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Polyploidy in Two Species of the *Allium ampeloprasum* Complex (Amaryllidaceae): New Evidence and Cytogenetic Insights

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Abstract

Providing new cytogenetic results, this study analyzes the karyotype of *Allium polyanthum* and *A. atroviolaceum* (Amaryllidaceae sect. *Allium*) using classical cytogenetic techniques (Feulgen staining, Ag-NOR, C-banding), while explicitly excluding meiotic analyses. Both species show a stable tetraploid chromosome complement (2n = 4x = 32) across all examined populations, with predominantly symmetrical karyotypes dominated by metacentric chromosomes; quantitative asymmetry parameters (M_{CA}, CV_{CL}, CV_{CI}) are very similar between the two species. Notably, a single specimen of *A. atroviolaceum* from Albania represented an exception: the chromosome number was variable within the meristematic tissue, exhibiting somatic numerical instability. The main differences concern the organization of nucleolar chromosomes: both species show secondary constrictions in a sub-telomeric position (i.e., "scorodoprasum" type) and secondary constrictions near the centromere (i.e., "sativum" type), but with different numbers and distributions. *Allium polyanthum* shows a maximum of 4 nucleoli per interphase nucleus (corresponding to 2 pairs of homologous chromosomes with secondary constrictions), while *A. atroviolaceum* shows up to 7, often smaller in size. The distribution of constitutive heterochromatin (C-banding) is also species-specific, despite an overall similarity in chromosome morphology. The authors interpret these features (normal chromosomal rearrangement, karyotype symmetry, heterochromatin organization) as indicative of a relatively recent evolutionary event, which may have contributed to the cytogenetic stabilization and diversification of these taxa. The study also confirms the usefulness of conventional cytogenetic markers, when integrated with NOR and heterochromatin analysis, for studying chromosome evolution in polyploid species of the genus *Allium*.

Keywords: *Allium atroviolaceum*; *Allium polyanthum*; classical cytogenetics; karyotype asymmetry; nucleoli; secondary constrictions

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Academic Editor: Jian Zhang

Received: 9 September 2026
Revised: 17 October 2026
Accepted: 18 October 2026
Published: 21 October 2026

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1. Introduction

The genus *Allium* L. (Amaryllidaceae) [1] comprises approximately 1090 currently accepted species distributed throughout the temperate and subtropical regions of the Northern Hemisphere and extending into southern Africa [2]. The genus exhibits remarkable biological diversity, particularly with respect to vegetative morphology, including bulbous

and rhizomatous growth forms, as well as ecological adaptations [3]. Owing to its wide geographical distribution, *Allium* encompasses several centers of diversification characterized by considerable morphological, ecological, and cytogenetic variability [4–14].

The genus *Allium* has long been the subject of extensive karyological [4,15–23] and biosystematic investigations [24]. These studies have demonstrated substantial variability among taxa and have highlighted the important role of polyploidy in morphological differentiation, ecological adaptation, and speciation processes [23,25]. Cytogenetic analyses have shown that the predominant basic chromosome number in the genus is x = 8. Nevertheless, additional basic chromosome numbers (x = 7, 9, 10, and 11) have also been reported, together with multiple ploidy levels, including tetraploid, hexaploid, and octoploid cytotypes [26]. These investigations have been fundamental for understanding the evolution and cytogenetic organization of economically important species such as *Allium sativum* L., *A. cepa* L., *A. porrum* L., and *A. ampeloprasum* L. [27].

In particular, the *Allium ampeloprasum* complex [4], belonging to subgenus *Allium*, section *Allium*, and including numerous subspecies, varieties, and taxonomic synonyms [2], comprises several taxa of major importance in human nutrition, such as *A. ampeloprasum* L. and *A. porrum* L. [6,27,28].

A variety of classical and molecular cytogenetic techniques have been applied to the study of the *A. ampeloprasum* complex, including Feulgen staining, Giemsa banding, fluorochrome staining with DAPI, Hoechst 33258, and CMA_3, as well as fluorescence in situ hybridization (FISH). These approaches have provided valuable information on chromosome number, karyotype structure, heterochromatin distribution, and genome organization [3,4,15,21,23,24,29–41].

The cytogenetics of the *A. ampeloprasum* complex has been investigated extensively, with most studies reporting a basic chromosome number of x = 8 and diploid complements of 2n = 2x = 16. In addition, several polyploid cytotypes have been described, including triploids (3x = 24), tetraploids (4x = 32), pentaploids (5x = 40), hexaploids (6x = 48), and octoploids (8x = 64) [4,6,16,17,23,31,38,40–44]. It should be noted that these represent variations in ploidy level rather than true cases of aneuploidy, the latter involving chromosome numbers that deviate from exact multiples of the basic set.

Karyomorphological studies in many species of the genus *Allium* using some morphometric parameters (e.g., M_{CA}, CV_{CL}, CV_{CI}) have highlighted interchromosomal and intrachromosomal karyotypic asymmetry [45–48], important in understanding the evolution of the karyotype [49,50].

Fundamental insights into the origin and evolution of polyploid taxa within the *Allium ampeloprasum* complex, including *A. ampeloprasum*, *A. porrum*, and *A. atroviolaceum* Boiss., have been obtained through cytogenetic analyses of mitosis and meiosis, complemented by detailed karyotype reconstructions [3,4,9,17,31,36–38,41,51,52]. Regarding meiotic studies, Kollmann [16], building on previous work [42], proposed that the localization of chiasmata in proximal chromosome regions restricts multivalent formation during meiosis in polyploid *A. ampeloprasum*. This species may exhibit both bivalent and multivalent chromosome associations, depending on the degree of homoeology among chromosome sets [17,37]. The predominance of bivalents generally indicates a certain degree of diploidization or preferential chromosome pairing, whereas the occurrence of trivalents and tetravalents reflects residual homoeologous interactions among related chromosome sets [31]. Such meiotic configurations are commonly observed in species that have undergone relatively recent polyploidization, including cultivated leek (*A. porrum*) [52]. Consequently, meiotic pairing behavior provides valuable insights into genome organization and evolutionary history. The frequent occurrence of diverse meiotic configurations within the *A. ampelopra-*

sum complex likely reflects its considerable genetic diversity and evolutionary flexibility and may have contributed substantially to the diversification of the group [17,41,53].

Allium polyanthum Schult. & Schult.f. and *A. atroviolaceum* Boiss. are two distinct evolutionary models within the *A. ampeloprasum* complex, both sharing a base chromosome number of x = 8. The inclusion of *A. polyanthum* and *A. atroviolaceum* within the *Allium ampeloprasum* complex is justified by a combination of morphological, evolutionary, and ecological factors. From a morphological standpoint, both species share the foundational traits of the group (e.g., tunicated bulbs with lateral bulbils, robust scapes, and dense umbels), while exhibiting continuous and graded transitions in features like tepal proportions and exserted stamens with *A. ampeloprasum* s.l. [7]. Furthermore, molecular phylogeny based on chloroplast DNA markers and ITS regions reveals complex alloploid origins, historical gene flows, and high genetic similarity, demonstrating that these entities form a closely cohesive, actively evolving species complex rather than isolated lineages [11,25,54]. Finally, their frequent occurrence in synanthropic and cultivated habitats, where human pressure and vegetative propagation foster intermediate forms, further blurs traditional taxonomic boundaries and supports their aggregation into a single complex.

A. polyanthum was long confused with, or treated as a subspecies or synonym of, *A. ampeloprasum* s.l. (e.g., *A. ampeloprasum* subsp. *polyanthum*) due to shared key traits of the section, including a globose umbel inflorescence structure, the presence of bulbils, and general leaf morphology. Mathew [5] recognizes *A. polyanthum* as a distinct yet closely related species, firmly placing it within the phylogenetic and taxonomic framework of the "*A. ampeloprasum* complex" and distinguishing it primarily by the number and length of its bulbils and subterranean, stalked secondary bulbils. While the baseline cytotype is tetraploid (2n = 4x = 32) [38], it frequently forms unstable pentaploid (2n = 40) and hexaploid (2n = 48) series [54].

A. atroviolaceum is another representative of the *A. ampeloprasum* group. It shares the same basic structural organization (a bulbous geophyte with basal/stoloniferous bulbils and tricuspidate inner stamens). Within the complex, *A. atroviolaceum* is distinguished by the characteristic coloration of its tepals (ranging from dark purple to blackish-violet) and by specific cytogenetic profiles (often associated with diploid and tetraploid forms); however, it displays such close genetic and morphological affinity that various botanists and taxonomists treat it as a core species within the *A. ampeloprasum* aggregate. Distributed primarily in the harshest and most arid environments of the Mediterranean–Turanian and Caucasian regions, *A. atroviolaceum* exhibits a stable tetraploid constitution (2n = 4x = 32) [25,38,55,56]. In contrast, within its Italian range, which is rare and highly fragmented (concentrated mainly in Puglia), specific cytogenetic data remain scarce, and local research is largely limited to phytogeographical analyses and conservation assessments [57]. In the present study, we conducted karyosystematic investigations of *Allium polyanthum* and *A. atroviolaceum* based on previously unexplored wild populations from their native habitats in Italy (Puglia and Basilicata). The study aimed to expand current knowledge of the natural karyological variability of these species through the analysis of chromosome number and morphology, including secondary constrictions, constitutive heterochromatin distribution, and chromosomal variations.

2. Materials and Methods

2.1. Materials

The material studied consisted of bulbs or bulbils from some populations of *Allium polyanthum* Schult. & Schult.f. and *A. atroviolaceum* growing in their natural habitat in the southern Puglia and Basilicata regions, in Italy (five specimens per site), and seed accession (one specimen) of *A. atroviolaceum* from Himara (S of Vlorë), in Albania (Table 1, Figure 1).

Table 1. Collecting sites of the *Allium polyanthum* and *A. atroviolaceum* samples investigated in the present study.

Taxon	Collecting Sites
Allium polyanthum Schult. & Schult.f.	Southern Italy: Molfetta (Bari), Bari, Cassano delle Murge (Bari), Casamassima (Bari).
A. atroviolaceum Boiss.	Southern Italy: Molfetta (Bari), Bari, Casamassima (Bari), Monopoli (Bari), Metaponto (Matera). Albania: Himara (S of Vlorë).

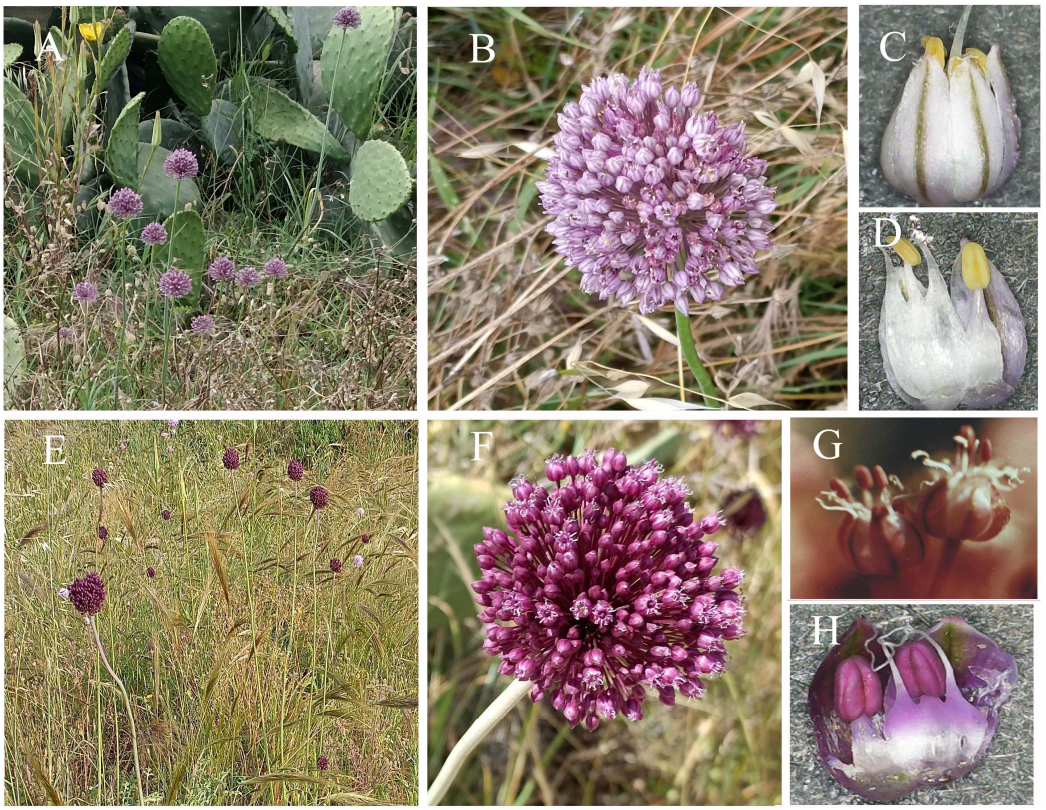


Figure 1. (**A**–**D**) *Allium polyanthum*. (**A**) Habit; (**B**) Inflorescence; (**C**) Flower; (**D**) Detail of the stamens with yellow anthers. (**E**–**H**) *A. atroviolaceum*. (**E**) Habit; (**F**) Inflorescence; (**G**) Flower; (**H**) Stamens with red-purple anthers.

2.2. Cytological Analysis

Root tips of bulbs, bulbils or germinated seeds were pre-treated 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. Hydrolysis was performed at 20 °C in 5.5 N HCl for 20 min [58]. The material was then stained with freshly prepared Feulgen stain. Chromosome counts and karyotype analyses were performed on a total of 30 specimens per species across six sampled sites in the Puglia and Basilicata regions. To ensure accurate characterization and assess chromosomal stability, a minimum of ten of the highest-quality metaphase spreads were examined for each specimen, with karyotypes constructed from the most well-spread metaphase plates.

For C-banding, root tips were fixed in 3:1 (*v*/*v*) ethanol-glacial acetic acid and stored in the deep freezer for up to several months. Subsequently, they were squashed in 45% acetic acid; coverslips were removed by the dry ice method, and the preparations were air-dried overnight. 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. The stain used was 3–4% Giemsa (BDH) at pH 7 [59].

For identification of the nucleoli, $AgNO_3$ (silver nitrate) precipitation was used. Root tips of bulbs or bulbils were pre-treated with 0.3% colchicine at room temperature for 2 h, then fixed in 3:1 (*v*/*v*) ethanol-glacial acetic acid and stored in a deep freezer. Subsequently, they were squashed in 45% acetic acid, coverslips were removed by the dry ice method, the preparations were air-dried overnight, and then they were incubated in $AgNO_3$ (1 gr/1 mL) at 37 °C for 10–30 min, modified after Lacadena & Cermeño [60].

2.3. Chromosome Numbers and Karyotype Parameters

Chromosome pairs were identified and arranged based on their length. However, it is important to note that the identification and placement of nucleolar chromosome pairs in karyotyping may be subject to variation due to the morphological similarity of the chromosomes. Therefore, the numerical designation of these pairs is intended only as a provisional reference and should not be interpreted as a unique assignment.

The nomenclature for secondary constrictions follows [15]. *Scorodoprasum* type: "secondary constriction in a sub-telomeric position, between the centromere and the telomere"; this location delimits an isolated distal segment called a satellite. *Sativum* type: "secondary constriction near the centromere".

The nomenclature used for describing karyotype composition follows Levan et al. [61], 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 [45–47].

Chromosome measurements were conducted using IdeoKar version 1.2 freeware (http://agri.uok.ac.ir/ideokar/index.html, accessed on 30 October 2025).

Preliminary screening revealed no chromosomal variation or intraspecific polymorphism among the six sampled localities; therefore, data from all populations were pooled and analyzed cumulatively at the species level.

To construct the karyotype, images obtained using the Feulgen and C-banding methods were processed with free photo-editing software, with all adjustments applied uniformly to the entire image.

2.4. Nomenclature

The nomenclature used for the species of *Allium* follows POWO [2].

3. Results

3.1. Allium polyanthum from S Italy

Our results confirm the chromosome number 2n = 4x = 32, previously described by numerous authors [11,23,38,62–64], in all specimens analyzed (Figures 2C and 3A). It is noteworthy that all individuals from the six populations studied exhibited similar karyomorphology, C-banding patterns, and nucleolar chromosomes, highlighting the absence of significant population-level variation. The karyotype exhibited 28 metacentric and 4 submetacentric chromosomes. Following the homology of most chromosomes, the karyotype was constructed with 16 pairs each. Pair number 7 showed a secondary "scorodoprasum-type" constriction in one submetacentric chromosome. Pair 13 showed a "sativum-type" secondary constriction in a metacentric chromosome (Figure 4A). The karyotype is symmetric, with both a low intrachromosomal asymmetry index (M_{CA}) (average 14.59) and interchromosomal asymmetry index (CV_{CL}) (average 22.59) (Table 2).

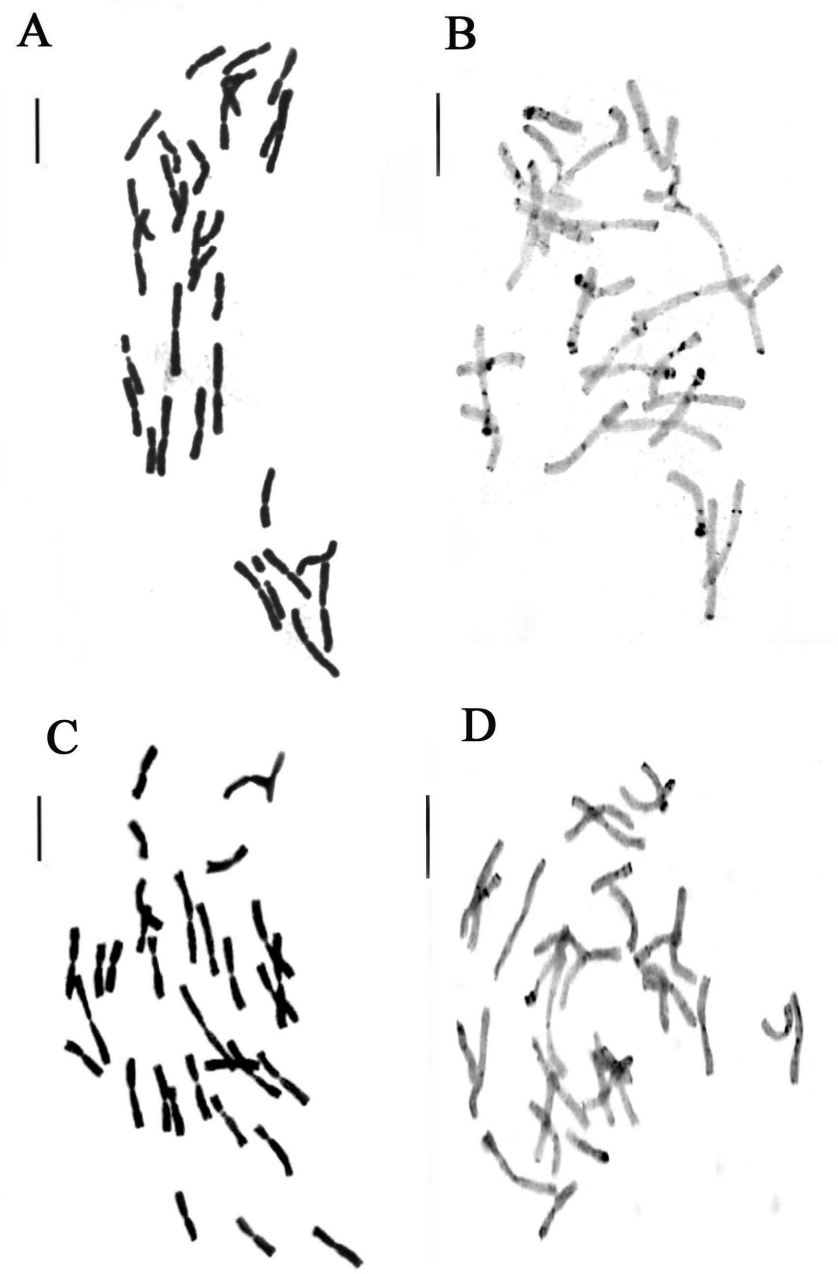


Figure 2. (**A**,**B**) Mitotic metaphase chromosomes of *A. atroviolaceum* (2n = 4x = 32) from the collection site of Casamassima (Bari, Italy), where the species was first recorded in Italy [65]: (**A**) Feulgen technique; (**B**) C-Banding. (**C**,**D**) *A. polyanthum* (2n = 4x = 32) from the collection site of Casamassima: (**C**) Feulgen technique; (**D**) C-Banding. All scale bars = 10 μm.

C-banding revealed a centromeric heterochromatin band in all chromosomes (Figure 3B). The chromosomes showed approximately 68 major bands, including centromeric, telomeric, and subterminal bands. Some chromosomes exhibited telomeric bands of varying sizes. Indeed, only pairs 10, 14, 15, and 16 showed conspicuous telomeric bands. An intercalated band was observed in chromosomes 1, 6, 9, 11, and 13, while a double band (visible as a dot) was observed in pair 10 (Figures 4B and 5A). Silver nitrate staining revealed a maximum of four nucleoli (Figure 6A,B).

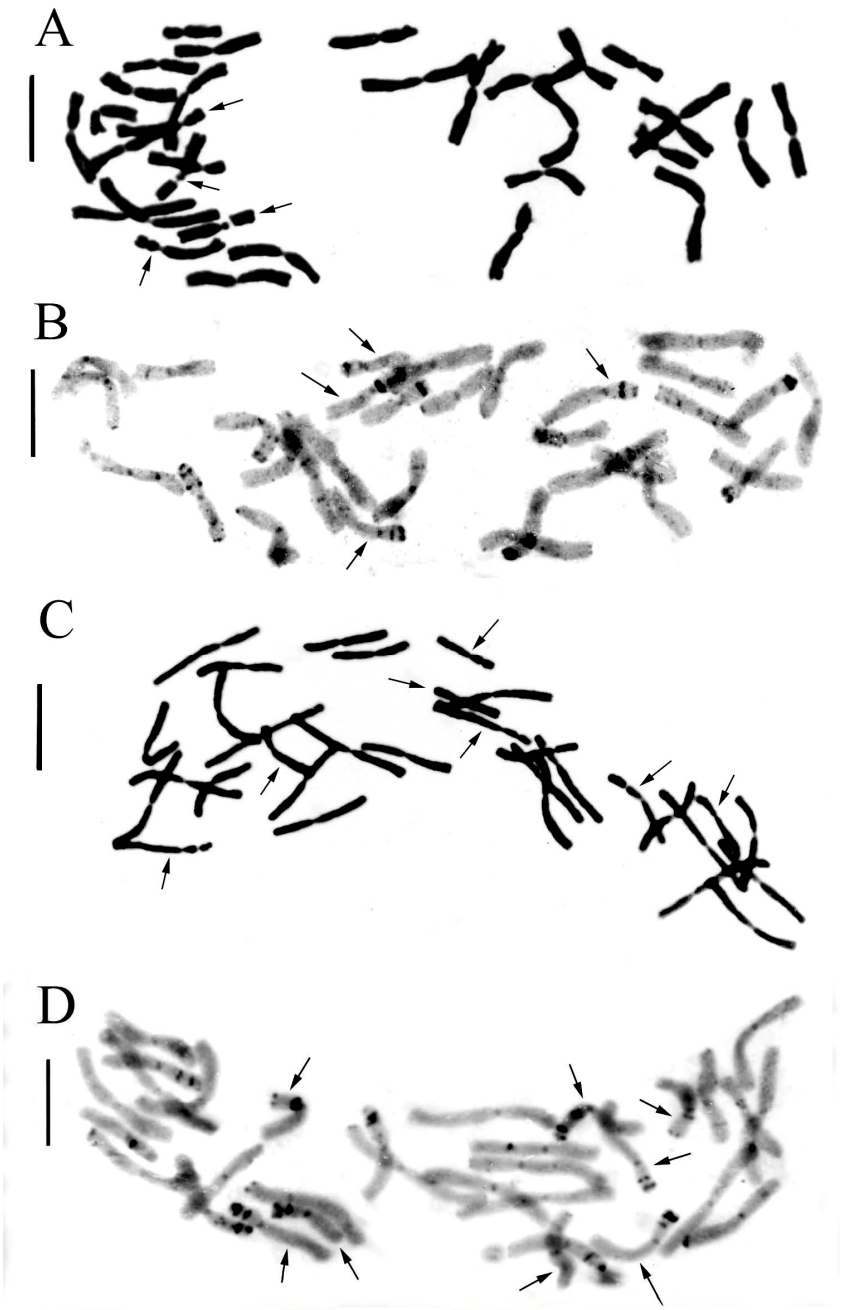


Figure 3. (**A**,**B**) *Allium polyanthum* (2n = 4x = 32). (**A**) Mitotic metaphase plate stained with the Feulgen technique; (**B**) Mitotic metaphase plate stained with C-banding. (**C**,**D**) *A. atroviolaceum* (2n = 4x = 32). (**C**) Feulgen technique; (**D**) C-Banding. Arrows indicate satellite chromosomes. All scale bars = 10 µm.

Table 2. Taxon name, chromosome number, formula, and morphometric parameters in *Allium polyanthum* and *A. atroviolaceum*. Values are expressed as mean ± Standard error calculated from the analyzed karyotypes per species. THL = total chromosome length of the haploid complement; M_{CA} = Mean Centromeric Asymmetry; CV_{CL} = Coefficient of Variation of Chromosome Length; CV_{CI} = Coefficient of Variation of the Centromeric Index. Chromosome abbreviations: m, metacentric; sm, submetacentric.

Taxon	Chromosome Number	Formula	THL (µm)	M_{CA}	CV_{CL}	CV_{CI}
Allium polyanthum	2n = 4x = 32	28 m + 4 sm	168.90 (±7.06)	14.59 (±1.44)	22.59 (±1.35)	13.16 (±0.77)
A. atroviolaceum	2n = 4x = 32	26 m + 6 sm	179.48 (±4.07)	14.05 (±0.27)	18.22 (±0.65)	12.73 (±0.77)

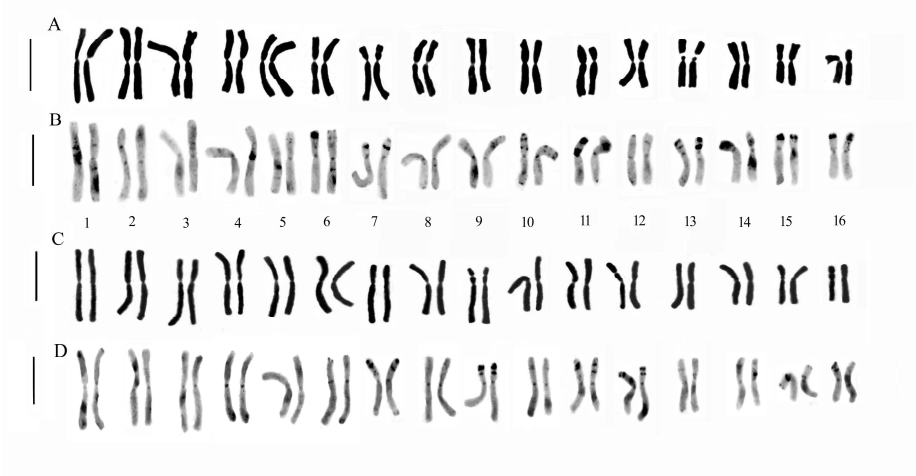


Figure 4. (**A**,**B**) *Allium polyanthum*. (**A**) Feulgen karyotype; (**B**) C-banded karyotype. (**C**,**D**) *A. atroviolaceum*. (**C**) Feulgen karyotype (note that in pairs 12, 15 and 16 it is not always possible to observe the secondary constraints in the two homologues); (**D**) C-banded karyotype. All scale bars = 10 μm.

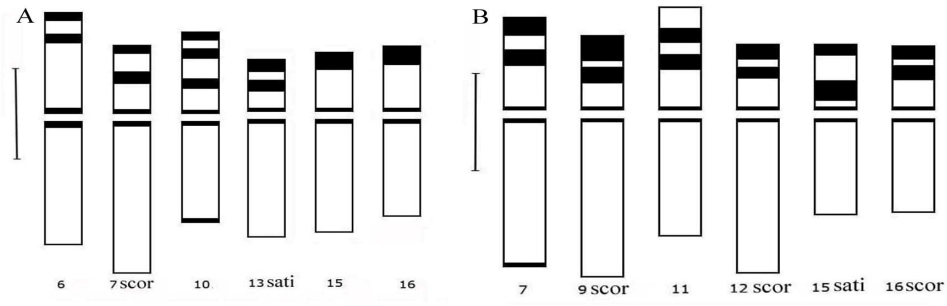


Figure 5. Partial haploid ideograms and heterochromatin distribution in: (**A**) *Allium polyanthum*; (**B**) *A. atroviolaceum*. Abbreviations: scor = scorodoprasum; sati = sativum. Scale bars = 10 μm.

3.2. Allium atroviolaceum from S Italy

The chromosome number 2n = 4x = 32 is confirmed [38] (Figures 2A and 3C). The karyotype of this species showed a karyological formula consisting of 26 metacentric and six submetacentric chromosomes. In this species, as in *A. polyanthum*, the karyotype was assembled into 16 pairs (Figure 4C). A chromosome with a "sativum-type" secondary constriction (number 15) and three pairs with a "scorodoprasum-type" secondary constriction (pairs 9, 12, and 16) were shown. As reported by D'Emerico and Pignone [38], in the "sativum-type" pair, the nucleolar constriction is not always visible in the metaphase plates using the Feulgen method. The karyotype is symmetrical, having a low intrachromosomal asymmetry (M_{CA}) index (average 14.05) and a low interchromosomal asymmetry (CV_{CL}) index (average 18.22) (Table 2). In specimens of this species, C-banding also revealed a centromeric heterochromatic band on all chromosomes (Figure 3D). The chromosomes showed approximately 63 major bands between centromeric, telomeric and subterminal. Some chromosomes showed heterochromatic bands or dots at the telomere. Pair number 7 showed two conspicuous heterochromatic bands at the telomere and intercalary positions. Pair number 9, with secondary subtelomeric constriction, showed two conspicuous bands, telomere and intercalary, close together. Pair number 11 showed two clear intercalary bands. Pairs number 12 and 16 showed a conspicuous telomere band. Pair number 15 showed a thin telomere band (Figures 4D and 5B). Silver nitrate staining revealed a maximum of seven nucleoli (Figure 6C,D). It is important to emphasize that it has not always been possible to identify the secondary constrictions on both chromosomes in the NOR (nucleolus organizer regions) homologs of pairs nos. 12, 15, and 16.

3.3. *Allium atroviolaceum from Himara (S of Vlorë), Albania*

For the accession used in our study, the results were obtained using the C-banding method alone (Figure 7A–C). The chromosome number was variable within the meristematic tissue, exhibiting somatic numerical instability with counts of 40 (Figure 7A), 44 (Figure 7B), and 48 (Figure 7C). The chromosomes showed a significant number of distinct bands (approximately 210) distributed across the centromeric, telomeric, and subterminal regions. Numerous chromosomes showed conspicuous telomeric bands, compared to the subtelomeric bands, which in many cases were highlighted as dots. In Figure 8, we have arranged the chromosomes in order of length. The last four chromosomes show a modest distribution of heterochromatin compared to the others.

Within the mitotic karyotype, where chromosomes are arranged individually, some elements exhibit similar C-banding patterns in identical positions, suggesting a potential morphological correspondence between them.

The substantial difference in the total number of bands observed between the Albanian (~210 bands) and the Italian (~63 bands) accessions can be explained by distinct biological features. Firstly, the Albanian material possesses a higher chromosome number (2n = 48) compared to the Italian accession (2n = 32). Secondly, C-banding revealed a significantly greater content and wider distribution of constitutive heterochromatin across the genome of the Albanian material, leading to a higher number of detectable bands. Because identical preparation protocols and counting criteria were applied to both accessions, these results reflect a genuine chromosomal and genomic divergence between the two geographical lines rather than technical variations.

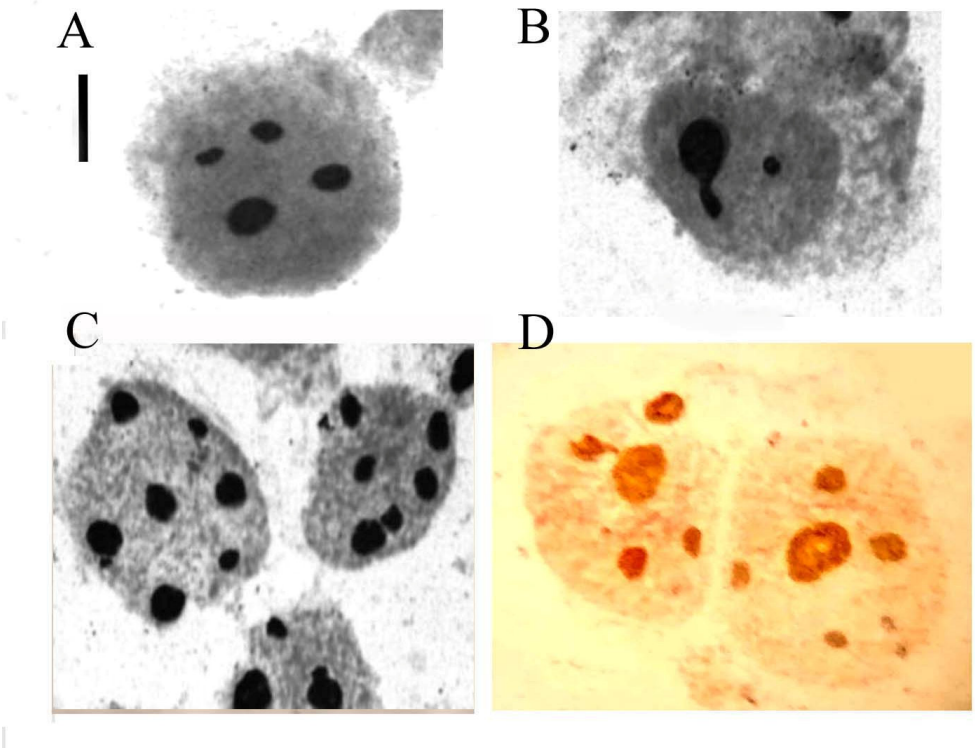


Figure 6. Silver staining. (**A**,**B**) *Allium polyanthum*, Nucleoli of interphase nuclei cells with three to four nucleoli. (**C**,**D**) *A. atroviolaceum***.** Nucleoli of interphase nuclei cells with five to seven nucleoli of equal or different size. Scale bar = 10 μm.

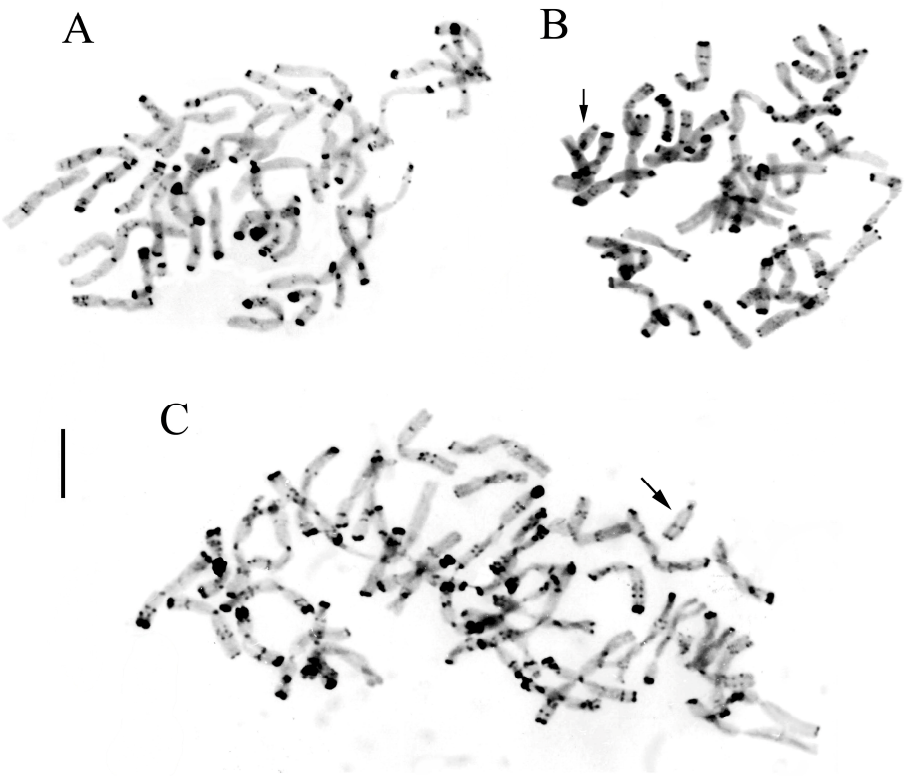


Figure 7. (**A**–**C**) Metaphase chromosomes of *Allium atroviolaceum* from Himara (S of Vlorë), Albania, after C-banding. (**A**) 40; (**B**) 44; (**C**) 48 chromosomes. Arrows: probable B-chromosome. Scale bar = 10 μm.

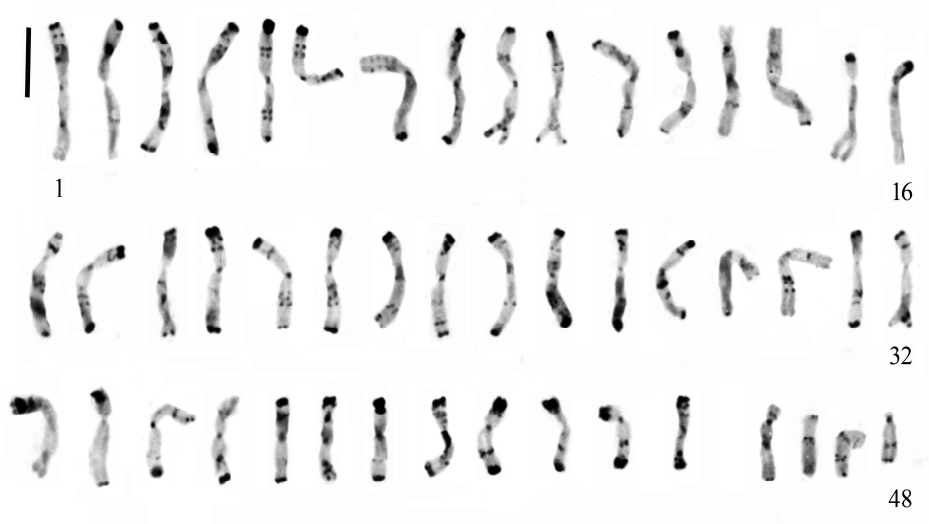


Figure 8. *Allium atroviolaceum* (48 chromosomes); C-banded karyotypes. Note the last four chromosomes with little constitutive heterochromatin. Scale bar = 10 μm.

3.4. Asymmetry Indices

Following the karyotype asymmetry proposed by Peruzzi & Eroğlu [47]. The Mean Centromeric Asymmetry (M_{CA}) values for *A. polyanthum* (14.59 ± 1.44) and for *A. atroviolaceum* (14.05 ± 0.27) lean heavily toward the symmetric lower bound of the (M_{CA}) scale (0–100), reflecting the overwhelming prevalence of metacentric chromosomes (28 m) and (26 m), respectively. Similarly, the Coefficient of Variation of Chromosome Length (CV_{CL}) values, 22.59 (±1.35) and 18.22 (±0.65), fall strictly into the lower tier of interchromosomal variation. This quantitative profile demonstrates a conserved and symmetric chromosome complement shared between these two closely related *Allium* species.

3.5. Statistical Basis

The karyometric indices computed via STATS 2.0 (Table 2) revealed a striking similarity in chromosomal symmetry between the two tetraploid taxa (2n = 4x = 32). Specifically, the mean values for the Mean Centromeric Asymmetry (M_{CA}), Coefficient of Variation of Chromosome Length (CV_{CL}), and Coefficient of Variation of the Centromeric Index (CV_{CI}) were extremely close between *A. polyanthum* and *A. atroviolaceum*. Furthermore, their respective standard errors showed a substantial overlap (e.g., 14.59 ± 1.44 vs. 14.05 ± 0.27 for M_{CA}). Due to this evident numerical and biological proximity, a formal interspecific statistical comparison was deemed redundant, as the data descriptively confirm a highly conserved and homogeneous karyotype structure shared by both species.

4. Discussion

Based on numerous studies reported in the literature, we re-examined previous statements by other authors regarding the polyploidy of *Allium polyanthum* and *A. atroviolaceum*. We also analyzed the karyomorphology of these species in populations occurring in two Italian regions, namely Puglia and Basilicata. In the Puglia region, *A. polyanthum* and *A. atroviolaceum* are widespread. The karyotypes of specimens from this area were previously investigated using Feulgen staining and C-banding techniques by D'Emerico and Pignone [38]. In the present study, we reconsidered, in the karyotypes of both species, the presence of satellite chromosomes with secondary constrictions located median or proximal to the centromere [15].

4.1. Some Considerations on the Techniques Used in the Two Species

In *Allium polyanthum* and *A. atroviolaceum*, Feulgen-stained chromosomes appear relatively homogeneous in morphology and can be distinguished primarily based on their length, centromere position, and the presence of secondary constrictions [15,20,38]. In contrast, C-banding revealed distinct dark bands corresponding to constitutive heterochromatin. Several putative chromosome pairs displayed distinguishable and reproducible C-banding patterns. These banding profiles provide information on the distribution of constitutive heterochromatin, which in *Allium* species is frequently localized in pericentromeric regions and, in some cases, also in the satellite regions [21,29,35,38,41]. Together, the three staining techniques employed in this study provide complementary information on chromosome organization in the two species examined, each highlighting different levels of structural and functional organization. Silver nitrate staining detects possible transcriptionally active nucleolar organizer regions (NORs). In Feulgen-stained chromosomes, these NORs correspond morphologically to the secondary constrictions observed on specific chromosome pairs [60]. Giemsa banding complements this characterization by revealing the distribution of constitutive heterochromatin associated with these regions [35,38].

4.2. Allium polyanthum

Allium polyanthum, previously confused with or included in *A. ampeloprasum* [66], is a highly variable Mediterranean species for which considerable cytogenetic variation has been reported. Published chromosome numbers include 2n = 16, 24, 32, 40, 48, and 56 [67], although some historical records may reflect the taxonomic complexity of the *A. ampeloprasum* complex and should therefore be interpreted with caution. In the populations examined in the present study, all analyzed individuals consistently showed a tetraploid chromosome complement of 2n = 4x = 32, in agreement with the observations of Verlaque et al. [63] and D'Emerico and Pignone [38]. The chromosomes of *A. polyanthum* are relatively large, and their frequent overlap at metaphase may obscure small secondary constrictions and satellites. These structures can nevertheless provide useful cytological characters for chro-

mosome identification and comparison, as previously demonstrated in *Allium* species [23]. The combined application of conventional Feulgen staining and C-banding was therefore particularly useful for assessing chromosome morphology and heterochromatin distribution. In the populations examined from Puglia, the 32 chromosomes could consistently be arranged into 16 morphologically recognizable pairs. This arrangement indicates a high degree of structural differentiation among chromosomes and facilitates the identification of corresponding chromosome pairs. A comparable organization has been described in *Allium iranicum* (Wendelbo) Wendelbo (≡ *A. ampeloprasum* subsp. *iranicum* Wendelbo), in which Ghaffari [9] reported predominantly bivalent configurations at meiotic metaphase I and interpreted the chromosome complement as consisting of two sets of 16 chromosomes. In that case, the meiotic evidence provided an important basis for inferring preferential chromosome pairing. Similar diploid-like organizations have also been discussed in other tetraploid *Allium* taxa, including *A. valdecallosum* Maire & Weiller (2n = 4x = 32) [12] and *A. rouyi* Gaut. (=*A. reconditum* Pastor, Valdés & J.M. Muñoz; 2n = 6x = 48) [68]. In the present study, however, no meiotic material was available; therefore, the organization observed in somatic metaphases can only be interpreted as evidence of a diploid-like karyotypic structure and does not provide direct evidence of preferential homologous pairing or diploidization. The C-banding pattern provided additional characters for chromosome identification and supported the reproducibility of the karyotype organization observed in Feulgen-stained preparations. In agreement with Bothmer [4], but differing from the observations of Guern et al. [23] and D'Emerico and Pignone [38], we consistently identified only one pair of satellite chromosomes corresponding to the so-called "scorodoprasum-type" (pair 7) and one pair corresponding to the "sativum-type" (pair 13). These chromosome categories are primarily distinguished by the position of their secondary constrictions [15]. The differences among previous reports may reflect genuine population-level variation in NOR number or organization, structural chromosomal polymorphism, or differences in the cytological visibility of secondary constrictions. Variation in NOR activity may also contribute to differences in their expression, although an environmental regulatory effect cannot be established from the present data.

The occurrence of satellite chromosomes is consistent with the presence of NORs, although the identification of a satellite chromosome should not be considered equivalent to direct molecular identification of an rDNA locus. This interpretation was further supported by silver staining, which revealed up to four nucleoli in interphase nuclei. Secondary constrictions are commonly associated with NORs containing tandemly repeated 35S rDNA units encoding the 18S, 5.8S and 28S ribosomal RNAs. During metaphase, active NORs may remain relatively decondensed and therefore appear as secondary constrictions [69]. Nevertheless, the number and visibility of secondary constrictions may vary according to chromosome condensation, orientation and the level of NOR expression.

4.3. Allium atroviolaceum

Although considerable cytogenetic information is available for *A. ampeloprasum* and *A. polyanthum*, data concerning *A. atroviolaceum* remain comparatively scarce and geographically fragmented, with several records originating from regional investigations [8,11,38,40,41,70]. In all populations of *A. atroviolaceum* examined in Puglia and Basilicata, the chromosome number was consistently 2n = 4x = 32, according to the observations of D'Emerico and Pignone [38]. Other chromosome numbers, including 2n = 16 and 2n = 24, have been reported from populations in Türkiye [71], indicating that cytogenetic variation may occur within the species or, alternatively, that some historical records require taxonomic reassessment. Feulgen staining and C-banding allowed the 32 chromosomes of the Puglia and Basilicata populations to be arranged consistently into 16 morphologically

recognizable pairs. As in *A. polyanthum*, this arrangement indicates a well-differentiated tetraploid karyotype in which individual chromosome pairs can be distinguished based on chromosome size, morphology and heterochromatin distribution. The presence of 16 recognizable chromosome pairs may be regarded as compatible with a diploid-like structural organization of the tetraploid complement. The alternative possibilities of an allotetraploid, segmental allopolyploid or structurally differentiated autotetraploid organization therefore remain open. The C-banding pattern provided additional evidence for the reproducibility of the chromosome morphology and revealed a characteristic distribution of constitutive heterochromatin. In contrast to *A. polyanthum*, *A. atroviolaceum* consistently exhibited three pairs of satellite chromosomes of the "scorodoprasum-type" and one pair of the "sativum-type" [15], according to the observations of D'Emerico and Pignone [38]. This difference in the number and morphology of satellite chromosomes represents a potentially informative cytogenetic distinction between the two taxa.

The *Allium atroviolaceum* specimen collected near Vlorë, Albania, deserves particular consideration because it exhibited an unusual distribution of constitutive heterochromatin, marked structural differences, and distinct somatic numerical instability within the examined meristematic tissues compared with the stable karyotypes observed in the Puglia populations examined both in the present study and by D'Emerico and Pignone [38]. Cytogenetic analysis of the meristematic tissue revealed a pattern of somatic mixoploidy, although the low frequency of optimal metaphases and technical difficulties in chromosome spreading, associated with the absence of Feulgen staining, warrant caution regarding exact counts. Alongside the main lineage estimated at 2n = 48 chromosomes, plates with 44 and 40 chromosomes were observed. Assuming a basic chromosome number of x = 8 for this group, the counts of 2n = 40 and 2n = 48 represent euploid pentaploid (5x) and hexaploid (6x) complements, respectively, whereas only the 2n = 44 count is not an exact multiple of the base number and represents a true aneuploid condition or another specific cytogenetic anomaly requiring further verification. Notably, the 48-chromosome complement includes four markedly smaller elements; the potentially unstable nature of these minor chromosomes (such as B-chromosomes) could account for their preferential mitotic elimination, thereby explaining the recorded somatic variability. The observed variations may indicate substantial population-level chromosomal polymorphism or a more complex genomic organization. Alternatively, they could be associated with structural chromosome rearrangements or mitotic instability, which is often reflected by such intra-individual numerical variation in somatic tissues. More speculatively, these features could stem from interspecific hybridization or introgression involving another *Allium* species, phenomena well known to induce genomic shock and numerical chromosomal aberrations. Because additional individuals from the same population were not available, it is currently impossible to determine whether this mosaicism and these structural features are characteristic of the population or represent an individual somatic cytogenetic variant. The Vlorë specimen should therefore be regarded as preliminary evidence of complex cytogenetic differentiation requiring confirmation through the screening of wider population samples and a detailed analysis of both somatic and germline stability.

4.4. Nucleolar Constrictions

The analysis of secondary constrictions and nucleoli provided additional information on the organization and activity of putative nucleolar organizer regions in the two species. In *A. polyanthum*, up to four nucleoli were observed in interphase nuclei, whereas up to seven nucleoli, including several markedly smaller nucleoli, were observed in *A. atroviolaceum*. In the corresponding metaphase karyotypes, two chromosome pairs bearing visible secondary constrictions were identified in *A. polyanthum*, whereas four chromosome

pairs with secondary constrictions were recognized in *A. atroviolaceum*. Nucleoli develop around transcriptionally active nucleolar organizer regions containing tandem arrays of 35S rDNA, which encode the 18S, 5.8S and 28S ribosomal RNAs [72]. NOR activity results in the transcription of precursor rRNA and the recruitment of nucleolar proteins, leading to the formation of nucleolar domains. Nucleolar size and morphology may vary according to several factors, including the number of active NORs, rDNA copy number, transcriptional activity, developmental stage and the fusion of adjacent nucleoli [73–75]. Consequently, nucleolar number cannot be interpreted directly as an exact measure of the number of NOR-bearing chromosomes. Several active NORs may contribute to a single nucleolus following nucleolar fusion, whereas different levels of NOR activity may result in nucleoli of different sizes. The observation of up to four nucleoli in *A. polyanthum* is therefore compatible with the activity of the four chromosomes represented by the two pairs showing secondary constrictions, although a direct one-to-one correspondence cannot be assumed. In *A. atroviolaceum*, the occurrence of up to seven nucleoli is particularly noteworthy because only four chromosome pairs with visible secondary constrictions were identified. This discrepancy may reflect differences in NOR activity, the dynamic behavior and partial separation of nucleolar domains, or limitations in the cytological detection of very small or weak secondary constrictions. It should not, however, be interpreted as definitive evidence for the presence of seven NOR-bearing chromosomes.

The Feulgen reaction provides excellent visualization of chromosomal DNA but does not necessarily reveal all secondary constrictions, particularly when these structures are extremely narrow, weakly stained or located in an unfavorable orientation. Conventional chromosome staining may therefore underestimate the number of chromosomes carrying NORs.

4.5. Evidence for a Diploid-like Organization of the Tetraploid Karyotype

The populations of *A. polyanthum* and *A. atroviolaceum* investigated in Puglia and Basilicata consistently exhibited a tetraploid chromosome number of 2n = 4x = 32. In both species, somatic metaphase chromosomes could be reproducibly arranged into 16 morphologically distinguishable pairs rather than eight groups of four chromosomes. This karyotypic organization indicates substantial structural differentiation among the chromosomes, permitting the recognition of corresponding pairs based on morphology and heterochromatin distribution, which is compatible with a diploid-like genome organization.

Such a structure is highly relevant to the complex evolutionary history documented within the *A. ampeloprasum* complex. Hirschegger et al. [11] provided evidence consistent with an allopolyploid origin for its tetraploid members, while the preferential bivalent formation observed in *A. iranicum* was interpreted by Ghaffari [9] as evidence of preferential pairing. Comparable diploid-like features have also been reported in other polyploid *Allium* taxa, including *A. valdecallosum* [12] and *A. reconditum* [68].

However, the present observations differ from these examples because meiotic chromosome-pairing data are currently unavailable for the studied populations. Consequently, somatic karyotype morphology alone cannot establish whether these chromosomes preferentially pair during meiosis, represent two distinct sets, or reflect structural differentiation within an autotetraploid or segmental allopolyploid genome.

Therefore, these results demonstrate a diploid-like karyotypic organization rather than direct evidence of an active diploidization process, a complex evolutionary phenomenon where polyploids progressively acquire diploid genetic and meiotic traits. Confirming diploidization in *A. polyanthum* and *A. atroviolaceum* would require meiotic analyses tracking bivalent, multivalent, and univalent frequencies to conclusively determine the degree of preferential pairing.

4.6. Cytogenetical Comparison Between the Two Analyzed Species

Despite several morphological and cytogenetic differences, the results obtained in the present study indicate that *Allium polyanthum* and *A. atroviolaceum* share several important cytogenetic features. Both species consistently exhibited a tetraploid chromosome complement of 2n = 4x = 32 in the populations examined, and in both taxa the chromosomes could be arranged into 16 morphologically recognizable pairs. The two species also possessed satellite chromosomes and secondary constrictions, together with broadly comparable chromosome morphology and C-banding organization [38]. These shared features indicate a substantial degree of karyotypic similarity and are consistent with a close cytogenetic relationship. However, similarity in chromosome morphology and heterochromatin distribution alone cannot establish recent divergence or directly demonstrate common ancestry; such evolutionary inferences require corroborating evidence from meiotic, molecular cytogenetic and genomic data. A notable difference concerned the number of chromosome pairs bearing visible secondary constrictions. In the present material, *A. polyanthum* consistently exhibited two pairs of satellite chromosomes, whereas *A. atroviolaceum* exhibited four pairs. This difference contrasts with some previous observations for *A. polyanthum* [38] and may indicate variation in the number or organization of NOR-associated regions. However, the reduced number of visible secondary constrictions in *A. polyanthum* should not be interpreted directly as evidence of transcriptional silencing of specific NORs. Secondary constrictions are affected by chromosome condensation, orientation and the cytological expression of NORs, whereas nucleolar activity cannot be reliably inferred from their absence alone. Differences in NOR number or visibility could reflect structural polymorphism, variation in NOR activity, or technical limitations in the detection of small secondary constrictions. Molecular cytogenetic mapping, particularly FISH with 35S rDNA probes, would be required to determine whether the observed differences correspond to differences in the number, position or activity of rDNA loci [74–78]. Traditional C-banding proved particularly informative for distinguishing chromosomes and characterizing the distribution of constitutive heterochromatin in both taxa. The observed patterns included relatively small centromeric bands on many chromosomes, together with variable telomeric and subtelomeric bands and heterochromatic regions associated with secondary constrictions [79,80]. Constitutive heterochromatin is generally enriched in repetitive DNA and may include satellite DNA and other repetitive sequence families [39,81,82]. Its distribution can vary among closely related taxa as a consequence of the amplification, deletion or redistribution of repetitive sequences, transposable-element accumulation, unequal recombination and structural chromosome rearrangements [82,83]. Consequently, the differences in C-banding patterns observed between *A. polyanthum* and *A. atroviolaceum* provide evidence of cytogenetic differentiation despite their overall karyotypic similarity. The cytogenetic similarity between the two taxa, combined with differences in chromosome morphology, satellite chromosomes and heterochromatin distribution, suggests that they share a broadly conserved chromosomal framework while exhibiting detectable structural differentiation. The morphometric indices M_{CA}, CV_{CL}, CV_{CI}, and THL calculated via Stats 2.0 provided a robust framework to differentiate the otherwise highly conservative karyotypes of the two studied taxa. Due to the intrinsic similarity of chromosome numbers (2n = 4x = 32) and morphological formulas, evaluating the absolute error pooled at the species level was critical to isolate true biological divergence from potential technical noise (e.g., uneven chromosome condensation or operational measurement errors).

The statistical resolution highlights that the inter-specific variations outpace the intra-specific error margins. Notably, the Total Haploid Length (THL) increases from 168.90 ($\pm$7.06) µm in *A. polyanthum* to 179.48 ($\pm$4.07) µm in *A. atroviolaceum*, indicating a net divergence in total chromatin content (Table 2). This macro-structural difference is tightly

linked with a rearrangement in chromosome homogeneity, as evidenced by the sharp drop in the chromosome length variation index (CV_{CL}) from 22.59 (±1.35) to 18.22 (±0.65). Conversely, the centromeric index variation (CV_{CI}) remains stable, 13.16 (±0.77) vs. 12.73 (±0.77), demonstrating that while total length and chromosome size distribution shifted during the evolution of these species, the internal symmetry of the chromosome arms was strictly preserved. These distinct morphometric signatures confirm that the identified differences are fixed genomic features rather than technical artifacts.

Only a limited number of cytogenetic studies have investigated *A. polyanthum* and, particularly, *A. atroviolaceum* in Italy. Since its first record in Puglia by Garbari [65], *A. atroviolaceum* has been reported from additional localities in the region [84–87], suggesting a capacity for establishment in a range of Mediterranean habitats. However, an apparent increase in distribution should not by itself be interpreted as evidence of recent range expansion, ecological competitiveness or adaptation, because differences in historical sampling effort and detection probability may also contribute to the observed distribution pattern. The occurrence of *A. atroviolaceum* in warm and relatively dry Mediterranean environments may indicate ecological tolerance, but the present cytogenetic data do not demonstrate physiological adaptation to drought or high temperature. Consequently, although *A. atroviolaceum* may represent a potentially interesting genetic resource for future studies of stress tolerance in *Allium*, claims concerning its direct value for crop improvement should be regarded as prospective rather than demonstrated. Such applications would require experimental evaluation of physiological traits, genetic diversity, crossability with cultivated *Allium* species and the inheritance of potentially useful characters. If confirmed, these characteristics could eventually be relevant to pre-breeding and introgression programs aimed at improving environmental resilience.

At present, molecular information for the Italian populations of both *A. polyanthum* and *A. atroviolaceum* remains limited. DNA sequencing, molecular-marker analyses and genome-wide approaches could substantially improve our understanding of their evolutionary relationships and genomic differentiation. In particular, comparative FISH mapping of 35S and 5S rDNA loci would provide an important complement to the present Feulgen, C-banding and Ag-NOR observations. While our sampling of six populations per species demonstrated that these cytogenetic differences represent stable, species-specific characteristics within the investigated range, an even broader geographic sampling covering the entire Euro-Mediterranean distribution would help determine if further macro-geographical structuring or regional polymorphisms exist. Such studies could further test hybridization or introgression in areas where the distributions of related *Allium* taxa overlap.

5. Conclusions

The populations of *Allium polyanthum* and *A. atroviolaceum* investigated in this study share a conserved tetraploid chromosome number (2n = 4x = 32) and several features of chromosome morphology and heterochromatin distribution, indicating substantial cytogenetic similarity. In both taxa, the chromosomes could be consistently arranged into 16 morphologically recognizable pairs, reflecting a diploid-like structural organization of the tetraploid karyotype. However, this pattern does not, in itself, demonstrate preferential homologous pairing or an ongoing diploidization process. As meiotic chromosome behavior was not investigated, the present data cannot distinguish among allotetraploid, segmental allopolyploid or structurally differentiated autotetraploid genomic organizations. Accordingly, morphological correspondence between chromosomes in somatic metaphases should not be interpreted as evidence of genetic homology or preferential meiotic pairing. Further meiotic analyses, particularly of bivalent, multivalent and univalent frequencies, will be necessary to assess the extent of diploid-like meiotic behavior.

Despite their overall karyotypic similarity, the two taxa exhibited distinct patterns of chromosomal differentiation. *A. polyanthum* displayed two chromosome pairs with visible secondary constrictions, compared with four in *A. atroviolaceum*. Differences in the distribution of constitutive heterochromatin revealed by C-banding further indicate that karyotypic conservation coexists with structural differentiation. The discrepancy between the number of visible secondary constrictions and the number of nucleoli, particularly in *A. atroviolaceum*, also shows that nucleolar number cannot be considered a direct indicator of the number of active nucleolar organizer regions (NORs). Similarly, differences in NOR-associated chromosome morphology require molecular cytogenetic confirmation before they can be attributed to variation in rDNA organization or activity.

The unusual heterochromatin pattern observed in the *A. atroviolaceum* specimen collected near Vlorë deserves particular attention. Although it may reflect population-level cytogenetic variation or a more complex genomic constitution, the available evidence does not allow these possibilities to be distinguished. Hybridization or introgression may also be considered as potential explanations, but this hypothesis remains tentative and requires investigation of additional individuals and independent molecular markers.

Overall, the results indicate that *A. polyanthum* and *A. atroviolaceum* possess broadly conserved tetraploid karyotypes while differing in chromosome structure, heterochromatin distribution and NOR-associated features. Rather than providing direct evidence of ongoing diploidization, these findings highlight the coexistence of karyotypic conservation and measurable chromosomal differentiation. Integrating classical cytogenetics with meiotic analysis, FISH-based mapping of rDNA loci and genome-wide molecular approaches will be essential to clarify the genomic relationships, evolutionary history and degree of differentiation of these Mediterranean *Allium* taxa.

Author Contributions: Conceptualization, A.T., R.P.W. and S.D.; methodology, A.T. and S.D.; collection and identification of plants, A.T., A.A., P.M., R.P.W. and S.D.; analysis of data, A.T., R.P.W. and S.D.; writing—original draft preparation, A.T., R.P.W. and S.D.; writing—review and editing, A.T., A.A., P.M., R.P.W. and S.D.; supervision, A.T., S.D. and R.P.W.; project administration, A.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors thank Domenico Pignone for his assistance in the field and with materials. The authors are also grateful to the four reviewers, for their valuable comments and suggestions.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

NOR	Nucleolus organizer regions
DAPI	4′,6-diamidino-2-phenylindole
CMA_3	Chromomycin A3
FISH	Fluorescence In Situ Hybridization
$AgNO_3$	Silver-staining
M_{CA}	Mean Centromeric Asymmetry
CV_{CL}	Coefficient of Variation of Chromosome Length
CV_{CI}	Coefficient of Variation of the Centromeric Index

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