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RESEARCH ARTICLE

A Metagenomic Study of Primate Insect Diet Diversity

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Descriptions of primate diets are generally based on either direct observation of foraging behavior, morphological classification of food remains from feces, or analysis of the stomach contents of deceased individuals. Some diet items (e.g. insect prey), however, are difficult to identify visually, and observation conditions often do not permit adequate quantitative sampling of feeding behavior. Moreover, the taxonomically informative morphology of some food species (e.g. swallowed seeds, insect exoskeletons) may be destroyed by the digestive process. Because of these limitations, we used a metagenomic approach to conduct a preliminary, "proof of concept" study of interspecific variation in the insect component of the diets of six sympatric New World monkeys known, based on observational field studies, to differ markedly in their feeding ecology. We used generalized arthropod polymerase chain reaction (PCR) primers and cloning to sequence mitochondrial DNA (mtDNA) sequences of the arthropod cytochrome b (*CYT B*) gene from fecal samples of wild woolly, titi, saki, capuchin, squirrel, and spider monkeys collected from a single sampling site in western Amazonia where these genera occur sympatrically. We then assigned preliminary taxonomic identifications to the sequences by basic local alignment search tool (BLAST) comparison to arthropod *CYT B* sequences present in GenBank. This study is the first to use molecular techniques to identify insect prey in primate diets. The results suggest that a metagenomic approach may prove valuable in augmenting and corroborating observational data and increasing the resolution of primate diet studies, although the lack of comparative reference sequences for many South American insects limits the approach at present. As such reference data become available for more animal and plant taxa, this approach also holds promise for studying additional components of primate diets. Am. J. Primatol. 74:622–631, 2012.

Key words: metagenomics; neotropical primates; dietary ecology

INTRODUCTION

The study of primate diet and foraging ecology is fundamental to understanding primates' impact on and function in their ecological communities. Although most modern primates eat primarily fruit and other plant parts, arthropods are also an important component in the diets of many species [Harding, 1981; Lambert, 2011], and the proportion of arthropod prey included in the diet can vary widely both between species and seasonally [Richard, 1985]. Currently, most descriptions of the diet of wild primates are based on either direct observation of foraging behavior, morphological classification of food remains passed in feces [e.g. Moreno-Black, 1978], and/or analysis of the stomach contents of deceased animals [e.g. Milton & Nessimian, 1984].

However, these traditional methods are limiting for several reasons when trying to identify arthropod prey. First, the forest canopy often obscures direct observation of primates during field studies, making it difficult to identify specific food items being consumed [Moreno-Black, 1978]. Observational studies are particularly impractical for studying the consumption of arthropods, given that observers are often unable to discern whether or not a prey item was caught and eaten. Second, although broad

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generalizations about diet can be made from the characterization of prey fragments found in feces, taxonomically informative morphology (e.g. insect exoskeletons) may be destroyed by the digestive process [Moreno-Black, 1978]. This is particularly problematic for soft-bodied prey items, which will be underrepresented, if present at all, in fecal material [Deagle et al., 2005]. Finally, stomach analyses provide information only about what the animal ate just before death and cannot be used for long-term diet sampling from the same individuals [Moreno-Black, 1978]. Given these impediments, a metagenomic approach to diet analysis, using DNA from fecal samples to assess prey remains, is a promising alternative [Deagle et al., 2005; Hofreiter et al., 2010; Symondson, 2002].

A typical metagenomic study employs the polymerase chain reaction (PCR) to selectively amplify DNA sequences unique to a particular taxon or set of taxa from a mixture of complex genetic material [Jarman et al., 2004]. This is ideal for the selective amplification of prey DNA present in fecal samples, which typically contain DNA from intestinal microbes and ingested prey and plants, as well as the animal itself. Once sequences have been selectively amplified, they may then be identified taxonomically based on a "DNA barcode" [Hebert et al., 2003], a standardized region of DNA used for species identification [Valentini et al., 2009].

Typical DNA barcoding studies often use mitochondrial DNA (mtDNA) loci for selective amplification and subsequent identification. The use of mitochondrial rather than nuclear loci improves the probability of amplifying prey DNA from a fecal sample since the number of copies of mtDNA per cell typically exceeds that of nuclear DNA by several orders of magnitude. Additionally, published sets of PCR primers are already available for many mtDNA genes [King et al., 2008]. For animals, the standard region of mtDNA proposed for DNA barcoding is an approximately 648-bp region of the cytochrome c oxidase I (*COI*) gene [Ratnasingham & Hebert, 2007]. Beyond *COI*, however, a number of other genes have also been used for DNA-based identification of prey, including *cytochrome c oxidase II* (*COII*), *12s* and *16s* rRNA genes [King et al., 2008], and the cytochrome b (*CYT B*) gene [Pons, 2006].

Since the first application of PCR to animal dietary analysis from feces by Höss et al. [1992], similar approaches have been used to characterize the diets of various animal groups, including marine carnivores [Jarman et al., 2004], insectivores [Clare et al., 2009; Zeale et al., 2010], and even extinct ground sloths (using DNA amplified from ancient fecal material) [Poinar et al., 1998]. However, despite the successes of metagenomics and DNA barcoding in dietary analyses, the method has yet to be used extensively by primatologists. Bradley et al. [2007] were the first to publish a DNA-based primate diet analysis in which they amplified plant-specific DNA from the fecal samples of western gorillas (*Gorilla gorilla*) and black and white colobus monkeys (*Colobus guereza*). More recently, Hofreiter et al. [2010] used a molecular approach to study the diets of gorillas and bonobos (*Pan paniscus*), focusing on the consumption of vertebrate prey.

This study is the first to evaluate the feasibility of a metagenomic approach in the comparative study of New World primate diets and the first to focus on arthropod prey. Using the same general method as the studies reviewed above, taxon-specific primers corresponding to the *CYT B* gene of arthropods [Pons, 2006] were employed to preliminarily investigate insect diversity in the diets of six New World monkey genera living sympatrically in a lowland Amazonian rainforest site.

METHODS

Fecal samples were collected during the course of long-term ecological and behavioral fieldwork on New World monkeys at the Tiputini Biodiversity Station in the Yasuní National Park and Biosphere Reserve in eastern Ecuador. Ten species of nonhuman primates occur at the site, and the six that were chosen for this study—squirrel monkeys, *Saimiri sciureus macrodon*; white-fronted capuchins, *Cebus albifrons*; red woolly monkeys, *Lagothrix poeppigii*; equatorial sakis, *Pithecia aequatorialis*; red titis, *Callicebus discolor*; and white-bellied spider monkeys, *Ateles belzebuth*—include two genera from each of the three major groups of platyrrhine primates. From observational studies, these primates are known to differ considerably in the degree to which they focus on insect prey, and we chose these species specifically to sample that range of variation in insectivory. For example, foraging for insect prey comprises close to half of the activity budget of *Saimiri* [Lima & Ferrari, 2003; Terborgh, 1983], and during months when fruit is less available, insect foraging may represent as much as 80% of foraging time [Lima & Ferrari, 2003]. By contrast, *Ateles* devotes minimal time to insect foraging, and insect prey constitutes a negligible portion of the annual diet [Di Fiore et al., 2010].

Fecal samples were collected fresh into RNALater™ nucleic acid preservation buffer (Ambion), with a volumetric ratio of feces-to-buffer of roughly 1:1. Samples were stored at room temperature for up to 6 months before export to the United States and, in the laboratory, were kept at −20°C to 4°C until extraction. For this preliminary study, we selected one sample from three different individuals of each taxon, each from a different social group (i.e. 18 samples total) and separated in time by about 2–16 weeks, in order to encompass some of the possible sources of variation in insectivory that might be

expected across individuals, across space, and across time.

DNA was extracted from each sample using QIAGEN Stool Mini kits, following the manufacturer's protocol for "Isolation of DNA from Stool for Human DNA Analysis" with minor modifications. These included incubating the stool samples at room temperature (~22°C) for 30–60 min after the addition of Buffer ASL and incubating the samples for an extra 20 min at 70°C in Buffer AL. Additionally, for the final elution step, 100 μL of prewarmed AE buffer (70°C) was added to the QIAamp spin column membrane, which was then left to incubate at room temperature for 20 min prior to centrifugation. The manufacture's estimated DNA yield for the protocol is 75–300 ng/μL.

For all samples, we amplified an approximately 400-bp segment of the mitochondrial *CYT B* gene using conserved primers designed for arthropods, CB3: 5′-GAGGAGCAACTGTAATTACTAA-3′ and CB4: 5′-AAAAGAAARTATCATTCAGGTTGAAT-3′ [Pons, 2006]. PCR amplifications were carried out in 26 μL reactions consisting of 3 μL DNA template, 5 μL 5× Flexi reaction buffer (Promega), 2.0 mM $MgCl_2$, 200 μM each dNTP, 0.48 μM each primer, and 1 Unit GoTaq Flexi (Promega). In order to minimize the chance of contaminating amplification reactions with exogenous arthropod DNA, all amplification mixtures were prepared in a HEPA-filtered hood, which was disinfected before and after use with bleach and UV exposure. This area was separate from the lab space designated for fecal DNA extractions. All reactions were prepared using barrier pipet tips and pipets dedicated exclusively to PCR-setup to prevent cross contamination through aerosols.

Thermal cycling conditions followed a touchdown protocol, including (i) initial denaturation at 94°C for 3 min 30 sec; (ii) eight cycles of 94°C for 30 sec, 52°C for 30 sec decreasing 1° per cycle, 72°C for 1 min; (iii) 42 cycles of 94°C for 30 sec, 44°C for 30 sec, and 72°C for 1 min; and (iv) a final extension at 72°C for 10 min. All reactions were carried out in Bio-Rad thermal cyclers (MyCycler or iCycler). PCR amplification products were visualized via gel electrophoresis on 1.8% agarose gels.

Successful amplification products from each sample were then cloned directly into pSC-A-amp/kan cloning vectors (Stratagene), following the manufacturer's instructions. Colonies were grown at 37°C for at least 17 hr and then stored at −4°C. DNA was extracted from bacterial colonies by boiling in 100 μL of TE buffer (10 mM Tris, 0.1 mM EDTA) at 95–100°C for 10 min. Colony PCR with T3/T7 primers was then carried out in a total volume of 25 μL, consisting of 2 μL of boiled colony DNA template, 5 μL 5× Flexi reaction buffer (Promega), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.48 μM each primer, and 1 Unit GoTaq Flexi (Promega). Colony PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. As before, all reactions were carried out in Bio-Rad thermal cyclers (MyCycler or iCycler). Colony PCR amplification products were again visualized via gel electrophoresis on 1.8% agarose gels.

Colony PCR products were subject to an exonuclease/phosphatase (ExoSAP) reaction consisting of 7 μL PCR product, 1.0 μL 10× SAP Reaction Buffer (USB), and 2.0 μL enzyme mix (20 μL SAP [1 unit/μL] and 2.0 μL Exo1 [10 units/μL]) (USB). The ExoSAP reaction involved incubating the samples at 37°C for 1 hr followed by deactivation of the enzymes by heating at 80°C for 15 min.

Both the light and heavy strands of each colony PCR amplicon were then sequenced using BigDye Terminator v3.1 (Applied Biosystems) cycle sequencing chemistry. Cycle sequencing reactions were performed in a total volume of 10 μL consisting of 1.0 μL DNA template, 1.0 μL BigDye Terminator v3.1 reaction mix, 1.5 μL 5× Sequencing Buffer (Applied Biosystems), and 1.2 μM of either T3 or T7 primer. Thermal cycling conditions were as follows: 35 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. DNA fragments were separated and visualized on an ABI Prism 3730 DNA Analysis System (Applied Biosystems).

Sequence reads were imported into the software Sequencher v4.7 and base calls were verified by eye. Sequence similarity was determined via multiple alignment using ClustalW2 [Chenna et al., 2003] and uncorrected pairwise distance matrix generation with PAUP* [Swofford, 2003]. A cloned sequence or cluster of sequences was considered an "operational taxonomic unit" (OTU) if it differed from all others recovered by more than 2% sequence divergence across approximately 400 bp of *CYT B* sequenced. This threshold allowed for some within-taxon variation as well as for sequencing errors introduced during PCR and cloning steps [Jarman et al., 2004].

This working cutoff was determined based on the distribution of pairwise distance comparisons for all insect sequences. A histogram of this data revealed a large and clear natural break in the pairwise distance comparisons at 6% divergence, with only a handful of pairwise divergences in the 2–6% range (Fig. 1). Upon closer examination, however, we found that a small group of recovered sequences inflated this threshold, and when excluding these sequences, the natural break fell at 2% divergence. Given the estimated diversity of insect populations determined in other studies [Hebert et al., 2004; Smith et al., 2005], the 6% diversity threshold for OTUs seemed inappropriately high. For example, a barcoding study of lepidopterans showed that a 3% threshold revealed approximately 98% of lepidopteran species identified through conventional morphological taxonomy [Hebert et al., 2003]. Since the change in

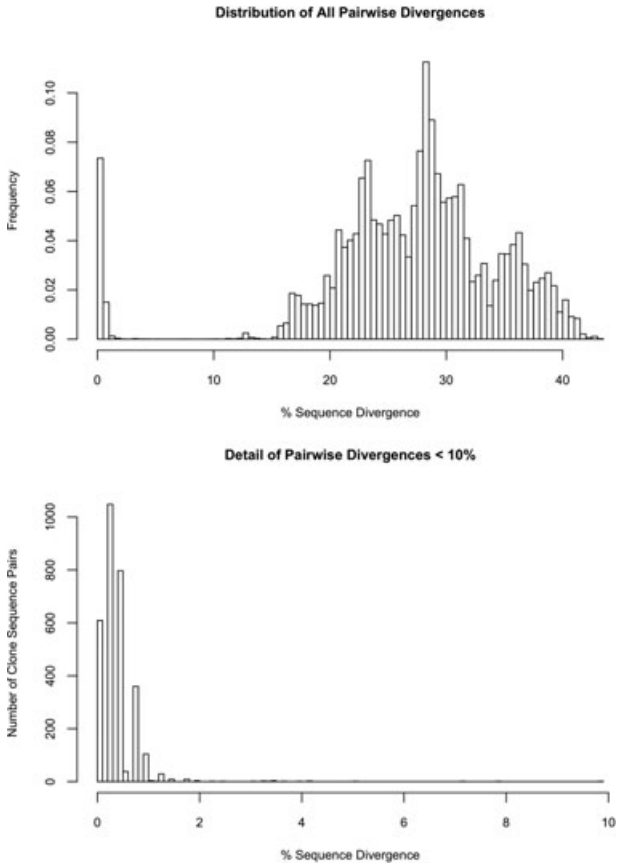

Fig. 1. Distribution of pairwise divergences among all arthropod *CYT B* clone sequences (top panel) and detail of the pairwise divergences in the 0–10% range (bottom panel). Most sequences diverged by more than 2% with only a few between 2% and 6%. Sequences diverging by less than 2% were considered as belonging to the same OTU.

cutoff criteria affected only the OTUs determined for *Saimiri*, our OTU assignments are reported at both 2% and 6% divergence.

The relative variation at each codon position was examined to verify that all amplified *CYT B* sequences were consistent with true arthropod mitochondrial genes, as opposed to being nuclear mtDNA ("numt") insertions [Zhang & Hewitt, 1996]. As expected for a protein-coding gene, the second codon position exhibited the least nucleotide variation, while the third position exhibited the highest variation. All sequences were submitted to NCBI GenBank under accessions JF960968-JF961332.

Finally, we identified the closest match in GenBank to each of the recovered sequences using the *blastn* algorithm [Altschul et al., 1990]. Working taxonomic identifications were assigned for each sequence according to the BLAST result with the lowest E-value, which is the same as the default output of the NCBI web interface. The E-value indicates the statistical significance of a sequence match [Altschul et al., 1990], essentially representing the number of other sequence hits equally good or better than the current alignment that can be expected to arise due to randomness in the comparative database. The E-value is considered to be a better metric than sequence identity alone for determining the appropriateness of the results for a BLAST hit. E-value takes into account the alignment itself, the length of the query sequence, and the length of the database, which are important considerations due to the chance of randomly getting a false match for an input. A Python script written for this project (using BioPython modules) compared each sequence to all arthropod sequences in NCBI GenBank's nonredundant nucleotide database [Cock et al., 2009] and ranked these by E-value.

This research follows the American Society of Primatologists principles for the ethical treatment of primates and was conducted with institutional approval by the University Animal Welfare Committee of New York University. The research was done in full agreement with all Ecuadorian legislation. Samples were collected and exported with the permission from the Ecuadorian Ministerio de Ambiente and were imported with permission of the U.S. Center for Disease Control and Prevention.

RESULTS

A total of 480 clones were sequenced from three individuals of each primate genus (16–32 clones per individual). For all analyses, we pooled clones from different individuals of the same primate taxon together. Using the 2% sequence divergence criterion for grouping sequences into OTUs, the number of OTUs recovered for each genus ranged from three (for titi monkeys, *Callicebus*) to 15 (for squirrel monkeys, *Saimiri*), with a total of 49 OTUs identified by the BLAST analysis (Table I). Using the program EstimateS, we plotted the number of sampled OTUs against the number of clones selected from each primate genus' clone library to generate a diversity accumulation curve (Fig. 2) [Colwell, 2000]. Using a 6% divergence criterion, the results remain unchanged for all primate taxa, except *Saimiri*, in which four OTUs, identified as belonging to the same family, were consolidated into one, decreasing the total number of inferred OTUs from 15 to 11.

Although the total number of clones selected for sequencing was standardized for all individuals, some sequencing reactions yielded low-quality sequence data that were unsuitable for analysis, or showed closer similarity to nonarthropod taxa in GenBank ($n = 121$ of 480 sequences). These sequences were excluded from further analysis, leaving 359 clones identified as being arthropod *CYT B* sequences. Additionally, the presence of stop codons mid-gene was observed for 12 sequences with apparent indel mutations, which may have been an artifact of the PCR and cloning process as many

TABLE I. Arthropod Taxa in the Diets of Six New World Monkeys, Identified Using Metagenomic Methods

Primate	Primate sample number	OTU sample number	Number of clones	Putative taxon	Lowest assigned taxonomic level	Common name
Saimiri	1	**1**	1	Agaonidae	Family	Fig wasps
	1	**2**	1	Agaonidae	Family	Fig wasps
	1	**3**	1	Agaonidae	Family	Fig wasps
	1	**4**	2	Agaonidae	Family	Fig wasps
	1	**5**	1	Agaonidae	Family	Fig wasps
	1	6	11	Neolepidoptera	Infraorder	Moths
	1	7	1	Nymphalidae	Family	Brush-footed butterflies
	3	8	32	Tettigoniidae	Family	Katydids
	2	9	26	Neoptera	Subclass	Winged insects
	1	10	5	Elmidae	Family	Riffle beetles
	1	11	1	Elateridae	Family	Click beetles
	2	12	1	Ectobiidae	Family	Cockroaches
	2	13	1	Noctuidae	Family	Owlet moths
	2	14	1	Saturniidae	Family	Emperor moths
	1	15	1	Nephilidae	Family	Spiders
Average number of OTUs per sample	5					
Number of insect *CYTB* sequences recovered/total number of clones sequenced	86/96					
Cebus	3	1	17	Sepsidae	Family	Black flies
	2	2	10	Tettigoniidae	Family	Katydids
	3	3	14	Curculionidae	Family	Weevils
	2	4	6	Polyphaga	Suborder	Beetles
	1	5	3	Chironomidae	Family	Nonbiting midges
	2	6	1	Scarabaeidae	Family	Scarab beetles
	2	7	1	Tephritidae	Family	Fruit flies
	2	8	3	Neoptera	Subclass	Winged insects
	2	9	4	Muscomorpha	Infraorder	Flies
	2	10	1	Formicidae	Family	Ants
	1	11	8	Apidae	Family	Bumble/honey bees
	1	12	14	Agaonidae	Family	Fig wasps
Average number of OTUs per sample	4					
Number of insect *CYTB* sequences recovered/total number of clones sequenced	82/96					
Lagothrix	1	1	25	Agaonidae	Family	Fig wasp
	3	2	30	Calliphoridae	Family	Blowflies
	2	3	13	Chrysomelidae	Family	Leaf beetles
	2	4	1	Curculionidae	Family	Weevils
	1	5	2	Chrysomelidae	Family	Leaf beetles
	1	6	1	Chrysomelidae	Family	Leaf beetles
	2	7	12	Formicidae	Family	Ants
Average number of OTUs per sample	2.3					
Number of insect *CYTB* sequences recovered/total number of clones sequenced	84/96					
Pithecia	1	1	3	Psychodidae	Family	Sandflies/mothflies
	2	2	1	Psychodidae	Family	Sandflies/mothflies
	1	3	1	Aphididae	Family	Aphids
	1	4	1	Aphididae	Family	Aphids

TABLE I. Continued

Primate	Primate sample number	OTU sample number	Number of clones	Putative taxon	Lowest assigned taxonomic level	Common name
	2	5	18	Saturniidae	Family	Emperor moths
	3	6	12	Tettigoniidae	Family	Katydids
	2	7	3	Formicidae	Family	Ants
	2	8	1	Formicidae	Family	Ants
Average number of OTUs per sample	2.7					
Number of insect *CYTB* sequences recovered/total number of clones sequenced	40/96					
Callicebus	1	1	11	Formicidae	Family	Ants
	3	2	12	Bacillidae	Family	Stick insects
	2	3	10	Salticidae	Family	Jumping spiders
Average Number of OTUs per sample	1					
Number of insect *CYTB* sequences recovered/total number of clones sequenced	33/48					
Ateles	2	1	7	Formicidae	Family	Ants
	3	2	9	Neoptera	Subclass	Winged insects
	1	3	15	Endopterygota	Infraclass	Insects that complete metamorphosis
	2	4	3	Elateroidea	Superfamily	Beetles
Average number of OTUs per sample	1.3					
Number of insect *CYTB* sequences recovered/total number of clones sequenced	34/48					

The maximum divergence among clones within an OTU is 2%. OTUs shown in bold (#s 1–5 for *Saimiri*) represent those that would be consolidated into a single OTU using a 6% sequence divergence criterion.

followed homopolymer runs. For three sequences, mid-gene stop codons were observed without indel-caused frameshifts, and it is not clear whether these few sequences are indeed authentic arthropod mitochondrial sequences. Of these 15 sequences containing premature stop codons, 14 were grouped into OTUs with multiple other sequences and therefore do not inflate the diversity of sequences recovered.

Taxonomic identifications were assigned for each OTU according to the best BLAST result (lowest E-value) for each sequence (Table I). The most specific taxonomic grouping common to all of the sequences grouped into an OTU was considered to be that OTU's "working" taxonomic assignment. For example, if the best BLAST results for the set of sequences within a single OTU corresponded to multiple, different species belonging to a single genus, then their common genus name was used as the "working" taxonomic identity recorded for that OTU. However, due to the incompleteness of the comparative insect sample in GenBank, percent sequence identity for the recovered sequences was often not high enough for confident OTU assignment at the genus level. We therefore assigned identities to OTUs (Table I) at the level of family or higher.

Insect orders that were identified as diet items in the current study are denoted by shaded cells in Table II, for comparison to those reported in prior studies that did not employ metagenomic methods.

DISCUSSION

In this study, insect *CYT B* sequences were successfully amplified from the feces of six New World primate genera. Using the criteria described above, we were able to assign a working identification to the family level for most OTUs, although more detailed taxonomic assignments were limited by the incompleteness of the comparative arthropod sequence sample in GenBank. Moreover, while many of the orders to which prey items were assigned in this study had been recorded in previous observational

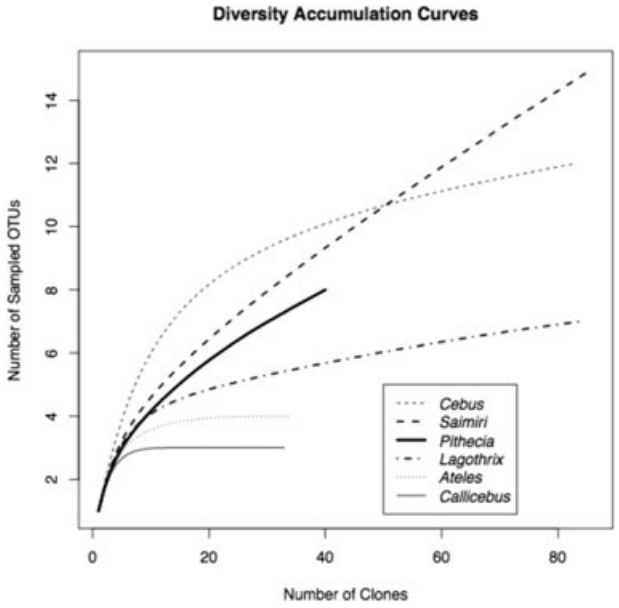

Fig. 2. Diversity accumulation curve for insect OTUs sampled from the clone libraries of six Neotropical primate genera, generated using the software EstimateS [Colwell, 2000]. Clone libraries with higher diversity do not plateau, indicating that more clones would need to be sequenced in order to accurately represent all of the insect diversity present in the library.

studies, novel arthropod prey orders were discovered for *Pithecia* (Diptera, Hemiptera), *Callicebus* (Phasmatodea), and *Ateles* (Hymenoptera, Coleoptera) using this metagenomic approach. Table II outlines the overlap between our study's findings and the results of traditional studies on the arthropod components of the diets of these six genera, based on field observations of feeding behavior, morphological analysis of insect remains passed in feces, and analysis of the stomach contents of deceased animals.

The number of OTUs sampled from clone libraries and the corresponding diversity accumulation curves provide a proxy for the insect diversity consumed by each primate genus, at least in this very small sample from each taxon. Based on the samples used for this study, capuchin monkeys (*Cebus*), followed by squirrel monkeys (*Saimiri*), appear to consume the greatest variety of insects. The number of OTUs sampled from the woolly monkey (*Lagothrix*) clone library is lower than those of the cebines (Fig. 2). This is consistent with observed woolly monkey foraging behavior: these primates allocate less time to insect foraging and focus more on fruit consumption, and, therefore, the insect portion of their diet is likely to be less diverse [Defler & Defler, 17; Peres, 1994; Stevenson et al., 1994].

Based on the total number of OTUs recovered, the insect diversity consumed by saki monkeys (*Pithecia*) was lower than that of squirrel and capuchin monkeys, but slightly higher than that of woolly monkeys. Given that observational studies have found that the major components of saki monkey diets are fruits and other plant parts [Di Fiore and Fernandez-Duque, unpublished data; Kinzey, 1992; Norconk, 2007], the insect diversity found in this study is higher than expected. Additionally, because observational studies suggest that insects make up a similar percentage of both the titi (*Callicebus*) and saki monkey diets [Norconk, 2007], we expected similar levels of insect sequence diversity to be present in their feces. This is not supported

TABLE II. Summary of Arthropod Consumption by Six Genera of New World primates. Orders of Arthropod Prey Known to be Consumed by the Primate Taxa Under Study, Either Through Field Observation or Analysis of Morphological Remains of Prey in Feces or Stomach Contents (Key to References Below Table). Shaded Cells Indicate Orders of Arthropod DNA Sequences that were Identified as Diet Items in the Current Study.

	Cebids		Pitheciids		Atelids	
Arthropod order	*Cebus*	*Saimiri*	*Pithecia*	*Callicebus*	*Ateles*	*Lagothrix*
Lepidoptera	5, 6, 7, 9, 13, 17	**1, 7, 9, 12, 20**	**7, 15**	14, 16	21	3, 14, 19
Hymenoptera	**5, 6, 7, 8, 9, 13, 17**	**7, 9, 12, 20**	**7, 11, 15**	**7, 10, 14**		**2, 3, 4, 14, 19**
Diptera	**7**			14		**19**
Coleoptera	**8, 9, 13**	**7, 9, 12**		7, 10, 14, 16		**3, 14, 19**
Blattodea	7, 9, 13	**7, 9**				
Mantodea	7, 9	7, 9				19
Isoptera	7, 8, 9, 13, 17	7, 9	11		3, 21	2, 3, 4, 14
Phasmatodea	9, 13	9	11			
Orthoptera	**5, 6, 7, 8, 9, 13, 17**	**1, 7, 9, 12, 20**	**11**	7, 16		2, 3, 18, 19
Hemiptera	9	7, 9				3, 14
Homoptera	7, 9, 13	9, 12, 20				14
Araneae	6, 7, 9,	**1, 7, 9, 12**	15	**16**		2, 3, 18, 19
Phalangida		9				

[1] Boinski, 1988. [2] Defler & Defler, 1996. [3] Dew, 2005. [4] Di Fiore, 25. [5] Fragaszy & Boinski, 1995. [6] Galetti & Pedroni, 1994. [7] Izawa, 1975. [8] Izawa, 1979. [9] Janson & Boinksi, 1992. [10] Kinzey, 1977. [11] Kinzey & Norconk, 1993. [12] Lima & Ferrari, 2003. [13] Melin et al., 2007. [14] Milton & Nessimian, 1984. [15] Norconk, 1996. [16] Palacios et al., 1997. [17] Panger et al., 2002. [18] Peres, 1994. [19] Stevenson et al., 1994. [20] Stone, 2007. [21] Symington, 1988.

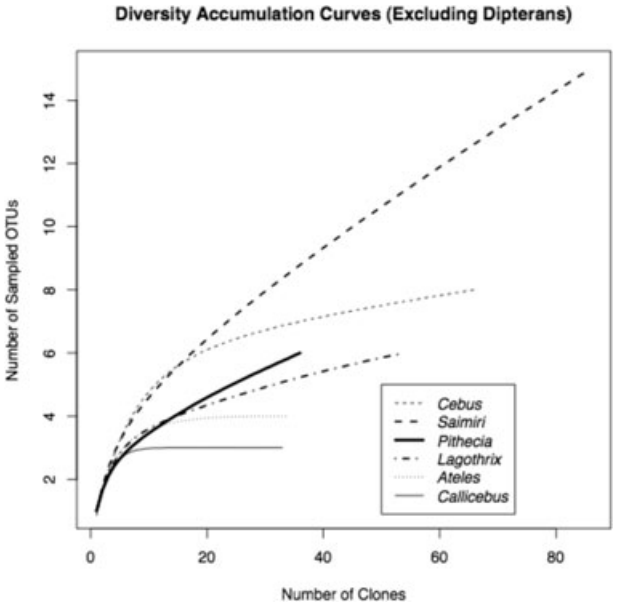

Fig. 3. Diversity accumulation curve for insect OTUs sampled from the clone libraries of each primate genus, recalculated after excluding those OTUs identified as dipterans.

by our analyses, however, as the insect variety consumed by titis (*Callicebus*) appears to be less than half that of the saki monkeys. While it is possible that saki monkeys consume a greater diversity of insects, the finding may be a sampling artifact based on the time when the fecal samples were collected from each individual and the small number of samples was used for this preliminary study.

It should be noted that because the curves corresponding to *Saimiri*, *Cebus*, *Lagothrix*, and *Pithecia* do not yet plateau (Fig. 2), sequencing of additional clones would be necessary to fully represent the insect sequence diversity present in the clone libraries derived from pooling single fecal samples from each of three individuals. The curves generated for the *Callicebus* samples, however, do plateau, suggesting that the sampled OTUs reasonably represent the insect diversity present in the clone libraries created from this small set of samples.

The small number of OTUs found in the spider monkey (*Ateles*) clone library and the fact that the diversity accumulation curve reaches a clear plateau (Fig. 2) together suggest that we have captured a reasonable representation of the total insect diversity present in the three fecal samples from this genus. These findings are consistent with observational studies, which suggest that spider monkeys consume only a few insect species, if they forage for insects at all [Dew, 2005; Di Fiore et al., 2008; Klein & Klein, 1977; Link, 2003].

While the metagenomic approach is promising, a number of limitations to the method remain to be resolved in future studies. First, the propensity for contamination presents an important limitation of the approach, particularly when sequences belonging to unlikely prey items are identified. Fruit flies, for example, lay eggs in primate food items, including fruits and flowers [Throckmorton, 1975]; therefore, it is possible that fruit fly sequences could be identified in the feces of a primate consuming these items in large quantities. More significantly, fruit flies, as well as many other dipterans (e.g. blow flies), also lay eggs in feces. The behavior of these insects can result in local DNA contamination that is not easy to prevent, except by collecting fecal samples as rapidly as possible following defecation. In this study, all fecal samples were collected within seconds or minutes of deposition. Nevertheless, due to the possibility of dipteran oviposition in feces, we also recalculated the diversity accumulation curves, excluding those OTUs identified as dipteran insects in our *blastn* search (Fig. 3). Excluding these taxa reduced the overall number of OTUs for saki, woolly, and capuchin monkeys and altered the diversity accumulation curves for these primates. Qualitatively, however, the major results of the study remain largely unchanged: squirrel monkeys and capuchins remained the primate genera with the greatest diversity of insects in the diet, as reflected in the small set of fecal samples analyzed, followed by saki and woolly monkeys, and the diversity accumulation curves for all four of these species still do not seem to approach a clear asymptote.

Beyond local DNA contamination, additional factors that might affect arthropod DNA amplification should be noted. These include variable rates of digestion as well as potential biases in the DNA extraction, PCR amplification, and cloning steps, all of which could limit the detection of certain prey items found in a fecal sample [Braley et al., 2010].

Finally, the most significant limitation of this study (and of using a metagenomic approach to study the insect diets of tropical vertebrates generally) is the scarcity of South American *CYT B* insect sequences currently archived in NCBI GenBank. While the region of *CYT B* targeted in this study was readily amplified for a wide variety of insects, our taxonomic assignments based on BLAST results must be considered tentative, rather than as precise identifications. Given the mega-diversity of Amazonian insects and the paucity of Amazonian insect sequences in GenBank, this is perhaps not unexpected, but this fact constitutes a major limitation for the purposes of this dietary study. Taxonomic assignments might be improved through the use of an alternative DNA barcoding gene, such as *COI*, and as DNA barcoding databases such as the Barcode of Life Database (BOLD) [Ratnasingham & Hebert, 2007] grow, these may come to include a more extensive collection of reference sequences from South American insects. Still, amplification of insect DNA using *COI* primers was attempted in this study with little success.

These limitations aside, metagenomics may be a valuable method for primate dietary research. As many researchers are already collecting fecal samples for behavioral studies and DNA analyses, a DNA-based dietary component could be a relatively easy and inexpensive addition to field studies [Bradley et al., 2007]. Additionally, with ongoing advances in DNA sequencing technology (e.g. various "next-generation sequencing," or NGS, methods), it is possible that extensive cloning—the most expensive and time-consuming step in our approach—might be eliminated altogether, allowing more complete recovery of arthropod DNA and more accurate estimates of prey taxon diversity. Finally, with the expansion of reference sequence databases, DNA-based identification of prey from feces could provide a robust and noninvasive means of studying the dietary ecology of elusive primates and may have significant implications for future conservation planning. In the meantime, however, a metagenomic approach will be most effective if conducted in tandem with other methods of studying primate diet, including direct observation of foraging behavior and morphological classification of food remains from feces.

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DATA ACCESSIBILITY

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