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Interspecific hybridization in oysters: Restriction Enzyme Digestion Chromosome Banding confirms *Crassostrea angulata* × *Crassostrea gigas* F1 hybrids

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Abstract:

The taxonomic status of the two commercially important cupped oysters, *Crassostrea angulata*, the Portuguese oyster (Lamarck, 1819) and *Crassostrea gigas*, the Japanese oyster (Thunberg, 1793) has long been in question. The recent observation of the hybridization between *C. gigas* and *C. angulata* and the production of fertile F1s led us to search for cytogenetic evidence of both parental genomes in the interspecific hybrids. The cytogenetic characterization of the hybrids was performed by the use of restriction endonuclease treatments. This technique has recently shown the potential for individual chromosome identification by banding in oysters. Chromosomes of *C. gigas*, *C. angulata* and their hybrids were treated with two different restriction enzymes (*Apa*I and *Hae*III), stained with Giemsa, and examined for banding patterns. These chromosome markers allowed the parental haploid sets to be identified in the hybrids. The analysis of the banded karyotypes of the interspecific hybrids showed that for each chromosome pair, one of the homologues presented a banding pattern consistent with that of *C. gigas* and the other homologue presented a banding pattern consistent with that of *C. angulata*. These cytogenetic results substantiate the reported interspecific hybridization between *C. gigas* and *C. angulata*. In view of these results and taking into account the present expansion of *C. gigas* aquaculture in southern Europe, the question of the need for preservation of pure *C. angulata* stocks should be raised as only a few populations remain in the south of Spain and Portugal. Recently, changes in the genetic composition of populations in southern Portugal have indeed been observed, showing that human activities have created contact zones between the two taxa while no natural sympatric zones exist in Europe.

Keywords: Chromosome banding; *C. angulata*; *Crassostrea gigas*; In situ restriction enzyme banding; Interspecific hybrids

1. Introduction

The Pacific oyster Crassostrea gigas and the Portuguese oyster Crassostrea angulata have often been considered as the same species (Menzel, 1974). Recently however, differences between these two species have been observed at several levels. These include differing ecophysiological characteristics (His, 1972; Goulletquer et al., 1999; Haure et al., 2003) and growth rate, where several studies have concluded that the Pacific oyster has a greater growth potential than the Portuguese oyster (eg. Bougrier et al., 1986; Parache, 1989; Soletchnick et al., 2002). Furthermore, genetic differences have been observed at several levels, through studies of the mitochondrial cytochrome oxidase subunit I (COI) gene (Boudry et al., 1998; O'Foighil et al., 1998; Boudry et al., 2003), and microsatellite analysis (Huvet et al., 2000). Karyotype analysis highlighted the close genetic similarity of these two taxa in comparison with other cupped oyster species (Leitão et al., 1999a), although differences between their respective karyotypes were observed using G- banding, notably on chromosome pair 7 (Leitão et al., 1999b). The comparative analysis of restriction enzyme (RE) ideograms have recently revealed different restriction in situ banding patterns for these two species with each of three different REs (Leitão et al., 2004). Chromosome 10 however showed similar longitudinal banding in the two species, suggesting that this chromosome is the most conserved between C. gigas and C. angulata. On the other hand, the general dissimilarity between the restriction in situ banding patterns of C. gigas and C. angulata suggested that these two species are two different cytotypes.

In marine bivalves, several cases of interspecific hybridization between close species have been reported, these include clams (Mercenaria spp: Bert et al., 1993) and mussels (Mytilus complex spp: eg. Rawson et al., 1999; Bierne et al., 2002). In oysters, fertilisation in crosses between C. gigas and C. virginica appeared to be normal *per se*, however subsequent larval development ceased before the umbo stage (e.g. Stiles, 1973, 1978; Gaffney and Allen, 1993). In C. gigas x C. sikamea, the crosses presented a clear asymmetry in fertilization success. Eggs from C. sikamea were readily fertilized by *C. gigas* sperm, yet the reciprocal cross resulted in little or no fertilization (see Gaffney and Allen, 1993 for review). In contrast, crosses between C. gigas and C. rivularis and the C. gigas x C. iredalei cross had limited fertilization success (Menzel, 1987; see Gaffney and Allen, 1993 for a review). While there were high fertilization rates in C. gigas x C. rhizophorae crosses, no larvae survived to metamorphosis. Although there are many reports of the successful production of hybrids between C. angulata and C. gigas (Imai and Sakai, 1961; Menzel, 1974; Huvet et al., 2002), until now no cytogenetic confirmation of the hybridization between these taxa has been made. Moreover there is a lack of nuclear species-diagnostic markers, since only mitochondrial diagnostic markers were used by Boudry et al. (1998) and "pseudospecific" nuclear markers by Huvet et al. (2004) to differentiate between C. angulata and C. gigas. In Southern Europe, the recent transplantation of C. gigas for aquacultural purposes (Ruano, 1997) has created a contact zone where the two taxa have apparently produced viable F1 hybrids (Huvet et al., 2004). This introduction could endanger the few remaining populations of C. angulata present in the south of Portugal and Spain

One of the most useful applications of the cytogenetic analysis in aquaculture involving interspecific hybridization is the identification of elements of the haploid sets of the parental species in the chromosome complement of the hybrid products (Philipps and Reed, 1996). Until now however, only standard karyotypes have been made with hybrid oyster chromosome complements to show pairing of the parental haploid complements in some experimental interspecific hybrids: C. virginica × C. corteziensis (Rodriguez-Romero and Montes de Oca, 1995) and C. virginica × C. rhizophorae (Rodriguez-Romero and Montes de Oca, 1998).

As mentioned above, the identification of the ten individual chromosome pairs of three species of oysters by G-banding (Leitão et al., 1999b) allowed a accurate comparative analysis of the karyotype of these species. However the classical cytogenetic technique of G-banding has some disadvantages such as limited reproducibility, large time investment required, and the fact that the banding is often lost during any subsequent in situ hybridization (FISH) procedure. More recently the application of restriction enzyme chromosome banding (RE banding) to four species of oysters (Leitão et al., 2004), including C.gigas and C. angulata, provided 3 new different patterns of chromosomes identification (one for each enzyme) for all species studied. RE banding also has the major advantage of being compatible with FISH (Chaves et al, 2002).

In this study we applied the restriction enzyme digestion technique to karyotypes of F1 interspecific hybrids of C. gigas and C. angulata, in order to characterize their karyotypes and thus provide a cytogenetic verification of hybridization between the two taxa. This will aid prediction of their future co-evolution in the recently created hybrid zones.

2. Material and Methods

Biological material

Specimens of C. gigas (Thunberg) were collected from the Seudre estuary, where this species was introduced from Japan in the 1970s (Grizel and Héral, 1991), and is currently farmed on a large scale. Specimens of C. angulata (Lamarck) were collected in Setúbal bay (Portugal), and then acclimated at the IFREMER hatchery, where reciprocal crosses between these two taxa were made to obtain F1 interspecific hybrids.

Chromosome preparation

Whole juvenile animals (ca. 2.5 cm length) were incubated for 7-9 h in a 0.005 % solution of colchicine in seawater. Because cell cultures are not yet available for molluscs, we used growing somatic tissues from the gills as a source of mitoses. After dissection, the gills were treated for 30 min in 0.9 % sodium citrate in distilled water. The material was fixed in a freshly prepared mixture of absolute alcohol and acetic acid (3:1) with three changes of 20 min each. Fixed pieces of gill from each individual were dissociated in solution of 50 % acetic acid in distilled water. The suspension was dropped onto heated slides at 44º C and air-dried (Thiriot-Quiévreux and Ayraud, 1982). The slides were kept at -20º C until they were needed.

In Situ Restriction Endonuclease Digestion

Slides were aged for 6 h, in a dry incubator at 65º C, before the restriction endonuclease treatment. The restriction enzymes used (ApaI and HaeIII) were diluted in the buffers indicated by the manufacturer (Invitrogen, Life Technologies), and final concentrations of 30 U were obtained per 100 µl. 100 µl of the appropriate solution were placed on the slides and covered with coverslips. These slides were incubated in a humid chamber for 16h at 37º C. Control slides were submitted to the same treatment as described above but incubated with the buffer alone. The slides were then washed in distilled water, air dried and stained with Giemsa (1% solution, diluted in phosphate buffer at pH 6.8).

Microscopy and Image processing

RE-banded metaphase images were acquired with a CCD camera (Axiocam, ZEISS) coupled to a ZEISS Axioplan 2 Imaging microscope. Digitised photos were printed from Adobe Photoshop (version 5.0) using only contrast and colour functions that optimised entire images.

Karyotypes organization

A total of 20 hybrid karyotypes banded with Apa I and 18 hybrid karyotypes banded with HaeIII were observed. The karyotypes of the F1 interspecific hybrids were organised following their length and centromeric index, but equally by following the banding pattern obtained with the restriction endonucleases ApaI and HaeIII.

3. Results

The diploid chromosome number of the F1 interspecific hybrids was 20 as in all Crassostrea species. The two REs used yielded specific banding patterns. The in situ RE experiments performed with the two REs (ApaI, GGGCC/C and HaeIII GG/CC) were compared with control (buffer only) treatments on slides from the same F1 interspecific hybrids. In all control slides, there was no banding pattern induced in the chromosomes, and all chromosomes showed a Giemsa standard staining.

Examples of banded metaphases of the F1 interspecific hybrids with the two REs are given in figure 1. The complete results are brought together and summarised in figure 2 which shows each one of the ten chromosome pairs of the F1 interspecific hybrids as well as an example of the haploid set of both parental species, for each of the two enzymes used.

To compare each homologue of each chromosome pair in the F1 interspecific hybrid complement with the haploid set of the parental species, only the number and relative position of the

major bands were taken into account; the intensity of the bands was not considered. The intensity of the bands in RE treatments appears to be related to the type of counterstaining used (e.g. Giemsa or fluorochromes) (Gosálvez et al, 1991). Furthermore, there is no agreement with the correlation between the loss of DNA extraction (after RE treatment) and the reduction in the staining (Gosálvez et al., 1991). Several authors demonstrate that the loss of DNA after a RE digestion can increase the capacity of the stain to bind to a specific chromosome region (Gosalvez et al., 1991; Nieddu et al., 1999).

The comparison of the ten pairs of banded chromosomes (cf. figure 2) from hybrids with those from the parental species, revealed that one of the homologues in each pair presented the same general restriction in situ banding pattern as C. angulata, and the other homologue presented a pattern like C. gigas. An example of this, is clearly shown by chromosome 1 where the ApaI banding produces three major bands (near centromeric, near telomeric and central arm band) in the long arm of one of the homologues. This pattern is similar to C. angulata. In the long arm of the other homologue five major bands can easily be observed which are distributed longitudinally along the long arm, in this case the pattern is similar to that of *C. gigas*.

This is also evident for the long arm of chromosome 3 where the HaeIII banding produced one major median band in one of the homologues, similar to C. angulata, where the other homologue presents three major bands (near centromeric, near telomeric and central arm band) similar to the pattern of C. gigas. Chromosome 2 is also a good example, where the HaeII banding pattern produced two major bands in the short arm, and one pericentromeric and three major bands in the long arm of one of the homologues, this pattern is similar to C. angulata. Whereas the other homologue presented one major band in the short arm and one major band in the long arm, in this case the pattern being similar to C. gigas.

There are some chromosomes where the general chromosome banding pattern of C. angulata (and corresponding hybrid chromosome) is particularly different from that of C. gigas (and corresponding hybrid chromosome). The most obvious case can be observed in chromosome 7 of the hybrids with the restriction enzyme ApaI (Figure 2). One of the homologues of the interspecific hybrid presents two major bands in the short arm (in a near centromeric and near telomeric region), and one near centromeric band and one major band in the long arm (in a near telomeric region), like C. angulata. In C. gigas though, and the corresponding hybrid homologue, there is only one major central band in the short arm, and two major bands in the long arm (in a near centromeric and medium position).

4. Discussion

According to the statistics of the Food and Agriculture Organization (FAO, 1999) only Portugal produces C. angulata oysters (618 tons in 1997). Nevertheless, the presence of C. gigas was detected in three sites along the southern European coasts in Tavira, Ria Formosa and Barrinha, Faro (Fabioux et al., 2002). Hybrid zones provide unique opportunities to study evolutionary processes that maintain reproductive isolation between species. The expansion of C. gigas aquaculture to southern Europe has put these two taxa in contact, creating a putative hybrid zone. Hybridization issues are complex and especially problematic for rare species that come into contact with other species that are more abundant (Allendorf et al. 2001), as seen in this case. Huvet et al., (2000) suggested for the first time that natural hybridization may occur in the south of Portugal between C. gigas and C. angulata. Furthermore, Huvet et al., (2001, 2002) showed that minimal hybridization occurred between C. angulata and C. gigas in nature, despite the sympatric occurrence of both taxa and the successful production of viable hybrids in laboratory conditions. More recently, Huvet et al., (2004) provided the first genetic data showing natural hybridization between these two taxa, however using for this a "pseudospecific" nuclear marker marker together with an mtDNA marker.

REs have been used on chromosomes of several species (from plants to animals) to produce in situ cleavage of the DNA molecule housed in the chromosome. This is visible as a longitudinal differentiation of the chromosomes or a banding pattern (in situ restriction banding pattern; for a review, see Gosálvez et al., 1997). In bivalves, this technique has been applied to only 6 species: Mytilus galloprovincialis (Martinez-Lage et al., 1994), Argopecten purpuratus (Gajardo et al., 2002), C. angulata (Leitão et al., 2004, Cross et al., 2005), C. gigas, Ostrea edulis and O. conchaphila (Leitão et al., 2004). In all cases, specific chromosome bands were obtained after digestion with REs. This technique has also been applied in a chromosomal evolution study within the Ostreidae family (Leitão et al., 2004). The dissimilarity in longitudinal differentiation of chromosomes between species karyotypes

reflects a different gene distribution (Verma and Babu, 1995). It is well known, for example, that R-bands are relatively rich in genes (Sumner, 2003), and a different R-banding pattern between different species karyotypes could be correlated with a different gene pattern distribution. In situ RE banding could also be correlated with the pattern of gene distribution, because the REs are base-specific. It seems that the integrity of each separate chromosome/gene pool was maintained in the F1 hybrids which had one chromosome from each parent (C. gigas and C. angulata), in each pair.
The application of the REs ApaI and HaeII to the F1 interspecific hybrids in this study showed that for each pair of chromosomes, one of the homologues presents the banding pattern consistent with that of C. gigas and the other homologue presents the banding pattern consistent with that of C. angulata. This chromosomal evidence substantiates the interspecific hybridisation between the two taxa. Some of the chromosome pairs in the interspecific hybrids showed greater differences between banding patterns of the homologues, than did other pairs. This was the case with chromosome 7, which had already shown the highest dissimilarity in G-banding pattern between C. gigas and C. angulata in a previous study (Leitão et al., 1999b). The present study, is the first cytogenetic confirmation of the hybridization between these two species through identification of complete parental genomes on the karyotypes of F1 interspecific hybrids.

The next step to improve our understanding of the taxonomic relationship between these two closely related species should be to analyse RE chromosome banding of the F2 interspecific hybrids, and meiosis of the F1s (formation of bivalents, genetic recombination/admixture). Such studies will help in making realistic predictions about the co-evolution of these two taxa in this zone of southern Europe. Differences observed between homologous chromosomes may lead to pairing difficulties and be important in generating infertility barriers. Hybridization might have begun only recently and is probably geographically restricted to the recently created hybrid zones, but if conservation measures are not taken, this situation might be problematic especially because C. angulata is a rare species. Accurate identification of hybrids is important not only for sustainable aquaculture development, guiding aquaculture domestication efforts and identifying useful crosses, but also for a better understanding of biodiversity issues (Bartley et al., 2001).
The application of restriction enzyme chromosome banding to closely related species (such as those in this study) constitutes an additional tool in hybrid recognition and has been demonstrated to be a more reliable and more expeditious for oyster chromosome banding than classical banding techniques (Leitão et al., 2004). It also has the advantage that it can be used simultaneously with FISH techniques (Chaves et al., 2002), supporting the development of gene mapping in oysters.
Besides its value as a new approach to specific problems in oyster taxonomy (this study, Leitão et al., 2004), this technique may also be very useful in other studies of more economic or ecological importance. The use of REs has for instance, provided a rapid method for the identification of the missing chromosomes in the study of the economically important aneuploidy phenomenon reported in oysters (Bouilly et al., 2005) and could also offer a valuable technique for chromosome segregation studies on commercially important triploid or tetraploid oysters (Guo and Allen, 1997).

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Figures

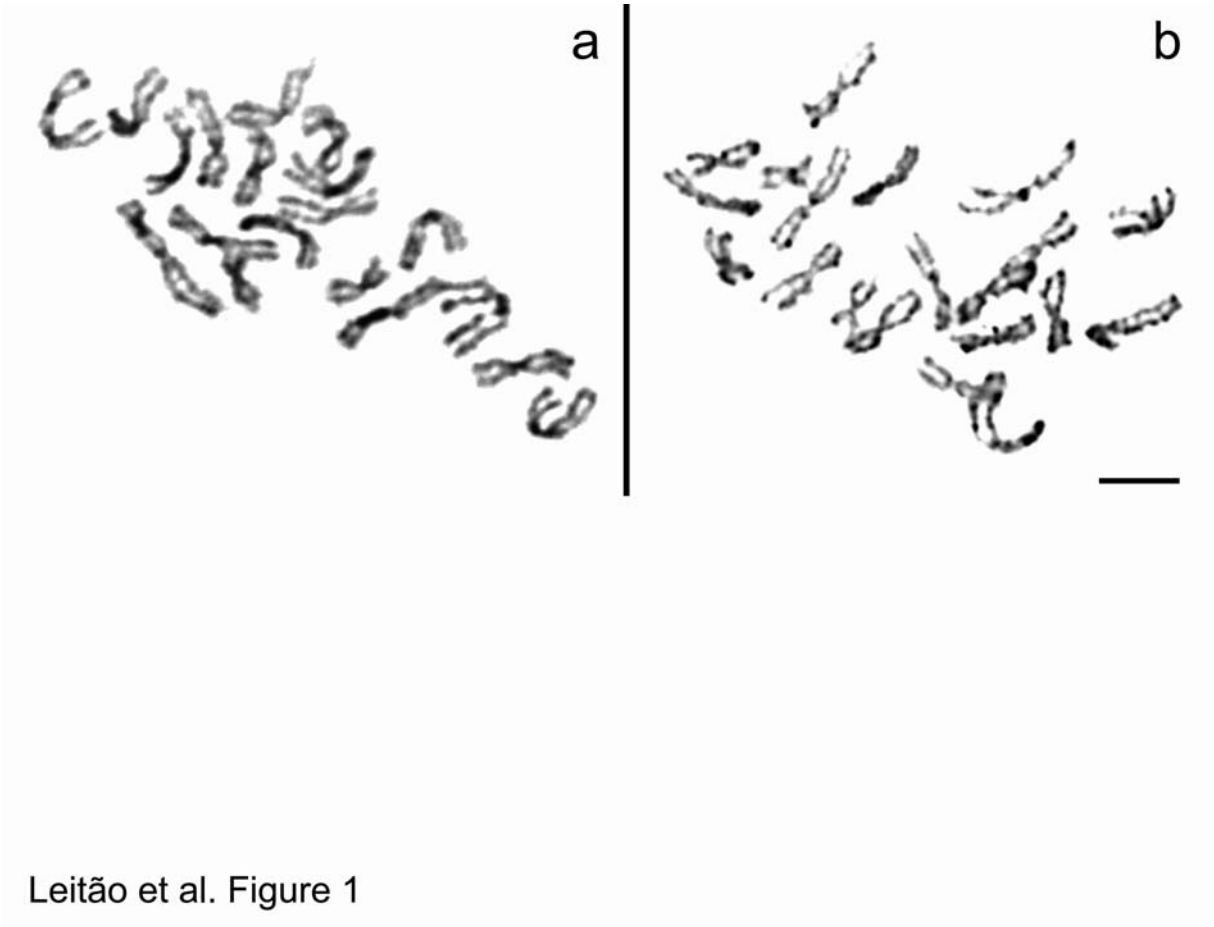
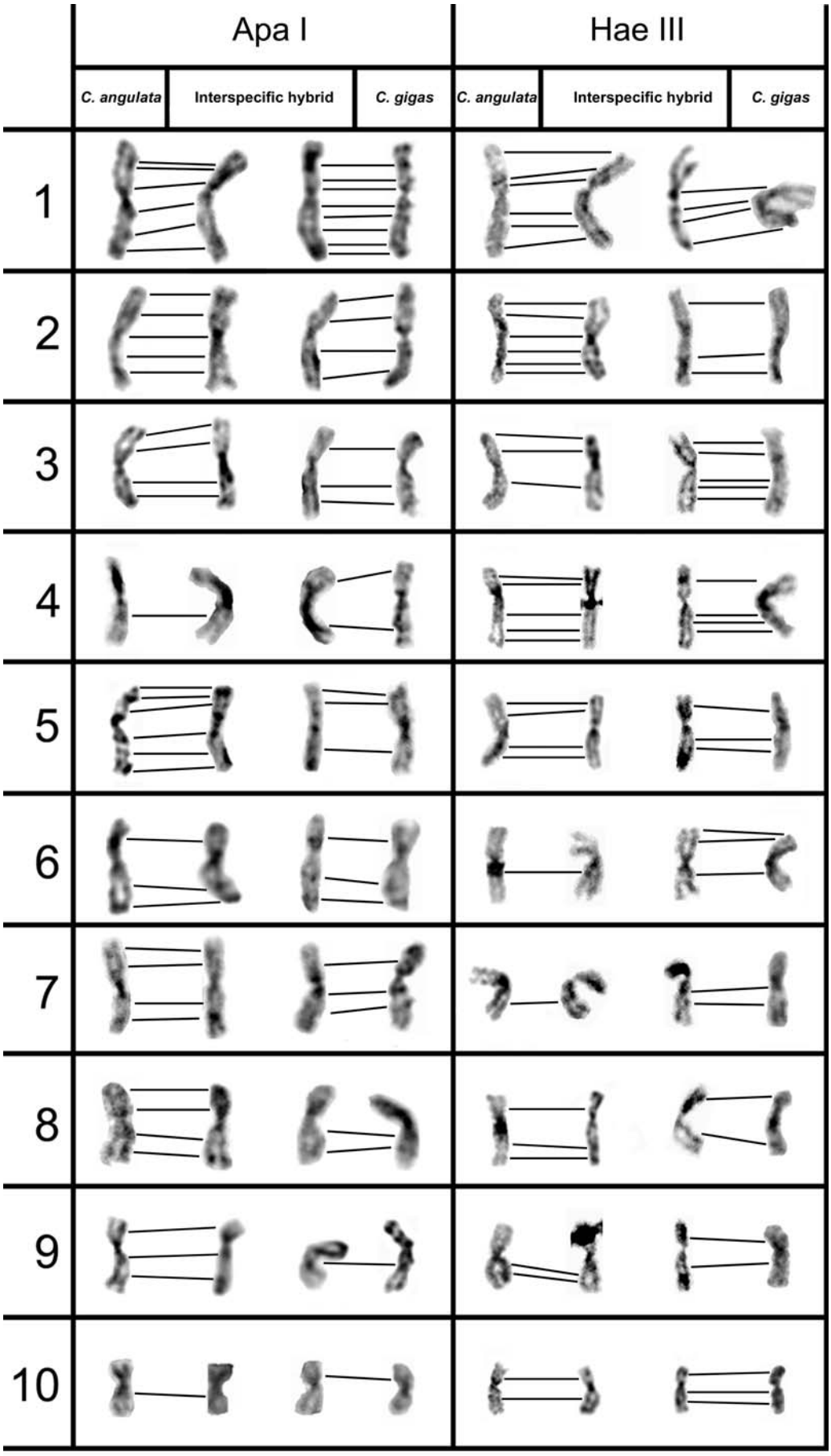


Figure 1. Examples of metaphases banded with the two REs. (a) metaphase of F1 interspecific hybrid C. angulata x C. gigas with ApaI; (b) metaphase of F1 interspecific hybrid C. angulata x C. gigas with HaeIII. Scale bar = 5 µm.



Leitão et al. Figure 2

Figure 2. Diploid distribution of chromosomal bands in the F1 interspecific hybrid and haploid distribution of chromosomal bands in each of the parental species: C. angulata and C. gigas. Dark lines indicate only the major bands that allow the inference of similarities between each hybrid homologue and the respective parental chromosome.