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Prolactin Receptor Gene Diversity in Azara's Owl Monkeys (*Aotus azarai*) and Humans (*Homo sapiens*) Suggests a Non-Neutral Evolutionary History among Primates

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Abstract Although paternal care is rare in mammals, males of several primate taxa exhibit high degrees of this behavior. Studies a number of vertebrate species found a positive correlation between prolactin (PRL) levels and paternal care. Studies of maternal care in knockout mice also indicate that the prolactin receptor (PRLR) plays a critical role in the neural regulation of parental care. To better understand the extent of *PRLR* genetic variation within primates, we characterized intraspecific coding variation in Azara's owl monkeys (*Aotus azarai*) from northern Argentina, a species with intensive paternal care. We then examined *PRLR* variation in 1088 humans (*Homo sapiens*) from the 1000 Genomes Project. Lastly, we assessed interspecific variation in *PRLR* in 4 different *Aotus* spp. and 12 phylogenetically (and behaviorally) disparate primate taxa. Our analyses revealed that the coding region of *PRLR* exhibits significant variation in all species of primates, with nonsynonymous amino acid substitutions being enriched in the intracellular domain, a region responsible for activation of downstream targets in the PRL pathway. In addition, several species exhibit entire codon deletions in this region. These results suggest a non-neutral evolutionary history of the *PRLR* locus within

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different primate lineages, and further imply that the translated PRLR protein has undergone considerable change throughout primate evolution. Such changes may be driven by selection for different behaviors and physiologies resulting from modulations of the pleiotropic prolactin pathway. Yet, the genetic variants in *PRLR* among primate taxa do not discretely cluster with species-level differences in paternal care behaviors. These observations imply that other mechanisms must be involved in the regulation of paternal care in primates.

Keywords Behavioral genetics · Night monkey · Paternal care · Platyrrhini · *PRLR*

Introduction

Vertebrate species exhibit strikingly diverse patterns of parenting behaviors and strategies (Clutton-Brock 1991; Maynard-Smith 1977). Many avian species engage in biparental care, where both males and females routinely and consistently perform behaviors that are associated with increasing the survival of infants (Burley and Johnson 2002). By contrast, biparental care is relatively rare among mammals (Fernandez-Duque *et al*. 2009). It is hypothesized that paternal care evolved in species in which it increases the fitness of parental males, either by increasing survival probability of their own offspring or by increasing the males' mating success (Fernandez-Duque *et al*. 2009; Gubernick and Teferi 2000; Wright 1990).

At present, little is known about the genetic mechanisms or genetic polymorphisms that underlie biparental care patterns. There is growing evidence that the physiological basis of paternal care behavior is modulated by hormones, e.g., prolactin, arginine vasopressin, cortisol, and oxytocin, that also regulate maternal care behavior (Gordon *et al*. 2010; Neuman and Insel 2003; Schradin and Anzenberger 1999; Storey *et al*. 2000; Wynne-Edwards and Reburn 2000). For example, increased levels of the polypeptide pituitary hormone prolactin (PRL) are correlated with paternal care behaviors in males of Djungarian hamsters (*Phodopus campbelli*) and striped mice (*Rhabdomys pumilio*) (Gubernick and Nelson 1989; Reburn and Wynne-Edwards 1999; Schradin 2008; Schradin and Pillay 2004). Among primates, a similar association between PRL and paternal care in common marmosets (*Callithrix jacchus*) and cotton-top tamarins (*Saguinus oedipus*) further hints at a highly conserved role for PRL in the regulation of paternal care in mammals (Dixson and George 1982; Mota and de Sousa 2000; Mota *et al*. 2006; Schradin *et al*. 2003; Ziegler *et al*. 1996, 2004).

Even when there seems to be a strong relationship between PRL and paternal care, one must consider that PRL is pleiotropic in its effects. It is linked to >300 biological functions among vertebrates, and plays a key role in reproduction, lactation, growth and metabolic pathways, tumor formation, and immunoregulation (Ben-Jonathan *et al*. 2008; Bole-Feysot *et al*. 1998). To carry out these various functions, PRL binds to a dimeric receptor complex that is comprised of two prolactin-receptor (PRLR) protein subunits (Bole-Feysot *et al*. 1998). The "long" isoform of a PRLR protein is composed of a 234-amino-acid (AA) ligand-binding extracellular domain (ECD), a 24 AA transmembrane domain (TMD), and a 364 AA intracellular domain (ICD) that is responsible for the activation of downstream targets in the PRL pathway (Dalrymple *et al*. 2000). Many isoforms of PRLR exist owing to extensive modifications of the ICD

through numerous alternative splicing pathways (Goffin *et al*. 2002; Hu *et al*. 2002; Qazi *et al*. 2006). Similarly, the heterogeneous physiological roles of PLR are explained in part by the *PRLR* gene having multiple promoters, which regulate tissue expression differences and ostensibly result in diverse cellular effects (Hu *et al*. 2002).

Previous studies of the physiological roles of PRL and PRLR have relied on gene knockout experiments in mice. Female mice null for $PRLR^{-/-}$ have been shown to display severe deficits in maternal care behaviors when compared to $PRL^{-/-}$ null mice, suggesting a more significant role for the PRLR receptors than the PRL hormone in the regulation of those behaviors (Horseman *et al*. 1997; Lucas *et al*. 1998; Ormandy *et al*. 1997). Further, in biparental species such as Djungarian hamsters and common marmosets, PRL suppression produces a negligible effect on both paternal and maternal care (Almond *et al*. 2006; Brooks *et al*. 2005), whereas increased *PRLR* mRNA levels correlate positively with paternal care behaviors in taxa as divergent as hamsters and discus fish (Khong *et al*. 2009; Ma *et al*. 2005).

Several species of platyrrhine primates consistently display high degrees of paternal care. Owl monkeys (*Aotus* spp.), which inhabit forests from Panama to Argentina, are one taxon in which paternal care has been extensively documented both in captivity and in the wild (Fernandez-Duque 2012; Wolovich *et al*. 2008; Wright 1990, 1994). Paternal care has been observed in nearly all species within the genus, regardless of the species' particular ecological context (Fernandez-Duque 2012). As such, owl monkeys represent an excellent model for studying the origin and evolution of paternal care behaviors.

To understand better the extent of coding sequence variation that exists within primates, we explored *PRLR* sequence diversity in a wild population of Azara's owl monkeys (*Aotus azarai*) and across a broad range of primate taxa. First, we framed our comparative analyses at the level of the population (*Aotus azarai* and *Homo sapiens*) to reveal intraspecific variation and to detect any signatures of recent species-specific selection. Next, we characterized *PRLR* variation in the genus *Aotus* to identify lineage-specific genetic variants. Finally, we assessed interspecific variation in *PRLR* in 12 phylogenetically disparate primate taxa exhibiting diverse mating systems and varying degrees of paternal care behaviors to pinpoint any variants that coincided with such behaviors. Given the highly conserved function of PRLR across taxa, we predicted that there would be minimal lineage-specific changes, and that any observed changes resulting in important structural and functional modifications of the mature protein may be attributable to some form of selection.

Methods

Focal Population

The wild focal population of *Aotus azarai* is located in the northern province of Formosa, Argentina in the South American Gran Chaco region. Owl monkeys are socially monogamous, and social groups generally comprise two to six individuals, with only one pair of reproductive adults (Fernandez-Duque and Huck 2013; Fernandez-Duque *et al*. 2001). Male owl monkeys typically become the primary carriers of their offspring when their infants are 1 week old, and infants generally return to the mothers only to nurse (Huck and Fernandez-Duque 2012; Rotundo *et al*. 2005; Wolovich *et al*. 2008).

Samples

We isolated DNA from 25 individuals in the study population of *Aotus azarai* using tissue, blood, feces, hair, and placental samples (Babb *et al*. 2010). The panel included 21 residents of the study site population in Estancia Guaycolec and 4 captive individuals from the Saenz-Peña Municipal Zoo. These owl monkeys represent diverse mitochondrial lineages in the population (Babb *et al*. 2011), and do not include related individuals (Table I).

To examine intraspecific variation of *PRLR* in humans (*Homo sapiens*), we accessed the most recent release of the 1000 Genomes Project Phase 1 genotype data set (sequence index 20101123). This data set features 1092 individuals from 14 populations genotyped at >37 million loci (1000 Genomes Project Consortium 2010, 2012) (Table I). We downloaded the .vcf file for chromosome 5 from NCBI (ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/), and filtered the data to include only 2980 single-nucleotide polymorphism (SNP) genotypes for 1088 unrelated individuals in a 200,000 base pair (bp) window surrounding the *PRLR* locus (human genome build hg19, chr5: 35,065,975-35,092,482). We performed all text manipulation on a Unix platform using custom Perl scripts.

To assess interspecific variation, we analyzed samples from four disparate taxa of *Aotus* (*A. lemurinus*, *A. l. griseimembra*, *A. nigriceps*, *A. nancymaae*), along with two titi monkey individuals (*Callicebus donacophilus*), two saki monkey individuals (*Pithecia pithecia pithecia*), and two squirrel monkey individuals (*Saimiri sciureus sciureus*) (Table I). All non-*Aotus azarai* samples were obtained from the Zoological Society of San Diego. In addition, we acquired published sequences of the protein-coding region of *PRLR* for eight additional primate taxa (*Cebus apella*, *Callithrix jacchus*, *Nomascus leucogenys*, *Gorilla gorilla gorilla*, *Pongo abelii*, *Pan troglodytes*, *Macaca mulatta*, *Homo sapiens*), and one rodent (*Mus musculus* [outgroup]) using GenBank (NCBI) and the University of California-Santa Cruz Genome Browser (UCSC GB) (Table I). These sequences, representing platyrrhine and catarrhine primates, included published mRNA transcripts, as well as our own BLAT searches conducted against whole genome sequences (Kent *et al*. 2002; Karolchik *et al*. 2008). In addition, we obtained the *PRLR* sequence for *Homo neanderthalensis* from the ENSEMBL browser (Green *et al*. 2010).

DNA Sequencing

To assess intraspecific variation, we sequenced the exonic content related to the "long" *PRLR* mRNA isoform (1866 bp, 8 exons, Dalrymple *et al*. 2000). We identified the exonic targets by importing *PRLR* sequences for all primate taxa and assembling contigs in Sequencher v4.9 (Gene Codes). We designed primers based on conserved regions that flanked either side of each putative *PRLR* exon using the oligo software programs NetPrimer (Premier BioSoft) and Primer3 (Rozen and Skaletsky 2000) (Fig. 1). From each sample, we generated eight amplicons that directly interrogated 3986 bp spanning 26 kilobases (kb) inclusive of the entire 1866 bp of *PRLR* mRNA coding sequence. Samples were cycle sequenced on an ABI 3130xl Gene Analyzer as described in Babb *et al*. (2010). We assessed read quality for each sequence using ABI Sequencing Analysis v5.4 and aligned them (avg. coverage ~4x) using Sequencher v4.9 and

Table I Samples investigated at the *PRLR* locus

ID	Species	Common name	Sex	Locale
AA008	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA014	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA015	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA021	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA032	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA034	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA037	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA053	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA057	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA063	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA067	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA071	*Aotus azarai*	Azara's owl monkey	M	Downstream, Formosa, AR
AA082	*Aotus azarai*	Azara's owl monkey	M	Downstream, Formosa, AR
AA087	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA092	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA108	*Aotus azarai*	Azara's owl monkey	F	Upstream, Formosa, AR
AA109	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AA114	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA122	*Aotus azarai*	Azara's owl monkey	F	Core area, Formosa, AR
AA123	*Aotus azarai*	Azara's owl monkey	M	Core area, Formosa, AR
AAF1	*Aotus azarai*	Azara's owl monkey	F	Saenz-Pena Zoo, AR
AAF1B	*Aotus azarai*	Azara's owl monkey	F	Saenz-Pena Zoo, AR
AAF2	*Aotus azarai*	Azara's owl monkey	F	Saenz-Pena Zoo, AR
AAM2	*Aotus azarai*	Azara's owl monkey	M	Saenz-Pena Zoo, AR
AAPLunk	*Aotus azarai*	Azara's owl monkey	Unknown	Core area, Formosa, AR
CD01	*Callicebus donacophilus*	White-eared titi monkey	M	San Diego Zoo/CRES
CD02	*Callicebus donacophilus*	White-eared titi monkey	M	San Diego Zoo/CRES
PP01	*Pithecia pithecia*	White-faced saki monkey	M	San Diego Zoo/CRES
PP02	*Pithecia pithecia*	White-faced saki monkey	F	San Diego Zoo/CRES
SS01	*Saimiri sciureus*	common squirrel monkey	M	San Diego Zoo/CRES
SS02	*Saimiri sciureus*	Common squirrel monkey	F	San Diego Zoo/CRES
AL001	*Aotus lemurinus*	Lemurine owl monkey	M	San Diego Zoo/CRES
ALG002	*Aotus lemurinus griseimembra*	Gray-handed night monkey	F	San Diego Zoo/CRES
ANA001	*Aotus nancymaae*	Nancy Ma's night monkey	F	San Diego Zoo/CRES
ANI001	*Aotus nigriceps*	Black-headed night monkey	M	San Diego Zoo/CRES
CJ01	*Callithrix jacchus*	Marmoset	M	Genome build: calJac3[a]
CA01	*Cebus apella*	Capuchin	Unknown	Accession number: AY227708.1[b]
MC01	*Macaca mulatta*	Rhesus macaque	F	Genome build: rheMac2[a]

Table I (continued)

ID	Species	Common name	Sex	Locale
NL01	*Nomascus leucogenys*	Gibbon	F	Genome build: NLeu1.0[c]
PA01	*Pongo albeii*	Orangutan	F	Genome build: ponAbe2[a]
PT01	*Pan troglodytes*	Chimpanzee	M	Genome build: panTro2[a]
GG01	*Gorilla gorilla*	Gorilla	F	Genome build: gorGor3[c]
HN001	*Homo neanderthalensis*	Neandertal	Unknown	Accession number: PRLR-001[d]
MM10	*Mus musculus*	Mouse	M + F	Genome build: mm10[a]
HS01	*Homo sapiens*	Human	M + F	Genome build: hg19[a]
ASW	*Homo sapiens*	Human	37 F, 24 M	Americans of African ancestry in SW USA[e]
LWK	*Homo sapiens*	Human	48 F, 47 M	Luhya in Webuye, Kenya[e]
YRI	*Homo sapiens*	Human	45 F, 43 M	Yoruba in Ibadan, Nigera[e]
CLM	*Homo sapiens*	Human	31 F, 29 M	Colombians from Medellin, Colombia[e]
MXL	*Homo sapiens*	Human	34 F, 30 M	Mexican ancestry from Los Angeles[e]
PUR	*Homo sapiens*	Human	27 F, 28 M	Puerto Ricans from Puerto Rico[e]
CHB	*Homo sapiens*	Human	53 F, 44 M	Han Chinese in Bejing, China[e]
CHS	*Homo sapiens*	Human	50 F, 50 M	Southern Han Chinese[e]
JPT	*Homo sapiens*	Human	39 F, 50 M	Japanese in Tokyo, Japan[e]
CEU	*Homo sapiens*	Human	40 F, 45 M	Utah residents of European ancestry[e]
FIN	*Homo sapiens*	Human	58 F, 35 M	Finnish in Finland[e]
GBR	*Homo sapiens*	Human	48 F, 41 M	British in England and Scotland[e]
IBS	*Homo sapiens*	Human	7 F, 7 M	Iberian population in Spain[e]
TSI	*Homo sapiens*	Human	48 F, 50 M	Toscani in Italia[e]

The core study area is located in Formosa Province, Argentina (lat = 25°, 59.4′ S; long = 58′, 11.0′ W).

Data sources: [a] UCSC Genome Browser (Karolchik *et al*. 2008; Kent *et al*. 2002); [b] Rojas-Garcia *et al*. 2003; [c] Ensembl Genome Browser (Flicek *et al*. 2011); [d] Ensembl Neandertal Genome Browser (Green *et al*. 2010); [e] 1000 Genomes Project Consortium 2010, 2012: ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/

Geneious Pro v5.6 (Drummond *et al*. 2010). In cases where nonspecific amplifications occurred, we excised gel slices containing target amplicons and subjected them to cycle sequencing. All optimized PCR parameters and recipes are listed in Table II.

Population Genetic Analyses

For each individual, we assembled *PRLR* genic sequences by aligning the overlapping forward and reverse fragments using strict (95%) agreement thresholds. We created alignments for a total of 44 *PRLR* sequences representing 17 primate species. We

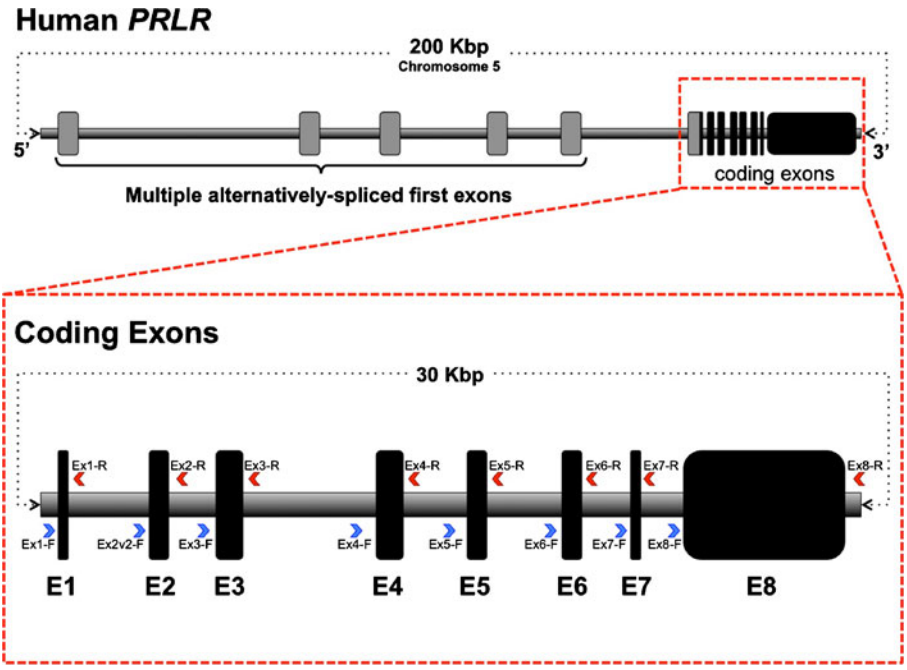


Fig. 1 A schematic diagram of the human *PRLR* gene. The coding region has been boxed in red and enlarged in the diagram of coding exons (labeled E1–E8). The locations of the oligonucleotide primers used in this study are shown as arrows at approximate positions given the diagrammatic scale. [Color version is available online].

generated summary statistics using Arlequin v3.11 (Excoffier and Lischer 2010), Geneious, and R (R Core Team 2012, pegas package: Paradis 2010) for four separate data sets: 1) population of *Aotus azarai*; 2) population of *Homo sapiens* (1000 Genomes data at three tiers: global, continental [EUROPE], subpopulation [CEU]); 3) platyrrhine vs. catarrhine (+ hominoid species); and 4) all primate species. To explore potential demographic and evolutionary pressures that may have influenced the observed changes in the coding region of *PRLR* in our population-based intraspecific comparisons, we calculated Tajima's *D* (Tajima 1989a, b) using R:pegas. For the data on *Homo sapiens*, we calculated minor allele frequencies (MAF) for all 1000 Genomes SNPs found in the 200-kb region encompassing *PRLR* (2980 SNPs), as well as all *PRLR* coding SNPs in both humans and owl monkeys, and recorded all transitions (TI), transversions (TV), deletions, and amino acid substitutions.

Phylogenetic Analyses

Data Alignment To avoid biasing the range of sequence variation toward the species *Aotus azarai*, we restricted the number of sequences of *A. azarai* used to the most frequent haplotype in the focal population. We aligned the sequence of *Aotus azarai* with the other 19 primate *PRLR* sequences, and pruned the resulting matrix of 20 sequences (17 species) to 1866 bases to remove the terminal TGA stop codon, thereby representing the "long" isoform of *PRLR* mRNA. We further divided this mRNA

Table II Primers and PCR conditions for PRLR sequence analysis

Region	Exon length (bp)	Primer	Primer sequence (5′–3′)	Amplicon length (bp)	PCR (time[c])	T_A (°C)	$MgCl_2$ (μl)	DNA (5 ng/μl)
Exon 1	69	PRLR-Ex1-F	AAAGTGGTGGATGTGCCTGAC	226	30cyc (:30, :30, :30)	56.4	2.5	4
		PRLR-Ex1-R	GACAAACACCCCAGGCAATG					
Exon 2[a]	135	PRLR-Ex2-F	CAGCAGAAGGGTCAAATGG	469	35cyc (:30, :30, :30)	55	3.75	4
		PRLR-Ex2-R[b]	GGTTTCATAGGAGAGAAGGGA					
Exon 3	168	PRLR-Ex3-F	GGCCAGTGGTATTGATCTATG	347	35cyc (:30, :45, 1:30)	56.4	2.5	4
		PRLR-Ex3-R	TCCATCCAAAACCCAAGAAG					
Exon 4	171	PRLR-Ex4-F	AAAGGTGCAAGCAATGAGTG	341	30cyc (:30, :30, :30)	56.4	2.5	4
		PRLR-Ex4-R	GTCACAACTGCATTGGAGGC					
Exon 5	141	PRLR-Ex5-F	GTTCAGCCTAACTCCACCGTAG	353	30cyc (:30, :45, 1:30)	56.4	3.75	4
		PRLR-Ex5-R	TCTATTGTTCTGGCTAAGGCTC					
Exon 6[a]	96	PRLR-Ex6-F	GATGGAGGAAAACACTCTTGG	337	TD: 10cyc+35cyc (:30, :45, 1:00)	61.2-51.2	3.5	6
		PRLR-Ex6-R	GGCAAACACACTACATCTAATGG					
Exon 7	75	PRLR-Ex7-F	GGAGCTGCCAAACTCTAAGTC	297	30cyc (:30, :30, :30)	51.2	3.75	4
		PRLR-Ex7-R	GCTGAAACTACCAGGCTGAAC					
Exon 8	1014	PRLR-Ex8-F	GCACTGTGGGTTTAGAAGTTTC	1616	35cyc (:30, :45, 1:30)	59.1	3.5	6
		PRLR-Ex8-R	GTCCCTCAAGAATACTAAGCAG					

Touchdown PCR (TD; Don *et al*. 1991; Korbie and Mattick 2008) was used for this region to apply an initial segment of 10 cycles at T_A°C + 5°C and lowering 1°C in each successive cycle. The second segment is run at T_A°C, and the annealing temperature is kept static.

Components for all PCR reactions (unless otherwise noted): 2.5 μl of buffer II (ABI), 2.5 μl of $MgCl_2$ (ABI), 1.25 μl of 10 mM dNTPs premix (ABI), 2 μl of forward primer, 2 μl of reverse primer, 0.5 μl of AmpliTaq Gold (ABI), 4 μl of DNA (5 ng/μl), and 10.25 μl of ddH_20 to bring the total volume to 25 μl.

[a] Indicates exons that required gel excision because of primer amplification of nonspecific products.

[b] PRLR-Ex2-R was redesigned after weak amplification and sequencing. PRLR-Ex2v2-R has a sequence (5′–3′) of GGAGAATGTGGGCATGGACC.

[c] Indicates timing of different temperature cycles in seconds: 95°C denaturation, T_A°C annealing, 72°C extension.

alignment into domain-specific matrices (ECD: positions 1–702, TMD: positions 703–774, ICD: positions 775–1866). We formatted and annotated all matrices in Geneious for calculations in PAUP* 4.0b10 (Swofford 2002), PAML/codeML (Yang 2007), and MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003).

Network Analyses We generated multistate median joining (MJ) networks for all sequences of *Aotus azarai* using Network v4.6.1.0 (Bandelt *et al*. 1999; Bandelt and Parson 2008). We included only variable exonic *PRLR* characters generated in a variance table using Sequencher and Geneious. To describe the degree of amino acid variation across broader evolutionary distances, we produced similar MJ networks for the full-length amino acid sequences (622 AA) of the *PRLR* coding region from all primate taxa.

Phylogenetic Model Selection To select the most appropriate model for our analyses, we ran the program jModelTest v2.1.1 (Darriba *et al*. 2012; Felsenstein 2005; Guindon and Gascuel 2003) using 203 substitutions patterns to survey 1624 models of nucleotide substitution (+F base frequencies, rate variation of +I and +G with nCat = 4). We implemented the Akaike Information Criterion (AIC) setting, and conducted parallel searches using Bayesian Information Criterion (BIC) and performance-based Decision Theory (DT). The base tree for our likelihood calculations was optimized for Maximum Likelihood (ML) phylogenetic analysis.

Analysis of Adaptive Evolution We conducted ML analysis in PAUP* 4.0b10 to estimate the most likely gene tree based on the alignments of *PRLR* nucleotides and amino acid codons and using the nucleotide substitution model(s) specified by jModelTest. We initiated separate ML runs for the full-length *PRLR* mRNA (minus TGA stop codon, 1866 bp), ECD (702 bp), and ICD (1092 bp). Next, to investigate signatures of selection along phylogenetic branches, we analyzed the ratio of nonsynonymous (d_N) to synonymous (d_S) amino acid changes for *PRLR* mRNA and domain-specific regions independently using the topology of the ML gene tree. We then used the codeML program within PAML to calculate the relative rates of change along different phylogenetic branches. We subsequently applied a likelihood ratio test (LRT) to estimate the accuracy ranges of two phylogenetic models, M0 (variable branch lengths/substitution rates) and M1 (homogeneous branch lengths/substitution rate).

Bayesian Coalescent Estimation To map the *PRLR* mutation rate onto the primate phylogeny, we imported 21 sequences into the annotation program, BEAUti v1.5.4 for analysis in the program BEAST v1.5.4 (Drummond *et al*. 2006; Drummond and Rambaut 2007). We set temporal priors based on log-normally distributed radiometric fossil dates to approximate coalescent ages of primate taxa (Online Resource 1, 2). We used the TN93 substitution model with a gamma site heterogeneity model (the TPM3 model selected by jModelTest was unavailable in BEAST) with three partitions for codon positions and equal base frequencies, a relaxed lognormal clock model, and a randomly generated starting tree and chose the Yule Process speciation parameter as the tree prior. The Markov Chain Monte Carlo (MCMC) search was run with four chains for 4,000,000 generations, with trees sampled every 200 generations.

We assessed the level of convergence (<0.05) using the mean standard deviation in split frequencies among the four chains before accepting the post-convergence tree likelihoods of our runs. For each run, we discarded the first 2000 trees as "burn-in" to remove extraneous pre-convergence probability values (Altekar *et al*. 2004). We further analyzed the results generated by BEAST in TRACER v.1.5 (Rambaut and Drummond 2007) to evaluate the accuracy of the estimations based on the effective sample sizes (ESS) of the data. Finally, we summarized the output files from the ML and BI phylogenetic calculations using TreeAnnotator v1.5.3 (Drummond and Rambaut 2007) to construct a consensus tree (mean node heights and maximum clade credibility values) for each analysis. We imported summary consensus trees into FigTree v1.3.1 (Drummond and Rambaut 2007: [http://beast.bio.ed.ac.uk/FigTree]) for tree visualization.

Genomic Comparisons

To investigate the genomic structure of *PRLR*, we compared the exonic sequences of *Aotus* to those in the marmoset (*Callithrix jacchus*, calJac3), human (hg19), chimpanzee (*Pan troglodytes*, panTro3), orangutan (*Pongo albeii*, ponAbe2), and rhesus macaque (*Macaca mulatta*, rheMac2) genomes using the UCSC Genome Browser (Karolchik *et al*. 2008; Kent *et al*. 2002). We also measured the conservation present among taxa by evaluating >110,000 nucleotides encompassing the *PRLR* locus within the five curated primate genomes for the occurrence of single nucleotide polymorphisms (SNPs), repetitive elements, polyadenylation (poly(A)) sites, and chromosomal rearrangements. To detect the occurrence of copy number variants (CNVs) or segmental duplications near *PRLR*, we further interrogated the Database of Genomic Variants (DGV) (Iafrate *et al*. 2004) for evidence of structural variation. We performed all genomic alignments and statistics using Geneious.

Results

Population Genetic Analyses

Our analysis of 25 *PRLR* sequences of *Aotus azarai* revealed 10 polymorphic sites that were variable within the population (Table I; Online Resource 3), with the bulk of variants being singletons and doubletons. The majority (9 of 10) of owl monkey *PRLR* variants were located within the ICD. The nucleotide diversity estimate (π) for the entire region was low (0.0002), with 99.9% pairwise identity between sequences. The negative values for the selective neutrality index Tajima's *D* (−2.6999, $P < 0.0069$) indicated an excess of low-frequency variants at this locus (Table III).

The *PRLR* locus in 1088 individuals of *Homo sapiens* presented 16 polymorphic sites that were largely singletons, with the majority of these variants also occurring in the ICD (11 of 16). Nucleotide diversity, π, was extremely low (<0.0001), and sequences of *Homo sapiens* sequences had a mean pairwise identity of 99.99%. The Tajima's *D* for human *PRLR* sequences was also negative (−0.8091, $P = 0.4185$). These calculations

Table III Statistics for *PRLR* coding region sequences in *Aotus azarai*, *Homo sapiens*, and two primate infraorders

	Aotus azarai	*Homo sapiens*	Platyrrhines	Catarrhines
Summary statistics:				
No. of individuals (*N*)	25	1088	13	7
Nucleotides (bp)	1866	1869	1869	1869
Polymorphic sites	10	16	212	137
Transitions (TI)	6	10	137	91
Transversions (TV)	4	6	60	37
Insertion/deletions (indels)	0	0	15[a]	9[b]
Domain variants:				
ECD polymorphic sites	1	4	65	60
TMD polymorphic sites	0	1	7	1
ICD polymorphic sites	9	11	140	76
Nucleotide diversity:				
Nei's gene diversity (π)	0.0002	< 0.0001	0.0311	0.0223
Sequence diversity:				
Pairwise identity (%)	99.9	99.99	96.6	97.6
Selective neutrality:				
Tajima's *D* (*p*)	−2.6999 ($P < 0.0069$)	−0.8091 ($P = 0.4185$)	—	—

[a] Codon deletions in one *Aotus* and four *Saimiri.*

[b] Codon deletions in three *Macaca.*

ignore sites with ambiguous calls (e.g., an individual heterozygous "C/T" at a position receives an IUPAC call "Y"), meaning that our results probably underestimate the total amount of variation present.

The *PRLR* protein coding sequence for each genus within the platyrrhines or catarrhines exhibited a substantial number of lineage-specific nucleotide changes that distinguished it from other primate genera. The TI:TV ratio was largely equivalent between platyrrhines (2.283:1) and catarrhines (2.459:1), suggesting a similar mutation rate for *PRLR* in both infraorders. Consistent with both intraspecific analyses, *PRLR* nucleotide variants among platyrrhines and catarrhines were also predominantly located in the ICD (platyrrhines: 140 of 212 polymorphic sites, catarrhines: 76 of 137 sites).

Phylogenetic Analyses

Network Analyses The network of *PRLR* coding sequences of *Aotus azarai* revealed a single distinct clade that consists of a central haplotype occurring at high frequencies in the population, and eight unique derivative haplotypes extending from it (Fig. 2a). Derivative haplotypes were separated from the central haplotype by one to three mutations. In noting a reticulation at nucleotide site 1689 (perhaps due to the presence of only A/G heterozygotes at that site), we down-weighted this character position in the owl monkey *PRLR* network for clarity of presentation.

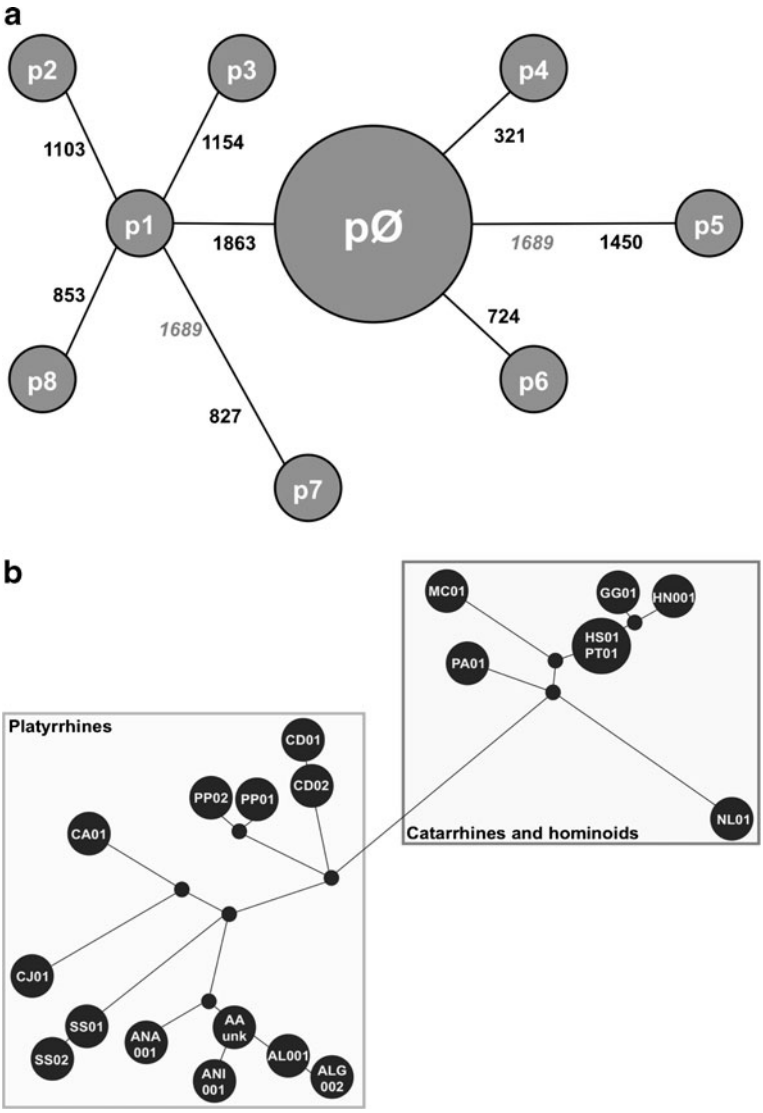


Fig. 2 Median-joining networks of *PRLR* coding region sequences from 25 individuals of *Aotus azarai* (**a**) and another 17 primate taxa (**b**). Node size is relative to the number of individuals that share a particular sequence. Reticulating nucleotide positions are italicized and shown in gray. The branches leading to the different clades are proportional to their actual mutational distances, but have been shortened for the full representation of *PRLR* diversity in this network. Refer to Table I for the key to node labels.

In the interspecific network of 21 translated *PRLR* amino acid sequences, 42 amino acid changes separated the platyrrhines from the catarrhines and hominoids, which exhibited considerably less mutational substructure than the platyrrhines (Fig. 2b). *Callicebus* and *Pithecia* were differentiated from other platyrrhines by the absence of seven shared, derived mutations accumulated in the lineage leading to *Aotus*, *Saimiri*, *Callithrix*, and *Cebus*. Among the catarrhines and hominoids, *Pan troglodytes* and *Homo sapiens* coalesced to the same node, indicating a complete

conservation of the *PRLR* amino acid sequence in these two species. In addition, *Gorilla gorilla* and *Homo neanderthalensis* appeared to share a single amino acid substitution, but were otherwise separated by five Neanderthal-specific and three gorilla-specific changes.

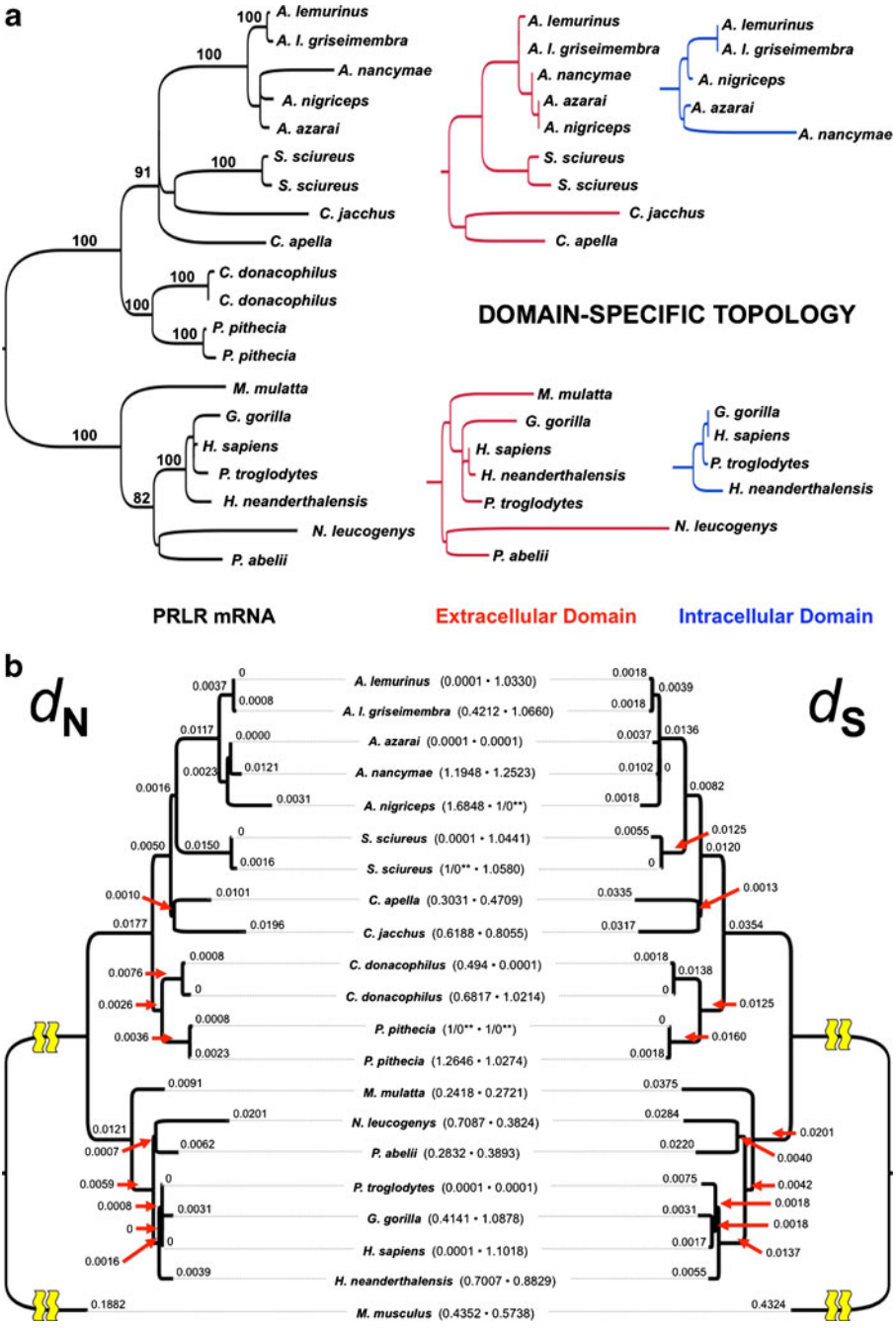
In a direct pairwise amino acid sequence comparison between *Homo neanderthalensis* and *Homo sapiens* we detected five nonsynonymous substitutions, all of which occurred in the ICD region of *PRLR*. BLAT comparisons indicated that two of the five changes resulted in dissimilar amino acids on the basis of hydrophobicity and polarity (BLOSUM 62 matrix, Henikoff and Henikoff 1992). If errors due to deamination are considered, none of the nonsynonymous changes could be attributed to C→T transitions, although two G→A transitions were responsible for amino acid changes (Briggs *et al*. 2007). However, it should be noted that sequence errors likely exist in the *PRLR* sequence of *Homo neanderthalensis* due to the inherent difficulties in ancient DNA sequencing.

Major mutational differences in primate *PRLR* sequences also existed in the form of entire codon deletions within the ICD of the gene. Among the platyrrhines, the *Aotus* and *Saimiri* sequences possessed one (1585–1587 bp; 529 AA) and four codon deletions (1462–1473 bp; 488–502 AA), respectively. All five taxa *Aotus* and both individuals of *Saimiri* that we sequenced exhibited these lineage-specific deletions. In addition, the sequence for *Macaca* had three successive codon deletions (1258–1266 bp; 420–422 AA). Such changes likely truncate the length of the ICD in these taxa, as well as the overall length of the mature *PRLR* mRNA.

Phylogenetic Model Selection Each of the three types of searches (AIC, BT, DT) that we ran in jModelTest selected the TPM3 model (Kimura 1981) for use in our *PRLR* phylogenetic reconstructions. Under the AIC searches, the TPM3uf+G model was selected as the most appropriate for our data set, and possessed a likelihood score ($-ln$ L) of 5350.57. For the BIC and DT searches, the TPM3+G model (Kimura 1981) was selected with identical likelihood scores of –lnL = 5357.40. We applied both variations of the model in all phylogenetic calculations and observed negligible differences in the results.

Analysis of Adaptive Evolution The likelihood ratio test indicated that the M1 (variable) model was only slightly more likely (ln L = −6433.4307) than M0 (homogenous) model (ln L = −6458.7099), with this result being nonsignificant ($P > 0.1224$). Therefore, we present individual branch values to specifically highlight lineage diversity in our analyses of adaptive evolution of *PRLR* (Fig. 3a, b). The resulting d_N/d_S ratio values for the majority of the branches further emphasized that a large degree of diversity at *PRLR* exists on the codon/amino acid level across different primate taxa.

The ML tree topology also reflected variable domain evolution, as the ECD topology and ICD topology were both distinct from the full-length mRNA ML tree topology (Fig. 3a). We observed variable, d_N/d_S ratios for different primate taxa when analyzing full-length *PRLR* mRNA (Fig. 3b). Notably, 11 of 13 *Aotus*-specific nonsynonymous substitutions occurred within the ICD (Fig. 4a; Table III). This disproportionate rate of nonsynonymous substitutions in the ICD was characteristic of all primate taxa (Fig. 4b), and is also reflected in the slightly higher d_N/d_S ratios we observed when assessing adaptive evolution in only the ICD region (Fig. 3b).



Bayesian Coalescent Estimation The topology of the phylogenetic chronogram exhibited taxonomic arrangements similar to those seen in both the ML and median network analyses (Fig. 5). In addition, age estimations and their associated error

◀ **Fig. 3** (**a**) A comparison of ML phylograms depicting the taxonomic relationships and relative distances among 20 primate taxa. Discrepancies between the ML phylogenetic arrangements are shown for the *PRLR* coding region, with full mRNA on the left, and the alternative ECD and ICD arrangements shown on the right. Numbers below the branches represent bootstrap values based on 10,000 replicates. (**b**) Oppositional phylogram depicting the different forms of amino acid substitutions at *PRLR* in 20 primates and the outgroup of *Mus musculus*. Branch lengths are based on *PRLR* coding sequence variation and are exhibited by separate d_N and d_S trees. The d_N/d_S ratios for each terminal taxonomic branch are listed next to the taxonomic names in the center of the figure in parentheses: (mRNA d_N/d_S ratio • ICD d_N/d_S ratio). The outgroup branch lengths of both oppositional trees have each been truncated for clarity. [Color versions are available online].

ranges generated at each phylogenetic node were consistent with those of other recent studies of molecular data, although divergence estimates for the catarrhines and hominoids were slightly later than estimates from other studies (Hodgson *et al*. 2009; Perelman *et al*. 2011). High mutation rates were evident for branches leading to the entire primate clade (from mouse), the entire platyrrhine clade, the *Aotus–Saimiri* clade, *Pongo pygmaeus abelii*, and *Gorilla gorilla*.

Genomic Comparisons

The upstream and downstream regions flanking the *PRLR* locus in the five curated primate genomes exhibited relatively high levels of nucleotide conservation, particularly when compared to the *PRLR* locus itself, which had a considerable amount of intronic sequence diversity due to large indels (Fig. 6). We observed 65,720 identical sites (58.8% pairwise identity [pw. id.]) across the entire 110,138 bp *PRLR* five-species alignment. The 5′ upstream region (50,032 bp) possessed 28,154 identical sites (56.3% pw. id.), whereas the 3′ downstream region (32,702 bp) exhibited 23,412 identical sites (71.6% pw. id.). Within the 27,404 bp region encompassing the eight exons of *PRLR*, the five primate genomes had only 13,154 sites in common (48% pw. id.). The bulk of this variation is attributable to more than 50 large (>50 bp) insertion and deletions. For example, *Callithrix* possessed three different complex deletions (~ 4 kb, 1.1 kb, and 2.4 kb) between *PRLR* exons 3 and 4. Within this same intronic region, the hominoids (*Homo*, *Pan* and *Pongo*) exhibited a common insertion that is >1.2 kb in length. The longest genomic indel (6894 bp) belonged to *Callithrix*, and was found in the 5′ upstream region of *PRLR*. In fact, of the 37 insertions and 33 deletions in the five primate genomes, 12 insertions and 19 deletions belonged to *Callithrix*. Many of these events coincided with the absence of poly(A) sites in *Callithrix* that are present in other primate species. Each of the four catarrhine primate species possessed 11 poly(A) sites within the 110 kb *PRLR* locus window, whereas *Callithrix* possesses only seven.

Although few intraspecific CNV data sets exist for the nonhuman primate genomes, four structural variants in human populations were located proximate to *PRLR*. They included one CNV (DGV #44206, Bentley *et al*. 2008) and three large indels (DGV #42114, Wang *et al*. 2008; #40748, Wheeler *et al*. 2008; and #12674, Mills *et al*. 2006) located >75 kb upstream (5′) from the first exon of *PRLR*, with none being functionally associated with the locus or its transcription. No segmental duplications were reported for the 110-kb window surrounding *PRLR* in any primate genome, and cross-primate alignment nets displayed no breakpoints (fission/fusion events) of chromosomes in any of the primates throughout their cytogenetic evolution.

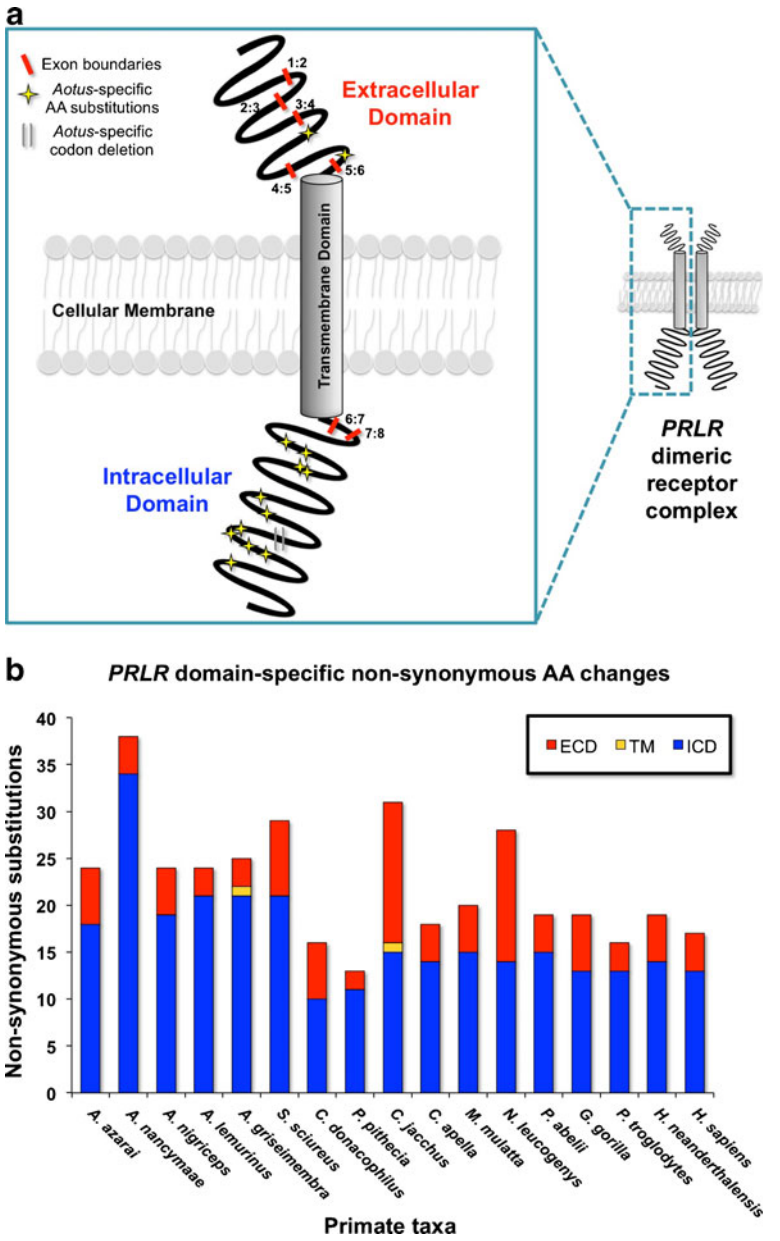


Fig. 4 (**a**) A two-dimensional projection of nonsynonymous amino acid changes specific to the *PRLR* coding sequence of the genus *Aotus*. Only one unit of the dimeric receptor complex is depicted, and salient features are designated as follows: exon boundaries (dark gray bars), *Aotus* specific amino acid changes (white stars), and the location of the codon deletion (two light gray parallel bars). (**b**) A stacked bar graph displaying the number of nonsynonymous amino acid changes of the coding sequence for *PRLR* in 17 different species, relative to a translated consensus DNA sequence (assessed by plurality [50% majority]). Substitutions that distinguish all catarrhines and hominoids from all platyrrhines have been excluded due to a sample bias toward platyrrhines, which results in an overrepresentation of catarrhine-specific changes. Amino acid changes have been partitioned by domain: extracellular (ECD, gray [red]), transmembrane (TMD, white [yellow]), intracellular (ICD, black [blue]). [Color versions are available online].

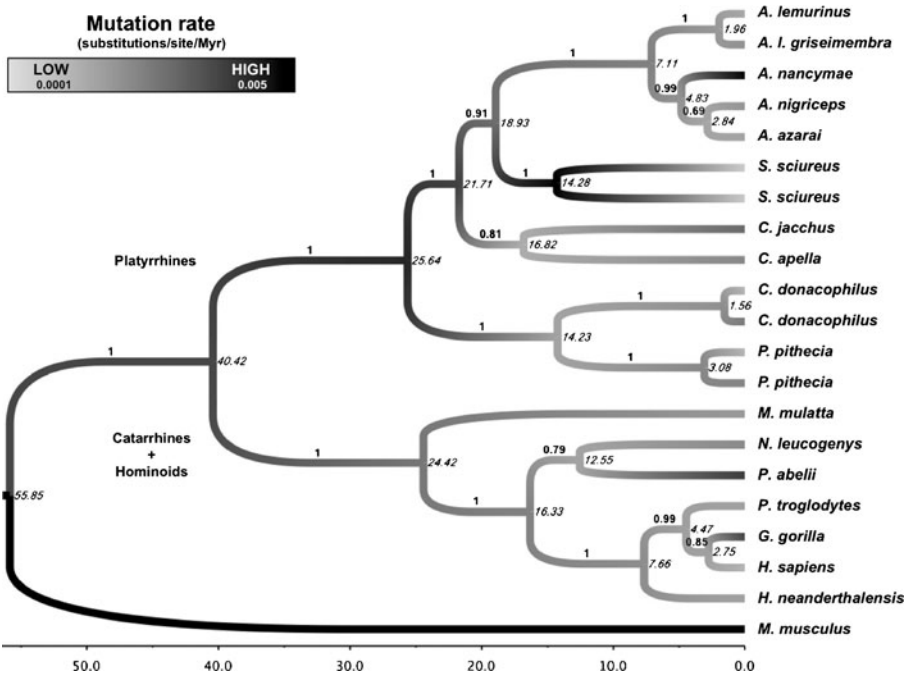


Fig. 5 A chronogram depicting the Bayesian coalescence age estimation of *PRLR* coding region sequences, as calculated in BEAST. Age estimates for each node are italicized and positioned to the right of them. Posterior probabilities (>50%) are indicated above each branch. The mutation rate (substitutions per site per million years) of *PRLR* along the different evolutionary lineages is displayed as a black-gray gradient across taxa. Platyrrhine primates are represented in the top portion of the chronogram, while catarrhine primates and hominoids are found in the lower portion of the chronogram. The mouse sequence (mm10) is located at the very bottom of the tree. The horizontal scale below the tree is in millions of years [Mya].

Discussion

Our results reveal considerable *PRLR* coding region variation within the population of *Aotus azarai* and the genus *Aotus*, and extensive nucleotide, codon, and amino acid variation among different primate taxa. Although it is tempting to suggest that variation at the *PRLR* locus is being maintained by the positive selection of different mRNA transcripts in different primate lineages, it is well known that demographic effects, such as population expansions and founder events, can also produce similar patterns of genetic variation (Tajima 1989a, b). Further, we should note that, due of the pleiotropic functionality of the PRL pathway, the observed variation at the *PRLR* locus could also derive from selection on a number of different phenotypes that are associated with this system, and that paternal care may not be the phenotype under selection. In fact, the observed differences in *PRLR* among primate taxa cannot be explained by species-level differences in paternal care.

PRLR Sequence Diversity in Populations of *Aotus azarai* and *Homo sapiens*

The 25 individuals of *Aotus azarai* exhibit considerable coding region diversity for the *PRLR* locus, which is unusual for a pleiotropic gene (He and Zhang 2006). Yet,

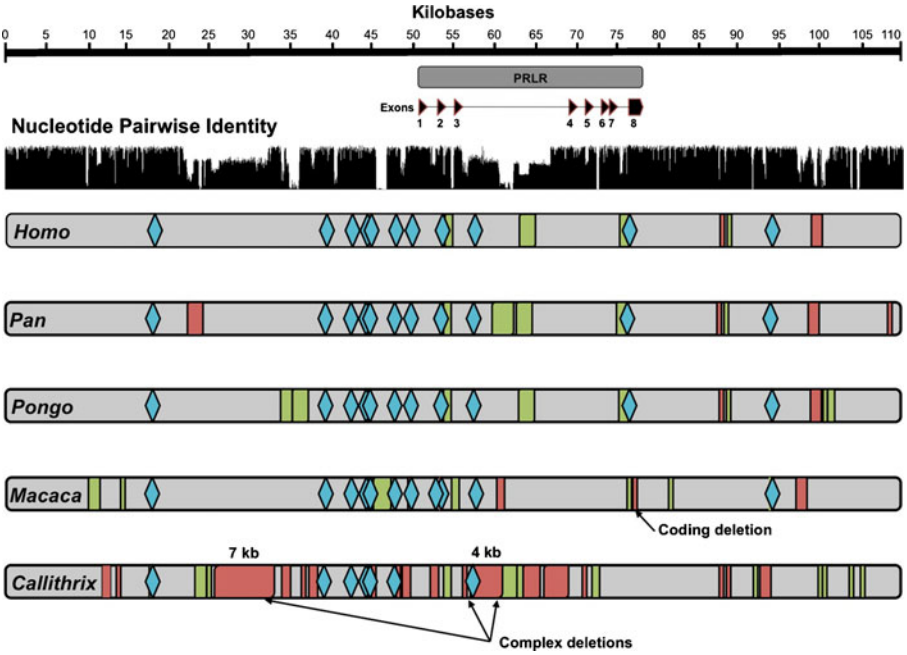


Fig. 6 Primate genomic variation flanking the *PRLR* locus. Nucleotide sequence conservation is plotted as a continuous black histogram at the top of the figure, calculated from pairwise identity of nucleotide positions in the 5-way multiple sequence alignment. The 5′ upstream, exonic, and 3′ downstream regions spanning 110 kb from five curated primate genomes (UCSC) are shown with deletions (white [red] bars), insertions (black [green] bars), and poly-adenylation sites (dark gray [blue] diamonds). [Color version is available online].

the majority of observed variants were singletons, suggesting that either the population is expanding or that the locus is experiencing positive selection (Tajima 1989a). The former explanation seems more likely, as the pattern of mtDNA variation in *Aotus azarai* also implies that the focal population has recently begun a demographic expansion (three major mitochondrial haplogroups) within the gallery forests of the region (Babb *et al*. 2011). However, the fact that many of these changes result in nonsynonymous amino acid substitutions and predominantly occur in the ICD intimates that *PRLR* is experiencing some deviation from neutral evolution (Table III; Online Resource 3).

The situation is similar for humans. Many low-frequency coding variants are observed at global, continental and local population scales (Online Resource 3). The demographic explanation is probably applicable here, as patterns of rare genomic variants indicate that human populations have been experiencing explosive growth for the last 10,000 years (Keinan and Clark 2012). However, like *Aotus azarai*, a surprising number of the human *PRLR* variants generate nonsynonymous substitutions (Table IV). The majority falls within the ICD, again underscoring some level of domain-specific departure from neutrality, although this observation could also be explained by lineage-specific relaxed selection. Of the 64 amino acids that directly distinguish owl monkey from human orthologs of PRLR, 41 occur in the ICD (Online Resource 4). Interestingly, nucleotide positions in *PRLR* that are variable in one species appear to be fixed in the

other, whereas a screening of MAF along 200kb of human chromosome 5 suggests that complex intraspecific variation exists throughout the entirety of *PRLR*'s genomic footprint (Online Resource 5).

PRLR Phylogenetic Variation

Members of the genus *Aotus*, the platyrrhines, and the catarrhines all exhibit numerous nonsynonymous changes in the ICD of *PRLR*. In addition, three primate taxa possess genus-specific codon deletions in the ICD, which presumably yield an abbreviated mature protein that is functionally altered. Among the hominoids, *Pan troglodytes* and *Homo sapiens* have identical amino acid sequences, suggesting that purifying selection may have produced a strictly conserved amino acid sequence in these two lineages over the past 6–7 million years (Chen and Li 2001; The Chimpanzee Sequencing and Analysis Consortium 2005). By contrast, there is a surprising lack of *PRLR* sequence conservation between *Homo sapiens* and *Homo neanderthalensis* despite their close evolutionary relationship (Green *et al*. 2006, 2010; Noonan *et al*. 2006; Noonan 2010; Serre *et al*. 2004), even when some mutational differences could be attributable to DNA sequencing errors.

The contrasting pattern of sequence diversity in the ECD and the ICD may be explained by their different functions. Given the promiscuity of the prolactin receptor (ligands include prolactin, growth hormones, and lactogens), it is possible that small coding changes in the ECD could adversely affect binding affinities with one or multiple ligands (Rozakis-Adcock and Kelly 1991, 1992; Somers *et al*. 1994). By contrast, the ICD is responsible for cellular signaling responses after a ligand–receptor interaction has been established. Site-directed mutational studies of *PRLR* ICD have shown differential recruitment properties and interactions with tyrosine kinase JAK-2 (binds to *PRLR* amino acid residues 267–274, 312–324) and STAT5 (binds to residues 404–448) proteins involved in transcriptional activities (Goffin *et al*. 2002; Lebrun *et al*. 1995a, b; Pezet *et al*. 1997a, b). Although intraspecific non-synonymous variants do not exist in any of these binding sites, seemingly fixed species-level differences are present, including the deletion of three entire codons in *Macaca* (residues 421–423). An accelerated rate of evolution in the ICD could therefore result in structural or tissue-specific functional differences that affect downstream cellular events. Therefore, it is possible that these particular nonsynonymous changes could affect the functional activity of PRL receptor neurons, and thus somehow influence the manifestation and frequency of paternal care behaviors.

Despite observing a host of nucleotide and amino acid changes in *PRLR*, we found that the genomic regions surrounding the *PRLR* locus in five of those taxa (*Homo*, *Pan*, *Pongo*, *Macaca*, and *Callithrix*) were surprisingly well conserved. In fact, the area bounded by the first and eighth exon of the *PRLR* gene exhibited a greater amount of sequence variation than either its 5′ or 3′ flanking regions. Nevertheless, the content of some of the large interspecific intronic insertions and deletions may have functional ramifications for the splicing and transcription of the *PRLR* locus. For example, the deletions of at least four poly(A) sites in the *Callithrix* genome point toward a putatively differential capacity for *PRLR* mRNA splice variants in platyrrhines compared to catarrhines and hominoids. Considering that multiple splice-variants of the PRLR protein are known, there may be a wide range of functional

Table IV Interspecific comparison of intraspecific variation of the *PRLR* coding region

Domain	Exon	Codon	Human variants	MAF $N = 1088$	Conserved amino acid?	Owl monkey variants	MAF $N = 25$
ECD	2	26	rs184625944, (ref_T>alt_C) rc: A>G, synonymous	0.0005	**No**		—
		65	rs182263490, (ref_T>alt_C) rc: A>G, His>Arg, nonsynonymous	0.0005	Yes		—
	3	100	rs78705921, (ref_T>alt_C) rc: A>G, Ile>Val, nonsynonymous	0.0508	**No**		—
		107		—	Yes	C>G, Asn>Lys, nonsynonymous	0.09
	4	170	rs72478580, (ref_T>alt_G) rc: A>C, Ile>Leu, nonsynonymous	0.0183	Yes		—
TMD	6	236	rs187422736, (ref_G>alt_A) rc: C>T, synonymous	0.0005	Yes		—
		242		—	Yes	G>C, Ala>Pro, nonsynonymous	0.04
ICD	7	276		—	Yes	T>A, Ile>Lys, nonsynonymous	0.04
		285		—	Yes	G>A, Glu>Lys, nonsynonymous	0.04
	8	298	rs140947849, (ref_G>alt_A) rc: C>T, synonymous	0.0005	Yes		—
		344	rs146442340, (ref_C>alt_A) rc: G>T, Asp>Tyr, nonsynonymous	0.0005	Yes		—
		357	rs184252713, (ref_G>alt_C) rc: C>G, Pro>Arg, nonsynonymous	0.0009	Yes		—
		368		—	**No**	G>A, Arg>Gln, nonsynonymous	0.06
		385		—	Yes	A>T, Asn>Ile, nonsynonymous	0.04
		390		0.0005	Yes		—

Table IV (continued)

Domain	Exon	Codon	Human variants	MAF $N = 1088$	Conserved amino acid?	Owl monkey variants	MAF $N = 25$
			rs192767214, (ref_T>alt_C) rc: A>G, His>Arg, nonsynonymous				
		409	rs189248053, (ref_C>alt_G) rc: G>C, Gly>Ala, nonsynonymous	0.0009	Yes		—
		470	rs62355478, (ref_C>alt_T) rc: G>A, synonymous	0.0027	Yes		—
		484		—	Yes	T>C, Ser>Pro, nonsynonymous	0.04
		538	rs148096787, (ref_C>alt_T) rc: G>A, Gly>Arg, nonsynonymous	0.0005	**No**		—
		541	rs192521410, (ref_C>alt_G) rc: G>C, Glu>Asp, nonsynonymous	0.0005	Yes		—
		564		—	Yes	G>A, synonymous	0.26
		568	rs76500088, (ref_G>alt_A) rc: C>T, synonymous	0.0037	Yes		—
		578	rs146459777, (ref_C>alt_G) rc:G>C, Glu>Gln, nonsynonymous	0.0009	Yes		—
		584		—	Yes	A>G, synonymous	0.08
		611	rs142058010 (ref_G>alt_A) rc:C>T, synonymous	0.0014	Yes		—
		622		—	Yes	T>C, synonymous	0.50

isoforms within primate taxa, underscoring the pleiotropic biological functions of the PRL pathway in these organisms.

The lack of intraspecific CNVs, segmental duplications and interspecific chromosomal rearrangements in the region implies that structural variation may not be altering *PRLR* gene dosage in different primate species. However, as no formal measurement of structural variation exists for different species of primates, we cannot argue conclusively that this is the case. Similarly, as our current study does not highlight the regulatory elements involved in *PRLR* transcription, we are not in a position to make claims about transcriptional variation in primates. Yet, our data do suggest that variation has differentially accumulated in the different domains of the *PRLR* locus at the level of the population, the species, the genus, and the infraorder, and that this variation could be due to some combination of demographic and selective events.

Role of *PRLR* in the Evolution of Paternal Care in Primates

The emergence and maintenance of paternal care is undoubtedly driven by a complex combination of genetic and ecological factors. In pair-bonded species, such as *Aotus azarai*, high-quality paternal care may be associated with future mating opportunities and/or increased survival of biologically related offspring (Fernandez-Duque *et al*. 2008; Gubernick and Teferi 2000; Smuts and Gubernick 1992). The hypothetical reproductive fitness gained by paternal care could potentially be enough to drive the selection of associated genetic variants. However, the pleiotropic nature of the prolactin–PRLR pathway makes it difficult to delineate which phenotypes are the targets of selective pressures that result in the maintenance of diversity at a genetic locus. Further, the differences we observed in *PRLR* among primate taxa do not discretely cluster with species-level differences in paternal care behaviors.

We have characterized sequence variation at the *PRLR* locus in *Aotus azarai* and many other primate taxa, and found that the ICD exhibits extensive diversity compared to the rest of the gene. This observation, in turn, suggests that variation in PRLR intercellular communication may be a modulating factor in the evolution of the PRL pathway in primates, and provide genetic propensities that, in combination with other neurogenetic and ecological factors, could contribute to the emergence of paternal care behaviors in certain primate taxa.

Currently, little is known about the complexities of *PRLR* expression, regulation, and receptor distribution, particularly in the brain. Alternative splicing, heterodimeric receptor complexes, and receptor promiscuity may produce a range of distinct physiological and behavioral phenotypes (Goffin *et al*. 2002; Hu *et al*. 2002; Perrot-Applanat *et al*. 1997). Exploring the structure, sequence diversity and signatures of selection within the regulatory region will undoubtedly inform our understanding of the way that *PRLR* functions in different primate taxa. In addition, a broader focus on the PRL neuroendocrine pathway and expression patterns of both PRL and *PRLR*, as well as their relationships with other signaling pathways and molecular systems, should aid the pursuit of understanding functional variation and the evolution of complex behaviors in primates.

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