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Localization of rDNA loci by Fluorescent *In Situ* Hybridization in some wild orchids from Italy (Orchidaceae)

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Abstract — The 18S-5.8S-25S (pTa71) and 5S (pTa794) rDNA were used as probes for *in situ* hybridization to reveal the physical localisation of ribosomal genes in some species of orchids. *Earlia robertiana (2n* = 36) revealed one chromosome pair with both pTa71 and pTa794 signals; *Anacamptis papilionacea (2n* = 32) showed two pairs of 5S rDNA and one pair of 18S-25S sites; *A. morio (2n* = 36) had two pairs of 18S-25S rDNA and one pair of 5S sites; the natural hybrid *A. x gennarii (A. morio x A. papilionacea,* 34 chromosomes) showed three 18S-25S and three 5S rDNA sites; finally, *A. collina (2n* = 36) showed only one pair of 18S-25S rDNA and one pair of 5S rDNA sites. This preliminary contribution indicates that repetitive DNA sequences in orchids may prove very useful for the understanding of evolutionary trends.

Key words: *Anacamptis, Barlia,* FISH, ribosomal genes.

INTRODUCTION

In recent years different methodological approaches have been used to elucidate systematic relationships and evolutionary pathways in orchids: isozyme polymorphism (ARDUINO *et al.* 1989; Rossi *et al.* 1994), karyomorphology (BIANCO *et al.* 1991; D'EMERICO *et al.* 1992, 1996a) and ITS length variation (PRIDGEON *et al.* 1997; BATEMAN *et al.* 1997; ACETO *et al.* 1999).

Molecular cytogenetic methods, and fluorescent *in situ* hybridisation (FISH) *in primis,* used to identify highly repetitive sequence families and their distribution on plant chromosomes, have prove to be powerful tools to characterise chromosomes and to investigate phyletic relationships in some plant groups (MALUSZYNSKA and HESLOP-HARRISON 1993 a; GALASSO *et al.* 1995,1997; ZOLDAS *et al.* 1999). To the authors' best knowledge, no data have been produced until now on the application of FISH to study European orchids.

The genes coding for the ribosomal RNAs constitute an abundant class of repeated sequences. In plants the 5S and the 18S-5.8S-25S rRNA genes are present as many hundreds of tandemly repeated units at one or more loci within the genome. The rDNA sequences have been used as chromosome-specific markers to map and to tag particular chromosomes (MALUSZYNSKA and HESLOP-HARRISON 1993 a, b and references therein; OSUJI *et al.* 1998; TAKETA *et al.* 1999). In this paper, the physical distribution of 18S-5.8S-25S and 5S rDNA sequences on the chromosomes of five Orchidaceae taxa, all of Italian origin is reported for the first time.

MATERIALS AND METHODS

The taxa examined and their collecting sites are shown in Table 1.

In all cytological techniques, immature ovaries were pre-treated with 0.3% colchicine at room tern-

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Table 1 — List of the materials used in the present study, and their collecting sites.

Taxon	Sites *
Barlia robertiana	Cassano Murge (Ba); Gargano promontory (Fg)
Anacamptis papilionacea	Adelfia (Ba); Gargano promontory (Fg)
A. morio	Adelfia (Ba); Gargano promontory (Fg)
A. x gennarii	Adelfia (Ba); Gargano promontory (Fg)
A. collina	Cassano Murge (Ba); Gargano promontory (Fg)

* Ba = province of Bari; Fg = province of Foggia

perature tor 2h, then hxed in etnanol: acetic acid (3:1) and squashed in 45% acetic acid. The coverslips were removed after freezing on dry ice and the slides were allowed to dry in air before use.

For fluorescent *in situ* hybridisation the ribosomal sequences 18S-5.8S-25S (in short, 18S-25S) (pTa71) and 5S (pTa794) were used as probes. The former contains a 9kb EcoRI repeat unit of the 18S-25S rDNA and the intergenic spacer regions, isolated from *Triticum aestivum* (GERLACH and BEDBROOK 1979), the latter corresponds to a complete 410 bp 5S gene unit, containing the 5S gene and intergenic spacers, isolated from *Triticum aestivum* (GERLACH and DYER 1980). The slides were hybridised using both probes simultaneously: pTa71 labelled with rhodamine-4-dUTP and pTa794 with digoxigenin-11 -dUTP.

The slides pre-treatment and FISH procedure followed the protocol of HESLOP-HARRISON *et al.* (1991). The chromosomes and DNA probe were denatured together at 70°C for 5 min and hybridisation carried out at 37°C overnight. After hybridisation, the coverslips were removed in 2 x SSC at room temperature and then given a stringent wash for 10 min in 20% (v/v) formamide in 0.1 x SSC at 42°C, to remove sequences with less than 85% homology; the slides were then incubated in immunoflorescent reagents. For the detection of digoxigenin-labelled probe, slides were equilibrated in 4 x SSC/0.1% (v/v) Tween 20 and blocked in 5% (w/v) bovine serum albumin in 4x SSC/0.1% (v/v)Tween 20 for 5 min.

Slides were incubated with sheep anti-digoxigenin antibody conjugated with FITC in a moist chamber at 37°C for 1 h. The slides were washed in 4 x SSC/Tween 20 for 3 x 5 min and subsequently counterstained with DAPI prior to observation. They were finally mounted in antifade solution (AFj, Citifluor) and examined with Leitz epifluorescence microscope with single and triple band-pass filters.

Photographs were taken on Fujcolor Super HG 400 colour print film. Relevant images were then digitalized using a Polaroid SprintScan 35 film scanner and digitally elaborated with Adobe Photoshop software, using only functions applying to the whole image at a time. Colour prints were obtained using both a Mitsubishi CP-D1E photo printer and an Epson Stylus Colour 1520 with photo-quality paper.

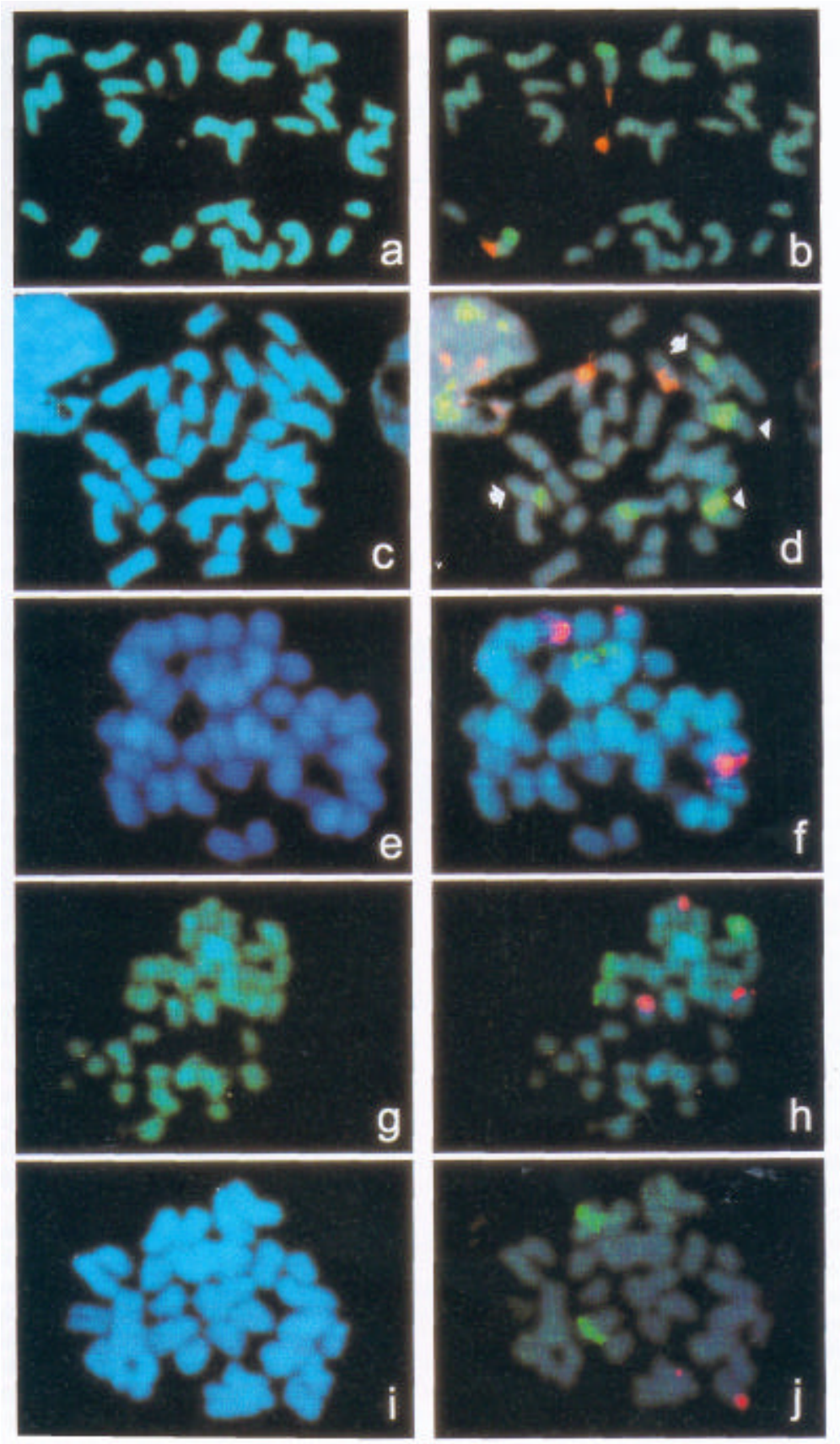
RESULTS

The classification of Orchidinae used followed BATEMAN *et al.* (1997).

Earlia robertiana (Loisel.) W. Greuter. The chromosome number is *2n* = 36. The karyotype consist of $26m+4m^{sc}+2m^{sc}_{s}+-4sm^{sc}$ (D'EMERico *et al.* 1992). Double-target *in situ* hybridization on *B. robertiana* chromosomes revealed one chromosome pair — no. 5 according to D'EMERICO *et al.* (1992) — carrying both the pTa794 and the pTa71 signals on opposite arms: the 5S rDNA sequences were located at telomeric position on the short arm, whereas the 18S-25S rDNA loci in the telomeric domain of the long arm (Figures la, b; 2a).

Anacamptis papilionacea (L.) Bateman, Pridgeon & Chase. The chromosome number is *2n* = 32. The karyotype formula of the species consist of $16m+2m^{sc}+10sm+2sm^{sc}_{s}+2st$ (D'EMERico *et al.* 1996a; 1996b). After FISH, the signals of the pTa71 probe localised at the telomeric region of the long arm of the satellited chromosome (pair 8). The 5S rDNA sequences were present on two pairs of chromosomes: a major 5S rDNA site was localized close to the centromere on the long arm of a long chromosome, while a minor site was paracentromerically located on the long arm of another chromosome pair. Rarely, another 5S rDNA signal

Figure 1 — *In situ* hybridisation to chromosomes of orchids species. Blue DAPI staining shows chromosomal DNA (a, c, e, gi); red and green signals show sites of hybridization of 18S-25SrDNA and 5S rDNA respectively (b, d, f, h, j). *Barlia robertiana* (a, b) with both the pTa794 and the pTa71 signals on opposite arms; the apparent gap in the signal of one chromosome is due to NOR region stretching; *Anacamptis papilionacea (c,* d) with five sites of 5S rDNA (homologous chromosomes are indicated with arrows and arow-heads respectively), and two sites of 18S-25SrDNA; *A. mono* (e, f) with four sites of 18S-25S rDNA and two sites of 5S rDNA; *A. x qennarii* (g, h) with three 5S rDNA and three 18S-25S rDNA sites; *A. collina* (i, j) with two sites of 5S rDNA and two sites of 18S-25S rDNA.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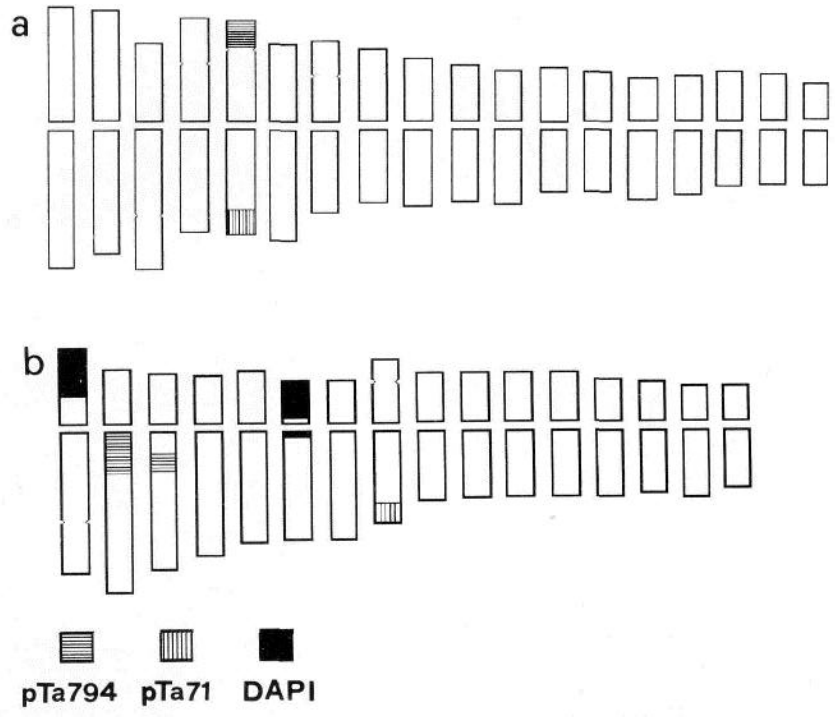

Figure 2 — Haploid idiograms of: a) *Barlia robertiana* with both the pTa794 and the pTa71 signals on opposite arms, b) *Anacamptis papilionacea* with two sites of 5S rDNA and one site of 18S-25S rDNA.

could be observed at the paracentromeric position on the long arm of one only of the two homologous chromosomes of an unidentified pair (Figures 1 c, d; 2 b).

Anacamptis morio (L.) Bateman, Pridgeon & Chase. The chromosome number is *2n* = 36. The karyotype qonsist of $26m+4m^{s}+4sm+2sm^{s}$ (D'EMERico *et al.* 1996a). In this species ribosomal sequences where present on three different chromosome pairs: two of them carried the 18S-25S rDNA hybridisation signals, and the third one showed the 5S rDNA sequences (Figure le, f).

Anacamptis x gennarii Reichenb. fil. This natural hybrid *A. morio* x *A. papilionacea* has 34 chromosomes (D'EMERico *et al.* 1996b). *In situ* hybridisation of ribosomal genes allowed the localisation of three 18S-25S rDNA signals, and three 5S rDNA sites. The 5S rDNA sites showed an evident variation in signal strength: one showed a major hybridisation signal, which resembled the one observed in *A. papilionacea,* while the other two ones had a lesser intensity (Figure Ig, h).

Anacamptis collina (Banks et Solander) Bateman, Pridgeon & Chase. The somatic chromosome number is *2n* - 36. This species possess $14m+4m^{sc}+8sm+2sm^{s}+2sm^{s}_{sc}+6sm_{sc}$ chromosomes (D'EMERico *etal.* 1996a). FISH revealed one pair of hybridisation sites for the 18S-25S-rDNA and one for the 5S rDNA sequences. One chromosomal site of the 18S-25S rDNA genes clusters was not always detectable, indicating a possible heteromorphism between homologous chromosomes (Figure li, j).

DISCUSSION

The regions coding for rRNA are organised in arrays of tandem repeats, where coding regions are separated by spacers (FEDOROFF 1979). The spacer regions can be highly variable, both among and within given species, while the coding regions are rather well conserved (APPELS and DVORAK 1982). The variability of the spacer region has been used in orchids to investigate phyletic relationships, leading to new insights into the understanding of the evolution within this group of species (PRIDGEON *et al.* 1997; BATEMAN *et al.* 1997; ACETO *et al.* 1999). This paper reports for the first time on the physical locations of rDNA loci on

the somatic chromosomes of Italian wild Orchidaceae.

Due to their small dimension an exact chromosomal localisation of pTa794 and pTa71 probes is very difficult. Notwithstanding the absence of an exact identification of the chromosome carrying the rDNA genes clusters, it was evident that the distribution of hybridisation signals among the analysed taxa was quite different.

It is interesting to notice that in *B. robertiana,* a chromosome pair showed a characteristic distribution of the 5S and 18S-25S rDNA sequences, being located at opposite telomeric regions respectively; possibly this chromosome corresponds to pair no. 5 described by D'EMERICO *et al.* (1992) as satellited pair. This specific chromosome, which is not present in the *Anacamptis* species studied, might be proposed as a further landmark specific to *Earlia.*

It is worth noticing that among *Anacamptis* species, only *A. papilionacea* showed two pairs of 5S rDNA sites, *A. morio* and A *collina* showing only one. In *A. papilionacea* the signals on one pair were much more intense than those on the other pair. Repeated sequences are subject to very rapid changes in copy number; in this connection REDDY and APPELS (1989) and SASTRI *et al.* (1992) suggested that in some plant groups (e.g. *Secale, Hordeum)* major sites, deriving from the overrepresentation of few ancestral ones, might denote a more recent origin of the major clusters in respect to the minor sites. In this light, the presence of a major site of 5S rDNA gene clusters could be regarded as a further indication of a recent origin of this species, reinforcing previous reports (D'EMERICO *et al.* 1996a). In addition, the occasional occurrence of a minor 5S site on only one homologous chromosome of an unidentified pair could be taken as an indication of unequal inter-chromosomal recombination events, or recent chromosomal restructuration (ABBO *et al.* 1994).

With respect to the 18S-25S rDNA sites, pTa 71 signals were present on one pair of chromosomes in *A. papilionacea* and *A. collina,* two pairs of sites were observed in *A. morio,* and three sites in *A. x gennarii* (the natural hybrid *A. morio x A. papilionacea).*

A final note regards the hybrid *A. x gennarii.* In this entity it was possible to observe that the number of 18S-25S and 5S ribosomal sites is exactly the sum of the haploid site numbers of the two parents *(A. morio* and .XL *papilionacea),* and that the chromosomal domains of the hybridisation sites correspond to those of the parental species.

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