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Article

Intraspecific Genetic Variability of *Brassica cretica* Lam. (Brassicaceae) Using SSR Markers

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Abstract: Unraveling the evolutionary history of *Brassica* L. crops and their wild relatives remains a key challenge in plant evolutionary biology. *Brassica cretica* is considered the closest living relative of the cultivated *B. oleracea*. It is mainly distributed in the Aegean phenotypic variability, obscuring its infraspecific classification. In this study, we analyzed five Greek populations of *B. cretica* and one *B. oleracea* botanical variety using SSR markers to assess genetic diversity and differentiation. High genetic diversity was detected within natural populations, with a mean of 21.9 alleles per locus and an expected heterozygosity of 0.647. Significant genetic differentiation ($F_{st} = 0.812$) revealed the presence of four distinct gene pools, partly supporting the current infraspecific classification of *B. cretica*. The cultivated plants cluster closely with *B. cretica* subsp. *cretica*, supporting the hypothesis of an Eastern Mediterranean origin. Our findings suggest that *B. cretica* subsp. *cretica* may have been introduced to suitable habitats or that cultivated plants may have reverted to a feral state in the Peloponnese, given the genetic similarity between populations from Crete and northern Peloponnese. The identified genetic diversity underscores the importance of *B. cretica* as a genetic resource for breeding programs and highlights the need for conservation, particularly for populations exhibited unique genetic traits.

Keywords: *Brassica cretica*; crop wild relative (CWR); natural populations; genetic variability; SSR markers

1. Introduction

Resolving the evolution of the *Brassica* L. (Brassicaceae) crops and their wild relatives probably represents one of the most fascinating tasks in plant evolutionary biology [1–4]. Darwin [5] already highlighted the parallels between his theory of natural selection and the cultivation practices that shaped the diverse forms of *Brassica oleracea* L. crops. The domesticated forms of *B. oleracea* include six major botanical varieties [broccoli (var. *italica*), Brussels sprouts (var. *gemmifera*), cabbage (var. *capitata*), cauliflower (var. *botrytis*), kale (var. *acephala*), and kohlrabi (var. *gongylodes*)], and 12 additional cultivated crop types [6], representing a unique example of diversification through domestication in the plant kingdom. Global production of *B. oleracea* crops exceeded 100 million metric tons in 2023, indicating its agricultural and economic significance [7].

The generic delimitation of *Brassica* remains unresolved. *Brassica oleracea* crops and their wild relatives, however, constitute the monophyletic B. sect. *Brassica* within the polyphyletic genus

Brassica [1]. The section includes about 13 species and several infraspecific taxa distributed across the Mediterranean Basin and the Atlantic coasts of Europe [8]. Several hypotheses on the progenitors of *Brassica oleracea* crops have been proposed. *Brassica montana* Pourr., *B. rupestris* Raf., *B. cretica* Lam., *B. incana* Ten., and wild *B. oleracea* have been nominated as single or multiple progenitor species [9–11]. Mabry et al. [6], using RNA-seq data of 224 accessions representing 14 *B. oleracea* crop types and nine potential wild progenitor species concluded that the Aegean endemic *B. cretica* is the closest living relative of cultivated *B. oleracea*, supporting an Eastern Mediterranean origin of cultivation. They also suggested that cultivated plants of this species can revert to a wild-like (feral) state with relative ease. A combination of those results with the evolutionary history of *B. oleracea* may contribute to a growing body of knowledge on *B. oleracea* crop domestication that will facilitate continued breeding efforts including adaptation to changing environmental conditions.

Brassica cretica is distributed in the Eastern Mediterranean (Albania, Greece, Türkiye, Lebanon, and Israel), with most of its populations concentrated in the Aegean Islands and the coastal regions of mainland Greece and Anatolia. The extensive phenotypic variability of *B. cretica* has led to the classification of multiple subspecies and varieties; however, there is no consensus regarding their taxonomic status. Gustafsson et al. [12] identify two subspecies: *Brassica cretica* subsp. *cretica* and subsp. *nivea*. In contrast, Snogerup et al. [11] recognize three subspecies: *B. cretica* subsp. *aegaea* subsp. *cretica*, and subsp. *laconica*. The disjunct distribution of *Brassica cretica* in the Middle East has been attributed either to early East Mediterranean trade [13] or to introductions into suitable natural habitats without cultivation [11]. Some populations within the natural range of the species in the Aegean may have also resulted from human-mediated dispersal. According to Mabry et al. [6], early forms of *B. cretica* may have played underappreciated roles in the domestication of *B. oleracea* crops. Therefore, resolving the domestication story of *B. cretica* is fundamental for fully understanding the evolutionary history of *B. oleracea*.

Brassica cretica is an extremely diverse wild relative of cultivated cabbage (*B. oleracea*), with genetic distances between its populations often exceeding those found between distinct species in the *B. villosa*/*B. rupestris*/*B. macrocarpa* group [14]. This diversity likely results from ‘isolation by distance’ processes, which have shaped significant morphological differences among local populations [11,15]. As it is a diploid ($2n = 18$) species, partially self-incompatible, hermaphroditic, and insect-pollinated, the geographical isolation of its populations, along with potential pollinator-driven selection based on flower color differences [16], may contribute to its high genetic diversity. Understanding this diversity is crucial for assessing the species’ adaptability and its potential use in breeding programs aiming at enhancing crop resilience [17].

Breeders of cole crops (*B. oleracea* L.) have an interest in utilizing current and emerging PCR-based marker systems to differentiate elite germplasm. Among various systems available for genetic analysis in plants, molecular markers are more efficient, precise and reliable in discriminating closely related species or cultivars [18–20]. SSRs are co-dominant, highly polymorphic PCR-based markers and thus expected to be a very powerful tool in cultivar discrimination. Polymorphic SSR markers have been recognized as a reliable tool for assessing genetic diversity and population structure in *Brassica* species in many cases [21–23] as well as identifying salinity tolerant genotypes [24]. Furthermore, SSR markers identified in cabbage can be applied to ten other wild and cultivated species within the Brassicaceae family. This highlights their potential value in advancing *Brassica oleracea* research, including genetic mapping, marker-assisted selection (MAS), and comparative genome analyses [20].

In this study, 10 SSR molecular markers were used to analyze the genetic diversity and population structure of five natural populations of *Brassica cretica* and one *B. oleracea* botanical variety to reveal genetic diversity at *B. cretica* population levels, reveal the distribution of genetic variation within and between the five natural populations, and identify the closest relative among the five *B. cretica* populations to cultivated species. The genetic information obtained will help to exploit the most promising population in terms of genetic diversity and similarity to cultivated species and thus to explore its future implication in breeding programs.

2. Materials and Methods

2.1. Plant Material & DNA Extraction

This study includes five natural *B. cretica* populations (two *B. cretica* subsp. *aegaea* populations originated from Evia and Ymittos, two *B. cretica* subsp. *cretica* populations originated from Akrokorinthos and Crete, and one *B. cretica* subsp. *laconica* population from Leonidio) from different regions of Greece and one commercial botanical variety of *B. oleracea* (population F) (Table 1; Figure 1). Ripe seeds from each population were collected on their natural habitats (Table 1; Figure 1) during physiological ripening period. Seedling preparation was conducted at the greenhouse facilities of the Laboratory of Vegetable Production at Agricultural University of Athens. Specifically, 20 ripe seeds per population were sown on rockwool sheets (AO Plug, Grodan, Roermond, the Netherlands), covered with a thin layer of vermiculite. During germination, seedlings were exposed to ambient light conditions and temperatures ranging from 25 °C to 27 °C during the day, and 17 °C to 19 °C during the night. Additionally, the seedlings were irrigated regularly with a nutrient solution containing 16.4 mM N, 6.6 mM K⁺, 4.4 mM Ca²⁺, 2.8 mM Mg²⁺, 2.75 mM SO₄²⁻, 1.3 mM H₂PO₄, 20 μM Fe (EDDHA), 10 μM Mn²⁺, 6 μM Zn²⁺, 0.8 μM Cu²⁺, 30 μM B, 0.7 μM Mo, resulting in an electrical conduc-tivity and pH of 2.4 dS/m and 5.6, respectively. One hundred and twenty (120) newly developed seedlings (20 per population) were analyzed in total.

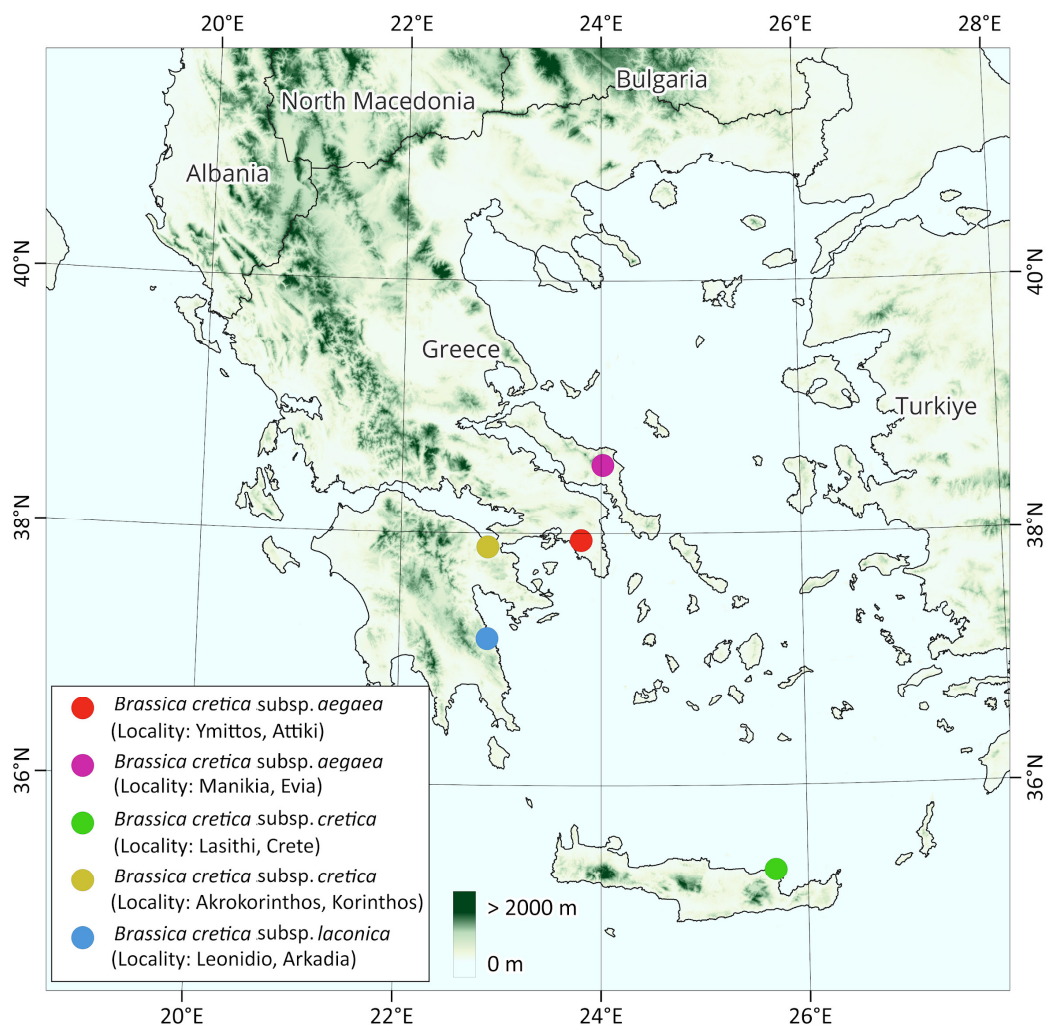


Figure 1. Geographical locations of the five natural populations of *Brassica cretica* included in this study.

Table 1. Collection localities of the studied populations, coordinates, and seed collection dates.

CODE	Taxon	Locality	Country	Seed collection date	Latitude	Longitude
A	<i>Brassica cretica</i> subsp. <i>aegaea</i>	Manikia, Evia	Greece	June 2022	38° 32.582'	24° 1.052'
B	<i>Brassica cretica</i> subsp. <i>aegaea</i>	Ymittos, Attiki	Greece	June 2022	37° 56.671'	23° 48.030'
C	<i>Brassica cretica</i> subsp. <i>cretica</i> or <i>nivea</i>	Akrokorinthos, Korinthos	Greece	May 2022	37° 53.363'	22° 52.138'
D	<i>Brassica cretica</i> subsp. <i>cretica</i>	Lasithi, Crete	Greece	May 2021	35° 19.772'	25° 41.458'
E	<i>Brassica cretica</i> subsp. <i>laconica</i>	Leonidio, Arkadia	Greece	June 2022	37° 9.940'	22° 52.255'
F	<i>Brassica oleracea</i> var <i>Rubra</i>	commercial variety of cabbage as outgroup				

Total genomic DNA was extracted from fresh plant tissue which was stored in deep freeze (–80°C) before DNA isolation. DNA was extracted according to classical CTAB protocol [25] with minor technical modifications. DNA concentrations and purity ratios were measured with Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Then, all individual DNA samples were diluted at final concentration 50 ng/μl with molecular grade water and stored at freeze –20 °C for PCR analysis.

2.2. Molecular Analysis; PCR Amplification & SSR Markers

The present study used ten SSR markers (Table 2) to analyze genetic variation namely, Ol10B11, Ol10B01, Ol09A01, Ol10F11, Ni4-B10, sORA26, BN12A, Na10-F06, nga111, MB4. Seven SSR primers were studied by [26]; Ol10F11, Ni4-B10, sORA26, BN12A, Na10-F06, nga111, MB4 and five markers described by [23]: Ol10B11, Ol10B01, Ol09A01, Ol10F11, sORA26.

PCR parameters were identical to previous studies with some technical modifications. Selection of those primers was based after an initial screening of a larger number of 14 primers during preparative experiments standardizing PCR conditions according to previously used protocols [23,26].

Table 2. Loci of populations studied, sequences of SSR primers, repeat motifs, PCR products (expected) size range (bp), and annealing temperatures (Ta) for each primer pair.

Locus	Forward & reverse primer sequences (5'→3')	Repeat Motif	Size range (bp)	Ta (°C)
Ol10B11	AAAATGTGAGGCTGTTTGGG TTTCGCAGCAGTAAACATGG	(GA) ₂₅	76–180	52,5
Ol10B01	CCTCTTCAGTCGAGGTCTGG AATTTGGAAACAGAGTCGCC	(GA) ₂₀	160–280	56
Ol09A01	TTCGAAGCTCATTATCGCAG CCGGGCTCTCTCTCTCTCTC	(GA) ₇₅	120–340	56,5
Ol10F11	TTTGGAACGTCCGTAGAAGG CAGCTGACTTCGAAAGGTCC	(GGC) ₇	139–184	56
Ni4-B10	GTCCTTGAGAACTCCACCG CCGATCCCATTCTAATCCC	(CT) ₂₀	170–200	56
sORA26	TGTTTACCTGTTGGAGAT AACCTAAGCATCTGCGA	(GA) ₅	62–76	49

BN12A	GCCGTTCTAGGGTTTGTGGGA GCCGTTCTAGGGTTTGTGGGA	(GA) ₁₁ (AAG) ₄	250–330	59
Na10-F06	CTCTTCGGTTCGATCCTCG TTTTTAACAGGAACGGTGGC	(CCG) ₆	84–126	54,5
nga111	TGTTTTTTAGGACAAATGGCG CTCCAGTTGGAAGCTAAAGGG	(GA) ₁₆	120–150	54,5
MB4	TGTTTTGATGTTTCCTACTG GAACCTGTGGCTTTTATTAC	(TG) ₁₀	57–69	50

PCR reactions were performed in 96-well plates Nippon Genetics thermal cycler (FastGene Ultra Cycler Gradient) where different PCRs could be performed at the same time in the same gradient machine using different SSR loci with different T_m (Table 2). The conditions applied for PCRs agreed with corresponding literature [23,26]. The annealing temperature (T_a) was kept 2–3 °C below the melting temperature (T_m) of that primer sequence.

For SSR genetic analysis, PCR amplifications were performed in a total volume of 20 µL for each molecular marker (Eurofins Genomics Custom DNA oligos) separately containing 50 ng/µl of DNA template, 1X reaction buffer (5X KAPA Taq reaction buffer with no magnesium), 2 mM of MgCl₂ (Kapa Biosystems) in the case of Ol10B11, Ol10B01 and Na10-F06, 2,5 mM of MgCl₂ and 0,25 µM of each dNTP (Enzyquest dNTP mix, 10 mM each), 0.4 µM each of the reverse and forward primer, except Na10-F06 and nga111 where we added 0.6 µM of each (Eurofins Genomics Custom DNA oligos) and 0.5 unit KAPA Taq DNA polymerase (Kapa Biosystems, 5u/µl). The first amplification step consisted of initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 35 s, primer annealing at 50 °C - 59 °C (varying with SSR primer pair) for 35 s, primer extension at 72 °C for 35 s and a final extension step at 72 °C for 7 min.

Three technical independent PCR reactions were performed for each SSR marker. The amplified fragments were resolved on 2.2% agarose gel running in 0.5X TBE electrophoresis buffer on device Mupid One Electrophoresis System using 50 bp DNA step ladder (Sigma Aldrich) as size standard reference. DNA bands were visualized under UV light using the gel illumination system Mini Bis Pro (DNRBio-Imaging Systems) connected with software Gel capture.

2.3. Data statistical Analysis

Utilizing binary data generated by SSR primers, the software GenAlex vs. 6.5 was used for the analysis of the results [27].

2.3.1. Variation Within Population

The effective numbers of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), total Expected Heterozygosity (H_t), and Shannon’s Information Index (I) were determined using GenAlex software.

2.3.2. Variation Among Populations

To estimate among-populations genetic variation, F_{st} (Fixation Index) was calculated to measure the genetic differentiation between the populations based on allele frequencies. For allelic richness (AR) and private allelic richness (pAR) calculations software HP-RARE was used [28]. Means and significant values over the loci and populations were obtained by bootstrapping to assess the variability and accuracy of the estimates. The hierarchical distribution of genetic variation (AMOVA) among and within the populations for the ten markers was assessed with GenAlEx software [27], allowing for a partition of the total genetic variation into among-group and within-group components. The effective size of each population (N_e) can be used in the calculation of the Shannon Indices (Shannon’s diversity index) (I) to quantify the richness and evenness of genetic diversity, as well as in the estimation of the effective number of migrants (N_m), which provides insights into gene

flow between populations. Nei’s unbiased genetic distance and significance among populations were also estimated, providing a measure of genetic divergence based on allele frequencies. Moreover, genetic distance for each pair of populations was calculated to investigate the relatedness between populations. FreeNA software [29] was used to estimate Nei’s unbiased genetic distance and significance among populations [30], incorporating methods for correcting null alleles. Cavalli-Sforza and Edward’s chord genetic distances (DC) [31], were also estimated for each pair of populations, utilizing the computed methods ENA (DCENA) and INA (DCINA) [29].

The dendrogram among populations was designed with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) based on Nei’s genetic distance using R software. The most likely number of clusters (K) was defined using DELTA Evanno’s method 9 [32] which evaluates the most likely number of genetic clusters based on a model of population structure, using.

3. Results

3.1. Genetic Variation Within Populations

A total of 219 alleles were obtained from the ten SSR loci analysed (Table 3). SSRs, Ol10B11, Ol10B01and Ol09A01 detected >25 alleles. The (total) number of alleles per locus (Na) varied from 16 (minimum Na10-F06) to 32 (maximum; Ol09A01). Respectively, effective number of alleles (Ne) ranged from minimum 2.413 (Na10-F06) to maximum 4,107 (Ol09A01), with a mean value of 3.050 per locus. The number of individuals per population was twenty (20), and the effective number of alleles was 21.9. Expected heterozygosity (He) within population for all SSR loci studied was 0.65.

Table 3. Measured parameters of ten SSR loci across populations.

Locus	Ol10B11	Ol10B01	Ol09A01	Ol10F11	Ni4-B10s	ORA26	BN12A	Na10-F06	nga111	MB4	MEAN
Na	27	30	32	19	19	18	18	16	20	20	21,9
Ne	3,606	3,985	4,107	2,489	2,844	2,759	2,629	2,413	2,805	2,859	3,049
I	1,374	1,438	1,418	0,977	1,081	1,052	1,005	0,907	1,082	1,114	1,145
Ho	0,075	0,35	0,308	0,308	0,175	0	0	0,05	0,017	0	0,128
He	0,720	0,734	0,705	0,583	0,638	0,634	0,61	0,568	0,631	0,645	0,647

Na = (total) number of alleles, Ne = number of effective alleles, I = Shannon’s information index, Ho = observed heterozygosity, He = Nei’s expected heterozygosity, Mean F-Statistics represent arithmetic averages. p<0,05.

3.2. Genetic Variation Between and Within Populations

The average number of alleles (Na) per population ranged from 3.200 (population C) to 4.400 (population A), with a mean of 3.650 alleles per individual population. The effective number of alleles (Ne) ranged between 2.747 in population E and 3.519 in the population A, with a mean of effective 3.050 alleles per individual population. Population A, obviously, indicates a higher genetic stability. The expected heterozygosity (He) varied between 0.617 (minimum) (population F) and 0.678 (maximum) (population B). Observed heterozygosity (Ho) was generally noticed in low levels, ranging from 0.060 (population C) to 0.220 (population F). The overall average allelic richness (AR) was 3.26; the highest (3.69) was noticed in population A, whereas the lowest (3.05) was in populations C & E. Private allelic richness (pAR) varied between 0.09 (population D) and 0.86 (population B) with a mean value 0.62 (Table 4).

Table 4. Genetic diversity parameters at population level based on ten SSR loci: Na = (mean) number of alleles, Ne = (mean) number of effective alleles, I = Shannon’s information index, He = expected heterozygosity, Ho =observed heterozygosity, AR= allelic richness, pAR =private allelic richness, F =Wright’s fixation index. Mean F-Statistics represent arithmetic averages.

Population	Na	Ne	I	He	Ho	AR	pAR	Fst
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A	4.400	3.519	1.279	0.677	0140	3.69	0.82	0.808
B	3800	3.191	1.218	0678	0.095	3.44	0.86	0.862
C	3200	2.917	1.091	0645	0.060	3.05	0.62	0.886
D	3700	3.043	1.131	0.636	0.125	3.25	0.09	0.840
E	3.300	2.747	1.075	0.629	0.130	3.05	0.60	0.792
F	3500	2.879	1.075	0.617	0.220	3.09	0.72	0.682
Mean	3.650	3.050	1.145	0.647	0.128	3.26	0.62	0.812

The genetic differentiation between populations was high ($F=0.812$; $p>0.05$). AMOVA test proved that differentiation within-population was 80% and among populations was 20% (Figure 2).

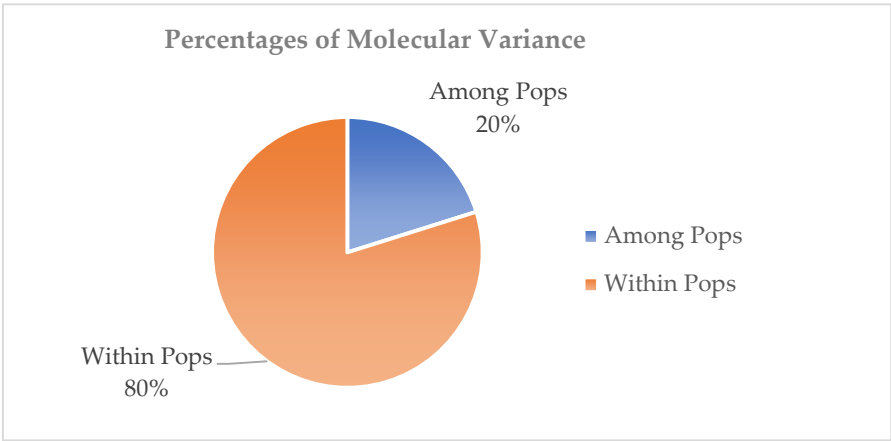


Figure 2. Analysis of molecular variance (AMOVA) among and within five *Brassica cretica* populations.

The DELTA method of Evanno [32] was employed to determine the optimal number of clusters (K) (Figure 2), which revealed the presence of four distinct gene pools (K=4) (Figure 3) in the Structure analysis performed using Clumpak software [33].

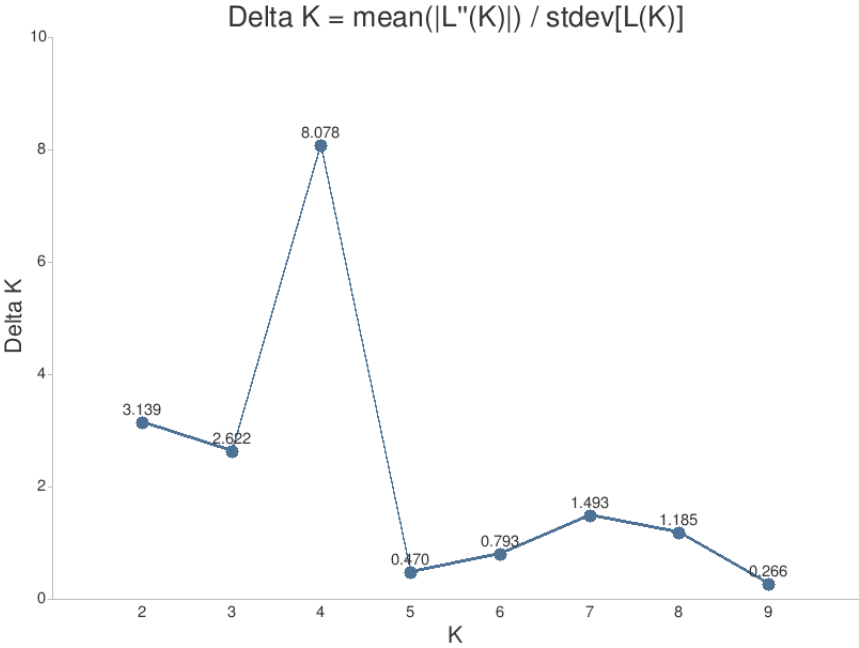


Figure 3. Structure analysis results of studied populations when K=4.

The STRUCTURE analysis results revealed (optimum) four gene pools in Greece (Figure 3). Group I included *B. cretica* subsp. *aegaea* populations from Evia and Ymittos. At group II, populations

of *B. cretica* subsp. *cretica* from Crete and northern Peloponnese were included. Groups III and IV were formed by *B. cretica* subsp. *laconica* and *B. oleracea*, respectively (Figure 4).

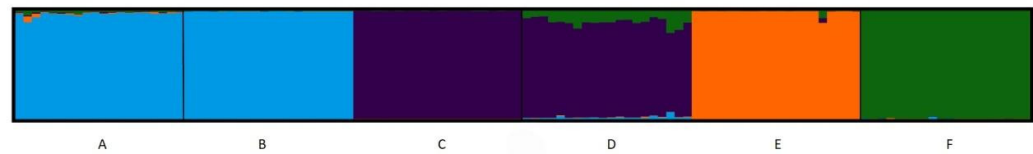


Figure 4. Results from Structure analysis of studied populations (K=4) using Clumpak software. Population codes according to Table 1.

To further examine relationships among populations the INA and ENA methods were used to calculate Pairwise populations Fst Values. (Table 5). The greatest genetic distance was observed between populations C and F (Fst= 0.236) indicating a high genetic divergence, while the smallest genetic distance between populations C and D (Fst= 0.106), indicating their close genetic relatedness.

Table 5. Corrected coefficient of genetic differentiation (Fst) between populations, with INA method (above the diagonal; 0.000) and ENA method (below the diagonal) [29].

A	B	C	D	E	F	
0.000	0.081	0.147	0.094	0.145	0.125	A
0.122	0.000	0.128	0.123	0.156	0.148	B
0.203	0.172	0.000	0.074	0. 179	0,177	C
0.131	0.175	0.106	0.000	0.151	0.129	D
0.200	0.212	0.234	0.212	0.000	0.154	E
0.172	0.199	0.236	0.179	0.215	0.000	F

A UPGMA dendrogram (Figure 4), based on Nei’s (unbiased) genetic distance matrix [30], was constructed using R software and the Ape and Phangorn packages [34,35], to illustrate the genetic relationships among the six studied populations. Populations of *B. cretica* subsp. *aegaea* and *B. cretica* subsp. *cretica* form two distinct clades. The *B. oleracea* cultivar forms a clade that is sister to the clade comprising these two subspecies. Notably, *B. cretica* subsp. *laconica* appears genetically distinct from the other *B. cretica* subspecies, forming an entirely separate clade. These findings are largely consistent with the results of the STRUCTURE analysis.

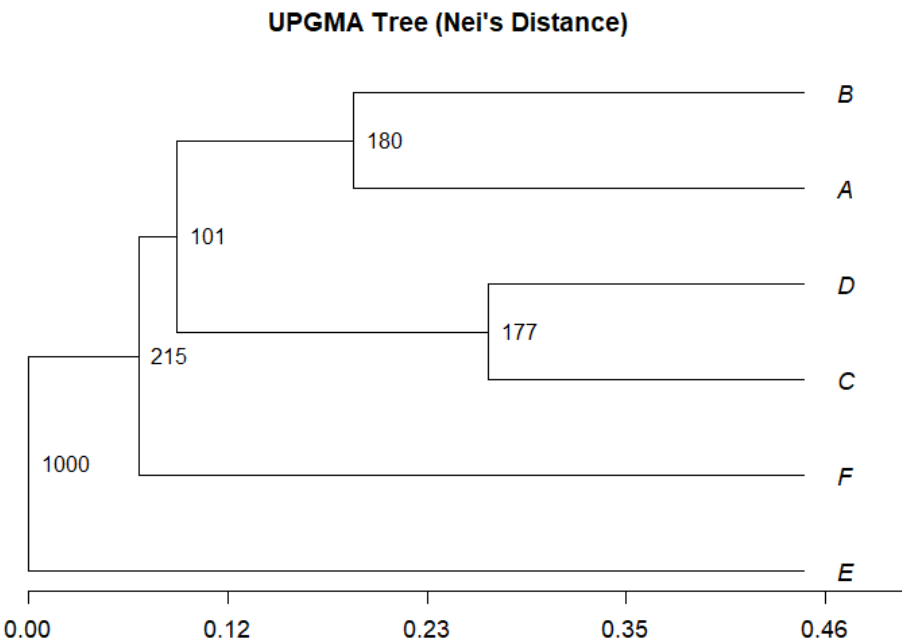


Figure 4. UPGMA tree using Nei’s genetic distance. Bootstrap values are presented at the branch intersections.

4. Discussion

In the Mediterranean basin, the fragmentation of species into genetically isolated populations has significant implications for diversification, adaptation, and speciation. The rich Aegean flora is a product of the region’s complex geological history, which has shaped a fragmented insular landscape. Many plant species exist in small, isolated populations with limited migration [36–38], contributing to the characteristic mosaic variation of Aegean plant taxa [11,39,40]. Herein, ten SSR markers were used to assess the genetic diversity and population structure of five *Brassica cretica* populations and one *B. oleracea* botanical variety. This study aimed to evaluate genetic diversity within and genetic differentiation among *B. cretica* populations, examine the distribution of genetic variation, and identify the closest relative to the cultivated plants. We found high genetic diversity, with the number of alleles per locus (N_a) ranging from 18 (sORA26 and BN12A) to 32 (OI09A01), with a mean value of 20. Notably, the observed N_a was higher than that reported by [26] ($N_a=5.4$) for several shared loci (OI10-F11, Ni4-B10, sORA26, BN12A, Na10-F06, nga111 and MB4). Furthermore, our study recorded a mean number of private alleles of 0.62, significantly higher than the 0.141 reported in the previous study, indicating a unique gene pool to our study populations.

The average number of alleles per population (N_a) in our study was 3.65, which is higher than the 1.9 reported for seven *B. cretica* populations from Crete [26] and greater than the values found in 25 *B. oleracea* accessions from Ireland using similar molecular markers [41]. This higher allele number may be attributed to the isolation by distance among our populations compared to the cultivated species studied by El-Esawi et al. [41]. Additionally, the wider geographical distribution of our sampled populations across long-established biogeographical barriers, compared to the more geographically restricted Cretan populations examined by [26], may further account for the observed increase in genetic diversity.

The populations of all *B. cretica* subspecies showed similar heterozygosity (H_e) values. The average expected heterozygosity (H_e) across the studied populations was 0.647, significantly higher than the values reported in previous studies. Edh et al. [26] and Maggioni et al. [42] found a H_e of 0.211, while Watson-Jones et al. [43] reported a H_e of 0.26 in their analyses of nine wild *Brassica* populations, respectively. Allelic richness was 3.26 and private allelic richness was pAR (0.62) higher

than the value (0.141) reported by Edh et al. [26]. This higher number of heterozygosity and private allelic richness can be partly attributed to outcrossing breeding systems or the methodology used.

The mean F_{ST} value for our studied populations was 0.81, indicating a high level of differentiation among them. Similar results were reported by Edh et al. [26] using nuclear and chloroplast markers, which revealed exceptionally high levels of population diversity (overall F_{ST} = 0.628 and 1.000, respectively). In addition, although Watson-Jones [43] reported lower F_{ST} values (0.2257), they also detected considerable differentiation among populations. These results suggest that *Brassica* species, particularly *B. cretica* populations, are fragmented across various habitats and have adapted to varying environmental conditions, including soil type, temperature, and moisture levels. Such environmental differences may drive local adaptation and contribute to genetic divergence between populations [26].

Genetic distance analysis and UPGMA dendrograms consistently reveal genetic divergence among *Brassica* samples, classifying them into four major genetic groups. This grouping is further supported by population structure analysis. Three key conclusions emerge from these findings. *Brassica cretica* subsp. *cretica* and subsp. *aegaea* are genetically distinct but monophyletic, partly supporting the intraspecific classification proposed by Snogerup et al. [11]. While this classification is widely accepted by sources like POWO [44], Euro+Med [45], and the Flora of Greece web (2025), <https://portal.cybertaxonomy.org/flora-greece/intro>, among others, the classification into two subspecies (i.e., *B. cretica* subsp. *cretica* and subsp. *nivea*) proposed by Gustafsson et al. [12]) is still followed in some studies, e.g., [46,47]. Our results indicate that the population from southern Peloponnese (Leonidio), classified as *B. cretica* subsp. *laconica*, significantly deviates from the other two subspecies of *B. cretica*. The flavonoid composition of *B. cretica* subsp. *laconica* also contrasts to that of the other two subspecies [48], suggesting a distinct differentiation in southern Peloponnesian populations. Their taxonomic re-evaluation could provide insights into the evolutionary history of *B. oleracea*/*B. cretica* complex, advancing our understanding on the domestication of *B. oleracea* crops.

The STRUCTURE analysis reveals a high degree of genetic similarity between the populations from northern Peloponnese (Akrokorinthos Castle) and eastern Crete. According to Snogerup et al. [11], populations from both regions belong to *Brassica cretica* subsp. *cretica*. Notably, the Akrokorinthos Castle population, located at the acropolis of ancient Corinth with a continuous human presence for millennia, was designated as the type locality for this subspecies [11]. These authors also suggested that the disjunct distribution of *B. cretica* subsp. *cretica* in Crete and northern Peloponnese likely reflects remnants of a once more continuous range rather than human introduction. This conclusion was supported by observed chromosomal rearrangements and reduced interfertility between populations. However, our analyses indicate high genetic similarity between the Akrokorinthos and the Cretan populations and reduced genetic variability in the Akrokorinthos population ($H_o=0.060$), suggesting that an early human introduction to northern Peloponnese cannot be ruled out. Chromosomal rearrangements and reduced interfertility among populations may have resulted from a founder effect, leading to reduced genetic diversity and the fixation of chromosomal mutations, as well as genetic drift, selection, or local adaptation. Interestingly, the Akrokorinthos population has rather low genetic similarity to *B. oleracea* cultivar, possibly indicating an introduction to suitable habitats without cultivation as suggested by Snogerup et al. [11].

The *Brassica oleracea* cultivar clusters closely with *B. cretica* subsp. *cretica*, particularly with the Cretan populations, which exhibit the highest genetic similarity. This observation agrees with previous findings by Mabry et al. [6], who identified *B. cretica* as the closest extant relative of domesticated *B. oleracea*, lending further support to the hypothesis of an eastern Mediterranean origin for cultivated cabbages and kales. Historical evidence also suggests an eastern Mediterranean origin for *B. oleracea*. Descriptions of kales, a form of *B. oleracea*, first appear in the works of the Greek scholar Theophrastus (370–285 BC), whereas earlier texts from 800–600 BC make no mention of such plants. This temporal context implies that domestication likely took place in this region during that period [49].

Using SSR markers we were able to detect the genetic diversity in five *B. cretica* populations. Our findings indicate the need for additional molecular and phenotypic studies to clarify the taxonomic classification of *B. cretica* in the Aegean region. These investigations should include a broader range of populations from different regions of Greece and the eastern Mediterranean. Our results also revealed high genetic diversity and allelic richness in *B. cretica* populations, making them a valuable genetic resource for breeding with *Brassica* crops.

Climate change threatens both the survival of native Brassica populations and the sustainability of Brassica crop production. Rising temperatures disrupt flowering times, pollination, and seed viability, while extreme heat stress reduces germination and biomass production [49]. Drought and water stress further hinder seed germination, early development, and overall yield, with wild populations facing habitat loss due to desertification [50]. Extreme weather events further damage plants, displace seeds, and accelerate soil erosion, while habitat fragmentation and genetic erosion reduce adaptive potential. Climate-induced pollinator declines and shifts in flowering periods disrupt reproduction [51]. To mitigate these threats, conservation efforts must focus on preserving genetic diversity, protecting natural habitats, and implementing sustainable agricultural practices. To our knowledge, very few accessions of *B. cretica* currently exist in genetic banks in Greece. Therefore, establishing ex situ conservation measures for Greek populations, including seed collection and vegetative propagation, should be a high priority.

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References

1. Arias, T.; Pires, J.C. A fully resolved chloroplast phylogeny of the brassica crops and wild relatives (*Brassicaceae: Brassicaceae*): Novel clades and potential taxonomic implications. *Taxon* 2012, 61, 980-988.
2. Lysak, M.A.; Koch, M.A.; Pecinka, A.; Schubert, I. Chromosome triplication found across the tribe *Brassicaceae*. *Genome research* 2005, 15, 516-525.
3. Schranz, M.E.; Lysak, M.A.; Mitchell-Olds, T. The ABC's of comparative genomics in the *Brassicaceae*: building blocks of crucifer genomes. *Trends in plant science* 2006, 11, 535-542.
4. Tatout, C.; Warwick, S.; Lenoir, A.; Deragon, J.-M. SINE insertions as clade markers for wild crucifer species. *Molecular Biology and Evolution* 1999, 16, 1614-1614.
5. Eiseley, L.C.; Grote, A. Charles Darwin, Edward Blyth, and the Theory of Natural Selection. *Proceedings of the American Philosophical Society* 1959, 103, 94-158.

6. Mabry, M.E.; Turner-Hissong, S.D.; Gallagher, E.Y.; McAlvay, A.C.; An, H.; Edger, P.P.; Moore, J.D.; Pink, D.A.; Teakle, G.R.; Stevens, C.J. The evolutionary history of wild, domesticated, and feral *Brassica oleracea* (*Brassicaceae*). *Molecular Biology and Evolution* 2021, 38, 4419-4434.
7. Ndong, J.T.K. Review of the Food and Agriculture Organisation (FAO) Strategic Priorities on Food Safety 2023. In *Food Safety-New Insights*; IntechOpen: 2023.
8. Sciandrello, S.; Brullo, C.; Brullo, S.; Giusso Del Galdo, G.; Minissale, P.; Salmeri, C. A new species of *Brassica* sect. *Brassica* (*Brassicaceae*) from Sicily. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology* 2013, 147, 812-820.
9. Hodgkin, T. Cabbages, kales, etc. 76-82. In J. Smartt and N. Simmonds (ed.). *Evolution of crop plants*. 1995.
10. Maggioni, L.; von Bothmer, R.; Poulsen, G.; Lipman, E. Domestication, diversity and use of *Brassica oleracea* L., based on ancient Greek and Latin texts. *Genetic Resources and Crop Evolution* 2018, 65, 137-159.
11. Snogerup, S.; Gustafsson, M.; Von Bothmer, R. *Brassica* sect. *Brassica* (*Brassicaceae*) I. taxonomy and variation. *Willdenowia* 1990, 271-365.
12. Gustafsson, M.; Bentzer, B.; Bothmer, R.v.; Snogerup, S. Meiosis in Greek *Brassica* of the *oleracea* group. 1976.
13. Dixon, G. Origins and diversity of *Brassica* and its relatives. In *Vegetable brassicas and related crucifers*; CABI Wallingford UK: 2006; pp. 1-33.
14. Lázaro, A.; Aguinagalde, I. Genetic Diversity in *Brassica oleracea* L. (*Cruciferae*) and Wild Relatives ($2n=18$) using RAPD Markers. *Annals of Botany* 1998, 82, 829-833.
15. Widén, B.; Andersson, S.; Rao, G.Y.; Widén, M. Population divergence of genetic (co) variance matrices in a subdivided plant species, *Brassica cretica*. *Journal of Evolutionary Biology* 2002, 15, 961-970.
16. Grant, V. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences* 1994, 91, 3-10.
17. Frankham, R. Inbreeding and extinction: a threshold effect. *Conservation Biology* 1995, 9, 792-799.
18. Hu, S.; Yu, C.; Zhao, H.; Sun, G.; Zhao, S.; Vyvadilova, M.; Kucera, V. Genetic diversity of *Brassica napus* L. Germplasm from China and Europe assessed by some agronomically important characters. *Euphytica* 2007, 154, 9-16.
19. Mishra, P.; Singh, N. Allelic diversity among short duration maize (*Zea mays* L.) genotypes using SSR markers. *Madras Agricultural Journal* 2012, 99, 1.
20. Xu, Y.; Xing, M.; Song, L.; Yan, J.; Lu, W.; Zeng, A. Genome-wide analysis of simple sequence repeats in cabbage (*Brassica oleracea* L.). *Frontiers in Plant Science* 2021, 12, 726084.
21. Allender, C.; Allainguillaume, J.; Lynn, J.; King, G.J. Simple sequence repeats reveal uneven distribution of genetic diversity in chloroplast genomes of *Brassica oleracea* L. and ($n=9$) wild relatives. *Theoretical and Applied Genetics* 2007, 114, 609-618.
22. Shi, J.; Huang, S.; Zhan, J.; Yu, J.; Wang, X.; Hua, W.; Liu, S.; Liu, G.; Wang, H. Genome-wide microsatellite characterization and marker development in the sequenced *Brassica* crop species. *DNA research* 2014, 21, 53-68.
23. Thakur, A.K.; Singh, K.H.; Singh, L.; Nanjundan, J.; Khan, Y.J.; Singh, D. SSR marker variations in *Brassica* species provide insight into the origin and evolution of *Brassica* amphidiploids. *Hereditas* 2018, 155, 1-11.
24. Kumar, M.; Choi, J.-Y.; Kumari, N.; Pareek, A.; Kim, S.-R. Molecular breeding in *Brassica* for salt tolerance: importance of microsatellite (SSR) markers for molecular breeding in *Brassica*. *Frontiers in Plant Science* 2015, 6, 688.
25. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical bulletin* 1987.
26. Edh, K.; Widén, B.; Ceplitis, A. Nuclear and chloroplast microsatellites reveal extreme population differentiation and limited gene flow in the Aegean endemic *Brassica cretica* (*Brassicaceae*). *Molecular Ecology* 2007, 16, 4972-4983.
27. Peakall, R.; Smouse, P.E. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 2006, 6, 288-295.
28. Kalinowski, S.T. hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular eEcology Notes* 2005, 5, 187-189.

29. Chapuis, M.-P.; Estoup, A. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 2007, 24, 621-631.
30. Nei, M. Analysis of gene diversity in subdivided populations. *Proceedings of the national academy of sciences* 1973, 70, 3321-3323.
31. Cavalli-Sforza, L.L.; Edwards, A.W. Phylogenetic analysis. Models and estimation procedures. *American Journal of Human Genetics* 1967, 19, 233.
32. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 2005, 14, 2611-2620.
33. Kopelman, N.M.; Mayzel, J.; Jakobsson, M.; Rosenberg, N.A.; Mayrose, I. Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular ecology resources* 2015, 15, 1179-1191.
34. Schliep, K.; Potts, A.A.; Morrison, D.A.; Grimm, G.W. Intertwining phylogenetic trees and networks; 2167-9843; *PeerJ Preprints*: 2016.
35. Paradis, E.; Schliep, K. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 2019, 35, 526-528.
36. Higgins, S.I.; Lavorel, S.; Revilla, E. Estimating plant migration rates under habitat loss and fragmentation. *Oikos* 2003, 101, 354-366.
37. Neilson, R.P.; Pitelka, L.F.; Solomon, A.M.; Nathan, R.; Midgley, G.F.; Fragoso, J.M.; Lischke, H.; Thompson, K. Forecasting regional to global plant migration in response to climate change. *Bioscience* 2005, 55, 749-759.
38. Snogerup, S. The Mediterranean islands. *Plant Conservation in the Mediterranean area* 1985, 159-173.
39. Bittkau, C.; Comes, H.P. Molecular inference of a Late Pleistocene diversification shift in *Nigella s. lat. (Ranunculaceae)* resulting from increased speciation in the Aegean archipelago. *Journal of Biogeography* 2009, 36, 1346-1360.
40. Runemark, H. Reproductive drift, a neglected principle in reproductive biology. 1969.
41. El-Esawi, M.A.; Germaine, K.; Bourke, P.; Malone, R. Genetic diversity and population structure of *Brassica oleracea* germplasm in Ireland using SSR markers. *Comptes rendus biologiques* 2016, 339, 133-140.
42. Maggioni, L.; von Bothmer, R.; Poulsen, G.; Härnström Aloisi, K. Survey and genetic diversity of wild *Brassica oleracea* L. germplasm on the Atlantic coast of France. *Genetic Resources and Crop Evolution* 2020, 67, 1853-1866.
43. Watson-Jones, S.; Maxted, N.; Ford-Lloyd, B. Population baseline data for monitoring genetic diversity loss for 2010: a case study for Brassica species in the UK. *Biological Conservation* 2006, 132, 490-499.
44. POWO. Plants of the world online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet. 2023.
45. Euro+ Med, E. Euro+ Med PlantBase—the information resource for Euro-Mediterranean plant diversity. Available online at: 2006.
46. Rakow, G. Species origin and economic importance of *Brassica*. In *Brassica*; Springer: 2004; pp. 3-11.
47. Kioukis, A.; Michalopoulou, V.A.; Briers, L.; Pirentos, S.; Studholme, D.J.; Pavlidis, P.; Sarris, P.F. Intraspecific diversification of the crop wild relative *Brassica cretica* Lam. using demographic model selection. *BMC genomics* 2020, 21, 1-14.
48. Aguinalde, I., Gómez-Campo, C., & Sanchez-Yelamo, M. D. A chemosystematic survey on wild relatives of *Brassica oleracea* L. *Botanical journal of the Linnean Society*, 1992, 109, 57-67.
49. Maggioni, L.; von Bothmer, R.; Poulsen, G.; Branca, F. Origin and Domestication of Cole Crops (*Brassica oleracea* L.): Linguistic and Literary Considerations1. *Economic Botany* 2010, 64, 109-123, doi:10.1007/s12231-010-9115-2.
50. Palmgren, M.; Shabala, S. Adapting crops for climate change: regaining lost abiotic stress tolerance in crops. *Frontiers in Plant Science* 2024, 2, 1416023.
51. Batool, M.; El-Badri, A.M.; Hassan, M.U.; Haiyun, Y.; Chunyun, W.; Zhenkun, Y.; Jie, K.; Wang, B.; Zhou, G. Drought stress in *Brassica napus*: effects, tolerance mechanisms, and management strategies. *Journal of Plant Growth Regulation* 2022, 1-25.

52. Trunschke, J.; Junker, R.R.; Kudo, G.; Alexander, J.M.; Richman, S.K.; Till-Bottraud, I. Effects of climate change on plant-pollinator interactions and its multitrophic consequences. *Alpine Botany* 2024, 134, 115-121.

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