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Advances in the Study of Orchidinae Subtribe (Orchidaceae) Species with 40,42-Chromosomes in the Mediterranean Region

Alessio Turco [1], Robert Philipp Wagensommer [2,*], Pietro Medagli [1], Antonella Albano [1] and Saverio D'Emerico [3]

[1] Department of Biological and Environmental Sciences and Technologies, University of the Salento, 73100 Lecce, Italy; alessio.turco@unisalento.it (A.T.); pietro.medagli@unisalento.it (P.M.); antonella.albano@unisalento.it (A.A.)
[2] Faculty of Education, Free University of Bozen-Bolzano, 39042 Brixen-Bressanone, Italy
[3] "Aldo Moro" University of Bari, 70125 Bari, Italy; sdeme@yahoo.it
* Correspondence: robertphilipp.wagensommer@unibz.it

Abstract: This study presents an updated analysis of cytogenetic data for several species within the 40,42-chromosome genera of the subtribe *Orchidinae*. The research includes insights into the distribution of heterochromatin obtained using C-banding and fluorochrome techniques. Our investigation confirmed variation in the distribution of heterochromatin and repetitive DNA sequences among species pertaining to *Neotinea* s.l. and *Orchis* s.str. These variations also potentially contribute to the diversification of these species. Cytogenetic analyses of the *Neotinea* group demonstrated that both H33258 and DAPI staining result in blocks of fluorescent regions on numerous chromosomes. Particular attention was paid to the cytological composition of the polyploid *Neotinea commutata*, focusing on its potential origin. Based on the karyological results acquired, a hypothesis concerning the origin of *N. commutata* is proposed. The most noteworthy revelations regard the *O. mascula* complex. In these species, the telomeric areas of all chromosome sets display extensive heterochromatin. Fluorochrome staining revealed telomeric blocks on many chromosomes that were not seen with Giemsa staining. This highlighted a distinct feature of *O. mascula*, where particularly large C-bands surrounding the centromeric regions of multiple chromosomes were found. However, in *O. mascula*, *O. provincialis*, *O. pauciflora*, and *O. patens*, C+ chromatin may not show a significant response to fluorochrome Hoechst or DAPI+ staining. The unique cytomorphological arrangement observed in the *O. mascula* species, unlike other members of the *O. mascula* complex, suggest epigenetic phenomena. Additional data are presented for the genera *Dactylorhiza* and *Gymnadenia*. A deeper understanding of the diversity of chromosomal structures among these orchids promises to shed light on the mechanisms underlying speciation, adaptation, and the remarkable diversity characteristic of the Orchidaceae family.

Keywords: *Chamorchis*; cytogenetics; *Dactylorhiza*; *Gymnadenia*; Karyosystematic; *Neotinea* s.l.; *Orchis* s.str.; *Platanthera*

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1. Introduction

Several groups within the Orchidinae subtribe, namely *Anacamptis* Rich., *Dactylorhiza* Neck. ex Nevski, *Gymnadenia* R. Br., *Himantoglossum* Spreng., *Ophrys* L., *Orchis* Tourn. ex L., *Platanthera* Rich. and *Serapias* L., have been the subject of systematic studies [1–12].

The above-mentioned genera, known for their high degree of chromosomal diversity and complexity, are among the most extensively studied groups in orchid cytogenetics [13–24]. These studies have revealed significant variation among species and lineages in the distribution and abundance of repetitive DNA, which may reflect different modes of chromosomal evolution, including genome duplication and rearrangement [25]. Studies of orchid cytogenetics employ various techniques to analyze chromosome structure, behavior, and function. Traditional methods such as chromosome counting and karyotype

and meiotic analyses have been extensively used to describe chromosomal characteristics and variation in orchids [26]. However, these methods have limitations when it comes to detecting subtle chromosomal differences and identifying specific genes or sequences associated with particular traits. The karyotypes of these genera commonly exhibit considerable variation in chromosome size, with some species possessing very small and highly condensed chromosomes.

Traditional cytogenetic studies based on Giemsa staining, as well as fluorochrome Hoechst 33258, DAPI, and CMA3, have shed light on the organization and evolution of repetitive DNA sequences in the aforementioned orchids [27–29].

A key mechanism in this regard is polyploidization, which involves the duplication of entire genomes. Polyploidization can enhance genetic diversity by generating new combinations of genes and functional redundancy [30–32]. Some species within the above-mentioned genera have been reported to exhibit polyploidy, which is believed to have played a role in their adaptive radiation and speciation [33–35]. Thus, orchid cytogenetics is a rapidly advancing field that offers exciting opportunities for interdisciplinary research and practical applications.

Regarding the genera belonging to the subtribe Orchidinae, extensive analyses of *Orchis* s.l. have yielded interesting results. Indeed, several decades ago, this group displayed a wide range of features, encompassing variations in chromosome numbers and arrangements, as well as differences in the quantity and distribution of heterochromatin. Based on cytogenetic research [15] and subsequent molecular investigations [1,3,36,37], the *Orchis* genus was classified into three distinct taxonomic groups: *Anacamptis* s.l., consisting of taxa with 2n = 32, 36 chromosomes and *Neotinea* s.l. and *Orchis* s.str., characterized by 42 chromosomes. Based on this new taxonomic subdivision, numerous species belonging to the genera *Anacamptis* s.l., *Neotinea* s.l., and *Orchis* s.str. have been studied extensively with reference to morphological, cytogenetic, and molecular aspects [3,12,23,24,34,38–43].

Various studies have been conducted within these groups, employing multidisciplinary methods to investigate species belonging to *Neotinea* s.l. and *Orchis* s.str. [34,38,40,42,44]. Recently, certain authors have employed methods such as flow cytometry (for measuring genome size), amplified fragment length polymorphism (AFLP), and internal transcribed spacer (ITS) analysis, reporting interesting data on the species *Neotinea tridentata*, *N. ustulata*, *N. lactea*, and *N. commutata* [12,45]. However, despite the improvements achieved utilizing the extensive research, in order to elucidate the origin of certain species, additional cytogenetic data are needed.

In this paper, we present the current state of orchid cytogenetics in selected species belonging to *Neotinea* s.l., *Orchis* s.str., *Platanthera*, *Chamorchis*, *Dactylorhiza*, and *Gymnadenia* characterized by chromosome number 2n = 40, 42, presenting, also, new data and highlighting the main results.

In this context, in addition to DAPI fluorochrome being used for chromosome counting in many cases, we reconsidered Hoechst 33258. Despite its limited usage in recent cytogenetic studies, Hoechst 33258 exhibited distinct brilliance in our analyses, particularly emphasizing heterochromatic segments rich in A-T. Therefore, we revisited certain species, particularly those belonging to *Neotinea* s.l. and *Orchis* s.str., aiming to comprehend specific cytological differences identified using Giemsa banding. Based on our findings, we propose new insights into the origin of these species.

2. Materials and Methods

2.1. Cytological Analysis

As plant material, immature ovary tissues from 142 specimens belonging to the following species were used: *N. lactea*, *N. tridentata*, *N. ustulata* and *N. maculata*, all belonging to *Neotinea* s.l.; *O. mascula*, *O. provincialis*, *O. pauciflora*, *O. patens*, *O. anthropophora* and *O. purpurea*, all belonging to *Orchis* s.str.; *D. romana*, *D. saccifera*, *D. sambucina* and *D. phoenissa*, all belonging to the *Dactylorhiza* genus; *G. conopsea*, *G. rhellicani* and *G. austriaca*, all belonging to the *Gymnadenia* genus (some in Figure 1). The list of species, provenance,

and number of plants analyzed is provided in Table 1. The karyotype and associated parameters of all taxa included in the study (see Figure 1) are presented in Table 2.

Figure 1. Representative species of *Neotinea* s.l., *Orchis* s.str., *Platanthera*, *Dactylorhiza* s.l. and *Gymnadenia* s.l. discussed in the present cytogenetic study. (**A**) *Neotinea lactea*; (**B**) *N. tridentata*; (**C**) *N. ustulata*; (**D**) *Orchis mascula*; (**E**) *O. provincialis*; (**F**) *O. pauciflora*; (**G**) *O. anthropophora*; (**H**) *O. purpurea*; (**I**) *Platanthera chlorantha*; (**J**) *Dactylorhiza romana*; (**K**) *D. phoenissa*; (**L**) *Gymnadenia conopsea*.

Table 1. Taxon, provenance, number of plants, and chromosome numbers of taxa analyzed in the study.

Taxon	Provenance	No. Plants	2n	No. of Plants with B-Chromosome
Neotinea commutata (Tod.) R.M. Bateman	Italy	8	84	
N. lactea (Poir.) R.M. Bateman, Pridgeon & M.W. Chase	Italy	12	42 42+1B	1
N. maculata (Desf.) Stearn	Italy	4	42	
N. tridentata (Scop.) R.M. Bateman, Pridgeon & M.W. Chase	Italy	15	42 42+1B	1
N. ustulata (L.) R.M. Bateman, Pridgeon & M.W. Chase	Italy	9	42 42+1B	1
Orchis anthropophora (L.) All.	Italy	5	42	
O. italica Poir.	Italy	12	42 42+1B	1
O. mascula (L.) L.	Italy	10	42 42+1B	2
O. patens Desf.	Italy	2	84	
O. pauciflora Ten.	Italy	5	42	
O. provincialis Balb. ex Lam. & DC.	Italy	10	42	
O. purpurea Huds.	Italy	5	42	
Platanthera chlorantha (Custer) Rchb.	Italy	5	42	
Dactylorhiza romana (Sebast.) Soó	Italy	15	40 40+1B, 40+2B, 40+3B	3
D. phoenissa (B. Baumann & H. Baumann) P. Delforge	Lebanon	3	80 80+4B	1
D. saccifera (Brogniart) Soó	Italy	5	40	
D. sambucina (L.) Soó	Italy	10	40	
Gymnadenia austriaca (Teppner & E. Klein) P. Delforge	Italy	3	80	
G. conopsea (L.) R.Br.	Italy	2	40	
G. rhellicani Teppner & E. Klein	Italy	2	40	

Table 2. Taxon, code, formula, and morphometric parameters in *Neotinea* s.l., *Orchis* s.str., *Platanthera*, *Chamorchis*, *Dactylorhiza* s.l., and *Gymnadenia* s.l. (average values). THL = Total chromosome length of the haploid complement; MCA = Mean Centromeric Asymmetry; CVCL = Coefficient of Variation of Chromosome Length; CVCI = Coefficient of Variation of Centromeric Index. Chromosome abbreviations: m, metacentric; sm, submetacentric, st, subtelocentric.

Taxon	Code	Formula	THL	M_{CA}	CV_{CL}	CV_{CI}
Neotinea lactea (Poir.) R.M. Bateman, Pridgeon & M.W. Chase	Nla	16m+26sm	61.72	26.82	23.73	14.91
N. tridentata (Scop.) R.M. Bateman, Pridgeon & M.W. Chase	Ntr	24m+18sm	39.57	24.52	35.49	16.46
N. ustulata (L.) R.M. Bateman, Pridgeon & M.W. Chase	Nus	28m+14sm	42.89	20.38	24.63	14.38
Orchis anthropophora (L.) All.	Oan	36m+6sm	32.33	14.47	22.65	11.88
O. italica Poir.	Oit	28m+14sm	40.43	23.43	25.01	15.44
O. mascula (L.) L.	Oma	28m+14sm	46.60	21.41	32.67	15.40
O. provincialis Balb. ex Lam. & DC.	Opr	10m+24sm+8st	44.43	33.75	35.27	25.53
O. purpurea Huds.	Opu	34m+8sm	51.72	20.47	26.36	12.29
Platanthera chlorantha (Custer) Rchb.	Pch	30m+12sm	58.23	19.59	21.25	11.75
Chamorchis alpina (L.) Rich.	Cal	24m+18sm	46.48	26.22	20.73	18.30
Dactylorhiza romana (Sebast.) Soó	Dro	14m+20sm+6st	44.50	31.08	29.65	20.22
D. saccifera (Brogniart) Soó	Dsa	36m+4sm	46.78	16.50	15.18	07.69
Gymnadenia conopsea (L.) R.Br.	Gco	22m+12sm+6st	44.93	27.77	24.96	25.41
G. rhellicani Teppner & E. Klein	Grh	14m+26sm	35.23	30.37	18.90	19.84

At least ten metaphases were examined, and the karyotype was constructed from well-spread-out metaphase plates. Immature ovary tissues were pre-treated with 0.3% colchicine at room temperature for 2 h. For Feulgen staining, they were fixed in 3:1 (*v*/*v*) ethanol–glacial acetic acid and stored in deep-freeze for up to several months. Hydrolysis was performed at 20 °C in 5.5 N HCl for 20 min [46]. The material was then stained in freshly prepared Feulgen stain.

For C-banding, immature ovaries were fixed in 3:1 (*v*/*v*) ethanol–glacial acetic acid and stored in the deep-freeze for up to several months. Subsequently, they were squashed in 45% acetic acid; coverslips were removed using the dry-ice method, and the preparations were air-dried overnight. 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 2× SSC at 60 °C for 1 h. They were then stained using 3–4% Giemsa (BDH) at pH 7.

For Hoechst 33258 staining, squash preparations were made up as they were for C-banding, and then were stained in a 2 µg/mL dye solution in pH 7 McIlvaine buffer for 5 min, rinsed, and mounted in 1:1 *v*/*v* buffer–glycerol [47].

For DAPI (4–6-diamidino-2-phenylindole) staining, ovaries were treated as they were for C-banding and stained using a buffered DAPI solution (0.6 mg/mL) for 5 min, after which, they were rinsed and mounted in 1:1 (*v*/*v*) buffer and glycerol.

For chromomycin A3 (CMA) staining, slides were stained with 0.5 mg/mL CMA for 1 h and mounted in 1:1 (*v*/*v*) McIlvaine's pH 7.0 buffer–glycerol.

2.2. Chromosome Numbers and Karyotype Parameters

Chromosome pairs were identified and arranged on the basis of their length. The nomenclature used for describing karyotype composition follows Levan et al. [48], who denote centromeric positions using the terms "median (arm ratio 1.0–1.7)", "submedian (a.r. 1.7–3.0)", "subterminal (a.r. 3.0–7.0)", and "terminal (a.r. 7.0–∞)". Morphometric karyotype characters were evaluated by calculating haploid complement length, while M_{CA} (Mean Centromeric Asymmetry) and CV_{CL} (Coefficient of Variation of Chromosome Length) were used for the evaluation of karyotype asymmetry [49,50].

Chromosome measurements were conducted using IdeoKar version 1.2 freeware (http://agri.uok.ac.ir/ideokar/index.html, accessed on 20 March 2023).

A diagram of karyotype M_{CA} and CV_{CL} values was generated using Open Office 4.1.14.

2.3. Nomenclature

Regarding species nomenclature, we followed Delforge [51] and in some cases POWO [52].

3. Results

In this study, several species (Figure 1) yielded new karyological information, while for others, existing data was revised, expanded, and re-evaluated using IdeoKar software version 1.2. Specifically, the Hoechst and DAPI staining methods were found to produce different results regarding heterochromatin composition. Notably, Hoechst rendered the bands significantly more discernible.

3.1. Genus Neotinea s.l.

In the *Neotinea* group, cytological investigations confirmed diploid species with chromosome number 2n = 2x = 42. We confirm, here, the chromosome number 2n = 42 for *N. maculata*. However, an exception was observed in *N. commutata*, which has a chromosome count of 2n = 4x = 84. The species *N. lactea*, *N. tridentata*, and *N. ustulata* also exhibited specimens with a chromosome count of 2n = 42 + 1B (Table 1 and Figures 1 and 2).

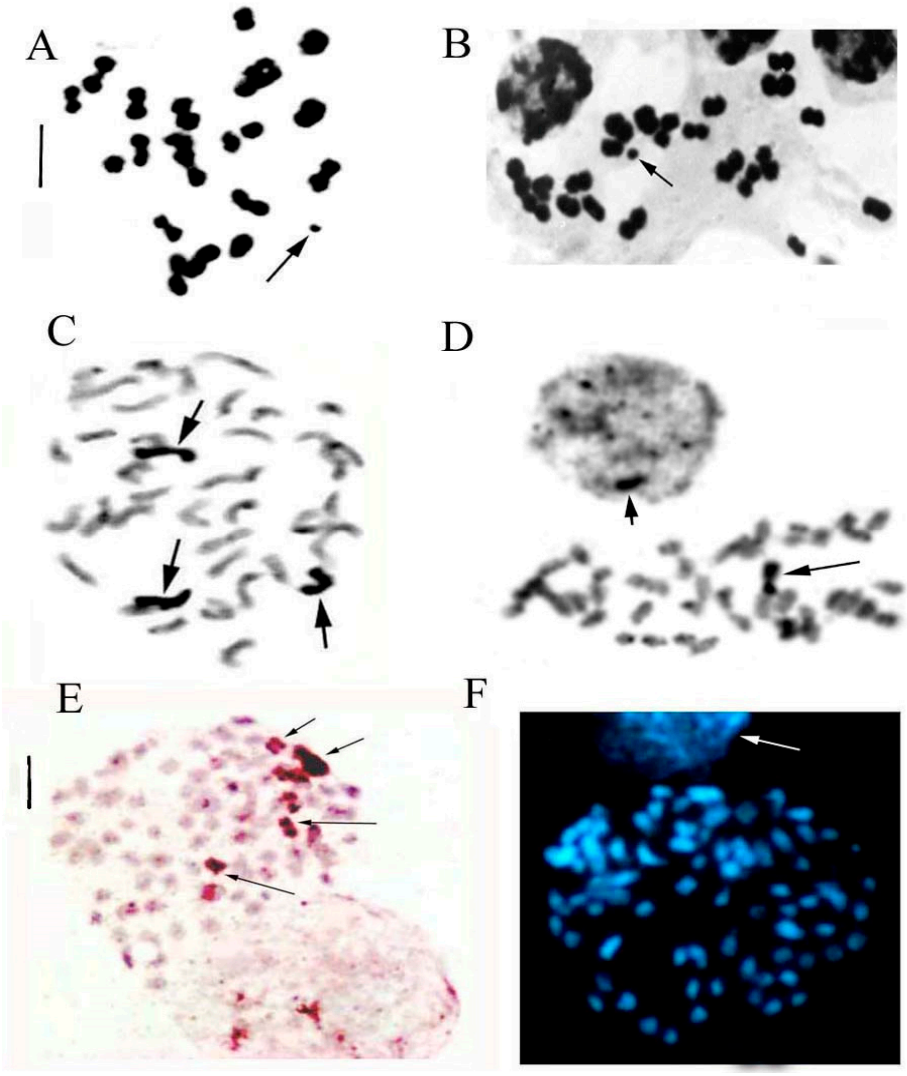

Figure 2. (**A**) *Neotinea lactea*, metaphase I of meiosis showing 21 bivalents + 1B; the arrow indicates the B-chromosome. (**B**) *N. ustulata*, metaphase I of meiosis showing 21 bivalents + 1B; the arrow indicates the B-chromosome. (**C**,**D**) *Dactylorhiza romana*, Giemsa C-banding. Somatic pro-metaphase; the arrows indicates the three heterochromatic B-chromosomes (**C**), and somatic metaphase showing one heterochromatic B-chromosome; the long arrow indicates the B-chromosome, and the short arrow indicates an interphase nucleus containing chromocentres (**D**). (**E**,**F**) *D. phoenissa*. Somatic metaphase stained with Giemsa C-banding; the arrows indicate heterochromatic B-chromosomes (**E**), and somatic metaphase stained with DAPI; the arrow indicates an interphase nucleus containing chromocentres (**F**). Scale bar = 5 µm.

The construction of karyotypes was achievable only for specific species, such as *N. tridentata*, *N. lactea*, and *N. ustulata*. The karyotype of these species is primarily made up of metacentric chromosomes, although *N. lactea* demonstrates additional submetacentric chromosomes and a greater level of karyotype asymmetry than *N. tridentata* (Figure 3).

In *N. lactea*, Giemsa C-banding analysis revealed the presence of centromeric heterochromatin and prominent bands located in telomeric positions on numerous chromosomes. Hoechst and DAPI staining displayed vivid blocks in telomeric regions, corresponding to the Giemsa C-bands (Figure 4A). Conversely, *N. tridentata*, following Hoechst and DAPI staining, predominantly displayed bands at the centromeric regions. Additionally, double or triple bands were observed in certain chromosomes (Figure 4B,C). Analyzed using traditional methods such as Feulgen, Giemsa, and DAPI dye, *N. commutata* showed 42 bivalents in metaphase I (Figure 4D). Giemsa C-banding analysis showed centromeric

heterochromatin and some chromosomes with telomeric bands (Figure 4E). DAPI staining detected centromeric and telomeric bands (Figure 4F).

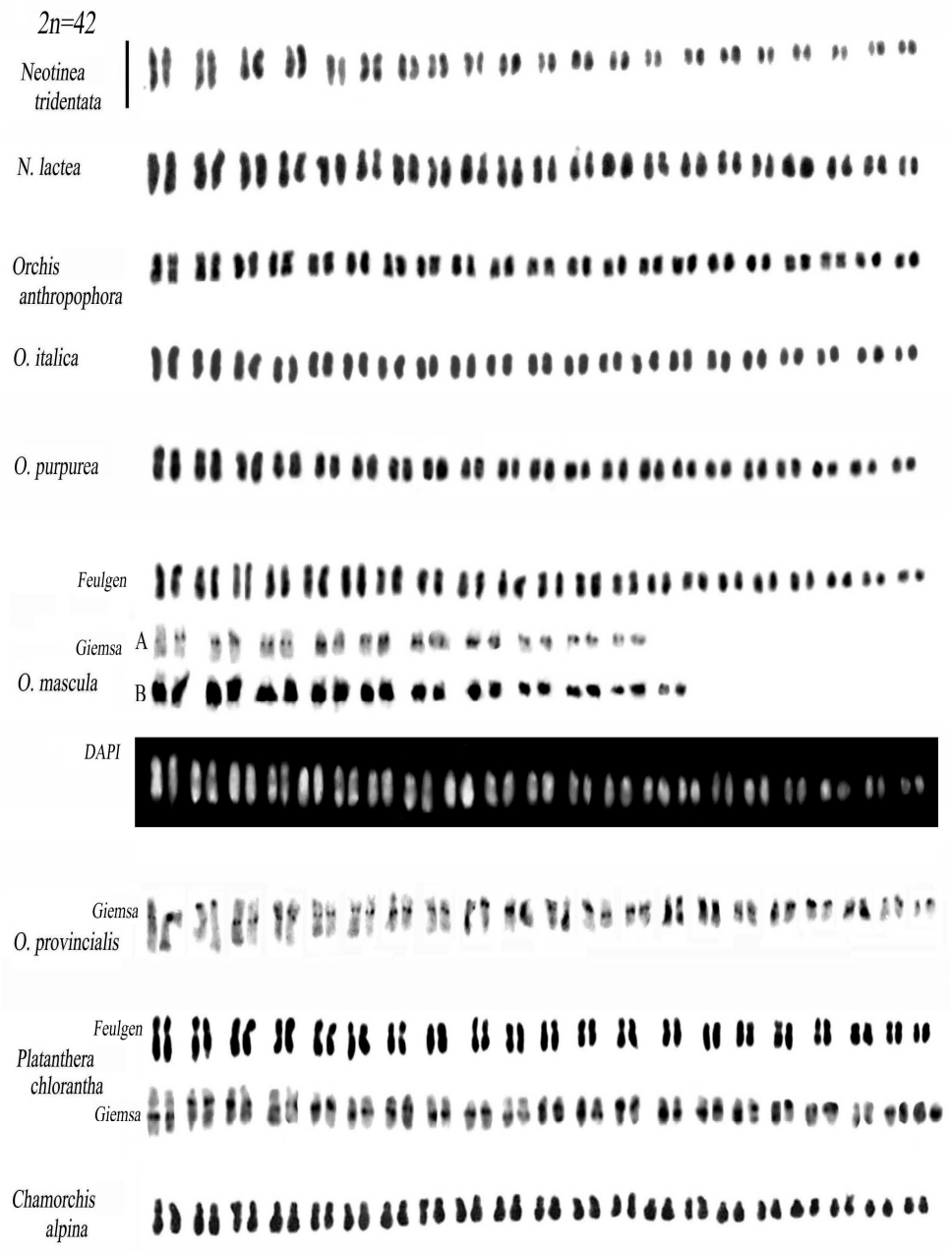

Figure 3. Karyotypes of *Neotinea* s.l., *Orchis* s.str., *Platanthera*, and *Chamorchis* species. In *Orchis mascula*, the karyotype obtained using the Feulgen method, Giemsa banding, and DAPI is characterized by the subdivision into two distinct groups (A, B). The karyotype of *Orchis provincialis*, obtained using Giemsa banding, has numerous centromeric bands and a limited number of chromosomes with terminal bands. In *Platanthera chlorantha*, numerous chromosomes exhibit centromeric bands, while a few display subtelomeric bands. Scale bar = 5 μm.

In karyological analyses of the examined taxa in the *Neotinea* group, both H33258 and DAPI staining result in bright-fluorescent regions on numerous chromosomes, with staining seen in regions that overlap perfectly. In addition, DAPI heterochromatin consistently co-localizes with C bands.

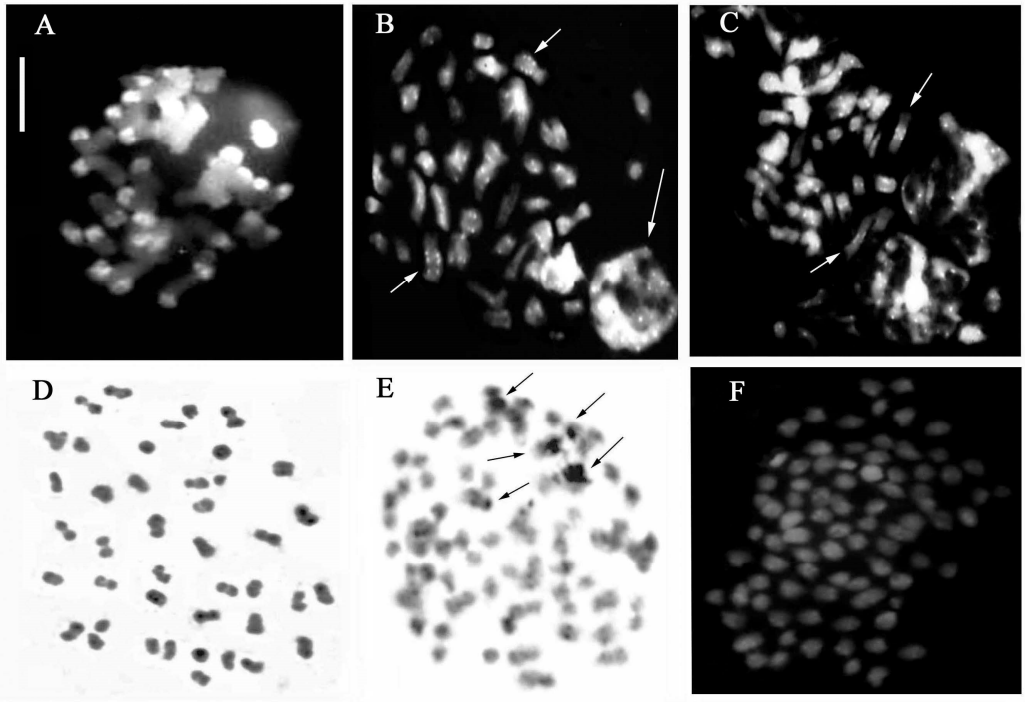

Figure 4. (**A**) *Neotinea lactea*, in a somatic metaphase stained with Hoechst 33258; there is a notable presence of chromosomes featuring extensive blocks of telomeric heterochromatin. (**B**,**C**) *N. tridentata* stained with Hoechst 33258; the short arrows indicate chromosomes with double or triple bands (observed as dots), and the long arrow shows an interphase nucleus with numerous chromocentres. (**D**–**F**) *N. commutata*. (**D**) Metaphase I of meiosis stained with Feulgen, showing 42 bivalents. (**E**) Somatic metaphase stained with the Giemsa banding technique (arrows indicate chromosomes showing terminal bands). (**F**) Somatic metaphase stained with DAPI dye. Scale bar = 5 µm.

3.2. Genus Orchis s.str.

In this study, we present the karyotypes of *Orchis anthropophora* and *O. purpurea* for the first time (Figures 1 and 3). Additionally, we conducted a thorough examination of the karyotypes of *O. mascula*, *O. provincialis*, *O. pauciflora* Ten., and *O. patens* Desf. species using the Giemsa banding technique. Moreover, we employed Hoechst, DAPI, and CMA3 dyes to facilitate a more comprehensive analysis (Figures 3 and 5).

Cytological investigations within *Orchis* s.str. yelded a consistent chromosome count of 2n = 2x = 42, with the noteworthy exception of *Orchis patens*, which exhibited a count of 2n = 4x = 84. The karyotypes of these species are predominantly composed of metacentric chromosomes. A notable resemblance in karyomorphology is observed between the *Neotinea* s.l. and *Orchis* s.str. groups (Figure 3).

The C-banding patterns of *O. provincialis* and *O. mascula* reveal interesting features. Indeed, *O. provincialis* displayed a considerable number of chromosome pairs characterized by thin centromeric heterochromatin (often appearing as two dots), together with two pairs characterized by smaller telomeric bands (Figure 5E). In contrast, *O. mascula* displayed chromosomes marked by extensive centromeric heterochromatin, as well as chromosomes that possessed thin centromeric bands (Figures 3 and 5A). In many cases, these heterochromatic regions extended across the entire length of the chromosomes, with euchromatin confined to telomeric positions or restricted to the short arm, while the long arm remained heterochromatic. Following Giemsa staining of the bands observed in bivalents during metaphase I of meiosis in *O. mascula*, we formulated the karyotype by arranging the corresponding chromosomes into pairs based on their centromeric bands (Figures 3 and 5A,B). It was thereby possible to divide the chromosomal complement of *O. mascula* into genome A, consisting of chromosomes with thin centromeric bands, and genome B, consisting of chromosomes with wide centromeric bands (Figure 3).

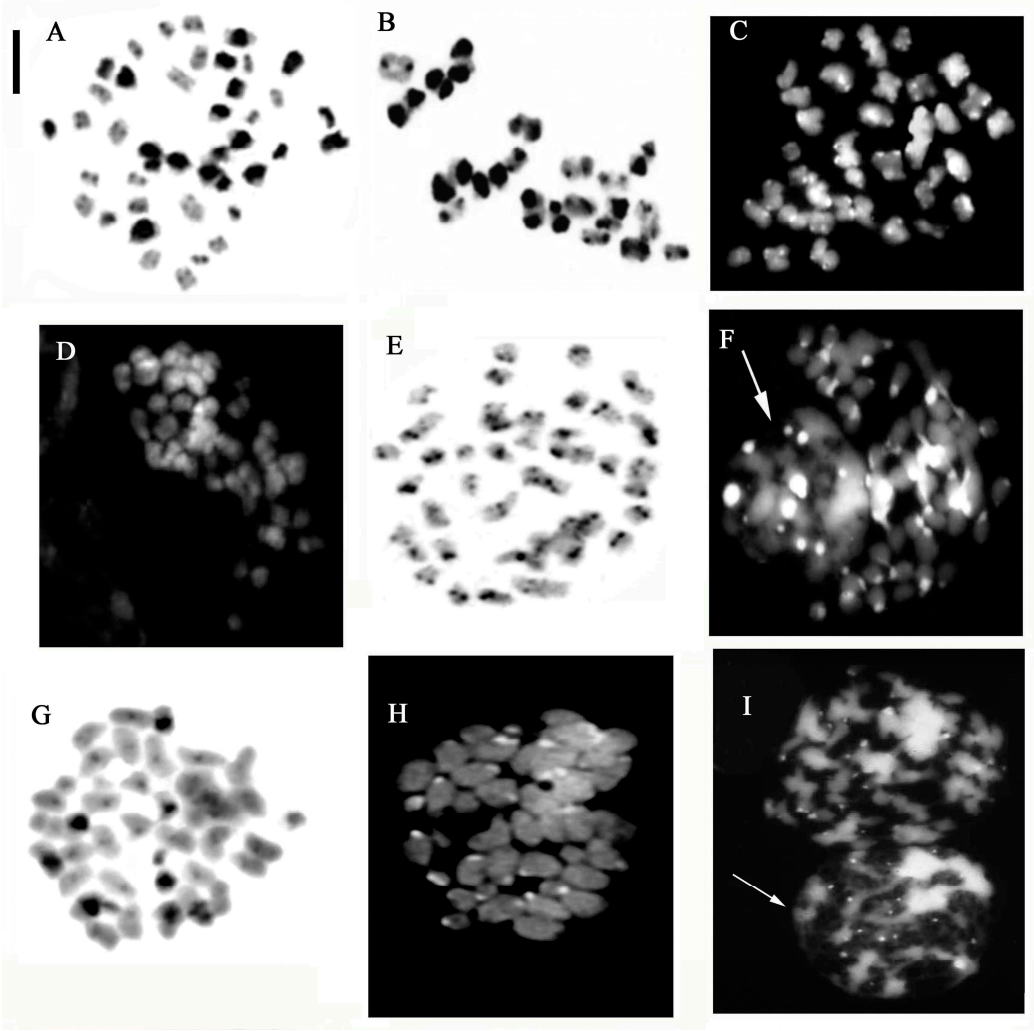

Figure 5. (**A**,**B**,**E**,**G**), with Giemsa C-banding; (**A**,**B**) *Orchis mascula*. (**A**) Somatic metaphase: half of the set, consisting of chromosomes with broad centromeric bands, can be seen. (**B**) Metaphase I of meiosis showing 21 bivalents; 11 bivalents with large blocks of constitutive heterochromatin are visible. (**E**) *O. provincialis*; chromosomes are characterized by thin centromeric heterochromatin, a few exhibiting terminal bands. (**G**) *O. pauciflora*, a few chromosomes with broad centromeric bands are observed. C, F, H, I with fluorochrome Hoechst 33258; numerous chromosomes are characterized by terminal bands. (**C**) *O. mascula*, (**F**) *O. provincialis*, (**H**) *O. pauciflora*, and (**I**) O. patens. (**D**) Fluorochrome CMA3, *O. mascula*. The arrows in F and I indicate interphase nuclei containing chromocentres. Scale bar = 5 μm.

Analyses conducted on *O. pauciflora* indicate an intermediate genome between the species *O. mascula* and *O. provincialis*. Specifically, application of Giemsa banding unveiled three pairs of chromosomes distinguished by prominent centromeric heterochromatic bands. Regarding the chromosomal complement of *O. patens*, C-banding with Giemsa reveals a cytogenetic situation similar to what is observed in *O. provincialis*.

In all the species examined, the results obtained by fluorochrome staining revealed a distribution of bands rich in A-T base pairs that notably differed from what was observed with Giemsa staining. In this context, the application of Hoechst fluorochrome exhibited a higher staining intensity in the heterochromatic bands than DAPI. It is noteworthy that in *O. provincialis* and *O. pauciflora*, all sets of chromosomes exhibited significant regions of telomeric heterochromatin (Figure 5F,H). In contrast, *O. mascula* displayed less-extensive telomeric bands, with certain chromosomes having heterochromatic blocks on both arms

(Figure 5C). *O. patens* yielded similar results to *O. provincialis* when studied using both Giemsa and fluorochrome staining techniques (Figure 5I).

In the above-mentioned species, application of Hoechst 33258 and DAPI staining revealed numerous chromosomes with telomeric bands that were not detectable with Giemsa staining (Figures 3 and 5). This is exemplified by the substantial centromeric regions of heterochromatin of *O. mascula*, which show a uniform response after Hoechst staining (Figure 5C). However, the fluorescence data suggest that in *O. mascula*, *O. provincialis*, *O. pauciflora*, and *O. patens*, C+ chromatin may not show a reactive response to DAPI+ staining.

In *O. mascula*, using the CMA3 fluorochrome, we detected some chromosomes with telomeric bands (Figure 5D).

3.3. Genus Dactylorhiza

In this study, we present new data for *D. sambucina* (L.) Soò (2n = 2x = 40), investigated with Feulgen and DAPI techniques. After DAPI staining, centromeric and telomeric bands were observed, and interphase nuclei exhibited numerous chromocentres (Figure 6A). Additional new data were acquired by analyzing material from *D. romana* specimens (Figures 1J and 2C,D) originating from a station in Puglia. Giemsa and DAPI fluorochrome banding methods were employed, confirming the existence of the B chromosomes previously observed. Similar results were obtained for samples of *D. phoenissa* from Lebanon (Figures 1H and 2E,F).

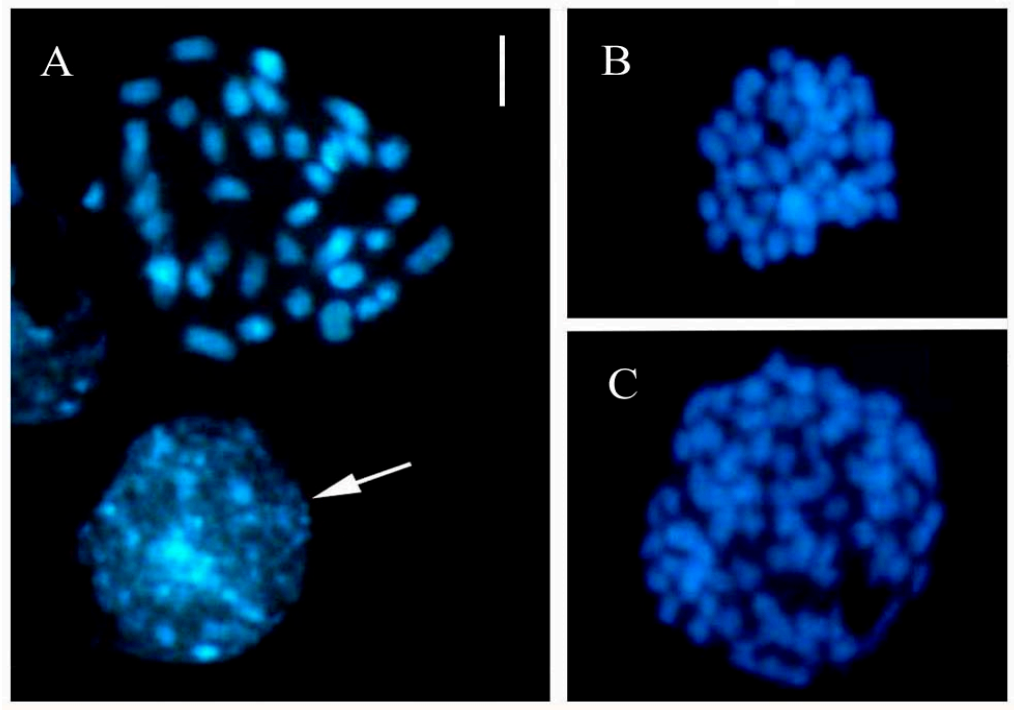

Figure 6. (**A**–**C**) Somatic metaphases stained with DAPI dye. (**A**) *Dactylorhiza sambucina*, 2n = 40; the arrow indicates an interphase nucleus containing numerous chromocentres. (**B**) *Gymnadenia rhellicani*, 2n = 40; (**C**) *G. austriaca*, 2n = 80. Scale bar = 5 μm.

3.4. Genus Gymnadenia

Further data, limited to Italian specimens, were obtained for some species of the *Gymnadenia* genus. We confirm the chromosome numbers 2n = 2x = 40 for *G. conopsea* (Figures 1L and 7) and *G. rhellicani*, 2n = 4x = 80 for *G. austriaca* (Teppner & E. Klein) P. Delforge and 2n = 5x = 100 in *G. buschmanniae* (Teppner & Ster) Teppner & E.Klein.

In this study, we present the karyotype obtained for the species *G. austriaca* using the Feulgen technique and Giemsa banding. It is possible to observe numerous chromosomes that present bands of constitutive heterochromatin like those noted in *G. rhellicani* (Figure 7).

Here, we report new cytological data relating to *G. rhellicani* and *G. austriaca* obtained using the DAPI fluorescence technique which found weak centromeric and telomeric bands in both species (Figure 6B,C).

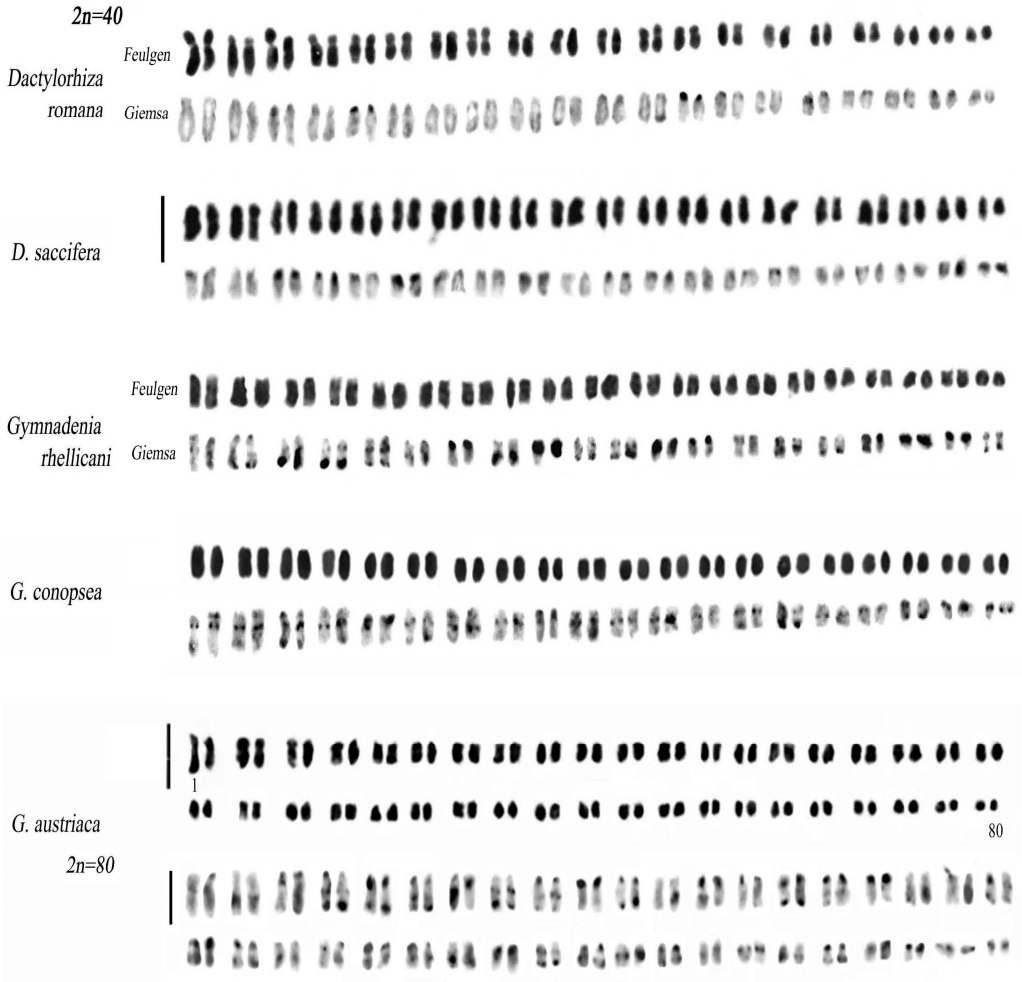

Figure 7. Karyotypes in *Dactylorhiza* and *Gymnadenia* species. Following Feulgen and Giemsa staining, chromosomal complements reveal the presence of constitutive heterochromatin. Scale bar = 5 µm.

3.5. B-Chromosomes

Supernumerary chromosomes have been confirmed in some specimens of *Neotinea s.l.*, *Orchis s.str.*, and *Dactylorhiza* (Table 1 and Figure 2).

3.6. Diagram of the Morphometric Parameters

We used the asymmetry indices MCA and CVCL to produce the diagram in Figure 8 which highlights selected species, each represented by a distinct color.

The diagram displays a partial degree of homogeneity among the examined species. However, *Orchis anthropophora* has a lower MCA, and *Orchis provincialis* a higher MCA index. These differences are also to be ascribed to the complex karyomorphology of these species, knowledge of which is important for good measurement of the indices.

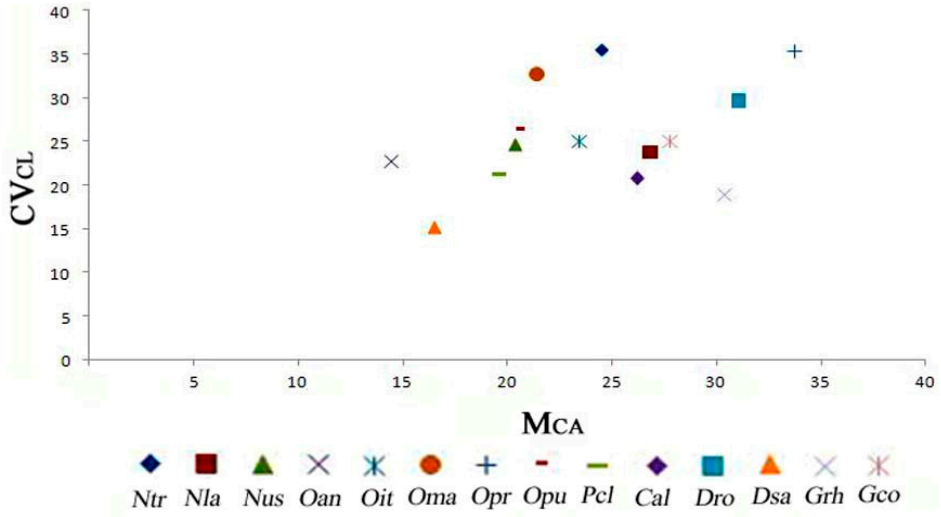

Figure 8. Diagram of morphometric parameters M_{CA} (Mean Centromeric Asymmetry) and CV_{CL} (Coefficient of Variation of Chromosome Length).

4. Discussion

Neotinea s.l. is predominantly distributed across the Mediterranean region [51]. Comparative investigations of the karyotype between the 36-*Anacamptis* group and the 42-*Neotinea* group have shown that the latter group has smaller chromosomes and a more intricate karyomorphology [15,24]. Nevertheless, the karyotype of *N. lactea* displays greater asymmetry than *N. tridentata*, the latter exhibiting a karyotype primarily composed of metacentric chromosomes ([13,53] and this study).

Analysis using Giemsa C-banding confirmed the presence, in *N. lactea*, of centromeric heterochromatin and highly distinct bands located at telomeric locations on numerous chromosomes. This C-banding pattern was corroborated by Hoechst 33258 and DAPI fluorochrome staining. On the other hand, *N. lactea* exhibited more pronounced heterochromatin bands than *N. tridentata*.

Along with the polyploid *N. commutata*, characterized by a chromosome count of 2n = 84, *N. lactea* and *N. tridentata*, both possessing a chromosome count of 2n = 42, yielded interesting findings. Mazzola [54] initially proposed that *N. commutata* originated as an autopolyploid from *N. tridentata*, as both taxa were present in Sicily, including some sympatric populations. However, a more comprehensive investigation incorporating morphology, cytogenetics, and molecular techniques indicated that *N. commutata* is an allotetraploid species resulting from the hybridization of *N. tridentata* and the closely related *N. lactea*. Moreover, the polyploid *N. commutata* exhibited 42 bivalents in metaphase I ([34] and this study), suggesting that its formation could be attributed to allopolyploidization.

Conversely, Giemsa C-banding and DAPI staining validated the absence for *N. commutata* chromosomes of the telomeric heterochromatic blocks previously identified in various *N. lactea* chromosomes. In the genome of *N. commutata*, one can distinctly observe numerous chromosomes exhibiting centromeric or subtelomeric bands or dots, characteristic of those observed in *N. tridentata*.

Recently, based on additional data (e.g., flow cytometry), Trávníček et al. [12] suggested an autotetraploid origin for *N. commutata*.

Some observations can thus be made regarding the polyploid origin of the species *N. commutata*. Indeed, based on further karyological findings, it is feasible to propose a new pathway for the emergence of *N. commutata*. *N. lactea* and *N. tridentata* are two closely related orchid species within the *Neotinea* genus. These species share specific morphological and molecular traits owing to their intimate evolutionary connection; yet, they also manifest notable distinctions [34], to such an extent that previously *N. lactea* was classified as *Orchis tridentata* subsp. *lactea* (Poir.) K.Richt. However, subsequent DNA-sequence-based molecular analyses led to its reclassification as part of the *Neotinea* group, naming it *N.*

lactea (Poir.) R.M. Bateman, Pridgeon & M.W.Chase. Indeed, the genetic sequences of both *N. lactea* and *N. tridentata* exhibit similarities or close relatedness, attributed to their recent shared ancestral lineage. This genetic resemblance reasonably explains their presence in molecular analyses involving the species *N. commutata*. As a result, a plausible hypothesis can be formulated, i.e., that in *N. tridentata* an initial stage of autopolyploidy was followed by allopolyploidization, giving rise to *N. commutata*. This progression might have led to the displacement of autopolyploid forms by the subsequent allopolyploids, which proved to be well adapted to the island's ecological conditions. Supporting this proposition, the techniques employed reveal the absence in *N. commutata* of chromosomes featuring conspicuous telomeric heterochromatic bands, which in contrast are observed in *N. lactea*. This suggests a transition in karyotype traits and constitutes evidence for a complex evolutionary process involving autopolyploidy and subsequent allopolyploidization in the origin of *N. commutata*.

It should be emphasized that as a general trend within the *Neotinea* group, the heterochromatin regions stained with DAPI consistently align with C-banding patterns. Curiously, this pattern extends to other taxa such as *Anacamptis papilionacea* (L.) R.M.Bateman, Pridgeon & M.W.Chase and *A. coriophora* (L.) R.M.Bateman, Pridgeon & M.W.Chase, which exhibit similar characteristics upon C-banding, Hoechst, and DAPI staining [15,24]. Consequently, in the *Neotinea* group, the heterochromatin regions that stained positively with Hoechst and DAPI consistently coincide with the C-positive bands. This indicates an organizational pattern that differs notably from what is observed in the *Orchis mascula* complex.

Orchis s.str. comprises a collection of terrestrial orchid species, totaling around 20 in number. These orchids are predominantly distributed across Europe, North Africa, and western Asia [55]. Research into the chromosome composition of *Orchis* plants has revealed that the standard chromosome count is 2n = 42, except for *O. patens*, which exhibits a chromosome count of 2n = 84 [39]. A noteworthy anomaly has been documented in *O. italica*, wherein a triploid condition occurs, resulting in a chromosome number of 2n = 3x = 63 [14]. Within the *Orchis* s.str. grouping, the Masculae and Provinciales subgroups, which encompass species such as *O. mascula*, *O. provincialis*, *O. pauciflora* Ten., *O. patens* and the subspecies *O. mascula* subsp. *ichnusae* (Corrias) Devillers-Tersch. & Devillers, exhibit notable cytogenetic variability when contrasted with the Militares group (including species such as *O. militaris* L., *O. simia* Lam., *O. italica*, etc.). Specifically, the Militares group displays limited heterochromatin, as identified through Giemsa banding techniques, while the Masculae and Provinciales groups show additional constitutive heterochromatin [39,40,42].

An interesting observation is the substantial resemblance in karyomorphology between *Orchis* s.str. and *Neotinea* s.l. Nonetheless, the former group displays intriguing divergences in the arrangement and allocation of heterochromatin. An investigation encompassing *O. italica*, *O. militaris*, *O. purpurea*, and *O. simia* found analogous responses to C-banding. These species exhibited relatively minimal constitutive heterochromatin, at least based on the employed methodologies. In stark contrast, the C-banding patterns of *O. provincialis* and *O. mascula* were particularly captivating.

O. mascula and *O. provincialis* share morphological and phylogenetic similarities. Notably, a study focused on ribosomal spacer sequences (ITS) revealed that despite their close phylogenetic relationship, *O. mascula* and *O. provincialis* were clearly discernible from a molecular standpoint [42]. Indeed, even though *O. mascula* and *O. provincialis* both belong to the *O. mascula* subgroup, they demonstrated distinct chromatin arrangements. When subjected to Giemsa C-banding, *O. provincialis* displayed multiple chromosome pairs featuring delicate centromeric heterochromatin and two pairs with smaller terminal bands. In contrast, *O. mascula* showed chromosomes with notably extensive centromeric heterochromatin and chromosomes carrying slender centromeric bands [40]. Conversely, the use of certain fluorochromes (Hoechst 33258, DAPI) revealed different distributions of bands from what was observed with Giemsa staining. The findings concerning the size of heterochromatin blocks in the two species were particularly intriguing. *O. provincialis* displayed substantial blocks of telomeric heterochromatin, whereas *O. mascula* exhibited more slen-

der telomeric bands. Interestingly, these heterochromatin blocks were not detected using Giemsa staining in either species. Notably, in the case of *O. mascula*, the C-banding pattern presented notably large C-bands encircling the centromeres of numerous chromosomes. The few other instances in which such a distribution of heterochromatin has been observed occurred in *Serapias* [56]. The same heterochromatin distribution pattern was observed in *O. mascula* subsp. *ichnusae*, an endemic subspecies found in Sardinia. Furthermore, comprehensive examinations encompassing morphological characteristics, karyological analyses, and molecular investigations of intermediate traits corroborated the presence of hybridization between the insular *O. mascula* subsp. *ichnusae* and *O. provincialis*, leading to the formation of the *Orchis* x *penzigiana* A. Camus hybrid. This hybrid displays an intermediary heterochromatin distribution [42]. Conversely, within *O. provincialis*, constitutive heterochromatin is predominantly localized in distinct centromeric regions, often appearing as dual dots, and only four chromosomes exhibited telomeric bands. Fluorescence data indicated that both *O. mascula* and *O. provincialis* might not exhibit a DAPI+ reaction in their C+ chromatin. This is particularly the case for *O. mascula*'s sizable centromeric heterochromatin zones, which exhibited an indifferent response to DAPI staining. It is possible that these "centromeric bands" potentially signify an alternative kind of heterochromatin within the centromeric region, as observed in *Cymbidium* sp. [57].

Similar findings have been documented in *O. pauciflora*. In this species, the application of Giemsa banding techniques revealed the existence of three pairs of chromosomes bearing expansive heterochromatic bands, one pair featuring both a centromeric and a subtelomeric band, and several pairs with slender centromeric bands. Bands were also observed with Hoechst fluorochrome. Additionally, the overall arrangement of heterochromatic blocks in *O. pauciflora* is similar to that of *O. mascula*. Moreover, with respect to the putative species *O. clandestina*, comprehensive karyological analyses using both Giemsa and fluorochrome banding methods, along with molecular investigations involving rDNA and ITS sequences, indicate a hybrid origin for the taxon. This analysis also enables the recognition of *O. patens* and *O. provincialis* as the parent species [39].

Taken together, these discoveries highlight substantial cytogenetic variability within the *O. mascula* complex, especially when contrasted with other species categorized under the *Orchis* s.str. group. As a result, the distinct cytomorphological arrangement observed in *O. mascula*, as opposed to the other species in the *O. mascula* complex, raises interesting questions regarding its origins. The likelihood of encountering epigenetic phenomena is greater, exhibiting a wide centromeric band of constitutive heterochromatin along almost their entire length which make up half of the genome. This is especially the case when applying the Giemsa banding technique, except for certain instances where the euchromatic telomeric portion is involved. Similar results have been observed in *Avena sativa* L. subsp. *byzantina* (K.Koch) Romero Zarco [58]. Epigenetics is primarily concerned with the study of inheritable changes in phenotype that transpire without modification of DNA sequences [29,59]. DNA methylation, histone modification, and the involvement of non-coding RNA are among the mechanisms responsible for epigenetic regulation in both animals and plants [60]. In terms of plant chromosomes, heterochromatic regions can undergo modification at the DNA-sequence level via DNA methylation [61].

In this study, based on the outcomes of the Giemsa banding technique, we categorize the chromosomal makeup of *O. mascula* into groups A and B. Currently, the factors contributing to this distinction remain unclear, underscoring the need for additional research addressing ecological, morphological, cytogenetic, and molecular aspects. Speculation concerning the cytogenetics of this species raises several questions that warrant exploration. The specimens of *O. mascula* that were previously examined, including *O. mascula* subsp. *ichnusae*, as well as those from various locations in Italy that are studied here, exhibit consistent cytological traits across a range of banding techniques for staining.

In *O. mascula*, the wide centromeric bands, which are rendered prominent by the Giemsa banding technique, contain a substantial amount of constitutive heterochromatin. The eukaryotic genome contains various categories of repetitive DNA sequences, which

can exert significant influence on the evolution of genomes and chromosomes [62–65]. Nevertheless, when employing Hoechst and DAPI dyes, all chromosomes exhibit telomeric bands rich in A-T content on either a single arm or both arms. Hence, the centromeric bands identified using C-banding consist of distinct sequences. This is supported by the limited presence of chromosomes displaying G-C-rich bands when examined using the fluorochrome CMA3. Conversely, the FISH technique, which is valuable for detecting additional repetitive DNA sequences, has not been employed thus far.

In summary, despite lacking knowledge of the specific types of heterochromatin, we can propose two potential origins for *O. mascula*: (1) A hybrid in which one of the parental species possessed a genome abundant in constitutive heterochromatin. (2) The result of a substantial environmental change that had a pronounced effect on the DNA, leading to the current genome structure.

Notably, epigenetic responses to diverse ecological and environmental conditions have been documented in agricultural contexts (e.g., maize, wheat, barley, rice) [66–69].

Aside from potential identifications of the repetitive heterochromatin sequences, the difference between the genomic characteristics of *O. mascula* and, for instance, *O. provincialis* remains unresolved. Therefore, further studies are needed to clarify the nature of the constitutive heterochromatin highlighted in the present study.

Platanthera, one of the largest genera in the Orchideae tribe, has species in Europe and North Africa [9,53]. Interestingly, in previous studies the karyotype of the species *P. chlorantha* and *P. algeriensis* Batt. & Trab. showed similarities in size and structure with that of the *Anacamptis* s.l. and *Himantoglossum* s.l. groups (2n = 36) [15]. Furthermore, C-banding analysis showed constitutive heterochromatin in the subtelomeric and centromeric regions of several chromosomes. Furthermore, with DAPI fluorochrome, numerous *P. chlorantha* chromosomes showed marked fluorescence in the centromeric and subtelomeric positions. However, to understand this interesting genus, further data are required.

The genus *Chamorchis* represented by the single species *C. alpina* (L.) Rich. shows a moderately asymmetric karyotype with a clearly observable centromeric position in all chromosomes. In fact, the karyomorphology of this species differs considerably from those of other genera with 42 or 40 chromosomes, such as *Neotinea* s.l., *Orchis* s.str., *Dactylorhiza*, and *Gymnadenia* [18,40]. This species is characterized by a distinct karyotype and a notable amount of constitutive heterochromatin [70]. There are currently no data based on staining methods. However, the results in our possession suggest notable structural rearrangements in its chromosomal complement.

Studies of the karyotype of *Dactylorhiza* species have uncovered various structural variations in the morphology of their chromosomes and heterochromatic bands. One particularly fascinating aspect of *Dactylorhiza* cytogenetics is the occurrence of hybridization and polyploidization events, which result in the creation of hybrid species and polyploid complexes. These events have played a significant role in the diversification and adaptive radiation of *Dactylorhiza* species, but, to date, they have not been extensively studied using cytogenetic techniques. *Dactylorhiza* encompasses diploid (2n = 40) and polyploid (2n = 80) species, with a basic number of x = 20 [71,72]. However, there are exceptions within the genus. For instance, *D. insularis* and *D. maculata* subsp. *meyeri* exhibit a chromosome number of 2n = 3x = 60, although this is typically seen in hybrid taxa [73,74]. In Southern Italy, a cytological analysis of *D. romana* revealed some individuals with B-chromosomes, such as 2n = 40+1B, 2n = 40+2B, and 2n = 40+3B.

The chromosome of the genus *Dactylorhiza* is relatively small, like the 42-chromosome *Neotinea* and *Orchis* groups. Of the numerous species of *Dactylorhiza*, only *D. romana*, *D. saccifera* [75], *D. phoenissa* (B.Baumann & H.Baumann) P.Delforge [76], and *D. sambucina* (this study) have been subjected to a range of karyological investigations. In the *Dactylorhiza* species studied, C-banding of chromosomes revealed centromeric heterochromatin and chromosomes with telomeric heterochromatin. The C-banding and DAPI proved useful in identifying B chromosomes [75,76]. In individuals carrying the B chromosome, they displayed complete heterochromatin.

The genus *Gymnadenia* consists of diploid species with 2n = 2x = 40, and polyploid 2n = 4x = 80 are found, alongside 2n = 3x = 60 and 2n = 5x = 100 [77–79]. These chromosome numbers indicate that polyploidy has played a role in the evolution of various species in this genus [80]. Karyotype morphology in *Gymnadenia* is highly complex, with the centromeric position visible only in long chromosomes. Therefore, it was only with the use of Giemsa banding that further data for understanding the chromosomal complement could be obtained. C-banding analysis of *Gymnadenia* species revealed the presence of small centromeric bands in all of them. However, a comparison of chromosomes between species showed some distinctive characteristics. In *G. rhellicani* (2n = 40), C-banding revealed the presence of centromeric and telomeric heterochromatin in numerous chromosome pairs. *G. conopsea* (2n = 40) showed a close karyomorphological resemblance to *G. rhellicani*, although some interesting variations in the distribution and content of heterochromatin were also observed. In *G. conopsea*, all chromosomes exhibited evident centromeric heterochromatin, while fewer chromosomes displayed telomeric C-bands. *G. austriaca* (2n = 80) exhibited a high similarity in the distribution and content of heterochromatin along the chromosomes compared to the diploid *G. rhellicani*. Both species displayed similar banding patterns, including telomeric and intercalary bands located on the long or short arms of numerous chromosomes ([70] and this study). Using the DAPI dye, both *G. rhellicani* and *G. austriaca* showed some bands in centromeric and telomeric positions.

Therefore, cytogenetic studies have provided valuable insights into chromosomal diversity and evolution within the genera *Neotinea* s.l., *Orchis* s.str., *Platanthera*, *Chamorchis*, *Dactylorhiza* s.l., and *Gymnadenia* s.l. Understanding karyotype variations and chromosomal rearrangements in these orchids can shed light on the processes underlying speciation, adaptation, and the generation of the Orchidaceae family's remarkable diversity.

These findings call for cross-disciplinary investigations concerning the significance of constitutive heterochromatin in the evolutionary dynamics of these plant species. Cytogenetic explorations have demonstrated their worth as a means of unravelling the intricate chromosomal attributes and evolutionary pathways present within the *Neotinea* s.l. and *Orchis* s.str. groupings. A more profound comprehension of the observed differences in karyotype and chromosomal structures among these orchids offers the promise of shedding light on the mechanisms underpinning speciation, adaptation, and the emergence of the remarkable diversity evident within the Orchidaceae family.

Our study used classical methods, but the incorporation of comparative cytogenetics, aided by fluorescence techniques such as FISH, will undoubtedly enhance the examination of chromosomal evolution across species [81]. Indeed, comprehending alterations in the structure and arrangement of chromosomes contributes significantly to our under-standing of evolutionary processes. Fluorescence In Situ Hybridization (FISH) is a widely employed technique involving the use of fluorescent probes that selectively bind to specific DNA sequences on chromosomes. This enables researchers to visualize the precise location and distribution of these sequences within the cell [82].

5. Conclusions

In this study, we revisited the cytogenetics of *Neotinea* s.l., *Orchis* s.str., *Platanthera*, *Chamorchis, Dactylorhiza*, and *Gymnadenia* species with 40,42 chromosomes, focusing on karyotype organization, chromosomal evolution, and possible implications for speciation. Our findings shed light on the complex evolutionary relationships among these species and provide valuable insight for future research into orchid cytogenetics. Our analysis revealed variation in the distribution of heterochromatin and repetitive DNA sequences that may contribute to the diversification of these species.

Our comparative karyotype analysis, supported by fluorescence experiments, revealed several instances of chromosomal rearrangements that have probably played a role in the differentiation of these species. Therefore, the cytogenetic data gathered in this study provide valuable insights into the speciation of Orchidinae taxa with 40,42 chromosomes.

These findings contribute to the growing body of knowledge on orchid cytogenetics and provide a foundation for future research in this fascinating group of plants.

In conclusion, the study of orchid cytogenetics has uncovered a remarkable level of complexity and variability within this plant group. Classical cytogenetic techniques have proven invaluable in elucidating taxonomic relationships, characterizing individual chromosomes, and clarifying the mechanisms underlying polyploidy.

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