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RESEARCH ARTICLE

Molecular Cytogenetic Analysis of One African and Five Asian Macaque Species Reveals Identical Karyotypes as in Mandrill

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Abstract: ***Background*:** The question how evolution and speciation work is one of the major interests of biology. Especially, genetic including karyotypic evolution within primates is of special interest due to the close phylogenetic position of Macaca and *Homo sapiens* and the role as *in vivo* models in medical research, neuroscience, behavior, pharmacology, reproduction and Acquired Immune Deficiency Syndrome (AIDS).

***Material & Methods*:** Karyotypes of five macaque species from South East Asia and of one macaque species as well as mandrill from Africa were analyzed by high resolution molecular cytogenetics to obtain new insights into karyotypic evolution of old world monkeys. Molecular cytogenetics applying human probes and probe sets was applied in chromosomes of *Macaca arctoides*, *M. fascicularis*, *M. nemestrina*, *M. assamensis*, *M. sylvanus*, *M. mulatta* and *Mandrillus sphinx*. Established two- to multicolor-fluorescence *in situ* hybridization (FISH) approaches were applied. Locus-specific probes, whole and partial chromosome paint probes were hybridized. Especially the FISH-banding approach multicolor-banding (MCB) as well as probes oriented towards heterochromatin turned out to be highly efficient for interspecies comparison.

***Conclusion*:** Karyotypes of all seven studied species could be characterized in detail. Surprisingly, no evolutionary conserved differences were found among macaques, including mandrill. Between the seven here studied and phenotypically so different species we expected several *via* FISH detectable karyoypic and submicroscopic changes and were surprised to find none of them on a molecular cytogenetic level. Spatial separation, may explain the speciation and different evolution for some of them, like African *M. sylvanus*, *Mandrillus sphinx* and the South Asian macaques. However, for the partially or completely overlapping habitats of the five studied South Asian macaques the species separation process can also not be deduced to karyotypic separation.

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1. INTRODUCTION

The question what distinguishes human from other animals and especially from other primates [1] is one of the driving forces of the scientific interest in evolution in general. There are many ways to approach this question, like comparison of anatomy, etiology, behavior, or genetics, to mention only a few possibilities [2]. The genetics of different species can be compared on different levels of resolution, like classical and banding cytogenetics, molecular cytogenetics or molecular genetics. While (molecular) cytogenetics leads to resolution levels of 2-10 megabasepairs, molecular genetics can go down to the DNA- and basepair level. However, molecular genetics, esp. sequencing approaches, cannot analyze repetitive regions of genomes, constituting bog parts of genomes, also being considered as potentially important for speciation. Thus (molecular) cytogenetic and molecular genetic data complement each other and both are needed for deep understanding of evolutionary changes [3, 4].

1.1. Cytogenetics and Molecular Cytogenetics

Classical and banding cytogenetic data is available for most Old World Monkeys (OWMs), while detailed molecular (cyto)genetic data is in general sparse. Here, seven OWM-species were studied by means of fluorescence *in situ* hybridization (FISH)-banding [5] and locus-specific probes and compared to each other and with data from the literature.

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Those were from Africa *Macaca sylvanus* (MSY) and *Mandrillus sphinx* (MSP) and from South East Asia *Macaca arctoides* (MAR) *M. fascicularis* (MFA), *M. nemestrina* (MNE), *M. assamensis* (MAS) and *M. mulatta* (MMU).

For macaques (Catarrhini; Ceropithecoidae) being a morphologically highly diverse group, a quick radiation during the last 3-5 million years in Africa and especially Asia is suggested [6]. Cytogenetic data was available for them as summarized in Table **1**, indicating for 20 autosome pairs and two heteromorphic gonosomes in males of these species [7]. Also, most important (FISH) and molecular genetic studies previously available for the seven studied species are summarized in Table **1**.

Here the first comparative molecular cytogenetic study for the characterization of the karyotype of six macaque species and mandrill using human multicolor banding combined with locus-specific and heterochromatin-specific probes is presented.

2. MATERIAL AND METHODS

2.1. Cell Culture and Chromosomal Preparation

Immortalized male and female lymphoblast cell lines derived from stump-tailed macaque (*Macaca arctoides*, MAR), crab-eating macaque (*Macaca fascicularis*, MFA), southern pig-tailed macaque (*Macaca nemestrina*, MNE), Assam macaque (*Macaca assamensis*, MAS), barbary macaque (*Macaca sylvanus*, MSY), rhesus macaque (*Macaca mulatta*, MMU) and mandrill (*Mandrillus sphinx*, MSP) were cultivated according to standard techniques. Chromosomes were prepared following standard protocols [36].

2.2. Fluorescence *In Situ* Hybridization (FISH)

FISH was done as previously reported using human derived MCB probe sets or locus-specific bacterial artificial chromosomes (BAC) probes, in parts combined as subcentromere/subtelomere-specific multicolor (subCTM-)FISH probe sets [37, 38]. Additionally, three *Homo sapiens* (HSA) derived homemade microdissection probes were utilized: a probe specific for the short arm of all human acrocentric chromosomes, and others for 1q12 and 9q12, 9p12/ 9q13, 16q11.2 and Yq12 [39].

Images were captured by an Axioplan II microscope (Carl Zeiss Jena GmbH, Germany) equipped with filter sets for DAPI, FITC, TR, SO, Cy5 and DEAC. Image analysis was performed *via* pseudocolor banding and fluorochrome profile analyses using the ISIS digital FISH imaging system (MetaSystems Hard & Software GmbH, Altlussheim, Germany). A total of 10 up to 20 metaphases per species and probe were taken into account.

3. RESULTS

The karyotypes of all here studied seven species were on molecular cytogenetic identical. The detected changes compared to human karyotype are summarized in Table **2** [40]. Also Fig. (**1**) summarizes the results obtained for all species exemplified for MAR.

Overall, 11 inversions, 10 neocentromere formations and two translocation events were observed with respect to the human karyotype. Besides, chromosomes being homologous to human chromosomes 3, 6, 9, 17 and 21 had highly complex rearrangements not simply to explain or describe by

Table 1. Previous studies done in the here studied OWM-species.

Methods Species	Cytogenetics	Molecular Cytogenetics	Molecular Genetics
Macaca arctoides **MAR**	[7-10]	n.a.	SAS [11]
Macaca fascicularis **MFA**	[7, 12-15]	LSP [16-20] WCP [21] FB [22]	COPOG [23] SAS [11, 24]
Macaca nemestrina **MNE**	[7]	FB [25-26]	SAS [11]
Macaca assamensis **MAS**	n.a.	n.a.	SAS [27-28]
Macaca sylvanus **MSY**	[29]	WCP [29] FB [30]	SAS [11]
Macaca mulatta **MMU**	[7, 13]	LSP [31-33] WCP [34]	SAS [3, 11, 33] NGS [3]
Mandrillus sphinx **MSP**	[15]	LSP [19]	SAS [35]

Abbreviations: COPOG = Cloning of Parts of Genome; FB = FISH-banding; LSP = Locus Specific Probes; n.a. = Not Available; NGS = Next Generation Sequencing; SAS = Sanger Sequencing; WCP = Whole Chromosomes Paints.

Table 2. Breakpoints of macaques according to MCB and molecular data from Ventura *et al.* [40-41].

HSA-MCB-probe	MAR / MNE / MAS / MFA / MMU / MSP / MSY	Breakpoint position [NCBI36/ hg18]	BACs
1	inv(1)(q23.3q42.13)	160,918,751-161,225,664	RP11-572K18 + RP11-331H2
	cen in 1q42.13	226,810,735-226,866,653	RP4-621015
2	**inv(2)(q11.1q14.1)**	89,772,752 - 95,469,732	RP11-468G5/ RP11-316G9
	in bold acc. to online resource Uni Bari	114,076,736-114,076,791	n.a.
	cen in 2p11.2 in M-13	86,622,638-86,827,260	RP11-722G17
	inv(2)(q14.1q21.1)	114,076,736-114,076,791	n.a.
	in bold acc. to online resource Uni Bari	131,799,777-131,995,056	RP11-109E12
	cen in 2q22.1 in M-12	138,730,526-138,830,121	RP11-846E22 RP11-343I5
3	der(3)(qter->q27.3::**p22.3->p24**::q22.1->q27.3::p22.3->p12.3::p26.3->p24::q22.1->p12.3:)	0-4,328,222	RP11-183N22
		15,045,785-15,213,797	RP11-616M11
		36,506,239-36,658,135	RP11-240N7
		75,628,601-75,698,634	RP11-634L22/ RP11-413E6
	in bold acc. to online resource Uni Bari	131,347,36-131,354,303	RP11-787P10/ RP11-924M2
		187,819,875-187,998,697	RP11-177B11
	cen in 3q26.1	164,122,697-164,539,723	RP11-355I21/ RP11-418B12
4	inv(4)(p15.3q10)	86,039,028-86,261,868	RP11-367P3
		48,773,495-52,354,875	RP11-317G22/ RP11-365H22
	cen identical	-	-
5	no change	-	-
	cen: identical	-	-
6	inv(6)(p24q25.2) and inv(6)(q21q25.2)	0-213,636	subtelomeric probe (Vysis)
		108,439,777-108,647,294	RP11-815N24
		158,977,778-159,193,482	RP11-507C10
	cen in 6q24.3	145,651,644-145,845,896	RP11-474A9

(Table 2) contd….

HSA-MCB-probe	MAR / MNE / MAS / MFA / MMU / MSP / MSY	Breakpoint position [NCBI36/ hg18]	BACs
7	der(7)(21qter->21q11.2::**7p22.3->7p22.1::7q21.3->7q22.1**::7q11.23->7p**21.3::7p21.3->7q11.23::7q22.1-**>7qter)	6,613,748-7,043,428	RP11-108O03/ RP11-1061P7
		76,490,507-76,700,668	RP11-606M6
		97,263,693-97,536,166	RP11-652L7/ RP11-150J17
	in bold acc. To online resource Uni Bari	101,859,446-103,221,699	RP11-163E9/ RP11-418B19
	cen identical	-	-
8	no change	-	-
	cen identical	-	-
9	der(9)(9qter->9q34::?->?::9q34->9p24.3::9q21.11->9q22.33☺	0-615,148	RP11-3J10
		-	-
		70,000,000-70,488,561	HAS band 9q12/ RP11-203L2
		98,602,467-98,954,600	RP11-330M2 + RP11-520B13
	del(9)(q12q12)	-	-
	cen in 9q33.2	124,189,785-124,493,134	RP11-542K23/ RP11-64P14
	unknown material in 9q33.2	-	-
10	inv(10)(q11.23q22.3)	52,020262-52,248,654	RP11-591H22
		88,943,287-89,105,572	RP11-322M19
	cen identical	-	-
11	inv(11)(p15.4q13.4)	3,455,204-3,501,436	RP11-650F7/ RP11-749O23
		71,060,796-71,133,202	RP11-684B2/ RP11-483L13
	cen in 11p15.4	5,864,725-5,865,181	RP11-625D10/ RP11-6661M13
12	no change	-	-
	cen: identical	-	-
13	no change	-	-
	cen in 13q21.31	61,282,357-61,709,544	RP11-1043D14 + RP11-539I23
14	der(15)t(14;15)(q11.2;q26.3)	prox. From 18,806,381	RP11-324B11
	cen see 15	-	-

(Table 2) contd….

HSA-MCB-probe	MAR / MNE / MAS / MFA / MMU / MSP / MSY	Breakpoint position [NCBI36/ hg18]	BACs
15	der(15)t(14;15)(q11.2;q26.3)	18,400,000-22,905,050	centromere RP11-441B20
	cen in 15q25	82,835,478-83,006,963	RP11-182J1
16	**inv(16)(q22.1q22.3)**	68,394,830-68,894,008	RP11-779G13/ RP11-155G24
	in bold acc. to online resource Uni Bari	72,719,303-73,147,016	RP11-339I16/ RP11-236J9
	dim(16)(q11.2)	-	-
	cen identical	-	-
17	der(17)(pter->q12::**q23.3->q21.32::q12->q21.32**::q23.3->qter)	33,322,352-33,713,298	RP11-115K3/ RP11-923C2
		42,866,560-43,587,728	RP11-671B19/ RP1142F20
	in bold acc. to online resource Uni Bari	57,597,398-57,765,687	RP11-42F20/ RP11-50G1
	unknown material in 17p10 and 17q24 inserted	-	-
	cen identical	-	-
18	no change	-	-
	cen in 18q21.2	50,313,129-50,360,135	RP11-61D1/ RP11-289E15
	unknown material in 18q21.1	-	-
19	no change	-	-
	cen identical	-	-
20	der(20)(22qter->22p13::20p11.21->20p13::20q11.21->20qter)	25,522,225-29,667,570	RP11-694B14/ RP5-854E16
		0-659,205	RP11-530N10
	cen see 22	-	-
21	der(7)(21qter->21q11.2::**7p22.3->7p22.1::7q21.3->7q22.1**::7q11.23->**7p21.3::7p21.3->7q11.23::7q22.1-**>7qter) in bold acc. to online resource Uni Bari	13,200,000 14,822,550	centromere RP11-641G16
	cen see 7	-	-
22	der(20)(22qter->22p13::20p11.21->20p13::20q11.21->20qter)	14,430,000-16,159,326	centromere CTA-115F6
	cen identical with HSA22	-	-
X	no change	-	-
	cen identical	-	-
Y	del(Y)(q12q12)	-	-
	cen identical	-	-
	unknown material in Yqter	-	-

Abbreviations: cen = centromeric position.

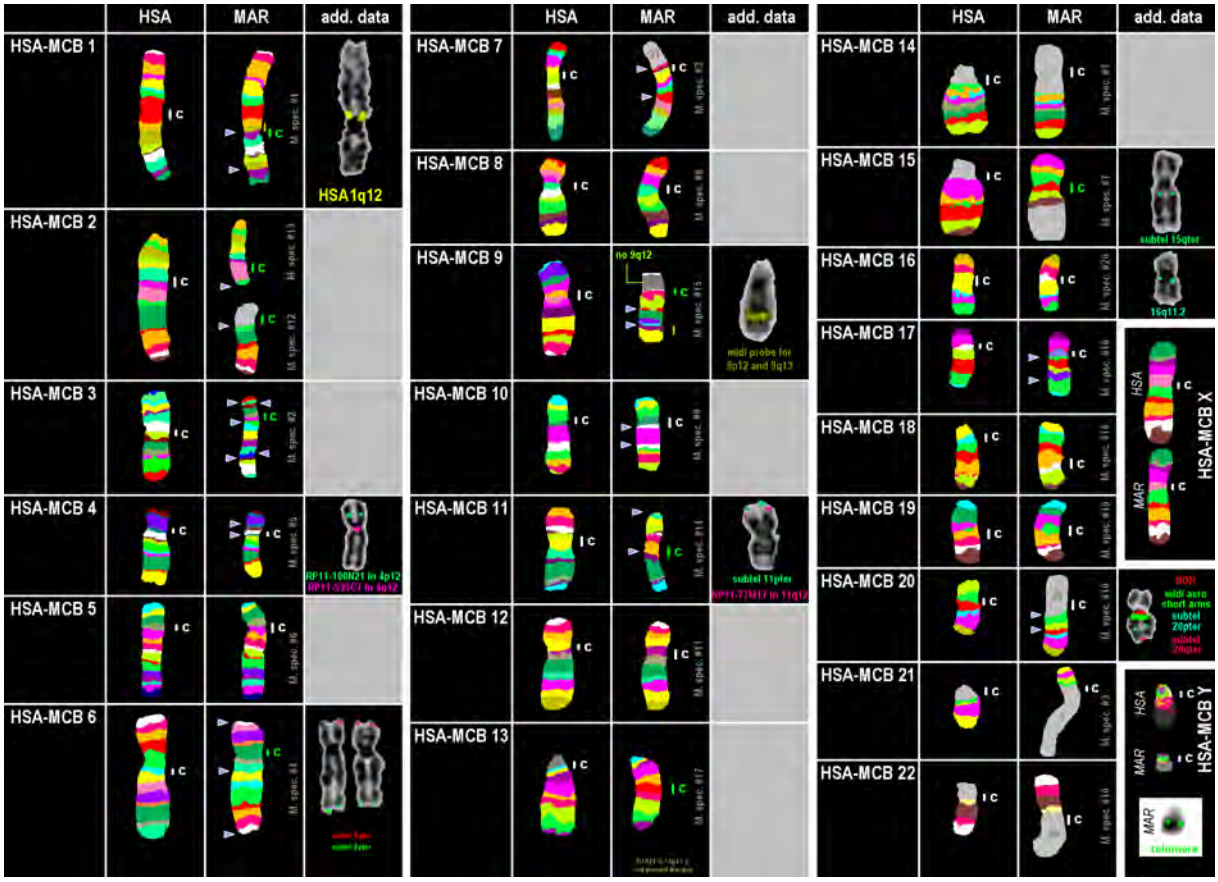

Fig. (1). Results of MCB and selected locus- and heterochromatin-specific probes are depicted here. Macaque chromosomes are numbered according to Morescalchi *et al.* [29].

inversions or insertions. Furthermore, repetitive DNA was identified as follows:

- repetitive sequence D1Z5 located in HSA in 1q11-q12 was present in all studied species at the corresponding homologous region on their chromosome 1;
- the human hemiheterochromatic region 9p12/ 9q13 is located on long arm of monkey chromosomes 15, while D9Z3 in from HSA 9q12 is not detectable in the studied species;
- the region being present in human as band 16q11.2 (D16Z3) could also be found in the studied OWMs at the homologous region on monkey chromosome 20;
- the region being present in human 10 times at the short arms of the acrocentric chromosomes can only be found at the long arm of chromosome 10 distal to the Nucleolus Organizer Region (NOR) and the centromere of this chromosome; and
- unknown, monkey specific DNA was amplified and located in regions homologous to HSA 9q33.2, 17p10, 17q24, 18q21.1 and Yqter, distal to the telomeric sequences. Repetitive DNA as present in human male in Yq12 was not observed in the studied OWMs.

According to Table **3**, 33 of 51 evolutionary conserved breakpoints appearing in the seven studied species, *i.e.* 65% colocalize with fragile sites.

4. DISCUSSION

The present study is another good example for suitability of molecular cytogenetics, especially MCB combined with locus-specific and heterochromatin-specific probes, to get new insights into chromosomal evolution of primates. Previous comparable studies were done in *Gorilla gorilla* [36], *Hylobates lar* [43] and *Trachypithecus cristatus* [38]. In those studies more or less unique karyotypic features were observed, while in the present one surprisingly the identical karyotype was found in seven species of OWM. Overall, this result is in concordance with previous cytogenetic studies at lower resolution (Table **1**). The here described evolutionary conserved inv(4)(p15.3q10) was initially only reported by Karere *et al.* [44], however with other suggested breakpoints. Furthermore, the karyotypic uniformity of the studied species is confirmed also by the fact that for some of them interspecies crossing was reported in captivity [45, 46] and also in common ancestors as recent sequence analysis between macaque species groups imply [47].

Compared *e.g.* to *Hylobates lar* [43], there are only few evolutionary conserved breakpoints and rearrangements present in macaques and mandrill compared to HSA. Still the 'complex rearranged' chromosomes homologous to human chromosomes 3, 6, 9, 17 and 21, being afterwards stable during evolution is striking and completely different than observed *e.g.* in New World Moneys (own unpublished data).

Repetitive elements may also play their role in speciation – here macaque and mandrill specific DNA-amplifications

Table 3. Evolutionary conserved breakpoints in the seven studied species compared with human fragile sites (FS). Data on FS localizations are listed acc. to Mrasek *et al.* [42].

Breakpoint in Studied OWMs	Fragile Site	Breakpoint in Studied OWMs	Fragile Site
1q23.3	FRA1P	7q22.1	FRA7F
1q42.13	FRA1H	9p24.3	FRA9H
2p11.2	FRA2L	9q21.11	FRA9D
2q11.1	FRA2R	9q22.33	n.a.
2q14.1	n.a.	9q33.2	FRA9M
2q21.1	n.a.	9q34	FRA9N
2q22.1	n.a.	10q11.23	FRA10J
3p26.3	FRA3E	10q22.3	n.a.
3p24	FRA3A	11p15.4	FRA11J
3p22.3	FRA3G	11q13.4	FRA11E
3p12.3	FRA3I	13q21.31	n.a.
3q22.1	FRA3N	14q11.2	FRA14D
3q26.1	FRA3O	15q25	FRA15F
3q27.3	FRA3C	15q26.3	FRA15G
4p15.3	FRA4D	16q22.1	FRA16C
4q10	n.a.	16q22.3	n.a.
6p24	n.a.	17q12	FRA17D
6q25.2	n.a.	17q21.32	n.a.
6q21	FRA6F	17q23.3	n.a.
6q24.3	n.a.	18q21.2	FRA18B
6q25.2	FRA6M	20p13	FRA20C
7p22.3	FRA7B	20p11.21	n.a.
7p22.1	n.a.	20q11.21	n.a.
7p21.3	FRA7L	21q11.2	FRA21
7q11.23	FRA7J	22p13	n.a.
7q21.3	n.a.	--	--

could be found for previously described regions like that being homologous to human 18q21.1 [48]. Also observations on seeding of neocentromeric regions preferentially in gene deserts [49] fit to the here described data (Table **2**). As others suggested before [50] we could also confirm a high degree of colocalization of fragile sites with the here reported evolutionary conserved breakpoints.

In this special group of OWMs karyotypic evolution cannot be the driving force of speciation. Thus one can expect submicroscopic genetic changes in the genomes of the seven here studied and phenotypically so different species as described *i.e.* by Yan *et al.* [51] for MMU subspecies and MFA. Among the evolutionary forces leading to annidation of the studied species, spatial separation, may be an explanation for a part of the speciations, like for African *M. sylvanus*, *Mandrillus sphinx* on the one and the South Asian macaques on the other end. However, for the five studied South Asian macaques partially or completely overlapping in habitats the species separation process might must have other reasons. One idea for a driving force comes from recent paper of Zhou *et al.* [52] suggesting niche separation of *M. assamensis* and *M. mulatta* based on adaptation to reduce resource competition.

CONCLUSION

Even though karyotypic evolution plays a major role in speciation and species separation this seems to be unimportant between the seven here studied Catarrhini-species. Sub-

microscopic changes, like gene mutations, activation of pseudogenes, *etc*. seem to be the main reasons for the phenotypic differences of those species.

LIST OF ABBREVIATIONS

AIDS	=	Acquired Immune Deficiency Syndrome
BAC	=	Bacterial Artificial Chromosomes
cen	=	Centromeric position
COPOG	=	Cloning of Parts of Genome
DNA	=	Deoxyribonucleic acid
FB	=	FISH-banding
FISH	=	Fluorescence *in situ* hybridization
HSA	=	*Homo sapiens*
LSP	=	Locus Specific Probes
MAR	=	*Macaca arctoides*
MAS	=	*Macaca assamensis*
MCB	=	Multicolor-banding
MFA	=	*Macaca fascicularis*
MMU	=	*Macaca mulatta*
MNE	=	*Macaca nemestrina*
MSP	=	*Mandrillus sphinx*
MSY	=	*Macaca sylvanus*
NGS	=	Next Generation Sequencing
NOR	=	Nucleolus Organizer Region
OWM	=	Old World Monkey
SAS	=	Sanger sequencing
subCTM-FISH	=	Subcentromere/subtelomere-specific multicolor fluorescence *in situ* hybridization
WCP	=	Whole Chromosomes Paints

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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