

Chromosomal evolution and phylogenetic analyses in *Tayassu pecari* and *Pecari tajacu* (Tayassuidae): tales from constitutive heterochromatin

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Abstract

The mammalian family Tayassuidae (peccaries) is confined to the New World and comprises three recognized extant species, white-lipped (*Tayassu pecari*), collared (*Pecari tajacu*) and chacoan (*Catagonus wagneri*) peccaries, which exhibit distinct morphological and chromosomal features. The phylogenetic relationships among the tayassuids are unclear and have instigated debate over the palaeontological, cytogenetic and molecular aspects. Constitutive heterochromatin analysis can be used in understanding the phylogenetic relationships between related species. Here we describe, for the first time, the constitutive heterochromatin (C-positive heterochromatin) of two tayassuid species, *Tayassu pecari* and *Pecari tajacu*. We demonstrate that *in situ* restriction endonuclease digestion with sequential C-banding could be a complementary tool in the study of constitutive heterochromatin heterogeneity in chromosomes of the Tayassuidae. Our characterization of peccary chromosomes suggests that the *Pecari tajacu* autosomal karyotype is more primitive and has accumulated great diversity in its constitutive heterochromatin. This idea is supported by several other studies that analysed nuclear and mitochondrial sequences of the living peccary species. Finally, the tayassuid X chromosome primitive form seems to be the one of *Tayassu pecari*.

Introduction

Constitutive heterochromatin (CH) represents a substantial proportion of eukaryotic genomes. The ubiquity and persistence of heterochromatin in these genomes suggest that this distinctive genomic component has an adaptive value, besides being considered a genomic compartment carrying out important functions with an unconventional structural and functional basis (Dimitri *et al.* 2004). Among other repetitive sequences, this genomic fraction comprises satellite DNAs that usually reside in the centromeric regions of chromosomes (Chaves *et al.* 2000). Identification of CH and its size differences is made feasible by the simple but extremely useful C-banding technique. But other analytical methods are indispensable when the

molecular nature of CH is the central concern. Among these, use of restriction endonucleases (which cleave DNA at specific sequences) and use of base-specific fluorochromes (which delimit chromosome regions rich in different types of DNA bases) are techniques that have proven to be very useful in improving understanding of the mechanisms involved in the evolution of CH in different genomes (reviewed in Gosálvez *et al.* 1997; Pieczarka *et al.* 1998). In addition, restriction enzyme treatment followed by C-banding is useful in demonstrating C-heterochromatin heterogeneity (Rocco *et al.* 2002; Schmid *et al.* 2002; Chaves *et al.* 2004; Adega *et al.* 2005).

The number, morphology and G-banding pattern (e.g. Pirtle 1967; Hufty *et al.* 1973; Giannoni *et al.* 1982a,b; Benirschke and Kumamoto 1989; Zijlstra *et al.* 1997) of the peccaries *Pecari tajacu* (collared peccary) and *Tayassu pecari* (white-lipped peccary) are well known, and even

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Keywords. constitutive heterochromatin; C-positive heterochromatin; *in situ* restriction endonuclease digestion; karyotype evolution; phylogeny; *Pecari tajacu*; *Tayassu pecari*.

molecular cytogenetic studies, such as rRNA gene assignments in *T. pecari* (Bosma *et al.* 2004) and comparative chromosome painting with pig (Bosma *et al.* 2004; Adega *et al.* 2006) and sheep paint probes in both species (Adega *et al.* 2006; and unpublished data) are available, but their CH was only analysed by classical C-banding techniques (Pirtle 1967; Benirschke and Kumamoto 1989).

In this work, we describe in detail heterochromatin heterogeneity of the two peccary species *P. tajacu* and *T. pecari* using a panel of eight restriction endonucleases (RE) followed by the classical C-banding technique. The *in situ* RE-banding revealed in the chromosomes was demonstrated to be suitable for studying chromosome organization. RE digestion with sequential C-banding allowed detailed characterization of CH in terms of its localization, detection of different CH subclasses, and molecular composition.

Since these two species are related, and the syntenic regions as well as the chromosomal evolutionary rearrangements (relative to another suiform genome, the pig) are known (Bosma *et al.* 2004; Adega *et al.* 2006), it was possible to track the C-positive heterochromatin along the chromosomes of the two species, and hence to delineate a hypothesis on the evolutionary remodelling of heterochromatin in these species. Further, this approach is a contribution to the understanding of the chromosomal evolutionary process and phylogenetic relationships within this family.

Materials and methods

Chromosome preparation and karyotype description

The material analysed consists of chromosome preparations of the tayassuid species *T. pecari* (three individuals) and *P. tajacu* (four individuals) and were made from short-term lymphocyte cultures of whole blood samples using standard protocols (Chaves *et al.* 2002).

The karyotype of *T. pecari* (individuals from South America) has 26 chromosomes, with the two largest chromosome pairs being submetacentric, nine pairs metacentric/submetacentric, the smallest autosomal pair acrocentric, the sex chromosome X a medium acrocentric, and the Y a small acrocentric.

The *P. tajacu* karyotype (individuals from Arizona, USA, North America) is characterized by a diploid chromosome number of 30, with eight metacentric/submetacentric autosomal pairs, six autosomal chromosome pairs acrocentric, the sex chromosome X a medium submetacentric, and the Y a small acrocentric. The nomenclature used for the chromosomes of *T. pecari* is that of Bosma *et al.* (2004), and for *P. tajacu* that of Adega *et al.* (2006).

GTD-banding

Air-dried slides were aged at 65°C for 5 h, or overnight, and then were submitted to standard G-banding procedures with trypsin (Verma and Babu 1995). As the chromosome slides proceeded sequentially to C-banding, they were not

stained at this stage. Instead, the slides were first fixed with paraformaldehyde (Chaves *et al.* 2002). Briefly, dry slides were placed in a 1× PBS solution (2 × 5 min) before fixation in 4% paraformaldehyde / 1× PBS at room temperature. After a 10-min fixation, slides were dehydrated for 2 min each in 70, 90 and 100% chilled ethanol, and air dried. Slides were stained with DAPI for better contrast (Chaves *et al.* 2002). Inversion of the DAPI colour in Adobe Photoshop software revealed the chromosomes G-banding pattern (GTD-banding, G-bands by trypsin with DAPI).

In situ RE digestion

Air-dried slides were aged at 65°C for 6 h and then were submitted to *in situ* RE digestion. The eight REs used (*AluI*, *ApaI*, *BamHI*, *DraI*, *HaeIII*, *MboI*, *PstI* and *RsaI*) were diluted in buffers as indicated by the manufacturer (Invitrogen, Life Technologies, Stratchclyde, UK), and final concentrations of 30 U per 100 µl were obtained. One hundred µl of each of these solutions was placed on slides and covered with coverslips. The slides were incubated in a moist chamber for 16 h at 37°C. Control slides were given the same treatment as described above but incubated only with buffer. The slides were then washed in distilled water and air dried. As the chromosome slides proceeded to sequential C-banding techniques, they were fixed with paraformaldehyde (Chaves *et al.* 2002), as described above for GTD-banding. Finally, the slides were stained with DAPI (inversion of the DAPI colour revealed the RE-banding). The residual bands obtained after RE digestion were suitable for chromosome identification.

CBP-banding sequential to G-bands or RE-bands

The C-banding technique was performed sequentially to G-bands, or to RE-banding, and after destaining the slides. CBP-banding (C-bands by barium hydroxide using propidium iodide) was done using the standard procedure of Sumner (1972) but with propidium iodide as a counterstain. The use of either Giemsa or propidium iodide counterstain produces results similar to C-band identification. However, there are some technical limitations that must be considered. For example, the intensity of the bands in RE treatments seems related to the type of counterstain used (e.g. Giemsa or fluorochromes) (Gosálvez *et al.* 1991). Therefore, it is not advisable to compare results obtained by using different counterstains. Briefly, the slides were submitted to routine C-banding with classical treatment times reduced, approximately, to half: hydrochloric acid (0.1 M) 20 min, barium hydroxide (5% solution) 7 min and 2× saline sodium citrate (2× SSC: 0.3 mol/l NaCl, 0.03 mol/l sodium citrate) at 60°C for only 40 min. The slides were counterstained with 1.5 µg/ml propidium iodide. The images in figures 1 and 2 are representative of at least 35 metaphases observed (for each RE used), in no less than five experiments carried out at different times.

Chromosome observation

Chromosomes were observed with a Zeiss Axioplan 2 imaging microscope coupled to an Axiocam digital camera with AxioVision software version 2.0.5 (Zeiss, Göttingen, Germany). Digitized photos were prepared for printing in Adobe Photoshop version 5.0; contrast and colour optimization were the functions used, and they affected the whole of the image equally.

Results and discussion

We used eight different REs in chromosome preparations of *T. pecari* and *P. tajacu*. The action of all eight REs and RE+C-banding on peccary chromosomes are presented in figures 1 and 2. In the left column of figures 1 and 2, we present control G-banding and C-banding of the chromosomes, which are the comparative controls for the analysis of the eight RE actions. The G-banding technique was used for the identification of chromosomes in the control experiment of C-banding (without previous *in situ* RE digestion).

The residual RE bands (figures 1 and 2, left column in each RE presented) are mainly G-like and suitable for chromosome identification. One would expect as many banding patterns as there are REs available, since the DNA cleavage should be different for each RE. However, most of the banding patterns obtained by treatment with different REs overlap. Some REs (e.g. *ApaI*, *PstI*, *RsaI*) produced a higher banding contrast. The banding patterns demonstrated for each RE are reproducible and can be used in sequential experiment procedures without loss of chromosome morphology.

The C-positive heterochromatin (figures 1 and 2, right chromosome in each column, control C-band and RE+C-band presented) is mainly found at the centromeres of most chromosomes, although some C-bands are observed at interstitial locations. In the individuals analysed, some heterochromatin polymorphisms of minor significance were detected, as observed for pig chromosomes (Adega *et al.* 2005), but these chromosome polymorphisms did not interfere with the heterochromatin patterns presented and discussed in the present work.

Constitutive heterochromatin (C-positive heterochromatin) characterization in T. pecari

In figure 1, the C-positive heterochromatin (C-bands) is presented for all *T. pecari* chromosomes. All the autosomal chromosomes exhibit centromeric C-bands, except chromosome 4 that shows no detectable C-positive heterochromatin at this location. At the centromere location of chromosomes 5 and 6, it is possible to observe large C-positive heterochromatin blocks. Chromosomes 1, 2 and 4 show interstitial C-positive heterochromatin. Telomeric C-bands can be seen on chromosomes 1, 2, 3 and 4. The X and Y chromosomes also present C-positive heterochromatin regions. The acrocentric X chromosome exhibits centromeric, interstitial and telomeric C-blocks.

The Yq-arm appears heterochromatic with no detectable C-positive heterochromatin at the centromeric region.

When we applied REs and sequential C-banding to the chromosomes, it was possible to verify the C-band heterogeneity (figure 1), similarly to what has been observed in other species (Chaves *et al.* 2004; Adega *et al.* 2005).

The arrowheads in figure 1 indicate the C-bands revealed only after the RE treatment. These bands were not disclosed by classical C-banding procedures. These C-positive bands, only disclosed by previous RE treatments, could be explained by the fact that RE digestion might increase the capacity of the stain (in the present work, propidium iodide) to bind to a specific chromosome region (Gosálvez *et al.* 1997; Nieddu *et al.* 1999; Chaves *et al.* 2004). Most of these bands are interstitial. Chromosome 1 is the autosome revealing the highest number of C-positive heterochromatin bands. Also interesting is the clear intra-C-band revealed on the X chromosome after *MboI*+C-banding. It seems that the X centromeric C-band (figure 1) with this RE+C treatment split into two evident bands.

In *T. pecari* chromosomes (cf. figure 1), it was possible to identify at least three major classes of CH: (peri)centromeric, interstitial, and telomeric. With the RE+C-banding treatment, these major C-positive heterochromatin blocks could be discriminated in at least 21 C-positive heterochromatin subclasses (figure 1; and evaluation of the RE action in the major C-positive heterochromatin bands, data not shown), which are indicative of a different C-positive heterochromatin composition reflected by a different RE action on the respective chromosome bands. Clearly, the RE+C treatments are different when comparing centromeric, interstitial or telomeric bands. Furthermore, inside each major C-positive heterochromatin class, for instance centromeric, it is possible to discriminate at least 10 autosome centromeric subclasses.

Constitutive heterochromatin (C-positive heterochromatin) characterization in P. tajacu

In figure 2, we present the C-positive heterochromatin (C-bands) for the *P. tajacu* chromosomes. On the majority of the autosomal chromosomes, it is possible to observe centromeric C-bands. However, that is not the case on chromosomes 5 and 11. Autosomes 1, 2, 3, 4, 6, 7, 8, 10 and 12 reveal interstitial C-bands, and chromosomes 1, 2, 5, 6, 7 and 8 show telomeric C-bands. The submetacentric chromosome 2 is the autosome that presents the highest number of C-positive heterochromatin bands. The sex chromosomes also show C-positive heterochromatin blocks. The X chromosome presents conspicuous centromeric and interstitial C-bands, but on the Y chromosome only a telomeric C-band is detected.

The RE+C-banding on *T. pecari* chromosomes revealed CH heterogeneity, and this was also observed with this approach for *P. tajacu* C-positive heterochromatin

The arrowheads in figure 2 indicate interstitial and telomeric C-bands revealed only by previous RE treatment. Inside each major CH class—(peri)centromeric, interstitial and telomeric—for each different morphological chromosome type, it was possible to discriminate at least 32 C-positive heterochromatin subclasses (figure 2; and evaluation of RE action in the major C-positive heterochromatin bands, data not shown), suggesting that this species also presents a karyotype highly rearranged in respect of its CH.

Interspecies constitutive heterochromatin (C-positive heterochromatin) relationship

The comparative chromosome painting results between the domestic pig and white-lipped and collared peccaries clearly revealed the high level of reshuffling between these two karyotypes (Bosma *et al.* 2004; Adegá *et al.* 2006). Based on these two works, and on the assumption that the presence of CH facilitates the occurrence of chromosomal rearrangements (Yunis and Yasmineh 1971; Peacock *et al.* 1982; John 1988; Chaves *et al.* 2004), we made a comparative analysis between *T. pecari* and *P. tajacu* CH localization and the breakpoints in each chromosome (figure 3). In figure 3, the corresponding breakpoints (assigned by the pig paint probes) in *T. pecari* and *P. tajacu* were named with letters in the G-banded karyotype. It was possible to assign 22 breakpoints (letters A to V). The location of the CH bands is indicated to the right of each chromosome. In *P. tajacu*, it was possible to correlate CH with 18 breakpoints. In *T. pecari* it was not possible to determine the exact correspondence for a few equivalent breakpoints (e.g. *T. pecari* chromosome 2, breakpoint F/G). Therefore, in *T. pecari*, only four of the 20 breakpoints did not present CH. These results suggest that CH could somehow be involved in the chromosome rearrangements that have occurred during the evolution of these species. Moreover, some of the breakpoints where it was not possible to assign CH are the same in the two species (*P. tajacu* chromosome 1 and *T. pecari* chromosome 3, breakpoint C; *P. tajacu* chromosome 5 and *T. pecari* chromosome 5, breakpoint N), suggestive of a further ancient rearrangement, considering the hypothesis of CH elimination during the process of chromosomal rearrangement.

The general comparison of the distribution and characterization of C-positive heterochromatin in the two tayassuid species suggests that the karyotypes of these species are extremely divergent. This can be seen in the present work by analysing the different actions of the same RE in the two karyotypes: e.g. *AluI*+C treatment generates conspicuous C-positive heterochromatin blocks in *T. pecari*, in contrast to the near nonexistence of C-positive heterochromatin blocks on *P. tajacu* chromosomes. Another important feature is that

the CH variation seems more pronounced for *P. tajacu* than *T. pecari* karyotype, suggesting not only differences in the nature of CH in the two karyotypes, but also considerable heterogeneity within each karyotype. Evidence for this comes from the higher number of C-positive heterochromatin subclasses identified for *P. tajacu* (32), compared to *T. pecari* (21), and the large number of C-positive heterochromatin subclasses detected in both species.

According to the fossil record, stratigraphy, and nuclear and mitochondrial DNA-based evolutionary analyses (Gongora and Moran 2005), the divergence of collared and white-lipped peccaries could have taken place before the emergence and the completion of the land bridge at the Isthmus of Panama that occurred some time in the late Pliocene, 2.5 to 3 million years ago. Thus, our data suggest that the radiation of the two species was extremely fast, and the great dissimilarity between the species is also in agreement with the results from the comparative chromosome data with pig probes (Bosma *et al.* 2004; Adegá *et al.* 2006). The higher number of C-positive heterochromatin classes/subclasses on *P. tajacu* autosomes suggests that this karyotype is more primitive, that is it did not go through a high level of homogenization due to concerted evolution of chromosomes. This karyotype resembles the ancestral form, displaying a high diversity of CH, which is thought to facilitate the occurrence of chromosomal rearrangements (Yunis and Yasmineh 1971; Peacock *et al.* 1982; John 1988; Chaves *et al.* 2004).

Finally, the analysis of the X chromosome C-positive heterochromatin in both species demonstrated that the acrocentric form of *T. pecari* contains a higher amount and number of C-positive heterochromatin blocks, in comparison with the submetacentric *P. tajacu* X chromosome; as well as a different CH molecular nature. In the *T. pecari* acrocentric X chromosome it was possible to identify three major C-positive heterochromatin bands, and the *MboI*+C treatment revealed an extra block at the pericentromeric region of the X chromosome. The *P. tajacu* submetacentric X chromosome shows only two major C-positive heterochromatin blocks. These results suggest that the acrocentric X chromosome form from *T. pecari* is the more primitive, assuming again that the presence of higher CH diversity is an ancestral condition resembling the ancestral karyotype form, not having undergone the characteristic heterochromatin homogenization of concerted evolution (Elder and Turner 1995).

General remarks and conclusions

Although the constitutive heterochromatin of the tayassuid species *P. tajacu* and *T. pecari* have been previously described by classical C-banding procedures (Pirtle 1967

Benirschke and Kumamoto 1989), the characterization of CH heterogeneity is documented here for the first time for both species.

The present work demonstrated that *in situ* RE digestion with sequential C-banding (figures 1 and 2) could be an alternative tool for the study of CH heterogeneity in tayassuid chromosomes, especially when other techniques, such as fluorescence *in situ* hybridization with repetitive sequences (from the species in analysis), are not available.

The C-positive heterochromatin characterization of *P. tajacu* and *T. pecari* autosomes suggests that the *P. tajacu* autosomal karyotype is more primitive, and has accumulated great diversity in its CH. This idea is also supported by Gongora and Moran (2005), who performed nuclear and mitochondrial evolutionary analyses in collared, white-lipped and chacoan peccaries. These authors even suggest that the collared peccary should be classified in a separate genus within the family Tayassuidae, which is also consistent with previous analyses of cytochrome *b* by Theimer and Keim (1998). The chromosome painting studies (Bosma *et al.* 2004; Atega *et al.* 2006) also reported an extensive karyotypic variation among the living Tayassuidae, which is in accordance with the great variation found in the C-positive heterochromatin of *P. tajacu* and *T. pecari* karyotypes in the present study. Finally, the tayassuid X chromosome primitive form seems to be the one of *T. pecari*. This finding is not surprising, since the evolution of sex chromosomes is often independent of that of the autosomal complement (Ohno 1967; Graves and Shetty 2000). Further studies regarding CH characterization, chromosome painting and other molecular analyses in tayassuid species will certainly contribute to a better understanding of the evolutionary processes and phylogenetic relationships within this family.

Acknowledgements

We sincerely express our gratitude to Anneke Bosma for kindly providing the fixed cell cultures of *T. pecari* used in this work. We thank Lisa Bates from Tucson Wildlife Center, Inc., and Donald DeYoung from The University of Arizona, for their kind supply of *P. tajacu* samples. This work was supported by a PhD grant (SFRH/BD/3280/2000) of the Science and Technology Foundation (FCT) of Portugal, and INVOTAN and FCT (SFRH/BSAB/329/2003) sabbatical grants.

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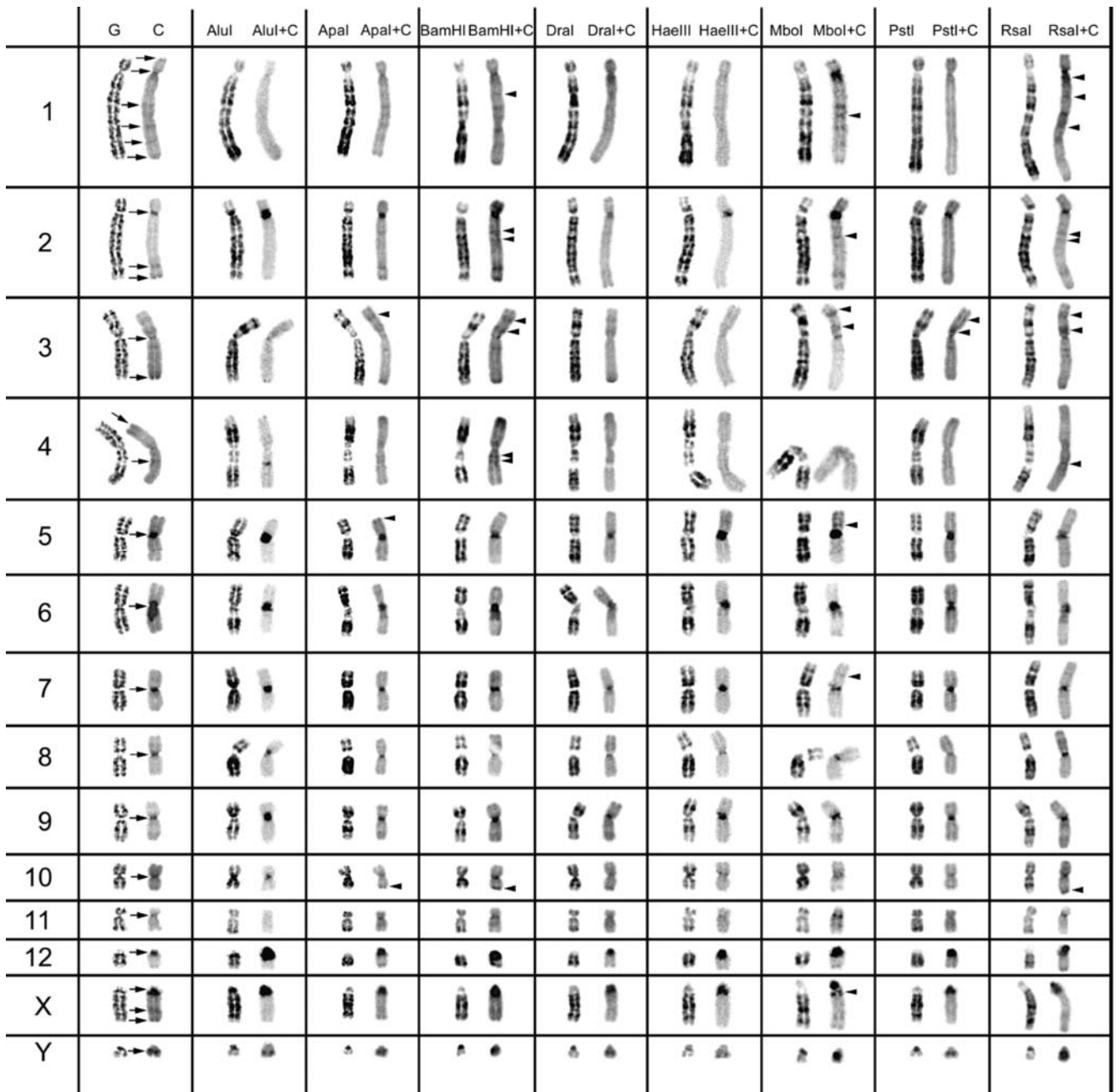


Figure 1. Control G-banding and C-banding of *Tayassu pecari* chromosomes are shown on the left, with the classical C-bands indicated by arrows. The other columns show the bands produced by the eight different restriction endonucleases, before and after C-banding (arrowheads indicate C-positive heterochromatin bands only revealed by previous RE treatment).

	G	C	AluI	AluI+C	ApaI	ApaI+C	BamHI	BamHI+C	DraI	DraI+C	HaeIII	HaeIII+C	MboI	MboI+C	PstI	PstI+C	RsaI	RsaI+C
1																		
2																		
3																		
4																		
5																		
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11																		
12																		
13																		
14																		
X																		
Y																		

Figure 2. Control G-banding and C-banding of *Pecari tajacu* chromosomes are shown on the left, with the classical C-bands indicated by arrows. The other columns show the bands produced by the eight different restriction endonucleases, before and after C-banding (arrowheads indicate C-positive heterochromatin bands only revealed by previous RE treatment).

PTA	Chr	TPE
Breakpoint CH A ——— B ——— C ——— D ——— (-) E ——— F ——— G ———	1	Breakpoint CH R ——— I ———
H ——— I ———	2	H ——— F/G ——— Q ———
J ——— K ——— (-)	3	A ——— B ——— C ——— D ——— (-) E ———
L ———	4	K/S ——— (-) M ———
M ——— N ——— (-)	5	P/M ——— N ——— (-)
O ———	6	O/U ———
P ———	7	Q/L ———
Q ———	8	P/L ———
R ———	9	O ——— U ——— (-)
S ———	10	V/T ———
T ——— (-)	11	
U ———	12	
V ———	14	

Figure 3. Homologous breakpoints in *Pecari tajacu* (PTA) and *Tayassu pecari* (TPE) (assigned by comparative chromosome painting with pig probes) named with letters (A to V) in the G-banded karyotypes. The location of the CH bands is indicated to the right of each chromosome by a dash.