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Population Genetic Structure of *Convolvulus persicus* L. in the Western Black Sea Region (Romania and Bulgaria) and Its Restricted Distribution

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Abstract: *Convolvulus persicus* L. is an endangered narrow-range taxon, characteristic of the habitats along the coastal regions of the Caspian and the Black Seas. The aims of our research were to update the actual distribution area and the genetic evaluation of three representative populations from the western coastline of the Black Sea located in Sulina, Agigea, and Durankulak. ISSR amplifications were used to assess the genetic intrapopulation diversity and the genetic differentiation among populations. The average genetic polymorphism was 57.8 ± 16.03%. The intrapopulation genetic diversity parameters indicated that the Agigea population exhibits a higher genetic diversity, with this small population being part of the Agigea Marine Dunes Reserve. Although the interpopulation genetic distance was reduced (0.176–0.223) and the distribution of the total variation (AMOVA) was 57% within the population and 43% among the populations, the interpopulation genetic differentiation (PhiPT) was high (0.428, $p < 0.001$), probably due to the large geographical distances between the remaining populations. The populations' genetic structures showed a lower genetic distance between the Agigea and Sulina samples. The clonability test supported the vegetative multiplication on the Durankulak and Sulina beaches. Our results showed that the genetic diversity and the distance among the populations in *C. persicus* were influenced by habitat conditions, destruction, and fragmentation, but also by conservation measures.

Keywords: genetic diversity; *Convolvulus persicus*; genetic differentiation among populations

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1. Introduction

Convolvulus persicus L. is an endemic species typically found in sandy habitats along the coastal regions of the Caspian and Black Seas. Due to increasing anthropogenic pressures in these areas during the second half of the 20th century, these habitats have been dramatically affected. As a result, *C. persicus* has become a threatened species within its characteristic geographic range [1]. It is protected by national legislation in each of the countries where it still occurs as a native species: South-East Romania, Eastern Bulgaria, European Turkey, North-West Anatolia, Georgia, Russia (Republic of Dagestan), Azerbaijan, Iran, and Turkmenistan [2].

Recent studies have reported *C. persicus* on the Black Sea coastline only in Romania, Bulgaria, and Turkey [3]. The largest populations are found in the Delta of the Danube

River, specifically on the sandy shores between the mouths of the Danube's distributaries, between the Sulina and Sfântu Gheorghe branches.

C. persicus is a pioneer species that colonizes nascent dunes. It was first described by Carl Linné in 1753 as "Convolvulus foliis ovalibus tomentosis, pedunculis unifloris". This medicinal, also called fisherman's tea, is used by the Delta herds to treat cough [4]. Twenty different compounds with antioxidant activity have been identified in the literature [5,6], and extracts from its aerial parts have been investigated for their potential in the treatment of Alzheimer's disease [7].

C. persicus is a diploid perennial species (2n = 26), with vegetative reproduction by the elongated rhizomatous stems bearing numerous nodes that give rise to aerial shoots. These shoots initiate extensively around the nodes and mature individuals can generate clones with either branched or unbranched stems. Sometimes several clones coexist in close proximity [8,9]. The plant is well adapted to harsh environmental conditions such as exposure to sandstorms, saltwater spray, and intense solar radiation. It has white hermaphrodite flowers that produce a fruit capsule containing 2–4 seeds after insect pollination. Although the seeds are fertile, the presence of seedlings from germinated seeds is rarely observed in the field; instead, the propagation is predominantly vegetative, stimulated by the burial in the sand [1].

Due to the anthropogenic influences, this taxon has become endangered within its native range. It is protected by national regulations in all the countries where it occurs and it is included within various types of protected areas in Iran, Azerbaijan, Romania, Bulgaria, and Turkey [1].

In Romania, the sites of *C. persicus* populations are not included in the protected areas, but the Danube Delta was designated as a Site of Community Importance (SCI) according to the Habitats Directive 92/43/EEC (ROSCI0065). Although the *C. persicus* taxon was not listed in Annex II as a species of community interest, it occurs in two types of habitats of community importance: "1210 Annual vegetation of drift lines" and "2110 Embryonic shifting dunes", as a characteristic species of embryonic dunes [10].

Due to its rarity, small fragmented local populations, and vulnerability to human activity, *C. persicus* has been included in the Red Book of Vascular Plants in Romania and Bulgaria. In the Red List of Vascular Plants [11] and the Critical List of Vascular Plants in Romania [12], *C. persicus* was considered as rare and later reclassified as critically endangered [13]. In Bulgaria, the species is listed as critically endangered in the Red List of Bulgarian Vascular Plants [14], is protected by the Biodiversity Act, and its habitats have been included in the Bulgarian Natura 2000 network [15].

Anthropogenic influences have led to the fragmentation of the distribution of *C. persicus*. A reduction in the population size may lead to inbreeding and a decrease in the genetic diversity within the population [1,3]. The study of genetic variation is crucial for the development of effective conservation strategies.

Previous studies on the species have examined the intrapopulation variability using alloenzyme marker systems, detecting isoenzymes such as catalase, peroxidase, alkaline phosphatase, and esterase [16]. However, the limitations of this technique led us to analyze genetic variation using ISSR markers. ISSR analysis is proven to be a reliable method for assessing intrapopulation variability by amplifying inter-repetitive DNA sequences that are prone to mutations. This method also offers advantages such as simplicity, speed, and low cost, and it has a high polymorphism [17]. Moreover, ISSR molecular markers require minimal amounts of DNA and do not require knowledge of primer annealing sequences [18]. In contrast, the RAPD technique has a low reproducibility, AFLP is expensive, and SSR markers require knowledge of flanking sequences [19–21].

In this context, our research aimed to update the distribution map of the species, to conduct a comparable diagnosis of the genetic diversity, and to assess the genetic differentiation between populations of the *C. persicus* taxon in three populations—Sulina (SP), Agigea (AP), and Durankulak (DP)—of the western coastline of the Black Sea. These populations comprise the last remaining sites of *C. persicus* that can be found on the western coast of the Black Sea, namely the Danube Delta, the Marine Sand Dunes Agigea, and the Nature 2000 site of Durankulak.

This study provides new insights into fragmented distribution and restriction population, thereby helping to take appropriate conservation decisions for this species.

2. Materials and Methods

2.1. Convolvulus persicus Mapping

For mapping *C. persicus*, we used data from the literature and the iNaturalist platform [1,2,8,14,22–32]. The data sources were used to gather an actual distribution of our species and are available in the Supplementary Materials Table S1. From the platform, the only data considered were those that had geographical coordinates, species images, and community validations.

The distribution map was created using Google Earth Pro, version 7.3 [33]. Overlapping records were merged into a single record.

2.2. Plant Material

Samples consisted of leaf material, collected from 15 individuals from three populations situated on Sulina Beach (45°08′28.35″ N, 29°40′45.45″ E), Agigea (44°5′18.53″ N, 28°38′31.31″ E) and Durankulak Beach (43°41′42.43″ N, 28°33′53.77″ E) in July 2024. The areas of the studied populations were about 2400 m^2 in Durankulak, 25 m^2 in Agigea, and 3000 m^2 in Sulina. The chosen individuals were situated at a distance of at least 5 m from each other to avoid the collection of clones. A voucher for the plant from Sulina Beach was introduced in the BUCA Herbarium Institute of Biology Bucharest with the registration number 163.225.

2.3. DNA Extraction

The fresh plantlets of about 100 mg weight were used for the DNA extraction. For the extraction of genomic DNA, the NucleoSpin Plant II kit (Machererey-Nagel, Dueren, Germany) was used, and then the DNA quality and quantity were estimated using a BioDrop Duo spectrophotometer (BioDrop, Cambridge, UK).

2.4. PCR Amplification and ISSR Profiling

Ten ISSR primers similar to those from UBC (The University of British Columbia Biotechnology Laboratory, Vancouver, BC, Canada) provided by Integrated DNA Technologies B.V., Leuven Belgium, were initially tested. From these primers, only seven had clear scorable and reproducible bands. The selected primers were comprised of 16–18 bases and their sequences are presented in Table 1. Polymerase chain reaction (PCR) amplifications were performed in a 25 µL reaction volume containing 10 ng of template DNA, 2.5 µL of primer at a concentration of 10 µM, and 12.5 µL of the GoTaq Green Master Mix (Promega, Madison, WI, USA). Each sample was then diluted according to the concentration ascertained following the extraction process. The amplifications were achieved using a Mastercycler Gradient thermal cycler (Eppendorf, Wesseling-Berzdorf, Germany). The PCR amplifications were performed by adjusting the annealing temperature according to the melting point (Tm) of each primer (Table 1). The cycling conditions were the initial denaturation step for 2 min at 94 °C, followed by 30 cycles: 30 s for denaturing DNA at

94 °C, 30 s at the specific annealing temperature of primers, 40 s at 72 °C for the extension, and finally the extension step for 5 min at 72 °C. The amplified products were analyzed using gel electrophoresis [34–36]. Electrophoretic separation was performed with 10 µL of amplified products on 1.8% agarose gel in a TBE buffer, run for 2 h at 140 V, using a Midi Horizontal HU13 Unit (Scie-Plas, Cambridge, UK).

Table 1. The analysis of the ISSR amplified bands and generated loci in three populations of *C. persicus* from the Black Sea western coast.

Primer Cod	Sequence (5′-3′)	T_m (°C)	Amplify Range (pb)	No. of Loci	No. of Polymorphic Loci			No. Total of Amplified Bands		
					SP	AP	DP	SP	AP	DP
MC1	$(AC)_8G$	53	250–2715	13	10	11	5	118	135	163
MC2	$(ATG)_6$	43	280–2285	11	4	10	4	95	118	76
MC3	$(GA)_8YG$	49	105–1405	10	5	9	3	128	120	68
MC4	$(GACA)_4$	47	261–2265	10	7	10	4	118	89	105
MC5	$(GGAGA)_3$	48	223–1145	7	4	7	3	58	40	75
MC6	$(GA)_8T$	45	227–2085	11	3	10	4	104	119	97
MC7	$VHV(GT)_7$	52	185–1545	15	7	11	7	163	196	187

DNA amplification products were subjected to staining with ethidium bromide at a concentration of 0.02 µg/mL This was carried out in a gel, which was then visualized under ultraviolet light using a GENi gel documentation system (Syngene, Cambridge, UK) The analysis was then performed using the Gene Tools software (SynGene, Cambridge, UK, version 4.02). The weight molecular marker (Mg) employed was the BenchTop 1 Kb DNA Ladder (Promega, Madison, WI, USA).

2.5. Data Analysis for ISSR Polymorphism

ISSR markers are considered the dominant multilocus DNA fragments. As such, the scoring system employed for the analysis involved assigning a value of 1 to each band that was present, and a value of 0 to each band that was absent. The amplified patterns of all the analyzed primers were transformed into a binary character matrix.

Three types of genetic analysis were generated with the GenAlEx 6.5 software: frequency-based population analysis, interpopulation analysis as Nei's genetic distance, and analysis of molecular variance (AMOVA) and spatial genetic analysis as principal coordinate analysis (PCoA) [37].

The basic population genetic diversity parameters were calculated as a percentage of the polymorphic loci, the band frequency, the allele frequency (p, q) considering complete outcrossing [38], the number of alleles (Na), the effective number of alleles (Ne), Shannon's Information Index (I), and the expected and unbiased expected heterozygosity (He and uHe). These calculations were calculated assuming Hardy–Weinberg equilibrium using the GeneAlEx software for dominant markers using the following formulas:

$$Ne = 1/(p^2 + q^2), \quad (1)$$

$$I = -1 \times (p \times Ln(p) + q \times Ln(q)), \quad (2)$$

$$He = 2 \times p \times q, \quad (3)$$

$$uHe = (2N/(2N - 1)) \times He, \quad (4)$$

where $q = \sqrt{(1 - \text{Band Frequency})}$, $p = 1 - q$, and N = sample size.

From the diploid binary data, a pairwise (individual-by-individual) genetic distance matrix (GD) was generated using the method of [39].

The genetic structure and variability among the population were investigated using the AMOVA test based on a pairwise genetic distance matrix. The AMOVA calculated the PhiPT parameter, an F_{ST} analogue, to estimate the genetic interpopulation differentiation. The variance was statistically calculated using randomization with 999 permutations. The GeneAlEx software allows the calculation of the genetic distance using the formula:

$$\text{PhiPT} = \text{AP}/(\text{WP} + \text{AP}) = \text{AP}/\text{TOT} \quad (5)$$

where AP = estimated variance among populations, WP = estimated variance within populations, and TOT = total estimated variance.

The average Nei's genetic distance between populations (Nei D) and pairwise population Nei Unbiased Genetic Distance (uNei D) was calculated based on Nei's genetic identity (Nei I) and the estimated allele frequencies data:

$$\text{Nei D} = -1 \times \text{Ln (Nei I) and uNei D} = -1 \times \text{Ln (uNei I)} \quad (6)$$

Also, the pairwise genetic distance was used for a multivariate analysis PCoA, which reveals separation among multiple loci and multiple samples data set and clustering analysis as a Neighbor-joining tree. The Neighbor-joining dendrograms were generated using the MEGA software version 11 [40].

3. Results

3.1. Distribution Area of Convolvulus persicus L.

The impact of anthropogenic activity led to a decline in the habitat of *C. persicus*. A comprehensive review of the literature survey supplemented by information gathered from the iNaturalist platform gives an overview of this species distribution (Figure 1).

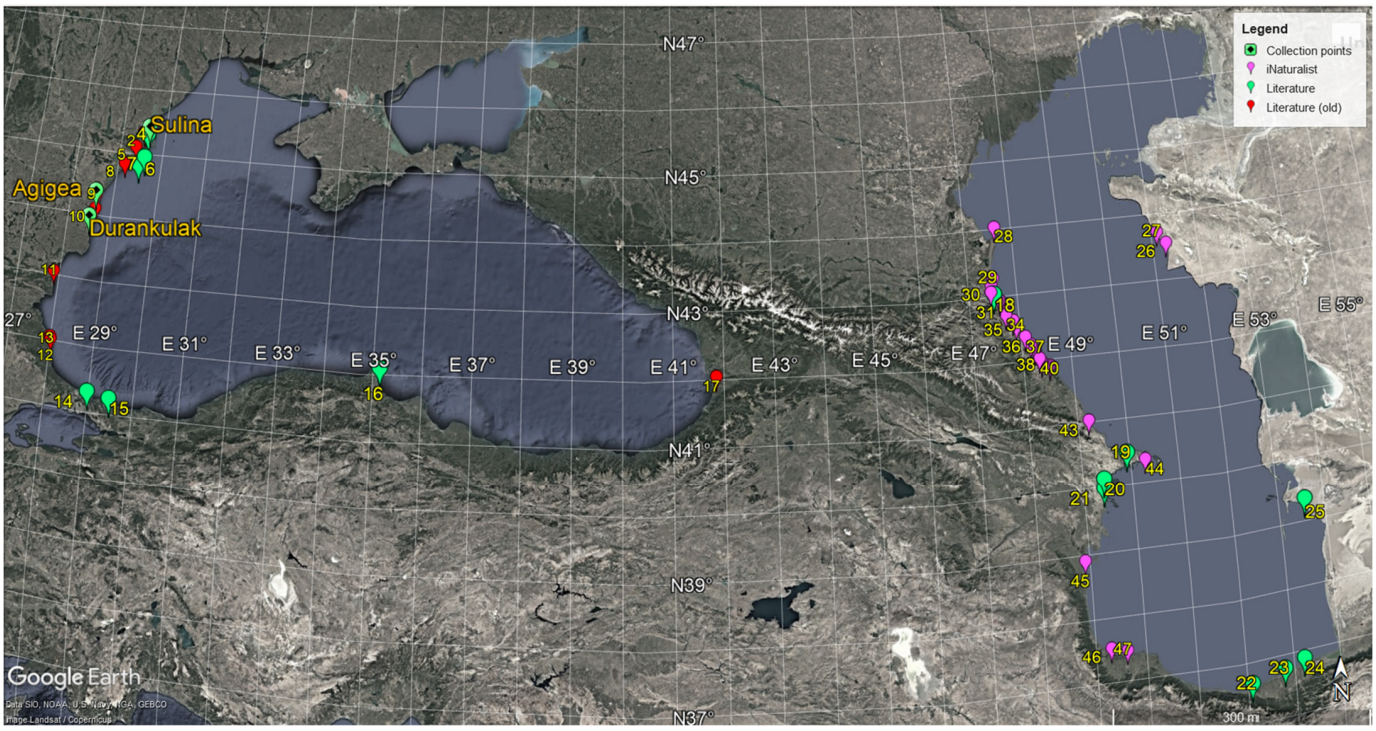


Figure 1. Distribution map of *C. persicus* in area of the Black and Caspian Seas. The numbers represent the distribution sites. The name and coordinates of these sites are listed in Supplementary Materials Table S1.

Currently, the literature reported this species in the Caspian Sea coast; in the coast zone of Azerbaijan (32); in Iran, in sites such as Rostam Rood Nour, Goharbaran Sari, and Naftchal Babolsar from the Mazandaran region [2,23,24]; and in the Republic of Dagestan, on the western coast, in Makhachkala [25].

A number of 190 observations (some overlapping) were registered for *C. persicus* in the iNaturalist platform, from 2006–2024, from which 51.8% were in the past two years. A detailed list of these observations is given in Supplementary Material Table S1. Most of the observations (144) were from the Republic of Dagestan, followed by Azerbaijan (33 observations). In Kazakstan, two locations were recorded (Aktau and South of Aktau) in the iNaturalist platform. No specific locations for *C. persicus* have been identified in the literature or on the iNaturalist platform in Turkmenistan.

In the seaside area of the Black Sea, *C. persicus* was spread on the eastern coast of the North Caucasus, on the southern coast of Turkey, and on the western coast of Romania and Bulgaria. Actually, in Turkey it was cited only from Sarıyer, Gümüşdere beach and Şile, Sofular village, on sandy dunes, and others at Sinop [26,27]. In Georgia, however, *C. persicus* is considered extinct in the wild [28].

In Romania, *C. persicus* was discovered in 1915, and subsequently mentioned in "Flora Romaniae" in four locations: three in the South of the Coast, one in the North and the South of Constanța and one in the Delta, at Sf. Gheorghe on the seafront at the discharge of Sf. Gheorghe's branch [8]. In 2009, the taxon was cited in 14 sites: four around Constanta at Mamaia, Agigea, Eforie, and Techirghiol Movila; two at Sackhalin, Perisor; and eight in the Danube Delta at Sf. Gheorghe, Mila 15, Rosetti, Sfiștofca, Cardon, Letea, Caraorman, and Sulina [29,30]. Currently, the presence is confirmed in five sites: Agigea, Sf. Gheorghe (Sărăturile beach), Sackhalin Island, Sulina beach, and Cardon [1,2].

In Bulgaria, its distribution included sites such as the Black Sea coast near the mouth of the Rezovska River, Silistar Bay, the Kamchia River estuary, and the Durankulak Lake [2,14]. Over the past decade, only two subpopulations have been recorded, along the beach of Durankulak Lake, with few individuals in each subpopulation [14]. Subsequently, one of the subpopulations has disappeared. Efforts to confirm the species' presence in other reported localities have been unsuccessful since 1990.

The iNaturalist platform includes 29 observations regarding *C. persicus* in Romania. The data are overlapping and do not bring new information beyond those already published in the literature.

Along the seaside area of the Caspian Sea and Black Sea, the species was recorded in two places outside the coast area. The first location was in Lake Hazar in Turkey [26] and the second was in Dealul Gîlmeia Seciu, Ploiesti, Romania (Herbarium BUCA, no. 29290, 1947).

3.2. Variation in Amplified Bands and Polymorphism Level

The genetic diversity in three populations representative of the western limit of the spreading area of the taxon *C. persicus* was assessed using a dominant multilocus system of ISSR markers. Ten ISSR primers were used to amplify the regions between the repetitive microsatellite sequences, but only seven primers produced well-reproducible, scorable patterns. The other three primers generated too many bands, which cannot be clearly distinguished using separation gel electrophoresis.

PCR amplification with these seven types of primers with lengths between 15 and 18 bp generated 784 fragments for the SP, 817 fragments for AP, and 771 fragments for DP across 15 samples for each population. The number of amplified bands per primer ranged between 7 and 15 for all the populations (Table 1). The molecular lengths of these amplicons varied from 105–2715 bp. A number of 76 loci was obtained by aligning the amplified bands from all the populations according to the molecular lengths. The number

of polymorphic loci varied between 3–11/primer; for SP and AP, the highest number of polymorphic loci was detected for the MC 1 and MC 7 primers, while in DP, between 3–7 polymorphic loci/primer were detected with a maximum for the MC 7 primer. From all 76 loci scored, 68 were polymorphic in AP, 40 in SP, and only 31 in DP. The polymorphic loci percentage was quite different: 85.53% in AP, 47.37% in SP, and 38.16% in DP. The polymorphism average among the three populations was 57.02 ± 14.5%.

3.3. Intrapopulational Genetic Diversity

The genetic diversity parameters differed significantly between the three populations ($p < 0.001$). In AP, higher values were registered for all the genetic diversity parameters (Table 2). The main parameter, expected heterozygosity (He) or genetic diversity within the population, was 0.308 ± 0.015 in AP, 0.237 ± 0.028 in SP, and lower in DP (0.168 ± 0.026). Taking into account the number of samples from each population, the genetic diversity slightly increased for all the populations, being expressed in the form of the uHe parameter (Table 2). Another important parameter for intrapopulation genetic diversity, the Shannon Information Index (I), the equivalent of the Shannon–Weaver index in ecology, and it was higher in AP and SP, having values of 0.461 ± 0.020 and 0.341 ± 0.040, respectively. The number of different alleles (Na) provides an arithmetic mean across the loci and recorded a maximum value for AP (1.935 ± 0.021). A number of effective alleles (Ne) represents an estimate of the equally frequent alleles in the ideal population having lower values than the number of observed alleles for all the populations.

Table 2. Genetic diversity data generated using the GeneAlEx software for Sulina, Agigea, and Durankulak natural population of *C. persicus*: number of observed alleles (Na), number of effective alleles (Ne), Shannon's information index (I), expected heterozygosity (He), and unbiased expected heterozygosity (uHe).

Population	Na	Ne	I	He	uHe
Sulina	1.508 ± 0.078	1.432 ± 0.054	0.341 ± 0.040	0.237 ± 0.028	0.245 ± 0.029
Agigea	1.935 ± 0.021	1.537 ± 0.031	0.461 ± 0.020	0.308 ± 0.015	0.318 ± 0.016
Durankulak	1.349 ± 0.082	1.298 ± 0.050	0.247 ± 0.037	0.168 ± 0.026	0.173 ± 0.027
Average	1.597 ± 0.044	1.422 ± 0.025	0.350 ± 0.019	0.237 ± 0.013	0.245 ± 0.014

Data presented as mean ± standard error (SE).

3.4. Genetic Differentiation Among Populations

The AMOVA revealed highly significant populations differentiation with PhiPT, of 0.428 ($p < 0.001$). The PhiPT parameter is an equivalent of F_{st}, an inbreeding coefficient for codominant markers, which provides a measure of genetic differentiation among populations. The highest genetic differentiation was registered between SP and DP of 0.525, while between AP and DP, a PhiPT of 0.428 was registered, and a PhiPT of less than 0.346 was registered between AP and SP. The distribution of total variation by AMOVA was partitioned between within the population (57%) and among populations (43%) (Table 3).

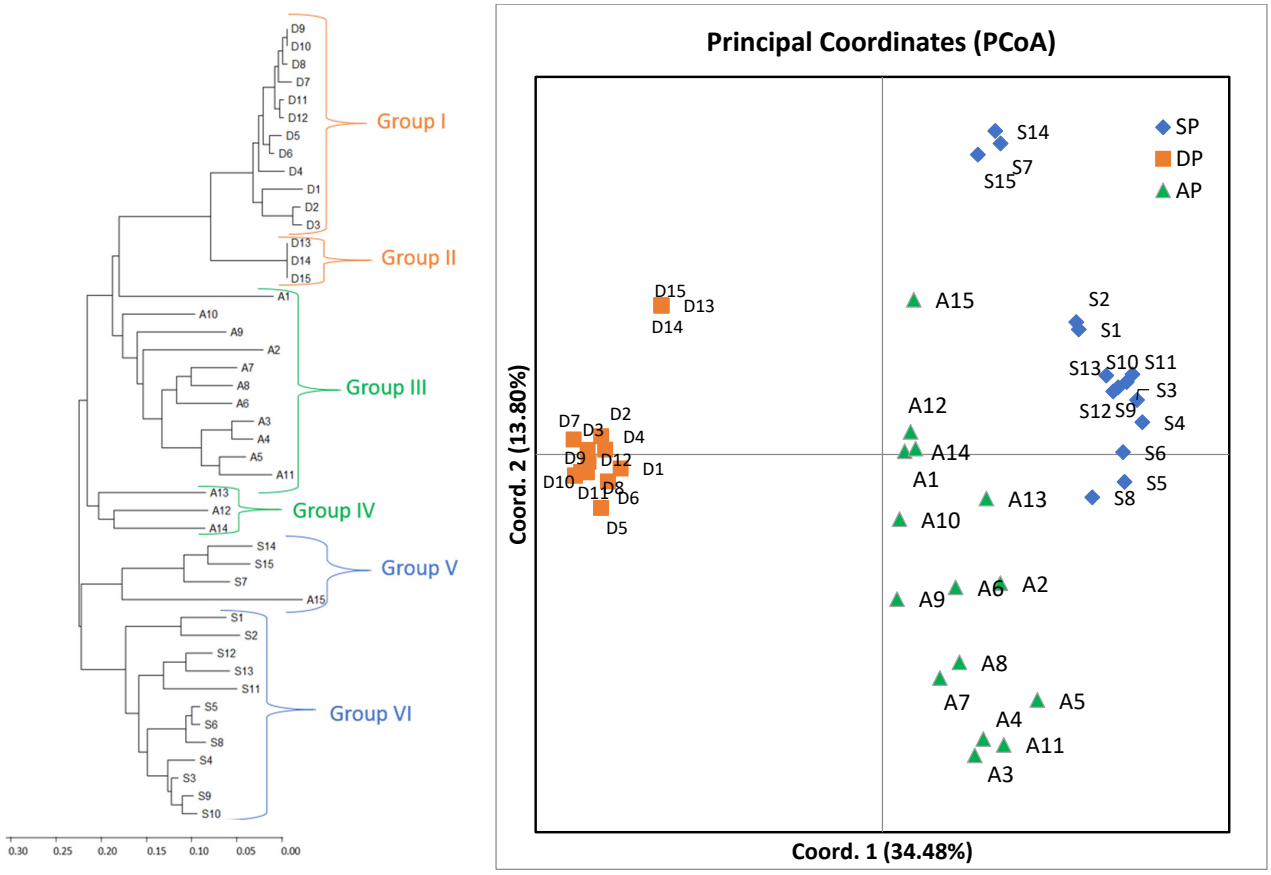
Table 3. Distribution of genetic variability within and among the Sulina (SP), Agigea (AP), and Durankulak (DP) natural populations of *C. persicus*.

Source	Degrees of Freedom	Sum of Square	Mean Square	Estimated Variance	% Total Variance
SP		84.800			
AP		162.267			
DP		64.533			
Among populations	2	181.156	90.578	5.544	43%
Within populations	42	311.600	7.419	7.419	57%
Total	44	492.756		12.963	100%

Based on the ISSR allele frequencies, Nei I closed values of 0.800–0.839 were calculated between the three populations and for further Nei D, the low genetic distances were 0.176–0.223. The pairwise population Nei unbiased genetic distance analysis calculated values of 0.810–0.844 for uNei I and 0.169–0.211 for uNei D.

3.5. Genetic Structure of Populations by Clustering Analysis

A pairwise genetic distance matrix generated using ISSR binary data was used for the Neighbor-joining dendrogram construction. The Neighbor-joining clustering algorithm generated using the MEGA software grouped 45 genotypes into six clusters (Figure 2a). Three main clusters comprised the majority of samples from each population, while three samples from each population were separated by the population's main clusters. Thus, DP comprised two clusters: group I, which included the majority of samples (D1–D12), and group II (D13–D15). AP displayed another two clusters: the main group III (A1–A11) and group IV (A12–A14), while the A15 individual clustered with the three different individuals, S7, S14, and S15 from SP, forming the group V. The last group VI included the rest of the samples from SP (S1–S6, S8–S13). The genetic distance between the groups from the same population was small in DP and AP, at 0.158 between groups I and II, and 0.177 between groups III and IV. A low genetic distance was also calculated between AP and SP (groups III and VI). The highest genetic distance was determined between SP and DP, which varied from 0.527 to 0.568 between the groups of these populations.



(**a**) (**b**)

Figure 2. Genetic structure of three *C. persicus* populations: (**a**) Neighbor-joining dendrogram and (**b**) Principal Coordinates Analysis (PCoA) showing the genetic distance between samples (S1–S15 from SP, A1–A15 from AP, D1–D15 from DP).

The biplot of PCoA demonstrated the separation of three major groups, which correspond to the three populations, but also confirmed the partitioning of the same samples from the populations (Figure 2b). Also, both cluster analyses displayed a larger dispersion of samples from AP and SP by the analysis of the multivariate data set as multiple loci and multiple samples. These confirmed a higher genetic diversity in AP and SP, than DP, and showed close genetic relationships between certain individuals from the populations.

The repeated matching multilocus genotypes test, which calculated the clonality probability, showed that samples S5, S6, S9, and S10 from SP were genetically similar and may represent the ramets of the same clone. In DP, many sets of genetically similar samples were listed. The first set contains the three samples D13, D14, and D15, which were grouped separately from the majority population, and then pairs like D2 with D9, D8 with D6, and D11 with D3. The Neighbor-joining and PoCA analyses showed, also, that the samples in DP and SP were genetically closely related.

4. Discussion

4.1. Analysis of Intra- and Inter-Populational Genetic Diversity and Structure

Several previous studies showed that the threats of *C. persicus* would be due to several factors: limited distribution area (south-western coast of the Black Sea in Europe and west-southeastern coastal regions of the Caspian Sea in Asia); anthropogenic activities, especially tourism activities; loss of dune-specific habitats by coastal erosion, landscaping transformations, and environmental climate changes; and few conservation measures from the past that resulted in distribution area fragmentation, restriction, and deterioration [1,3]. For these reasons, the majority of the populations reported in the past century on the western Black Sea coast disappeared or survived only in a protected area as the Danube Delta Biosphere Reserve, the natural reservation of Marine Sand Dunes Agigea, and the Nature 2000 protected site BG0000154 Ezero Durankulak [2].

Based on our review, many populations reported in the past have not been observed recently (since 2006) in the Black Sea basin. In the Caspian Sea area, other localities have been mentioned on the iNaturalist platform, providing new and important information on the distribution of *C. persicus*. In the North Caucasus, Georgia, and Turkmenistan, information on certain locations of *C. persicus* was difficult to find in the older literature, and to the best of our knowledge, no new locations have been published in the literature or on the iNaturalist platform.

An important consequence in population deterioration is a reduction in the population size, which determines inbreeding and a reduction in the intrapopulation genetic diversity, and the study of genetic variation is the first step in developing a conservation strategy.

ISSR markers have been widely used in population genetic analyses of plant species, to estimate genetic structure and population differentiation for rational conservation decisions [19,41–44]. In our case, the polymorphism generated by the seven used ISSR primers was 57.02 $\pm$ 14.5%, while the MC1 and MC7 primers showed the highest polymorphism of 11 from 13 loci being polymorphic, followed by the MC7 primer with a polymorphism of 11 from 15 loci in AP. This type of marker, ISSR, proved to be useful for the detection of polymorphism in our populations. The disadvantage of these dominant markers would be the lack of software that can calculate the genetic distance in the absence of some data sets. Therefore, the option for interpolating missing individual-by-individual pairwise is necessary. The majority of population genetic analysis software is very restricted, with dominant markers rather than codominant ones [45,46]. Also, in the case of dominant markers, not all genetic diversity parameters can be calculated, as for codominant ones, so the analysis software must offer alternative methods and formulas.

Besides ISSR markers, a series of other molecular markers have been proven to be useful to determine the genetic diversity in *Convolvulus* genus, as ITS fragments [47], RAPD markers [48,49], and the direct amplification of the microsatellite region (DAMD-CPR) cleaved amplified polymorphic sequence (CAPS) [50].

Our research showed that the registered values for the intrapopulation genetic diversity parameters in both studied populations were related to the results obtained by other authors in five populations of bindweed (*Convolvulus arvensis* L.), using RAPD dominant markers [51]. In AP and SP, all the genetic diversity parameters—Na, Ne, He, and I—were above or comparable with the *C. arvensis* population Oltu, with the highest level of genetic diversity (Na = 1.6056, Ne = 1.4208, H = 0.2381, and I = 0.3488), while for DP, the recorded parameters were similar or below the level of the Erzurum population with the lowest diversity (Na = 1.4296, Ne = 1.289, H = 0.1672, and I = 0.2463) from all five *C. arvensis* populations. Take into account that this species belongs the same genus, being the second largest taxa within the *Convolvulacee* family, one of the common weeds with a wide-spreading area in many habitats and many populations, we can state that the genetic diversity in our populations was comparable with the non-threatened populations without a restricted distribution area being a characteristic of the genus. Some populations of *C. arvensis* appear to propagate entirely by vegetative propagation [52]. If the rhizome can be observed at soil level for *C. arvensis*, in case of *C. persicus*, the individuals are hard to detect, with the rhizome being deeply buried in the sand.

On the other hand, numerous studies have demonstrated that species with narrow distributions or endangered species, such as *Hagenia abyssinica* [53], *Lilium pumilum* [54], and *Hedysarum theinum* [55], continue to maintain significant genetic diversity. Moreover, the genetic diversity of another species with a restricted range belonging to the same genus, such as *Convolvulus argyrothamnos*, is quite similar to our results recorded in populations from the Black Sea of *C. persicus*. These authors obtained high values of genetic diversity using ISSR primers (Na = 1.582, Ne = 1.412, I = 0.24, H = 0.352, He = 0.241, uHe = 0.322) for an endemic species of *Convolvulus* that is specific to the Greek island [56], comparable with other extremely narrow species of the Mediterranean zone. Despite the extremely restricted range, many of these species show moderate to high diversity [57], which suggests a high adaptation to the conditions offered by the restricted area.

The Nei's genetic distance between these studied populations of *C. persicus* was 0.176–0.223, comparable with Nei's gene diversity value for all five populations of *C. arvensis* of 0.2053. Our results showed a significant population differentiation with a PhiPT of 0.428 ($p < 0.001$), while the genetic differentiation among *C. arvensis* populations calculated as GST was 0.293 [51]. In population genetics, gene differentiation based on Gst or Fst values is classified as low for values < 0.05, medium for values between 0.05–0.15, high for values between 0.15–0.25, and very high for values > 0.25. From this point of view, the PhiPT parameter, an analog to Fst, calculated using AMOVA for dominant markers, showed a very high genetic differentiation between our populations, especially SP and DP. This increased inter-populational divergence can be a result of restricted gene flow among fragmented habitats and the long geographical distance of about 200 km between SP and DP.

The AMOVA presented 53.8% variation within populations, and 32.9% among populations, showing a high variation within *C. arvensis* populations [51]. A similar distribution pattern of total variation by AMOVA was also registered in our population, with a higher difference among populations of 43%, which can be explained by the higher inter-populational geographical distance.

While genetic diversity can arise through mutation, genetic drift, gene flow, and selection, it is widely recognized that factors such as reproductive patterns, life forms, historical

events, and geographic distribution play a crucial role in determining the extent of genetic differentiation among populations [58,59]. The breeding system is the primary factor influencing genetic diversity and its distribution within and among populations [59,60]. RAPD and ISSR analyses have revealed that species with widespread outcrossing tend to exhibit low levels of diversity between populations, but a high differentiation within populations [61,62]. In our case, the difference in genetic variation within and among the population is lower, 57% and 43%, respectively. Outcrossing plant species typically show between 10% and 20% genetic variation among populations [63]. It seems that self-crossing species exhibit higher levels of genetic diversity between populations, such as *Senecio vulgaris* (40%) [64] and *Antirrhinum subaeticum* (46.8%) [65]. The proportion of 43% genetic differentiation among the population suggested a preponderance of self-crossing plant breeding in our populations. The genetic structure of two of three of the populations, the neighbor-joining tree and the PCoA sustained this self-crossing behavior, because of the closed genetic distances between certain samples. Additionally, the clonal probability test showed the possibility of the existence of some clones, although the samples were collected at a distance of at least 5 m from each. All of these factors supported the predominantly clonal reproduction of this species, which is known to spread primarily using lateral roots and root buds [1]. The vegetative reproduction through the rhizomes is predominant, after burial in the sand, the rhizome differentiates adventitious roots, which expand into the new sand layer. The underground rhizomes can vary from 15 cm to 3.5 m, while the extensive roots can reach up to 5.5 m in length, penetrating deeply into the sand or sandy soil [3]. Furthermore, field surveys conducted along the Iranian coast of the Caspian Sea revealed significantly lower seed production compared with flower production. Germination rates were found to be less than 10% across the three populations studied on the southern coast of Caspian Sea [24].

4.2. Implications for C. persicus Conservation

The *C. persicus* species has been under threat in recent years because of the destruction of its habitats, mainly due to anthropogenic activities that led to habitat fragmentation and the disappearance of many populations, which might play an important role in creating this species' current endangered status.

Generally, genetic diversity in the studied populations was comparable with its relative *C. arvensis* and considerable differentiation was also observed among the three populations, so the genetic variation loss is not the cause of its endangered status.

However, a slight decrease in the genetic diversity of DP, lower than *C arvensis* populations, indicates the need to take on new protective measures for this population. Taking into account the reduced number of individuals in this population, the great distance from neighboring populations, isolation, and reduced gene flow, but also the prevalence of asexual reproduction that emerges from the analysis of the genetic structure, an urgent plan of action would be necessary. The current in situ conservation area for reducing the impact of human activities to increase the population through natural regeneration was not enough to reach genetic diversity. A potential action plan's measure for the conservation of this species in Bulgaria, as recommended by the Red List of Bulgarian Vascular Plants [14], would be its introduction from Romanian populations. Introgression through the hybridization of genetically diverse populations across different geographic regions can play an important role in the genetic enhancement of populations [66], when the descendants are fitter than the parents, but it can also have a negative outcome when the descendants manifest the outbreeding depression [67]. Accordingly, a plan for increasing the genetic variability by hybridization with genetically diverse populations across different geographic regions can be risky, less fitted individuals as a result of outbreeding depression

in a population can cause further genetic diversity degradation and the loss of locally adapted genotypes [65,68]. Alternatively, plants can be multiplied through proper propagation and seedling management to enhance the number of individuals in the population [64]. This management can involve ex situ seed germination followed by transplantation or the establishment of a regeneration system through tissue cultures. Recent studies have provided valuable information regarding the seed's dormancy in this species and some recommendations for breaking dormancy through warm stratification treatment [69]. Also, some of the regeneration methods using direct or indirect morphogenesis from different types of explants, such as stem, roots, hypocotyl, petiole, or leaves, were reported in *C. persicus* [70].

The higher genetic diversity in AP would suggest that the conservation measures had effects, but the small size of the population and its limited area (about 25 m^2) within the protected zone indicated that other factors can affect the genetic diversity of this population. Compared with the other two larger populations from Durankulak (about 2400 m^2) and the Sulina (about 3000 m^2) beach area, with predominantly vegetative multiplication, this small remaining population presented the preponderance of seed propagation, probably due to the favorable conditions offered by the Marine Sand Dunes Reserve. In fact, it is not directly exposed to environmental factors acting at the beach level, but also it is protected by the natural plant associations of which it is part of within this zone [2].

On Sulina beach, the main factors threatening the species are tourist activities that can affect the forming of dunes and the associated vegetation, as well as urbanization and infrastructure development that have led to population fragmentation. From this point of view, tourist education and involvement of the local people in the community to sustain conservation programs for these species would be necessary. Because the majority of samples from this population presented leaves with fungi infection spots, another limiting factor would be the biotic one. The parasitism with phytopathogens of the type *Septoria convolvuli*, *Diplodina convolvuli*, and *Didymosporium convolvuli-peaches*, but also with *Cuscuta* sp. plants [13] can be incriminated.

The clear genetic differentiation between populations that tends to increase in the absence of gene flow contributed to the identification of those populations as an evolutionary significant unit. Preserving these sites maximizes the potential for the future evolutionary success of species or population groups of species [71–73]. The frequent anthropization affected many characteristics of the biome, leaving small fragments of the population surrounded by a landscape that is highly modified and degraded. Further, the increased edge effect has led to changes in both abiotic and biotic factors, resulting from the sharp transition between adjacent ecosystems, which in turn affects population dynamics [74,75].

The small populational fragments often represent the last remnants of the biota and can be essential for the conservation of some species [76]. The use of evolutionarily significant units as a target for conservation suggests the need for inclusion in future studies of all the existing populations in the Sulina area, but also those from adjacent regions, such as Sf. Gheorghe, Sacalin, or Cardon from the Danube Delta, where *C. persicus* populations have still been reported. However, the genetic information alone will not provide a sufficient solution, demographic and ecological perspectives will complete the drawing of a conservation strategy.

5. Conclusions

The research has provided an updated and detailed map of the *C. persicus* distribution.

The genetic diversity of the Sulina, Agigea, and Durankulak remaining populations of *C. persicus* of the western Black Sea coastline reflected patterns that were comparable with those of non-threatened species within the genus, in spite of its limited distribution

and endangered status. Sulina and Agigea exhibited higher genetic diversity parameters relative to Durankulak, and the genetic differentiation between the three populations (PhiPT = 0.428) is significant due to habitat fragmentation and geographic isolation. Genetic structure analysis and clonal probability tests indicated a predominant clonal reproduction in SP and DP by expanded rhizomes with limited sexual reproduction due to low seed production and germination rates. This reproductive strategy for exceeding the conditions offered by sandy beach habitats contributed to the detected genetic variation and inter-population divergence.

Conservation measures are required, including in situ and ex-situ strategies, and population consolidation through assisted propagation and reintroduction. Habitat disturbance, tourism, urbanization, fungal infections, and parasitism also necessitate local community involvement and further studies to integrate genetic, ecological, and demographic data to develop a holistic conservation strategy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ecologies6010018/s1, Table S1: *Convolvulus persicus* distribution points.

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Abbreviations

The following abbreviations are used in this manuscript:

SP	The Sulina population in Romania
DP	The Durankulak population in Bulgaria
AP	The Agigea population in Romania

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