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"CHROMOSOME AND GENOTYPE CHARACTERIZATION IN SOME RICE BIOTYPES SHOWING GRAIN SHATTERING"

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DOTTORANDO

CHIARA RUINI

DOCENTE GUIDA

Prof. LIVIA MARTINETTI

DOCENTI DI SUPPORTO

Prof. ANTONIO CESARE SPARACINO
Prof. CARLOTTA HALFER
Prof. FILIPPO GEUNA
Dr. PIETRO PARMA

COORDINATORE DEL DOTTORATO

Prof. DANIELE BASSI

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ABSTRACT

CHROMOSOME AND GENOTYPE CHARACTERIZATION IN SOME RICE BIOTYPES SHOWING GRAIN SHATTERING

Red rice (*Oryza sativa var. silvatica* L.) is a common weed in rice crop, very troublesome to control due to high shattering. The aim of the present research was to analyse the genome of some biotypes of red rice in order to promote innovatives methods for its control.

In the first year, morphological and karyotypical analysis of some phenotypically different red rice biotypes in comparison with the rice cultivar ' Loto' were carried out. The seeds of rice and red rice were sown in greenhouse and afterwards the root tips of the seedlings were employed for the cytological analysis. The chromosome preparations were made by the standard air-drying technique, after enzymatic maceration, and stained with a 4% Giemsa solution for 20 min., according to Fukui and Iijima' s method (1992).

The analysis, by means of a computerized chromosome image method (CHIA– EA), showed that in red rice various translocations occur, which always involve a chromosome of the first pair together with other elements of the set. Previous results clearly indicated that each red rice biotype is characterized by a specific translocation, showing a relationship among different phenotypes and karyotypes. It is important to note that in *Oryza sativa* one gene for shattering maps to the long arm of chromosome 1, which is always involved in the translocations. The project is divided into three phases: the field growing and observations, the laboratory analysis and the computer analysis. The first results that we have obtained support the proposed assumption. An increase in the dose of the genes frequently alters the phenotype, depending upon the effect of the genes involved.

In the second year, the research was based on the molecular analysis. Since the shattering gene is located on the long arm of the chromosome 1, which is involved in the translocation process, I have analyzed this genomic locus by both cytogenetic and molecular biology techniques.

The shattering character relies on the presence of a single-nucleotide polymorphism (SNP) which can explain about 70% of the non-shattering character associated to this gene whereby this SNP probably affects a regulatory region. In my experimental results, the CDS of the eight biotypes of *Oryza sativa* var. silvatica coincides with a rice genome

presenting a high non- shattering character, except for the case of the qSH1-SNP, located outside the CDS, coinciding with the low non-shattering genome rice.

In the third year, in order to ascertain if the shattering gene is displaced, breaks or remains on chromosome 1, we made use of the FISH method. The FISH cytogenetic method is employed in order to identify specific sequences of nucleic acids in the chromosome. It represents a significant contribution to standard cytogenetic for the identification of chromosome numerical and structural anomalies. It led however to an encouraging result for two different biotypes: the discovery that the shattering gene does not undergo translocation. Other QTLs for shattering character were noticed on chromosomes 1, 3, 4, 7, 8 and 11, in other cultivated rice species which partially explain the shattering character, although the responsible gene was identified only in three of these QTLs: 1, 4 and 7.

The analysis of the involved genes of chromosomes 4 and 7 in our eight red rice biotypes has revealed that the QTLs found on these chromosomes are probably not involved in the shattering character.

These analysis provide a wide overview of a possible correlation between the different phenotypes of the eight biotypes, shattering level, karyotypic differences connected with a specific translocation for each biotype and the fact that both these translocations and the gene responsible for the shattering of the eight biotypes are always present in chromosome 1. The investigation of the relationship between the various translocations and the expression of the shattering gene would require further and more sophisticated analyses.

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INTRODUCTION

THE ORIGIN AND TAXONOMY OF RICE

Rice is the most important cereal in the world and the main crop in almost all tropical and sub-tropical regions. Twenty-two species belonging to the genus *Oryza* (fam. *Poaceae*) are known nowadays, but only two of them (*Oryza sativa* L. and *Oryza glaberrima* Steud) are currently cultivated. Because of its economic and social importance, rice is one of the most studied plant, both from the genetic and from the cytological standpoint.

The wide botanical variability of rice is the result of an evolution process due both to natural factors and to the long selection performed by the growers.

The two cultivated annual species are supposed to derive from a common perennial progenitor (*Oryza perenne* Moench). It is believed that the basic differentiations occurred in South-Eastern Asia (*Oryza sativa*) and in the inner delta of Niger, in West Africa (*Oryza glaberrima*), while *Oryza sativa* evolved in quite different environments and gave rise to ecological forms belonging to three sub-species: *indica, japonica, javanica*.

The sub-species *indica* is widespread in South-Eastern Asia. The *indica* varieties present tall size, wide and drooping leaves, latent seeds and tendency to grain shattering (i.e. to drop the kernel before maturing).

The sub-species *japonica* evolved in China and spread in Korea, Japan and temperate countries, where genotypes with short grains, low amylose content, low grain shattering and tolerance to low temperatures were selected.

The sub-species *javanica* evolved in Indonesia and spread in Philippines, Japan and Formosa. Its characteristics are intermediate between the previously mentioned ones: tall size, low grain shattering, wide and upright leaves and seeds with awn (Bandino and Russo., 1989).

Rice needs a hot, humid climate and a large availability of water: rice crops are usually flooded during its entire period of growth.

Because of the steady increase of the world population, the future food production efficiency per unit area must be improved (Labrada., 1996). The yield of rice crop is strongly conditioned by the presence of weeds, which may reduce it even by 10-20 %. One of the main weeds is *Oryza sativa* subspecies *japonica* var. *sylvatica*, commonly called “red rice”. In the whole of the Korean peninsula, 22 red rice varieties are found and is also found in the Philippines, Vietnam, Nepal, Cambodia and other Asian countries. In Korea, most of red rice varieties/germplasms are classified by categories, such as plant type, panicle type and rice color. Many studies suggested that temperature is an important factor controlling the germination characteristics of red rice and it could be useful to classify red rice cultivars (Cho, Y.S., 2010).

RED RICE

GENERAL FEATURES

Red rice is an annual weedy form of *Oryza sativa*, classified as *Oryza sativa* L. var. *sylvatica*. It's morphological characteristics before the grain ripening phase are quite similar to the cultivated rice, and it is hardly distinguishable.

Red rice presents in general a greater size and heading earliness than the cultivated rice.

Among its main peculiarities we recall the tendency to grain shattering, the red colour of the pericarp and the presence of awns of different colour and length.

GRAIN SHATTERING

The tendency to drop kernels before maturity is called "shattering", and it is associated to a "shattering gene". Plants homozygous for the recessive allele (shsh) have no shattering aptitude, while plants heterozygous (Shsh), or homozygous for the dominant allele (ShSh) show a grain shattering phenotype. The sh gene affects the development of the three abscission layers between kernel and rachilla. In fact, this gene causes the suberization and the subsequent lignification of the abscission layers, leading to the grain shattering phenomenon (Nagao and Takahashi., 1963).

A pool of seeds is therefore created, from which red rice quickly spreads. Nevertheless, its reproduction is mainly due to self-pollination and cleistogamy, and only a small part gives rise to hybridation with the cultivated rice. In the process of rice domestication, human selection was likely to have favored mutations that reduced grain shattering but did not eliminate the formation or function of the abscission layer. In this way, grain loss due to shattering was largely prevented during harvest while a certain level of grain abscission was maintained so that the yield increase was not offset by creating difficulties in threshing. Rice was domesticated from wild grass species. Because wild grasses naturally shed mature grains, a necessary early step toward rice domestication was to select plants that could hold on to ripe grains to allow effective field harvest. The selection process might have been mainly unconscious because grains that did not fall as easily had a better chance of being harvested and planted in the following years. Consequently, nonshattering alleles had an increased frequency and eventually replaced the shattering alleles during domestication.

RED PERICARP

Figure 1. Kernels of red

A frequently observed character of red rice is the presence of a red pericarp of the grains, causing the name of the rice itself. The kernel colour, however, may range between red

and brown-black, and may even be white (Ferrero., 1994) **(Fig. 1)**.

The pericarp coloration is due to the concurrence of two complementary dominant genes, **Rc** and **Rd** (Bandino and Russo., 1989). **Rc** determines the basic pigment, while **Rd** increases its action, and controls the pigment distribution over the surface of the pericarp. The usual condition of cultivated varieties is determined, however, by the double recessive genes (**rcrd**), deleting the surface pigment of the pericarp. The brown pericarp kernels have a **Rcrd** genotype.

The red kernel colour may also appear in some cultivated rice varieties, because of a spontaneous retro-mutation phenomenon (**rd** → **Rd**), or as a consequence of the hybridation with species bearing such trait.

AWNS

Figure 2. Red rice awns

When awns are present, their colour may range between white and black-violet, and they may have a variable length (**Fig. 2**)

The awn formation is due to some polygenes with additive effect (An1, An2, An3).

THE PHYSIOLOGY OF RED RICE SEEDS

The biologic characteristics of red rice are quite different from the ones of cultivated rice.

The weed control is helped by the knowledge of the seed biology. Beyond, in fact, the aforementioned morphologic characteristics (that make red rice a dangerous weed), we must take into account:

- the timing and the persistence of the germinative capacity of the seeds,
- the duration of seed dormancy after harvesting (almost absent in cultivated varieties),
- the grain shattering level.

The high variability of these characters found in the various ecotypes of red rice, may have important agronomic consequences.

The knowledge of the dynamics of the germinative capacity and shattering of grains of red rice is fundamental for pointing out the correct period to control such weedy rice.

According to some studies carried out in Italy , the red rice seeds may already germinate 9 days after blooming. Three weeks after blooming, the number of seeds able to germinate ranges between 55% and 70% of the total amount (Balsari and Tabacchi, 1997; Ferrero *et al.*, 1998).

Unlike cultivated rice, the seeds of red rice, as well as those of other spontaneous species, present a more or less high dormancy level (Russo, 1995). From an agronomic

standpoint, this phenomenon allows red rice with delayed germination to escape both chemical and mechanical control (Ferrero and Vidotto, 1997). The strong selective pressure exerted by the control strategies applied for many years may even favour the diffusion of delayed emergence.

Dormancy is progressively lost by the seeds after maturing, at a rate depending on the biotype (Cohn and Hughes, 1981).

The mechanisms determining the appearance or loss of dormancy are still not clear. The break of dormancy is always accompanied by a pH reduction of the tissues of the seeds, especially of the embryo (Footitt and Cohn, 1992).

The dormancy is influenced by growing conditions (Delatorre, 1999), and the humidity and temperature during storage (Leopold *et al.*, 1988; Ferrero, 1994).

The planning of any weed control strategy requires the knowledge of the seed vitality persistence, i.e. the rate at which the stock of seeds in the soil gradually loses its germinative capacity. This information is fundamental when (as in the case of red rice) control criteria based also on crop rotation are adopted. In a series of long lasting experiments (more than 7 years) performed in the USA on pools of red and cultivated rice seeds conserved in flooded or in dry soil, a germination rate larger than 90% (for certain sites and populations) was observed for the red rice seeds. An even longer longevity, reaching 12 years (Diarra *et al.*, 1985), was found in more recent works (Ferrero and Finassi, 1995).

EMERGENCE CAPACITY

The emergence capacity is another feature making red rice more competitive than cultivated rice. In fact, the number of seeds giving rise to seedlings is inversely proportional to the depth where the seeds are placed (Roberts and Feast, 1972), and to the layer of water on the surface of the soil (Ferrero and Finassi, 1995; Saldain *et al.*, 1996; Saldain *et al.*, 1997; Gealy *et al.*, 2000).

In the cultivated species, the seedlings may emerge only when the depth is not higher than 3-4 cm, while in red rice they may emerge from quite deep layers, since its growing capacity may reach even 12-14 cm.

CROP DAMAGES

Red rice raises a serious problem in almost all the rice crops around the world, and even more where a direct sowing is performed.

The damage due to weeds mainly consists in the yield lowering caused by their competitive absorption of light and nutrients and in the higher processing cost.

The weed kernels not yet fallen down before rice threshing, in fact, are harvested with rice itself and pollute the commercial lots.

The elimination of these kernels in the rough crop requires a great effort, leading to a longer processing and to a greater reject at sorting. Because of the competition affecting the crop, the observed processing losses range, in fact, between 40 and 50%.

In order to provide a feeling of the weed aggressiveness and diffusion potential we recall that in the usual rice growing conditions weedy rice produces at least 3-4 fertile culms by plant, with 80-100 seeds for each panicle; in case of low competition capacity by the cultivated varieties (because of their small size, low density and so on) even 6-8 fertile culms per plant may occur.

DIFFUSION IN ITALY

Red rice has always worried Italian farmers. As we know, such a problem is certainly present in Italian rice crops since the beginning of the XIX century (Biroli, 1807).

The Experimental Institute of Rice Growing of Vercelli (which successively became the CRA-RIS) organized contests, since 1911, for the production of red rice-free seeds, aiming to limit the diffusion of this noxious weed.

One of the factors favouring the weed diffusion is certainly given by a permissive seed legislation. Red rice is present, with various degrees of infestation, in all the rice crops.

DIFFUSION IN EUROPE

Red rice represents a problem in all European countries where rice is grown.

In France, it infests about 70% of the rice crops and is faced with the so-called “false sowing” (to be described later on).

In Spain it is limited to the Catalan region, where it infests 60-70% of the 20000 ha dedicated to rice growing. The most common control method is the “puddling”, which is a kind of false-sowing, with a final phase performed by tractors endowed with cage wheels.

In Portugal the control is performed by means of false-sowing (and a final treatment with glyphosate), hand weeding and crop rotation (mainly with tomato and sunflower).

In Greece no specific control method is employed, because of the low diffusion of this weed.

Figure 3. Red rice experimental plots

RED RICE CONTROL

Red rice control is quite harder than that of other weeds because of its high morphologic variability and its affinity with cultivated rice.

Neglecting transgenic rice cultivars improved to overcome this difficulty, selective herbicides would protect red rice as well as cultivated rice.

The control is further entangled by the continuous emergence of red rice, although its main germination flux occurs between half april and half may (Ferrero and Vidotto, 1996).

The high adaptability of the germination process enhances the competitive aggression power of the red rice forms emerging earlier (or later) than the cultivated varieties (Ferrero and Vidotto, 1997).

Because of the impossibility to rely on chemicals alone, and the absence of a specific control method allowing to keep the weed below an acceptable level, red rice must be faced by means of an integrated approach (**Fig. 3**). No single method, in fact, appears to be decisive by itself.

PREVENTIVE CONTROL

Such a control consists of all the devices employed to reduce as much as possible the provision of seeds of the weed due to human activities. It is mainly obtained by using certified selected seeds and careful cleaning of machinery and farm implements employed during the harvesting, in order to avoid the weed diffusion into the weed-free lots.

AGRONOMIC CONTROL

Soil tilling

In an integrated control of red rice the choice of the soil tilling method depends on the seed bank (both overall and at different depths) and the other control method to be applied. Weedy rice control methods that can be applied in rice crops are expensive,

time-consuming and usually do not lead to a total eradication of the weed infestation. Incomplete control of the weed for a given year could lead to eliminating the results of several years of good control. Weedy rice escapes of 5 percent or less can produce enough seeds to restore original soil seed bank population levels.

Water management

The presence of a water layer on the field surface strongly affects the emergence and the development of rice weeds. Just like other weeds, as green bristle-grass (*Echinochloa* spp.), red rice is fostered by a very thin water layer.

Also for water, the best management method must be selected in relation to the other ones.

In the case of false seedbed, for instance, in order to stimulate emergence, the rice field is flooded only at the beginning of the seed dressing, leaving then the water level to be gradually lowered by percolation and evaporation.

When on the contrary herbicides are employed before sowing, a high water level is employed, in order to restrain the emergences.

Use of early varieties

The application of false seedbed, delaying by about 30 days the usual sowing time, requires the use of early varieties.

Crop rotation

Crop rotation is the most efficient agronomic technique for a long lasting weed abatement. In fact, one of the main causes of the dramatic diffusion of red ice is the almost complete absence of crop rotations in the Italian rice fields. When the infestation is particularly serious, crop rotation is the only possible solution.

Corn and soya are the most usually employed cultivations for rice crop rotation. Red rice may then be controlled either by means of chemical weeding (before or after the red rice emergence) or mechanical techniques.

False seedbed

False seedbed is probably the most widespread technique in Italian rice crops. Its aim is to induce a plentiful and possibly uniform emergence of the weed before rice sowing.

The emergent seedlings are then removed by means of mechanical or chemical methods. The main factors for the success of the practice are:

- the type of soil preparation: the less the soil is tilled, the larger is the emergence rate;
- water dose during the false seedbed: although the lowering of the water level causes a larger emergence rate, the seedlings grown in a thick water layer appear more sensitive to chemicals;

- false seedbed duration: it must be a compromise between the need of a high emergence rate of red rice seeds and the need to conclude early the crop cycle.

Hand weeding

Figure 4. Red rice hand removal.

Red rice hand removal (**Fig. 4**) presents very high costs, it is nowadays applicable only in case of low infestation levels, and requires skilled seasonal labour (Cerina *et al.*, 1994).

Chemical weeding

In red rice control, because of the high affinity between weed and cultivation, chemical treatments are applicable almost only before sowing (**Fig. 5**).

Figure 5. Total weeding.

The use of chemical products after rice sowing is limited to assistance by bar wetting.

The treatments to be performed before sowing are red rice pre-emergence,

red rice post-emergence, together with false sowing.

Genetic control

Biotechnologies are widely applied to rice improvement, mainly aiming at inducing resistance to adverse climatic conditions (such as drought), to insects (Fujimoto *et al.*, 1993) and to herbicides (Rathore *et al.*, 1993). The researches concerned mainly the resistance induced by plant transformation. Transgenic varieties are mostly cultivated in the USA.

The possibility of an efficient red rice control in transgenic rice cultivars was soundly demonstrated (Sujatha *et al.*, 1997; Wheeler *et al.*, 1997; Wheeler *et al.*, 1998; Chambers and Childs, 1999), but it presents a few potential risks, whose most dangerous one is the passage of the herbicide resistance-inducing gene to red rice itself. Resistant offsprings were indeed observed in situations of crossbreeding between red rice and transformed variety.

Mechanical control

The mechanical control of red rice is always matched to false seedbed, it is applied by means of harrows of different kinds, but its results are less satisfactory than the chemical weeding. In spite of these limitations, it has the advantage of a low cost and a lower dependance on climatic conditions.

RICE KARYOTYPICAL ANALYSIS

Kuwada in 1910 analyzed the rice karyotype, defining its chromosomic number (2n=24). The analysis and identification of the chromosomes were hindered, however, by the following factors:

-the small size of chromosomes at the metaphasic stage: chromosomes, in fact, appear in a quite contracted form, and are similar one to another, so that their identification is impossible, in spite of their different length;

-the presence of thick cell walls hinders to obtain efficient preparations, because of the absence of dispersion of the chromosomes on the slide by means of the usual crushing techniques;

-the absence of reproducible bands, such as the ones employed in animal cytogenetics: the bands obtained on each chromosome, in fact, turned out to be heterogeneous, and therefore unfit to ensure the chromosome identification.

More suitable methods were therefore employed in order to get good preparations, such as enzymatic maceration and air-drying (Fukui and Mukai, 1988).

Rice chromosomic analysis must be performed, moreover, on prometaphasic chromosomes, because in this phase they are more elongated, showing therefore different lengths and chromatin condensations.

Since the usual stains carmine and acetic orcein turned out to give unsatisfactory results, a new dye was adopted: the Giemsa dye, that allows to get a colour varying along the chromosome, according to the different chromatin condensation level. Euchromatin leads to zones with low intensity colour, while eterochromatin leads to a more intensely coloured zones (Fukui, 1986 b).

Many authors analyzed the rice karyotype both in germinal tissues at the pachitene stage (Shastry *et al*., 1960; Kurata *et al*., 1981 b; Chen *et al*., 1982; Chung and Wu, 1987), and in prometaphases of somatic cells (Nandi, 1936; Hu, 1958; Kurata and Omura, 1978; Kurata *et al*., 1981 b; Fukui, 1986 a; Chung and Wu, 1987).

The analyses of the rice karyotype were mainly based on the differences about chromosome length, centromere position, chromatin density along the chromosome and the arm ratio (Kurata and Omura, 1978; Wu and Chung, 1989; Fukui and Iijima, 1991). Because of the subjectivity of the chromosome identification, there were many disagreements, and therefore did not existe an encoded chromosome identification system.

In order to balance the absence of an objective chromosome classification method, Iijima *et al.* (1991) developed a new method, called CHIAS (Chromosome Image Analysis System), based on the image analysis of chromosomes, and allowing an objective characterization of each chromosome. This system may provide quantitative information on each chromosome in a standard and fast way, and it is based on fixed

procedures, concerning chromosome length, arm ratio and, above all, "Condensation Pattern" (CP), i.e. the density profile of chromatin distribution along the chromosome.

CHIAS ANALYSIS

CHIAS is an interactive analysis system of the chromosome image, which was mainly employed for the “Condensation Pattern" (CP). CP is the third parameter (besides chromosome length and arm ratio) employed by Fukui (1986 a) for the chromosome identification.

This system was mainly developed for the plant chromosomes, in order to obtain a standard method for the karotype analysis. Plants, in fact, are often characterized by a very large and variable number of chromosomes presenting also cases of polyploidy and aneuploidy.

Three versions were worked out for CHIAS.

The first one (CHIAS I, Fukui, 1986) makes use of a system operating only with images having a specific format.

The second one (CHIAS II, Fukui and Najayama, 1996) makes use of images in a format compatible with the main operative systems, but requires a large amount of memory in the system.

The third version (CHIAS III, Fukui and Kato, 1998) was contrived to bypass the previous difficulties.

CHIAS IDENTIFICATION OF THE CHROMOSOMES OF A HAPLOID VARIETY OF JAPANESE RICE: KOSHIHIKARI , n = 12

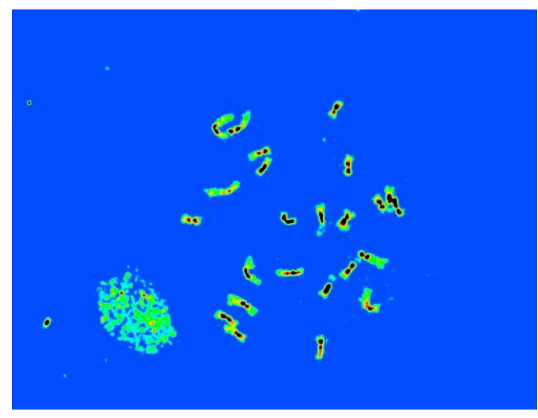

Figure 6. Image pseudocoloring (CHIAS III Manual Online).

The CHIAS chromosome identification system was applied to the prometaphases of a haploid rice variety ('Koshihikari' var. *japonica*), obtained by means of enzymatic maceration and air-drying, with a final colouring by means of the Giemsa 4% solution (Fukui and Mukai, 1988; Fukui *et al.*, 1988; Iijima and Fukui,1990; Fukui and Iijima,1991; Fukui and Iijima,1992; Fukui, 1998). Prometaphases were selected with straight chromosomes and no superposition.

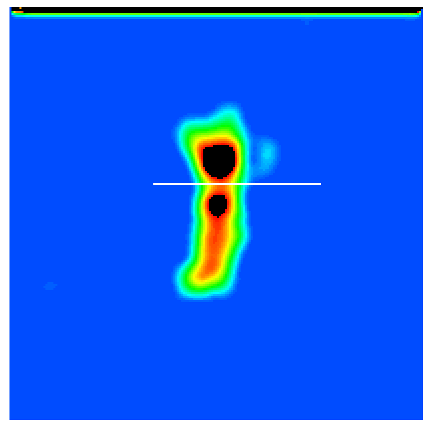

Figure 7. Centromere identification (CHIAS III Manual Online).

Microscope pictures were taken with three different exposures: plain, overexposed, and underexposed.

Plain and overexposed pictures, processed with CHIAS, are employed to extract the chromosome profiles.

Underexposed pictures are employed, in their turn, to sharpen the cromatin density distribution along the chromosomes.

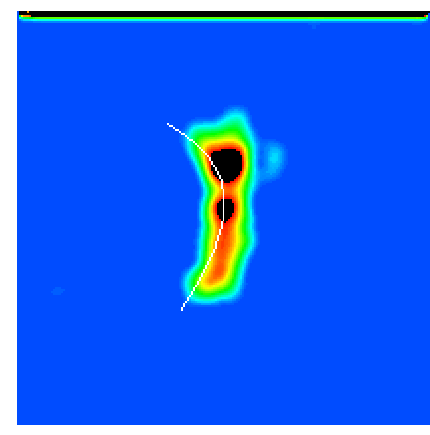

Figure 8. Middle axis of chromatid. (CHIAS III Manual Online)

The picture is saved in the computer memory by means of a TV camera. It is then handled with CHIAS, thus obtaining a digital image with 256 grey ranges. The grey range, in its turn, is transposed into a fictitious coloration, so that a different colouring (defined "pseudocolouring") is obtained for each different coloration range, making the chromatin identification easier also to human eye (**Fig. 6**).

The narrowest chromosome region (corresponding to the centromere) is successively identified, and a line dividing the chromosome into two arms is traced and assigned a zero grey value (**Fig.7**).

A median line is finally traced along each chromatid, along which the chromatin density distribution (CP) is determined for each chromosome (**Fig. 8**).

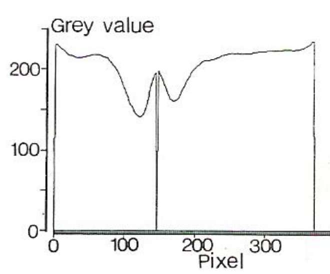

Figure 9. Condensation Pattern of a rice chromosome (Fukui and Iijima, 1990).

Data concerning chromosome length, arm ratio and chromatin density along the chromosome are therefore acquired. This information is graphically represented by plotting the grey values (on a scale from 0 to 256) versus the chromosome length (expressed in pixels), showing also the centromere position and the arm length.

The regions with higher grey intensity are the less condensed ones, while the more condensed ones correspond to the minima of the plot (**Fig. 9**).

By means of the CHIAS analysis of each chromosome, we get four different images, each leading to different data (**Fig. 10**):

- the first image displays the chromosome in white and black;

- the second (pseudocolored) image allows to visualize the most condensed parts;

- the third is a 3-D image;

-the fourth image graphically represents the CP, which is unique and typical for each chromosome, because it defines the real chromatin density distribution along the chromosome, which would be hardly perceived from the white and black image alone.

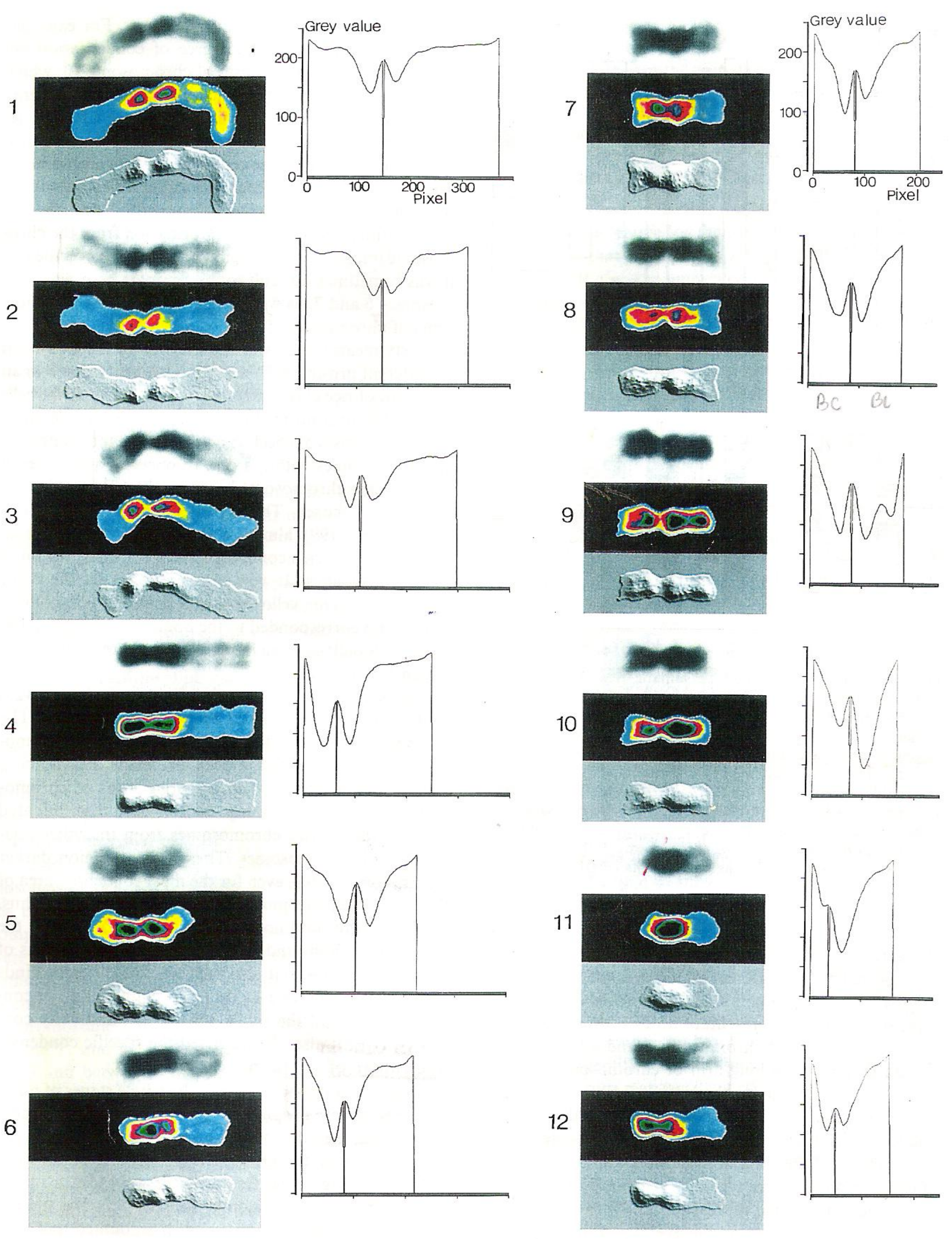

Figure 10. Chromosome images, digitally handled with the CHIAS system (Fukui K. e Iijima K., 1991).

Starting from the CP plot, we may build the chromosome ideogram (**Fig.11**) in three different chromatin condensation intensities along the chromosome.

Grey ranges with values between 0 and 123 represent te most condensed regions, shown in black in the figure.

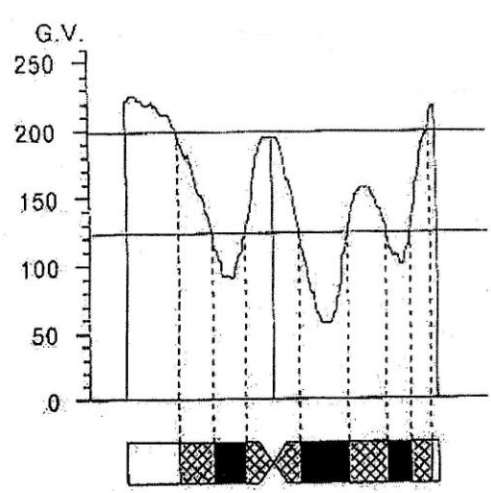

Figure 11. CP plot, and ideogram of the chromosome n°. 9 (Kamisugi *et al.*, 1993)

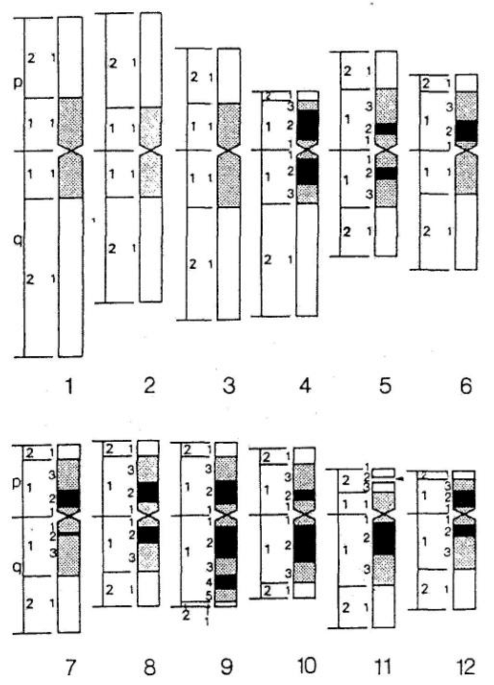

Figure 12 Cytologic map of the chromosomes of the haploid cv 'Koshihikari' of *Oryza sativa* var. *japonica* (Fukui and Iijima, 1991).

Grey ranges between 123 and 199 are zones with an intermediate condensation, checked in the **figure 11**.

Human perception of different colours ranges between 123 and 199. No difference is perceived above and below these limits.

The CHIAS method allowed the identification and characterization of all the prometaphase chromosomes, providing therefore the

possibility of building a complete cytologic map for the cv ‘Koshihikari’ of rice **(Fig. 12)**.

DESCRIPTION OF RICE CHROMOSOMES

By means of CHIAS and direct observation of prometaphases, some key characters were found for the identification of rice chromosomes (Iijima *et al.*, 1991; Fukui and Iijima, 1992).

The twelve rice chromosomes are numbered in order of decreasing length, and are subdivided into metacentric, submetacentric and acrocentric.

Chromosome 1

It is the longest one, and it is submetacentric. The two arms present wide euchromatic zones, and the zones of the long arm are about twice as long as those of the short one. The pericentromeric regions are mildly condensed.

Chromosome 2

It is a large metacentric; its two arms are symmetric, and have little condensed pericentromeric zones of average length. The tips of the two chromatids are often separate.

Chromosome 3

It is a large submetacentric. The euchromatic zone of the long arm is twice as long as the short one. The pericentromeric regions are mildly condensed and symmetric.

Chromosome 4

It is the largest acrocentric. The euchromatic zone on the long arm is very extended, while the short arm is almost entirely condensed, with a small distal euchromatic zone. The pericentromeric zones are strongly condensed and of uniform length.

Chromosome 5

It's a small metacentric, with symmetric arms. Both the extended pericentromeric condensed zones and the euchromatic zones have the same arm structure.

Chromosome 6

It is submetacentric. The pericentromeric zone is more extended and condensed in the short arm. The euchromatic zone of the long arm is five times as long as that of the short arm. The chromatids often converge in the distal zone.

Chromosome 7

It is quite similar to chromosome 6, since it is almost metacentric, and has eterochromatic symmetric zones, with an analogous condensation level. The euchromatic zone in the long arm is almost four times larger than in the short arm.

Chromosome 8

It is a small metacentric. The pericentromeric eterochromatic zones are symmetric and extend to a large part of the chromosomic arms. The short arm is almost entirely eterochromatic, with a small euchromatic zone, while the long arm presents an euchromatic zone almost double with respect to the short one.

Chromosome 9

It is almost metacentric. Most of the chromosome is condensed: three strongly condensed blocks may be seen, in fact, one of them on the long arm and one on the short one. The euchromatic zones are quite small.

Chromosome 10

It is almost metacentric. The pericentromeric zones are quite extended and condensed, especially on the long arm. The euchromatic zones on both arms are quite small.

Chromosome 11

It is acrocentric. It is endowed with a probe (seat of the nucleolar organizer) attached to the short arm, or apparently detached from it. The (very small) short arm, and more than half of the long arm, are strongly condensed, especially in the pericentromeric zones. A small euchromatic zone may be seen on the long arm.

Chromosome 12

It is the smallest acrocentric. Its morphology is analogous to that of chromosome 4, but the euchromatic zone is quite smaller.

METHODS FOR CHROMOSOME IDENTIFICATION

Iijima, Kakeda. and Fukui (1991) have established the following sequence of 11 steps for the identification of the 12 rice chromosomes.

1. Can visual inspection alone allow to identify chromosomes 4,11,12 ?

   In case of negative answer, stop here!
2. In case of positive answer, select chromosomes 4, 11, 12.
3. Identify the longest remaining chromosomes, i.e. chromosomes 1, 2 and 3.
4. Find the most metacentric of the three longest chromosomes. It's chromosome 2.
5. Select the chromosome with the smallest short arm, selecting it between the longest remaining ones: it's chromosome 3.
6. In case chromosomes 1,2,3 could not be completely identified, the analysis must be interrupted.
7. Select a chromosome with three condensed blocks: two on the long arm and one on the short arm: it is chromosome 9.
8. Select, between the three shortest chromosomes of the remaining five, that with a condensation on the long arm greater than on the short arm: it is chromosome 10.
9. Discard now, from the remaining four chromosomes, the one with the shortest long arm, and remove the chromosome with the longest short arm from the other ones: the last ones are chromosomes 5 and 8.
10. The longest of the two chromosomes is number 5; the other one is chromosome 8.

11. Select finally a chromosome with very different condensation levels between long and short arm: it is chromosome 6. The last one is chromosome 7. These steps are summarized in **Figure 13**.

The number of the relevant step is placed inside the rectangles and rhombuses, while the numbers pointed out by the arrows are the ones of the chromosomes selected at each step.

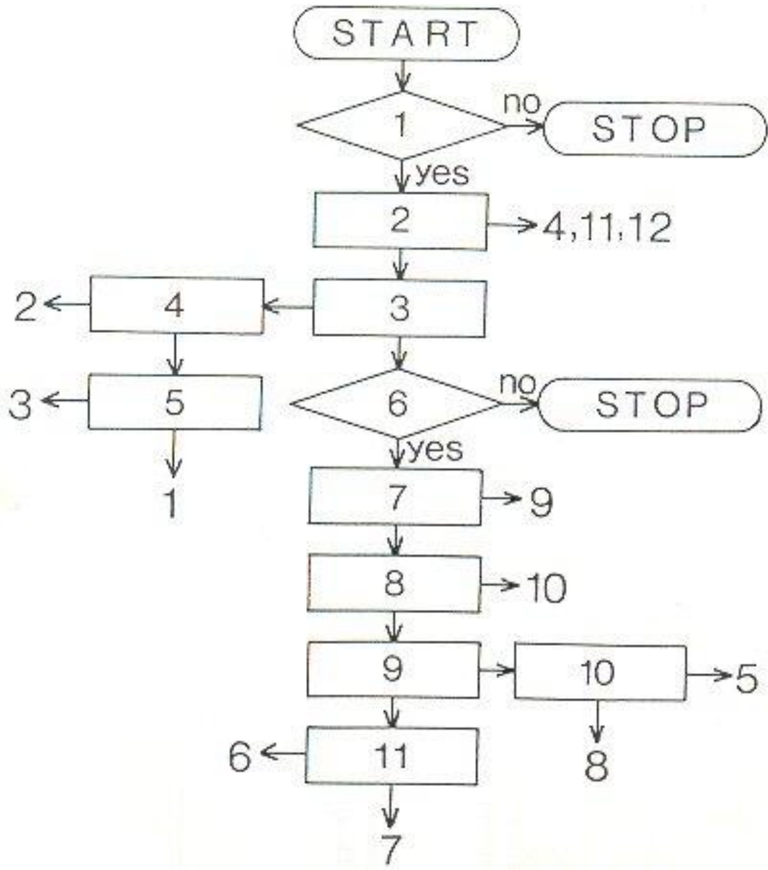

Figure 13. Scheme of the chromosome identification sequences (Fukui and Iijima, 1992).

KARYOTYPES OF ITALIAN CULTIVARS OF RICE

The first analyses of diploid Italian rice karyotypes based on methods analogous to the CHIAS III system first employed by Fukui and Kato in 1998 were performed by Sparacino *et al.* (2003, 2004) at the Faculty of Agriculture of the University of Milan.

Such a method, based on the Visual Basic programming environment for the Application of Microsoft Excel (VBA), was called CHIA-EA (Chromosome Image Analysis - Excel Application).

The karyotype analysis was applied to somatic chromosomes of two varieties of *Oryza sativa* subsp. *japonica*:

- the cultivar 'Loto' (2n=24), employed as control;
- the varietas *sylvatica* (2n=24), generally defined "*red rice*".

The results of this research, compared with the aploid cv 'Koshihikari' (var. *japonica*, x = 12) - analyzed with the CHIAS system - pointed out no substantial difference concerning the chromosome characteristics in terms of morphology, arm ratio and eterochromatin density distribution **(Fig. 14)**.

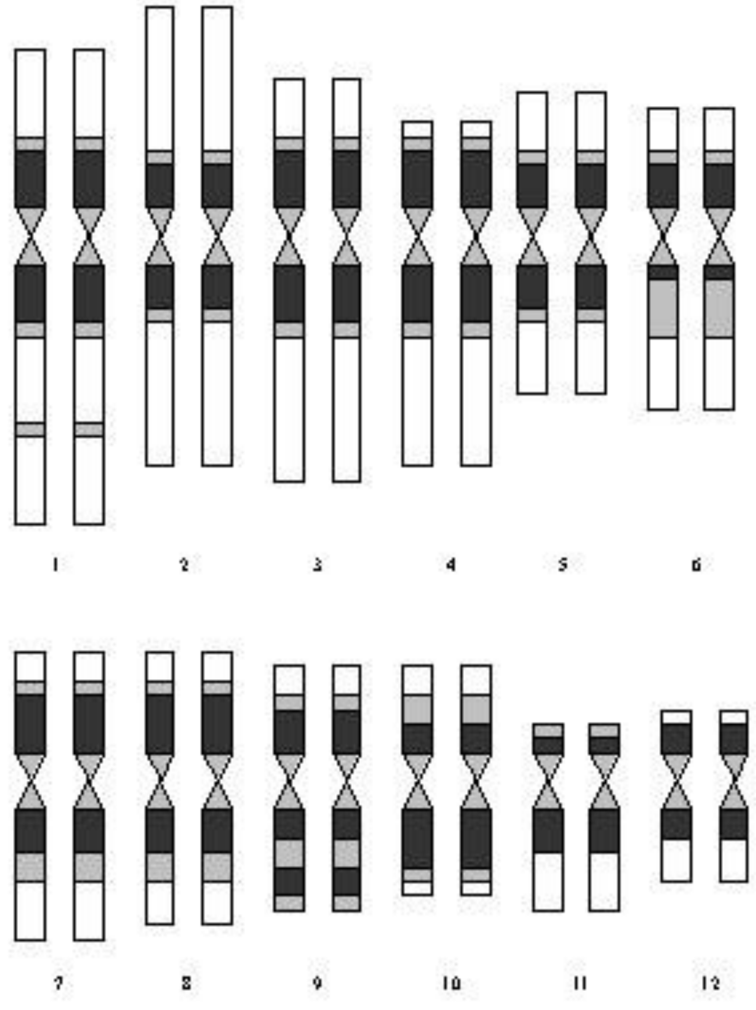

Figure 14. Quantitative ideogram of the somatic chromosomes of the 'Loto' variety (Sparacino et al., 2004). The condensed regions are shown in black; the mildly condensed ones, in grey; the euchromatic ones, in white.

The analysis of the red rice karyotype has shown, however, the presence of mutations both at a chromosomic and at a genomic level.

As far as chromosome mutations are concerned, in fact, different translocations were observed, always involving both a chromosome pertaining to the first pair (presenting a marked deficiency on the long arm) and another element of a pair of the entire set: either 2, or 3, or 4, or 5, or 6, or 7, or 8, or 9, or 12.

Also the second chromosome involved in the translocation turns out to be anomalous, because of an additional segment on one of the two arms, either the long or the short one, modifying therefore its arm ratio. All the translocations observed so far are summarized in the quantitative ideogram of **Figure 15**.

As far as genomic mutations are concerned, on the other hand, eteroploid cells were occasionally observed with 23 chromosomes (2n-1) and with 25 chromosomes (2n+1), due to a non-disjunction at the meiosis.

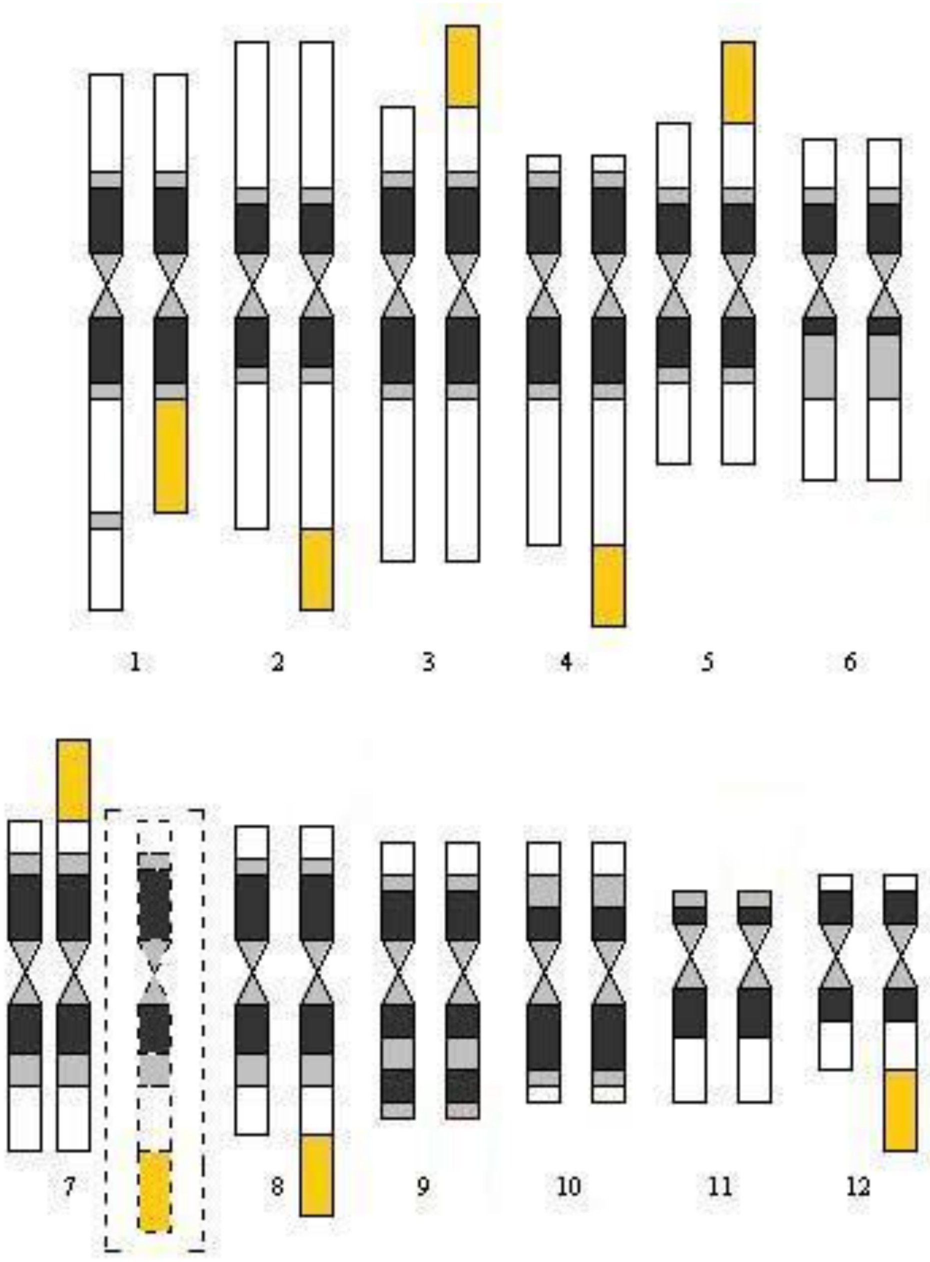

Figura 15. Quantitative ideogram of the somatic chromosomes of red rice.

The most condensed regions are marked in black; the mildly ones, in grey and the euchromatic ones in white. The long arm of the anomalous chromosome of the first pair and the additive segment of chromosomes involved in anomalies, finally, are marked in yellow (Sparacino *et al.*, 2004).

RICE MOLECULAR ANALYSIS

Linkage studies revealed the presence of rice shattering loci on chromosome 11 (Nagao and Takahashi, 1963), chromosome 1 (Oba *et al.*, 1990), chromosome 4 (Eiguchi and Sano, 1990; Nagai *et al.*, 2002) and chromosome 3 (Fukuta and Yagi, 1998). Moreover, rice shattering QTLs have been reported on chromosomes 1, 3, 4, 7, 8 and 11 (Xiong *et al.*, 1999; Cai and Morishima, 2000; Bres-Patry *et al.*, 2001; Thomson *et al.*, 2003). However, only two rice shattering genes have been identified through map-based quantitative trait loci (QTL) cloning. The qSH1 gene, a major QTL on chromosome 1 causes loss of expression only at the abscission layer.

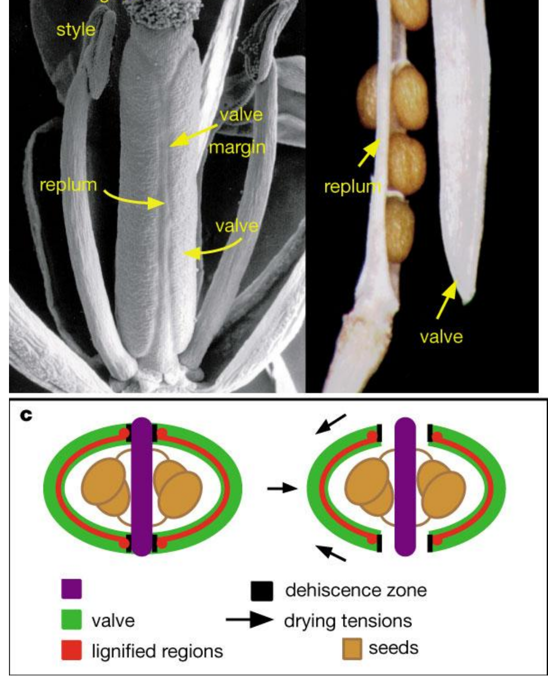

Figure 16. The abscission layer alongside the valve of *Arabidopsis* fruit.

A work published on Science (Konishi et al., 2006), reported the existence of a QTL region proven by the analysis both of the shattering-type *indica* cultivar 'Kasalath' and of a nonshattering-type *japonica* cultivar 'Nipponbare'. Located at 12 kb from SNP there is one ORF similar to the RPL gene of the *Arabidopsis* involved in the abscission layer alongside the valve of the *Arabidopsis* fruit. This ORF represents the gene of the non- shattering degree (**Fig.16)**.

A single-nucleotide polymorphism (SNP) in the 5' regulatory region of the qSH1 gene caused loss of seed (i.e. shattering), due to the absence of abscission layer formation. The Authors, Konishi et al, also found other SNP and AT repeat, A repeat and T repeat. The qSH1-SNP responsible for the shattering is a T nucleotide in the non-shattering-type *japonica* cultivar 'Nipponbare', and a G nucleotide in the shattering-type *indica* cultivar 'Kasalath'. The Authors also represented the non-shattering degree for different rice typologies in association with the relevant SNP, as shown in **Figure 17**.

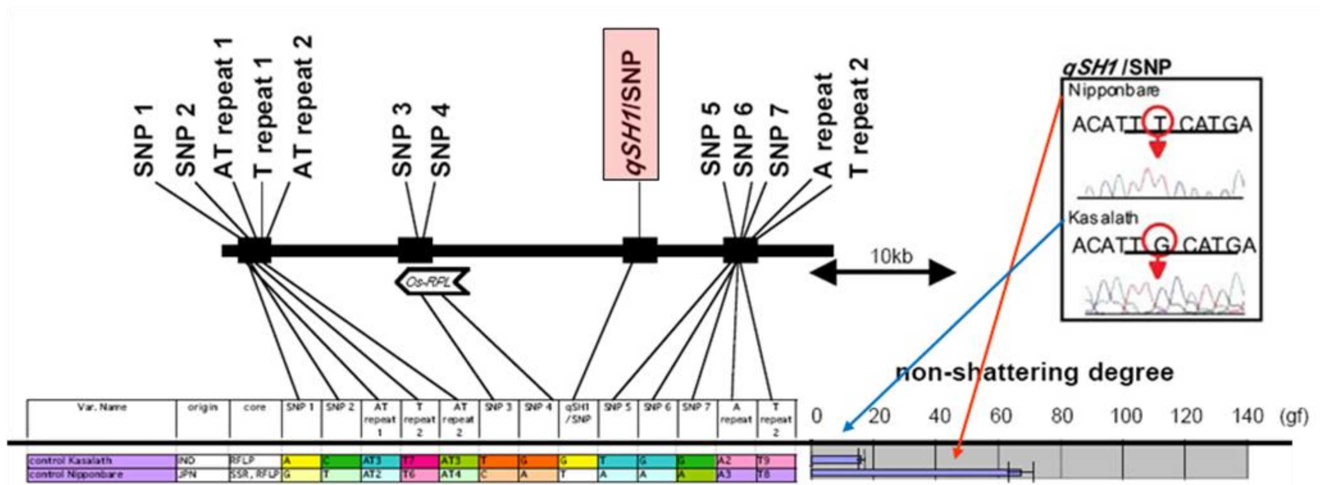

Figure 17: The SNP of Kasalath and Nipponbare found on chromosome 1 by Konishi et al. (2006) are shown in the upper part of the figure; the results of Kasalath (first line) and Nipponbare (second line) are shown in the lower part. The column on the right shows the relevant shattering degrees.

They also represented in a table the non-shattering level for different rice typologies in association with the relevant SNP (**Fig. 18**).

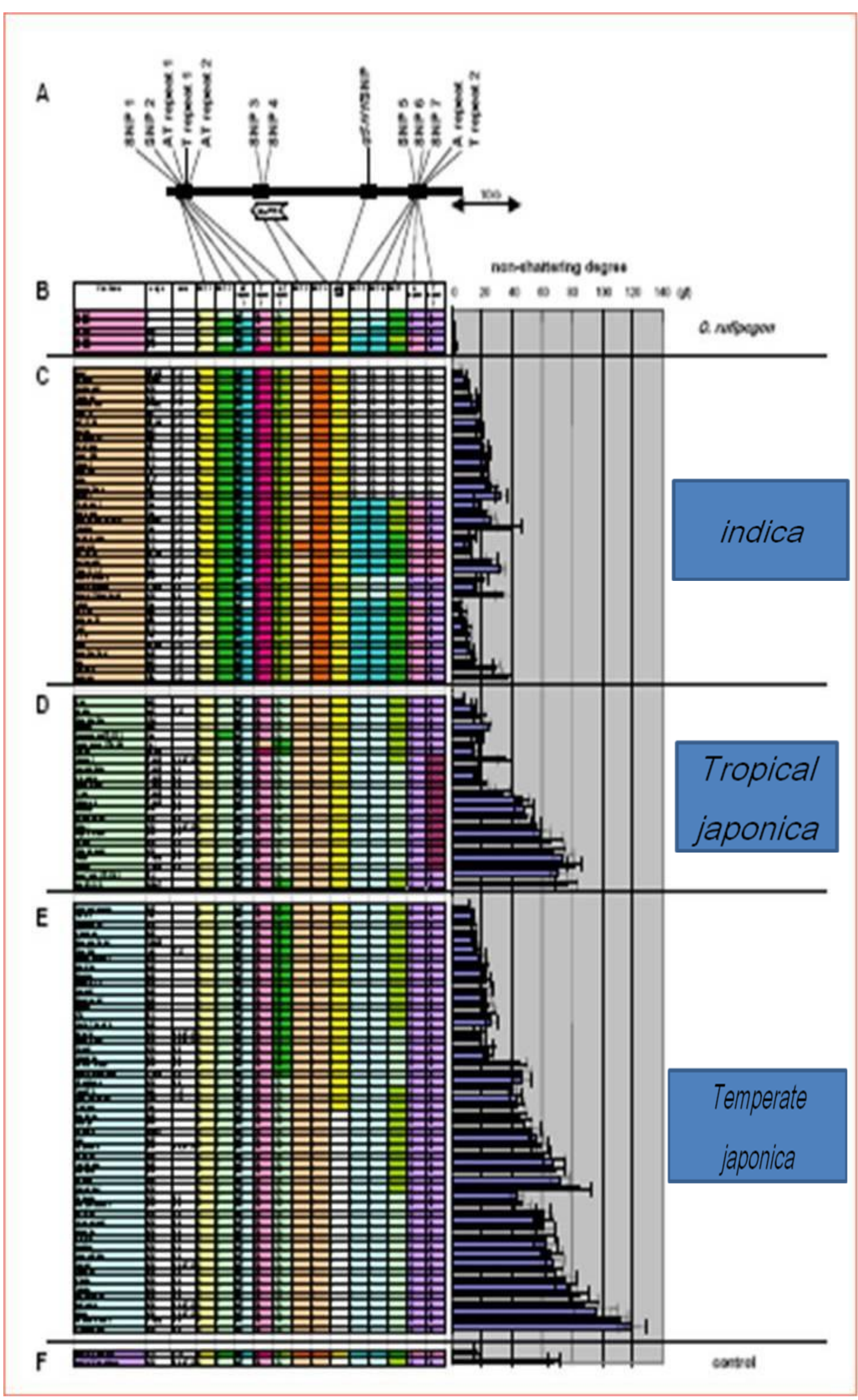

Figure 18. The non-shattering level for different rice typologies in association with the relevant SNP.

Figure 19 presents the sequenced regions containing the qSH1- SNP and all the SNP, AT, T and A repeats found by Konishi et al.

Chromosome 1

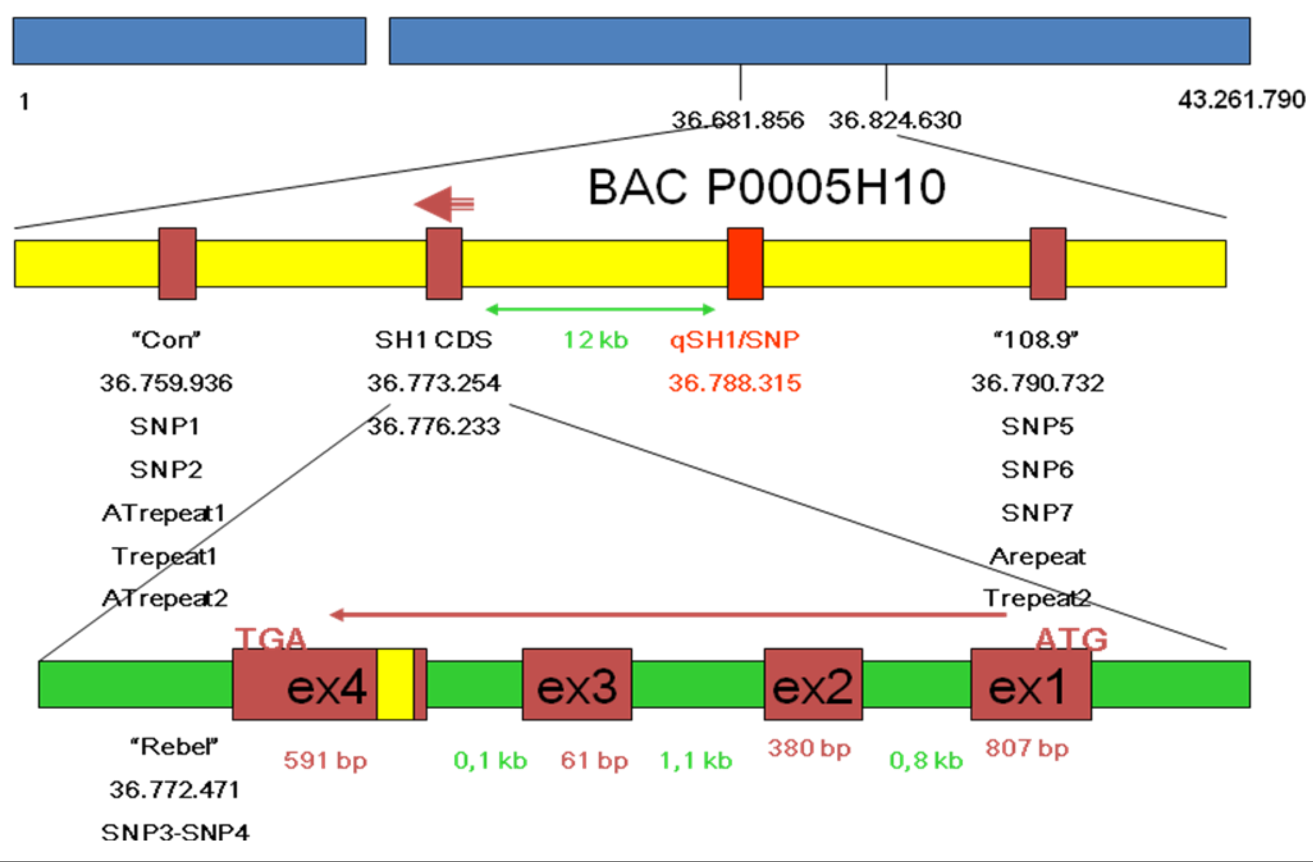

Figure 19. From up to down:

blue: chromosome 1;

yellow and brown: the 4 sequenced zones in the region containing the shattering gene;

green and brown: the 4 exons into which the region containing SNP3 and SNP4 was subdivided.

Hyeonso et al. (2010) identified a recessive shattering locus sh-h in the artificial obtened mutant line Hsh of shattering rice, that carries an enhanced abscission layer. They mapped sh-h in a 34 kb region on chromosome 7 by analyzing 240 F_2 plants and five F_3 lines from the cross between Hsh and 'Blue&Gundil'. The Hsh has a SNP where A becomes T (**Fig. 20**). This causes the production of a mutant protein, shorter by 5 amino-acids than the 'Hawacheong' wild type in the acceptor site of 7th intron of the OsCPL1 gene, responsible for the shattering character (QTL). These results demonstrate that OsCPL1 represses differentiation of the abscission layer during panicle development.

Moreover, the insertion of transferred DNA (T-DNA) into a mutant line has a SNP where G changes into T in the 8th exon of OsCPL1 gene; this mutation leads to the substitution of an amino-acid, so that a serine is replaced by an isoleucine. The obtained protein abolishes the phosphatase activity, thus enhancing the abscission and seed shattering.

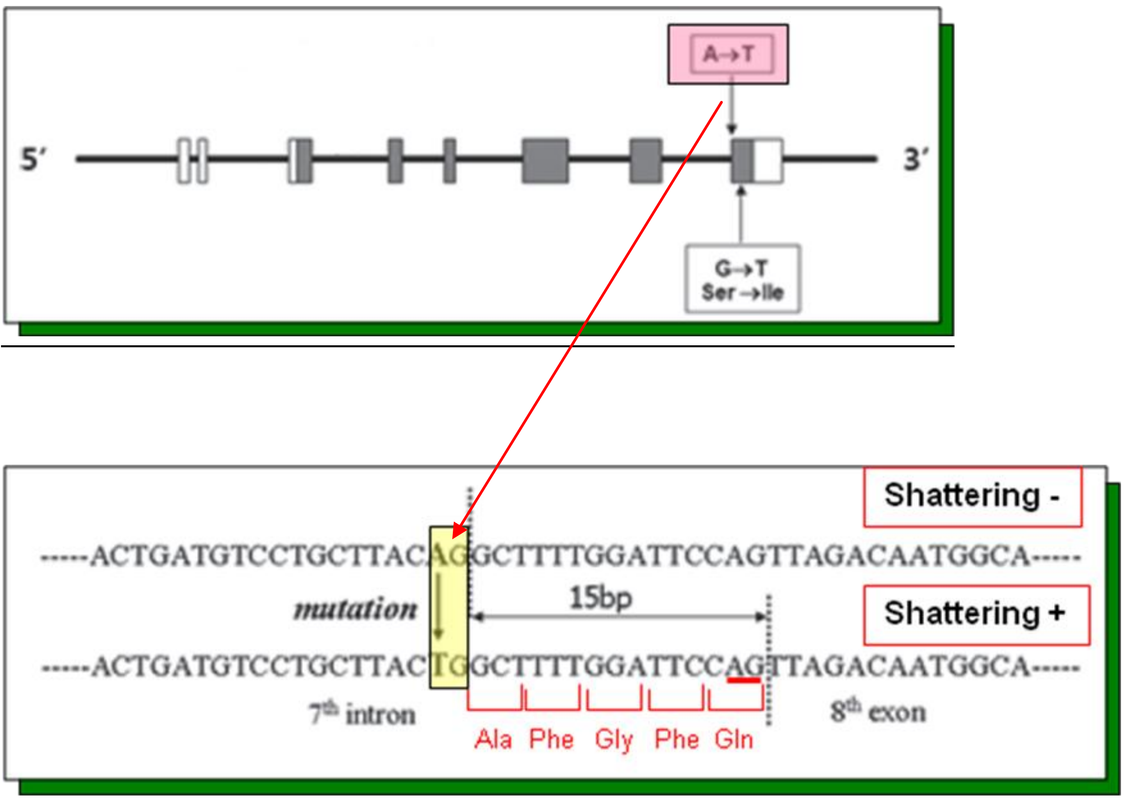

Figure 20. A→T and G→T mutations in the OsCPL1 gene of the Hwacheong and Hsh lines.

Another major QTL on chromosome 4, named sh4 is described by Changbao Li et al., (2006) which found that human selection of an amino acid substitution in the predicted DNA binding domain encoded by a gene of previously unknown function was primarily responsible for the reduction of grain shattering in rice domestication.

They mapped a region of Chr4, 1.7kb wide, where they found mutations responsible for the non-shattering character in some cultivars of rice. The comparison of two such sequences in rice cultivars revealed seven mutations, one of which is located in an intron. The important mutations turn out to be located in an exon, and are represented in **Figure 21**. Here (b) represents the insertion (or deletion) of 5 amino-acids (TGGAA); (c) represents the insertion (or deletion) of the amino-acid Valine, and (d) represents an amino-acid substitution where T mutates to G, so that Asparagine mutates to Lysine. The substitution undermined the gene function necessary for the normal development of an abscission layer that controls the separation of a grain from the pedicel. The increased expression of sh4 in the late stage of seed maturation suggests that the gene may also play a role in the activation of the abscission process.

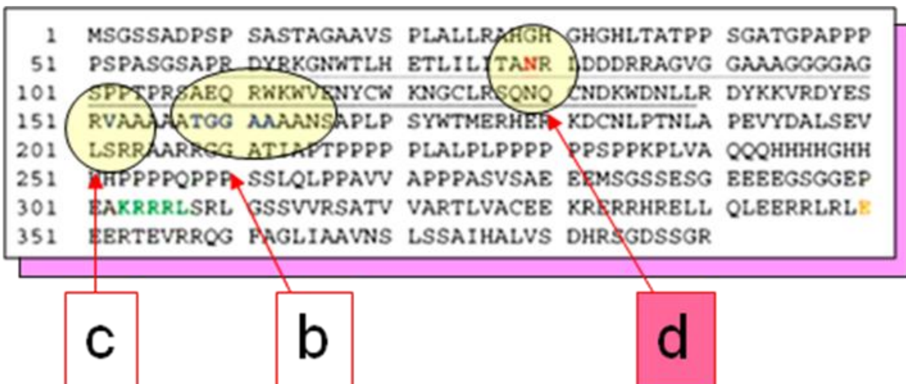

Figure 21. This figure shows the sh4 protein sequence of *Oryza sativa*. Mutations between the mapping parents are indicated by (b), (c) and (d). For *Oryza sativa* (non-shattering rice) N = AAT; for *Oryza nivara* (shattering rice) N is replaced by K = AAG. The mutations (c) and (b) are present only in *Oryza nivara*.

AIM OF THE WORK

One of the main factors affecting the rice yield is the presence of weeds, one of which is represented by *Oryza sativa* sub. *japonica* var. *sylvatica*, commonly called 'red rice'.
Kuwada (1910) first reported the chromosome number of cultivated rice, using both mitotic and meiotic cells, while no information about red rice genome is reported in literature.
The cytologic study of red rice is important because it allows:
- to study the anomalies in the chromosome structure and number;
- to try to establish the relation between karyotype and phenotype;
- to explore the main points to be deepened by molecular methods.

The red rice peculiarity is the grain shattering before its full physiological maturity: this phenomenon is regulated by a gene (Konishi et al., 2006). Genetic studies reveal the existence of several QTLs presenting shattering character. These QTLs are localized on rice chromosomes 1, 3, 4, 7, 8, 11 (Hyeonso Ji et al., 2010). The responsible genes have been identified for three of them: the chromosomes 1 (Konishi et al., 2006), 4 (Changbao et al., 2006) and 7 (Hyeonso et al., 2010) . Previous studies (Sparacino et al., 2003) have pointed out that chromosome 1 is implied in some chromosome anomalies and we wanted to clear this point by applying the FISH technique.
The aim of the present work is to perform a comparative analysis of the morphological and karyotypical characteristics of different biotypes of red rice. This comparison could give more information about the determinism of the grain shattering character and help both the biotype classification and the control of red rice. The location of the grain shattering gene in different biotypes of red rice could indicate a correlation between the translocation phenomenon and the activation of the shattering gene.
I considered eight biotypes of *Oryza sativa* var. *sylvatica* that show a high degree of shattering. In the eight biotypes I investigated both the chromosome complement, and the genome sequence around the non-shattering locus.

The expected results may be summarized as follows:

- information about possible chromosomic differences between red rice biotypes and the cultivated rice;
- information about possible genomic differences between the red rice biotypes and the cultivated rice varieties bearing the grain shattering character;
- genetic analysis of the shattering gene on the chromosomes 1, 4 and 7.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

The experiments were carried out on eight phenotypically different weedy rice biotypes (*Oryza sativa* subsp. *japonica* var. *silvatica*, 2n=24) which were collected in 92 Italian rice fields. The Italian cv 'Loto' of rice (*Oryza sativa* subsp. *japonica*, 2n=24) was considered as control.

Morphological characterization of the biotypes was primarily based on the following seed traits: length, shape, weight, pericarp and kernel colour, and the presence and colour of awns. In addition, plant height was measured at maturity from the surface of the soil to the tip of the panicle. In order to assess the shattering degree, failing to provide a valid scientific method, we limited ourselves to the simple visual observation. Every year, in May, germination of seeds occurred in a growth chamber, and seedlings were then transplanted into pots in a peat-based substrate in a greenhouse (**Fig. 22**).

Figure 22. Red rice biotypes in greenhouse.

At the 3rd leaf stage, the young plants were transplanted into containers (100x50x50 cm) filled with a medium texture soil, arranging six plants of the same biotype per container, in open air (**Fig.23**).

Figure 23. Red rice biotypes into large containers.

At flowering, panicles of each biotype were isolated with cellulose bags in order to prevent the cross pollination (**Fig.24**)

Figure 24. Isolation of panicles at flowering

The irrigation was performed according to the Italian rice growing system, consisting of periodical flooding.

CYTOLOGICAL ANALYSIS

The cytological preparations were made by using the root tips of red rice biotypes and cv 'Loto' of rice, performing their enzymatic maceration, then the standard air-drying technique, and finally staining with 4% Giemsa solution (pH 6.8) for 20 min (Fukui and Iijima, 1992).

At the prometaphase the chromosomes were elongated and identifiable.

Detailed analyses of images of chromosomes at prometaphase, which is the best stage to detect morphological differences, were performed using the computerized methodology named Chromosome Image Analysis-Excel Application-CHIA-EA (Sparacino *et al.*, 2004). The prometaphasic chromosome images were imported into the application. First each chromosome image appeared in black and white, then it was converted into a pseudo-coloured image. This fact enabled us to determine all the parameters necessary to characterize each chromosome, that is: a) length, b) centromere position, c) arm ratios, d) distribution of chromatin density.

The CP (Condensation Pattern) profiles identify and characterize the somatic chromosomes; the vertical axis gives the grey values from 0 to 255 defining the chromatin density distribution along the length of the chromosome, and the horizontal axis gives the chromosome length, the centromere position and the arm ratios by means of the pixel number.

The data were converted into graphs, or CP profiles, which enabled the characterisation of each chromosome and the construction of the corresponding cytological map.

DNA ANALYSIS

DNA EXTRACTION FOR PCR (Polymerase Chain Reaction)

The leaves from 8 biotypes were crumbled in liquid nitrogen with mortar and pestle, preserving then the obtained material in a few test tubes for 24 hours at -70°C.

The DNA extraction was performed by means of a DNeasy Plant Kit, which is a mini-kit for the mini-prep purification of total cellular DNA (www.qiagen.com). The DNeasy Plant

Kit uses advanced silica-gel–membrane technology and simple spin procedures to isolate highly pure total cellular DNA from plant tissues and cells or fungi. The DNA is conserved at -4°C.

DNA was extracted from 100 mg of leaves, thus obtaining 150 µl of DNA, with a average concentration of 50 ng/µl.

PCR

Choice of the Primer

The primers for the genetic analysis of qSH1, qSH4 and qSH7 are shown in **Table 1**.

Table 1 - Primer used

Loci	Name FP	Forward Primer (5' – 3')	Name RP	Reverse Primer (5' – 3')	Amplified region
qSH1	qSH1-F 106.5K-U	TTACTTGGCGGCTTTGAAGT	qSH1-R 107.2K-L	TATGGTTGGATTGGGACGAT	qSH1-SNP
qSH1	RBEL-E1-U	ATCATGCAGCAAGTGACCAC	RBEL-E2-L2	TCACAACCTAGAGATGAGGC	SNP3-SNP4
qSH1	con24-13U	CAATGGAAAAGCCGCTGATG	con24-13L	CGTTGCATGAATTGTAGCAC	SNP1-SNP2-(AT)1-(AT)2-(T)1
qSH1	108.9K-U	ACAGGGTGATCCCAACAGTT	109.8K-L	TAACCGGTGATGGTTGTGCA	SNP5-SNP6-SNP7-SNP8-(A)-(T)2
qSH4	qSH4-F	GGACTACCGCAAGGGGAAC	qSH4-1R	AGAGCGCGTCGTAGACCTC	qSH4-SNP (d) - b - c
qSH4	qSH4-2F	GGGCGGAGTGAGTAATTGAT	qSH4-2R	TACATCGATCGTCCTTGCTG	Exon 2
qSH7	qSH7-F	TTGCTATTGGCTTTCACTGG	qSH7-R	TGGGATCATCAAACCAGCTT	qSH7-SNP (Exon 8)
qSH7	qSH7-1F	TGATCCAGATGGTTTGGAGA	qSH7-1R	TGATTTTGCATTAACATACACATCAT	Exon 3
qSH7	qSH7-2F	TGGCTGTATTTGGTTCTTTGC	qSH7-3R	GGCATCAAAAGATGAATTCAAAA	Exon 4
qSH7	qSH7-4F	TTTTGCTCTCCATACCATGTTG	qSH7-4R	TGGAACAGAAGGCTCCTTAG	Exon 5
qSH7	qSH7-5F	CGAAATTGTCTTTTCCTTTCTCC	qSH7-5R	GAATACAAACCACTAAAACCAGGA	Exon 6
qSH7	qSH7-F	TTGCTATTGGCTTTCACTGG	qSH7-6R	CATACCAGCCCTTCTGCATT	Exon 7

PCR amplification

PCR amplifications were performed in a final volume reaction of 30 µl (**Table 2**).

Table 2 - PCR Standard Mix

Reagent	Concentration	Volume
Template DNA	50 ng/µl	1 µl
Forward primer	5 µM	1 µl
Reverse primer	5 µM	1 µl
dNTPs mix	150 µM	3 µl
PCR Buffer	10x	3 µl
Taq gold polymerase	5U/µl	0.3 µl
Nuclease free water	Water DEPC	up to 30 µl

PCR purification

The PCR products were purified with UltraClean DNA BloodSpin Kit of MO BIO Laboratories.

DNA EXTRACTION FOR THE C_0T PRODUCTION

The C_0t value is the product of C_0 (the initial concentration of DNA), t (time in sec), and a constant that depends on the concentration of cations in the buffer.

The DNA was extracted from 12 g of leaves, thus obtaining 1600 µl, with a concentration of 0.64 µg/µl. The leaves were crumbled with mortar and pestle, adding liquid nitrogen.

The material was conserved in a few Eppendorf tubes, at -70°C, for a few days.

Two different extraction methods were employed:

1) DNeasy Plant Maxi Kits uses advanced silica-gel–membrane technology and simple spin procedures to isolate highly pure total cellular DNA from plant tissues and cells or fungi (www.qiagen.com);

2) The protocol reported by Dellaporta *et al.* (1983) modified as follows:

- Samples in groups of 300mg of milled leaves
- Pre-heating of the buffer (Solution 1) at 65 °C for 20’
- Adding 0.6 ml of buffer, and incubating at 65 °C for 15’ and at 4°C for 1h
- Centrifuging at 5200 rpm for 25’ at 15°C
- Recovering the supernatant
- Adding 5 µl RNAsi (100mg/ml) and incubating at 37 °C for 1h
- Adding 1 vol of phenol : chloroform (500-600µl) and centrifuging at 5200 rpm for 15’
- Recovering the supernatant
- Adding 1 vol of chloroform (500µl) and centrifuging at 5200 rpm for 15’
- Recovering the supernatant
- Adding 0.6 vol of isopropanol (300µl) and 1/10 vol of NaAc (50µl) 3M pH 5.2, and precipitating at -80 °C either for 90’ or overnight
- Centrifuging at 5200 rpm for 30’ at 4 °C
- Throughing away the supernatant
- Washing pellet with 500 µl per sample of cold EtOH 70%
- Centrifuging at 5200 rpm for 5’ at 4 °C
- Throughing away the supernatant
- Re-suspending in water DEPC up to 100µl
- Storing at -20 °C

Extraction buffer for each sample:

- 100mM Tris HCl pH 8
- 100mM NaCl
- 5mM EDTA
- 1% SDS
- 2.5% ß-mercaptoethanol

C_0T PREPARATION

This preparation was carried out according to Zwick *et al.* (1997):

- Step 1. Quantify with care the concentration of genomic DNA: 0.64 µg/ul.
- Step 2. Dilute the genomic DNA at a concentration of 470 ng/µl with 5 M NaCl and H_2O, reaching a final concentration of 0.3 M NaCl and a final DNA volume of 1.089 ml.
- Step 3. Aliquot 1 ml of DNA in tubes with screw cap.
- Step 4. Autoclave thrice for 5' in liquid cycle.
- Step 5.Take 500 ng and put on agarose gel 1% (the DNA must range between 100 and 1000 bp: otherwise, autoclave again in order to reach this range).
- Step 6. Put all samples in a Falcon tube of 15 ml, and keep in ice.

Reannealing

1) The time required by the reannealing phase is provided by the formula:

$$C_0t = 1 = \text{mol/L} \times T_s$$

where the initial concentration (C_0) is expressed in nucleotid moles per liter, and time (T_s) in seconds.

An average of 339 g/mol of a deoxynucleotid monophosphate is assumed.

The fragmented DNA is at a concentration of 470 ng/µl = 0.470 g/L.

Since 1 mole of dNTP = 339 g/mol, the moles of fragmented DNA are given by:

$$0.470 \text{ g/L} / 339 \text{ g/L} = 13.8 \times 10^{-4} \text{ mol/L}.$$

The value $C_0t = 1$ is therefore obtained if the reaction lasts 721 sec (about 12 min).

2) From the volume of DNA, we obtain 5.1 µl of S1 nuclease, with a concentration of 1 U/µg DNA.
3) The DNA must be now denatured by putting the Falcon 15 at 95 °C for 10 min.
4) Place first the Falcon in ice for 10 sec, and then at 65 °C for the time computed by means of the formula given above (reannealing time), i.e. for 12 min.

Digestion with S1 nuclease

1. After the reannealing time, add to the tube 5 µl of S1 10X nuclease and 218.8 µl of buffer 5X and put at 37 °C for 8 min.
2. Stop the reaction, add 30 µl of EDTA, put at -70 ° C for 10 minutes, keep in ice for 10 min and storage at -20°C.
3. Re-precipitate with sodium acetate 0.3 M and a volume of isopropanol amounting to 0.8 of the total at -20°C, for 2 hours.
4. Centrifuge at 13000 GPM for 25 min.
5. Remove the liquid, while keeping the pellet, and dry at 37 °C.
6. The following day add 100 µl of DEPC water, and make use of vortex.
7. Make an agar gel 1%.
8. The concentration of the C_OT is 0.8 µg/µl.

S1 10x Buffer Solution:

- 0.5 M NaO Ac pH 4.5
- 45 mM $ZnSO_4$
- sterilized filter

S1 Nuclease:

- Produced by Boeringher man, 10.000 U [400 U/µl].

FISH METHOD

We followed the Fish method reported by De Lorenzi *et al.* (2007).

FISH analysis makes use of a rice-specific BAC clone provided by NIAS (National Institute of Agrobiological Sciences) of the Genome Resource Center. This BAC is denominated P0005H10, and it overlaps the shattering gene region of chromosome 1.

The required methodology is the following:

- [P0005H10] = 66,7 µl = 667 ng/µl
- Prepare in an Eppendorf this mixture of bases:

- 2.5 µl buffer 10x
- 2.5 µl ß-mercaptoethanol (0,1M)
- 0.5 µl other bases
- 0.3 µl Cy3 – dUTP (1mM)

- Prepare the probe with 1 µl DNA_{bac} and 16,2 µl H_2O
- Add 2 µl mix enzymes and vortex (V_{tot} = 25 µl)
- Incubate at 15 °C for 45'
- Add 1 µl EDTA and put in the refrigerator
- The probe is precipitated by adding 9 µl COT, 3 µl ssDNA (salmon sperm) and 3.7 µl of sodium acetate. Then, vortex
- Add 130 µl of frozen EtOH, and vortex
- Put Eppendorf at -20 °C for 4h
- Pre-heat hybridation at 37 °C
- Centrifuge the probe at 13000 cycles per minute for 30'
- Dry the liquid and put it at 37 °C for 10'
- Add 8 µl of bases and keep at 37 °C for 30'
- Pass the slide through alcohol bowls at 70%-80%-95%, 6 min for each step
- Leave the slide to dry in air
- Vortex the probe
- Pre-heat the slides for 2' at 60 °C on the plate
- Place a drop of the probe at the center of the slide
- Place the slide on the plate at 75 °C for 4' and wind with parafilm
- Place in moist chamber at 37 °C for a night
- Flush three times for 5' in SSC kept at 60 °C
- Put on the slide 250 µl mix (BSA, SSC, Tween), and cover with coverslip, and put in moist chamber at 37 °C for 30'
- Remove coverslip
- Put on slide 100 µl detection solution, add 5 µl avidin FITCH and cover with coverslip
- Put at 37 °C for 30'

- Gently remove coverslip and flush three times for 5' in the solution 4x SSC, 0.1 Tween 20 at 42 °C
- Wash with PBS for 1'
- Dip in 100µl DAPI for 5'
- Quick flush of PBS
- Quick flush in distilled H_2O
- Dry in the dark
- Place coverslip with Vectashield Mounting Medium and keep in dark

Employed solutions:

- Buffer 10x:
  - 0,5 M TRIS HCl pH 7,2
  - 0,1 M $MgSO_4$
  - 1 mM DTT
  - 500 µg/µl BSA
- Other bases:
  - 0,5 mM dATP
  - 0,5 mM dGTP
  - 0,5 mM dCTP
- Detection: 1% BSA, 4x SSC, $TWEEN_{20}$ 0.1
- SSC 0.1x
- EDTA 0.5M pH 8
- SSDNA 10 µg/µl
- NaAc (sodium acetate) 3M
- EtOH (ethanol) 100%
- BSA 3% e 1%
- $TWEEN_{20}$ 0.1
- DAPI 1µl [0,2mg/ml] + 1ml SSC 2x

MICROSCOPES

The chromosomic analysis was performed by means of two microscopes:

1. LEICA DM-RB Microscope with LEICA DC 300 Camera
2. LEICA DMR Microscope with LEICA DC 250 Camera

RESULTS

MORPHOLOGY

The main qualitative traits of the grains of red rice biotypes are summarized in **Table 3.** We may observe that only in biotype 1 the awn was absent. Biotype 2 differs from the other ones both for the awn length and for the color of the caryopsis. The seed color is the same for all biotypes, while some differences may be noticed both in the form of the caryopsis and in the awn color and length.

Biotypes		1	2	3	4	5	6	7	8
Caryopsis	Color	White	Dark Spotted	White	White	White	White	White	White
	Shape	Semi-rounded	Oval	Semi-oval	Semi-oval	Oval	Semi-oval	Oval	Semi-oval
Seed		Dark red, light striate	Dark red, light striate	Dark red, light striate	Dark red, light striate	Dark red, light striate	Dark red, light striate	Dark red light striate	Dark red, light striate
Awns		Absent	Dark brown and long	Red and medium	Red and medium	Dark brown and medium	Light brown and short	Light brown and long	Light brown and short

Table 3. Main traits of the eight red rice biotypes. The awns are considered short when they range between 0-2 cm; medium for 2-3 cm; long > 4 cm.

Average values and standard deviations concerning seed length, width and thickness of red rice biotypes are reported, respectively, in **Figure 25**, **26** and **27**.

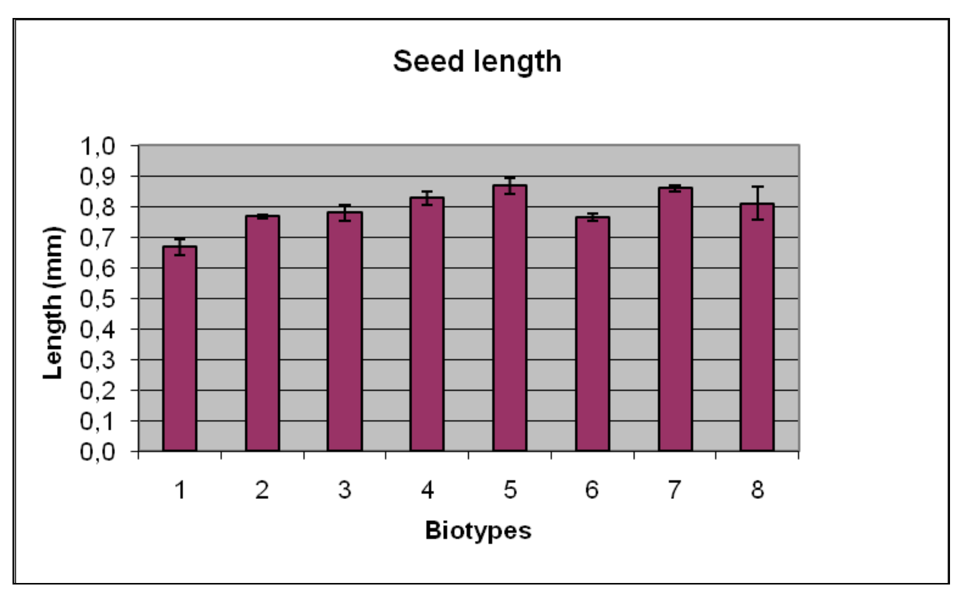

Figure 25. Average seed length (with standard deviation) in different biotypes.

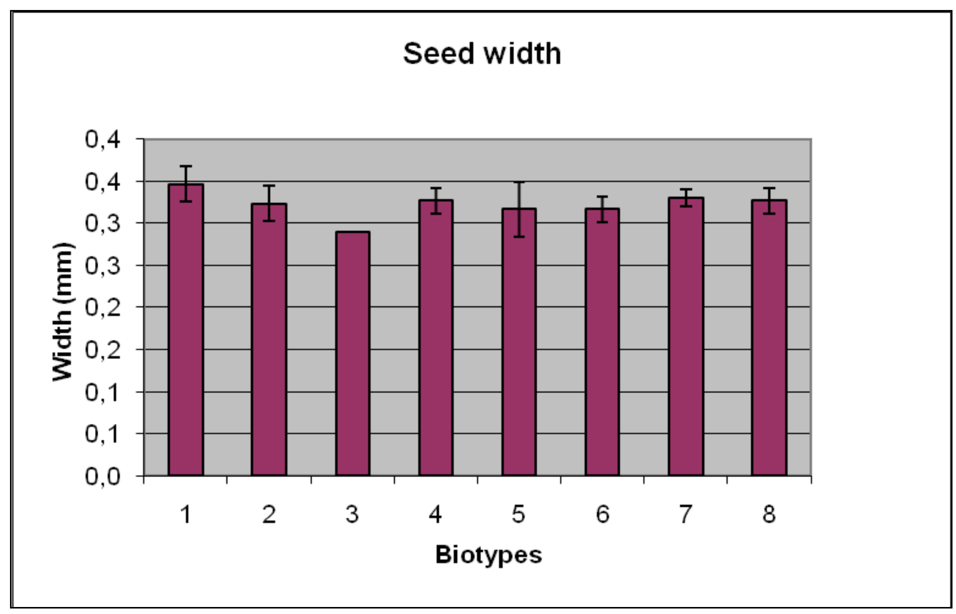

Figure 26. Average seed width (with standard deviation) in different biotypes.

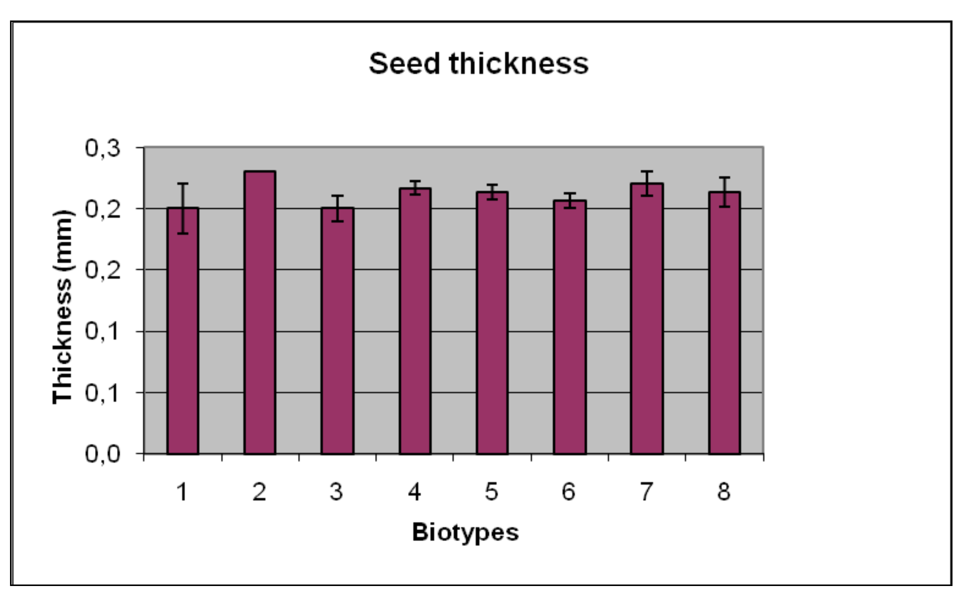

Figure 27. Average seed thickness (with standard deviation) in different biotypes.

A remarkable difference may be observed about the biotype lengths (biotype 1 is the shortest and biotype 5 the longest one). Biotype 3 is the narrowest, while the others have quite similar widths. The thickness, on the other hand, is almost the same for all biotypes.

About the shattering level, results are reported in **Figure 28.** We observe that the shattering degree is proportional to the length (including awns) of the seeds, ranging from 50% in biotype 1 to 90% in biotype 2.

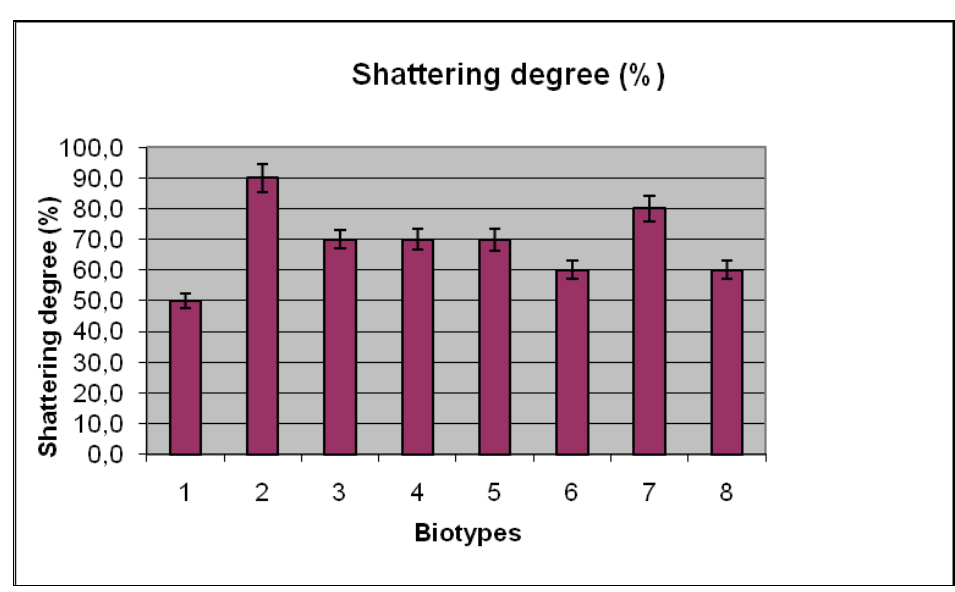

Figure 28. Shattering degree (with standard deviation) in different biotypes.

As far as qualitative parameters is concerned (**Table 4**), they turned out to remain unchanged during the three years of the trial, and we may observe the differences between biotype 6 and all the others. Biotype 8, in its turn, has a different color of the collar.

Biotypes	Culm color			
	Collar	Nodes	Internodes	Sheath
1	light red	green	green	green
2	light red	green	green	green
3	dark red	green	green	green
4	light red	green	green	green
5	light red	green	green	green
6	red	red	red	green
7	light red	green	green	green
8	red	green	green	green

Table 4. Culm color of the biotypes

Figure 29, on the other hand, shows that the average size of all the biotypes was higher in the first year. Probably climatic and growing conditions caused these differences.
Biotypes 1 and 8 were the lowest and the tallest, respectively, while biotypes 3, 5, 6 and 7 had similar heights and biotypes 2 and 4 differed from all the others.

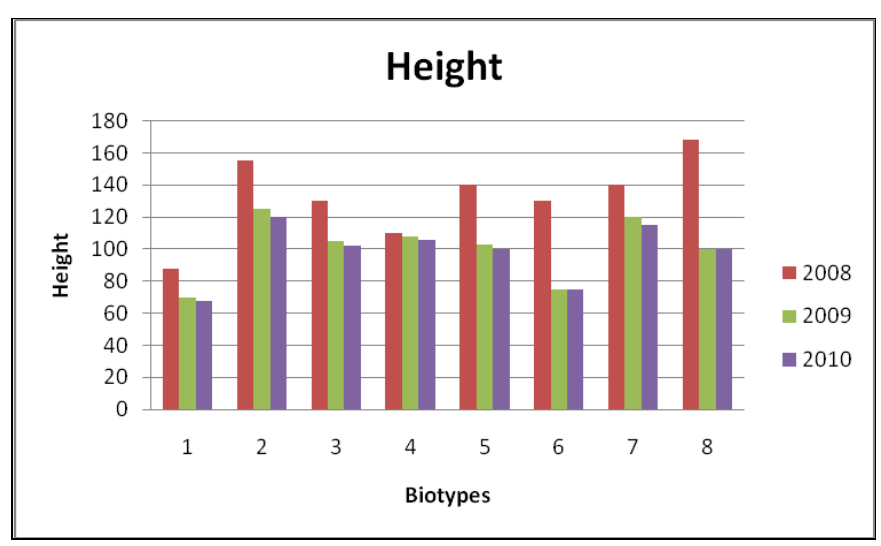

Figure 29. Average plant height at maturity stage during the three years of the trial.

CYTOGENETICS

At the prometaphase the chromosomes were elongated and easily identifiable (**Fig. 30**).

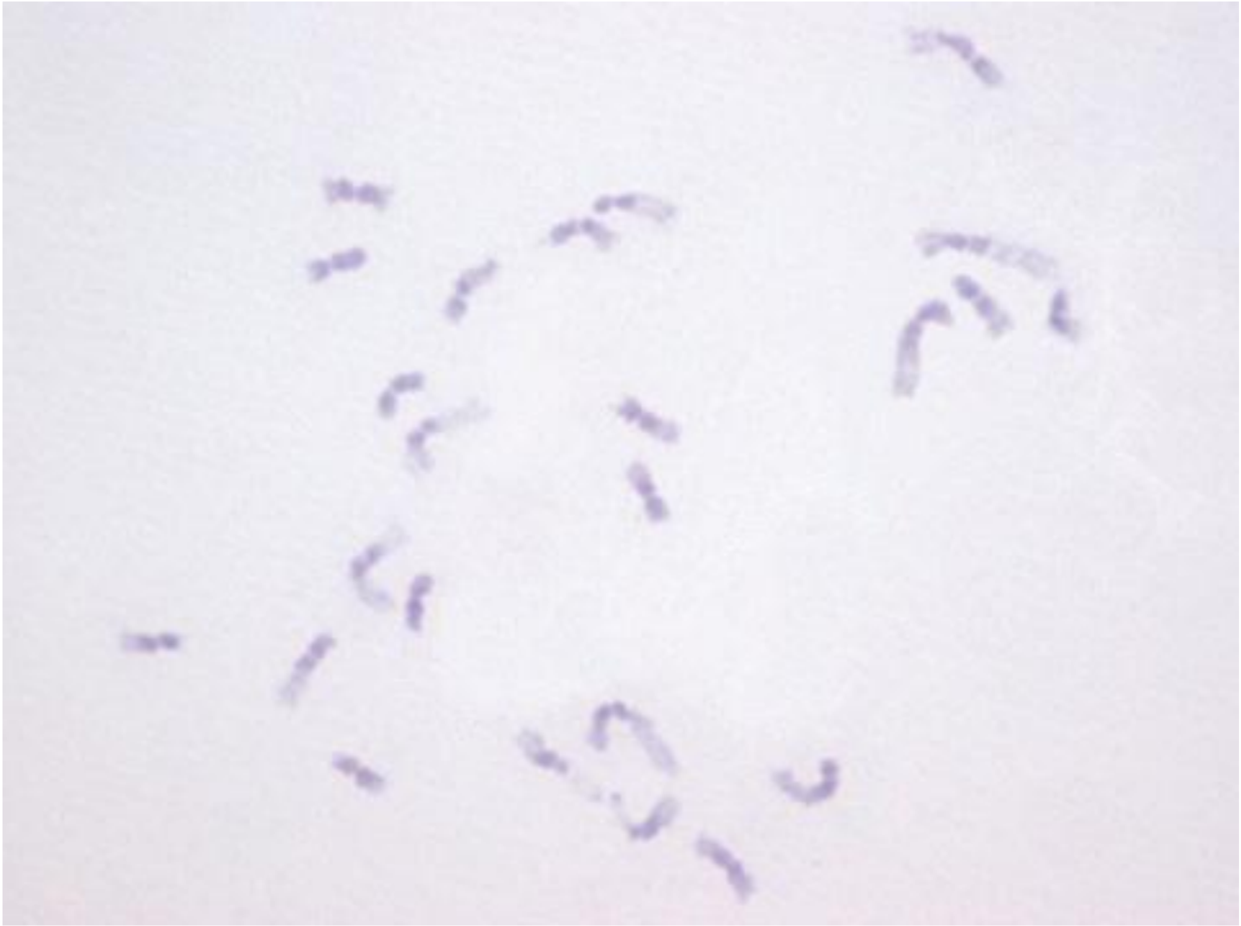

Figure30. Prometaphase in cv 'Loto'

In **Figure 31** is reported the pseudo-color image obtained from the black and white image. In this way we have determined all the parameters necessary to characterize each chromosome, i.e.: 1) length differences, 2) centromere position, 3) arm ratios, 4) distribution of chromatin density.

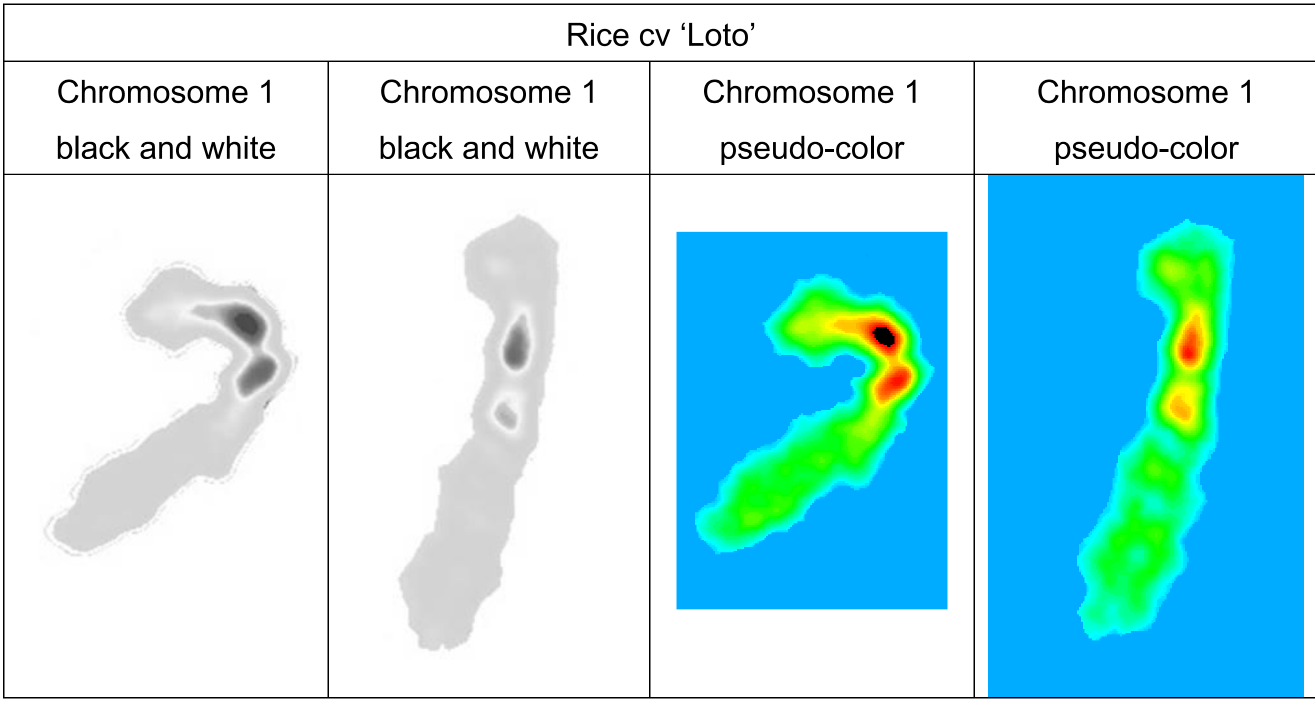

Figure 31. Image analysis of chromosome 1 of cv 'Loto'

The Condensation Pattern (CP) profile identifies and characterizes the somatic chromosomes; the vertical axis gives the grey values from 0 to 255 defining the chromatin density distribution along the length of the chromosome in pixels, and the horizontal axis gives the chromosome length in pixels, the centromere position and the arm ratios by means of the pixel number (**Fig. 32**).

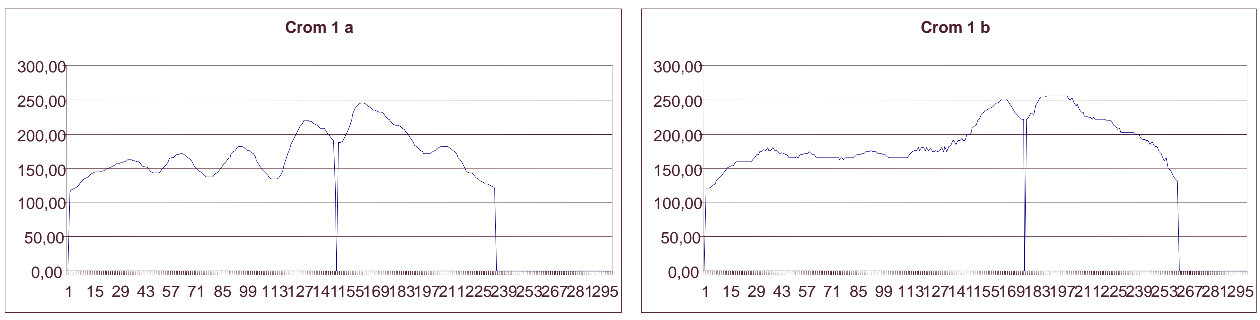

Figure 32. CP profiles of the first pair of 'Loto' chromosomes (a and b).

The cytogenetic map (**Fig. 33**) of the Italian rice cultivar 'Loto' (*Oryza sativa* ssp. *japonica*) does not reveal any substantial difference in chromosomal characteristics between it and the other rice cultivars belonging to the ssp. *japonica*.

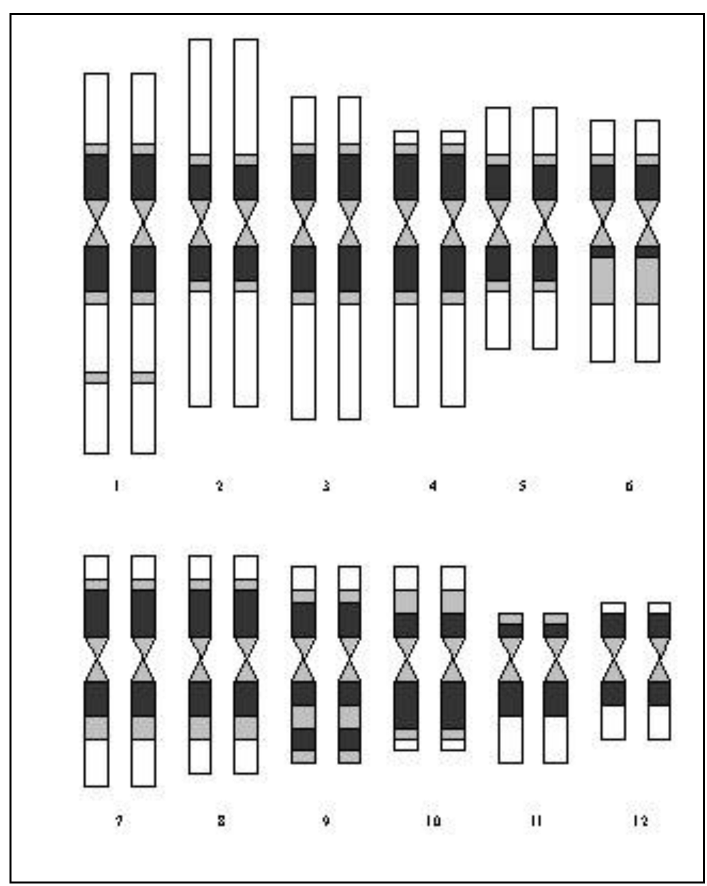

Figure 33. Cytogenetic map of the Italian rice cv 'Loto'. The three different colors correspond to different condensation degrees of chromatin along the chromosome. The black regions are the most condensed ones; the grey regions feature an intermediate condensation; the white regions are the less condensed ones.

Chromosome images and CP profiles of 10 prometaphase karyotypes, which were produced using the CHIA-EA method, provide evidence of the presence of many translocations in weedy red rice. In addition, this method clearly demonstrates that each biotype is characterised by a specific translocation. Given that chromosome 1 is involved in all the observed translocations, all the karyotypes share the same anomaly, i.e. deletion of an element of chromosome pair 1, which is shorter than its homologue, because of a considerable deletion of the distal segment on its long arm. In addition, taking into account that the distal segment of chromosome 1 may be displaced by translocation to the

terminus of either the short (S) or the long (L) arm of another chromosome, a second anomalous chromosome containing an additional segment at one of its two extremities is present in all karyotypes. Both anomalies are observable in the CP profiles of three prometaphases for each biotype (**from Figure 34 to 81**), where we can see the pixel extent of each chromosome arm, as well as an altered ratio of the length of the chromosome arms.

Biotype 1

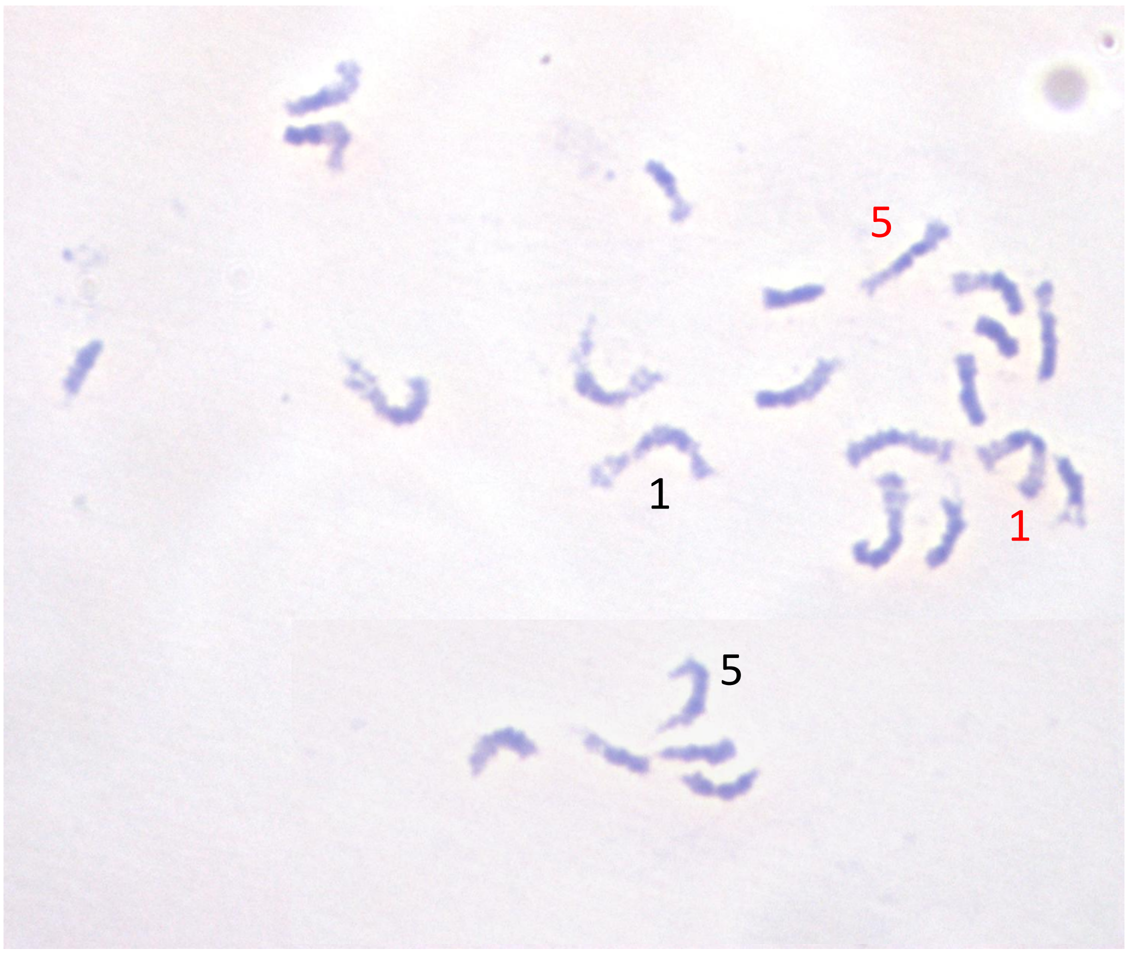

Figure 34. Prometaphase of red rice biotype 1. The chromosome couples involved in the translocation are 1 and 5. The red numbers label anomalous chromosomes.

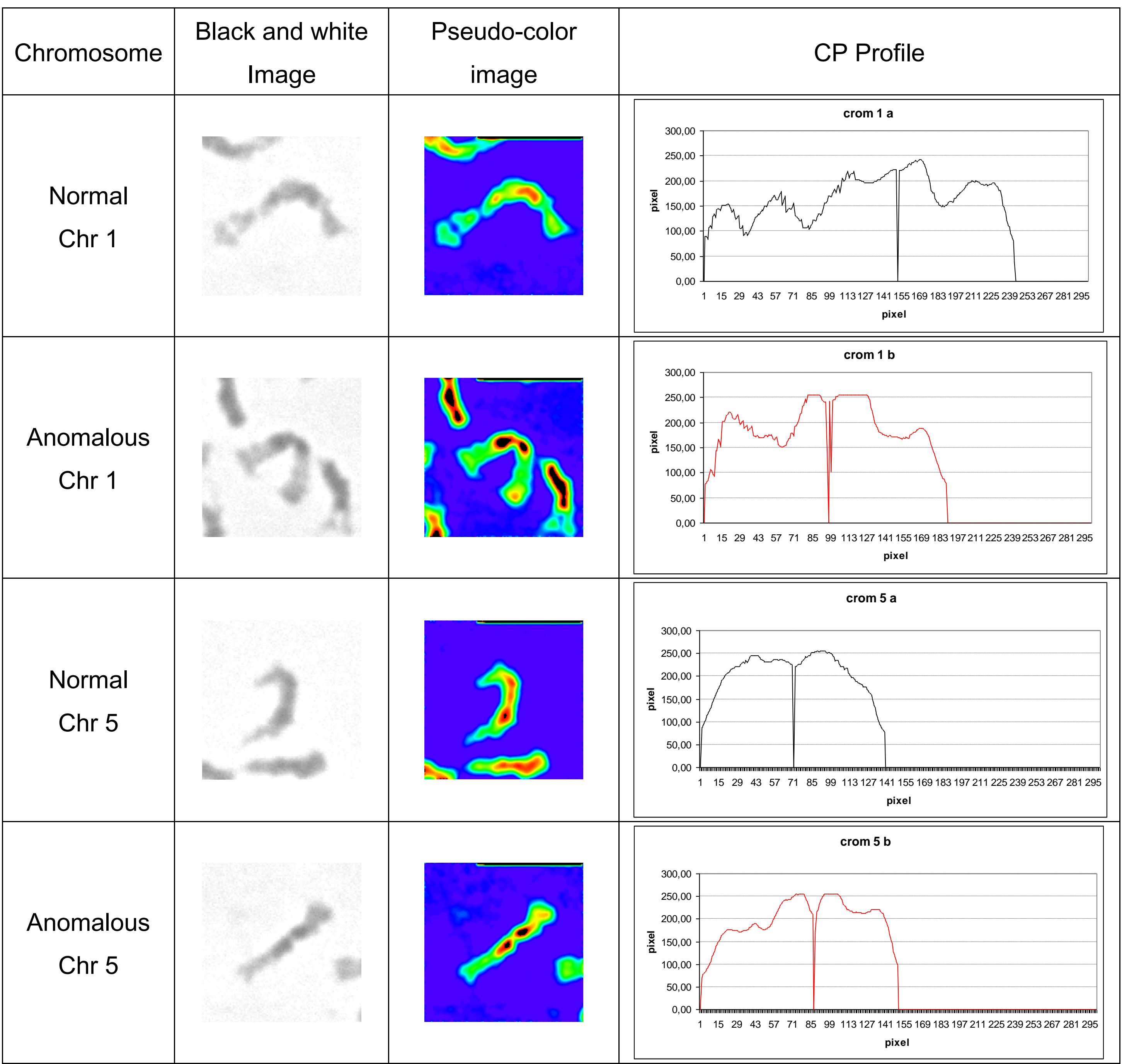

Figure 35. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 5 (a and b) from biotype 1. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The Pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

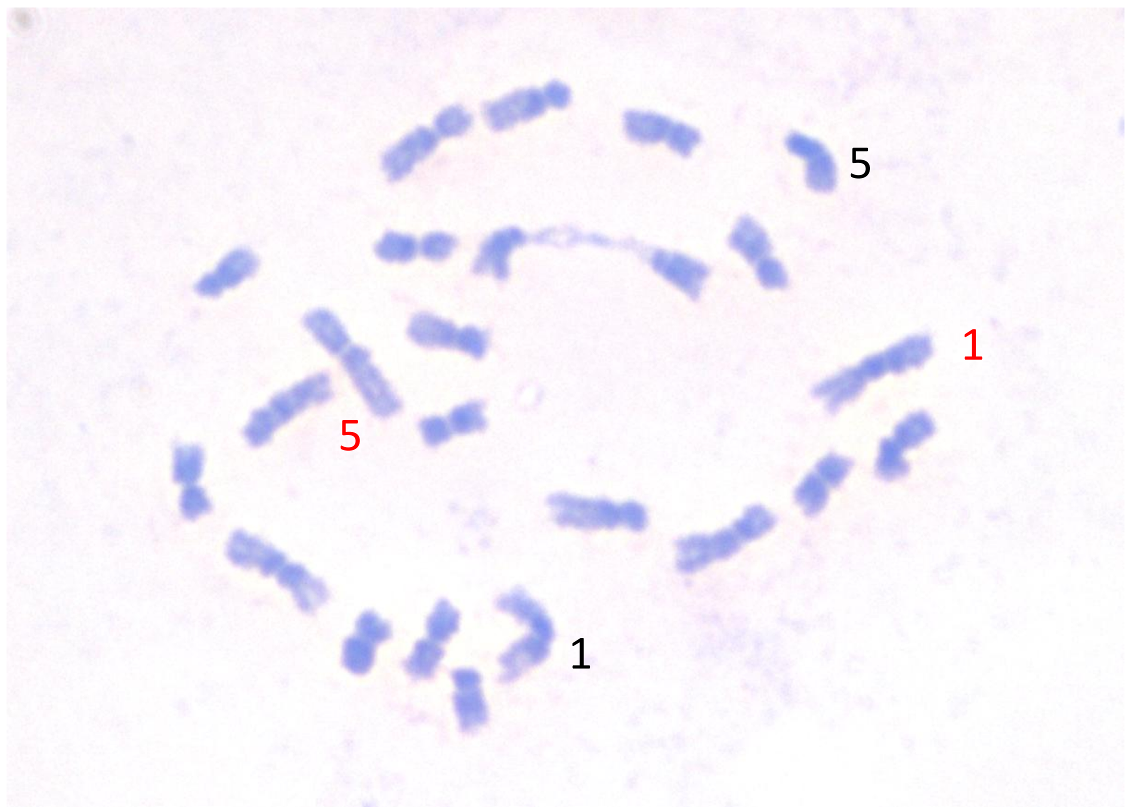

Figure 36. Prometaphase of red rice biotype 1. The chromosome couples involved in the translocation are 1 and 5. The red numbers label anomalous chromosomes.

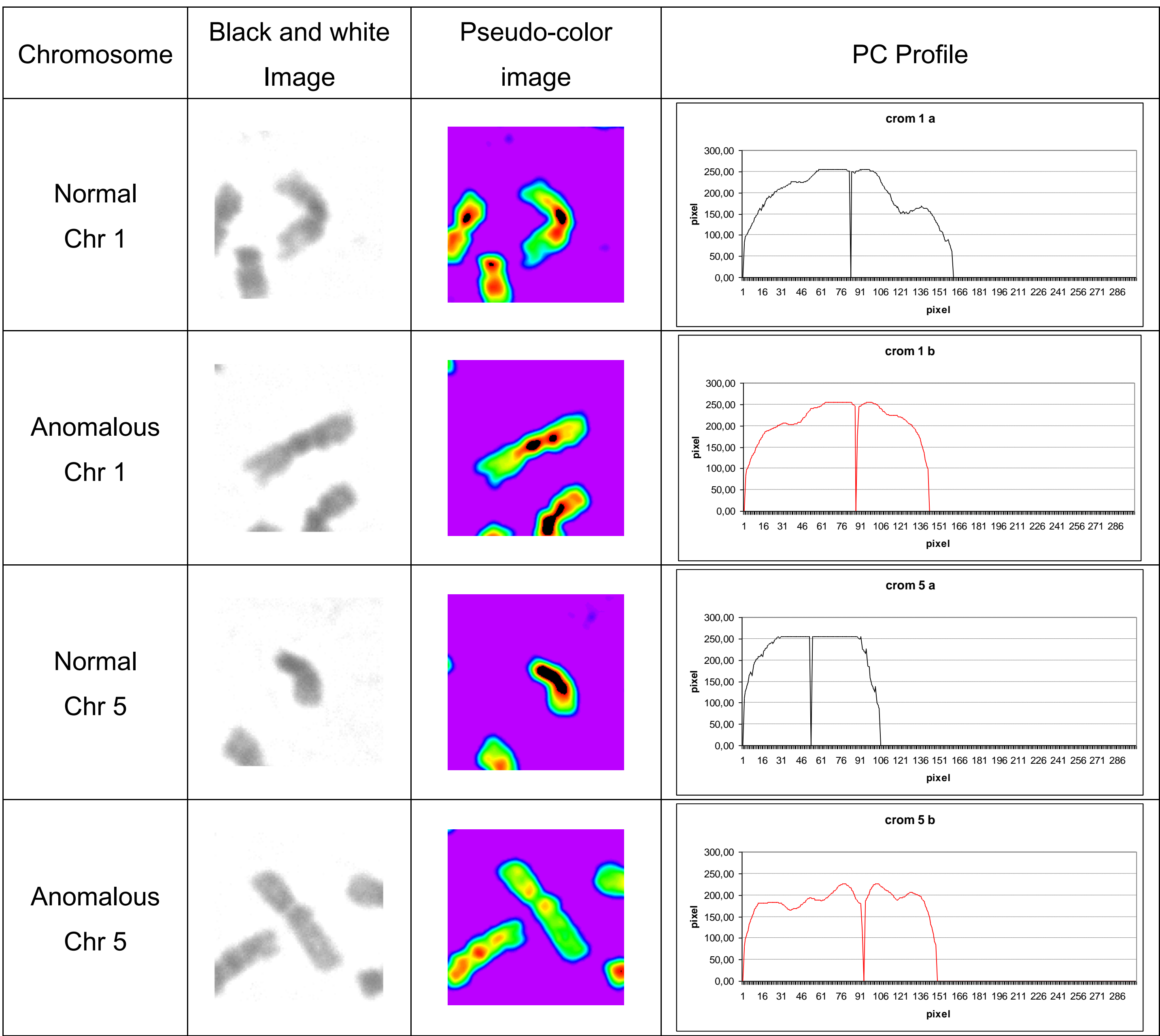

Figure 37. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 5 (a and b) from biotype 1. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a**; black) and for the anomalous chromosome (**b**; red) are shown, and the differences in long arm length and centromere position are clearly visible.

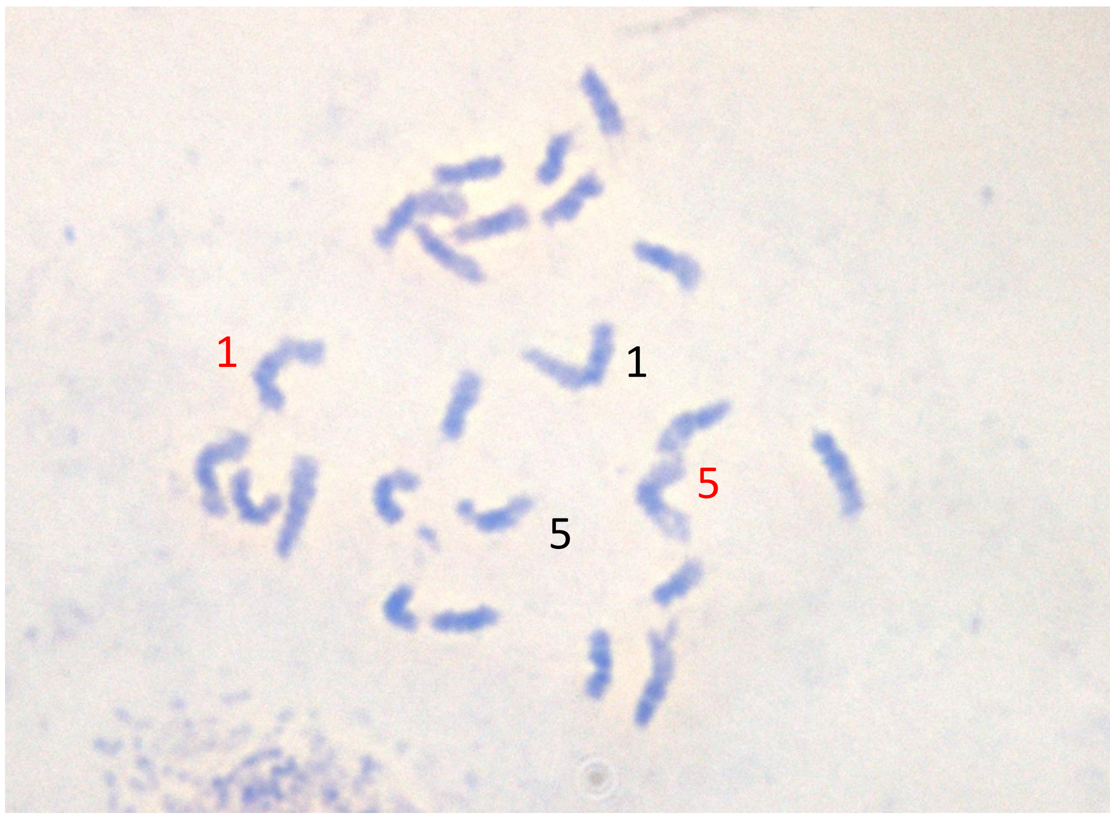

Figure 38. Prometaphase of red rice biotype 1. The chromosome couples involved in the translocation are 1 and 5. The red numbers label anomalous chromosomes.

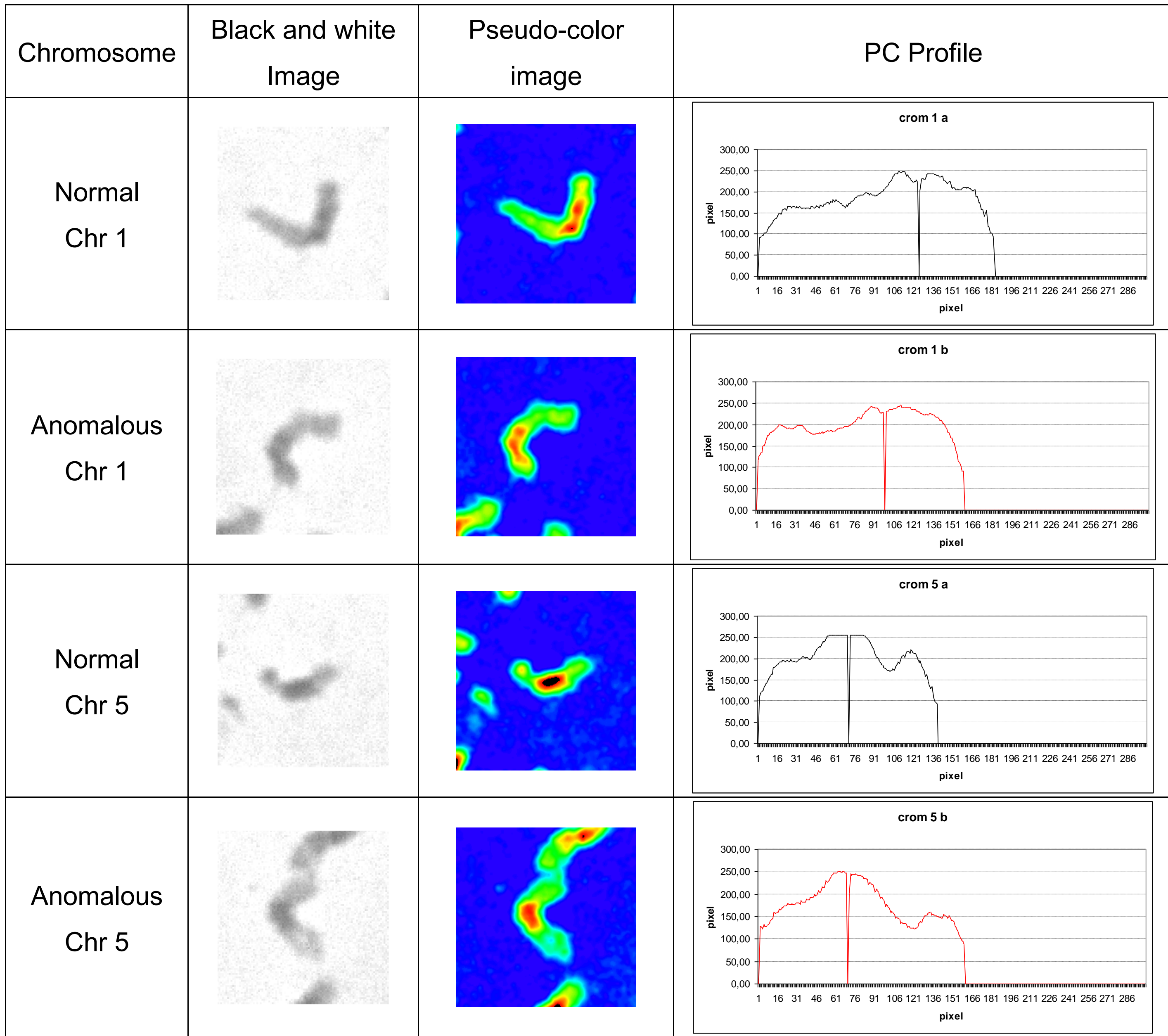

Figure 39. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 5 (a and b) from biotype 1. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

Biotype 2

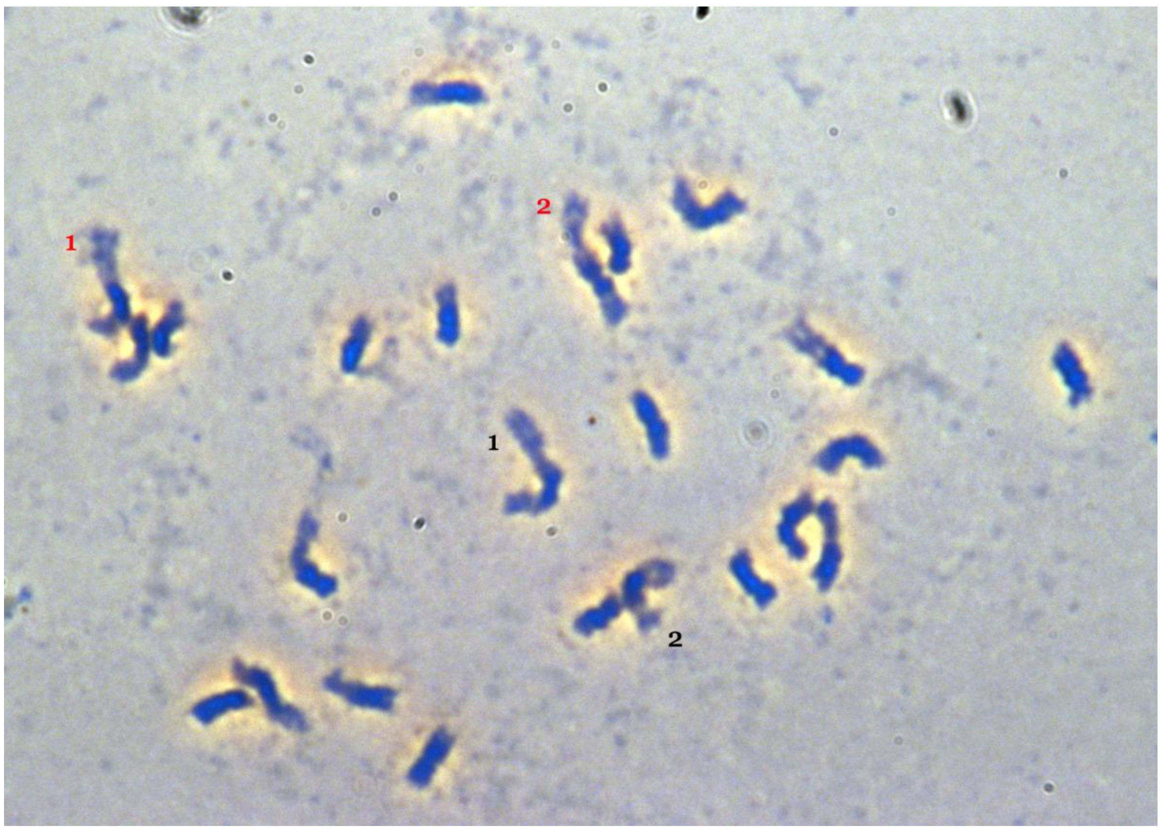

Figure 40. Prometaphase of red rice biotype 2. The chromosome couples involved in the translocation are 1 and 2. The red numbers label anomalous chromosomes.

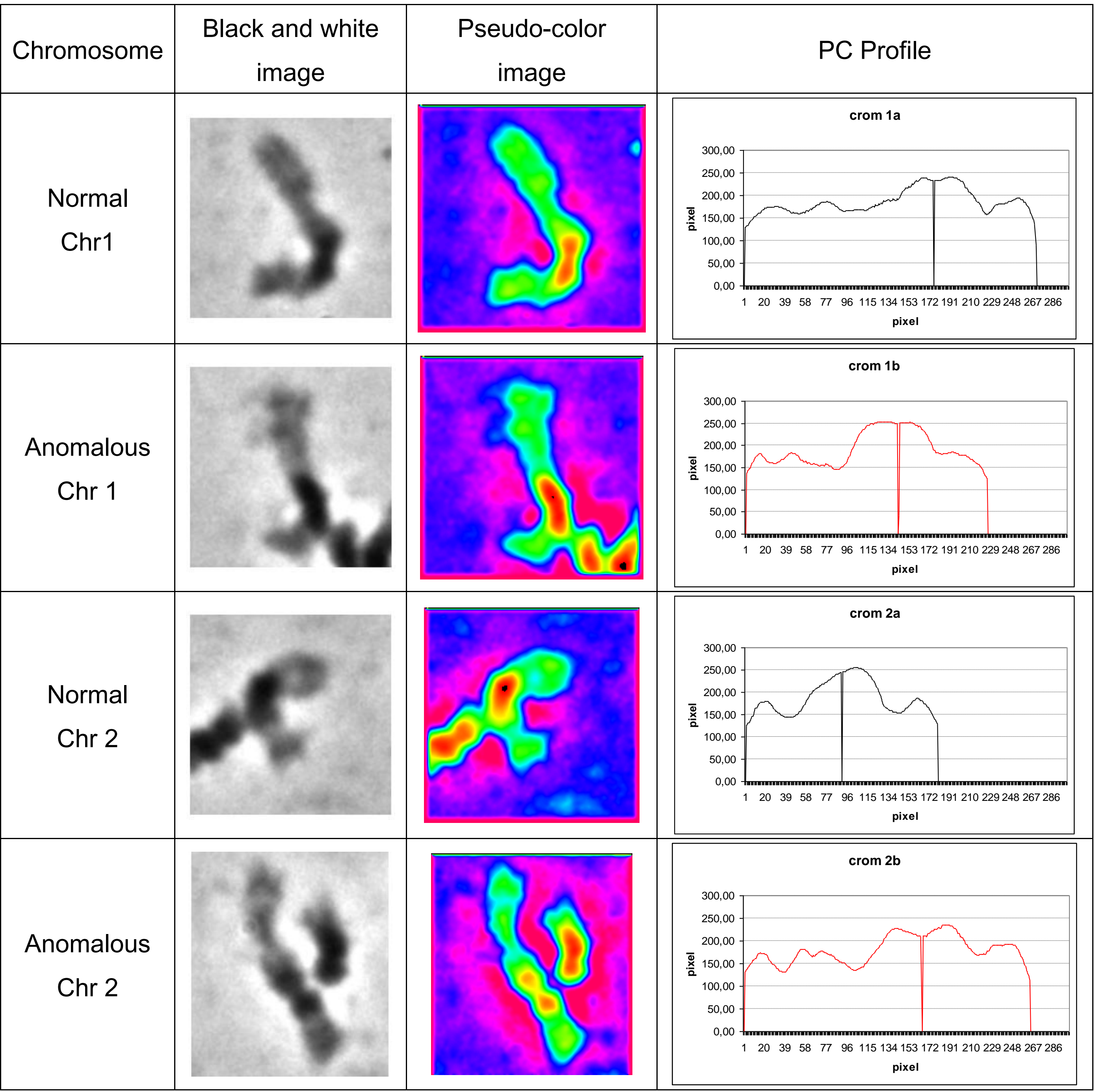

Figure 41. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 2 (a and b) from biotype 2. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

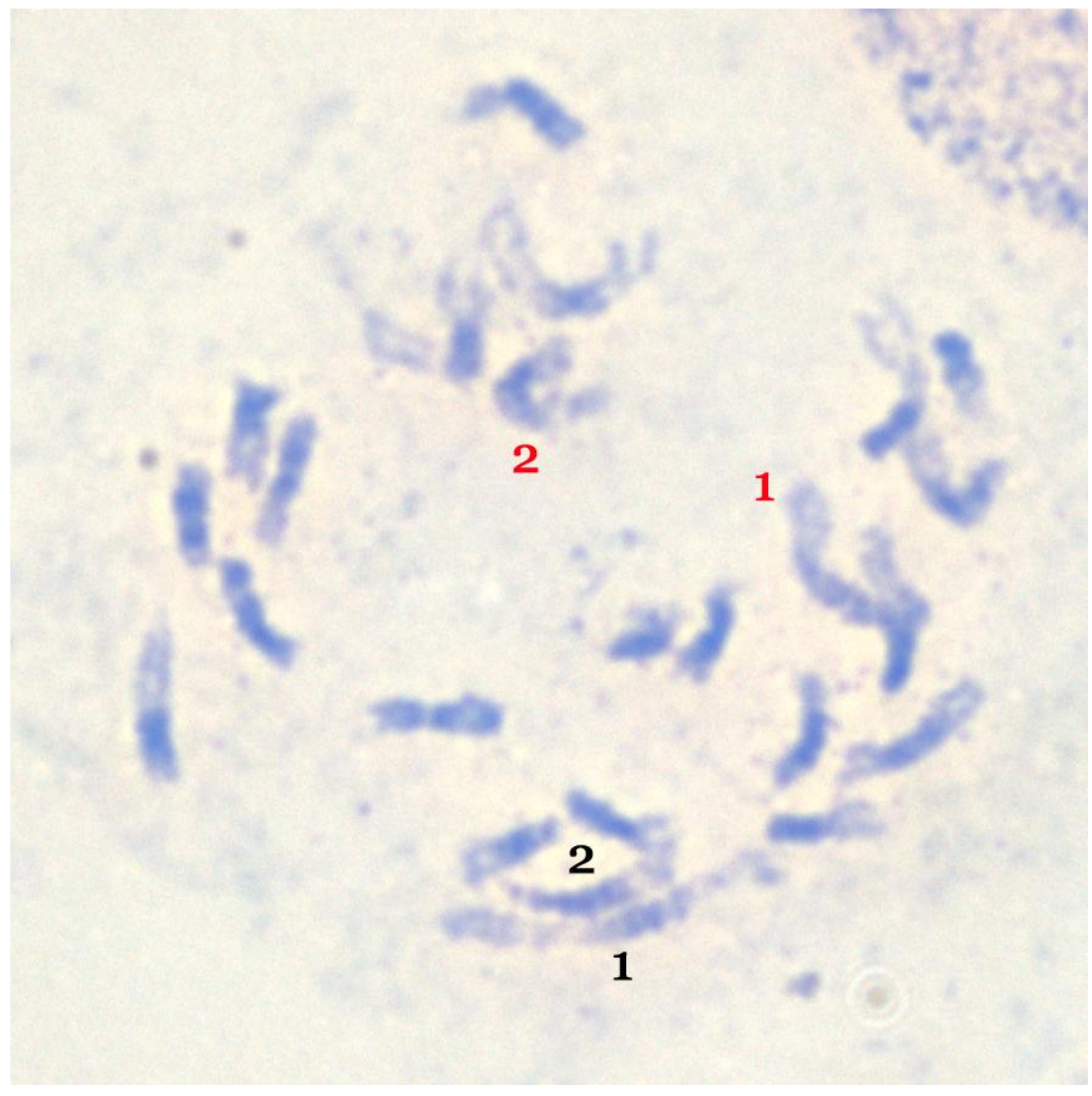

Figure 42. Prometaphase of red rice biotype 2. The chromosome couples involved in the translocation are 1 and 2. The red numbers label anomalous chromosomes.

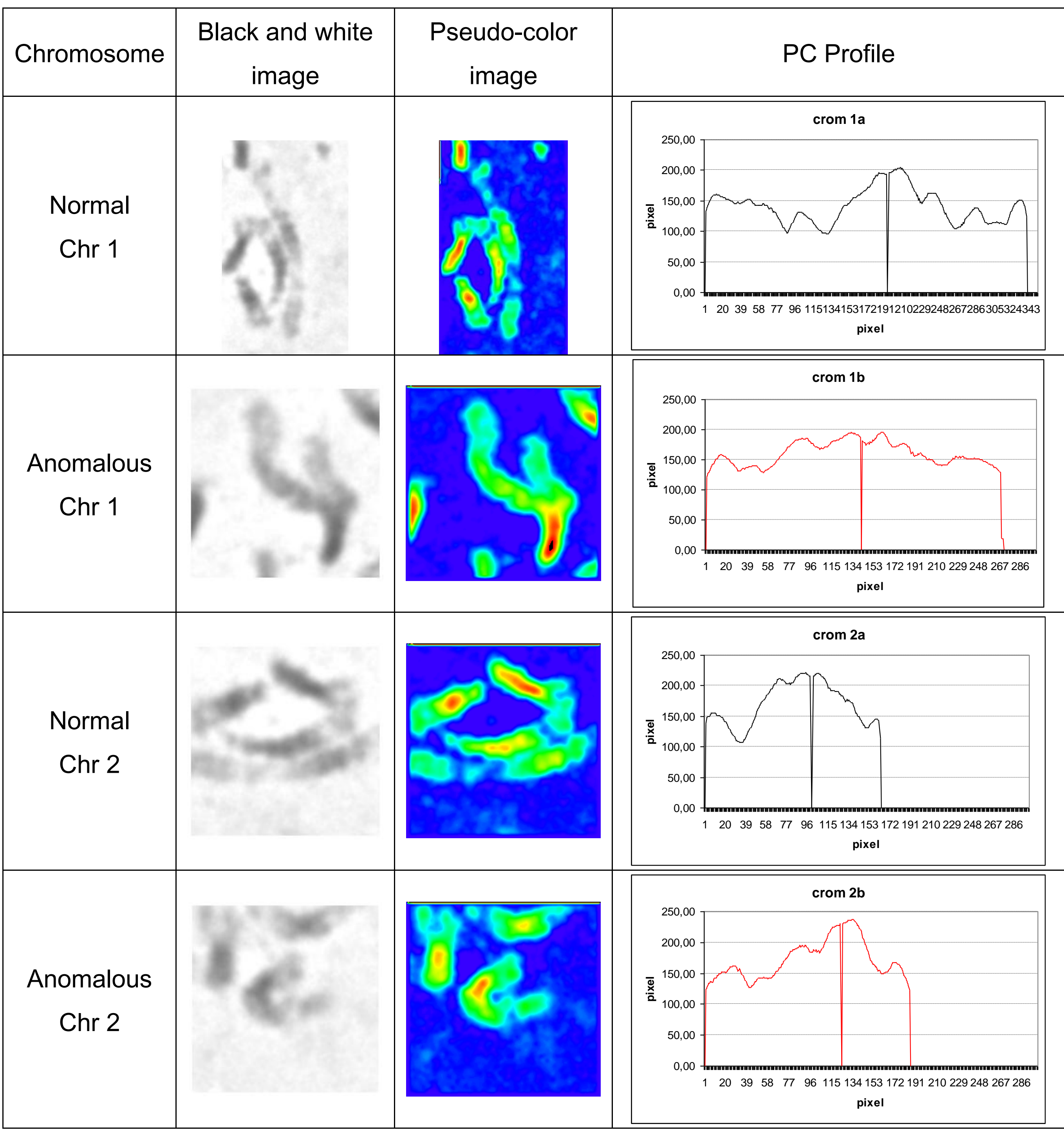

Figure 43. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 2 (a and b) from biotype 2. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

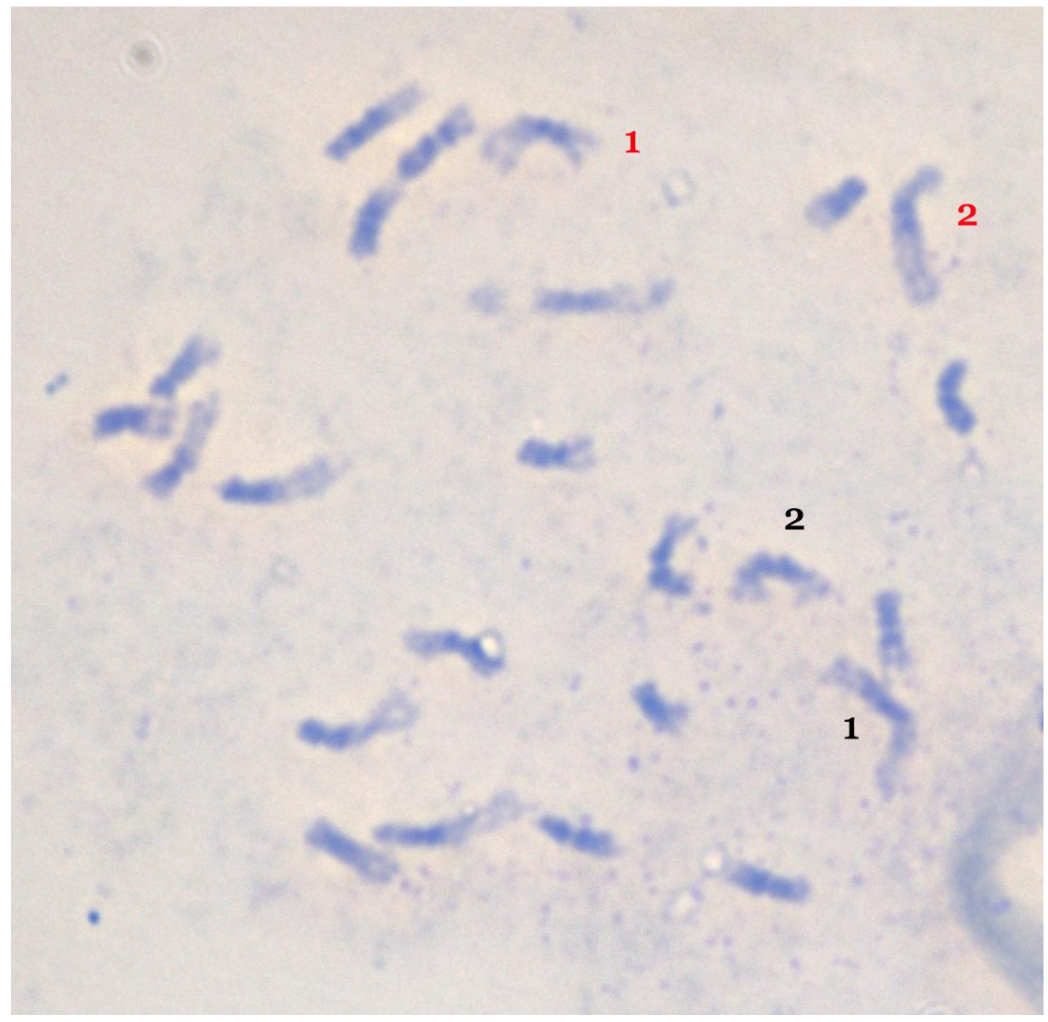

Figure 44. Prometaphase of red rice biotype 2. The chromosome couples involved in the translocation are the ones of chromosomes 1 and 2. The red numbers label anomalous chromosomes.

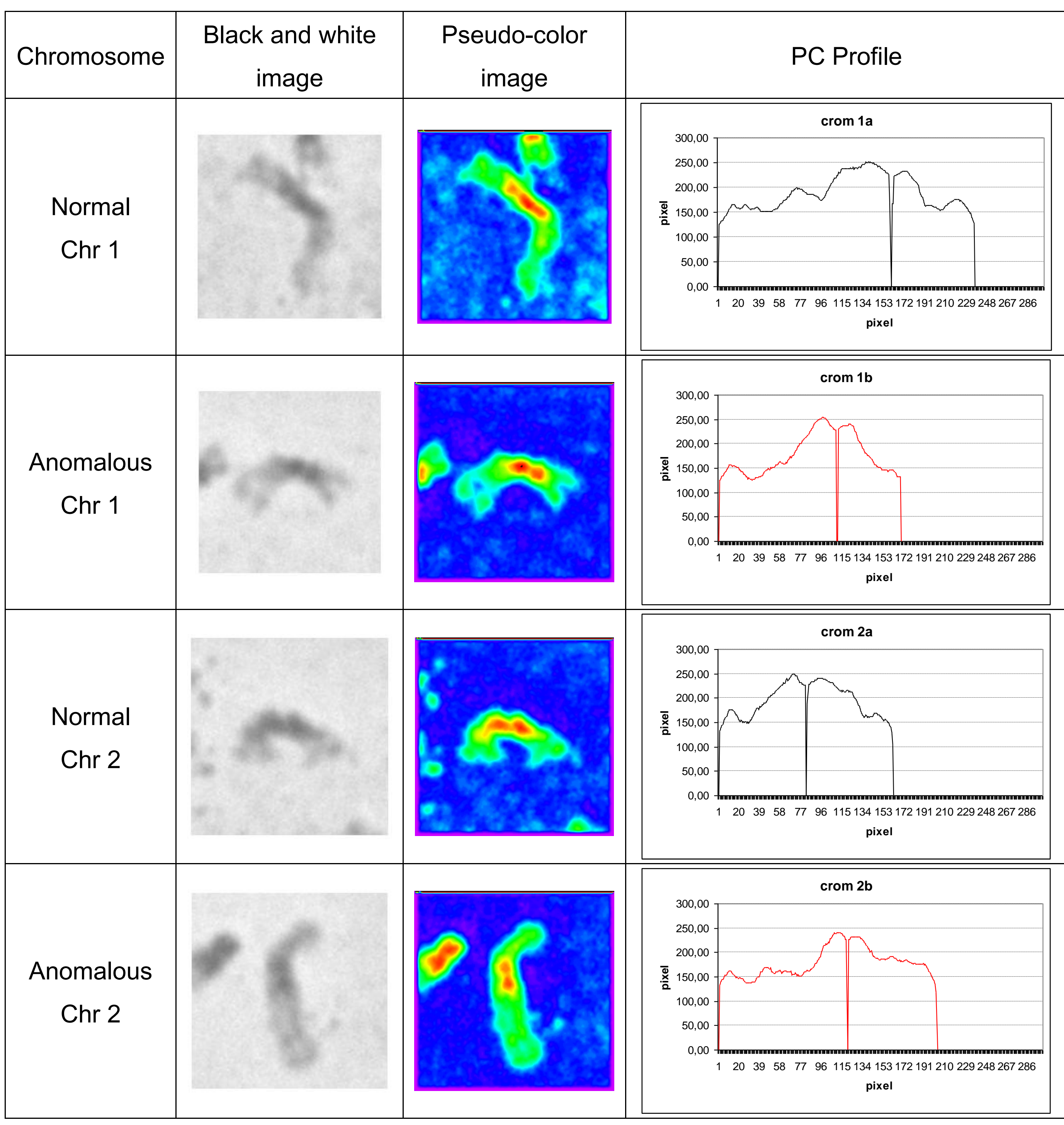

Figure 45. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 2 (a and b) from biotype 2. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

Figure 46. Prometaphase of red rice biotype 3. The chromosome couples involved in the translocation are 1 and 12. The red numbers label anomalous chromosomes.

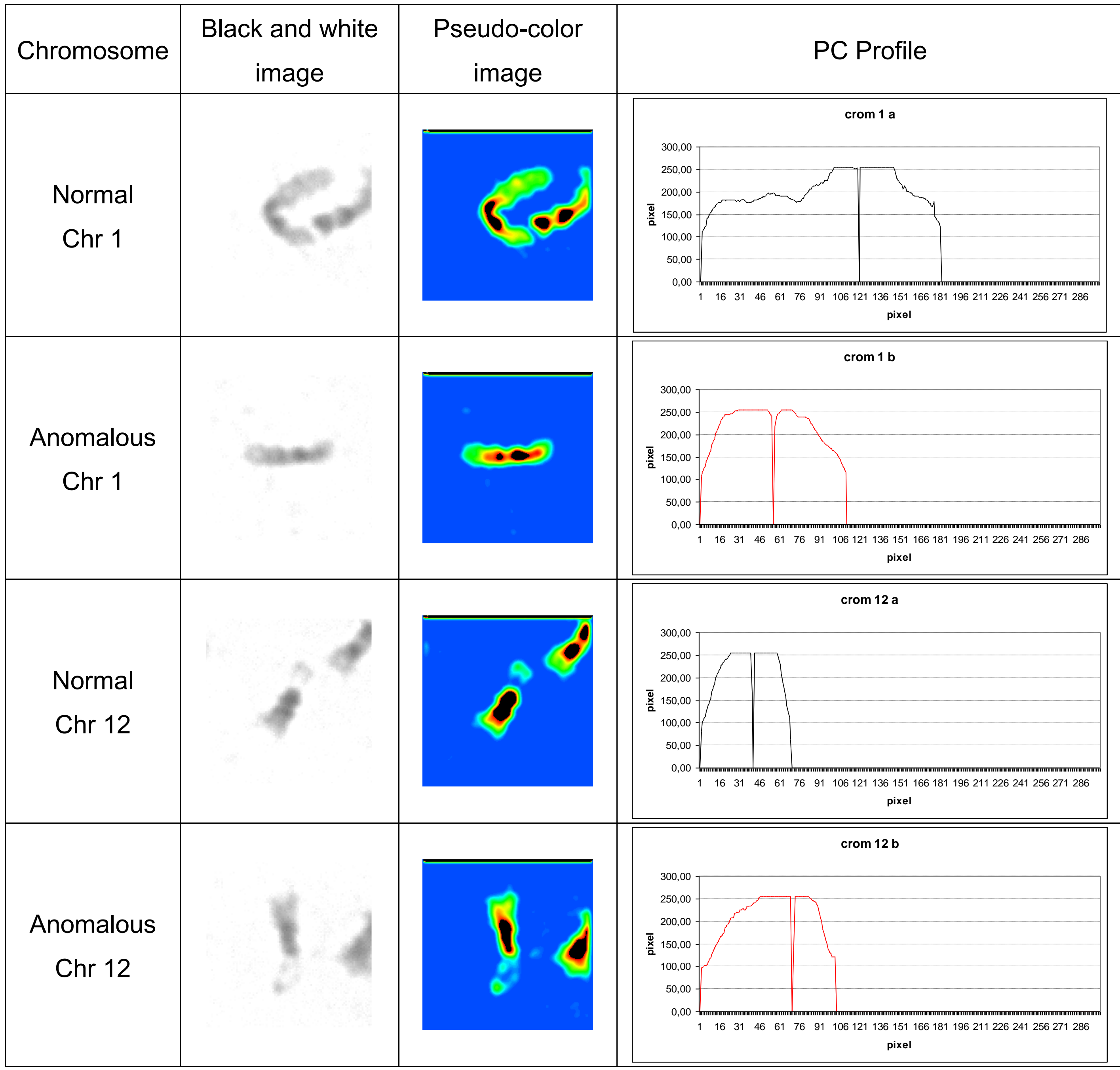

Figure 47. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 12 (a and b) from biotype 3. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a**; black) and for the anomalous chromosome (**b**; red) are shown, and the differences in long arm length and centromere position are clearly visible.

Figure 48. Prometaphase of red rice biotype 3. The chromosome couples involved in the translocation are 1 and 12. The red numbers label anomalous chromosomes.

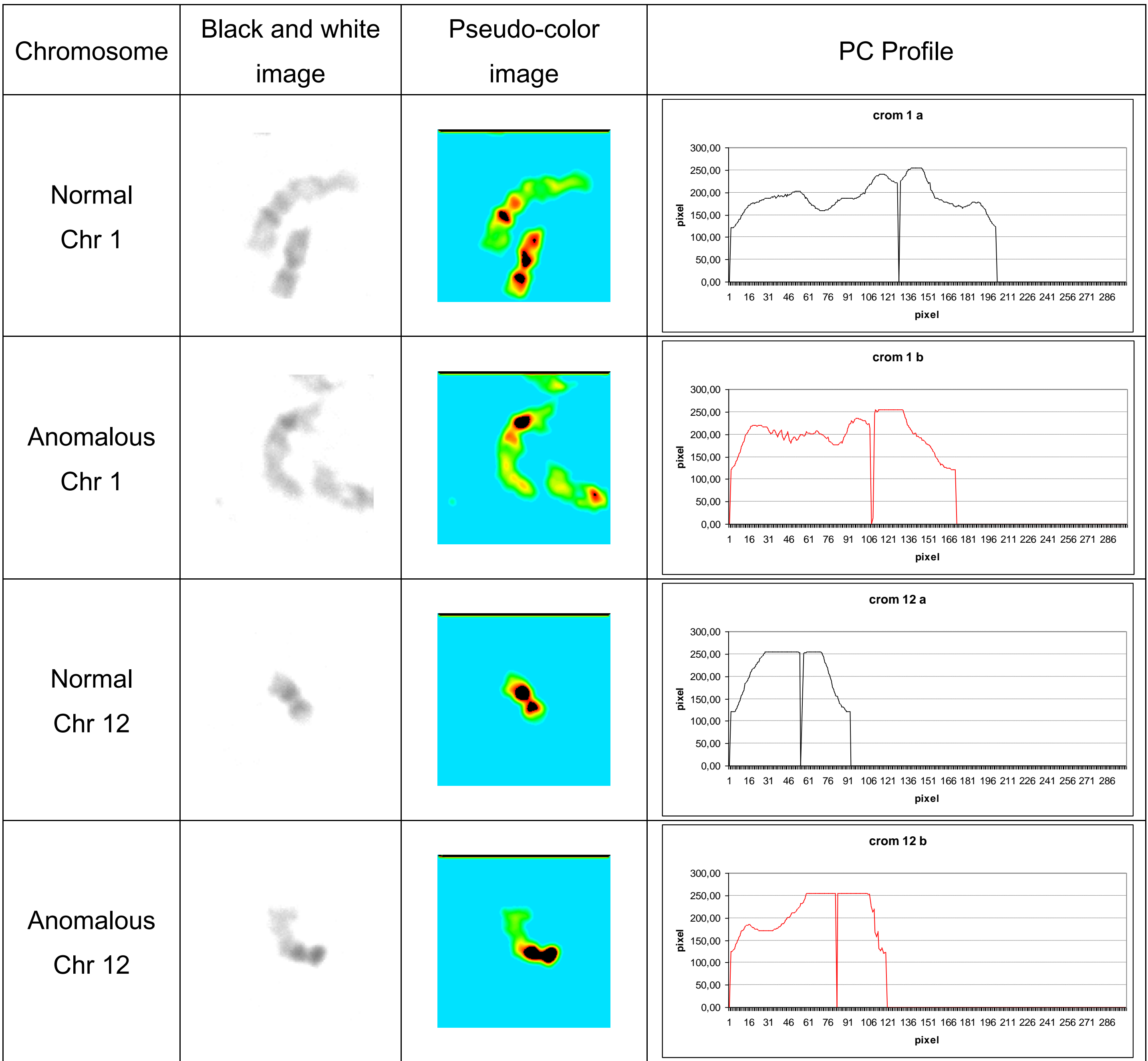

Figure 49. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 12 (a and b) from biotype 3. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

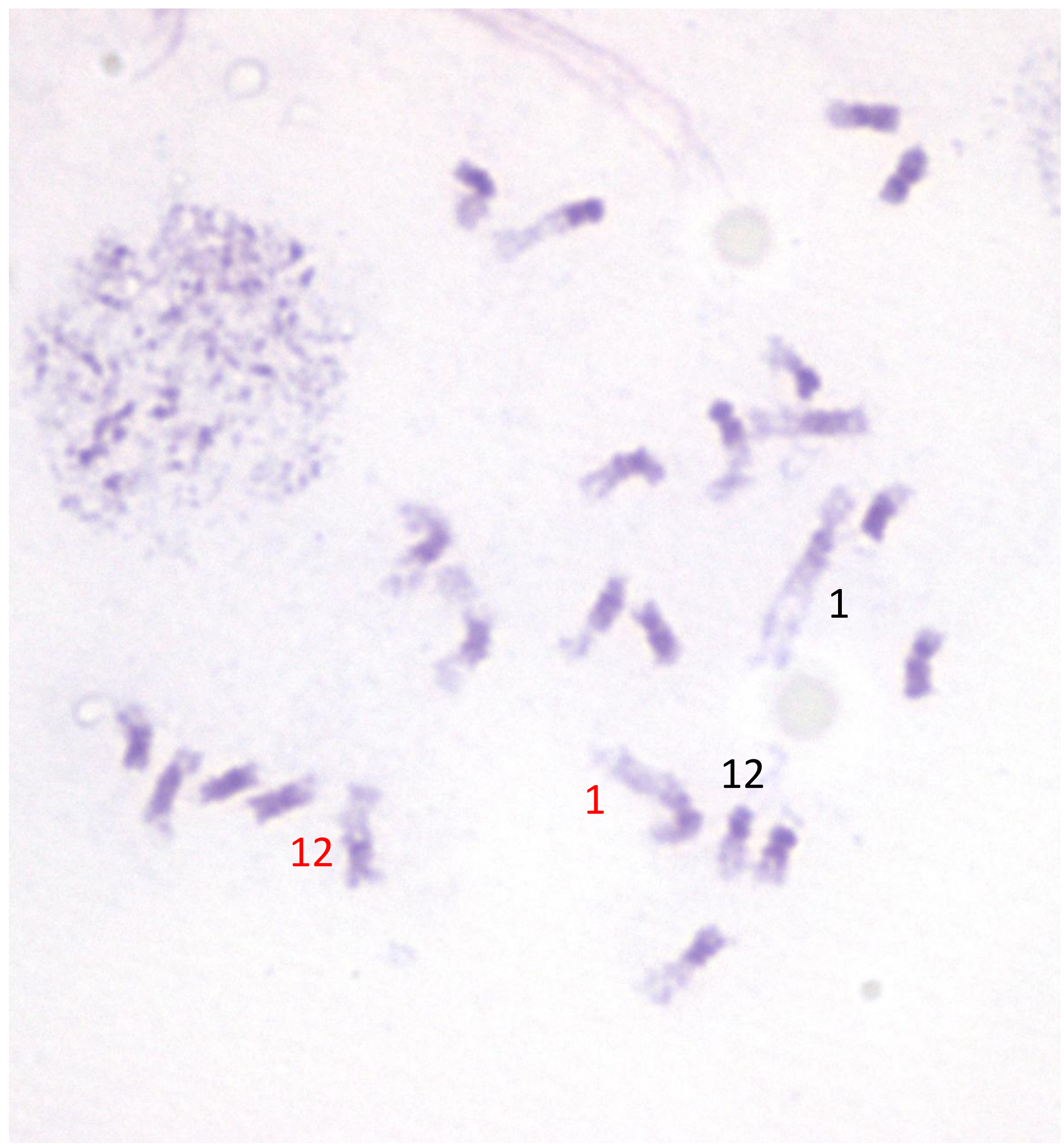

Figure 50. Prometaphase of red rice biotype 3. The chromosome couples involved in the translocation are 1 and 12. The red numbers label anomalous chromosomes.

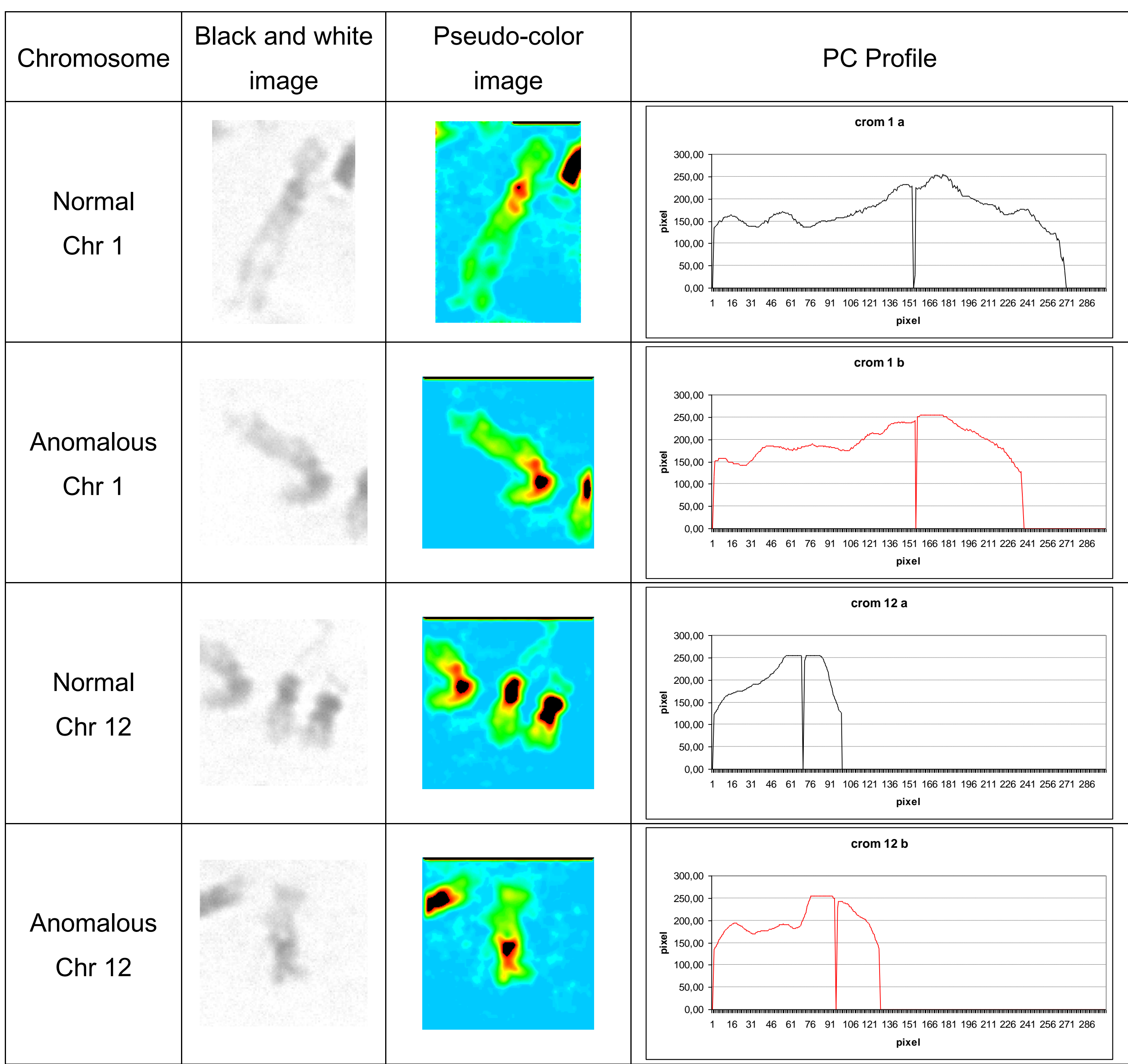

Figure 51. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 12 (a and b) from biotype 3. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

Biotype 4

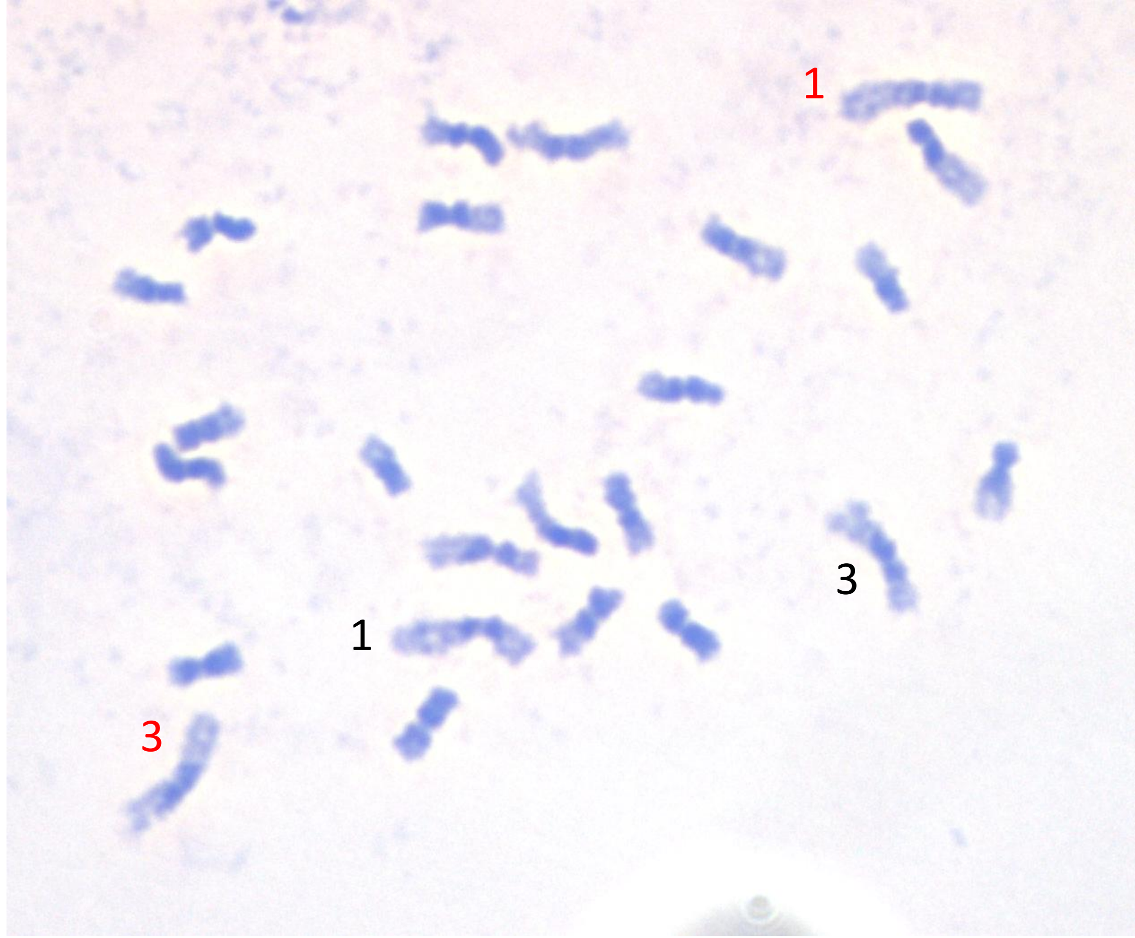

Figure 52. Prometaphase of red rice biotype 4. The chromosome couples involved in the translocation are 1 and 3. The red numbers label anomalous chromosomes.

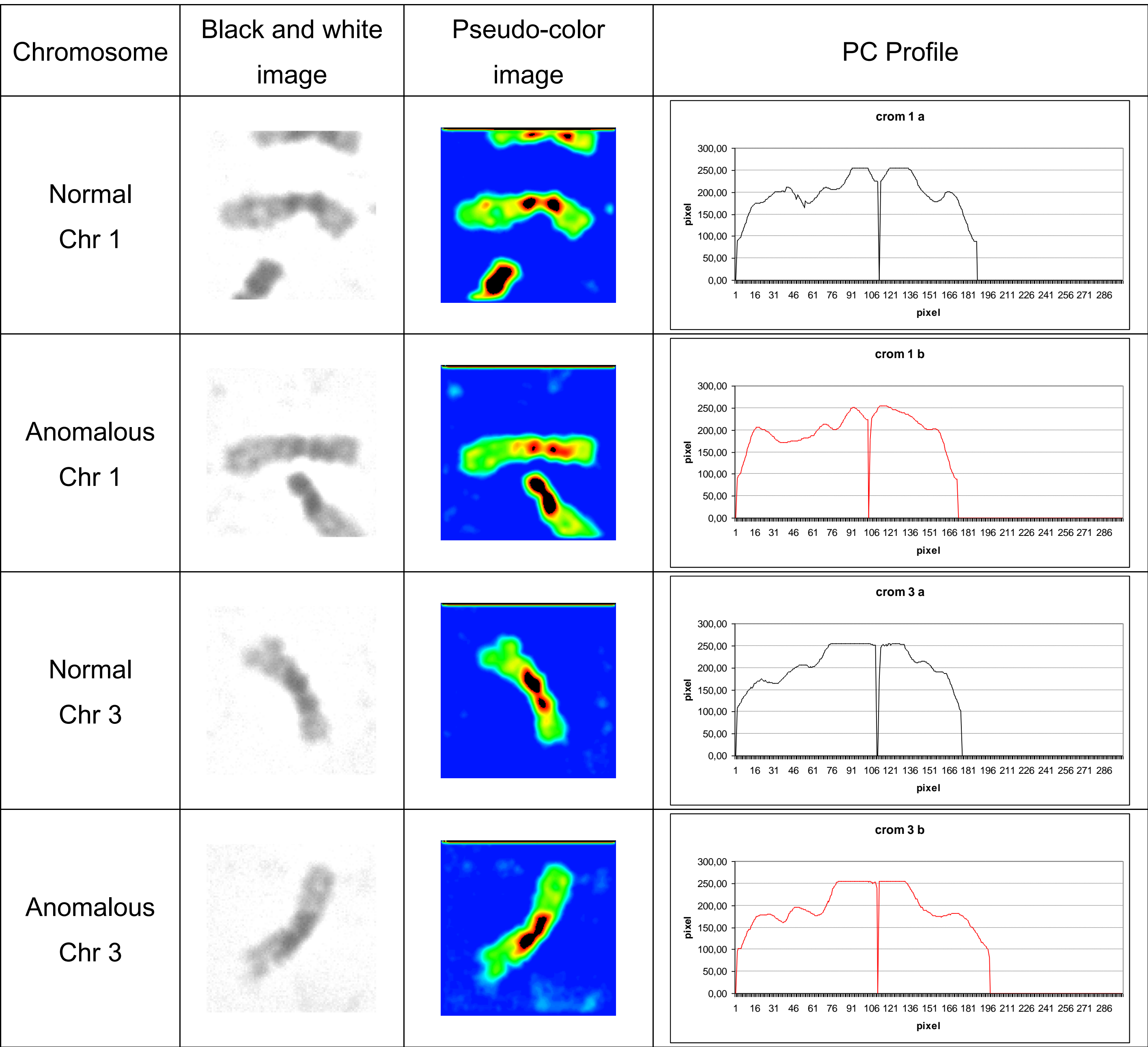

Figure 53. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 3 (a and b) from biotype 4. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

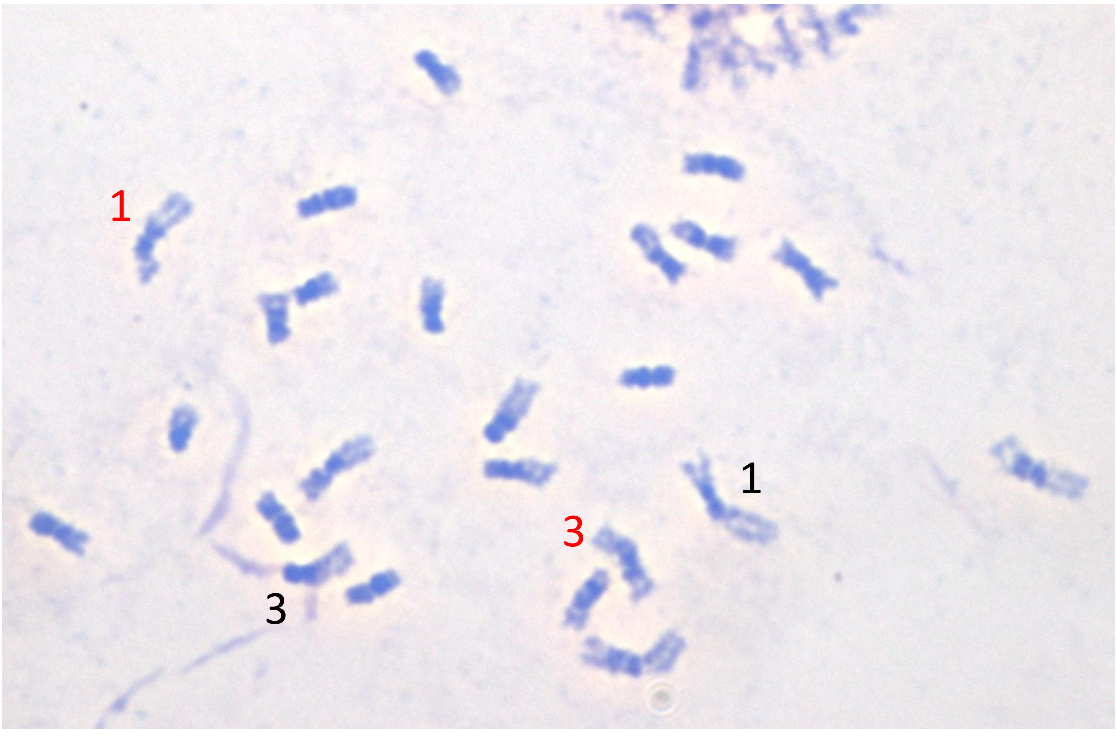

Figure 54. Prometaphase of red rice biotype 4. The chromosome couples involved in the translocation are 1 and 3. The red numbers label anomalous chromosomes.

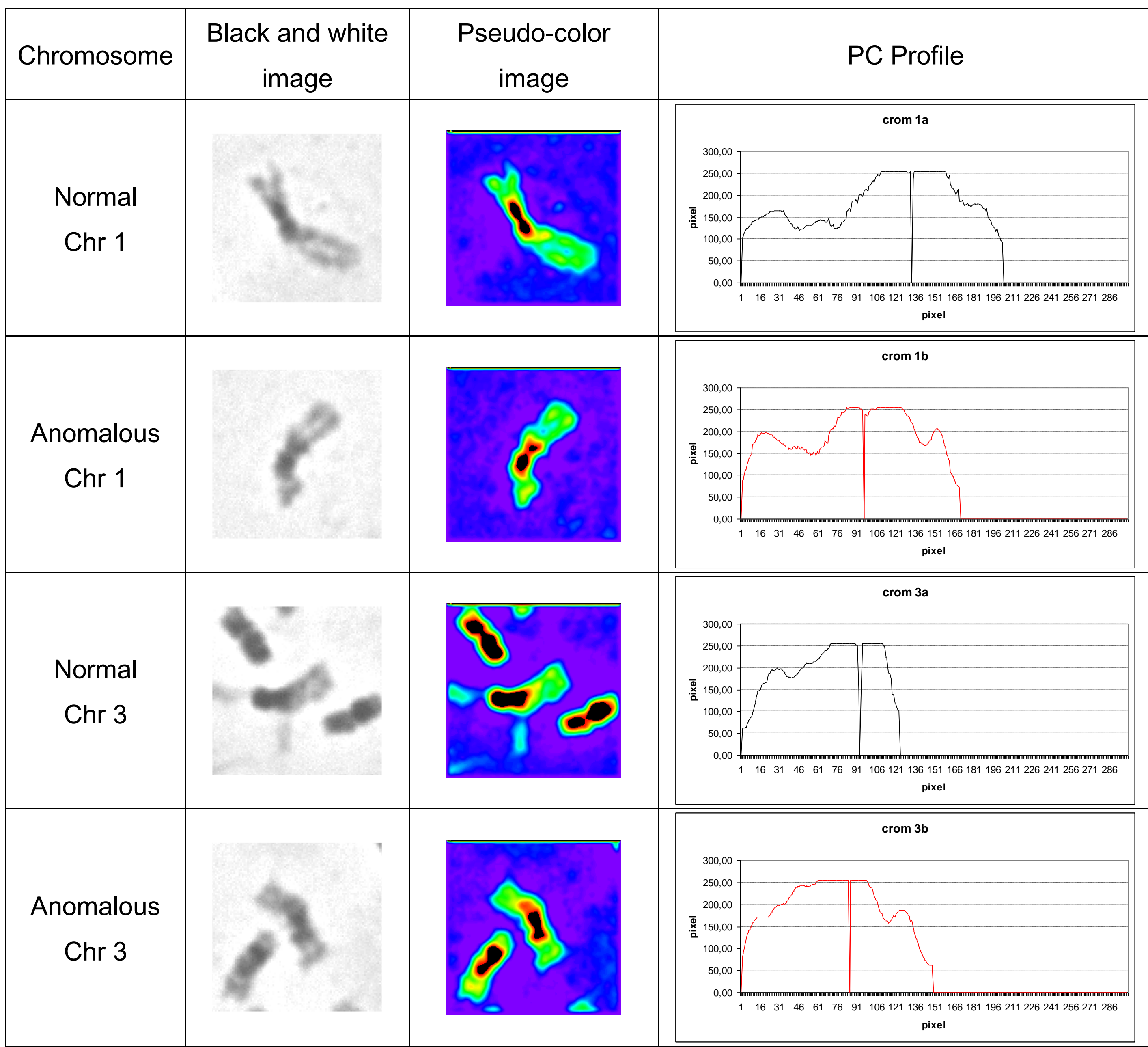

Figure 55. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 3 (a and b) from biotype 4. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a**; black) and for the anomalous chromosome (**b**; red) are shown, and the differences in long arm length and centromere position are clearly visible.

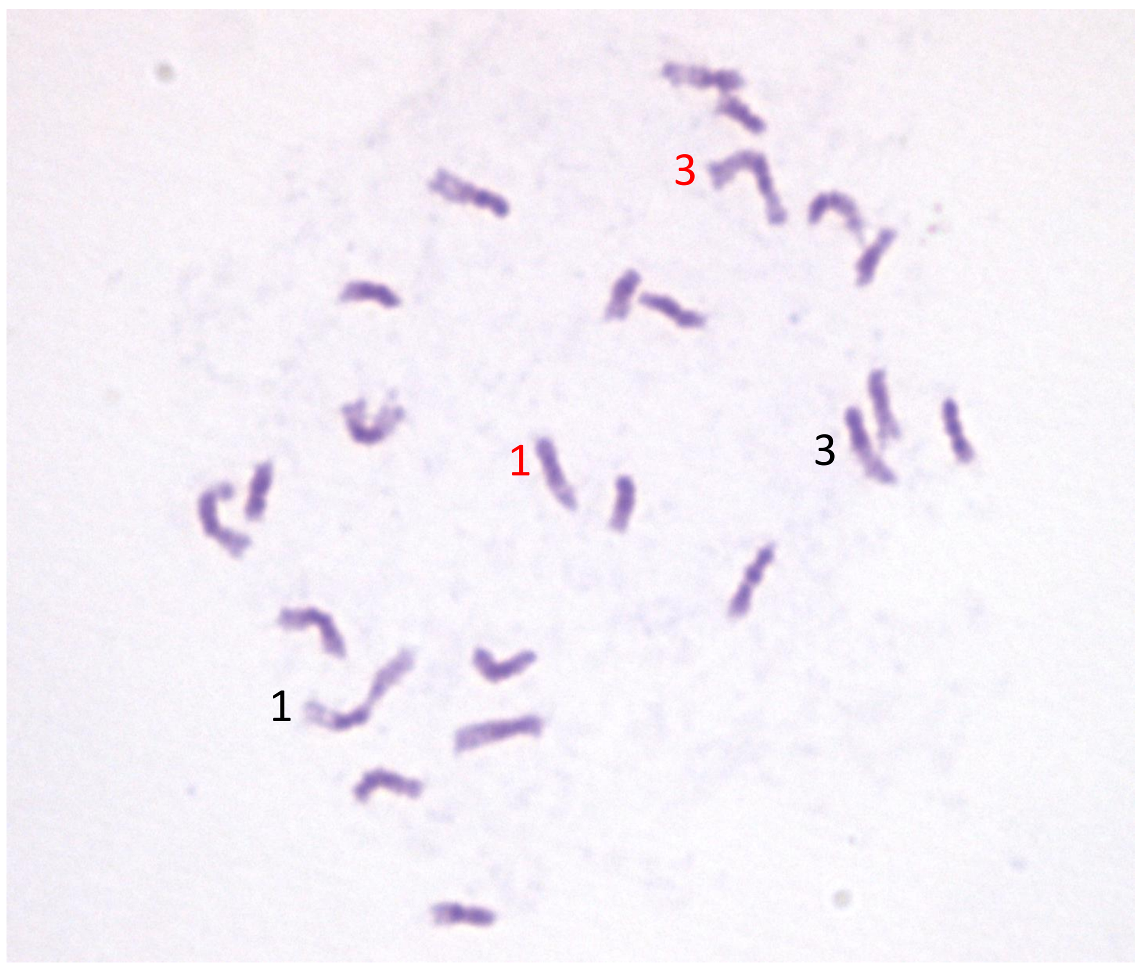

Figure 56. Prometaphase of red rice biotype 4. The chromosome couples involved in the translocation are 1 and 3. The red numbers label anomalous chromosomes.

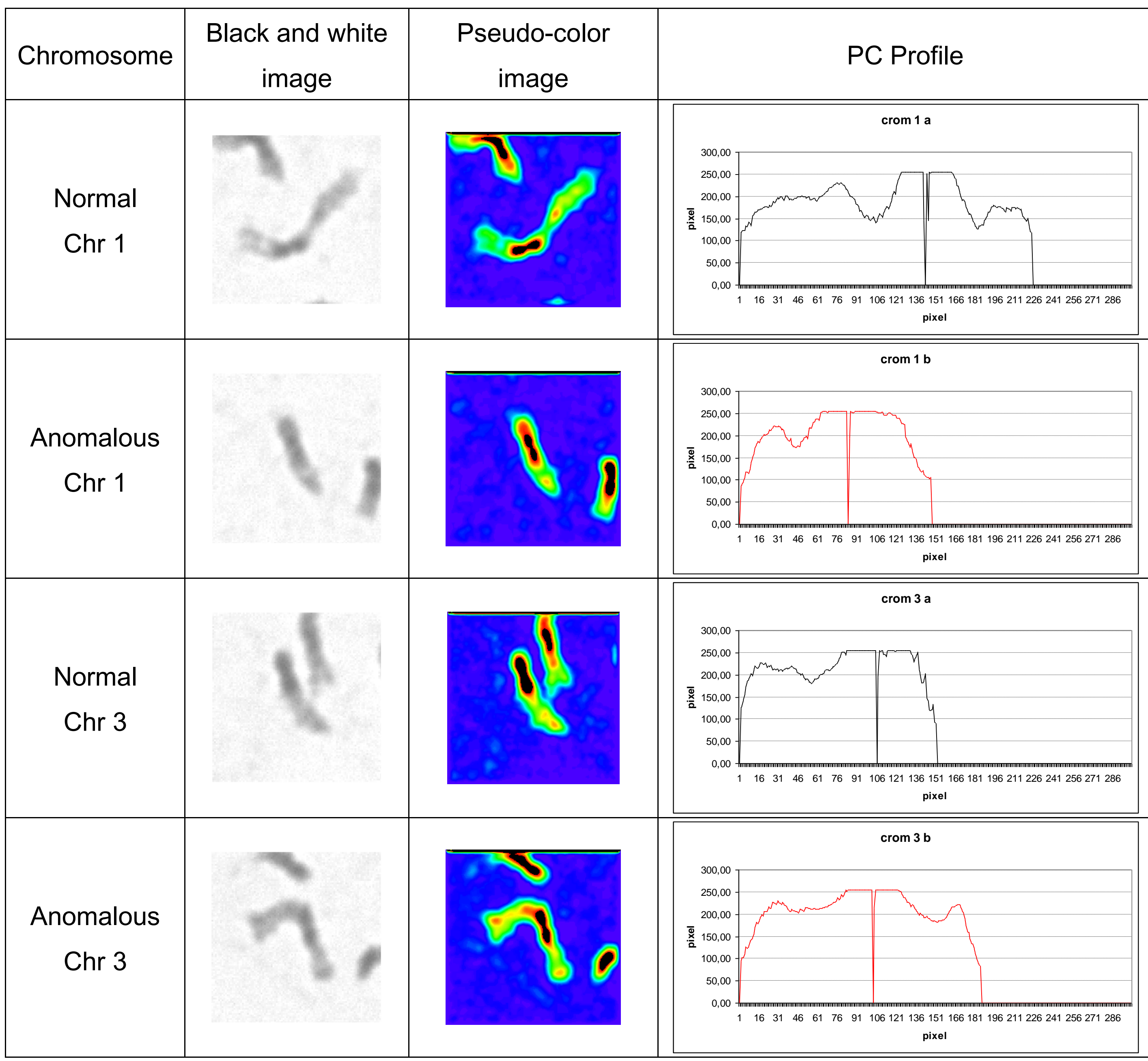

Figure 57. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 3 (a and b) from biotype 4. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

Biotype 5

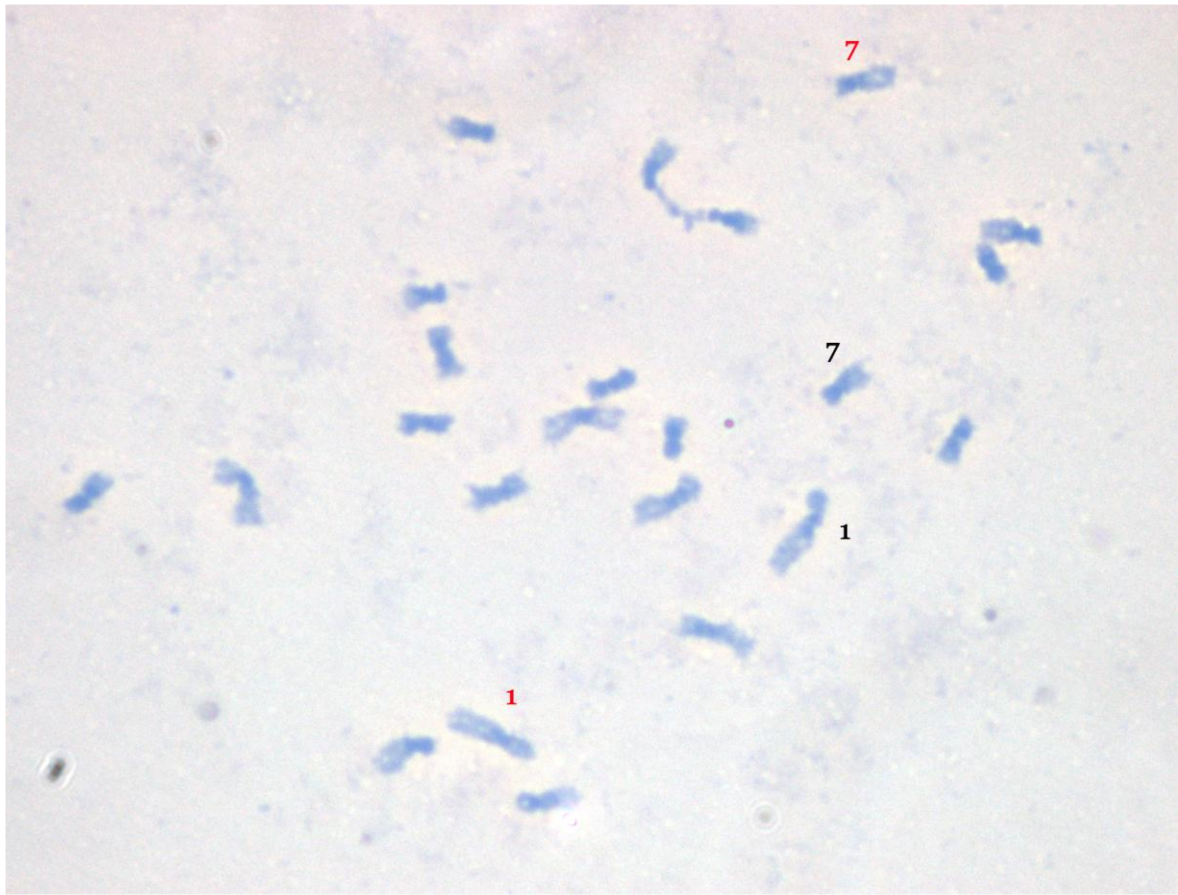

Figure 58. Prometaphase of red rice biotype 5. The chromosome couples involved in the translocation are 1 and 7. The red numbers label anomalous chromosomes.

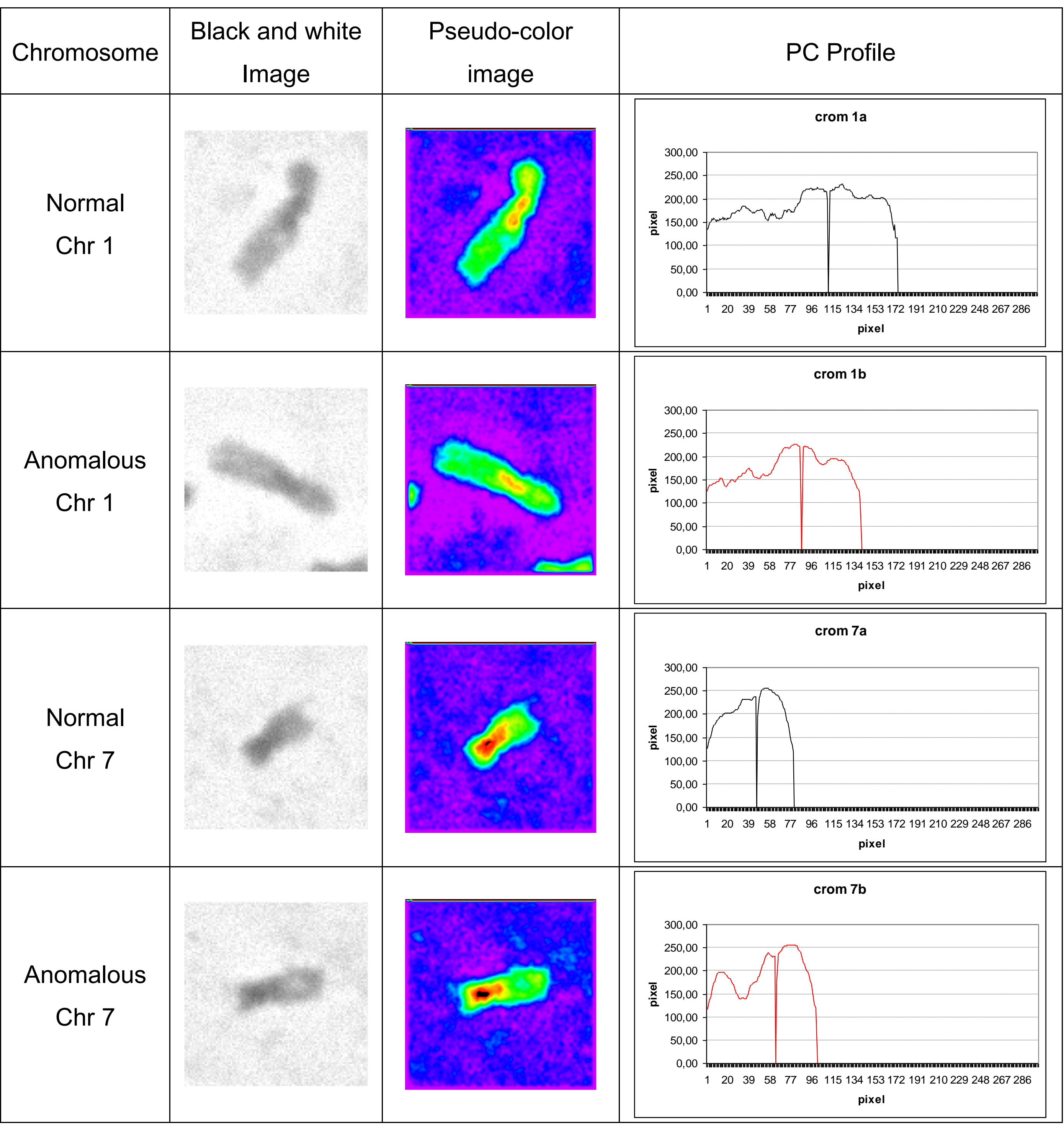

Figure 59. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 7 (a and b) from biotype 5. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

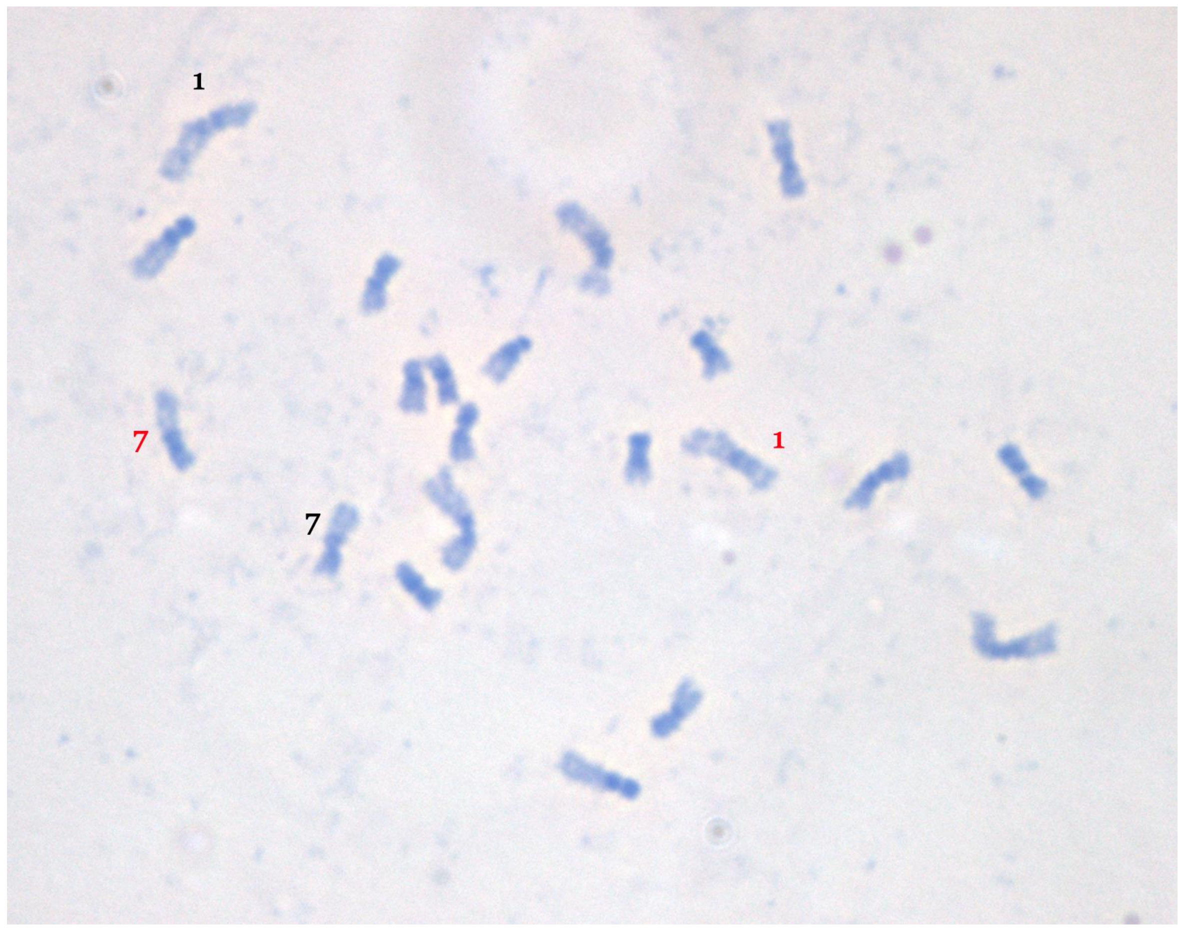

Figure 60. Prometaphase of red rice biotype 5. The chromosome couples involved in the translocation are 1 and 7. The red numbers label anomalous chromosomes.

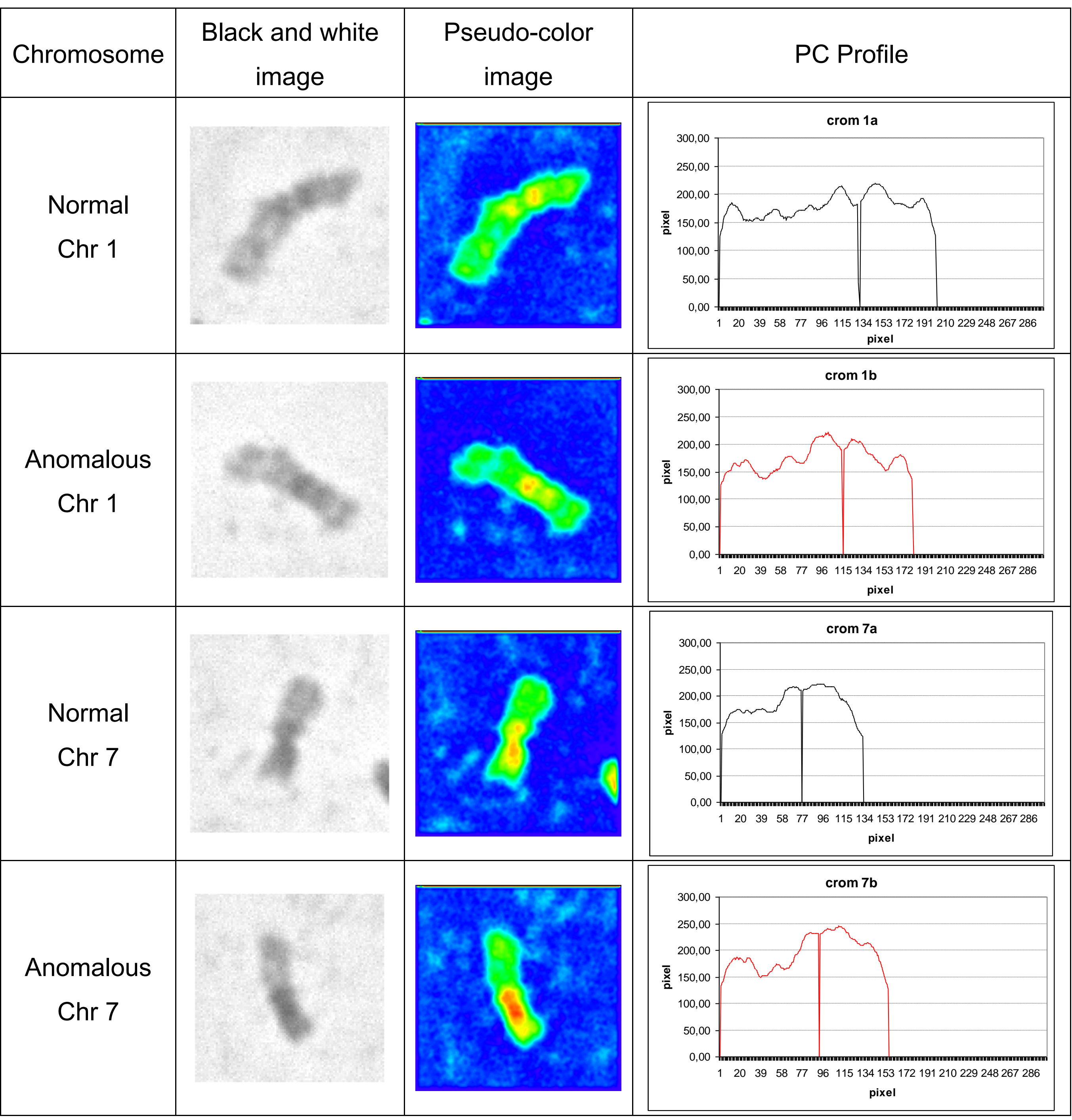

Figure 61. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 7 (a and b) from biotype 5. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

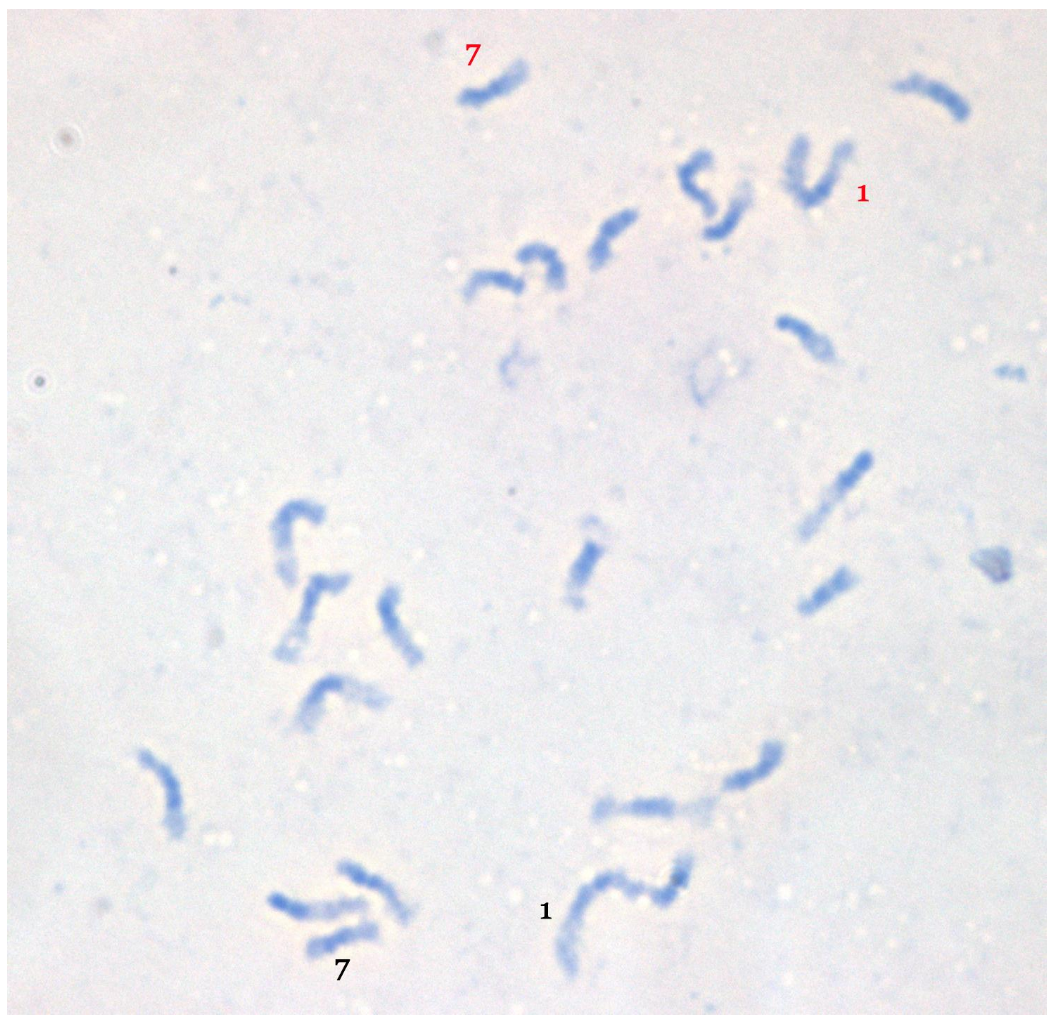

Figure 62. Prometaphase of red rice biotype 5. The chromosome couples involved in the translocation are 1 and 7. The red numbers label anomalous chromosomes.

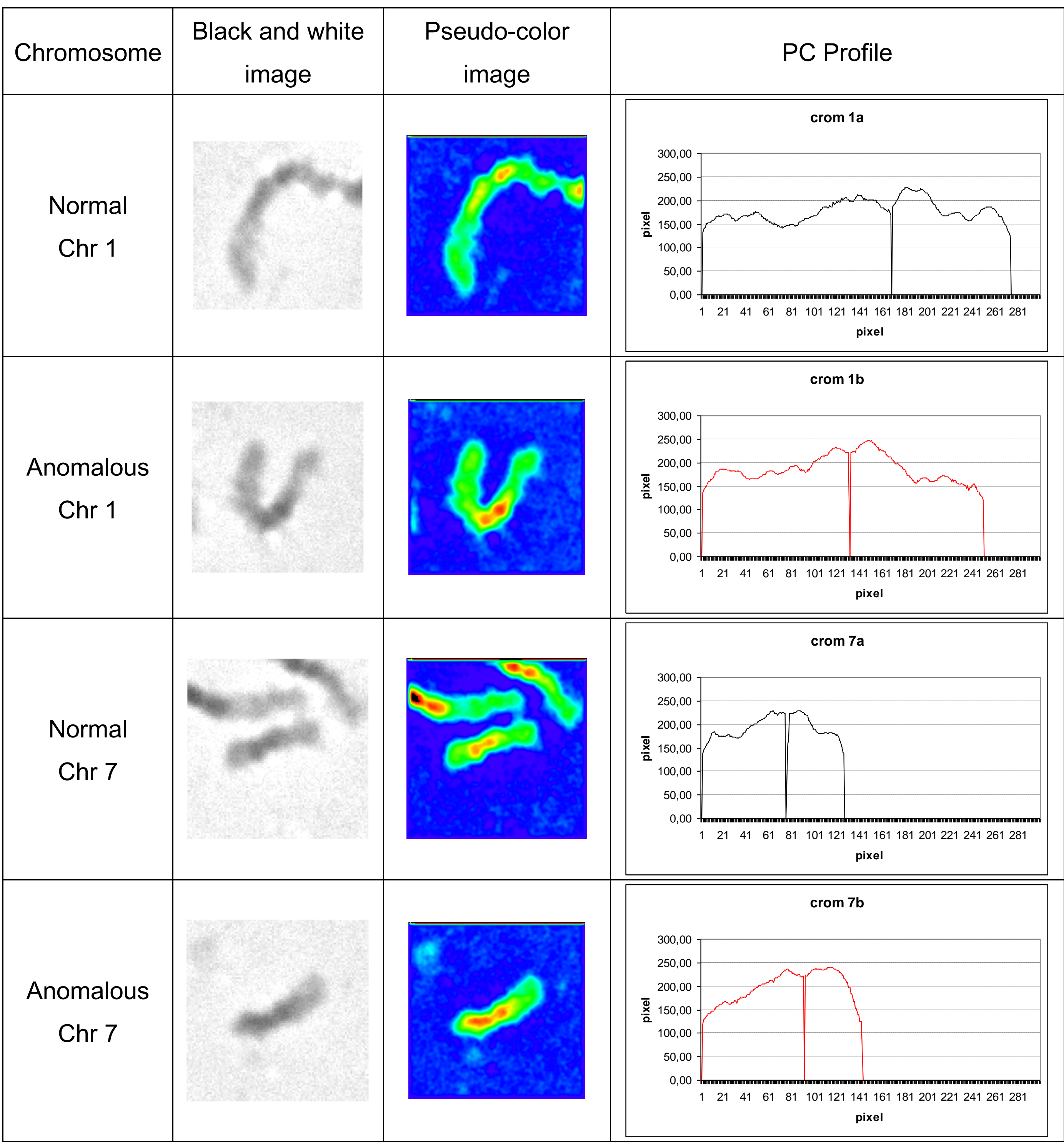

Figure 63. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 7 (a and b) from biotype 5. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

Biotype 6

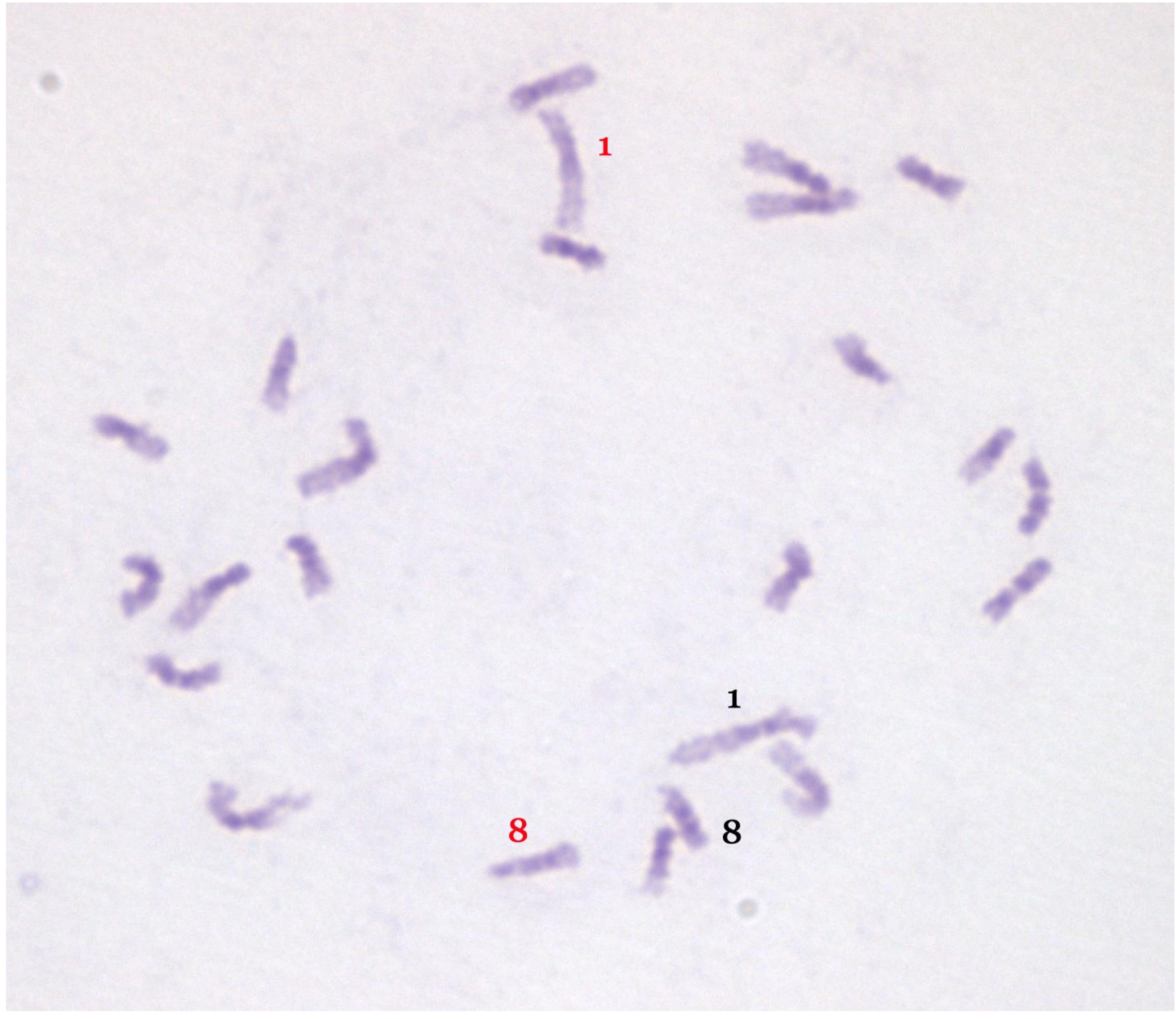

Figure 64. Prometaphase of red rice biotype 6. The chromosome couples involved in the translocation are 1 and 8. The red numbers label anomalous chromosomes.

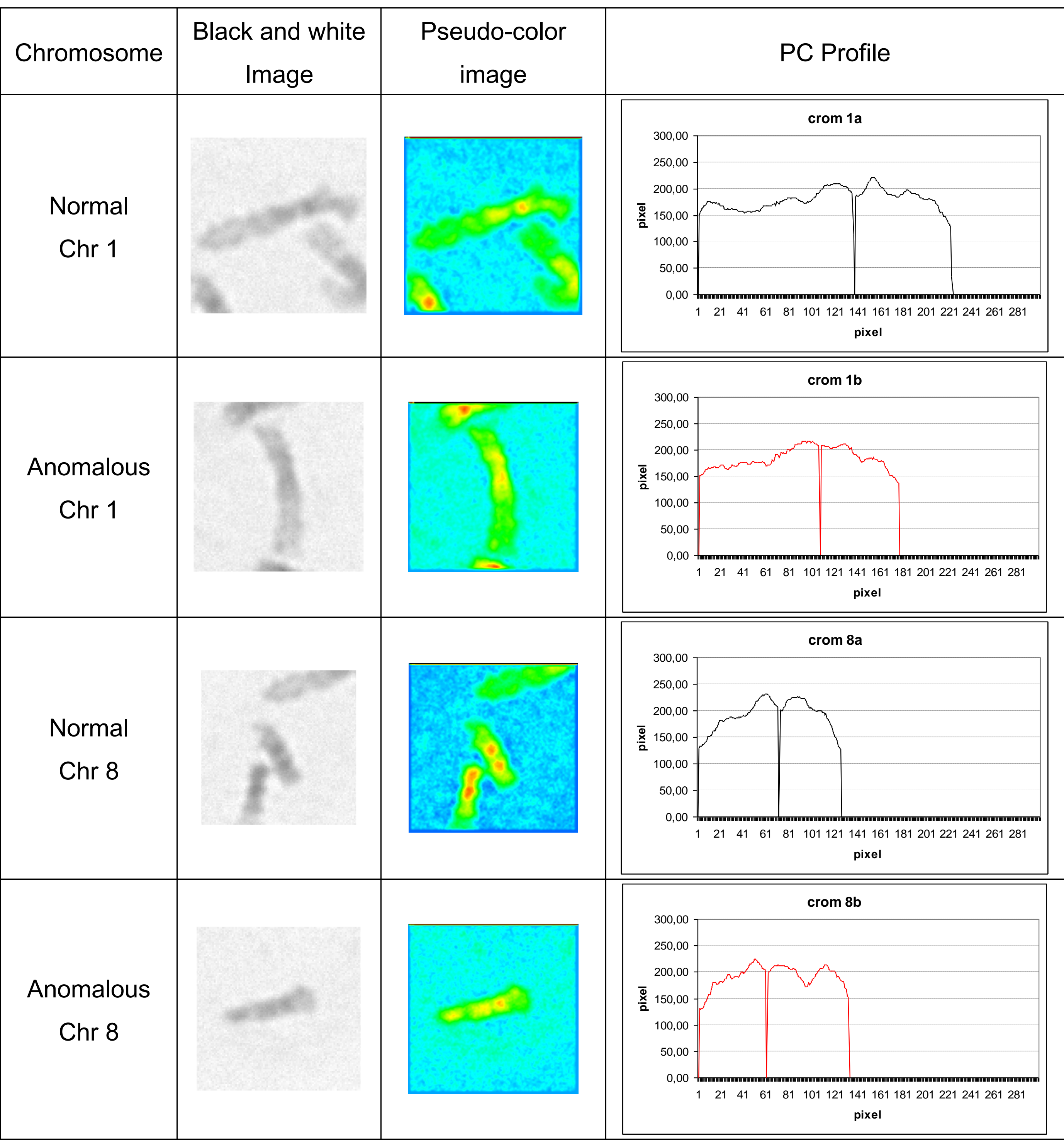

Figure 65. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 8 (a and b) from biotype 6. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a**; black) and for the anomalous chromosome (**b**; red) are shown, and the differences in long arm length and centromere position are clearly visible.

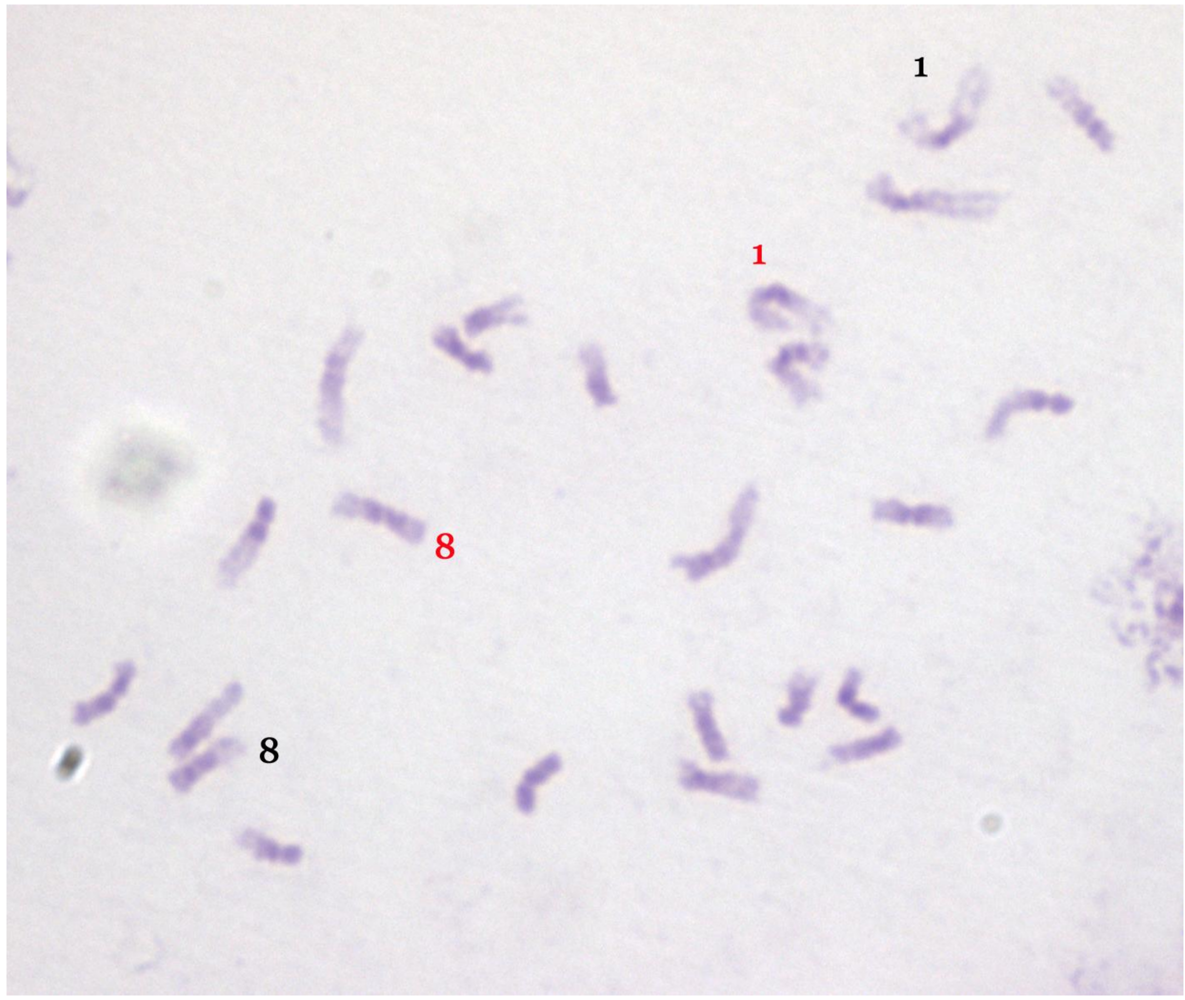

Figure 66. Prometaphase of red rice biotype 6. The chromosome couples involved in the translocation are 1 and 8. The red numbers label anomalous chromosomes.

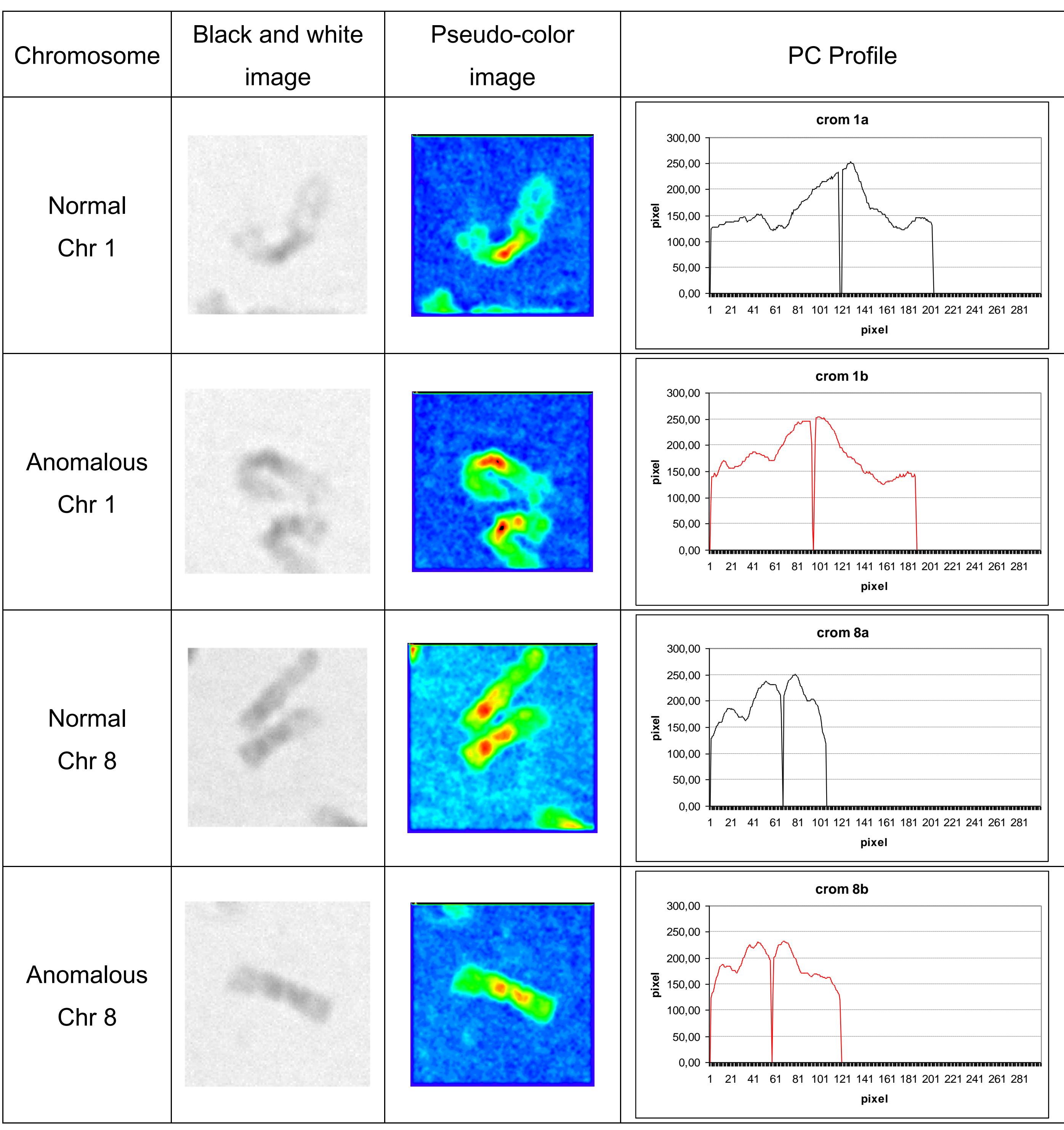

Figure 67. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 8 (a and b) from biotype 6. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

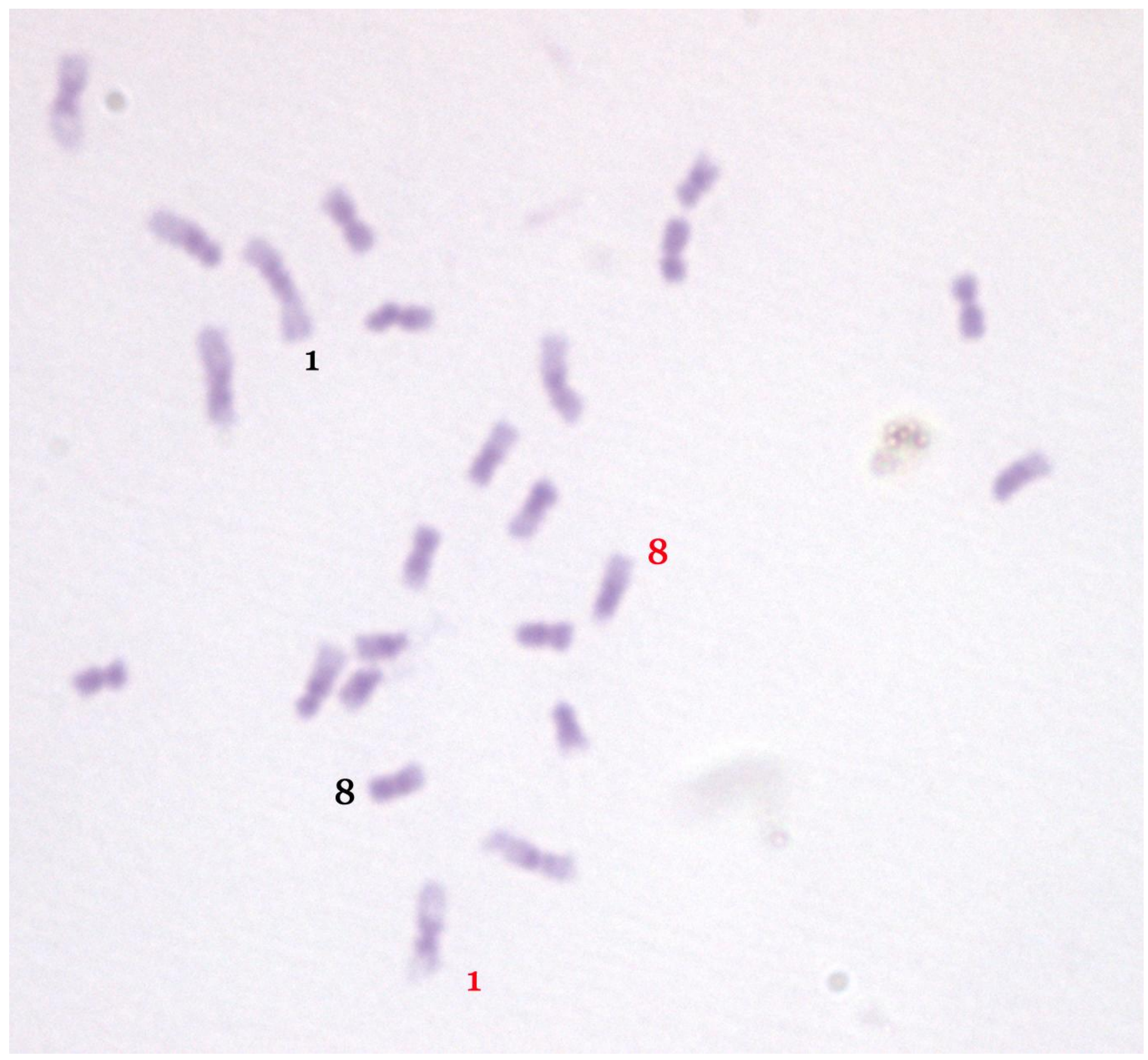

Figure 68. Prometaphase of red rice biotype 6. The chromosome couples involved in the translocation are 1 and 8. The red numbers label anomalous chromosomes.

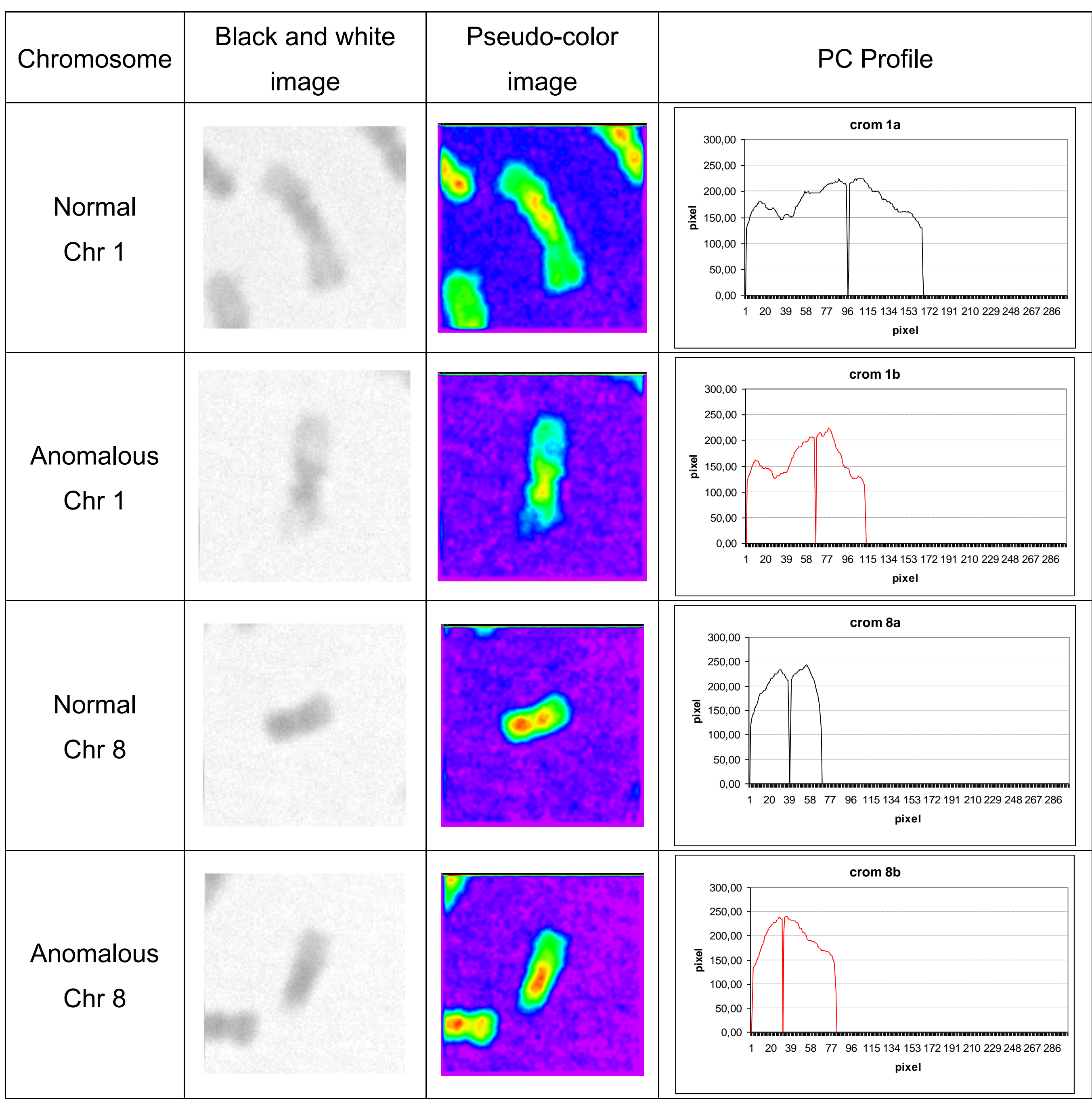

Figure 69. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 8 (a and b) from biotype 6. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a**; black) and for the anomalous chromosome (**b**; red) are shown, and the differences in long arm length and centromere position are clearly visible.

Biotype 7

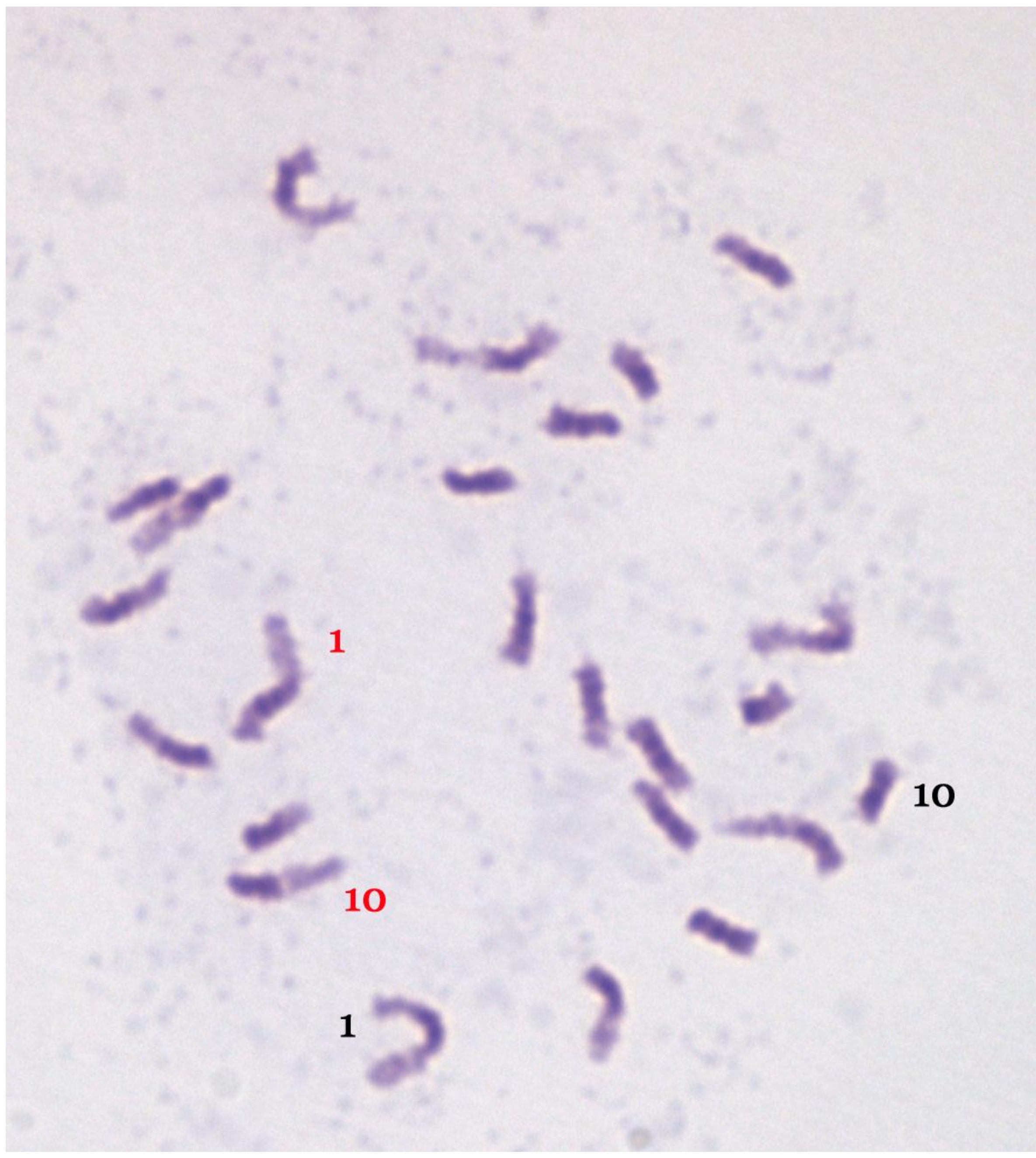

Figure 70. Prometaphase of red rice biotype 7. The chromosome couples involved in the translocation are 1 and 10. The red numbers label anomalous chromosomes.

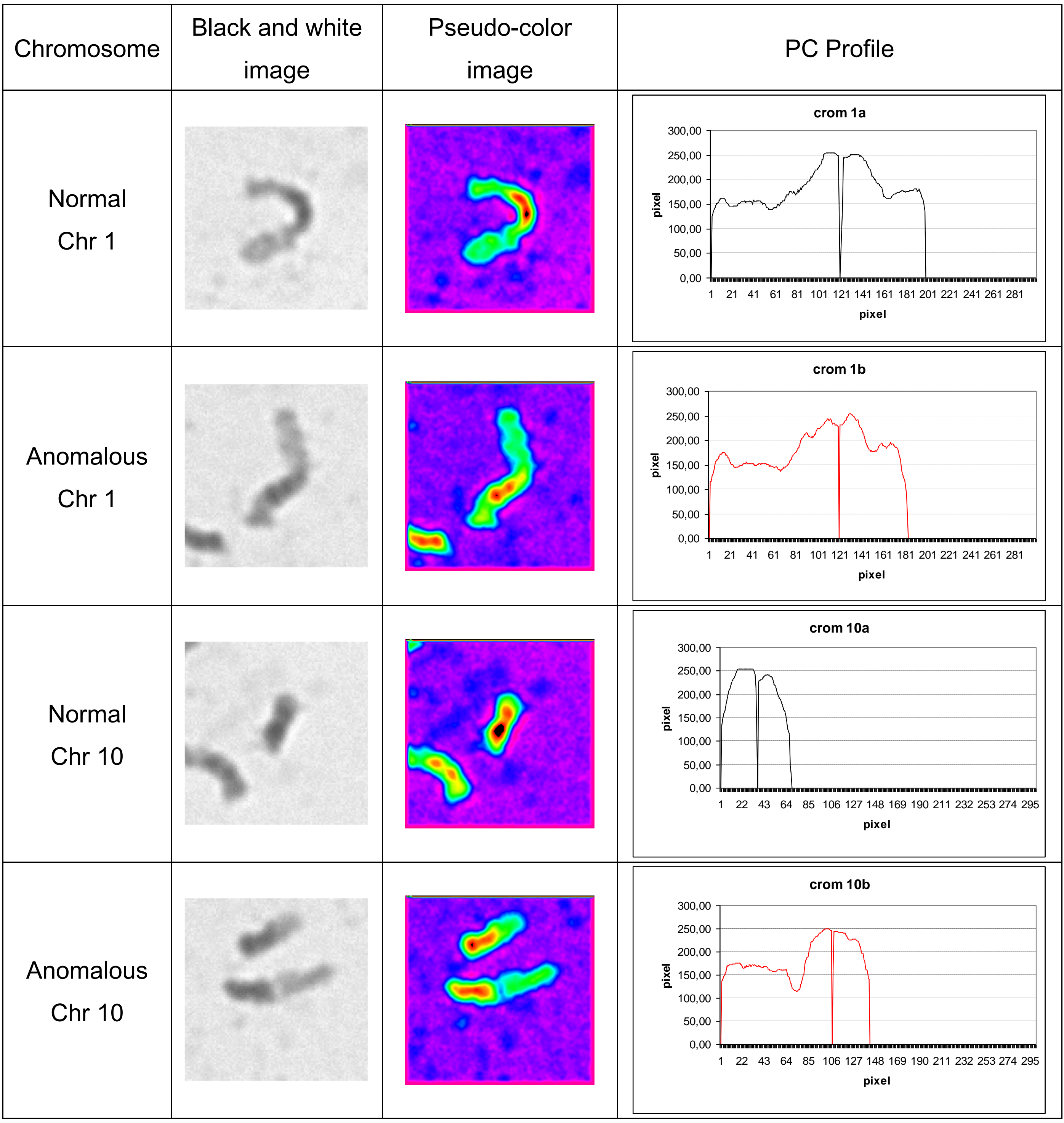

Figure 71. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 10 (a and b) from biotype 7. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

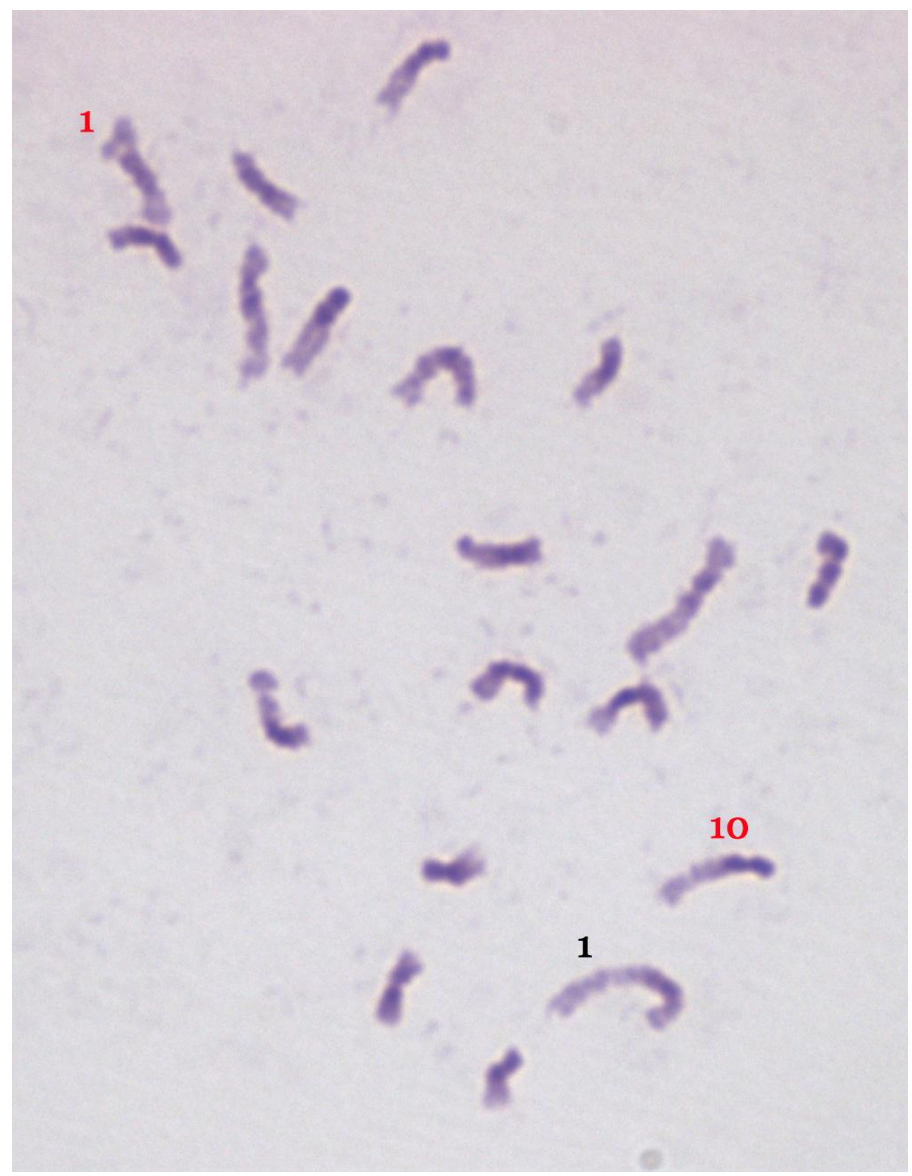

Figure 72. Prometaphase of red rice biotype 7. The chromosome couples involved in the translocation are 1 and 10. The red numbers label anomalous chromosomes.

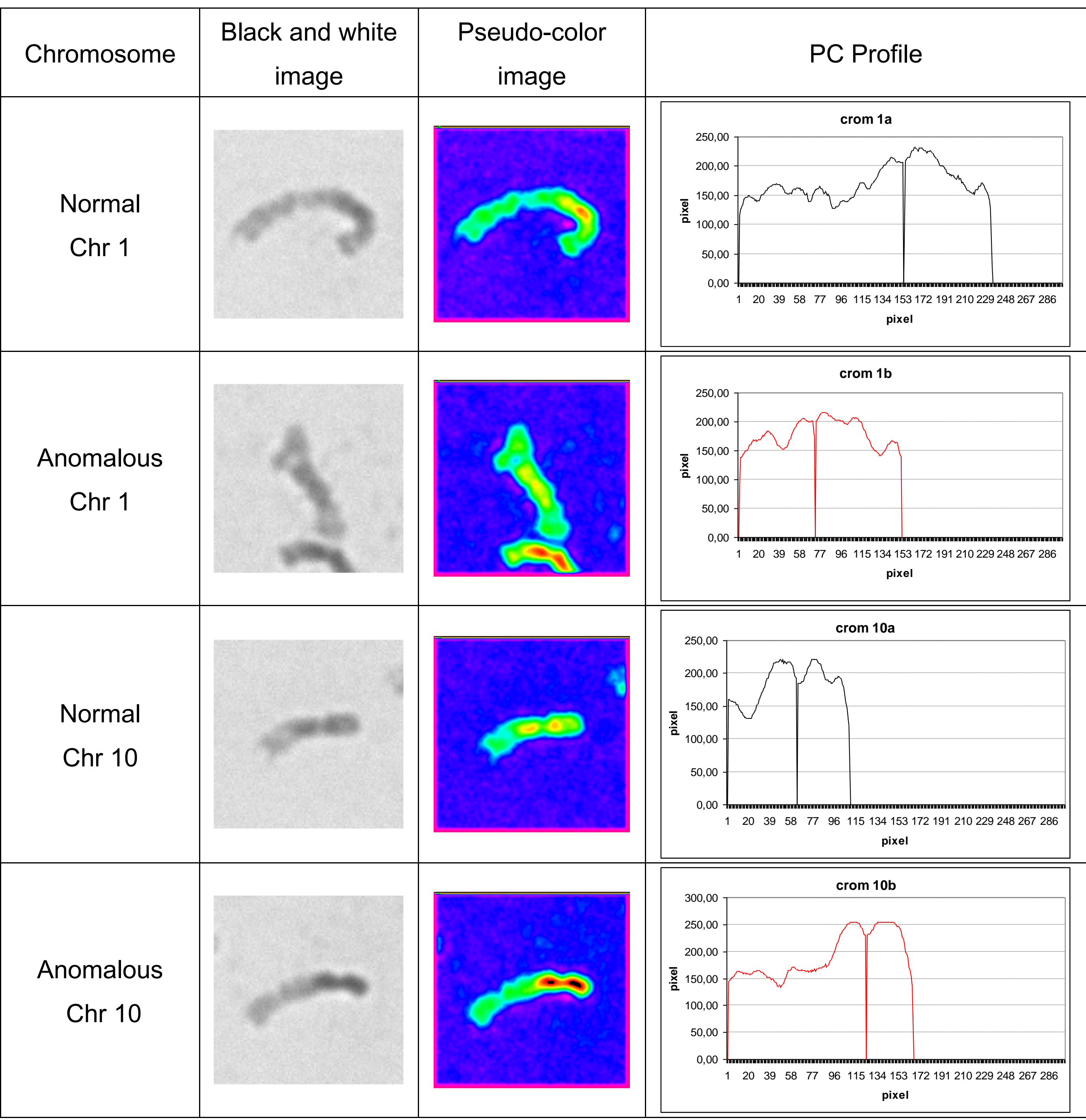

Figure 73. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 10 (a and b) from biotype 7. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

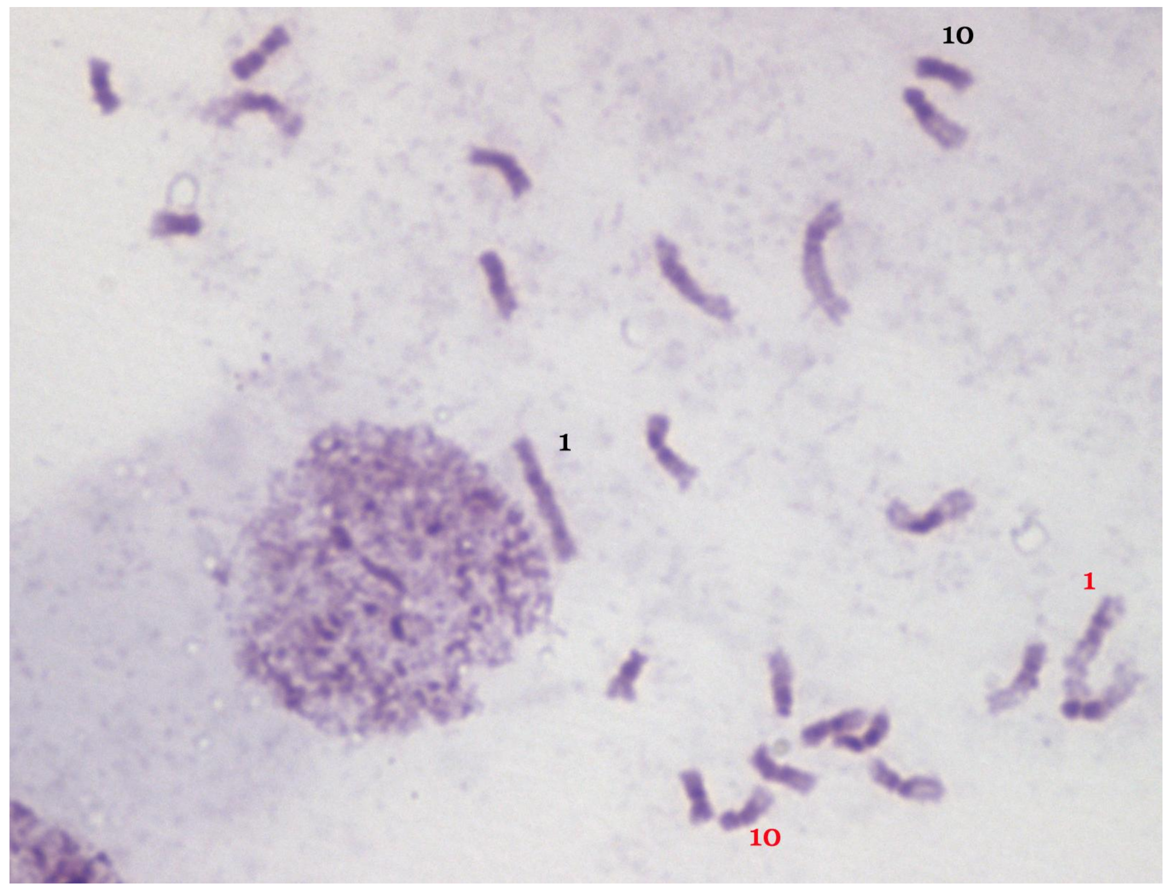

Figure 74. Prometaphase of red rice biotype 7. The chromosome couples involved in the translocation are 1 and 10. The red numbers label anomalous chromosomes.

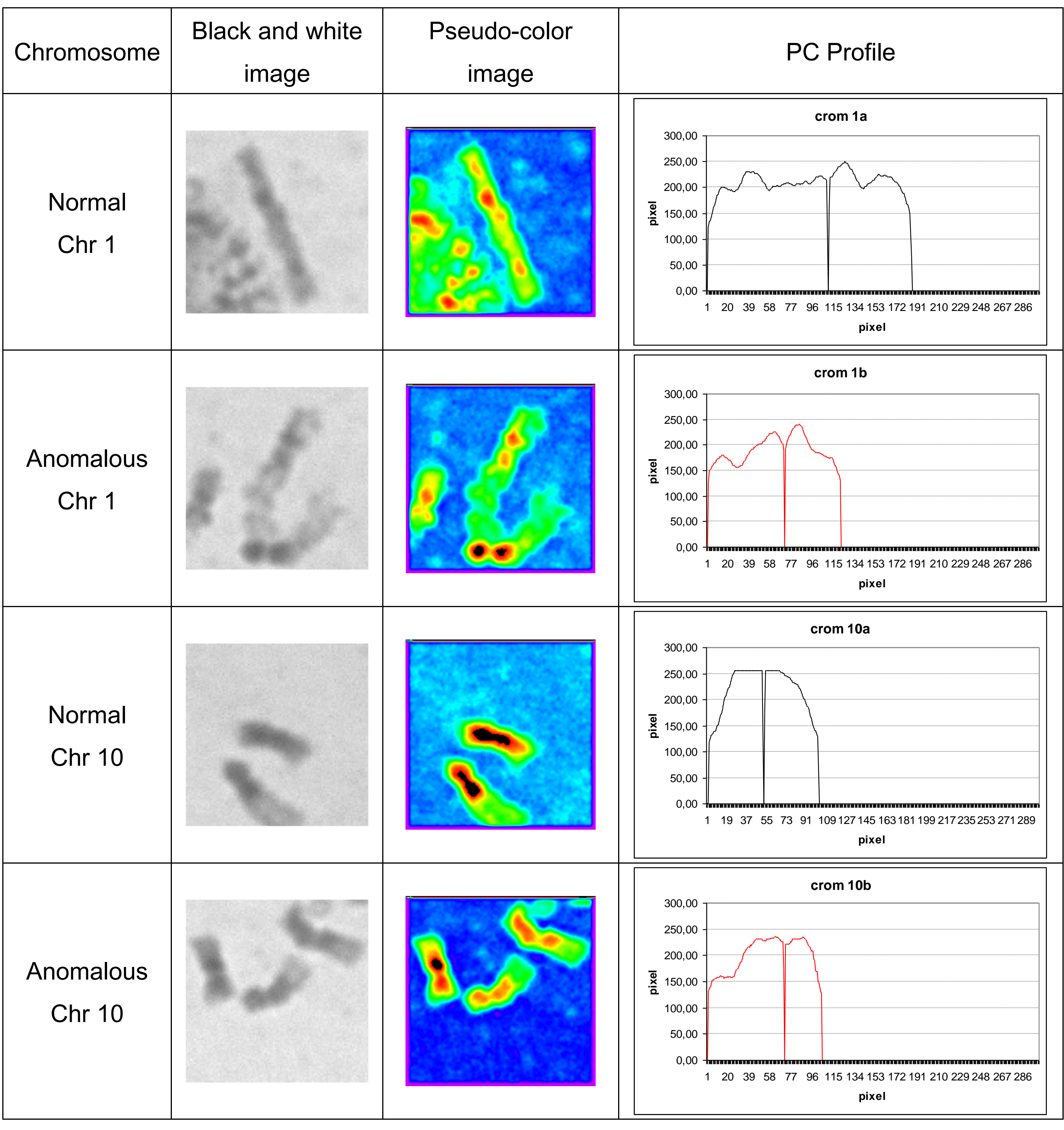

Figure 75. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 10 (a and b) from biotype 7. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

Biotype 8

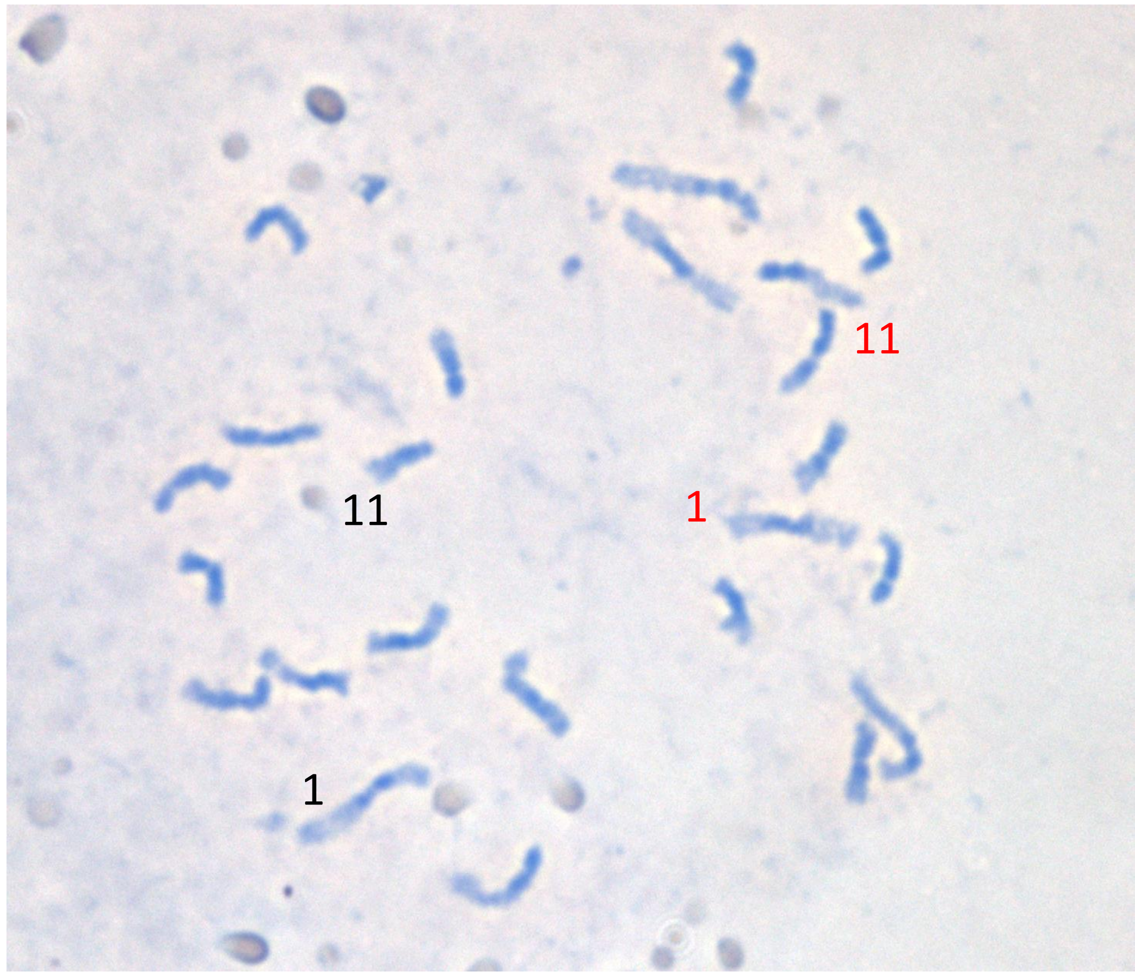

Figure 76. Prometaphase of red rice biotype 8. The chromosome couples involved in the translocation are 1 and 11. The red numbers label anomalous chromosomes.

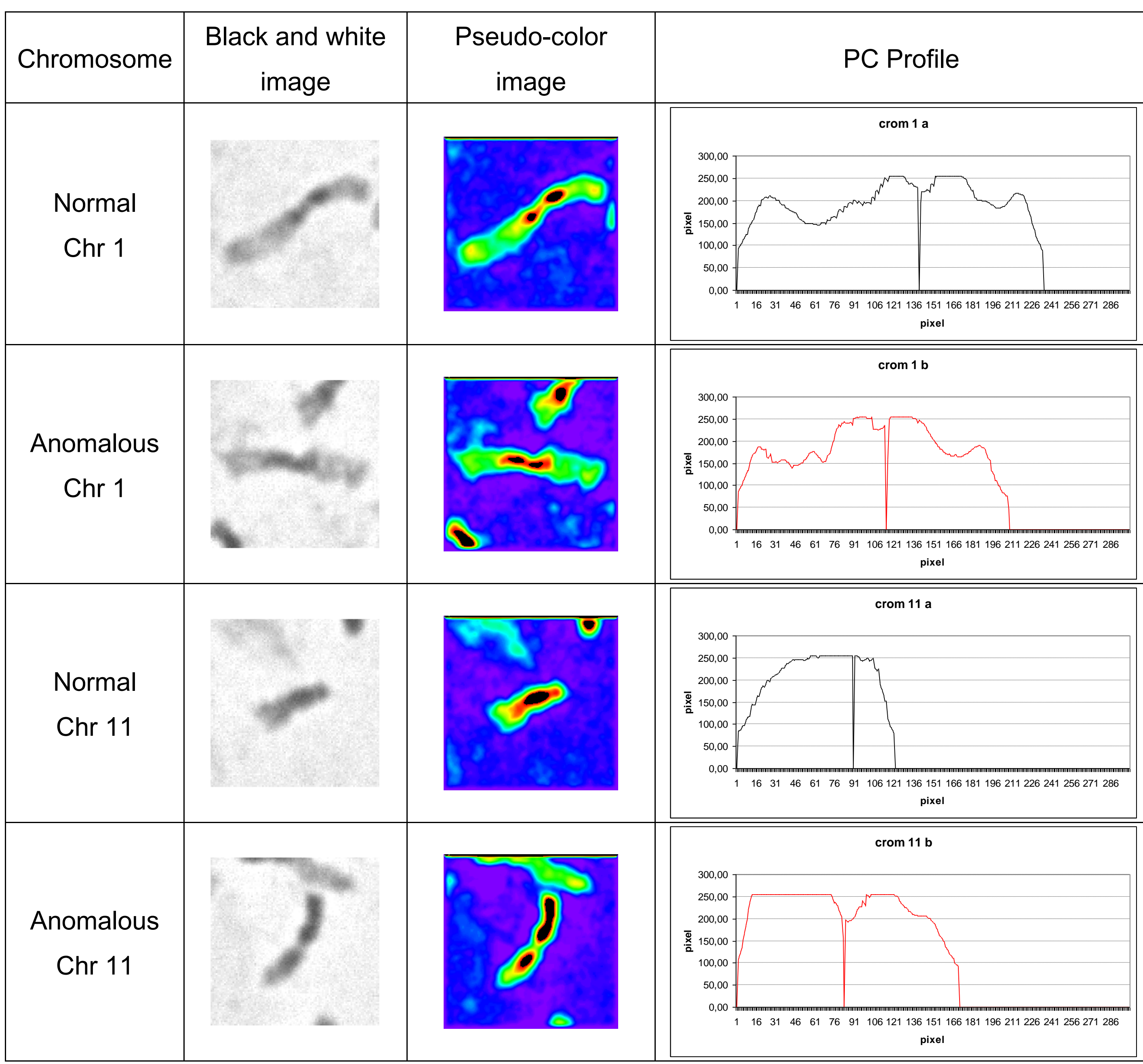

Figure 77. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 11 (a and b) from biotype 8. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

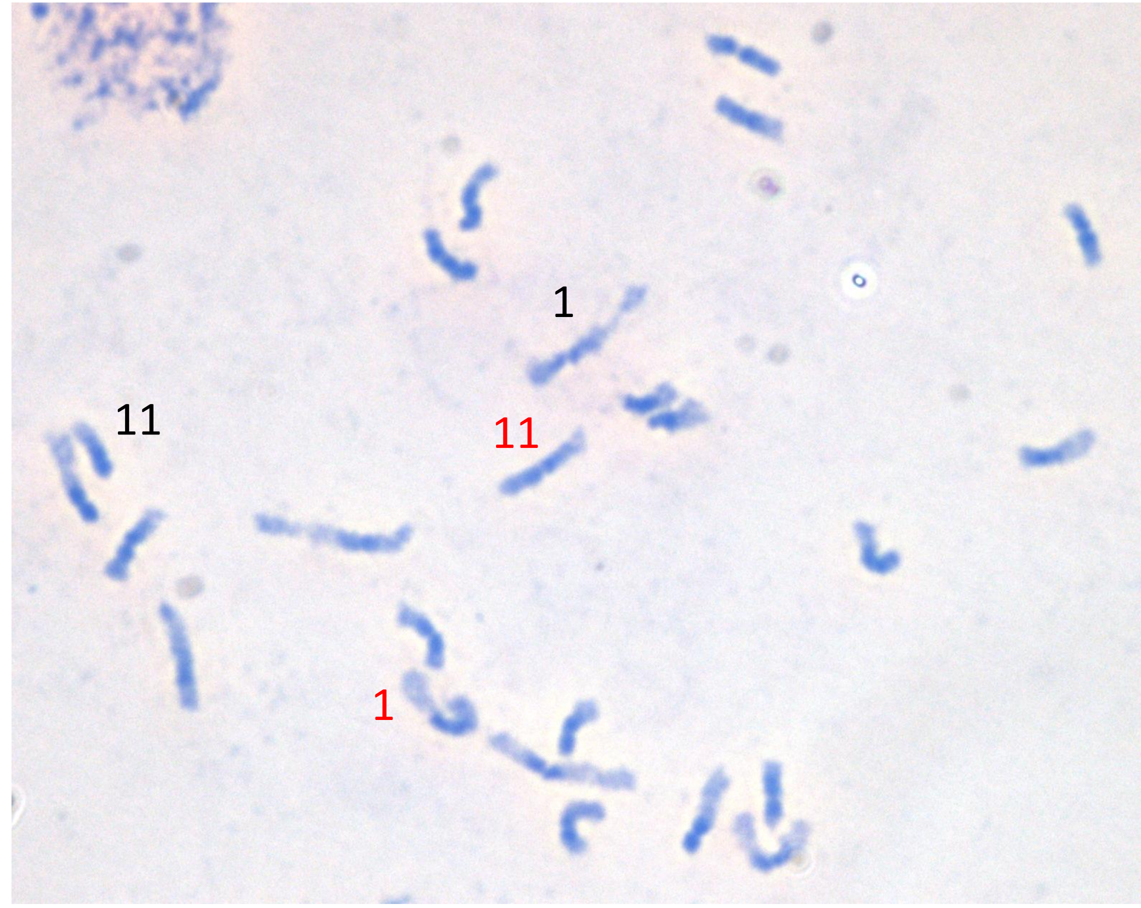

Figure 78. Prometaphase of red rice biotype 8. The chromosome couples involved in the translocation are 1 and 11. The red numbers label anomalous chromosomes.

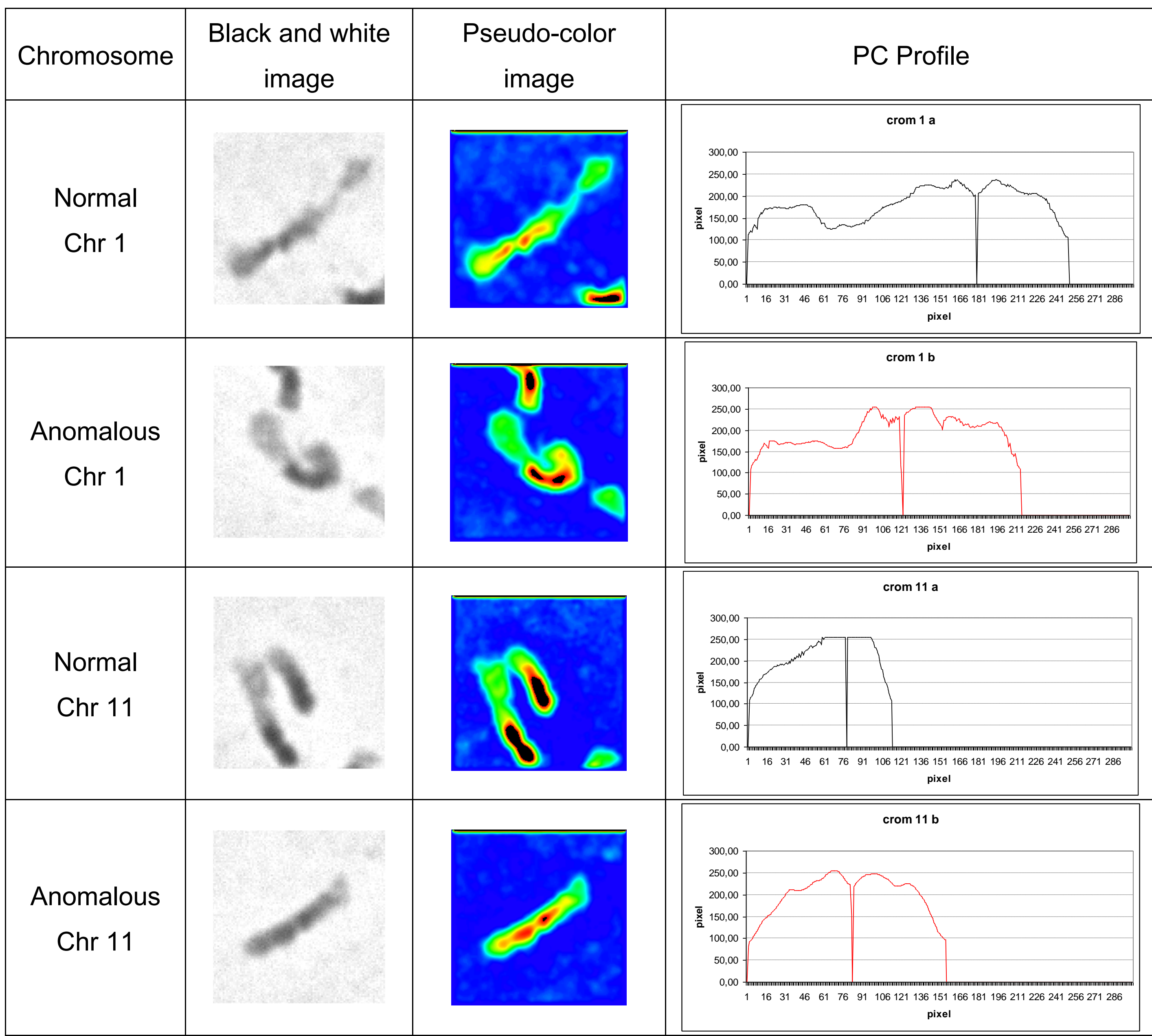

Figure 79. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 11 (a and b) from biotype 8. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

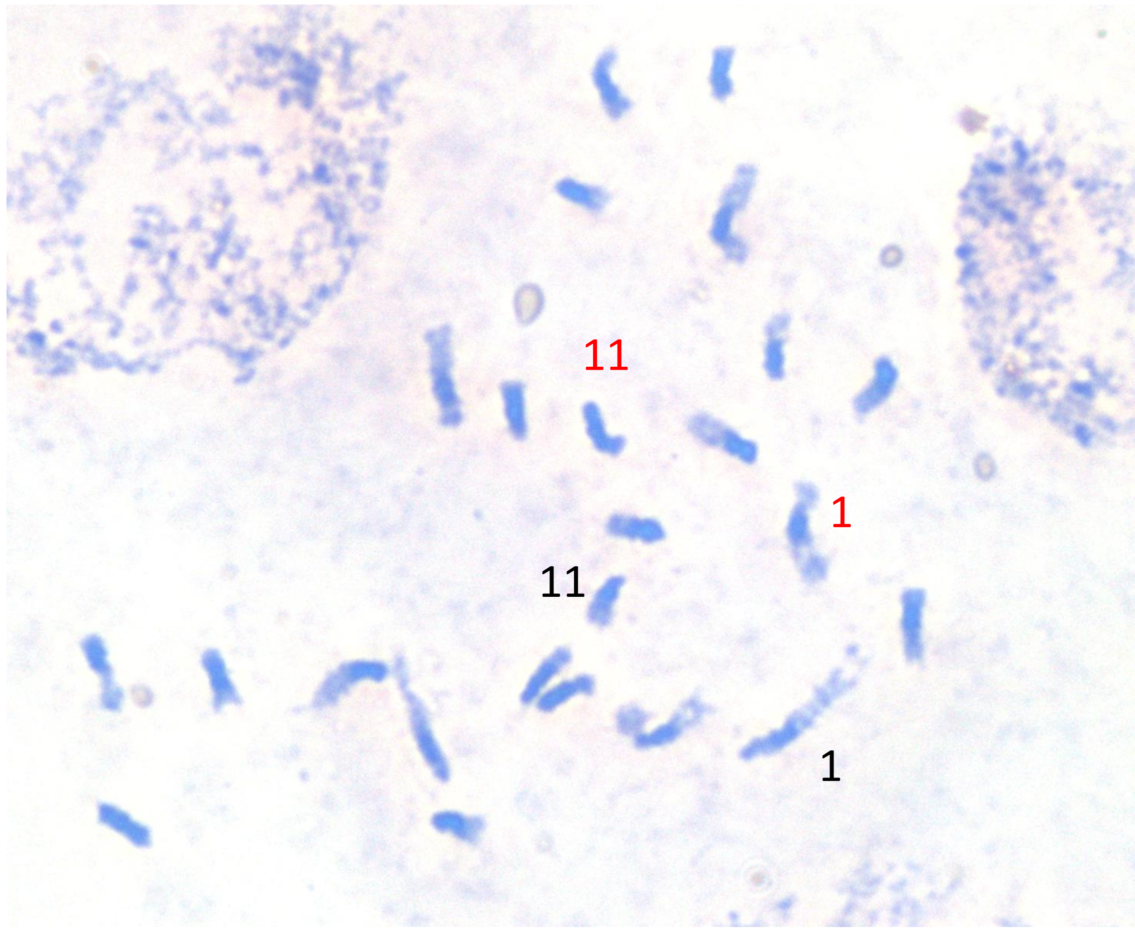

Figure 80. Prometaphase of red rice biotype 8. The chromosome couples involved in the translocation are 1 and 11. The red numbers label anomalous chromosomes.

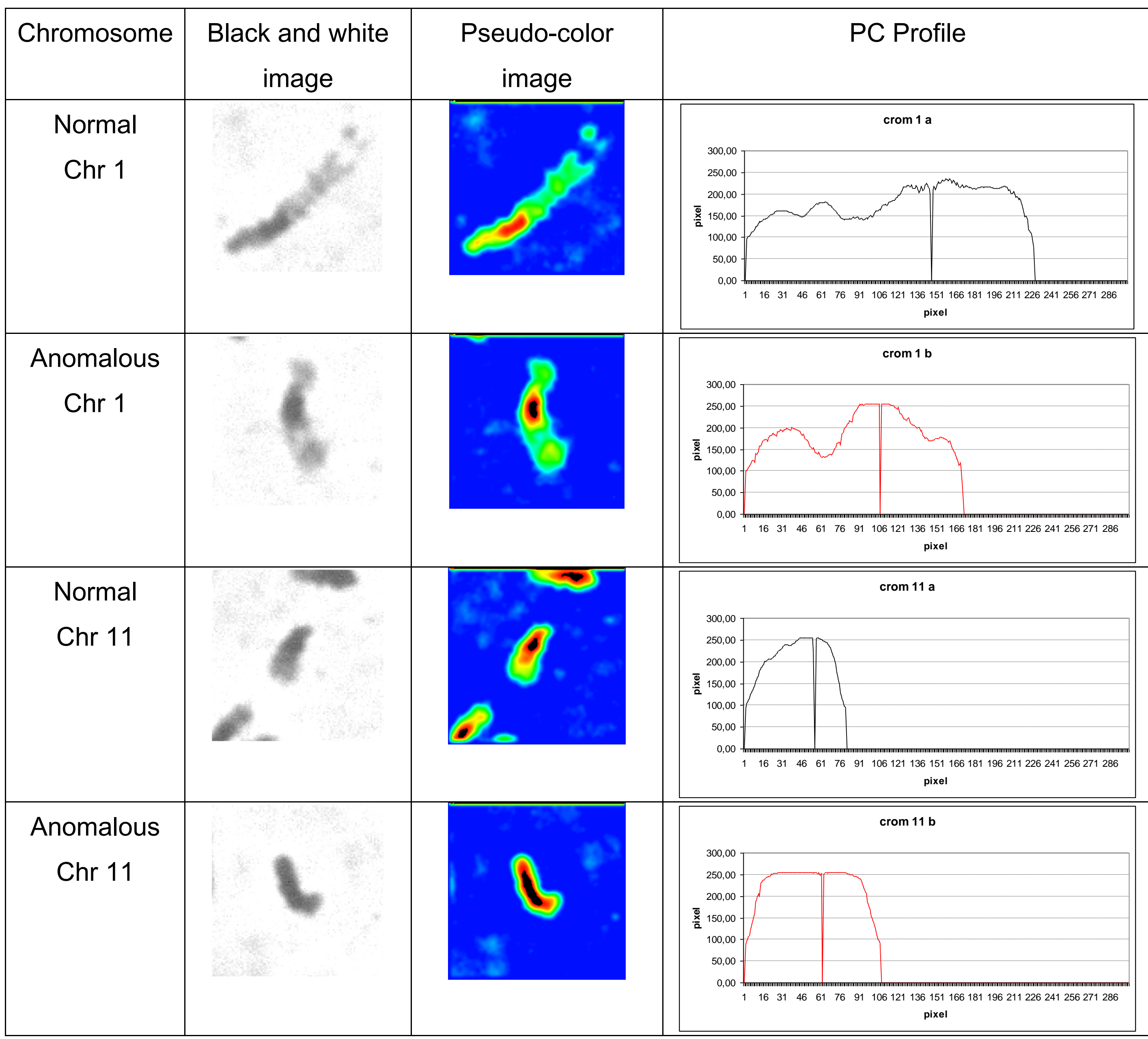

Figure 81. Computer-aided characterization of a pair of homologues of chromosome 1 (a and b) and chromosome 11 (a and b) from biotype 8. The grey images (left) show a normal (up) and an anomalous (down) Giemsa-stained chromosome. The pseudo-color images (centre) given by CHIA-EA are shown for the same chromosomes. Moreover, the CP profiles (right) for the normal chromosome (**a;** black) and for the anomalous chromosome (**b;** red) are shown, and the differences in long arm length and centromere position are clearly visible.

The CP profiles of the normal (black) and anomalous (red) chromosomes reveal an additional segment on the arm of the anomalous chromosome. The translocation involves (**Table 5**):

- for biotype 1, the long arm of one element of chromosome pair 1 and the short arm of one element of chromosome pair 5;
- for biotype 2, the long arm of one element of chromosome pair 1 and the long arm of one element of chromosome pair 2;
- for biotype 3, the long arm of one element of chromosome pair 1 and the long arm of one element of chromosome pair 12;
- for biotype 4, the long arm of one element of chromosome pair 1 and the short arm of one element of chromosome pair 3;
- for biotype 5, the long arm of one element of chromosome pair 1 and the long arm of one element of chromosome pair 7;
- for biotype 6, the long arm of one element of chromosome pair 1 and the short arm of one element of chromosome pair 8;
- for biotype 7, the long arm of one element of chromosome pair 1 and the long arm of one element of chromosome pair 10;
- for biotype 8, the long arm of one element of chromosome pair 1 and the short arm of one element of chromosome pair 11.

Biotypes	Phenotypic shattering degree (%)	Chromosome involved in translocation	Arm anomalous
1	50%	T1-5	Short
2	90%	T1-2	Long
3	70%	T1-12	Long
4	70%	T1-3	Short
5	70%	T1-7	Long
6	60%	T1-8	Short
7	80%	T1-10	Long
8	60%	T1-11	Short

Table 5. Shattering degree observed on the plants, chromosome number and anomalous arm involved in the translocations.

The following **Table 6** summarizes the arm ratios of normal and anomalous somatic chromosomes from weedy red rice.

Chromosome	Arm ratio	
	Normal	Anomalous
1	2.02 ± 0.99	1.40 ± 0.39
2	1.40 ± 0.37	1.55 ± 0.29
3	2.73 ± 0.66	1.72 ± 0.57
4	2.92 ± 0.59	--
5	1.05 ± 0.01	0.67 ± 0.08
6	1.98 ± 0.42	--
7	1.46 ± 0.25	1.62 ± 0.17
8	1.28 ± 0.22	0.77 ± 0.15
9	1.36 ± 0.19	--
10	1.29 ± 0.18	2.96 ± 0.01
11	1.73 ± 0.82	0.75 ± 0.44
12	1.72 ± 0.53	2.46 ± 0.34

Table 6. The average arm ratios (with their standard deviations) are shown only for the chromosomes that are involved in the translocations. 10 normal and 10 anomalous chromosomes were measured for each biotype.

All the observed translocations are summarized in **Figure 82**.

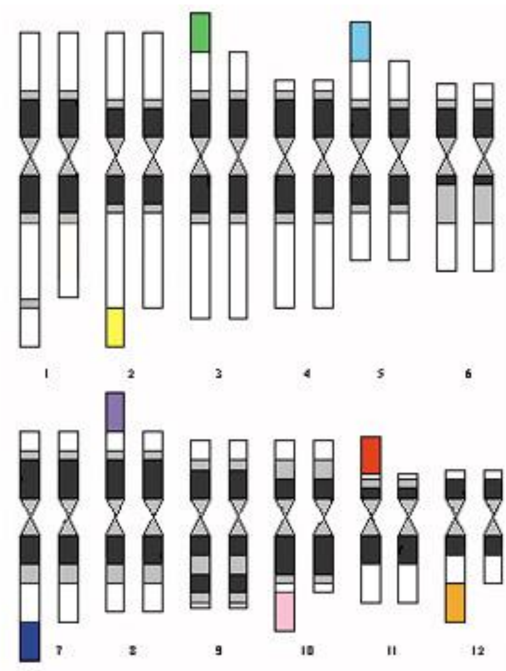

Figure 82. Quantitative ideogram of the somatic chromosomes of weedy red rice, showing the eight different types of translocation between chromosome 1 and other chromosomes in the genome: biotype 1 (light-blue); biotype 2 (yellow); biotype 3 (orange); biotype 4 (green); biotype 5 (blue); biotype 6 (violet); biotype 7 (pink); biotype 8 (red).

EVALUATION OF THE BREAK POINT POSITION ON CHROMOSOME 1

The length of the **normal** Chr1 is of 43261 Kb, and since we found (see **Fig. 83 a**) that it is measured by 253 px, we deduce that 1 px corresponds to 171 Kb.

Moreover, since we found for the **anomalous** Chr1 a length measured by 218 px (see **Fig. 83 b**), it obviously turns out to correspond to about 37278 Kb.

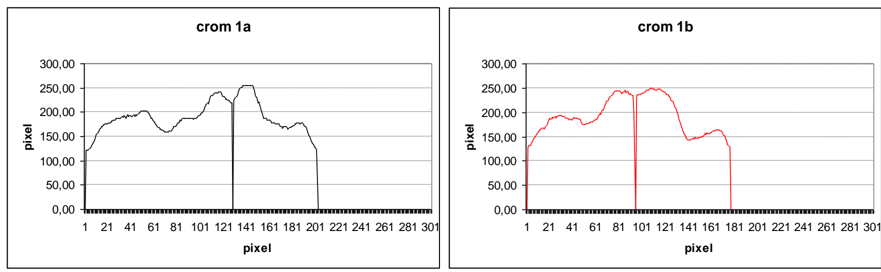

Figure 83. a) CP of Normal Chr1; b) CP of anomalous Chr1

It is well known, on the other hand, that the shattering locus qSh1 is approximately located at 36.7 Mb from the telomere region of the short arm of chromosome 1: the qSH1 locus appears to be very close to the estimated break point on chromosome 1.
It was important, therefore, to know if the genomic region containing the shattering locus on chromosome 1 is altered or not by the translocation. In order to make this point clear we applied the FISH technique, which required a probe BAC, covering the region containing the shattering gene (**Fig. 84**).

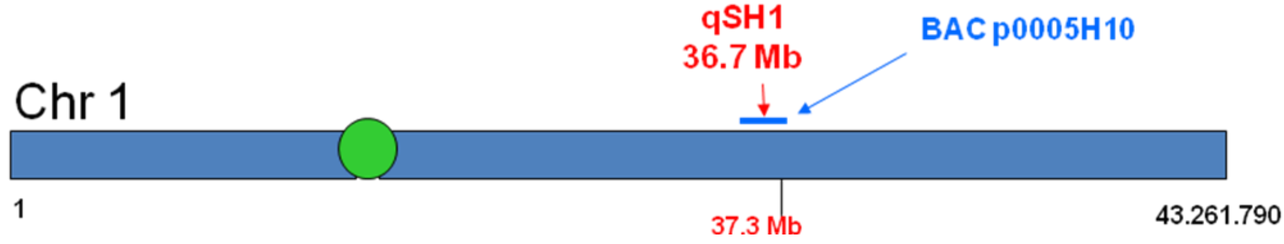

Figure 84. BAC, qSH1 and break point positions along Chr1.

We encountered various problems:
1) because of the small and condensed size of the rice chromosomes, and of the difficulty to get mitoses, a new and more complex methodology was assessed to improve both the quality and the quantity of mitotic prometaphases (i.e. 40 prometaphases per slide at least);
2) because of the large number of the FISH signals, the DNA competitor (C_0t) was employed in order to suppress stray signals. The main problems were due to the use of a new methodology; moreover, about 2 mg of DNA were required to produce rice C_0t, and rice turned out to allow a quite low yield (0.6 ug/ul for 600 g of leaves);
3) a FISH methodology originally developed for other species was transferred to rice.
The FISH analysis of the red rice biotypes 3 (**Fig. 85**) and 5 (**Fig. 86**) provided many pictures, the most representative of which are the following.

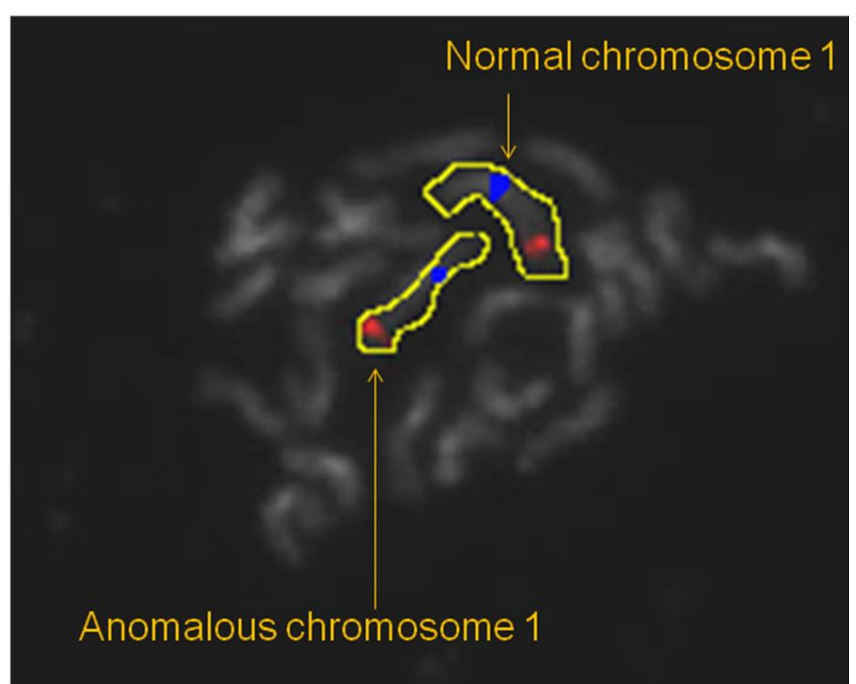

Figure 85. Biotype 3. The yellow line identifies the couple of Chromosomes 1; the red points represent the BAC probe; the blue points show the centromere position of Chr1.

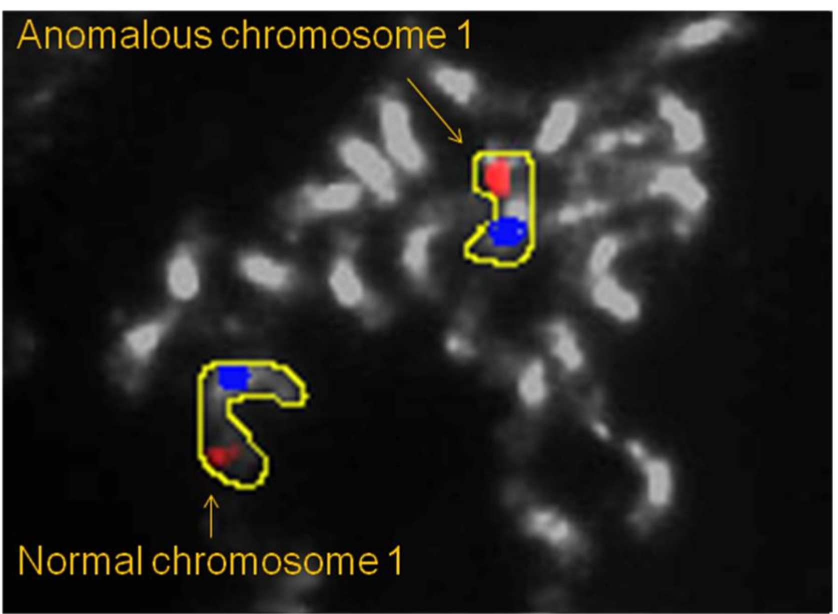

Figure 86. Biotype 5. The yellow line identifies the couple of Chromosome 1; the red points represent the BAC probe; the blue points show the centromere position of Chr1.

These FISH analyses clearly show that the shattering gene does neither break nor translocate, but remains located on chromosome 1.

GENOMICS

Genetic studies revealed the existence of several QTLs presenting shattering character. These QTLs are localized on rice chromosomes 1, 3, 4, 7, 8, 11.
The responsible genes have been identified for three of them: qSH1 (Konishi et al., 2006), qSH4 (Changbao et al., 2006), qSH7 (Hyeonso et al., 2010)

CHROMOSOME 1 – qSH1

The red rice peculiarity is the shattering before the full physiological maturity. This character is controlled by a gene.
The non-shattering character is under the major control of a locus located on the chromosome 1, and the responsible gene is now well known (qSH1).
In recent years it was realized that one of the shattering gene activation on chromosome 1 is due to a single-nucleotide polymorphism (SNP) which can explain about 70% of the non-shattering character associated to this gene whereby this SNP probably affects a regulatory region.
Konishi et al. (2006) proved the existence of a QTL region by means of the analysis both of the shattering-type *indica* cultivar ('Kasalath') and of a non-shattering-type *japonica* cultivar ('Nipponbare').
It may be observed that the CDS of the eight biotypes of *Oryza sativa* sub. *japonica* var. *silvatica* exactly coincides with the genome of the non-shattering rice cv 'Nipponbare' (ssp. *japonica*) except for the qSH1-SNP, coinciding with the shattering-type *indica* cv 'Kasalath'. Konishi et al. in their paper on Science (2006) observed a shattering process in cv 'Nipponbare' occurring when the genomic fragment of cv 'Kasalath' containing qSH1-SNP was inserted into its chromosome 1. As far as AT repeat 2 ('(AT)2') is concerned, the red rice biotypes differ both from 'Nipponbare' and from 'Kasalath', because they have an (AT)5.
Morover, in the biotypes 1 and 4 I found a new SNP (SNP8) which turns out to be different both from *japonica* and from *indica* subspecies.
In my experiment two other cultivar of rice were also employed as control: the non-shattering cv 'Perla' and the shattering cv 'Sprint'. 'Perla' gave the same results as

'Nipponbare', while the characteristics of 'Sprint' coincide with those of red rice, except for the absence of SNP8.

These experimental results are summarized in **Table 7**.

Primer1	Primer2	PCR product size	Polymorphism	Start position	Non shattering	Shattering	Shattering								Non shattering	Shattering
					japonica variety 'Nipponbare'	*indica* variety 'Kasalath'	B1	B2	B3	B4	B5	B6	B7	B8	'Perla'	'Sprint'
con24-13L	con24-13U	876bp	SNP1	417	G	A	G	G	G	G	G	G	G	G	G	G
			SNP2	437	T	C	T	T	T	T	T	T	T	T	T	T
			AT repeat1	448	(AT)2	(AT)3	(AT)2	(AT)2	(AT)2	(AT)2	(AT)2	(AT)2	(AT)2	(AT)2	(AT)2	(AT)2
			T repeat1	454	T6	T7	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6
			AT repeat2	549	(AT)4	(AT)3	(AT)5	(AT)5	(AT)5	(AT)5	(AT)5	(AT)5	(AT)5	(AT)5	(AT)4	(AT)5
RBEL-E1-U	RBEL-E1-L2	520bp	SNP3	213	C	T	C	C	C	C	C	C	C	C		
			SNP4	370	A	G	A	A	A	A	A	A	A	A		
106.5k-U	107.2k-L	718bp	qSH1/SNP	380	T	G	G	G	G	G	G	G	G	G	T	G
108.9k-U	109.8k-L	862bp	SNP5	462	A	T	A	A						A	A	A
			SNP6	581	A	G	A	A	A	A	A	A	A	A	A	A
			SNP8	636	A	A	G	A	A	G	A	A	A	A	A	A
			SNP7	645	A	G	A	A	A	A	A	A	A	A	A	A
			A repeat	703	A3	A2	A3	A3	A3	A3	A3	A3	A3	A3	A3	A3
			T repeat2	706	T8	T9	T8	T8	T8	T8	T8	T8	T8	T8	T8	T8

Table 7. From the left: primers employed; mutations looked for; observed results for: 'Nipponbare' (non-shattering), 'Kasalath' (shattering), eight biotypes (shattering), 'Perla' (non-shattering), 'Sprint' (shattering).

Konishi et al. (2006) also represented in a table the non-shattering degree for different rice typologies in association with the relevant SNP, AT repeat, A repeat and T repeat.

The eight analyzed biotypes are shown (red points) in the following **Figure 87**, according to the SNP, AT repeats, A repeat and T repeats discovered. The biotypes show a low non-shattering degree. Moreover, cv 'Perla' (blue points) presents a **high** non-shattering degree, whereas cv 'Sprint' (black points) shows a **low** non-shattering degree and is placed, as expected, near the red rice.

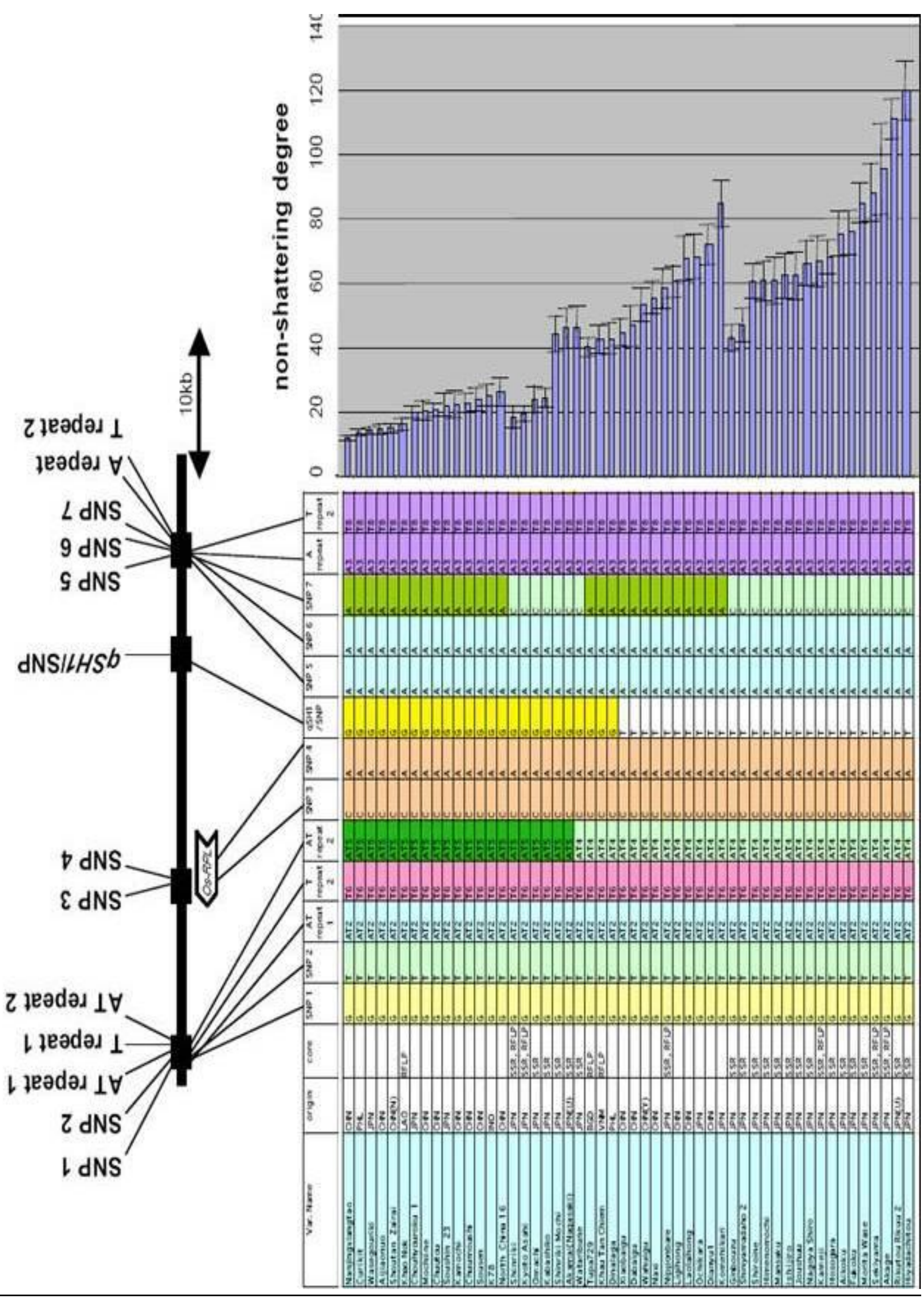

Figure 87. Classification of some cultivars of rice according to the non-shattering degree, adapted from Konishi et al., 2006.

CHROMOSOME 7 – qSH7

Table 8 summarizes our results and shows that the QTLs of qSh7 is not involved in the shattering character of the eight biotypes analyzed in our experiment.

Biotypes	A→T	G→T
1	A	G
2	A	G
3	A	G
4	A	G
5	A	G
6	A	G
7	A	G
8	A	G
cv ‘Perla’	A	G

Table 8. The QTLs of qSH7 in each biotype and in the cv ‘Perla’.

CHROMOSOME 4 – qSH4

We didn't observe any mutation in red rice biotypes and the cv 'Perla', without observing any mutation, since they all shared the same sequence of the non-shattering cultivar (**Fig. 88**).

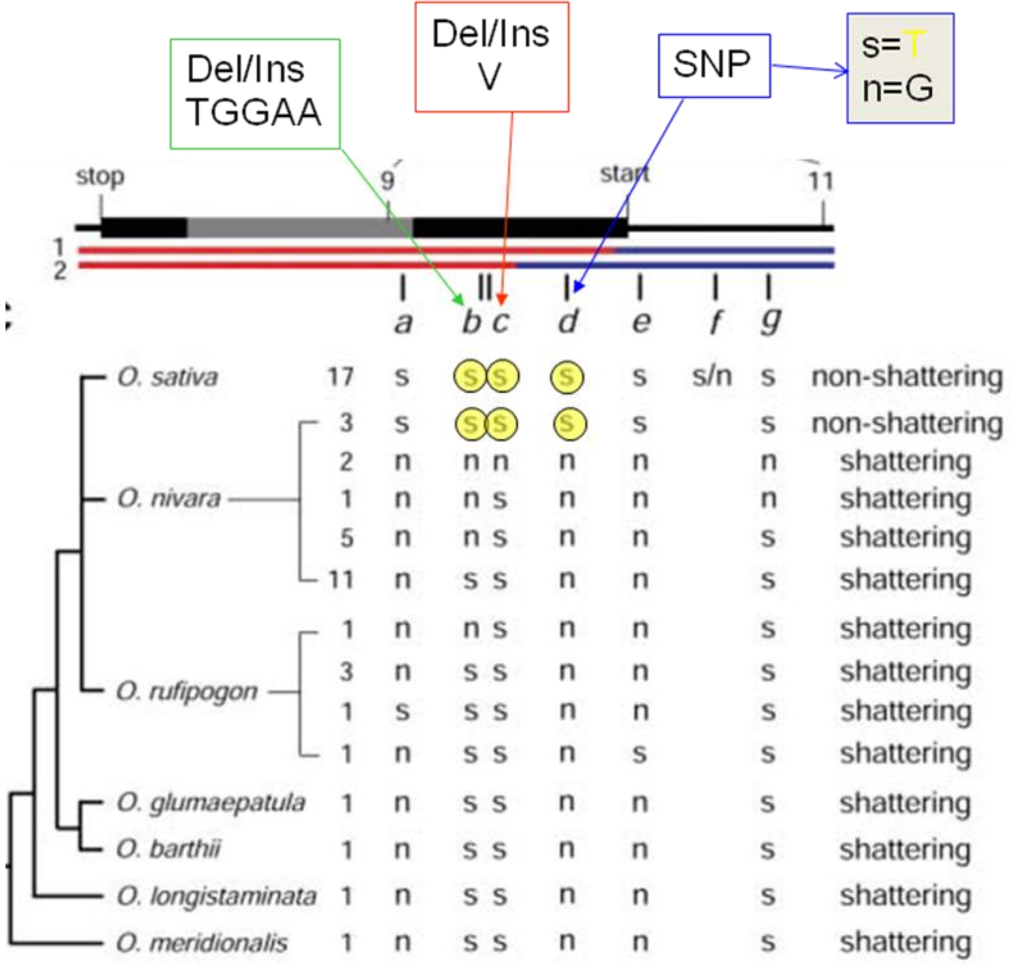

Figure 88. Seven mutations found among the mapping parents. These mutations were scored in different rice cultivars and wild species. Sequences of *Oryza sativa* and *Oryza nivara* parents were labeled by 's' and 'n', respectively. The yellow circles mark the eight biotypes and 'Perla' sequences adapted from Changbao et al., 2006

DISCUSSION AND CONCLUSIONS

One of the main factors affecting the yield of rice is the presence of weeds, and red rice is a particularly dangerous weed in Italian rice crops.

Red rice (*Oryza sativa* subspecies *japonica* var. *silvatica*, 2n = 24) is a spontaneous form of cultivated rice (2n = 24) presenting a wide spread of morphologic and biologic characteristics with a negative impact on growth rate and quality of cultivated rice.

The grain shattering, the presence of awns, and red color of seeds are the main peculiarities of red rice.

A previous research (Sparacino et al., 2004), performed by the analysis of the computerized chromosome image (CHIA-EA = Chromosome Image Analysis – Excel Application) has revealed the presence of chromosomal anomalies, including the occasional presence of cells with 23 chromosomes (2n - 1) and 25 chromosomes (2n + 1) was observed as the result of a meiotic nondisjunction.

Morphologic and genotypic differences could turn out to be a useful diagnostic tool for red rice classification and biology understanding.

Moreover information about the genetic differences among red rice biotypes could provide suitable techniques for the limitation of the weed growth.

In order to investigate this problem, first, we employed seeds from single plants of different biotypes, for the karyotypic analysis. Then, we started a comparative and systematic analysis aiming at identifying morphologic characteristics correlated with karyotypic differences.

The present work represents a morphologic, karyotypic and genomic characterization of eight phenotypically distinct red rice biotypes, in comparison with the rice cultivars employed as control.

In the three years of experiment, we concluded that the biotypes differ for many parameters.

As far as the length of the seed is concerned, biotype 1 is the shortest one, and biotypes 2, 3, 6 turned out to be shorter than 5 and 7.

Concerning width, biotype 3 is quite narrow, while the others are larger and very similar.

Concerning thickness, biotype 2 is thicker than the others.

Because of these quantitative differences, the shape of the seeds is quite various - from the semi rounded shape of biotype 1 to the oval shape of biotype 2.

These quantitative data, together with the qualitative ones - such as color and awn length - show a considerable phenotypic variability in the eight biotypes.

About the qualitative traits of plants at full maturity, biotype 6 differs from the others for its red collar, nodes and internodes, biotype 8 has only red collar and the other biotypes have green collar, nodes and internodes.

Concerning quantitative features, biotype 1 is the lowest one, biotype 8 is the tallest one, biotypes 3, 5, 6, 7 are similar and biotype 4 is taller than biotype 1 and lower than all the others.

During the harvest we observed that the shattering degree appears to be related to the seed length (including its awn).

Biotype 1 (presenting the shortest seed) has a shattering degree about 50%, while biotypes 2 and 7 (with the longest seeds) have a shattering level about 90% and 80%, respectively.

Both biotypes 6 and 8 feature around 60% of shattering, and biotypes 3, 4, 5 around 70%.

The analysis of karyotype compared biotypes and the rice cv ‘Loto’, employed as control.

The homogeneous results obtained within each biotype agree with previous observations of various translocations in red rice, which always involve the distal segment of the long arm of chromosome 1 and one of the other chromosomes (Sparacino et al. 2004). In addition, it has been demonstrated that each phenotypic red rice biotype is characterized by a specific translocation. In fact, a detailed longitudinal examination of the structure of prometaphase chromosomes, which are still quite elongated, by the use of a computerized image method (CHIA-EA), revealed two types of anomalous chromosomes in each biotype, each of which showed a variation in size.

Evidence for the differences in length between two homologous chromosomes is unequivocally shown in the CP profiles, which allow individual members of a pair to be distinguished. The first anomaly, which is always present, involves chromosome 1. In this case, the anomalous chromosome 1 appears somewhat shorter than the normal one, due to a deletion of the distal segment of the long arm. In contrast, the second structural anomaly, which exhibits a remarkable difference in chromosome length, varies among biotypes. This is due to the addition of the distal segment, which has been displaced by translocation to the extremity of either the long or the short arm of another chromosome. In

this case, the two arms of the rearranged chromosomes vary in their relative length according to the position of the centromere, and are characterized by a different arm ratio.
Both chromosomal and genomic plant mutations, as it is well known, are quite widespread, both in nature and in induced mutations, provided they are compatible with life, because they do not involve the loss of genetic material and cause therefore even striking phenotypic effects.
These phenomena are well known in the subspecies *indica*, *japonica* and *javanica* of *Oryza sativa*, and may even involve all the twelve chromosomes pairs.
These data, however, do not allow to assess if the red rice translocations are simple or reciprocal.
Reciprocal translocations are the most frequent ones: two non-homologous chromosomes exchange portions of their arms, whose length depends on the break points.
Heterozygous translocations not involving a considerable loss of genetic material should not cause, in general, phenotypic effects in plants, except for semi-sterility.
A phenotypic effect could be due, however, to the formation of new association groups for those genes, belonging to the translocated segment, which are displaced from their original position.
A break close to or within a gene (with loss of a DNA segment), or a gene displacement close to the heterochromatic zone, may cause the change of the functions of one or more genes ("position effect").
In this connection, in order to ascertain if one of the shattering gene is displaced, breaks or remains on chromosome 1, the FISH cytogenetic method was used.
FISH is employed in order to identify specific sequences of nucleic acids in the chromosomes. It represents a significant contribution to standard cytogenetics for the identification of chromosome numerical and structural anomalies.
Its application to rice chromosomes presented various problems, at first due to the small and condensed size of the rice chromosomes, and the difficulty to get mitoses. To this goal a new and more complex methodology was employed to improve both the quality and the quantity of mitotic prometaphases (in order to get at least 40 prometaphases per slide).
Moreover, due to the large number of the FISH signals, a competitor DNA (C_0t) procedure was employed in order to suppress stray signals (Zwick M.S. et al., 1997). The main

problems were related to the use of a new methodology and to the fact that large amounts of genomic DNA were required for each hybridization experiment.
Nevertheless, we obtained an encouraging result for two different biotypes (3 and 5): the discovery that the shattering gene does not undergo translocation.
This induces to think, first of all, that the phenotypic effect is due to the formation of new "association groups" of the genes situated in the translocated segment, which change their position with respect to the original one. The translocation should therefore be reciprocal.
Further researches by using FISH method could confirm the reciprocity of the translocation.
In recent years it was realized that one of the shattering gene activation on chromosome 1 is due to a single-nucleotide polymorphism (SNP) which can explain about 70% of the non-shattering character associated to this gene whereby this SNP probably affects a regulatory region (Konishi et al., 2006).
The genotype of our eight shattering red rice biotypes was compared with the rice cultivars 'Nipponbare' (shattering), 'Kasalath' (non shattering), 'Perla' (non shattering) and 'Sprint' (shattering).
The genotype of our biotypes was found to coincide with that of 'Nipponbare', except for the SNP of the non-shattering character, which turned out to coincide with that of cv 'Kasalath'. These results lead to important conclusions.
Since the eight biotypes, as well as 'Nipponbare', 'Perla' and 'Sprint', belong to the subspecies *japonica*, an overall genotype similarity was expected.
Nevertheless, while the 'Nipponbare' and 'Perla' genotypes completely coincide at all the SNP loci considered in this work, the genotypes of red rice and 'Sprint' differ for a single SNP causing the activation of the shattering gene qSH1.
These considerations led to another logical conclusion, i.e. that the qSH1 SNP is largely responsible for the shattering character also for the red rice biotypes.
Other variants associated to the AT repeat 2 and SNP8 loci were noticed in the red rice genome during this research, but no correlation was found, up to now, between genotype and phenotype of the biotypes involved.
Other QTLs for shattering character were noticed on chromosomes 1, 3, 4, 7, 8 and 11, in other cultivated rice species which partially explain the shattering character, although the

responsible gene was identified only in three of these QTLs: 1, 4 and 7 (Hyeonso et al., 2010).

That is why we decided to take into account only these three QTLs of chromosomes 1, 4 and 7 (Konishi et al., 2006; Changbao et al., 2006; Hyeonso et al., 2010).

The analysis of the involved genes of chromosomes 4 and 7 in our eight red rice biotypes has revealed the QTLs found on these chromosomes are problaby not involved in the shattering character of the red rice biotypes.

We may conclude, on the basis of the results obtained so far, that shattering is largely (if not totally) dependent, both for our biotypes and for some cultivated rices (‘Perla’ and ‘Sprint’), on the mutation (qSH1-SNP) present on chromosome 1, and that the differences between the phenotypes of the eight red rice biotypes could be due to translocations causing the association of new groups of genes contained in the translocated segment.

Our analysis provides a wide overview of a possible correlation between the different phenotypes of the eight biotypes, shattering level, karyotypic differences connected with a specific translocation for each biotype and the fact that both these translocation and the gene responsible for the shattering of the eight biotypes are always present in chromosome 1.

This basic research could open the way to further studies which are currently in progress, and could reveal other karyotypic variants characterizing additional phenotypically distinct biotypes.

The identification of a pattern of chromosomal, morphological and genotypic diversification within the same variety could promote the development of suitable techniques limiting the expansion of the weedy red rice.

We may conclude that the investigation of the relationship between the various translocations and the expression of the shattering gene would require further and more sophisticated analyses.

REFERENCES

BANDINO M., RUSSO S. (1989) - Genetica dei cereali. Edagricole, 561-591.

BALSARI P., TABACCHI M. (1997) - Lotta meccanica di soccorso al riso crodo. L'Informatore Agrario, 53, 56-60.

BRES-PATRY C., LORIEUX M., CLEMENT G., BANGRATZ M. and GHESQUIERE A. (2001) - Heredity and genetic mapping of domestication-related traits in a temperate japonica weedy rice. Theor. Appl. Genet. 102, 118–126.

CAI H.W., MORISHIMA H. (2000) - Genomic regions affecting seed shattering and seed dormancy in rice. Theor. Appl. Genet. 100, 840–846.

CHANGBAO LI, AILING ZHOU, TAO SANG (2006) - Rice Domestication by Reducing Shattering. Science 311, 1936.

CHEN JT., LAI HC., CHUNG MC., WU HK. (1982). Identification of rice reciprocal translocation and the location of lazy gene. Bot. Bull. Acad. Sin., 23, 71-87.

CHO Y.S. (2010) - Germination characteristics of Korean and Southeast Asian red rice (*Oryza sativa* L.) seeds as affected by temperature. Asian J. Plant Sci., 9, 104-107.

CHUNG MC,WU HK. (1987) - Karyotype analysis of IR36 and two trisomic line of rice. Bot. Bull. Acad. Sin., 28, 289-304.

DELLAPORTA S. L., WOOD J., HICKS J.B. (1983) - A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1, 19–21.

DE LORENZI L., DE GIOVANNI A., MOLTENI L., DENIS C., EGGEN A., PARMA P. (2007) Characterization of a balanced reciprocal translocation, rcp(9;11)(q27;q11) in cattle. Cytogenetic and genome research, 119 (3-4), 231–234.

DIARRA A., SMTTH RJ, Jr., TALBERT RE (1985) - Growth and morphological characteristics of red rice (Oryza sativa) Biotypes. Weed Science: 33, 310-314.

EIGUCHI M., SANO Y. (1990) - A gene complex responsible for seed shattering and panicle spreading found in common wild rices. Rice Genet. Newsl. 7, 105–107.

FERRERO A. (1994) - Caratteristiche biologiche del riso crodo. Proceedings "Progetto Riso Crodo" 1° Convegno riso crodo, 9/11 Novara.

FERRERO A., VIDOTTO F. (1997) - Comportamento del riso crodo nell'emergenza – L'Informatore Agrario, 53, 49-52.

FONTENOT HA. (1973) - Wild ducks fight red rice. Rice Joumal, 76, 14.

FUKUI K. (1998) - Analysis and Utility of Chromosome Information by Using the Chromosome Image Analyzing System, CHIAS. Bull. Natl. Inst. Agrobiol., 4: 153-176.

FUKUI K. (1986a) - Standardization of Karyotyping plant Chromosomes by newly developed Chromosome Image Analyzing System (CHIAS). Theor Appl. Genet., 72, 27-32.

FUKUI K. (1986b) - Comparison between Giemsa and orceina staining methods in rice chromosomes. La Kromosomo II, 43-44, 1398-1404.

FUKUI K., IUIMA K. (1990) - Clarification of the Conditions for the Image Analysis of Plant Chromosomes. Bull. Natl. Inst. Agrobiol., 6: 1-58.

FUKUI K., IUIMA K. (1992) - Manual on Rice Chromosome. Misc. Publ. Inst. Agrobiol. Resour., 4, 1-25.

FUKUI K., KAKEDA K., IUIMA K and ISHIKI (1988) - Computer-aided identification of rice chromosomes. Rice Genetics Newsletters, 5: 31-34.

FUKUI K., KATO S. (1998) - Condensation Pattern (CP) Analysis of plant Chromosomes by an improved Chromosome Image Analysing System, CHIAS III. Chromosome Research, 6,473-479.

FUKUI K., MUKAI Y. (1988) - Condensation pattern as new image parameter for identification of small Chromosomes in plants. Jpn. J. Genet., 63, 359-366.

FUKUTA Y. and YAGI T. (1998) - Mapping of a shattering resistance gene in a mutant line SR-5 induced from an indica rice variety, Nan-jing11. Breed. Sci. 48, 345–348.

HOLM L.G., PLUCKNETT D.L., HERBERGER J.P. (1997) - The world's worst weeds: distribution and biology. University press of Hawaii, 32-46.

HU CH. (1958) - Karyological studies in haploid rice II. Analysis of karyotype and somatic pairing. Jpn. J. Genet., 33, 296-301.

HYEONSO JI, SUNG-RYUL KIM, YUL-HO KIM, HAKBUM KIM, MOO-YOUNG EUN, IL-DOO JIN, YOUNG-SOON CHA, DOH-WON YUN, BYUNG-OHG AHN, MYUNG CHUL LEE, GANG-SEOB LEE, UNG-HAN YOON, JUNG-SOOK LEE, YEON-HEE LEE, SEOK-CHEOL SUH,WENZHU JIANG, JUNG-IL YANG, PING JIN, SUSAN R. MCCOUCH, GYNHEUNG AN, AND HEE-JONG KOH (2010) - Inactivation of the CTD phosphatase-like gene OsCPL1 enhances the development of the abscission layer and seed shattering in rice. The Plant Journal 61, 96.

IUIMA K., KAKEDA K., FUKUI K. (1991) - Identification and Characterization of Somatic rice chromosomes by Imaging Methods. Theor. Appl. Genet., 81, 597-605.

KAMISUGI Y., FUKUI K. (1990) - Automatic Karyotyping of Plant Chromosomes by Imaging Techniques. Biotechniques, 3, 290-295.

KAMISUGI Y., FURUYA N., IIJIMA K., FUKUI K. (1993) - Computer-aided Automatic identification of rice chromosomes by image parameters. Chromosome research, 1, 189-196.
SAEKO KONISHI, TAKESHI IZAWA, SHAO YANG LIN, KAWORU EBANA, YOSHIMICHI FUKUTA, TAKUJI SASAKI, MASAHIRO YANO (2006) - An SNP Caused Loss of Seed Shattering During Rice Domestication. Science 312,1392.

KURATA N., OMURA T. (1978) - Karyotype analysis in rice 1. A new method for identifying all chromosome pairs. Jpn. J. Genet., 53, 251-255.

KURATA N., OMURA T., IWATA N. (1981 b) - Studies on centromere, chromomere, and nucleolus in pachytene nuclei of rice, Oryza sativa, microsporocytes. Cytologia, 46, 791-800.

LABRADA L. (1996) - Weed control in rice. Pp3-5 in B.A. Auld and K-U Kim, eds Weed Management in Rice. Rome : Food and Agriculture Organization of the United Nations.

NAGAI Y.S., SOBRIZAL P.L., SANCHEZ T., KURAKAZU K.D., KURAKAZU A.Y. (2002) - Sh3, a gene for seed shattering, commonly found in wild rices. Rice Genet. Newsl. 19, 74–75.

NAGAO S., TAKAHASHI M. (1963) - Trial construction of twelve linkage groups in Japanese rice. J. Fac. Agr. Hokkaido Univ. 53, 72–130.

NANDI H. K. (1936) - The chromosome morphology, secondary association, and origin of cultivated rice. Jpn. J. Genet. 33, 315-336.

OBA S., KIKUCHI F. and MARUYAMA K. (1990) - Genetic analysis of semidwarfness and grain shattering of Chinese rice [*Oryza sativa*] variety ‘‘Ai-Jio-Nan-Te’’. Jpn. J. Breed. 40, 13–20.

OKA H. I., WU H. K. (1988) - Comparison of data rice Chromosomes presented by different authors. Rice Genetics Newsletters, 5, 34-41.

RATHORE K. S., CHOWDHURY V. K., HODGES T. K. (1993) - Use of bar as a selectable marker gene and for the production of herbicide-resistant rice plant from protoplasts. Plant Molecular Biology, 21, 871-884.

ROBERTS H. A., FEAST P. M. (1972) - Fate of seeds of some annual weeds in different depths of cultived and undisturbed soil. Weed Research, 12, 316-324.

RUSSO S. (1995) - Strategie di lotta al riso crodo. L’Informatore Agrario, 51, 89-95.

SCHWARZACHER, WOLF (1974) - Methods in human cytogenetics. Berlin/ Heidelberg/New York, Springer-Verlag, pp 78–81.

SHASTRY S., RANGA R., MISTRA R. (1960) - Pachitene analysis in *Oryza*, I. Chromosome morphology in *Oryza sativa*. Indian J. Genet. Plant Breed, 20, 15-21.

SPARACINO A.C., FERRO R., MANNINO M.R. (1985) - Evoluzione della flora infestante nelle risaie del Pavese. Atti Convegno “Erbe infestanti graminacee nella moderna agricoltura: dinamica, problemi e possibili soluzioni”. Verona, 14 novembre .

SPARACINO A.C., HALFER C., TANO F., DITTO D. (2003): Identification and characterization of somatic chromosomes of red rice (*Oryza sativa* var. *sylvatica*) by means of a computerized chromosomal imaging method (CHIA-EA). 3rd. Temperate Rice Conference, 10-13/3,. Punta del Este (Uruguay).

SPARACINO A.C., SGATTONI P. (1993) - Le erbe infestanti il riso. L'informatore agrario 49, 37-55.

THOMSON M.J., TAI T.H., MCCLUNG A.M., LAI X.H., HINGA M.E., LOBOS K.B., XU Y., MARTINEZ C.P., McCOUCH S.R. (2003) - Mapping quantitative trait loci for yield, yield components and morphological traits in an advanced backcross population between Oryza rufipogon and the Oryza sativa cultivar Jefferson. Theor. Appl. Genet. 107, 479–493.

TOGASHI K. (1942) - A survey of plant disease of cultivated plants in Iwate Prefecture in 1941. Bulletin 8. Iwate Agricoltural Experiment Station. Morioka, 25 pp.

VIDOTTO F., FERRERO A., DUCCO G. (2001) - A mathematical model to predict Oryza sativa var. sylvatica population dynamics. Weed Research, 41**,** 407-420.

VIGGIANI P., ANGELINI R. - Erbe spontanee e infestanti: tecniche di riconoscimento. Edagricole.

WU H. K., CHUNG M. C., CHEN M. H. (1958) - Karyotype analysis of cultivar IR 36. Rice Genetics Newsletters, 2, 54-57.

WU H. K., CHUNG M. C. (1989) - The interchanged chromosomes involved in Nishimura's translocation lines. in “Breeding research: The key to the survival of the earth.” Proceedings 6th International Congress of SABRAO, Tsukuba, Japan, p. 183-186.

XIONG L.Z., LIU K.D., DAI X.K., XU C.G., ZHANG Q. (1999) - Identification of genetic factors controlling domestication-related traits of rice using an F2 population of a cross between Oryza sativa and O. rufipogon. Theor. Appl. Genet. 98, 243–251.

ZWICK M.S., HANSON R.E., ISLAM-FARIDI M.N., STELLY D.M., WING R.A., PRICE H.J., MCKNIGHT T.D. (1997) - A rapid procedure for the isolation of *C0t-1* DNA from plants. Genome, 40, 138-142.

WEB SITE

http://www.agroselviter.unito.it/weed/weedyrice/weedyriceit.htm

http://www.regione.piemonte.it/agri/ita/news/pubblic/quaderni/num15/art11-15.htm