Comparative Analysis of Plant Morphological Features and Molecular Genetic Data for Polymorphism Study in Genus *Tulipa* L. and Determining the *Tulip* Species

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Posted Date: 1 June 2023

doi: 10.20944/preprints202306.0008.v1

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Article

Comparative Analysis of Plant Morphological Features and Molecular Genetic Data for Polymorphism Study in Genus *Tulipa* L. and Determining the *Tulip* Species

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Abstract: Morphological features and composition of ISSR DNA fragments were studied in species of the genus *Tulipa* L. A comparative analysis of the data obtained was carried out and an attempt was made to clarify the species belonging of plants. Plants obtained from various sources were studied. It was found that representatives of the genus *Tulipa* L. from the two subgenera *Tulipa* and *Eriostemone*s are well separated not only morphologically, but also by the composition of ISSR fragments. At the intra- and interspecific level, the results for morphological traits and molecular data differed. Interspecific and intraspecific differences were more clearly traced in the complex analysis of morphological features and ISSR PCR data. All samples obtained in the form of bulbs and renewed vegetatively had an identical set of ISSR fragments. Plants grown from seeds were characterized by a significant variety of molecular markers and had both species-specific and individual genetic variability. It was found that the samples obtained and registered as the *Tulipa urumiensis* Stapf are the yellow-flowered form of the *Tulipa tard*a Stapf, and a sample received as the *Tulipa turkestanika* Regel is the *Tulipa bifloriformis* Vved. Thus, a comprehensive study of both the data of fragmentary DNA analysis and morphological features, provided a sufficient number of DNA samples, makes it possible to clarify the species of tulip plants, and also allows us to assess the genetic diversity of the genus *Tulipa*.

Keywords: *Tulipa*; tulip; species; identification, DNA, PCR, ISSR analysis, morphological features

1. Introduction

Genus *Tulipa* L. includes a large number of species, both diverse in their morphological characteristics, and morphologically similar and difficult to identify. The number of species in the genus differs according to different researchers, currently there are from 76 species [1] to 87 species [2]. In some cases, it is difficult to accurately identify closely related species of the *Tulipa* L. In this regard, difficulties arise in determining the types of tulip entering the botanical collections.

In order to clarify the species, as well as to study the polymorphism of plants within a taxon, in addition to analyzing morphological features, it may be useful to use molecular markers, which are currently widely used in taxonomic studies and for studying biodiversity. Previously, the study of DNA polymorphism in varieties and species, as well as tulip populations, was carried out using AFLP markers [3–5]. Later, microsatellite sequences (SSR), such as EST-SSR and NBS-LRR markers [6,7], as well as intermicrosatellite sequences (Inter Simple Sequence Repeat—ISSR), were used to study genetic diversity, population structure and phylogenetic studies in the genus Tulipa, and the latter were used to study intra- and interspecific polymorphism [8–11].

T. tarda is widespread in horticulture and is described in detail in a number of sources, the species grows naturally in Central Asia [12–16], etc. In the description of *T. tarda*, researchers mention that the perianth can be not only white-yellow with white tips of the perianth leaves, but also completely yellow, which is much rarer [13,14]. The following description is given for *T. urumiensis* in the literature: a species from the subgenus *Eriostemones*, close to *T. australis*, grows in Northwestern Iran, is difficult to cultivate. It differs in dwarf sizes: the height of the plant is 5–8 cm, and most of the

 doi:10.20944/preprints202306.0008.v1

stem is underground, the height of the flower is 3–4 cm, the length of the lower leaf is up to 12 cm, the leaves (2–3) are close directed upwards, the flower is elongated-bell-shaped, yellow [13]. *Tulipa urumiensis* also describe in the International Register of Names of Tulips of the Royal Association of Bulb Producers KAVB [15]: origin—northwestern Iran; Lake Rezai (Urmia), perianth is cleaer yellow, outer segments are pigmented on reverse with olive and red, anthers are yellow, multi-flowered, height 10 cm. Zonneveld [2] combined both species under the name T. tarda, while Christenhusz et al. [1], on the contrary, suggested using the name T. urumiensis for the combined species.

The purpose of this work was to study the complex of morphological parameters, as well as DNA polymorphism in the composition of ISSR fragments and to clarify the species belonging of tulip plants from two subgenera *Tulipa L. и Eriostemones (Boiss.) Hall*.

2. Materials and Methods

The following species from the subgenus were included in this study*:*

Tulipa vvedenskyi Z. Botsch., *Tulipa kaufmanniana* Regel, *Tulipa biflоriformis* Vved., *Tulipa turkestanica* Regel., *Tulipa biflora* Pall., *Tulipa tarda* Stapf., *Tulipa urumiensis* Stapf.

Table 1 provides a description of the samples taken for the study and the source of the origin of the material. To study the morphological features and composition of ISSR fragments, plants from the collection of tulips of the Department of Ornamental Plants of the Main Botanical Garden Russian Academy of Sciences (GBS RAS), Moscow, Russia were taken. The study included both plants grown from seeds and vegetative clones. The plants for the study were taken in the flowering phase. We studied 2–3 plants for vegetative clones and 5–8 plants for samples grown from seeds. Species identification was carried out using the key [12,13] and morphological descriptions [16–18], and plants taken for study were compared with herbarium samples stored in the Herbarium of the Main Botanical Garden RAS (MHA).

Table 1. Description Taxon of the samples taken for the study.

Name of samples	Taxon	Method of reproduction	Number of plants for study	Source of the material
Ur 1a, 1b	*T. urumiensis*	seed	2	grown in the collection of the Main Botanical Garden of the RAS, Moscow, Russia, from plants Ur 4
Ur 2a, 2b	*T. urumiensis*	seed	2	received by delectus
Ur 3a-3f	*T. urumiensis*	seed	6	grown in the collection of the Main Botanical Garden of the RAS, Moscow, Russia, from plants Ur 4
Ur 4a, 4b	*T. urumiensis*	vegetative	2	obtained from a commercial source, the Netherlands
Tar 1a, 1b	*T. tarda*	seed	2	received by delectus
Tar 2a, 2b	*T. tarda*	seed	2	grown in the collection of the Main Botanical Garden of the RAS, Moscow, Russia, from plants Tar 4
Tar 3a-3j	*T. tarda*	seed	10	grown in the collection of the Main Botanical Garden of the RAS, Moscow, Russia, from plants Tar 4
Tar 4a, 4b	*T. tarda*	vegetative	2	obtained from a commercial source, Russia
Tar 5a, 5b	*T. tarda* cv. *Soln'ishko*	vegetative	2	received from the Botanical Garden of Komarov Botanical Institute of the RAS, St. Petersburg, Russia
Bifl 1, 2	*T. biflora*	vegetative	2	received from the Central Botanical Garden of the National Academy of Sciences of Belarus, Minsk, Republic of Belarus
Bfrs 1, 2	*T. bifloriformis*	vegetative	2	received from the Central Botanical Garden of the National Academy of Sciences of Belarus, Minsk, Republic of Belarus
Bfrs 3a-g	*T. bifloriformis*	seed	7	grown in the collection of the Main Botanical Garden of the RAS, Moscow, Russia, from plants Bfrs 1, 2
Turk 1a, 1b	*T. turkestanika*	vegetative	2	obtained from a commercial source, Russia
Vved 1a, 1b	*T. vvedenskyi*	vegetative	2	received from the Tashkent Botanical Garden of the Academy of Sciences of Uzbekistan, Tashkent, Republic of Uzbekistan
Kaufm 1a, 1b	*T.kaufmanniana*	seed	2	received by delectus from South Siberian Botanical Garden of Altai State University, Barnaul, Russia

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The study of morphological features. Morphological signs were studied from herbarium samples collected at the experimental site of the Laboratory of Plant Physiology and Immunity and the Laboratory of Ornamental Plants of the GBS RAS. The following signs were studied: plant height; length, color and pubescence of the stem, length; width, color, fringing and pubescence of the leaf; number of flowers; length, width, color of the outer and inner tepals; color of anthers and staminate filaments, pubescence at the base of the staminate filaments. The selected features are used to describe a species of *Tulipa*, as well as when compiling keys for determining the types of tulip.

Study of the composition of molecular markers. DNA was isolated from fresh leaves using CTAB method [19]. The conditions of PCR were described in detail earlier [20]. For ISSR-PCR, primers synthesized and purified in PAAG by Syntol Ltd. (Moscow, Russia) were used. 10 primers were selected for PCR, their description is given in Table 2. Each amplicon, which was visualized as a strip in an electrophoretic agarose gel, was considered as a counting feature and taken into account as a binary code (1/0). Obscure bands were considered missing.

Table 2. List of ISSR primers used for PCR formulation.

Primer	Sequence	Annealing temperature of primers, °C	Number of polymorphic ISSR fragments
M2	(AC)8(C/T)G	50,0	10
M4	(AG)8(C/T)C	50,0	12
M7	(CAG)5	52,7	11
M8	(GTG)5	52,7	7
M9	(GACAC)4	50,0	10
M12	(CA)6(A/G)(C/T)	50,0	14
M13	(AGC)4(C/T)	50,0	15
UBC 825	(AC)8T	58,0	9
UBC 847	(CA)8RC	45,0	8
UBC 855	(AC)8CYT	50,0	7

Statistical processing of the results. Based on the results obtained, two matrices were compiled: one for morphological features, the second for molecular genetic data. Next, cluster analysis and data processing was carried out by the method of main coordinates in the PAST program [21]. To assess the stability of the resulting dendrogram, a bootstrap analysis was performed with 1000 replicas. For morphological data, the matrix included quantitative and qualitative features, cluster analysis was performed using the Gover distance. The presence/absence matrix of ISSR fragments was analyzed by cluster analysis using the Jacquard similarity measure.

3. Results and Discussion

3.1. Analysis of morphological features

The study of morphological features showed that two subgenera *Tulipa* L. and Eriostemones Boiss. They are clearly separated from each other, but the results obtained do not always allow us to distinguish species according to their systematic position. So, as a result of cluster analysis, the samples were distributed as follows (Figure 1).

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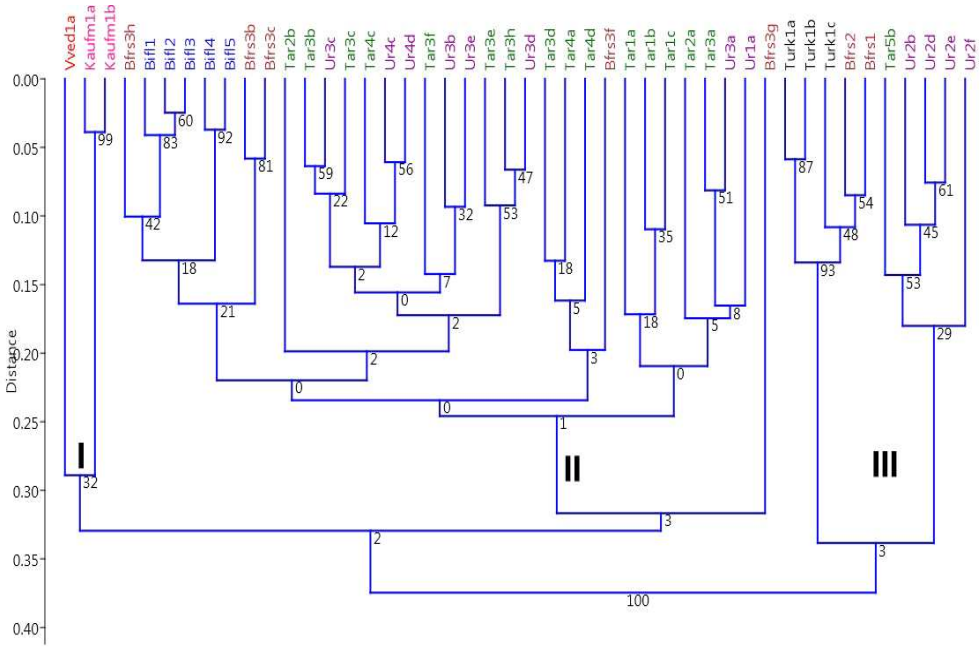

Figure 1. Results of morphological data cluster analysis.

Plants from the subgenus Tulipa formed a separate cluster I, and the neighboring cluster II—plants from the subgenus Eriostemones. Cluster III also consists of representatives of Eriostemones, but samples were grouped rather randomly and not according to their species and systematic position. The bootstrap support for large clusters is low. At the same time, the samples of Tulipa bifloriformis and T. turkestanica formed a single subcluster in subcluster III with a high similarity measure and a bootstrap support coefficient of 93%. This may indicate that these samples belong to the same species. The analysis of morphological features by the method of principal coordinates (PCoA) showed similar results (Figure 2).

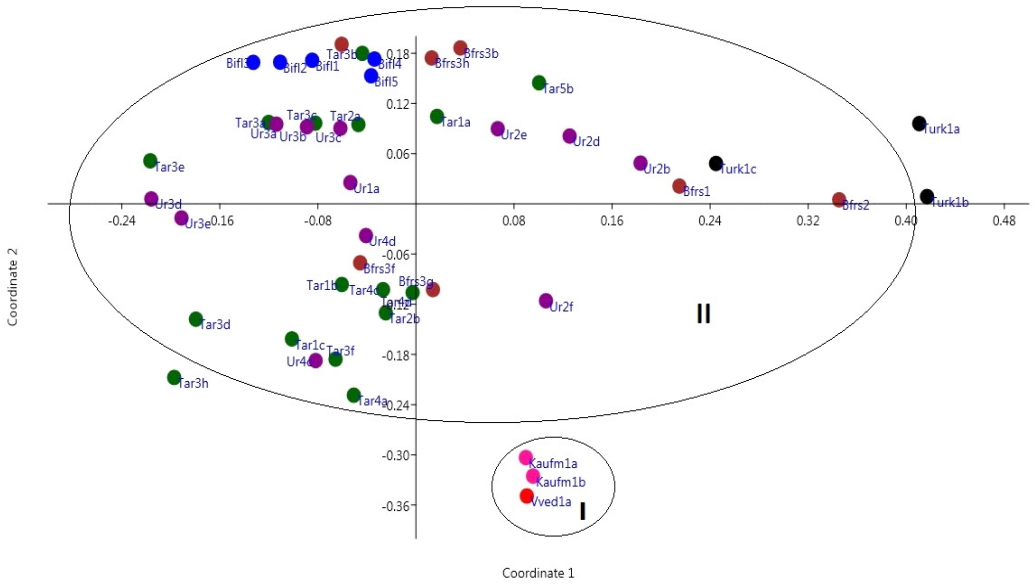

Figure 2. Distribution of samples as a result of the analysis of morphological features by the method of principal coordinates.

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The method of main coordinates also failed to separate the studied samples according to species, but all plants were divided into two clouds corresponding to the subgenera *Tulipa* and *Eriostemones*. It is not possible to distinguish species within a subgenus by the complex of selected morphological features of PCoA. Thus, the results of the analysis of only morphological features using cluster analysis and the method of principal coordinates not being informative in this case.

Khaleghi et al. analyzing the complex of morphological features by the method of principal components also obtained the separation of samples according to the subgenera to which they belonged [22]. In turn, Dekhkanov. et al. carried out cladistic analysis of a complex of morphological features of 48 tulip species from Central Asia and obtained a tree [23], which (despite low bootstrap support) agrees mainly with the molecular data of Zonneveld [2] and Christenhusz et al. [1]. In this study, the species were mostly divided not only into subgenera, but also into sections [23].

3.2. Analysis of molecular data

As a result of PCR, we obtained 102 polymorphic fragments. The data of the cluster analysis of the obtained matrix are presented in Figure 3. In this case, a clearer distribution of samples was observed according to their taxonomic position. The specimens were also divided into two main groups according to two subgenera: *Tulipa* and *Eriostemones*. Plants grown from seeds were distinguished by significant genetic diversity, while those propagated vegetatively represented one clone and had the same composition of ISSR fragments. Two species from the subgenus *Tulipa*, *T. vvedenskyi* and *T. kaufmanniana* formed one cluster with a high similarity measure and 99% bootstrap support. Within the second group, corresponding to the subgenus *Eriostemones*, the samples were grouped into cluster II and III.

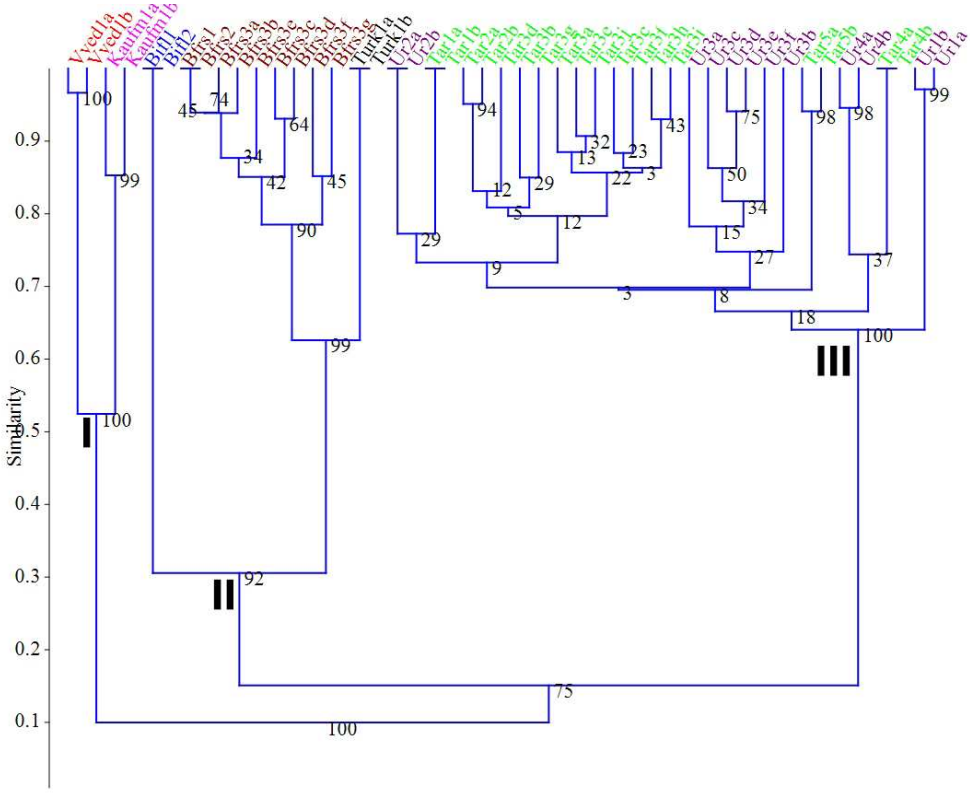

Figure 3. Results of cluster analysis of PCR-ISSR data.

Within cluster II, *T. biflora* plants formed a subcluster sister to the subcluster uniting *T. turkestanica* Regel with *T. bifloriformis* Vved. Since, according to the results of ISSR-PCR analysis, the above species are combined into one cluster (II) with a high degree of bootstrap support (92), this may indicate their closer relationship with each other than with *T. tarda* (cluster III) The *T. tarda* and

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the *T. urumiensis* also formed a common subcluster, isolated from the other *Eriostemones* species. In this group, the samples of *T. tarda* and *T. urumiensis* from different sources were practically inseparable and had a high degree of similarity. Thus, a significant similarity in the composition of ISSR fragments indicates that the plants received as *T. urumiensis* are actually the yellow-flowered form of *T. tarda*. Based on the above, it can be concluded that the studied types of tulips have a polymorphism of ISSR markers, which allows for a more thorough identification of varieties.

As a result of statistical processing of data from fragment analysis by the method of principal coordinates, a more significant picture was obtained than when analyzing morphological data by this method. In Figure 4, you can see three well-separated clouds, in addition, *T. biflora* samples occupied a separate position. The first cloud was formed by *T. vvedenskyi* and *T. kaufmanniana*, the second by *Tulipa bifloriformis* and *T. turkestanica*, the third by *T. tarda* and *T. urumiensis*.

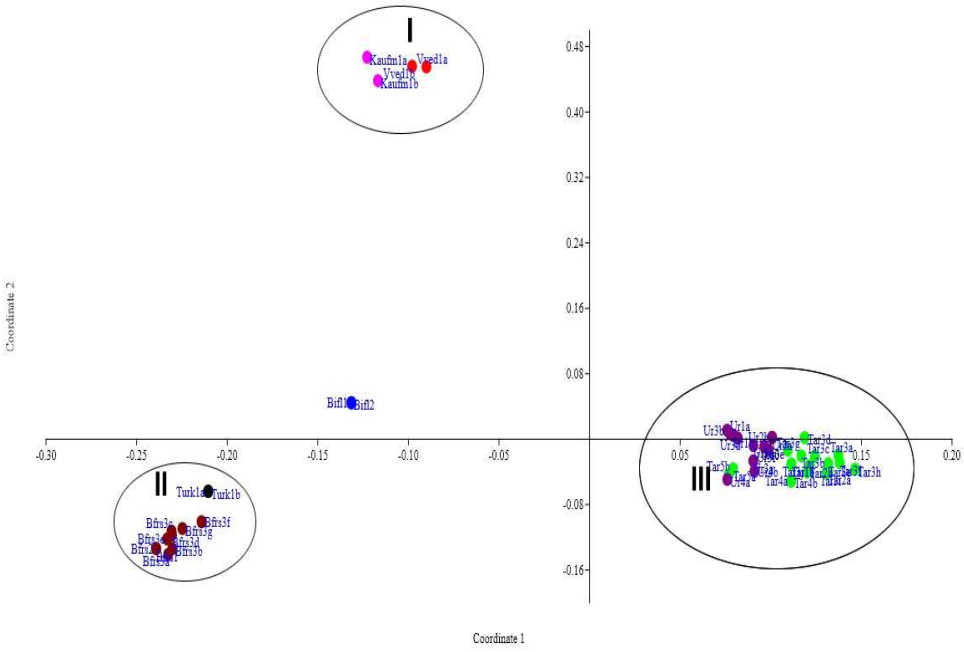

Figure 4. Distribution of samples as a result of PCR-ISSR data analysis by the principal coordinates method.

The samples of *T. tarda* and *T. urumiensis* formed a single group both in the analysis of morphological features and molecular data, and also when we determined by the key, all were assigned to *T. tarda*. In our experience, fully formed flowering plants of *T. urumiensis* have an average plant height of 20–21 cm (sometimes up to 31 cm, in *T. tarda* it is 18–20 cm), leaves are usually 5 (in *T. tarda*—5–6), the length of the lower leaf is 19–21 cm (in *T. tarda*—21–28 cm). Thus, *T. urumiensis* plants are significantly larger than the plants described in the literature [13,15] and morphologically similar to *T. tarda*.

Summing up the data obtained, it can be concluded that the samples obtained from different sources as the *Tulipa urumiensis* Stapf are in fact *T. tarda* Stapf., i.e., its yellow-flowered form. This is evidenced also by the data of the analysis of ISSR fragments and morphological features of plants Ur 1–3 and Tar 1–3, which were grown from seeds obtained from free pollination of yellow-flowered plants *T. urumiensis* and white-flowered plants *T. tarda*, respectively. Among the plants from both clones, there were both plants with yellow and white flowers, otherwise having signs of *T. tarda* (Figure 5). Recently, Christenhusz and Wilson proposed to classify cultivated *Tulipa urumiensis* plants as *T. tarda* [24], which in our opinion is more correct, since the morphological features of all *T. urumiensis* specimens correspond to *T. tarda* and are supported by the results of molecular genetic studies. The species *Tulipa urumiensis* Stapf was described from a plant grown in the Van Tubergen nursery from a bulb, which is known to have been brought from Urmia (Iran) and this species has

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never been rediscovered in Iran, it may have died out there or the bulb was mistakenly labeled when shipped to the Netherlands [24].

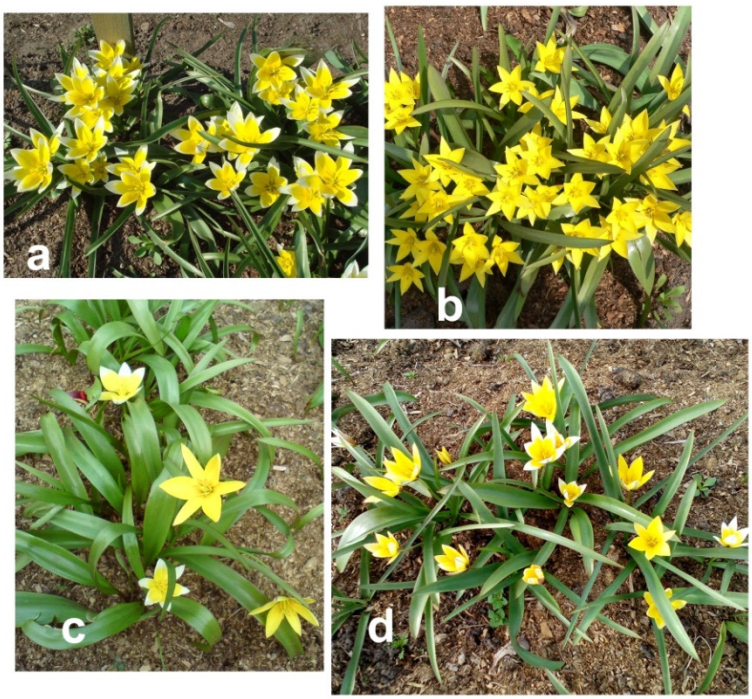

Figure 5. *Tulipa tarda* (**a**—vegetative propagation, **c**—seed propagation) and *Tulipa urumiensis* (**b**—vegetative propagation, **d**—seed propagation).

As a result of our research, we found that the plants received as the *T. turkestanika* Regel and *T. bifloriformis* Vved. with a high degree of probability, they belong to the same species—*T. bifloriformis* Vved and are two clones of this species, because they are similar in composition of ISSR-markers and morphologically. According to Zonneveld [2], *T. bifloriformis* is synonymous with *T. turkestanika*, but in all likelihood, this conclusion applies only to those cultivated clones that come from European sources. To determine the boundaries of these two species, an additional study of the complex of morphological features and polymorphism of plant DNA from natural habitats is necessary.

Thus, a comprehensive analysis of morphological features and ISSR markers in the presence of a sufficient number of plants makes it possible to more fully analyze samples and clarify the species of tulip plants.

Author Contributions: methodology, analysis of results, writing of the article—Semenova M.V.; data collection—Olekhnovich L.S.; data processing and visualization—Enina O.L. All authors have read the published version of the manuscript and agreed with it.

Funding: This research was funded by Institutional research project № 122042700002-6 of the Tsitsin Main Botanical Garden of the RAS.

Data Availability Statement: Not applicable.

Acknowledgments: The authors express their sincere gratitude N.N. Danilina for the plant material and the staff and I.A. Shantzer, head of the Main Botanical Garden Laboratory of Molecular Genetic Systematics for the opportunity to conduct molecular genetic research. We thank the Ministry of Science and Higher Education of the Russian Federation for the support of the Center of Collective Use "Herbarium MBG RAS", grant 075-15-2021-678.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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