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Karyotype structure, supernumerary chromosomes and heterochromatin distribution suggest a pathway of karyotype evolution in *Dactylorhiza* (Orchidaceae)

SAVERIO D'EMERICO[1*], SALVATORE COZZOLINO[2], GIUSEPPE PELLEGRINO[3], DOMENICO PIGNONE[4] AND ANTONIO SCRUGLI[5]

[1]*Dipartimento di Biologia e Patologia Vegetale, Università di Bari, Via Orabona, 4 I-70125 Bari, Italy*
[2]*Orto Botanico, Università Federico II-Napoli, Italy*
[3]*Dipartimento di Ecologia, Università della Calabria-Rende, Italy*
[4]*C.N.R. Istituto del Germoplasma, Bari, Italy*
[5]*Dipartimento di Scienze Botaniche, Università di Cagliari, Italy*

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The relationships between *Dactylorhiza romana* and *D. saccifera* from southern Italy were analysed. These two species, both with $2n = 2x = 40$ chromosomes and belonging to different sections of the genus, were distinguishable on the basis of karyotype structure and heterochromatin amounts and distribution. Their C-banded karyotypes differed considerably. *D. saccifera* showed most chromosomes with banded regions in the short arms, whereas in *D. romana* the bands were located mostly at telomeric regions of longer arms. Several individuals of *D. romana* had one or two very large heterochromatic supernumerary chromosomes. Based on evidence resulting from karyotype structure and heterochromatin distribution in the two species and on the genetic distances derived from the comparison of ITS sequences, it is suggested that *D. romana* represents a primitive form with respect to *D. saccifera* and is a possible intermediate step in the evolution of the genus *Dactylorhiza* from the 42-chromosome *Orchis* group.
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ADDITIONAL KEYWORDS: – chromosome morphology – *Dactylorhiza romana* – *D. saccifera* – heterochromatin banding – phyletic relationships.

INTRODUCTION

Dactylorhiza Necker ex Nevski is a genus of Orchidinae (Orchidaceae) that includes about 50 taxa (Quentin, 1995). The genus has a basic chromosome number of $x = 20$ and consists mostly of diploid ($2n = 2x = 40$) and tetraploid ($2n = 4x = 80$) species (Soó, 1980; Moore, 1982), the exceptions being *D. sambucina* with $2n = 40$ or 42 (Cauwet-Marc & Balayer, 1984 and references therein) and *D. insularis* with $2n = 3x = 60$ (Scrugli, 1977; Gathoye & Tyteca, 1989), which is an apomictically reproducing allotriploid derived from *D. romana* and *D. sambucina* (Bullini *et al*., 2001). Chromosomes in the genus are small in size and similar to those of the 42-chromosome *Orchis* species (D'Emerico *et al.*, 1993). The karyotype structure of the genus is known from only three taxa, namely *D. maculata* ssp. *meyeri* (Vöth & Greilhuber, 1980), *D. sambucina* (Cauwet-Marc & Balayer, 1984) and *D. insularis* (D'Emerico *et al.*, 1992).

Since chromosome number changes via auto- or allopolyploidy seem to represent one of the main sources of speciation within the genus (Averyanov, 1990; Hedrén, 1996), preliminary karyological analyses of diploid species represent a fundamental step towards the understanding of the evolutionary trends in the genus.

In this paper we report on the karyomorphology and chromosomal heterochromatin distribution in *Dactylorhiza romana* and *D. saccifera*, sampled from different sites in southern Italy. In order to derive a better insight into karyotype evolution, the karyological

*Corresponding author.
E-mail: demerico@botanica.uniba.it

Table 1. Taxa examined in the present study, their collecting sites, number of investigated specimens and chromosome number

Taxon	Sites	Number of investigated specimens and flower colour	Chromosome number (2*n*)
Dactylorhiza romana	Martina Franca (Taranto); Gargano Peninsula (Foggia), Apulia; near Catania (Sicily)	Yellow: 18	40
		Purple: 6	40
		Purple: 10	40 + 1B
		Purple: 2	40 + 2B
D. saccifera	Gargano Peninsula (Foggia), Apulia; Cilento (Salerno), Campania; Madonie (Sicily)	20	40

results will be discussed in the light of the genetic similarity between taxa as disclosed by the analysis of the internal transcribed spacer (ITS) sequences of the ribosomal DNA (Bateman *et al.,* 1997; Aceto *et al*., 1999).

MATERIAL AND METHODS

Mitotic chromosomes were prepared from immature ovaries, pretreated with 0.3% colchicine at room temperature for 2 h.

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

For C-banding, ovaries were fixed in ethanol–glacial acetic acid (3 : 1 v/v) 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 air-dried overnight. The slides were then immersed in 0.2 N 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 1 h, 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 the same buffer (Perrino & Pignone 1981).

For DAPI (4-6-diamidino-2-phenylindole) staining, ovaries were treated as for H33258 and stained using a buffered (pH 7 McIlvaine) DAPI solution (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).

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

RESULTS

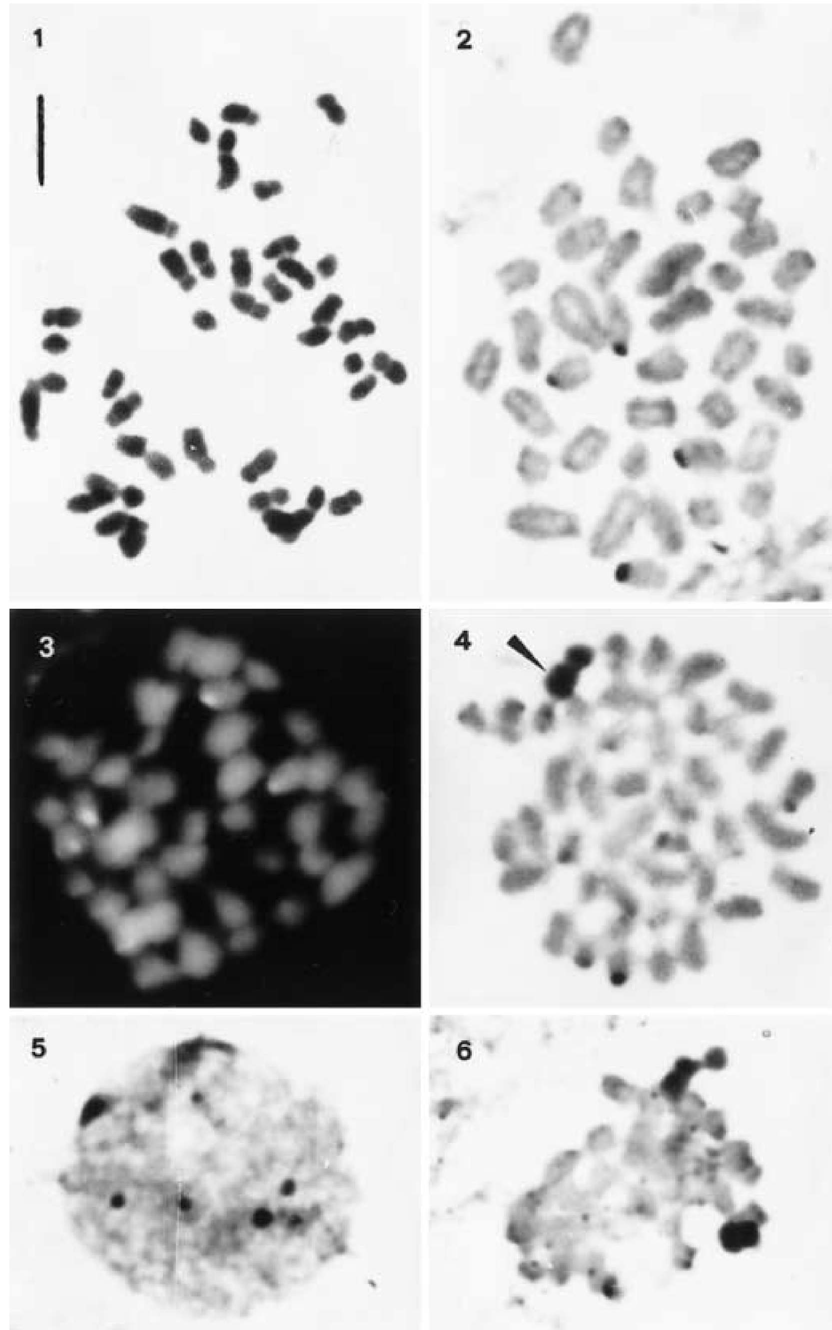
Dactylorhiza romana (Sebastiani) Soó

Plants from Apulia and Sicily show 2*n* = 2*x* = 40 chromosomes (Fig. 1), in agreement with previous reports (Del Prete *et al.,* 1980; Bianco *et al*., 1987). However, many individuals with purple flowers from Martina Franca (Apulia) show 2*n* = 41 chromosomes (40 + 1 B metacentric). In addition, two specimens with purple flowers have 2*n* = 42 (40 + 2 B metacentric). The karyotype structure of this species is 16m + 22sm + 2st chromosomes. Individual chromosomes vary in length from 1.00 to 3.33 μm.

Five chromosome pairs show evident telomeric heterochromatin after Giemsa C-banding. Four large bands are located at the long arm telomere of pairs 7, 13, 15, and 16, while a small but clear band is on the short arm telomere of pair 10. Inconstant centromeric bands are seen on all chromosomes (Figs 2 and 11a). Interphase nuclei exhibit a number of chromocentres that equals that of the constant bands (Fig. 5). The supernumerary chromosomes are heterochromatic, as evidenced by the C-banding (Figs 4 and 6). After DAPI or H33258 staining the major C-bands exhibit a degree of fluorescence, while the centromeric faint ones do not show any differential reaction (Fig. 3). Unfortunately, DAPI staining in individuals with 2n = 41 chromosomes failed to identify the supernumerary chromosomes.

Dactylorhiza saccifera (Brogn.) Soó

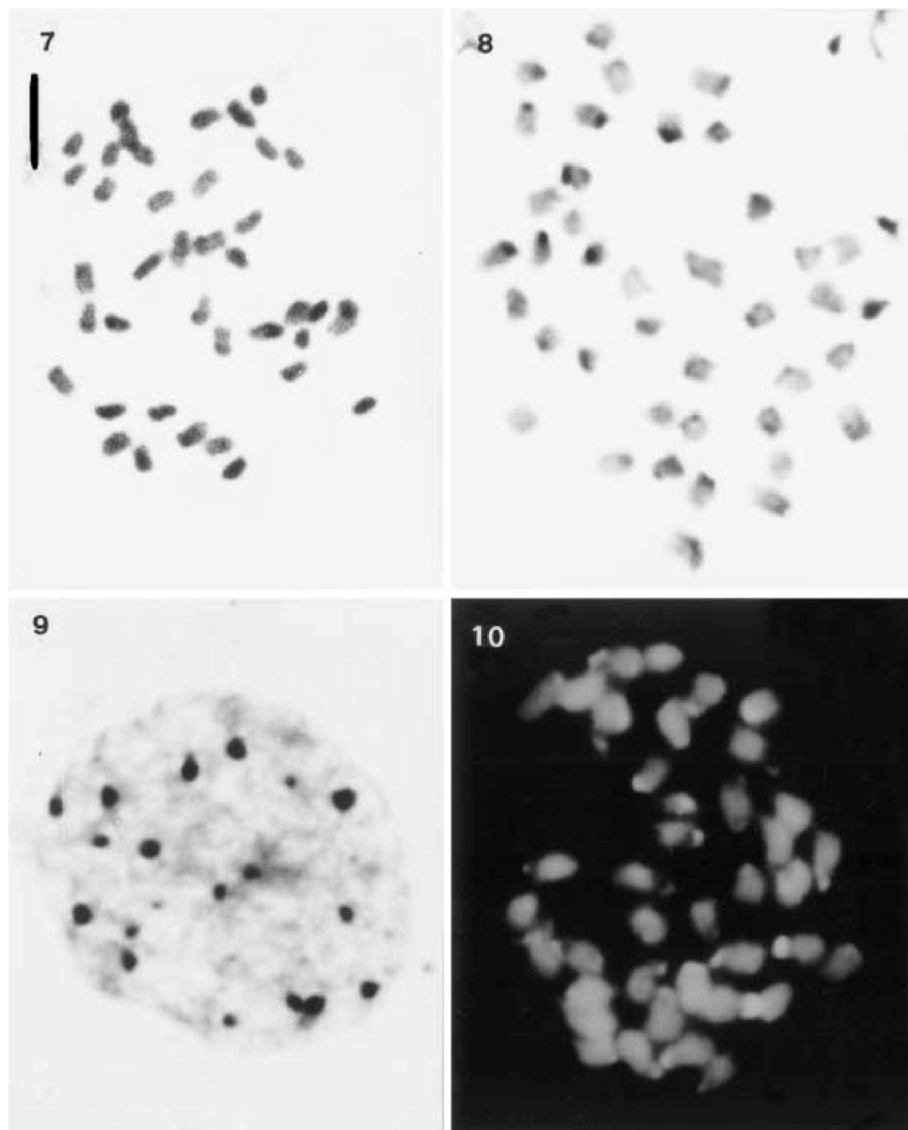
Plants from the Gargano Peninsula (Apulia), Cilento (Campania) and Madonie (Sicily) areas have 2*n* = 40 (Fig. 7), in agreement with reports by Cauwet-Marc &



Figures 1–6. *Dactylorhiza romana*. All figures to same scale, bar = 5 µm. Fig. 1. Feulgen staining, mitotic metaphase, 2n = 40. Fig. 2. Giemsa C-banding, metaphase. Fig. 3. H33258 stained, mitotic metaphase. Fig. 4. Giemsa C-banding, metaphase showing one heterochromatic supernumerary chromosome. It is interesting to note the main heterochromatic blocks joined by poorly stained small gap (arrow). Fig. 5. Giemsa C-banding, interphase nucleus. Fig. 6. C-banding, metaphase showing two heterochromatic B-chromosomes.

Balayer (1984) and Gathoye & Tyteca (1989). The karyotype is 32m + 8sm. Chromosome lengths vary from 1.00 to 2.17 µm.

Most chromosomes have telomeric C-bands located mainly on the short arms. The largest bands are those on pairs 2, 6, 8, 9, 12 and 18 (Figs 8 and 11b). When chromosomes are stained with Hoechst 33258 or DAPI (Fig. 10), positive bands on the short arms can be detected in the same domains where C-bands are located. Interphase nuclei have 12 chromocentres,



Figures 7–10. *Dactylorhiza saccifera*. All figures to same scale, bar = 5 μm. Fig. 7. Feulgen staining, mitotic metaphase, 2n = 40. Fig. 8. Giemsa C-banding, mitotic metaphase. Fig. 9. C-banding, interphase nucleus. Fig. 10. H33258 stained, mitotic metaphase.

distinguished from the remaining spots by their larger size/intensity (Fig. 9).

DISCUSSION

The karyotypes of *Dactylorhiza romana* and *D. saccifera* proved to be quite different. *D. saccifera* has smaller chromosomes, a more uniform karyotype, a higher average content of C-banded heterochromatin and a larger number of heterochromatic domains than *D. romana*. Chromosome uniformity in *D. saccifera* makes it difficult to identify homologous chromosomes solely on the basis of centromere position, whereas the chromosomes of *D. romana* are identified more easily.

In earlier classifications, the two species were allocated to two different sections of *Dactylorhiza*, namely, *D. romana* in *Sambucinae* and *D. saccifera* in *Dactylorhiza* (Soó, 1968). Nevertheless, Nei's indices of genetic distance based on isozyme data (Nei, 1972) indicate a low degree of phyletic separation (Rossi *et al.*, 1995). The observed differences in chromosomal morphology and amount and distribution of C-banded heterochromatin suggest that the two species are separated quite widely. Additionally, the two taxa display different ITS sequences that might imply a mecha-

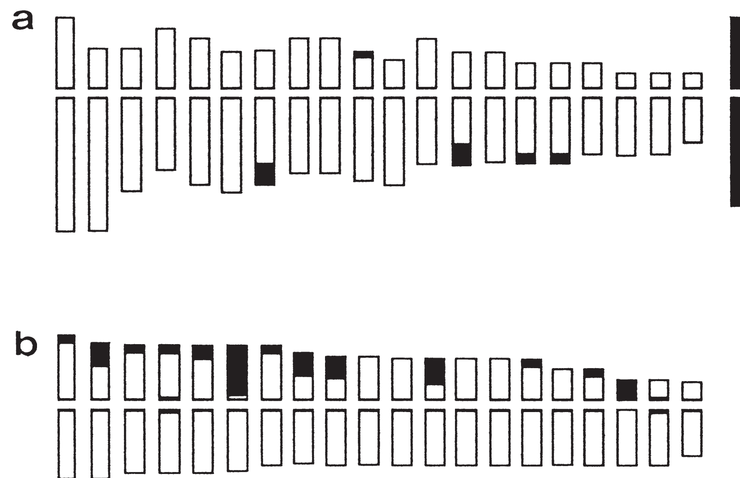


Figure 11. Idiograms showing basic chromosome sets and heterochromatin distribution. (a) *Dactylorhiza romana*. (b) *D. saccifera*.

nism of reproductive isolation. In fact, *D. romana* (European Molecular Biology Laboratory accessions DRZ94065 and DRZ94066) and *D. saccifera* (Cozzolino *et al.*, in prep.) showed ITS1 sequences 246 and 247 bp long and ITS2 sequences 241 and 237 bp long, respectively. When aligned, the ITS1 and ITS2 sequences of the two taxa showed 9 base substitutions (2 transitions and 7 transversions), 3 insertion/deletions and a sequence identity of 97.4%. In this regard, even if the two species are close from a phylogenetic point of view, they display levels of genetic identity (and, of course, of genetic diversity) comparable to those of other distinct (pairs of) orchid species. For instance, the same range of identity between ITS sequences (97.4%) has been found between two pairs of close and clearly distinct *Orchis* species, namely in *O. militaris*/*O. simia* and in *O. mascula*/*O. provincialis* (Aceto *et al.*, 1999).

Previous karyomorphological studies have suggested that *D. romana* is related closely to the 42-chromosome *Orchis* species (D'Emerico *et al.*, 1990, 1992, 1993; Bianco *et al.*, 1991). Moreover, Cauwet-Marc & Balayer (1984) and Gathoye & Tyteca (1989) pointed out that *D. sambucina* with 40 or 42 chromosomes possesses the most ancestral karyotype in *Dactylorhiza*, in terms of chromosome number and karyotype structure. These data suggest that the *Sambucinae* group (Soó, 1968), including *D. sambucina* and *D. romana*, is probably the primitive group from which the whole *Dactylorhiza* genus evolved. Additionally, the presence in *D. romana* and *D. sambucina* of several individuals with $2n = 41$ (40 + 1 B) and $2n = 42$ (40 + 2 B) chromosomes reinforces the idea that the $2n = 40$ karyotypes originated from an ancestral $2n = 42$ through chromosome number reduction and heterochromatinization of a chromosome pair. The supernumerary chromosomes, in this hypothesis, could represent an intermediate step in which the chromosomes are progressively depleted of functional genes and are eventually lost. These data further support the basal position of the *Sambucinae* clade. In this perception, the karyotype of *D. saccifera* (which seems to be a more derived clade) represents a secondary arrangement that occurred during the rapid radiation of the genus. In this context, molecular studies on nucleotide sequences of the ITS1 and ITS2 from a wide selection of Orchidinae (Pridgeon *et al.*, 1997; Aceto *et al.*, 1999) have assessed the basal position of *D. romana* within the genus *Dactylorhiza*. These data indicated a close relationship between this genus and the $2n = 42$ *Orchis* clade, from which *Dactylorhiza* probably evolved.

Dactylorhiza romana has individuals with yellow or purple flowers. Most samples with purple flowers from Martina Franca have one or, rarely, two heterochromatic supernumerary chromosomes (Table 1), which are always absent in the analysed yellow-flower individuals and were not detected in purple-flower individuals from the other stations. The association between purple flowers and supernumerary chromosome(s) is thus established for only the Martina Franca population. Nevertheless the association does not appear to be merely stochastic, since no yellow-flower individuals ever showed supernumerary chromosomes. It can be argued either that (a) this association is present only in one station as a consequence of specific ecological/adaptive conditions, or (b) the frequency of the association in the other stations is lower, and it will be observed when more purple-flower individuals will be analysed.

It is interesting to point out that the supernumerary chromosomes found in the Martina Franca population of *D. romana* are large and have morphology and size similar to the longest A-chromosomes of the complement. This is quite unusual, since previous reports of supernumerary chromosomes in the Orchidaceae have always referred to small heterochromatic B-chromosomes (Aoyama & Tanaka, 1988; Teppner *et al*., 1994). The heterochromatin structure of the supernumerary chromosomes appeared to be nonhomogeneous. Less-contracted chromosomes comprised two main heterochromatic blocks joined by a poorly stained small gap. This pattern is similar to that of the A-chromosomes and might substantiate an internal origin of the supernumerary chromosome due to a rapid process of genomic turnover (Stark *et al*., 1996) and therefore support the specificity of this population as specified in the former hypothesis.

As a final note, it must be pointed out that the specimens carrying the supernumerary chromosomes were found at the extreme edge of the species range. It has been suggested that supernumerary chromosomes might be related to geographical or adaptive radiation of a species (Rosato *et al*., 1997; Ding *et al*., 1998). It has been observed that *Neotinea ustulata* (= *Orchis ustulata*) and *N. tridentata* (= *Orchis tridentata*) also have supernumerary chromosomes in samples from the edge of their geographical range (D'Emerico *et al*., 1990). It is possible that the occurrence of the supernumerary chromosomes in wild Orchidaceae is related to adaptation and might be the result of a rapid genome restructuring as a response to adaptation (Stark *et al*., 1996).

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REFERENCES

Aceto S, Caputo P, Cozzolino S, Gaudio L, Moretti A. 1999. Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. *Molecular Phylogenetics and Evolution* **13:** 67–76.

Aoyama M, Tanaka R. 1988. Notable chromosome numbers in *Cymbidium lancifolium*, *C. javanicum* and *C. nipponicum*. *Journal of Japanese Botany* **63:** 329–333.

Averyanov LV. 1990. A review of the genus *Dactylorhiza*. In: Arditti, J, ed. *Orchid biology: review and perspectives*, V. Timber Press. Portland, Oregon, pp. 159–206.

Bateman RM, Pridgeon AM, Chase MW. 1997. Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 2. Infrageneric relationships and reclassification to achieve monophyly of *Orchis sensu stricto. Lindleyana* **12:** 113–141.

Battaglia E. 1957a. A new '5 minutes-fixation' using cold hydrolysis. *Caryologia* **9:** 368–370.

Battaglia E. 1957b. A simplified Feulgen method using cold hydrolysis. *Caryologia* **9:** 372–373.

Bianco P, D'Emerico S, Medagli P, Ruggiero L. 1991. Polyploidy and aneuploidy in *Ophrys*, *Orchis* and *Anacamptis* (Orchidaceae). *Plant Systematics and Evolution* **178:** 235–245.

Bianco P, Medagli P, D'Emerico S, Ruggiero L. 1987. Numeri cromosomici per la Flora Italiana. *Informatore Botanico Italiano* **19:** 322–332.

Bullini L, Cianchi R, Arduino P, De Bonis L, Mosco MC, Verardi A, Porretta D, Corrias B, Rossi W. 2001. Molecular evidence for allopolyploid speciation and a single origin of the western Mediterranean orchid *Dactylorhiza insularis* (Orchidaceae). *Biological Journal of the Linnean Society* **72:** 193–201.

Cauwet-Marc AM, Balayer M. 1984. Les genres *Orchis* L., *Dactylorhiza* Necker ex Nevski, *Neotinea* Reichb. et *Traunsteinera* Reichb: caryologie et proposition de phylogénie et d'évolution. *Botanica Helvetica* **94:** 391–406.

Del Prete C, Garbari F, Giordani A. 1980. Numeri cromosomici per la Flora Italiana: 690–695. *Informatore Botanico Italiano* **12:** 117–120.

D'Emerico S, Bianco P, Medagli P. 1992. Karyological studies on Orchidaceae. Tribe Ophrydeae, subtribe Serapiadinae. *Caryologia* **45:** 301–311.

D'Emerico S, Bianco P, Medagli P. 1993. Considerazioni citotassonomiche su alcuni generi di Orchidaceae. *Informatore Botanico Italiano* **25:** 86–89.

D'Emerico S, Bianco P, Medagli P, Ruggiero L. 1990. Karyological studies of some taxa of the genera *Himantoglossum*, *Orchis*, *Serapias and Spiranthes* (Orchidaceae) from Apulia (Italy). *Caryologia* **43:** 267–276.

Ding KY, Ge S, Hong DY, **Yu ZH. 1998.** Cytotype variation and cytogeography of *Scilla sinensis* (Louriro) Merrill (Hyacinthaceae) in China. *Hereditas-Landskrona* **129:** 151–160.

Gathoye JL, Tyteca D. 1989. Contribution à l'Etude Cytotaxonomique des *Dactylorhiza* d'Europe Occidentale. *Mémoires de la Société Royale de Botanique de Belgique* **11:** 30–42.

Hedrén M. 1996. Genetic differentiation, polyploidization and hybridization in northern European *Dactylorhiza* (Orchidaceae): evidence from allozyme markers. *Plant Systematics and Evolution* **201:** 31–55.

Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for centromeric position on chromosomes. *Hereditas* **52:** 201–220.

Moore RG. 1982. *Flora Europaea. Check-List and Chromosome Index*. Cambridge University Press, Cambridge.

Nei M. 1972. Genetic distance between populations. *American Naturalist* **106:** 283–292.

Perrino P, Pignone D. 1981. Contribution to the taxonomy of *Vicia* species belonging to the section Faba. *Kulturpflanze* **29:** 311–319.

Pridgeon AM, Bateman RM, Cox AV, Hapeman JR, Chase MW. 1997. Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphyly of *Orchis sensu lato. Lindleyana* **12:** 89–109.

Quentin P. 1995. *Synopsis Des Orchidées Européennes. Cahiers de la S.F.O., No. 2*. Paris.

Rosato M, Chiavarino AM, Naranjo CA, Poggio L. 1997. B chromosomes in native races of maize from Argentina. II. Genome size and altitude. *Maize Genetics Cooperation Newsletter* **71:** 47–48.

Rossi W, Arduino P, Cianchi R, Piemontese N, Bullini L. 1995. Genetic divergence between *Dactylorhiza romana* and *D. saccifera* (Orchidaceae), with description of their natural hybrid. *Webbia* **50:** 1–15.

Scrugli A. 1977. Numeri cromosomici per la flora italiana. *Informatore Botanico Italiano* **9:** 116–125.

Soó R De.,. 1968. Die Geschichte der Erforschung der Gattung *Orchis* (sensu lato), besonders von *Dactylorhiza*. *Jahresberichte des Naturwissenschaftlichen Vereins in Wuppertal* **21/22:** 7–19.

Soó R De.,. 1980. Genus *Dactylorhiza*. In: Tutin, TG, Heywood, VH, Burges, NA, Moore, DM, Valentine, DH, Walters, SM, Webb, DA, eds. *Flora Europea*, 5. Cambridge University Press. Cambridge.

Stark EA, Connerton I, Bennett ST, Barnes SR, Parker JS, Forster JW. 1996. Molecular analysis of the structure of the maize B-chromosome. *Chromosome Research* **4:** 15–23.

Teppner H, Klein E, Drescher A, Zagulskij M. 1994. *Nigritella carpatica* (Orchidaceae-Orchideae) – a relic endemic of the Eastern Carpathians. Taxonomy, distribution, karyology and embryology. *Phyton-Horn.* **34:** 169–187.

Vöth W, Greilhuber J. 1980. Zur Karyosystematik von *Dactylorhiza maculata* s.1. und ihrer Verbreitung, Insbesondere in Niederösterreich. *Linzer Biologische Beiträge* **12:** 415–468.