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Investigation of marmoset hybrids *(Cebuella pygmaea* × *Callithrix jacchus)* and related Callitrichinae (Platyrrhini) by cross-species chromosome painting and comparative genomic hybridization

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Abstract. We report on the cytogenetics of twin offspring from an interspecies cross in marmosets (Callitrichinae, Platyrrhini), resulting from a pairing between a female Common marmoset (*Callithrix jacchus*, 2n = 46) and a male Pygmy marmoset (*Cebuella pygmaea*, 2n = 44). We analyzed their karyotypes by multi-directional chromosome painting employing human, *Saguinus oedipus* and *Lagothrix lagothricha* chromosome-specific probes. Both hybrid individuals had a karyotype with a diploid chromosome number of 2n = 45. As a complementary tool, interspecies comparative genomic hybridization (iCGH) was performed in order to screen for genomic imbalances between the hybrids and their parental species, and between *Callithrix argentata* and *S. oedipus*, respectively. These genomic imbalances were confined to centromeric and telomeric heterochromatin, while euchromatic chromosome regions appeared balanced in all species investigated. When comparing marmosets and tamarins, sequence divergence of centromeric heterochromatin was already clearly noticeable. In the *C. argentata* and *C. pygmaea* genomes numerous subtelomeric regions were affected by amplification of different repetitive sequences. Cross-species FISH with a microdissection-derived *C. pygmaea* repetitive probe revealed species specificity of this repetitive sequence at the molecular cytogenetic level of resolution.

Few reports have been published about interspecies hybrids among primates and hardly any about intergeneric hybrids. Hybrid animals have been observed in the wild and in captivity between some taxa of Old World monkeys and gibbons, for example intergeneric hybrids between *Papio* × *Macaca* (Moore et al., 1999), *Cercopithecus pogonias* × *Cercopithecus ascanius* (Dugoujon et al., 1982), *Symphalangus* × *Hylobates* (Pellicciari et al., 1988). Among Prosimians, hybrids between species or subspecies were most commonly described in lemurs (Rumpler and Dutrillaux, 1976; Ratomponirina et al., 1982), for example *Eulemur albocollaris* × *Eulemur fulvus rufus* and *Eulemur f. fulvus* × *Eulemur m. macaco*. In New World Monkeys interspecies hybrids both in the wild and in captivity have been documented in several genera, for example within genus *Callithrix* (reviewed by Coimbra-Filho et al., 1993), between squirrel monkey (sub-) species (Moore et al., 1990) and within genus *Aotus* (Yunis et al., 1977).

In the present study we report on the cytogenetics of the twin offspring from an interspecies cross in marmosets (Callitrichinae, Platyrrhini), resulting from a pairing between a female Common marmoset *(Callithrix jacchus)* and a male Pygmy marmoset *(Cebuella pygmaea)*. They were born in 1998, developed normally and are still alive. Whether the *C. jacchus* × *C. pygmaea* hybrid individuals investigated here have to be regarded as intergeneric or interspecies hybrids is a

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Table 1. Labeling scheme and false color assignment of *L. lagothricha* and *S. oedipus* chromosome paint probes used in the 31-color multiplex probe for M-FISH karyotyping of the *C. jacchus* × *C. pygmaea* interspecies hybrids

Probe subset	Probe pool	*L. lagothricha* probe (LLA)																									*S. oedipus* probe (SOE)					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	20+21	22	23	25	26	27	28	29	30	7	9	11	17	X	Y
L1	2.1red			X		X			X		X		X		X		X			X	X	X	X		X		X	X				X	X
	2.2green									X	X	X		X	X	X	X	X		X	X		X	X	X	X			X				
	2.3blue				X		X	X	X			X	X	X	X			X			X	X		X	X			X		X			
L2	1.1red	X		X	X			X	X	X	X	X							X			X	X	X	X	X	X						
	1.2green		X			X	X	X		X			X	X		X				X	X	X	X	X		X	X				X		

matter of the taxonomic position of *Cebuella*, which is still disputed (reviewed by Rylands, 2000). On the basis of morphological studies, Rosenberger and Coimbra-Filho (1984) and Natori (1994) argued in favor of including *Cebuella* in the genus *Callithrix*. This opinion was supported by molecular genetic evidence (Barroso et al., 1997; Porter et al., 1997; Canavez et al., 1999). Based on DNA sequence comparisons, Rylands et al. (2000) proposed a closer relationship of *Cebuella* to the Amazonian marmosets (the so-called "argentata group") than to the Atlantic forest marmosets (the "jacchus group"). Despite these lines of evidence, Rylands et al. (2000) suggested that *C. pygmaea* deserves generic status in order to recognize the distinctiveness of this taxon.

Recently, Neusser et al. (2001) studied several Callitrichinae by multi-directional chromosome painting using human and *Saguinus oedipus* chromosome-specific DNA probes, among them *Callithrix jacchus*, *C. argentata* and *Cebuella pygmaea*. The results revealed that *C. jacchus* shares identical chromosomal syntenies with *C. pygmaea*, with the exception of a single Robertsonian type fission involving segments homologous to human chromosomes 2 and 16. The karyotype of *C. jacchus* thus differs from *C. pygmaea* by a diploid chromosome number of 46, compared to 44 chromosomes in *C. pygmaea*.

To investigate whether structural inter- and intrachromosomal changes occurred in these *C. jacchus* × *C. pygmaea* interspecies hybrids, we performed multi-directional chromosome painting employing human, *S. oedipus* and *Lagothrix lagothricha* chromosome-specific probes. As a complementary tool to chromosome painting, interspecies comparative genomic hybridization (iCGH, Toder et al., 1998) and cross-species FISH with a microdissection-derived *C. pygmaea* repetitive probe was performed in order to screen for genomic imbalances between the interspecies hybrids, their parental species and other Callitrichinae. Unfortunately, studies on meiosis or spermatogenesis in the hybrid individuals could not yet be performed, since an invasive approach would intervene with ongoing behavioral studies.

Materials and methods

Cell samples, tissue culture and chromosome preparation

Metaphases from two *Callithrix jacchus* × *Cebuella pygmaea* hybrid individuals (CJA × CPY, a male and female twin, described by Anzenberger et al., 2001), a female *C. jacchus* individual (CJA, mother of the twin hybrids) and one male and female *C. pygmaea* individual (CPY, mother and son, individual 1 and 2), kept at the primate station of the Anthropological Institute, University of Zurich, Switzerland, were prepared from PHA-stimulated blood cells. A tissue sample from the deceased father *(C. pygmaea)* of the interspecies hybrids was obtained from the same institution. *S. oedipus* (SOE) metaphases were prepared from the same individual as described in Müller et al. (2001). *C. goeldii*, *C. pygmaea* (individual 3) and *C. argentata* metaphase preparations were the same as described in Neusser et al. (2001).

Chromosome painting and interspecies comparative genomic hybridization (iCGH)

Human, *S. oedipus* and *L. lagothricha* chromosome-specific painting probes were the same as described before (Müller et al., 2001; Stanyon et al., 2001). Human six-color paint probe sets H1-H4 were described in Müller et al. (2001). For 31-color M-FISH with *L. lagothricha* and *S. oedipus* chromosome-specific probes, a combinatorial labeling scheme was followed (Table 1) as described for sequential hybridization experiments (Müller et al., 2002). Some *L. lagothricha* (LLA) probes were replaced or complemented by *S. oedipus* (SOE) homologs, because they could not be resolved in the flow karyogram or were not of sufficient quality. Probe labeling was performed by DOP-PCR in the presence of biotin, digoxigenin, dinitrophenol (DNP) or TAMRA-conjugated dUTPs as described earlier (Roberts et al., 1999).

For iCGH experiments genomic DNA from cultured cells and blood samples was prepared according to standard protocols. Interspecies CGH was carried out according to Toder et al. (1998), with modifications. Each 500 ng of DOP-PCR or nick-translation labeled genomic DNA from test (biotinylated) and reference species (digoxigenin labeled) was mixed. No competitor DNA (Cot-1 DNA) for the suppression of repetitive sequences was added.

Characterization of Callitrichinae with a microdissection-derived Cebuella repetitive probe

Chromosomal microdissection of a heterochromatic region present in the *C. jacchus* × *C. pygmaea* interspecies hybrid genome was performed as described by Weimer et al. (2000), with modifications. Prior to microdissection, metaphase preparations of the interspecies hybrid were hybridized with 200 ng biotinylated *S. oedipus* (SOE) chromosome 21-specific probe. The SOE 21 homologous target chromosome was then visualized using a colorimetric technique. Microdissection was performed with a glass needle on an inverse microscope (Axiovert 135, Zeiss). Approximately five fragments were collected and amplified by DOP-PCR. The resulting repetitive probe was combined with the *L. lagothricha* multi-color probe subset L1 (Table 1). The four probes were labeled with digoxigenin, biotin, dinitrophenol and TAMRA-conjugated dUTPs.

In situ hybridization and probe detection

In situ hybridization and probe detection were carried out as described by Neusser et al. (2001). In iCGH experiments or when hybridizing the repetitive probe, probes were applied immediately after denaturation. Sequential hybridization of the 31-color *L. lagothricha*/*S. oedipus* multiplex probe subsets L1 and L2 was performed according to Müller et al. (2002). Biotinylated DNA probes were detected by Avidin-Cy5 (Jackson Immuno Research) or Avidin-AMCA (Roche) and digoxigenin-labeled probes by sheep anti-digoxigenin-FITC (Roche) or mouse anti-Dig-Cy5 (Jackson Immuno Research). DNP-labeled probes were detected by goat anti-DNP and rabbit

anti-goat-FITC (Sigma) antibodies. When performing FISH prior to microdissection the colorimetric detection technique employing avidin-alkaline phosphatase (Roche) and NBT/BCIP (Gibco) described in Schwarzacher and Heslop-Harrison (2000) was applied.

Microscopy and image analysis

Metaphases were captured with a cooled CCD camera (Photometrics C250/A equipped with a KAF1400 chip, Kodak) coupled to a Zeiss Axiophot microscope. Camera control and digital image acquisition was performed using SmartCapture VP software (Digital Scientific, Cambridge, UK). CGH experiments were evaluated with Quips v3.0.1 software (Vysis).

Results and discussion

The karyotypes of one male and one female *C. jacchus* × *C. pygmaea* hybrid individual were analyzed by multi-directional chromosome painting in order to determine structural rearrangements. Interspecies CGH was performed to visualize genomic imbalances between the interspecies hybrids, their parental and other Callitrichinae species. The chromosomal localization and distribution of *C. pygmaea* repetitive genomic elements was assessed by in situ hybridization with a microdissection-derived probe in the two interspecies hybrids, three *C. pygmaea* individuals and in other Callitrichinae species. Figure 1 illustrates representative metaphases after hybridization with these probes.

Multi-directional chromosome painting

The 31-color probe set composed of combinatorially labeled *L. lagothricha* and *S. oedipus* chromosome-specific probes served as whole karyotype screening tool for structural chromosome rearrangements in the male and the female *C. jacchus* × *C. pygmaea* hybrid individuals (see Table 1 for probe composition). Sequential hybridization (Re-FISH, Müller et al., 2002) of the two probe subsets L1 and L2 allowed us to differentiate all chromosomes of the interspecies hybrids in a single experiment (Fig. 1A and B). The evaluation of 20 cells per individual revealed a constant diploid chromosome number of 2n = 45. These results were in agreement with previous studies of the parental species (Neusser et al., 2001; Stanyon et al., 2001). Neither human (Fig. 1C) nor *L. lagothricha* chromosome-specific probes detected any further inter- or intrachromosomal rearrangements in the two hybrid individuals.

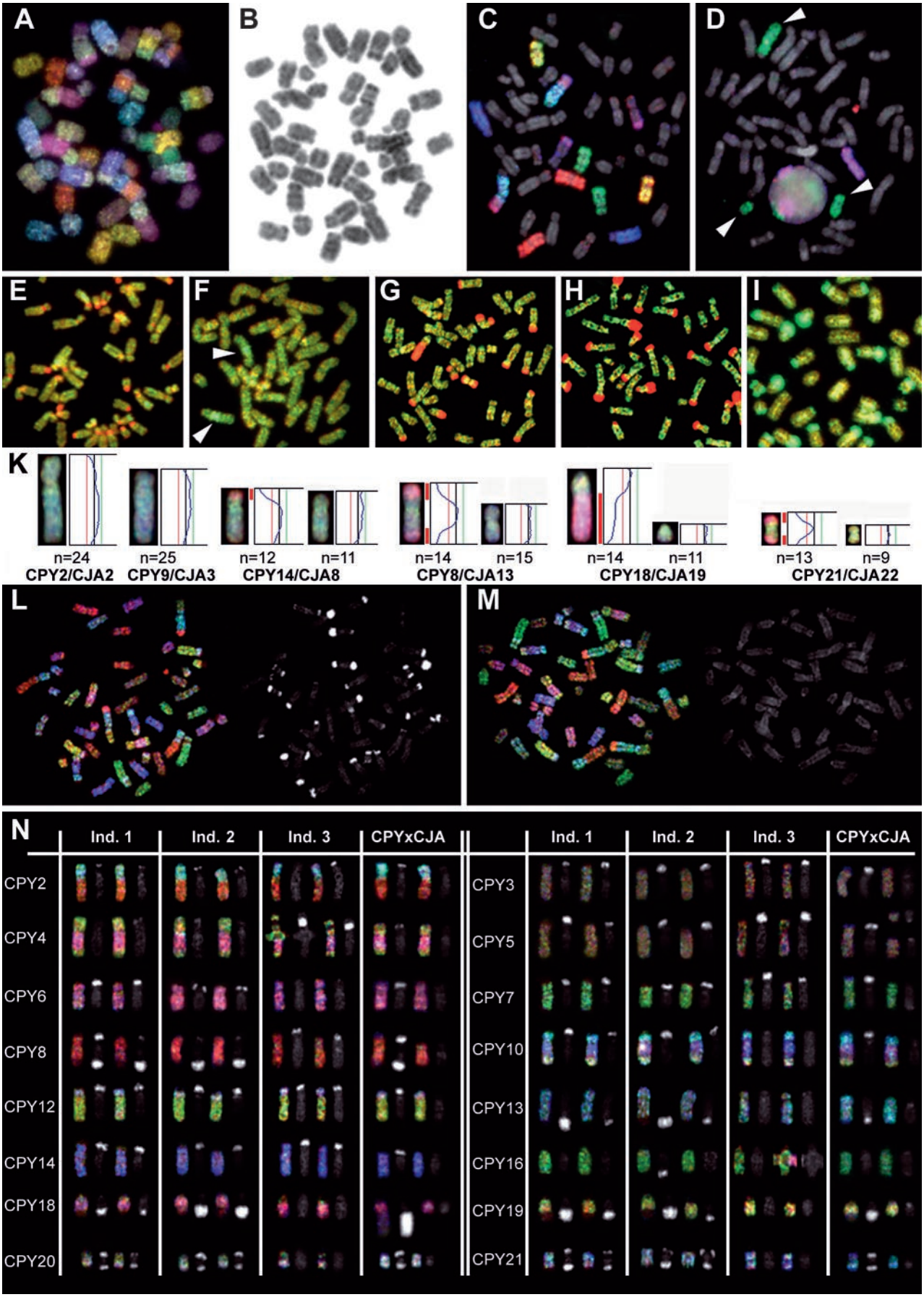
An asymmetric hybridization pattern on chromosomes of the interspecies hybrids with human chromosome 2 and 16 probes detected the cause of the intermediate diploid chromosome number of 2n = 45: A single acrocentric chromosome of medium size and the long arm of a sub-metacentric chromosome were painted by the human chromosome 2-specific probe. The human chromosome 16 paint probe hybridized to the short arm of the same sub-metacentric chromosome and a single small acrocentric chromosome. The haploid state of the sub-metacentric and the two acrocentric chromosomes was confirmed by *S. oedipus* chromosome 10-specific probe (Fig. 1D). Since the karyotype of *C. jacchus* is composed of a diploid number of 46 chromosomes and is evolutionarily derived from the karyotype of *C. pygmaea* (2n = 44) by a single Robertsonian-type fission involving human homologous chromosome segments 2pter→q12 and 16q (Neusser et al., 2001), the interspecies hybrids show an intermediate karyotype of the parental species with a diploid chromosome number of 45 chromosomes.

Interspecies CGH (iCGH)

The hybridization of differentially labeled genomic marmoset *(C. jacchus)* and tamarin *(S. oedipus)* DNA to *C. jacchus* metaphases (Fig. 1E) showed a completely balanced karyotype, except for most *C. jacchus* centromeric regions, where the *C. jacchus* genome appeared overrepresented. When the same experiment was performed on *S. oedipus* metaphases, *S. oedipus* centromeric regions appeared equally amplified (data not shown) indicating that these color changes are not caused by copy number differences. If this was the case, in both experiments the same species would have appeared overrepresented. Instead, the divergence of centromeric sequences between both species is clearly noticeable and much higher as compared to euchromatin.

A quantitative analysis of 15 cells (data not shown) after CGH between *C. pygmaea* and *C. jacchus* (parents of the interspecies hybrids) on metaphases of *C. jacchus* revealed no genomic imbalances, except for the Xq pericentromeric region, which is overrepresented in the female *C. jacchus* genome (Fig. 1F). When the same experiment was performed on metaphases of the *C. pygmaea* × *C. jacchus* interspecies hybrid (Fig. 1G), the densitometric analysis of 15 cells (gain threshold: 1.2, loss threshold: 0.8) revealed high overrepresentation of *C. pygmaea* sequences in terminal regions of chromosomes CPY 3, 5–8, 10, 12–14 and 18–21. Figure 1K illustrates representative chromosomes with and without genomic imbalances. Taken together, these two iCGH visualized several unbalanced chromosome regions comprised of sequences specific for *C. pygmaea*. If they were shared with *C. jacchus*, they would have been overrepresented as well on *C. jacchus* chromosomes (Fig. 1F). By inference, the occurrence of *C. pygmaea*-specific sequences allowed the identification of the paternal origin of 13 homologs of the interspecies hybrid.

Reciprocal interspecies CGH between *C. argentata* and *C. pygmaea* (individual 2) on metaphase chromosomes of *C. argentata* (Fig. 1H) and *C. pymaea* (Fig. 1I) visualized unbalanced regions on various chromosome ends in both species. *C. pygmaea* sequences were highly overrepresented when hybridized to *C. pygmaea* chromosomes and vice versa *C. argentata* sequences on *C. argentata* chromosomes. These regions correspond to chromosome segments that were not hybridized with any chromosome paint probes from other species (see Fig. 1A–D and Neusser et al., 2001) and that were previously identified to be C-band positive (Nagamachi et al., 1992; Alves et al., 1995). Our data indicate that these unbalanced regions are comprised of constitutive heterochromatin with sequence motives absent in human, *L. lagothricha* and *S. oedipus*, respectively. Moreover, the direct comparison of *C. pygmaea* and *C. argentata* genomes revealed no sequence homology of sub-telomeric heterochromatin in the two species. *C. pygmaea* repetitive sequences exclusively hybridized to *C. pygmaea* chromosomes and vice versa. Therefore, at the resolution of CGH these sub-telomeric heterochromatin blocks present in both species appear to comprise species-specific sequences.


A
B
C
D
E
F
G
H
I
K
n=24
CPY2/CJA2
n=25
CPY9/CJA3
n=12
n=11
CPY14/CJA8
n=14
n=15
CPY8/CJA13
n=14
n=11
CPY18/CJA19
n=13
n=9
CPY21/CJA22
L
M
N
Ind. 1
Ind. 2
Ind. 3
CPYxCJA
CPY2
CPY4
CPY6
CPY8
CPY12
CPY14
CPY18
CPY20
Ind. 1
Ind. 2
Ind. 3
CPYxCJA
CPY3
CPY5
CPY7
CPY10
CPY13
CPY16
CPY19
CPY21

Chromosomal microdissection

A FISH probe specific for *C. pygmaea* repetitive sequences certainly provides a more detailed insight into presence, absence or chromosomal distribution of these repetitive sequences as compared to iCGH. By chromosomal microdissection we therefore established a DNA probe for the highly overrepresented region present on the distal end of the paternally *(C. pygmaea)* inherited chromosome CPY18 of the male *C. jacchus* × *C. pygmaea* hybrid individual. Hybridization of this probe to *C. pygmaea* individual 1 revealed extensive cross-hybridization on regions of several other chromosomes which in previous C-banding studies were shown to be comprised of *C. pygmaea* constitutive heterochromatin (Fig. 1L). We therefore conclude that this probe comprises repetitive sequences. In order to screen for the occurrence and distribution pattern of these sequences in different Callitrichinae species, this probe was hybridized to metaphases of *C. jacchus*, *C. argentata*, *S. oedipus* and *C. goeldii*. The probe was combined with multi-color *L. lagothricha* subset L1 (Table 1, Materials and methods), which allowed a secure localization of all homologous regions to the microdissected probe on other chromosomes (Fig. 1L and M). In no other Callitrichinae species hybridization above background level was observed. The results demonstrated at the molecular cytogenetic level of resolution that the repetitive sequences present in the microdissected region are species specific for *C. pygmaea*.

The different *C. pygmaea* individuals investigated showed a considerable variability in the distribution pattern of the respective repeat sequence, while the male and the female *C. jacchus* × *C. pygmaea* hybrids were very similar to each other. Figure 1N presents an overview of all chromosomes of the investigated individuals showing hybridization. Individuals 1 and 2 (mother and son) showed a rather similar hybridization pattern on chromosomes 6, 10, 13 and 18–21, which was absent in individual 3 belonging to a different breeding group. On the other hand, in individual 3 a large interstitial hybridization signal was detected on chromosome 4, which was absent in the other individuals and the interspecies hybrid. Similarly, individual 2 showed hybridization on chromosome 16 not found in the other individuals. Size polymorphisms of the repeat block were also observed between homologous chromosomes of the same individual, as well as between homologs of different individuals. Differences between homologs in the same individual are most remarkable on chromosome 5 and 8 of individual 1 and on chromosome 13 and 19 of individual 1 and 2. A prominent example for size variations of repetitive regions between homologous chromosomes of different individuals is CPY 18 of the interspecies hybrid. These polymorphisms are representing potentially useful markers for future population studies. The investigation of the interspecies *C. jacchus* × *C. pygmaea* hybrid individuals revealed the paternal origin of two further chromosomes (CPY 2 and 4) by the occurrence of minor heterochromatic blocks, not visible by iCGH.

These findings complement the reports by Alves et al. (1995) on the isolation of a 1,528-bp *C. argentata*-derived CarB repeat only found in the *C. argentata* group of species, which was mapped to the same chromosomal regions that in our study were identified by iCGH to contain *C. argentata*-specific repetitive sequences. Reciprocally, the *C. pygmaea*-derived repeat probe we established did not hybridize to chromosomes of other Callithrichidae, including *C. argentata*. Hence, we extended the Alves et al. (1995) study by showing that *C. pygmaea* repeat sequences are not present in *C. argentata* either at the level of resolution of FISH. Our results imply that the addition of heterochromatin in *C. argentata* and *C. pygmaea* may have had a different evolutionary history and that this chromosomal trait does not represent a synapomorphism, which would phylogenetically link the two species.

By conclusion, the existence of *C. jacchus* × *C. pygmaea* interspecies hybrids with a balanced karyotype and a healthy morphology, may add an argument against a classification of *Cebuella* and *Callithrix* in separate genera. To address these questions in more detail, further studies on the fertility status and meiosis of the interspecies hybrids would appear promising. Chromosome regions composed of large heterochromatic blocks, which were only present in one paternal species and which were inherited by the hybrid offspring in haploid state, further indicate that the presence of significant amounts of heterochromatin in the interspecies hybrids at least did not affect their viability. These findings are in agreement with King (1993) who summarized several lines of evidence leading to the

Fig. 1. (**A–D**) FISH experiments with human, *S. oedipus* and *L. lagothricha* chromosome-specific painting probes on metaphase chromosomes of the *C. jacchus* × *C. pygmaea* interspecies hybrid. (**A**) M-FISH karyotyping by sequential hybridization of *L. lagothricha* subsets L1 and L2, resulting in 31 distinguishable color combinations (overlay image of L1 and L2 is shown) and (**B**) chromosomal counter stain (inverted DAPI) of the same cell. (**C**) FISH with a 6-color probe set, composed of human chromosome 4 (blue), 6 (red), 9 (cyan), 11 (yellow), 12 (green) and 13 (magenta) specific probes. (**D**) Triple hybridization with *S. oedipus* chromosome 10 (green), X (magenta) and Y (red) specific probes, revealing an asymmetric hybridization pattern with *S. oedipus* chromosome 10-specific probe (arrowheads) and the chimeric status of blood cells common to Callitrichinae (XY in the metaphase and XX in the interphase displayed). (**E–K**) Interspecies CGH experiments with differentially labeled genomic DNAs from two Callitrichinae species each. (**E**) *S. oedipus* (green)/*C. jacchus* (red) on a *C. jacchus* metaphase, (**F**) *C. jacchus* (green)/*C. pygmaea* (red; mother and father of the interspecies hybrids) on a *C. jacchus* metaphase (arrowheads indicate the Xq pericentromeric region, overrepresented in the female *C. jacchus* genome) and (**G**) *C. jacchus* (green)/*C. pygmaea* (red) on a metaphase of the male *C. jacchus* × *C. pygmaea* interspecies hybrid. (**H**) Hybridization of *C. argentata* (red) and *C. pygmaea* (green) DNA on *C. argentata* metaphase chromosomes and (**I**) *C. argentata* (red) and *C. pygmaea* (green) on a *C. pygmaea* metaphase. (**K**) Display of selected chromosomes after quantitative evaluation of 15 cells from the experiment illustrated in (**G**), together with the respective fluorescence intensity ratio profiles (threshold for gains 1.2 and losses 0.8). Chromosome regions comprised of repetitive elements are significantly overrepresented in the paternal homolog of some chromosomes, whereas euchromatic regions appear balanced (CPY = *C. pygmaea*, CJA = *C. jacchus*). (**L–N**) Characterization of different Callitrichinae individuals with the *C. pygmaea* microdissection-derived repeat probe (right), together with *L. lagothricha* probe subset L1 (left) to facilitate chromosome identification on (**L**) *C. pygmaea* (individual 1) and (**M**) *C. jacchus* metaphases. (**N**) Alignment of all chromosomes (rows) with homologous regions to the repeat probe in different *C. pygmaea* (CPY) individuals (= Ind. 1–3) and the *C. jacchus* × *C. pygmaea* (CPY × CJA) interspecies hybrid after the hybridization of the probe set illustrated in **L** and **M**. For each individual (columns) both homologs are shown, on the left the hybridization pattern of the multi-color *L. lagothricha* subset L1, on the right the *C. pygmaea* repeat probe. The chromosome nomenclature of *C. pygmaea* follows Canavez et al. (1996).

assumption that heterochromatin does not necessarily trigger speciation processes, since it may not have the potential to act as a profound reproductive barrier. Our investigation contributed examples of genomic imbalances caused by rapidly yet independently evolving repetitive genome elements in closely related species. Their function remains unclear, but may rather be associated with adaptation than with speciation processes.

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