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Morphological, Anatomical, Physiological and Genetic Studies of *Iris aphylla* L. Wild Species Conservation in "Ex Situ" Conditions

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Abstract: Wild *Iris* plants are usually found in spontaneous flora, but due to their ornamental characteristics, they can also be used for ornamental purposes, which means that it is very important to find the perfect conditions for plant growth. This research aimed to evaluate the ornamental value and adaptive behavior of wild *Iris aphylla* L. in "ex situ" conditions. Plants from wild flora were cultivated experimentally in the Floriculture field at the Faculty of Horticulture, IULS, Iași, Romania. The biometric determinations revealed the significantly higher ornamental value of conserved plants grown in "ex situ" conditions compared to wild plants. In "ex situ" conditions, the plants displayed more vigorous growth (~100%) and had a higher number of flowers per stem (5–9 flowers), whereas, in wild conditions, this species has from two to a maximum of five flowers. Given the absence of anatomical studies in the literature, detailed anatomical investigations of the leaf structure were performed, complemented by analyses of the photosynthetic pigment content to assess the plant's physiological performance. Additionally, the molecular phylogenetic analyses conducted using two plastid markers (*rbcL* and *trnL-F*) confirmed the taxonomic classification of the native *I. aphylla* L. species. To the best of our knowledge, this is the first report on the molecular phylogeny of the wild Iridaceae species in Romania. These findings provide insights into the taxonomy, morphology, cultivation potential, and ornamental value of the species, supporting future conservation and horticulture development programs.

Keywords: *Iris aphylla* L.; molecular identification; genetic markers; plant anatomy; photosynthetic pigments

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1. Introduction

The preservation of the ecological conditions needed for the ongoing existence and economic development of human society is based on biological diversity and the benefits offered by natural ecosystems, respectively. Therefore, in the global context in which

researchers have documented a fast reduction, and even the disappearance, of many plant species, the conservation of plant species diversity, both "in situ" and "ex situ", becomes a priority, supported by aesthetic, scientific, and ethical considerations [1–3]. The genus *Iris* L., one of the largest and most complex genera in the family Iridaceae, is the subject of many studies of this type, with numerous taxa considered to be in vulnerable categories.

The Iridaceae family includes approximately 1800–2000 species and 60–85 genera [4,5], which are widespread across almost all of the globe, except for most of the Arctic, the taiga in the extreme north of Eurasia, and in some areas of deserts and tropical forests [2].

The genus *Iris* includes from 200 to 400 herbaceous perennial species with rhizomes or bulbs [5–10]. It is also mentioned that the number of officially registered varieties reaches almost 100,000 [11]. The distribution of the species in the genus *Iris* is limited to temperate regions in the Northern Hemisphere, mainly in Eurasia, North America, and North Africa [8–12]; however, Central Asia can also be considered one of the largest biodiversity centers of the genus [13].

A large number of *Iris* taxa have ornamental value due to the diversity of the shapes and colors of the flowers [14]. The presence in various organs of secondary metabolites (alkaloids, flavonoids, and their derivatives, quinones, terpenes, and steroids, etc.) with anticancer, antioxidant, antiplasmodial, immunomodulatory, and/or anti-inflammatory properties, etc., makes it possible to use these plants both in traditional medicine and in modern pharmacology [8,11,15–18]. The rhizomes of some species of *Iris* are used in food, and the cosmetics and perfume industries also use raw materials obtained from the *Iris* species [8,9].

Although it has been the subject of extensive studies, including morphological, anatomical, cytological, ecological, and phylogenic analyses [15,19], the genus *Iris*, which has a high level of variability both within and between species and populations [20], still lacks clarity and is considered taxonomically problematic [6,9–11,21]. These shortcomings have been caused by ambiguous gender evolution, population polymorphism, and species hybridization [11,17,22].

DNA barcoding, using standardized regions of nuclear DNA (e.g., ITS—internal transcribed spacer region) and chloroplast DNA (e.g., *rbcL*—ribulose bisphosphate carboxylase large subunit gene; *trnL* intron and *trnL-trnF*—intergenic spacer; *matK*—maturase K; *psbA*—photosystem II protein D; *rpl20*—50S ribosomal subunit protein L20; *rpoB*—beta subunit of the RNA polymerase gene; *rpoC*—DNA-directed RNA polymerase subunit beta gene) offer a powerful approach to address these challenges by enabling accurate plant species identification, especially when the morphological differences are subtle [17,23–27]. These markers have proven effective in resolving phylogenetic relationships within the genus *Iris* [17,23,28–39].

In regard to spontaneous flora in Romania, there are numerous species of *Iris*, native and naturalized, with most of them being xerophytes or mesoxerophytes. In a study conducted by Oprea [40], the author mentions the presence of 23 species and subspecies and five hybrids of *Iris*, with some taxa being classified into different sociological categories (not threatened—NT; vulnerable—VU) [40].

Therefore, in this paper, we present a series of morphological, anatomical, physiological, and genetic studies carried out on *I. aphylla* L., one of the vulnerable (VU) *Iris* species, which can be found in the wild flora of Romania [40–42]. It is a European steppe forest species with a wide distribution in Ukraine, the central and southern European part of Russia, the Caucasus, and Asia Minor, but with less common populations in other European countries, such as Albania, the Czech Republic, France, Germany, Romania, Hungary, Italy, Serbia and Montenegro, Poland, etc. [42,43]. The populations found in Poland, Belarus, Germany, the Czech Republic, Slovakia, Hungary, and Romania are considered to be at the limit of their geographical range [44]. The species prefers well-drained soils and sunny places in meadows and thickets or on rocks in the subalpine area [42]. The literature also mentions an interspecific hybrid of this species with *I. pumila* L., which could possibly be found in the central part of Romania [41].

The numerous synonyms and subspecies documented in the literature indicate that *I. aphylla* is a species with high morphological variability [43], the division into subspecies being often controversial [45]. Cytology studies have also highlighted variable chromosome counts, with ploidy levels in *I. aphylla* being the subject of discussion among researchers [44]. The tetraploid forms, with 2n = 48 chromosomes, are the most widespread in northern, central, and southern Europe [45,46], and there have also been reported forms with 2n = 24 and 40 chromosomes [42,45]. In order to clarify certain taxonomic aspects and evolutionary trends of the *Iris* species, including *I. aphylla*, studies have also been carried out on pollen morphology [47].

I. aphylla is is characterized by protandrous flowering, cross-pollination, and a flower structure that limits access to pollinating insects, resulting in rare flowering and fruiting and dominant vegetative reproduction [48]. In addition, the decline in *I. aphylla* L. populations in many European areas is attributed, among other things, to low competition with other plants in natural habitats due to deep seed dormancy and poor seed germination [49]. Aspects regarding the flowering phenology of *I. aphylla* L. were analyzed in the conditions of the Cluj area [50].

At the same time, data on changes in the plant pigment complex along global latitudinal gradients are limited [51]. Most of the known works deal with the content of plant pigments under extreme environmental conditions [52].

To our knowledge, the current study represents the first attempt to assess the ornamental characteristics of *I. aphylla* from northeastern Romania under "ex situ" conditions. In this context, this research focuses on the following: (i) The physiological response of the plant in "ex situ" culture, aiming to investigate its anatomical structure and genetically identify the species. Due to the unique ornamental features of this species, studies have been carried out on its suitability for adaptation to cultivation technology in view of recommending it for inclusion in ornamental plant assortment in landscaping. Chlorophyll concentration data provide valuable information about the photosynthetic potential of the plant under cultivation conditions and complement all other studies within this manuscript. The other condition is (ii) the molecular identification and classification of the native *I. aphylla* specimen through phylogenetic analysis, which will be performed based on two selected plastid markers: the *rbcL* gene and the *trnL-F* region. Therefore, the interdisciplinarity of our study leads to valuable conclusions and recommendations based on physiological, histo-anatomical, and molecular studies, as well as on morphological observations and determinations of taxa grown under "ex situ" culture conditions.

2. Materials and Methods

2.1. Site Description and Sampling

The biological material is represented by the *Iris aphylla* species identified in the meadow of Vulturi, Popricanicommune, Iasi County, Romania (located by GPS coordinates 47°26′79.01″ N and 27°54′75.1″ E; Figure 1).

In order to study the ornamental potential of the species in cultivation conditions, the collection was established within the didactic field of the Floriculture discipline (located by GPS coordinates: 47°11′37.4″ N and 27°33′16.1″ E) at the Faculty of Horticulture, Iasi University of Life Sciences (IULS), Romania. The growing area in which *I. Aphylla* has been cultivated is located in an area with an excessive temperate-continental climate of transition with aridity nuances. The warm season has a hot and dry climate, while the cold season is characterized by abundant rainfall and very low temperatures. Evaporation is emphasized; drought and aridity phenomena are suspended by heavy downpours that are accompanied by hail, thunderstorms, and gales, accentuated by very active local thermal ascent. The winter season is usually accompanied at the beginning and end of the cold season by early and late frosts, fogs, and snowfalls caused by horizontal movements of cold and very cold air of polar or arctic origin.

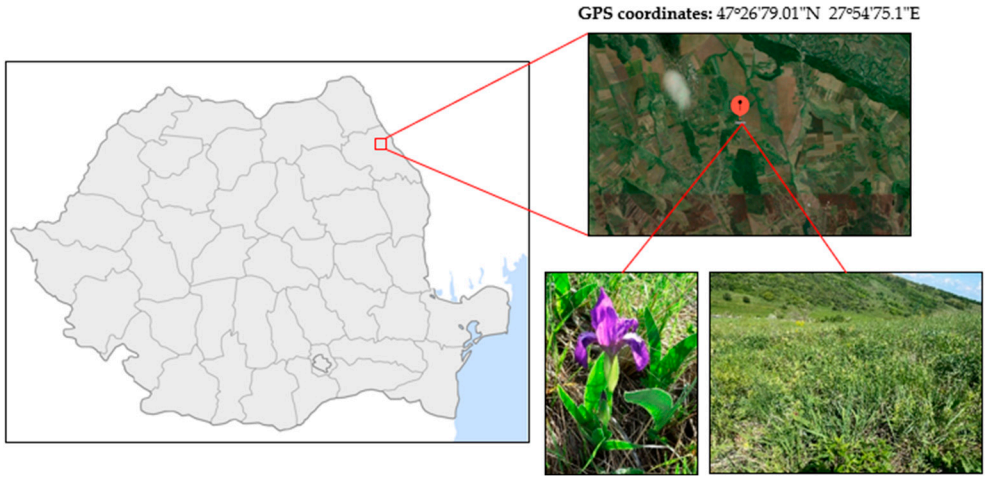

Figure 1. Location of the site where *Iris aphylla* L. was identified in the wild flora of Romania (Google maps).

The properties of the soil on which the species were cultivated are characterized by a topsoil layer of 25 mm with a soil granulometry size consisting of 9.9% coarse sand, 36.6% soft sand, 22.4% silt, and 31.1% clay. The analytical determinations showed the following values: pH 7.8, 3% carbonates, 4.2% humus content, 29.5% cation exchange capacity, 1.08 me/100 g complex Na, and Na/T*100 = 3.66. The macronutrient values recorded were 0.219% total N, 246 ppm accessible P, and 429 ppm accessible K. In terms of microelements, they had the following results: zinc 15 ppm, copper 5.0 ppm, boron 0.32 ppm, and manganese 51 ppm. Laboratory determinations on the soil analyses were carried out at the Centre for Pedological and Agrochemical Studies Iasi, Romania.

The mean annual temperature in the area is 10.95 °C, the maximum temperature per month in summer is ~23.5 °C, and the average annual precipitation was 578.7 mm (during 2017–2021), based on data from the Meteorological Station of the Wine Research Station Iasi, Romania (Table 1).

During the period of study, large differences in the thermal regime could be observed in the winter of 2020, when in January, the average temperature was 1.1 °C (3 °C higher than in 2019), and in February of the same year, the average temperature was 4.3 °C (2.5 °C higher than in 2019). In all months of 2020, there was an increase in monthly temperatures, as well as in the annual average (about 1.53 °C higher than in 2018), the first year of plant growth. In the months of May and April, the highest temperatures were recorded in 2018, when the difference from the average was 4.3 °C in April and 2.2 °C in May. From the studied period, the year 2019 stood out with a very low amount of precipitation in March (9.80 mm), the month in which the start of vegetation of the studied species occurs. The values of precipitation by year suggest that 2019 was the year with the lowest annual amount. Analyzing the values during the vegetation period, it can be observed that 2019 has values close to 2020 (with a total amount of annual precipitation being 547.4 mm). During the study period, 2019 is characterized by two months with low luminosity (August with 198.4 h and September with 199.9 h). The year 2020 is characterized by higher brightness in January, with 99.6 h of sunlight, and the lowest brightness in December, with 25.5 h.

The sampling of rhizome fragments from the plants, identified in the spontaneous flora for the "ex situ" cultivation of the species, was carried out in September 2017. For the collection of biological material, several field trips were carried out, where plants in different stages of development were identified: Juveniles (individuals with a single leaf fan, comprising more than two leaves and having a poorly developed rhizome), vegetative adults or immatures (non-flowering individuals with more than two leaf fans and a fully developed root system, which has a developed rhizome), and generative adults (individuals bearing flowers). Biological material was collected from the colonies of generative adults, and two rhizome fragments were collected from five rhizome colonies 12 m apart.

Table 1. Environmental conditions in the field during 2017–2021.

Average monthly temperatures (°C)													
Months/ Years	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Means
2017	−5	−1.1	7.4	9.7	16.5	21.4	21.8	22.8	17.1	10.8	5.2	2.9	10.7
2018	−1	−2.2	0.8	15.3	19.1	20.7	21.2	22.9	16.7	12.3	2.5	−1.4	10.5
2019	−2.9	1.8	7.1	10.4	16	22.4	21.5	22.5	17.4	11.4	8.1	3.2	11.5
2020	1.1	4.3	7	11.3	14	20.9	22.7	23.5	19.6	13.6	4.5	1.8	12.03
2021	0.1	−0.9	3.1	8.1	15.4	19.7	23.4	20.9	14.7	9.5	6.7	0.2	10.0
Monthly precipitation (mm)													
Months/ Years	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Sum
2017	18.10	22.70	64.00	78.40	47.80	49.00	67.60	24.00	26.60	64.20	37.00	47.20	546.6
2018	38.8	37.0	72.2	9.2	13.6	219.6	184.2	3.0	30.4	2.6	64.6	52.6	727.8
2019	50.6	32.8	9.8	46	98.6	63	33.8	43.2	38.8	30.6	10.2	21.3	478.7
2020	3.6	43.2	18.2	8.4	102.2	108.4	42	9.2	29.8	104.8	22.8	54.8	547.4
2021	28.4	24.6	50.4	53.2	68.2	93.6	87.6	95.4	10.4	2.8	8.8	69.2	593.0
Sunlight duration (hours)													
Months/ Years	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Sum
2017	53.6	42	128	221.6	290.5	291	293.4	278.4	205.5	147.7	101	85	2137.7
2018	53.8	57.5	214	192.7	328.5	231.2	214.1	284.8	252.7	140.8	64.2	93.5	2127.8
2019	83.1	72.5	151.1	239.2	285.5	282.8	276.5	198.4	199.9	190.6	96.4	73.6	2149.6
2020	99.6	116.4	191	279.8	178.2	235.7	275.6	295.3	259.5	123.1	65.1	25.5	2144.8
2021	65.2	107.1	163.4	183.7	212.8	218	283.7	265.1	188.3	195.9	116.4	30.1	2029.7

Since 2018, an extensive study has been carried out on plant development, preservation of ornamental value, and adaptation to "ex situ" conditions.

2.2. Identification of Morphological Characters

The biometric measurements were carried out from the start of plant vegetation in March 2018 (7 months after crop establishment) until the plants entered their dormancy period in October 2021. The measurements of the morphological characters studied focused on the following biometric indicators: the number of flowers per plant (pc), flower stem length (cm), number of leaves (pc), leaf length, and width (cm). All biometric indicator results were statistically processed using scatter plots and mathematical modeling by linear regression.

In the case of the linear regression model, the general equation is Equation (1):

$$\bar{Y} = b_1 + b_2X \quad (1)$$

where b_1 and b_2 represent the regression parameters.

Parameter b_2 is the expected change in response Y that is associated with a one-unit increase in X.

The correlations were carried out with MS EXCEL in MS Office 2019.

2.3. Anatomical Study

Anatomical studies on leaf structure were obtained using two techniques: (1) the tissue freezing technique and (2) the resin embedding technique. To perform tissue freezing sections, a part of the samples (leaves) was cut into 20–30 µm thick sections using a freezing microtome (CM 1325; Leica, Wetzlar, Germany). The sections were stained for 5 min using FSA (Basic Fuchsin, Safranin, and Astra Blue), after which they were washed with water and mounted for analysis. The study of the obtained sections was carried out by

observation using a set of equipment consisting of an optical microscope (OLYMPUS BX50 optical microscope, Tokyo, Japan) equipped with an Axiocam 208 digital color camera and analyzed with the ZEN 3.0 software (Carl Zeiss Microscopy Gmbh, Munich, Germany). To obtain the anatomical structures by using the resin embedding technique, another set of leaf samples was fixed in FAA (formaldehyde, alcohol, acetic acid), washed in three steps for 15 min each with 0.01 M PBS (phosphate buffered saline), with pH 7.4. The next step was to dehydrate the samples at room temperature for 20–30 min each, passing them through a graded series of ethanol, starting at 50% and increasing it to 70%, 95%, and 100%.

The incorporation of fixed and dehydrated samples into Spurr resin was performed by observing the manufacturer's protocol [53]. For light microscopy analysis, 1–2µm sections were made from the samples embedded in the Spurr resin and cut with an ultramicrotome (Ultratome Nova LKB Bromma, Stockholm, Sweden) equipped with a diamond knife (DIATOME Histo 45°). The obtained sections were stained with 1% toluidine blue and studied under an OLYMPUS BX50 optical microscope equipped with an Axiocam 208 digital color camera. The analysis and imaging of anatomical structures were performed using the ZEN 3.0 software (Carl Zeiss Microscopy Gmbh, Germany).

2.4. Determination of Photosynthetic Pigments

The biological material used to determine the assimilatory pigments consisted of leaves harvested before the floral stems emerged, both during flowering and after flowering. The extracts for the analysis of photosynthetic pigments from *I. aphylla* leaves were prepared according to the method presented by Lichtenthaler and Buschmann [54]. Photosynthetic pigments were analyzed by spectrophotometry analysis, using the UV–VIS spectrophotometer (T70 UV/VIS Spectrophotometer PG Instruments Ltd., Leicestershire, UK). For photosynthetic pigment extraction, fresh material was weighed to 0.03–0.05 g. The tissue was ground, and complete homogenization of the plant material with the solvent was achieved by adding 2–3 mL of pure acetone. The resulting liquid was then transferred to a graduated cylinder, and the process was repeated until a colorless filtrate was obtained. Finally, the volume of the filtrate was brought to 10 mL and centrifuged for 10 min at 10,000 rpm. The extracts were measured at wavelengths of 661.6 nm for chlorophyll a, 644.8 nm for chlorophyll b, and 470 nm for the carotenoid pigments [54]. The determinations regarding the content in assimilating pigments were conducted within the Horticultural Research Center of the Faculty of Horticulture, IULS, Romania.

2.5. DNA Extraction, Amplification and Sequencing

Genomic DNA was extracted from fresh leaf tissue using a modified CTAB method [31]. The purity, integrity, and concentration of genomic DNA were checked by NanoDrop spectrophotometer (Thermo ScientificNanoDrop 2000 UV/Vis Spectrophotometer, Waltham, MA, USA) and electrophoresis on 1.0% agarose gel. PCR reactions were performed using a C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA), using 2×MyTaq^{TM}Red Mix (Bioline, USA), 10µM of each primer (Generi Biotech, Hradec Kralove, Czech Republic), and 10–25 ng of genomic DNA as the template. Two plastid regions were amplified: the ~700 bp fragment of the *rbcL* gene and the ~900 bp region encompassing the *trnL* intron and *trnL-trnF* intergenic spacer (together referred to as the *trnL-F* region), using specific primers, and optimized conditions [23,55]. The type of oligonucleotide used, PCR conditions, and references are detailed in Table 2. PCR products were purified using the PureLink^{TM} PCR purification/gel extraction kit (Invitrogen, Carlsbad, CA, USA) and directly sequenced on both strands with the same primers as in the amplification step (CEMIA, Larisa, Greece).

Table 2. Oligonucleotide, PCR conditions, and references.

Primers	Sequences (5′-3′)	cpDNA	PCR Conditions	Reference
S-523	AAACCAAAATTGGGATTATCCGCAAAAAATTA	*rbcL*	95 °C for 5 min, 35 cycles × 95 °C for 45 s, 57 °C for 1 min, 72 °C for 1 min; 72 °C for 10 min	[23]
Z-1204R	CCCTAAGGGTGTCCTAAAGTTTCTCCACC			
trnL2 c	CGAAATCGGTAGACGCTACG	*trnL-F*	95 °C for 5 min, 35 cycles × 95 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min; 72 °C for 10 min	[55]
trnF f*	ATTTGAACTGGTGACACGAG			

2.6. Phylogenetic Analysis and Plant Genotyping

For the phylogenetic analysis, raw DNA sequences were edited and assembled using the DNA Baser v. 3.5.4 program, and the resulting consensus sequences were submitted to the NCBI database. The acquired sequences, along with the related reference sequences retrieved from the GenBank database, were aligned with the ClustalW algorithm in the MEGA X 10.2.2 software package [56], trimmed to the same length, and used for phylogenetic analysis. Phylogenetic trees were constructed using the neighbor-joining (NJ) method based on Kimura's two-parameter model and the maximum likelihood method (ML) under the best-fit substitution model for each DNA marker: (LG + G + F) model for the *rbcL* and (T92 + G) Tamura 3-parameters for the *trnL-F* region. Bootstrap support values were calculated with 1000 replicates. The DNA sequences determined for the selected markers were deposited in GenBank [57].

3. Results

3.1. Species Description and Distribution

Iris aphylla L. (Syn.: *I. hungarica*Waldst. et Kit.; *I. aphylla* L. ssp. *hungarica* (Waldst. et Kit.) Asch. et Graebn.) is characterized by a rhizome of 18–22 mm in diameter; a stem that is 15–30 cm tall and branched below the middle; leaves up to 2–3 cm in width, with 5–6 ribbed and about the same length as the stems, usually falcate and acuminate at the apex; inflated spathes, ovate to oblong in shape, herbaceous, sometimes purple-tinted, and with very narrow membranous margins; 2–5 flowers; an ovary 9–14 mm long; a perigone tube (hypanthium) 14–22 mm; purple-violet petals, narrowly obovate and of 40–65 × 20–25 mm, with the outer ones deflexed in the lower half with multi-cellular whitish hairs along the middle area (bearded), and the inner ones erect, glabrous; three stamens under the petaloid stigma lobes; a brown capsule that is elongated-cylindrical and of 30–50 × 13–20 mm, with a 6-winged long rostrate; and seeds of 4–5 × 3 mm, ± pyriform, and is reddish brown, rugose. It is a Central-Eastern European species. In Romania, it is sporadic in dry meadows, thickets, in sandy-rocky sunny areas, from the forest-steppe zone up to the mountain belt.

3.2. Morphological Characters

The morphological studies aimed to identify two very important aspects: both the adaptation of this species to "ex situ" culture conditions and the application of cultivation technology, as well as the decorative impact of the plants during the growing season.

Considering that this species is decorative not only by its flowers but also by its beautiful foliage, the studies comprised measurements of the characteristics of the flowers and the flower stem (the height of the flower stem, length of the inflorescences, petal size, number of flowers per inflorescence) as well as the number and size of the leaves.

The results obtained from the measurements of the morphological characteristics were compared with those in the literature (Table 3), and it was found that the results of the "ex situ" culture have higher values than those specified in the literature.

Table 3. The main morphological characteristics of the studied species (compared to other data found in literature).

Morphological Characteristics	*Iris aphylla* L.	
	"Ex Situ" (Personal Results)	"In Situ" [58,59]
Height of the floral stems (cm)	25–50	15–30
Number of flowers per stem (pc)	5–9	2–5
Length of tepals (mm)	40–64	40–65
Width of tepals (mm)	20–26	20–25
Flowers color	violet	violet
Number of leaves (pc)	2–6	2–4
Length of leaves (cm)	27–48	15–30
Width of leaves (cm)	2–5	2–3

From the measurements of morphological characteristics, correlations between different morphological decorative characteristics were obtained, and correlated linear regressions were constructed. Based on the obtained data, correlation coefficients were calculated, and regression equations were obtained. Regarding the plants grown "ex situ", the results of the morphological characteristics that showed different values from those of the same characteristics of the plants grown "in situ" were statistically processed using linear regression analysis.

When considering pairs of characteristics, such as the height of the floral stems and the number of flowers, very strong direct correlations were identified, showing r = 0.897 (Figure 2).

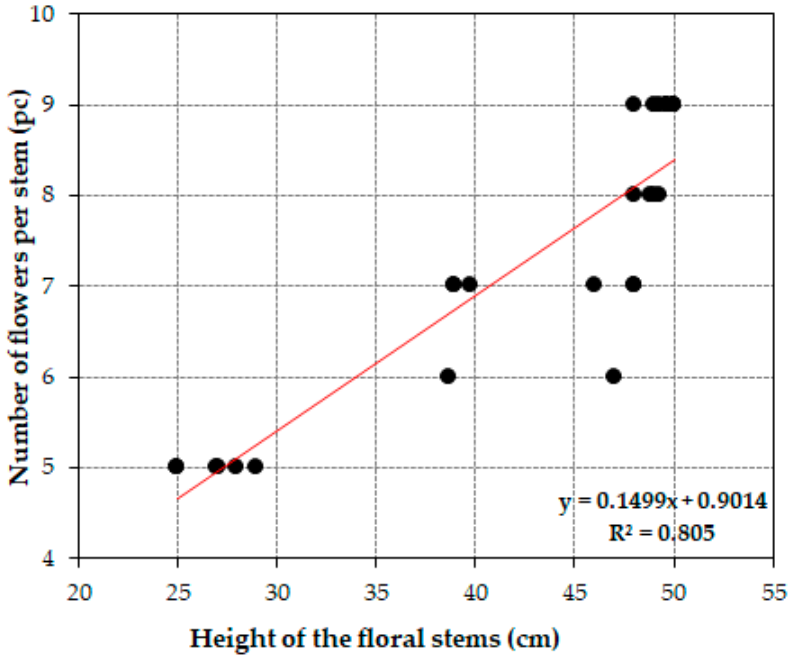

Figure 2. Correlation between the number of flowers per stem (pc—pieces) and height of the floral stem (cm).

According to the data obtained, we can observe the development of the species under "ex situ" conditions, which emphasize the following: the increase in the number of flowers in the inflorescence with up to four flowers causes both an increase in the height of the

flower stalk, with an average of 15 cm, and an increase in the length of the inflorescence, with an average of 9.35 cm.

For two other pairs of characteristics, where the correlation between the inflorescence length and number of flowers (Figure 3) was realized, it was found to be direct, with a calculated r = 0.795.

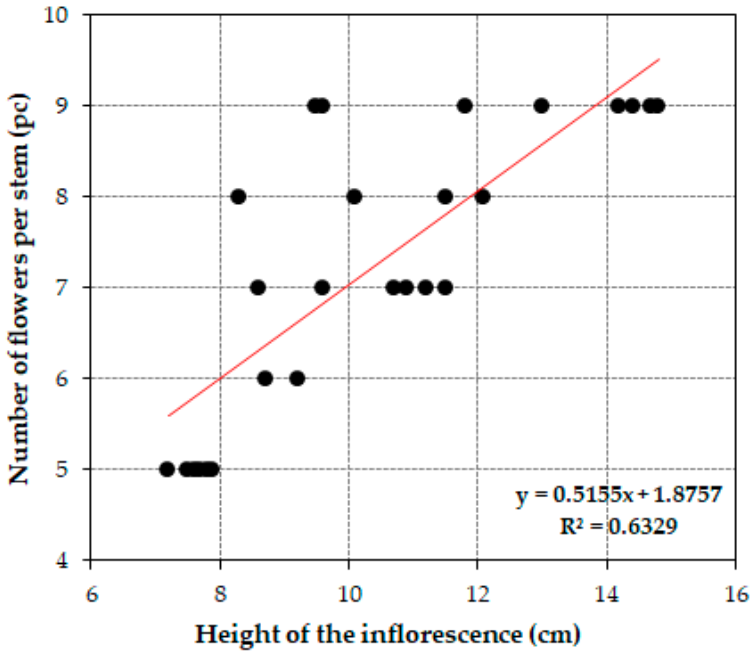

Figure 3. Correlation between the number of flowers (pc—pieces) and height of the inflorescence (cm).

The obtained result highlights that the inflorescence length increases with the increase in the number of flowers in the inflorescence.

In the case of the pairs of leaf length/number of leaves per plant (Figure 4), the correlations were mean indirect (r = −0.577).

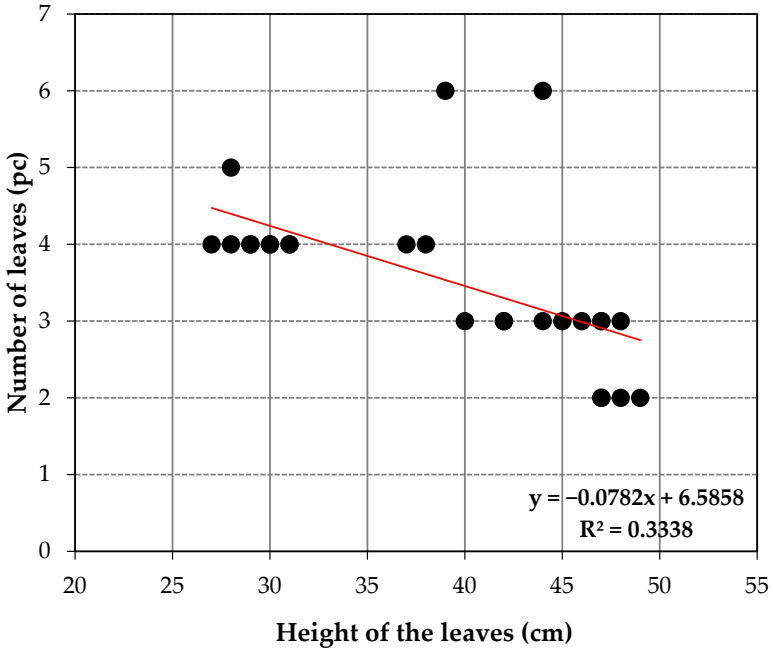

Figure 4. Correlation between the number of leaves per plant (pc—pieces) and height of the leaves (cm).

For the characteristics of leaf width/number of leaves per plant (Figure 5), the correlations obtained were strong indirect (r = −0.698), indicating that the leaf width depends on the number of leaves per plant.

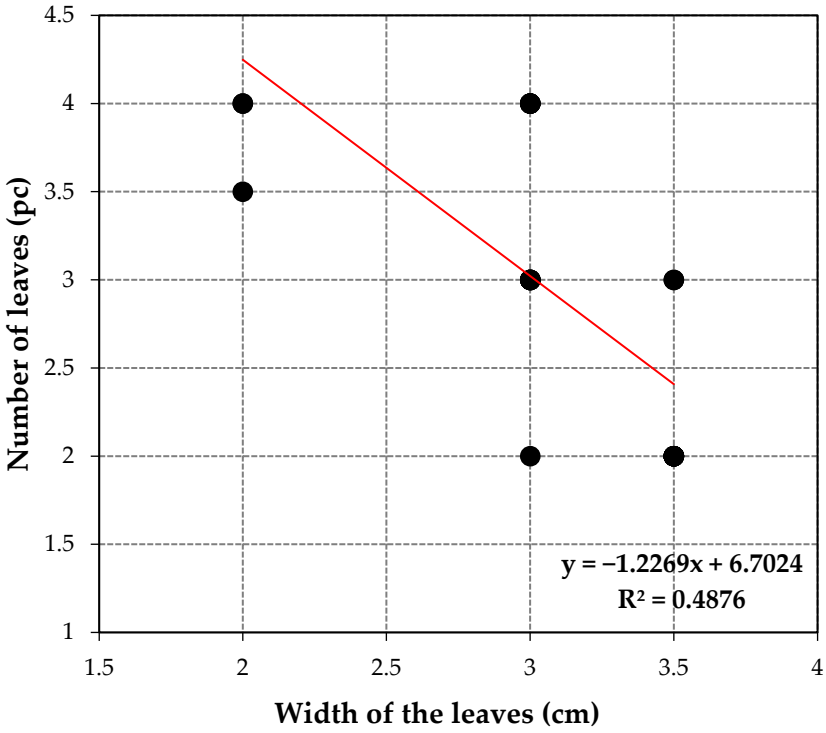

Figure 5. Correlations between the number of leaves per plant (pc—pieces) and width of the leaves (cm).

The results obtained show that when the number of leaves per plant increases, the leaf width decreases.

Also, according to the model, in the event of the appearance of more leaves on the plant, the leaf width decreases.

3.3. Study of Anatomical Structure

Leaves (Figure 6a) are amphistomatic (Figure 6b) and of an isofacial type, with both sides having an abaxial epidermis. The cells of the epidermis form a single layer with large square-shaped cells (Figures 6b,c and 7a–c). They do not have a very thick cuticle (Figures 6d,e and 7b,c), nor do they have papillae or micropapillae. The sunken stomata are numerous and of the anomocytic type and are located transversely to the longitudinal axis of the leaf. The shape of the stomata cells is reniform (rounded-oval in cross-section) (Figure 7b,c,e), of the anomocytic type. The mesophyll is isolateral, and the cross-section is spongy (Figure 6b,d,e and Figure 7a,b), presenting several rows of spongy cells on both sides of the leaf, as well as an intermediate zone (Figures 6e,f and 7a,b,d) with some air spaces and cells without chloroplasts (transparent cells). The spongy cells of the outermost layer of the mesophyll seen paradermally are frequently elongated transversely and parallel to the epidermis (Figures 6d–f and 7b,c). Palisade cells are absent. At their ends, the leaves present accumulations of a V-shaped sclerenchyma zone (Figure 6c).

The vascular bundles are present in two rows near the epidermis, alternating larger bundles with smaller ones (Figure 6a,b and Figure 7a). The xylem of the vascular bundles is oriented towards the center of the leaf, while the phloem is directed towards the epidermis (Figures 6f and 7a,b,f). The elements of the phloem and xylem are clearly visible (Figures 6f and 7f). The sheath of the bundle is clear, especially in the vascular bundles with a larger diameter (Figures 6f and 7b,f). The xylem vessels have a wide lumen, and above them, in

the larger vascular bundles, collenchyma cells are abundant (Figures 6f and 7a,b). Above the phloem, there is a very evident sclerenchyma zone (Figures 6f and 7a,b); the phloem is represented by sieve tubes with clearly visible accompanying cells (Figure 7f).

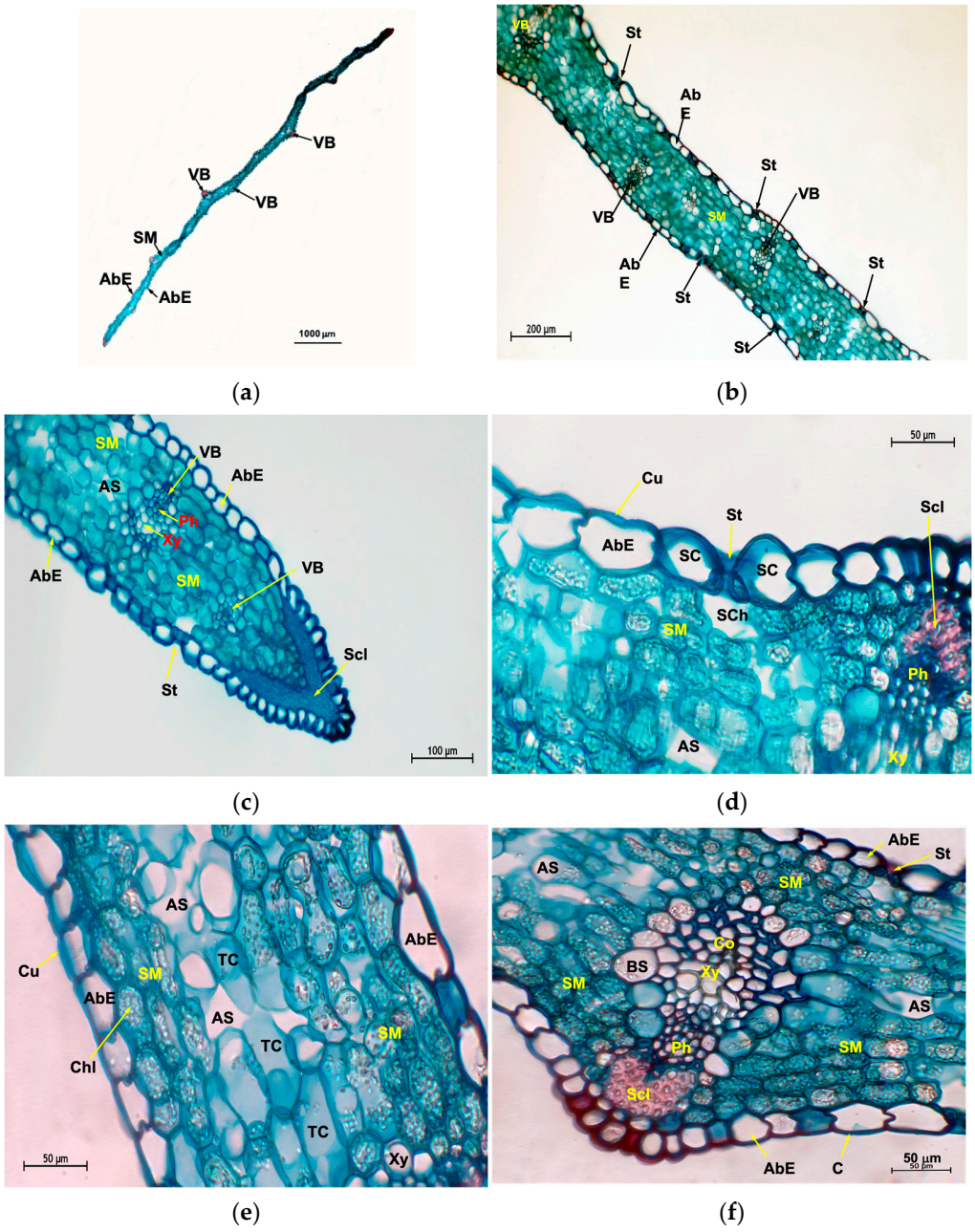

Figure 6. Cross-sections of *Iris aphylla* L. leaf. Sections were made with a freezing microtome and stained with FSA. (**a**) General view of the leaf (×20); (**b**) detail of the leaf blade (×100); (**c**) detail of the tip of the leaf blade (×200); (**d**) detail of the epidermis and the spongy mesophyll (×400); (**e**) detail of the spongy mesophyll and the 2 epidermis (×400); and (**f**) detail of a vascular bundle (×400). Abbreviations: AbE: abaxial epidermis; AS: air space; Chl: chloroplast; Co: collenchyma; Cu: cuticle; Ph: phloem; SC: subsidiary cells; SCh: stomata chamber; Scl: sclerenchyma; SM: spongy mesophyll; St: stomata; TC: translucent cells; VB: vascular bundle; Xy: xylem.

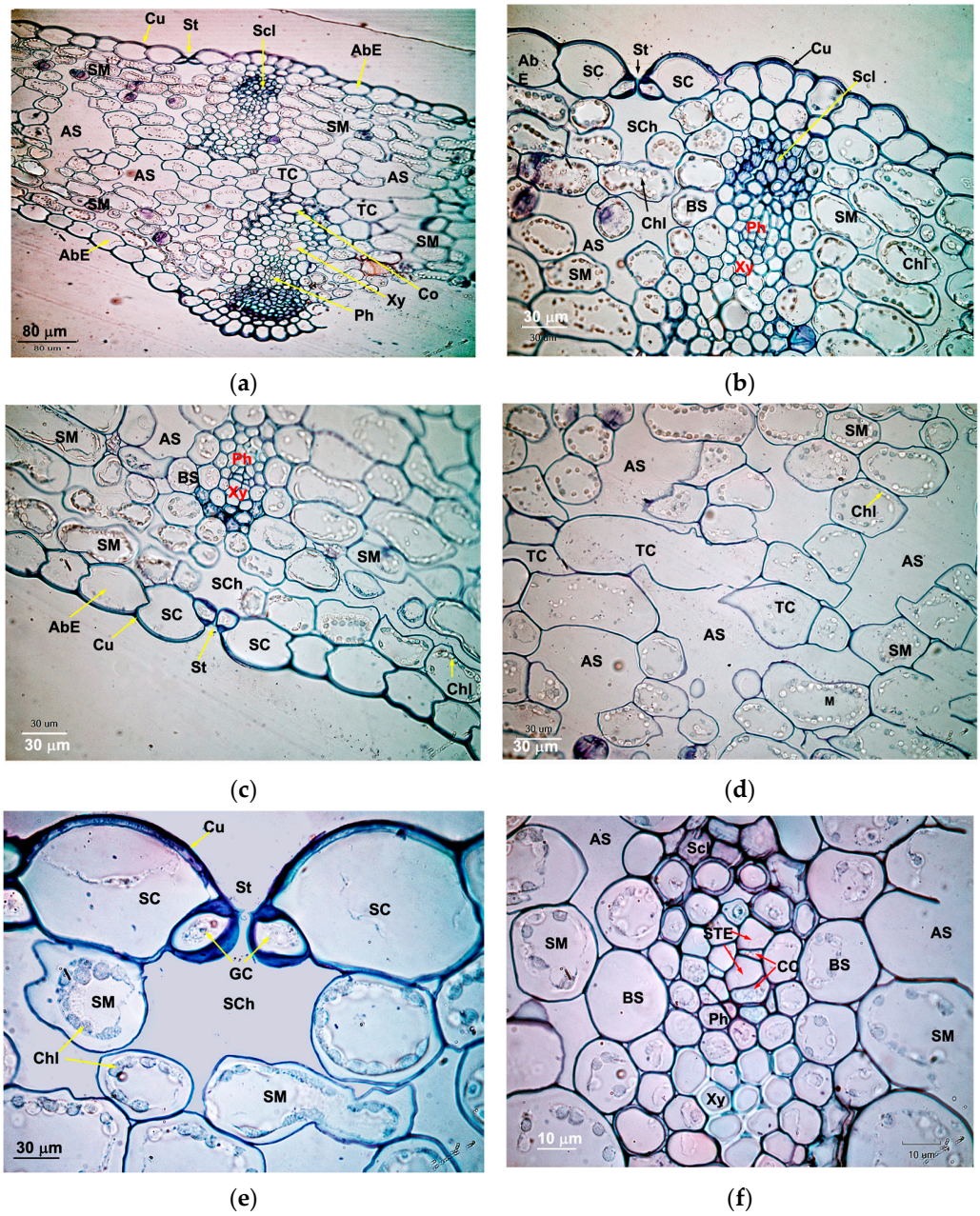

Figure 7. Cross-sections of *Iris aphylla* L. leaf. Semi-thin sections were made with a resin microtome and stained with toluidine blue. (**a**) General appearance of the spongy mesophyll and two vascular bundles (×200); (**b**,**c**) detail of a vascular bundle and the epidermis and spongy mesophyll (×400); (**d**) detail of the area of transparent cells (×1000); (**e**) detail of a stoma (×1000); (**f**) detail of a vascular bundle showing the phloem and xylem (×1000). Abbreviations: AbE: abaxial epidermis; AS: air space; BS: bundle sheath; CC: companion cell; Chl: chloroplast; Cu: cuticle; GC: guard cells; Ph: phloem; SC: subsidiary cells; SCh: stomata chamber; Scl: sclerenchyma; SM: spongy mesophyll; St: stomata; STE: sieve tube element: TC: translucent cells; Xy: xylem.

3.4. Photosynthetic Pigments

The main pigments of the leaves are chlorophylls (a and b) and carotenoids.

Among these pigments, chlorophylls are unique in that they play an essential role in photosynthetic activity by absorbing light and producing the biochemical energy needed to complete the Calvin–Benson cycle [60–62]. The pigment system efficiency depends not only on the concordance between its structure and function but also on the stage of plant's development and the ecological conditions in which the studied plants develop [51,52,63–65].

The main indicators of physiological stress in plants are photosynthetic pigments. In the case of high temperatures and the presence of drought, there is not only a decrease in the total content of photosynthetic pigments but also an increase in the content of carotenoid pigments in leaves.

Studies carried out on *I. aphylla* have shown that the value of different pigments (chlorophyll a (Chl.a), chlorophyll b (Chl.b), total chlorophyll (TC), and carotenoids (Cx) is influenced by the vegetation phenophase; the results obtained show significant differences between these parameters. Regardless of the phenophase of harvesting, in *I. aphylla*, it was highlighted that the plants always showed a higher content of chlorophyll a as compared to levels of chlorophyll b.

Within the framework of physiological determinations, the content of assimilatory pigments in leaves was studied at different growth phases of plants grown "ex situ" (before the occurrence of flower stems, during the flowering period, and post-flowering).

The results of the physiological analyses carried out in the three vegetative phenophases showed the highest values of assimilatory pigment content in the flowering phenophase and the lowest values of assimilatory pigment content in the post-flowering phenophase (Table 4).

Table 4. Average photosynthetic pigment content of *Iris aphylla* (mg/g FW).

Vegetation Phenophase	Chl. a mg/g FW	Chl. b mg/g FW	x + c mg/g FW	TC Σ	Chl.a/Chl.b	Chl./ Car.
Before the occurrence of flowering stems	1.65 ± 0.03	0.56 ± 0.02	0.54 ± 0.05	2.75	2.95	4.1
At/during flowering	1.97 ± 0.02	0.61 ± 0.04	0.57 ± 0.02	3.22	2.90	4.6
Post-flowering	1.45 ± 0.03	0.51 ± 0.02	0.65 ± 0.04	2.61	2.84	3.0

Each value is shown as the mean ± S.D.; FW—fresh weight.

The total chlorophyll pigment content ranged from 3.22 mg/g FW in the vegetative phenophase when the plants were flowering to 2.61 mg/g FW in the post-flowering phenophase. Studies on the chlorophyll pigment content of plants grown under normal ecophysiological conditions indicate chlorophyll a/chlorophyll b ratios of about 3:1 [66].

The light intensity causes the chlorophyll a/chlorophyll b ratio to increase or decrease, so plants exposed to intense light show an increase in the values of this ratio, while plants grown in shade show a decrease in the values of the ratio.

In the case of *I. aphylla* plants, which carry out their flowering period in the months with a high light intensity, the values of this ratio were higher in this vegetation phenophase (3.0). The values obtained in the other two vegetative phenophases, when plants benefited from lower light intensity, showed lower values (2.95 before flowering and 2.84 after flowering), which correlates with the results of studies on other plant species, which under high light conditions showed values between 3.2–4.0 [67].

By comparing the results obtained for the chlorophyll b content with those in the carotenoid pigment content, a more significant increase in the values of carotenoid pigments was observed in the phenophase after plant flowering (0.65 mg/g FW). These values confirm the results of other studies on other plant species in which it was observed that abiotic stress induces an increase in carotenoid pigments [63,68].

In the case of variations in climatic factors, this causes changes in the photosynthetic processes at the physiological level, which can be evidenced by the values obtained in the ratio of chlorophyll pigments to carotenoids [69]. Within the three vegetative phenophases, the highest value of the chlorophyll/carotenoid pigment ratio was registered during plant flowering (4.60), and the lowest value was after plant flowering (3.0). The comparison of the chlorophyll pigment/carotenoid ratio values obtained in this study with the literature results indicates values lower than 4.8:1, which is the chlorophyll pigment/carotenoid ratio value of the plants grown under normal ecophysiological conditions. The lower values of the ratio of chlorophyll and carotenoid pigments in the post-flowering phenophase

when the plants are preparing to enter the dormancy period suggest that *I. aphylla* plants physiologically show stress caused by changes in the intensity of abiotic factors such as temperature, light, and humidity.

These variations in climatic factors induce the onset of physiological stress in plants, which causes not only a decrease in chlorophyll pigment content but also a decrease in the chlorophyll a/chlorophyll b ratio [61].

3.5. Molecular Identification of Native Iris aphylla Specimen

To assess the taxonomic identity and relationships of the native *Iris aphylla* specimen with previously classified *Iris* species, molecular phylogenetic analyses were conducted using two plastid markers: the *rbcL* gene and the *trnL-F* region. Partial sequences of both markers were obtained from the *I. aphylla* sample and analyzed separately due to their different rates of evolution and nucleotide composition. The sequences were aligned and trimmed to equal lengths (705 nt for the *rbcL* gene and 818 nt for the *trnL-F* region), and phylogenetic trees were generated for each marker (Figures 8 and 9). Maximum likelihood (ML) and neighbor-joining (NJ) analyses were employed to reconstruct the phylogenetic relationships of the native *I. aphylla* specimen. The resulting tree topologies were mostly consistent across the two methods, with minor differences in species' placement within clusters. ML trees, which provided slightly higher bootstrap support values, were selected for interpretation for both markers. In the *rbcL* gene phylogeny (Figure 8), the *I. aphylla* specimen (referred to as *Iris aphylla* voucher REF01) was positioned in an independent lineage in a cluster containing *I. germanica* L., *I. foetidissima* L., I. forrestii Dykes, and *I. unguicularis* Poir. Pairwise comparisons revealed that the *I. aphylla*REF01 sample exhibited the highest sequence similarity (99.29%) with these species and only had similarities ranging from 98.58% to 97.02% with other *Iris* species in the *rbcL* phylogeny.

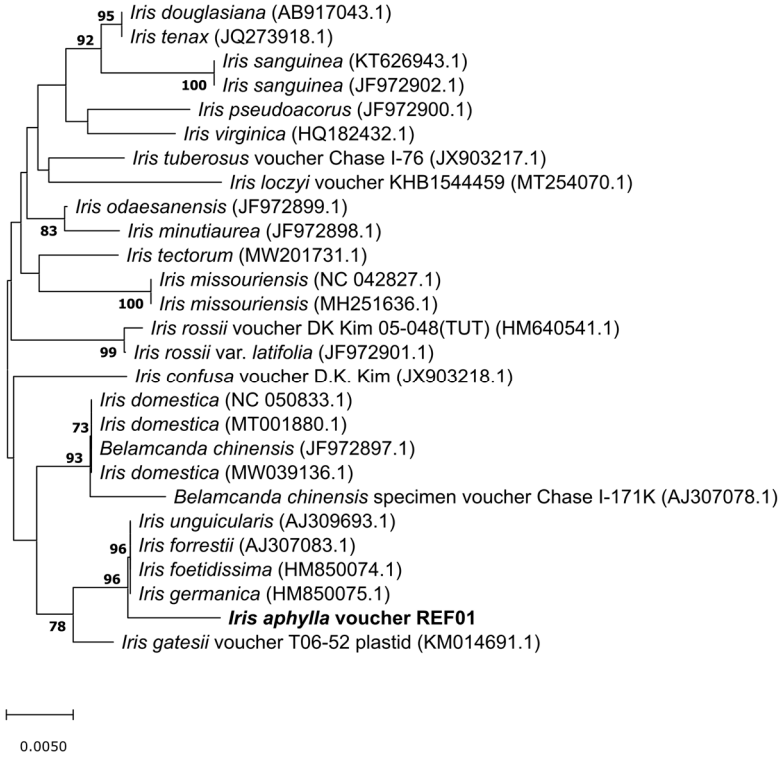

Figure 8. Maximum likelihood (ML) tree inferred under the best-fit substitution model (LG + G + F), based on *rbcL* gene (705 nt), showing the relationship of *Iris aphylla* L. voucher REF01 plant specimen, compared to related *Iris* species obtained from NCBI database. Bootstrap values (calculated for 1000 replicates) >70% are shown on the branches. Scale bar = 0.5% substitutions per site.

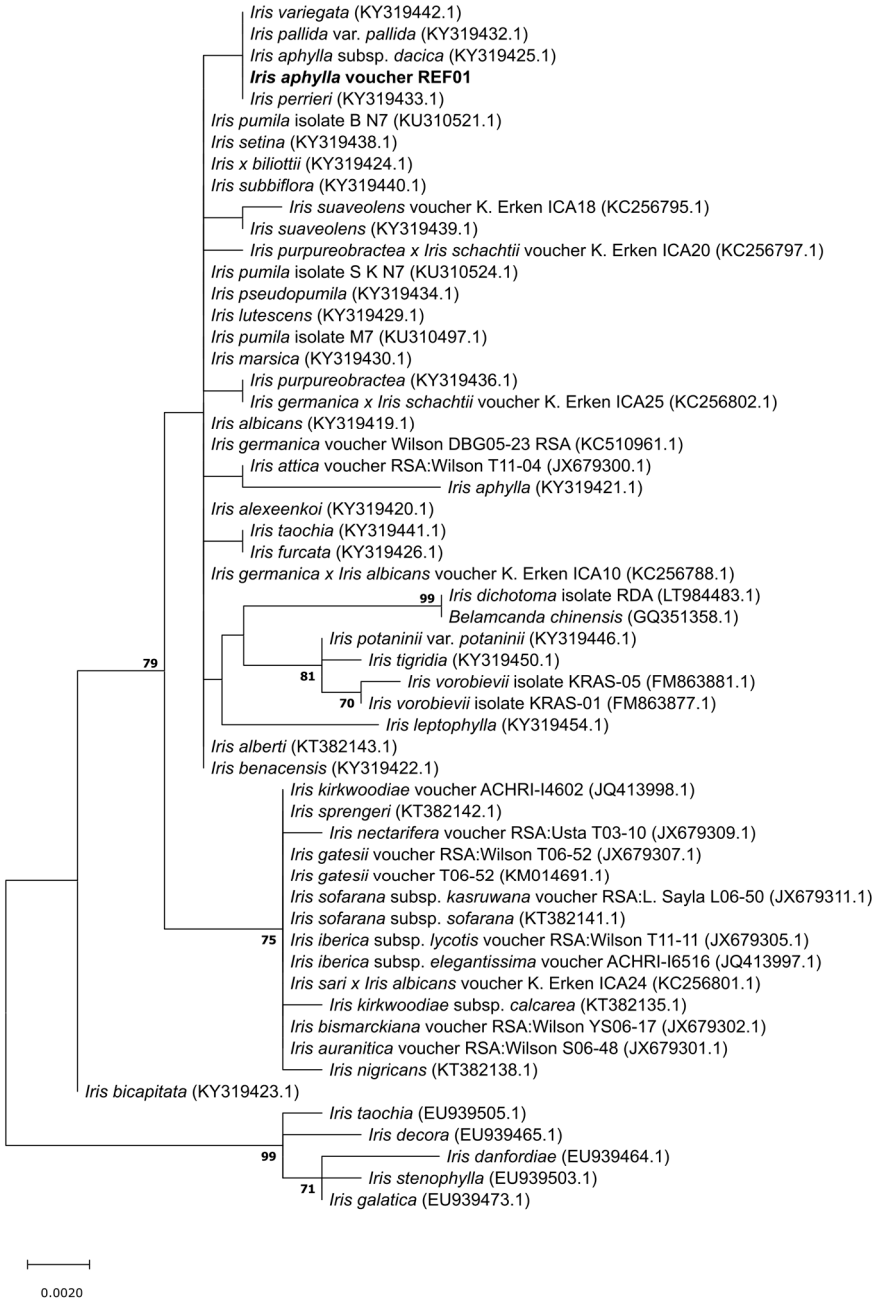

Figure 9. Maximum likelihood (ML) tree inferred under the best-fit substitution model (T92 + G), based on *trnL-F* region (*trnL* intron and partial *trnL-trnF* intergenic spacer) (818 nt), showing the phylogenetic relationships of *Iris aphylla* voucher REF01 specimen with closely related *Iris* species retrieved from NCBI database. Bootstrap values (calculated for 1000 replicates) >70% are shown on the branches. Scale bar = 0.2% substitutions per site.

For the *trnL-F* region, the generated ML tree (Figure 9) placed the native *I. aphylla* specimen in a cluster with its closest relative, *I. aphylla* subsp. *dacica* (Beldie) Soó (specimen Wilson LB05-38UC), with which it shared 100% sequence similarity. However, it was more distantly related to another *I. aphylla* specimen (Wilson G99-09 UC), with only 97.76% sequence similarity. Across the *trnL-F* phylogeny, the *I. aphylla* REF01 specimen shared similarities ranging from 96.66% to 99.13% with other species in the genus *Iris*.

4. Discussion

The evolution of the average temperatures registered for the period 2017–2021 shows large temperature differences from the normal average recorded in March 2017, 2019, and 2020. The thermal regime varied greatly in the experimental years so that the monthly total values in almost every year were significantly different from those recorded in the same months of other years. In the cold season, the highest values were registered in 2020, and the lowest thermal values were recorded in January of the years 2017 and 2019. The year with the highest thermal value was 2020, with an average annual value of 12.03 °C. As for the precipitation regime, it varied strongly over the study period. Very large variations in precipitation can be observed in the months of April and June of 2018 and 2020 as compared to the months of other years. The mean monthly sunshine duration showed high fluctuations from year to year, but the total annual value over the experimental period did not differ greatly. Normally, these lower values were recorded in the cold season, when the degree of insolation is lower. From the analysis of the contrasts found in the thermal and rainfall data, it is confirmed that the area where the plants were cultivated is part of a forest-steppe zone, with an excessive temperate-continental climate of transition, with aridity nuances [70,71]. Although the results reflect a range of risk factors, both the studied species and other species of Iridaceae, Liliaceae, and Asphodelaceae showed good adaptation, materialized by resistance to unfavorable conditions and outstanding ornamental traits [70–72]. Since the adaptation of plants to new environmental conditions is evidenced by the vigor of plant growth and the preservation of ornamental characters, mathematical modeling was used in the study through statistical analysis of the variables.

The differences between the cold and warm seasons are accentuated, both in terms of temperature and rainfall, by horizontal movements of cold and very cold air of polar or arctic origin, which cause climatic accidents in the form of frosts, fogs, and snowfalls, both early and late.

Statistical analysis of variables is an important part of data analysis as it represents a good way of determining correlations between variables. This correlation between the analyzed parameters provides us clues about the direction, power, and mode of connection between them. In order to obtain a series of indications regarding the direction, strength, and connection between the analyzed morphological characters, an analysis of the correlations between these characters was also performed. Grouping the measured data into pairs leads to a first estimate of the common distribution.

Mathematical modeling using statistical analysis on the variables completes the research developed in this paper and details how the number of flowers depends on the height of the floral stem and the length of the inflorescence, while the number of leaves depends on the length and width of the leaf. In the case of this study, the number of flowers depends on the height of the stem and the length of the inflorescence, with the grouping of the measured data in pairs highlighting the direct correlations, such as very strong and strong. Furthermore, when grouping the data in pairs for characteristics such as the number of leaves with the length and width of the leaves, strong indirect correlations were obtained in both cases. Superior results on the morphological characteristics obtained by other Iris species under "ex situ" compared to "in situ" conditions have been highlighted in other studies [73]. The same vigor and ornamental mite growth trends were also evident in other vulnerable Iridaceae species [74].

In this paper, data were statistically analyzed using simple linear regression as mathematical modeling. The linear regression modeling of the parameters for the number of leaves and leaf length, as well as the stem diameter and stem height, shows an increase in the percentage of linkage between the parameters, with R^2 values = 0.805 in the case of correlation between the number of flowers per stem and floral stem height, R^2= 0.633 in the case of correlation between the number of flowers and inflorescence length in the inflorescence, R^2= 0.333 in the case of correlation between the number of leaves per plant, and R^2= 0.487 in the case of correlation between the number of leaves per plant and width of leaves. Checking for violations of the assumptions of linearity, constant variance, and error

independence in a linear regression model is usually conducted by plotting the residuals against the predicted values (or against each of the individual predictors) [75]. The study of the correlations between different morphological characteristics shows the existence of a positive correlation in most cases, with the stronger being between the height of flower stems, number of flowers, and number of flowers and inflorescence length. By analyzing the correlations obtained within the pairs of characteristic flower stem heights and the number of flowers, it can be concluded that the results are influenced by favorable microclimatic conditions in the experimental field. The model used in the statistical interpretation is a relevant one for highlighting the correlations between the pairs of characteristics analyzed, since each parameter X represents the expected change in parameter Y associated with a 1-unit increase in X. In order to evaluate the performance of the regression model, the R^2 value was used, which measures the proportion of the variation in the dependent variable, which is explained by the independent variable. The use of linear regression analysis to determine a relationship between two variables in order to derive information about one of them from the values of the other has also been used for other floricultural species [73,74,76–78].

Superior results on the morphological characteristics obtained by other *Iris* species under "ex situ" compared to "in situ" conditions have been highlighted in other studies [73,74]. The same vigor and ornamental plant growth trends were also evident in other vulnerable Iridaceae species [74,79]. Comparative analysis of the decorative potential of different floristic species of wild flora, cultivated ex situ, showed variations in plant and inflorescence characteristics. It has been found that the cultivation of *Physalis alkekengi*, *Allium atroviolaceum*Boiss, *Pancratium maritimum* L., and *Crocus tommasinianus* Herb. species under "ex situ" conditions can provide, in addition to the decorative aspect of green spaces, high-quality decorative elements, superior to those of native individuals, which can be used in landscape design [78–81].

Knowledge of the structural and functional changes associated with abiotic stress conditions can be used to observe the adaptability of the species to new culture conditions. Therefore, anatomical investigations at the foliar level were performed. The results of the leaf anatomical studies provided important data on the anatomical characterization of *I. aphylla*, confirming the structures identified within the genus, which have been studied in other species. At the present moment, studies on the anatomical characterization of this species, highlighting the unique anatomical differences of the species, were not performed. Studies on the following numerous species within the genus are found in the literature: *I. peshmeniana* Güner & T. Hall. and *I. aucheri* (Baker) Sealy [16], *I. croatica* Horvat & M.D. Horvat [47], and *I. masia* [82]. Some studies have compared the anatomical leaf structures of three species endemic to Uzbekistan (*I. sogdiana* Bunge, *I. korolkowii* Regel, and *I. Svetlanae* (Vved.) T.Hall & Seisums) [2], while others have studied the anatomical structure and micromorphology of sepals in several species of *Iris*, to highlight the differences between species and explain the interaction between flowers and pollinating insects [83]. The study of Kandemir and Çelik [82] focused on a comparison of the morphological and anatomical properties of two endemic species found in Turkey, the *I. pamphylica* Hedge and *I. masia*Leichtlin ex Dykes, and the degrees of kinship between the species was also determined. Moreover, the morphological and anatomical properties of the two subspecies of *I. masia* (*I. masia* subsp. *masia* and *I. masia* subsp. *dumaniana* Güner) were analyzed at the root, stem, and leave levels [82]. The importance of using plant anatomy is also essential for morphologically similar species. In this case, morphological and anatomical features are compared to differentiate species (*I. peshmeniana* Güner & T. Hall. and *I. aucheri* (Baker) Sealy) of the subgenus *Scorpiris* Spach (*Juno iris*). Root, stem, and leave cross-sections were also analyzed (the number of layers of exoderm and cortex, the marginal structure of cortex parenchyma cells, the structure of the central root cylinder, conditions of the micropapillae in the lower epidermis, and the number of layers of palisadic tissue and cancellous parenchyma) [16].

The leaves of *I. aphylla* have a different leaf structure compared to other species. In this species, the leaves are amphistomatic and isolateral, while in *I. unguicularis* subsp. *carica* var. carica, the leaves are ensiform and isobilateral, and in *I. unguicularis* subsp. *carica* var. syriaca, the leaves are isobilateral [4] but similar to that of other species such as *I. masia* subsp. *dumaniana*, *I. pamphylica*, and *I. masia* subsp. *masia* [82]. As far as the cells of the epidermis are concerned, they are present in a single layer as in other species *(I. aucheri*, *I. peshmeniana*, *I. unguicularis* subsp. *carica* var. *carica,* and *I. unguicularis* subsp. *carica* var. *syriaca*) [4,16]. In *I. aphylla*, the shape of the epidermal cells is similar to *I. unguicularis*, which is formed by large square-shaped cells and is different from *I. aucheri* (the epidermis is formed by small rectangular-shaped cells), *I. peshmeniana* (large rectangular-shaped epidermal cells), *I. masia* subsp. *dumaniana* (the epidermis is small and square-shaped), and *I. masia* subsp. *masia* (the epidermis is single-layered, small, and square-shaped) [4,16,82]. The epidermis of the *I. aphylla*, unlike other species such as *I. masia* subsp. *dumaniana*, *I. pamphylica*, or *I. masia* subsp. *masia*, does not have micropapillae in the outer area. The epidermis also does not present bulliform cells, such as in *I. masia* subsp. *dumaniana* and *I. pamphylica*. The mesophyll of *I. aphylla* is only of the spongy type, not presenting palisade parenchyma, as occurs in *I. sogdiana* [2] and *I. pamphylica* [82]. In *I. aphylla*, vascular bundles are arranged in two alternating rows, as also occurs in *I. pamphylica* and *I. masia* subsp. *masia*, whereas vascular bundles have only one row in *I. masia* subsp. *Dumaniana* [82]. This situation has been observed in other species of the Iridaceae family. In *I. aphylla*, the phloem has a dense sclerenchyma cap at the phloem poles of the vascular bundles and at the leaf corner, as in *I. masia* subsp. *masia* and *I. pamphylica*. This cap of sclerenchyma deforms the epidermis in *I. aphylla*, forming rounded keels, a characteristic that also appears in *I. masia* subsp. *masia* but not in *I. masia* subsp. *dumaniana* or in *I. pamphylica* [82]. According to Kandemir and Çelik [82], we believe that the presence and absence of the sclerenchyma cap and keels in both epidermis have a taxonomic value between species. The phloem is always located towards the outside, while the xylem is located towards the inside of the mesophyll and protected by collenchyma cells. In the major vascular bundles, the phloem and xylem have between 8 and 10 vessels, large and small.

The anatomy of the leaf of *I. aphylla* does not show special adaptations to extreme climates (very thick cuticles, very sunken stomata, abundant trichomes, very compact mesophylls, etc.), which coincide with other *Iris* species from similar habitats. This explains why this plant loses its leaves during the winter season.

As the evaluation of photosynthetic processes during different phenological stages is essential for optimizing the conditions necessary for plant adaptation, the content of assimilatory pigments was determined. Chlorophyll is a pigment that gives green character to plants and occupies a unique role in photosynthetic activity by absorbing light and producing biochemical energy for use in the Calvin–Benson cycle [60,61]. Chlorophylls a and b mainly capture light in the antenna complex through photosystem II and, consequently, initiate electron transport [62]. The efficiency of the pigment system depends on the conformity between its structure and function and environmental conditions (primarily on the intensity of light) [63,64]. Latitudinal changes in the rate of solar radiation must affect the pigmentary system of leaves, the efficiency of which directly influences the photosynthetic productivity of plants [52]. Chlorophyll metabolism is affected by the developmental stage of the plants, the light and hormone levels, and other factors [65]. At the same time, data on the changes in the plant pigment complex along global latitudinal gradients are not numerous [51]. The chlorophyll (Chl) content in plant leaves changes throughout the stages of plant development. The pigment content is affected by exposure to terrestrial vegetation to various types of natural and anthropogenic stress [84]. The chlorophyll content of the leaves is an indicator of the physiological state of the plants and is closely related to the photosynthetic capacity of the plant [85,86]. The chlorophyll content of leaf tissue is affected by the degree of soil fertility [87] and by the stress caused by the adaptation of species to different environmental conditions compared to the area of origin [88], the presence of

drought, salinity and the presence of diseases and pests [89,90]. Most of the known works deal with the content of plant pigments under extreme environmental conditions [52].

Considering the lack of previous phylogenetic studies on the native *I. aphylla*, molecular analyses were conducted to confirm the identity of our specimen and provide additional insights into its relationships within the subgenus *Iris*. While genetic divergence and phylogenetic relationships among various species in the subgenus *Iris* have been explored [17,23,28–39], studies specifically focusing on *I. aphylla* are limited [30,34]. Previous research has demonstrated the effectiveness of markers such as trnL-F across different sections of *Iris* [34], and combining it with other markers like matK has produced robust phylogenetic trees [30].

To confirm the taxonomic classification of our native *I. aphylla* L. specimen, we performed phylogenetic analyses using the *rbcL* and *trnL-F* plastid markers, which are widely recognized for their effectiveness in resolving phylogenetic relationships within the Iridaceae family, particularly among *Iris* species [23,30–35,37–39,91]. Their frequent use is attributed to their universality, ease of amplification, and reliability in distinguishing between genera and species [91,92]. The *rbcL* gene is known for its conserved nature, allowing reliable amplification across a wide range of plant taxa, making it a standard DNA barcode in molecular phylogenetics [92,93]. Despite its limited variability, *rbcL* effectively resolves phylogenetic relationships at the genus level and higher taxonomic levels [23]. The *trnL-F* region, which includes the trnL intron and a partial trnL-trnF intergenic spacer, is a non-coding plastid marker known for its relatively high sequence variability. The *trnL-trnF* intergenic spacer is considered a universal plastid marker, widely used in plant systematics and phylogeography [91]. Despite its slow molecular evolution, the plastid *trnL* intron has conserved sites that are valuable for evolutionary studies at higher taxonomic levels [24,92]. Its sequences have been effectively used to reconstruct phylogenies among closely related species and identify plant taxa [39]. Primers developed by Taberlet et al. [94] have successfully amplified DNA from diverse plant groups, including algae, bryophytes, pteridophytes, gymnosperms, and angiosperms. Its non-coding nature allows for greater evolutionary flexibility and enhanced resolution at the species level [28,34,94]. Previous research has consistently demonstrated that the trnL-F region, whether used alone or in combination with other DNA barcodes, produces robust, well-supported phylogenies that effectively resolve species-level relationships and clarify complex taxonomic boundaries [28,34,35,37–39,91]. For instance, one study [28] highlighted its utility in generating well-supported phylogenies when combined with markers such as matK and ITS. Similarly, Makarevitch et al. [23] reported its effectiveness in distinguishing closely related taxa within the Iridaceae family. Recent studies [34,37] further confirmed its role in elucidating phylogenetic relationships within *Iris*, emphasizing its capability for species-level resolution.

One challenge in the fine-scale classification of *I. aphylla* is the limited availability of reference sequences in public databases such as GenBank, which complicates the assignment of exact taxonomic status. Consequently, we analyzed the *rbcL* and *trnL-F* regions separately rather than concatenating them. This approach allowed us to assess their individual contributions to phylogenetic resolution.

The *rbcL* gene phylogeny revealed a close phylogenetic relationship between our *I. aphylla* REF01 specimen and species such as *I. germanica*, *I. foetidissima* [95], *I. forrestii* [35], and *I. unguicularis* [4]. This finding aligns with previous research on evolutionary relationships within the genus *Iris*, which highlighted the reliability of the *rbcL* gene for higher-level taxonomic classification. The high sequence similarity (99.29%) between *I. aphylla* voucher REF01 and these species suggests a recent common evolutionary ancestry. Similar observations were made by Boltenkov et al. [21] and Wilson (Wilson, 2009), who noted that the *Iris* species often cluster tightly in plastid DNA-based phylogenies while maintaining distinct evolutionary lineages. Despite this genetic closeness, the independent lineage of *I. aphylla* in the *rbcL* tree suggests that it retains distinct genetic features.

The phylogeny based on the *trnL-F* region showed the *I. aphylla* REF01 specimen clustered with *I. aphylla* subsp. *dacica* (Wilson LB05-38UC), exhibiting 100% sequence

similarity. This finding suggests that these two may represent very closely related populations or subspecies with minimal genetic differentiation. However, the more distant relationship between *I. aphylla* REF01 and another *I. aphylla* specimen (Wilson G99-09 UC), which showed 97.76% sequence similarity, indicates an intraspecific variation that may be attributed to geographic separation, environmental adaptations, or historical events, leading to genetic divergence. Similar intraspecific variability has been documented in other studies on *Iris* species [17,30], which highlight the genetic complexity within this genus. A recent study revising the taxonomy of *Iris scariosa*, based on combined chloroplast DNA sequence data, also addressed the classification within subgenus *Iris*, placing *I. aphylla* in a moderately supported cluster alongside *I. germanica* and *I. reichenbachii* [34]. Key studies have contributed to our understanding of these markers in Iris systematics, including works that explored various plastid and nuclear regions to resolve taxonomic relationships within the genus [23,28,34,37]. Research involving the trnL-F region has yielded variable results: some studies report low divergence, while others identify considerable nucleotide variability [23,37]. Despite these discrepancies, its role in generating consensus within phylogenetic trees remains well-supported [28]. Notably, our findings highlighted the effectiveness of the trnL-F marker in distinguishing closely related subspecies and emphasizing genetic diversity within *I. aphylla*. Likewise, the conserved nature of *rbcL* has facilitated large-scale phylogenetic studies across angiosperms, serving as a cornerstone for plant molecular systematics [91,92].

The phylogenetic analyses utilizing both *rbcL* and *trnL-F* markers provided significant insights into the relationships of the native *Iris aphylla* specimen. These analyses confirmed its close genetic connections to other *Iris* species while also revealing unique genetic differences within the species. The *rbcL* marker established a broad phylogenetic framework, whereas the *trnL-F* region allowed for more detailed resolution at the species level and proved to be a reliable molecular marker within the *Iris aphylla* group.

The results encourage further investigation into other populations of *I. aphylla* and related taxa, utilizing additional molecular markers as suggested by Kress [24] and Saddhe and Kumar [27], to assess genetic diversity and relationships comprehensively. Combining chloroplast markers with nuclear data is known to improve species delineation accuracy, particularly when genetic variation exists within species or populations. Such research could lead to more refined taxonomic classifications and a better understanding of the evolutionary history of this group.

To prevent intermixing or physical proximity of *Iris* plants originating from different areas, it is recommended to either grow them “ex situ“ alone or remove immature fruits to prevent spontaneous hybridization. The literature studies propose to reduce the risk of inbreeding depression and self-incompatibility in “ex situ” conservation by dividing the collection of living plants on a regional basis.

To prevent any of the above-mentioned situations, the *I. aphylla* species in this study was taken from a single location. It was the only *Iris* species cultivated in the experimental field, and its fruiting bodies were removed before maturation. By applying these measures confirmed in other literature studies, the risks of genetic homogenization or ecological displacement were eliminated [96]. Considering that the genetic analyses were performed in the first year after introduction into cultivation, the risk of genetic adaptation to cultivation and a loss of adaptations to native wild conditions was not considered, as these only manifest after a long period of cultivation, as stated by Ensslin and Godefroid [97]. The study of “ex situ” conservation complements “in situ” conservation in that it involves maintaining genetic variations far from their original location. In “ex situ“ conservation, sample specimens representing populations of species or subspecies are collected and conserved either in gene banks as living collections of field-grown plants or in botanical gardens and arboreta, or as samples of seeds, underground organs, tissue explants, pollen, or DNA extract under special artificial conditions. “Ex situ” conservation techniques are ideal for many plant species in rare or vulnerable categories originating from native loca-

tions threatened by genetic erosion, as they provide easy access to the biological material needed in exploitation work [98].

The study carried out in this paper on the growth of *I. aphylla* species under "ex situ" and "in situ" conditions in the northeastern part of Romania is unique at the national and international levels. For this reason, other comparative data from other regions or climates could not be included for wider relevance. The only studies at a national level were carried out in the Cluj area and included only the species' presentation, phenological data, medicinal properties, and biometric measurements on wild flora plants.

The process of plant conservation involves two essential strategies: translocation and introduction [96]. Translocation conservation is the intentional relocation of plant species from one location to another in order to generate conservation benefits at the population, species, or ecosystem level. Within this conservation strategy, two key strategies are distinguished: reinforcement and reintroduction into the native range of the species. The conservation strategy of introduction is achieved outside the native range of the species by facilitating colonization and ecological replacement [99]. Thus, introduction activities can be implemented not only in the area close to the area from which they were taken but also away from the current range [96]. The underlying principles underlying the growth or improvement of rare plant species are to increase the size of the population and restore it or restore it by releasing newly obtained individuals into an area where they were previously found [100]. The ecological implications of the new area are of paramount importance in the successful introduction of *Iris* species, as it must exhibit sufficient carrying capacity to support the growth of the reintroduced population as well as the capacity to sustain the long-term viability of the population. In different taxa, the success of reintroduction has been shown to be dependent on numerous aspects, such as the propagation method, ecological conditions, human intervention, and permanent monitoring of the plant after reintroduction [15,99,100]. Since the *Iris* species are known to be either exogenously and/or endogenously dormant species that may restrict seed germination [52,101] or cause limited seed production, it is more favorable that propagation is achieved naturally by rhizomes or by somatic embryogenesis techniques. As such, plant regeneration of *I. pallida* L. and *I. germanica* L. via somatic embryogenesis from leaves, apices, and young flowers can occur [102]. At present, micropropagation is of great importance for the realization of clonal propagation. It is of great importance because it makes it possible to regenerate plants using a small part of the parent plant, which makes even clonal propagation from a single valuable specimen affordable. In "ex situ" conservation, in vitro techniques are used for the multiplication of endangered plant species, both as micropropagation methods and as long-term preservation methods. These plants can represent valuable biological material for research, living collections, and plant reintroduction programs [96,103].

5. Conclusions

The primary objective of this study was to assess the ornamental value and behavior of *I. aphylla* under "ex situ" conditions, employing both morpho-anatomical and biochemical approaches. Additionally, phylogenetic analyses using plastid markers, specifically the *rbcL* and the *trnL-F* regions, were conducted to support taxonomic classification.

The data regarding the morpho-decorative characteristics analyzed within the *I. aphylla* L. species grown "ex situ" indicates greater vigor compared to the plants in the spontaneous flora, expressed by higher values of the height of the floral stems, the number and size of the leaves, and the number of flowers from the inflorescences.

Leaves of *I. aphylla* L. are amphistomatic and of isofacial type, with the stomata being rounded, oval, sunken, numerous, and located transversely to the longitudinal axis of the leaf. The mesophyll presents several rows of spongy cells and an intermediate zone with some air spaces and cells without chloroplasts. The vascular bundles are present in two rows near the epidermis; the xylem of the vascular bundles is oriented towards the center of the leaf, and the phloem is directed towards the epidermis, and above the phloem, there is a very evident sclerenchyma zone.

A higher content of assimilatory pigments was observed during flowering; in this vegetative phenophase, the value of total content increased by 17.09% compared to the values obtained before the occurrence of flower stems, and by 23.37% compared to the values obtained in the post-flowering period.

The values of the chlorophyll and chlorophyll/carotenoid pigment ratios showed values within the theoretical limits; the chlorophyll a/b ratio results ranged from 2.95 before the occurrence of flower stems to 2.84 post-flowering, and the chlorophyll/carotenoid ratio results ranged from 4.6 during flowering to 3.0 post-flowering.

The values obtained in the three phenophases of vegetation show a good adaptation to the ecological conditions in the culture.

This study also provides new insights into the taxonomic assignment and phylogenetic relationships of the native *I. aphylla* L. using the plastid markers, *rbcL* and *trnL-F* regions. Our analysis confirmed the taxonomic identity of the *I. aphylla* voucher REF01, revealing its close genetic relationship with other *Iris* species while highlighting unique genetic features that distinguish it as a distinct lineage. The *trnL-F* region serves as a powerful molecular marker for resolving species-level relationships within the *Iris* genus. Its high-resolution phylogenetic data help distinguish closely related taxa, uncover intraspecific variations, and clarify complex taxonomic classifications. This makes it a valuable tool for advancing molecular phylogenetics, supporting accurate species identification, and informing conservation strategies aimed at preserving the genetic diversity and evolutionary heritage of this ecologically and botanically significant group.

Although climatic conditions characterize the area as one of thermal and rainfall contrasts, which imply a number of risk factors, the species *I. aphylla* has shown a good adaptation, reflected by its resistance to unfavorable conditions and its outstanding ornamental qualities. These results showed good adaptability of the plants and maintenance of the ornamental characteristics under "ex situ" conditions, with the possibility of their recommendation for landscaping or as cut flowers.

In subsequent studies, we aim to assess how specific cultivation conditions influence the growth, development, and key traits of the *I. aphylla* species.

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