

Advances in chromosomal studies in Neottieae (Orchidaceae): constitutive heterochromatin, chromosomal rearrangements and speciation

BARTOLO^{1*} GIUSEPPINA, CRISTIAN BRULLO¹, SANTA PULVIRENTI¹, ANTONIO SCRUGLI², MARIA CARMEN TERRASI¹ and SAVERIO D'EMERICO^{1,3}

¹Dipartimento di Botanica, Università di Catania, via A. Longo 19, 95125, Catania, Italia

²Dipartimento di Scienze Botaniche, Università di Cagliari, viale S. Ignazio 13, 09123, Cagliari, Italia

³Dipartimento di Biologia e Patologia Vegetale, Università di Bari, via E. Orabona 4, 70126, Bari, Italia

Abstract — In this work, we describe a karyomorphological study on three taxa of the tribe Neottieae (Orchidaceae). *Epipactis aspromontana* and *E. schubertiorum* are characterized by a chromosome complement of $2n = 2x = 38$. Significant differences in heterochromatin distribution were found between them. Similarities in the karyotype structure and C-banding of *E. schubertiorum* and *E. helleborine* group have been observed. A specimen of *E. aspromontana* showed a triploid chromosome number. The meiosis are characterized by univalent, bivalent and trivalent forms and in some somatic metaphase cells has been possible to observe a series of aneuploid numbers with 46, 47, 48, 49, 50, 51, 52 and 53 chromosomes. The largest differences can be emphasized between the *Epipactis* species and *Neottia nidus-avis*, mainly in the the karyomorphology and heterochromatin distributions. In *Neottia nidus-avis* the evolution process seems to be determined by reversing Robertsonian mutations.

Key words: Chromosome banding, *Epipactis*, karyotypes, *Neottia*, Robertsonian mutations.

INTRODUCTION

The tribe Neottieae (*Orchidaceae*) comprises about 100 species of autotroph or saprophytic orchids grouped in two subtribes: *Limodoriinae* with 4 genera (*Aphyllorchis*, *Cephalanthera*, *Epipactis* and *Limodorum*) and *Listerinae* with 2 genera (*Listera* and *Neottia*), distributed in northern hemisphere and in the tropical Africa and Asia (DRESSLER 1993).

The chromosome complement in the species belonging to this tribe is extremely variable ($2n = 20, 32, 34, 36, 38, 40, 44, 48, 56, 60$ and 64). The most species of this tribe have been karyologically studied by many authors (COUTINHO

1957; KLIPHUIS 1963; MEILI-FREI 1966; MEHRA and KASHYAP 1983; D'EMERICO *et al.* 1999). Several genera are characterized by asymmetrical chromosome complements with a bimodal distribution as concerns the chromosome length. STEBBINS (1971) suggested that the bimodality is an extreme and specialized form of karyotype asymmetry, arising from a combination of centric fissions, pericentric inversions and unequal translocations.

Disploidy was already indicated as mechanism of karyotype evolution in some orchid genera, such as *Epipactis*, *Limodorum* and *Listera* (D'EMERICO *et al.* 1999; D'EMERICO *et al.* 2000). It is likely that the mechanism involved in the dispoloid differentiation of chromosomes is the centric fusion or fission (SCHWARZACHER and SCHWEIZER 1982; COX *et al.* 1998; RYAN *et al.* 2000).

Giemsa C-banding technique, which stains constitutive heterochromatin, was used to identify individual chromosomes in many species, as

*Corresponding author: phone: 00-39-095430901; fax: 00-39-095-441209; e-mail: giuseppinabrullo@gmail.com

well as for investigating taxonomic relationships among different species (VOSA 1975; FLAVELL 1986; GILL and SEARS 1988; D'EMERICO *et al.* 1996). Moreover, staining techniques represent additional tools for studying genome evolution among closely related diploid and polyploid species (MORRIS and GILL 1987). In previous studies, the analysis of the distribution of heterochromatin in some chromosome pairs revealed to be homogeneous in *Cephalanthera* and *Epipactis*, thus supporting a possible palaeo-polyploid origin (D'EMERICO *et al.* 1999).

Aim of this paper is to provide an overview on the recent researches concerning the Italian species belonging to the genera *Epipactis* and *Neottia*.

MATERIALS AND METHODS

A list of the examined specimens is given in Table 1. Voucher specimens or photographs have been deposited in the Herbarium of the Department of Botany of Catania (CAT), Department

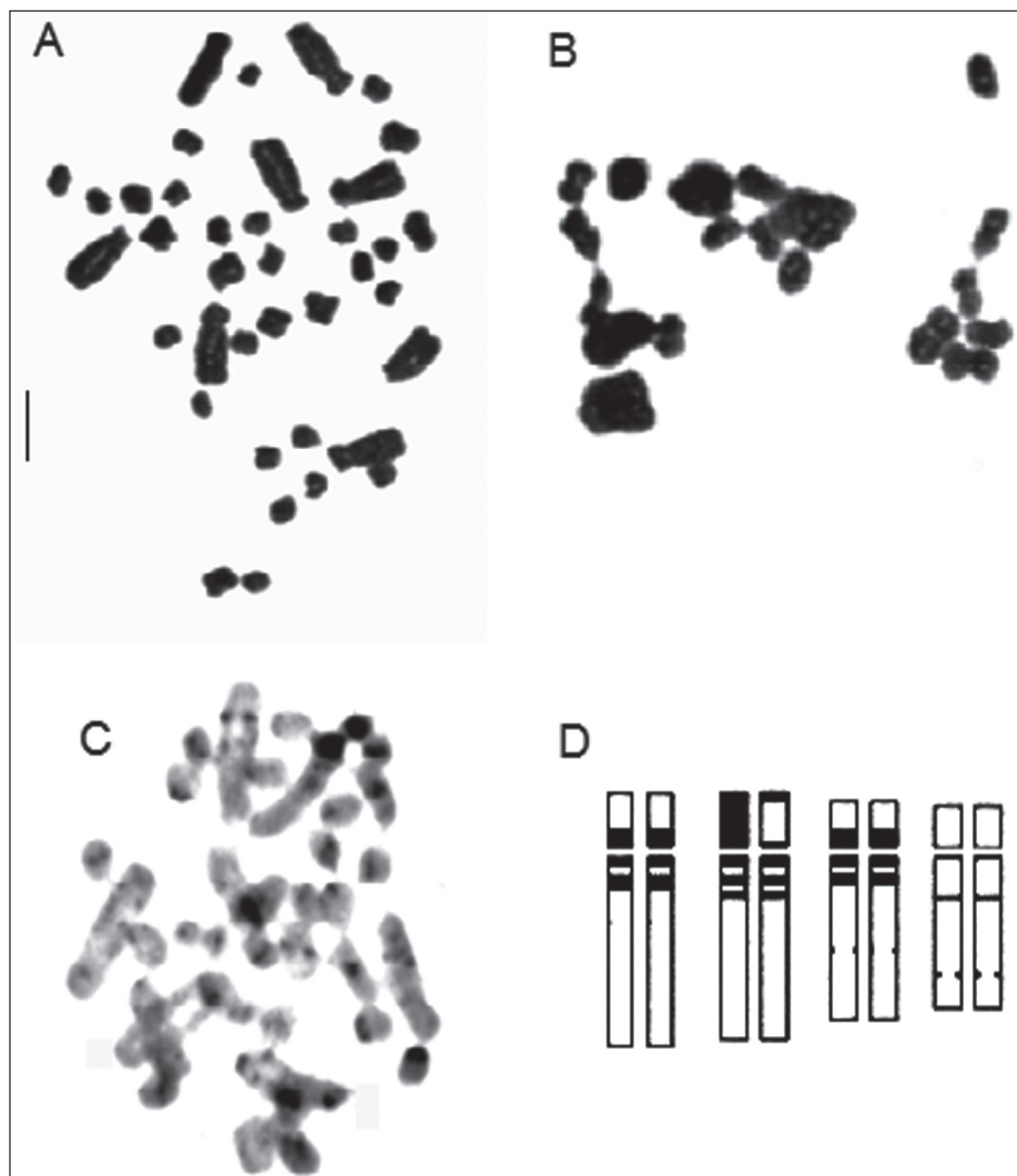


Fig. 1 — Diploid *Epipactis aspromontana*. (A) Feulgen staining, somatic metaphase, $2n=38$. (B) Feulgen staining, metaphase I, 19 bivalents. (C) Giemsa C-banded somatic metaphase. (D) Partial idiograms showing heterochromatin distribution in long chromosome pairs. Bar = $5\mu\text{m}$

of Botany of Cagliari (CAG), Department of Plant Biology of Bari (BI).

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 5min in 5:1:1:1 (v/v) absolute ethanol, chloroform, glacial acetic acid and formalin, hydrolysed at 20°C in 5.5N HCl for 20min (BATTAGLIA 1957) 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 state temperature for 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 3min, 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.

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

RESULTS

The investigated species are the following:

Epipactis aspromontana Bartolo, Pulvirenti et Robatsch.

The chromosome number of this species observed on individuals coming from Aspromonte (S Calabria) is $2n = 2x = 38$, and is reported for the first time (Figure 1A). Meiotic studies from embryo sac mother cells (E.M.C.s) revealed 19 bivalents at metaphase I (Figure 1B). The karyo-

type is bimodal and consists of 4 large and 15 small chromosome pairs, of which 6 are metacentric, 11 submetacentric and 2 subtelocentric. All chromosomes show centromeric bands. Pairs 1 and 3 are characterized by a centromeric band and one intercalary band on the long arm. Characteristic of this species is the occurrence of an evident C-band, occupying almost the whole short arm of pair 2 of long chromosomes. Several small chromosome pairs show heterochromatic short arms (Figs. 1C,D).

In the present study, we found one individual where has been possible to observe a series of aneuploid numbers, with 46, 47, 48, 49, 50, 51, 52 and 53 chromosomes (Figs. 2A,B). Metaphase I from E.M.C.s revealed univalent, bivalent and trivalent forms (Figure 2C). Staining with C-banding technique showed centromeric, telomeric and intercalary C-bands. A large number of chromocentres with a wide ranging size was observed in interphase nuclei.

Epipactis schubertiorum Bartolo, Pulvirenti et Robatsch.

At beginning, this species was found at 900-1000 m a.s.l. in the pine-woods or beech-woods of Serra San Bruno (Calabria, S Italy). The chromosome number of the investigated populations is diploid with $2n = 2x = 38$ chromosomes (Figure 3A). Metaphase I in E.M.C.s revealed 19 bivalents (Figure 3B). The karyotype of *E. schubertiorum* consists of 4 large and 15 small chromosome pairs. Interphase nuclei show 2 large chromocentres corresponding to the number of bands detected on pair 3. Pairs 1 and 2 show a medium-large band on the short arm proximal to the centromere and an intercalary band on the long arm. Pair 3 shows an intercalary band on the short arm and a large band on the long arm (Figure 3C). In addition, this pair shows a

TABLE 1 — Neottieae taxa investigated, origin of samples, voucher specimens and chromosome numbers.

Taxon	Locality	Collector/voucher	Chromosome number
<i>Epipactis aspromontana</i>	Calabria: Aspromonte, Torrente Listi, 24/07/2001	Bartolo et al./017922/CAT	$2n = 2x = 38$
<i>E. schubertiorum</i>	Calabria: Serra San Bruno, 25/07/2001	Schubert et al./KL; Bartolo et al./018040/CAT	$2n = 2x = 38$
<i>Neottia nidus-avis</i>	Puglia: Gargano, Foresta Umbra, 30/05/2007	D'Emérico/Medagli/BI	$2n = 2x = 36$
	Basilicata: Muro Lucano, 24/05/2008	D'Emérico/Medagli/BI	
	Sardegna: Laconi, Casa Broccu, 27/05/1998	Scrugli/CAG	

great similarity in C-banding to *E. helleborine* group. Pair 4 shows only a thin intercalary band on the long arm. Some small chromosome pairs show terminal bands on the short arm.

Neottia nidus avis (L.) L.C.M. Richard.

This species occurs throughout Europe and

partly in Asia (Korea and Japan). The chromosome number detected for the individuals collected in different localities is $2n=2x=36$ (Figure 4A), and coincides with other previous investigations (SCRUGLI 1977; CAPINERI and ROSSI 1987; RUIZ 1995). This species has a karyotype consisting of 11 metacentric, 1 submetacentric,

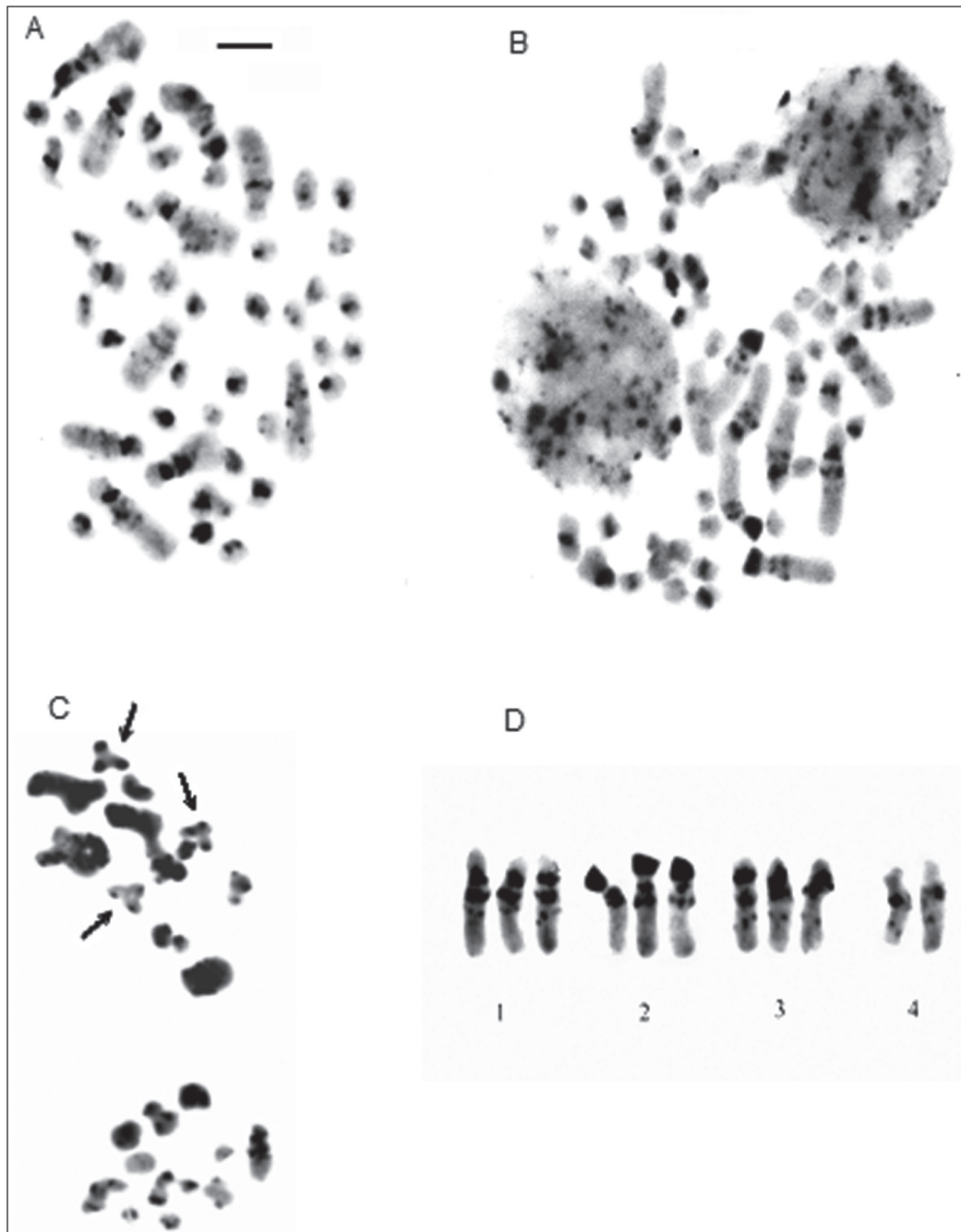


Fig. 2 — Triploid *Epipactis aspromontana*. (A) Giemsa C-banded somatic metaphase with 46 chromosomes. (B) Giemsa C-banded somatic metaphase with 52 chromosomes. (C) Giemsa C-banded, metaphase I, (The arrow points to the trivalents). (D) Partial Giemsa C-banded karyotype showing significantly C-banded chromosomes. Bar = $5\mu\text{m}$

2 submetacentric and 4 telocentric pairs. (Figure 4E). Chromosome pair 1 is long. Pairs 2 to 18 are progressively shorter. Pair no. 11 possess a small satellite on the short arm. All chromosomes have centromeric C-bands (Figure 4D). Pair 2 shows terminal C-band in the long arm. Pairs 3, 5 and 18 have heterochromatic short arms.

Occasionally, in somatic metaphases two or three telocentric chromosomes showed association between them (Figs. 4B,C).

DISCUSSION

Previous cytogenetical reports on the genera *Cephalanthera* (SCHWARZACHER and SCHWEIZER 1982; D'EMERICO *et al.* 1999; D'EMERICO *et al.* 2000; MOSCONE *et al.* 2007), *Epipactis* (D'EMERICO *et al.* 1999), *Limodorum* (BARTOLO *et al.* 2002), and *Listera* (VOSA and BARLOW 1972; D'EMERICO *et al.* 2000), have showed some cases of chromosome alterations generated by Robertsonian fission and quantitative heterochromatin distribu-

tion mainly located in the chromosomes of the large group.

The species of the genus *Epipactis* surveyed in this study are *E. aspromontana* and *E. schubertiorum* occurring in southern Apennine (Italy). *E. aspromontana* is a rare endemic orophyte known from Aspromonte and Serre Calabre (S-Italy), growing on deep soils along mountain streams in the undergrowth of beech-woods at an elevation of 1100-1440 m of altitude. Its chromosome number $2n = 2x = 38$ is quoted for the first time and the karyomorphology is rather similar to that observed in other species of *Epipactis*, as *E. palustris*, *E. gracilis*, *E. distans*, *E. helleborine* group, *E. microphylla*, *E. atrorubens* (D'EMERICO *et al.* 1999). On the other hand, *E. aspromontana* is quite different from the species belonging to the *E. helleborine* group. The main difference concerns the C-band heterochromatin (known to contain highly repetitive DNA) distribution, in fact some C-bands are absent on the larger chromosomes pairs of *E. aspromontana*, a feature that distinguishes it quite well from the other

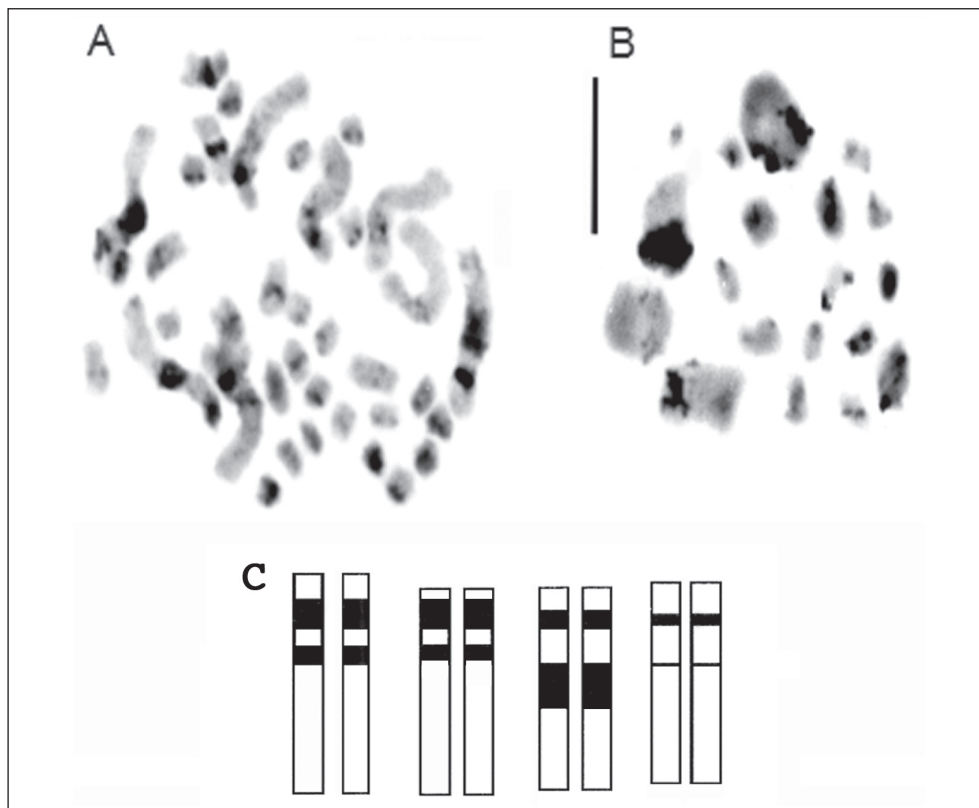


Fig. 3 — *E. schubertiorum*. (A) Giemsa C-banded somatic metaphase, $2n=38$; (B) Giemsa C-banded, metaphase I, 19 bivalents; (C) Partial idiograms showing heterochromatin distribution in long chromosome pairs. Bar = $5\mu\text{m}$

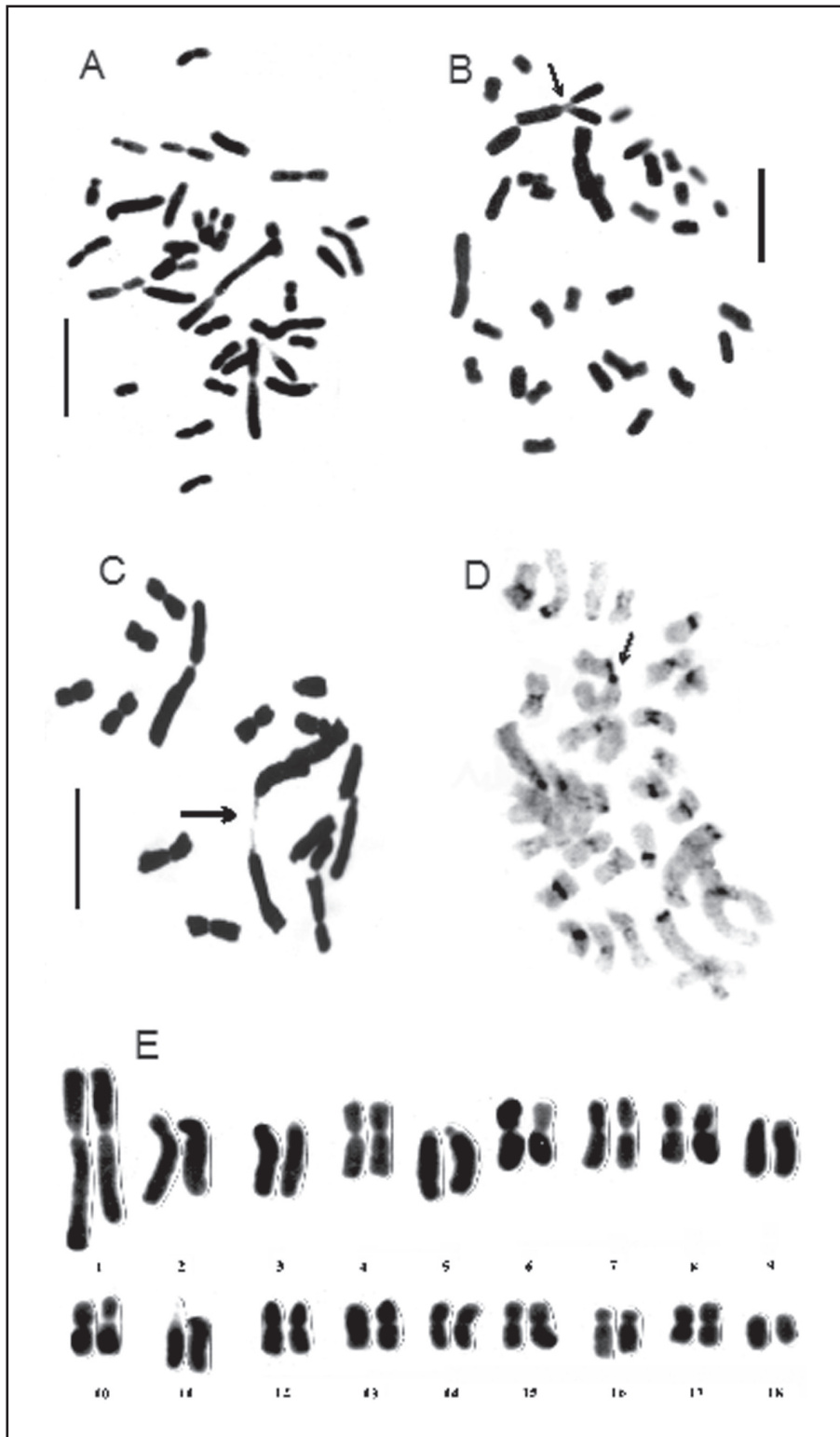


Fig. 4 — *Neottia nidus-avis*. (A) Feulgen staining, somatic metaphase, $2n=36$. (B) Feulgen staining, somatic metaphase. Note that there are three telocentric chromosomes associated between them. (C) Feulgen staining, somatic metaphase. Note that there are two telocentric chromosomes associated between them. (D) Giemsa C-banded somatic metaphase. Note that there are two telocentric chromosomes associated between them. (E) Diploid Feulgen karyotype. Bar = $5\mu\text{m}$

species of the *E. helleborine* group. In this context, *E. helleborine*, *E. placentina*, *E. tremolsii*, *E. robatschiana* and *E. meridionalis* (D'EMERICO *et al.* 1999; BARTOLO *et al.* 2003; BARTOLO *et al.* 2006) are characterized by conspicuous bands in the large chromosome pair 3, while *E. aspromontana* shows a large band that occupies entirely the short arm of pair 2 of long chromosome. However, in some individuals of the latter species pair 2 constantly shows a high cytogenetic heterogeneity in the quantity of heterochromatin among homologous chromosomes (Figure 1D).

A complex situation is observed in a triploid individual of *E. aspromontana*. There were no significant morphological difference between triploid and diploid individuals. Moreover no tetraploid were found. Triploid individuals may appear sometimes in diploid populations as a consequence of non-reduction at meiosis, thus producing diploid gametes (BRANDHAM 1982). Trivalents, few bivalents and univalents have been detected in the meiotic plates. On the other hand, numerous mitotic metaphase plates show a very variable chromosome number (46, 47, 48, 49, 50, 51, 52 and 53). In these metaphase plates, it was possible to recognize three sets of base (Figure 2D). In this context, in somatic chromosome number cells was previously reported by NAIR (2007).

In spite of *E. aspromontana*, the C-band of *E. schubertiorum* appears to be more related to that observed in the species of the *E. helleborine* group. In fact, in pair 3 *E. schubertiorum* shows a large band on the long arm near the centromere, and some pairs characterized by the occurrence of heterochromatic short arms. As already emphasized by D'EMERICO (1999), C-banding technique shows that all the surveyed *Epipactis* species have some chromosome pairs characterized by heterochromatic short arms. The origin of this C-banding is probably due to the centromeric dissociation occurring in some metacentric or submetacentric chromosomes followed by heterochromatin amplification. The data indicate that centric fission produces variation in chromosome number within populations, thus increasing the probability of the genetic isolation and speciation (PERRY *et al.* 2004).

The subtribe *Listerinae* is considered as derived from an *Epipactis*-like ancestor (DRESSLER 1993). Based on the karyotype morphology, it is possible to differentiate quite well *Neottia nidus-avis* from the *Epipactis* species. Also noteworthy is the high number of large telocentric chromosome pairs in comparison with *Epipactis*

species. In some specimens of *Neottia nidus-avis* from Muro Lucano (Potenza, Italy), associations among telomeric chromosomes are frequently observed in metaphase.

Centric fusion has been inferred in *Cephalanthera* and *Epipactis*, also based on the observation of quadruple structures in the centromeric regions as revealed by C-banding (SCHWARZACHER and SCHWEIZER 1982; D'EMERICO *et al.* 1999). In our work, C-banding technique evidenced association between short arms completely heterochromatic of two telocentric chromosomes. Heterochromatin is considered to be responsible for maintaining important structural features of chromosomes. Moreover, alterations in the amount of heterochromatin can be induced by structural rearrangements (JOHN and MIKLOS 1979). Among the possible hypotheses regarding the chromosomal evolution of *Neottia nidus-avis*, we suggest that some chromosomal telocentric pairs in this species have arisen from an ancestral karyotype via fission. These pairs could then be transformed via fusions in meta/subcentromeric chromosomes and therefore generate a new cytotype.

Finally, based on our data it seems that both reversing Robertsonian mutations (fission fusion) and heterochromatin variation are extremely important in the karyotype differentiation of Neottieae species.

REFERENCES

- BARTOLO G., D'EMERICO S., PULVIRENTI S., SCRUGLI A. and TERRASI M.C., 2002 — *Karyotype structure and chromosome banding in Limodorum brulloi Bartolo & Pulvirenti (Orchidaceae)*. J. Eur. Orchideen, 34: 87-96.
- BARTOLO G., D'EMERICO S., PULVIRENTI S., TERRASI M.C. and STUTO S., 2003 — *Epipactis robatschiana (Orchidaceae), a new species from Calabria (Southern Italy)*. Caryologia, 56: 439-445.
- BARTOLO G., D'EMERICO S., PULVIRENTI S. and TERRASI M.C., 2006 — *Chromosomal structure and heterochromatin distribution in Epipactis meridionalis Baumann & Lorenz (Orchidaceae)*. J. Eur. Orchideen, 38(1): 33-38.
- BATTAGLIA E., 1957 — *A new e5 minutes-fixation fusing cold hydrolysis*. Caryologia, 9: 368-370.
- BRANDHAM P.E., 1982 — *Inter-embryo competition in the progeny of autotriploid Aloineae (Liliaceae)*. Genetica, 59: 29-42.
- CAPINERI R. and ROSSI W., 1987 — *Numeri cromosomici per la flora Italiana*. Informatore Botanico Italiano, 19: 314-318.
- CLARK M.S. and WALL W.J., 1996 — *Chromosomes, the complex code*. Chapman and Hall, London. pp. 237-243.

- COX A.V., ABDELNOUR G.J., BENNETT M.D. and LEITCH I.J., 1998 — *Genome size and karyotype evolution in the slipper orchids (Cypripedioideae: Orchidaceae)*. American Journal of Botany, 85: 681-687.
- D'EMERICO S., PIGNONE D. and BIANCO P., 1996 — *Karyomorphological analyses and heterochromatin characters disclose phyletic relationships among $2n=32$ and $2n=36$ species of Orchis (Orchidaceae)*. Plant Systematics and Evolution, 200: 111-124.
- D'EMERICO S., GRÜNANGER P., SCRUGLI A. and PIGNONE D., 1999 — *Karyomorphological parameters and C-bands distribution suggest phyletic relationship within the subtribe Limodorinae Bentham (Orchidaceae)*. Plant Systematics and Evolution, 217: 147-161.
- D'EMERICO S., PIGNONE D. and SCRUGLI A., 2000 — *Karyomorphology and evolution in italian populations of three Neottieae species (Orchidaceae)*. Cytologia, 65: 189-195.
- DRESSLER R.L., 1993 — *Phylogeny and classification of the orchid family*. Portland, Oregon, Dioscorides Press., 314 pps.
- FLAVELL R.B., 1986 — *Repetitive DNA and chromosome evolution in plants*. Phil. Trans. R. Soc. Lond. B, 312: 227-242.
- GILL B.S. and SEARS R.G., 1988 — *The current status of chromosome identify chromosomes of diploid B. riparius and it analysis in wheat*. In J.P. Gustafson and R. Appelsb (ed.) Chromosome structure and function. Plenum, New York., pp. 299-321.
- JOHN B. and MIKLOS G.L.G., 1979 — *Functional aspects of satellite DNA and heterochromatin*. Intern. Rev. Cytology, 58: 1-14.
- KLIPHUIS E., 1963 — *Cytological observations in relations to the taxonomy of the orchids of the Netherlands*. Acta Bot. Neerl., 12: 172-194.
- LEVAN A., FREDGA K. and SANDBERG A.A., 1964 — *Nomenclature for centromeric position on chromosomes*. Hereditas, 52: 201-220.
- MEILI-FREI E., 1966 — *Cytogenetik und Cytotaxonomie einheimischer Arten von Epipactis, Listera, Neottia (Orchidaceae)*. Berichte der Schweizerischen Botanischen Gesellschaft, 75: 219-292.
- MOSCONE E.A., SAMUEL R., SCHWARZACHER T., SCHWEIZER D. and PEDROSA-HARAND A., 2007 — *Complex rearrangements are involved in Cephalanthera (Orchidaceae) chromosome evolution*. Chromosome Research, 15: 931-943.
- NAIR R.R., 2007 — *Aneuploid variation of chromosome number in the somatic cells of Piper magnificum Trel*. Cytologia, 72: 239-242.
- PERRY J., SLATER R.H. and CHOO H.K., 2004 — *Centric fission-simple and complex mechanism*. Chromosome Research, 12: 627-640.
- RAMSEY J. and SCHEMSKE D.W., 1998 — *Pathways, mechanisms and rates of polyploid formation in flowering plants*. Annual Review of Ecology and Systematics, 29: 477-501.
- RUIZ L., 1995 — *Mediterranean chromosome number reports 5*. Flora Mediterranea, 5: 363-373.
- SCHWARZACHER T. and SCHWEIZER D., 1982 — *Karyotype analysis and heterochromatin differentiation with Giemsa C-banding and fluorescent counterstaining in Cephalanthera (Orchidaceae)*. Plant Systematics and Evolution, 141: 91-113.
- SCRUGLI A., 1977 — *Numeri cromosomici per la flora italiana*. Informatore Botanico Italiano, 9: 116-125.
- STEBBINS G.L., 1971 — *Chromosomal evolution in higher plants*. Edward Arnold, London, UK.
- VOSA C.G. and BARLOW P.W., 1972 - *Meiosis and B chromosomes in Listera ovata (Orchidaceae)*. Caryologia, 25: 1-8.
- VOSA C.G., 1975 — *The use of Giemsa and other staining techniques in karyotype analysis*. Curr Adv. Plant Sci., 14: 495-510.

Received February 4th 2010; accepted June 16th 2010