	1	6	1
M. assamensis	1	1	1	1	1
M. cyclopis	1	1	1	1	1
M. nigra	1	1	1	1	1
M. silenus	1	1	1	1	1
C. aethiops	1	1	0	1	0
Total	67	59	52	67	**46**

Table 5.2. Recombination estimate, *C* (and *C* scaled per base pair), obtained for *SLC6A4*. In addition to overall recombination found across all regions sequenced (Total), estimates for each individual region were obtained. Recombination estimates were calculated using just *M. mulatta* samples as well as over all samples available.

Region	*M. mulatta*		All samples	
	C	per bp	*C*	per bp
5'	0	0	0	0
Exonic	21.053	0.006	23.945	0.007
3'	0	0	2.369	0.003
Coding	0	0	0	0
Total	21.438	0.004	20.745	0.004

Table 5.3. Indices of within-species genetic diversity found in *SLC6A4* for four species of macaques. Number based on all areas sequenced. Indices include the number of polymorphisms found within each species and two estimates of theta. The polymorphisms found within each species are separated into two types: SNPs and indels. In addition, the location of the polymorphisms indicated (UTR, Exon, or Intron), as are the number of nonsynonymous (NS) polymorphisms

	M. mulatta			*M.*	*M.*	*M.*
	India	China	**Total**	*fascicularis*	*fuscata*	*nemestrina*
Polymorphic sites	40	41	**53**	**30**	**12**	**30**
SNP	33	36	**45**	**19**	**12**	**29**
Indel	7	5	**8**	**1**	**0**	**1**
UTR	9	12	**14**	**5**	**2**	**4**
Introns	30	27	**36**	**15**	**10**	**23**
Exons	1	3	**3**	**0**	**0**	**3**
NS polymorphisms	1	1	**1**	**0**	**0**	**1**
Theta (S)	8.84	10.85	**10.58**	**6.29**	**3.49**	**10.25**
Theta (Pi)	8.71	11.93	**11.05**	**7.67**	**3.54**	**11.82**

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Table 5.4. Genetic distance within and among species, average of all loci, for *SLC6A4*. Numbers based on all areas sequenced. The diagonal elements show the average number of pairwise differences within species and the off-diagonal elements show the avareage number of pairwise differences among species.

	M. mulatta	India	China	*M. fascicularis*	*M. fuscata*	*M. nemestrina*
M. mulatta	0.0022					
India	NA	0.0017				
China	NA	0.0025	0.0024			
M. fascicularis	0.0032	0.0033	0.0030	0.0015		
M. fuscata	0.0035	0.0038	0.0031	0.0032	0.0007	
M. nemestrina	0.0043	0.0044	0.0041	0.0038	0.0041	0.0024

Table 5.5. Results of five selection tests for *SLC6A4*. Tests are based on all areas sequenced. MK: McDonald-Kreitman test. The number for MK represents the G-value obtained by the trapezoidal method of numerical integration in DNAsp. The numbers for the HKA test represent the sum of deviations calculated in the HKA program. For all tests but Tajima's D, *M. sylvanus* was used as the outgroup. Tajima's D does not require an outgroup.

Species	Fay and Wu's H	p-value	Tajima's D	p-value	MK	p-value	ω	p-value	HKA	p-value
M. mulatta	0.049	0.424	-0.459	0.386	0.288	0.591	0.442	0.280	11.471	0.322
Indian	0.015	0.418	-0.557	0.341	0.079	0.779	0.513	0.295	11.216	0.341
Chinese	2.183	0.720	-0.244	0.418	0.156	0.693	0.356	0.194	13.842	0.180
M. fascicularis	0.545	0.548	0.746	0.814	NA	---	0.324	0.257	11.731	0.303
M. fuscata	2.013	0.942	0.050	0.600	NA	---	0.162	0.113	8.733	0.558
M. nemestrina	2.133	0.669	0.641	0.748	0.067	0.795	0.324	0.217	8.742	0.557

Table 5.6. Results of Fay and Wu's H looking at only the 5' region.

Species	Fay and Wu's H	p-value
M. mulatta	-1.882	0.053
Indian	-1.711	0.008
Chinese	-0.283	0.246
M. fascicularis	0.294	0.600
M. fuscata	0.416	0.821
M. nemestrina	1.455	0.888

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CHAPTER 6

REVIEW AND CONCLUSION

A REVIEW OF OBJECTIVES AND DISCUSSION OF FINDINGS

This dissertation explores the evolutionary history of the serotonin system in macaques. Serotonin, which plays a primary role in regulating behavior, is the subject of an exceptionally large body of research, due in large part to its connection with mental and physical health. As such, much of this work has been restricted to the psychological and medical fields. This has lead to an overwhelming focus on understanding the proximate mechanisms of the serotonin system, while discussion of ultimate questions about serotonin has largely been relegated to a side note. This is unfortunate, since the serotonin system has the potential to inform our understanding of behavioral evolution.

On the other hand, behavioral studies within anthropology that use an evolutionary framework are also lacking. The study of behavioral evolution largely focuses on explanations that emphasize the current adaptational value of a behavior, particularly in terms of ecological pressures (Rendall and Di Fiore, 2007), such as in the socio-ecological model (Wrangham, 1980; Van Schaik, 1989; Sterck et al., 1997). However, these models are insufficient on their own, as they tend to underestimate the effects of phylogeny, drift, and the underlying genetic structure of a behavior (Grafen, 1984; Owens, 2006), despite evidence that these forces may shape behavioral evolution (Spuhler and Jorde, 1975; de Queiroz and Wimberger, 1993; Di Fiore and Rendall, 1994; Chan, 1996; Owens, 2006; Rendall and Di Fiore, 2007). Moreover, these studies often fail to consider the biological aspects of the behavior (i.e., proximate mechanisms), when examining its evolution. Thus, studies on the evolution of behavior must go beyond simple

adaptationist explanations to include considerations of the evolutionary history of the species, the underlying biology of a behavior, and the multiple evolutionary forces that can affect behavior (Lewontin, 1979; Owens, 2006).

It is with these concerns in mind that I framed this project. Because serotonin provides a biological mechanism through which evolution may act to shape behaviors, the examination of the genes underlying the serotonin system presents one way to explore behavioral evolution. In contrast to observational studies that focus on the fitness outcomes of a behavior, an approach using molecular evolutionary theory allows for explicit testing of multiple evolutionary forces, including random genetic drift. Thus, I examined the genetic variation within and between several species of macaques for four serotonin related genes – *HTR1A*, *HTR1B*, *TPH2*, and *SLC6A4* – and inferred the role of selection in their evolutionary history.

For three out of the four genes, I found that selection – specifically positive and balancing selection – has *not* played a significant role in shaping genetic variation. Instead, the incredibly low number of mutations occurring within the coding regions suggest that purifying selection is acting to preserve the protein structure for serotonin receptor 5-HT_{1B} (coded by *HTR1B*), the second isoform of tryptophan hydroxylase (coded by *TPH2*), and the serotonin transporter (coded by *SLC6A4*). In terms of variation in potential regulatory regions, namely the introns and untranslated regions, neutral evolution predominates. While Fay and Wu's H for *M. mulatta* was significant for both *TPH2* and the 5' region of *SLC6A4*, this is best explained as an artifact of demographics – specifically, this is likely due to the presence of population structure. Nevertheless, Fay and Wu's H do indicate significant evolution, if not selection. That is, the Chinese and Indian populations of *M. mulatta* have a number of derived alleles with higher than expected frequencies for *TPH2* and *SLC6A4*, respectively. Because this dissertation examines

genes underlying the serotonin system from a statistical viewpoint only, the phenotypic effects of these alleles are unknown. However, if these alleles do have a significant effect on behavior, then the results presented here indicate that behavioral differences observed for these two populations are likely an artifact of demographics, and not selection. This suggests that not all behaviors are adaptive, a prospect not often considered in studies of primate behavior.

This is not to say that the serotonin system is completely unaffected by positive selection. In fact, the results obtained for one gene – *HTR1A* – argue against this. For this gene, which codes for serotonin receptor 5-HT_{1A}, I obtained strong evidence for selective sweeps occurring within the macaque genus. Compared to every other locus analyzed, *HTR1A* shows extremely large interspecific differences, especially relative to intraspecific variation, as indicated by the HKA test. Moreover, a subset of the macaque species all share a codon loss, an extremely rare event in gene evolution, and an approach that examined the rate of coding evolution from a phylogenetic framework (PAML) gave significant results. That none of the other genes examined showed evidence for selection only highlights the unusual genetic pattern found in *HTR1A*, and strengthen the interpretation of positive selection. The results of this research raise the question, why has positive selection acted on *HTR1A*, to the exclusion of the other genes analyzed? Put another way, if selection is acting on the serotonin system, why is evidence of selection only found in *HTR1A*? I suggest four possible explanations.

One of the most challenging aspects of addressing a complex trait such as behavior from a genetic framework is the knowledge that even very strong selection is likely to leave a genetic signature that is "diluted" over several genes, and therefore difficult to detect with current methods (Lande, 1975). However, Robertson (1967) suggested that for quantitative traits, the distribution of allelic effects is expected to be exponential. That is, most of the variation of a

trait, such as behavior, will be explained by a few alleles with large effect, while the remaining variation is attributable to an increasingly larger number of alleles of smaller effect. Thus, one interpretation of the results is that, for macaques, *HTR1A* might be classified as a gene of major effect. Accordingly, selection acting on phenotypes regulated by serotonin, such as dispersal, will predominantly affect *HTR1A* but show only "diluted" signals in other genes of minor effect. While this certainly fits the results, it suggests that *TPH2*, *HTR1B*, and *SLC6A4* have only minor effects on phenotypes, and this seems to contradict what much of research in psychology has indicated. For example, in examining *SLC6A4* in relation to anxiety, Lesch et al. (1996) found that the polymorphism in the promoter region alone explained 3-4% of total variance, and 7-9% of genetic variance. While this is not a particularly large contribution to genetic variance, it is certainly not negligible either. However, these estimates were based on humans, and it is possible that in macaques that the contribution of *SLC6A4* to genetic variance is actually much smaller.

A second, closely related explanation for the results obtained here is that selection on one gene – *HTR1A* – was sufficient to produce adaptive change. In order to evolve a set of behaviors, there are multiple proximate routes that a species can take. For example, serotonin is only one neurological system that has been associated with behavior, and it is possible to create behavioral changes through modification of the oxytocin (Kirsch et al., 2005) or dopamine (Garcia et al., 2010) systems. Even within the serotonin system, selection could have acted on any number of different components, as demonstrated by the genes examined here. Thus, even though genes such as *TPH2* and *SLC6A4* are important in regulating behaviors, it is possible that the genetic variation present in *HTR1A* at the time of selection was such that it allowed behaviors to move towards their optimum without necessitating concurrent changes in the other genes. Put another

way, the genetic variation necessary to evolve adaptive behavior might not have been present in *TPH2*, *HTR1B*, or *SLC6A4*, but was present in *HTR1A*. Thus, selection acted along the only path (or gene) available.

A third explanation is that these genes, while extremely important in regulating behaviors, are largely shielded by environmental or genetic "buffers." Certainly, many of these studies have demonstrated a significant gene by environment interaction (e.g., Bennett et al., 2002; Champoux et al., 2002; Caspi et al., 2003; Wasserman et al., 2006). Therefore, it may be that outside of, for example, captive environments, the circumstances under which negative behaviors might develop are exceedingly uncommon, and thus selection will only rarely have the chance to act on genes that contribute to the maladaptive behavior. Closely connected to this is the idea that these genes are best thought of as plasticity genes (Belsky et al., 2009; Homberg and Lesch, 2010; Chapter 2) with certain genetic variants showing a stronger gene by environment interaction (Figure 2.6). The presence of genetic variants that increase plasticity will result in a lowered correlation between genotype and phenotype. If plasticity itself is not under selection, but a particular behavior or set of behaviors are, "plasticity" genes will act to diminish the effects of selection. Hence, these genes will evolve in a neutral fashion. If this is the case, the results for *HTR1A* suggest that this gene is somehow less "buffered" than the other genes. Future studies that compare the relative influence of environment on each of these genes would help to address this hypothesis.

Finally, it is possible that I detected selection on *HTR1A*, and not *TPH2* or *SLC6A4*, because it is a low complexity gene, with no introns and decreased recombination rates. These features certainly allowed me to obtain more complete sequencing data for *HTR1A* than for *TPH2* or *SLC6A4*, and because recombination can lessen the signal of selection (Hudson et al.,

1987; Przeworski, 2002) it is likely that selection on "simpler" genes is easier to detect. While this does not explain the non-significant results for *HTR1B*, it would explain why *SLC6A4*, which is so widely studied and so well connected to behavior, did not show signs of selection.

These explanations are not mutually exclusive, and it is likely that a combination of all of these factors contributed to results described here. Regardless, it is clear that, although drift, demography, and purifying selection are important evolutionary forces, positive selection has acted on at least one attribute of the serotonin system. Previous studies have theorized on the role of selection in the evolution of serotonin functioning, but very few have actually tested these theories (but see Claw et al., 2010). By using a molecular genetic approach, this research provides empirical evidence for the importance of the serotonin system in macaque evolution. Furthermore, by highlighting 5-HT_{1A} as an important feature in macaque evolution, the results of this dissertation provide a guide for future studies. Serotonin functions as part of an incredibly complex neurological system, with many different components and levels of interactions. We can address some of this complexity by narrowing investigations to the most critical components of this system. This dissertation provides such a focus with 5-HT_{1A}, and lays the groundwork for a large body of exciting research.

FUTURE RESEARCH

The primary aim of this dissertation was to explore the evolutionary history of the serotonin system, a neurological structure with strong connections to behavior. The results of this work have contributed to our understanding of evolution, but they have also raised several important questions. Thus, this dissertation represents a starting point. For the remainder of this

chapter, I shall outline potential avenues of future study that I wish to explore, which will build on the research established here.

One question that has been raised concerns *HTR1A*. Past genetic studies have shown that the Sulawesi macaques are a subset of *M. nemestrina* genetic variation (e.g., Evans et al., 1999), and thus it seems highly unusual for *HTR1A* to show no association between the Sulawesi samples and the *M. nemestrina* samples. I postulated that one potential explanation for this pattern is that *HTR1A* regulates dispersal behavior, and divergent haplotypes would be expected to be present in species with a large geographic range or in species that occupy the outskirts of the macaque range, such as Sulawesi macaques (Chapter 3). If this is true, a wider sampling of *M. nemestrina* – specifically from the areas of Borneo and Sumatra, which border Sulawesi – might reveal *M. nemestrina* individuals who possess the Sulawesi-like haplotypes of *HTR1A*. If I can show that, relative to other genes, *HTR1A* is unique in segregating geographically, this would provide evidence for the dispersal hypothesis. Thus, I am currently working on a follow-up study, in collaboration with D. Melnick at Columbia University, to examine the genetic variation of *HTR1A* in *M. nemestrina* on a geographic scale.

Additionally, another line of research involves the promoter region of *SLC6A4*. I was unable in this dissertation to fully address the evolutionary history of this locus. However, given that it is a major source of research and has been repeatedly implicated in behavior studies (Chapter 2), it would be worthwhile to fully sequence this region and apply a more thorough analysis. While the genetic variation of *SLC6A4* did not indicate positive selection, the promoter region is approximately 15 kilobases upstream of *SLC6A4*, and appears to be unlinked to the gene (Chapter 5). Consequently, a separate analysis might produce very different results.

While these two potential studies are interesting and can help to address some of the gaps in this dissertation, they are largely an extension of what I have already carried out. Studies using different approaches and methodologies are needed to complement and enhance the work in this dissertation. Key to this process is identifying the phenotypic outcomes of the polymorphisms identified in these studies. While evolution ultimately works on a genetic level, and was therefore the focus of this dissertation, selection, and therefore adaptation, works at the phenotypic level. Thus to understand, for example, why selection seems to have acted on *HTR1A* and not any other gene studied here, the phenotypic effects of *HTR1A* must first be known.

One way to examine the effects of mutations occurring within and around genes is to look at differences in expression patterns. This would allow us to see if a certain polymorphism changes the activity level of a gene and would narrow the focus to a few key mutations. The next step would be to examine the effect on serotonin genetic variation and behavior. In terms of looking at *M. mulatta*, the California National Primate Research Center (CNPRC) at UC Davis has behavioral data over 3,000 individuals *M. mulatta* individuals and most of these individual's DNA has been banked and is available for sequencing (Capitanio, personal communication). Furthermore, their population consists of pure Chinese individuals, pure Indian individuals, and Chinese-Indian hybrids. This provides an amazing opportunity to obtain a more detailed understanding of the effects of serotonin genetic variation on behavior and to relate this to the results obtained here. For example, I found that Indian *M. mulatta* are differentiated from Chinese *M. mulatta* by the presence of two large indels in Intron 1 of *SLC6A4*, but concluded that past demographic events, rather than selection, were more likely to have caused the evolution of this inter-population difference (Chapter 3). Establishing a connection between

these indels and behavior would provide a clear example of how demography can shape behavioral evolution and would deepen our understanding of macaque evolution.

However, for *HTR1A*, much of the interesting genetic variation is occurring between species. This makes assessment of genetic effects somewhat difficult. A critical starting point is to sample and observe animals in hybrid zones. Along these lines, work being done on baboons might provide a useful analog. While not a direct extension of this research, much of the techniques utilized throughout this study could be used as a basis for work in these species. Previous evidence has shown that serotonin profiles in two species of baboons (*Papio anubis* and *P. hamadryas*) are distinct, and that naturally occurring hybrids are intermediate between the two species (Jolly et al., 2009). Moreover, these differing profiles reflect the behavioral differences between the two species, as hybrids with more "hamadryas-like" serotonin levels exhibited more "hamadryas-like" behaviors. The presence of a hybrid zone provides a natural experiment to look at the genetic profiles of these two species and identify genes that may underlie the species-level differences in behavior. Furthermore, by studying primates in natural settings, we can begin to address other, related questions. For example, how does environment interact with genetics? Or, how do behavioral differences, and the genetic architecture underlying behavior, contribute to the formation and maintenance of species? Addressing these questions will help to further situate the evolutionary importance of the serotonin system.

Studying behavior from a genetic point of view is incredibly challenging; at any given moment, there are a thousand different factors – biological and otherwise – that contribute to a behavior. To try to pinpoint the role of genetics, and evolution, within the myriad of other influences is a large task to carry out, and we are only beginning to develop the tools necessary. However, that very same aspect of behavioral and evolutionary genetics creates an incredible

wealth of potential research, and by continuing to pursue questions about evolution, genetics, and behavior, we may one day be much closer to understanding why we do the things we do.

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APPENDIX A

ADDITIONAL DISCUSSION OF OUTLIER *M. FASCICULARIS* HAPLOTYPE

Although most haplotypes found for each species tended to group together, one *Macaca fascicularis* haplotype provided an outlier for *HRT1A*. Despite there being evidence of recombination occurring around the gene, this individual did not share any of the polymorphisms that distinguished the other *M. fascicularis* samples and instead showed greater similarity to the *M. nemestrina*, *M. assamensis*, and *M. silenus* samples. I took this as evidence of either substructure within *M. fascicularis*, which can affect selection tests, or introgression. If the individual outlier is due to population substructure, this violates many of the assumptions of the selection tests. In such situations, the effect of substructure can be addressed by analyzing each subpopulation separately. If the outlier is due to introgression, then this *M. fascicularis* individual is not representative of the entire *M. fascicularis* species. In either case, removal of the outlier from analyses is warranted.

Inclusion or exclusion of the sample does not have an effect on most of the analyses. Specifically, for either gene examined, the results of the McDonald-Kreitman and the maximum likelihood analyses carried out in PAML do not differ whether or not this sample is included. Results for **ω** do differ, but only slightly. Specifically, when the outlier is included, the *M. fascicularis* and *M. sylvanus* comparison becomes significant and the *M. fascicularis* and *M. fuscata* comparison becomes nonsignificant. However, **ω** remains similar and all results for **ω**, both with and without the outlier, have p-values less than 0.077. That is, all results approach significance, and therefore the interpretation of the results remains unchanged.

However, its inclusion does affect the outcome of the HKA tests for *HTR1A*; the results obtained when *M. fascicularis* is compared to the other macaque species are no longer

significant. This is primarily because the HKA program employed (http://genfaculty.rutgers.edu/hey/software) uses the number of polymorphic sites to determine the level of within-species variation. When the outlier is included, this estimate of within-species variation is increased such that its proportion to between-species variation more closely matches that seen in the non-coding regions. That is, the high level of between-species variation seen for *HTR1A* for *M. fascicularis* no longer appears anomalous. However, the number of polymorphic sites does not allow for unequal frequencies of haplotypes; in this case, a single individual accounted for approximately 75% of the number of polymorphic sites.

It should be noted, though, that even if I did include this outlier, there were still significant results in the analyses carried out in PAML and for the HKA tests that compared *M. nemestrina* to the mulatta group. Therefore, there remains evidence for selection occurring on some of the macaque lineages for *HTR1A*.

ADDITIONAL DISCUSSION OF POSSIBLE EFFECT OF NON-SELECTIVE EVOLUTIONARY FORCES

It is possible that several non-selective forces could have affected the results, such as demography, increased mutation rate, and recombination. However, this is unlikely. Demographic events, such as a rapid population expansion, are expected to have a similar effect on all genes, and I did not see a similar pattern in either *HTR1B* or the five non-coding loci that were sequenced. In addition, a demographic event is unlikely to produce the results found in the likelihood ratio tests (LRTs), as tests based on **ω** do not depend on demographic assumptions (Nielsen, 2005).

It is also unlikely that an increase in mutation rate for the *HTR1A* gene produced the pattern found. One major cause of increased mutation rate in mammals is the methylation of

CpG sites, which causes an increase in C to T mutations (and G to A mutations if on the opposite strand). I examined the possibility that this was the case for *HTR1A*. While a large proportion (24%) of the polymorphisms occurred in the expected direction at CpG sites in *HTR1A*, this was also true for *HTR1B* (22%). Furthermore, the polymorphisms at the CpG sites were more likely to be the cause of within-species variation, rather than among-species variation. Thus, mutation induced by CpG methylation does not explain the unique pattern found in *HTR1A*. While there are other causes of increased mutation rate, these also seem unlikely as the HKA test is designed to account for these factors, even if it does so imperfectly. The HKA test is based on the premise that the neutral mutation rate will drive both the within-species variation and the among-species divergence in a proportional manner (Hudson et al., 1987). Although the among-species divergence is higher for *HTR1A* than for *HTR1B*, an elevation in mutation should also cause an increase in within-species variation as well. A look at Table 5 shows that, except for *M. nemestrina*, this is not the case. Furthermore, as with demography, this would not explain the significant results from the LRTs.

Finally, I found evidence for recombination, which can affect analyses. This is a conservative assumption for the HKA test, but its presence can lead to an increase in false positives for both MK and LRT comparing various sites models. Since there were nonsignificant p-values in the MK test and significant p-values in the HKA test, these results were probably not due to the presence of recombination. In contrast, the significant results achieved using the LRT could be the result of recombination, rather than selection (Anisimova et al., 2003). However, this is unlikely for two reasons. First, the recombination estimates are based on the entire region sequenced, but the LRTs used in PAML are based on the coding region alone. Estimates of the recombination rate (C) using only the coding region are zero. Second, estimates of C for the

entire region were low. Anisimova et al. (2003) showed that a LRT comparing M7 to M8 showed no difference in the rate of false positives when $C = 0.001$ than when $C = 0$. While the estimate of recombination is higher than this, p-values were well below a significance level of 5% or even 1%. Thus, a mild increase in the rate of false positives due to recombination seems unlikely to be responsible for the results, although this cannot be entirely ruled out.

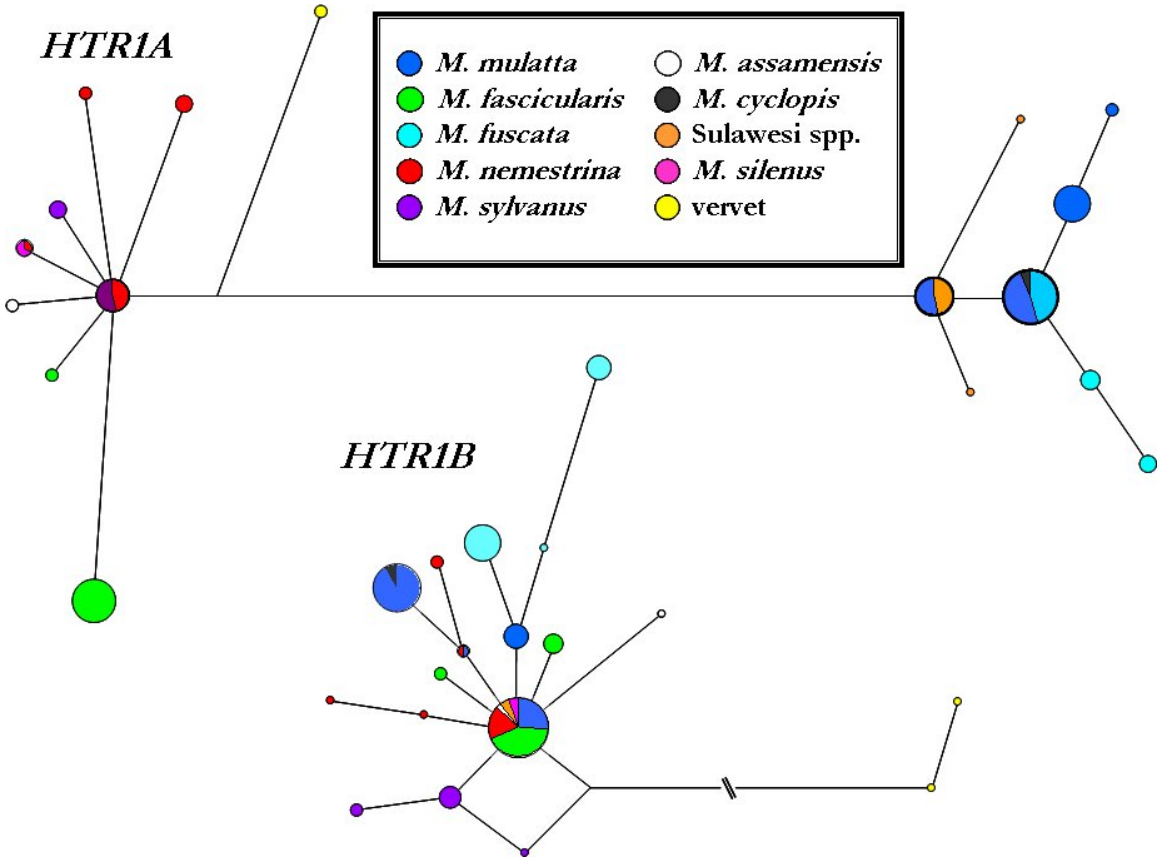

Figure A.1. Haplotype networks for the coding regions only of *HTR1A* (top) and *HTR1B* (bottom). Each circle represents a haplotype whose size is proportional to the frequency of the haplotype. The lengths of the lines connecting the circles are proportional to the number of mutations that separate each haplotype. To provide scale, a few of the lines are labeled to show the number of mutations between haplotypes. Because of the larger number of mutations separating the vervet from the macaques in *HTR1B*, this line is not drawn to scale.

Table A.1. List of sources for species samples. Number of samples indicates the number of individuals for which complete sequence data were obtained for both genes. NINPRC = New Iberia National Primate Research Center. *See Ferguson et al. (2009).

Species	# of samples	Extracted from	Source
M. mulatta	20	blood	DG Smith and J Satkoski, UC Davis
Indian	11	blood	DG Smith and J Satkoski, UC Davis
Chinese	9	blood	DG Smith and J Satkoski, UC Davis
M. fascicularis	11	blood	DG Smith and J Satkoski, UC Davis
M. fuscata	11	blood	Oregon National Primate Research Center*
M. nemestrina	6	blood	DG Smith, UC Davis; NINPRC
M. sylvanus	4	blood	DG Smith, UC Davis; A. Deinard
M. assamensis	1	blood	NINPRC
M. cyclopis	1	blood	New England National Primate Research Center
M. nigra	1	blood	Baltimore Zoo
M. silenus	1	blood	Woodland Park Zoo
C. aethiops	1	blood	NINPRC
Total	57		

Table A.2. List of primers used to sequence *HTR1A* and *HTR1B*. For both genes, the number in the primer name represents the position of the primer relative the start of the coding region, with +1 marking the first nucleotide of the start codon. For *HTR1A*, these numbers are based on the rhesus macaque reference genome. For *HTR1B*, I followed the numbering system of Cigler et al. (2001), which is based on the human genome. For the source column: a = primers designed for this project; b = primers published Cigler et al. (2001).

Gene	Primer ID	Sequence	Type	Source
HTR1A	HTR1A_-433F	5'-ACA GAG TGA CCG TGG AGG ATG-3'	PCR and Sequencing	a
	HTR1A_-309F	5'-AGC GAC AGA CAG ACG TTC C-3'	Sequencing	a
	HTR1A_18F	5'-TGG TCA GGG CAA CAA CAC-3'	Sequencing	a
	HTR1A_272R	5'-ATG GGC AGC ACT AAC ACC-3'	Sequencing	a
	HTR1A_-107R	5'-TCG GAG GAA GGG AAT GCA G-3'	Sequencing	a
	HTR1A_621R	5'-CAG CGG GAT GTA GAA AGC-3'	Sequencing	a
	HTR1A_328F	5'-CTG TTT ATC GCC CTG GAC-3'	Sequencing	a
	HTR1A_479F	5'-GGC TTA TTG GCT TCC TCA TC-3'	Sequencing	a
	HTR1A_1074R	5'-AAG CCA GCA GAG GAT GAA GG-3'	Sequencing	a
	HTR1A_1262R	5'-TGG CGG CAG AAC TTA CAC-3'	Sequencing	a
	HTR1A_942F	5'-TTG TGC CTC CGC CTC TTT-3'	Sequencing	a
	HTR1A_1523R	5'-GCA AAG TCT GAG CCA ATG TC-3'	Sequencing	a
	HTR1A_1187F	5'-CGG TCA TTT ACG CAT ACT TC-3'	Sequencing	a
	HTR1A_1364F	5'-CTT CTT CTC TGT CTC TCT GCT C-3'	Sequencing	a
	HTR1A_1954F	5'-CCC TGC TTC CTT TGT TTC-3'	Sequencing	a
	HTR1A_2212R	5'-GCC TCC CGC AGT AAG TAA GTG-3'	PCR and Sequencing	a
	HTR1A_-1071F	5'-AGT GCA GTG GCG CGA GAA-3'	PCR and Sequencing	a
	HTR1A_-1059F	5'-CGA GAA CGG AGG GAG GTA AC-3'	Sequencing	a
	HTR1A_-660R	5'-AGT GCC TCT TTC CTC TGG-3'	Sequencing	a
	HTR1A_-744R	5'-TCA GAA CTC ACT TAC ACA CAC C-3'	Sequencing	a
	HTR1A_-530R	5'-CAC TTG CCT TCC CTT TCA GT-3'	PCR and Sequencing	a
HTR1B	HTR1B_1247R	5'-TTC GAC CTA CCT GTG GAA CC-3'	PCR and Sequencing	b
	HTR1B_-174F	5'-GGC TGC CGC ACC CAT GAC CT-3'	Sequencing	b
	HTR1B_864F	5'-CCA AGT CAA AGT GCG AGT CT-3'	Sequencing	b
	HTR1B_-40R	5'-ATG GAG CGG ACG AAG GAG A-3'	Sequencing	b
	HTR1B_493R	5'-TCT TGG GAG TCC TTT TAG C-3'	Sequencing	b
	HTR1B_317F	5'-GCA CCA TGT ACA CTG TCA CC-3'	Sequencing	a
	HTR1B_-595F	5'-CAG CGC TGC TCC TAG ACT TC-3'	PCR and Sequencing	b
	HTR1B_957F	5'-TTT GGG AGC CTT TAT TGT G-3'	PCR and Sequencing	a
	HTR1B_1385R	5'-TGG GCA GGG AAG TTC TAC-3'	Sequencing	a
	HTR1B_1231F	5'-TCC ACA GGT AGG TCG AAT C-3'	Sequencing	a
	HTR1B_1536R	5'-TGG TTC TAG TGG GCA TTA TC-3'	PCR and Sequencing	a

Table A.3. Additional non-coding loci sequenced. The nearest feature was identified by blasting the consensus sequence against Build 1.1 (annotated) of the rhesus genome on the NCBI website.

Chromosome	Name	Fragment Length (base pairs)	Nearest Feature (base pairs)
4	Chr04-2	478	69,159 at 5' side: similar to Eukaryotic translation elongation factor 1 epsilon-1
6	Chr06-5	428	77,112 at 5' side: hypothetical protein
9	Chr09-2	526	None within 1Mb
14	Chr14-2	462	68,130 at 5' side: CD82 molecule isoform 1
20	Chr20-1	400	20,964 at 3' side: similar to xylosyltransferase I

Table A.4. Genetic distance within and between species for the five non-coding regions sequenced. The diagonal elements show the nucleotide diversity within species and the off-diagonal elements show the nucleotide diversity between species. For ease of comparative purposes to *HTR1A*, all areas where the nucleotide diversity of *HTR1A* was relatively higher than in the non-coding regions are highlighted, which most commonly occurred between species (off-diagonal) and tend to reflect the results of the HKA test. For the ratio of nucleotide diversity of *HTR1A* to a non-coding region, x, light gray = $1 < x \leq 1.5$; medium gray = $1.5 < x \leq 3$; blue/dark grey = $3 < x$. Ratios could not be calculated where nucleotide diversity of the non-coding region equaled 0. Ratios were determined using the nucleotide diversity calculated when outlier was removed. Mul = *M. mulatta*; Fas = *M. fascicularis*; Fus = *M. fuscata; Nem = M. nemestrina*; Syl = *M. sylvanus*.

Chr04-2

	Mul	Fas	Fus	Nem	Syl
Mul	**0.0031**				
Fas	**0.0037**	**0.0036**			
Fus	**0.0051**	**0.0059**	**0.0020**		
Nem	**0.0019**	**0.0021**	**0.0038**	**0.0000**	
Syl	**0.0122**	**0.0119**	**0.0142**	**0.0104**	**0.0000**

Chr14-2

	Mul	Fas	Fus	Nem	Syl
Mul	**0.0042**				
Fas	**0.0062**	**0.0049**			
Fus	**0.0043**	**0.0038**	**0.0006**		
Nem	**0.0029**	**0.0044**	**0.0027**	**0.0004**	
Syl	**0.0063**	**0.0078**	**0.0068**	**0.0045**	**0.0000**

Chr06-5

	Mul	Fas	Fus	Nem	Syl
Mul	**0.0034**				
Fas	**0.0043**	**0.0039**			
Fus	**0.0023**	**0.0032**	**0.0009**		
Nem	**0.0038**	**0.0045**	**0.0024**	**0.0037**	
Syl	**0.0019**	**0.0026**	**0.0006**	**0.0019**	**0.0000**

Chr20-1

	Mul	Fas	Fus	Nem	Syl
Mul	**0.0024**				
Fas	**0.0037**	**0.0043**			
Fus	**0.0017**	**0.0029**	**0.0009**		
Nem	**0.0023**	**0.0036**	**0.0015**	**0.0018**	
Syl	**0.0183**	**0.0198**	**0.0180**	**0.0185**	**0.0000**

Chr09-2

	Mul	Fas	Fus	Nem	Syl
Mul	**0.0065**				
Fas	**0.0080**	**0.0050**			
Fus	**0.0077**	**0.0083**	**0.0007**		
Nem	**0.0070**	**0.0049**	**0.0068**	**0.0034**	
Syl	**0.0279**	**0.0251**	**0.0305**	**0.0267**	**0.0000**

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APPENDIX B

ADDITIONAL TABLES

Table B.1. List of primers used to sequence *TPH2*. The number in the primer name represents the position of the primer relative the start of the coding region, with +1 marking the first nucleotide of the start codon. These numbers are based on the rhesus macaque reference genome. For the source column: a = primers published by Chen et al. (2006); b = primers designed for this project; a, b = primers based on Chen et al. (2006), but modified for this project.

Primer ID	Sequence	Type	Gene Region	Source
TPH-1312F	5'-TGC ATG TCA AGT TGC TGG AT-3'	PCR and Sequencing	5'UTR and Exon 1	a
TPH214R	5'-TTG AGG TGT GCG TGC TTA C-3'	PCR and Sequencing	5'UTR and Exon 1	a, b
TPH-777F	5'-GGC TGA TAG GAG GAA TAA GA-3'	Sequencing	5'UTR and Exon 1	a
TPH-319R	5'-GGG TGG TGG AGA ACA ATA CAT AA-3'	Sequencing	5'UTR and Exon 1	a
TPH-271F	5'-CAG ATA ACC CCA GGC TTC AG-3'	Sequencing	5'UTR and Exon 1	a
TPH-230R	5'-TGG CCA CAG TCA GAT TAC AGA C-3'	Sequencing	5'UTR and Exon 1	a
TPH-974R	5'-TTG ATT TAG CCA CAG GGA GTT T-3'	Sequencing	5'UTR and Exon 1	a
TPH-1209F	5'-CCT CAC CAC ATA ACA CAC AG-3'	Sequencing	5'UTR and Exon 1	b
TPH-1211R	5'-GGA GGC AGA GGT TAA GAG TAC-3'	Sequencing	5'UTR and Exon 1	b
TPH-707F	5'-CTG AAA GAG TGG AAT TGG AAT G-3'	Sequencing	5'UTR and Exon 1	b
TPH-378R	5'-GAC GTT AGC TCT CTC ATC TCT C-3'	Sequencing	5'UTR and Exon 1	b
TPH-387R	5'-TCT CTC ATC TCT CAA GCC TG-3'	Sequencing	5'UTR and Exon 1	b
TPH3435R	5'-TTT GTA AGG GAG GGT GTT TG-3'	PCR and Sequencing	Exon 2	b
TPH2224F	5'-TGA TTC AAT GAC GCT AAG AG-3'	PCR and Sequencing	Exon 2	b
TPH2921R	5'-GCC ACA GCT TAT ACA ATT CT-3'	Sequencing	Exon 2	b
TPH2620F	5'-TGA CGA CAA AGG CAA CAA G-3'	Sequencing	Exon 2	b
TPH3035F	5'-AAA CAC AAG AGG AGC CAA AC-3'	Sequencing	Exon 2	b
TPH2558R	5'-TAG TTA GGG TTC CAG AAT CC-3'	Sequencing	Exon 2	b
TPH2244R	5'-TCT CTT AGC GTC ATT GAA TC-3'	Sequencing	Exon 2	b
TPH2978F	5'-TAT TGC CAG GTT AGG AGG TC-3'	Sequencing	Exon 2	b
TPH3306R	5'-GCC TGA GAA GAT GCT ACG AC-3'	Sequencing	Exon 2	b
TPH5128F	5'-CTC TCT GGG AAA TGA TGA TG-3'	PCR and Sequencing	Exons 3-4	b
TPH6536R	5'-TGG GTG CAT TGC TTA CTT CT-3'	PCR and Sequencing	Exons 3-4	b
TPH5462R	5'-CCG AGA TTT CCT GGA TTC-3'	Sequencing	Exons 3-4	b
TPH5309F	5'-CCT CTT GCA TGG GTA CTT G-3'	Sequencing	Exons 3-4	b
TPH5606F	5'-GGG TGG TCT TAG CTT GTT G-3'	Sequencing	Exons 3-4	b
TPH6141F	5'-GAT CAT GCC TCT GGG AAA C-3'	Sequencing	Exons 3-4	b
TPH6337R	5'-TCA GGG TAG ATG TGG GAA G-3'	Sequencing	Exons 3-4	b
TPH6003R	5'-CCA TCC TTC TAA CCT GCT TC-3'	Sequencing	Exons 3-4	b
TPH5224R	5'-ACT TCT GTG TAT AGG TTG TCC-3'	Sequencing	Exons 3-4	b
TPH5192F	5'-TTA TAT GAG GCA GGA CAA CC-3'	Sequencing	Exons 3-4	b
TPH5248F	5'-ACA GAA ATA ATG GTT GGA AA-3'	Sequencing	Exons 3-4	b
TPH5366R	5'-TGC CAT CAG CTT TTC TTG-3'	Sequencing	Exons 3-4	b
TPH5983F	5'-GGA AGC AGG TTA GAA GGA TG-3'	Sequencing	Exons 3-4	b

Table B.1 (cont).

Primer ID	Sequence	Type	Gene Region	Source
TPH89706F	5'-CAT TCA TTG TTC CCT GCT G-3'	PCR and Sequencing	Exons 10-11 and 3'UTR	b
TPH91891R	5'-AGG CAT TAT TTC CCA TCT CC-3'	PCR and Sequencing	Exons 10-11 and 3'UTR	b
TPH91502F	5'-GCA TTG ACC TTG TAG AAC CTG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91522R	5'-CAG GTT CTA CAA GGT CAA TGC-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91249F	5'-AGC CTT TCC TCT GTG TTC G-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91367R	5'-AAG TTT CCT ACA ATT CTG GG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91007R	5'-TTA GCC AAG CCA TGA CAC AG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH90603R	5'-CCT CAT CTT TTC TTT GGC TTC-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH90767F	5'-ACT TCA ATC CCT ACA CGC AGA G-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH90045R	5'-TGC GTG GAG AGG TAA TTG AG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH89941F	5'-TAC AGG CCC ATT CCA GAC-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH90300F	5'-CGT GTC CAA TTT ACC CTG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH89884F	5'-ATT GCT CTG CTG TAG TTG CTG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH89813F	5'-GCT CTT GGC ATA ACT CTA ACC-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH90255R	5'-GTA CTG TGG GAG AAA ATG AGA-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91846R	5'-CCA GGA CGA ATT TAT CAG G-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91862R	5'-TTC ACA CAT GCA CAC ACC AG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91566F	5'-CAG AAA GGA CAT TAG GAA AGA C-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91640F	5'-AAA TTA TCG TGC TTA GGA GGT-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH90406F	5'-GAT GTC ACG GCA CTT TGG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91147R	5'-TAA GGA GAC TAA GCA GGA ATG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH91250R	5'-ACG AAC ACA GAG GAA AGG C-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH89750F	5'-CTG AAC GGA GCT AAT GAT GG-3'	Sequencing	Exons 10-11 and 3'UTR	b
TPH90423R	5'-CCA AAG TGC CGT GAC ATC-3'	Sequencing	Exons 10-11 and 3'UTR	b

Table B.2. Interspecific differences of TPH2. The sites listed indicate the position of the mutation realtive to the start of the coding region with +1 marking the first nucleotide of the start codon and -1 marking the nucleotide just prior to the first nuceotide of the start codon. Site numbers are based on the alignment of all 10 species listed. Areas shaded in grey represent sites within in exons. The pink shading prepresents a nonsynmous mutation (54Lys>Arg). Only sites that showed fixed differences between species are shown here (no intraspecific variation), although intraspecific polymorphisms are indicated if they occur at the same site as a fixed difference. For information on intraspecific variation, refer to Table 4.4. Indels are not listed here.

	M. mulatta	*M. fascicularis*	*M. fuscata*	*M. nemestrina*	*M. sylvanus*	*M. assemensis*	*M. cyclopis*	*M. nigra*	*M. silenus*	*C. aethiops*
-1268	A	A	A	A	A	A	A	A	A	G
-1255	T	T	T	C	T	T	T	T	T	T
-1229	C	C	C	C	C	Y	C	C	C	C
-1205	C	C	C	C	A	C	C	C	C	C
-1168	T	T	T	T	T	T	T	T	T	C
-1155	G	G	G	G	A	G	G	G	G	G
-1058	T	T	T	T	T	Y	T	T	T	T
-1054	C	C	C	C	C	C	C	C	C	T
-998	G	G	G	G	G	G	G	G	G	A
-985	G	G	G	G	G	G	G	A	G	G
-954	A	A	A	A	A	A	A	A	R	A
-946	T	T	T	T	T	T	T	T	G	T
-921	A	A	A	A	A	A	A	A	A	C
-898	C	C	C	C	C	C	Y	C	C	C
-896	T	T	T	T	T	T	T	T	K	T
-821	G	G	G	G	A	G	G	G	G	A
-819	T	T	T	T	T	T	T	T	T	A
-816	T	T	T	T	T	T	T	T	T	G
-815	T	T	T	T	T	T	T	T	T	G
-795	C	C	C	Y	C	C	C	T	T	C
-774	G	G	G	G	G	G	S	G	G	G
-766	G	G	G	G	G	G	A	G	G	G
-676	A	A	A	A	A	A	G	A	A	A
-647	A	A	A	A	A	G	A	A	A	A
-634	C	C	C	C	C	S	C	C	C	C
-603	A	A	A	A	A	A	A	A	A	G
-591	C	C	C	C	C	C	C	T	C	C
-561	G	G	G	G	G	G	G	G	G	A

	M. mulatta	*M. fascicularis*	*M. fuscata*	*M. nemestrina*	*M. sylvanus*	*M. assemensis*	*M. cyclopis*	*M. nigra*	*M. silenus*	*C. aethiops*
-542	A	A	A	A	A	A	A	A	A	G
-507	C	C	C	C	C	C	C	T	C	C
-413	Y	C	T	C	C	C	T	C	C	C
-407	C	C	C	C	C	C	C	C	Y	C
-399	A	A	A	A	A	A	A	R	A	A
-380	T	T	T	T	T	T	T	T	T	C
-356	A	A	A	R	A	A	A	A	A	A
-277	T	T	T	T	T	T	T	T	Y	T
-179	G	G	G	G	G	G	G	G	G	A
-139	T	T	T	T	C	T	T	T	T	T
-122	T	T	T	T	T	T	T	T	T	G
-54	C	C	C	C	C	C	C	C	C	S
81	G	G	G	G	G	G	G	G	G	A
119	A	A	A	A	A	A	A	A	A	G
120	C	C	Y	C	C	C	C	C	C	T
137	A	A	A	R	G	G	A	G	G	G
139	A	A	A	A	A	R	A	A	A	A
150	C	C	C	C	C	C	C	C	C	T
165	C	C	C	C	C	C	C	C	C	Y
2279	C	C	C	C	C	C	C	C	C	T
2281	G	G	G	G	C	G	G	G	G	G
2297	G	G	G	G	G	G	G	G	G	A
2325	T	T	T	T	T	T	T	W	T	T
2342	C	C	C	Y	C	C	C	C	C	T
2361	T	T	T	T	T	K	T	T	T	T
2404	C	C	C	T	T	T	C	T	T	T
2424	A	A	A	A	A	A	A	A	A	G
2452	G	G	G	G	G	R	G	G	G	G

Table B.2. (cont).

	M. mulatta	*M. fascicularis*	*M. fuscata*	*M. nemestrina*	*M. sylvanus*	*M. assemensis*	*M. cyclopis*	*M. nigra*	*M. silenus*	*C. aethiops*
2456	C	C	C	C	C	C	C	C	C	Y
2493	G	G	G	G	G	G	G	R	G	G
2505	T	T	T	T	T	T	T	T	T	K
2565	C	C	C	C	C	C	C	C	T	C
2566	R	G	G	G	G	A	G	G	G	G
2649	A	A	A	A	A	A	A	A	A	G
2722	G	G	G	A	A	R	G	A	A	A
2783	G	G	G	G	G	G	G	G	G	A
2845	G	G	G	G	G	G	G	G	G	A
2872	C	C	C	T	T	T	C	T	T	T
2892	G	G	G	G	G	G	G	K	G	G
2894	G	G	G	G	G	G	G	S	G	G
2896	C	C	C	C	C	C	C	C	C	T
2975	A	A	A	A	A	A	A	A	A	G
3046	G	G	G	G	G	G	G	G	A	G
3056	A	A	A	A	G	A	A	A	A	A
3083	A	A	A	A	A	A	A	A	A	G
3084	R	G	G	G	G	G	G	G	G	A
3140	A	A	A	A	A	A	G	A	A	A
3141	M	C	C	C	C	C	A	C	C	C
3248	A	A	A	A	A	A	A	A	A	G
3260	G	G	G	G	G	R	G	G	G	G
3340	T	T	T	T	T	T	T	T	T	C
3371	G	G	G	G	G	G	G	G	G	S
3393	C	C	C	C	C	C	C	C	C	A
3394	C	C	C	C	C	C	C	C	C	G
5164	A	A	G	A	A	A	A	A	A	A
5252	M	C	A	A	A	A	A	A	A	A
5264	G	G	G	G	G	G	G	R	G	G
5277	G	G	G	G	G	G	A	G	G	G
5301	T	T	T	T	T	T	T	T	K	T
5317	A	A	A	A	A	A	A	A	A	C
5362	T	T	T	T	T	T	T	Y	T	T
5385	R	G	G	G	G	G	G	G	A	G
5426	C	C	C	C	C	C	C	C	C	T

	M. mulatta	*M. fascicularis*	*M. fuscata*	*M. nemestrina*	*M. sylvanus*	*M. assemensis*	*M. cyclopis*	*M. nigra*	*M. silenus*	*C. aethiops*
5468	G	G	G	G	G	G	G	G	G	A
5673	T	T	T	T	T	T	T	Y	T	T
5829	A	A	A	A	A	A	A	A	A	C
5839	C	C	C	C	C	C	C	C	C	T
5860	A	A	A	A	A	A	A	A	A	C
5945	C	C	C	C	C	C	C	C	M	C
5971	C	C	C	C	C	C	C	C	C	G
5972	A	A	A	G	G	G	G	G	G	G
5973	C	C	C	C	C	C	C	Y	C	C
5974	G	G	G	G	G	G	G	G	G	A
6012	C	C	C	C	C	C	C	T	C	C
6108	A	A	A	A	A	A	A	A	A	T
6145	C	C	C	C	C	C	T	C	C	C
6151	T	T	T	T	T	T	T	W	T	T
6252	T	T	T	T	T	A	T	T	T	T
6307	G	G	G	G	G	G	R	G	G	G
6308	G	G	G	G	G	G	G	G	G	A
6331	T	T	T	T	T	T	T	Y	T	T
6333	C	C	C	C	C	C	C	S	C	C
6334	C	Y	C	C	C	C	C	C	C	T
6378	T	T	T	T	T	Y	T	T	T	T
89785	C	C	C	Y	C	C	C	C	T	C
89803	T	T	T	Y	T	T	T	T	C	T
89833	T	T	T	T	C	T	T	T	T	T
89838	K	T	T	K	G	G	T	G	G	G
89855	C	C	C	C	T	C	C	C	C	C
89879	Y	T	C	T	T	T	T	T	T	T
89882	A	A	A	A	A	A	A	A	A	T
89902	C	C	C	C	G	C	C	C	C	G
89912	A	A	A	A	A	C	A	A	A	A
89919	G	G	G	K	G	G	G	G	T	G
89949	C	C	C	C	T	C	C	C	C	C
90019	T	T	T	T	T	T	T	T	K	T
90058	T	K	T	T	T	T	G	T	T	T
90100	T	T	T	T	T	T	T	T	Y	T

Table B.2. (cont).

	M. mulatta	*M. fascicularis*	*M. fuscata*	*M. nemestrina*	*M. sylvanus*	*M. assemensis*	*M. cyclopis*	*M. nigra*	*M. silenus*	*C. aethiops*
90137	C	C	C	C	C	C	C	C	C	T
90171	G	G	G	G	G	R	G	G	G	G
90186	C	C	C	C	C	C	C	C	C	A
90222	C	C	C	C	C	C	C	C	C	A
90304	Y	C	C	C	C	C	C	C	C	A
90363	G	G	G	G	G	G	G	G	G	A
90369	C	C	C	M	A	A	C	A	A	A
90372	T	T	T	T	C	T	T	T	T	T
90416	A	A	A	M	A	A	A	C	C	A
90427	A	A	A	A	A	G	A	A	A	A
90428	Y	C	C	C	C	C	C	C	C	T
90441	G	G	G	G	G	K	G	G	G	G
90663	C	C	C	C	C	C	C	C	C	T
90699	A	A	A	A	A	A	A	A	A	C
90707	G	G	G	G	G	G	G	G	G	A
90717	T	T	T	T	T	C	T	T	T	T
90741	G	G	G	G	G	G	G	T	G	G
90781	A	A	A	A	A	A	A	A	A	G
90799	G	R	G	G	G	G	G	G	G	A
90874	A	A	A	A	A	A	A	A	A	G
90959	R	G	G	G	G	G	G	G	G	A
90975	C	C	C	C	C	C	C	C	C	T
91014	G	G	G	G	G	G	G	G	G	A
91170	G	G	K	G	G	R	G	G	G	G
91177	C	C	C	C	C	C	C	C	C	G
91229	C	C	C	C	C	S	C	C	G	C
91246	C	C	C	C	C	C	C	C	C	T
91283	G	G	G	G	G	G	G	G	G	A
91307	A	A	A	A	A	A	A	A	A	G
91313	A	A	A	A	G	A	A	A	A	C

	M. mulatta	*M. fascicularis*	*M. fuscata*	*M. nemestrina*	*M. sylvanus*	*M. assemensis*	*M. cyclopis*	*M. nigra*	*M. silenus*	*C. aethiops*
91323	T	T	T	T	T	T	T	T	T	Y
91352	T	T	T	T	T	T	T	T	T	C
91380	C	C	C	C	C	C	C	C	C	A
91459	K	G	G	K	G	G	G	G	T	G
91478	C	C	C	C	C	C	C	C	C	G
91494	T	T	T	T	T	T	T	T	T	C
91496	T	T	T	T	T	T	T	T	T	C
91497	G	G	G	G	G	G	G	G	G	A
91531	A	A	A	A	A	A	A	A	A	G
91558	C	C	C	C	C	C	C	C	C	T
91571	A	A	A	A	A	R	A	A	A	A
91736	T	T	T	T	T	T	T	T	T	C
91770	C	C	C	C	C	C	C	C	C	G
91815	T	T	T	T	T	T	T	Y	T	T
91819	G	G	G	G	G	G	G	G	G	A
91830	G	G	G	G	G	G	G	G	G	T
91839	T	T	T	T	C	T	T	T	T	T
91848	G	G	G	G	G	G	G	G	G	T
91869	C	C	C	C	C	C	C	A	C	C
91901	T	T	T	T	T	T	T	T	T	A
91945	K	K	G	G	G	G	T	G	G	G
91950	K	G	T	G	G	G	G	G	G	G
91956	K	K	G	T	T	T	G	T	T	T
91976	C	C	C	C	C	C	C	C	C	A
92001	T	T	T	T	T	T	T	T	T	C
92006	A	A	A	A	A	A	A	A	A	G
92012	T	T	T	T	T	G	T	T	T	T
92013	C	C	C	C	C	C	C	C	C	Y
92019	G	G	G	R	G	G	G	G	A	G
92030	G	G	G	G	G	G	G	G	G	A

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APPENDIX C

ADDITIONAL TABLES

Table C.1. List of primers used to sequence *SLC6A4*. The number in the primer name represents the position of the primer relative to a reference sequence obtained from the UCSC genome browser and does not indicate position relative to coding regions. For the source column: a = primers designed for this project; b = primers published by Wendland et al. (2006); c = primers published by Vallender et al. (2008).

Primer ID	Sequence	Type	Gene Region	Source
SLC_16250F	5'-CCA TAA GGT GAG ATT TCC AG-3'	PCR and Sequencing	5' Region	a
SLC_17259R	5'-AGT CTA AGC CAT CAT GTT CC-3'	PCR and Sequencing	5' Region	a
SLC_16632F	5'-TCC AAG CAC CCA GAG TTC-3'	Sequencing	5' Region	a
SLC_16873F	5'-TAC ACG GCA CTC TAT CCC-3'	Sequencing	5' Region	a
SLC_16751R	5'-CAT CCT TAC ACG CTG AGA G-3'	Sequencing	5' Region	a
SLC_18999F	5'-TTG TGG AAG TGT AGC CAA ATA TCA AT-3'	PCR and Sequencing	Exonic	a
SLC_24982R	5'-GCC ATC TGT GAT CAA TCA AAA TTA AG-3'	PCR and Sequencing	Exonic	a
SLC_18787F	5'-CAT AGC TGA CCC CCA CTT TAG G-3'	Nested Primers	exonic	a
SLC_21551R	5'-GGT GAA GAG AGA GAG GGT GCA T-3'	Nested Primers	exonic	a
SLC_21079F	5'-GGT GTT AGC CAA ATG GGC TTT-3'	Nested Primers	Exonic	a
SLC_23460R	5'-AGG AGC ACT GAC AAA ACC GAA G-3'	Nested Primers	Exonic	a
SLC_19450R	5'-GAG GTT TCA GAG GAG GCC A-3'	Sequencing	Exonic	a
SLC_19150F	5'-TTG CAT CAG AAT CAC CTT AG-3'	Sequencing	Exonic	a
SLC_19724F	5'-GGG ACA GTA CCA CCG AAA TG-3'	Sequencing	Exonic	a
SLC_20314F	5'-CCT CAT CTC CTC CTT CAC GG-3'	Sequencing	Exonic	a
SLC_20894F	5'-TGC TGG AAT TTC AGG CAA TG-3'	Sequencing	Exonic	a
SLC_19883R	5'-CTG ACT GAT TCC AGG AGA AG-3'	Sequencing	Exonic	a
SLC_19973R	5'-CTT CTC ATT GCC ATT TAT TCT G-3'	Sequencing	Exonic	a
SLC_20422R	5'-GAG GGT CCA GGT GAT GTT GT-3'	Sequencing	Exonic	a
SLC_21014R	5'-CTC CTT TGC CAA TGT GAC TG-3'	Sequencing	Exonic	a
SLC_21688R	5'-GAG GTT TCG ACA TGT TGG CC-3'	Sequencing	Exonic	a
SLC_22470F	5'-AAG AGA CAC TGA CGT CCA TC-3'	Sequencing	Exonic	a
SLC_22477F	5'-ACT GAC GTC CAT CCA CCC AC-3'	Sequencing	Exonic	a
SLC_22551F	5'-TCC CCT CCC TGG AAC AGC -3'	Sequencing	Exonic	a
SLC_22517F	5'-TGC TCC CCT GCT CCC CTC -3'	Sequencing	Exonic	a
SLC_1139F	5'-GCC TGG CGT TGC CGC TCT GAA T-3'	PCR	Promoter	b
SLC_1539R	5'-CAG GGG AGA TCC TGG GAG GGA-3'	PCR	Promoter	b
SLC_41166F	5'-GTC AAA TTC CAA CTC GCT G-3'	PCR and Sequencing	3' Region	a
SLC_41921R	5'-AGC TTC TTA CAT CTT CCT TTC CTG-3'	PCR and Sequencing	3' Region	a
SLC_41372F	5'-CAC ACT CAA TGA GAG GAA AAA GG-3'	PCR and Sequencing	3' Region	c
SLC_41963R	5'-CAC AGA CTC ACA TGC TTA CAT GG-3'	PCR and Sequencing	3' Region	c

Table C.2. Results of analyses for the 5' region. *C. aethiops* is used here as the outgroup for Fay and Wu's H and HKA.

Species	Fay and Wu's H	p-value	Tajima's D	p-value	HKA	p-value
M. mulatta	-1.882	0.053	-0.956	0.214	3.333	0.912
M. fascicularis	0.294	0.600	0.121	0.594	4.659	0.793
M. fuscata	0.416	0.821	0.166	0.666	5.335	0.721
M. nemestrina	1.455	0.888	0.384	0.653	11.956	0.153

Table C.3. Results of analyses for the 3' region. *C. aethiops* is used here as the outgroup for Fay and Wu's H and HKA.

Species	Fay and Wu's H	p-value	Tajima's D	p-value	HKA	p-value
M. mulatta	-0.377	0.250	0.272	0.680	4.721	0.787
M. fascicularis	0.529	0.599	0.780	0.805	6.383	0.604
M. fuscata	0.017	0.345	1.113	0.849	6.090	0.637
M. nemestrina	-1.182	0.089	-0.128	0.435	12.599	0.126

Table C.4. Results of analyses for the "exonic" region. *M. sylvanus* is used here as the outgroup for Fay and Wu's H and HKA.

Species	Fay and Wu's H	p-value	Tajima's D	p-value	HKA	p-value
M. mulatta	0.008	0.436	-0.530	0.329	11.361	0.330
M. fascicularis	-0.791	0.282	0.610	0.775	15.171	0.126
M. fuscata	1.673	0.965	-0.292	0.426	11.949	0.288
M. nemestrina	0.089	0.457	0.796	0.832	9.177	0.515

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