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Seed Phenotyping and Genetic Diversity Assessment of Cowpea (*V. unguiculata*) Germplasm Collection

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Abstract: Cowpea is a nutrient-rich staple legume and climate-resilient crop for vulnerable agroecosystems. However, the crop still remains underutilized, mainly due to its narrow genetic base, and the production is often ravaged by aphid infestation outbreaks. Thus, genetic diversity assessment and the detection of defense-related alleles are fundamental to germplasm management and utilization in breeding strategies to support food safety in climate change times. A germplasm collection of 87 cowpea landraces sourced from Greece was subjected to seed phenotyping, SSR genotyping and to screening for the presence of aphid-resistance-conferring alleles. Significant diversity in the species' local germplasm was revealed. The landraces were grouped in metapopulations based on their broader geographical origin. High amounts of variation and statistically significant differences were detected among the landraces regarding the seed morphological traits, the seed color and eye color according to MANOVA (Wilk's $\lambda = 0.2$, $p < 0.01$) and significant correlations were revealed among these features according to Pearson's test ($p < 0.05$). High levels of genetic polymorphism were detected for the metapopulations, ranging from 59% (VuPop3) to 82% (VuPop4). The AMOVA revealed that 93% of the molecular diversity was distributed among the landraces of each metapopulation. Further population structure analysis presumed the existence of two inferred populations, where in population A, 79% of the landraces have a cream/cream-brown seed coat, whereas in population B, 94% of the landraces are brown-ochre to black-seeded. Molecular screening for alleles conferring aphid resistance revealed the correspondence of 12 landraces to the resistant genotype of TVu-2876. The study highlights the importance of cowpea germplasm collection genetic diversity, as a source of important agronomic traits, to support breeding efforts and expand cowpea cultivation to foster food security and agriculture sustainability and diversification in climate change.

Keywords: cowpea; germplasm; seed; phenotyping; diversity; molecular markers; aphid resistance; breeding

1. Introduction

Ongoing human population growth places great pressure on the production of agronomic resources, threatening food security, that is exacerbated by quickly escalating climate change. Furthermore, the recent pandemic crisis had an indirect impact in intensifying food insecurity, affecting disproportionally susceptible groups of the population [1]. In order to encounter undernourishment and the risk of a potential food crisis in vulnerable ecosystems, such as the Mediterranean, viable staple food production schemes and agriculture diversification are essential. Pulses have a key role in agriculture sustainability, diversification and food security.

Grain legumes, commonly known as pulses, are protein-rich crops cultivated in rainfed and low-input farming systems, promoting sustainable farming and enhancing soil nutrient composition and structure via biological nitrogen fixation [2]. Among pulses, cowpea holds an exceptional position due to its adaptability to sandy soils and drought, which have made it an important crop in semiarid regions. Cowpea (*Vigna unguiculata* L. Walp.) is a diploid (2n = 22) herbaceous, warm-season, predominantly self-pollinated (approx. 95%) annual

crop of the Leguminosae family, domesticated in Western and Eastern Africa. Recent studies indicated that by 400 BCE, cowpea cultivation spread throughout the Mediterranean basin, sub-Saharan Africa and Southern Asia, and was transferred to the New World during colonization [3,4]. Its continuous cultivation in the Mediterranean region since antiquity is closely linked to the its socio-economic and cultural value for local communities [3,5], as depicted also by the broad phenotypic and genotypic diversity in the local germplasm [6].

Cowpea is cultivated in Southern Europe, subtropical Asia and America, while most of the world's grain production share comes from sub-Saharan Africa (>95%). Its grains consist of up to 33% protein, 63% carbohydrates, 4.6% lipids, minerals, vitamins and fiber [7,8] and it constitutes a cheap protein source of energy for food and fodder, hence, demonstrating enormous potential for mitigating malnutrition [9] and supporting a plant protein diet. However, it is considered an underutilized crop, being inferior in yield, compared to other legumes that are cultivated in intensive farming schemes. Globally, dry seed yield remains below the potential of the crop (8.9 M tons in 2020), with an average yield of 5912 hg/ha in 2020, as it is grown predominantly by smallholder farmers in non-intensive, resource-poor traditional systems [10,11]. On the other hand, farmers prefer higher-yielding crops that provide increased income, such as dry peas (20,364 hg/ha), green peas (78,477 hg/ha), chickpeas (10,163 hg/ha), lentils (13,049 hg/ha) and soybeans (278,42 hg/ha) (FAOSTAT, 2020) [12]. Compared to other legumes, cowpea thrives in a range of soil types, pH and temperatures, while it is well adapted to drought-related conditions; therefore, it represents a promising climate-smart crop [13]. Thus, among other orphan crops, cowpea has lately gained researchers' interest and efforts for the promotion of its cultivation in many countries. Yet, extensive breeding remains crucial to improve the crop's yield and the food-value chain in a changing environment. Evaluation of the genetic diversity in the cowpea germplasm is a precondition for constructive breeding of the species. Cowpea's molecular genetic diversity has already been examined with various marker systems, indicatively RAPDs, AFLPs, ISSRs, SNPs [14] and SSRs [15–18]. Among them, microsatellites comprise a robust genotyping method, extensively used in population genetic studies in various plant species. SSRs are multi-allelic, co-dominant, highly reproducible markers, with coverage extending along the entire genome. This marker system is equally proposed for genotype identification, pedigree analysis, genetic mapping of qualitative and quantitative traits, as well as for marker-assisted selection breeding strategies [19–21]. Taking into account the scarcity of genomic information on Greek cowpea accessions, SSR molecular markers constitute a functional approach for a thorough evaluation of untapped diversity.

Climate change and global warming adversely affect legume cropping systems, especially in drought-prone regions in the temperate zone, including the Mediterranean basin. Increased temperatures and altered precipitation patterns have significant impacts on cowpea production and on insect pests and pathogens. Specifically, changes in climate can result in an increased number of aphid generations, increased survival during overwintering, expansion of their geographic distribution, emergence of new biotypes, increased incidence of aphid-transmitted plant diseases and reduced effectiveness of biological control, especially natural enemies [22]. Globally, up to 20 viruses have been reported to infect cowpea. Many of them are seed borne, such as *cowpea mosaic virus* (CMV), and occur in several host species. Insect-borne viral transmission from diseased to healthy plants within fields and across neighboring fields may result in severe epidemics and crop damage. Among them, *cowpea aphid-borne mosaic virus* (CABMV) is responsible for crop losses of up to 87%, depending on the cultivar and the developmental stage of the plant [23]. Thus far, aphid population management relies mostly on prophylactic application of insecticides to properly secure the cowpea crop production. However, global warming drivers accounting for increased temperatures and a decrease in relative humidity may cause synthetic insecticides and management approaches to be less effective. Further, the use of synthetic pesticides further exacerbates the carbon and energy footprint and the environmental impact, raising concerns over field-evolved resistance of aphid populations to chemical

formulations [24,25]. Therefore, breeding for aphid resistance is the most effective approach to support cowpea production in a climate-changing environment.

In this respect, evaluation of species genetic diversity in the local germplasm could further the discovery of valuable genes/alleles that contribute to yield and crop tolerance. Landraces constitute a major part of the local germplasm, encompassing substantial diversity levels, conserved both naturally and by farmer's selection [26,27]. In Greece, cowpea comprises a valuable pulse crop of marginal lands and an important genetic pool of local landraces still in use for plant protein production and forage [28]. So far, molecular genetic characterization of the European cowpea germplasm is scarce and limited to Spanish and Italian accessions [29–31]. On the other hand, the Balkan peninsula located at the crossroads of three continents, Africa, Asia and Europe, is considered a diversity hotspot for several species, including cowpea; however, reports on cowpeas' local germplasm molecular diversity are scarce. To address this knowledge gap, the present study aimed to explore the genetic diversity of 87 local cowpea accessions, through the assessment of important seed morphological traits and imaging as well as genotyping with SSR molecular markers. Additionally, the presence of aphid-resistance-conferring alleles was also investigated using linked molecular markers for further breeding strategies.

2. Materials and Methods

2.1. Plant Material

A germplasm collection of 87 landraces provided by the Institute of Plant Breeding and Genetic Resources of the Hellenic Agricultural Organization-DEMETER was collected from local farmers' fields in Greece (Figure 1), where the seed progeny was recycled for every consecutive growing season. Details on plant material are demonstrated in Supplementary Table S1, according to IPGRB [32].
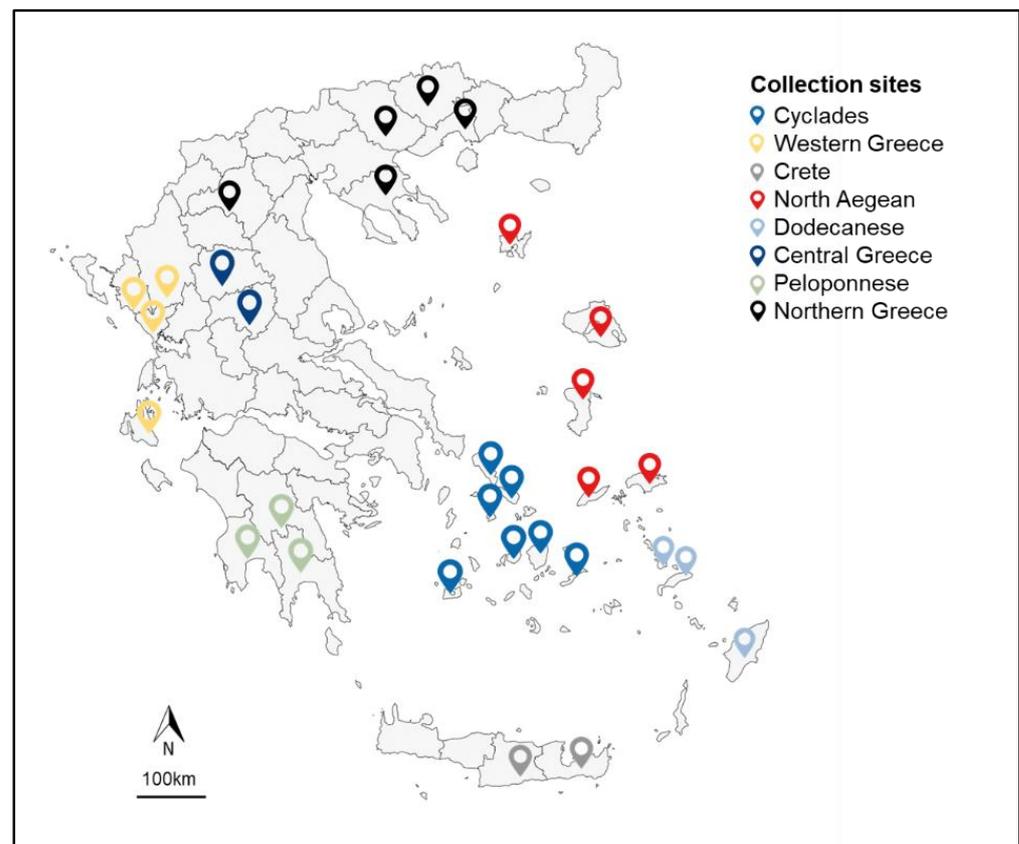

Figure 1. Map of the collection sites of 87 *V. unguiculata* landraces, grouped in 8 metapopulations as to geographic origin.

2.2. Genetic Diversity Assessment

2.2.1. Seed Morphological Diversity Analysis

We estimated the Hundred-Seed Weight (HSW) by weighing 100 seeds in three independent replicates. The seed morphological diversity was assessed by subjecting 10 seeds of every sample to visual examination, according to IBPGR 1981 standard criteria, applying image analysis, as previously described [33]. Multivariate Analysis of Variance (MANOVA) was employed to detect statistically significant differences among the measured variables, according to Wilks' Lambda test. Multiple-Factor Analysis (MFA) was used to cluster the 87 accessions in groups according to the Gower's distance. The UPGMA dendrogram distinguished the accessions in different clusters. The aforementioned analyses were performed with R software version 4.2 [34].

2.2.2. Molecular Genetic Diversity Analysis

For every accession, the molecular genetic diversity was assessed on the bulked samples of 10 individuals. Genomic DNA was extracted from seeds, applying the QIAGEN DNeasy Plant Pro Kit (Qiagen, Germany). Qbit 4 Fluorometer (Thermo Fisher Scientific, USA) was used in order to estimate the yield of the extracted DNA for every sample, which was further normalized to 10 ng/μL for downstream applications. For the examination of the genetic diversity, we initially used 20 SSR molecular markers that were previously found to be polymorphic in cowpea population genetic studies [20,35]. In this germplasm collection, four of them (VM9, VM35, VM74, VM78) were not capable of amplifying all the accessions, while two (VM37, VM71) of them were monomorphic. Thus, those markers were excluded from the analysis (online Supplementary Table S2). We also screened the accessions with molecular markers KAD-61 and CP-171-172, in order to investigate the presence of alleles linked to aphid resistance [36]. PCR amplification reactions were carried out on a Veriti™ 96-Well Thermal Cycler, using KAPA Taq ReadyMix PCR Kit, in a total reaction volume of 15 μL, containing 1X KAPA Taq ReadyMix (1.5 mM $MgCl_2$), 0.2 μM of each primer, PCR-grade H_2O and 10 ng genomic DNA template.

The PCR amplification program constitutes an initial denaturation step at 94 °C/4 min, followed by 35 cycles of denaturation at 94 °C/30 s, primer annealing at the optimal T_a for each marker, extension at 72 °C/40 s and final extension at 72 °C/4 min. The amplified products of KAD-61 and CP-171-172 (5 μL of each PCR) were electrophoresed onto 2% agarose gel in 1x TAE buffer, stained with ethidium bromide and visualized by illumination with ultraviolet light. The amplified products of 14 SSR markers were further subjected to fragment analysis (CeMIA SA, Larissa, Greece) and data were analyzed using OSIRIS software [37].

For every sample, the SSR amplicons were classified as present (1) or absent (0) and were further used for the construction of a binary matrix. Landraces are cultivated germplasms, continually evolving in agricultural environments within a defined eco-geographical area and under the influence of local human culture [38]. Based on their geographical origin, the accessions were grouped into 8 metapopulations, in order to assess the genetic diversity in the cowpea collection [39]: Cyclades (VuPop1), Western Greece (VuPop2), Crete (VuPop3), North Aegean (VuPop4), Dodecanese (VuPop5), Central Greece (VuPop6), Peloponnese (VuPop7) and Northern Greece (VuPop8); they were subjected to estimation of their genetic diversity parameters, such as the number of total, effective and private alleles, the Shannon's Information (I) and the diversity (h) indices. Analysis of Molecular Variance (AMOVA) was performed in order to explore the proportion of polymorphism distributed among and within the cowpea metapopulations, using GenAlEx 6.5 [40].

Genetic distances between the 8 metapopulations were calculated according to Nei's genetic distance [41] and a dissimilarity matrix was generated, which was subjected to tree construction, under Unweighted Pair Group Method Analysis (UPGMA), using R software version 4.2 [34]. The Polymorphic Information Content of the SSR markers was calculated according to Smith et al. (1997) [42].

STRUCTURE v. 2.3.4 software was used to establish the metapopulation structure formed by 87 cowpea landraces, under the Bayesian clustering method [43]. The lengths of the burn-in and Markov Chain Monte Carlo (MCMC) were both set at 10,000 iterations [44]. Thirty runs for each K-value were performed with K ranging from 1 to 10, in order to obtain an accurate estimation of the number of populations. STRUCTURE Harvester was used to calculate Delta-K and the appropriate K value was chosen [45].

3. Results

3.1. Seed Morphological Diversity

The seed morphological measurements were statistically analyzed and summary statistics are presented in Table 1. Details on the seed coat color, eye color and testa texture are provided in Supplementary Table S1. According to the MANOVA, statistically significant differences were detected among the 87 accessions (Wilk's lambda = 0.2, $p < 0.01$). More specifically, a wide variation was observed within VuPop1, VuPop2, VuPop3, VuPop4 and VuPop8 regarding the HSW and the seed area, contrary to VuPop5, VuPop6 and VuPop7, where most of the accessions approximate the mean value (Figure 2a).

Table 1. Summary statistics of the seed morphological characteristics.

Variable	Observations	Minimum	Maximum	Mean	Standard Deviation
HSW (g)	87	6.8	31	19.2	5.3
Area (mm^2)	"	27.9	80.5	54.3	11.2
Perimeter (mm)	"	20.8	40.8	31.3	3.8
Circularity *	"	0	0.1	0.1	0
Height (mm)	"	3.3	5.6	4.6	0.5
Width (mm)	"	4.6	8	6.4	0.7
Gray Value * (Min)	"	3	15	9.2	1.6
Gray Value * (Max)	"	122	254	158.3	23
Gray Value * (Mean)	"	15.6	109.8	62.9	28.2
Gray Value * (Median)	"	13	119	66.7	33.7
Integrated Density *	"	12,724,700	916,154,682	65,281,757	97,469,132

* Circularity is morphometric and Gray value and integrated density are densitometric parameters that have no units.

Partitioning of the variance and grouping of the assumed populations with respect to their quantitative (hundred-seed weight, seed area, perimeter, circularity, width, Gray value, integrated density) and qualitative (seed color, seed shape, eye color, testa texture) seed morphological attributes were estimated by Factorial Mixed Data Analysis (FMDA). The quantitative traits include the hundred-seed weight (HSW), seed area (A), perimeter (P), circularity (C), height (H), width (W), Gray value (Gv) and integrated density (ID), whereas the qualitative traits include the seed color (SC), shape (SS), eye color (EC) and the testa texture (TT). All populations were grouped in a single cluster in the 3D MFA plot, where the first three dimensions represent 73.5% of the observed variability (Figure 2b).

A Pearson's correlation test revealed a significantly very strong positive correlation between HSW and seed area (0.83), between area and perimeter (0.84) and between Gray value (mean and median) and cream seed color (0.85), suggesting that measurement of a single feature is sufficient to represent other highly correlated characteristics. Medium positive correlations were detected between seed height and area (0.6), perimeter (0.59) and weight (0.47), between seed width and HSW (0.5), area (0.63) and perimeter (0.56), as well as between Gray value and B eye color (0.53) and rough testa texture (0.6). Note-worthily, medium positive correlations were demonstrated between cream seed color and HSW (0.58), area (0.5), B eye color (0.64) and rough testa texture (0.64). Contrarily, moderate negative correlations were detected among brown seed color and HSW (−0.54), area (−0.49) and width (−0.4). Those data indicate the preference of local cowpea germplasm cultivation and breeding in Greece, which is directed towards cream seeded landraces (Figure 3).
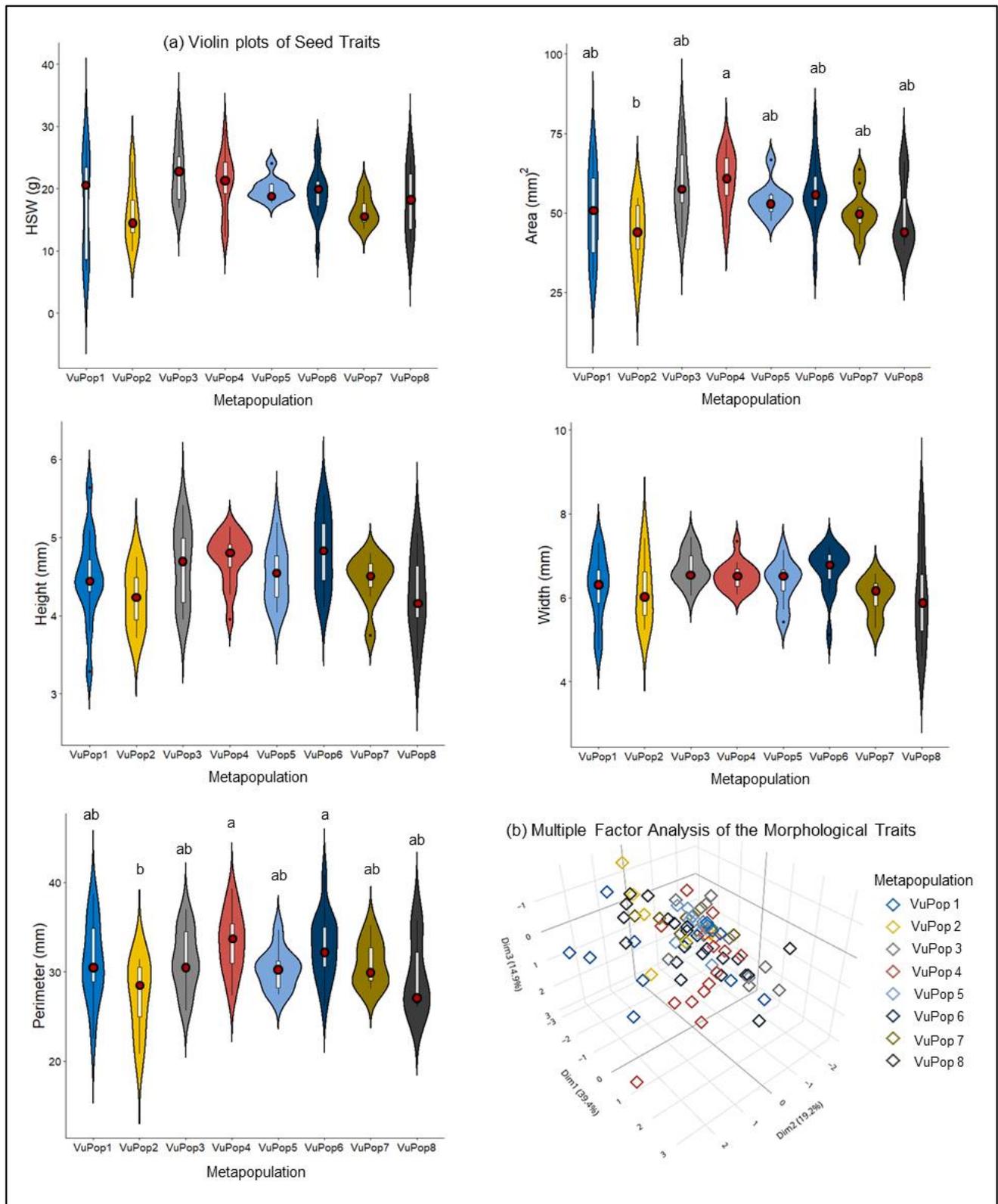
Figure 2. (**a**) Violin plots of the HSW and seed geometrical features for the 8 cowpea metapopulations. Metapopulations that share no common letter are significantly different according to Tukey's HSD ($p < 0.05$); (**b**) Multiple Factor Analysis plot of the cowpea metapopulations' seed features, where the first three axes explain 73.5% of the observed variability.
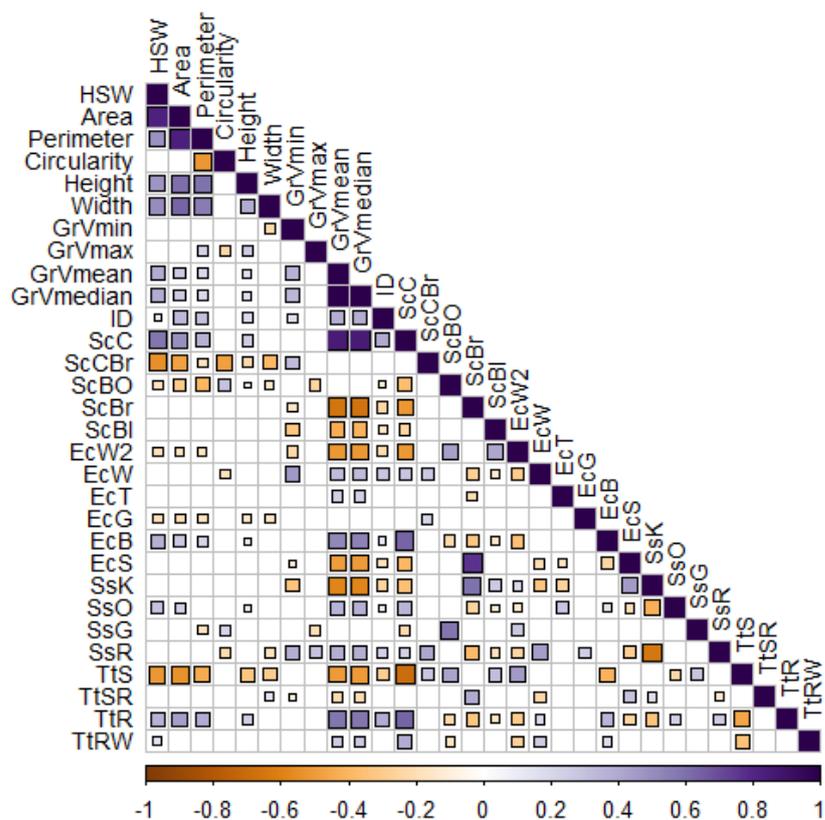
Figure 3. Pearson's corrplot of the seed morphological traits. Non-significant correlations were omitted from the plot (*p* > 0.05). Seed trait abbreviations are explained in the Abbreviations.

3.2. Molecular Genetic Analysis

Genotyping of the eight cowpea metapopulations with 14 polymorphic microsatellites generated, in total, 78 different alleles. The number of alleles per SSR primer set ranged from 2 (VM22, VM36, VM39, SSR1, SSR6421, SSR6243) to 16 (SSR6239), with an average of 5.5. The polymorphic information content (PIC), a measure indicative of the allelic diversity for a specific locus, varied from 0.1 (SSR6819) to 0.8 (SSR6807), with an average of 0.35 (Supplementary Table S4). The microsatellite loci that exhibited the highest PIC values were SSR6807, SSR6239 and SSR6866, indicating their usefulness in discriminating different genotypes. A summary of the molecular diversity components for the eight cowpea metapopulations is provided in Table 2.

Table 2. Summary of the genetic diversity statistics over 14 SSR loci.

Metapopulation	No. of Landraces	P%	Na	Ne	Np	I	h	uh
VuPop1	13	77	1.58 ± 0.09	1.32 ± 0.04	1	0.33 ± 0.03	0.21 ± 0.02	0.23 ± 0.02
VuPop2	6	63	1.28 ± 0.11	1.39 ± 0.04	-	0.35 ± 0.03	0.23 ± 0.02	0.28 ± 0.03
VuPop3	5	59	1.22 ± 0.11	1.36 ± 0.04	-	0.33 ± 0.03	0.22 ± 0.02	0.27 ± 0.03
VuPop4	23	82	1.64 ± 0.09	1.33 ± 0.03	-	0.35 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
VuPop5	9	73	1.47 ± 0.1	1.38 ± 0.04	-	0.36 ± 0.03	0.24 ± 0.02	0.26 ± 0.02
VuPop6	15	69	1.42 ± 0.1	1.32 ± 0.04	2	0.32 ± 0.03	0.2 ± 0.02	0.22 ± 0.02
VuPop7	9	63	1.27 ± 0.11	1.37 ± 0.04	1	0.33 ± 0.03	0.22 ± 0.02	0.25 ± 0.02
VuPop8	7	69	1.14 ± 0.1	1.36 ± 0.04	3	0.35 ± 0.03	0.23 ± 0.02	0.26 ± 0.02
Mean	11	69 ± 3	1.4 ± 0.1	1.4 ± 0.04	1.75	0.3 ± 0.03	0.2 ± 0.02	0.3 ± 0.02

P% = Percentage of polymorphic loci; Na = Number of different alleles; Ne = Number of effective alleles, Np = Number of private alleles, I = Shannon's Information Index, h = Diversity, uh = unbiased Diversity.

The Analysis of Molecular Variance (AMOVA) revealed that 93% of the observed variability was assigned within each cowpea metapopulation (Supplementary Table S5)

and the UPGMA arranged the metapopulations into two distinct clusters (Figure 4a,b). Cluster I is comprised of the landraces that originate in Dodecanese islands, whereas cluster II is formed by the rest of the metapopulations.
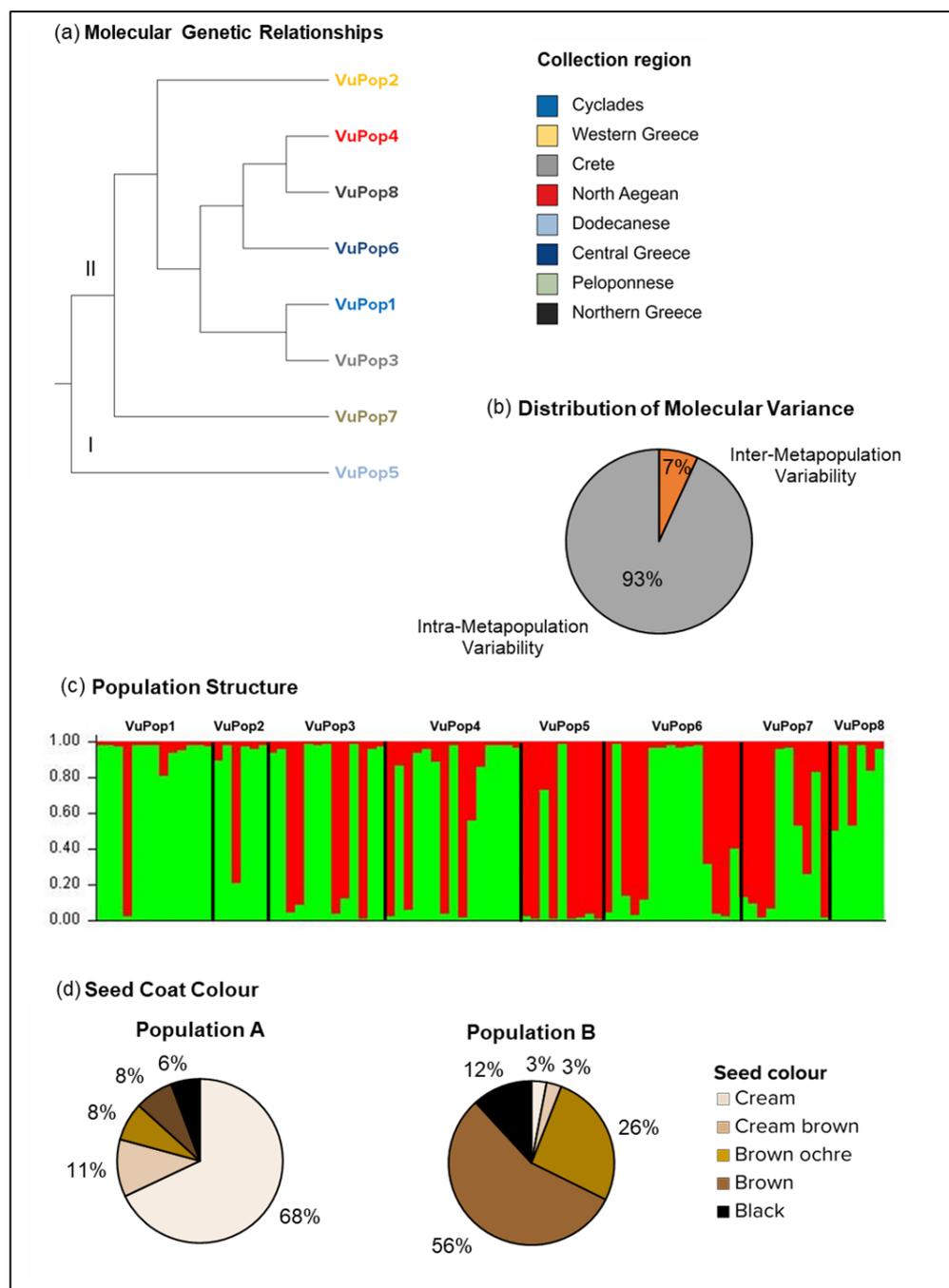

Figure 4. (**a**) SSR-generated dendrogram of the 8 cowpea metapopulations, distributing the landraces in two main clusters; (**b**) distribution of variance according to AMOVA; (**c**) population structure graph of the estimated membership of the 87 cowpea landraces, based on 14 SSR markers (K = 2), indicating the emergence of two subpopulations; and (**d**) seed coat color distribution among the two populations.

According to the ΔK population structure analysis, the 87 accessions were grouped into two assumed populations (A, B) (Figure 4c). Population A comprises the majority of the accessions (61%), while population B gathered 39% of the accessions (likelihood threshold 0.55). Both populations were mixed in composition, in that they included accessions from

all regional territories. The majority of the accessions from Cyclades (93.2%), Crete (60%), Western Greece (83,3%), North Aegean (69.5%) and Northern Greece (71.4%) are arranged in population A, whereas the majority of the accessions from the Dodecanese (77.7%) and Peloponnese (66.6%) are grouped in population B. The accessions from Central Greece are distributed equally in both populations. Regarding the seed coat color, population A comprises mostly cream/cream brown landraces (79%), while a smaller part concerns brown/brown ochre (16%) and black (6%) seeded landraces. Contrarily, population B includes almost exclusively dark seeded landraces (brown ochre to brown seed coat at 82%, black seed coat at 12%), and only 6% of the landraces are a cream-brown color (Figure 4d). We estimated the F_{st} values for both populations (F_{stA} = 0.23, F_{stB} = 0.16), deducing moderate differentiation levels of one another (F_{stmean} = 0.2), and calculated the average distances among the individuals in each population (H_{eA} = 0.23, H_{eB} = 0.26).

Molecular marker KAD-61 was successfully detected in all accessions; however, the amplicon size of 414 bp in all accessions was larger than suggested in the literature (up to 200 bp). Sequence analysis of the amplicon from all accessions revealed 95% coverage and 99.5% identity to the corresponding nucleotide sequence coding for the LRR receptor-like serine/threonine-protein kinase FLS2, which signals for plant innate immunity responses. However, due to the observed ambiguity in the amplicon size, the use of this marker requires further investigation to confirm, in vivo, the assessed genetic resistance in the given germplasm collection.

Further molecular screening of the germplasm for the presence of resistance-conferring alleles to *Aphis craccivora* (Koch) with the microsatellite CP-171-172 revealed that 12 accessions were associated with the resistant genotype (VuGR3, VuGR33, VuGR35, VuGR41, VuG47, VuGR49, VuGR56, VuGR76, VuGR77, VuGR78, VuGR85, VuGR86). However, this marker was detected only in 35 out of the 87 accessions.

4. Discussion

Orphan crops such as cowpea are vital in reassuring food security in vulnerable regions worldwide, underlining their continuous cultivation and untapped diversity. Despite their importance to agriculture, only recently has the species gained breeders' attention, in order to increase plant-protein production to meet food crisis challenges due to climate change [46]. Cultivated cowpea's germplasm genetic base is narrow because of its highly self-pollinating nature and to precedent breeding programs, as improved elite lines were constantly used as parents in crosses to generate segregating populations, exhibiting limited gene flow. On the other hand, the genetic diversity in the local germplasm remains untapped, constraining the effective incorporation of locally adapted landraces and alleles in breeding strategies.

Landraces are defined as "geographically or ecologically distinctive populations, which have evolved under cultivation and become locally adapted to various environments" [47]. Contrary to modern varieties, landraces have not been exposed to systematic plant-breeding procedures and are diverse in their genetic composition, both within and between populations. They provide a broad representation of the natural variation that is present in a species and constitute the primary gene pool available to breeders for improvements in crops. Hence, landraces constitute a dynamic component in the cultivated species genetic diversity. Screening of eight Greek metapopulations, composed of 87 landraces with 14 microsatellites, revealed a range of 2 to 16 alleles per locus. Among others, some of those molecular markers were previously employed for genotyping a plethora of cowpea cultivars, breeding lines, landraces and wild relatives, and reported similar [21,48–50] and more limited allele ranges [16,19,51]. The number of alleles per locus is highly associated with the number of accessions subjected to SSR genotyping, the selected markers and the type of the germplasm, and is indicative of the polymorphic content of the germplasm under evaluation. Allele ranges similar to our results were previously detected in up to 7-fold more multitudinous study populations of African origin [49]. It is, therefore, deducible that the accessions that were evaluated in the present study hold a significant

amount of untapped diversity, which should be taken into consideration for incorporation in breeding strategies seeking the expansion of the species germplasm genetic base.

PIC values and gene diversity (also called expected heterozygosity) are both estimates of the genetic polymorphism present in populations and are used for the assessment of the quality of a marker [52]. The PIC values represent the informativeness of molecular markers, which is categorized as low (PIC < 0.25), moderate (0.25 < PIC < 0.5) and high (PIC > 0.5), respectively. In this study, we used 14 SSR markers to evaluate the genetic diversity of 87 cowpea accessions. Most SSRs exhibited moderate to high PIC values, ranging from 0.1 to 0.8, with an average of 0.35 (Supplementary Table S4). The low PIC values observed in some of the SSRs are explained by the frequencies of the alleles' presence in those loci. Disproportionate frequency distribution of a locus' alleles results in the generation of low PIC values (e.g., VM36, SSR6819, SSR6215, SSR6243) (Supplementary Table S3). However, most of the microsatellites used in our study detected a great number of alleles in the different metapopulations, indicating the existence of a highly polymorphic germplasm, and also revealing highly variable loci (SSR6807, SSR6866 and SSR6239 detected 11, 12 and 16 alleles, respectively). Additionally, the comparatively low levels of gene diversity observed in our study (0.2) could be explained by the predominant self-pollinating nature of cowpea. The cowpea's genetic base is considered narrow and the inherent self-pollination mechanism further contributes to its maintenance. However, landraces from the Mediterranean basin have demonstrated significant diversity [6,19,28,53,54]. Noteworthily, the metapopulations collected in the Aegean islands (Cyclades, Dodecanese and North Aegean) hold the highest amounts of polymorphism among the germplasm under evaluation (73–82%). This is most likely attributed to the geographical position of the Aegean islands. Closely neighboring both Europe and Asia, these insular complexes have served as intermediate docking stations for maritime traders for millennia [55]. As previously proposed, cowpea was probably transferred through Asia minor, constantly enriching the islands' gene pool with new alleles, forming a crucible cowpea germplasm [3]. The AMOVA proposed that the detected variability is distributed within each metapopulation by 93%. This probably highlights the allocation of several accessions with preferable traits in various parts of Greece, through trading networks, and hybridization with local germplasm, subsequently giving formation to variable cowpea germplasms throughout the different regions [56]. In support of this claim, Xiong et al. (2016), having performed a robust SNP genotyping analysis on a world cowpea collection from 56 countries, reported that the majority of the diversity exists within given geographic regions, rather than among the different regions [57]. Moreover, agriculture in the Aegean islands is confined in small terrace fields that are scattered amid steep slopes [58]. Considering the prevalent self-pollinating nature of cowpea, the emergence of isolated genetic pools is likely.

To further investigate the heterogeneity among different metapopulations, analysis of population structure presumed the emergence of two groups, where most of the accessions from Cyclades, Crete, Western Greece, North Aegean and Northern Greece are arranged in one homogeneous population and the majority of the accessions from the Dodecanese and Peloponnese in another, making evident the germplasm expansion throughout Greece, the potential homogeneity of landraces of distant territories and the heterogeneity in germplasms cultivated in neighboring regions (Cyclades–Dodecanese). Interestingly, the accessions from the Dodecanese islands seem more distinct from the rest of the accessions (Figure 4a). Central Greece is a nodal champaign in the middle of Greece concentrated with intensive agricultural production, characterized by a variety of microclimatic conditions between the northern–southern and the eastern–western parts of its territory. Based on the structure analysis results, accessions that originate from Central Greece were equally divided in both the aforementioned populations, suggesting a diverse cowpea germplasm hotspot.

Legumes, as a milestone staple in the Mediterranean diet, are strongly connected to the socio-economic profile of a region [59]. Different cowpea productive regions cultivate certain varieties more than others, in terms of consumer-desired seed traits, such as the

coat color and pattern, features determinant of quality and use. Usually, light-colored seeds are destined for flour, while darker seeds are used as whole beans. Every year, Greek islands are visited by a vast number of tourists, of varicolored gastronomical backgrounds, hence, determining the culinary profile of these regions and, consequently, defining the regional agriculture [60]. In the Dodecanese islands, Crete and Peloponnese, most of the accessions are dark-seeded (Figure 5). Dark-seeded varieties were found to be more resilient under water deficit and extreme heat conditions, with little chlorophyl reduction and high levels of proline, an osmolyte compound, contributing to the cells' osmotic adjustment and stabilization of subcellular compartments [61]. Therefore, potential selection of adaptive accessions with dark seed coats would justify the cultivation of mostly brown-seeded landraces, as in these areas, irrigated lands are limited and are further decreased as the regional climate is characterized by increased temperatures, which will further rise due to climate change [62].
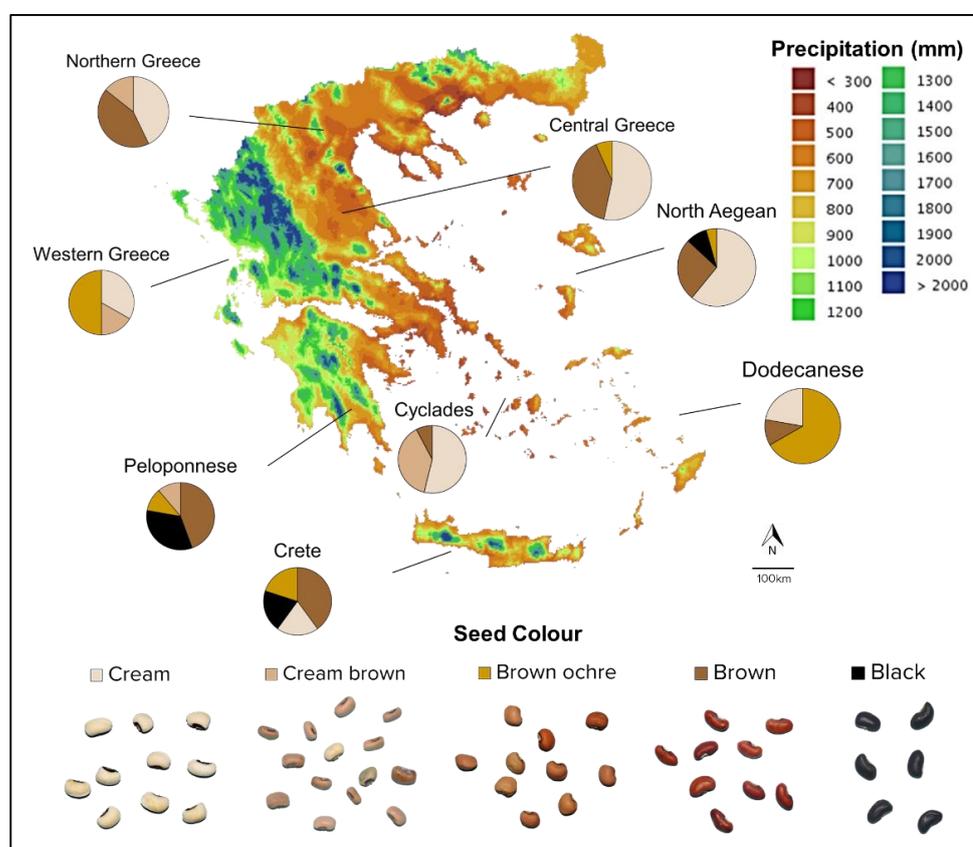

Figure 5. Map of Greece showing average annual precipitation (mm) and the composition of the different cowpea populations regarding their seed color (http://climatlas.hnms.gr/, accessed on 30 September 2022).

On the other hand, every year from mid-May to mid-September, the North Aegean and Cyclades receive strong Etesian northern winds, also known as "meltemia", which contribute to the establishment of milder summer temperatures, benefiting completion of seed filling and protecting from abrupt temperature increases, compared to the mainland and the Dodecanese [63]. Taking into account that cowpea is a warm season crop and harvesting occurs in early summer, this phenomenon buttresses the cultivation of white-seeded cowpea accessions in those regions.

Dark seed color is also strongly associated with overaccumulation of several secondary metabolites, such as flavanols (catechin, catechin glucoside and epicatechin) and anthocyanins (delphinidin), compounds of pronounced nutraceutical, pharmaceutical and medicinal interest [64–66]. Apart from that, the seeds' composition on functional proteins

justifies the need to transform cowpea from cash crop to industrial crop [67]. Protein content in cowpea is reported to reach up to 40% of the seed dry weight [68] and is found to be most abundant in cream-colored varieties [69]. The seed color and pattern along with seed size are determinants of the cowpea's market price and have been considered as major breeding targets [70]. Cowpea is characterized by high intra-plant variability regarding the seed weight and protein content, which is attributed to indeterminate flowering and pod position. Development of high-yielding, uniformly flowering cultivars, with constant numbers of seeds per pod would increase the seed size and protein content homogeneity and would promote the industrialization of the crop [71]. Many Greek accessions examined in this study are considered highly productive, as they present HSW values up to 31 g (VuGR23), higher than in previously described commercial and local germplasms from Africa and India, indicating their potential for further breeding into elite cultivars [72–76]. Similar results were reported in Southern European landraces from Italy and the Iberian peninsula that further support the claim of germplasm migration inside the Mediterranean basin through trade from Asia minor [29]. The landraces collected in the Northern Greek territories demonstrate slightly higher values regarding the overall seed size and weight. Both features are considered significantly heritable and this discrimination may be attributed to the more favorable average climatic conditions in northern regions, where the crop can receive adequate precipitation and irrigation, promoting sufficient seed filling prior harvest, in contrast to the southern territories, which are more drought-prone. Moreover, Central and Northern Greece are run by several streams, rivers and aquifers, and intensive irrigated agriculture allows for safeguarding production that would otherwise be lost in non-irrigated cultivation schemes, under drought waves.

Cowpea, being mostly cultivated in semiarid areas, is well adapted to abiotic and biotic stresses. However, infestation with *Aphis craccivora* is challenging for the crop and may result in major yield losses. Cowpea aphid is phytotoxic and damages the crop, regardless of pest densities. Along with the direct damage caused by the sap imbibition, *A. craccivora* is responsible for the transmission of more than 14 viruses to cowpea and other pulses, rendering the need for successful aphid population management in cowpea cultivation systems imperative [77]. Despite the use of insecticides, cultural and integrated pest management for controlling aphid-induced damage to crops, methods have not been very effective, as smallholder farmers growing cowpea in marginal areas cannot afford their cost, in addition questioning their environmental footprint. Thus, development of cultivars with enhanced endogenous resistance is the most efficient and sustainable alternative to chemical pest control, sustaining crop production for longer periods. Conducting resistance bioassays in large breeding populations is a time-consuming and wearisome procedure. Development of molecular markers perfectly linked to the resistant phenotype would promote rapid selection of the preferred genotypes. Thus far, efforts have been made in detecting resistant genotypes and elucidating the molecular mechanism underlying aphid resistance [77]. Antibiosis is proposed as the principal defense response of the African-resistant germplasm, in blocking sap ingestion by cowpea aphids, secondarily supported by antixenosis mechanisms [78–80]. Investigation of the genetic mechanism regulating aphid resistance in TVu-2876 indicated the action of a single dominant gene [36]. Molecular screening of the Greek germplasm revealed the presence of the resistant TVu-2876 genotype in 12 landraces, highlighting a promising source of aphid genetic resistance that needs to be further examined. The majority of them originated in Peloponnese, Central Greece and North Aegean regions. Out of them, seven are dark-seeded, while five of them are cream black-eyed seeds. These landraces could be incorporated in gene pyramiding assays in cowpea breeding strategies. Dark seed coats are rich in secondary metabolites that contribute to defense responses against biotic stresses [81]. Varieties with a high abundance of seed secondary metabolites are predisposed to biosynthesize high amounts of such compounds when needed. Previously, positive correlation has been detected between the plant flavonoid content and abundance and the resistance to aphids. More specifically, kaempferol, quercetin and isorhamnetin cowpea chemotypes

were associated with resistance responses, with quercetin and isorhamnetin demonstrating a direct involvement in the resistance mechanism [82,83]. Sequencing and alignment of the KAD-61 amplicon to the *V. unguiculata* genome detected absolute homology to the leucine-rich repeat (LRR) receptor-like serine/threonine-protein kinase (RLK) FLS2 gene of *V. unguiculata* ssp. *sesquipedialis* cultivar Xiabao 2 genome [84]. The transmembrane receptor kinase FLAGELLIN SENSITIVE2 (FLS2) is essential for flagellin perception and is integral in plant defense responses against pathogens [85]. With respect to the germplasm under evaluation, no nucleotide sequence variations were detected among the cowpea landraces in the aforementioned locus. Moreover, the amplicon size of KAD-61 was the same among all landraces, divergent to that proposed in the literature. Hence, KAD-61 is considered unreliable for discriminating among resistance and susceptibility conferring alleles in the given accessions. More profound genomic analyses are needed for the detection of resistant alleles in the present germplasm and the development of linked molecular markers that would accelerate selection for aphid resistance. Further research is also suggested regarding the in vivo demonstration of aphid resistance in VuGR3, VuGR33, VuGR35, VuGR41, VuG47, VuGR49, VuGR56, VuGR76, VuGR77, VuGR78, VuGR85 and VuGR86, as depicted, presaged by marker CP-171-172 to harbor the resistant TVu-2876 genotype. Contemporary biotechnological approaches providing insect and pathogen resistance through molecular network engineering have already been successfully employed in cowpea and would assist cowpea breeding for increased plasticity, subsequently promoting cowpea productivity [86].

5. Conclusions

Cowpea is considered a staple, resilient and underutilized legume, cultivated in low-input agricultural systems and a promising industrial protein-rich crop. Cowpea experienced two bottleneck effects during domestication and the breeders' tendency of using improved elite lines as parental material for the generation of segregating populations in breeding programs resulted in limiting the crop's genetic base. Therefore, scrutinization of unexploited local germplasms is substantial for species gene pool enrichment. Significant polymorphism was detected among 87 Greek landraces, regarding seed morphological characteristics, seed color and molecular genetic diversity. Many of them are considered high-producing, when compared to other cultivated accessions, and 12 of them possess the *A. craccivora* resistance-conferring CP-171-172 allele, which would be highly favorable for incorporation in cowpea breeding programs, towards pyramiding aphid resistance to elite germplasms. Furthermore, future genome-wide association studies are proposed for the germplasm evaluated in this study, for the detection of desirable alleles regarding biotic and abiotic stress resilience and their potential introgression to elite germplasms, as climate change imposes the development of adaptive cultivars for safeguarding plant–protein production in vulnerable Mediterranean agro-ecosystems.

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Abbreviations

ScC	Seed color Cream
ScCBr	Seed color Cream-Brown
ScBO	Seed color Brown-Ochre
ScBr	Seed color Brown
ScBl	Seed color Black
EcW2	Eye color W^2
EcW	Eye color W
EcT	Eye color T
EcG	Eye color G
EcB	Eye color B
EcS	Eye color S
SsK	Seed shape Kidney
SsO	Seed shape Ovoid
SsG	Seed shape Globose
SsR	Seed shape Rhomboid
TtS	Testa texture Smooth
TtSR	Testa texture Smooth to Rough
TtR	Testa texture Rough
TtRW	Testa texture Rough to Wrinkled

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