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The problems and promise of DNA barcodes for species diagnosis of primate biomaterials

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The Integrated Primate Biomaterials and Information Resource (www.IPBIR.org) provides essential research reagents to the scientific community by establishing, verifying, maintaining, and distributing DNA and RNA derived from primate cell cultures. The IPBIR uses mitochondrial cytochrome *c* oxidase subunit I sequences to verify the identity of samples for quality control purposes in the accession, cell culture, DNA extraction processes and prior to shipping to end users. As a result, IPBIR is accumulating a database of 'DNA barcodes' for many species of primates. However, this quality control process is complicated by taxon specific patterns of 'universal primer' failure, as well as the amplification or co-amplification of nuclear pseudogenes of mitochondrial origins. To overcome these difficulties, taxon specific primers have been developed, and reverse transcriptase PCR is utilized to exclude these extraneous sequences from amplification. DNA barcoding of primates has applications to conservation and law enforcement. Depositing barcode sequences in a public database, along with primer sequences, trace files and associated quality scores, makes this species identification technique widely accessible. Reference DNA barcode sequences should be derived from, and linked to, specimens of known provenance in web-accessible collections in order to validate this system of molecular diagnostics.

Keywords: primate; DNA barcoding; mtDNA

1. INTRODUCTION

The advent of molecular techniques has opened new possibilities for taxonomic research, which is important given that the vast majority of all extant species are not well characterized morphologically. Taxonomies based on morphological analyses can be problematic due to either convergence in phenotype among unrelated species or the failure to identify 'cryptic species' where morphologic divergence has not kept pace with genetic divergence.

In an effort to standardize the approach to species identification using molecular techniques it has been proposed that as many species as possible be characterized for the same genetic markers (Blaxter 2004). The mitochondrial gene, cytochrome *c* oxidase subunit I (*cox1*) has been proposed as a candidate locus given its conserved sequence allows for 'universal' primers to be used across multiple divergent taxa and its high degree of phylogenetic signal relative to other mtDNA loci that have been used for interspecific analysis (e.g. 12s or 16s ribosomal DNA). This feature is perhaps due to heavy stabilizing selection within species for mitochondrial/nuclear cytochrome protein complexes (Hebert *et al.* 2003*a*,*b*). Thus a sequence of several hundred nucleotides in length acts as a unique identifier for members of a given species, hence the analogy to a computerized barcode label, although the analogy is imperfect given the existence of intraspecific variation so that not all members of a species are expected to be completely identical. Nonetheless the degree of intraspecific variation compared to the degree of interspecies variation would be expected to be low enough such that sequences from polymorphic species would cluster together in a genetic distance based cluster analysis.

Early studies demonstrated the efficacy of using universal primers to amplify and sequence a variety of taxa from divergent phyla (Hebert *et al.* 2003*a*), but the use of *cox1* sequences as species identifying barcodes has been limited to a study of North American birds (Hebert *et al.* 2004*a*) and one complex species of neotropical butterflies (*Astraptes fulgerator*) from Costa Rica (Hebert *et al.* 2004*b*); but see e.g. Janzen *et al.* (2005), Saunders (2005), Smith *et al.* (2005) and Armstrong & Ball (2005) for recent examples. To date, the efficacy of molecular barcoding has not been determined within mammalian taxa.

Primates is a mammalian order with worldwide distribution, the members of which are important in conservation, evolutionary and biomedical studies. The taxonomic classification of extant species of the primate order has been agreed upon for several decades with a few interesting exceptions (Le Gros Clark 1954). The placement of tarsiers and the demarcation of the groupings among the hominoidea (including the genus *Homo*) are two of those exceptions that have been the focus of extensive taxonomic reorganization. Even with the overall structure of primate taxonomy in place there remains much work to be done in understanding the relationships of closely related taxa within many of the major groupings (Ruiz-Garcia & Alvarez 2003; Singer *et al.* 2003).

In addition, to elucidating relationships among the lower taxonomic levels of primates, there are practical

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aspects to DNA barcoding. The bush meat trade is threatening many wild populations of primates and other endangered species (Brashares *et al.* 2004). To effectively prosecute poachers trafficking in meat it would be beneficial to law enforcement and conservation officials to have access to a forensic database of primate *cox1* samples so that positive identification of seized contraband could be made. Given that many non-human primates are important in biomedical research (Vandeberg & Williams-Blangero 1996, 1997) it has become increasingly desirable to genetically characterize the various species used in research. Thus far, there are species-specific differences (and even strain differences within species) to pathological aetiology and temperamental differences that may be important in behavioural research (Champoux *et al.* 1994; Champoux *et al.* 1997). In addition biomaterial repositories would have a simple and universal means of verifying the species identity of samples submitted to them for inclusion in their collections. Finally, the ability to identify or verify the source of biomaterials from field-collected specimens may be a useful tool to conservation and range scientists as well as a means of identifying the geographical provenience of captive born animals.

Past efforts to collect, store and develop genetic resources have largely been uncoordinated efforts scattered over different institutions and countries (Savolainen & Reeves 2004). Primates are a target group for scientific and technological development due to their importance in biomedicine and conservation biology, especially given their evolutionary proximity to humans. Within this context, the collection and storage of primate resources covering all branches of their taxonomy is an urgent need to boost primate molecular biology. Such resources include living and preserved collections, tissues, DNA, frozen viable cells and cell lines. The storage of complementary information on the origin, morphology, physiology, ecology, demography or behaviour of the specimens is also crucial to explore the link between gene and function. The coordinated development of these resources will prevent repeated sampling of wild populations, reduce the number of animals used in research, and help to standardize molecular tools and protocols.

The Integrated Primate Biomaterials and Information Resource (www.IPBIR.org) provides essential research reagents to the scientific community by establishing, verifying, maintaining and distributing DNA and RNA derived from primate cell cultures. Proper quality assurance/quality control requires the ability to verify the identity of samples as they move through the accession, culture and extraction processes. At present IPBIR has 97 of the approximately 200 different species (Cheney *et al.* 1986) of primates representing most of the major taxonomic divisions of the order. Given the taxonomic breadth of the samples in the resource it is important to choose a molecular marker that would work in as many species as possible.

IPBIR uses DNA sequences for routine identification of non-human biomaterials by simple sequence matching. The GenBank public-access database provides a working archive of available sequences, forming a valuable resource for such studies. However, the accuracy and reliability of sequences deposited in GenBank have been questioned, especially when the sequences are not linked to a voucher specimen. The identity of some DNA sequences deposited in public databases is being contested and there is a need to determine if such reports reveal a widespread phenomenon (Bridge *et al.* 2003).

The publicly available *cox1* DNA sequences are distinct for each primate species, but represent a very incomplete data set. Moreover, sequence information can be difficult to interpret for several reasons. First, different levels of variation may occur in the same DNA region in different taxa, making generalized comparisons between taxa problematic. Second, amplification of non-target DNA from contaminants, or numts (nuclear mitochondrial DNA sequences), constitutes a danger. It has been proposed that as many as 500 copies of translocated mtDNA exist in the human nuclear genome (Richly & Leister 2004), ranging in length from 47 to 14 654 base pairs (bp) (Ricchetti *et al.* 2004). Hence, caution must be taken when amplifying any mitochondrial segment short of the entire mitochondrial genome. Third, sequences derived from unspecified reference materials cannot be validated.

In this study we investigate the feasibility of using *cox1* sequence 'molecular barcode' data to verify the species designation of 225 individuals representing 56 species of primates (table 1) using both 'universal' *cox1* primers identified in earlier molecular barcoding studies (Hebert *et al.* 2003*a*) as well as primers developed specifically for primate taxa.

2. MATERIAL AND METHODS

(a) *DNA Extraction*

Genomic DNA was extracted from cell pellets obtained from lymphoblastoid cell cultures, fibroblastoid cell cultures or buffy coats obtained from whole blood using standard salting-out techniques (Miller *et al.* 1988) or from tissue biopsies or plasma/serum using QiaAmp DNA Blood Kits (Qiagen).

(b) *PCR Amplification*

A region approximately 727-bp long near the 5′ terminus of the *cox1* gene was amplified using one of three primer sets (table 2). PCR reactions were done in a total volume of 25 μL and consisted of 2.5 μL of 10× PCR II buffer (Applied Biosystems), 2.5 μL of 25 mM $MgCl_2$, 2.0 μL of 10 mM dNTP mix (2.5 mM each dNTP), 0.2 μL of each primer (25 μM stock) and 0.2 μL of 5 U/μL TaqGold DNA polymerase (Applied Biosystems), 2.0 μL of DNA template (∼50 ng) and dH_2O to 25 μL. The thermocycling conditions were as follows: 96.0 °C for 10 min to activate the TaqGold and then 35 cycles of 96.0 °C for 1 min, 50.0 to 58.0 °C for 1 min and 72.0 °C for 1 min followed by a final hold of 72.0 °C for 10 min. PCR products were visualized on 6% polyacrylamide minigels and the PCR product was purified using QiaQuick 96 PCR Purification Kit (Qiagen). The purified PCR product was eluted into 45 μL buffer AE (Qiagen).

(c) *DNA Sequencing*

Cycle sequencing reactions were carried out in 10 μL total volume. A forward and reverse reaction was performed for each sample consisting of 5.4 μL of the purified PCR

Table 1. Taxonomic distribution of the samples used in this study.

infraorder	suborder	family	subfamily	genus	Species	number in this study
Haplorhini	Catarrhini	Cercopithecidae	Cercopithecinae	*Allenopithecus*	*nigroviridis*	2
				Cercocebus	*torquatus*	1
				Cercopithecus	*ascanius*	2
					cephus	1
					lhoesti	1
					mitis	6
					neglectus	7
					petaurista	1
					wolfi	1
				Chlorocebus	*aethiops*	27
				Erythrocebus	*patas*	1
				Lophocebus	*albigena*	1
				Macaca	*fascicularis*	1
					fuscata	1
					nemestrina	1
					mulatta	2
					nigra	1
					thibetana	1
				Mandrillus	*leucophaeus*	1
					sphinx	3
				Miopithecus	*talapoin*	1
				Papio	*anubis*	6
					cynocephalus	54
					hamadryas	2
				Theropithecus	*gelada*	2
			Colobinae	*Colobus*	*guereza*	2
					polykomos	1
				Nasalis	*larvatus*	2
				Semnopithecus	*entellus*	1
				Trachypithecus	*francoisi*	1
		Hylobatidae		*Hylobates*	*gabriellae*	2
					agilis	1
					lar	2
					leucogenys	1
					pileatus	1
					syndactylus	3
		Hominidae		*Pan*	*paniscus*	7
					troglodytes	25
				Gorilla	*gorilla*	19
				Pongo	*pygmaeus*	9
	Platyrrhini	Callitrichidae		*Callimico*	*goeldii*	1
				Leontopithecus	*rosalia*	4
				Saguinus	*fuscicollis*	1
					midas	1
		Cebidae	Atelinae	*Ateles*	*geoffroyi*	1
				Lagothrix	*lagotricha*	1
			Aotinae	*Aotus*	*nancymaae*	1
			Pithecinae	*Pithecia*	*pithecia*	1
			Cebinae	*Saimiri*	*bolivensis*	1
					oerstedii	1
Strepsirhini		Daubentoniidae		*Daubentonia*	*madagascariensis*	1
		Lemuridae		*Eulemur*	*fulvus*	1
					mongoz	3
				Varecia	*variegata*	1
		Galagonidae		*Galago*	*moholi*	1
				Otolemur	*garnettii*	1
total						225

product, 4 μL of Big Dye Terminator Ready Reaction Mix, v1.1(Applied Biosystems) and 0.6 μL of 2.5 μM primer (same as PCR primer). Cycling conditions were 96.0 °C for 1 min and then 25 cycles of 96.0 °C for 10 s, 50.0 °C for 5 s, 60.0 °C for 4 min. Unincorporated fluorescent dye terminators were removed from the cycle sequencing reactions using SigmaSpin 96-well plate (Sigma Aldrich), dried at 37 °C for 20 min and resuspended in 10 μL of HiDi formamide (ABI). The cycle sequencing product was detected using an ABI 3730 DNA analyser.

(d) *Sequence analysis*

The forward and reverse sequence files for each sample were analysed and a consensus sequence for each sample was

Table 2. Primer set success by genus.
(Primer Set 1 comprised LCOI1490: ggt caa caa atc ata aag ata ttg g and HCOI2198: taa act tca ggg tga cca aaa aat ca. Primer Set 2 comprised VERTCOIf1: ttc tca acc aac caa caa aga cat tgg and VERTCOIr1: tag act tct ggg tgg cca aag aat ca. Primer Set 3 comprised OWMCO-If: (A/G)CT (G/C)TT TTC AAC AAA (C/T)CA (C/T)AA AGA C and OWMCO-Ir: GTA (A/G)AC TTC (G/C)GG GTG (A/G)CC (A/G)AA GAA TC.)

taxa	primer set 1	primer set 2	primer set 3
Pan	yes	no	yes
Gorilla	yes	no	yes
Pongo	no	yes	
Hylobates	yes	no	
Papio	no	yes	yes
Cercopithecus	no	yes	yes
Macaca	yes	no	yes
Mandrillus	yes	no	yes
Chlorocebus	no	no	yes
Allenopithecus	yes	yes	yes
Leontopithecus	yes	yes	
Saimiri	yes	yes	
Pithecia	yes		
Varecia	yes		
Daubentonia	yes	no	no

created using 'Sequencher' (GeneCodes). The consensus sequences were in turn aligned using 'Sequencher' and exported into a NEXUS file for distance analysis and cluster analysis using PAUP 4.0b10 (Swofford 1998). Mean pairwise differences were computed for all species and genera. In order to validate the *cox1* sequences obtained in this study we compared them with *cox1* sequences from primate species for which the whole mitochondrial DNA sequence has been established and deposited in GenBank (http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/9347.html) as well as previously archived *cox1* sequences (Andrews & Easteal 2000; Wu *et al.* 2000). In addition we constructed neighbour-joining trees using PAUP and based on Kimura 2p distances to determine whether the sequences cluster as would be expected based on overall taxonomic affinity.

(e) *rtPCR*

In the case of several old world monkey species, PCR products obtained using the different primers yielded different sequences. The paucity of whole mitochondrial sequences for cercopithecines limited our ability to determine which of the *cox1* sequences were derived from mitochondrial *cox1* and which were derived from numts. Reverse transcriptase PCR (rtPCR) was performed on *Papio anubis* DNA obtained from lymphoblasts to ensure amplification of the transcribed mitochondrial *cox1* (Collura *et al.* 1996). Reverse transcription products were amplified in three separate reactions using the three primer sets. The RNA extractions were also amplified with AmpliTaq in place of reverse transcriptase to identify any DNA contamination and a positive control rtPCR reaction was also performed using primers for phosphoglycerate kinase (PGK).

3. RESULTS

All samples in this study amplified with one or more primer sets (table 2). Generally, if samples representing a given species amplified with Primer set 1 (Hebert *et al.* 2003*a*) and gave 'phylogenetically credible' results they were not assayed with the other two primer sets. In some cases where a species did not amplify with one primer but did with another they were tested with the third primer set in order to increase the probability the sequence was not obtained from numts (Thalmann *et al.* 2004 and references therein) or other spurious amplicons. In addition to checking clustering for an expected phylogenetic signal to determine whether a sequence was derived from the actual *cox1* and not a numt we checked to see that the fragments were identical to, or at least clustered with, the appropriate whole mitochondrial sequences obtained from GenBank. Also, sequences which yielded amino acid transcription interrupted by stop codons were not included in the analysis as they would not likely be derived from the functional mitochondrial *cox1* gene. This did occur in a handful of cercopithecine species but the design of primer set 3 did eliminate amplification of spurious *cox1* in those species.

The results of the reverse transcription PCR experiment showed that primer sets 2 and 3 amplified *cox1* from RNA extracts of *P. anubis* lymphoblasts (figure 1). Primer set 1 did not amplify a fragment from the RNA preparation indicating that the Primer set 1 fragment amplified from genomic DNA is not from the mitochondrial genome but is possibly from a numt.

The results of the sequencing analysis for each of the samples were submitted to GenBank (Accession numbers: AY544148–62, AY632376–7, AY671787–98, AY673675, AY972630–808). The sequences were aligned with *cox1* sequences obtained from primate whole mitochondrial genomes obtained from GenBank as well as primate *cox1* sequences from other studies that have been deposited in GenBank. Sequences less than 400 bp long were excluded from the analysis. A neighbour-joining tree (figure 2) based on Kimura 2p distances (Kimura 1980) was calculated using PAUP, bootstrap values are based on 1000 replicates.

The neighbor-joining tree generally agrees with the commonly accepted primate phylogeny with platyrrhine sequences clustering together 99% of the time and catarrhines cluster 95%. The strepsirhines, however, do not form a cohesive cluster; this is not unexpected given the relatively short fragment used in the analysis. There are 17 *cox1* sequences from previous studies that were retrieved from GenBank. In ten of the cases the sequences from this study clustered with *cox1* sequences obtained from GenBank at the species level (table 3). In four cases the sequences from this study were not represented in GenBank by the same species, however they did cluster together with GenBank derived sequences at the generic level (*Saimiri*, *Macaca sylvanus*, *Trachypithecus* and *Galago*). In the remaining three cases the GenBank sequences did not cluster with the sequences derived from the same species in this study: i.e. *Colobus polykomos* and *C. guereza* did not cluster separately, *Papio hamadryas* from GenBank clustered with *P. anubis* in this study and *Theropithecus gelada* from GenBank did not cluster with *T. gelada* from this study nor did it cluster with any of the cercopithecine *cox1* sequences.

In the cases for which there are no previously reported data to which we can compare the sequences generated in this study we tallied the extent to which

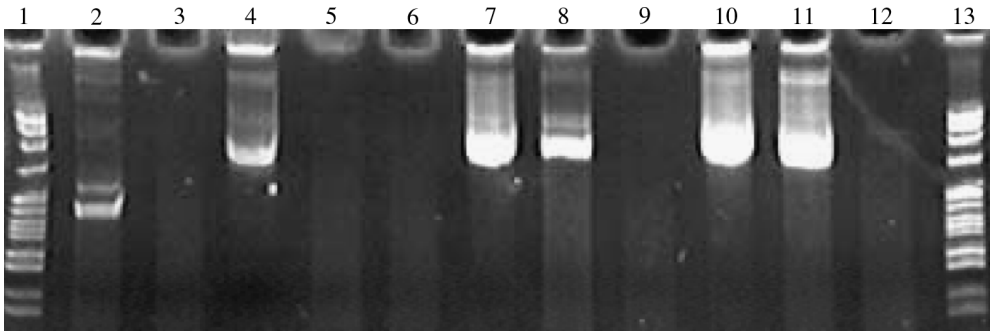

Figure 1. Results of rtPCR performed on RNA extracted from *Papio anubis* liver. Lanes 1 & 13; *Msp* I digested pBR322 ladder. Lane 2; PGK positive control. Lane 3; PGK DNA control (no RNA). Lane 4; amplification of genomic DNA using primer set1. Lane 5; rtPCR with primer set 1. Lane 6; 'mock' rtPCR using AmpliTaq instead of reverse transcriptase and primer set 1. Lane 7; amplification of genomic DNA using primer set2. Lane 8; rtPCR with primer set 2. Lane 9; 'mock' rtPCR using AmpliTaq instead of reverse transcriptase and primer set 2. Lane 10; amplification of genomic DNA using primer set 3. Lane 11; rtPCR with primer set 3. Lane 12; 'mock' rtPCR using AmpliTaq instead of reverse transcriptase and primer set 3.

our sequences cluster with members of the same species (table 4). There are 10 clusters supported in 100% of the bootstrap replicates in which all members of a given species cluster together. In fact, except for *Papio* and *Colobus* as mentioned above, there are no cases where we have multiple specimens of a species in which the sequences do not cluster together.

4. DISCUSSION

Using a segment of the 5′ region of *cox1* we are able to identify the appropriate species from which a biomaterial submitted to the Integrated Primate Biomaterials and Information Resource was derived. This ability to generate a 'molecular barcode' is useful in our case for quality control and the management of the IPBIR. It allows us to verify the identity of samples, as reported by the submitter, as they move through each stage of the accession, cell culture, DNA extraction and aliquoting processes. Since the samples comprising the IPBIR collection are from identified specimens of known species, the *cox1* sequences derived from the IPBIR resource have the potential to serve as a forensic database for the identification of primate biomaterials such as those seized in the bush meat trade.

The success of DNA barcoding depends on the amount of intraspecific variation relative to the amount of interspecific variation present among species across their range. The amount of intraspecific variation, measured as mean pairwise difference, in the present study varies (figure 3) from none (*Eulemur mongoz*, *Leontopithecus rosalia*, *Cercopithecus neglectus*) to 0.038 for *Pongo pygmaeus* (mean for all species $=0.011$, s.e. $=0.004$). The amount of intraspecific variation at *cox1* will depend on the degree to which the samples reflect the geographic diversity of widely dispersed species (e.g. *Pan troglodytes* and *Chlorocebus aethiops*), the amount of gene flow among subpopulations and also whether the species is perhaps an amalgam of multiple species. For example it has been argued that the Sumatran (*P. pygmaeus abelii*) and Bornean orangutans (*P. pygmaeus*) actually are distinct species (Xu & Arnason 1996; Warren *et al.* 2001); in fact the orangutans in this study cluster robustly by subspecies (100% of bootstrap replicates) and the degree of genetic divergence is comparable to that that exists between *Pan paniscus* and *P. troglodytes*, which lends support for the case that the genus *Pongo* includes two separate species.

It is interesting to note that there are three cases where the *cox1* sequences derived from the complete mitochondrial genome sequences did not cluster with the same species from this study. In the first case GenBank sequence gi:4049475 is listed as being obtained from *P. hamadryas* but the two *P. hamadryas* from this study PR00440 and PR00559 cluster together outside of the *P. cynocephalus* / *P. anubis* group whereas gi:4049475 clusters with *P. anubis* samples. Since it is known that *P. anubis* and *P. hamadryas* do interbreed (Szmulewicz *et al.* 1999) it is possible that the GenBank sample represents a hybrid individual. In any event this study supports Newman *et al.*'s (2004) finding that *P. cynocephalus* and *P. anubis* are not monophyletic clades but rather cluster together.

The second case involves *C. polykomos* (gi:4239860) and *C. guereza* (gi:60392100). These two *cox1* sequences were submitted independently to GenBank; they differ at only 8 positions out of the 1545 bp that constitute the whole *cox1* sequence, in fact in the first 700 bp which constitute the region used for molecular barcoding they differ at only a single position. Clearly these two individuals fall within the range of variation of a single species. One of the samples from this study (PR00655, *C. guereza*) clusters 100% of the time with these two GenBank sequences. Two other samples (PR00597, *C. polykomos* and PR00980, *C. guereza*) also cluster together 100% of the time. The two *Colobus* samples cluster together as well 100% of the time. Thus we will need to determine whether the species named associated with these samples are indeed correct or whether *cox1* will not differentiate these species as in the *P. anubis*/*P. cynocephalus* case.

In the third case a specimen from GenBank (gi: 12484065, listed as *T. gelada)* does not cluster with the *T. gelada* from this study nor does it even cluster with other African cercopithecids, perhaps representing a numt, contamination or a misidentification of the original sample. This sample points out the importance of having barcode sequences linked to a morphologically vouchered specimen so that such anomalies can

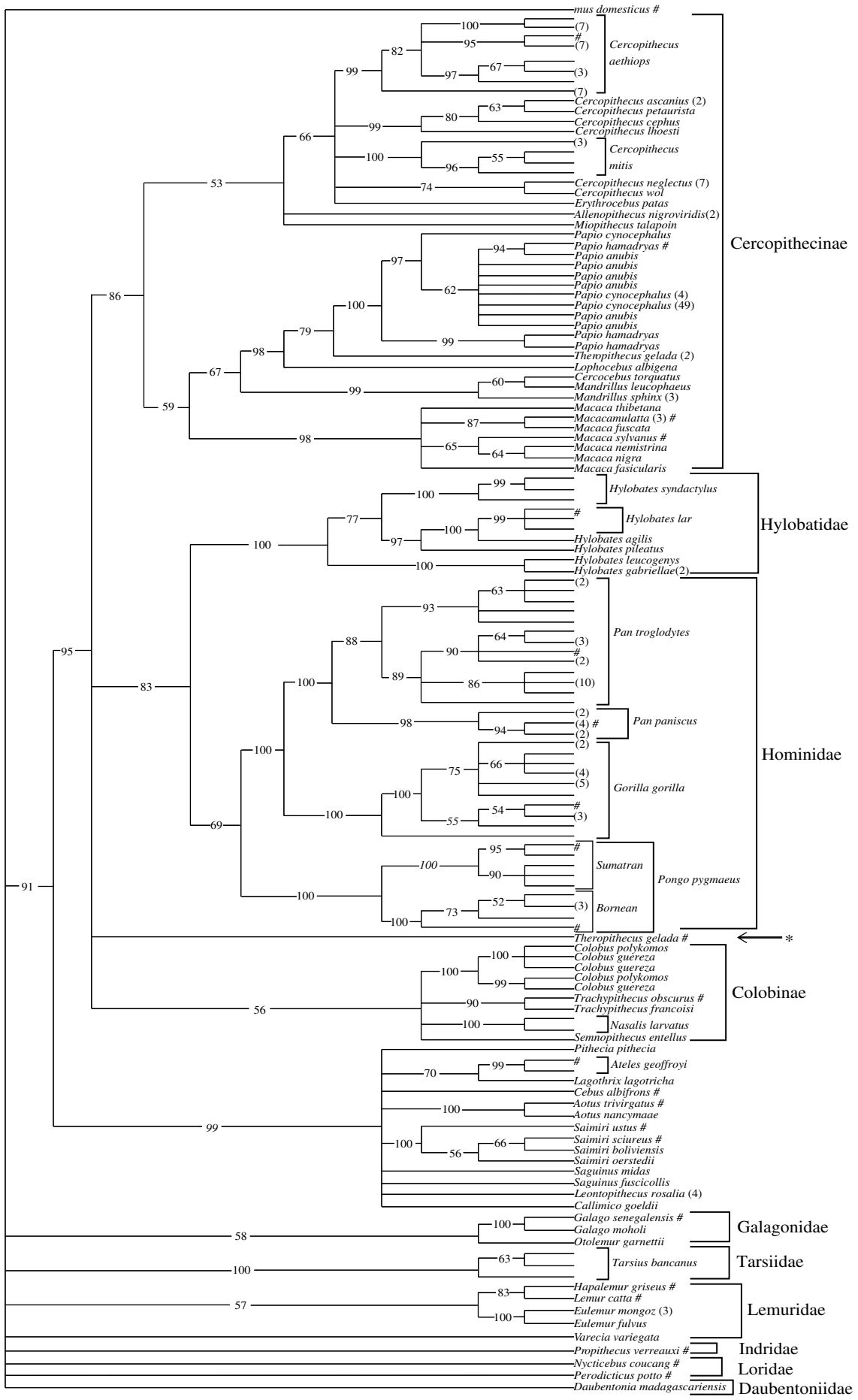

Figure 2. Bootstrapped neighbour-joining tree calculated from Kimura 2p distances and based on 1000 replicates. The number in parentheses indicates the number of samples that share identical sequences at that position; #, indicates the position of *cox1* sequences obtained from GenBank.

Table 3. Samples from this study that cluster with cox1 sequences from fully sequenced mtDNAs and cox1 sequences from previous studies obtained from GenBank.

GenBank acc no.	species	IPBIR sample number	species
gi:4239858	*Ateles geoffroyi*	PR00134	*Ateles geoffroyi*
gi:12484071	*Saimiri sciureus*	PR00741	*Saimiri oerstedii*
gi:12484069	*Saimiri ustus*	PR00474	*Saimiri boliviensis*
gi:4239860	*Colobus polykomos*	PR00655	*Colobus guereza*
gi:60392100	*Colobus guereza*	PR00597	*Colobus polykomos*
		PR00980	*Colobus guereza*
gi:60392086	*Trachypithecus obscura*	PR01099	*Trachypithecus francoisi*
gi:49146236	*Macaca mulatta*	PR00112	*Macaca mulatta*
		PR00408	
gi:14010693	*Macaca sylvanus*		with all macaques
gi:5835638	*Papio hamadryas*	PR00041	*Papio anubis*
gi:12484067	*Cercopithecus aethiops*	BP00219	*Chlorocebus*
		BP00214	*(Cercopithecus) aethiops*
		BP00211	
		BP00221	
		BP00220	
		BP00213	
		BP00215	
gi:5835820	*Hylobates lar*	PR00495	*Hylobates lar*
		PR00715	
gi:5835834	*Pongo pygmaeus abelii*	PR00253	*Pongo pygmaeus*
		PR00841	Sumatran
		PR00054	
		PR01003	
gi:5835163	*Pongo pygmaeus*	PR00276	*Pongo pygmaeus*
		PR01011	Bornean
		PR00002	
		PR00648	
		PR00488	
gi:5835121	*Pan troglodytes*	PR00744	*Pan troglodytes*
		PR00643	
		PR00660	
		PR00512	
		PR00226	
		PR00953	
gi:5835135	*Pan paniscus*	PR00092	*Pan paniscus*
		PR00446	
		PR00111	
		PR00367	
		PR00366	
gi:5835149	*Gorilla gorilla*	PR00573	*Gorilla gorilla*
		PR00265	
		PR01054	
		PR00807	
gi:12484065	*Theropithecus gelada*		does not cluster with cercopithecines
gi:21449875	*Lemur catta*	PR00126	*Lemur catta*
		PR00715	
gi:4239864	*Galago senegalensis*	PR00519	*Galago moholi*

be resolved by returning to the original sample (Ruedas *et al.* 2000).

The generation of incorrect sequences appears to happen for several reasons, not the least of which being the misidentification of the original material. Other problems involve primer specificity and the amplification or co-amplification of numts. Still other problems for the repository involve contamination of the cell culture in the laboratory. Non-target DNA from contaminants or numts can easily result in the extraction or amplification of incorrect or chimeric DNA sequences.

For molecular barcoding to have forensic value, reference barcode sequences should be derived from, and linked to, voucher specimens in web-accessible collections. NCBI maintains a number of databases (including GenBank, PubMed, Taxonomy and others) that are linked together in the Entrez indexing and retrieval engine. The LinkOut program allows outside groups to maintain sets of hotlinks from objects in Entrez back to specific locations on their web sites. The Integrated Primate Biomaterials and Information Resource has indexed holdings in the taxonomy domain of Entrez and indexed barcode sequences derived from the repository specimens in the sequence domain of Entrez (GenBank). This is a simple and practical approach to the problem of linking biological specimens with the biological data and research that

Table 4. Samples from this study that cluster consistently in a neighbour-joining bootstrap analysis based on Kimura 2p distances.

bootstrap value	IPBIR sample number	species
100	PR00786	*Leontopithecus rosalia*
	PR00960	
	PR00961	
	PR00963	
100	PR00232	*Mandrillus leucophaeus*
	PR00295	
100	PR00398	*Mandrillus sphinx*
	PR01048	
	PR00718	
100	PR00566	*Cercopithecus ascanius*
	PR00634	
100	PR00710	*Cercopithecus mitis*
	PR00993	
	PR00995	
	PR00987	
	PR00991	
	PR00997	
100	PR01121	*Cercopithecus neglectus*
	PR00981	
	PR00983	
	PR00985	
100	PR00100	*Allenopithecus nigrovirids*
	PR00198	
100	PR00969	*Hylobates syndactylus*
	PR00598	
	PR00721	
100	PR00381	*Hylobates gabriellae*
	PR00652	
100	PR00370	*Eulemur mongoz*
	PR00338	
	PR00288	

are derived from them. The individual barcodes validate the species identification of specimens submitted to the repository and collectively form a publicly available reference database for primate molecular diagnostics.

Although GenBank encourages the separate submission of identical sequences obtained from multiple specimens, current practice in phylogenetics research often involves reporting only the variable haplotypes. For barcoding to assume a quantitative approach to species diagnosis, barcode sequences have been submitted for all specimens in the study. Moreover, data quality is of crucial importance to barcoding if it is to develop into a forensic tool. The Consortium for the Barcode of Life (CBOL; www.barcoding.si.edu) Database Working Group is calling for the deposition of barcode sequences in GenBank together with the primers that were used to generate them, their trace files and associated quality scores.

With the characterization of the IPBIR collection, we have expanded the number of primate barcodes from about a dozen sequences from unvalidated source material in GenBank (derived from primate whole mtDNA sequences), to include 56 species of the approximately 200 species in the order primates. This work sheds light on the reliability of the existing data and represents a significant increase in the potential for

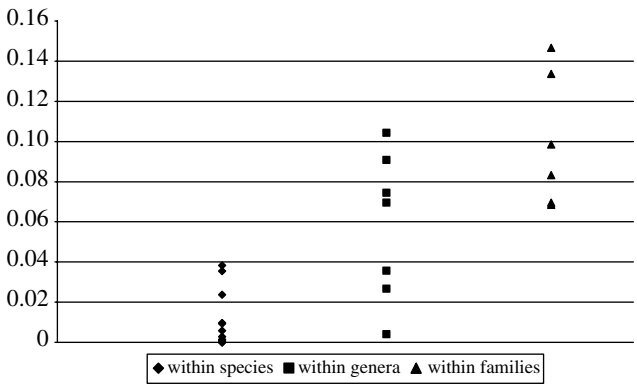

Figure 3. Mean pairwise differences (Kimura 2p) at various taxonomic levels.

DNA barcoding to be employed as a tool for molecular diagnostics of primates.

Prior to this study, a major practical concern for DNA barcoding was the relatively few sequences deposited for which a specimen was available for re-examination. The deposition of materials in IPBIR, a public-access collection, provides a mechanism to allow verification of potentially problematic data and the re-examination of source material as advocated by Ruedas *et al.* (2000). The DNA barcode data generated by IPBIR represents sequences not previously found in GenBank. Thus, we hope the quality control efforts performed on the samples provided to the IPBIR will contribute significantly to the expansion of data available for the study of primate genetic diversity.

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