Cytogenetic Studies in *Psilanthus ebracteolatus* Hiern., a Wild Diploid Coffee Species

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Summary The genus *Psilanthus* is taxonomically close related to *Coffea* and its cytogenetic presents few studies due to the symmetry of its small chromosomes. In this work we analyzed cytogenetic features of *Psilanthus ebracteolatus*, a wild diploid coffee native from Ivory Coast included in the Coffee Germplasm Bank of the Instituto Agronômico de Campinas, Brazil. For heterochromatic banding assays the fluorochromes CMA₃ and DAPI were used. For FISH assays the rDNA probes pTa71 and pScT7 were used. This species presented no DAPI positive bands, two CMA₃ positive band pairs and one pair of NOR and one of 5S rDNA sites. Although we observed cytological similarity between the studied species and the few data presented for *Psilanthus* in literature, the analysis based on cytogenetic markers contributes to karyological characterization of this genus.

Key words FISH, NOR, *Psilanthus*, rDNA site.

The genera *Psilanthus* Hook.f. and *Coffea* L. constitute the tribe Coffeae, which belongs to family Rubiaceae, subfamily Cinchonoideae (Wrigley 1988). These 2 close related genera are distinguished on the features of flower and flowering characteristics (Bridson 1987). While *Psilanthus* presents a long corolla tube associated with inserted anthers and a short style, *Coffea* presents a short corolla tube associated with exerted anthers and a long style (Couturon *et al.* 1998).

The genus *Psilanthus* presents about 30 species distributed in Africa, Asia and New Guinea (Charrier and Berthaud 1985, Bridson 1987). Although the species of this genus presents no commercial use and apparently little breeding potential (Wrigley 1988), the study of the cytogenetic characteristics of its species may provide new useful data to understand the karyotype evolution occurred in the subfamily Cinchonoideae.

Cytogenetic studies in *Psilanthus* and *Coffea* were limited by the relatively small size of their chromosomes (1–3 μ m) and the symmetrical karyomorphology observed in the studied species of these genera (Charrier and Berthaud 1985, Lombello and Pinto-Maglio 2003). There are few cytomolecular studies for *Psilanthus* and *Coffea*. In fact the cytological literature brings for *Psilanthus* basically chromosomal number reports of some representative species. Recently our research group presented cytogenetic data for 2 species of *Psilanthus*: *P. bengalensis* and *P. travancorensis* (Lombello and Pinto-Maglio 2003). Due to its economic importance *Coffea* has chromosome number accountings for most species of the genus, a few data of banding techniques, as those presented by Pierozzi *et al.* (1999), and more recently some cytomolecular information (Pinto-Maglio *et al.* 2000, Barbosa *et al.* 2001, Lombello and Pinto-Maglio 2002, 2003). For *Psilanthus* the chromosome number accountings present $2n=2x=22$ for all the studied species (Charrier and Berthaud 1985, Lombello and Pinto-Maglio 2003). All the studied species of both genera presented symmetrical karyomorphology. In such cases staining techniques with the fluorochromes DAPI and CMA₃, that evidence heterochromatic regions rich in AT and GC bases respectively, are often used in order to characterize the species cytogenetically (Gupta *et al.* 1990, Yen-Yu *et al.* 2001). Fluorescent *in situ* hybridization (FISH) assays with rDNA probes are also an important tool for karyotype charac-

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terization (Heslop-Harrison 1991, Raina *et al.* 2001).

In the present paper we bring cytogenetic data for *Psilanthus ebracteolatus* Hiern, a wild diploid species that belong to the Coffee Germplasm Bank of Instituto Agronômico de Campinas (IAC). This species is originated from Ivory Coast, at humid and semidry forest of low altitude (Couturon *et al.* 1998). There are no previous cytomolecular data for this species. This study is part of a project that intends to characterize cytogenetically all the coffee species of this germplasm bank.

Materials and methods

Plant material and slide preparations

Seeds of *Psilanthus ebracteolatus* were collected at Coffee Germplasm Bank of the Instituto Agronômico de Campinas (IAC) in Southeastern Brazil. These seeds were germinated in Petri dishes. The root tips were excised and pre-treated with the anti-mitotic agent PDB (*p*-dichlorobenzene) in saturated solution at 16°C for 2 h and 30 min. The material was fixed in Carnoy's solution (alcohol 3 : 1 acetic acid, v/v) for 24 h and stored at -20°C. For the slide preparation root tips were digested in a solution of citrate buffer with 2% of cellulase and 20% of liquid pectinase for 1 h at 37°C and squashed in 45% acetic acid.

Fluorochrome staining and in situ hybridization

For DAPI (4',6-diamino-2-phenylindole) and CMA₃ (chromomicin) banding we followed the protocols of Schweizer (1976). In the hybridization we followed the protocol of Pendas *et al.* (1993). The probes used were pTa71, which contains one unit of 18S-5.8S-26S rDNA (9 kb) derived from common wheat (Gerlack and Bedbrook 1979), and pScT7, containing an insert of 5S rDNA with 300–500 pb isolated from *Secale cereale*. The probe pTa71 were labeled with biotin-16-dUTP and pScT7 with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation, following the manufacturer's instructions. The hybridized sites were detected with Avidin-Rhodamine for biotinylated probe pTa71 and IgG-FITC for digoxigenin labeled probe pScT7. The slides were counterstained with DAPI as showed by Heslop-Harrison *et al.* (1991) and mounted with anti-fading Vectashield (Vector Laboratories). Slides were examined with an Olympus BX50 epifluorescent microscope. Chromosome images were captured by an Optronics DEI-750 camera and processed in Image-Pro Plus 3.0 program only using contrast and lightning adjustments, functions that affect the whole image equally.

Results and discussion

The diploid chromosome number observed for *Psilanthus ebracteolatus* was $2n=22$ and the karyomorphology observed was regular and symmetrical. The DAPI staining did not evidence positive AT rich bands (Fig. 1a). The CMA₃ staining evidenced 2 band pairs located at terminal position in chromosomes. One pair presents stronger signal than the other (Fig. 1b). The hybridization with pTa71 probe evidenced one pair of 18S-5.8S-26S sites of rDNA (Fig. 1c) and the hybridization with pScT7 probe evidenced one pair of signals related to 5S rDNA (Fig. 1d).

The chromosome number observed *P. ebracteolatus* agrees with the numbers presented in literature for the genus *Psilanthus* (Mangenot and Mangenot 1958, 1962, Couturon *et al.* 1998). The karyomorphology regular and symmetrical reinforces the trend observed for the genus by Bouharmont (1959, 1963).

The absence of DAPI positive AT rich bands agrees with the results presented by Lombello and Pinto-Maglio (2003) for *Psilanthus bengalensis* and *P. travancorensis*. In coffee group at this time only *Coffea humilis* presented positive DAPI bands (Lombello and Pinto-Maglio 2003), what

Fig. 1. Mitotic cells of *Psilanthus ebracteolatus* ($2n=22$). A) Stained with DAPI, B) stained with CMA, with arrows showing terminal CMA positive bands, C) stained with rhodamine and counter-stained with DAPI, with arrows showing sites of 45S rDNA, D) stained with FITC and counter-stained with DAPI, with arrows showing sites of 5S rDNA. Bar=5 μ m.

seems to be an exception in this group. Pinto-Maglio *et al.* (2000) studying *C. arabica*, Barbosa *et al.* (2001) studying *C. canephora*, *C. dewevrei* and *C. eugenioides* and Lombello and Pinto-Maglio (2002, 2003) studying *C. brevipes*, *C. kapakata*, *C. sp. Moloundou*, *C. racemosa*, *C. salvatrix*, *C. sessiliflora*, *Psilanthus bengalensis* and *P. travancorensis* observed a uniform DAPI staining with no chromosomal bands.

The number of CMA₃ positive bands observed here for *P. ebracteolatus* was the same of that observed in our earlier study (Lombello and Pinto-Maglio 2003) for *P. travancorensis*, although these bands present different features. In *P. travancorensis* there is one pair of terminal and one pair of intercalary signals, with the terminal sites presenting stronger signals than the intercalary ones (Lombello and Pinto-Maglio 2003) instead 2 pairs of terminal sites in *P. ebracteolatus*. In *P. bengalensis* only one pair of CMA₃ positive bands were observed in terminal chromosome regions (Lombello and Pinto-Maglio 2003).

The number of 45S rDNA sites evidenced by FISH with pTa71 probe observed for *P. ebracteolatus* is the same of the other 2 species with cytological data. The features of these sites are similar between these species, with a strong terminal signal observed. The number and the features of the 5S rDNA sites evidenced by FISH with pScT7 probe is the same between the 3 *Psilanthus* studied species. The signals are located in intercalary regions of the chromosomes and are weaker than those observed for 45S rDNA. The 45S rDNA signal observed for *P. ebracteolatus* in FISH assay may refer to the strongest terminal signal observed in CMA₃ staining. The 5S rDNA signal observed cannot refer to the other CMA₃ band, due to its intercalary position.

This low numerical rDNA site variation between coffee species was expected based on the lit-

the genetic polymorphism observed in this group, as showed by Lashermes *et al.* (1996) in their study with restriction fragment length polymorphism (RFLP) of chloroplast DNA in 25 taxa of coffee species. Cytogenetic studies seem to confirm this trend for coffee species (Barbosa *et al.* 2001, Lombello and Pinto-Maglio 2002, 2003). Moreover low numerical rDNA site variation no remarkable divergence in length signal was observed in FISH assays between *P. ebracteolatus* and the other two studied species *P. bengalensis* and *P. travancorensis*.

Lashermes *et al.* (1997) analyzed the phylogenetic relationships of some coffee-trees species based on variation of the sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. With these data the authors organized coffee species in groups with similarity for these sequences. These groups present strong correspondence with geographical distribution. That work places *P. ebracteolatus* in a sister group to a clade consisting of East-African *Coffea* species as *Coffea racemosa* and *C. sessiliflora*, despite its West-African origin, and distant from *P. travancorensis*, placed in Madagascar group. This incongruence may be explained by the occurrence of introgressive hybridization between species of these clades, as pointed out by Cros *et al.* (1998) that analyzed the variation in chloroplast DNA between 23 taxa of coffee and observed several inconsistencies among West-African taxa. The cytogenetic data reinforces the arrangement of coffee species in biogeographical clades as proposed by Lashermes *et al.* (1997) and Cros *et al.* (1998).

Even though the studied species of *Psilanthus* present none numerical nor morphological rDNA site variation, the features of CMA₃ bands of *P. ebracteolatus* are similar to those presented for *C. sessiliflora* (Lombello and Pinto-Maglio 2002) and *C. racemosa* (Lombello and Pinto-Maglio 2003), 2 species of its sister clade (Lashermes *et al.* 1997), and different of those presented for *P. travancorensis* (Lombello and Pinto-Maglio 2003), which is one terminal and one intercalary pair of CMA₃ positive bands (Lombello and Pinto-Maglio 2003). The present data also agree with the results presented by Combes *et al.* (2000), that studied the polymorphism of microsatellite sequences in 13 coffee taxa and observed close genetic relation between species of *Psilanthus* and *Coffea*. These results reinforce the importance of chromosome mapping using cytogenetic techniques like fluorochromes banding and *in situ* hybridization, in studies that intend to compare related taxa with similar karyological characteristics.

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