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Contribution to the study of wild *Orchidaceae*, genus *Platanthera* L.C.M. Richard. Karyotype and C-banding analysis of two species from Italy

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Abstract. This study examined the chromosome numbers and karyotypes of two *taxa* of the genus *Platanthera* (*Orchidaceae*) from Italy. Cytological analyses showed 2n = 2x = 42 in *P. chlorantha* and *P. algeriensis*. Karyotype analysis revealed similarity between the species. The karyotypes are as follows: *P. chlorantha* consists of 34 metacentric + 8 submetacentric pairs and *P. algeriensis* consists of 36 metacentric + 6 submetacentric pairs. Both species possess a rather symmetrical karyotype. *P. chlorantha* has a very similar C-banding pattern to *P. algeriensis*. DAPI bright blocks were observed in *P. chlorantha*. These analyses also show the close relationship between the studied species.

Keywords: Chromosome number, C-Banding, heterochromatin content, karyotypes, *Orchidaceae*, *Platanthera algeriensis, P. chlorantha*.

INTRODUCTION

The genus *Platanthera* Rich., also known as "butterfly orchids", belongs to the subtribe *Orchidinae* (subfamily *Orchidoideae*) and consists of 100 to 200 species (Wood 2001; Delforge 2016; Efimov 2016 and references therein). The geographical distribution of *Platanthera* species covers most of the temperate areas of Europe, North Africa, Asia, New Guinea and North and Central America (Hulténand Fries 1986; Wood 2001; Efimov 2016), with 12 species widespread in Europe, six of which are found in Italy (Delforge 2016).

This genus is divided into 5 sections (Efimov, 2016), three of which are found in Europe: *P. hyperborea*, the most ancient clade, which has a Far-Eastern and North-American distribution, with only *P. hyperborea* (L.) Lindl. present in Iceland; the *P. oligantha* clade, with a circumpolar distribution, and finally the Eurasian section *Platanthera* (Delforge, 2016). *Platanthera* species are terrestrial, photosynthetic and – in very few cases – epiphytic or lithophytic. They are found in a variety of habitats, including meadows, temperate and boreal forests, bogs, fens, marshes and prairies. European *Pla-*

tanthera species are geophytes, characterised by a broad anther, 2 elongated root-tuberoids, a dorsal sepal and petals combining to form a helmet, a stigma without processes and an enlarged receptive surface with a nectariferous spur.

In cytological analyses performed on taxa of the genus *Platanthera*, the chromosome number was found to be 2n = 2x = 42 (Cauwet-Marc and Balayer 1986; Yokota 1987; Yang and Zhu 1988; Dalgaard 1989; D'Emerico 2001 and references therein). To date however, only four taxa exhibit polyploidy: *Platanthera hyperborea* (Dalgaard 1989) and *P. huronensis* (Nutt.) Lindl. (Sheviak and Bracht 1998), both with 2n = 4x = 84 chromosomes; *P. obtusata* (Banks ex Pursh) Lindl., which may be triploid in some populations with 2n = 63 (Tanaka and Kamemoto 1984); and the Nordic–Siberian *P. oligantha* Turcz. (= *P. obtusata* subsp. *oligantha*), which according to Webb (1980) is hexaploid (2*n* = 126).

As already mentioned above, six of these species are found in Italy: *Platanthera algeriensis* Batt. & Trab. 1892, *P. bifolia* subsp. *bifolia* (L.) Rich. 1817, *P. bifolia* subsp. *osca* Lorenz, Romolini, Romano & Soca 2015, *P. bifolia* subsp. *subalpine* Brügger, *P. chlorantha* (Custer) Rchb. and *P. kuenkelei* subsp. *kuenkelei* var. *sardoa* Lorenz, Akhalk, Baumann, Cortis, Cogoni & Scrugli 2012.

In this study, karyotype morphology and the distribution of heterochromatin in Italian specimens of *P. chlorantha* and *P. algeriensis* were studied for the first time. The aim of this study was to verify chromosome numbers and to compare the heterochromatin pattern of the above-mentioned species in order to verify similarities between them.

MATERIALS AND METHODS

The material studied in this investigation was gathered from natural populations of *Platanthera chlorantha* and *P. algeriensis* in Apulia and Sardinia (Table 1).

Mitotic 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 5.5 N HCl for 20 min (Battaglia 1957) and stained in freshly prepared Feulgen solution.

For C-banding, ovaries were fixed in 3:1 (v/v) ethanol–glacial acetic acid and stored in a 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 were 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 (D'Emerico et al. 1996). For DAPI (4–6-diamidino-2-phenylindole) staining, ovaries were treated as for C-banding and stained using a buffered DAPI solution (0.6 mg/mL) for 5 min, followed by rinsing and mounting in glycerol buffer (1:1 v/v).

Chromosome pairs were identified and arranged on the basis of their length and any other evident karyomorphological feature. Heterochromatin content was assessed using MicroMeasure 3.3, a freeware program from Colorado State University (Reeves 2001). Karyotype symmetry indices – Mca (Mean Centromeric Asymmetry) and CVcl (Coefficient of Variation of Chromosome Length) – were used for the evaluation of karyotype asymmetry (Peruzzi et al. 2009).

The nomenclature used for describing karyotype composition followed Levan et al. (1964). A list of the examined specimens and their sampling locations is given in Table 1.

RESULTS AND DISCUSSION

Analysis of the somatic metaphases showed that the diploid chromosome number is 2n = 2x = 42 in both *Platanthera chlorantha* and *P. algeriensis*.

P. chlorantha, known as the "Greater Butterfly Orchid" (Lima-de-Faria 2020) was found to be diploid with 2*n* = 2*x* = 42 chromosomes (Fig. 1a), in agreement with previous reports (Scrugli 1980; Averyanov et al. 1985; Cauwet-Marc and Balayer 1986), with chromo-

Table 1. Taxon, sites, chromosome number, formula and percent heterochromatin in set of the chromosomes of species *Platanthera chloranta* and *P. algeriensis*. m, metacentric; sm, submetacentric.

Taxon	Site	Chromosome number (2n)	Formula	% Het in set
P. chlorantha	Martina Franca (TA) Balvano (PZ)	42	34m+8sm	25.50
P. algeriensis	Aritzo (NU)	42	34m+2m(sm)+6sm	22.43

Figure 1. Mitotic metaphase with Feulgen staining of *Platanthera chlorantha* (a) and *P. algeriensis* (b); 2n = 2x = 42. Bar = 5 μm.

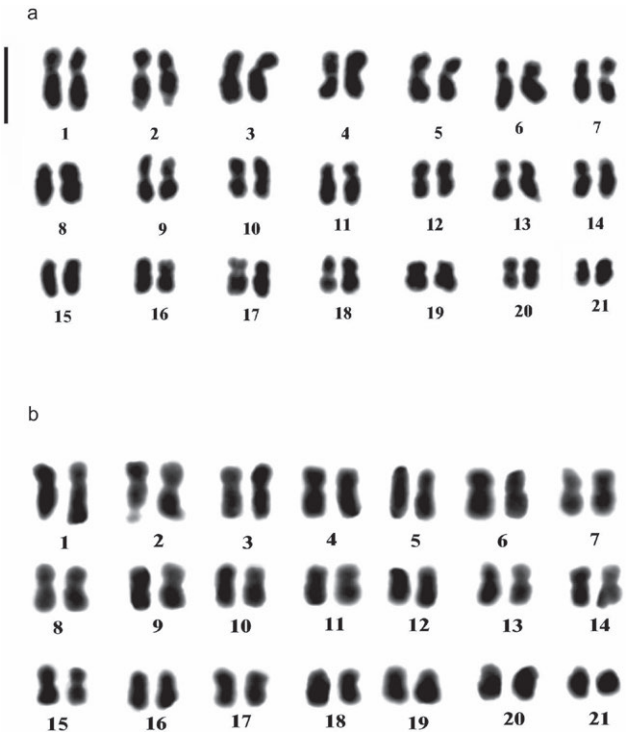

Figure 2. Diploid karyotypes of *Platanthera chlorantha* (a) and *P. algeriensis* (b). Bar = 5 μm.

somes that range in size from 4.04 to 1.8 μm at metaphase, and the arm length ratio was from 1.03 to 3.00. Six well spread-out metaphases were paired on the basis of chromosome size and centromere position and used for chromosome measurements. The karyotype consisted of 34 metacentric and 8 submetacentric chromosome pairs (Fig. 2a). The complement showed two chromosome pairs with secondary constrictions on the long arm (pair 2) and the short arm (pair 8).

This species possesses a fairly symmetrical karyotype (Mca = 18.50±1.09 and CVcl = 21.53±0.29), with metacentric chromosomes being the most frequent. It is interesting to note that this species has similarities in terms of dimensions and structure (such as the visibility of centromeres) with karyotypes of the *Anacamptis* group (2n = 2x = 36) (D'Emerico et al. 1996). Similarities with *Chamorchis alpina* (L.) Rich. (2n = 2x = 42) (D'Emerico and Grünanger 2001) and *Dactylorhiza romana* (Sebast.) Soò (2n = 2x = 40) (D'Emerico et al. 2002) can also be observed.

The C-banding analysis shows that constitutive heterochromatin was located in the centromeric regions of numerous chromosomes (Fig. 3a). One pair of chromosomes had the subtelomeric C-bands only on the short arm. Interphase nuclei exhibited a number of chromocentres equal to that of the constant bands (Fig. 3b). The centromeric regions of numerous chromosomes had bright fluorescence after staining with DAPI (Fig. 3c).

In *Platanthera algeriensis*, somatic cells showed 2n = 2x = 42 chromosomes (Fig. 1b). This species is similar to *P. chlorantha* apart from its greener flowers and its very different preferred habitat. In Europe this species is restricted to a few sites in Corsica, Sardinia, mainland Italy and Spain, but it also occurs in Algeria. In this species, similarities to the karyotype structure and C-banding of *P. chlorantha* were observed. Chromosome lengths were found to be between 3.90 and 2.18 μm. The karyotype consisted of 34 metacentric, 2 metacentric/submetacentric and 6 submetacentric chromosome pairs (Fig. 2b). In addition, this species possesses a symmetrical karyotype (Mca = 21.21±0.83 and CVcl = 18.19±0.60). Constitutive heterochromatin was also detected in the centromere regions of numerous chromosomes (Fig. 3d).

The present analysis of chromosome evolution showed that the species *P. chlorantha* and *P. algeriensis* are very close. Indeed, specimens of the two species in the present study exhibited practically the same karyotype and C-banding pattern. However, *P. algeriensis* seems to differ from *P. chlorantha* in that it has lower heterochromatin content (Fig. 3-d vs. Fig. 3-a). The small

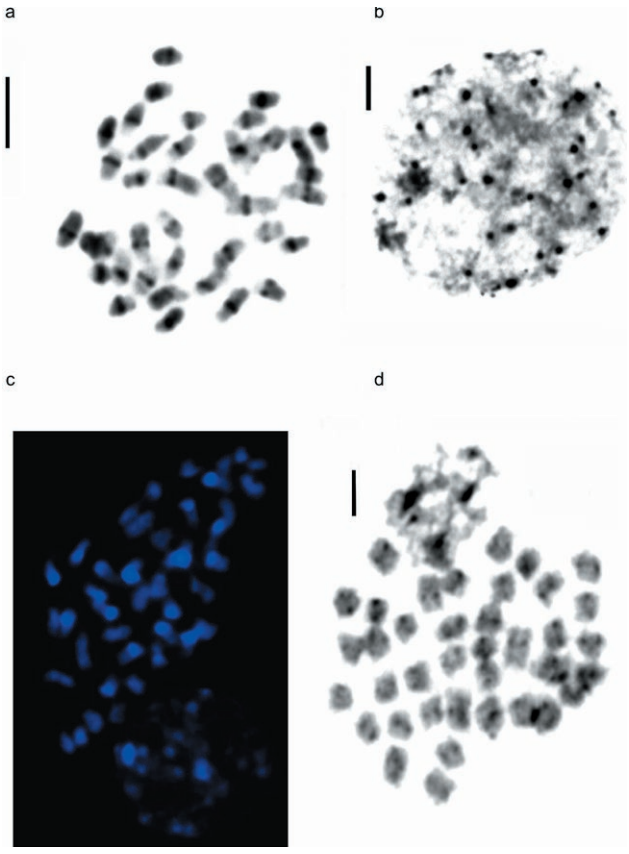

Figure 3. Giemsa C-banded mitotic metaphase of *Platanthera chlorantha* (a) and *P. algeriensis* (d); in *P. chlorantha*, interphase nuclei exhibit numerous chromocentres (b); *P. chlorantha* DAPI stained mitotic metaphase (c). Bar = 5 μm.

differences in the C-banding patterns found between the two species seem to indicate limited rearrangement of constitutive heterochromatin during their evolution.

In a study based on plastid DNA sequence variation, Pavarese et al. (2011) found that *Platanthera algeriensis* was characterized by haplotypes A and B, both of which are shared with *P. chlorantha*. In another study, Italian *Platanthera chlorantha* were found to form a single group with *P. algeriensis* from Tunisia and Sardinia (Bateman et al. 2012).

CONCLUDING REMARKS

The chromosome number 2n = 2x = 42 has been reported in 13 of the 17 genera for which data are available, although the subtribe *Orchidinae* includes about 50 genera (D'Emerico 2001; Felix and Guerra 2005). In this subtribe, the Internal Transcribed Spacer (ITS) phylogenies (Bateman et al. 2001; Bateman et al. 2003; Jin et al. 2017) show that the group *Pseudorchis-Amerorchis-Galearis-Platanthera* s.l. includes genera with the chromosome number 2n = 2x = 42 (Löve and Simon 1968; Löve 1981; Cauwet-Marc and Balayer 1986). Also it is suggested that *Dactylorhiza* s.l. and *Gymnadenia* s.l., which have 2n = 2x = 40, probably derive from 42 chromosomes (Pridgeon et al. 1997; Bateman et al. 2009).

As pointed out in the discussion, it is interesting to note the remarkable karyomorphological similarity in the species *Platanthera chlorantha*, *P. algeriensis* (this work), *Chamorchis alpina* (D'Emerico and Grunanger 2001), *Galearis diantha* (Schltr.) P. F. Hunt (Luo 2004), which have 2n = 2x = 42 chromosomes and *Dactylorhiza romana* (D'Emerico et al. 2002), which has 2n = 2x = 40 chromosomes.

Last but not least, in spite of the extensive cytogenetic literature that has built up over the years, little is known about the karyotype structure in other species of the genus *Platanthera* and related genera. Furthermore, to our knowledge, there are few studies of constitutive heterochromatin content, despite the fact that C-banding patterns provide extra information useful in assigning genomes.

We have a limited quantity of data on the *Platanthera* genus but it is possible to make some considerations on heterochromatin content. Indeed, *Platanthera chlorantha* and *P. algeriensis* show centromeric and subtelomeric heterochromatin, with a higher percentage in the former. On this basis, an evolutionary comparison is possible with the genera *Dactylorhiza* and *Gymnadenia*. Previous cytological studies using the traditional Giemsa C-banding technique have shown significant heterochromatin content in some species of these two genera (D'Emerico et al. 2002; D'Emerico and Grunanger 2001; Baumann et al. 2012). For example, in the genus *Gymnadenia*, *G. rhellicani* (Teppner and Klein) Teppner and Klein, *G. conopsea* and *G. odoratissima* (L.) Rich. have been found to possess numerous chromosomes with centromeric and telomeric heterochromatin. Similar banding patterns were previously observed in three species of the genus *Dactylorhiza*, including two diploids (*D. romana*, *D. saccifera* (Brogn.) Soò) and one polyploid (*D. urvilleana* subs. *phoenissa* B. Baumann and H. Baumann). Moreover, the distribution of C-banding patterns in *Dactylorhiza* karyotypes is of great cytological interest, although its nature can only be conjectured for the time being. Specifically, *D. romana* specimens have shown the chromosome numbers 2n = 40+1B and 2n = 40+2B, with one or two heterochromatic supernumerary chromosomes, which seems to suggest a possible evolutionary trend from 2n = 42 to 2n = 40 (D'Emerico et al. 2002).

For a better understanding of phylogenetic relationships within the genus *Platanthera*, cytogenetic analysis should be extended to other species. Moreover, new chromosome methods such as fluorescent *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) will help to solve these problems using chromosomal analysis techniques.

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