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Multiple Displacement Amplification for Generating an Unlimited Source of DNA for Genotyping in Nonhuman Primate Species

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We evaluated a whole genome amplification method—multiple displacement amplification (MDA)—as a means to conserve valuable nonhuman primate samples. We tested 148 samples from a variety of species and sample sources, including blood, tissue, cell-lines, plucked hair and noninvasively collected semen. To evaluate genotyping success and accuracy of MDA, we used routine genotyping methods, including short tandem repeat (STR) analysis, denaturing gradient gel electrophoresis (DGGE), Alu *repeat analysis, direct sequencing, and nucleotide detection by tag-array minisequencing. We compared genotyping results from MDA products to genotypes generated from the original (non-MD amplified) DNA samples. All genotyping methods showed good results with the MDA products as a DNA template, and for some samples MDA improved genotyping success. We show that the MDA*

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procedure has the potential to provide a long-lasting source of DNA for genetic studies, which would be highly valuable for the primate research field, in which genetic resources are limited and for other species in which similar sampling constraints apply.

KEY WORDS: *Alu*-SINE; minisequencing; multiple displacement amplification; short tandem repeat; single nucleotide polymorphism.

INTRODUCTION

Advances in molecular ecology and an increased amount of genomic sequence information from different species available in the public databases have allowed the design of genotyping methods for molecular studies on relatedness, population structure, and demographic history of wild-living populations. However, the ethics of invasive sampling from many populations are often debated and challenged (Goossens *et al.*, 2000; Taberlet *et al.*, 1996). Primates, in particular, are sensitive in this respect (Constable *et al.*, 2001; Morin *et al.*, 1994) because the potentially traumatic effects of immobilization and sampling from individuals living in complex, flexible, and highly responsive societies can jeopardize behavioral and ecological studies in the group (Anthony *et al.*, 2003).

At the same time, there is also an increasing demand on high-quality DNA from primate taxa from the scientific community for benchmarking in studies in areas such as medical genetics (Holbrook *et al.*, 2000), systematics (Weiss and Goodman, 2001), biodiversity assessment (Meijaard and Nijman, 2003) and wildlife forensics. Primates remain model study systems for human diseases (Olanow, 2004), and recent efforts to sequence chimpanzee (Olson and Varki, 2003) and rhesus macaque genomes (http://www.genome.gov/11008262) imply that DNA samples from primates will remain crucial in comparative genomic studies. Many primates are also threatened with extinction (Harcourt and Parks, 2003), and there is a need both to augment the systematics of many primate taxa using DNA sequence data and to study genetic diversity at the population level to assist in conservation management programs. There are, therefore, many calls on the limited primate genetic resources currently available for such studies. The formation of key groups of researchers and institutions that are involved in the collection, curation, and distribution of the valuable material, such as the Integrated Primate Biomaterial Information Resource in the United States (IPBIR: www.ipbir.org) and the INPRIMAT consortium in Europe (www.inprimat.org) has recognized the demand globally. It seems likely that ethical considerations will in the future lead to maintaining fewer primates in captivity both in zoos and biomedical institutions (Balmford *et al.*,

1996), which will inevitably further decrease the availability of fresh material for genetic studies involving primates.

Though for decades researchers have successfully practiced immortalization of primate genetic material through lymphocyte transformation, which is a valuable source of material, the approach also has limitations because it requires fresh blood and does not work well for some species. Genomic rearrangements are a regular occurrence in other types of cell transformation, complicating genomic studies in particular. There is an urgent need to find methods to augment currently available ones to conserve and maintain primate genetic resources, and to take advantage of noninvasive procedures to obtain samples for genetic studies.

Whole genome amplification methods, such as multiple displacement amplification (MDA) (Dean *et al.*, 2002), are a potential approach to augment cell-line immortalization to produce viable primate genetic resources in perpetuity for the research community. MDA allows generation of thousands or even millions of copies of whole genomes in fragments of >10 kb pairs in length from small sources of genomic DNA (Dean *et al.*, 2002). The isothermal MDA reaction utilizes the highly processive bacteriophage phi29 DNA polymerase and its DNA strand-displacing activity (Blanco *et al.*, 1989). In the MDA reaction, random hexamer primers annealed to denatured genomic DNA are extended by the phi29 DNA polymerase to form products up to 100 kb (Dean *et al.*, 2002). As the DNA polymerase encounters another newly synthesized DNA strand downstream, it displaces it and thus creates a new single-stranded DNA template for priming. Strand displacement leads to hyperbranched primer extension reactions that may yield milligram amounts of DNA product from just a few nanograms of genomic DNA (Lasken and Egholm, 2003). Owing to its 3′–5′ proofreading activity, the fidelity of the phi29 DNA polymerase is very high with an error rate of $<10^{-6}$ (Blanco and Salas, 1985; Esteban *et al.*, 1993), which in turn requires exonuclease-protected primers to achieve a high yield (Dean *et al.*, 2001). As the reaction involves no thermal cycling and high molecular weight copies of genomic DNA are produced, the genomic coverage of MDA products is higher than that of the polymerase chain reaction (PCR)-based whole genome amplification methods degenerate oligonucleotide-primed PCR (DOP-PCR) and primer extension preamplification (PEP) (Dean *et al.*, 2002).

We evaluated MDA as a means to increase the amount of rare primate DNA for genotyping. We analyzed 148 DNA samples extracted from various biological materials from 22 different primate genera. We compared original DNA and MDA products via analyses of MHC class II loci, short tandem repeat markers, the mitochondrial control region and 12S

ribosomal RNA gene, an *Alu* repeat, and single nucleotides in 5 nuclear genes, using standard genotyping techniques.

MATERIALS AND METHODS

Samples

We analyzed 148 DNA samples from blood, cultured cells, semen, hair, and other tissues from 22 primate genera (Table I). The Primate Research Institute, Kyoto University, Japan, provided 15 Japanese macaque DNA samples extracted from blood. We extracted 19 noninvasively collected semen samples from Japanese macaques (Domingo-Roura *et al.*, 2004). We stored the 34 Japanese macaque samples as DNA extracts at 4°C for >10 yr. The Institute of Zoology, London, UK, kindly provided 11 ape samples extracted from blood. Cardiff School of Biosciences, Cardiff University, UK, provided 15 samples from apes and galagos. Of these, we extracted 5 gorilla samples from blood and the rest of the samples from plucked hair as described by Goossens *et al.* (1998). We extracted 10 samples of 6 species, including macaque, gibbon, baboon, and apes, from blood (B. Crouau-Roy, Université Paul Sabatier Toulouse, France). The Biomedical Primate Research Center, Rijswijk, The Netherlands, provided 57 samples from 34 species. Of these, we extracted 16 samples including macaque, tamarin, marmoset, langur, baboon, and African green monkey, from EDTA-blood or from immortalized B lymphocytes, and 3 orangutan samples from 1–5 hair follicles as described by Allen *et al.* (1998). We extracted the rest of the 57 samples, representing both Old and New World monkeys, from blood and cell lines. The German Primate Center, the Universities of Zürich and Freiburg, and the Institute of Zoology, London, provided 21 chimpanzee blood and tissue samples from muscle and liver biopsies obtained during diagnostic necropsies. We extracted DNA from blood, cell lines, or tissues using standard phenol-chloroform or salting-out methods as described by Sambrook *et al.* (1989; Table I).

Multiple Displacement Amplification

The concentration of the DNA samples ranged from <1 ng/μl to 50 ng/μl, and we subjected 1-μl aliquots of them to MDA. We performed the MDA reactions via the GenomiPhi^{TM} DNA Amplification kit (Amersham Biosciences, Uppsala, Sweden). Briefly, we added 1 μl of the DNA samples to 9 μl of sample buffer, and denatured at 95°C for 3 min

Table I. Species, DNA source, and marker analysis of samples

Species	Common name	No. of samples	DNA source	Marker
Allenopithecus nigroviridis	Allen's swamp monkey	1	Blood	12S
Ateles paniscus	Black spider monkey	1	EDTA-blood	12S
Callithrix jacchus	Common marmoset	2	EDTA-blood or immortalized B lymphocytes	STRa & MHC
Cebus apella sp.	Tufted capuchin	1	EDTA-blood	12S
Cercocebus torquatus	White-collared mangabey	1	Blood	12S
Cercopithecus aethiops pygerythrus	Vervet monkey	1	PBMC	12S
C. aethiops sp.	African green monkey	1	EDTA-blood or immortalized B lymphocytes	STRa & MHC
C. mitis albogularis	Blue monkey	1	PBMC	12S
C. mitis kolbi	Blue monkey	1	Cell-line	12S
C. mona	Mona monkey	1	PBMC	12S
Colobus polykomus	King colobus monkey	1	EDTA-blood	12S
Galago senegalensis	Senegal galago	1	Plucked hair	STRb
Galago sp.	Bush baby	1	Plucked hair	STRb
Gorilla gorilla	Gorilla	4	Blood	STRb
G. gorilla	Gorilla	2	Blood	STRc
G. gorilla gorilla	Western lowland gorilla	5	Blood	STRb
Hylobates lar	White-handed gibbon	1	Blood	STRc
H. symphalangus	Siamang	1	PBMC	12S
Leontopithecus chrysomelas	Golden-headed lion tamarin	1	EDTA-blood or immortalized B lymphocytes	STRa & MHC
Lophocebus albigena	Gray-cheeked mangabey	1	Frozen blood	12S
L. aterrimus	Black-crested mangabey	1	PBMC	12S
Macaca arctoides	Stump-tailed macaque	1	EDTA-blood	12S
M. fascicularis	Long-tailed macaque	4	EDTA-blood or immortalized B lymphocytes	STRa & MHC
M. fuscata	Japanese macaque	15	Blood	HVRI
M. fuscata	Japanese macaque	1	PBMC	12S
M. fuscata	Japanese macaque	19	Semen	HVRI
M. maura	Moor macaque	1	Cell-line	12S
M. mulatta	Rhesus macaque	4	EDTA-blood or immortalized B lymphocytes	STRa & MHC

Table I. Continued

Species	Common name	No. of samples	DNA source	Marker
M. nemestrina	Pig-tailed macaque	1	PBMC	12S
M. nigra	Celebes black macaque	1	Frozen blood	12S
M. silenus	Lion-tailed macaque	1	EDTA-blood	12S
M. sylvanus	Barbary macaque	4	Blood	STRc
M. sylvanus	Barbary macaque	1	PBMC	12S
M. thibetana	Tibetan macaque	1	EDTA-blood	12S
Mandrillus leucophaeus	Drill	1	Blood	12S
M. sphinx	Mandrill	1	Blood	12S
Pan troglodytes	Chimpanzee	4	Blood	STRb
P. troglodytes	Chimpanzee	21	Blood or tissue	Alu
P. troglodytes troglodytes	Central common chimpanzee	5	Plucked hair	STRb
Papio anubis	Olive baboon	1	Blood	12S
P. cynocephalus	Yellow baboon	1	PBMC	12S
P. papio	Guinea baboon	1	Blood	12S
P. papio	Guinea baboon	1	EDTA-blood or immortalized B lymphocytes	STRa & MHC
P. ursinus	Chacma baboon	1	Blood	12S
Papio sp.	Baboon	1	Blood	STRc
Pongo abelii	Sumatran orangutan	3	Hair	12S
P. abelii	Sumatran orangutan	2	SDS-conserved blood	12S
P. pygmaeus	Borneo orangutan	3	Blood	STRb
P. pygmaeus	Borneo orangutan	1	Blood	STRc
P. pygmaeus	Borneo orangutan	6	Blood	12S
P. pygmaeus	Borneo orangutan	3	Plucked hair	STRb
Saguinus oedipus	Cotton-top tamarin	2	EDTA-blood or immortalized B lymphocytes	STRa & MHC
Semnopithecus entellus	Hanuman langur	1	EDTA-blood or immortalized B lymphocytes	STRa & MHC
S. entellus	Hanuman langur	1	EDTA-blood	12S
Theropithecus gelada	Gelada baboon	2	EDTA-blood	12S
Trachypithecus johnii	Nilgiri langur	1	EDTA-blood	12S

Note. We performed nucleotide detection by tag-array minisequencing on all samples, except 5 chimpanzee samples for which we performed only analysis of *Alu* repeats. 12S: sequencing of the mitochondrial 12S rRNA gene; STRa & MHC: analysis of STR marker set a (Table II), sequencing and DGGE analysis of MHC class II loci; STRc: analysis of STR marker set c (Tables II and III); STRb: analysis of STR marker set b (Table II); HVRI: sequencing of the mitochondrial control region; *Alu*: analysis of *Alu*-SINE; PBMC: peripheral blood mononuclear cells.

before adding 9 μl of reaction buffer and 1 μl of enzyme mix to a final volume of 20 μl. We then incubated the reaction mixtures at 30°C for 17 h. The MDA yield in 20-μl reaction volumes varied from 1.5 μg/μl to 4 μg/μl. We measured the concentrations of the DNA samples and MDA products via the PicoGreen® reagent kit (Molecular Probes, Leiden, The Netherlands).

Analysis of the Major Histocompatibility Complex (MHC) Class II Loci

We amplified exon 2 from each of the *DQA1*, *DQB1*, *DPB1*, and *DRB* genes by PCR (Doxiadis *et al.*, 2003) from 250–500 ng of original DNA or 5–10 μl of a 1:100 dilution of the MDA products. We sequenced the amplified exon 2 of *DQA1*, *DQB1*, and *DPB1* (Doxiadis *et al.*, 2003), and analyzed the sequences using the Sequence Navigator program (Applied Biosystems, Foster City, CA). The primer pair for *DRB* amplification was 5′MDRB and 3′MDRB1 + GC (Knapp *et al.*, 1997). We analyzed the amplified exon 2 of *DRB* by denaturing gradient gel electrophoresis (DGGE) as described by Knapp and co-workers with minor modifications (Doxiadis *et al.*, 2000; Knapp *et al.*, 1997; Otting *et al.*, 2000). Briefly, we electrophoresed GC-clamped PCR products in a 9.5% acrylamide-gel with a 40–65% parallel denaturing gradient of formamide and urea in a BIORAD D-Gene apparatus (BIORAD, Richmond, CA) at constant temperature of 56°C and at 120 V for 16 h, followed by staining the gel using SYBR-green (Invitrogen Life Technologies, Carlsbad, CA).

Analysis of Short Tandem Repeat Markers

Cecilia Penedo, University of California, Davis, CA, donated primers for 7 STR markers (marker set a in Table II). The sequences for 5 human primer pairs (D8S1106, D3S1768, D18S72, D4S2365, and D13S765) are available at the Genome Database (http://www.gdb.org/) and we developed primers for MFGT21 and MFGT22 from *Macaca fuscata* sequences (Domingo-Roura *et al.*, 1997). We performed PCR in a final volume of 25 μl containing 20–100 ng of genomic DNA or 2 μl of a 1:100 dilution of the MDA product and 5′primers fluorescently labeled with either Fam, Ned, or Vic (Applied Biosystems) (Andrade *et al.*, 2004). We ran 1 μl of each PCR product on an ABI 310 Genetic Analyzer (Applied Biosystems) and determined the fragment sizes via STRand (http://www.vgl.ucdavis.edu/STRand). We amplified an additional STR

marker set (marker set b in Table II), using the human PCR primers given in the Genome Database (http://www.gdb.org/). We used PCR conditions for D8S1106 as described by Lathuilliere *et al.* (2001) and adapted conditions for the other markers from Bradley *et al.* (2000; D7S2204 and D10S1432), Clifford *et al.* (1999; D1S548, D3S1768, and D5S820), Nurnberg *et al.* (1998; D5S820), and Smith *et al.* (2000; D4S243 and D10S1432). We ran the PCR fragments on an ABI PRISM® 377 Sequencer (Applied Biosystems) and performed size analysis via Genotyper® 3.6 (Applied Biosystems). We amplified 9 additional STR markers (marker set c in Table II and all markers in Table III) by human primers given in the Genome Database (http://www.gdb.org/) in an optimized multiplexed reaction (Jeffery, 2003). The loci analyzed included D1S550, D2S1326, D4S1627, D5S1457, D10S1432, D16S2624, D21S11, Amelogenin, and HUMFIBRA. We ran the PCR fragments on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) and performed size analysis using GeneScan® Analysis 3.5.1 (Applied Biosystems).

Analysis of *Alu* Short Interspersed Elements (SINEs)

We tested 4 *Alu* elements termed Yc1JXPan4_2, Yc1JXPan7, Yc1JXPan8, and Yc1JxPan24 derived from GenBank accession nos. AC092764, AC096849, AC119571, and AC097330, respectively, for insertion presence or absence polymorphisms of the *Alu* elements. We applied PCR primers and conditions according to Salem *et al.* (2003), and subjected 300 ng of genomic DNA or 1 μl of MDA product to 30 cycles of a wax-mediated hot start PCR using the Qiagen *Taq* PCR Core Kit (Qiagen GmbH, Hilden, Germany). We analyzed the PCR products on 1% agarose gels stained with ethidium bromide.

Sequencing of Mitochondrial DNA

We amplified a 392-bp fragment of the hypervariable segment I of the mitochondrial control region via PCR and sequenced the fragment using the human primers L15996 and H16401 (Vigilant *et al.*, 1989) as described in Marmi *et al.* (2004), but using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). We amplified a 389-bp fragment of the mitochondrial 12S rRNA gene via PCR and sequenced the fragment using the conserved primers L1091 and H1478, and conditions essentially according to published methods (Kocher *et al.*, 1989). We isolated the PCR products from agarose gels using the QIAquick Gel Extraction Kit (Qiagen GmbH),

Table II. STR genotyping in original DNA and MDA products from blood or cell lines

Marker	No. of samples	Genotyping success		
		DNA = MDA	DNA > MDA	MDA > DNA
MFGT21[a]	16	15 (8)	1[d]	—
D8S1106[a]	16	14 (8)	2[d]	—
D3S1768[a]	16	16 (10)	—	—
D18S72[a]	16	15 (7)	—	1
D4S2365[a]	16	12 (2)	3	1
D13S765[a]	16	12 (5)	3[d]	1
MFGT22[a]	16	12 (6)	2[d]	2[d,e]
D3S1768[b]	10	9	1	—
D4S243[b]	10	5	5	—
D7S2204[b]	10	7	3	—
D10S1432[b]	10	9	1	—
D1S548[b]	10	8	2	—
D5S820[b]	10	9	1	—
D8S1106[b]	10	8 (1)	2	—
Amelogenin[c]	10	10	—	—
D1S550[c]	10	10	—	—
D2S1326[c]	10	10	—	—
D4S1627[c]	10	10	—	—
D5S1457[c]	10	9	1[d]	—
D10S1432[c]	10	10	—	—
D16S2624[c]	10	10	—	—
D21S11[c]	10	10	—	—
HUMFIBRA[c]	10	10	—	—

Note. DNA = MDA: Identical genotyping success of original DNA and MDA products, in parentheses are no. of samples that failed for both templates; DNA > MDA: Original DNA was successfully genotyped while MDA products were not. MDA > DNA: MDA products were successfully genotyped while original DNA was not.

[a]STR marker set a.

[b]STR marker set b.

[c]STR marker set c.

[d]We observed allelic dropout in 5 cases. Three of these cases involved MDA product from the same sample.

[e]In 1 case we observed 2 alleles different from the original allele for the MDA product.

sequenced the products via the BigDye® Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems), and analyzed the sequences via SeqMan II (DNASTAR, Madison, WI).

Nucleotide Detection by Tag-Array Minisequencing

In short, the tag-array minisequencing method (Lindroos *et al.*, 2002; Lovmar *et al.*, 2003) comprises the following steps: We amplify genomic DNA or MDA products by multiplexed PCR. We use the PCR products

Table III. STR genotyping in original DNA and MDA products from hair

Marker	No. of samples	DNA = MDA	Allelic dropouts[a]	False alleles[a]
Amelogenin	8	8	—	—
D1S550	8	7	1	—
D2S1326	8	3	2	3
D4S1627	8	5	2	1
D5S1457	8	6	2	—
D10S1432	8[b]	6	1	—
D16S2624	8	8	—	—
D21S11	5	4	1	—
HUMFIBRA	8	7	1	—

Note. DNA = MDA: Identical genotyping success of original DNA and MDA products.
[a]We observed allelic dropouts and false alleles in MDA products only.
[b]One MDA product failed to genotype.

from each sample as templates in a multiplexed single base primer extension (minisequencing) reaction in solution, in which minisequencing primers designed to anneal immediately adjacent to the nucleotide position to be detected are extended by a DNA polymerase with 1 out of 4 fluorescently labeled ddNTPs. The 5′-end of each minisequencing primer contains a unique 20-bp tag sequence from the Affymetrix GeneChip® Tag collection (Affymetrix, Santa Clara, CA) (Fan *et al.*, 2000), and hybridization to complementary oligonucleotides that are immobilized on a microarray (glass-slide) in an array-of-arrays conformation captures the extended minisequencing primers (Pastinen *et al.*, 2000). The conformation allows detection of all 9 nucleotide positions in both polarities in 80 samples simultaneously on each slide.

We chose 5 universal primer pairs for the genes encoding the brain-derived neurotrophic factor (BDNF), estrogen receptor 1 (ESR1), nerve growth factor B (NGFB), tumor necrosis factor-α (TNF-α), and prodynorphin (PDYN; Jiang *et al.*, 1998) to design PCR primers in conserved exon DNA sequences. We downloaded all available primate DNA sequences for the 5 genes from GenBank® databases (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and aligned them via MultAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html). We then redesigned the universal PCR primers to match the primate genomic sequences better. All PCR products gave 1 unique hit to the available genomic sequences according to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). We designed minisequencing primers for each gene in both DNA polarities via NetPrimer (http://www.premierbiosoft.com/netprimer/). Integrated DNA Technologies (Coralville, IA) synthesized the oligonucleotides. We will provide the oligonucleotide sequences on request.

Table IV. PCR success of 4 MHC class II loci in original DNA and MDA products

		PCR success		
MHC class II loci	No. of samples	DNA = MDA	DNA > MDA	MDA > DNA
DRB	16	10 (2)	3 (2)	3
DQA1	16	15 (1)	1 (1)	—
DQB1	16	12	3 (1)	1
DPB1	16	14 (5)	1	1

Note. DNA = MDA: Identical PCR amplification success of original DNA and MDA products; in parentheses are no. of samples that failed for both templates. DNA > MDA: Original DNA amplified more efficiently than MDA products; in parentheses are no. of samples that failed for MDA products. MDA > DNA: MDA products amplified more efficiently than original DNA.

We subjected 4 μl of the original DNA samples (concentration range <1 ng/μl–50 ng/μl) or of 1:100 dilutions of MDA products to 5-plex PCRs using the AccuPrime™ *Taq* DNA Polymerase System (Invitrogen Life Technologies). We performed the minisequencing reactions and subsequent hybridization to the c-tag oligonucleotides on the microarray slides as in Lovmar *et al.* (2003), and scanned the slides via ScanArray® Express (Perkin-Elmer Life Sciences, Boston, MA).

RESULTS

We analyzed 148 DNA samples from various types of biological material from 22 primate genera. We compared original DNA and MDA products from each sample by DGGE analysis and sequencing of MHC class II loci, analysis of 21 STR markers, sequencing of the mitochondrial control region and 12S ribosomal RNA gene, analysis of an *Alu* repeat, and nucleotide detection by tag-array minisequencing of 9 single nucleotides in 5 nuclear genes (Table I).

Analysis of the MHC Class II Loci

We amplified exons 2 of the polymorphic MHC class II loci *DRB*, *DQA1*, *DQB1*, and *DPB1* from 16 blood and cultured cell samples (Table I). As estimated by agarose gel electrophoresis, the quantities of the PCR products from original DNA and MDA products were comparable for most samples and loci (77%). We observed 8 cases (14%) when amplification of original DNA was more efficient than that of MDA product, and 5 cases (9%) when the opposite was true (Table IV).

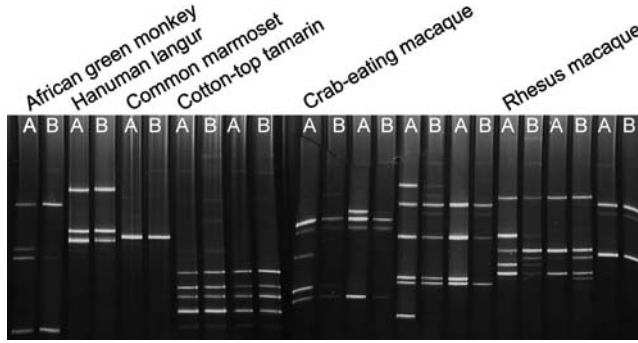


Fig. 1. Genotyping the MHC class II *DRB* loci using denaturing gradient gel electrophoresis. We loaded 12 successfully PCR-amplified samples in pairs, with MDA products in lanes A and original DNA in lanes B. Samples are, from left to right: African green monkey, Hanuman langur, common marmoset, 2 cotton-top tamarins, 4 long-tailed macaques, and 3 rhesus macaques.

When we subjected PCR products of exon 2 of *DPB1*, *DQA1*, and *DQB1* to direct sequencing, all amplicons were identical to the respective MHC sequence. For the homozygous genotypes, MDA products and original DNA always showed the same DNA sequence. However, in the case of heterozygosity, sequencing of original DNA mostly showed an equal quantity of both alleles, whereas in amplicons originating from MDA products, we observed imbalanced amplification of 1 allele for some of the samples. For the multicopy MHC-DRB gene, we applied DGGE for genotyping. In DGGE, PCR products are separated according to sequence-specific differences in denaturation, and therefore usually 1 allele is represented by 1 band in the gel. In all samples, products from original DNA and MDA products showed concordant migration in electrophoresis, though we observed some differences in band intensities. Even heteroduplexes with slower migrating and weaker bands than the actual *DRB* alleles showed similar banding patterns. In Fig. 1 the allelic banding patterns of the Hanuman langur sample, all 3 samples of New World monkeys, and the right-most rhesus macaque sample were identical and equally strong, whereas the bands from MDA products of all 4 long-tailed macaque samples were stronger. In contrast, in 2 rhesus macaque samples and in the African green monkey sample the *DRB* alleles appeared to be more efficiently amplified from original DNA in some cases, and in other cases MDA products performed better. The differences in amplification efficiency for the *DRB* loci do not seem to be dependent on haplotype and may be due to stochastic effects during amplification.

Analysis of Short Tandem Repeat Markers

In total, we analyzed 21 STR loci in DNA samples originating from blood, cultured cells, or hair (Table I). We analyzed 7 STR markers (marker set a in Table II), located on different chromosomes, in the same set of 16 samples as the MHC loci above. The results are 92% concordant between original DNA and MDA products, with one 1 spurious allele and 4 allelic dropouts detected in the MDA products. Three of the allelic dropouts occurred in the same rhesus macaque sample. We achieved successful amplification of both genomic DNA and MDA products in 76% of the cases. Comparable to the PCR amplification of the MHC region, amplification of some loci was successful only from the original DNA. For 4 samples, the MDA products amplified better than the original DNA (Table II).

We analyzed another set of 7 STR markers (marker set b in Table II), from 7 different chromosomes, in another sample set (Table I). The 10 samples, originating from blood, showed no allelic dropout or genotype discrepancy when we compared the genotypes in original DNA to MDA products. In 78% of the cases the genotyping was successful in both original DNA and MDA products, and in 22% of the cases the alleles were amplified only in original DNA (Table II).

We analyzed a set of 9 STR markers in 2 sample sets originating from blood (marker set c in Table II) or hair (Table III), respectively (Table I). Ten of the samples originating from blood showed fully concordant results for the original DNA and MDA products, except for 1 allelic dropout in a single MDA product. In 78% of the cases, the results from hair samples were fully concordant between original DNA and MDA products. We observed, however, 10 cases of allelic dropout and 4 spurious alleles in MDA products from 5 of the samples. In 1 case we obtained no amplification product from the MDA products.

Analysis of *Alu* Short Interspersed Elements

We found the *Alu*-SINEs Yc1JXPan4_2, Yc1JXPan7, Yc1JXPan8, and Yc1JXPan24 all to be present in chimpanzees, whereas they were absent at the orthologous loci in humans and other great apes. We screened original DNA and MDA products from 21 chimpanzee blood and tissue samples for the insertion presence or absence of *Alu* polymorphisms. Only the locus Yc1JXPan7 proved to be polymorphic, owing to an *Alu*-integration giving rise to a fragment size polymorphism with allelic size differences in the range of a complete 300-bp *Alu* element. The genotyping success in

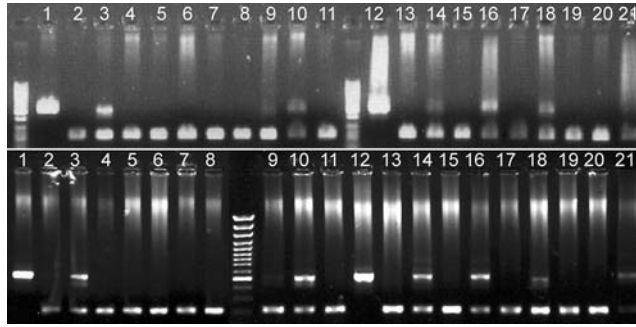


Fig. 2. Agarose gel electrophoresis of the amplified polymorphic *Alu*-SINE Yc1JXPan7 in 21 chimpanzee samples. We observed insertion presence or absence of the 300-bp fragment. PCR products from original DNA are in the upper panel and those from MDA products in the lower panel. We ran both gels with a 100-bp ladder.

original DNA and MDA products was similar, though the bands from the MDA products were more distinct. All results are concordant (Fig. 2).

Sequence Analyses of Mitochondrial DNA

We sequenced a 392-bp PCR product from the control region of mitochondrial DNA in 28 Japanese macaque samples, of which 12 originated from blood and 16 from semen. The sequences from the blood samples showed a high nucleotide identity between original DNA and MDA product, ranging from 98% to 100% for each sample within the common read length, which was on average 329 bp. Similarly, the sequences from semen samples showed a high nucleotide identity ranging from 98% to 100% for each sample. All but 3 sequences from the semen samples were 100% identical within the common read length, which was on average 178 bp. We calculated the overall sequence identity for each sample source as the percentage of all nucleotides concordant between original DNA and MDA products, when summarizing all sequenced nucleotides within the common read lengths (Table V).

In addition, we sequenced a 389-bp PCR product in the mitochondrial 12S rRNA gene in 35 samples (Table I), of which 3 originated from hair and 32 from blood or cell lines. The common read length from original DNA and MDA products was on average 375 bp. Within the region, the nucleotide identity of the sequences from original DNA and MDA products was 99–100% in almost all samples. One of the 3 Sumatran orangutan hair samples had a lower nucleotide identity of 95%, and in the Hanuman

Table V. Sequence analysis of 2 mitochondrial DNA regions

Sample source	No. of samples	Average common read length	Sequence identity (%)[a]
Control region			
Blood	12	329	99.4
Semen	16	178	99.8
12S rRNA gene			
Blood/cell line	32	373	99.3
Hair	3	386	98.3

[a]Total number of identical nucleotides over all common read lengths in all samples.

langur blood sample the nucleotide identity was 90%. As for the sequences from the control region, we calculated an overall sequence identity for each sample source (Table V).

Nucleotide Detection by Tag-Array Minisequencing

Finally, to analyze the whole sample collection with the same method, we analyzed 2 conserved nucleotide positions in each of the *BDNF*, *ESR1*, *NGFB*, and *TNF-α* genes, and 1 nucleotide position in the *PDYN* gene by tag-array minisequencing. We genotyped 143 samples for the 9 nucleotide positions in both DNA polarities in parallel (Table I). On the same microarray slide and in the same experiment, we analyzed 18 minisequencing products from both the original DNA and the corresponding MDA products. An example of nucleotide detection in original DNA and MDA product from 1 chimpanzee sample is in Fig. 3.

The reproducibility of nucleotide detection was >99.9% based on repetition of 70% of the assays. The overall genotyping success rate was 93% (2397/2574 genotypes) for the MDA products and 94% (2424/2574 genotypes) for the original DNA. The concordance rate between nucleotides that we successfully genotyped in both original DNA and MDA products was >99.9%. In Table VI we summarize the genotyping success rate for MDA products from varying amounts of DNA of different sample type, compared to the original DNA samples.

The noninvasively collected semen samples had a success rate of 100% for nucleotide detection in both MDA products and original DNA. We successfully detected all 18 nucleotides in both original DNA and the corresponding MDA products in 81 (57%) of the samples. We observed a difference of ≥ 3 in number of successfully detected nucleotides between original DNA and the corresponding MDA product in 24 (17%) samples. For 13 samples (9%) the success rate was higher for the MDA products than the

Table VI. Success rate of nucleotide detection using tag-array minisequencing

	DNA sample source		
DNA input[a]	Blood or cell line ($n = 111$)	Hair ($n = 13$)	Semen ($n = 19$)
>10 ng ($n = 44$)	No. of nucleotides successfully detected		
MDA product	642 (94%)	34 (94%)	72 (100%)
Original DNA	671 (98%)	35 (97%)	72 (100%)
1–10 ng ($n = 66$)			
MDA product	825 (96%)	29 (54%)	270 (100%)
Original DNA	827 (96%)	35 (65%)	270 (100%)
<1 ng ($n = 33$)			
MDA product	409 (91%)	116 (81%)	
Original DNA	375 (83%)	139 (97%)	

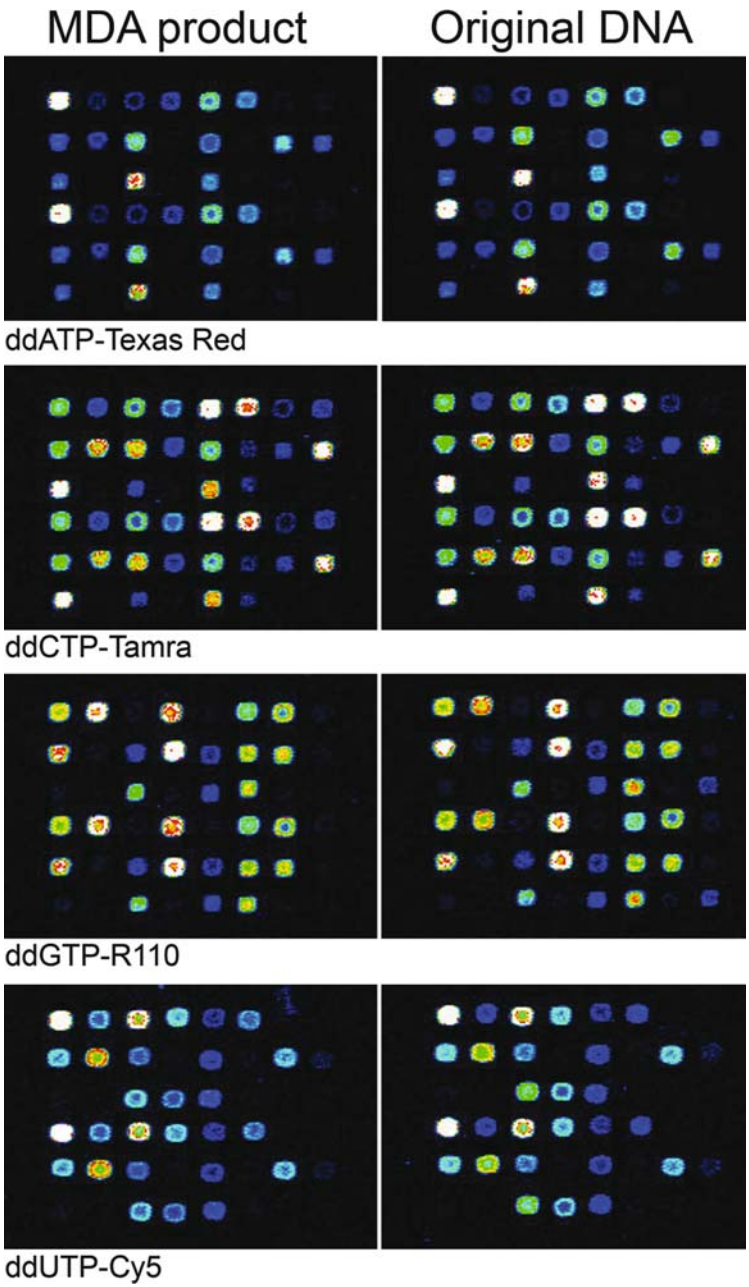
[a]Amount of DNA subjected to MDA.

original DNA. We observed no systematic correlation between the amount of DNA subjected to MDA and the genotyping success. The amount of DNA subjected to the MDA reaction was <1 ng in 32 samples. Of these samples 8 were hair samples and 3 of them had a higher success rate in the original DNA. In contrast, for 10 samples with <1 ng DNA derived from blood or cell lines, the success rate was higher for the MDA products (Table VI).

Phylogeny-Specific Nucleotide Signatures

Five of the 9 nucleotide positions varied between the analyzed species, and formed characteristic nucleotide signatures that follow the primate phylogeny (Table VII). We successfully genotyped the strepsirrhines,

Fig. 3. Example of results from detection of 9 nucleotide positions in MDA products (left) and original DNA (right) from 1 chimpanzee sample by tag-array minisequencing. Images are shown for 2 of the 80 subarrays on a microarray slide, obtained by scanning the slide at 4 wavelengths for detecting Texas Red-labeled ddATP, Tamra-labeled ddCTP, R110-labeled ddGTP, and Cy5-labeled ddUTP, respectively. We detected each nucleotide position in both DNA polarities and in duplicate, seen as identical patterns of 18 spots in the upper and lower halves of each subarray. The signal strength of the spots is visualized by a rainbow scale, where blue indicates a weak signal and white a strong signal. Each subarray also includes spots corresponding to positive controls for the minisequencing reaction, spotting density, and hybridization efficiency, and to negative controls (Lovmar *et al.*, 2003).


MDA product
Original DNA
ddATP-Texas Red
ddCTP-Tamra
ddGTP-R110
ddUTP-Cy5

Continued

represented by 2 galagos, at 8 nucleotide positions, displaying a characteristic nucleotide signature. Analogously, the genotypes of New World monkeys formed 1 nucleotide signature, with only 1 genotype that deviated from the signature in 2 cotton-top tamarin samples. The genotypes of all Old World monkeys, mainly represented by 55 macaques, formed 1 nucleotide signature, with the only exception of 2 mangabey samples that had a different nucleotide variant at 1 position. Genotypes of the gibbons and great apes together formed one nucleotide signature. We also genotyped humans, represented by 4 Swedish males, forming an exclusive nucleotide signature. We confirmed all the nucleotide signatures by the genotypes determined in the original DNA (Table VII).

DISCUSSION

We aimed to evaluate MDA as a method to perpetuate primate genetic material using as wide a variety of sample types and molecular approaches commonly applied in genetics laboratories today. This method is potentially relevant not only for perpetuating the scarce remains of once plentiful DNA samples that are irreplaceable, but also could be applied to the increasingly important class of samples collected noninvasively from the field. Currently, noninvasive genetic analysis is problematic for multiple reasons. In particular, the low quantity and quality of DNA extracts commonly available from noninvasively collected samples can result in problems during PCR amplification. Issues include the production of erroneous genetic data such as through DNA sequence artefacts (Dalen *et al.*, 2004), stochastic nonamplification of alleles (McKelvey and Schwarz, 2004), generation of false alleles, e.g., in microsatellite typing (Valiere *et al.*, 2002), problems of contamination, especially from human sources (Bayes *et al.*, 2000) or simply an absence of successful PCR altogether. It is potentially possible to address many of the problems by application of whole genome amplification methods such as MDA. A commonly used source of genetic material in primatology is noninvasively collected fecal samples, though, apart from degradation, it might present problems owing to the presence of large quantities of DNA from ingesta and gut flora, which would make it difficult to estimate the amount of DNA subjected to MDA. Fecal samples may also contain inhibitors of the phi29 DNA polymerase, causing failure of MDA, analogously to failure of PCR.

Researchers have already described a wide range of applications for MDA. One of the useful applications, and also an advantage over PCR-based whole genome amplification methods, is direct amplification of cell lysates, as, e.g., buccal swabs and whole blood. Hosono *et al.* (2003) showed that the MDA products from the cells were indistinguishable from

Table VII. Phylogeny-specific nucleotide signatures consisting of 9 nucleotide positions in 5 different genes

	No. of samples	Gene and nucleotide position								
		BDNF-1	*BDNF-2*	*ESR1-1*	*ESR1-2*	*NGFB-1*	*NGFB-2*	*PDYN-1*	*TNF-α-1*	*TNF-α-2*
Human	4	T	A	G	A	A	G	C	C	C
Apes and gibbons	56	C	A	G	G	A	G	C	C	C
Old world monkeys	78	C	G[a]	G	A	G	G	C	C	C
New world monkeys	7	T[b]	G[b]	G	G	G	G	C	C	T
Galago	2	T	G	G	A	A	G	C	C	n.d.[c]

Note. BDNF: Brain derived neurotrophic factor; ESR1: estrogen receptor 1; NGFB: nerve growth factor B; PDYN: prodynorphin; TNF-α: tumor necrosis factor-α.

[a] Of the Old World Monkeys, 2 mangabey species, *Lophocebus albigena* and *Lophocebus aterrimus*, had a nucleotide A instead of a G at *BDNF-2*.

[b] Of the New World Monkeys, 2 cotton-top tamarins, *Saguinus oedipus*, had a nucleotide C instead of a T at *BDNF-1*, and determination of *BDNF-2* failed.

[c] In the galagos, determination of *TNF-α-2* failed.

the original genomic DNA in both SNP and STR DNA assays. Researchers have also successfully performed MDA directly on mosquito larva and adult legs (Gorrochotegui-Escalante and Black, 2003) and on residual cells left by incidental contact as fingerprints (Sorensen *et al.*, 2004). In our study the MDA reaction was always performed on purified genomic DNA from a variety of sample sources, though the MDA was advantageous when genotyping a sample with a high level of blood proteins left. MDA yield is dependent on the reaction volume, and not on the amount of DNA starting material (Dean *et al.*, 2002; Hosono *et al.*, 2003), and thus MDA gives a stable yield and eliminates the need for DNA concentration measurements and subsequent dilutions. We used MDA product directly after a 1:100 dilution and thus saved both sample material and time.

The success of MDA varied somewhat according to the species/tissue of origin/marker combination. It is reasonable to suppose that in principle most primate species present the same challenges of genomic complexity and marker availability, so our discussion of the value of MDA is confined to issues of DNA quality as a function of tissue origin and marker type. We analyzed 5 classes of DNA marker (4 nuclear markers and mitochondrial DNA) using 3 DNA sequence-based technologies and 3 fragment analysis approaches.

MHC gene products play an essential role in adaptive immunology and exhibit extensive polymorphism. Exon 2, which codes for the beta-1 domain of the peptide presenting molecule (*DRB*), is the most polymorphic. Some Old World monkeys are polygenic for *DRB* and variable numbers of copies of the gene may be present. Therefore, simple typing techniques, such as direct sequencing, are not always applicable. DGGE is as an appropriate prescreening method for multicopy genes, as usually 1 allele is represented by 1 band. For all samples analyzed, DGGE from original DNA and MDA products provided consistent patterns. In cases in which differences arose, only the intensity of the bands varied between MDA products and those generated from the original DNA. Further, direct sequencing of other variable class II loci, *DPB1, DQA1*, and *DQB1* produced concordant results between original DNA and MDA products.

We tested 3 different STR marker sets with DNA samples from different sources: blood, cultured cell samples, or hair. Therefore, the results are not directly comparable and amplification rates of original DNA and MDA products varied, as did the concordance of original DNA and MDA products. For example, we compared genotypes of 7 gorillas, 6 orangutans, and 5 chimpanzees for extracted DNA and DNA from the same extraction that had undergone MDA. We genotyped each pair of samples multiple times for 8 microsatellite loci and the amelogenin locus. In half of the samples MDA genotypes agreed with the original template DNA at all loci and

for 1 sample we observed allelic dropout at 1 locus for 1 of the original samples but not for the MDA product. Another individual showed no amplification across multiple loci for the original DNA but failed to amplify only at 1 locus in the MDA product. However, in contrast, MDA samples showed allelic dropout compared to the original extracts at multiple loci for 5 individuals and further, in 3 cases, alleles appeared in the MDA samples that were not detected in the original extracts. In general, however, allelic dropout/lack of amplification was more frequent for the MDA samples.

For STR analysis, as expected, samples extracted from hair showed more allelic dropout than samples from blood. We extracted from blood 8 of the 9 samples in which MDA DNA matched the original extracted DNA. All 5 samples in which the MDA samples showed allelic dropout compared to the original DNA extracts were from plucked hair. One blood and 1 plucked hair sample showed better results for MDA than for the original DNA extract. In general, it appears that performing MDA on good quality DNA yielded high-quality genotyping results. However, poor-quality samples provided MDA-generated genotypes of more variable quality than from original extracts, though the ability to produce large numbers of repeat amplifications with MDA template allows greater opportunity to produce accurate consensus genotypes.

One of the *Alu*-SINEs that we analyzed was polymorphic in the chimpanzee. The 21 DNA samples were of good quality and we used >10 ng DNA as input to the MDA reaction. Fragment size analysis of the presence or absence polymorphism was equally successful, and always showed concordant results, using the original DNA or the MDA products. The analysis demonstrates the stability and accuracy of MD amplification of high-quality DNA.

The high sequence identities shown between 392 bp of the mtDNA hypervariable I region from original DNA and WGA products demonstrate that it is possible to use MDA to obtain large amounts of DNA and then do the sequencing from the amplified product with the same results as from original DNA. Moreover, we have demonstrated that it is possible to use the technique even with samples of poor quality, such as DNA from semen samples, and obtain a very high sequence identity. This raises the possibility of using MDA with samples such as museum specimens for mtDNA, in which routine amplification and sequencing of DNA from such material can be problematic.

The array of arrays format of tag-array minisequencing permits genotyping of 80 samples for all nucleotide positions in parallel, and thus is well suited for comparative studies such as this. For all samples, we could genotype MDA products and original DNA in the same experiment, which facilitates simultaneous and accurate comparisons of the results.

In general, the success rate of nucleotide detection in MDA products was similar to that for the original DNA. We observed a tendency toward a lower success rate for hair samples of <10 ng DNA input amount, in agreement with the STR data, which is probably attributable to degraded starting material. Encouragingly, samples originating from blood or tissues of <1 ng DNA input amount were considerably more successful for MDA products than the original DNA. It therefore seems possible to conserve DNA samples of low concentration to a certain degree, bearing in mind that any imbalanced amplification of alleles would not be detected here, because the analyzed nucleotides positions are not polymorphic. It has previously been shown that 3 ng of DNA in the MDA reaction should be used to be certain of balanced amplification (Lovmar *et al.*, 2003).

Because the 9 nucleotide positions were well conserved and could easily be divided into nucleotide signatures that are specific for suborders or families in the primate order, we considered genotypes following this phylogeny correct. Only 3 species in this study, *Lophocebus albigena*, *L. aterrimus*, and *Saguinus oedipus*, deviated from the nucleotide signature, all at the same nucleotide position. Therefore, in cases in which original DNA failed to genotype while MDA products were successful, one could consider the genotypes observed as the correct ones. All genotypes of the 9 nucleotide positions corresponded to the nucleotides at the positions in the available primate sequences in public databases.

In conclusion, MDA seems an ideal method to replenish DNA in high-quality but almost exhausted DNA extracts. Further, though not eliminating the genotyping errors that affect DNA profiling in primates, especially for DNA extracted from noninvasively collected material, MDA shows promise as a method of perpetuating DNA samples with which one should apply the usual caution, replication, and experimental rigor. The rigorous methods are now well established (Valiere *et al.*, 2002) and thus MDA provides an opportunity to study samples that are usually rapidly exhausted and cannot be replaced. Thus, though the in-lab cost of DNA profiling may not necessarily be reduced for noninvasive samples (especially when adding the cost of the MDA reaction itself), substantial savings in time and money are possible on collecting fresh material in the field.

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