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Heterochromatin distribution in selected taxa of the 42-chromosomes *Orchis s. l.* (Orchidaceae)

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Abstract - In six 42-chromosomes taxa belonging to genus *Orchis s. l.* heterochromatin location and distribution and staining properties were analysed by means of C-banding and of the fluorochromes 4'-6-diamidino-2-phenylindole-2HCl (DAPI) and Hoechst 33258. Most species could be distinguished on the basis of heterochromatin amounts and distribution. In the species *O. mascula* and *O. provincialis* most DAPI-positive sites did not co-localize with C-bands. DAPI revealed bright fluorescence at telomeric or subtelomeric regions of numerous chromosomes of *O. mascula* and particularly large/bright blocks at the telomeres of *O. provincialis*. In *O. x penzigiana* (*Orchis mascula* ssp. *ichnusae* x *O. provincialis*) overall heterochromatin distribution followed that of the parental species. In *Neotinea* group all DAPI positive bands co-localize with C-bands, but have different distribution in the taxa analysed. Present and literature data indicate a high level of plasticity of heterochromatin organization in *Orchis s. l.*, and suggest evolutionary pathways in agreement with recent molecular data.

Key words: Heterochromatin banding, karyotype structures, *Neotinea lactea*, *N. tridentata*, *Orchis mascula*, *O. mascula* ssp. *ichnusae*, *O. provincialis*, *O. x penzigiana*.

INTRODUCTION

The genus *Orchis s. l.* is an important and controversial group of the tribe Orchideae (Orchidaceae). Previous investigations based on karyomorphology and heterochromatin distribution indicated a possible grouping of some taxa (D'EMERICO *et. al.* 1996a, b). Later, BATEMAN *et al.* (1997) on the basis of the nucleotide sequences of ribosomal gene spacers (ITS1 and ITS2) divided the genus into three groups, namely *Anacamptis s. l.*, including all species with *2n*=36 chromosomes, *Neotinea s. l.* and *Orchis s. s.*, distinguished by the presence of *2n*=42 chromosomes taxa.

In *Orchis s. s.*, *O. mascula* (L.) L. and *O. provincialis* Balbis ex Lam. et DC., belonging to subgroup *Orchis mascula*, are two species which exhibit much morphological similarity and close phylogeny (PRIDGEON *et al.* 1997; ACETO *et al.* 1999). In this regard it is notable that in Sardinia is present *O. mascula* ssp. *ichnusae* and intermediate phenotypes between this taxon and *O. provincialis*, classified as *O. x penzigiana* A. Camus nothosubsp. *sardoa* Scrugli et Grasso (SCRUGLI *et al.* 1988). Recently, karyomorphological and molecular data have been reported for these and some related taxa suggesting their specificity within of the *Orchis s. s.* (PELLEGRINO *et al.* 2000).

In the *Neotinea s. l.*, *N. lactea* (Poir.) R.M. Bateman, Pridgeon, & M.W. Chase blossoms approximately two weeks before *N. tridentata*

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Table 1 – List of the taxa examined in the present study, their collecting sites, and chromosome numbers.

Taxa	Sites	Chromosome number (2*n*)
Orchis mascula	Basilicata, Liguria, Sardegna	42
O. mascula ssp. *ichnusae*	Sardegna	42 42+1B
O. provincialis	Basilicata, Liguria, Sardegna, Sicilia	42
O. x penzigiana	Sardegna	42
Neotinea lactea	Puglia, Sardegna	42
N. tridentata	Puglia, Sardegna	42

(Scop.) R.M. Bateman, Pridgeon, & M.W. Chase and exhibits morphology and karyotype structure similar to the latter (D'EMERICO *et al.* 1990, 1992). The taxonomy of *N. lactea* has been controversial; in fact, this taxon was first placed as *Orchis tridentata* subsp. *lactea* (SUNDERMANN 1980) and successively, always on morphological basis, as *Orchis lactea*.

In the present contribution, cytogenetical analysis was used to characterize *Neotinea lactea*, *N. tridentata*, *Orchis mascula*, *O. mascula* ssp. *ichnusae*, *O. provincialis* and *O. x penzigiana*, and to investigate the heterochromatin distribution by means of C-banding and fluorochromes.

MATERIALS AND METHODS

A list of the examined specimens is reported in Table 1.

Mitotic and meiotic chromosomes were prepared from immature ovaries, pre-treated with 0.3% colchicine at room temperature for 2h.

For Feulgen staining they were fixed for 5 min in 5:1:1:1 (v/v) absolute ethanol, chloroform, glacial acetic acid, and formalin; hydrolysed at 20°C in 5.5N HCl for 20 min (BATTAGLIA 1957a, b) and finally stained in freshly prepared Feulgen stain.

For C-banding, ovaries were fixed in ethanol-glacial acetic acid (3:1 v/v) and stored in the deep-freeze for up to several months. Subsequently, they were squashed in 45% acetic acid; coverslips were removed by the dry ice method and the preparations air-dried overnight. The slides were then immersed in 0.2N HCl at 60°C for 3 min, thoroughly rinsed in distilled water and then treated with 4% $Ba(OH)_2$ at 20°C for 4 min. After thorough rinsing they were incubated in 2xSSC at 60°C for 1h, and then stained in 3-4% Giemsa (BDH) at pH 7.

For Hoechst 33258 staining, squash preparations were made as for C-banding and then stained in a 2 µg/ml dye solution in pH 7 McIlvaine buffer for 5 min, rinsed and mounted in buffer-glycerol v/v 1:1 (PERRINO and PIGNONE 1981).

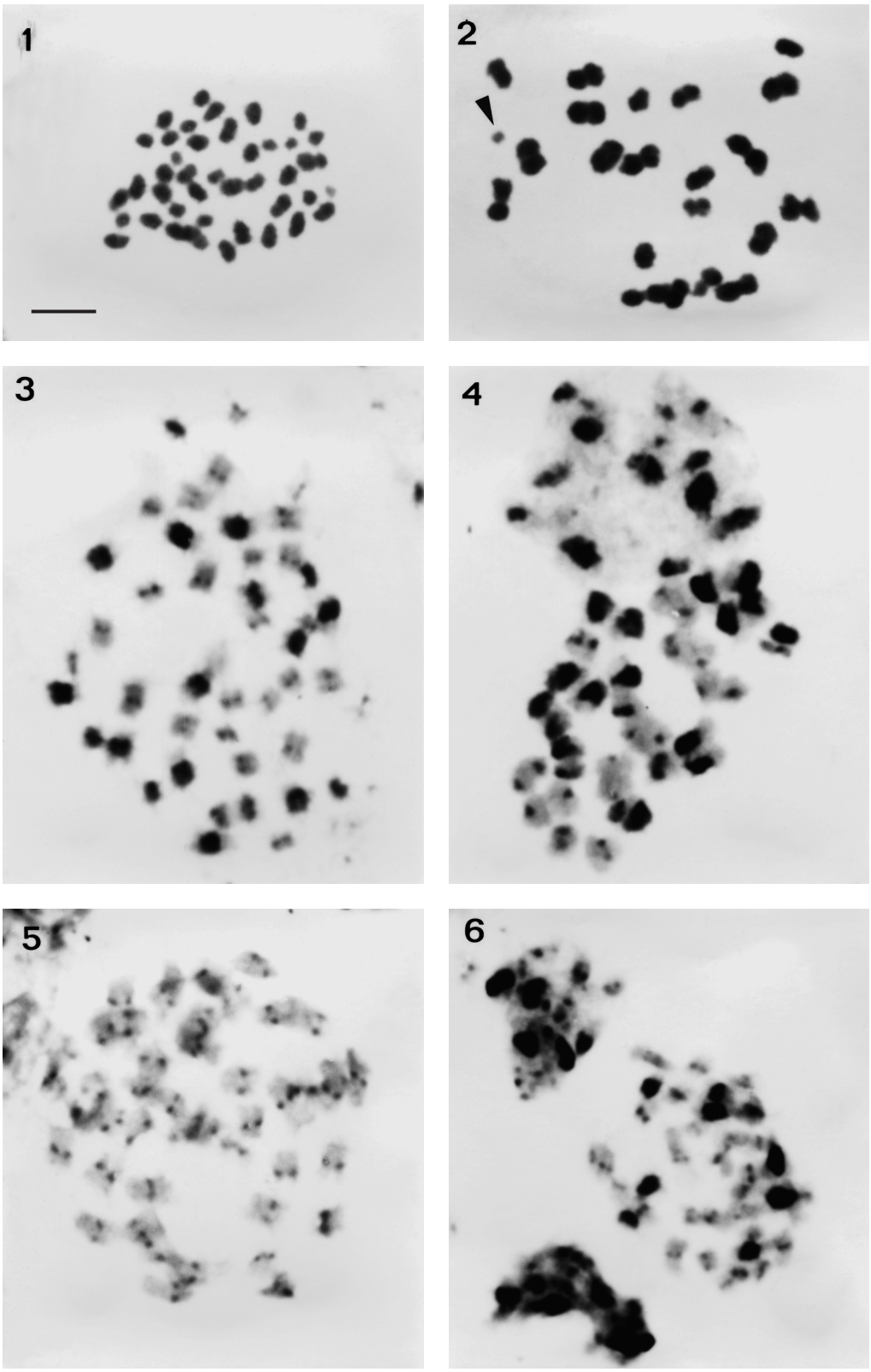
For DAPI (4-6-diamidino-2-phenylindole) staining, ovaries were treated as for H33258 and stained using a buffered DAPI solution of 0.6 µg/ml for 5 min, followed by rinsing and mounting in buffer-glycerol v/v 1:1.

Chromosome pairs were identified and arranged on the basis of their length and any other evident karyomorphological feature. The nomenclature used for describing karyotype composition followed LEVAN *et al.* (1964).

Table 2 – List of the taxa and their chromatin constitution.

Taxa		$C^+/DAPI^=$ Large Centrom.	$C^+/DAPI^=$ Small Centrom.	$C^+/DAPI^+$ Small Centrom.	$C^+/DAPI^=$ Telom.	$C^+/DAPI^+$ Telom.	$C^=/DAPI^+$ Telom.
Group *Orchis s. s.*							
Subgroup *Orchis mascula*							
	O. mascula	+	+				+
	O. mascula ssp. *ichnusae*	+	+				+
	O. provincialis		+		+*	+*	+
	O. x penzigiana	+	+		+*	+*	+
Group *Neotinea s. l.*	*N. lactea*		+#			+	
	N. tridentata			+			

* Few chromosomes only, # All dot-like, = Neutral



Figs. 1-4 – *Orchis mascula*. (1) Feulgen staining, mitotic metaphase, $2n = 42$; (2) Feulgen staining, metaphase I with 21 bivalents plus 1 B (arrowed); (3) Giemsa C-banding, somatic metaphase; (4) Giemsa C-banding, metaphase I with 21 bivalents. Fig. 5 – *Orchis provincialis*, Giemsa C-banding, somatic metaphase. Fig. 6 – *Orchis x penzigiana*, Giemsa C-banding, somatic metaphase. Bar 5 µm.

RESULTS

The results of heterochromatin reacting properties per each taxon are reported in Table 2.

All the taxa analysed were diploids with *2n* = 42 chromosomes. The karyotypes of both *O. mascula* and *O. mascula* ssp. *ichnusae* were very similar and consisted of 24 metacentric, 14 submetacentric and 4 subtelocentric chromosomes (Fig. 1). Metaphase I in embryo sac mother cells (E.M.C.s) revealed 21 bivalents. In *O. mascula* ssp. *ichnusae* one specimen showed a very small supernumerary chromosome in addition to the 21 bivalents (Fig. 2). After C-banding, somatic metaphase chromosomes in both taxa showed a particular heterochromatin distribution. In ten pairs heterochromatin was distributed on most of the chromosome length with the exclusion of the telomeric regions; the remaining eleven pairs were mostly euchromatic with the exception of thin centromeric bands (Fig. 3). C-banded Metaphase I showed ten bivalents containing the large heterochromatic bands and eleven with thin bands (Fig. 4). Interphase nuclei showed very large chromocentres.

The chromosomes of *O. mascula* after staining with both Hoechst 33258 and DAPI showed bright fluorescence at the telomeric or subtelomeric regions of many chromosomes (Fig. 7). These bands did not correspond to any C-band. Blocks appeared larger in two chromosome pairs. Interphase nuclei showed chromocentres, variable in number and dimension, in relation with the number of bright blocks observed after DAPI (or H33258) staining.

22 metacentric, 14 submetacentric and 6 subtelocentric chromosomes composed karyotype structure of *O. provincialis*. After C-banding, many chromosomes displayed thin centromeric bands; in primary constrictions this heterochromatin may be observed as two dots (Fig. 5). Two chromosome pairs exhibited heterochromatic short arm and chromosome pair 1 showed a telomeric band on long arm. Interphase nuclei showed numerous medium-small chromocentres. After staining with Hoechst 33258 or DAPI all chromosomes of *O. provincialis* showed very distinct bright blocks at telomeric regions (Fig. 9). Most of these blocks did not correspond to C-bands.

Individuals of *Orchis mascula* ssp. *ichnusae x O. provincialis* exhibited a chromosome number of *2n*=42 as both the parental species (SCRUGLI *et al.* 1976; SCRUGLI 1977). C-banding revealed 10 mostly heterochromatic and 32 mostly euchromatic chromosomes (Fig. 6). Some parental chromosomes, that are the highly heterochromatic ones deriving from the *O. mascula* parent, could be easily distinguished. After staining with DAPI showed large amounts of bright fluorescent blocks located mainly at the telomeres (Fig . 8). The general distribution of the bands appears similar to that of the parental species.

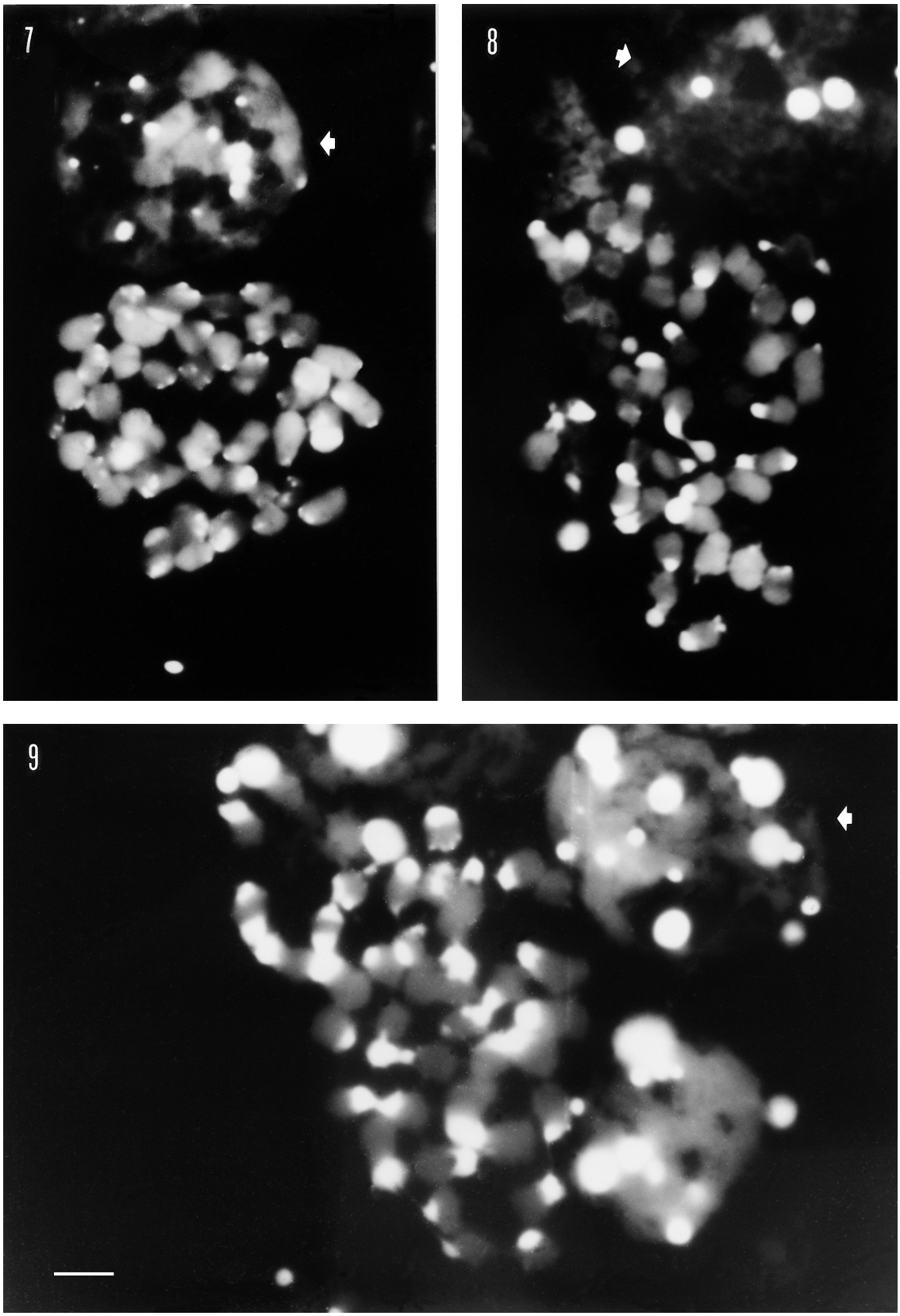
Neotinea tridentata showed a karyotype mainly of metacentric chromosomes (D'EMERICO *et al.* 1990). All chromosomes have small centromeric C-bands. After DAPI staining centromeric bands are present (data no shown).

Neotinea lactea showed a chromosome complement rather similar to those of *N. tridentata*, but with a more asymmetrical karyotype (D'EMERICO *et al.* 1992). *N. lactea* showed stronger heterochromatin bands than in *N. tridentata*. In *N. lactea*, Giemsa C-banding analysis showed centromeric heterochromatin and very conspicuous bands located at telomeric positions on many chromosomes (Fig. 10). Staining with DAPI showed bright blocks at the telomeric regions corresponding to Giemsa C-bands (Fig. 11).

DISCUSSION

Karyological analyses have evidenced that in the examined taxa both H33258 and DAPI staining produce bright fluorescent regions on many chromosomes; in fact, the two fluorochromes produce staining in regions that perfectly co-localize. For this reason, from now we will refer to chromatin with bright fluorescence reaction to DAPI and H33258 as to DAPI positive ($DAPI^+$) heterochromatin. It is interesting to point out that part of this $DAPI^+$ heterochromatin does not give positive reaction, demonstrated by dark staining, to C-banding as in other chromatin regions that are from now on indicated as C-bands or C^+ chromatin (JOHN *et al.* 1985; D'AMATO 1986; BELLA and GOSÁLVEZ 1991).

The present study demonstrates that *O. mascula* and *O. provincialis* belonging to *Orchis mascula* subgroup possess different chromatin organization. The C-banding pattern of *Orchis mascula* (such as of *Orchis mascula* ssp. *ichnusae*) displayed the peculiar presence of very large

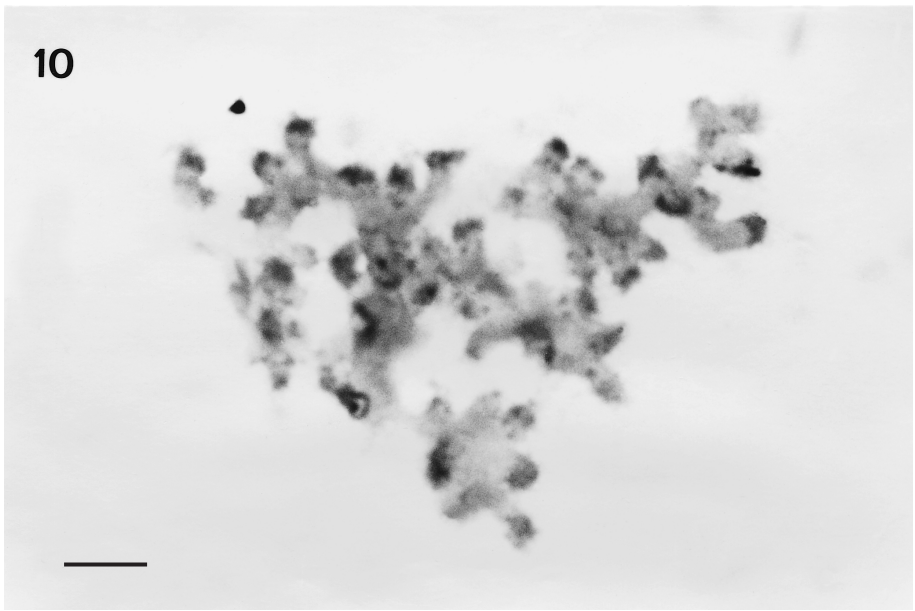


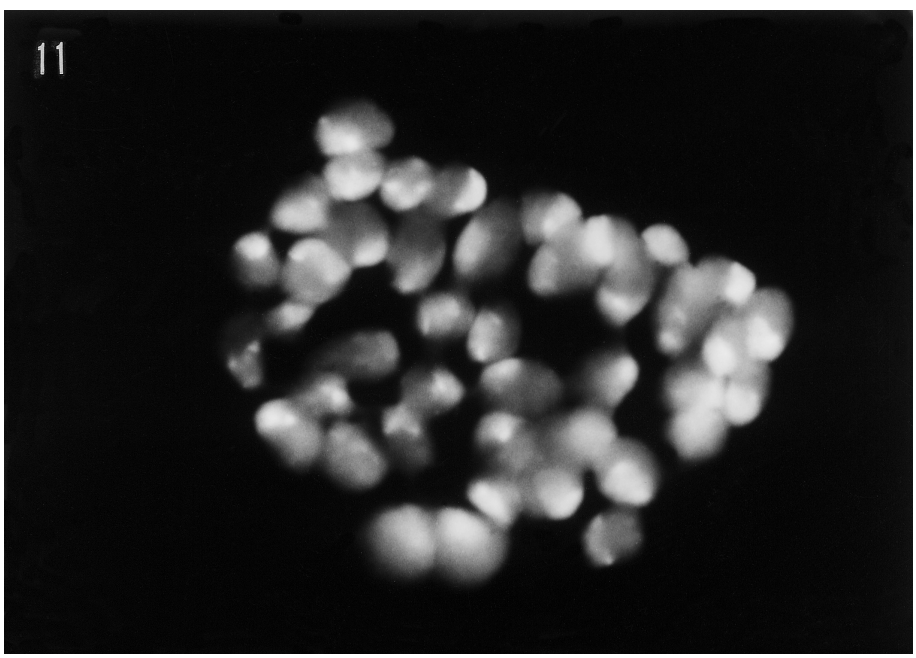
Figs. 7-9 – DAPI stained mitotic metaphase; (7) *O. mascula* ssp. *ichnusae*; (8) *Orchis x penzigiana*; (9) *Orchis provincialis*. Interphase nuclei are indicate with arrows. Bar 5 µm.

C-bands around the centromere of several chromosomes; similar heterochromatin distribution has been observed in few cases only, e.g. in *Cicer*, *Cymbidium*, *Melipona*, or *Serapias* (SCHWEIZER and NAGL 1976; GALASSO *et al.* 1996; ROCHA and POMPOLO 1998; D'EMERICO *et al.* 2000). Conversely, in *O. provincialis* C^+ chromatin is mostly located at strict centromeric domains (often seen as two dots), and only six chromosomes show telomeric bands.

Fluorescence data indicate that in both *O. mascula* and *O. provincialis* C^+ chromatin may not show $DAPI^+$ reaction. This is the case of the large centromeric heterochromatin regions of *O. mascula*, which show indifferent reaction after DAPI staining.

The heterochromatin regions observed in *O. mascula* ssp. *ichnusae* and *O. provincialis* can easily be detected in the interspecific hybrid *O. x penzigiana*. In fact, this hybrid shows 10





Figs. 10-11 – *Neotinea lactea*. (10) Giemsa C-banding; (11) DAPI stained mitotic metaphase. Bar 5 μm.

distinct chromosomes with large C+ centromeric chromatin blocks of clear *Orchis mascula* ssp. *ichnusae* origin, as well as 32 chromosomes which cannot be distinguish after C-banding (21 from *O. provincialis* and 11 from *O. mascula* ssp. *ichnusae*). After staining with DAPI, many chromosomes display bright telomeric bands as in both parental taxa. This might indicate that in the hybrid no interaction occurs among the chromatin organization of the two parental genomes.

In *Neotinea* group DAPI+ heterochromatin always co-localized with C+ bands. This indicates an organization that is quite different from that observed in the *Orchis mascula* subgroup. In the two analysed species *Neotinea lactea* and *N. tridentata* distribution of heterochromatin was different, but its organization did not differ. It is noteworthy that other taxa of *Orchis s. l.* such as *Anacamptis papilionacea* (L.) R.M. Bateman, Pridgeon, & M.W. Chase (=*Orchis papilionacea* L., *2n*=32) and *A. coriophora* (L.) R.M. Bateman, Pridgeon, & M.W. Chase (=*Orchis coriophora* L., *2n*=36) exhibit similar banding patterns when stained with C-banding and DAPI as *Neotinea lactea* (D'EMERICO *et al.* 1996 a, b). If this might be considered a junction between the 36-chromosomes *Anacamptis* species and the 42-chromosome ones is questionable. Further investigation is needed to interpret this finding.

In this connection, in other 42-chromosomes *Orchis s. s.* species such as *O. italica*, *O. purpurea* and *O. simia*, belonging to *Orchis militaris* subgroup, preliminary karyological analyses revealed that all the chromosomal chromatin had neutral reaction to both C-banding (D'EMERICO 2001) and DAPI staining (unpublished data).

Based on heterochromatin reacting properties of the considered taxa, two main clade of taxa can be identified in agreement molecular studies (BATEMAN *et al.* 1999), namely *Orchis s. s.* and *Neotinea s. l.* On the other hand, within them it is possible to distinguish particularly regarding the amounts of heterochromatin distribution.

In conclusion, the observed plasticity for heterochromatin organization in *Orchis s. l.* appears to reflect the perspective outlined by molecular data (PRIDGEON *et al.* 1999; BATEMAN *et al.* 1999). Taken altogether cytogenetical and molecular data shed a novel light on understanding the phyletic relationships among the 42-chromosomes *Orchis* species.

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